



**HAL**  
open science

# Production d'ingrédients laitiers fonctionnalisés par des microorganismes producteurs de composés antifongiques

Lucille Garnier

## ► To cite this version:

Lucille Garnier. Production d'ingrédients laitiers fonctionnalisés par des microorganismes producteurs de composés antifongiques. Microbiologie et Parasitologie. Université de Bretagne occidentale - Brest, 2017. Français. NNT : 2017BRES0074 . tel-02116505

**HAL Id: tel-02116505**

**<https://theses.hal.science/tel-02116505v1>**

Submitted on 1 May 2019

**HAL** is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.



université de bretagne  
occidentale

UNIVERSITE  
BRETAGNE  
LOIRE

THÈSE / UNIVERSITÉ DE BRETAGNE OCCIDENTALE  
*sous le sceau de l'Université Bretagne Loire*

pour obtenir le titre de  
DOCTEUR DE L'UNIVERSITÉ DE BRETAGNE OCCIDENTALE  
*Spécialité: Microbiologie, virologie et parasitologie*  
École Doctorale EGAAL

présentée par

**Lucille Garnier**

Préparée au Laboratoire Universitaire de  
Biodiversité et Ecologie Microbienne (EA 3882)

# Production d'ingrédients laitiers fonctionnalisés par des microorganismes producteurs de composés antifongiques

**Thèse soutenue le 2 octobre 2017**

devant le jury composé de :

**Monique ZAGOREC**

Directrice de recherche, INRA, Nantes / *Rapporteur*

**Henry Eric SPINLER**

Professeur, AgroParisTech / *Rapporteur*

**Laurence ABRAHAM**

Responsable laboratoire RD, Sodiaal Fromages/ *Examinatrice*

**Jean-Luc JANY**

Maître de Conférences, Université de Brest / *Examinateur*

**Florence VALENCE**

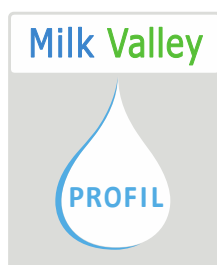
Docteur/Ingénieur, INRA, Rennes / *Encadrante de thèse*

**Jérôme MOUNIER**

Professeur, Université de Brest / *Directeur de thèse*



Cette thèse, réalisée en co-tutelle entre le Laboratoire Universitaire de Biodiversité et Ecologie Microbienne (LUBEM) et l'Institut National pour la Recherche en Agronomie (INRA) – Sciences et Technologie du Lait et de l'Œuf (STLO), s'intègre dans le projet interrégional « Assemblages protéiques multifonctionnels pour l'innovation en industrie laitière » (PROFIL) monté par l'association Bretagne biotechnologies alimentaires (Bba), l'INRA –STLO et le Pôle Agronomique Ouest (PAO), avec le soutien financier des régions Bretagne et Pays de la Loire.





*« Et demain... tout commence. »*

*Samuel, Demain tout commence – Hugo G lin*



# Remerciements

*Je tiens tout d'abord à remercier les membres du jury, qui m'ont fait l'honneur d'accepter d'évaluer ce travail. Je leur suis reconnaissante du temps et de l'énergie qu'ils y ont consacrés.*

*Je remercie également Emmanuel Coton, directeur du LUBEM, pour m'avoir accueillie au sein de son laboratoire et pour l'aide et les conseils qu'il a su m'apporter au cours de ces trois années.*

*Je souhaite également remercier les financeurs et organisateurs du projet PROFIL cités ci-avant pour m'avoir permis de réaliser mes travaux de recherche dans les meilleures conditions possibles et pour m'avoir permis également de participer à des congrès ou journées thématiques en France comme à l'étranger.*

*Jérôme et Florence, merci pour tout. Merci pour la confiance que vous m'avez accordée en me permettant de travailler sur ce projet. Merci pour votre aide, vos conseils, votre accompagnement et votre soutien. Merci pour le temps que vous m'avez consacré. Merci pour votre investissement dans l'écriture des articles, de la revue scientifique et dans la correction de mon manuscrit. J'ai beaucoup appris grâce à vous et travailler avec vous a été un réel plaisir. Je vous souhaite beaucoup de réussite dans la suite de votre travail.*

*Je suis également extrêmement reconnaissante envers l'ensemble du personnel du LUBEM et de l'ESLAB pour leur accueil chaleureux. Et je pense à Audrey en particulier : merci pour ta disponibilité, pour ton aide généreuse et pour tous tes conseils judicieux. Un grand merci à Franck également pour tes nombreux conseils sur les champignons et à Amélie pour m'avoir sorti et entré d'innombrables souches de la collection! Je tiens également à exprimer toute ma gratitude aux locataires du bureau voisin : merci Gaëtan pour toutes tes remarques et interventions très subtiles qui m'ont fait beaucoup rire. Et surtout, un grand merci à Karim pour toutes les fois où « Tu-sais-qui » a essayé de te joindre quand tu n'étais pas là...! Stella, Christophe, Sylvie, Dorothée, Florian et Elisabeth, je vous remercie pour votre gentillesse et pour m'avoir toujours rendu service quand je vous sollicitais. Mes remerciements vont également à MHT pour ta gentillesse à mon égard et pour le super surnom que tu as trouvé: Samoussette !*

*Je pense également à ceux avec qui j'ai partagé le bureau. Guillaume, malgré ta semi-surdité tu as su m'écouter et me comprendre, ou presque... :). Nicolas, ou le digitabuphiliste du bureau, j'espère que tu es fier de la collection que tu as maintenant, en tout cas, moi je suis fière d'y avoir contribué ! Et enfin, Fabienne, Annie, Marcia, Laura et Guillaume je vous souhaite le meilleur pour la suite de votre thèse et pour la suite de votre travail.*



*Une pensée également pour tous les stagiaires qui ont mis le pied à un moment ou à un autre dans ce projet. Dans l'ordre chronologique, Nicolas, Mégane, Mathilde, Marine et Charles, merci pour votre aide, votre gentillesse et votre bonne humeur quotidienne avec une mention spéciale pour Marine qui m'a énormément aidée ces 6 derniers mois.*

*J'aimerais aussi remercier toute l'équipe du STLO de Rennes, en particulier Nico, Béné, Manon, Victo, Jessica, Paulette, Marie-No, Julien, Marie B., Nadine, Marielle, Gilles et Anne, qui m'ont accueilli les bras ouverts et qui ont été d'une grande aide pour l'avancement de ces travaux.*

*Un grand merci également à Morgane et Stéphan du PAO pour l'organisation des comités techniques et réunions avec les industriels, pour m'avoir permis de participer aux assemblées générales du PAO et de Bba et merci également pour la visite de l'usine Bel à Vendôme. J'aimerais aussi vous remercier pour votre soutien et votre bonne humeur toujours au rdv.*

*J'aimerais également faire une petite dédicace à l'équipe régionale féminine de volley de l'ESL avec qui j'ai passé de très bons moments ces 2 dernières années! Alors, Zaza, Laure, Sarace, Daphné, Camille, Fleur Caramel, Marie, Marine, Mallorie, Nono, Pauline, Soso et évidemment le(s) coach(es), merci pour tout!!*

*Je tiens aussi à remercier tout particulièrement Marlène et Marie-Caroline, ce sont deux amies que j'ai rencontré au LUBEM. Merci pour tous les moments qu'on a partagé, les pauses, les pique-niques, les soirées, les apéros, les repas, les restos, les journées, les week-ends au Futuroscope, au parc Astérix,... Merci aussi pour la via ferrata c'était top ! MC merci de m'avoir laissé ton poney quelques temps (je suis très fière d'être sa tata même si après de nombreux essais infructueux, il a réussi à me mettre par terre!!) et merci aussi pour tes blagues de rire et chanson et tes proverbes « à la con » ça aide à détendre l'atmosphère dans les moments critiques. Marlène, merci pour l'année de covoiturage, je sais que je peux compter sur toi, c'est ça quand on est frère et sœur. Bref, sans vous, je n'aurai pas su quoi faire alors merci pour tout et surtout ne changez pas! Vous allez me manquer!*

*J'aimerais aussi dire un grand merci à ma famille, qui, malgré les plus ou moins 10000km qui nous séparent, ont cru en moi, et m'ont soutenu pendant ces 8 années d'études. Si j'en suis là, c'est donc aussi et surtout grâce à vous. Vous m'apportez tous un petit quelque chose. Des encouragements, du réconfort, du rire, mais aussi des colipays, des cartes postales et des supers voyages (Rodrigues, Lisbonne et bien évidemment la Réunion même si c'est la maison... )!! Papa et maman, je sais que vous êtes là pour moi mais sachez que je serai là pour vous aussi, dans les bons comme dans les mauvais moments. Et enfin Alice, merci pour tous ces moments qu'on a partagé à la Réunion comme en Métropôle (à lire avec le même accent que le « Letchi amer »), tous ces moments sportifs, tous ces fou-*

*rires, tout simplement merci d'être là et je ne te souhaite que le meilleur pour la suite de tes aventures !  
Nartrouve bientôt!*

*Et Mathieu, tu sais ce que je pense de toi et de nous alors merci pour tout et surtout merci d'être là...*

*Pour finir, je dirai que ces 3 années passées en Bretagne ont été très enrichissantes, tant d'un point de vue humain, sportif, que scientifique mais aussi météorologique (oui... la Bretagne étant la seule région en France où il peut vraiment faire beau plusieurs fois par jour...) et que ces années resteront gravées dans ma mémoire... et dans mon manuscrit!*



# Avant-propos

Le projet PROFIL a pour objectif principal la création de produits laitier sans additifs répondant ainsi aux attentes intrinsèques de naturalité des consommateurs et correspondant aux nouveaux usages et à de nouvelles habitudes de consommation et ceci via la valorisation des protéines lactières. Le projet est divisé en 4 grands axes concernant (i) les propriétés antifongiques, dans lequel s'insère cette thèse, (ii) les propriétés émulsifiantes, (iii) les propriétés texturantes et (iv) les propriétés de vectorisation et d'encapsulation.

Le texte est structuré en quatre parties. La première partie, intitulée « **Introduction générale** », pose la problématique de la recherche et résume les connaissances actuelles sur l'histoire et la diversité des produits laitiers, sur la diversité et la taxonomie des bactéries lactiques, propioniques et des champignons, sur la diversité des contaminations fongiques dans les produits laitiers et enfin sur les moyens de lutte mis en place pour lutter contre ces contaminations et l'intérêt des microorganismes et de leurs activités antifongiques, en biopréservation. Le dernier chapitre de cette revue bibliographique fait l'objet d'une revue scientifique publiée dans le journal *Microorganisms* dans le cadre d'un numéro spécial « *Food and Microorganisms* » et s'intitule « ***Diversity and control of spoilage fungi in dairy products : an update*** ». Suite à cette revue bibliographique, des hypothèses sont formulées, les objectifs principaux de la recherche sont posés et les différentes étapes du travail sont détaillées.

Le seconde partie, intitulée « **Résultats** », est divisée en quatre études, qui correspondent aux quatre grandes étapes du projet de recherche. Les quatre études faisant l'objet d'articles scientifiques sont rédigées en anglais et précédées d'un résumé détaillé en français.

Dans la première étude, intitulée « **Diversité des contaminations fongiques des produits laitiers** », la biodiversité des contaminations fongiques des produits laitiers et de leur environnement de fabrication a été étudiée. Ce travail a permis la sélection de 10 cibles fongiques représentatives sur lesquelles la résistance aux conservateurs, actuellement utilisés dans le commerce, a été testée. Cette première étude a fait l'objet d'un article scientifique, intitulé « ***Diversity of spoilage fungi associated with a variety of French dairy products and their resistance to chemical preservatives*** », publié dans *The International Journal of Food Microbiology*.

Dans la deuxième étude, intitulée « **Développement d'une méthode de criblage haut-débit pour détecter des activités antifongiques dans un modèle mimant le fromage** », une nouvelle méthode de criblage haut débit a été mise en place pour détecter des activités antifongiques dans un modèle fromage et a permis la sélection de fermentats/ingrédients actifs qui ont ensuite été testés sur produits réels. Cette deuxième étude fait l'objet d'une publication soumise dans *Journal of Dairy Sciences* et s'intitule « **Technical note : High-throughput screening method for antifungal activity in a cheese-mimicking model** ». Il s'agit d'un article méthodologique décrivant cette nouvelle méthode de criblage et ses différentes applications possibles.

Dans la troisième étude, la méthode de criblage développée est utilisée pour rechercher les activités antifongiques d'une collection de 698 souches bactériennes et fongiques cultivées dans des milieux laitiers dans le but de produire des ingrédients laitiers antifongiques. Les fermentats/ingrédients d'intérêt ont ensuite été testés sur produits réels après optimisation de leur activité antifongique et les résultats obtenus suite à ces essais font partie intégrante de cet article. Cette étude s'intitule « Développement d'ingrédients laitiers antifongiques : du criblage *in vitro* aux applications à l'échelle pilote », fait l'objet d'une publication intitulée « **Development of antifungal ingredients for dairy products : from in vitro screening to pilot scale applications** », à soumettre dans *Food Microbiology* dans le cadre d'une édition spéciale « *Microbial Spoilers in Food 2017 Symposium* ».

Dans la quatrième étude, intitulée « **Identification des composés antifongiques et des mécanismes d'action** », les molécules et les mécanismes impliqués dans l'activité antifongique des ingrédients sélectionnés ont été étudiés. Ce troisième chapitre s'intitule « **Antifungal activity of fermented dairy ingredients: identification of antifungal compounds and effects on fungal growth.** », et fait l'objet d'un article à soumettre dans *Food Microbiology*.

La troisième partie de ce manuscrit, intitulée « **Discussion générale** », reprend les résultats les plus marquants de cette étude, les discute, évalue leur impact sur la biopréservation des produits laitiers et décrit les perspectives pour des travaux futurs.

Enfin, la quatrième et dernière partie, intitulée « **Valorisation du travail de thèse** » met en avant les communications affichées et orales effectuées au cours de ces trois années de thèse.

# Sommaire

<b>TABLE DES ILLUSTRATIONS</b> .....	15
<b>LISTE DES ABREVIATIONS</b> .....	17
<b>INTRODUCTION GENERALE</b> .....	19
Problématique .....	21
Synthèse bibliographique .....	25
<b>Chapitre 1~Histoire et diversité des produits laitiers</b> .....	27
1. Origine et histoire des produits laitiers .....	29
2. Diversité des produits laitiers.....	32
<b>2.1. Le lait</b> .....	32
<b>2.2. La crème</b> .....	33
<b>2.3. Le beurre</b> .....	34
<b>2.4. Les yaourts et laits fermentés</b> .....	34
<b>2.5. Le fromage</b> .....	36
<b>2.6. Les desserts lactés</b> .....	37
3. Les produits laitiers : un écosystème complexe.....	37
<b>3.1. Bactéries lactiques acidifiantes</b> .....	38
<b>3.2. Microorganismes d'affinage</b> .....	38
<b>Chapitre 2~Les bactéries lactiques et propioniques</b> .....	41
1. Origine et diversité des bactéries lactiques .....	43
2. Métabolisme des bactéries lactiques .....	46
<b>2.1. Métabolismes homofermentaires et hétérofermentaires</b> .....	46
<b>2.2. Production de composés aromatiques</b> .....	48
3. Origine et métabolisme des bactéries propioniques.....	49
4. Innocuité des bactéries lactiques et propioniques .....	50
<b>Chapitre 3~Taxonomie et métabolisme des champignons</b> .....	53
1. Taxonomie fongique .....	55
<b>1.1. Champignons supérieurs : Ascomycota et Basidiomycota</b> .....	58
<b>1.2. Le phylum des Mucoromycota</b> .....	58
2. Caractérisation de la diversité .....	59
3. Métabolisme des champignons .....	61
<b>3.1. Effets sur l'apparence et l'affinage des fromages</b> .....	61
<b>3.2. Production de composés antimicrobiens</b> .....	63
<b>Chapitre 4 ~Diversité et contrôle des contaminations fongiques dans les produits laitiers</b> ....	65
Objectifs et démarche expérimentale .....	103
<b>RESULTATS</b> .....	109
Etude 1 ~ Diversité des contaminations fongiques des produits laitiers.....	111
Etude 2 ~ Développement d'une méthode de criblage haut-débit pour détecter des activités antifongiques dans un modèle mimant le fromage.....	125
Etude 3 ~ Développement d'ingrédients laitiers antifongiques : du criblage <i>in vitro</i> en modèle fromage aux applications à l'échelle pilote.....	147
Etude 4 ~ Identification des composés antifongiques et des mécanismes d'action .....	215
<b>DISCUSSION, CONCLUSIONS ET PERSPECTIVES</b> .....	285
<b>REFERENCES BIBLIOGRAPHIQUES</b> .....	285
<b>VALORISATION DU TRAVAIL DE THESE</b> .....	303



# Table des illustrations

## Figures

- Figure 1.** Photo et reconstitution d'une peinture murale observée dans la grotte de Teshuinat II au Sud-Ouest de la Lybie. (a) peinture murale et (b) reconstitution montrant des agriculteurs Sahariens avec leur pots et leur vaches (Dunne *et al.*, 2012). .....30
- Figure 2.** Influence monastique sur la fabrication du Maroilles. (Gauche) Illustration d'un marchand de Maroilles au XVIIIème siècle. (Droite) Illustration de l'Abbaye de Maroilles en 1598. ....31
- Figure 3.** Schéma didactique présentant les différentes variétés de fromage issu du lait, en termes de technologie et de caractéristiques microbiologiques (adaptée de Almena-Aliste and Mietton, 2014). .....37
- Figure 4.** Arbre phylogénique des bactéries lactiques et positionnement par rapport aux genres *Listeria*, *Staphylococcus* et *Bacillus* (d'après Axelsson, 2004). .....44
- Figure 5.** Voies métaboliques des hexoses et des pentoses chez les bactéries lactiques.(a) Métabolisme homofermentaire des hexoses via la voie de Emden-Meyerhoff. (b) Métabolisme hétérofermentaire des hexoses via la voie de la phosphoketolase. (c) Métabolisme homofermentaire des pentoses via la voie des pentoses phosphate. (d) Métabolisme hétérofermentaire des pentoses via la voie des phosphoketolase (Gänzle, 2015). .....47
- Figure 6.** Principales étapes du métabolisme du citrate chez les bactéries lactiques citrate-positives (d'après Monnet *et al.*, 2008).....48
- Figure 7.** Cycles de la fermentation propionique d'après Wood (1981).....50
- Figure 8.** Classification du règne des champignons. (A) Classification jusqu'aux années 2000, (B) classification actuelle, (C) classification proposée par Spatafora *et al.* en 2016 (adaptée de la thèse de A. Hermet, 2014 et de Spatafora *et al.*, 2016). .....57
- Figure 9.** Récapitulatif des différentes étapes des travaux de thèse..... 108
- Figure 10.** Retard de croissance (en jour) de *P. commune* UBOCC-A-116003 et *M. racemosus* UBOCC-A-116002 à la surface des fromages blancs pendant une incubation à 10°C .....208
- Figure 11.** Dénombrement de *R. mucilaginosa* UBOCC-A-216004 après 7 et 14 jours d'incubation à 10°C (ufc/g de fromage) .....209
- Figure 12.** Retard de croissance (en jour) de *P. commune* UBOCC-A-116003 et *M. racemosus* UBOCC-A-116002 à la surface des pâtes pressée à croûte morgée pendant une incubation à 12°C et 95% d'humidité relative. ....212



## **Tableaux**

- Tableau 1.** Composition du lait de différentes espèces animales (adapté de Jandal, 1996 et Barłowska *et al.*, 2011) .....33
- Tableau 2.** Schémas technologiques et bactéries lactiques et propioniques associés aux différentes typologies de produits laitiers fermentés (www.produits-laitiers.com, Corrieu and Luquet, 2008). .....35
- Tableau 3.** Classements phylogénétiques des genres *Lactobacillus* et *Pediococcus* selon Felis and Dellaglio (2007) et Salvetti *et al.* (2012). Les groupes sont ordonnés selon le nombre d'espèce inclus. Les nouvelles espèces sont soulignées. Les espèces reclassées sont entre guillemets. ....45
- Tableau 4.** Activité antifongique d'une sélection de 22 fermentats issus de bactéries lactiques et propioniques contre 11 cibles fongiques après un test *in vitro* sur le modèle fromage. ....205

# Liste des abréviations

**ADN** : Acide Désoxyribonucléique  
**AOP** : Appellation d'Origine Protégée  
**ARNr** : Acide RiboNucléique ribosomique  
**ATP** : Adénosine Triphosphate  
**BBA** : Bretagne Biotechnologie Agroalimentaire  
**CE** : Conformité Européenne  
**CEE** : Communauté Economique Européenne  
**CIRM-BIA** : Centre International de Ressources Microbiennes – Bactéries d'Intérêt Alimentaire  
**CMI** : Concentration minimale inhibitrice  
**CNIEL** : Centre National Interprofessionnel de l'Économie Laitière  
**CO<sub>2</sub>** : Dioxyde de Carbone  
**DGGE** : Denaturing Gradient Gel Electrophoresis  
**DLC** : Date Limite de Consommation  
**EFFCA**: European Food and Feed Culture Association  
**EFSA**: European Food Safety Authority  
**FAO**: Food and Agricultural Organization  
**GC**: Chromatographie en Phase Gazeuse  
**GC/MS**: Chromatographie en phase gazeuse couplée à la spectrométrie de masse  
**GRAS**: Generally Recognize As Safe  
**HACCP** : Hazard Analysis Critical Control Point  
**HPLC**: High Performance Liquid Chromatography  
**IGP** : Indication Géographique Protégée  
**INRA** : Institut National de le Recherche en Agronomie  
**ISO**: International Organization for Standardization  
**ITS**: Internal Transcribed Spacer (espaceur interne transcrit)  
**LC-MS/MS** : Liquid Chromatography coupled to tandem mass spectrometry  
**LDH** : Lactate Déshydrogénase  
**LH**: Lait Low Heat  
**LUBEM** : Laboratoire Universitaire de Biodiversité et Ecologie Microbienne  
**NGS** : Next-Generation Sequencing (Séquençage de Nouvelle Génération)  
**O<sub>2</sub>** : Dioxygène  
**OMS** : Organisation Mondiale de la Santé  
**PAO** : Pôle Agronomique Ouest  
**PCR** : Polymerase Chain Reaction (Réaction de polymérisation en chaine)  
**Poly-Ph** : Poly-phosphate  
**PPi** : Pyrophosphate  
**PROFIL** : Assemblages PROtéiques multi-Fonctionnels pour l'Innovation en industrie Laitière  
**QPS** : Qualified Presumption of Safety  
**STLO** : Sciences et Technologies du Lait et de l'Œuf  
**TRFLP** : Terminal Restriction Fragment Lengh Polymorphism  
**TTGE** : Temporal Temperature Gradient Gel Electrophoresis  
**UBOCC** : Université de Bretagne Occidentale Culture Collection  
**UE** : Union Européenne  
**UF** : Perméat d'ultrafiltration  
**UHT** : Ultra-High temperature (Ultra-Haute Température)



# **Introduction générale**



# **Problématique**



La production mondiale de laits s'élève à 802,2 millions de tonnes en 2014 (Centre National Interprofessionnel de l'Économie Laitière [CNIEL], 2017). Les enjeux économiques associés aux produits laitiers se situent donc à l'échelle mondiale. En France, l'industrie laitière, un marché national important avec une consommation de produits laitiers parmi les plus fortes au monde (environ 87 kg/habitant/an en 2014) mais également à une activité exportatrice en forte expansion (CNIEL, 2017), représente le deuxième plus gros chiffre d'affaires de l'industrie agroalimentaire. Les produits laitiers, qui existent depuis des millénaires, sont consommés à travers le monde entier, pour leur qualité gustative et pour leurs effets positifs sur la santé.

Dans l'industrie laitière, la sensibilité des produits laitiers aux contaminations fongiques est une problématique récurrente. En effet, bien que la stabilité et la qualité microbiologique des produits laitiers soient assurées par le respect de bonnes pratiques d'hygiène et d'autres moyens de prévention et de contrôle (système Hazard Analysis Critical Control Point [HACCP], filtration de l'air, traitements thermiques etc.), certains champignons sont susceptibles de les contaminer. Ces contaminations fongiques, qui peuvent survenir à toutes les étapes de production, de la fourche à la fourchette, sont responsables d'importantes pertes économiques pour les entreprises du secteur laitier (Mayoral *et al.*, 2005). En effet, le développement de champignons d'altération peut entraîner une détérioration importante des propriétés organoleptiques (aspect visuel, texture, arômes) des produits entraînant des retraits de lots, des rejets de la part des consommateurs ainsi que des pertes de produits. Elle peut limiter en outre leur durée de vie, et par conséquent leur capacité d'exportation.

Différents conservateurs chimiques tels que les acides lactique (E270), acétique (E260), propionique (E280), sorbique (E200) et leur sels, certains esters d'acide gras (monolaurine) ou certains composés phénoliques sont parfois utilisés afin d'améliorer la stabilité microbiologique et augmenter la date limite de consommation (DLC) des produits laitiers (Gould *et al.*, 1996 ; Davidson *et al.*, 2001 ; Smith and Hong-Shum, 2003). Leur utilisation (molécule autorisée, dose, type de produits) est règlementée par le règlement Union Européenne (UE) n°1129/2011. L'utilisation de la natamycine (E235), un antibiotique antifongique produit par *Streptomyces natalensis*, sous sa forme purifiée, est quant à elle également autorisée dans certains fromages. Malheureusement, certaines cibles fongiques peuvent développer des résistances à ces conservateurs (Brul and Coote, 1999 ; Viljoen *et al.*, 2001). De plus, la législation européenne (Directives 89/107/Communauté Économique Européenne [CEE] et 95/2/Conformité Européenne [CE]) tend à réduire leur utilisation en raison de la nocivité potentielle associée à la consommation de certains additifs chimiques tels que le benzoate



de sodium (McCann *et al.*, 2007). Enfin, les additifs alimentaires véhiculent une mauvaise image auprès des consommateurs qui désirent des produits dépourvus de conservateurs chimiques tout en privilégiant la praticité de produits alimentaires possédant de longues durées de conservation. Ces différents facteurs favorisent la recherche de nouvelles stratégies pour améliorer la stabilité microbiologique de ces aliments ou remplacer les méthodes existantes comme l'utilisation de conservateurs.

La biopréservation apparaît comme une alternative prometteuse susceptible de répondre aux exigences imposées par le marché des produits laitiers. En effet, ces dernières années, on assiste à un développement conséquent des recherches de cultures bioprotectrices permettant la biopréservation des aliments. La biopréservation, définie par Stiles en 1996, est un moyen d'étendre la durée de vie des aliments grâce à l'utilisation des microorganismes et/ou de leurs métabolites antimicrobiens. Depuis quelques années, le potentiel antifongique de nombreuses souches de bactéries lactiques et propioniques a ainsi été mis en évidence et révèle toute l'étendue de la capacité d'inhibition de ces microorganismes (Schnürer and Magnusson, 2005, Leyva Salas *et al.*, 2017). Celle-ci repose sur la biosynthèse d'une ou plusieurs molécules (acides organiques, dipeptides cycliques, composés de nature protéique,...) qui peuvent agir en synergie. Moins étudiés en agroalimentaire que leurs homologues bactériens, les champignons ont également des activités antifongiques qui constituent un champ d'investigation d'intérêt. Dans tous les cas, la biodiversité microbienne est telle qu'elle constitue une réserve très importante de potentialités antifongiques d'intérêt à des fins de biopréservation des produits laitiers.

Dans ce contexte, notre intérêt s'est porté sur la recherche et le développement d'ingrédients antifongiques 100% laitiers obtenus par la fermentation de matrices laitières par des bactéries ou des champignons potentiellement producteurs de composés antifongiques (que nous appellerons fermentats) ainsi que sur l'exploration des mécanismes et des molécules impliqués dans l'activité antifongique. Pour ce faire, nous avons dans un premier temps (i) étudié la diversité des contaminants fongiques présents dans les produits laitiers ou leur environnement de fabrication et étudié leur résistance aux conservateurs chimiques puis (ii) recherché des bactéries lactiques, propioniques et des champignons présentant une activité antifongique dans un milieu laitier permettant de générer des fermentats actifs. Nous avons ensuite (iii) évalué l'activité antifongique de ces fermentats en produits réels et (iv) identifié les molécules et les mécanismes impliqués dans l'activité antifongique de ces microorganismes.

# **Synthèse bibliographique**



# **Chapitre 1**

~

## **Histoire et diversité des produits laitiers**



## 1. Origine et histoire des produits laitiers

Le lait ou les aliments transformés ou obtenus à partir de laits, communément appelés produits laitiers, sont connus depuis des millénaires. En effet, ils sont vraisemblablement apparus dans le « Croissant fertile » entre la fin du Mésolithique qui marque le début de l'élevage caprin (Dubeuf and Boyazoglu, 2009), puis ovin et bovin, et le début du Néolithique qui est marqué par la naissance de la première laiterie à Sumen (McSweeney *et al.*, 2017). Certains historiens suggèrent que les premières transformations fromagères appelées aujourd'hui caillé ou faisselle pourraient dater du VI<sup>ème</sup> millénaire av. J.-C., et seraient issues de la conservation du lait dans les estomacs des animaux abattus contenant encore de la présure. En effet des traces de fabrication fromagère sont retrouvées dans l'iconographie antique mais aussi à travers des moules à caillés vieux de 5000 ans avant notre ère (Salque *et al.*, 2013). Des traces archéologiques de fabrication du fromage datant du début de l'ère Sumérienne (4000 av. J.-C) ont également été retrouvées, notamment sur des peintures murales dans des grottes (**Figure 1**) ou des tombes (Dunne *et al.*, 2012 ; Farnworth, 2003).

La découverte du caillage du lait via l'action, probablement accidentelle, de la présure et des bactéries lactiques marque également le début de la production fromagère procurant ainsi de nombreux avantages aux populations. En effet, la conversion du lait en fromage est un moyen de stabilisation et de stockage du lait, facilitant aussi son transport. De plus, une étude de Vigne and Helmer (2007) a montré qu'avant le néolithique, l'homme ne consommait pas de lait à l'âge adulte et l'étude de squelettes nord-européens de près de 6000 ans a montré qu'il ne synthétisait pas encore la lactase; l'enzyme permettant de digérer le lactose. Des mutations génétiques auraient eu lieu ultérieurement à cette date et se sont propagées favorablement dans les régions où l'on consommait du lait à l'âge adulte. Ainsi, le fromage permettait d'une part, l'amélioration de la digestibilité du lait grâce à sa teneur réduite en lactose et d'autre part, la diversification du régime alimentaire humain (Fow and McSweeney, 2017).

Le XV<sup>e</sup> siècle av. J.-C est marqué par la naissance des premiers fromages en Mésopotamie et en Inde avant de se développer dans le reste du monde, si bien qu'à partir du I<sup>er</sup> millénaire av. J.-C. en Europe, aussi bien dans la Rome antique qu'en Grèce, les fromages de lait de chèvre et de brebis étaient des aliments quotidiens (Fox and McSweeney, 2017). C'est au Moyen-Âge que s'est diffusé le savoir-faire fromager dans toute l'Europe, notamment grâce aux grandes migrations des peuples et des pèlerins (Fox and McSweeney, 2017). C'est à cette période et sous l'influence monastique que le fromage va connaître son essor, marqué par l'amélioration de la qualité des

produits laitiers mais aussi par leur diversification. En effet, à cette époque, certains monastères deviennent d'importants centres de productions fromagères et vont jouer un rôle important dans l'essor du fromage, notamment grâce à la fabrication et à l'affinage des fromages tels que le Maroilles (**Figure 2**), le fromage de Tamie, le Saint Paulin, le Port du Salut (aujourd'hui Port-Salut) ou encore le Munster qui sont parvenus jusqu'à nous (Fox and McSweeney, 2017).



**Figure 1. Photo et reconstitution d'une peinture murale observée dans la grotte de Teshuinat II au Sud-Ouest de la Lybie. (a) peinture murale et (b) reconstitution montrant des agriculteurs Sahariens avec leur pots et leur vaches (Dunne *et al.*, 2012).**

De même c'est parce que ces communautés indépendantes et isolées avaient des conditions de communication limitées que beaucoup de variétés de fromage ont été produites dans des régions

géographiques limitées. C'est ainsi qu'aujourd'hui, la production de certaines variétés de fromages est toujours localisée et, dans certains cas, protégée par des appellations reconnues telles que l'Appellation d'Origine Protégée (AOP) ou l'Indication Géographique Protégée (IGP). L'influence monastique sur la diversité et la qualité des fromages fut donc déterminante pour le développement de ces produits et c'est donc en Europe et plus particulièrement en France que les moines mirent au point des recettes variées et commencèrent à composer ce qui deviendra, avec plus de 1200 variétés de fromages, le plus grand plateau de fromage du monde (CNIEL, 2017). Les déplacements des moines d'un monastère à un autre ont également contribué à la dissémination des différentes variétés de fromage et probablement aussi à la création de nouvelles variétés hybrides (Fox and Mc Sweeney, 2017).



**Figure 2. Influence monastique sur la fabrication du Maroilles. (Gauche) Illustration d'un marchand de Maroilles au XVIIIème siècle. (Droite) Illustration de l'Abbaye de Maroilles en 1598.**

C'est à partir du XVe siècle que les paysans se mettent à fabriquer leur fromage pour conserver le lait le plus longtemps possible et ainsi résister à la famine qui gagnait les campagnes. C'est seulement à partir du XVIIIe siècle que les paysans commencèrent à exporter leur fromage dans les



villes en mettant au point des recettes pour optimiser la conservation et faciliter le transport. Enfin, l'ultime étape dans l'expansion du savoir-faire fromager à travers le monde est marquée par l'apport des compétences et connaissances des européens en matière de production fromagère lors de la colonisation de l'Afrique, de l'Océanie et des Amériques. En effet, le fromage, et les produits laitiers plus généralement, qui n'étaient pas fabriqués dans ces régions avant la colonisation (Fox and McSweeney, 2017), sont aujourd'hui des produits d'importance économique majeure dans certains des pays concernés tels que le Canada ou les États-Unis qui ont produit respectivement 530 000 et 7 071 000 tonnes de produits laitiers (fromages, beurres, lait en poudre) en 2014 (CNIEL, 2017).

C'est avec les travaux de Louis Pasteur et d'Émilie Duclaux, au XIX<sup>ème</sup> siècle, que l'industrialisation et la diversification des produits laitiers fermentés tels que nous les connaissons à l'heure actuelle deviennent possibles. La sécurisation des procédés de fabrication, grâce à la pasteurisation et à la création de ferments stables, va permettre le transport au long cours des produits laitiers. Au XX<sup>ème</sup> siècle, les progrès industriels et agricoles, notamment dans le domaine de la conservation, continuent et la consommation augmente. De plus, les connaissances sur la chimie et la microbiologie des produits laitiers se renforcent et permettent la mise en place de réglementations, notamment les normes d'hygiène, qui ont pour but de protéger le consommateur. Après la Seconde Guerre mondiale, la production explose. Dans les années 1970, le cracking du lait, c'est-à-dire la séparation des différents éléments qui le constituent, permet à l'industrie laitière d'innover en créant de nouveaux produits et au début du XXI<sup>e</sup> siècle, la Chine commence, elle aussi, à produire et consommer des produits laitiers. Ainsi, les produits laitiers sont consommés à travers le monde entier et, à l'heure actuelle, 1778 variétés de fromage ont été référencées ([www.cheese.com](http://www.cheese.com)) mais de nombreuses variétés sont très proches et devraient plutôt être considérées comme des variantes que comme de véritables variétés (McSweeney *et al.*, 2017). Cela montre tout de même l'existence d'une très grande diversité de fromages mais aussi, plus généralement, de produits laitiers.

## **2. Diversité des produits laitiers**

### **2.1. Le lait**

Le lait entre dans la catégorie des produits laitiers et peut être consommé sous différentes formes (entier, demi-écrémé, écrémé, concentré...). Mais le lait est également la matière première à l'origine de nombreux produits laitiers et le choix du lait joue un rôle essentiel dans leur diversité. En effet, les

saveurs, les propriétés nutritives et la durée de conservation varient selon le type de lait utilisé, d'où l'obtention de produits laitiers aux goûts bien différents (Barłowska *et al.*, 2011 ; Smiddy *et al.*, 2012 ; Devle *et al.*, 2012). Le premier facteur influençant les propriétés organoleptiques du produit fini est la composition du lait, qui dépend elle-même de l'espèce animale dont il est issu (**Tableau 1**). En effet, chaque lait possède une composition et des caractéristiques aromatiques qui sont ensuite transférées au produit laitier lui donnant ainsi un caractère distinctif (Jandal, 1996 ; Barłowska *et al.*, 2011).

**Tableau 1. Composition du lait de différentes espèces animales (adapté de Jandal, 1996 et Barłowska *et al.*, 2011)**

Constituants	Chèvre	Brebis	Vache
Matière grasse (%)	4,07	6,99	4,09
Lactose (%)	4,51	4,75	4,82
Protéine (%)	3,26	5,73	3,42
Caséine (%)	2,41	5,16	2,63
Calcium (mg/100g)	134	195 à 200	122
Potassium (mg/100g)	152	136 à 140	152
Sodium (mg/100g)	59,4	44 à 58	58

Le lait cru contient également un microbiote naturel (Giannino *et al.*, 2009 ; Delavenne *et al.*, 2011 ; Quigley *et al.*, 2013 ; Montel *et al.*, 2014) ainsi que des enzymes natives (Fox and Kelly, 2006 ; McSweeney *et al.*, 2017). Ainsi, la fabrication des produits laitiers, commence avec la sélection d'un lait de haute qualité microbiologique et chimique.

Parmi les produits laitiers, on distingue les produits non fermentés (crèmes desserts, crème crue et crème Ultra High Temperature [UHT] liquide) des produits laitiers fermentés (beurre, crème fraîche, fromages, yaourts et laits fermentés). Chaque catégorie de produits présente un schéma technologique différent et une flore bactérienne particulière lorsqu'il s'agit de produits fermentés (**Tableau 2**).

## 2.2. La crème

La crème est un concentré issu du lait riche en matière grasse, elle est obtenue mécaniquement par centrifugation ou naturellement par décantation du lait cru. La crème est aussi définie comme une émulsion résultant de la forte concentration en globules de matière grasse laitière (Panchal *et al.*,

2017). Les crèmes se distinguent selon leur texture et leur teneur en matière grasse pouvant aller de 10 à 80% ou parfois plus (Panchal *et al.*, 2017). La crème crue (30 à 40% de matières grasses) est la crème issue de la séparation du lait et de la crème, directement après l'écémage et sans passer par l'étape de pasteurisation. L'obtention de la crème (crue maturée ou fraîche) peut également se faire avec l'ajout de ferment (**Tableau 2**) ([www.produits-laitiers.com](http://www.produits-laitiers.com)).

### **2.3. Le beurre**

Le beurre est un produit laitier produit par l'agitation, après maturation, de la crème (Findik and Andiç, 2017), issue de l'écémage du lait (**Tableau 2**). Sa saveur varie en fonction de son taux de sel mais reste douce. Le beurre doux est obtenu simplement après barattage de la crème. Pour le beurre salé (3% de sel) ou demi-sel (entre 0,5 et 3%), il est ensuite mélangé à une proportion précise de sel ([www.produits-laitiers.com](http://www.produits-laitiers.com)).

### **2.4. Les yaourts et laits fermentés**

Selon la définition du *Codex alimentarius* de 2003, on parle de yaourt ou de yoghourt (les deux termes sont reconnus par l'UE), pour désigner les produits laitiers transformés à partir de ferments lactiques spécifiques, *Lactobacillus delbrueckii* subsp. *bulgaricus* et *Streptococcus thermophilus* qui doivent êtreensemencés simultanément et se trouver vivants dans le produit fini (**Tableau 2**). Le remplacement de l'une de ces espèces ou l'ajout d'espèce(s) supplémentaire(s) ne permettent plus de qualifier le produit de « yaourt » (*Codex Alimentarius* Commission, 2003) et le terme de « lait fermenté » est alors utilisé. Il existe une grande diversité de laits fermentés à travers le monde, dont les laits fermentés concentrés (labneh), acides et alcoolisés (kéfir, koumis), peu acide (lait ribot) ou épaissis (viili) (Béal and Sodini, 2003). Les différents types de laits fermentés et de yaourts sont classés selon leur saveur (nature, sucré, aromatisé, aux fruits), leur texture (ferme, brassé, liquide, concentré), leur taux de matière grasse (lait entier, standard, maigre), le type de lait (vache, brebis, chèvre,...) et les ferments qui y sont ajoutés (Béal and Sodini, 2003).

**Tableau 2. Schémas technologiques et bactéries lactiques et propioniques associés aux différentes typologies de produits laitiers fermentés ([www.produits-laitiers.com](http://www.produits-laitiers.com), Corrieu and Luquet, 2008).**

<b>Produit laitier</b>	<b>Schéma technologique</b>	<b>Flore bactérienne associée</b>
Beurre	Pasteurisation, Écrémage, Ensemencement, Maturation, Barattage, Malaxage, Conditionnement	<i>Leuconostoc mesenteroides</i> subsp. <i>cremoris</i> , <i>Leuconostoc dextranicum</i> , <i>Lactococcus lactis</i>
Crème fraîche épaisse	Écrémage, Pasteurisation, Ensemencement, Maturation, Conditionnement	<i>L. lactis</i> , <i>L. lactis</i> subsp. <i>lactis</i> biovar <i>diacetylactis</i> , <i>L. mesenteroides</i> subsp. <i>cremoris</i>
Fromages frais ou blancs	Écrémage, Pasteurisation ou non, Caillage, Moulage, Égouttage, Conditionnement	<i>L. lactis</i> subsp. <i>lactis</i> , <i>cremoris</i> ou <i>diacetylactis</i> , <i>L. mesenteroides</i> subsp. <i>cremoris</i>
Fromages à pâtes molles et à croûte fleurie ou lavée	Écrémage, Pasteurisation ou non, Caillage, Moulage, Égouttage, Démoulage, Salage, Ensemencement, Affinage/(Lavage)/ Affinage	<i>L. lactis</i> , <i>Streptococcus thermophilus</i>
Fromages à pâtes pressées non cuites	Écrémage, Pasteurisation ou non, Caillage, Émietter, Moulage, Démoulage, Salage, Ensemencement, Affinage	<i>S. thermophilus</i> , <i>L. lactis</i> , <i>Lactobacillus helveticus</i> , <i>Lactobacillus casei</i> , <i>Lactobacillus plantarum</i> , <i>Lactobacillus rhamnosus</i> , <i>Lactobacillus delbruecki</i> , <i>Enterococcus faecium</i> , <i>Enterococcus durans</i>
Fromages à pâtes persillées	Écrémage, Pasteurisation ou non, Caillage, Moulage, Pressage, Démoulage, Salage, Ensemencement, Perçage, Affinage	<i>L. lactis</i> , <i>L. mesenteroides</i>
Fromages à pâtes pressées cuites	Écrémage, Pasteurisation ou non, Caillage, Trancher le caillé, Chauffage, Moulage, Presse, Démoulage, Salage, Ensemencement, Affinage (8-15°C puis 16-23°C)	<i>L. delbruecki</i> subsp. <i>lactis</i> , <i>L. helveticus</i> , <i>Leuconostoc</i> spp., <i>S. thermophilus</i> , <i>Propionibacterium</i> spp.
Fromages à pâtes filées	Écrémage, Pasteurisation ou non, Caillage, Trancher le caillé, Mélange caillé et petit-lait, Chauffage, Etirement de la pâte, Coupage, Pressage, Conditionnement	<i>S. thermophilus</i> , <i>L. delbruecki</i> subsp. <i>bulgaricus</i> , <i>L. helveticus</i> , <i>E. durans</i> , <i>Enterococcus faecalis</i>
Yaourts	Écrémage, Pasteurisation, Ensemencement, Maturation, Conditionnement	<i>L. delbrueckii</i> subsp. <i>bulgaricus</i> et <i>S. thermophilus</i>
Laits fermentés	Écrémage, Pasteurisation, Ensemencement, Maturation, Conditionnement	<i>L. delbrueckii</i> subsp. <i>bulgaricus</i> et <i>S. thermophilus</i> , <i>Lactobacillus</i> spp., <i>Bifidobacterium</i> spp., <i>Leuconostoc</i> spp., <i>S. thermophilus</i>

## 2.5. Le fromage

Le fromage est défini par la norme Food and Agricultural Organization (FAO)/Organisation Mondiale de la Santé (OMS) n°A-6 (1978, modifiée en 1990) comme « le produit frais ou affiné, solide ou semi-solide, dans lequel le rapport protéines de lactosérum/caséine n'excède pas celui du lait obtenu :

- par coagulation du lait, lait écrémé, lait partiellement écrémé, crème de lactosérum ou babeurre, seul ou en combinaisons, grâce à l'action de la présure ou d'autres agents coagulant appropriés et par égouttage partiel du lactosérum résultant de cette coagulation ;
- par l'emploi de techniques de fabrication entraînant la coagulation du lait et/ou des matières obtenues à partir de lait, présentant des caractères physiques, chimiques et organoleptiques similaires à ceux du produit défini plus haut. »

En France, l'article 1 du décret n°2013-1010 du 12 novembre 2013 accorde et réserve la dénomination « fromage » à « un produit fermenté ou non, affiné ou non, obtenu à partir des matières d'origine exclusivement laitières suivantes : lait, lait partiellement ou totalement écrémé, crème, matière grasse, babeurre, utilisées seules ou en mélange et coagulées en tout ou partie avant égouttage ou après élimination partielle de la partie aqueuse.»

Seules les étapes de coagulation et d'égouttage sont communes à tous les types de fromages selon ces deux définitions juridiques. Néanmoins, la préparation du lait, le salage et l'affinage sont des étapes communes à un très un grand nombre de fromages (**Tableau 2** et pour plus de détails sur ces étapes voir Goudédranche *et al.*, 2002, 2001a, 2001b).

Il existe une multitude de variétés de fromages qui comprennent tout ou seulement une partie de ces étapes qui peuvent être classés dans 8 grandes familles : les fromages à pâte fraîche, à pâte filée, à pâte pressée cuite, à pâte pressée semi-cuite, à pâte pressée non cuite, à pâte molle persillée, à pâte molle à croûte fleurie ou lavée, et à pâte fondue (mélange de fromages fondus).

La **Figure 3**, ci-après, répertorie l'éventail des différentes technologies à l'origine de la diversité fromagère, ayant le lait comme matière première d'origine. Chacune des familles et des variétés qui les composent ont donc un procédé de fabrication particulier ainsi qu'un microbiote qui leur est propre, conférant ainsi aux fromages des caractéristiques organoleptiques uniques.

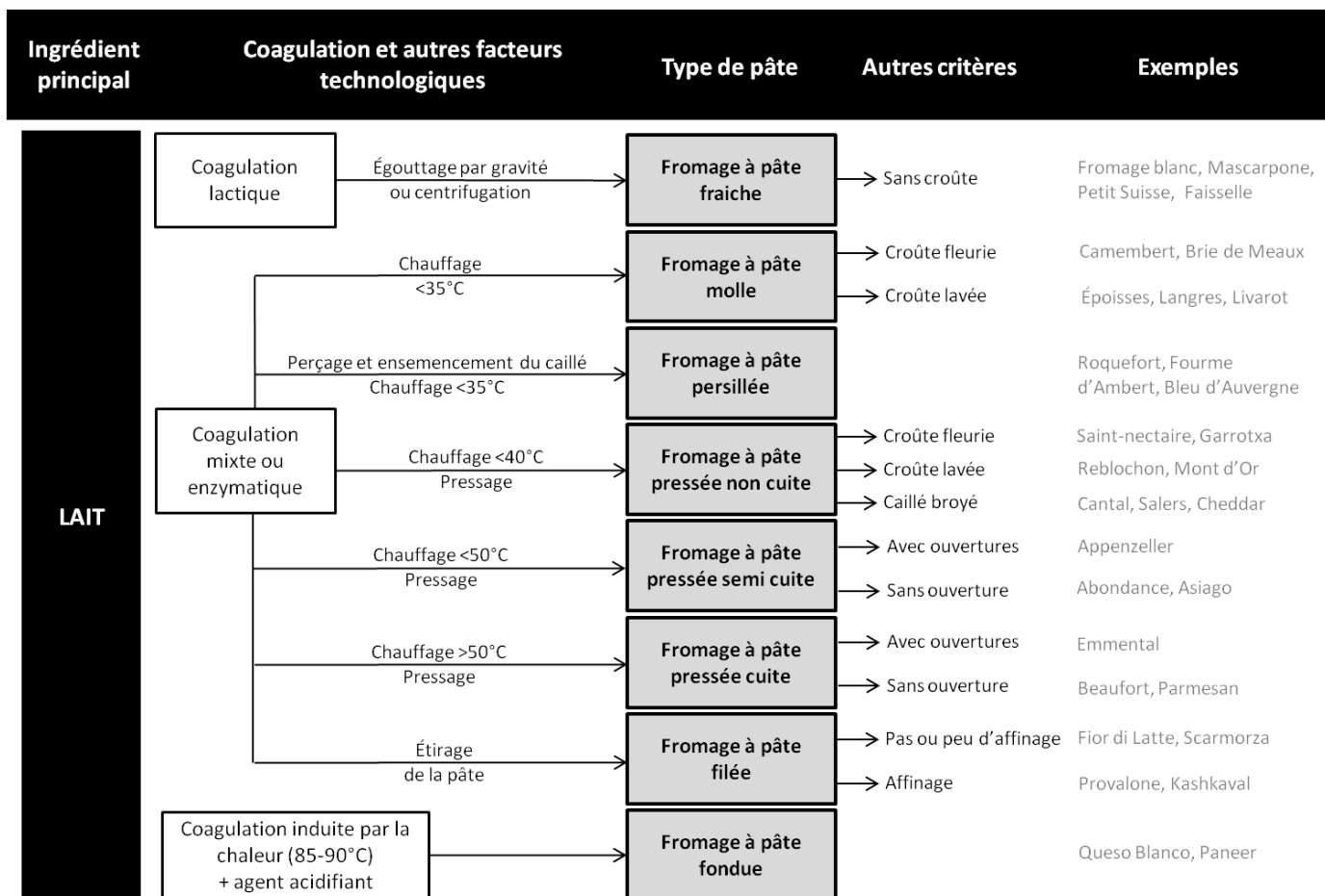


Figure 3. Schéma didactique présentant les différentes variétés de fromage issu du lait, en termes de technologie et de caractéristiques microbiologiques (adaptée de Almena-Aliste and Mietton, 2014).

## 2.6. Les desserts lactés

Les desserts lactés sont des produits laitiers non fermentés proposant des variétés très diverses (flan, riz au lait, mousses à base de lait, glaces, crèmes desserts etc) qui doivent toutes répondre aux mêmes points communs, à savoir être (i) composés d'au moins 50% de lait, (ii) être sucrés ou parfumés et généralement (iii) ne pas contenir de ferments lactiques, car la préparation est souvent cuite, détruisant ainsi tout ferment encore présent ([www.produits-laitiers.com](http://www.produits-laitiers.com)).

## 3. Les produits laitiers : un écosystème complexe

Les micro-organismes (*i.e.* bactéries lactiques acidifiantes et les microorganismes d'affinage) jouent un rôle important lors de la fabrication des produits laitiers et lors de l'affinage des fromages (Montel *et al.*, 2014 ; Fox and McSweeney, 2017). Les bactéries lactiques acidifiantes, telles que *Lactococcus*

*lactis*, *S. thermophilus*, *Lactobacillus helveticus* et *L. delbrueckii* peuvent être utilisées individuellement ou en association selon le type et/ou la variété de produits laitiers (**Tableau 2**). Ces bactéries, qui peuvent être un mélange de souches définies ou non, sont ajoutées au début de la fabrication ou sont naturellement présentes dans le lait et/ou l'environnement fromager (Johnson and Law, 1999 ; Quigley *et al.*, 2013 ; Montel *et al.*, 2014). Quant au microbiote d'affinage, il est composé de bactéries, de levures et de champignons filamenteux formant ainsi un écosystème complexe et spécifique de chaque variété de fromage, au sein duquel des interactions microbiennes ont lieu (Irlinger and Mounier, 2009 ; Montel *et al.*, 2014 ; Dugat-Bony *et al.*, 2016).

### **3.1. Bactéries lactiques acidifiantes**

Les bactéries lactiques acidifiantes, ou bactéries levains, appartenant le plus souvent aux genres *Lactococcus*, *Lactobacillus*, *Streptococcus*, *Leuconostoc* et *Enterococcus*, sont définies comme des souches produisant suffisamment d'acide lactique à partir du lactose pour abaisser le pH du lait à moins de 5,3 en 6h à 30-37°C (Corrieu and Luquet, 2008). Elles permettent donc l'acidification du lait et contribuent également à l'affinage via la protéolyse et la transformation des acides aminés en composés aromatiques par leur système enzymatique (Fox and Wallace, 1997). Certaines produisent aussi du diacétyl à partir du citrate et du dioxyde de carbone (CO<sub>2</sub>) à partir du lactose (hétérofermentaires strictes) et du citrate (homofermentaires et hétérofermentaires). Le choix des genres/espèces de bactéries lactiques est donc clé au regard du produit final recherché et des fonctionnalités désirées (arôme, texture, couleur,...). Selon les températures intervenant pendant le processus de fabrication, des espèces particulières sont nécessaires et des cultures mésophiles (température de croissance optimale entre 26 et 30°C) ou thermophiles (température optimale de croissance aux alentours de 42°C) seront utilisées (Corrieu and Luquet, 2008).

### **3.2. Microorganismes d'affinage**

Les microorganismes d'affinage, aussi appelés « flore secondaire », sont spécifiques de la phase de maturation lors de la fabrication des fromages. Ces micro-organismes proviennent essentiellement d'un ensemencement dirigé du lait, de l'environnement ou du matériel et sont aussi à l'origine des qualités organoleptiques du fromage (aspect, texture, arômes propres). La flore secondaire est composée notamment de bactéries lactiques non levain (lactobacilles mésophiles et pédiocoques), de bactéries propioniques ou de bactéries corynéformes (Corrieu and Luquet, 2008 ; Chamba and

Irlinger, 2004 ; Coeuret *et al.*, 2004). Les principales espèces de bactéries lactiques non levains retrouvées sont *Lactobacillus casei*, *Lactobacillus plantarum*, *Lactobacillus brevis* et *Lactobacillus buchneri* (Dugat-Bony *et al.*, 2016 ; Quigley *et al.*, 2013 ; Hoier *et al.*, 1999). Les bactéries propioniques, telles que *Propionibacterium freudenreichii*, *Propionibacterium acidipropionici*, *Propionibacterium jensenii* et *Propionibacterium thoenii* (Beresford *et al.*, 2001) dont certaines participent à la formation d'ouvertures dans les fromages tels que le Gruyère, font également partie des microorganismes d'affinage.

Quant au microbiote de surface des fromages, il est composé de bactéries appartenant aux actinobactéries (*Arthrobacter arilaitensis*, *Brevibacterium aurantiacum*, *Brevibacterium linens*, *Corynebacterium casei*), aux firmicutes (*Staphylococcus xylosus*), aux  $\gamma$ -protéobactéries (*Halomonas* et *Vibrio* spp.), à la famille des *Enterobacteriaceae* (*Proteus*, *Hafnia*, *Serratia*, *Morganella* spp.) ainsi que des bactéries lactiques halophiles et alcalophiles telles que *Marinilactibacillus psychrotolerans* ou *Alkalibacterium olivoapovliticus* (Dugat-Bony *et al.*, 2016 ; Coton *et al.*, 2012 ; Mounier *et al.*, 2009 ; Ishikawa *et al.*, 2007).

Si les bactéries sont une composante essentielle de la flore secondaire des fromages, il ne faut pas négliger l'importance des champignons dans cet écosystème. Au sein des champignons, on distingue les levures et les champignons filamenteux, plus communément appelés moisissures. Les levures ont la capacité de se développer à basse température, à faible pH et, pour certaines espèces, à de fortes concentrations en sel (Pitt and Hocking, 2009). Elles sont donc particulièrement adaptées à l'environnement des produits laitiers (Fleet, 1990). En consommant l'acide lactique et en produisant des métabolites alcalins (NH<sub>3</sub>), les levures vont participer à la désacidification du caillé rendant ainsi possible la croissance des bactéries d'affinage acido-sensibles. Elles vont également participer au développement des arômes grâce à leurs activités lipolytiques et protéolytiques. Les principales levures utilisées dans la fabrication des fromages appartiennent aux genres *Candida*, *Pichia*, *Cyberlindnera*, *Debaryomyces*, *Kluyveromyces*, *Saccharomyces*, *Torulasporea* et *Yarrowia*. Elles appartiennent toutes à la classe des Saccharomycotina au sein du phylum des Ascomycota (Bourdichon *et al.*, 2012). Quant aux champignons filamenteux, appartenant principalement aux classes des Sordariomycotina (*Lecanicillium*, *Scopulariopsis* et *Fusarium*), des Saccharomycotina (*Galactomyces*) et des Eurotiomycotina (*Penicillium*, *Sphaerosporium* et *Sporendonema*) au sein du phylum des Ascomycota ou encore au genre *Mucor* au sein du phylum des Mucoromycota, sont associés à une grande variété de fromages et jouent un rôle important dans leur affinage (Bourdichon *et al.*, 2012 ; Hermet *et al.*, 2012 ; Ropars *et al.*, 2012 ; Dugat-Bony *et al.*, 2016). En effet, en



métabolisant l'acide lactique, les champignons filamenteux neutralisent la pâte et en produisant de nombreuses enzymes, ils vont également participer activement à la maturation du fromage et au développement d'arômes caractéristiques.

*Ainsi, les bactéries lactiques et propioniques jouent un rôle important dans les processus de fabrication des produits laitiers. Leur diversité et leur métabolisme sont développés dans le chapitre suivant. Quant aux champignons, ils ont également un rôle important dans certaines technologies fromagères, notamment dans l'affinage des fromages à croûte lavée ou fleurie, ou encore à pâte persillée. Leur taxonomie et leurs rôles dans ces processus seront détaillés dans le chapitre 3 de la synthèse bibliographique.*

## **Chapitre 2**

~

### **Les bactéries lactiques et propioniques**



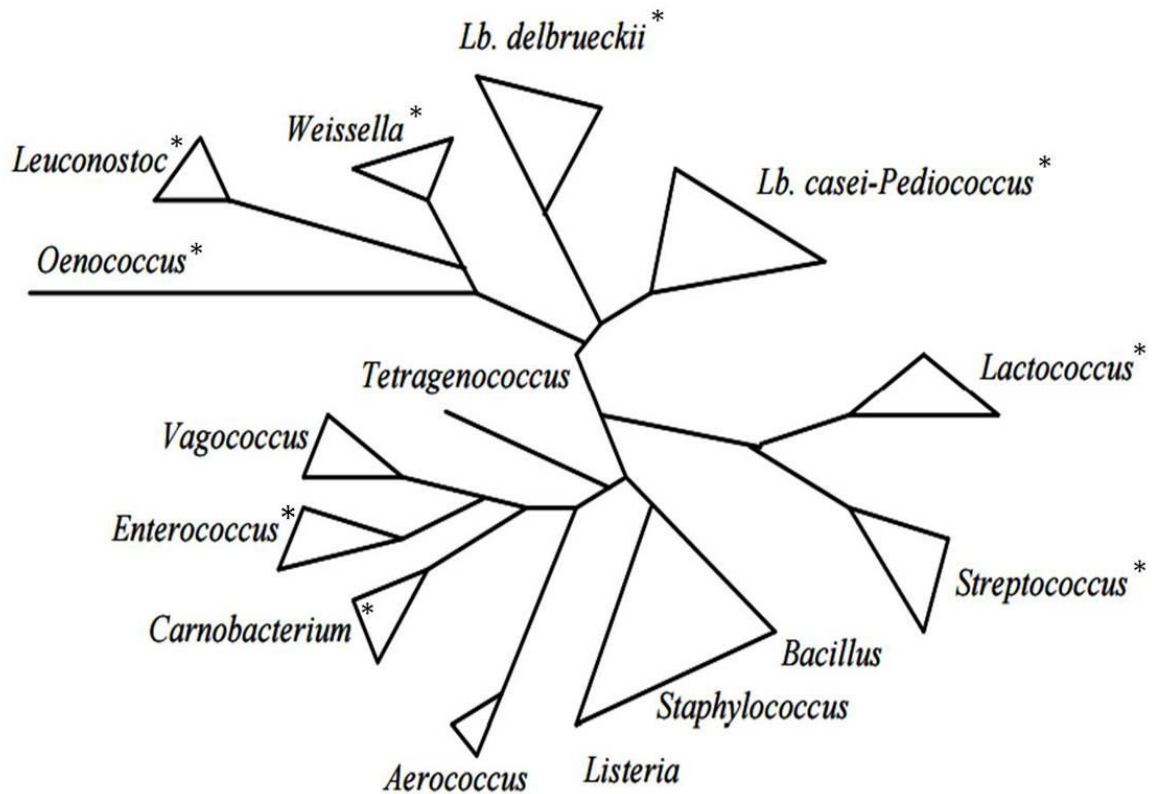
C'est en 1919 que Orla-Jensen définit pour la première fois le terme de bactéries lactiques. Ce terme n'a pas de signification taxonomique stricte mais correspond à un ensemble de bactéries à Gram positif, oxydase négative, catalase négative, bacilles ou de forme coccoïde, non sporulantes, immobiles et capable de produire de l'acide lactique par la fermentation de composés glucidiques (Desmazeaud, 1983 ; Pfeifer and klaenhammer, 2007).

## 1. Origine et diversité des bactéries lactiques

Apparues il y a 3 milliards d'années, la diversité génétique des bactéries lactiques a connu une rapide expansion il y a 65 millions d'années avec l'apparition des mammifères (Corrieu and Luquet, 2008). Il y a 8000 ans, cet essor s'est encore accentué avec le début de l'élevage du bétail (Taillez, 2001). Les bactéries lactiques entretiennent donc une relation étroite avec les humains et les animaux puisqu'elles colonisent le tractus gastro-intestinal et les muqueuses des mammifères ainsi que leur environnement. Elles sont notamment retrouvées dans le lait et les produits laitiers fermentés et dans de nombreux aliments fermentés tels que le saucisson, la choucroute, le pain et les produits laitiers, dans les matières végétales et dans les ensilages. Les bactéries lactiques jouent, au sein de ces produits, un rôle de conservation ou d'altération (Stiles and Holzapfel, 1997 ; Bernadeau *et al.*, 2006).

Les bactéries lactiques sont des microorganismes dont le génome présente un faible pourcentage en guanine et en cytosine (33-55%), elles font partie des *Firmicutes*. Ces bactéries englobent plusieurs genres tels que *Aerococcus*, *Alloiococcus*, *Atopobium*, *Carnobacterium*, *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Oenococcus*, *Pediococcus*, *Streptococcus*, *Tetragenococcus*, *Vagococcus* et *Weissella* (**Figure 3**) parmi lesquels neuf sont utilisés dans les industries agroalimentaires (Stiles and Holzapfel, 1997 ; Pot, 2008).

Anciennement, les espèces du genre *Lactobacillus* étaient classés en trois grands groupes phylogénétiques (**Figure 4**): (i) *L. delbrueckii*, (ii) *L. casei* - *Pediococcus* et (iii) le groupe des *Leuconostoc* qui contenait aussi quelques lactobacilles (Collins *et al.*, 1991). Aujourd'hui ces groupes et les espèces qu'ils comprennent ont été réévalués grâce aux nouvelles méthodes taxonomiques pour aboutir finalement à une nouvelle classification comportant plus d'une dizaine de groupes (Felis and Dellaglio, 2007 ; Pot, 2008) (**Tableau 2**).



**Figure 4. Arbre phylogénique des bactéries lactiques et positionnement par rapport aux genres *Listeria*, *Staphylococcus* et *Bacillus* (d'après Axelsson, 2004). Les distances évolutives sont calculées par comparaison des séquences du gène codant l'ARNr 16S. \*bactéries lactiques utilisées dans l'agroalimentaire.**

Aujourd'hui, le terme de bactéries lactiques décrit les organismes de l'ordre des *Lactobacillales*, suggérant ainsi que les bactéries lactiques constituent un groupe homogène d'un point de vue phylogénétique (Gänzle, 2015). Au sein de cet ordre, le genre *Lactobacillus* comprend 152 espèces et est très proche phylogénétiquement du genre *Pediococcus*. Selon Salvetti *et al.* (2012), ces genres peuvent être classés en 15 groupes (d'au moins 3 espèces), 4 couples et 10 espèces seules (**Tableau 3**).

**Tableau 3. Classements phylogénétiques des genres *Lactobacillus* et *Pediococcus* selon Felis and Dellaglio (2007) et Salvetti *et al.* (2012). Les groupes sont ordonnés selon le nombre d'espèce inclus. Les nouvelles espèces sont soulignées. Les espèces reclassées sont entre guillemets.**

Groupes	Felis and Dellaglio (2007)	Salvetti <i>et al.</i> (2012)
<i>L. delbrueckii</i>	<i>L. acetotolerans</i> , <i>L. acidophilus</i> , <i>L. amylolyticus</i> , <i>L. amylophilus</i> , <i>L. amylotrophicus</i> , <i>L. amylovorus</i> , <i>L. crispatus</i> , <i>L. delbrueckii</i> , <i>L. fornicalis</i> , <i>L. gallinarum</i> , <i>L. gasseri</i> , <i>L. hamsteri</i> , <i>L. helveticus</i> , <i>L. iners</i> , <i>L. intestinalis</i> , <i>L. jensenii</i> , <i>L. johnsonii</i> , <i>L. kalixensis</i> , <i>L. kefiranofaciens</i> , <i>L. kitasatonis</i> , <i>L. psittaci</i> , “ <i>L. sobrius</i> ”, <i>L. ultunensis</i>	<i>L. acetotolerans</i> , <i>L. acidophilus</i> , <i>L. amylolyticus</i> , <i>L. amylophilus</i> , <i>L. amylotrophicus</i> , <i>L. amylovorus</i> , <i>L. crispatus</i> , <i>L. delbrueckii</i> , <u><i>L. equicursoris</i></u> , <i>L. fornicalis</i> , <i>L. gallinarum</i> , <i>L. gasseri</i> , <u><i>L. gigeriorum</i></u> , <i>L. hamsteri</i> , <i>L. helveticus</i> , <u><i>L. hominis</i></u> , <i>L. iners</i> , <i>L. intestinalis</i> , <i>L. jensenii</i> , <i>L. johnsonii</i> , <i>L. kalixensis</i> , <i>L. kefiranofaciens</i> , <i>L. kitasatonis</i> , <u><i>L. pasteurii</i></u> , <i>L. psittaci</i> , <u><i>L. taiwanensis</i></u> , <i>L. ultunensis</i>
<i>L. salivarius</i>	<i>L. acidipiscis</i> , <i>L. agilis</i> , <i>L. algidus</i> , <i>L. animalis</i> , <i>L. apodemi</i> , <i>L. aviarius</i> , <i>L. equi</i> , <i>L. mali</i> , <i>L. murinus</i> , <i>L. nagelii</i> , <i>L. ruminis</i> , <i>L. saerimmeri</i> , <i>L. salivarius</i> , <i>L. satsumensis</i> , <i>L. vini</i>	<i>L. acidipiscis</i> , <i>L. agilis</i> , <i>L. animalis</i> , <i>L. apodemi</i> , <i>L. aquaticus</i> , <i>L. aviarius</i> , <u><i>L. cacaonum</i></u> , <u><i>L. capillatus</i></u> , <u><i>L. ceti</i></u> , <i>L. equi</i> , <u><i>L. ghanensis</i></u> , <u><i>L. hayakitensis</i></u> , <u><i>L. hordei</i></u> , <i>L. mali</i> , <i>L. murinus</i> , <i>L. nagelii</i> , <u><i>L. oeni</i></u> , <u><i>L. pobuzihi</i></u> , <i>L. ruminis</i> , <i>L. saerimmeri</i> , <i>L. salivarius</i> , <u><i>L. succicola</i></u> , <i>L. satsumensis</i> , <u><i>L. uvarum</i></u> , <i>L. vini</i>
<i>L. reuteri</i>	<i>L. antri</i> , <i>L. coleohominis</i> , <i>L. fermentum</i> , <i>L. frumenti</i> , <i>L. gastricus</i> , <i>L. ingluviei</i> , <i>L. mucosae</i> , <i>L. oris</i> , <i>L. panis</i> , <i>L. pontis</i> , <i>L. reuteri</i> , <i>L. secaliphilus</i> , <i>L. vaginalis</i>	<u><i>L. alvi</i></u> , <i>L. antri</i> , <i>L. coleohominis</i> , <i>L. fermentum</i> , <i>L. frumenti</i> , <u><i>L. equigenerosi</i></u> , <i>L. gastricus</i> , <i>L. ingluviei</i> , <i>L. mucosae</i> , <i>L. oris</i> , <i>L. panis</i> , <i>L. pontis</i> , <i>L. reuteri</i> , <i>L. secaliphilus</i> , <i>L. vaginalis</i>
<i>L. buchneri</i>	<i>L. buchneri</i> , <i>L. diolivorans</i> , <i>L. farraginis</i> , <i>L. hilgardii</i> , <i>L. kefir</i> , <i>L. parabuchneri</i> , <i>L. parafarraginis</i> , <i>L. parakefir</i> , associée avec <i>L. acidifarinae</i> , <i>L. namurensis</i> , <i>L. spicheri</i> , <i>L. zymae</i>	<i>L. buchneri</i> , <i>L. diolivorans</i> , <i>L. farraginis</i> , <i>L. hilgardii</i> , <i>L. kefir</i> , <u><i>L. kisonensis</i></u> , <u><i>L. otakiensis</i></u> , <i>L. parabuchneri</i> , <i>L. parafarraginis</i> , <i>L. parakefir</i> , <u><i>L. rapi</i></u> , <u><i>L. sunkii</i></u>
<i>L. alimentarius</i>	<i>L. alimentarius</i> , <i>L. farciminis</i> , “ <i>L. kimchii</i> ”, <i>L. mindensis</i> , <i>L. nantensis</i> , <i>L. paralimentarius</i> , <i>L. tuceti</i> , <i>L. versmoldensis</i>	<i>L. alimentarius</i> , <u><i>L. crustorum</i></u> , <i>L. farciminis</i> , <u><i>L. futsaii</i></u> , <u><i>L. kimchiensis</i></u> , <i>L. mindensis</i> , <i>L. nantensis</i> , <u><i>L. nodensis</i></u> , <i>L. paralimentarius</i> , <i>L. tuceti</i> , <i>L. versmoldensis</i>
<i>L. brevis</i>	<i>L. brevis</i> , <i>L. hammesii</i> , <i>L. parabrevis</i>	<i>L. acidifarinae</i> , <i>L. brevis</i> , <i>L. hammesii</i> , <u><i>L. koreensis</i></u> , <i>L. namurensis</i> , <i>L. parabrevis</i> , <u><i>L. paucivorans</i></u> , <u><i>L. senmaizukei</i></u> , <i>L. spicheri</i> , <i>L. zymae</i>
<i>L. collinoides</i>	Associée avec <i>L. manihotivorans</i>	<i>L. collinoides</i> , <u><i>L. kimchicus</i></u> , <u><i>L. odoratitofui</i></u> , <i>L. paracollinoides</i> , <i>L. similis</i>
<i>L. fructivorans</i>	<i>L. fructivorans</i> , <i>L. homohiochii</i> , <i>L. lindneri</i> , <i>L. sanfranciscensis</i>	<i>L. florum</i> , <i>L. fructivorans</i> , <i>L. homohiochii</i> , <i>L. lindneri</i> , <i>L. sanfranciscensis</i>
<i>L. plantarum</i>	<i>L. plantarum</i> , <i>L. parapantarum</i> , <i>L. pentosus</i>	<u><i>L. fabifermentans</i></u> , <i>L. parapantarum</i> , <i>L. pentosus</i> , <i>L. plantarum</i> , <u><i>L. xiangfangensis</i></u>
<i>L. sakei</i>	<i>L. curvatus</i> , <i>L. fuchuensis</i> , <i>L. graminis</i> , <i>L. sakei</i>	<i>L. curvatus</i> , <i>L. fuchuensis</i> , <i>L. graminis</i> , <i>L. sakei</i>
<i>L. casei</i>	<i>L. casei</i> , <i>L. paracasei</i> , <i>L. rhamnosus</i> , “ <i>L. zeae</i> ”	<i>L. casei</i> , <i>L. paracasei</i> , <i>L. rhamnosus</i>
<i>L. coryniformis</i>	<i>L. bifermentans</i> , <i>L. coryniformis</i> , <i>L. rennini</i> , associée avec <i>L. composti</i>	<i>L. bifermentans</i> , <i>L. coryniformis</i> , <i>L. rennini</i>
<i>L. manihotivorans</i>	-	<i>L. manihotivorans</i> , <i>L. nasuensis</i> , <u><i>L. porcinae</i></u>
<i>L. perolens</i>	<i>L. harbinensis</i> , <i>L. paracollinoides</i> , <i>L. perolens</i>	<i>L. harbinensis</i> , <i>L. perolens</i> , <u><i>L. shenzhenensis</i></u>
<i>L. vaccinostercus</i>	-	<i>L. oligofermentans</i> , <i>L. suebicus</i> , <i>L. vaccinostercus</i>
<i>Pediococcus</i> 2 clusters non associés	<i>P. cellicola</i> , <i>P. damnosus</i> , <i>P. parvulus</i> , <i>P. inopinatus</i> <i>P. acidilactici</i> , <i>P. claussenii</i> , <i>P. pentosaceus</i> , <i>P. stilesii</i>	<i>P. acidilactici</i> , <u><i>P. argentificus</i></u> , <i>P. cellicola</i> , <i>P. claussenii</i> , <i>P. damnosus</i> , <u><i>P. ethanolidurans</i></u> , <i>P. inopinatus</i> , <u><i>P. lolii</i></u> , <i>P. parvulus</i> , <i>P. pentosaceus</i> , <u><i>P. siamensis</i></u> , <i>P. stilesii</i>
Couples	<i>L. rossiae</i> - <i>L. siliginis</i> <i>L. vaccinostercus</i> - <i>L. suebicus</i> <i>L. manihotivorans</i> - <i>L. collinoides</i>	<i>L. kunkeei</i> - <u><i>L. ozensis</i></u> <i>L. rossiae</i> - <i>L. siliginis</i> <i>L. concavus</i> - <i>L. dextrinicus</i> <i>L. pantheris</i> - <u><i>L. thailandensis</i></u>
Espèces seules	<i>L. kunkeei</i> , <i>L. malefermentans</i> , <i>L. pantheris</i> , <i>L. harpeae</i> , “ <i>Paralactobacillus selangorensis</i> ”	<i>L. algidus</i> , <i>L. brantae</i> , <i>L. camelliae</i> , <i>L. composti</i> , <i>L. floricola</i> , <i>L. malefermentans</i> , <i>L. saniviri</i> , <i>L. selangorensis</i> , <i>L. senioris</i> , <i>L. sharpeae</i>

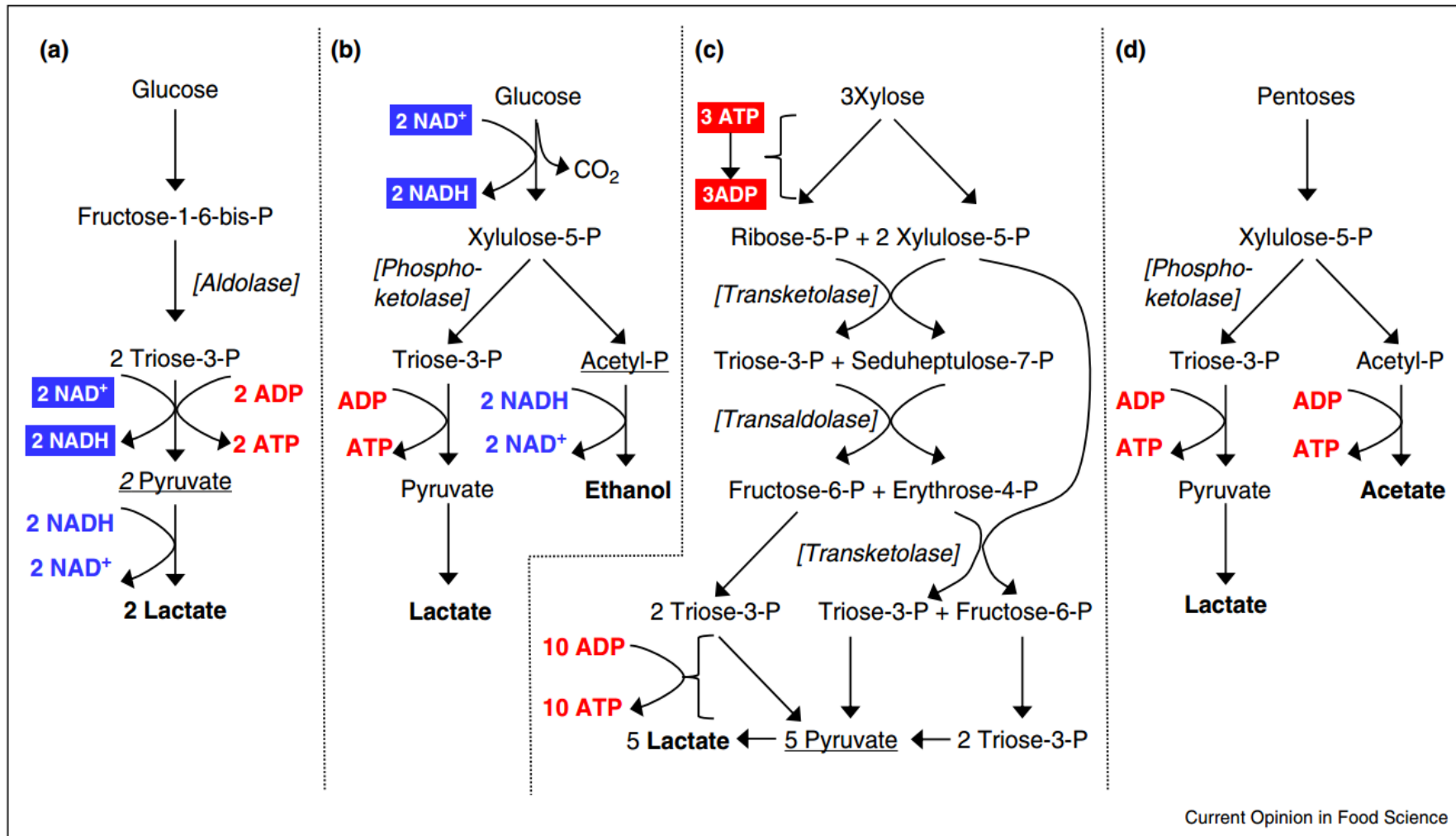
Les bactéries lactiques ont des besoins nutritionnels complexes. En effet, elles sont exigeantes en vitamines, bases azotées, minéraux, glucides et en dérivés d'acides nucléiques (Desmazeaud, 1983 ; Tailliez, 2001 ; Bernardeau *et al.*, 2006). Leur incapacité à synthétiser certains acides aminés ne leur permet de se développer que sur des milieux riches en acides aminés. Grâce à leurs protéinases, aminopeptidases ou exopeptidases, certaines sont capables d'utiliser des acides aminés, peptides ou protéines exogènes. Les bactéries lactiques se développent donc dans des biotopes riches où ces différents éléments sont disponibles. Ainsi, le lait, contenant une source importante d'azote, est un milieu de croissance favorable à certaines bactéries lactiques qui puisent dans la fraction azotée non soluble du lait les éléments indispensables à leur croissance (Desmazeaud, 1983).

## 2. Métabolisme des bactéries lactiques

### 2.1. Métabolismes homofermentaires et hétérofermentaires

Les bactéries lactiques sont classées en 3 groupes distincts selon les voies métaboliques utilisées pour fermenter les glucides: (i) les homofermentaires strictes dont le produit majeur de la fermentation du glucose est l'acide lactique (**Figure 5a**), (ii) les hétérofermentaires facultatives qui produisent de l'acide lactique à partir des hexoses (**Figure 5a**) et de l'acide lactique et acétique à partir des pentoses (**Figure 5d**) et (iii) les hétérofermentaires strictes qui produisent de l'acide lactique, du CO<sub>2</sub> et de l'acide acétique (en présence de dioxygène [O<sub>2</sub>]) ou de l'éthanol (en absence de O<sub>2</sub>) à partir des hexoses (**Figure 5b**) et de l'acide lactique et acétique à partir des pentoses (**Figure 5d**). Cependant, bien que présente, la voie des pentoses phosphate (**Figure 5c**) pour la fermentation homofermentaire des pentoses n'est pas prise en compte dans cette classification (Gänzle, 2015).

Les bactéries lactiques homofermentaires constituent souvent l'unique microbiote de fermentation dans de nombreux produits laitiers (Gänzle, 2015). Le lactate est le principal produit du métabolisme lorsque les carbohydrates fermentescibles sont abondants et le pyruvate, dont le sort dépend de la disponibilité de l'O<sub>2</sub> et des substrats, est le point clé de ramification du métabolisme des hexoses (**Figure 5**). Pendant le métabolisme homofermentaire des pentoses, via la voie des pentoses phosphates, une proportion significative du pyruvate est métabolisée en éthanol ou en acide acétique (Gänzle, 2015). Quant aux bactéries lactiques hétérofermentaires, elles emploient la voie de la phosphokétolase pour le métabolisme des carbohydrates, qui converti généralement le pyruvate, provenant des hexoses et pentoses en acide lactique (Gänzle, 2015).

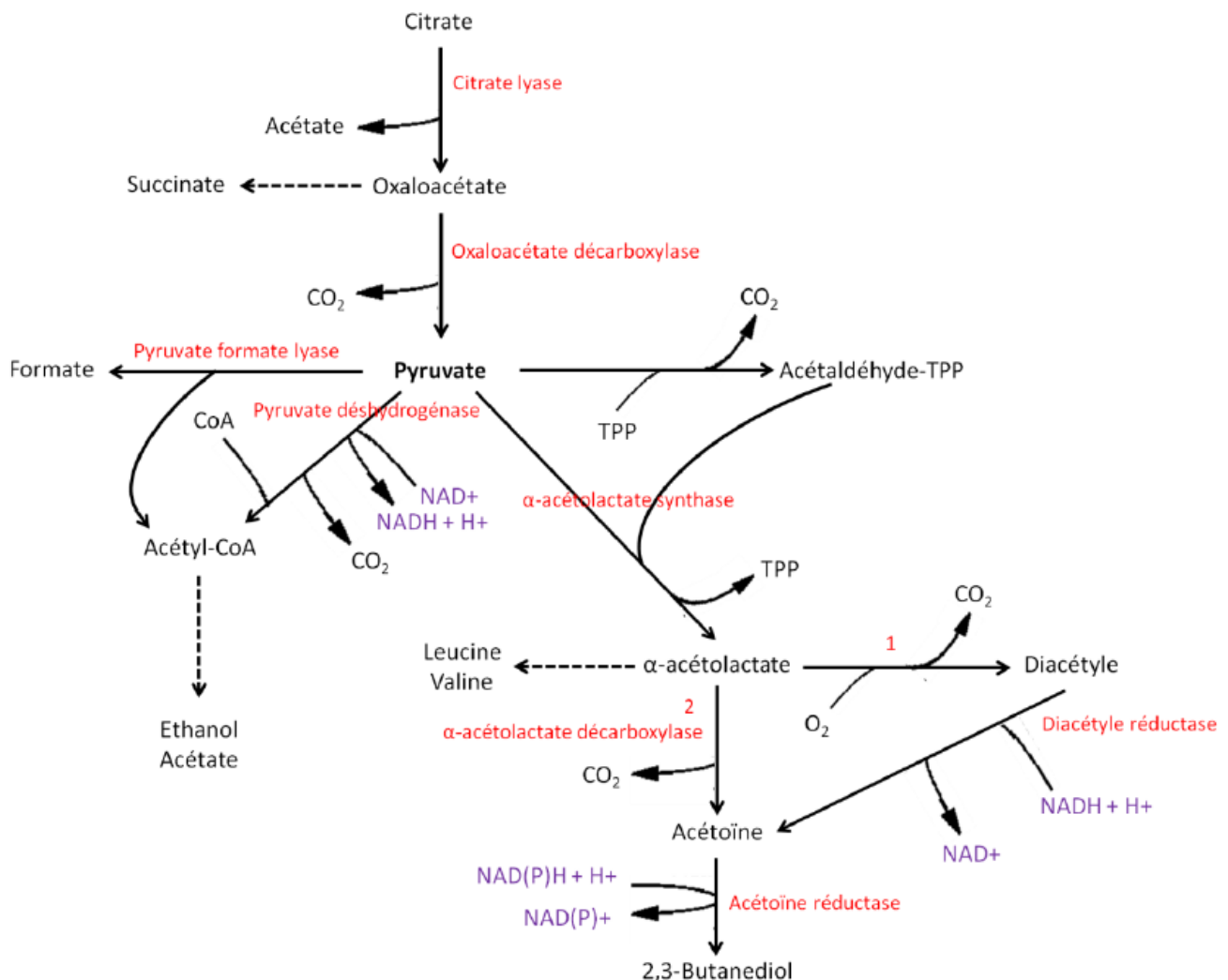


**Figure 5.** Voies métaboliques des hexoses et des pentoses chez les bactéries lactiques. Les produits majeurs du métabolisme sont inscrits en gras ; les points de ramification sont soulignés. La formation de co-facteurs réduits/oxidés (inscrits en blanc sur fond bleu ou en bleu respectivement) est indiquée si elle se passe en amont/aval de points de ramification importants ; la synthèse de l'Adénosine TriPhosphosphate (ATP), est inscrite en rouge. (a) Métabolisme homofermentaire des hexoses via la voie de Emden-Meyerhoff. (b) Métabolisme hétérofermentaire des hexoses via la voie de la phosphoketolase. (c) Métabolisme homofermentaire des pentoses via la voie des pentoses phosphate. (d) Métabolisme hétérofermentaire des pentoses via la voie des phosphoketolase (Gänzle, 2015).



## 2.2. Production de composés aromatiques

La formation des arômes dans les produits laitiers résulte en partie de la conversion du lactose, du citrate, des caséines et des lipides issus du lait, en composés aromatiques pendant la fermentation (Thierry *et al.*, 2015). Le lactose est converti en lactate via la glycolyse et le métabolisme du citrate permet la formation de diacétyle, acétoïne et acide acétique (Smit *et al.*, 2005) (**Figure 6**). Cette activité résulte de la présence, chez certaines souches, de gènes de transport du citrate tel que le gène codant la citrate perméase, et des gènes du métabolisme du citrate (McSweeney and Fox, 2017).



**Figure 6. Principales étapes du métabolisme du citrate chez les bactéries lactiques citrate-positives (d'après Monnet *et al.*, 2008). Les enzymes impliquées sont indiquées en rouge. 1, décarboxylation chimique oxydative ; 2, décarboxylation chimique non oxydative.**

Les composés aromatiques produits par les bactéries lactiques dérivent également du catabolisme des acides aminés tels que la méthionine, la phénylalanine, la thréonine et des

acides aminés à chaîne ramifiée, obtenus majoritairement par la protéolyse de la caséine. Parmi les molécules aromatiques issues du métabolisme des acides aminés on retrouve notamment le 3-méthyl-butanal (arôme puissant de fromage) et le méthanthiol (arôme fromage et sulfure) présent dans les fromages tels que le Gouda, le Cheddar et le Camembert (Smit *et al.*, 2005). De nombreuses enzymes, incluant des décarboxylases, des déhydrogénases, des peptidases, des protéases, des aminotransférases et des estérases, sont mises en jeu dans les diverses réactions impliquées dans la formation des arômes (Smit *et al.*, 2005). Or, d'une souche à l'autre, le contenu enzymatique varie considérablement, la production de composés aromatiques est donc un caractère souche-dépendant (Thierry *et al.*, 2015 ; Ampuero and Bosset, 2003). Pour une espèce considérée, le choix des souches ajoutées au produit, en fonction des composés aromatiques qu'elles sont capables de synthétiser, est donc très important pour la qualité sensorielle du produit.

### 3. Origine et métabolisme des bactéries propioniques

Il existe 2 grands groupes pour le genre *Propionibacterium* en fonction de leur origine : (i) les bactéries propioniques laitières et (ii) les bactéries propioniques cutanées qui font partie du microbiote commensal de la peau et des muqueuses humaines. Les bactéries propioniques sont des bactéries à Gram positif, bacilles ou forme coccoïdales, immobiles, anaérobies facultatives, catalase positive capables de produire de grande quantité d'acide propionique à partir du lactate (**Figure 7**). Les bactéries propioniques laitières comprennent 4 espèces : *P. acidipropionici*, *P. jensenii*, *P. thoenii* et *P. freudenreichii* actuellement divisé en 2 sous espèces selon leur capacité à fermenter le lactose et à réduire le nitrate (lac-/nit+ et lac+/nit- pour *P. freudenreichii* subsp. *freudenreichii* et *P. freudenreichii* subsp. *shermanii*, mais l'existence de souches inclassifiables (lac+/nit+ et lac-/nit-) remet en question cette subdivision (Freitas *et al.*, 2015).

Les bactéries propioniques laitières peuvent être utilisées comme ferment, notamment pour la fabrication de certains fromages à pâte pressée cuite tel que l'emmental, pour leurs propriétés aromatisantes mais aussi pour leur capacité à former des trous caractéristiques, du fait de la production de CO<sub>2</sub> durant leur développement (Chamba and Perreard, 2002).

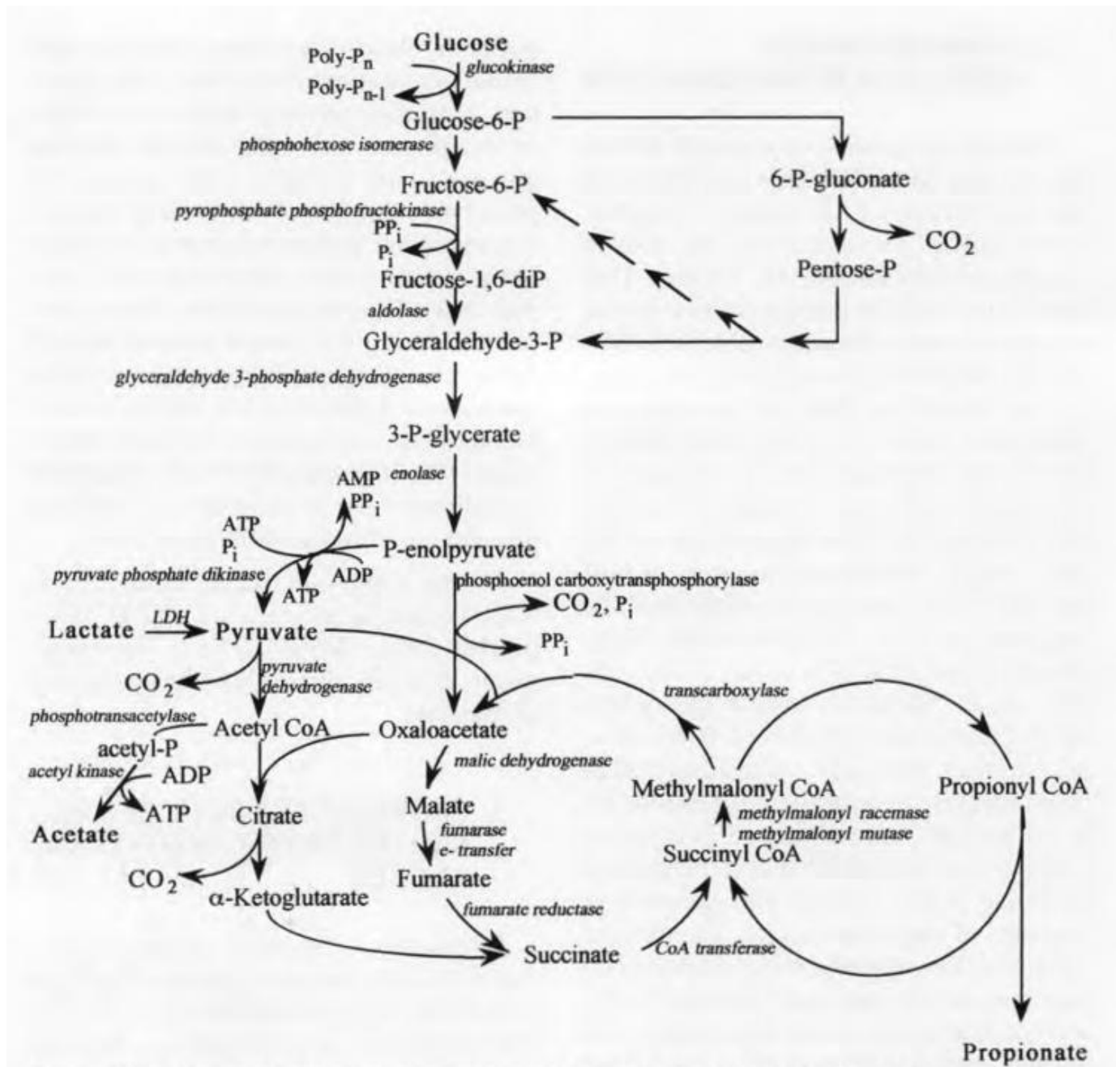


Figure 7. Cycles de la fermentation propionique d'après Wood (1981). LDH : lactate déshydrogénase ; Poly-Ph : polyphosphate ; PPI : pyrophosphate. Pour faciliter la lecture, seule la réaction de conversion du fructose-6-P en fructose-1,6-diP nécessitant des pyrophosphates est montrée. De même, la production d'ATP par le transfert d'électron est omise. Toutes les réactions sont dirigées vers la production de propionate, bien qu'elles soient réversibles.

#### 4. Innocuité des bactéries lactiques et propioniques

Le terme de culture microbienne alimentaire a été défini par l'European Food and Feed Culture Association (EFFCA) comme « des bactéries, levures ou champignons filamenteux vivant utilisés dans la production des aliments » (Bourdichon *et al.*, 2012).

Au delà de leur effet positif sur la préservation des aliments, les bactéries doivent respecter certains critères spécifiques :

- Elles ne doivent pas entraîner une altération des produits (Holzapfel *et al.*, 1995 ; Privat and Thonart, 2011).
- Elles ne doivent pas affecter la croissance et les propriétés fonctionnelles des souches endogènes de l'aliment et nuire aux processus de fermentation du produit, dans le cadre de produits fermentés (Holzapfel *et al.*, 1955).
- Elles doivent pouvoir être utilisées à l'échelle industrielle et survivre à lyophilisation et/ou à la congélation.
- Leur innocuité doit être garantie.

À l'heure actuelle, il n'existe pas, en France et en Europe, de réglementation pour l'autorisation de la mise sur le marché d'une culture microbienne pour l'alimentation humaine (Wessels *et al.*, 2004 ; Brillet *et al.*, 2005). Le professionnel qui la met sur le marché a donc la responsabilité de l'évaluation de son innocuité au même titre que les aliments mis sur le marché ne doivent pas présenter de danger pour la santé humaine.

Aux Etats-Unis et en Europe, l'innocuité des microorganismes est définie par deux approches distinctes. On distingue le statut Generally Recognised As Safe (GRAS) aux Etats-Unis (Drouault and Corthier, 2001), attribué sur la démonstration de l'innocuité d'une souche microbienne en association avec un usage donné, et le statut Qualified Presumption of Safety (QPS) en Europe (European Food Safety Authority [EFSA], 2009) qui évalue l'innocuité au niveau de l'espèce et a conduit à la mise en place d'une liste positive d'espèces mise à jour régulièrement par l'EFSA. Il existe cependant un inventaire de culture microbienne alimentaire autorisé. Actuellement, cet inventaire contient 195 et 69 espèces bactériennes et fongiques respectivement (Bourdichon *et al.*, 2012). Parmi les espèces bactériennes et fongiques on retrouve principalement des espèces appartenant aux genres *Lactobacillus*, *Staphylococcus*, *Leuconostoc*, *Acetobacter*, *Gluconacetobacter*, *Bifidobacterium* et *Propionibacterium* et *Candida*, *Penicillium*, *Pichia*, *Saccharomyces*, *Aspergillus*, *Mucor* et *Rhizopus* respectivement (Bourdichon *et al.*, 2012).

L'approche QPS prend en compte 4 critères :

- Identification taxonomique précise de la souche
- Bilan des connaissances scientifiques sur la souche
- Application envisagée et matrice alimentaire considérée
- Preuve de l'absence de pathogénicité et plus particulièrement de l'absence de production d'amines biogènes et de résistance acquise aux antibiotiques dans le cas des bactéries lactiques.

Les amines biogènes résultent principalement de la décarboxylation d'acides aminés et parfois de l'amination de cétones ou d'aldéhydes ou de l'hydrolyse de composés azotés par des enzymes endogènes ou exogènes. Leur production apparaît lors du processus de fermentation et les plus répandues sont l'histamine et la tyramine (Bernadeau *et al.*, 2006). Les amines biogènes ont un impact sur l'homme ; la tyramine peut provoquer de l'hypertension et de fortes migraines alors que l'histamine peut provoquer des nausées, des vomissements et des diarrhées (Gardini *et al.*, 2002).

L'utilisation intensive et excessive des antibiotiques dans différents domaines comme la médecine ou l'agriculture a entraîné une sélection de souches bactériennes résistantes à ces antibiotiques (Aarestrup, 2005 ; Toomey *et al.*, 2010). En effet, les bactéries lactiques peuvent présenter des résistances acquises aux antibiotiques, portés par des éléments génétiques mobiles tels que les plasmides (Ammor and Mayo, 2007). Le danger est donc le transfert de ces gènes de résistance à une bactérie pathogène de l'alimentation ou du système digestif du consommateur. Avant d'utiliser une souche dans l'alimentation, il est donc essentiel de vérifier l'absence de résistance acquise aux antibiotiques en suivant la méthode décrite dans la norme International Organization for Standardization (ISO) 10932 (2010).

## **Chapitre 3**

~

# **Taxonomie et métabolisme des champignons**



Comme les bactéries, les champignons présentent un incontestable intérêt alimentaire. D'une part, de nombreuses espèces ont des qualités organoleptiques et nutritives intéressantes. Certains de ces champignons peuvent d'ailleurs être consommés sans transformation, c'est le cas de nombreux basidiomycètes tel que *Agaricus bisporus* et de certains Ascomycètes tel que *Tuber melanosporum*, tout deux connus sous les noms vernaculaires de « champignon de Paris » et de « Truffe noire », respectivement. D'autre part parce qu'ils interviennent dans la fabrication de laits fermentés tels que les grains de kéfir auxquels sont associés *Kluyveromyces lactis*, *Saccharomyces turicensis* et *Pichia fermentans* (Wang *et al.*, 2008). Les champignons ont également un rôle important en production fromagère ou des espèces telles que *Penicillium roqueforti* ou *Penicillium camemberti* ont un rôle clé dans la fabrication de fromages tels que le Roquefort ou le Camembert (Ropars *et al.*, 2012).

## 1. Taxonomie fongique

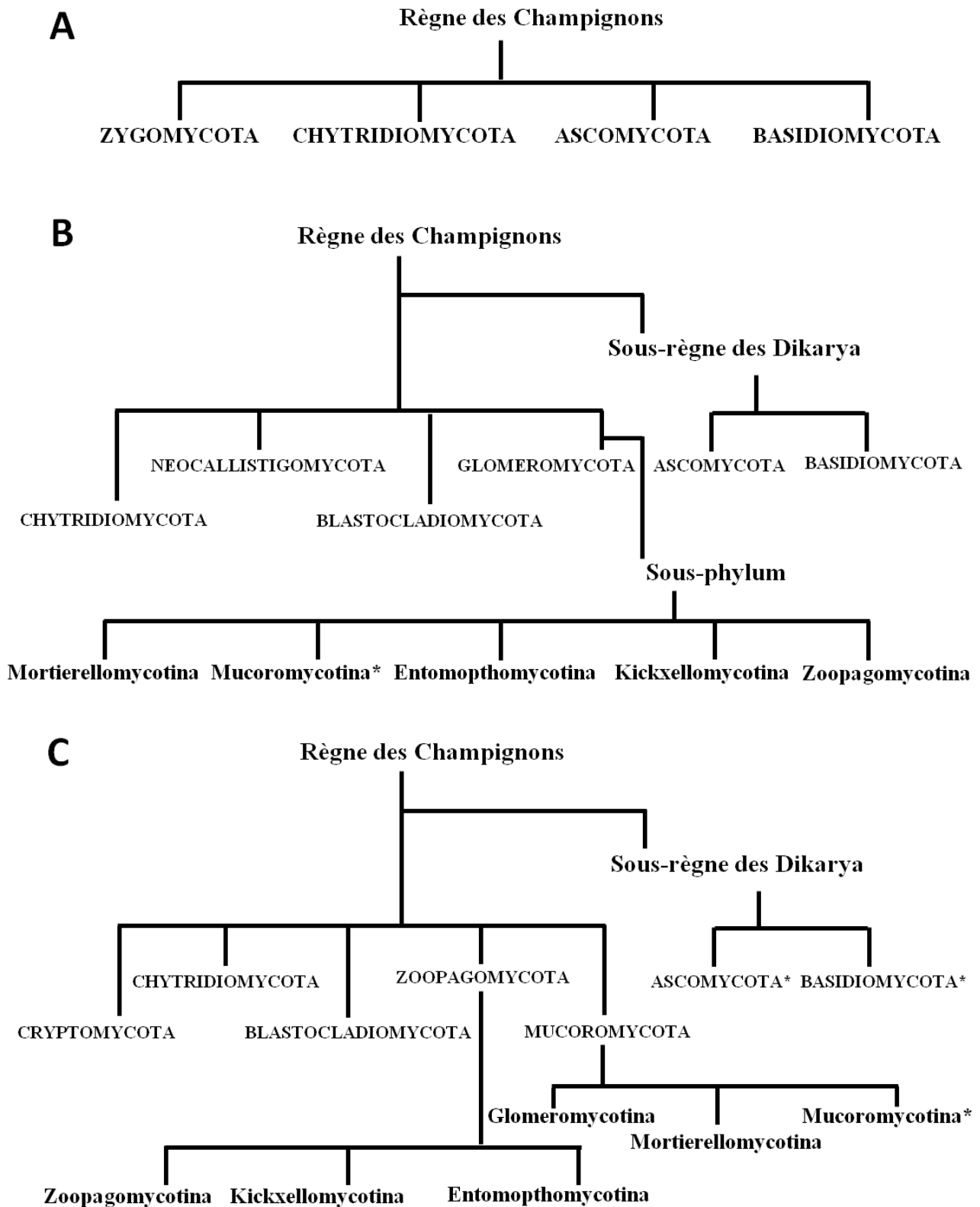
Les champignons filamenteux seraient apparus il y a environ 2,3 milliards d'années (Bengtson *et al.*, 2017) mais le règne des *Fungi* n'a été reconnu pour la première fois qu'en 1969 par Whittaker. À cette époque, trois classes étaient reconnues : les phycomycètes, les ascomycètes et les basidiomycètes. Les espèces où n'ont été observées que des spores asexuées ont été regroupées dans la « classe », artificielle, des deutéromycètes ou *Fungi Imperfecti* (Ainsworth, 1973). Comme les connaissances, grâce notamment à l'avènement de la biologie moléculaire, sur les champignons s'améliorent, la classification des champignons a subi d'importants changements. La classe des phycomycètes a été supprimée (Whittaker, 1969) et les membres appartenant à cette classe ont été regroupés dans 5 classes : les zygomycètes, les chytridiomycètes, les hypochytridiomycètes, les trichomycètes et les oomycètes (Ainsworth, 1973). Dans les classifications ultérieures, seuls les Chytridiomycota, les Zygomycota, les Ascomycota et les Basidiomycota étaient inclus dans le règne des *Fungi* (**Figure 8A**). Cette classification a été universellement acceptée jusqu'aux années 2000 et n'a cessé d'évoluer depuis grâce aux apports de la biologie moléculaire pour la classification.

Certains phyla comme les Myxomycota ou les Oomycota ont été exclus du règne des *Fungi*. En effet, Baldauf and Doolittle (1997) et Baldauf *et al.*, (2008) ont démontré l'appartenance des Myxomycètes au phylum des Amoebozoa du règne des Protista (Cavalier-Smith, 1998). En ce qui concerne les Oomycota, ils ont également été transférés dans le règne des Protista (Margulis and Schwartz, 1998). De plus, les Ascomycota et les Basidiomycota ont été



regroupés dans le sous-règne des Dikarya (**Figure 8B**). Concernant les Blastocladiomycota et les Neocallistigomycota, ils ont été séparés des Chytridiomycota et élevés chacun au niveau de phylum à part entière (James *et al.*, 2006 ; Hibbett *et al.*, 2007). Récemment, la nouvelle classification de Spatafora *et al.* (2016) propose 2 nouveaux phylums : les Mucoromycota et les Zoopagomycota qui remplacent le phylum des Glomeromycota, maintenant considéré comme un sous-phylum : les Glomeromycotina. Quant au phylum des Zoopagomycota, il comprend les Zoopagomycotina, les Kickxellomycotina et les Entomophthomycotina (**Figure 8C**).

Le règne des *Fungi* est aujourd'hui mieux délimité et la communauté scientifique, particulièrement les mycologues, s'accorde à dire que le règne des *Fungi* est composé de 1,62 millions d'espèces (Hawksworth, 1991). Néanmoins, comme Hawksworth le reconnaît lui-même, ainsi que Bass and Richards (2011) et Richards *et al.* (2012), le nombre réel d'espèce est certainement sous-estimé. En effet, une raison pour laquelle le manque de connaissance est important est que la plupart des champignons passe la majorité de leur cycle de vie (phase microbienne et donc invisible à l'œil nu) sous terre ou avec d'autres substrats (Grube *et al.*, 2017). De plus, une proportion importante de champignons ne produit pas de forme macroscopique (Grube *et al.*, 2017). Le nombre réel d'espèce serait donc très probablement un nombre à 7, voire 8, chiffres (Bass and Richards, 2011).



**Figure 8. Classification du règne des champignons. (A) Classification jusqu'aux années 2000, (B) classification actuelle, (C) classification proposée par Spatafora *et al.* en 2016 (adaptée de la thèse de A. Hermet, 2014 et de Spatafora *et al.*, 2016). \* phyla et sous-phylum retrouvés dans les produits laitiers.**

## 1.1. Champignons supérieurs : Ascomycota et Basidiomycota

Les champignons ascomycètes et basidiomycètes, qui auraient divergé il y a environ 642 millions d'années (Beimforde *et al.*, 2014), appartiennent au sous-règne des Dikarya et représentent la quasi-totalité des espèces décrites à ce jour et ayant une importance économique indéniable. Avec plus de 64 000 espèces décrites, appartenant à 6400 genres approximativement, les Ascomycota forment le plus grand phylum des *Fungi* (Kirk *et al.*, 2008, Blackwell, 2011). Les espèces de ce phylum sont présentes dans de très nombreuses niches écologiques et dans tous les écosystèmes terrestres et aquatiques (Schoch *et al.*, 2009) mais les principaux champignons ascomycètes retrouvés dans les produits laitiers, notamment fromagers, se retrouvent dans 2 classes : les Eurotiomycètes et les Sordariomycètes (Ropars *et al.*, 2012). Les champignons ascomycètes ont généralement une structure spécialisée, appelée asque, au sein de laquelle les spores méiotiques (ascospores) sont produites. Les Ascomycota sont majoritairement microscopiques mais comprennent également quelques champignons mycorhiziens macroscopiques tel que *Morchella angusticeps*, plus connu sous le nom de Morille. C'est également au sein de ce groupe que sont retrouvés les champignons filamenteux, aussi appelés moisissures, et les levures d'intérêts technologiques, mais aussi des champignons dits d'altération.

Avec presque 32 000 espèces décrites, les Basidiomycota constituent le deuxième plus grand phylum fongique (Kirk *et al.*, 2008). Considérés comme ubiquitaires, un certain nombre de basidiomycètes sont immédiatement reconnaissables par les fructifications élaborées qu'ils produisent. C'est en effet au sein de ce phylum que sont retrouvés la majorité des champignons macroscopiques comestibles tels que *Boletus edulis* (Cèpe de Bordeaux) ou *Craterellus cornucopioides* (Trompette de la mort), des champignons dits « vénéneux » tel que *Amanita verna* (Amanite printanière) ou *Boletus luridus* (Bolet blafard) mais également les rouilles et charbons, les levures, ainsi que certains pathogènes humains et des endophytes (Yang, 2011). Les champignons basidiomycètes sont généralement capables de produire des basides portant les spores sexuées appelées basidiospores.

## 1.2. Le phylum des Mucoromycota

Anciennement, les Mucoromycotina étaient placés dans le phylum des Zygomycota, groupe polyphylétique (James *et al.*, 2006) aujourd'hui redistribué parmi les Glomeromycota et 4

sous-phyla dont le placement est incertain : Mucoromycotina, Entomophthoromycotina, Kickxellomycotina et Zoopagomycotina. Récemment, les Mortierellales ont été séparés des Mucoromycotina et placés dans un sous-phylum distinct, les Mortierellomycotina (Hoffmann *et al.*, 2011) pour obtenir la classification actuelle (**Figure 8B**). Une étude très récente, de Spatafora *et al.* (2016), propose une nouvelle classification dans laquelle les Mucoromycotina font maintenant partie, avec les Mortierellomycotina et les Glomeromycotina (anciennement Glomeromycota), du phylum des Mucoromycota (**Figure 8C**). Dans la production de produits laitiers, les Mucoromycotina ont, eux aussi, une grande importance économique. En effet, certaines espèces telles que *Mucor lanceolatus* ou *Mucor racemosus*, appartenant à l'ordre des Mucorales interviennent respectivement dans la production ou dans l'altération des produits laitiers.

## 2. Caractérisation de la diversité

Au-delà de la nécessité de définir parfaitement les espèces fongiques au sein d'une classification, il est également important de pouvoir les identifier au sein de communautés, notamment dans les aliments qui sont des écosystèmes microbiens complexes et dynamiques. La mycologie étant un domaine de la microbiologie, la diversité peut être évaluée par l'utilisation de méthodes culture-dépendante et/ou indépendante.

Les méthodes culture-dépendantes consistent à isoler et à cultiver des micro-organismes avant de les identifier selon leur caractéristiques morphologiques, biochimiques ou génétiques. Dans le cas d'une identification selon les caractères génétiques, les étapes d'isolement et de culture sont suivies d'une étape d'extraction de l'acide désoxyribonucléique (ADN) puis d'une étape d'amplification une réaction de polymérase en chaîne (PCR). Le choix du gène (ou de la portion de gène) clé(e) est important pour la robustesse de l'identification. Chez les levures, c'est le domaine D1-D2 du gène codant l'acide ribonucléique ribosomique (ARNr) 28S qui offre la meilleure résolution taxonomique (Anderson and Cairney, 2004). Pour l'ensemble des champignons filamenteux, c'est l'espaceur interne transcrit (ITS) qui a été proposé comme « DNA barcode » (Schoch *et al.*, 2012) bien qu'il existe néanmoins des gènes permettant une meilleure résolution taxonomique chez certains genres fongiques. Par exemple, c'est une partie des gènes de la  $\beta$ -tubuline et du facteur d'élongation  $\alpha$  qui sont utilisés pour identifier au mieux les espèces appartenant aux genres *Penicillium* et *Cladosporium*, respectivement (Glass and Donaldson, 1995 ; Carbone and Kohn, 1999). Un grand nombre d'études, basées sur

l'utilisation de méthodes culture-dépendantes ont été réalisées afin d'évaluer la diversité microbienne des fromages (Andrighetto *et al.*, 1998; Fitzsimmons *et al.*, 1999; Mannu *et al.*, 2000; Berthier *et al.*, 2001; Dasen *et al.*, 2003). Pour ce type de méthode, le choix du milieu et des conditions de culture sont donc importants. En effet, un milieu trop sélectif risque de réduire la vision globale de la diversité alors qu'un milieu trop permissif risque de surestimer cette diversité. De plus, ces méthodes sont souvent longues à mettre en œuvre en raison du temps de croissance des microorganismes ainsi qu'à la nécessité de les purifier avant de les identifier. De plus, les espèces présentes en faibles quantités sont souvent « masquées » par des espèces plus abondantes (Hugenholtz *et al.*, 1998) et certains microorganismes ne sont pas cultivables (Ward *et al.*, 1990, 1992 ; Head *et al.*, 1998). Par conséquent, l'utilisation de méthodes culturales est susceptible de biaiser la vision de la diversité d'un écosystème donné. Wolfe *et al.* (2014) ont cependant démontré la possibilité de reproduire complètement, *in vitro*, les communautés complexes issues de fromages pondérant ainsi le fait que la cultivabilité soit réellement un problème dans le cas l'étude des communautés microbiennes des aliments.

Depuis la fin des années 1990, des techniques moléculaires culture-indépendantes telles que la TTGE/DGGE (temporal temperature gradient gel electrophoresis / denaturing-gradient gel electrophoresis), la TRFLP (terminal restriction fragment length polymorphism) et plus récemment, des techniques de métagénomique (metabarcoding, whole genome shotgun sequencing) sont utilisées pour l'étude des écosystèmes microbiens. Ces méthodes ouvrent des perspectives intéressantes pour la caractérisation de communautés microbiennes telles que le sol, l'eau ou les aliments (Ercolini, 2004 ; Evans and Seviour, 2012 ; Dugat-Bony *et al.*, 2016). Actuellement, avec le séquençage de nouvelle génération de (NGS), le séquençage des amplicons d'ARNr est communément utilisé pour étudier l'écologie microbienne des aliments bien que cette technique requiert des compétences importantes en bio-informatique (Ercolini, 2013). Cependant, ces méthodes, couplées ou non à une amplification PCR, présentent également des biais potentiels se situant soit au niveau de l'extraction d'ADN, du choix de la cible à amplifier ou de l'amplification PCR elle-même. Les méthodes culture-indépendantes ne permettent pas en outre d'avoir accès aux souches sous forme d'isolats cultivables. Ces méthodes ont néanmoins permis aux scientifiques de changer leur manière de voir la microbiologie alimentaire. En effet, les microorganismes présents dans les aliments sont maintenant vus comme une communauté à part entière (Cocolin and Ercolini, 2015).

Grâce à ces outils, l'identification des espèces fongiques est donc, aujourd'hui, relativement précise et est une étape clé puisqu'elle donne accès aux connaissances accumulées sur un organisme. Du fait de leur importance au niveau économique, les champignons présentent un

intérêt majeur, en particulier dans l'agroalimentaire comme organisme technologique mais aussi d'altération, notamment dans le secteur laitier.

Ces différentes approches de caractérisation taxonomique permettent aux scientifiques de mieux comprendre les processus dynamiques microbiens tels que la fermentation mais aussi les dynamiques d'altérations microbiennes des aliments (Cocolin and Ercolini, 2015). Les avancées qui en résultent permettent aux industries de devenir plus compétitives en optimisant les activités microbiennes désirées et en réduisant les pertes alimentaires.

### **3. Métabolisme des champignons**

Depuis des siècles, l'homme utilise les microorganismes dans l'alimentation, notamment dans la fabrication de pain, de fromage ou de boissons alcoolisées mais ce n'est qu'au début du 20<sup>ème</sup> siècle que l'on commence à parler de biotechnologies pour se référer à l'utilisation de bactéries (telles que les bactéries lactiques), mais aussi de champignons, dans les produits alimentaires (Punt *et al.*, 2002).

Les champignons sont utilisés en tant que flore technologique dans la fabrication de certains fromages. Ils proviennent notamment du lait cru, de l'environnement fromager (équipement, locaux) ou sont intentionnellement inoculés lors de la fabrication du fromage et font ainsi partie de l'écosystème d'affinage de certains fromages (Montel *et al.*, 2014). Lors de l'affinage, les principaux changements biochimiques impliquent le catabolisme du lactose résiduel, du lactate, du citrate mais aussi la lipolyse et la protéolyse à l'origine des acides gras libres et des acides aminés respectivement qui jouent directement un rôle important dans les caractéristiques aromatiques des fromages ou en tant que précurseurs d'arômes.

#### **3.1. Effets sur l'apparence et l'affinage des fromages**

Les champignons contribuent à l'apparence de la surface ou du cœur des fromages. Le choix des souches fongiques s'avère d'une grande importance selon le type de fromage recherché (Boutrou and Guéguen, 2005). En effet, les souches de champignons filamenteux sont sélectionnées selon différents critères tels que leur capacité de colonisation ou encore la hauteur, la densité et la couleur du mycélium. Par exemple, dans le Gorgonzola et le Roquefort, les souches de *P. roqueforti* utilisées tirent du bleu clair au jaune et du vert au vert sombre respectivement (Chamba and Irlinger, 2004). Quant aux champignons du genre *Mucor*,

ils sont connus pour synthétiser des caroténoïdes (Papp *et al.*, 2009), un groupe de pigments (Chandi and Gill, 2011) qui participe à la couleur de certains fromages tels que les fromages à croûte lavée. Concernant les souches de levures, elles peuvent présenter un aspect plutôt visqueux ou plutôt duveteux (Boutrou and Guéguen, 2005). Elles interviennent également dans la mise en place de la morge de certains fromages tels que le Reblochon ou le Comté. En effet, la morge est un revêtement, présent à la surface de ces fromages, constitué essentiellement de levures appartenant aux genres *Candida*, *Kluyveromyces*, *Debaryomyces*, *Rhodotorula* et de bactéries telles que *Brevibacterium linens*. Ainsi, la morge est à l'origine de la typicité des fromages dits à croûte morgée, elle a également une fonction de protection contre le dessèchement, la perte d'arôme et la croissance de microorganismes indésirables par antagonisme (Wyder *et al.*, 2000).

Les champignons, intervenant également dans la désacidification, sont rencontrés dans le cœur et à la surface des fromages. Selon les souches et les espèces, ils possèdent des capacités différentes à métaboliser les sucres, le lactate, à l'origine de l'activité désacidifiante (Chamba and Irlinger, 2004), et le citrate (Eliskases-Lechner and Ginzinger, 1995). Ainsi, l'utilisation de *K. lactis* et de *Debaryomyces hansenii* comme cultures d'affinage est très commune car ces 2 levures sont capables d'assimiler le lactose résiduel et le lactate. De même, *Galactomyces geotrichum* qui métabolise le galactose et le lactate et *P. camemberti* et *P. roqueforti*, capables d'utiliser l'acide lactique comme source de carbone sont des espèces couramment rencontrées en technologie fromagère. La dégradation du lactate et la production de métabolites alcalins, tel que l'ammoniac, par les champignons entraînent donc l'augmentation du pH de la surface du fromage. Cette désacidification permet la croissance des bactéries de surface, telle que *B. linens*, plus acido-sensibles. Ainsi, l'activité métabolique fongique va permettre une augmentation globale du pH de la matrice fromagère qui, associé à la protéolyse, permettent un ramollissement du fromage au cours de l'affinage (Chávez *et al.*, 2011).

En contribuant de manière significative au développement de la flaveur des fromages, la protéolyse est une des réactions biochimiques importante qui se produit au cours de l'affinage des fromages (Upadhyay *et al.*, 2004). Les levures montrent une large diversité interspécifique mais également intra-spécifique d'activités protéolytiques, notamment caséinolytique, aminopeptidasique et carboxypeptidasique (Schmidt *et al.*, 1993). Avec leur activité protéasique, endopeptidasique et exopeptidasique, *P. camemberti* et *P. roqueforti* sont des contributeurs majeurs de la protéolyse du fromage (Gripon, 1999). Le catabolisme des acides aminés résultants permet ensuite la production d'ammoniac et de composés volatils tels que les composés soufrés volatils comme le disulfure de diméthyle ou le trisulfure de

diméthyle qui ont un impact aromatique très important (Sørensen *et al.*, 2011). De plus, la décarboxylation et la désamination oxydative des acides aminés mènent également à la formation d'alcools et d'acides qui interviennent également dans la mise en place de la flaveur des fromages (Molimard and Spinnler, 1996). Le développement de la flaveur des fromages résulte aussi de l'hydrolyse enzymatique des triglycérides en acides gras et glycérol, mono- ou di-glycérides (Collins *et al.*, 2004), les acides gras libres étant d'importants précurseurs d'arômes qui contribuent à la flaveur des fromages (Collins *et al.*, 2004). En effet, les acides gras sont des précurseurs de méthylketones, d'alcools, de lactones et d'esters qui sont importants dans l'arôme des fromages à croûte fleurie. De plus, les acides gras volatils à chaîne courte jouent aussi un rôle important dans la flaveur de fromages à croûte fleurie. Par exemple, les acides acétique et propionique ont une odeur de vinaigre et un goût âcre alors que l'acide butyrique a plutôt des saveurs rances et fromagères (Molimard and Spinnler, 1996). Parmi les levures, qui participent à la lipolyse dans les fromages, c'est *Yarrowia lipolytica* qui a la plus forte activité lipolytique (Schmidt *et al.*, 1993) mais c'est dans les fromages affinés par les champignons tels que *P. camemberti* et *P. roqueforti* que la lipolyse est plus forte (Chamba and Irlinger, 2004).

Les champignons contribuent donc de manière positive au développement des qualités organoleptiques des fromages mais ils peuvent également être responsables d'altérations, ou accidents de fabrication, quand leur présence n'est pas désirée. Ainsi par exemple, si *P. camembertii* est essentiel pour la fabrication du Camembert, sa présence non désirée dans un fromage frais ou une crème fraîche peut conduire à l'altération de ces derniers.

### **3.2. Production de composés antimicrobiens**

Les champignons sont, selon les typologies de fromages considérées, impliqués dans l'écosystème fromager où ils jouent un rôle dans sa résistance à diverses perturbations, telle que l'apparition d'une flore d'altération (Wyder *et al.*, 2000). Ainsi, les champignons sont, comme les bactéries, des producteurs potentiels de composés antimicrobiens, notamment les antibiotiques, qui sont les composés fongiques antibactériens les plus connus. De plus, de nombreuses études (Acosta *et al.*, 2009 ; Coda *et al.*, 2013 ; Geisen, 2000 ; Ignatova *et al.*, 2015 ; Nally *et al.*, 2015 ; Pesce *et al.*, 2017 ; Leyva Salas *et al.*, 2017) ont montré la capacité des champignons à produire des peptides, des protéines ou des acides organiques à activité antifongiques. Une revue récente (Liu *et al.*, 2013) reporte l'utilisation de levures pour lutter contre les contaminations fongiques des fruits après leur récolte. Ainsi, les levures et



champignons filamenteux sont également une source potentielle de cultures bioprotectrices pour lutter contre les contaminations fongiques des produits laitiers.

# **Chapitre 4**

~

## **Diversité et contrôle des contaminations fongiques dans les produits laitiers**



Ce chapitre fait l'objet d'une revue scientifique, écrite en anglais, ayant pour titre « *Diversity and control of spoilage fungi in dairy products : an update* » publiée dans le journal *Microorganisms* pour une édition spéciale « *Food and Microorganisms* ».

Elle a pour objet de faire un état de l'art sur la diversité des champignons responsables d'altérations des produits laitiers ainsi que sur les moyens de prévention et de contrôle mis en place pour lutter contre ces contaminations, y compris la biopréservation qui est en plein essor depuis quelques années.





Review

# Diversity and Control of Spoilage Fungi in Dairy Products: An Update

Lucille Garnier <sup>1,2</sup> , Florence Valence <sup>2</sup> and Jérôme Mounier <sup>1,\*</sup>

<sup>1</sup> Laboratoire Universitaire de Biodiversité et Ecologie Microbienne (LUBEM EA3882), Université de Brest, Technopole Brest-Iroise, 29280 Plouzané, France; lucille.garnier@univ-brest.fr

<sup>2</sup> Science et Technologie du Lait et de l'Œuf (STLO), AgroCampus Ouest, INRA, 35000 Rennes, France; florence.valence-bertel@inra.fr

\* Correspondence: jerome.mounier@univ-brest.fr; Tel.: +33-(0)2-90-91-51-00; Fax: +33-(0)2-90-91-51-01

Received: 10 July 2017; Accepted: 25 July 2017; Published: 28 July 2017

**Abstract:** Fungi are common contaminants of dairy products, which provide a favorable niche for their growth. They are responsible for visible or non-visible defects, such as off-odor and -flavor, and lead to significant food waste and losses as well as important economic losses. Control of fungal spoilage is a major concern for industrials and scientists that are looking for efficient solutions to prevent and/or limit fungal spoilage in dairy products. Several traditional methods also called traditional hurdle technologies are implemented and combined to prevent and control such contaminations. Prevention methods include good manufacturing and hygiene practices, air filtration, and decontamination systems, while control methods include inactivation treatments, temperature control, and modified atmosphere packaging. However, despite technology advances in existing preservation methods, fungal spoilage is still an issue for dairy manufacturers and in recent years, new (bio) preservation technologies are being developed such as the use of bioprotective cultures. This review summarizes our current knowledge on the diversity of spoilage fungi in dairy products and the traditional and (potentially) new hurdle technologies to control their occurrence in dairy foods.

**Keywords:** spoilage; fungi; dairy products; control; diversity

## 1. Introduction

Since people began producing and storing food products, spoilage and food losses and waste became important issues for human with regards to food safety and security. Nowadays, up to one third of all food is spoiled or squandered before consumption, which represents about 1.3 billion tons per year [1]. These losses are the results of one or more problems occurring in the supply chain, from initial agricultural production down to the consumer level [1]. Concerning food spoilage, a food product can be physically, chemically, or microbiologically spoiled. Parasites, bacteria, and/or fungi are the main agents causing microbial spoilage. Certain parasites and bacteria are of public health concern because they are common contaminants of foods and are often responsible for food outbreaks. Nonetheless, being ubiquitous in nature, fungi are also very common in food. For a long time, besides altering food properties, they were not regarded as particularly detrimental to human health, although *Claviceps purpurea* has been related to ergotism outbreaks in the Middle Ages more than 200 years ago [2]. It is only in recent times that several mycotoxins produced by certain fungal species have been regarded as a major threat to human and animal health, especially in developing countries, being responsible for different adverse health effects [3].

Yeasts and molds are able to grow in a large variety of food including raw materials such as cereals, vegetables, fruits, meat, and milk, as well as processed products [4]. Given this large variety,

fungus spoilage leads to severe economic losses for food manufacturers. Indeed, it is estimated that between 5% and 10% of the world's food production is lost due to fungal deterioration [2]. Dairy products are less susceptible to fungal spoilage than other products, such as fruits or vegetables, because they are usually kept at refrigerated temperatures, are often made with heat-treated milk, and some of them are fermented products meaning that they may possess a competitive microbiota, have an acidic pH, and naturally contain organic acids. Nonetheless, a significant number of fungal species are able to survive and grow in dairy products. This astonishing adaptation capacity may be explained by the ability of fungi to utilize numerous substrates including carbohydrates, organic acids, proteins, and lipids that are present in milk and its derived products [5]. Moreover, these fungi are acidotolerant, xerotolerant, and/or psychrotolerant, and to some extent can tolerate chemical preservatives, which are sometimes added to increase product shelf-life. It is worth mentioning that several fungal species such as *Debaryomyces hansenii*, *Candida catenulata*, *Galactomyces geotrichum*, *Kluyveromyces marxianus*, *Mucor lanceolatus*, *Penicillium roqueforti*, *Penicillium camemberti*, or *Saccharomyces cerevisiae* are deliberately added as technological adjunct cultures to manufacture dairy products including kefir-type products and many kinds of cheese varieties [6–8]. Concerning undesirable species, their presence in dairy products may result in several types of food spoilage, e.g., visible growth of the fungus at the product surface, and the production of metabolites causing off-odors and flavors, as well as visible changes in color and/or texture [9]. In addition to organoleptic properties' deterioration, spoilage molds such as *Penicillium* and *Aspergillus* spp. can also produce mycotoxins [4,5,10,11]. In milk and dairy products, aflatoxin M1 (AFM1), which is produced by certain *Aspergillus* species, is the only mycotoxin for which maximum levels (0.05 ppb in the European Union (EU)) have been established. The occurrence of AFM1 in milk results from the conversion by dairy animals, fed on aflatoxin B1 (AFB1)-contaminated feedstuffs, of AFB1 to AFM1, which then pass to their urine and milk. Other mycotoxins such as ochratoxin A, citrinin, roquefortin C, mycophenolic, and cyclopiazonic acids have also been detected in cheeses at various concentrations [10]. Except for AFM1, intake of mycotoxins from dairy products is generally considered of limited importance compared to cereals and their derived products, and no human case of food poisoning related to mycotoxins has been documented so far.

Control of fungal spoilage is a major concern for industrials and scientists that are looking for efficient solutions to prevent and/or limit fungal growth or development in dairy products. Different traditional methods, also called traditional hurdle technologies, are implemented to control such contaminations including air treatment, cleaning and disinfection procedures, heat treatment, water activity reduction by brining, refrigeration, modified atmosphere packaging [12], as well as the use of chemical preservatives which are considered as food additives. The spoilage frequency and rate of many dairy foods can be reduced or retarded by the application of one or more of these treatments. However, fungal spoilage is still an issue for dairy manufacturers. Indeed, increasing fungal resistance toward heat treatments or chemical preservatives [13,14] and consumers' demand for more "natural" products, as well as legislation evolution have led industrial dairy producers to find complementary control approaches. This situation has led to the development of new (bio) preservation technologies such as the use of bioprotective cultures [15].

This review summarizes our current knowledge on spoilage fungi in dairy products with a special emphasis on their diversity, as well as the traditional and (potentially) new hurdle technologies to control their occurrence in dairy products.

## 2. Diversity of Spoilage Fungi in Dairy Products

With the use of multilocus sequencing of DNA regions with taxonomical interest, fungal taxonomy has undergone important changes during the last fifteen years. Many species have been reclassified and new phylogenetic species are also being regularly recognized within so-called "species complexes," in which members only harbor few differences in their morphological characters or are even morphologically undistinguishable from each other. For fungal identification, the internal transcribed spacer region (ITS) has been chosen as the best universal barcode [16]. Concerning yeast

identification, the D1/D2 domain of the 28 S rRNA gene is also widely used. It is worth mentioning that these genes are not always sufficient to identify isolates at the species level, and sequencing of other genes can be required. This is especially true for members of the *Aspergillus*, *Cladosporium*, *Penicillium*, and *Phoma* genera.

Over the years, the microbial diversity of milk and fermented dairy products has received considerable attention, and the development of next-generation sequencing (NGS) technologies [17–22] offers novel and rapid methods to characterize food ecosystems. However, except for raw milk, these techniques are not widely used to investigate fungal spoilage of dairy products because spoilage is generally the consequence of the outgrowth of a few species at the same time and because contamination can also occur on a distinct spot of the product surface. In addition, because read length is still limited with the currently used NGS technologies and because the taxonomic resolution of a single barcode marker can vary among taxa, it can be very tricky to identify operational taxonomic units (OTUs) at the species level. Nevertheless, these techniques offer new possibilities to investigate the potential sources of fungi in the dairy environment, which constitutes an important source of spoilage microorganisms.

### 2.1. Sources of Fungal Contamination

Fungal contamination of dairy foods can occur at different stages, from dairy farms to dairy processing units and at consumers' homes. Independent of the animal species, raw milk generally contains between 3 to 5 log<sub>10</sub> CFU mL<sup>-1</sup> fungi with higher number of yeast cells than fungal spores [23,24]. As shown by a recent study [25], the stable and milking parlor environments at the farm are important sources of fungi in the milk. In addition, an important yeast source is the teat surface [25]. It should be mentioned that yeast growth during milk storage is rare, as yeasts are rapidly outnumbered by psychrotrophic bacteria such as *Pseudomonas* spp. [26,27]. Moreover, except for a few fungal species, yeast and molds are not heat-resistant and should be killed after pasteurization. Therefore, during manufacturing, fungal contamination generally occurs after milk heat-treatment. Mold spoilage is often due to airborne fungi because fungal spores are easily dispersed into the dairy plant air [28]. In a recent study undertaken in a Greek dairy plant, fungal counts of 362.3 CFU/m<sup>3</sup> consisting mainly of mold spores were reported in outdoor air samples while 69.8 CFU/m<sup>3</sup> and 266.2 CFU/m<sup>3</sup> were found in samples from two indoor locations [29]. Recent studies [28–33] investigated the fungal diversity of the dairy environment in different dairy plants, and mold contaminations were also shown to originate from the air. In the case of sliced cheese, the cheese rind itself can also be a source of spoilage fungi which are transferred during cheese slicing to the cheese slice surface and will grow during storage at the retailer or at the consumer's home [30]. In contrast, yeast causing spoilage generally originates from brine, surface, equipment, or ingredient contaminations [32,34–36], but can also be detected in the air [29]. The brine used for cheese salting/curing is one of the most significant sources of fungi. In one study [32], counts of 1.10<sup>9</sup> CFU/cm<sup>2</sup> were reported in a brining tank. Moreover, ingredients such as fruit preparations can also be important contamination sources for both yeast and molds [4,37]. In addition, certain fruit preparations such as blueberries and strawberries are even more at risk as they do not support extensive heat-treatment [38]. It is also worth mentioning that 50–100% fruit preparations including lemon cells, strawberries, and blueberries were recently reported to contain heat-resistant ascospore-forming fungi [39]. Finally, packaging materials may also be a source of spoilage molds, despite the fact that it has not been extensively studied.

Although there has been limited research on the subject, fungal contaminations are also likely to occur at the consumer's home after product opening. In a recent study on 586 surface samples collected from ~293 refrigerators in Italy [40], 15% and 5% of total samples contained fungal populations >1 log<sub>10</sub> CFU cm<sup>-2</sup> and >2.5 log<sub>10</sub> CFU cm<sup>-2</sup>, respectively. Besides refrigerator surfaces, cross-contamination from one product to another can also be responsible for fungal contamination. For example, mold- and smear-ripened cheeses as well as vegetables and fruits are potential yeast and mold contamination sources. Finally, the indoor air at a consumer's home is also a potent source of spoilage molds.



## 2.2. Spoilage Yeasts

Dairy food spoilage caused by yeast can result in visible alterations mainly due to their outgrowth on the product surface, such as the “toad skin” defect caused by *G. geotrichum* or browning reaction caused by *Yarrowia lipolytica*. The latter defect is due to the extracellular accumulation of homogentisic acid, an intermediate of tyrosine catabolism, capable of auto-oxidization and polymerization, leading to the formation of pyomelanin, a brown pigment [41,42]. Yeast spoilage may also lead to detectable but non-visible alterations resulting in off-odor and -flavor or texture alterations through the production of ethanol, CO<sub>2</sub>, and volatile organic compounds (primary and secondary alcohols, aldehydes, esters) as well as the production of lipolytic and proteolytic enzymes (glycolysis). One should keep in mind that the extent of spoilage depends on two parameters: the minimal spoilage level and the chemical spoilage index, which represent the concentrations of specific spoilage organism(s) and that of spoilage metabolites determined at the time of sensory rejection, respectively [43]. In relation to yeast spoilage in dairy products, little data are available on these values. It was defined that yeasty and fermented off-flavors were detected when yeasts grew at populations equal or above 10<sup>5</sup>-10<sup>6</sup> CFU/g [9].

It is also worth mentioning that certain yeasts species are able to produce biogenic amines (BA) including histamine and tyramine [44–46]. For example, *Y. lipolytica* was responsible for histidine, lysine, ornithine, phenylalanine, and tyrosine decarboxylation, leading to the production of putrescine, 2-phenylethylamine, tyramine, and cadaverine, respectively, in a traditional Italian cheese [44]. However, the main BA producers in dairy products are bacteria including lactic acid bacteria (LAB) and enterobacteria, particularly with the formation of histamine and tyramine [47]. Interestingly, it has already been shown that *G. geotrichum* was able to slightly degrade BA such as tyramine [48].

A significant number of studies have been performed to assess spoilage yeast diversity (Table 1). These contaminations can be due to one or several species. For example, a recent study showed that up to 14 different yeast species could be encountered in as low as six fresh cheese samples, including cases of multi-contaminations [49]. Until now, more than 60 yeast species have been identified as spoilage agents of dairy products (Table 1). They belong to the Ascomycota and Basidiomycota phylum represented by 20 and 10 genera, respectively. Among Ascomycota, all identified species belong to the Saccharomycotina subdivision and Saccharomycetes class, which is the only class of this subdivision. Among this class, the genus *Candida* is the most frequently reported, representing half of the Ascomycota diversity, and is also characterized by a high diversity at the species level. Indeed, 24 different *Candida* species have already been reported as responsible for dairy product spoilage. *Candida parapsilosis* is the most frequently isolated species, followed by *Candida lusitanae*, *Candida inconspicua*, and *Candida intermedia*. All these species can thus be considered common contaminants of dairy products. Table 1 also shows that dairy products spoiled by *Candida* are principally unripened products and hard or semi-hard cheeses such as Scamorza or Caciotta.

Besides *Candida* spp., *Debaryomyces*, *Kluyveromyces*, *Yarrowia*, *Galactomyces*, and *Saccharomyces* spp. are also frequent spoilers of fresh dairy products (fresh cheese, cream, and yoghurt) and heat-treated products (Table 1). The presence of spoilage yeasts in the latter products is principally due to post-heat-treatment contaminations, but certain yeast species show high resistance to pasteurization [50] (see Section 3.2.1). Contrary to the genus *Candida*, one or two species from each genus are considered dairy spoilage agents. Indeed, the *Kluyveromyces* genus is represented by *Kluyveromyces marxianus* and *Kluyveromyces lactis*. *Debaryomyces*, *Galactomyces*, *Saccharomyces*, and *Yarrowia* genera are represented by *D. hansenii*, *G. geotrichum*, *S. cerevisiae*, and *Y. lipolytica*, respectively. Other Ascomycota yeasts responsible for spoilage include *Meyerozyma* (formerly *Pichia*) and *Pichia*, *Geotrichum*, *Dekkera*, *Torulaspota*, *Wickerhamomyces*, *Blastodendron*, *Cyberlindnera*, *Kazachstania*, *Peterozyma*, *Priceomyces*, and *Torulopsis* spp., principally isolated from soft and unripened dairy products such as fresh cheese or yoghurt.

Among Basidiomycota, six genera and four genera belonging to the Agaromycotina and Pucciniomycotina subdivisions are regularly encountered in spoiled dairy products. Among Pucciniomycotina, the genus *Rhodotorula* (Microbotryomycetes class), represented by three different

species, i.e., *Rhodotorula diffluens*, *Rhodotorula glutinis*, and *Rhodotorula mucilaginosa*, is the most frequently cited and is essentially isolated from unripened products (Table1). The latter genus is followed by *Cryptococcus* (Tremellomycetes class), represented by four species, i.e., *Cryptococcus humicola*, *Cryptococcus laurentii*, *Cryptococcus pseudolongus*, and *Cryptococcus minuta* isolated from unripened products (fresh cheese and butter/margarine) and soft cheese (Table1). Other *Basidiomycota* genera include *Saitozyma*, *Naganishia*, *Cutaneotrichosporon*, *Sporobolomyces*, *Sporodiobolus*, *Trichosporon*, and *Vanjira* spp.

**Table 1.** Diversity of spoilage yeasts in dairy products.

Phylum	Species	Product Types	References
Ascomycota	<i>Atelosaccharomyces pseudotropicalis</i>	Yoghurt	[26]
	<i>Blastodendron arztii</i>	Fresh unripened cheese	[51]
	<i>Candida acidothermophilum</i>	Yoghurt	[26,36,52]
	<i>Candida apis</i>	Hard or semi-hard cheese	[53]
	<i>Candida beverwijckiae</i>	Hard or semi-hard cheese	[51]
		Yoghurt	[26]
	<i>Candida blankii</i>	Heat-treated milk and dairy product	[53]
	<i>Candida glabrata</i>	Soft and semi-soft cheese	[51]
	<i>Candida glabrosa</i>	Hard or semi-hard cheese	[54]
	<i>Candida inconspicua</i>	Buffalo, goat, or sheep cheese	[51]
		Fresh unripened cheese	[49,51]
		Soft and semi-soft cheese	[51]
	<i>Candida intermedia</i>	Hard or semi-hard cheese	[2,54]
		Other dairy products	[49]
		Yoghurt	[26]
	<i>Candida krusei</i>	Soft and semi-soft cheese	[26]
	<i>Candida lusitaniae</i>	Buffalo, goat, or sheep cheese	[51]
		Fresh unripened cheese	[51]
		Soft and semi-soft cheese	[51]
		Yoghurt	[26,52]
	<i>Candida multigemmis</i>	Hard or semi-hard cheese	[2,54]
	<i>Candida mycoderma</i>	Buffalo, goat, or sheep cheese	[51]
	<i>Candida norvegica</i>	Soft and semi-soft cheese	[11]
	<i>Candida parapsilosis</i>	Fresh unripened cheese	[51]
		Hard or semi-hard cheese	[2,51,54]
		Soft and semi-soft cheese	[11,49,51]
		Yoghurt	[2,49]
	<i>Candida pseudoglabrosa</i>	Raw milk	[27]
	<i>Candida rugosa</i>	Soft and semi-soft cheese	[11]
	<i>Candida saitoana</i>	Yoghurt	[26]
	<i>Candida sake</i>	Fresh unripened cheese	[55]
		Hard or semi-hard cheese	[54]
	<i>Candida sphaerica</i>	Fresh unripened cheese	[26]
	<i>Candida stellata</i>	Hard or semi-hard cheese	[53]
		Yoghurt	[26,53]
	<i>Candida versatilis</i>	Yoghurt	[26,36]
	<i>Candida zeylanoides</i>	Fresh unripened cheese	[51]
		Hard or semi-hard cheese	[51]
		Soft and semi-soft cheese	[51]
	<i>Candida</i> sp.	Mold ripened cheese	[26]
	<i>Cyberlindnera jadinii</i>	Soft and semi-soft cheese	[11]
	<i>Debaryomyces hansenii</i>	Blue-veined cheese	[26]
	Buffalo, goat, or sheep cheese	[51]	
	Fresh unripened cheese	[51,53,55]	
	Hard or semi-hard cheese	[26,49,51]	
	Heat-treated milk and dairy product	[26,52,53]	
	Other dairy products	[52,53]	
	Soft and semi-soft cheese	[11,51]	
	Mold ripened cheese	[26]	
	Yoghurt	[2,26,36,52,53]	
<i>Dekkera anomala</i>	Fresh unripened cheese	[55]	

Table 1. Cont.

Phylum	Species	Product Types	References
		Yoghurt	[26]
	<i>Exophiala</i> sp.	Fresh unripened cheese	[49]
	<i>Galactomyces geotrichum</i>	Butter and margarine	[2]
		Fresh unripened cheese	[2,49,55]
		Heat-treated milk and dairy product	[2]
		Other dairy products	[49]
	<i>Geotrichum capitatum</i>	Soft and semi-soft cheese	[51]
	<i>Geotrichum klebahnii</i>	Hard or semi-hard cheese	[51]
	<i>Geotrichum</i> sp.	Buffalo, goat, or sheep cheese	[56]
		Raw milk	[57]
	<i>Kazachstania unispora</i>	Soft and semi-soft cheese	[11]
	<i>Kluyveromyces lactis</i>	Fresh unripened cheese	[49,55]
	<i>Kluyveromyces marxianus</i>	Blue-veined cheese	[26]
		Fresh unripened cheese	[26,53]
		Hard or semi-hard cheese	[26,53,54]
		Heat-treated milk and dairy product	[26,53]
		Mold ripened cheese	[26]
		Other dairy products	[52]
		Raw milk	[27]
		Soft and semi-soft cheese	[49]
		Yoghurt	[2,26,36,53]
	<i>Meyerozyma guilliermondii</i>	Buffalo, goat, or sheep cheese	[51]
		Fresh unripened cheese	[49]
		Soft and semi-soft cheese	[11,51]
		Yoghurt	[49]
	<i>Naumovia dairenensis</i>	Soft and semi-soft cheese	[11]
	<i>Peterozyma toletana</i>	Yoghurt	[36]
	<i>Pichia fermentans</i>	Soft and semi-soft cheese	[11,49]
	<i>Pichia membranifaciens</i>	Fresh unripened cheese	[26]
		Soft and semi-soft cheese	[11]
	<i>Pichia norvegensis</i>	Soft and semi-soft cheese	[11]
	<i>Priceomyces carsonii</i>	Hard or semi-hard cheese	[54]
	<i>Saccharomyces cerevisiae</i>	Blue-veined cheese	[26]
		Buffalo, goat, or sheep cheese	[51]
		Fresh unripened cheese	[51,53]
		Hard or semi-hard cheese	[53]
		Heat-treated milk and dairy product	[26]
		Other dairy products	[52]
		Soft and semi-soft cheese	[11,51]
		Yoghurt	[26,36,53]
	<i>Torulaspora delbrueckii</i>	Fresh unripened cheese	[51]
		Soft and semi-soft cheese	[11]
	<i>Torulopsis</i> sp.	Yoghurt	[26]
	<i>Wickerhomomyces anomalus</i>	Soft and semi-soft cheese	[11]
		Yoghurt	[2]
	<i>Yarrowia lipolytica</i>	Blue-veined cheese	[26]
		Buffalo, goat, or sheep cheese	[51]
		Butter and margarine	[53]
		Fresh unripened cheese	[26,55]
		Hard or semi-hard cheese	[2,49,51,54]
		Mold ripened cheese	[2,26]
		Soft and semi-soft cheese	[11,49]
		Yoghurt	[2]
Basidiomycota	<i>Cryptococcus humicola</i>	Butter and margarine	[2]
		Hard or semi-hard cheese	[2]
	<i>Cryptococcus laurentii</i>	Butter and margarine	[53]
		Fresh unripened cheese	[26,53]
	<i>Cryptococcus pseudolongus</i>	Fresh unripened cheese	[49]
	<i>Cryptococcus</i> sp.	Heat-treated milk and dairy products	[52]
		Soft and semi-soft cheese	[11]
	<i>Cystobasidium minuta</i>	Soft and semi-soft cheese	[11]
	<i>Cutaneotrichosporon curvatus</i>	Raw milk	[26]
	<i>Cutaneotrichosporon cutaneum</i>	Hard or semi-hard cheese	[51]
		Fresh unripened cheese	[51]

Table 1. Cont.

Phylum	Species	Product Types	References
	<i>Naganishia albida</i>	Butter and margarine	[53]
		Fresh unripened cheese	[53]
		Other dairy products	[53]
		Yoghurt	[53]
	<i>Rhodotorula diffluens</i>	Butter and margarine	[53]
		Fresh unripened cheese	[53]
		Hard or semi-hard cheese	[26,53]
		Heat-treated milk and dairy products	[26,52,53]
		Yoghurt	[2,26,53]
	<i>Rhodotorula glutinis</i>	Butter and margarine	[53]
		Fresh unripened cheese	[53]
		Other dairy products	[52,53]
		Soft and semi-soft cheese	[11]
	<i>Rhodotorula mucilaginoso</i>	Butter and margarine	[53]
		Fresh unripened cheese	[51,53]
		Other dairy products	[52,53]
		Soft and semi-soft cheese	[49]
		Yoghurt	[2,36]
	<i>Rhodotorula sp.</i>	Heat-treated milk and dairy products	[26]
	<i>Saitozyma flava</i>	Fresh unripened cheese	[53]
		Hard or semi-hard cheese	[53]
		Heat-treated milk and dairy products	[26,53]
		Other dairy products	[53]
	<i>Sporobolomyces roseus</i>	Fresh unripened cheese	[26]
	<i>Sporodiobolus salmonicolor</i>	Hard or semi-hard cheese	[49]
	<i>Trichosporon asahii</i>	Soft and semi-soft cheese	[49]
	<i>Vanrija humicola</i>	Fresh unripened cheese	[51]

### 2.3. Molds in Dairy Products

Dairy product spoilage by molds also consists in visible alterations due to the outgrowth of a thallus on the product surface. In vacuum-packed cheeses such as cheddar cheese, another defect called thread mold defect is sometimes encountered and is characterized by mold growth in folds and wrinkles of the plastic film in which the cheese is packaged [54]. Mold spoilage can also lead to the formation of off-flavors. For example, *Moniliella suaveolens* and *Cladosporium herbarum* were shown to produce ketones causing rancidity defect in butter while *Penicillium glabrum*, *Penicillium expansum*, *Penicillium chrysogenum*, and *Cladosporium cladosporioides* produced off-flavors including 2-methylisoborneol and geosmin which possess strong musty and earthy notes [2]. Interestingly, a study [58] showed that certain fungal species were able to metabolize sorbate salts (used as preservatives) to produce metabolites such as *trans*-1,3-pentadiene or *trans*-piperylene, resulting in the formation of plastic-like or “kerosene” off-flavors.

Molds responsible for dairy product spoilage are highly diverse at the genus and species levels (Table 2). Indeed, up to 100 mold species have been identified so far as being responsible for dairy product spoilage. Most species belong to the Ascomycota and Mucoromycota phyla (Table 2). Both Ascomycota and Mucoromycota have been principally isolated from hard or semi-hard cheeses.

Among Ascomycota, all identified spoilage molds (88 species distributed in 26 genera) belong to the Pezizomycotina subdivision, with the exception of *Lecanicillium lecanii* [59]. Within the Pezizomycotina subdivision, the Eurotiomycetes class is the most represented (nine genera and 57 species), followed by the Dothideomycetes (10 genera and 20 species), the Sordariomycetes (11 species of six genera) and the Leotiomycetes (one species). Among Eurotiomycetes, *Penicillium* is by far the most frequently reported spoilage genus with ~40 species, followed by *Aspergillus* (10 species). Other genera include *Byssoschlamys*, *Eupenicillium*, *Eurotium*, *Exophiala*, *Hamigera*, *Neosartorya*, *Rhinochlaidiella*, and *Talaromyces* spp.

*Penicillium* spp. are mainly isolated from hard and semi-hard cheeses but are also found in all other product types including fresh, blue-veined, mold-ripened, soft- and semi-soft cheeses, butter, yoghurt, milk as well as in buffalo, goat, or ewe's milk cheeses. Based on the classification system recently proposed by Houbraken and Samson [60], *Penicillium* spp. responsible for spoilage mainly belong to the Fasciculata section (11 species) but also to the Chrysogena (three species), Biverticillium (four species), Brevicompacta (three species) and Aspergilloides (two species) sections. Among the Fasciculata section, *Penicillium commune*, *Penicillium solitum*, *Penicillium crustosum*, and *Penicillium verrucosum* are frequently reported, while within the Chrysogena section, *P. chrysogenum*, *Penicillium nalgiovense*, and *Penicillium griseofulvum* are quite common. Within Brevicompacta, *Penicillium brevicompactum* is frequently reported, in addition to *Penicillium bialowiezense* [49] and *Penicillium spathulatum* [61]. Nonetheless, Samson et al. [62] underlined that *P. bialowiezense* could have been misidentified in previous studies with the closely related species *P. brevicompactum*. The *Penicillium* species mentioned above are adaptive to low temperatures, and several of them are considered xerophilic species [2], therefore, their occurrence in spoiled dairy products is not surprising. *Penicillium* spp. is also well-adapted to the cheese environment and has been associated with cheese manufacturing for centuries. In addition, a recent study highlighted the presence of not previously mentioned xerophilic species such as *Penicillium charlesii*, *Penicillium fellutanum*, *Penicillium adametzioides*, and *Penicillium antarcticum* [49].

Fungi from the Dothideomycetes class are principally isolated from hard and semi-hard cheeses and constitute the second most frequently identified class after Eurotiomycetes. Among this class, *Cladosporium*, with seven species distributed in three different *Cladosporium* complexes, is the most frequently reported. *Cladosporium* spp. grow slowly but are very common airborne fungi and are quite psychrotolerant and xerotolerant [2].

Among the Mucoromycotina sub-phylum, three different genera have already been isolated from contaminated dairy products, i.e., *Mucor* (four species), *Rhizopus* (one species), and *Thamnidium* (one species), all belonging to the Mucorales order. As for other genera belonging to the Ascomycota phylum, these genera were mostly isolated from spoiled cheeses [56,62,63].

Molds can also contaminate heat-treated dairy products. Indeed, species belonging to *Aspergillus*, *Byssochlamys*, *Cladosporium*, *Eupenicillium*, *Fusarium*, *Hamigera*, *Neosartorya*, *Penicillium*, and *Talaromyces* genera have been isolated from pasteurized milk, cream cheese, and heat-treated dairy beverages [2,49,57]. The presence of mold in heat-treated milk or dairy products might be due to post-processing contaminations during bottling or packaging [57] or to the heat-resistance of mold spores. Indeed, Pitt and Hocking (2009) isolated heat-resistant species such as *Byssochlamys nivea* (anamorph *Paecilomyces niveus*), *Eupenicillium brefeldianum*, *Hamigera avellanea*, and *Neosartorya fischeri* (anamorph *Aspergillus fischeri*) from cream cheese, as well as *B. nivea*, *N. fischeri*, and *Talaromyces macrosporus* from heat-treated dairy beverage [2]. In a recent study, it was shown that the dormancy of *Talaromyces* and *Neosartorya* spp. ascospores was broken during thermal processing, for which heat-treatment was not optimized for ascospore inactivation [64].

In addition, fungal spoilage of dairy products obtained by Ultra-High Temperature (UHT) treatment can also occur, but it is generally due to post-processing contaminations. For example, *Fusarium oxysporum* was isolated on several occasions from UHT-flavored milk beverages [2].

**Table 2.** Diversity of spoilage filamentous fungi isolated from contaminated dairy products.

Phylum	Genera	Species	Product Types	References		
Ascomycota	<i>Acremonium</i>	Nd *	Buffalo, goat, or sheep cheese	[56]		
		<i>Alternaria</i>	<i>Alternaria alternata</i>	Blue-veined cheese Hard or semi-hard cheese	[63] [54,65]	
	<i>Aspergillus</i> section <i>Aspergillus</i>	Nd *	<i>Aspergillus glaucus</i>	Hard or semi-hard cheese	[66]	
				Raw milk	[25]	
		<i>Aspergillus section Candidi</i>	<i>Aspergillus candidus</i>	Heat-treated milk	[57]	
				Raw milk	[57]	
		<i>Aspergillus section Circumdati</i>	<i>Aspergillus ochraceus</i>	Buffalo, goat, or sheep cheese	[56]	
				Buffalo, goat, or sheep cheese	[56]	
		<i>Aspergillus section Flavi</i>	<i>Aspergillus flavus</i>	Hard or semi-hard cheese	[67]	
				Heat-treated milk	[57]	
		<i>Aspergillus section Fumigati</i>	<i>Aspergillus tamaritii</i>	<i>Aspergillus fumigatus</i>	Raw milk	[57]
					Blue-veined cheese	[63]
	<i>Aspergillus section Nidulantes</i>		<i>Aspergillus sydowii</i> <i>Aspergillus versicolor</i>	Blue-veined cheese	[63]	
				Hard or semi-hard cheese	[59,68]	
	<i>Aspergillus section Nigri</i>	<i>Aspergillus niger</i>	Semi-soft cheese	[4,59]		
			Buffalo, goat, or sheep cheese	[56]		
	<i>Aspergillus</i>	Nd *	<i>Aspergillus niger</i>	Blue-veined cheese	[63,67]	
				Hard or semi-hard cheese	[54,66]	
	<i>Aureobasidium</i>	<i>Aureobasidium pullulans</i>	Raw milk	[25]		
			Hard or semi-hard cheese	[69]		
	<i>Bipolaris</i>	<i>Bipolaris australiensis</i>	Blue-veined cheese	[63]		
	<i>Botrytis</i>	<i>Botrytis cinerea</i>	Hard or semi-hard cheese	[54]		
	<i>Byssoschlamys</i>	<i>Byssoschlamys nivea</i>	Heat-treated milk	[2]		
	<i>Cladosporium cladosporioides</i> complex	<i>Cladosporium cladosporioides</i>	Blue-veined cheese	[63]		
			Buffalo, goat, or sheep cheese	[56]		
	<i>Cladosporium herbarum</i> complex	<i>Cladosporium phyllophilum</i> <i>Cladosporium butyri</i> <i>Cladosporium herbarum</i> <i>Cladosporium macrocarpum</i>	Butter and margarine	[2]		
			Hard or semi-hard cheese	[2,54,65]		
			Buffalo, goat, or sheep cheese	[56]		
			Butter and margarine	[49]		
	<i>Cladosporium sphaerospermum</i> complex	<i>Cladosporium halotolerans</i> <i>Cladosporium sphaerospermum</i>	Butter and margarine	[2]		
			Hard or semi-hard cheese	[2,54]		
			Hard or semi-hard cheese	[54]		
	<i>Cladosporium</i>	Nd *	<i>Cladosporium halotolerans</i>	Heat-treated milk	[49]	
			<i>Cladosporium sphaerospermum</i>	Hard or semi-hard cheese	[49,54]	
	<i>Didymella</i>	<i>Didymella pinodella</i>	Hard or semi-hard cheese	[59,66]		
			Semi-soft cheese	[59]		
	<i>Epicoccum</i>	<i>Epicoccum nigrum</i>	Fresh unripened cheese	[49]		
	<i>Eupenicillium</i>	<i>Eupenicillium brefeldianum</i>	Other dairy product	[49]		
	<i>Eurotium</i>	<i>Eurotium herbariorum</i>	Hard or semi-hard cheese	[54,69]		
			Heat-treated milk	[2]		
	<i>Exophiala</i>	Nd *	Hard or semi-hard cheese	[65]		
			Raw milk	[25,68]		
	<i>Fusarium</i>	<i>Fusarium avenaceum</i> <i>Fusarium oxysporum</i>	Cream cheese	[49]		
			Buffalo, goat, or sheep cheese	[56]		
	<i>Fusarium</i>	<i>Fusarium solani</i>	Hard or semi-hard cheese	[54,59]		
Semi-soft cheese			[59]			
Heat-treated milk			[2]			
<i>Fusarium verticillioides</i>		Nd *	Hard or semi-hard cheese	[59]		
			Hard or semi-hard cheese	[59]		
			Hard or semi-hard cheese	[67]		
<i>Gliocladium</i>	<i>Gliocladium roseum</i> <i>Hamigera avellanea</i>	Hard or semi-hard cheese	[66]			
		Heat-treated milk	[57]			
<i>Hamigera</i>	<i>Hamigera avellanea</i>	Raw milk	[57]			
<i>Lecanicillium</i>	<i>Lecanicillium lecanii</i>	Hard or semi-hard cheese	[54]			
		Heat-treated milk	[2]			
<i>Naumovia</i>	<i>Naumovia dairenensis</i> <i>Neosartorya fischeri</i>	Hard or semi-hard cheese	[59]			
		Semi-soft cheese	[59]			
<i>Neosartorya</i>	<i>Neosartorya fischeri</i>	Soft cheese	[11]			
		Heat-treated milk	[2]			
<i>Penicillium</i> section <i>Aspergilloides</i>	<i>Penicillium glabrum</i>	Butter and margarine	[2]			
		Hard or semi-hard cheese	[2,54,70]			
<i>Aspergilloides</i>	<i>Aspergilloides</i>	Fresh unripened cheese	[2]			
		Yoghurt	[2]			

Table 2. Cont.

Phylum	Genera	Species	Product Types	References
		<i>Penicillium spinulosum</i>	Hard or semi-hard cheese	[65]
	<i>Penicillium</i> section	<i>Penicillium funiculosum</i>	Buffalo, goat, or sheep cheese	[56]
	<i>Bioverticillium</i>	<i>Penicillium minioluteum</i>	Hard or semi-hard cheese	[69]
		<i>Penicillium purpureogenum</i>	Hard or semi-hard cheese	[68]
		<i>Penicillium rugulosum</i>	Hard or semi-hard cheese	[54,59]
			Semi-soft cheese	[59]
	<i>Penicillium</i> section	<i>Penicillium bialowiezense</i>	Hard or semi-hard cheese	[49]
	<i>Brevicompecta</i>		Yoghurt	[49]
		<i>Penicillium brevicompactum</i>	Hard or semi-hard cheese	[2,49,54,59,65,69,70]
			Semi-soft cheese	[59]
			Buffalo, goat, or sheep cheese	[56]
	<i>Penicillium</i> section <i>Sclerotiora</i>	<i>Penicillium adametzoides</i>	Fresh unripened cheese	[49]
	<i>Penicillium</i> section	<i>Penicillium antarcticum</i>	Hard or semi-hard cheese	[49]
	<i>Canescentia</i>	<i>Penicillium arenicola</i>	Buffalo, goat, or sheep cheese	[56]
		<i>Penicillium canescens</i>	Hard or semi-hard cheese	[68]
		<i>Penicillium novae-zeelandiae</i>	Hard or semi-hard cheese	[54]
	<i>Penicillium</i> section <i>Charlesii</i>	<i>Penicillium charlesii</i>	Fresh unripened cheese	[49]
			Heat-treated milk	[49]
		<i>Penicillium dierckxii</i>	Fresh unripened cheese	[49]
	<i>Penicillium</i> section <i>Chrysogena</i>	<i>Penicillium chrysogenum</i>	Butter and margarine	[2]
			Blue-veined cheese	[63]
			Hard or semi-hard cheese	[54,59,62,65,69,70]
			Semi-soft cheese	[59]
			Buffalo, goat, or sheep cheese	[56]
			Fresh unripened cheese	[2]
			Yoghurt	[2]
		<i>Penicillium griseofulvum</i>	Hard or semi-hard cheese	[68]
		<i>Penicillium naligovense</i>	Hard or semi-hard cheese	[4,49,59]
			Semi-soft cheese	[4,59]
			Buffalo, goat, or sheep cheese	[56]
	<i>Penicillium</i> section <i>Citrina</i>	<i>Penicillium steckii</i>	Hard or semi-hard cheese	[69]
	<i>Penicillium</i> section <i>Exilicaulis</i>	<i>Penicillium corylophitum</i>	Hard or semi-hard cheese	[65]
	<i>Penicillium</i> section <i>Fasciculata</i>	<i>Penicillium aurantiogriseum</i>	Hard or semi-hard cheese	[65,69]
			Buffalo, goat, or sheep cheese	[56]
			Yoghurt	[2]
		<i>Penicillium camembertii</i>	Hard or semi-hard cheese	[68]
		<i>Penicillium commune</i>	Other dairy products	[49]
			Hard or semi-hard cheese	[2,4,49,54,59,65,69,70]
			Mold ripened cheese	[2]
			Semi-soft cheese	[4,59]
			Buffalo, goat, or sheep cheese	[56]
			Fresh unripened cheese	[2]
		<i>Penicillium crustosum</i>	Buffalo, goat, or sheep cheese	[56]
			Hard or semi-hard cheese	[54,59,62,65,69,70]
			Semi-soft cheese	[59]
			Buffalo, goat, or sheep cheese	[56]
		<i>Penicillium discolor</i>	Hard or semi-hard cheese	[49,59]
			Semi-soft cheese	[59]
		<i>Penicillium echinulatum</i>	Hard or semi-hard cheese	[59,65,68,69]
			Semi-soft cheese	[59]
		<i>Penicillium hirsutum</i>	Buffalo, goat, or sheep cheese	[56]
		<i>Penicillium nordicum</i>	Hard or semi-hard cheese	[49]
		<i>Penicillium palitans</i>	Fresh unripened cheese	[49]
			Hard or semi-hard cheese	[49,65,69]
		<i>Penicillium solitum</i>	Buffalo, goat, or sheep cheese	[56]
			Hard or semi-hard cheese	[2,49,54,59,65,69]
			Other dairy products	[49]
			Semi-soft cheese	[4,59]
			Yoghurt	[49]
		<i>Penicillium verrucosum</i>	Buffalo, goat, or sheep cheese	[56]
			Hard or semi-hard cheese	[54,59,68,69]
			Mold ripened cheese	[2]
			Semi-soft cheese	[4,59]
		<i>Penicillium viridicatum</i>	Hard or semi-hard cheese	[2,54,65,67–69]
	<i>Penicillium</i> section <i>Paradoxa</i>	<i>Penicillium atramentosum</i>	Blue-veined cheese	[63]
			Hard or semi-hard cheese	[59]
			Semi-soft cheese	[59]
	<i>Penicillium</i> section <i>Penicillium</i>	<i>Penicillium expansum</i>	Butter and margarine	[2]
			Hard or semi-hard cheese	[2,65,68,69]
	<i>Penicillium</i> section <i>Lanata-</i>	<i>Penicillium oxalicum</i>	Buffalo, goat, or sheep cheese	[56]
	<i>Divaricata</i>	<i>Penicillium simplicissimum</i>	Hard or semi-hard cheese	[57]
	<i>Penicillium</i> section	<i>Penicillium roquefortii</i>	Buffalo, goat, or sheep cheese	[56]
	<i>Roquefortum</i>		Hard or semi-hard cheese	[2,54,59,65,68,69]
			Mold ripened cheese	[2]
			Semi-soft cheese	[4,59]
	<i>Penicillium</i>	Nd *	Buffalo, goat, or sheep cheese	[56]

Table 2. Cont.

Phylum	Genera	Species	Product Types	References			
Basidiomycota Zygomycota			Hard or semi-hard cheese	[54,66]			
			Heat-treated milk	[57]			
			Raw milk	[25,57]			
			<i>Phaeramularia</i>	Nd *	Hard or semi-hard cheese	[54]	
			<i>Phoma</i>	<i>Phoma glomerata</i>	Hard or semi-hard cheese	[2,70]	
					<i>Phoma nebulosa</i>	Hard or semi-hard cheese	[69]
					Nd *	Buffalo, goat, or sheep cheese	[56]
					Hard or semi-hard cheese	[2,54,65,69]	
			<i>Rhinochadiella</i>	Nd *	Blue-veined cheese	[63]	
			<i>Sarocladium</i>	<i>Sarocladium strictum</i>	Hard or semi-hard cheese	[69]	
			<i>Scopulariopsis</i>	<i>Scopulariopsis brevicaulis</i>	Buffalo, goat, or sheep cheese	[56]	
					Hard or semi-hard cheese	[51,59]	
					Semi-soft cheese	[4,59]	
					Hard or semi-hard cheese	[54,67]	
			<i>Talaromyces</i>	<i>Talaromyces macrosporus</i>	Heat-treated milk	[2]	
			<i>Trichoderma</i>	<i>Trichoderma harzianum</i>	Hard or semi-hard cheese	[65]	
			<i>Ulocladium</i>	<i>Ulocladium chartarum</i>	Hard or semi-hard cheese	[69]	
			<i>Wallemia</i>	<i>Wallemia sebi</i>	Raw milk	[25]	
			<i>Mucor</i>	<i>Mucor circinelloides</i>	Hard or semi-hard cheese	[49,69]	
					Yoghurt	[2]	
				<i>Mucor hiemalis</i>	Buffalo, goat, or sheep cheese	[56]	
					Hard or semi-hard cheese	[2,65,70]	
					Yoghurt	[2]	
	<i>Mucor plumbeus</i>	Hard or semi-hard cheese	[65,69]				
	<i>Mucor racemosus</i>	Hard or semi-hard cheese	[49,59,65,68,69]				
		Semi-soft cheese	[59]				
		Yoghurt	[2]				
<i>Rhizopus</i>	<i>Rhizopus stolonifer</i>	Blue-veined cheese	[63]				
		Buffalo, goat, or sheep cheese	[56]				
<i>Thamnidium</i>	<i>Thamnidium elegans</i>	Hard or semi-hard cheese	[49]				

\* Nd: Not determined.

### 3. Prevention and Control of Fungal Spoilage in Dairy Products

Today, fungal spoilage continues to be a major challenge for industrials and, in modern practices, both preventive and control approaches are usually combined to reduce its incidence in dairy foods (Figure1). Preventive methods can be defined as methods which can avoid contamination or recontamination during product processing, including packaging in aseptic conditions, use of air filtration system, and good manufacturing practices such as good hygienic practices and Hazard Analysis and Critical Control Points (HACCP) system implementation [71]. Control methods involve the use of methods which will either slow down or inhibit microbial growth, such as the addition of chemical preservatives, use of modified atmosphere packaging, and low temperature storage, as well as methods which will inactivate fungi such as heat and high-pressure treatments [71].

When it comes to choosing the most appropriate preventive and control methods, different factors must be taken into account. One major factor is the characteristics of the product to be preserved (composition,  $a_w$ ) and those of the microorganisms of concern as well as the sanitation conditions along the manufacturing process and product storage conditions. Another important factor is also consumer perception and acceptance of food preservation methods as well as their impact on hygiene and safety and their associated cost [72,73]. That is probably the reason why, among emerging methods to combat fungal spoilage, the use of bioprotective cultures and fermentates to decrease the use of chemical preservatives is gaining more and more attention [74].



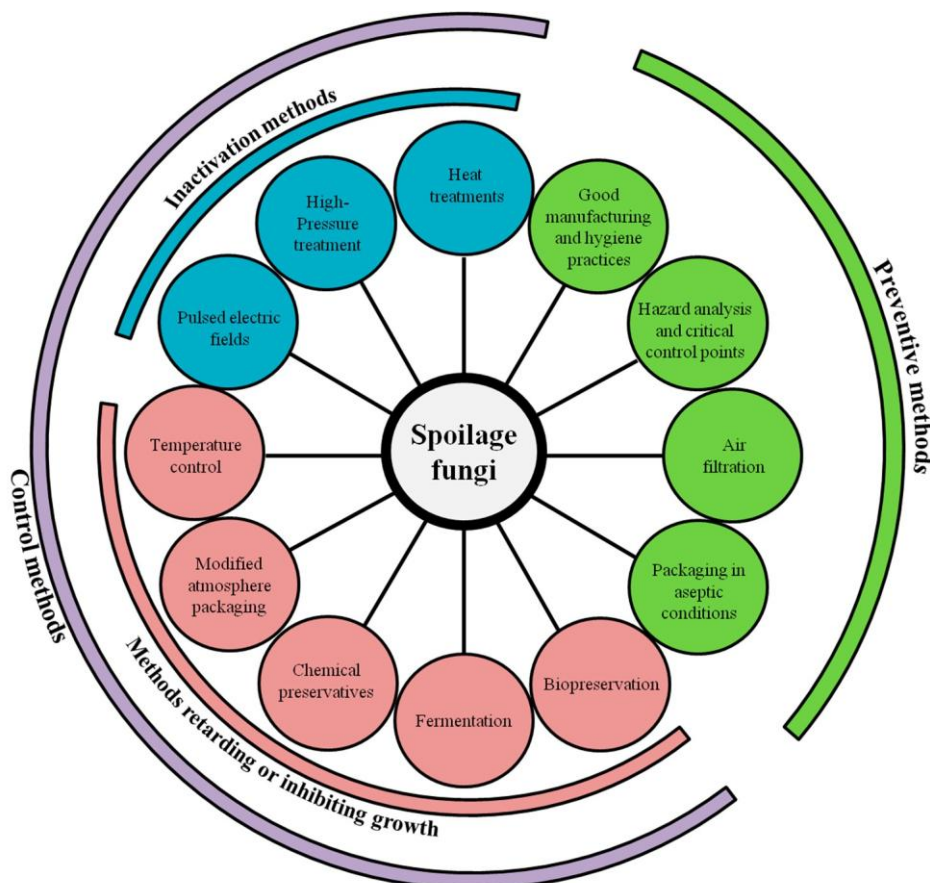


Figure 1. Preventive and control methods used in dairy products for preventing, inactivating, retarding, or inhibiting growth of spoilage fungi.

### 3.1. Preventive Methods

#### 3.1.1. Good Manufacturing and Distribution Practices

The success of any food preservation method depends on low initial levels of contamination, which in turn are achieved through the application of appropriate cleaning, sanitation, decontamination, and hygienic practices [73]. Dairy product manufacturing and packaging must also be done in the most hygienic and aseptic conditions possible to reduce the risk of biological contamination including that of fungal contamination [75,76]. In a code of good manufacturing and distribution practices (GMDPs), two main points are essential: (1) selecting good quality raw materials and monitoring the manufacturing process to control microbes while preventing cross-contamination; and (2) avoiding or retarding microbial growth [77,78]. GMDPs are an indispensable part of every food quality system. This code relies on the use of good hygiene practices and on the implemented hazard analysis critical control point (HACCP) systems from the production to the consumption of dairy products. In accordance with the *Codex Alimentarius* indications and Regulation 852/2004/CE, HACCP systems identify, evaluate, and control hazards that threaten food hygiene, in particular taking into account the microbiological risks [79,80]. Thus, the microbiological quality of dairy products is controlled at each step of the production process, from the raw materials to the finished product [81]. Prerequisite programs, such as Total Quality Management (TQM), a method including planning, organizing, and understanding each activity and involving each individual at each level, or Just-In-Time (JIT), a program designed to reduce waste by reducing flow times within the production system as well as response times from suppliers and to customers, are useful to improve product

quality [81,82]. These programs have significant costs and require important efforts, but will produce return in investment in the long term.

### 3.1.2. Air Filtration and Decontamination Systems

With specific regard to the fungal contamination risk, potential contamination sources include ambient air. Therefore, efficient air-filtration systems should be in place to reduce spore counts into the air where the product is vulnerable. For example, Beletstiotis et al. [29] showed that the operation of High Efficiency Particulate Air (HEPA) filters class 10.000, designed to withhold fungal spores, allowed a decrease in fungal loads by 30 times in the indoor air of a dairy plant. The direction of air flows and location of outlets should also be carefully examined in sensitive zones. Moreover, the control of air pressurization can be applied to prevent air flow from the dirtiest to cleanest areas. Cleanroom technologies (aseptic or ultraclean technologies) including minienvironments that protect equipment such as filling and packaging equipment, transport system tunnels, or protective enclosures can also be used.

For air decontamination, gaseous ozone was successfully applied for disinfecting empty cheese ripening and storage facilities with up to a 10-fold reduction in mold and yeast counts depending on the dose and treatment time [83,84], see for a review Varga and Szigeti, 2016 [85]. Finally, molds and yeasts are able to grow in humid zones (walls, ceiling, and floor) and on the equipment if these are not properly cleaned and disinfected.

Various disinfectants such as alcohol, peracetic acid, iodophors, aldehydes, quaternary amine compounds, chlorine-based agents, or hydrogen peroxide have been used in the dairy industry against fungal contaminations. Nonetheless, fungal response to such cleaning agents varies with disinfectant type and concentration. For example, Korukluoglu et al. [86] showed that *Aspergillus niger* was sensitive to alcohol but not to peracetic acid; moreover, *A. niger* was resistant to 0.5% iodophor but sensitive at 1%. In addition, Bundgaard-Nielsen and Nielsen (1995) demonstrated the existence of intraspecific variation in tolerance to quaternary ammonium compounds, especially in *P. commune* and *Penicillium nalgiovense* [87]. Finally, Martin et al. [88] revealed that the combination of hydrogen peroxide and formic acid had a synergistic action against *Scopulariopsis brevicaulis* and *G. geotrichum*, thus showing that a combination of different disinfectants is a possible solution to prevent fungal contaminations. More work is required to investigate the efficiency of disinfectant agents against fungi in the dairy product context, while taking into account the continuously evolving legislation on their use.

## 3.2. Control Methods

### 3.2.1. Inactivation Treatments

Concerning heat-treatments, thermization, pasteurization, and UHT sterilization are intended to partially or completely reduce milk microbial load while altering as little as possible milk organoleptic and nutritional properties [89]. During such heat-treatment, the product is subjected to a high temperature for a short period of time. In the EU, thermization, pasteurization, and UHT correspond to treatments at 57–68 °C for a minimum of 15 s, 71.7 °C for 15 s, and at least 135 °C for a few seconds, respectively [90]. According to Sakkas et al. [91], the efficacy of heat treatments are related to the temperature–time combinations, heating method utilized, and milk pre-treatment conditions (filtration, homogenization, standardization, etc.). Concerning milk thermization, the main objective is to reduce the population of spoilage microorganisms and to inactivate enzymes while minimizing heat damage to milk components. Milk pasteurization is much more efficient than thermization, but not all microorganisms can be destroyed and this treatment has to be followed by a rapid chilling (4 °C) to slow down subsequent microbial growth. Finally, UHT sterilization, used for milk and dairy drinks, is itself obtained by applying a continuous heat-flow for a short time-period which allows for the total destruction of microorganisms followed by milk aseptic packaging [87].

As mentioned earlier, certain fungal species, because of their considerable heat-resistance, are able to spoil heat-treated dairy products, in particular pasteurized fruit-based products. Heat-resistant species include *Byssoschlamys spectabilis*, *N. fischeri*, *H. avellanea*, and *Talaromyces bacillisporus* [92,93]. This feature is due to their heat-resistant ascospores which are activated when heat-treatments are not high enough to inactivate them. As an example, *N. fischeri* and *T. bacillisporus* were shown to possess decimal reduction times at 85 °C (D85) ranging from 15.0 to 30.1 min, and from 11.9 and 15.5 min, respectively [39,94]. *N. fischeri* and *T. bacillisporus* showed D85 ranging from 47 to 75 min in N-(2-Acetamido)-2-aminoethanesulfonic acid buffer as well as D90 ranging from 7.5 to 12.7 min and D95 ranging from 0.56 to 0.67 min in blueberry juice [95]. They showed D90 from 2.0 to 7.6 min and D95 from 1.7 to 2.3 min in juices. *N. fischeri* and *T. bacillisporus* showed D87 from 11.1 to 66.7 min, D90 from 4.7 to 13.5 min, and D95 from 0.43 to 1.52 min and ranged between 44.4 and 60.9 min at 82 °C and 2.7 and 4.1 min at 88 °C. As another example, *H. avellanea* ascospores are activated but not destroyed after heat-treatment for 20 min at 87 °C, 5 min at 90 °C, and 1 min at 95 °C, showing the importance of time-temperature conditions treatment [93].

Other techniques such as high-pressure (HP) treatments of dairy products can also be used to inactivate yeast cells and mold spores (among other microorganisms) and extend product shelf-life [95]. Mainville et al. [96] and Reys et al. [97] showed that yeasts from kefir were completely inactivated at 400 MPa for 15 min or 30 min without significant changes in protein and lipid structure of the product. Moreover, Evert-Arriagada et al. [98] recently demonstrated that HP processing under industrial conditions could extend the shelf-life of a commercial starter-free fresh cheese. Pressurized cheeses were firmer and more yellow than control cheeses, but these changes did not affect the panel preference for pressurized cheese. It is worth mentioning that there is an increasing number of HP-treated dairy products available on the market as this technology meets consumer's expectations for more "natural" and less-heavily processed food products.

Pulsed electric fields (PEF) treatment consists of pulsed electric fields of strong intensity (15 to 50 kV/cm) for only few seconds [99]; this represents another emerging technology for microbial inactivation, including that of yeast and molds, with potential application in fluid dairy foods (see the recent reviews of Buckow et al. [100] and Wan et al. [101]).

### 3.2.2. Temperature Control

Temperature control is critical for dairy food quality and shelf-life. Cold temperatures are used to minimize microbial growth in raw milk until processing and to extend the shelf-life of non-sterile dairy foods. Low temperatures, or even frozen storage, however, do not eliminate microorganisms [73]. Milk and dairy products are generally stored between 0 °C and 4 °C and at temperatures ranging from 4 to 10 °C at the consumer's home. However, most fungi are psychrotrophic and are thus able to grow at such temperatures. In a recent study, Gougouli et al. [102] evaluated the effect of temperature and inoculum size on the growth of one isolate each from 12 fungal species during yoghurt storage. The ability to grow at refrigerated temperature was species-dependent with estimated minimal growth temperatures of -6.7 and 9.6 °C for *P. commune* and *A. niger*, respectively. In addition, Gougouli et al. [102] also developed and validated a probabilistic model to predict the appearance of visible mycelia originating from single spores which could be used for choosing adequate conditions for challenge tests. It should be noted that besides storage temperature and the spoilage organism itself, fungal spoilage susceptibility depends on the spoiler initial population and the time required to form a visible thallus on the product surface, which can be predicted using predictive mycology tools.

### 3.2.3. Modified Atmosphere Packaging

Modified atmosphere packaging (MAP) is a commonly used method to control fungal spoilage and thus to extend dairy food shelf-life. MAP consists of the replacement of air by a defined gas mixture. In MAP, active agents such as carbon dioxide [103] or oxygen scavengers [104] can also

be utilized. During storage, passive modifications of the headspace gases can occur, resulting from product respiration and gas transfer through the film.

As extensively reviewed by Nguyen Van Long et al. [105] on a large number of fungal species, lowering the oxygen level below 1% will result in the partial growth inhibition of strict aerobic fungi, while it will slow down facultative anaerobes' growth as it enables O<sub>2</sub> use as a final electron acceptor. However, for many species including *Penicillium* and *Mucor* spp., only 0% O<sub>2</sub> concentration can totally inhibit growth [105]. It is worth mentioning that such an O<sub>2</sub> level will favor the growth of facultative or strict anaerobes and that it can have a detrimental effect on products such as mold-ripened cheeses, for which a certain oxygen level is necessary to maintain their organoleptic properties [106]. An increase of the CO<sub>2</sub> level in package atmosphere can have an inhibitory effect, thus further extending product shelf-life [105]. In their extensive review, Nguyen Van Long et al. [105] indicated that, independent of the fungal species, a CO<sub>2</sub> level above 50% was necessary to achieve fungal growth inhibition and that, depending on the fungal species, 50–90% were necessary to completely inhibit fungal growth in solid matrices. Three mechanisms have been described to explain the CO<sub>2</sub> antimicrobial effect. The first mechanism is the displacement of some or all of the O<sub>2</sub> available for growth of aerobic microorganisms. The second one is a pH decrease resulting from CO<sub>2</sub> dissolution into the product and carbonic acid formation. Carbonic acid will then behave as a weak organic acid provoking disturbances in pH homeostasis [107,108]. Nonetheless, high CO<sub>2</sub> concentration may sometimes affect product organoleptic properties. For example, high CO<sub>2</sub> levels (100%) led to very negative effects in the sensory qualities of cottage cheese, whereas for others, such as cheddar, packaging with 100% CO<sub>2</sub> is commonly used in dairy industries [109,110].

MAPs are often used for cheeses and different gas compositions have been suggested [109,111,112]. For example, in commercial sliced cheddar cheese, *P. commune* did not grow in atmospheres composed either of 20%, 40%, and 60% CO<sub>2</sub> and less than 0.5% O<sub>2</sub> [113]. *Eurotium chevalieri* and *Xeromyces bisporus* could not grow under atmosphere containing 80% CO<sub>2</sub> and 20% O<sub>2</sub> during incubation for 60 days, whereas *Mucor plumbeus* development was observed after 15 days [111]. Facultative anaerobic fungi are also susceptible to high CO<sub>2</sub> levels [113–115]. As an example, a 60% CO<sub>2</sub>/40% N<sub>2</sub> modified atmosphere was effective for inhibiting yeast growth and extending the shelf-life of whey cheese [113]. A good temperature control is necessary for effective MAP use because the CO<sub>2</sub> effect is enhanced as the temperature decreases. A combination of refrigeration and the storage of milk and milk products under modified gas atmospheres for the extension of shelf life has been reviewed extensively [108,116–118]. In general, most of the used atmospheres do not completely inhibit growth, but the spoilage time is delayed. Overall, more research is needed to improve our knowledge on O<sub>2</sub> and CO<sub>2</sub> effects on the germination and growth of spoilage fungi and, thus, to improve MAP efficiency.

### 3.2.4. Chemical Preservatives

In addition to the methods mentioned above, chemical preservatives are widely used in the dairy industry. Preservatives are food additives that help to prevent dairy products from spoilage by bacteria and/or fungi. Antifungal preservatives used in dairy products include weak organic acids, such as sorbic acid, benzoic acid, and propionic acid, and their salts, such as potassium sorbate, calcium sorbate, sodium benzoate, potassium benzoate, calcium benzoate, and sodium propionate, as well as natamycin, a polyethylene antibiotic (Table 3).

**Table 3.** List and regulations of chemical preservatives authorized in dairy products in the European Union (EU) [19] and the United States of America (USA) [120].

Preservatives	Dairy product	US Regulation	FDA Code	EU Legislation	EU Code
Natamycin (pimaricin)	Cheese	20 mg/kg	172.155	-	-
	Uncut hard, semi-hard and semi-soft cheese	20 mg/kg	172.155	1 mg/dm <sup>2</sup> , surface (not present at a depth of 5 mm)	E 235
Sorbic acid	Margarine	1000 mg/kg	182.3089	-	E 200
	Flavoured fermented milk	2000 mg/kg		1500 mg/kg	
	Non-heat treated dairy-based desserts	2000 mg/kg		300 mg/kg	
	Whey cheeses, Cheese products, Processed Cheeses	2000 mg/kg		2000 mg/kg	
	Curdled milk, unripened products, ripened products, pre-packed, sliced; layered ripened products	2000 mg/kg		1000 mg/kg	
Potassium sorbate and calcium sorbate	Cold-pack cheese, Cream cheese, Pasteurized process cheese food, Pasteurized process cheese spread, Semisoft part-skim cheeses	3000 mg/kg	182.3640 and 182.3225	-	E 202 and E 203
	Flavoured fermented milk products including heat-treated products	3000 mg/kg		1500 mg/kg	
	non-heat treated dairy-based desserts	3000 mg/kg		300 mg/kg	
	Whey cheeses, Cheese products, Processed Cheeses	3000 mg/kg		2000 mg/kg	
	Curdled milk, unripened products, ripened products, pre-packed, sliced; layered ripened products	3000 mg/kg		1000 mg/kg	
	Flavoured fermented milk products including heat-treated products	3000 mg/kg		1500 mg/kg	
	non-heat treated dairy-based desserts	3000 mg/kg		300 mg/kg	
	Whey cheeses, Cheese products, Processed Cheeses	3000 mg/kg		2000 mg/kg	
	Curdled milk, unripened products, ripened products, pre-packed, sliced; layered ripened products	3000 mg/kg		1000 mg/kg	
Sodium benzoate	Margarine	1000 mg/kg	184.1733	-	E 211
	Flavoured fermented milk products including heat-treated products	-		1500 mg/kg	
	Non-heat treated dairy-based desserts	-		300 mg/kg	
	Whey cheeses, Cheese products, Processed Cheeses	-		2000 mg/kg	
	Curdled milk, unripened products, ripened products, pre-packed, sliced; layered ripened products	-		1000 mg/kg	
Potassium benzoate and calcium benzoate	Flavoured fermented milk products including heat-treated products	-	184.1081	1500 mg/kg	E 212 and
	non-heat treated dairy-based desserts	-		300 mg/kg	E 213
	Whey cheeses, Cheese products, Processed Cheeses	-		2000 mg/kg	
	Curdled milk, unripened products, ripened products, pre-packed, sliced; layered ripened products	-		1000 mg/kg	
Propionic acid	Gruyere cheese, swiss cheese and emmentaler cheese	surface		-	E 280
	Ripened cheese	-		surface treatment	
Sodium propionate	Cheeses and related cheese products	surface	184.1784	-	E 281
	Ripened cheese	-		surface treatment	

Weak acids inhibit both bacterial and fungal growth. In addition, sorbic acid also inhibits spore germination in bacteria [121,122]. Preservatives such as benzoic and sorbic acid have an optimal inhibitory activity at pH between 4.5 and 5.5. Indeed, at such pH, the uncharged, undissociated acid form can diffuse freely across the cytoplasmic membrane and enter into the cell. The equilibrium between the undissociated and dissociated forms depends on the acid pKa and the medium pH. Once inside the cell ( $\text{pH}_i \sim 7$ ), the acid will dissociate and release charged anions and protons which cannot diffuse back across the membrane. Anions and protons accumulation into the cell is responsible for intracellular pH decrease and may cause membrane disruption, inhibition of essential metabolic reactions, stress in intracellular pH homeostasis, and/or accumulation of toxic anions which finally lead to cell death [14]. In a recent study, Garnier et al. [49] determined the minimal inhibitor concentration (MIC) of different weak acids including potassium sorbate, calcium propionate, and sodium benzoate for several fungal strains isolated from spoiled dairy products. They found that, independent of the studied species, calcium propionate was the least effective preservative followed by sodium benzoate and potassium sorbate. It should be emphasized that the inhibitory effect of weak acids on fungi is also influenced by other factors such as  $a_w$ . For example, the MIC of sorbic acid were 0.1% and 0.15% ( $w/w$ ) at 0.85  $a_w$  while they were 0.075% and 0.05% ( $w/w$ ) at 0.90  $a_w$ , for *Aspergillus flavus* and *P. roqueforti*, respectively [123]. Moreover, Stratford et al. [124] recently showed that, in contrast to acetic acid, sorbic acid did not decrease  $\text{pH}_i$  in *S. cerevisiae* but instead was a membrane-active compound, inhibiting the activity of a plasma-membrane  $\text{H}^+$ -ATPase proton pump. Most studies on the action mechanism of weak acids have been performed on *S. cerevisiae* and *Zygosaccharomyces bailli*. Therefore, more studies are necessary to understand the exact inhibition mechanism of weak acids against spoilage fungi encountered in dairy products. Finally, for hard and semi-hard smear cheeses, which are characterized by the development of a red-orange microbial mat on their surfaces, films permeable to moisture and  $\text{O}_2$  can be utilized to pack the cheeses in order to protect them from yeast and molds present in the dairy environment. Such films can also be coated with antifungal molecules such as natamycin. It is worth mentioning that a successful attempt has been made to replace synthetic film polymers by a chitosan coating containing natamycin for protecting semi-hard cheese from fungal contamination [125].

Fungal resistance or adaptation to weak organic acids rely on several mechanisms. In *S. cerevisiae* and other fungi, it includes maintenance of the cell wall structure, changes in plasma membrane or cell wall composition, metal metabolism, and activation of ATP-consuming membrane transporters to remove protons and anions [126]. Ullah et al. [127] showed that *S. cerevisiae* adaptation to sorbic or acetic acid resulted in a decreased diffusional entry of the molecule. They concluded that pre-exposed cells indeed decreased acid entry through alteration of either the plasma membrane structure or the cell wall composition or through an increase in intracellular buffering capacity. In addition, Brandao et al. [38] showed that the  $\text{H}^+$  internal concentration in *S. cerevisiae* was regulated by several systems, including plasma membrane  $\text{H}^+$ -ATPase, and that ENA1p, known for its involvement in saline or alkaline stress responses and regulation of the plasma membrane potential had an important but yet to be fully understood role in the cellular response to acid [126]. This study also demonstrated that acid stress response was dependent on calcium metabolism and was blocked by a calcineurin inhibitor. Other resistance mechanisms include the metabolization of weak acids into the cell. For example, Casas et al. [128] showed that *D. hansenii* could metabolize potassium sorbate into pentadiene, a volatile compound also responsible for off-odor, while *A. niger* was able to decarboxylate and detoxify sorbic acid thanks to a phenylacrylic acid decarboxylase [129].

Natamycin (also known as pimaricin) is a fungicide belonging to polyethylene antibiotics [130]. It is currently used to control fungal growth on cheese surfaces [131–133]. Produced by *Streptomyces natalensis*, this antibiotic acts by inhibiting the vacuole fusion process through specific interaction with ergosterol at the early priming stage of fusion, but does not permeabilize the membrane.

A World Health Organization (WHO) monograph on Food Additives states that obtaining resistance against natamycin is difficult, because of the action mode of these chemical agents [134]. Indeed, natamycin binds to ergosterol, an important component of the plasma membrane and of

the growing tips of germinating spores and vegetative hyphae [135,136]. In addition, natamycin MIC of different fungal species were reported to only differ negligibly, which is not in favor of resistance development [134]. For example, *C. parapsilosis*, *Rhodotorula* spp., and *Penicillium* spp. had mean MIC of 5.2 µg/mL, 2.3 µg/mL, and 2.3 µg/mL, respectively [137]. In contrast, Garnier et al. [49], who evaluated natamycin MIC after surface-treatment of one isolate each of 10 species from spoiled dairy products, observed that MIC ranged from 0.04 mg/dm<sup>2</sup> in *Cladosporium halotolerans* to >0.2 mg/dm<sup>2</sup> in *Y. lipolytica*. In addition, a recent study [138] showed that a continuous and prolonged incubation with natamycin induced a tolerance in individual strains.

The WHO [139], European Food Safety Authority [119], and Food and Drug Administration [120] have evaluated natamycin thoroughly and all list it as safe for human consumption. In the US, according to the Code of Federal Regulations, the final amount of natamycin must not exceed 20 ppm in cheese (20 mg/kg). In the EU, natamycin is only allowed for cheese surface-treatment with a maximum applied dose of 1 mg/dm<sup>2</sup>. Moreover, it must not be present at a depth >5 mm in the finished product [140].

### 3.2.5. Fermentation

Fermentation is one of the oldest preservation methods [141,142]. It is a biological process relying on the activity of specific microorganisms producing certain metabolites with antifungal and antibacterial activities [12,141]. Different microbial groups involved in dairy product manufacturing may suppress or retard the growth of spoilage fungi. LAB produce lactic acid as a major fermentation end-product, but may also produce other metabolites with antifungal activity (see also Section 4). Certain cheese types such as smear cheeses and mold-ripened cheeses also harbor on their surfaces complex microbiota composed of desirable aerobic bacteria and fungi which can compete with spoilage fungi for one or more limiting macro- and/or micronutrients and/or for space.

A complementary approach to reduce fungal spoilage in dairy products is gaining more and more interest: the use of bioprotective cultures and fermentates.

## 4. Bioprotective Cultures

Today, in more economically developed countries, there is a strong and increasing demand from consumers for foods that are more “natural”, i.e., less heavily processed and preservative-free [75,143]. That is the reason why a strong market demand exists for natural solutions to ensure both food safety and food shelf-life [144]. Biopreservation is not a new concept as it has been used for thousands of years in fermented foods. Also called biocontrol, it refers to the extension of food shelf-life and increase in food safety using natural or added microbiota and/or their antimicrobial compounds [145]. Food bioprotective cultures can thus be defined as food-grade bacterial or fungal strains that have been selected for their antimicrobial properties. They differ from starter or adjunct cultures which are primarily used for their technological functions (acid and aroma production, role in texture, color, etc.). Among microorganisms possessing antimicrobial properties, LAB, produce a large array of antimicrobial substances including organic acids such as lactic and acetic acids, fatty acids, reuterin, antifungal peptides, and bacteriocins [146, 147]. While research has mainly focused on screening for bacterial strains with antimicrobial activity against pathogenic or spoilage bacteria and fungi, fungi with antimicrobial activities also exist [148].

### 4.1. Lactic Acid Bacteria and Propionibacteria with Antifungal Activity

LAB and propionibacteria (PAB) can be used in food production as starter cultures to modify and improve nutritional and organoleptic food properties, or as protective cultures to improve product safety and/or shelf-life [144,149]. Indeed, LAB are used as starter cultures in the manufacturing of dairy products such as fermented milk, yoghurt, buttermilk, cottage cheeses, hard cheeses, and soft cheeses, among many others [150]. LAB have also been traditionally used as natural biopreservatives of food and feed, including milk and dairy products.

Antifungal LAB have been studied in a large food range including dairy products [15,151]. Today, several recent studies report the use of antifungal LAB to control dairy product spoilage [15,152,153].

LAB produce many antifungal metabolites [154] and most LAB are granted with a generally recognized as safe (GRAS) and qualified presumption of safety (QPS) status. Besides being safe for human consumption (absence of biogenic amine production and acquired antibiotic resistance), the main properties expected from antifungal LAB and PAB bioprotective cultures are: (1) an antifungal activity exhibited and maintained during manufacturing and storage; (2) no impact on starter cultures' functionalities; (3) no modification of product organoleptic properties; (4) an activity at the lowest possible inoculum to reduce the cost associated with their use; and (5) an easy propagation at high populations and resistance to lyophilization or freezing. There are currently several antifungal bioprotective cultures commercially available for dairy products, such as HoldBac series (DuPont Danisco), FreshQ® series (Dupont), and Befresh™ AF (Handary). Holdbac YM-B is a mixed culture of *Lactobacillus rhamnosus* and *Propionibacterium freudenreichii* subsp. *shermanii* and FreshQ® 2 is a single *L. rhamnosus* culture, while Befresh™ AF is a mixture of *Lactobacillus paracasei* and *P. freudenreichii* subsp. *shermanii*. Despite such bioprotective cultures being available on the market, research for new LAB with antifungal activities and identification of their associated metabolites is now the focus of many academic and industrial research groups.

Screening for antifungal strains is a critical step among those required to develop antifungal cultures. For example, a recent study described a high-throughput screening method to detect antifungal activities in *Lactobacillus* species cultivated in Man, Rogosa, and Sharpe (MRS) media [155]. This method allowed the detection of 154 strains with antifungal activities against *R. mucilaginosa* and to a lower extent against *Aspergillus tamari*, *Candida krusei*, and *K. marxianus*. However, as mentioned in several papers [156,157], screening in conventional MRS medium is not recommended as MRS contains acetate which may potentialize antifungal activity and artificially inflate the number of active isolates. Another interesting but labor-consuming approach, used by Delaverne et al. [158] to develop an antifungal isolate collection, was to plate raw milk samples on eight semi-selective media for LAB, and to systematically screen colonies for their antifungal activity against four spoilage fungi using the agar-overlay method. Among the ~72,000 tested colonies, >1200 colonies (i.e., 1.7% of tested colonies) had a detectable antifungal activity. However, one should keep in mind that laboratory media differ significantly in their physicochemical and microbiological characteristics with those of dairy foods and, thus, active strains in laboratory media may lose this ability in real products. Overall, further work is needed to develop high-throughput screening methods in dairy products mimicking models to increase the chances of finding suitable strains.

Among antifungal LAB, *Lactobacillus* and, to a smaller extent, *Lactococcus*, *Pediococcus*, *Weissella*, and *Leuconostoc* are the most frequently cited genera (Table 4). Indeed, many strains pertaining to species of the *Lactobacillus* genus including *Lactobacillus plantarum*, *Lactobacillus casei*, *Lactobacillus paracasei*, and *Lactobacillus brevis* were shown to possess antifungal activity against a large spectrum of fungal targets including *Penicillium*, *Candida*, *Kluyveromyces*, and *Rhodotorula* spp. as well as *Debaryomyces hansenii* and *Yarrowia lipolytica*, which are among the most important spoilage agents in dairy products [49]. Among *Propionibacterium*, *P. freudenreichii* is the major species reported to possess antifungal activity [157,159].

So far, besides lactic, acetic, and propionic acids, which are produced at g/L or g/kg levels, a very large variety of molecules has been reported to be responsible for antifungal activity. These molecules are generally produced at lower levels (mg/L or mg/kg), and include organic acids (2-pyrrolidone-5-carboxylic acid, 3-phenyllactic acid, 4-hydroxybenzoic acid, azelaic acid, DL-P-hydroxyphenyllactic acid, hydroxyphenyllactic, *p*-coumaric acid, phenyllactic acid (S)-(-)-2-hydrocinnamic acid, succinic acid, vanillic acid), fatty acids (3-hydroxydecanoic acid, decanoic acid, hydroxyisocaproic acid), cyclopeptides (cyclo(L-Pro-L-Pro), cyclo(L-Leu-L-Pro), cyclo(L-Tyr-L-Pro) cyclo(L-Met-L-Pro), cyclo(Phe-Pro), cyclo(Phe-OH-Pro), cyclo(L-Phe-L-Pro), cyclo(L-Phe-*trans*-4-OH-L-Pro) and cyclo(L-His-L-Pro)), reuterin [160], and volatile compounds such as diacetyl [153] (Table 4). Because these molecules are produced at quantities below their MIC, it is likely that they act in synergy. For example, in a study focusing on the effect of organic acids on molds, Dagnas et al. [161] showed that lactic acid alone had almost no inhibitory effect against several mold species while lactic and acetic acids could act in synergy.



**Table 4.** Lactic and propionic acid bacteria showing antifungal activities and their responsible compounds.

LAB and propionibacteria	Matrix	Metabolites	Targets	References
<b>Genus <i>Lactococcus</i></b>				
<i>Lc. lactis</i>	Lab-Lemco tryptone broth (LTB)	nd*	<i>Aspergillus flavus</i>	[163]
	LTB	Nisin	<i>Aspergillus parasiticus</i>	[164]
	Potatoe Dextrose Agar (PDA) + 0,1% Triton X	nd*	<i>Aspergillus fumigatus, Aspergillus parasiticus, Aspergillus flavus</i>	[165]
	PDA + 0,1% Triton X-100	Possibly proteinaceous compound(s)	<i>Fusarium spp., Aspergillus flavus, Aspergillus parasiticus</i>	[166]
	LTB and PDA	Possibly proteinaceous compound(s), lactic acid	<i>Penicillium expansum</i>	[167]
<b>Genus <i>Lactobacillus</i></b>				
<i>Lactobacillus spp.</i>	MRS agar	Acetic acid, propionic acid, lactic acid, peptides	<i>Penicillium candidum, Debaryomyces hansenii</i>	[168]
<b>Group <i>Lb. alimentarius /Lb. farciminis</i></b>				
<i>Lb. paralimentarius</i>	mSDB agar medium	Lactic acid, phenyllactic acid, acetic acid, peptides	<i>Aspergillus japonicus, Eurotium repens, Penicillium roseopurpureum</i>	[169]
<b>Group <i>Lb. brevis</i></b>				
<i>Lb. brevis</i>	PDA	Peptide	<i>P. roqueforti</i>	[170]
	wheat flour hydrolysate (WFH) broth	Acetic acid, phenyllactic acid, lactic acid	<i>F. graminearum</i>	[171]
	mMRS agar	Organic acids and proteinaceous compounds	<i>Fusarium spp.</i>	[172]
	MRS agar or PDA	Peptide	<i>Penicillium camemberti, Penicillium roqueforti, Aspergillus niger, Rhizopus oryzae, Kluyveromyces marxianus, Torulopsis candida, Meyerozyma guilliermondii, Saccharomyces cerevisiae</i>	[173]

Table 4. Cont

Group <i>Lb. casei</i>				
<i>Lb. casei</i>	PDA + 0,1% Triton X-100	Possibly proteinaceous compound(s)	<i>Aspergillus flavus, Aspergillus parasiticus, Fusarium sp.</i>	[166]
	PDA	Peptide	<i>Penicillium citrinum, Penicillium expansum, Aspergillus flavus</i>	[174]
	yoghurt	Lactic acid and cyclo-(Leu-Pro)	<i>Penicillium sp.</i>	[175]
	LTB and PDA	Possibly proteinaceous compound(s), lactic acid	<i>Penicillium expansum</i>	[167]
<i>Lb. paracasei</i>	yoghurt and cheese surface	Propionic acid, acetic acid, lactic acid, succinic acid, 2-pyrrolidone-5-carboxylic acid, 3-phynyllactic acid, hydroxyphenyllactic acid	<i>Candida pulcherrima, Candida magnoliae, Candida parapsilosis, Zygosaccharomyces bailii</i>	[152]
	MRS agar	Peptide	<i>Candida albicans, Candida blankii, Candida pseudointermedia</i>	[177]
	Chemically defined interaction medium	Diacetyl	<i>Penicillium solitum, Penicillium sp.</i>	[154]
	Yoghurt and acidified milk	Diacetyl, acetic acid, butanoic acid, 2,3-pentadione	<i>Penicillium solitum, Penicillium sp.</i>	[154]
<i>Lb. rhamnosus</i>	yoghurt	Acetic acid, lactic acid	<i>Rhodotorula mucilaginosa</i>	[15, 159]
Group <i>Lb. coryniformis</i>				
<i>Lb. coryniformis</i>	MRS agar or PDA	Peptide +/- 3KDa, phenyllactic acid, cyclo(Phe-Pro), cyclo(Phe-OH-Pro), reuterin	Broad spectrum	[148]
Group <i>Lb. delbrueckii</i>				
<i>Lb. acidophilus</i>	PDA + 0,1% TritonX	nd*	<i>Aspergillus fumigatus</i>	[165]
<i>Lb. amylovorus</i>	MRS agar	3-phenylpropanoic acid, p-coumaric, (E)-2-methylcinnamic acid, 3-phenyllactic acid, 3-(4-hydroxyphenyl)lactic acid, lactic acid, acetic acid, D-glucuronic acid, salicylic acid, cytidine and 2'-deoxycytidine, sodium decanoate,	<i>Aspergillus fumigatus, Aspergillus niger, Fusarium culmorum, Penicillium roqueforti, Penicillium expansum</i>	[177]
	MRS agar	Cyclo(L-Pro-L-Pro), cyclo(L-Leu-L-Pro), cyclo(L-Tyr-L-Pro) cyclo(L-Met-L-Pro) and cyclo(L-His-L-Pro)	<i>Aspergillus fumigatus, Aspergillus niger, Fusarium culmorum, Penicillium roqueforti, Penicillium expansum</i>	[177]
	Milk agar and cheese	DL- $\alpha$ -hydroxyphenyllactic acid, 4-hydroxybenzoic acid, (S)-(-)-2-hydroxyisocaproic acid, azelaic acid, phenyllactic acid, benzoic acid, hydrocinnamic acid, 3-hydroxydecanoic acid, DL- $\beta$ -hydroxylauric acid, decanoic acid, salicylic acid, 4-hydroxybenzoic, vanillic acid, (S)-(-)-2-hydroxyisocaproic acid	<i>Penicillium expansum</i>	[178]

Table 4. Cont

<i>Lb. delbrueckii</i>	LTB and PDA	Possibly proteinaceous compound(s), lactic acid	<i>Penicillium expansum</i>	[167]
Group <i>Lb. fructivorans</i>				
<i>Lb. sanfranciscensis</i>	malt-agar medium	Caproic acid, propionic acid, butyric acid, acetic acid and valeric acid	<i>Fusarium graminearum</i>	[179, 180]
Group <i>Lb. perolens</i>				
<i>Lb. harbinensis</i>	yoghurt	Acetic acid, lactic acid	<i>Yarrowia lipolytica</i> , <i>Penicillium expansum</i> , <i>Penicillium brevicompactum</i> , <i>Debaromyces hansenii</i> , <i>Rhodotorula mucilaginosa</i> , <i>Kluyveromyces lactis</i>	[15, 159]
Group <i>Lb. plantarum</i>				
<i>Lb. pentosus</i>	MRS	Peptide	<i>Aspergillus niger</i>	[181]
	MRS agar	Peptide, phenyllactic and hydroxyphenyllactic acid	<i>Penicillium nalgiovense</i> , <i>Aspergillus candidus</i>	[182]
<i>Lb. plantarum</i>	mMRS agar	(S)-(-)-2-hydroxyisocaproic acid, hydrocinnamic acid, phenyllactic acid, decanoic acid, azelaic acid, 4-hydroxybenzoic acid, p-coumaric acid, vanillic acid, DL-β-hydroxyphenyllactic acid, 3-hydroxydecanoic acid	<i>Microsporum canis</i> , <i>Microsporum gypseum</i> , <i>Epidermophyton floccosum</i>	[183]
	MRS broth	benzoic acid, 5-methyl-2,4-imidazolidinedione, tetrahydro-4-hydroxy-4-methyl-2H-pyran-2-one, 3-(2-methylpropyl)-2,5-piperazinedione, cyclo(glycyl-L-leucyl).	<i>Fusarium avenaceum</i>	[184]
	MRS agar	Lactic acid, PLA, cyclo(L-Leu-L-Pro), cyclo(L-Phe-L-Pro)	<i>Aspergillus niger</i> , <i>Fusarium graminearum</i> , <i>Fusarium culmorum</i> , <i>Fusarium oxysporum</i>	[185]
	Wheat flour hydrolysate (WFH)	Phenyllactic acid, 4-hydroxy-phenyllactic acid	<i>Eurotium repens</i> , <i>Eurotium rubrum</i> , <i>Penicillium corylophilum</i> , <i>Penicillium roqueforti</i> , <i>Penicillium expansum</i> , <i>Endomyces fibuliger</i> , <i>Aspergillus niger</i> , <i>Aspergillus flavus</i> , <i>Monilia sitophila</i> , <i>Fusarium graminearum</i>	[186]
	MRS broth	3-phenyllactic acid, cyclo(Phe-Pro), cyclo(Phe-OH-Pro), cyclo(L-Phe-L-Pro) and cyclo(L-Phe-trans-4-OH-L-Pro) dipeptides	<i>Fusarium sporotrichioides</i> , <i>Aspergillus fumigatus</i> , <i>Kluyveromyces marxianus</i>	[187]
	wheat flour hydrolysate (WFH)	acetic acid, phenyllactic acid, lactic acid	<i>Fusarium graminearum</i> , <i>Aspergillus niger</i>	[171]
	MRS agar	3-(R)-hydroxydecanoic acid, 3-hydroxy-5-cis-dodecanoic acid, 3-(R)-hydroxydodecanoic acid and 3-(R)-hydroxytetradecanoic acid	<i>Aspergillus fumigatus</i> , <i>Aspergillus nidulans</i> , <i>Kluyveromyces marxianus</i> , <i>Penicillium roqueforti</i> , <i>Penicillium commune</i> , <i>Penicillium anomala</i> , <i>Rhodotorula mucilaginosa</i>	[188]

Table 4. Cont

MRS agar plates	Acetic acid	<i>Aspergillus flavus</i> , <i>Fusarium graminearum</i> , <i>Rhizopus stolonifer</i> , <i>Sclerotium oryzae</i> , <i>Rhizoctonia solani</i> , <i>Botrytis cinerea</i> , <i>Sclerotinia minor</i>	[189]	
MRS agar	Peptide, phenyl-lactic and hydroxy-phenyllactic acid	<i>Penicillium nalgiovense</i> , <i>Penicillium camemberti</i> , <i>Penicillium verrucosum</i> , <i>Penicillium chrysogenum</i> , <i>Aspergillus candidus</i> , <i>Aspergillus flavus</i> , <i>Aspergillus ochraceus</i> , <i>Aspergillus fumigatus</i> , <i>Geotrichum candidum</i> , <i>Moniliella spp.</i> , <i>Mucor racemosus</i> , <i>Wallemia sebi</i> , <i>Eurotium herbariorum</i>	[182]	
Soybean	3,6-bis(2-methylpropyl)-2,5-piperazinedion	<i>Aspergillus flavus</i>	[190]	
MRS agar plates	3-3-phenyllactic acid (PLA), lactic acid, acetic acid	<i>Aspergillus fumigatus</i> , <i>Rhizopus stolonifer</i>	[191]	
MRS agar medium, apple-based agar growth medium	Lactic acid, acetic acid	<i>Penicillium expansum</i> , <i>Penicillium notatum</i>	[192]	
MRS agar medium	2-hydroxy-4- methylpentanoic acid	<i>Aspergillus niger</i> , <i>Aspergillus tubingensis</i> , <i>Penicillium crustosum</i>	[193]	
PDA plates	3-PLA, benzeneacetic acid, 2-propenyl ester	<i>Botrytis cinerea</i> , <i>Glomerella cingulate</i> , <i>Phytophthora drechsleri</i> <i>Tucker</i> , <i>Penicillium citrinum</i> , <i>Penicillium digitatum</i> , <i>Fusarium oxysporum</i>	[194]	
CMC broth	Lactic acid	<i>Fusarium avenaceum</i> , <i>Fusarium culmorum</i> , <i>Fusarium graminearum</i> , <i>Fusarium oxysporum</i>	[195]	
<b>Group <i>Lb. reuteri</i></b>				
<i>Lb. fermentum</i>	MRS	Peptide	<i>Aspergillus niger</i>	[181]
<i>Lb. reuteri</i>	wheat flour hydrolysate (WFH) broth	acetic acid, phenyllactic acid, lactic acid	<i>Fusarium graminearum</i> , <i>Aspergillus niger</i>	[171]
	mMRS agar	Vanillic acid, DL- <i>p</i> -hydroxyphenyllactic acid, 3-hydroxydecanoic acid, (S)-(-)-2-hydroxyisocaproic acid, hydrocinnamic acid, phenyllactic acid, decanoic acid, azelaic acid, 4-hydroxybenzoic acid, <i>p</i> -coumaric acid	<i>Microsporum canis</i> , <i>Microsporum gypseum</i> , <i>Epidermophyton floccosum</i>	[183]

Similar results were reported for other organic acids such as PLA, in which MIC decreased in the presence of lactic acid [185]. Only few studies [154,197] dealt with the action mode of LAB antifungal metabolites and the response of fungi at the physiological, transcriptomic, or proteomic levels. Therefore, further research should be undertaken in this area.

As shown in Table 3, most studies on LAB and PAB antifungal activity have been performed in (semi-)synthetic culture medium and it is obvious that important discrepancies can exist between in vitro and in situ tests. The main reasons for this are that intrinsic and extrinsic factors can affect the production of antifungal metabolites, their activity, and the susceptibility of the fungal target to these compounds. Intrinsic factors include medium composition in terms of macro- and micronutrients, pH,  $a_w$ , Eh, and food structure, while extrinsic factors are temperature, composition of the surrounding atmosphere, and humidity. Therefore, a second critical step in developing antifungal cultures is to test their efficiency in real products using challenge- and durability-tests and against one or several fungal targets. It should be noted that only a few publications have clearly shown in situ antifungal activity of selected LAB and PAB (Table 4). For example, Delavenne et al. [15,27] showed the antifungal activity of *Lactobacillus harbinensis* KV931Np against six fungal targets in yoghurt while Schwenninger and Meile (2004) demonstrated the antifungal activity of three mixed cultures of *Lactobacillus paracasei* subsp. *paracasei* SM20, SM29, or SM63 and *Propionibacterium jensenii* SM11, against *Candida pulcherrima*, *B. magnoliae*, *C. parapsilosis*, and *Zygosaccharomyces bailii* in yoghurt and on cheese surfaces [151]. More recently, Aunsbjerg et al. [153] proved the antifungal activity of *Lactobacillus paracasei* in chemically defined medium and in yoghurt against *P. solitum* and *Penicillium* sp.

#### 4.2. Fermentates

LAB and PAB may also be used to produce dairy fermentates containing antifungal metabolites, which are fermented dairy ingredients produced from milk via a fermentation process [144]. MicroGARD (DuPont Danisco) and DuraFresh (Kerry) are two currently available commercial fermentates. As an example, MicroGARD, which is FDA-approved, is produced by skimmed milk fermentation using *P. freudenreichii* subsp. *shermanii*. The use of this fermentate in cottage cheese partially inhibited *K. marxianus* and *P. expansum* and thus extended its shelf-life [197]. It may also be used in sour cream, yoghurt, and dairy desserts [198].

## 5. Conclusions

Despite technological advances, fungal spoilage is still a main issue in the dairy industry. Among the actual methods in use, a large focus concerns the replacement of traditional hurdle technologies such as chemical preservatives by new techniques to meet the increasing consumer demand for less-heavily processed and preservative-free dairy products. These new techniques include preventive methods such as a better management of air quality and non-thermal control methods such as modified atmosphere packaging and biopreservation using antifungal bioprotective cultures or fermentates.

**Acknowledgments:** We are grateful to the regional councils of Brittany (grant No. 13008651) and Pays de la Loire (grant No. 2014-07081) for the financial support and INRA for scientific coordination (J. Leonil) through the interregional project PROFIL, managed by BBA industrial association.

**Conflicts of Interest:** The authors declare no conflict of interest.

## References

1. FAO. Global FOOD Losses and Food Waste—Extent, Causes and Prevention. Rome. 2011. Available online: <http://www.fao.org/docrep/014/mb060e/mb060e00.pdf> (accessed on 5 July 2017).
2. Pitt, J.I.; Hocking, A.D. *Fungi and Food Spoilage*, 3rd ed.; Springer Science Business Media: New York, NY, USA, 2009; pp. 1–2, ISBN 978-1-4615-6391-4.
3. Shephard, G.S. Impact of mycotoxins on human health in developing countries. *Food Addit. Contam. A* **2008**, *25*, 146–151. [[CrossRef](#)] [[PubMed](#)]
4. Filtenborg, O.; Frisvad, J.C.; Thrane, U. Moulds in food spoilage. *Int. J. Food Microbiol.* **1996**, *33*, 85–102. [[CrossRef](#)]
5. Huis in't Veld, J.H.J. Microbial and biochemical spoilage of foods: An overview. *Int. J. Food Microbiol.* **1996**, *33*, 1–18. [[CrossRef](#)]
6. Prado, M.R.; Blandón, L.M.; Vandenberghe, L.P.S.; Rodrigues, C.; Castro, G.R.; Thomas-Soccol, V.; Soccol, C.R. Milk kefir: Composition, microbial cultures, biological activities and related products. *Front. Microbiol.* **2015**, *6*, 1177. [[CrossRef](#)] [[PubMed](#)]
7. Desmasures, N. CHEESE-mold-ripened varieties. In *Encyclopedia of Food Microbiology*, 2nd ed.; Batt, C.A., Ed.; Elsevier Inc.: Atlanta, GA, USA, 2014; pp. 409–415, ISBN 9780123847331.
8. Baroiller, C.; Schmidt, J.L. Contribution à l'étude de l'origine des levures du fromage de Camembert. *Lait* **1990**, *70*, 67–84. [[CrossRef](#)]
9. Ledenbach, L.H.; Marshall, R.T. Microbiological spoilage of dairy products. In *Compendium of the Microbiological Spoilage of Foods and Beverages, Food Microbiology and Food Safety*; Sperber, W.H., Doyle, M.P., Eds.; Springer: New York, NY, USA, 2010; pp. 41–67, ISBN 1441908269, 9781441908261.
10. Hymery, N.; Vasseur, V.; Coton, M.; Mounier, J.; Jany, J.L.; Barbier, G.; Coton, E. Filamentous fungi and mycotoxins in cheese: A review. *Compr. Rev. Food Sci. Food Saf.* **2014**, *13*, 437–456. [[CrossRef](#)]
11. Westall, S.; Filtenborg, O. Spoilage yeasts of decorated soft cheese packed in modified atmosphere. *Food Control* **1998**, *21*, 136–142. [[CrossRef](#)]
12. Ross, R.P.; Morgan, S.; Hill, C. Preservation and fermentation: Past, present and future. *Int. J. Food Microbiol.* **2002**, *79*, 3–16. [[CrossRef](#)]
13. Stratford, M.; Steels, H.; Nebe-von-Caron, G.; Avery, S.V.; Novodvorska, M.; Archer, D.B. Population heterogeneity and dynamics in starter culture and lag phase adaptation of the spoilage yeast *Zygosaccharomyces bailii* to weak acid preservatives. *Int. J. Food Microbiol.* **2014**, *181*, 40–47. [[CrossRef](#)] [[PubMed](#)]
14. Brul, S.; Coote, P. Preservatives agents in foods: Mode of action and microbial resistance mechanisms. *Int. J. Food Microbiol.* **1999**, *50*, 1–17. [[CrossRef](#)]
15. Delavenne, E.; Cliquet, S.; Trunet, C.; Barbier, G.; Mounier, J.; Le Blay, G. Characterization of the antifungal activity of *Lactobacillus harbinensis* K.V9.3.1.Np and *Lactobacillus rhamnosus* K. C8.3.1l in yoghurt. *Food Microbiol.* **2015**, *45*, 10–17. [[CrossRef](#)] [[PubMed](#)]
16. Schoch, C.L.; Seifert, K.A.; Huhndorf, S.; Robert, V.; Spouge, J.L.; Levesque, C.A.; Chen, W. Nuclear ribosomal internal transcribed spacer (ITS) region as a universal DNA barcode marker for fungi. *Proc. Natl. Acad. Sci. USA* **2012**, *109*, 6241–6246. [[CrossRef](#)] [[PubMed](#)]
17. Almeida, M.; Hébert, A.; Abraham, A.L.; Rasmussen, S.; Monnet, C.; Pons, N.; Delbès, S.; Loux, V.; Batta, J.M.; Leonard, P.; et al. Construction of a dairy microbial genome catalog opens new perspectives for the metagenomic analysis of dairy fermented products. *BMC Genom.* **2014**, *15*, 1257–1287. [[CrossRef](#)] [[PubMed](#)]
18. Callon, C.; Retureau, E.; Didienne, R.; Montel, M.-C. Microbial biodiversity in cheese consortia and comparative *Listeria* growth on surfaces of uncooked pressed cheeses. *Int. J. Food Microbiol.* **2014**, *174*, 98–109. [[CrossRef](#)] [[PubMed](#)]
19. Montel, M.-C.; Buchin, S.; Mallet, A.; Delbes-Paus, C.; Vuitton, D.A.; Desmasures, N.; Berthier, F. Traditional cheeses: Rich and diverse microbiota with associated benefits. *Int. J. Food Microbiol.* **2014**, *177*, 136–154. [[CrossRef](#)] [[PubMed](#)]
20. Wolfe, B.E.; Button, J.E.; Santarelli, M.; Dutton, R.J. Cheese rind communities provide tractable systems for in situ and in vitro studies of microbial diversity. *Cell* **2014**, *158*, 422–433. [[CrossRef](#)] [[PubMed](#)]
21. Quigley, L.; O'Sullivan, O.; Stanton, C.; Beresford, T.P.; Ross, R.P.; Fitzgerald, G.F.; Cotter, P.D. The complex microbiota of raw milk. *FEMS Microbiol. Rev.* **2013**, *37*, 664–698. [[CrossRef](#)] [[PubMed](#)]
22. Dugat-Bony, E.; Garnier, L.; Denonfoux, J.; Ferreira, S.; Sarthou, A.S.; Bonnarne, P.; Irlinger, F. Highlighting the microbial diversity of 12 French cheese varieties. *Int. J. Food Microbiol.* **2016**, *238*, 265–273. [[CrossRef](#)] [[PubMed](#)]
23. Lavoie, K.; Touchette, M.; St-Gelais, D.; Labrie, S. Characterization of the fungal microflora in raw milk and specialty cheeses of the province of Quebec. *Dairy Sci. Technol.* **2012**, *92*, 455–468. [[CrossRef](#)] [[PubMed](#)]
24. Marín, P.; Palmero, D.; Jurado, M. Occurrence of moulds associated with ovine raw milk and cheeses of the Spanish region of Castilla La Mancha. *Int. J. Dairy Technol.* **2015**, *68*, 565–572. [[CrossRef](#)]

25. Vacheyrou, M.; Normand, A.C.; Guyot, P.; Cassagne, C.; Piarrour, R.; Bouton, Y. Cultivable microbial communities in raw cow milk and potential transfers from stables of sixteen French farms. *Int. J. Food Microbiol.* **2011**, *146*, 253–262. [[CrossRef](#)] [[PubMed](#)]
26. Fleet, G.H. Yeasts in dairy products. *J. Appl. Bacteriol.* **1990**, *68*, 199–211. [[CrossRef](#)] [[PubMed](#)]
27. Von Neubeck, M.; Baur, C.; Krewinkel, M.; Stoeckel, M.; Kranz, B.; Stressler, T.; Fisher, L.; Hinrichs, J.; Scherer, S.; Wenning, M. Biodiversity of refrigerated raw milk microbiota and their enzymatic spoilage potential. *Int. J. Food Microbiol.* **2015**, *211*, 57–65. [[CrossRef](#)] [[PubMed](#)]
28. Kure, C.F.; Skaar, I.; Brendehaug, J. Mould contamination in production of semi-hard cheese. *Int. J. Food Microbiol.* **2004**, *93*, 41–49. [[CrossRef](#)] [[PubMed](#)]
29. Beletsiotis, E.; Guikas, D.; Kalantzi, K. Incorporation of microbiological and molecular methods in HACCP monitoring scheme of molds and yeasts in a Greek dairy plant: A case study. *Proc. Food Sci.* **2011**, *1*, 1051–1059. [[CrossRef](#)]
30. Temelli, S.; Anar, S.; Sen, C.; Akyuva, P. Determination of microbiological contamination sources during Turkish white cheese production. *Food Control* **2006**, *17*, 856–861. [[CrossRef](#)]
31. Oyugi, E.; Buys, E.M. Microbiological quality of shredded Cheddar cheese packaged in modified atmospheres. *Int. J. Dairy Technol.* **2007**, *60*, 89–95. [[CrossRef](#)]
32. Bokulich, N.A.; Mills, D.A. Facility-specific “house” microbiome drives microbial landscape of artisan cheesemaking plants. *Appl. Environ. Microbiol.* **2013**, *79*, 5214–5223. [[CrossRef](#)] [[PubMed](#)]
33. Radha, K.; Nath, L.S. Studies on the air quality in a dairy processing plant. *Ind. J. Vet. Anim. Sci. Res.* **2014**, *43*, 346–353. [[CrossRef](#)]
34. Schön, K.; Schornsteiner, E.; Dzieciol, M.; Wagner, M.; Müller, M.; Schmitz-Esser, S. Microbial communities in dairy processing environment floor-drains are dominated by product-associated bacteria and yeasts. *Food Control* **2016**, *70*, 210–215. [[CrossRef](#)]
35. Kaminarides, S.E.; Lakos, N.S. Yeasts in factory brine of feta cheese. *Aust. J. Dairy Technol.* **1992**, *47*, 68–71.
36. Suriyarachchi, V.R.; Fleet, G.H. Occurrence and growth of yeasts in yoghurts. *Appl. Environ. Microbiol.* **1981**, *42*, 574–579. [[CrossRef](#)] [[PubMed](#)]
37. Mayoral, M.B.; Martin, R.; Sanz, A.; Hernandez, P.E.; Gonzalez, I.; Garcia, T. Detection of *Kluyveromyces marxianus* and other spoilage yeasts in yoghurt using a PCR-culture technique. *Int. J. Food. Microbiol.* **2005**, *105*, 27–34. [[CrossRef](#)] [[PubMed](#)]
38. Penney, V.; Henderson, G.; Blum, C.; Johnson-Green, P. The potential phytopreservatives and nisin to control microbial spoilage of minimally processed fruit yoghurts. *Innov. Food Sci. Emerg. Technol.* **2004**, *5*, 369–375. [[CrossRef](#)]
39. Tranquillini, R.; Scaramuzza, N.; Berni, E. Occurrence and ecological distribution of heat resistant moulds spores (HRMS) in raw materials used by food industry and thermal characterization of two *Talaromyces* isolates. *Int. J. Food Microbiol.* **2017**, *242*, 116–123. [[CrossRef](#)] [[PubMed](#)]
40. Catellani, P.; Miotti Scapin, R.; Alberghini, L.; Radu, I.L.; Giaccone, V. Levels of microbial contamination of domestic refrigerators in Italy. *Food Control* **2014**, *42*, 257–262. [[CrossRef](#)]
41. Carreira, A.; Ferreira, L.M.; Loureiro, V. Brown pigments produced by *Yarrowia lipolytica* result from extracellular accumulation of homogentisic acid. *Appl. Environ. Microbiol.* **2001**, *67*, 3463–3468. [[CrossRef](#)] [[PubMed](#)]
42. Carreira, A.; Ferreira, L.M.; Loureiro, V. Production of brown tyrosine pigments by the yeast *Yarrowia lipolytica*. *J. Appl. Microbiol.* **2001**, *90*, 372–379. [[CrossRef](#)] [[PubMed](#)]
43. Dalgaard, P.; Gram, L.; Huss, H.H. Spoilage and shelf life of cod fillets packed in vacuum or modified atmospheres. *Int. J. Food Microbiol.* **1993**, *19*, 283–294. [[CrossRef](#)]
44. Gardini, F.; Tofalo, R.; Belletti, N.; Iucci, L.; Suzzi, G.; Torriani, S.; Guerzoni, M.E.; Lanciotti, R. Characterization of yeasts involved in the ripening of Pecorino Crotonese cheese. *Food Microbiol.* **2006**, *23*, 641–648. [[CrossRef](#)] [[PubMed](#)]
45. Roig-Sagués, A.X.; Molina, A.P.; Hernández-Herrero, M. Histamine and tyramine-forming microorganisms in Spanish traditional cheeses. *Eur. Res. Technol.* **2002**, *215*, 69–100. [[CrossRef](#)]
46. Suzzi, G.; Schirone, M.; Martuscelli, M.; Gatti, M.; Fornasari, M.E.; Neviani, E. Yeasts associated with Manteca. *FEMS Yeast Res.* **2003**, *3*, 159–166. [[CrossRef](#)]
47. Linares, D.M.; Martín, M.C.; Ladero, V.; Álvarez, M.A.; Fernández, M. Biogenic amines in dairy products. *Crit. Rev. Food Sci. Nutr.* **2011**, *51*, 691–703. [[CrossRef](#)] [[PubMed](#)]

48. Leuschner, R.G.; Heidel, M.; Hammes, W.P. Histamine and tyramine degradation by food fermenting microorganisms. *Int. J. Food Microbiol.* **1998**, *39*, 1–10. [[CrossRef](#)]
49. Garnier, L.; Valence, F.; Pawtowski, A.; Auhustsinava-Galerie, L.; Frotté, N.; Baroncelli, R.; Deniel, F.; Coton, E.; Mounier, J. Diversity of spoilage fungi associated with French dairy products. *Int. J. Food Microbiol.* **2017**, *241*, 191–197. [[CrossRef](#)] [[PubMed](#)]
50. Robinson, R.K. Dairy microbiology. In *The Microbiology of Milk*, 2nd ed.; Literary Cat Books: London, UK; New York, NY, USA, 1990; Volume 1, ISBN 978-0-471-38596-7.
51. Minervini, F.; Montagna, M.T.; Spilotros, G.; Monaci, L.; Santacroce, M.P.; Visconti, A. Survey on mycoflora of cow and buffalo dairy products from Southern Italy. *Int. J. Food Microbiol.* **2001**, *69*, 141–146. [[CrossRef](#)]
52. Jakobsen, M.; Narchus, J. Yeasts and their possible beneficial and negative effects on the quality of dairy products. *Int. Dairy J.* **1996**, *6*, 755–768. [[CrossRef](#)]
53. Fleet, G.H.; Mian, M.A. The occurrence and growth of yeasts in dairy products. *Int. J. Food Microbiol.* **1987**, *4*, 145–155. [[CrossRef](#)]
54. Hocking, A.D.; Faedo, M. Fungi causing thread mould spoilage of vacuum packaged Cheddar cheese during maturation. *Int. J. Food Microbiol.* **1992**, *16*, 123–130. [[CrossRef](#)]
55. Fadda, M.E.; Cosentino, S.; Deplano, M.; Palmas, F. Yeasts populations in Sardinian feta cheese. *Int. J. Food Microbiol.* **2001**, *69*, 153–156. [[CrossRef](#)]
56. Montagna, M.T.; Santacroce, M.P.; Spilotros, G.; Napoli, C.; Minervini, F.; Papa, A.; Dragoni, I. Investigation of fungal contamination in sheep and goat cheeses in southern Italy. *Mycopathologia* **2004**, *158*, 245–249. [[CrossRef](#)] [[PubMed](#)]
57. Jodral, M.; Liñan, E.; Acosta, I.; Gallego, C.; Roja, F.; Bentabol, A. Mycoflora and toxigenic *Aspergillus flavus* in Spanish milks. *Int. J. Food Microbiol.* **1993**, *36*, 171–174. [[CrossRef](#)]
58. Sensidoni, A.; Rondinini, G.; Peressini, D.; maifreni, M.; Bortolomeazzi, R. Presence of an off-flavor associated with the use of sorbates in cheese and margarine. *Int. J. Food Sci.* **1994**, *6*, 237–242.
59. Lund, F.; Filtenborg, O.; Frisvad, J.C. Associated mycoflora of cheese. *Food Microbiol.* **1995**, *12*, 173–180. [[CrossRef](#)]
60. Houbraken, J.; Samson, R.A. Phylogeny of *Penicillium* and the segregation of Trichocomoaceae into three families. *Stud. Mycol.* **2011**, *70*, 1–51. [[CrossRef](#)] [[PubMed](#)]
61. Frisvad, J.C.; Houbraken, J.; Popma, S.; Samson, R.A. Two new *Penicillium* species *Penicillium buchwaldii* and *Penicillium spathulatum*, producing the anticancer compound asperphenamate. *FEMS Microbiol. Lett.* **2013**, *339*, 77–92. [[CrossRef](#)] [[PubMed](#)]
62. Samson, R.; Frisvad, J.; Hoekstra, E.; Filtenborg, O. Introduction to food and airborne fungi. In *Mycopathologia*, 6th ed.; Centraal Bureau Voor Schimmelcultures: Utrecht, The Netherlands, 2002; p. 609, ISBN 10 9070351528.
63. Moubasher, A.H.; Abdel-Kader, M.I.A.; El-Kady, I.A. Toxigenic fungi isolated from Roquefort cheese. *Mycopathologia* **1978**, *66*, 187–190. [[CrossRef](#)]
64. Kikoku, Y.; Tagashira, N.; Nakano, H. Heat resistance of fungi isolated from frozen blueberries. *J. Food Prot.* **2008**, *71*, 2030–2035. [[CrossRef](#)] [[PubMed](#)]
65. Kure, C.F.; Wasteson, Y.; Brendehaug, J.; Skaar, I. Mould contaminants on Jarlsberg and Norvegia cheese blocks from four factories. *Int. J. Food Microbiol.* **2001**, *70*, 21–27. [[CrossRef](#)]
66. Bullerman, L.B.; Olivigni, L.B. Mycotoxin producing-potential of molds isolated from cheddar cheese. *J. Food Sci.* **1974**, *39*, 1166–1168. [[CrossRef](#)]
67. Hassanin, I.N. Detection of mycotoxigenic fungi and bacteria in processed cheese in Egypt. *Int. Biodeterior. Biodegrad.* **1993**, *31*, 15–23. [[CrossRef](#)]
68. Aran, N.; Eke, D. Mould mycoflora of Kaşar cheese at the stage of consumption. *Food Microbiol.* **1987**, *4*, 101–104. [[CrossRef](#)]
69. Kure, C.F.; Skaar, I. Mould growth on Norwegian semi-hard cheeses Norvegia and Jarlsberg. *Int. J. Food Microbiol.* **2000**, *62*, 133–137. [[CrossRef](#)]
70. Basílico, J.C.; deBasílico, M.Z.; Chiericatti, C.; Vinderola, C.G. Characterizaion and control of thread mould in cheese. *Lett. Appl. Microbiol.* **2001**, *32*, 419–423. [[CrossRef](#)] [[PubMed](#)]
71. Gould, G.W. *Mechanisms of Action of Food Preservation Procedures*; Gould, G.W., Ed.; Elsevier Applied Science: London, UK, 1989; ISBN 1851662936, 9781851662937.
72. Rahman, M.S. *Handbook of Food Preservation*, 2nd ed.; CRC Press: Boca Raton, FL, USA, 2007; ISBN 9781574446067.



73. Sofos, J.N. Current microbiological considerations in food preservation. *Int. J. Food Microbiol.* **1993**, *19*, 87–108. [CrossRef]
74. Dalié, D.K.D.; Deschamps, A.M.; Richard-Forget, F. Lactic acid bacteria—Potential for control of mould growth and mycotoxins: A review. *Food Control* **2010**, *21*, 370–380. [CrossRef]
75. Regulation (EC) No. 852/2004 of the European Parliament and of the Council of 29 April 2004 on the Hygiene and Foodstuffs. Available online: <http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2004:139:0001:0054:en:PDF> (accessed on 27 July 2017).
76. Rysstad, G.; Kolstad, J. Extended shelf life milk—Advances in technology. *Int. J. Dairy Technol.* **2006**, 85–96. [CrossRef]
77. Loureiro, V.; Malfeito-Ferreira, M. Spoilage yeasts in foods and beverages: Characterization and ecology for improved diagnosis and control. *Food Res. Int.* **2000**, *33*, 247–256. [CrossRef]
78. Loureiro, V.; Querol, A. The prevalence and control of spoilage yeasts in foods and beverages. *Trends Food Sci. Technol.* **1999**, *10*, 356–365. [CrossRef]
79. Odet, G. Qualité bactériologique des fromages au lait cru. *Cahiers de Nutrition et de Diététique* **1999**, *34*, 47–53.
80. Faye, B.; Loiseau, G. *Sources of Contamination in Dairy Supply Chains and Approaches to Quality Control*; Paper Read at International Cooperation in International Research for Agricultural Development (CIRAD)-FAO: Montpellier, France, 2002; ISBN 92-5-004787-8.
81. Sandrou, D.K.; Arvanitoyannis, I.S. Implementation of Hazard Analysis Critical Control Point (HACCP) to the dairy industry: Current status and perspectives. *Food Rev. Int.* **2000**, *16*, 77–111. [CrossRef]
82. De Steur, H.; Wesana, J.; Dora, M.K.; Pearce, D.; Gellynck, X. Applying value stream mapping to reduce food losses and wastes in supply chains: A systematic review. *Waste Manag.* **2016**, *58*, 359–368. [CrossRef] [PubMed]
83. Pinto, A.T.; Schmidt, V.; Raimundo, S.A.; Raihmer, F. Use of ozone to control fungi in a cheese ripening room. *Acta Sci. Vet.* **2007**, *35*, 333–337. [CrossRef]
84. Serra, R.; Abrunhosa, L.; Kozakiewicz, Z.; Venâncio, A.; Lima, N. Use of ozone to reduce molds in a cheese ripening room. *J. Food Prot.* **2003**, *66*, 2355–2358. [CrossRef] [PubMed]
85. Varga, L.; Szigeti, J. Use of ozone in the dairy industry: A review. *Int. J. Dairy Technol.* **2016**, *69*, 157–168. [CrossRef]
86. Korukluoglu, M.; Sahan, Y.; Yigit, A. The fungicidal efficacy of various commercial disinfectants used in the food industry. *Ann. Microbiol.* **2006**, *56*, 325–330. [CrossRef]
87. Bundgaard-Nielsen, K.; Nielsen, P.V. Fungicidal effect of 15 disinfectants against 25 fungal contaminants commonly found in bread and cheese manufacturing. *J. Food Prot.* **1995**, *59*, 268–275. [CrossRef]
88. Martin, H.; Maris, P. Synergism between hydrogen peroxide and seventeen acids against five agri-food-borne fungi and one yeast strain. *J. Appl. Microbiol.* **2012**, *113*, 1451–1460. [CrossRef] [PubMed]
89. Lado, B.H.; Yousef, A.E. Alternative food-preservation technologies: Efficacy and mechanisms. *Microbes Infect.* **2002**, *4*, 433–440. [CrossRef]
90. European Union Council Directive 92/46/EEC of 16 June 1992 Laying Down the Health Rules for the Production and Placing on the Market of Raw Milk, Heat-Treated Milk and Milk-Based Products. Available online: <https://www.fsvps.ru/fsvps-docs/ru/usefulinf/files/es9246.pdf> (accessed on 27 July 2017).
91. Sakkas, L.; Moutafi, A.; Moschopoulou, E.; Moatsou, G. Assessment of treatment of various types of milk. *Food Chem.* **2014**, *15*, 293–301. [CrossRef] [PubMed]
92. Houbraken, J.; Samson, R.A.; Frisvad, J.C. *Byssoschlamys*: Significance of heat resistance and mycotoxin production. In *Advances in Food Mycology*; Hocking, A.D., Pitt, J.I., Samson, R.A., Thrane, U., Eds.; Springer: New York, NY, USA, 2005; pp. 211–224.
93. Scaramuzza, N.; Berni, E. Heat-resistance of *Hamigera avellanea* and *Thermoascus crustaceus* isolated from pasteurized acid products. *Int. J. Food Microbiol.* **2014**, *169*, 63–68. [CrossRef] [PubMed]
94. Salomão, B.C.M.; Slongo, A.P.; Aragão, G.M.F. Heat resistance of *Neosartorya fischeri* in various juices. *LWT Food Sci. Technol.* **2007**, *40*, 676–680. [CrossRef]
95. Trujillo, A.J.; Capellas, M.; Saldo, J.; Gervilla, R.; Guamis, B. Applications of high-hydrostatic pressure on milk and dairy products: A review. *Innov. Food Sci. Emerg. Technol.* **2002**, *3*, 295–307. [CrossRef]
96. Mainville, I.; Montpetit, D.; Durand, N.; Farnworth, E.R. Deactivating the bacteria and yeast in kefir using heat treatment, irradiation and high pressure. *Int. Dairy J.* **2001**, *11*, 45–49. [CrossRef]

97. Reys, A.; Krzyzewska, A.; Laniewska-Moroz, L.; Iwanczak, M. Effect of high pressure on microflora of kefir. In Proceedings of the First International Conference on High Pressure Bioscience and Biotechnology HPBB-2000, Kyoto, Japan, 26–30 November 2000; Volume 19, pp. 1–652. [\[CrossRef\]](#)
98. Evert-Arriagada, K.; Hernández-Herrero, M.M.; Guamis, B.; Trujillo, A.J. Commercial application of high-pressure processing for increasing starter-free fresh cheese shelf-life. *Food Sci. Technol.* **2014**, *55*, 498–505. [\[CrossRef\]](#)
99. Ravishankar, S.; Zhang, H.; Kempkes, M.L. Pulsed electric fields. *Food Sci. Technol. Int.* **2008**, *14*, 429–432. [\[CrossRef\]](#)
100. Buckow, R.; Scott Chandry, P.; Ng, S.Y.; McAuley, C.M.; Swanson, B.G. Opportunities and challenges in pulse electric field processing of dairy products. *Int. Dairy J.* **2014**, *34*, 199–212. [\[CrossRef\]](#)
101. Wan, J.; Coventry, J.; Swiergon, P.; Sanguansri, P.; Viersteeg, C. Advances in innovative processing technologies for microbial inactivation and enhancement of food safety—Pulsed electric field and low temperature plasma. *Trends Food Sci. Technol.* **2009**, *20*, 414–424. [\[CrossRef\]](#)
102. Gougouli, M.; Kalantzi, K.; Beletsiotis, E.; Koutsoumanis, K.P. Development and application of predictive models for fungal growth as tools to improve quality control in yoghurt production. *Food Microbiol.* **2011**, *28*, 1453–1462. [\[CrossRef\]](#) [\[PubMed\]](#)
103. Sandhya. Modified atmosphere packaging of fresh produce: Current status and future needs. *Food Sci. Technol.* **2010**, *43*, 381–392. [\[CrossRef\]](#)
104. Ooraikul, B.; Stiles, M.E. *Modified Atmosphere Packaging of Food*; Ellis Horwood Publishing: New York, NY, USA, 1991; ISBN 978-0-7476-0064-0.
105. Nguyen Van Long, N.; Joly, C.; Dantigny, P. Active packaging with antifungal activities. *Int. J. Food Microbiol.* **2016**, *220*, 73–90. [\[CrossRef\]](#) [\[PubMed\]](#)
106. Rodriguez-Aguilera, R.; Oliveira, J.C.; Montanez, J.C.; Mahajan, P.V. Effect of modified atmosphere packaging on quality factors and shelf-life of surface mould ripened cheese: Part II varying storage temperature. *LWT Food Sci. Technol.* **2011**, *44*, 330–336. [\[CrossRef\]](#)
107. Sing, P.; Wani, A.A.; Karim, A.A.; Langowski, H.-C. The use of carbon dioxide in the processing and packaging of milk and dairy products: A review. *Int. J. Dairy Technol.* **2012**, *65*, 161–177. [\[CrossRef\]](#)
108. Hotchkiss, J.H.; Werner, B.G.; Lee, E.Y.C. Addition of carbon dioxide to dairy products to improve quality: A comprehensive review. *Compr. Rev. Food Sci. Food Saf.* **2006**, *5*, 158–168. [\[CrossRef\]](#)
109. Gonzalez-Fandos, E.; Sanz, S.; Olarte, C. Microbiological, physicochemical and sensory characteristics of Cameros cheese packaged under modified atmospheres. *Food Microbiol.* **2000**, *17*, 407–414. [\[CrossRef\]](#)
110. Taniwaki, M.H.; Hocking, A.D.; Pitt, J.I.; Fleet, G.H. Growth and mycotoxin production by fungi in atmosphere containing 80% carbon dioxide and 20% oxygen. *Int. J. Food Microbiol.* **2010**, *143*, 218–225. [\[CrossRef\]](#) [\[PubMed\]](#)
111. Khoshgozaran, S.; Azizi, M.H.; Bagheripoor-Fallah, N. Evaluating the effect of modified atmosphere packaging on cheese characteristics: A review. *Dairy Sci. Technol.* **2012**, *92*, 1–24. [\[CrossRef\]](#)
112. Taniwaki, M.H.; Hocking, A.D.; Pitt, J.I.; Fleet, G.H. Growth and mycotoxin production by food spoilage under high carbon dioxide and low oxygen atmospheres. *Int. J. Food Microbiol.* **2009**, *132*, 100–108. [\[CrossRef\]](#) [\[PubMed\]](#)
113. Dermiki, M.; Ntzimani, A.; Badeka, A.; Savvaadis, I.N.; Kontominas, M.G. Shelf-life extension and quality attributes of the whey cheese “Myzithra Kalathaki” using modified atmosphere packaging. *LWT Food Sci. Technol.* **2008**, *41*, 284–294. [\[CrossRef\]](#)
114. Eliot, S.C.; Vuilleumard, J.-C.; Edmond, J.-P. Stability of shredded Mozzarella cheese under modified atmospheres. *J. Food Sci.* **1998**, *63*, 1075–1080. [\[CrossRef\]](#)
115. Fedio, W.M.; Macleod, A.; Ozimek, L. The effect of modified atmosphere packaging on the growth of microorganisms in cottage cheese. *Milchwissenschaft* **1994**, *49*, 622–629.
116. Genigeorgis, C. Microbial and safety implications of the use of modified atmospheres to extend the storage life of fresh meat and fish. *Int. J. Food Microbiol.* **1985**, *1*, 237–251. [\[CrossRef\]](#)
117. Phillips, C.A. Review: Modified atmosphere packaging and its effects on the microbiological quality and safety of produce. *Int. J. Food Sci. Technol.* **1996**, *31*, 463–479. [\[CrossRef\]](#)
118. Rodriguez-Aguilera, R.; Oliveira, J.C.; Montanez, J.C.; Mahajan, P.V. Effect of modified atmosphere packaging on quality factors and shelf-life of surface mould ripened cheese: Part I constant temperature. *LWT Food Sci. Technol.* **2011**, *44*, 337–342. [\[CrossRef\]](#)

119. Blocher, J.C.; Busta, F.F. Multiple modes of inhibition of spore germination and outgrowth by reduced pH and sorbate. *J. Appl. Bacteriol.* **1985**, *59*, 469–478. [CrossRef] [PubMed]
120. Guynot, M.E.; Ramos, A.J.; Sanchis, V.; Marin, S. Study of benzoate, propionate, and sorbate salts as mould spoilage inhibitors on intermediate moisture bakery products of low pH (4.5–5.5). *Int. J. Food Microbiol.* **2005**, *101*, 161–168. [CrossRef] [PubMed]
121. Huang, Y.; Wilson, M.; Chapman, B.; Hocking, A.D. Evaluation of the efficacy of four weak acids as antifungal preservatives in low-acid intermediate moisture model food systems. *Food Microbiol.* **2009**, *27*, 6–33. [CrossRef] [PubMed]
122. Stratford, M.; Nebe-von-Caron, G.; Steels, H.; Novodvorska, M.; Ueckert, J.; Archer, D.B. Weak-acid preservatives: pH and proton movements in the yeast *Saccharomyces cerevisiae*. *Int. J. Food Microbiol.* **2013**, *161*, 164–171. [CrossRef] [PubMed]
123. Fajardo, P.; Martins, J.T.; Fuciños, C.; Pastrana, L.; Teixeira, J.A.; Vicente, A.A. Evaluation of a chitosan-based edible film as carrier of natamycin to improve the storability of Saloio cheese. *J. Food Eng.* **2010**, *101*, 349–356. [CrossRef]
124. EFSA. Scientific Opinion on the use of Natamycin (E 235) as a food additive. EFSA Panel on Food Additives and Nutrient Sources added to Food (ANS). *EFSA J.* **2009**, *7*, 1412. [CrossRef]
125. FDA (2015). ICFSAN/Office of Food Additive Safety. GRAS Notice Natamycin. Published: 6 November 2015. Response Letter GRAS Notice No. GRN 000578. Available online: <https://www.fda.gov/Food/IngredientsPackagingLabeling/GRAS/NoticeInventory/ucm484505.htm> (accessed on 27 July 2017).
126. Brandão, L.R.; Rosa, J.C.C.; Nicoli, J.R.; Almeida, M.V.S.; do Carmo, A.P.; Queiros, H.T.; Castro, I.M. Investigating acid stress response in different *Saccharomyces* strains. *J. Mycol.* **2014**, *12*, 1–9. [CrossRef]
127. Ullah, A.; Chandrasekaran, G.; Brul, S.; Smits, J.G. Yeast adaptation to weak acids prevents futile energy expenditure. *Front. Microbiol.* **2013**, *4*, 1–10. [CrossRef] [PubMed]
128. Casas, E.; Ancos, B.; Valderrama, M.J.; Cano, P.; Peinado, J.M. Pentadiene production from potassium sorbate by osmotolerant yeasts. *Int. J. Food Microbiol.* **2004**, *94*, 93–96. [CrossRef] [PubMed]
129. Plumridge, A.; Stratford, M.; Lowe, K.C.; Archer, D.B. The weak-acid preservative sorbic acid is decarboxylated and detoxified by a phenylacrylic acid decarboxylase, PadA1, in the spoilage mold *Aspergillus niger*. *Appl. Environ. Microbiol.* **2008**, *74*, 550–552. [CrossRef] [PubMed]
130. Kallinteri, L.D.; Kostoula, O.K.; Savvaidis, I.N. Efficacy of nisin and/or natamycin to improve the shelf-life of Galotyri cheese. *Food Microbiol.* **2013**, *36*, 176–181. [CrossRef] [PubMed]
131. De Oliveira, T.M.; De Fátima Ferreira Soares, N.; Pereira, R.M.; De Freitas Fraga, K. Development and evaluation of antimicrobial natamycin-incorporated film in Gorgonzola cheese conservation. *Packag. Technol. Sci.* **2007**, *20*, 147–153. [CrossRef]
132. Ollé Resa, C.P.; Gerschenson, L.N.; Jagus, R.J. Natamycin and nisin supported on starch edible films for controlling mixed growth on model systems and Port Salut cheese. *Food Control* **2014**, *44*, 146–151. [CrossRef]
133. Ollé Resa, C.P.; Jagus, R.J.; Gerschenson, L.N. Natamycin efficiency for controlling yeast growth in models systems and on cheese surfaces. *Food Control* **2014**, *35*, 101–108. [CrossRef]
134. WHO. *Safety Evaluation of Certain Food Additives and Contaminants; Natamycin (Pimaricin)*; WHO Food Additives Series 48; WHO: Geneva, Switzerland, 2002. Available online: [http://apps.who.int/iris/bitstream/10665/42578/1/WHO\\_TRS\\_909.pdf](http://apps.who.int/iris/bitstream/10665/42578/1/WHO_TRS_909.pdf) (accessed on 27 July 2017).
135. Van Leeuwen, M.R.; De Boer, W.; Smant, W.; Dijksterhuis, J. Filipin is a reliable in situ marker of ergosterol in the plasma membrane of germinating conidia (spores) of *Penicillium discolor* and stains intensively at the site of germ tube formation. *J. Microbiol. Methods* **2008**, *74*, 64–73. [CrossRef] [PubMed]
136. Van Leeuwen, M.R.; Van Doorn, T.; Golovina, E.A.; Stark, J.; Dijksterhuis, J. Water and air-distributed conidia exhibit differences in sterol content and cytoplasmic microviscosity. *Appl. Environ. Microbiol.* **2010**, *67*, 366–369. [CrossRef] [PubMed]
137. Stern, G.A. In vitro antibiotic synergism against ocular fungal isolates. *Am. J. Ophthalmol.* **1978**, *86*, 359–367. [CrossRef]
138. Streekstra, H.; Verkennis, A.E.E.; Jacobs, R.; Dekker, A.; Stark, J.; Dijksterhuis, J. Fungal strain and the development of tolerance against natamycin. *Int. J. Food Microbiol.* **2016**, *238*, 15–22. [CrossRef] [PubMed]

139. Joint FAO/WHO Expert Committee on Food Additives. *Evaluation of Certain Food Additives and Contaminants: 67th Report of the Joint FAO/WHO Expert Committee on Food Additives*; WHO Technical Report Series; WHO: Geneva, Switzerland, 2007; p. 25. Available online: <http://www.who.int/ipcs/publications/jecfa/reports/trs940.pdf> (accessed on 27 July 2017).
140. Bourdichon, F.; Casaregola, S.; Farrokh, C.; Frisvad, J.C.; Gerds, M.L.; Hammes, W.P.; Harnett, J.; Huys, G.; Laulund, S.; Ouwehand, A.; et al. Food fermentations: Microorganisms with technological beneficial use. *Int. J. Food Microbiol.* **2012**, *154*, 87–97. [[CrossRef](#)] [[PubMed](#)]
141. Caplice, E.; Fitzgerald, G.F. Food fermentations: Role of microorganisms in food production and preservation. *Int. J. Food Microbiol.* **1999**, *50*, 131–149. [[CrossRef](#)]
142. Brimelow, C.J.B.; Collins-Thompson, D. Food preservatives. *Food Control* **1992**, *3*, 218–219. [[CrossRef](#)]
143. Elsser-Gravesen, D.; Elsser-Gravesen, A. Biopreservatives. In *Biotechnology of Food and Feed Additives*; Zorn, H., Cermak, P., Eds.; Springer: Berlin, Germany, 2014; ISBN 978-3-662-43761-2.
144. Stiles, M.E. Biopreservation by lactic acid bacteria. *Antonie van Leeuwenhoek* **1996**, *70*, 331–345. [[CrossRef](#)] [[PubMed](#)]
145. Holzapfel, W.H.; Geisen, R.; Schillinger, U. Biological preservation of foods with reference to protective cultures, bacteriocins and food-grade enzymes. *Int. J. Food Microbiol.* **1995**, *24*, 343–362. [[CrossRef](#)]
146. Magnusson, J.; Schnurer, J. *Lactobacillus coryniformis* subs. *coryniformis* strain Si3 produces a broad-spectrum proteinaceous antifungal compound. *Appl. Environ. Microbiol.* **2001**, *67*, 1–5. [[CrossRef](#)] [[PubMed](#)]
147. Lassois, L.; de Lapeyre de Bellaire, L.; Jijakli, M.H. Biological control of crown rot of bananas with *Pichia anomala* strain K and *Candida oleophila* strain O. *Biol. Control* **2008**, *45*, 410–418. [[CrossRef](#)]
148. Zhang, H.; Wang, L.; Ma, L.; Dong, Y.; Jiang, S.; Xu, B.; Zheng, X. Biocontrol of major postharvest pathogens on apple using *Rhodotorula glutinis* and its effects on postharvest quality parameters. *Biol. Control* **2009**, *48*, 79–83. [[CrossRef](#)]
149. Carr, F.J.; Chill, D.; Maida, N. The lactic acid bacteria: A literature survey. *Crit. Rev. Microbiol.* **2002**, *28*, 281–370. [[CrossRef](#)] [[PubMed](#)]
150. Schwenninger, S.M.; Meile, L. A mixed culture of *Propionibacterium jensenii* and *Lactobacillus paracasei* subs. *paracasei* inhibits food spoilage yeasts. *Syst. Appl. Microbiol.* **2004**, *27*, 229–237. [[CrossRef](#)] [[PubMed](#)]
151. Aljewicz, M.; Cichosz, G. Protective effects of *Lactobacillus* cultures in Dutch-type cheese like products. *LWT Food Sci. Technol.* **2015**, *63*, 52–56. [[CrossRef](#)]
152. Aunbjerg, S.D.; Honoré, A.H.; Marcussen, J.; Ebrahimi, P.; Vogensen, F.K.; Benfeldt, C.; Skov, T.; Knøchel, S. Contribution of volatiles to the antifungal effect of *Lactobacillus paracasei* in defined medium and yoghurt. *Int. J. Food Microbiol.* **2015**, *194*, 46–53. [[CrossRef](#)] [[PubMed](#)]
153. Crowley, S.; Mahony, J.; van Sinderen, D. Current perspectives on antifungal lactic acid bacteria as natural bio-preservatives. *Trends Food Sci. Technol.* **2013**, *33*, 93–109. [[CrossRef](#)]
154. Inglin, R.C.; Stevens, M.J.A.; Meile, L.; Lacroix, C.; Meile, L. High-throughput screening assays for antibacterial and antifungal activities of *Lactobacillus* species. *J. Microbiol. Methods* **2015**, *114*, 26–29. [[CrossRef](#)] [[PubMed](#)]
155. Stiles, J.; Penkar, S.; Plocková, M.; Chumchalová, J.; Bullerman, L.B. Antifungal activity of sodium acetate and *Lactobacillus rhamnosus*. *J. Food Prot.* **2002**, *65*, 1188–1191. [[CrossRef](#)] [[PubMed](#)]
156. Le Lay, C.; Mounier, J.; Vasseur, V.; Weill, A.; Le Blay, G.; Barbier, G.; Coton, E. In vitro and in situ screening of lactic acid bacteria and propionibacteria antifungal activities against bakery product spoilage molds. *Food Control* **2016**, *60*, 247–255. [[CrossRef](#)]
157. Delavenne, E.; Mounier, J.; Deniel, F.; Barbier, G.; Le Blay, G. Biodiversity of antifungal lactic acid bacteria isolated from raw milk samples from cow, ewe and goat over one-year period. *Int. J. Food. Microbiol.* **2012**, *155*, 185–190. [[CrossRef](#)] [[PubMed](#)]
158. Fernandez, B.; Vimón, A.; Desfossés-Foucault, E.; Daga, M.; Arora, G.; Fliss, I. Antifungal activity of lactic and propionic acid bacteria and their potential as protective culture in cottage cheese. *Food Control* **2017**, *78*, 350–356. [[CrossRef](#)]
159. Axelsson, L.; Chung, T.C.; Debrogosz, W.J.; Lindgren, S. Production of a broad spectrum antimicrobial substance by *Lactobacillus reuteri*. *Microb. Ecol. Health Dis.* **1989**, *2*, 131–136. [[CrossRef](#)]
160. Dagnas, S.; Gauvry, E.; Onno, B.; Membré, J.M. Quantifying effect of lactic, acetic, and propionic acids on growth of molds isolated from spoiled bakery products. *J. Food Prot.* **2015**, *78*, 1689–1698. [[CrossRef](#)] [[PubMed](#)]

161. Coallier-Ascah, J.; Idziak, E.S. Interaction between *Streptococcus lactis* and *Aspergillus flavus* on production of aflatoxin. *Appl. Environ. Microbiol.* **1985**, *49*, 163–167. [[CrossRef](#)] [[PubMed](#)]
162. Luchese, R.H.; Harrigan, W.F. Growth of, and aflatoxin production by *Aspergillus paraciticus* when in the presence of either *Lactococcus lactis* or lactic acid and at different initial pH values. *J. Bacteriol.* **1990**, *69*, 512–519. [[CrossRef](#)]
163. Batish, V.K.; Roy, U.; Lal, R.; Grower, S. Antifungal attributes of lactic acid bacteria—A review. *Crit. Rev. Biotechnol.* **1997**, *17*, 209–225. [[CrossRef](#)] [[PubMed](#)]
164. Roy, U.; Batish, V.K.; Grover, S.; Neelakantan, S. Production of antifungal substance by *Lactococcus lactis* subsp. *lactis* CHD-28.3. *Int. J. Food Microbiol.* **1996**, *32*, 27–34. [[CrossRef](#)]
165. Florianowicz, T. Antifungal activity of some microorganisms against *Penicillium expansum*. *Eur. Food Res. Technol.* **2001**, *212*, 282–286. [[CrossRef](#)]
166. Voulgari, K.; Hatzikamari, M.; Delepoglou, A.; Georgakipoulos, P.; Litopoulou-Tzanetaki, E.; Tzanetakis, N. Antifungal activity of non-starter lactic acid bacteria isolates from dairy products. *Food Control* **2010**, *21*, 136–142. [[CrossRef](#)]
167. Garofalo, C.; Zannini, E.; Aquilanti, L.; Silvestri, G.; Fierro, O.; Picariello, G.; Clementi, F. Selection of sourdough lactobacilli with antifungal activity for use as biopreservatives in bakery products. *J. Agric. Food Chem.* **2012**, *60*, 7719–7728. [[CrossRef](#)] [[PubMed](#)]
168. Coda, R.; Rizello, C.G.; Nigri, F.; De Angelis, M.; Arnault, P.; Gobetti, M. Long-term fungi inhibitory activity of water-soluble extract from *Phaseolus vulgaris* cv. pinto and sourdough lactic acid bacteria during bread storage. *Appl. Environ. Microbiol.* **2008**, *74*, 7391–7398. [[CrossRef](#)] [[PubMed](#)]
169. Gerez, C.L.; Torres, M.J.; Font de Valdez, G.; Rollán, G. Control of spoilage fungi by lactic acid bacteria. *Biol. Control* **2013**, *64*, 231–237. [[CrossRef](#)]
170. Mauch, A.; Dal Bello, F.; Coffey, A.; Arendt, E.K. The use of *Lactobacillus brevis* PS1 to in vitro inhibit the outgrowth of *Fusarium culmorum* and other common *Fusarium* species found on barley. *Int. J. Food Microbiol.* **2010**, *141*, 116–121. [[CrossRef](#)] [[PubMed](#)]
171. Falguni, P.; Shilpa, V.; Mann, B. Production of proteinaceous antifungal substances from *Lactobacillus brevis* NCDC. *Int. J. Dairy Technol.* **2010**, *63*, 70–76. [[CrossRef](#)]
172. Gourama, H. Inhibition of growth and mycotoxin production of *Penicillium* by *Lactobacillus* species. *LWT Food Sci. Technol.* **1997**, *30*, 279–283. [[CrossRef](#)]
173. Li, H.; Liu, L.; Zhang, S.; Uluko, H.; Cui, W.; Lv, J. Potential use of *Lactobacillus casei* AST18 as bioprotective culture in yoghurt. *Food Control* **2013**, *34*, 675–680. [[CrossRef](#)]
174. Atanassova, M.R.; Fernández-Otero, C.; Rodríguez-Alonso, P.; Fernández-No, I.C.; Garabal, J.I.; Centeno, J.A. Characterization of yeasts isolated from artisanal short-ripened cows' cheeses produced in Galicia (NW Spain). *Food Microbiol.* **2016**, *53*, 172–181. [[CrossRef](#)] [[PubMed](#)]
175. Ryan, L.A.; Zannini, E.; Dal Bello, F.; Pawlowska, A.; Koehler, P.; Arendt, E.K. *Lactobacillus amylovorus* DSM 19280 as a novel food-grade antifungal agent for bakery products. *Int. J. Food Microbiol.* **2011**, *146*, 276–283. [[CrossRef](#)]
176. Lynch, K.M.; Pawlowska, A.M.; Brosnan, B.; Coffey, A.; Zannini, E.; Furey, A.; McSweeney, P.L.H.; Waters, D.M.; Arendt, E.K. Application of *Lactobacillus amylovorus* as an antifungal adjunct to extend the shelf-life of Cheddar cheese. *Int. J. Food Microbiol.* **2014**, *34*, 167–173. [[CrossRef](#)]
177. Corsetti, A.; Gobetti, M.; Rossi, J.; Damiani, P. Antimould activity of sourdough lactic acid bacteria: Identification of a mixture of organic acids produced by *Lactobacillus sanfrancisco* CB1. *Appl. Microbiol. Biotechnol.* **1998**, *50*, 253–256. [[CrossRef](#)] [[PubMed](#)]
178. Corsetti, A.; Settani, L.; van Sinderen, D.; Felis, G.; Dellaglio, F.; Gobetti, M. *Lactobacillus rossii* sp. nov., isolated from wheat sourdough. *Int. J. Syst. Evol. Microbiol.* **2004**, *55*, 35–40. [[CrossRef](#)]
179. Muhialdin, B.J.; Hassan, Z.; Sadon, S.K.; Zulkifli, N.A.; Azfar, A.A. Effect of pH and heat treatment on antifungal activity of *Lactobacillus fermentum* TE007, *Lactobacillus pentosus* G004 and *Pediococcus pentosaceus* TE010. *Innov. Romanian Food Biotechnol.* **2011**, *8*, 41–53. [[CrossRef](#)]
180. Coloretti, F.; Carri, S.; Armaforte, E.; Chiavari, C.; Grazia, L.; Zambonelli, C. Antifungal activity of lactobacilli isolated from salami. *FEMS Microbiol. Lett.* **2007**, *271*, 245–250. [[CrossRef](#)] [[PubMed](#)]
181. Guo, J.; Mauch, A.; Galle, S.; Murphy, P.; Arendt, E.K.; Coffey, A. Inhibition of growth of *Trichophyton tonsurans* by *Lactobacillus reuteri*. *J. Appl. Microbiol.* **2011**, *111*, 474–483. [[CrossRef](#)] [[PubMed](#)]

182. Niku-Paavola, M.L.; Laitila, A.; Mattila-Sandholm, T.; Haikara, A. New types of antimicrobial compounds produced by *Lactobacillus plantarum*. *J. Appl. Microbiol.* **1999**, *86*, 29–35. [[CrossRef](#)] [[PubMed](#)]
183. Dal Bello, F.; Clarke, C.; Ryan, L.; Ulmer, H.; Schober, T.; Strom, K.; Sjögren, J.; van Sinderen, D.; Schnürer, J.; Arendt, E.K. Improvement of the quality and shelf life of wheat bread by fermentation with the antifungal strain *Lactobacillus plantarum* FST 1.7. *J. Cereal Sci.* **2007**, *45*, 309–318. [[CrossRef](#)]
184. Lavermicocca, P.; Valerio, F.; Visconti, A. Antifungal activity of phenyllactic acid against molds isolated from Bakery products. *Appl. Environ. Microbiol.* **2003**, *69*, 634–640. [[CrossRef](#)] [[PubMed](#)]
185. Ström, K.; Sjögren, J.; Broberg, A.; Schnürer, J. *Lactobacillus plantarum* MiLAB 393 produces the antifungal cyclic dipeptides cyclo (L-Phe-L-Pro) and cyclo (L-Phe-trans-4-OH-L-Pro) and 3-phenyllactic acid. *Appl. Environ. Microbiol.* **2002**, *68*, 4322–4327. [[CrossRef](#)] [[PubMed](#)]
186. Sjögren, J.; Magnusson, J.; Broberg, A.; Schnürer, J.; Kenne, L. Antifungal 3-hydroxy fatty acids from *Lactobacillus plantarum* MiLAB 14. *Appl. Environ. Microbiol.* **2003**, *69*, 7554–7557. [[CrossRef](#)] [[PubMed](#)]
187. Sathe, S.J.; Nawani, N.N.; Dhakephalkar, P.K.; Kapadnis, B.P. Antifungal lactic acid bacteria with potential to prolong shelf-life of fresh vegetables. *J. Appl. Microbiol.* **2007**, *103*, 2622–2628. [[CrossRef](#)] [[PubMed](#)]
188. Yang, E.J.; Chang, H.C. Purification of a new antifungal compound produced by *Lactobacillus plantarum* AF1 isolated from kimchi. *Int. J. Food Microbiol.* **2010**, *139*, 56–63. [[CrossRef](#)] [[PubMed](#)]
189. Prema, P.; Smila, D.; Palavesan, A.; Immanuel, G. Production and characterization of an antifungal compound (3-phenyllactic acid) produced by *Lactobacillus plantarum* strain. *Food Bioprocess Technol.* **2008**, *3*, 379–386. [[CrossRef](#)]
190. Rouse, S.; Harnett, D.; Vaughan, A.; Sinderen, D.V. Lactic acid bacteria with potential to eliminate fungal spoilage in foods. *J. Appl. Microbiol.* **2008**, *104*, 915–923. [[CrossRef](#)] [[PubMed](#)]
191. Ndagano, D.; Lamoureux, T.; Dortu, C.; Vandermoten, S.; Thonart, P. Antifungal activity of 2 lactic acid bacteria of the *Weissella* Genus Isolated from Food. *J. Food Sci.* **2011**, *76*, 305–311. [[CrossRef](#)] [[PubMed](#)]
192. Wang, H.; Sun, Y.; Chen, C.; Sun, Z.; Zhou, Y.; Shen, F. Genome shuffling of *Lactobacillus plantarum* for improving antifungal activity. *Food Control* **2013**, *32*, 341–347. [[CrossRef](#)]
193. Laitila, A.; Alakomi, H.L.; Raaska, L.; Mattila-Sandholm, T.; Haikara, A. Antifungal activities of two *Lactobacillus plantarum* strains against *Fusarium* moulds in vitro and in malting of barley. *J. Appl. Microbiol.* **2002**, *93*, 566–576. [[CrossRef](#)]
194. Digaitiene, A.; Hansen, A.; Juodeikiene, G.; Eidukonyte, D.; Josephsen, J. Lactic acid bacteria isolated from rye sourdoughs produce bacteriocin-like inhibitory substances active against *Bacillus subtilis* and fungi. *J. Appl. Microbiol.* **2012**, *112*, 732–742. [[CrossRef](#)] [[PubMed](#)]
195. Lind, H.; Jonsson, H.; Schnürer, J. Antifungal effect of dairy propionibacteria—contribution of organic acids. *Int. J. Food Microbiol.* **2003**, *98*, 157–165. [[CrossRef](#)] [[PubMed](#)]
196. Mieszkin, S.; Hymery, N.; Debaets, S.; Coton, E.; Le Blay, G.; Valence, F.; Mounier, J. Action mechanism involved in the bioprotective effect of *Lactobacillus harbinensis* K.V9.3.1Np against *Yarrowia lipolytica* in fermented milk. *Int. J. Food Microbiol.* **2017**, *248*, 47–55. [[CrossRef](#)] [[PubMed](#)]
197. Al-Zoreky, N.; Ayres, J.W.; Sandine, W.E. Antimicrobial activity of Microgard against food spoilage and pathogenic microorganisms. *J. Dairy Sci.* **1991**, *74*, 758–763. [[CrossRef](#)]
198. Daeschel, A.M. Antimicrobial substances from lactic acid bacteria for use as food preservatives. *Food Technol.* **1989**, *43*, 91–94. [[CrossRef](#)]



© 2017 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (<http://creativecommons.org/licenses/by/4.0/>).



# **Objectifs et démarche expérimentale**





La finalité de ce projet de thèse était d'obtenir un ingrédient laitier, obtenu à partir de la fermentation d'un substrat laitier par une (ou plusieurs) bactérie(s) ou un (ou plusieurs) champignon(s), pouvant être utilisé pour la biopréservation des produits laitiers. Pour ce faire, nous avons émis l'hypothèse que les microorganismes se développant dans un milieu laitier, pourraient d'une part, potentialiser le substrat laitier considéré par le biais de leur métabolisme (fermentaire ou non) et d'autre part, produire *de novo* des molécules antifongiques.

La première étape de la thèse était d'évaluer la diversité des contaminants fongiques issus de produits laitiers contaminés et de leur environnement de fabrication. Il s'agissait en outre, sur les souches isolées représentantes de cette diversité, d'étudier leur résistance aux principaux conservateurs chimiques. En effet, si de nombreux articles ont mis en évidence la grande diversité de champignons filamenteux et de levures responsables d'altération des produits laitiers (Fleet, 1990 ; Kure *et al.*, 2003 ; Pitt and Hocking, 2009 ; Ledenbach and Marshall, 2010 ; Banjara *et al.*, 2015), très peu se sont intéressés à leur résistance aux conservateurs chimiques utilisés dans l'industrie laitière (Stratford *et al.*, 2013). Cette étape avait enfin pour objectif de constituer une collection de référence de champignons contaminants des produits laitiers et de définir les cibles fongiques les plus pertinentes à utiliser pour le reste de l'étude.

Nous avons commencé par réaliser une collecte de produits contaminés, de boîtes d'ambiance et d'isolats d'usine non identifiés auprès des partenaires industriels associés à ce projet. Les champignons ont ensuite été isolés puis identifiés par séquençage d'un ou plusieurs gènes. Cette première étape a permis la constitution d'une collection de référence composée de 170 souches (129 champignons filamenteux, 46 levures). Elle nous a également permis, pour 10 espèces de contaminants présentant la plus forte occurrence, d'étudier leur résistance aux principaux conservateurs chimiques (benzoate de sodium, propionate de calcium, sorbate de potassium et natamycine) ainsi que de sélectionner 4 isolats appartenant à 4 espèces fongiques (*G. geotrichum* UBOCC-A-216001, *M. racemosus* UBOCC-A-116002, *P. commune* UBOCC-A-116003 et *Y. lipolytica* UBOCC-A-216005), représentative de la diversité des contaminants fongiques des produits laitiers, pour l'étape de criblage de l'activité antifongique de bactéries et champignons en milieu laitier.

La seconde étape de la thèse a été de cribler le potentiel de bactéries et de champignons à générer des ingrédients laitiers antifongiques par la fermentation de fractions laitières d'intérêt. Pour ce faire, nous avons utilisé une collection de 500 bactéries et 198 champignons issus des collections du LUBEM, de la collection de culture de l'Université de Bretagne Occidentale (UBOCC) et du Centre International de Ressources Microbiennes – Bactérie

d'Intérêts Alimentaires (CIRM-BIA). Nous avons fait croître ces microorganismes dans 2 fractions laitières différentes : du lait « *low heat* » complémenté en matière grasse ou du perméat de lait ultra-filtré complémenté en extrait de levure pour produire les ingrédients potentiellement antifongiques. Ces ingrédients sont également appelés fermentats « LH » ou « UF » quand ils sont issus de lait « *low heat* » ou de perméat d'ultrafiltration de lait, respectivement. L'activité antifongique de ces fermentats a ensuite été évaluée sur une matrice modèle fromage permettant la croissance des cibles fongiques retenues. Ce criblage a été réalisé en plaques de 24 puits où chaque puits équivalait à un mini-fromage afin de disposer d'une méthode haut-débit (200 souches par semaine) compte tenu du nombre élevé de souches à cribler.

Les résultats de cette seconde étape nous ont permis de sélectionner 11 fermentats présentant des activités antifongiques intéressantes. Nous avons alors cherché à optimiser leur activité en faisant varier les températures et temps d'incubation, en réalisant des associations ou des co-cultures ou encore en ajoutant une étape de lyophilisation afin de concentrer ces fermentats mais aussi de faciliter leur conservation. Une fois les conditions de culture et de préparation des fermentats optimisées, les 7 fermentats les plus actifs, obtenus à partir de la fermentation d'un substrat laitier par *L. buchneri* L162 et L164, *Lactobacillus paracasei* L117, *Lactobacillus rhamnosus* CIRM-BIA 1952, *M. lanceolatus* UBOCC-A-109193, *P. jensenii* CIRM-BIA 1774 et la co-culture *Lactobacillus harbinensis* L172 et *S. thermophilus* CIRM-BIA 16, ont ensuite été testés sur deux produits laitiers (crème fraîche et fromage à pâte pressée à croûte morgée) afin de valider en condition réelle leur efficacité. Pour chaque produit laitier, 3 ingrédients ont été sélectionnés et leurs conditions d'applications ont été définies (dans la masse ou en traitement de surface) avant d'être testés contre 2 ou 3 cibles fongiques choisies selon le produit considéré (*M. racemosus* UBOCC-A-116002, *P. commune* UBOCC-A-116003 et *R. mucilaginosa* UBOCC-A-216004 pour la crème fraîche et les mêmes souches de *M. racemosus* et *P. commune* pour les fromages à pâte pressée à croûte morgée). L'efficacité de ces ingrédients a également été évaluée par la réalisation de tests d'usage et l'absence d'impact négatif sur les qualités organoleptiques des produits finaux vérifiée par des tests d'évaluation sensorielle.

La troisième et dernière étape de la thèse consistait à étudier les mécanismes d'action et les molécules impliquées dans l'activité antifongique des ingrédients laitiers sélectionnés, à savoir 2 fermentats LH issus de la fermentation de *L. rhamnosus* CIRM-BIA 1952 et *M. lanceolatus* UBOCC-A-109193 et un fermentat UF issu de la fermentation de *P. jensenii* CIRM-BIA 1774. Différentes méthodes ciblées (HPLC [High Performance Liquid

Chromatography] pour les acides organiques, LC-Q-TOF-MS [Liquid Chromatography-Quadrupole-Time Of Flight-Mass Spectrometry] pour les acides organiques et les acides gras, GC [Gas Chromatography] pour les acides gras libres, le head space-GC-MS pour les volatils) et non ciblées (nano LC-MS-MS [Liquid Chromatography coupled to tandem mass spectrometry] pour les peptides) ont été mises en place pour identifier et détecter le plus grand nombre de molécules connues pour leurs propriétés antifongiques d'une part, et d'autre part pour identifier des nouvelles molécules potentiellement antifongiques. La réponse physiologique de 2 champignons (*M. racemosus* UBOCC-A-109155 et *R. mucilaginosa* UBOCC-A-216004) au fermentat de *P. jensenii* CIRM-BIA 1774 a aussi été étudiée, incluant son effet sur la germination (*M. racemosus* UBOCC-A-109155) et la croissance (*M. racemosus* UBOCC-A-109155 et *R. mucilaginosa* UBOCC-A-216004) des cibles fongiques.

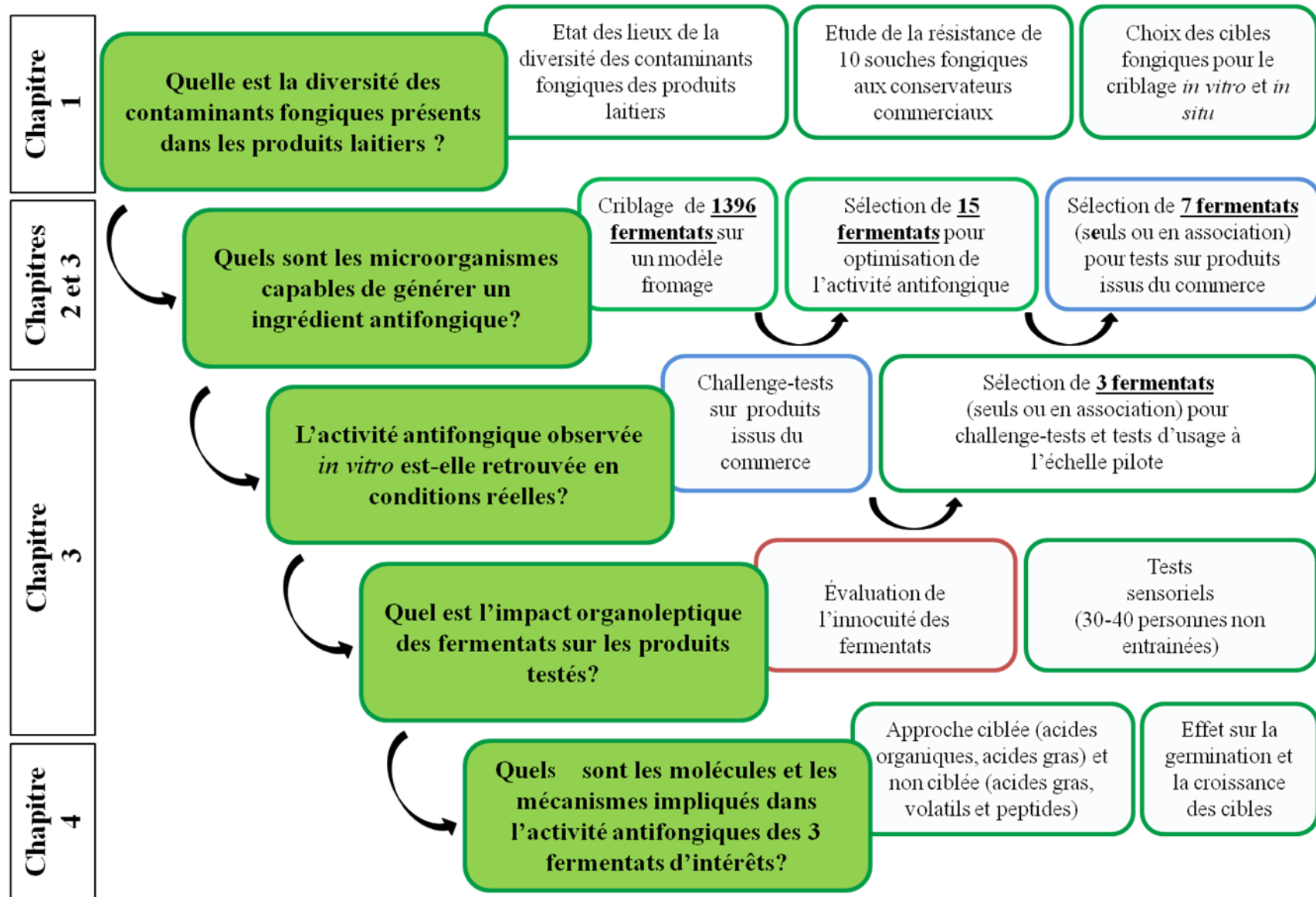


Figure 9. Récapitulatif des différentes étapes des travaux de thèse. (vert) Parties faisant l'objet d'articles scientifiques inclus dans cette thèse, (rouge) parties faisant l'objet d'un article scientifique présenté en annexe, (bleu) parties présentées dans la thèse mais ne faisant pas l'objet d'article scientifique.

# **Résultats**



# **Etude 1**

~

## **Diversité des contaminations fongiques des produits laitiers**





Dans cette étude, la diversité des contaminants fongiques associée à une grande variété de produits laitiers contaminés (beurre, crème fraîche, boissons lactées, fromages), mais aussi retrouvés dans l'air de l'environnement des industries laitières a d'abord été caractérisée. Dans un second temps, la résistance de 10 souches fongiques de 10 espèces différentes, représentatives de la diversité des contaminants fongiques observée, et isolées de ces produits laitiers contaminés, à 4 conservateurs communément utilisés dans les produits laitiers (propionate de calcium, benzoate de sodium, sorbate de potassium et natamycine) a été caractérisée.

### **Principaux résultats**

Cette étude a permis de mettre en évidence la très grande diversité des contaminants fongiques des produits laitiers. En effet, en dépit du nombre parfois limité de produits contaminés pour chaque catégorie de produit, sur 170 isolats identifiés, plus de 40 espèces de champignons différentes ont pu être mises en évidence.

#### **Champignons filamenteux**

Les champignons filamenteux (132 isolats identifiés) appartiennent aux phylums Ascomycota (5 genres, 22 espèces) et Mucoromycota (2 genres, 4 espèces). Au sein du phylum Ascomycota, un genre est particulièrement représenté, il s'agit du genre *Penicillium* qui représente à lui seul, près de 40% de la diversité totale, avec 18 espèces différentes. C'est donc le genre le plus abondant mais aussi le plus fréquemment isolé. En effet, il a été isolé dans une grande variété de produits (boissons lactées, crème, lait frais ou pasteurisé, yaourt, fromages). Au niveau de l'espèce, *Penicillium commune*, appartenant à la section Fasciculata et *Penicillium bialowiezense* de la section Brevicompecta sont les espèces dominantes au sein de la collection. *P. commune* a été fréquemment identifié en tant que contaminant des produits laitiers, en revanche, *P. bialowienzense* a très peu été identifié mais il peut être confondu avec l'espèce *Penicillium brevicompactum* et cela pourrait expliquer ce résultat. Pour les espèces du phylum Mucoromycotina, 3 espèces de *Mucor* (*M. racemosus*, *Mucor circinelloides* et *Mucor spinosus*) et 1 espèce de *Thamnidium* (*Thamnidium elegans*) ont été isolées de produits laitiers contaminés. Pour ces espèces, elles ont déjà été identifiées en tant que contaminants des produits laitiers, notamment dans les études de Kure, 2001 ; Kure and Skaar, 2000 ; Taniwaki *et al.*, 2001 et Dragoni *et al.*, 1997.

## **Levures**

Trente-huit levures ont été isolées à partir de produits laitiers contaminés, principalement rencontrées dans les produits frais. Les levures appartiennent aux phylums Ascomycota (8 genres) et Basidiomycota (4 genres). Au sein des Ascomycota, c'est le genre *Candida* qui a été le plus fréquemment isolé de produits laitiers contaminés, suivis des genres *Meyerozyma*, *Kluyveromyces* et *Yarrowia*. Au sein des Basidiomycètes, c'est le genre *Trichosporon* qui est le plus abondant, suivi des genres *Cryptococcus* et *Rhodotorula*. Au contraire des champignons filamenteux, toutes les espèces de levures identifiées dans cette étude ont déjà été identifiées en tant que contaminants des produits laitiers dans des études précédentes.

## **Air de l'environnement des industries laitières**

Au total, sur les 170 isolats identifiés, 70 isolats dont 65 champignons filamenteux étaient issus de l'environnement laitier. *Penicillium* spp. (44 isolats) est le champignon le plus représenté, suivi de *Cladosporium*, *Scopulariopsis*, *Microascus* et *Candida*. Comme observé dans les produits laitiers contaminés, le genre *Penicillium* montre une importante diversité spécifique avec un total de 11 espèces identifiées, dont *Penicillium solitum* qui est l'espèce la plus représentée (10 isolats). De plus, bien qu'attendus, ces résultats confirment que l'air ambiant est une source importante de contamination. En effet, une grande partie des espèces de champignons filamenteux isolées de boîtes d'ambiances a également été retrouvée dans les produits contaminés. Ce n'est en revanche pas le cas des levures qui proviennent probablement des équipements, aérosols ou des ingrédients ajoutés.

## **Résistance aux conservateurs chimiques**

La résistance *in vitro* de 10 souches fongiques, issues de produits laitiers contaminés, contre quatre conservateurs couramment utilisés en industrie fromagère a été étudiée: le propionate de calcium, le benzoate de sodium, le sorbate de potassium et la natamycine. Indépendamment de l'espèce étudiée, les concentrations minimales inhibitrices (CMI) du propionate de calcium étaient les plus élevées, suivies de celles du benzoate de sodium, du sorbate de potassium et de la natamycine. Dans les conditions testées, *Cladosporium halotolerans* UBOCC-A-116001 était le champignon le plus sensible aux conservateurs, suivi de *Dydimella pinodella* UBOCC-A-116004, qui était sensible aux sels d'acide mais légèrement plus résistant à la natamycine. Au

contraire, l'espèce *P. commune* UBOCC-A-116003 montrait une forte résistance aux sels d'acide mais avait seulement une résistance limitée à la natamycine. Cette étude a également permis de mettre en évidence la grande résistance de *Yarrowia lipolytica* UBOCC-A-216005 aux concentrations maximales autorisées par la réglementation, suivi de *Candida parapsilosis* UBOCC-A- 216002 et *Galactomyces geotrichum* UBOCC-A-216001.

*La totalité de ce travail est présenté dans l'article ci-après, publié dans « International Journal of Food Microbiology », et est donc rédigé en anglais.*





Contents lists available at ScienceDirect

## International Journal of Food Microbiology

journal homepage: [www.elsevier.com/locate/ijfoodmicro](http://www.elsevier.com/locate/ijfoodmicro)

## Diversity of spoilage fungi associated with various French dairy products



Lucille Garnier<sup>a,b</sup>, Florence Valence<sup>b</sup>, Audrey Pawtowski<sup>a</sup>, Lizaveta Auhustsinava-Galerie<sup>a</sup>, Nicolas Frotté<sup>a</sup>, Riccardo Baroncelli<sup>a</sup>, Franck Deniel<sup>a</sup>, Emmanuel Coton<sup>a</sup>, Jérôme Mounier<sup>a,\*</sup>

<sup>a</sup> Université de Brest, EA 3882, Laboratoire Universitaire de Biodiversité et Ecologie Microbienne, ESIAB, Technopôle Brest-Iroise, 29280 Plouzané, France

<sup>b</sup> STLO, Agrocampus Ouest, INRA, 35000 Rennes, France

## ARTICLE INFO

## Article history:

Received 2 June 2016

Received in revised form 3 October 2016

Accepted 20 October 2016

Available online 21 October 2016

## Keywords:

Dairy products

Fungi

Spoilage

Diversity

Preservative resistance

## ABSTRACT

Yeasts and molds are responsible for dairy product spoilage, resulting in significant food waste and economic losses. Yet, few studies have investigated the diversity of spoilage fungi encountered in dairy products. In the present study, 175 isolates corresponding to 105 from various spoiled dairy products and 70 originating from dairy production environments, were identified using sequencing of the ITS region, the partial  $\beta$ -tubulin, calmodulin and/or EFa genes, and the D1–D2 domain of the 26S rRNA gene for filamentous fungi and yeasts, respectively. Among the 41 species found in spoiled products, *Penicillium commune* and *Penicillium bialowiezense* were the most common filamentous fungi, representing around 10% each of total isolates while *Meyerozyma guilliermondii* and *Trichosporon asahii* were the most common yeasts (4.8% each of total isolates). Several species (e.g. *Penicillium antarcticum*, *Penicillium salamii* and *Cladosporium phyllophilum*) were identified for the first time in dairy products or their environment. In addition, numerous species were identified in both spoiled products and their corresponding dairy production environment suggesting that the latter acts as a primary source of contamination. Secondly, the resistance to chemical preservatives (sodium benzoate, calcium propionate, potassium sorbate and natamycin) of 10 fungal isolates representative of the observed biodiversity was also evaluated. Independently of the fungal species, natamycin had the lowest minimum inhibitory concentration (expressed in gram of preservative/l), followed by potassium sorbate, sodium benzoate and calcium propionate. In the tested conditions, *Cladosporium halotolerans* and *Didymella pinodella* were the most sensitive fungi while *Yarrowia lipolytica* and *Candida parapsilosis* were the most resistant towards the tested preservatives. This study provides interesting information on the occurrence of fungal contaminants in dairy products and environments that may help developing adequate strategies for fungal spoilage control.

© 2016 Elsevier B.V. All rights reserved.

## 1. Introduction

Food spoilage is a major problem for the food industry and consumers because it renders products unacceptable for consumption and consequently leads to significant food waste and economic losses. Such spoilage is frequently due to microbial activities. Indeed, because of their physico-chemical characteristics (pH,  $a_w$ , redox potential), food products, especially fresh products provide a favorable niche for the survival and growth during storage of certain undesirable microorganisms including bacteria and fungi.

In the context of dairy product spoilage, the presence and effect of spoilage or pathogenic bacteria is well documented (Brooks et al.,

2012; Canganella et al., 1998; Huis in't Veld, 1996; Ledenbach and Marshall, 2010). In contrast, despite the fact that yeasts and molds also cause important dairy food waste, only few studies (Banjara et al., 2015; Kure, 2001; Kure and Skaar, 2000) have been conducted on the diversity of spoilage fungi. Moreover, much more emphasis has been put on yeast than on mold diversity. According to the literature, the main genera involved in dairy product spoilage are *Candida*, *Galactomyces* and *Yarrowia* and, *Penicillium*, *Mucor* and *Cladosporium* for yeasts and molds, respectively (Deák, 2008; Pitt and Hocking, 2009). These contaminants mainly originate from the dairy environment including the air, the surfaces, the equipment and the personnel as well as raw materials and ingredients (Kure et al., 2004; Vacheyrou et al., 2011).

Fungal spoilage may be visible due mainly to the organism growth (colony or thallus), such as the “toad skin” or the “cat hair” defects caused by *Galactomyces geotrichum* and *Mucor* spp., respectively, or non-visible, via fungal metabolism resulting in production of off-odors and/or off-flavors, gas production or texture alteration (Ledenbach and Marshall, 2010). Furthermore, although spoilage yeasts have never been involved in food outbreaks, certain species are considered

\* Corresponding author at: Laboratoire Universitaire de Biodiversité et Ecologie Microbienne, Parvis Blaise-Pascal, Technopôle Brest-Iroise, 29280 Plouzané, France.

E-mail addresses: [lucille.garnier@univ-brest.fr](mailto:lucille.garnier@univ-brest.fr) (L. Garnier), [florence.valence@inra.fr](mailto:florence.valence@inra.fr) (F. Valence), [audrey.pawtowski@univ-brest.fr](mailto:audrey.pawtowski@univ-brest.fr) (A. Pawtowski), [liza.galerie2@orange.fr](mailto:liza.galerie2@orange.fr) (L. Auhustsinava-Galerie), [nicolas.frotte@univ-brest.fr](mailto:nicolas.frotte@univ-brest.fr) (N. Frotté), [riccardobaroncelli@gmail.com](mailto:riccardobaroncelli@gmail.com) (R. Baroncelli), [Franck.deniel@univ-brest.fr](mailto:Franck.deniel@univ-brest.fr) (F. Deniel), [emmanuel.coton@univ-brest.fr](mailto:emmanuel.coton@univ-brest.fr) (E. Coton), [jerome.mounier@univ-brest.fr](mailto:jerome.mounier@univ-brest.fr) (J. Mounier).

as opportunistic pathogens and may represent a risk for immuno-compromised people (Jacques and Casaregola, 2008). In addition, several mold spoilage species such as *Aspergillus* and *Penicillium* spp. are able to produce mycotoxins which may also be toxic for humans (Filtenborg et al., 1996; Huis in't Veld, 1996; Westall and Filtenborg, 1998a, 1998b). However, it is worth mentioning that no food poisoning cases related to the consumption of mycotoxin-contaminated dairy products have been documented so far (Hymery et al., 2014).

In order to avoid or delay fungal spoilage, and thus to extend product shelf life and safety, prevention methods combined with hurdle technologies are applied by dairy manufacturers. Prevention methods include the application of good manufacturing and hygiene practices, implementation of Hazard Analysis Critical Control Point (HACCP) system and the use of air filtration or decontamination equipment. The hurdle technologies can correspond to the use of heat treatment, modified atmosphere packaging, cold storage, addition of sodium chloride or sugar or fermentation with beneficial microorganisms, including bacteria and fungi (Bourdichon et al., 2012; Phillips, 1996; Sakkas et al., 2014). In certain dairy product types, the use of chemical preservatives (e.g. natamycin or organic acids such as acetic, lactic, propionic, sorbic, benzoic acids and their salts) is also permitted. For example, within the EU, propionate and sorbate, as well as natamycin can be used for the surface-treatment of hard, semi-hard and semi-soft cheese at *quantum satis* level and at a maximum level of 1 mg/dm<sup>2</sup> (Commission Regulation (EU) No 1129/2011, 2011). Moreover, benzoate can be added in non-heat-treated dairy-based desserts (at 300 mg/kg or mg/l) while sorbates can be added in a much larger range of dairy products (i.e. fermented milks, cheese and cheese-derived products) with maximum limits varying from 300 to 2000 mg/kg (Commission Regulation (EU) No 1129/2011, 2011). It is now well established that the intrinsic resistance of fungi to these preservatives is species-dependent (López-Malo et al., 2005; Rodrigues and Pais, 2000).

In the present study, we first investigated the biodiversity of spoilage fungi associated with a large range of dairy products (including butter, cream, fresh and semi-hard smear cheese, yoghurt drink and yoghurt) and in the air of dairy production environments. Then, we determined the resistance of 10 fungal isolates representative of the observed biodiversity to chemical preservatives commonly used in the dairy industry (e.g. calcium propionate, sodium benzoate, potassium sorbate and natamycin).

## 2. Materials and methods

### 2.1. Fungal isolates

In the present study, 175 fungal isolates, corresponding to 105 isolates from spoiled products and 70 isolates from air in dairy production environments were identified. Sixty-three isolates originating from contaminated products were directly provided by 4 dairy manufacturers over a period of 6 months and 32 fungi were isolated from spoiled products that were supplied by 3 dairy manufacturers over a period of 6 months. These products included cream cheese (7 samples), fresh cheese (6 samples), hard cheese (12 samples), yoghurt (4 samples) and yoghurt drink (3 samples). Sensorial properties of contaminated dairy products were not studied. In addition to these products, 70 isolates from the air of 3 industrial dairy plants were also identified after collection using an air sampler on yeast glucose chloramphenicol (YGC) agar.

### 2.2. Isolation of spoilage fungi from contaminated products

Contaminated pieces were removed with a sterile scalpel and deposited at the center of M2Lev (20 g/l malt extract (ME), 3 g/l yeast extract (YE), 15 g/l agar, 5 mg/l penicillin, 5 mg/l streptomycin) and M5S5 (50 g/l ME, 50 g/l sodium chloride, 15 g/l agar, 5 mg/l penicillin, 5 mg/l streptomycin) media prior to incubation for 2 to 7 days at

25 °C. For food materials with no visible fungal growth on their surfaces, isolation was also performed after successive dilution in physiological water supplemented with 0.005% Tween 80 and surface-plate on M2Lev medium incubated for 2 to 7 days at 25 °C. Isolates were then purified on the same agar medium and stored at –80 °C in 20% glycerol until further use.

### 2.3. Identification of fungal isolates

Isolates were preliminarily characterized at the genus level using phenotypic methods including macro- and microscopic observations. For species identification, barcode markers were sequenced. DNA was extracted from scraped colonies or mycelial plugs using the FastDNA SPIN Kit (MP Biomedicals, Illkirch, France) according to the manufacturer's instructions. DNA was then diluted to 50–100 ng/μl and kept at –20 °C until further analysis.

Depending on the studied fungal isolate, 1 to 3 DNA regions were amplified and sequenced. For filamentous fungi, the internal transcribed spacer (ITS) region (all isolates except presumptive *Penicillium* spp.), the partial β-tubulin gene (*Penicillium* spp.), the partial elongation factor α (EFα) (*Cladosporium* spp.) were PCR-amplified using primers ITS4 (5'-TCCCTCCGCTTATGATATGC-3') and ITS5 (5'-GGAAGTAAAAGTCGTAACAAGG-3') (White et al., 1990), Bt2a (5'-GGTAACCAAATCGGTGCTGCTTTC-3') and Bt2b (5'-ACCCTCAGTGTAGTGACCCTGGC-3') (Glass and Donaldson, 1995), EF1-728F (5'-CATCGAGAAGTTCGAGAAGG-3') and EF1-986R (5'-TACTTGAAGGAACCCCTACC-3') (Carbone and Kohn, 1999), respectively. In addition, the partial calmodulin gene was amplified using primers cmd5 (5'-CCGAGTACAAGGAGGCCTTC-3') and cmd6 (5'-CCGATAGAGGTCATAACGTGG-3') (Hong et al., 2005) for three *Penicillium* isolates related to *Penicillium antarcticum* in order to confirm their species identity. For yeasts, primers NL1 (5'-GCATATCAATAAGCGGAGGAAAAG-3') and NL4 (5'-GGTCCGIGITTCAGACGG-3') (Kurtzman and Robnett, 1997) were used to amplify the D1-D2 domain of the 26S rRNA gene. PCR amplifications were carried out in a FlexCycler thermocycler (Analytik Jena, Germany) and PCR products were checked by gel electrophoresis.

Sequencing was performed at the Biogenouest sequencing platform in the "Station Biologique de Roscoff" (<http://www.sb-roscoff.fr/SG/>) using the same primer pairs as those used for PCR amplifications. Sequences were assembled into contig using the DNA Baser software (Heracle software, Germany), and compared with the GenBank database using the "Basic Local Alignment Search Tool" (BLAST) (<http://www.ncbi.nlm.nih.gov/BLAST>). Phylogenetic analyses (multiple alignment using ClustalW and phylogenetic reconstruction using the neighbor-joining method) were performed with sequences retrieved from the NCBI database using the MEGA6 software (Tamura et al., 2013). Sequences have been deposited in GenBank under accession numbers KX928780 to KX928952.

### 2.4. Resistance to chemical preservatives

Resistance to calcium propionate, potassium sorbate, sodium benzoate and natamycin was evaluated for 10 isolates representative of the determined fungal species, namely, *Cladosporium halotolerans* UBOCC-A-116001, *G. geotrichum* UBOCC-A-216001, *Mucor racemosus* UBOCC-A-116002, *Penicillium commune* UBOCC-A-116003, *Didymella pinodella* UBOCC-A-116004, *Candida parapsilosis* UBOCC-A-216002, *Meyerozyma guilliermondii* UBOCC-A-216003, *Rhodotorula mucilaginosa* UBOCC-A-216004, *Trichosporon asahii* UBOCC-A-216005 and *Yarrowia lipolytica* UBOCC-A-216006. All chosen isolates were deposited at the Université de Bretagne Occidentale Culture Collection (UBOCC, Plouzané, France) and were collected from contaminated dairy products. Resistance to calcium propionate, potassium sorbate and sodium benzoate was evaluated *in vitro* at pH 5 for concentrations ranging from 0 to 3 g/l (tested concentrations: 0, 0.04, 0.08, 0.1, 0.15, 0.3, 0.5, 1, 1.5, 2, 2.5 and 3 g/l).

After addition to potato dextrose agar (PDA) medium of the required amount of each preservative, pH was adjusted with 1 M NaOH or HCl before sterilization at 121 °C for 20 min. pH was checked after sterilization. Then, agar medium (2 ml) was distributed into 24-well plates, left to solidify and centrally inoculated on the surface with the tested fungi using 10 µl of a  $5 \times 10^3$  spores or cells/ml suspension obtained as previously described by Delavenne et al. (2012), except that yeasts were cultured in potato dextrose broth (PDB) instead of yeast extract and malt-based medium (YEMA). Natamycin resistance was tested at pH 5 as described above for concentrations ranging from 0 to 0.2 g/l, corresponding to 1 mg/dm<sup>2</sup> (tested concentrations: 0, 0.01, 0.02, 0.04, 0.06, 0.08, 0.1, 0.12, 0.14, 0.16, 0.18 and 0.2 g/l) except that natamycin was added after distribution and solidification of PDA into 24-well plates using 100 µl of a stock solution at the required concentration. Plates were incubated at 25 °C in the dark and fungal growth was visually evaluated after 5 d. Two replicates were performed for each tested concentration and for each targeted fungus. The minimum inhibitory concentration (MIC) was determined as the concentration for which no visible growth occurred after the incubation period. A 2-way analysis

of variance (species × preservatives) was carried out and mean values were compared using the Fisher LSD test ( $p < 0.05$ ).

### 3. Results

#### 3.1. Fungal diversity in spoiled dairy products

##### 3.1.1. Mold diversity

Among the 105 isolates collected from dairy products, 60.0% were filamentous fungi (63 isolates) which represented a large species diversity (24 species). They belonged to the *Ascomycota* phylum (5 genera and 22 species) and *Mucoromycotina* subphylum (2 genera and 4 species). Among the *Ascomycota* phylum, *Penicillium* was the most frequently and abundantly isolated genus (46 isolates, 13 species) followed by *Cladosporium* and *Didymella* (3 isolates each, 3 and 2 species, respectively), 2 isolates of an unidentified *Sordariomycetes* and 1 isolate each of *Scopulariopsis* and *Exophiala* sp. from cream cheese (Table 1).

The *Penicillium* isolates were mainly isolated from semi-hard smear cheese samples (36 out of 46 isolates) but were also found to occur in all

Table 1  
Diversity of spoilage molds isolated from dairy products (n = number of samples) and factory air.

Species	Isolation source										Total in		
	Butter (n = 1)	Cream (n = 2)	Cream cheese (n = 8)	Fresh cheese (n = 6)	Hard cheese (n = 48)	Pasteurized milk (n = 2)	Yoghurt (n = 4)	Yoghurt drink (n = 3)	Unknown origin	Total in contaminated dairy products	Total in factory air		
<i>Acremonium</i> sp.	–	–	–	–	–	–	–	–	–	0 (0.0%)	1 (1.4%)		
<i>Alternaria infectoria</i>	–	–	–	–	–	–	–	–	–	0 (0.0%)	1 (1.4%)		
<i>Cladosporium cladosporioides</i>	–	–	–	–	–	–	–	–	–	0 (0.0%)	1 (1.4%)		
<i>Cladosporium halotolerans</i>	–	–	–	–	–	1	–	–	–	1 (1.0%)	2 (2.9%)		
<i>Cladosporium langeronii</i>	–	–	–	–	–	–	–	–	–	0 (0.0%)	1 (1.4%)		
<i>Cladosporium oxysporum</i>	–	–	–	–	–	–	–	–	–	0 (0.0%)	1 (1.4%)		
<i>Cladosporium phylophilum</i>	1	–	–	–	–	–	–	–	–	1 (1.0%)	0 (0.0%)		
<i>Cladosporium ramotenellum</i>	–	–	–	–	–	–	–	–	–	0 (0.0%)	3 (4.3%)		
<i>Cladosporium sphaerospermum</i>	–	–	–	–	1	–	–	–	–	1 (1.0%)	0 (0.0%)		
<i>Didymella heteroderae</i>	–	–	–	–	–	–	–	–	–	0 (0.0%)	1 (1.4%)		
<i>Didymella glomerata</i>	–	–	–	–	–	–	–	–	1	1 (1.0%)	0 (0.0%)		
<i>Didymella pinodella</i>	–	–	1	–	–	–	–	1	–	2 (1.9%)	0 (0.0%)		
<i>Epicoccum nigrum</i>	–	–	–	–	–	–	–	–	–	0 (0.0%)	1 (1.4%)		
<i>Exophiala</i> sp.	–	–	1	–	–	–	–	–	–	1 (1.0%)	0 (0.0%)		
<i>Fusarium merismoides</i>	–	–	–	–	–	–	–	–	–	0 (0.0%)	1 (1.4%)		
<i>Microascus croci</i>	–	–	–	–	–	–	–	–	–	0 (0.0%)	2 (2.9%)		
<i>Mucor circinelloides</i>	–	–	–	–	2	–	–	–	–	2 (1.9%)	0 (0.0%)		
<i>Mucor racemosus</i>	–	–	–	–	3	–	–	–	1	4 (3.8%)	0 (0.0%)		
<i>Mucor spinosus</i>	–	–	–	–	2	–	–	–	–	2 (1.9%)	0 (0.0%)		
<i>Penicillium adametzioides</i>	–	1	–	–	–	–	–	–	–	1 (1.0%)	0 (0.0%)		
<i>Penicillium antarcticum</i>	–	–	–	–	4	–	–	–	–	4 (3.8%)	0 (0.0%)		
<i>Penicillium bialowiezense</i>	–	–	–	–	8	–	1	–	–	9 (8.6%)	9 (12.9%)		
<i>Penicillium brevicompactum</i>	–	–	–	–	2	–	–	–	–	2 (1.9%)	2 (2.9%)		
<i>Penicillium charlesii</i>	–	1	–	–	–	1	–	–	–	2 (1.9%)	0 (0.0%)		
<i>Penicillium chrysogenum</i>	–	–	–	–	–	–	–	–	–	0 (0.0%)	2 (2.9%)		
<i>Penicillium commune</i>	–	–	–	–	10	–	–	1	–	11 (10.5%)	2 (2.9%)		
<i>Penicillium discolor</i>	–	–	–	–	4	–	–	–	–	4 (3.8%)	1 (1.4%)		
<i>Penicillium echinulatum</i>	–	–	–	–	–	–	–	–	–	0 (0.0%)	7 (10.0%)		
<i>Penicillium fellutanum</i>	–	–	2	–	–	–	–	–	–	2 (1.9%)	0 (0.0%)		
<i>Penicillium glabrum</i>	–	–	–	–	–	–	–	–	–	0 (0.0%)	3 (4.3%)		
<i>Penicillium nalgiiovense</i>	–	–	–	–	3	–	–	–	–	3 (2.9%)	0 (0.0%)		
<i>Penicillium nordicum</i>	–	–	–	–	1	–	–	–	–	1 (1.0%)	0 (0.0%)		
<i>Penicillium palitans</i>	–	–	1	–	1	–	–	–	–	2 (1.9%)	5 (7.1%)		
<i>Penicillium roqueforti</i>	–	–	–	–	1	–	–	–	–	1 (1.0%)	0 (0.0%)		
<i>Penicillium salamii</i>	–	–	–	–	–	–	–	–	–	0 (0.0%)	2 (2.9%)		
<i>Penicillium solitum</i>	–	–	–	–	2	–	1	1	–	4 (3.8%)	10 (14.3%)		
<i>Penicillium spathulatum</i>	–	–	–	–	–	–	–	–	–	0 (0.0%)	1 (1.4%)		
<i>Phaeosphaeria nodorum</i>	–	–	–	–	–	–	–	–	–	0 (0.0%)	1 (1.4%)		
<i>Pseudoscopulariopsis hibernica</i>	–	–	–	–	–	–	–	–	–	0 (0.0%)	1 (1.4%)		
<i>Scopulariopsis candida</i>	–	–	–	–	–	–	–	–	1	1 (1.0%)	2 (2.9%)		
<i>Stereum</i> sp.	–	–	–	–	–	–	–	–	–	0 (0.0%)	1 (1.4%)		
Unidentified <i>Sordariomycetes</i>	–	–	–	–	–	–	–	–	1	1 (1.0%)	0 (0.0%)		
<i>Thamnidium elegans</i>	–	–	–	–	1	–	–	–	–	1 (1.0%)	0 (0.0%)		
<i>Thanatephorus cucumeris</i>	–	–	–	–	–	–	–	–	–	0 (0.0%)	1 (1.4%)		
Total	1 (1.0%)	2 (1.9%)	5 (4.8%)	0 (0.0%)	45 (42.9%)	2 (1.9%)	2 (1.9%)	3 (2.9%)	4 (3.8%)	64 (61.0%)	65 (92.9%)		



other product types including cream, cream cheese, pasteurized milk, yoghurt drink, and yoghurt with the exception of butter. Based on the classification system recently proposed by Houbraeken and Samson (2011), *Penicillium* spp. isolates belonged to the section Brevicompecta (*Penicillium bialowiezense*, *Penicillium brevicompactum*), *Canescentia* (*P. antarcticum*), *Charlesii* (*Penicillium charlesii*, *Penicillium fellutanum*), *Chrysogena* (*Penicillium nalgiovense*), *Fasciculata* (*P. commune*, *Penicillium discolor*, *Penicillium nordicum*, *Penicillium palitans*, *Penicillium solitum*), *Sclerotiora* (*Penicillium adametzioides*) and *Roquefortorum* (*Penicillium roqueforti*). *P. commune* and *P. bialowiezense* were the most frequently isolated species (all except 2 isolates were obtained from semi-hard cheese). These 2 species were followed by *P. solitum*, *P. antarcticum* and *P. discolor* (4 isolates), *P. nalgiovense* (3 isolates), *P. brevicompactum*, *P. charlesii*, *P. fellutanum* and *P. palitans* (2 isolates), and *P. adametzioides*, *P. nordicum* and *P. roqueforti* with 1 isolate each.

Concerning other mold genera from the Ascomycota, 3 *Cladosporium* species (*C. halotolerans*, *Cladosporium phyllophilum* and *Cladosporium sphaerospermum*) and 2 *Didymella* species (*Didymella glomerata* and *D. pinodella*) were identified in 3 and 2 product types, respectively (Table 1).

For species of the Mucoromycotina subphylum, 3 *Mucor* species (*M. racemosus*, *Mucor circinelloides*, and *Mucor spinosus*) and 1 *Thamnidium* species (*Thamnidium elegans*) were found in semi-hard smear cheeses and an unknown product.

### 3.1.2. Yeasts spoilage of dairy products

Thirty-eight yeast isolates were collected from spoiled dairy products mostly represented by fresh dairy products (Table 2). They belonged to the Ascomycota or Basidiomycota phylum and were respectively represented by 8 and 4 genera within these two phyla. Among Ascomycota, *Candida* and *Meyerozyma* (formerly *Pichia*) (9 and 5 isolates obtained from 4 and 2 product types, respectively) were the most dominant genera followed by *Kluyveromyces* and *Yarrowia* (4 isolates each from 2 and 3 product types respectively), *Pichia* and *Galactomyces* (3 and 2 isolates from 1 and 2 product types) and, *Debaryomyces* (1 isolate from semi-hard smear cheese). Among Basidiomycota, *Trichosporon* (5 isolates obtained from fresh cheese) was the most dominant genus followed by *Cryptococcus* and *Rhodotorula* (3 isolates each, both from 2 product types), and *Sporidiobolus* (1 isolate from semi-hard smear cheese) (Table 2).

### 3.2. Fungal diversity in the air of dairy plants

A total of 70 isolates including 65 filamentous fungi and 5 yeasts were collected from the air of dairy plants (Tables 1 and 2). *Penicillium* spp. (44 isolates) were the most dominant fungi followed by *Cladosporium* (8 isolates), *Scopulariopsis*, *Microascus* and *Candida* (2 isolates each) and *Acremonium*, *Epicoccum*, *Fusarium*, *Alternaria*, *Phaeosphaeria*, *Pseudoscopulariopsis*, *Didymella*, *Rhodotorula*, *Stereum*, *Thanatephorus* and *Yarrowia* (1 isolate each).

As already observed for spoiled dairy products, the *Penicillium* genus showed an important diversity at the species level with a total of 11 species. *P. solitum* (10 isolates), was the most prevalent species followed by *P. bialowiezense* (9 isolates), *Penicillium echinulatum* (7 isolates), *P. palitans* (5 isolates), *Penicillium glabrum* (3 isolates), *Penicillium chrysogenum*, *P. brevicompactum*, *P. commune* and *Penicillium salamii* (2 isolates) and, *P. discolor* and *Penicillium spathulatum* (1 isolate each). Interestingly, 6 *Penicillium* species including *P. bialowiezense*, *P. brevicompactum*, *P. commune*, *P. discolor*, *P. palitans*, and *P. solitum* as well as *C. halotolerans*, *Scopulariopsis candida* and *Y. lipolytica* isolated from the air were also isolated from spoiled dairy products. In contrast, all the other species were only found in the ambient air.

### 3.3. Resistance to chemical preservatives

Independently of the fungal species studied, the MICs of calcium propionate were the highest, ranging from 2 to N 3 g/l, followed by those of sodium benzoate (MICs ranging from 0.3 to N 3 g/l), potassium sorbate (MICs ranging from 0.1 to 1 g/l) and natamycin (MICs ranging from 0.04 to N 0.2 g/l) as confirmed by ANOVA and Fisher LSD test. The same ranking was obtained with MICs calculated in molar concentration. In the tested conditions, *C. halotolerans* was the most sensitive to preservatives exhibiting the lowest MICs, followed by *D. pinodella* which was highly sensitive to organic acids but slightly resistant to natamycin (Table 3). On the contrary, *P. commune* showed a high resistance to organic acids but had only a limited resistance to natamycin as compared to other fungal species. As shown in Table 3, *Y. lipolytica* was the most resistant species with the highest MIC for potassium sorbate and calcium propionate, sodium benzoate and natamycin MICs exceeded the highest tested concentrations. *Y. lipolytica* was followed.

Table 2  
Diversity of spoilage yeasts isolated from contaminated dairy products (n = number of sample) and factory air.

Species	Isolation source										Total in contaminated dairy products	Total in factory air		
	Butter (n = 1)	Cream (n = 2)	Cream cheese (n = 8)	Fresh cheese (n = 6)	Hard cheese (n = 48)	Pasteurized milk (n = 2)	Yoghurt (n = 4)	Yoghurt drink (n = 3)	Unknown origin					
<i>Candida inconspicua</i>	–	–	4	–	–	–	–	–	–	–	4	(3.8%)	0	(0.0%)
<i>Candida intermedia</i>	–	–	–	–	–	–	–	2	–	–	2	(1.9%)	0	(0.0%)
<i>Candida parapsilosis</i>	–	–	–	1	–	–	2	–	–	–	3	(2.9%)	0	(0.0%)
<i>Candida zeylanoides</i>	–	–	–	–	–	–	–	–	–	–	0	(0.0%)	2	(2.9%)
<i>Cryptococcus curvatus</i>	–	–	–	–	–	–	–	–	–	–	0	(0.0%)	1	(1.4%)
<i>Cryptococcus diffluens</i>	–	–	–	–	–	–	–	–	1	–	1	(1.0%)	0	(0.0%)
<i>Cryptococcus pseudolongus</i>	–	–	1	–	–	–	–	–	–	–	1	(1.0%)	0	(0.0%)
<i>Cryptococcus uniguttulatus</i>	–	–	–	–	–	–	–	–	1	–	1	(1.0%)	0	(0.0%)
<i>Debaryomyces hansenii</i>	–	–	–	–	1	–	–	–	–	–	1	(1.0%)	0	(0.0%)
<i>Galactomyces geotrichum</i>	–	–	1	–	–	–	–	1	1	–	3	(2.9%)	0	(0.0%)
<i>Kluyveromyces lactis</i>	–	–	2	–	–	–	–	–	–	–	2	(1.9%)	0	(0.0%)
<i>Kluyveromyces marxianus</i>	–	–	–	2	–	–	–	–	–	–	2	(1.9%)	0	(0.0%)
<i>Meyerozyma guilliermondii</i>	–	1	–	–	–	–	4	–	–	–	5	(4.8%)	0	(0.0%)
<i>Pichia fermentans</i>	–	–	–	3	–	–	–	–	–	–	3	(2.9%)	0	(0.0%)
<i>Rhodotorula graminis</i>	–	–	–	–	–	–	–	–	–	–	0	(0.0%)	1	(1.4%)
<i>Rhodotorula mucilaginosa</i>	–	–	–	2	–	–	–	–	1	–	3	(2.9%)	0	(0.0%)
<i>Sporidiobolus salmonicolor</i>	–	–	–	–	1	–	–	–	–	–	1	(1.0%)	0	(0.0%)
<i>Trichosporon asahii</i>	–	–	–	5	–	–	–	–	–	–	5	(4.8%)	0	(0.0%)
<i>Yarrowia lipolytica</i>	–	–	–	1	1	–	–	–	2	–	4	(3.8%)	1	(1.4%)
	0 (0.0%)	1 (1.0%)	8 (7.6%)	14 (13.3%)	3 (2.9%)	0 (0.0%)	6 (5.7%)	3 (2.9%)	6 (5.7%)		41 (39.0%)		5 (7.1%)	

Table 3  
Minimal inhibitory concentrations of chemical preservatives against 10 representative strains of the spoilage fungal species.

Species	Strain number	Isolation source	Minimal inhibitory concentrations (g/l)			
			Calcium propionate	Potassium sorbate	Sodium benzoate	Natamycin (mg/dm <sup>3</sup> )
<i>Cladosporium halotolerans</i>	UBOCC-A-116001	Pasteurized milk	2	0.1	0.3	0.04 (0.2)
<i>Galactomyces geotrichum</i>	UBOCC-A-216001	Fresh cheese	N 3 <sup>a</sup>	1	1	0.1 (0.5)
<i>Mucor racemosus</i>	UBOCC-A-116002	Semi-hard cheese	N 3 <sup>a</sup>	0.5	1	0.1 (0.5)
<i>Penicillium commune</i>	UBOCC-A-116003	Semi-hard cheese	N 3 <sup>a</sup>	0.3	2	0.06 (0.3)
<i>Didymella pinodella</i>	UBOCC-A-116004	Cream cheese	3 <sup>a</sup>	0.15	0.5	0.1 (0.5)
<i>Candida parapsilosis</i>	UBOCC-A-216002	Yoghurt	N 3 <sup>a</sup>	1	2	0.1 (0.5)
<i>Meyerozyma guilliermondii</i>	UBOCC-A-216003	Cream cheese	N 3 <sup>a</sup>	0.5	1	0.08 (0.4)
<i>Rhodotorula mucilaginosa</i>	UBOCC-A-216004	Fresh cheese	N 3 <sup>a</sup>	0.15	1	0.1 (0.5)
<i>Trichosporon asahii</i>	UBOCC-A-216005	Fresh cheese	N 3 <sup>a</sup>	0.5	1	0.1 (0.5)
<i>Yarrowia lipolytica</i>	UBOCC-A-216006	Semi-hard cheese	N 3 <sup>a</sup>	1	N 3 <sup>a</sup>	N 0.2 <sup>a</sup> (N 1.0)

<sup>a</sup> Highest tested concentrations.

by *C. parapsilosis* and *G. geotrichum* which also showed relatively high resistances to all chemical preservatives.

#### 4. Discussion

The first part of this study provided an insight into the biodiversity of fungal contaminants associated with dairy products. To achieve this goal, we used phenotypic and genotypic approaches including sequencing of taxonomically relevant target genes to identify fungal isolates as accurately as possible. Whereas the D1–D2 domain of the 26S rRNA gene was sufficient to identify yeasts at the species level, the ITS region which is considered as the universal barcode for fungal identification (Schoch et al., 2012) had a limited taxonomic resolution for certain species as previously reported (Schoch et al., 2012; Le Lay et al., 2016). This was especially true for *Penicillium* and *Cladosporium* spp. for which partial  $\beta$ -tubulin or elongation factor  $\alpha$  genes were used instead and allowed for accurate identification. Despite the sampling limitation of the present study, i.e., a limited number of spoiled products from each category or isolates from these products, one striking result was the large species diversity of fungi encountered in spoiled dairy products. Indeed, N 40 species were identified including 24 mold species. However, this can be explained by the fact that numerous fungal species are acid-tolerant, grow well at reduced  $a_w$  and in the presence of NaCl, and are able to grow at reduced temperature.

The *Penicillium* genus was largely dominant and found to occur in all product types except butter and a large species diversity was also observed within this genus. Most species belonged to the Brevicompacta and Fasciculata sections. Within the Brevicompacta section, *P. brevicompactum* but not *P. bialowiezense* has been reported as a major contaminant in Italian (Montagna et al., 2004) and Spanish cheeses (Barrios et al., 1998) as well as the most frequently occurring species in the indoor environment of 3 cheese factories in Norway (Kure et al., 2004). The reason why *P. bialowiezense* was not reported in these studies remains unclear but as underlined by Samson et al. (2002), it could have been misidentified as the closely related species *P. brevicompactum* due to similar colony and micromorphological characteristics (restricted colonies, broad penicilli with inflated metulae). It is also worth mentioning that in dairy factory air, we detected 2 recently described species from the Brevicompacta section *P. salamii* and *P. spathulatum*, isolated from Italian dry-cured meats (Perrone et al., 2015) and moldy cheeses (Frisvad et al., 2013). Species from the Brevicompacta section grow well at refrigerated temperatures and are often considered as the most xerophilic penicillia (Pitt and Hocking, 2009). Therefore, their occurrence in dairy products and in particular in semi-hard cheese is not surprising. Like *P. brevicompactum*, species from the Fasciculata section such as *P. commune*, *P. discolor*, *P. nordicum*, *P. palitans*, *P. solitum* as well as other species such as *P. roqueforti* are well known spoilage agents of dairy products (Filtenborg et al., 1996; Kure, 2001; Ledenbach and Marshall, 2010; Pitt and Hocking, 2009). In contrast to

our best knowledge, xerophilic species such as *P. charlesii* and *P. fellutanum* (Pitt and Hocking, 2009) as well as *P. adametzioides* and *P. antarcticum* originally described in marine and subglacial environments (Park et al., 2014; Sonjak et al., 2006), are reported here for the first time in dairy products. It is worth mentioning that *P. antarcticum* isolates from dairy products have already been deposited in culture collections such as the Central Bureau for Fungal Cultures (CBS).

As previously reported (Kure, 2001; Kure and Skaar, 2000; Taniwaki et al., 2001), *M. racemosus*, *M. circinelloides* and *M. spinosus* (formerly *Mucor plumbeus*, Hermet et al., 2012) are also common spoilage fungi. In addition, *T. elegans*, a species that also pertains to the *Mucorales* order was identified. This species has been previously reported to spoil Taleggio cheese (Dragoni et al., 1997).

*Didymella* and *Cladosporium* spp. which were isolated in the present study, have also been previously isolated from various dairy products such as raw milk, cream, butter, margarine and cheeses (Pitt and Hocking, 2009). However, in the latter studies, identification at the species level was not performed or solely relied on the use of phenotypic methods and these genera have undergone important taxonomical changes in the last decade. In the present study, using molecular tools, we showed that the *Cladosporium* spp. spoilage agents belonged to 2 halotolerant *Cladosporium* species from the *sphaerospermum* complex (*C. halotolerans* and *C. sphaerospermum*) as well as *Cladosporium phylophilum* reported here for the first time in butter. Among *Didymella* spp., it is worth mentioning that *D. glomerata* was recently shown to be responsible for the abnormal coloration of Mozzarella cheese (Casalinuovo et al., 2015). It would be interesting to collect a higher number of *Cladosporium* and *Didymella* isolates from spoiled dairy products in order to identify which species are really problematic.

In contrast to mold species, all the yeast species that were identified in the present study, including the yeast-like fungus *G. geotrichum*, have been previously reported to spoil dairy products (Banjara et al., 2015; Deak, 2007; Fleet and Mian, 1987; Westall and Filtenborg, 1998a). Most species were isolated from fresh dairy products and are known to be well adapted to dairy matrices and able to grow at refrigerated temperatures (Fleet and Mian, 1987).

In addition to the analysis of spoiled dairy products, we also analyzed the fungal diversity in the ambient air of dairy factories. While it was not possible to relate occurring species with the spoiling agents encountered in the samples as spoiled dairy products and air samples did not automatically originate from the same plant, we showed that numerous mold species encountered in dairy products were also present in the ambient air, as previously demonstrated for Norwegian cheeses (Kure et al., 2004). This is associated with the fact that filamentous fungi have spore forms in their life cycle that are easily propagated via airborne dissemination. As yeast do not exhibit this trait, only few yeast isolates were obtained from ambient air suggesting that yeast contamination originates from other sources including surfaces and equipment as well as other reservoirs such as water spraying, ingredients and

the factory personnel. It would be interesting to perform in-depth studies of the yeast and mold occurrence and diversity within dairy food industrial facilities (air, equipment and surfaces), in order to evaluate the sources of contamination and increase the efficiency of cleaning/disinfection procedures and air quality management.

Despite the use of preservatives in dairy products, fungal contamination still occurs showing that fungi may present resistance to these antifungal compounds. The second objective of this study was thus to determine the actual resistance of selected spoilage fungi to preservatives used in the dairy industry. Concerning organic acid salts, independently of the studied species, calcium propionate was found to be the least effective preservative followed by sodium benzoate, which in turn was less effective than potassium sorbate. Natamycin, a fungicide belonging to the macrolide polyene antibiotics used to control fungal growth on the cheese surface (Kallinteri et al., 2013) was effective against all tested species (MICs ranging from 0.04 to 0.1 g/l) except against *Y. lipolytica*, the MIC of which exceeded the highest tested concentration. There were also important MIC differences among fungal species (up to 10 orders of magnitude) suggesting that the resistance behavior to organic acids and natamycin was species-dependent. Similar results have been reported in other studies for food spoilage molds and yeasts (Le Lay et al., 2016; Ollé Resa et al., 2014; Praphailong and Fleet, 1997; Romero-Gil et al., 2016). It would be of interest to include more strains from each species and to use dose-response models to assess the susceptibility and resistance of these fungal species.

Few species presented very high resistance to the different preservatives especially taking into account the maximum levels authorized within the EU. Among them, *Y. lipolytica* showed a significant resistance to natamycin, calcium propionate and sodium benzoate (MICs greater than the highest tested concentrations, corresponding to the maximum authorized concentrations in dairy products) while sorbate resistance was also very important but for concentrations still under the legal limits for industrial applications. In a previous study (Praphailong and Fleet, 1997), *Y. lipolytica* was shown to possess a comparable organic acid resistance to that of the well-known preservative-resistant spoilage yeast, *Zygosaccharomyces baillii*, hypothetically suggesting its ability to limit the entry of undissociated acid into the cell, to potentially degrade preservatives and/or to pump out intracellular protons and anions which accumulate after dissociation of the acid into the cell. For instance, *Y. lipolytica* is able to use propionic but not sorbic acid as a sole carbon source, possibly explaining why it was more sensitive to sorbate than propionate given that both acids have similar pKa (4.76 and 4.87) (Rodrigues and Pais, 2000). Concerning the high resistance of the tested strain to natamycin; no evident explanation can be given but it can be hypothesized that it may be related to its ability to decrease the ergosterol content of the cell membrane, which is the target compound for natamycin (Welscher et al., 2008).

In conclusion, this study has revealed new and interesting information on the diversity and preservative resistance of spoilage fungi encountered in dairy products. A first step to control fungal contamination should be the examination of the production lines of each plant to identify possible points in the processes where the dairy products are exposed to mold and yeast contamination. Then, a comprehensive study on the different spoilage fungi could be done to provide better knowledge of these undesirable microorganisms. Altogether, these data will help developing new strategies to decrease fungal spoilage occurrence and/or control their growth in dairy products and thus reduce food waste.

Acknowledgments

We are grateful to Regional Council of Brittany (grant no. 13008651) and Pays de la Loire (grant no. 2014-07081) for the financial support and INRA for scientific coordination (J. Leonil) through the interregional project PROFIL, managed by BBA industrial association.

## References

- Banjara, N., Suhr, M.J., Hallen-Adams, H.E., 2015. Diversity of yeast and mold species from a variety of cheese types. *Curr. Microbiol.* 70, 1–9.
- Barrios, M.J., Medina, L.M., Lopez, M.C., Jordano, R., 1998. Fungal biota isolated from Spanish cheeses. *J. Food Saf.* 18, 151–157.
- Bourdichon, F., Casaregola, S., Farrok, C., Frisvad, J.C., Gerds, M.L., Hammes, W.P., Harnett, J., Huys, G., Laulund, S., Ouwehand, A., Powell, I.B., Prajapati, J.B., Seto, Y., Ter Schure, E., Van Boven, A., Vankerckhoven, V., Zgoda, A., Tuijelaars, S., Hansen, E.B., 2012. Food fermentations: microorganisms with technological beneficial use. *Int. J. Food Microbiol.* 154, 87–97.
- Brooks, J.C., Martinez, B., Stratton, J., Bianchini, A., Krokstrom, R., Hutkins, R., 2012. Survey of raw milk cheeses for microbiological quality and prevalence of foodborne pathogens. *Food Microbiol.* 31, 154–158.
- Canganella, F., Ovidi, M., Paganini, S., Vetraino, A.M., Bevilacqua, L., Trovatielli, L.D., 1998. Survival of undesirable micro-organisms in fruit yoghurts during storage at different temperatures. *Food Microbiol.* 15, 71–77.
- Carbone, I., Kohn, L.M., 1999. A method for designing primer sets for speciation studies in filamentous ascomycetes. *Mycologia* 91, 553.
- Casalnuovo, F., Rodolfi, M., Ripa, P., Scognamiglio, A., Musarella, R., 2015. Abnormal colorations of mozzarella cheese caused by *Phoma glomerata* (Corda) Wollenw Hochapfel. *J. Food Res.* 4, 19.
- Commission Regulation (EU) No 1129/2011, 2011. Amending Annex II to Regulation (EC) No 1831/2003 of the European Parliament and of the Council by Establishing a Union List of Food Additives Text With EEA Relevance (11 November).
- Deák, T., 2008. Handbook of Food Spoilage Yeasts. 2nd ed. CRC Press, Boca Raton, FL, USA.
- Delavenne, E., Mounier, J., Deniel, F., Barbier, G., Le Blay, G., 2012. Biodiversity of antifungal lactic acid bacteria isolated from raw milk samples from cow, ewe and goat over one-year period. *Int. J. Food Microbiol.* 155, 185–190.
- Dragoni, L., Papa, A., Vallone, L., Milan Univ. (Italy) Istituto di Ispezione degli Alimenti di Origine Animale, 1997. Mycological quality standards of Italian cheese Taleggio. Typical and unacceptable surface moulds – mycological paste standard [Lombardy]. Arch. Vet. Ital. Italy.
- Filttenborg, O., Frisvad, J.C., Thrane, U., 1996. Moulds in food spoilage. *Int. J. Food Microbiol.* 33, 85–102 Specific Spoilage Organisms.
- Fleet, G.H., Mian, M.A., 1987. The occurrence and growth of yeasts in dairy products. *Int. J. Food Microbiol.* 4, 145–155.
- Frisvad, J.C., Houbraken, J., Popma, S., Samson, R.A., 2013. Two new *Penicillium* species *Penicillium buchwaldii* and *Penicillium spathulatum*, producing the anticancer compound asperphenamate. *FEMS Microbiol. Lett.* 339, 77–92.
- Glass, N.L., Donaldson, G.C., 1995. Development of primer sets designed for use with the PCR to amplify conserved genes from filamentous ascomycetes. *Appl. Environ. Microbiol.* 61, 1323–1330.
- Hermet, A., Méheust, D., Mounier, J., Barbier, G., Jany, J.-L., 2012. Molecular systematics in the genus *Mucor* with special regards to species encountered in cheese. *Fungal Biol.* 116, 692–705.
- Hong, S.-B., Go, S.-J., Shin, H.-D., Frisvad, J.C., Samson, R.A., 2005. Polyphasic taxonomy of *Aspergillus fumigatus* and related species. *Mycologia* 97, 1316–1329.
- Houbraken, J., Samson, R.A., 2011. Phylogeny of *Penicillium* and the segregation of *Trichocomaceae* into three families. *Stud. Mycol.* 70, 1–51.
- Huis in't Veld, J.H.J., 1996. Microbial and biochemical spoilage of foods: an overview. *Int. J. Food Microbiol.* 33, 1–18 Specific spoilage organisms.
- Hymery, N., Vasseur, V., Coton, M., Mounier, J., Jany, J.-L., Barbier, G., Coton, E., 2014. Filamentous fungi and mycotoxins in cheese: a review. *Compr. Rev. Food Sci. Food Saf.* 13, 437–456.
- Jacques, N., Casaregola, S., 2008. Safety assessment of dairy microorganisms: The hemiascomycetous yeasts. *Int. J. Food Microbiol.* 126, 321–326 Contribution to the safety assessment of technological microflora found in fermented dairy products.
- Kallinteri, L.D., Kostoula, O.K., Savvaidis, I.N., 2013. Efficacy of nisin and/or natamycin to improve the shelf-life of Galotyri cheese. *Food Microbiol.* 36, 176–181.
- Kure, C., 2001. Mould contaminants on Jarlsberg and Norvegia cheese blocks from four factories. *Int. J. Food Microbiol.* 70, 21–27.
- Kure, C.F., Skaar, I., 2000. Mould growth on the Norwegian semi-hard cheeses Norvegia and Jarlsberg. *Int. J. Food Microbiol.* 62, 133–137.
- Kure, C.F., Skaar, I., Brendehaug, J., 2004. Mould contamination in production of semi-hard cheese. *Int. J. Food Microbiol.* 93, 41–49.
- Kurtzman, C.P., Robnett, C.J., 1997. Identification of clinically important ascomycetous yeasts based on nucleotide divergence in the 5' end of the large-subunit (26S) ribosomal DNA gene. *J. Clin. Microbiol.* 35, 1216–1223.
- Ledenbach, L.H., Marshall, R.T., 2010. Microbiological spoilage of dairy products. In: Sperber, W.H., Doyle, M.P. (Eds.), *Compendium of the Microbiological Spoilage of Foods and Beverages*, Food Microbiology and Food Safety. Springer, New York, pp. 41–67.
- Le Lay, C., Mounier, J., Vasseur, V., Weill, A., Le Blay, G., Barbier, G., Coton, E., 2016. *In vitro* and *in situ* screening of lactic acid bacteria and propionibacteria antifungal activities against bakery product spoilage molds. *Food Control* 60, 247–255.
- López-Malo, A., Maris Alzamora, S., Palou, E., 2005. *Aspergillus flavus* growth in the presence of chemical preservatives and naturally occurring antimicrobial compounds. *Int. J. Food Microbiol.* 99, 119–128.
- Montagna, M.T., Santacroce, M.P., Spilotros, G., Napoli, C., Minervini, F., Papa, A., Dragoni, I., 2004. Investigation of fungal contamination in sheep and goat cheeses in southern Italy. *Mycopathologia* 158, 245–249.
- Ollé Resa, C.P., Jagus, R.J., Gerschenson, L.N., 2014. Natamycin efficiency for controlling yeast growth in models systems and on cheese surfaces. *Food Control* 35, 101–108.
- Park, M.S., Lee, E.J., Fong, J.J., Sohn, J.H., Lim, Y.W., 2014. A new record of *Penicillium antarcticum* from marine environments in Korea. *Mycobiology* 42, 109.

- Perrone, G., Samson, R.A., Frisvad, J.C., Susca, A., Gunde-Cimerman, N., Epifani, F., Houbraeken, J., 2015. *Penicillium salamii*, a new species occurring during seasoning of dry-cured meat. *Int. J. Food Microbiol.* 193, 91–98.
- Phillips, C.A., 1996. Review: Modified Atmosphere Packaging and its effects on the microbiological quality and safety. *Int. J. Food Sci. Technol.* 31, 463–479.
- Pitt, J.I., Hocking, A.D., 2009. *Fungi and Food Spoilage*. 3rd ed. Springer Science Business Media, New York, USA.
- Praphailong, W., Fleet, G.H., 1997. The effect of pH, sodium chloride, sucrose, sorbate and benzoate on the growth of food spoilage yeasts. *Food Microbiol.* 14, 459–468.
- Rodrigues, G., Pais, C., 2000. The influence of acetic and other weak carboxylic acids on growth and cellular death of the yeast *Yarrowia lipolytica*. *Food Technol. Biotechnol.* 12, 579–1006.
- Romero-Gil, V., García-García, P., Garrido-Fernández, A., Arroyo-López, F.N., 2016. Susceptibility and resistance of lactic acid bacteria and yeasts against preservatives with potential application in table olives. *Food Microbiol.* 54, 72–79.
- Sakkas, L., Moutafi, A., Moschopoulou, E., Moatsou, G., 2014. Assessment of heat treatment of various types of milk. *Food Chem.* 159, 293–301.
- Samson, R., Frisvad, J., Hoekstra, E., Filtenborg, O., 2002. Introduction to food and airborne fungi. Revised 6th edition. Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands 389 pp. Distributed in the United States by ASM Press., in: *Mycopathologia*. pp. 609–609.
- Schoch, C.L., Seifert, K.A., Huhndorf, S., Robert, V., Spouge, J.L., Levesque, C.A., Chen, W., 2012. Nuclear ribosomal internal transcribed spacer (ITS) region as a universal DNA barcode marker for fungi. *Proc. Natl. Acad. Sci. U. S. A.* 109, 6241–6246.
- Sonjak, S., Frisvad, J.C., Gunde-Cimerman, N., 2006. *Penicillium mycobiota in arctic subglacial ice*. *Microb. Ecol.* 52, 207–216.
- Tamura, K., Stecher, G., Peterson, D., Filipiński, A., Kumar, S., 2013. MEGA6: molecular evolutionary genetics analysis version 6.0. *Mol. Biol. Evol.* 30, 2725–2729.
- Taniwaki, M.H., Hocking, A.D., Pitt, J.I., Fleet, G.H., 2001. Growth of fungi and mycotoxin production on cheese under modified atmospheres. *Int. J. Food Microbiol.* 68, 125–133.
- Vacheyrou, M., Normand, A.-C., Guyot, P., Cassagne, C., Piarroux, R., Bouton, Y., 2011. Cultivable microbial communities in raw cow milk and potential transfers from stables of sixteen French farms. *Int. J. Food Microbiol.* 146, 253–262.
- Welscher, Y.M., Napel, H.H., Balagué, M.M., Souza, C.M., Riezman, H., de Kruijff, B., Breukink, E., 2008. Natamycin blocks fungal growth by binding specifically to ergosterol without permeabilizing the membrane. *J. Biol. Chem.* 283, 6393–6401.
- Westall, S., Filtenborg, O., 1998a. Spoilage yeasts of decorated soft cheese packed in modified atmosphere. *Food Microbiol.* 15, 243–249.
- Westall, S., Filtenborg, O., 1998b. Yeast occurrence in Danish feta cheese. *Food Microbiol.* 15, 215–222.
- White, T.J., Bruns, T., Lee, S., Taylor, J.W., 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis, M.A., Gelfand, D.H., Sninsky, J.J., White, T.J. (Eds.), *PCR Protocols: A Guide to Methods and Applications*. Academic Press, Inc., New York, pp. 315–322.

1  
2 En conclusion de cette Etude 1 ~ Diversité des contaminations fongiques associée à une  
3 variété de produits laitiers français:  
4

5 *Suite à ce travail, nous disposons d'une collection de contaminants de référence constituée*  
6 *de 132 et 38 souches de champignons filamenteux et de levures, respectivement. C'est dans ce*  
7 *panel de souches bien identifiées que 10 souches, appartenant aux espèces les plus*  
8 *fréquemment retrouvées dans des produits laitiers contaminés, à savoir M. racemosus*  
9 *UBOCC-A-116002, P. commune UBOCC-A-116003, G. geotrichum UBOCC-A-216001, Y.*  
10 *lipolytica UBOCC-A-216006, P. bialowiezense 5f22, Trichosporon asahii UBOCC-A-216005,*  
11 *Candida parapsilosis UBOCC-A-216002, Cryptococcus pseudolongus 1f7b, Rhodotorula*  
12 *mucilaginosa UBOCC-A-216004 et D. pinodella UBOCC-A-116004, ont été sélectionnées*  
13 *comme cibles pour le criblage de l'activité antifongique et l'étude du spectre d'action des*  
14 *ingrédients antifongiques les plus actifs.*

15

16

17

18

19

20

21

22

23

## **Etude 2**

24

~

25

**Développement d'une méthode de**

26

**criblage haut-débit pour détecter des**

27

**activités antifongiques dans un modèle**

28

**mimant le fromage.**

29



31 Ces dernières années, la demande des consommateurs pour plus de naturalité et l'évolution de  
32 la réglementation ont mené les industriels à explorer le potentiel de certains microorganismes  
33 à inhiber les contaminations fongiques dans le but de développer des cultures bioprotectrices.  
34 Il existe ainsi un certain nombre de cultures antifongiques disponibles sur le marché telles que  
35 FreshQ® (*L. plantarum* et *L. rhamnosus*) et HOLDBAC® (*L. plantarum*) et que nous avons  
36 utilisé comme référence dans le cadre de notre étude. Récemment, Inglin *et al.* (2015) ont  
37 développé une méthode de criblage haut-débit pour détecter rapidement des activités  
38 antifongiques en utilisant des plaques de 24 puits. Cette méthode permet le criblage de 2 000  
39 interactions antifongiques individuelles par jour. Néanmoins, ce criblage a été réalisé *in vitro*  
40 sur des milieux synthétiques très éloignés des matrices alimentaires et qui négligent les effets  
41 potentiels du milieu de criblage sur l'activité antifongique et l'expression de cette activité par  
42 les microorganismes. Nous présentons ici une méthode de criblage haut-débit pour détecter  
43 rapidement des activités antifongiques dans une matrice mimant le fromage, distribuée en  
44 plaque de 24 puits. Avec cette nouvelle méthode, nous sommes capables de tester l'activité  
45 antifongique de composés naturels, tels que les fermentats ou chimiques déposés à la surface  
46 du milieu, mais nous sommes aussi capables de tester des cultures bioprotectrices utilisées en  
47 tant que ferment.

48 Dans la première partie de ce travail, l'activité antifongique de 44 fermentats résultant de la  
49 fermentation d'un substrat laitier par des bactéries lactiques a été testée contre 4 cibles  
50 fongiques (*G. geotrichum* UBOCC-A-216001, *M. racemosus* UBOCC-A-116002, *P.*  
51 *commune* UBOCC-A-116003 et *Y. lipolytica* UBOCC-A-216006). Le milieu mimant le  
52 fromage utilisé est un retentat d'ultrafiltration (6X) emprésuré et inoculé avec un ferment  
53 lactique commercial. Les détails de la méthode sont présentés dans la publication associée au  
54 chapitre II.

55

## 56 **Principaux résultats**

### 57 **Fermentats**

58 Sur les fermentats testés issus de 47 souches de bactéries lactiques appartenant à 12 espèces,  
59 23 ont montré une activité antifongique contre au moins une des quatre cibles testées. Les  
60 fermentats actifs appartiennent principalement aux espèces *L. brevis*, *L. casei*, *L. paracasei* et  
61 *L. plantarum*. Pour une espèce donnée, les différentes souches testées ne montrent pas la  
62 même activité antifongique confirmant ainsi le caractère souche-dépendant de l'activité  
63 antifongique des microorganismes étudiés dans ces conditions.



## 64                   **Cultures bioprotectrices**

65    Parmi les 23 cultures testées, toutes celles qui présentaient une activité en tant que fermentat  
66    présentaient également une activité lorsqu'elles étaient utilisées en tant que cultures  
67    bioprotectrices associées au levain lactique commercial. Enfin, dans les conditions testées, *P.*  
68    *commune* UBOCC-A-116003 était la souche la plus fréquemment inhibée, suivie de  
69    *M. racemosus* UBOCC-A-116002. Quant aux souches *G. geotrichum* UBOCC-A-216001 et  
70    *Y. lipolytica* UBOCC-A-216006, leur croissance était peu affectée par les cultures  
71    bioprotectrices testées.

72

## 73                   **Résistance à la natamycine**

74    Nous avons également utilisé notre méthode pour déterminer la sensibilité de nos cibles  
75    fongiques à la natamycine. Nous avons ainsi montré que *M. racemosus* UBOCC-A-116002 et  
76    *T. asahii* UBOCC-A-216005 sont les champignons les plus sensibles à la natamycine, suivi de  
77    *P. commune* UBOCC-A-116003. Au contraire, la souche *Y. lipolytica* UBOCC-A-216006  
78    montre une forte résistance à la natamycine, avec une CMI très proche des concentrations  
79    maximales autorisées dans les produits laitiers en Europe. Les CMI observées sont plus  
80    faibles dans le milieu fromage que dans un milieu de laboratoire (Garnier *et al.*, 2017)  
81    indiquant que d'autres paramètres, de nature abiotique propre à la matrice comme son pH,  
82    l' $a_w$  ou encore sa composition physico-chimique mais aussi biotique tels que les métabolites  
83    (acide lactique, diacétyle) produits par le ferment lactique commercial utilisé pour la  
84    fabrication du fromage, potentialise leurs activités. Ces résultats soulignent l'importance  
85    majeure du « milieu de criblage » sur l'activité antifongique de microorganismes, de leurs  
86    métabolites ou encore de molécules purifiées.

87

88    Il est également important de noter que cette méthode de criblage permet de tester en duplicat  
89    environ 200 fermentats contre 4 cibles fongiques en 1 semaine, temps de croissance compris.  
90    En effet, environ 2000 essais peuvent être réalisés en une journée, à cette journée s'ajoutent  
91    les temps de croissance des cibles fongiques utilisées. Ces résultats montrent ainsi le caractère  
92    haut débit de la méthode.

93

94    *Ce travail fait l'objet d'un article soumis sous forme de « Technical Note » dans le « Journal*  
95    *of Dairy Science » et est présenté ci après en anglais.*

96 **Technical note: High-throughput method for antifungal activity screening in a cheese-**  
97 **mimicking model**

98

99 Lucille Garnier<sup>1, 2</sup>, Marcia Leyva Salas<sup>1, 2</sup>, Nicolas Pinon<sup>2</sup>, Norman Wiernasz<sup>1</sup>, Audrey  
100 Pawtowski<sup>1</sup>, Emmanuel Coton<sup>1</sup>, Jérôme Mounier<sup>1</sup> and Florence Valence<sup>2\*</sup>.

101

102 <sup>1</sup> Université de Brest, EA 3882, Laboratoire Universitaire de Biodiversité et Ecologie  
103 Microbienne, IBSAM, ESIAB, Technopôle Brest-Iroise, 29280 Plouzané, France.

104 <sup>2</sup> STLO, Agrocampus Ouest, INRA, 35000 Rennes, France

105

106 Lucille Garnier : [lucille.garnier@univ-brest.fr](mailto:lucille.garnier@univ-brest.fr)

107 [Marcia Leyva Salas : marcia.leyvasalas@univ-brest.fr](mailto:marcia.leyvasalas@univ-brest.fr)

108 Nicolas Pinon: [pinon\\_nicolas@orange.fr](mailto:pinon_nicolas@orange.fr)

109 Norman Wiernasz: [norman.wiernasz@oniris-nantes.fr](mailto:norman.wiernasz@oniris-nantes.fr)

110 Audrey Pawtowski: [audrey.pawtowski@univ-brest.fr](mailto:audrey.pawtowski@univ-brest.fr)

111 Emmanuel Coton : [emmanuel.coton@univ-brest.fr](mailto:emmanuel.coton@univ-brest.fr)

112 [Jérôme Mounier: jerome.mounier@univ-brest.fr](mailto:jerome.mounier@univ-brest.fr)

113

114 **\*Corresponding author:** Florence Valence

115 INRA, UMR1253 Science et Technologie du Lait et de l'Œuf

116 65 rue de Saint-Brieuc

117 35042 Rennes Cedex

118 Tel : +33(0)2 23 48 53 23

119 E-mail: [florence.valence-bertel@inra.fr](mailto:florence.valence-bertel@inra.fr)

120

121 Keywords: high-throughput screening, antifungal activity, cheese model

122 **Abstract**

123 In this study, a high-throughput antifungal activity screening method was developed using a  
124 cheese-mimicking matrix distributed in 24-well plates. This method allowed a fast antifungal  
125 activity screening of a large variety of antifungal agent candidates: bacterial fermented  
126 ingredients, bacterial isolates or preservatives. Using our method we characterized the  
127 antifungal activity of 44 lactic acid bacteria (LAB) milk-based fermented ingredients and 23  
128 LAB isolates used as protective cultures against 4 fungal targets (*Mucor racemosus*,  
129 *Penicillium commune*, *Galactomyces geotrichum* and *Yarrowia lipolytica*). We also used this  
130 method to determine the minimum inhibitory concentration (MIC) of a preservative, the  
131 natamycin, against 9 fungal targets. The obtained results underlined the strain-dependency of  
132 the antifungal LAB activity, the strong impact of the fermentation substrate on this activity as  
133 well as the impact of the screening medium on the natamycin MIC value. Our method could  
134 achieved a screening rate of 1.600 assays per week and can be implemented for various  
135 antifungal activity evaluations of microorganisms, fermentation products or purified  
136 compounds compatible with dairy technology.

137 Fungal spoilage of dairy products remains a major concern for dairy manufacturers  
138 despite the use of preventive and control approaches, including the use of chemical  
139 preservatives. Among methods that are increasingly used by industries for microbial  
140 contaminant control, biopreservation is gaining more and more attention due to a strong  
141 societal demand for preservative-free products (Gerez et al., 2013) and legislation evolution  
142 (Fuselli et al., 2012; Stratford et al., 2014). Biopreservation can be defined as a method using  
143 natural or added microbiota or their antimicrobial compounds allowing the preservation of a  
144 food product (in terms of safety and quality), and possibly the extension of its shelf-life (Stiles  
145 1996). Lactic acid bacteria (LAB) are well known for their ability to exhibit antifungal  
146 activity (Cheong, 2014; Wulijidelligen, 2011; Schillinger et al., 2010) and protective cultures  
147 containing LAB, such as FreshQ (consisting of *Lactobacillus plantarum* and *Lactobacillus*  
148 *rhamnosus* strains) or the HOLDBAC series (consisting of various lactobacilli and  
149 propionibacteria), are currently used in dairy products for their antifungal properties (Varsha  
150 et al., 2016). Recently, Inglin et al. (2015) developed a high-throughput screening method to  
151 detect antimicrobial (including antifungal) activities using an agar-spot assay in 24-well  
152 plates. This method enabled screening of 2.000 assays per day. However, despite being a  
153 useful primary high-throughput screening method for developing protective cultures, this  
154 antifungal screening assay used Mann, Rogosa and Sharpe (MRS) agar medium which can  
155 strongly impact expression of antifungal properties and/or impact antifungal molecule  
156 activity. Indeed, as mentioned in several papers (Stiles et al. 2002; Delavenne et al. 2012; Le  
157 Lay et al, 2016), MRS contains acetate which may reinforce LAB antifungal activity and  
158 artificially inflate the number of active isolates. However, assays applied to real food matrices  
159 (e.g. dairy product models such as yogurt) can rapidly become labor intensive thereby  
160 reducing the number of isolates that can be screened to detect strains with antifungal activity

161 (Delavenne et al., 2014; Lynch et al., 2014). In addition, these methods do not allow large  
162 scale and rapid screening of antifungal cultures or compounds.

163 In the present study, we present a high-throughput antifungal activity screening assay in  
164 a cheese-mimicking matrix distributed in a 24-well plate. The proposed method allowed  
165 testing the antifungal activity of a large variety of microbial protective compounds: bacterial  
166 fermentation products, bacterial cultures used as adjunct cultures and purified antifungal  
167 preservatives. To prepare a cheese-mimicking matrix, we used a standardized ultrafiltration  
168 retentate (6X) prepared as follows: whole raw cow milk was heated to 50 °C and skimmed  
169 using a cream separator (Westfalia, Handelsweg, Nederland). Skim milk was then micro-  
170 filtered (0.8 µm Sterilox GP membrane) at 50 °C using the pilot equipment GP7 (Brenet,  
171 Mamirole, France) and cream was heat-treated for 2 min at 95 °C followed by fat  
172 standardization (20g/kg final concentration). The retentate was ultrafiltered at 0.02 µm at  
173 50°C (T.I.A., Bollene, France), sterilized sodium chloride (Sogebul, Sainte-Maure de  
174 Touraine, France) was then added to reach 0.7% (w/w). The salted retentate was heat-treated  
175 for 2 min at 95°C (Microthermics, Raleigh, USA), distributed in 1L sterile bottles and kept at  
176 -20°C until use. Before plate preparation, 10 ml/L of a pH indicator (sterile solution of Litmus  
177 50 g/l), 10<sup>6</sup> colony forming unit (CFU)/L of commercial starters MA016 (*Lactococcus lactis*  
178 subsp. *cremoris* and *L. lactis* subsp. *lactis*, Elimeca, Thoissey, France) and 1.5 ml/L of 5X  
179 diluted and filtered (0.22 µm) rennet (Danisco, Dangé Saint-Romain, France) were added to  
180 the thawed retentate. After vigorous homogenization for 1 min, the retentate was distributed  
181 into 24-well plates (2 ml/well) and incubated for 1 h at 30 °C and for 3 days at 20 °C, leading  
182 to the formation of a "mini-cheese" in each well. The exudate was then removed from the  
183 surface of each mini-cheese and plates were stored at 12 °C until use. Using this cheese  
184 mimicking matrix, we have screened the antifungal activity of 44 LAB milk-based  
185 fermentation products and 23 LAB isolates used as protective cultures against 4 fungal

186 targets. LAB were obtained from the CIRM-BIA (Rennes, France) and LUBEM (Plouzané,  
187 France) culture collections. For fermentation product preparation, a 10%-reconstituted low  
188 heat milk supplemented with 45% anhydrous milk fat and 0.5% Litmus, named “LH”, was  
189 sterilized for 30 min at 110°C. Then, it was individually inoculated with a suspension of the  
190 LAB to be tested (1% v/v), obtained after 2 subcultures of 24 h at 30°C in MRS broth (Difco,  
191 Le Pont de Claix, France). LH medium inoculated with LAB was then incubated for 20 h at  
192 30°C. A hundred µL of the resulting fermentation products were then deposited on the surface  
193 of each “mini-cheeses”. After drying for 2h at room temperature under a laminar air flow,  
194 plates were then surface inoculated with one of 4 fungal targets, namely *Mucor racemosus*  
195 UBOCC-A-116002, *Penicillium commune* UBOCC-A-116003, *Galactomyces geotrichum*  
196 UBOCC-A-216001 and *Yarrowia lipolytica* UBOCC-A-216006, which were previously  
197 isolated from dairy products (Garnier et al. 2017) and were obtained from the Université de  
198 Bretagne Occidentale Culture Collection (UBOCC, Plouzané, France). Ten microliters of a  
199  $5.10^3$  spores or cells/ml, obtained as described previously (Delavenne et al., 2012), except for  
200 yeasts that were first precultured in PDB, were spotted at the center of each mini-cheese (1  
201 tested fungus/plate). Plates were then incubated at 12°C for 5, 6 and 8 days for *M. racemosus*,  
202 *G. geotrichum* and *P. commune* and *Y. lipolytica* respectively. Antifungal activity was then  
203 determined by visually evaluating fungal growth in comparison with a negative control  
204 without any fermentation product (Fig. 1). In order to test the antifungal activity of LAB  
205 isolates (n=23) for potential use as protective cultures, we applied the same methodology  
206 except that LAB isolates to be tested were individually suspended in sterilized milk to reach a  
207 final concentration of  $10^7$  CFU/ml of retentate and inoculated concomitantly with the  
208 commercial starter MA16, the pH indicator and the rennet before distribution in 24-well  
209 plates. In this context, 23 LAB cultures were tested in duplicate against the same fungal  
210 targets as those described above, after 2 pre-cultures for 24h at 30°C in MRS broth.

211 Incubation to obtain the “mini-cheeses” was performed as described above. Plates were then  
212 surface-inoculated with one of the 4 fungal targets, and the antifungal activity evaluated as  
213 described above.

214 All tested fungal species grew well in the cheese-mimicking model and the obtained results  
215 for both replicates were similar, confirming the suitability of this matrix to sustain fungal  
216 growth and the reproducibility of our screening method. Among the 46 tested LAB  
217 fermentation products, 25 showed antifungal activities against at least one fungal target while  
218 22 out of the 23 tested protective cultures showed antifungal activity against at least one  
219 target. More precisely, intermediate antifungal activities against at least one fungal target  
220 were observed for 14 (30%) fermentation products and 12 (55%) isolates, while complete  
221 inhibition against at least one fungal target, was only observed for LAB isolates (n=10; 45%)  
222 (Table 1). The most active (++ and +++) isolates or fermentation products corresponded to  
223 *Lactobacillus casei*, *L. plantarum*, *Lactobacillus brevis*, *Lactobacillus paracasei*,  
224 *Leuconostoc mesenteroides* and to a lesser extent to *L. rhamnosus*. Antifungal activity varied  
225 depending on the utilization mode (either as protective culture or as fermentation product).  
226 For example, for *L. plantarum* strains tested both for their fermentation products and as  
227 protective culture, fermentation products showed no activity against *M. racemosus* while they  
228 could inhibit the latter when used as protective cultures (Table 1). On the contrary, *L. brevis*  
229 CIRM- BIA 608 showed only activity against *M. racemosus* and *P. commune* when used as a  
230 fermentation product. None of the tested *Lactobacillus pentosus*, *Lactobacillus sakei* and  
231 *Leuconostoc citreum* isolates showed any activity (Table 1). As expected, among a same  
232 species, not all tested isolates showed the same antifungal activity. For example, each tested  
233 *L. casei* isolate showed antifungal activity but the inhibited fungal target and the obtained  
234 antifungal scores (+, ++ or +++) were different among them; thus confirming that the  
235 antifungal activity is a strain-dependent trait as suggested by Cortés-Zavaleta et al. (2014).



236 Finally, in the tested conditions, *P. commune* was the most frequently inhibited target,  
237 followed by *M. racemosus* and *G. geotrichum*. Concerning *Y. lipolytica*, none of the strains  
238 used to produce fermentation products were able to inhibit its growth while only 3 strains,  
239 used as protective cultures (*L. brevis* CIRM-BIA 128, *Lactobacillus harbinensis* L244 and  
240 *L. mesenteroides* CIRM-BIA 1187) showed intermediate antifungal activity against this target  
241 (Table 1). Overall, antifungal activity of the tested strains was higher when they were used as  
242 protective cultures; indeed, all isolate did show antifungal activity.

243 The proposed method was also used to evaluate the resistance to natamycin (a natural  
244 antibiotic produced by *Streptomyces natalensis* used for fungal control on cheese surface) of 9  
245 fungi, including those previously mentioned and *Phoma pinodella* UBOCC-A-116004,  
246 *Candida parapsilosis* UBOCC-A-216002, *Meyerozyma guilliermondii* UBOCC-A-216003,  
247 *Trichosporon asahii* UBOCC-A-216005 and *Rhodotorula mucilaginosa* UBOCC-A-216004  
248 (Garnier et al. 2017). Natamycin resistance was tested at a similar concentration range (from 0  
249 to 0.2 mg/dm<sup>2</sup>) as that used by Garnier et al. (2017). Natamycin was deposited on the mini-  
250 cheese” surface using 100 µl of a stock solution followed by fungal inoculation on the cheese  
251 surface as described above. The minimum inhibitory concentration (MIC) was determined as  
252 the concentration for which no visible growth occurred after the incubation period (Table 2).  
253 The obtained MIC values were then compared to those obtained by Garnier et al. (2017) in  
254 potato dextrose agar at pH 5. Independently of the fungal target, natamycin MICs were  
255 generally slightly lower in the cheese model than in PDA. This result may be explained by  
256 differences in the physiological status of the fungal targets as well as by the fact that other  
257 compounds present in the cheese may enhance natamycin activity. For example, lactic acid  
258 produced by lactococci starter cultures could act synergistically with natamycin.  
259 *M. racemosus* and *T. asahii* were the most sensitive fungi to natamycin with a MIC of 0.04  
260 mg/dm<sup>2</sup>, followed by *P. commune* (0.06 mg/dm<sup>2</sup>) (Table 2). *Y. lipolytica* showed the highest

261 resistance to natamycin with a MIC of 0.16 mg/dm<sup>2</sup> which is very close to the maximum  
262 authorized concentration (0.2 mg/dm<sup>2</sup>) that can be used in dairy products within the EU.

263 The main objective of this work was to develop a high-throughput screening method to  
264 evaluate antifungal activities of various antifungal agent candidates in a matrix as close as  
265 possible to the real dairy products, such as cheese, in terms of physical, chemical and  
266 biological composition. The proposed method, based on the use of a cheese mimicking-model  
267 distributed in 24-well plates, could achieve screening rate of 1600 assays per week, is easy to  
268 implement and did not require complex and high cost equipment. Such screening matrix,  
269 closer to an actual dairy product, makes antifungal activity screening more robust with  
270 regards to scale up validation which follows the screening step. It also allows antifungal  
271 activity screening of protective cultures, fermentation products or purified molecules and thus  
272 offers new perspectives for developing new biopreservation solutions.

273

#### 274 **Acknowledgments**

275 This work was financially supported by the Conseils Régionaux de Bretagne (grant  
276 N°13008651) and Pays de la Loire (grant N°2014-01081) in the framework of the PROFIL  
277 interregional project under the scientific coordination of INRA (J. Leonil) and managed by  
278 the Bretagne Biotechnologies Alimentaires industrial association.

279

280 **References**

281

282 Cheong, E. Y. L., Sandhu, A., Jayabalan, J., Kieu L., Thu T., Nhiep, Nguyen T., My H.,  
283 Huong T., Zwielehner, J., Bansal, N., Turner, M. S., 2014. Isolation of lactic acid bacteria  
284 with antifungal activity against the common cheese spoilage mould *Penicillium commune* and  
285 their potential as biopreservatives in cheese. Food Control, 46, 91-97.

286

287 Cortés-Zavaleta O., López-Malo A., Hernández-Mendoza A., García H.S. (2014). Antifungal  
288 activity of lactobacilli and its relationship with 3-phenyllactic acid production. Int. J. Food  
289 Microbiol. 173, 30–35

290

291 Delavenne, E., Cliquet, S., Trunet, C., Barbier, G., Mounier, J., Le Blay, G., 2014.  
292 Characterization of the antifungal activity of *Lactobacillus harbinensis* K.V9.3.1Np and  
293 *Lactobacillus rhamnosus* K.C8.3.1I in yogurt. Food Microbiol. 45, 10-7.

294

295 Delavenne, E., Mounier, J., Deniel, F., Barbier, G., Le Blay, G., 2012. Biodiversity of  
296 antifungal lactic acid bacteria isolated from raw milk samples from cow, ewe and goat over  
297 one-year period. Int. J. Food Microbiol. 155, 185-190.

298

299 Fuselli, F., Guarino, C., La Mantia, A., Longo, L., Faberi, A., Marianella, R.M., 2012. Multi-  
300 detection of preservatives in cheeses by liquid chromatography-tandem mass spectrometry. J.  
301 Chromatogr. B. 906, 9-18.

302

303 Garnier, L., Valence, F., Pawtowski, A., Auhustsinava-Galerie, L., Frotté, N., Deniel, F.,  
304 Coton, E., Mounier, J., 2017. Diversity of spoilage fungi associated with various French dairy  
305 products. *Int. J. Food Microbiol.* 241, 191-197.  
306  
307 Gerez, C.L., Torres, M.J., Font de Valdez, G., Rollan, R., 2013. Control of spoilage fungi by  
308 lactic acid bacteria. *Biol. Control* 64, 231-237.  
309  
310 Inglin, R.C., Stevens, M.J., Meile, L., Lacroix, C., Meile, L., 2015. High-throughput  
311 screening assays for antibacterial and antifungal activities of *Lactobacillus* species. *J.*  
312 *Microbiol. Meth* 114, 26-9.  
313  
314 Le Lay, C., Mounier, J., Vasseur, V., Weill, A., Le Blay, G., Barbier, G., Coton, E., 2016. *In*  
315 *vitro* and *in situ* screening of lactic acid bacteria and propionibacteria antifungal activities  
316 against bakery product spoilage molds. *Food Control* 60, 247-255.  
317  
318 Lynch, K. M., Pawlowska, A.M., Brosnan, B., Coffey, A., Zannini, E., Furey, A.,  
319 McSweeney, P.L.H., Waters, D.M., Arendt, E.K., 2014. Application of *Lactobacillus*  
320 *amylovorus* as an antifungal adjunct to extend the shelf-life of Cheddar cheese. *Int. Dairy J.*  
321 34, 167-173.  
322  
323 Schillinger, U., Villarreal, J.V., (2010). Inhibition of *Penicillium nordicum* in MRS medium  
324 by lactic acid bacteria isolated from foods. *Food Control* 21, 107–111.  
325  
326 Stiles, M.E., 1996. *Antonie van Leeuwenhoek* 70(2), 331-345.  
327

328 Stiles, J., Penkar, S., Plocková, M., Chumchalová, J., Bullerman, L.B., 2002. Antifungal  
329 activity of sodium acetate and *Lactobacillus rhamnosus*. J. Food Protect. 65(7), 1188-1191.  
330

331 Stratford, M., Steels, H., Nebe-von-Caron, G., Novodvorska, M., Hayer, K., Archer, D.B.,  
332 2013. Extreme resistance to weak-acid preservatives in the spoilage yeast *Zygosaccharomyces*  
333 *bailii*. Int. J. Food Microbiol. 166, 126-34.  
334

335 Varsha, K.K., Nampoothiri, K.M., 2016. Appraisal of lactic acid bacteria as protective  
336 cultures. Food Control. 69, 61-64.  
337

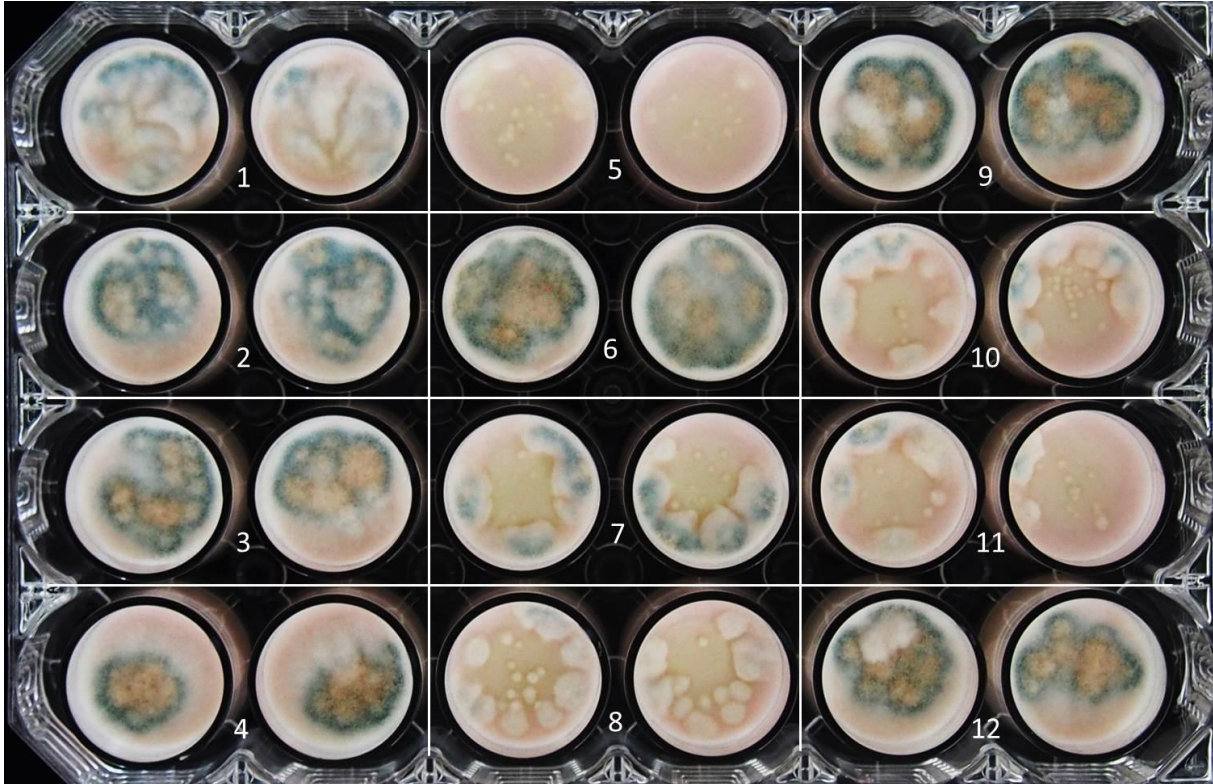
338 Wulijidigen, S. and Taku M., 2011. Screening and identification of lactic acid bacteria from  
339 Airag for antifungal activity. J. Anim.Vet. Adv. 10, 2751-2757.

340 **Table 1. Lactic acid bacteria used as dairy ingredient (fermentation product) or**  
 341 **protective culture for the antifungal assay**

Taxon	Strain number	Antifungal activity of fermentation product (F) or protective culture (Pc)			
		<i>Galactomyces geotrichum</i> (F / Pc)	<i>Mucor racemosus</i> (F / Pc)	<i>Penicillium commune</i> (F / Pc)	<i>Yarrowia lipolytica</i> (F / Pc)
<i>Lactobacillus casei</i>	L1	+ / -	++ / +	+ / ++	- / -
	L37	- / +	- / ++	- / ++	- / -
	L61	- / -	+ / +	+ / ++	- / -
	L95	- / nd	- / nd	+ / nd	- / nd
	L142	- / -	+ / +++	+ / +++	- / -
	L194	- / -	+ / ++	++ / +++	- / -
<i>Lactobacillus paracasei</i>	L93	- / -	++ / +	++ / ++	- / -
<i>Lactobacillus brevis</i>	L128	- / -	+ / +++	++ / +++	- / ++
	L31	- / -	- / ++	- / ++	- / -
	L30	- / nd	- / nd	++ / nd	- / nd
	CIRM-BIA 608	+ / nd	++ / nd	++ / nd	- / nd
	CIRM-BIA932	- / nd	- / nd	- / nd	- / nd
<i>Lactobacillus buchneri</i>	L233	- / -	+ / ++	++ / ++	- / +
	L56	- / nd	- / nd	- / nd	- / nd
	L84	- / nd	- / nd	++ / nd	- / nd
	L90	- / nd	- / nd	- / nd	- / nd
	L137	- / nd	- / nd	++ / nd	- / nd
<i>Lactobacillus harbinensis</i>	L244	- / -	+ / ++	- / +++	- / ++
<i>Lactobacillus rhamnosus</i>	CIRM-BIA 913	- / -	- / +	- / -	- / -
	CIRM-BIA 607	- / nd	- / nd	++ / nd	- / nd
	CIRM-BIA 909	- / nd	- / nd	++ / nd	- / nd
	CIRM-BIA 1107	- / +	- / +	- / +	- / -
	CIRM-BIA 1112	- / +	- / +	+ / +++	- / -
	CIRM-BIA 1436	- / -	- / +	- / +	- / -
<i>Lactobacillus reuteri</i>	CIRM-BIA 522	- / +	+ / -	++ / ++	- / ++
	L91	- / nd	- / nd	- / nd	- / nd
	L92	- / nd	- / nd	- / nd	- / nd
	L94	- / nd	- / nd	- / nd	- / nd
	L97	- / nd	- / nd	- / nd	- / nd
<i>Lactobacillus pentosus</i>	CIRM-BIA 853	- / nd	- / nd	- / nd	- / nd
	CIRM-BIA 1490	- / nd	- / nd	- / nd	- / nd
<i>Lactobacillus plantarum</i>	L11	- / -	- / +++	+ / ++	- / -
	L14	+ / +	- / +++	+ / ++	- / -
	L17	- / +	- / +++	+ / ++	- / -
	L63	- / -	- / +	++ / ++	- / -
	L89	- / nd	- / nd	+ / nd	- / nd
	L96	- / nd	- / nd	++ / nd	- / nd
<i>Lactobacillus sakei</i>	CIRM-BIA 892	- / nd	- / nd	- / nd	- / nd
	CIRM-BIA 1559	- / nd	- / nd	- / nd	- / nd
<i>Leuconostoc citreum</i>	CIRM-BIA 742	- / nd	- / nd	- / nd	- / nd
	CIRM-BIA 852	- / nd	- / nd	- / nd	- / nd
	CIRM-BIA 1453	- / nd	- / nd	- / nd	- / nd
	CIRM-BIA 1454	- / nd	- / nd	- / nd	- / nd
<i>Leuconostoc mesenteroides</i>	CIRM-BIA 1176	+ / +	- / -	- / -	- / -
	CIRM-BIA 1187	- / +	- / +++	+ / +++	- / +
	L126	- / -	- / +++	- / ++	- / -

342 (-) No antifungal activity; (+) Weak antifungal activity; (++) Intermediate antifungal activity; (+++) Complete  
 343 inhibition; (nd) Not determined (not tested as protective culture)

344  
 345



347

348 **Figure 1.** Visual evaluation of fungal growth to determine antifungal activity. Photograph of a  
 349 24-well plate showing the antifungal activity of 11 fermentation products from lactobacilli  
 350 strains (tested in duplicate) against *P. commune* UBOCC-A-116003 (50 spores/well in “mini-  
 351 cheeses” incubated for 8 days at 12°C. *Lactobacillus plantarum* L89 (1) and L96 (8),  
 352 *Lactobacillus buchneri* L90 (2), *Lactobacillus reuteri* L91 (3), L92 (4), L94 (6) and L97 (9),  
 353 *Lactobacillus casei* L95 (7), *Lactobacillus paracasei* L93 (5) and *Lactobacillus rhamnosus*  
 354 CIRM-BIA 607 (10) and CIRM-BIA 909 (11) fermentation products, negative control  
 355 (without fermentation product) (12). No antifungal activity (-) observation in positions 2, 3, 4,  
 356 6, 9, weak activity (+) in position 1, intermediate antifungal activities (++) in positions 7, 8,  
 357 10 and 5, 11. When complete inhibition was observed, a “+++” score was attributed to the  
 358 corresponding isolate.

359

360

**Table 2.** Minimal inhibitory concentrations of natamycin in model cheese versus PDA (Garnier et al., 2017) for 9 isolates of representative spoilage fungal species encountered in dairy products.

Species	Strain number	Isolation source	Minimal inhibitory concentrations (g/l)			
			Natamycin (mg/dm <sup>2</sup> ) cheese	Natamycin model	Natamycin (mg/dm <sup>2</sup> ) PDA (Garnier <i>et al.</i> , 2017)	
<i>Galactomyces</i>	UBOCC-A-	Fresh cheese	0.12	(0.6)	0.1	(0.5)
<i>Mucor racemosus</i>	UBOCC-A-	Semi-hard	0.04	(0.2)	0.1	(0.5)
<i>Penicillium</i>	UBOCC-A-	Semi-hard	0.06	(0.3)	0.06	(0.3)
<i>Phoma pinodella</i>	UBOCC-A-	Cream cheese	0.08	(0.4)	0.1	(0.5)
<i>Candida</i>	UBOCC-A-	Yoghurt	0.08	(0.4)	0.1	(0.5)
<i>Meyerozyma</i>	UBOCC-A-	Cream cheese	0.1	(0.5)	0.08	(0.4)
<i>Rhodotorula</i>	UBOCC-A-	Fresh cheese	0.08	(0.4)	0.1	(0.5)
<i>Trichosporon asahii</i>	UBOCC-A-	Fresh cheese	0.04	(0.2)	0.1	(0.5)
<i>Yarrowia lipolytica</i>	UBOCC-A-	Semi-hard	0.16	(0.8)	> 0.2*	(>1.0)*

(\*) Highest tested concentrations





En conclusion de cette Etude 2 ~ Développement d'une méthode de criblage haut-débit pour détecter des activités antifongiques dans un modèle mimant le fromage :

*Suite à ce travail, nous disposons d'une méthode de criblage haut-débit pour permettant de tester nos 1396 fermentats dans un modèle fromage et ce criblage (Résultats complets présentés dans l'étude 3) a permis la sélection de 11 fermentats bactériens et 1 fermentat fongique présentant une forte activité antifongique. Sur cette sélection réduite de fermentats, l'activité antifongique a été optimisée en faisant varier, dans un premier temps, le couple temps/température d'incubation, deux facteurs dont on sait qu'ils peuvent influencer la production de composés antifongiques. Dans un second temps, l'effet de la lyophilisation (facilitant la conservation, le stockage et la mise en oeuvre dans le cadre d'une utilisation industrielle) a été évalué sur ces mêmes fermentats bactériens.*

*Les fermentats optimisés les plus actifs seront ensuite testés sur produits réels (fromage blanc et fromage à pâte pressée) issus du commerce (résultats non inclus dans l'article 3 qui suit et présentés à la suite de ce dernier en données complémentaires) afin de tester une sélection réduite de fermentats (3) à l'échelle pilote sur fromage à pâte pressée et crème fraîche (résultats présentés dans l'article qui suit).*



## **Etude 3**

~

**Développement d'ingrédients laitiers  
antifongiques : du criblage *in vitro* en  
modèle fromage aux applications à  
l'échelle pilote**



Dans certains produits laitiers, l'utilisation de conservateurs chimiques, tels que le sorbate de potassium ou la natamycine, est autorisée pour lutter contre les contaminations fongiques. Cependant, la législation tend à limiter leur utilisation en corrélation avec une demande croissante des consommateurs pour des produits sans conservateur. La biopréservation représente ainsi une alternative intéressante aux conservateurs chimiques pour réduire les contaminations fongiques dans les produits laitiers. Les objectifs de cette étude étaient donc de cribler *in vitro* et *in situ* l'activité antifongique de fermentats dérivant de 2 substrats laitiers (LH et UF) fermentés respectivement par 23 espèces de bactéries lactiques, 4 espèces de bactéries propioniques et 87 espèces de levures et champignons filamenteux afin de développer des ingrédients antifongiques pour les produits laitiers.

Dans la première partie de ce travail, le potentiel antifongique de fermentats laitiers issus de 430 bactéries lactiques, 70 bactéries propioniques et de 198 champignons contre 4 cibles fongiques (*e.g.* *M. racemosus* UBOCC-A-116002, *P. commune* UBOCC-A-116003, *G. geotrichum* UBOCC-A-216001 et *Y. lipolytica* UBOCC-A-216006) a été évalué, *in vitro*, dans un milieu mimant un fromage, grâce à une méthode de criblage haut-débit. Ce criblage a permis la sélection de 11 fermentats antifongiques. L'activité antifongique de ces fermentats a ensuite été optimisée en faisant varier les conditions de culture (temps, température). Après optimisation, 3 fermentats ont été retenus et leur activité antifongique évaluée *in situ*, à l'échelle pilote, sur 2 matrices laitières (une pâte pressée non cuite à croûte morgée et une crème fraîche) par le biais de challenge-tests mais aussi de tests d'usage. De plus, des tests sensoriels ont été mis en place afin d'évaluer l'impact de ces fermentats sur les qualités organoleptiques des produits laitiers testés et valider ainsi leur potentielle utilisation à l'échelle industrielle.

Le détail des méthodes mises en œuvre est présenté dans l'article ci – après.

### **Principaux résultats :**

#### **Criblage *in vitro* de l'activité antifongique des fermentats**

Le nombre de fermentats présentant une activité antifongique varie largement selon le substrat de fermentation utilisé. Avec les fermentats issus de lait low heat, environ 17% des souches sont actives alors qu'avec le perméat d'ultrafiltration de lait, seulement 1% des souches montrent une activité.

Les cibles, quant à elles, ne montrent pas les mêmes sensibilités selon les substrats de fermentation. En effet, avec les fermentats issus du lait low heat, *P. commune* est la cible la plus sensible alors que *Y. lipolytica* est la cible la plus résistante. Au contraire, avec les fermentats issus du perméat d'ultrafiltration du lait c'est la souche de *M. racemosus* UBOCC-A-116002 qui est la plus sensible et les souches de *P. commune* UBOCC-A-116003 et *Y. lipolytica* UBOCC-A-216006 les plus résistantes. Sur les 27 espèces bactériennes testées, *L. buchneri* et *L. plantarum* représentent les espèces les plus actives dans le lait low heat.

Parmi les champignons testés, 4 isolats appartenant à *Aureobasidium pullulans* et *M. lanceolatus* et, *Actinomucor elegans* et *Verticillium dahliae* montrent des activités antifongiques intéressantes dans le lait low heat et le perméat d'ultrafiltration de lait respectivement.

Parmi les souches d'intérêt, nous avons sélectionné 22 souches bactériennes, appartenant à 7 espèces différentes, afin d'étudier leur spectre d'activité. Les résultats obtenus, ainsi que la méthode utilisée, sont présentés dans les données supplémentaires qui font suite à l'article ci-après. Cette étude, et les résultats du criblage nous ont ainsi permis la sélection, pour l'étape d'optimisation, de 11 fermentats issus de 11 souches (9 bactéries lactiques, 1 bactérie propionique et 1 champignon) présentant les activités antifongiques les plus intéressantes.

### **Optimisation de l'activité antifongique des fermentats**

Dans cette partie, différentes conditions d'incubation ont été appliquées aux 11 fermentats précédemment retenus afin d'optimiser leur niveau d'activité. Il n'y a pas de règle générale quant à l'effet des conditions de culture sur le niveau d'activité observé. Ainsi, les conditions de culture nécessaires pour atteindre une activité antifongique optimale dépendent de la souche testée.

Avant de réaliser le changement d'échelle, une étape intermédiaire était nécessaire afin de définir et valider les modalités d'application de nos fermentats dans les différentes typologies de produits. Ainsi, l'activité antifongique de 4 et 7 fermentats, seuls ou en association, a été testée en réalisant des challenge-tests sur des produits issus du commerce, un fromage blanc et un fromage à pâte pressée à croûte morgée respectivement. La liste des fermentats testés dans chaque produit, la méthode utilisée et les résultats obtenus sont présentés dans la partie « Données complémentaires » à la suite des résultats de l'évaluation du spectre d'activité

suivant l'article « *Development of antifungal ingredients for dairy products: from in vitro screening to pilot scale application* ».

Cette étape intermédiaire, ainsi que les résultats de l'étape d'optimisation, nous ont permis d'aboutir à la sélection de 3 fermentats (*L. rhamnosus* CIRM-BIA 1952 cultivé 48h à 30°C dans le lait low heat, *P. jensenii* CIRM-BIA 1774 cultivé 96h à 30°C puis 24h à 12°C dans le perméat d'ultrafiltration de lait et *M. lanceolatus* UBOCC-A-109193 cultivé 96h à 30°C puis 48h at 12°C dans le lait low heat) pour la validation à l'échelle pilote.

### **Tests *in situ* à l'échelle pilote : crème fraîche**

Dans cette partie de l'étude, l'activité antifongique des fermentats issus de *P. jensenii* et de *L. rhamnosus*, seuls ou en association, a été testée dans de la crème fraîche, contre 3 cibles fongiques sélectionnées, *P. commune* UBOCC-A-116003, *M. racemosus* UBOCC-A-116002 et *R. mucilaginosa* UBOCC-A-216004 en challenge test mais aussi en conditions de contaminations naturelles ambiantes lors de tests d'usage. Tous les fermentats contenant *P. jensenii* CIRM-BIA 1774 ont montré une très forte activité antifongique dans la crème fraîche. Au contraire, les fermentats contenant uniquement *L. rhamnosus* CIRM-BIA 1952 n'ont pas montré d'effet lors des challenge-test mais ont permis de réduire le niveau de contamination naturelle dans le cas des tests d'usage. Les analyses sensorielles ont montré un fort impact négatif du fermentat issu de *P. jensenii* CIRM-BIA 1774 sur les qualités organoleptiques de la crème fraîche. Des tests supplémentaires ont donc été nécessaires afin de trouver les concentrations pour lesquels l'impact organoleptique serait absent ou négligeable. Ainsi, à une concentration de 0,4%, l'activité antifongique en challenge-test est moindre mais cette concentration permettait de réduire les contaminations en conditions naturelles de manière significative.

### **Test *in situ* à l'échelle pilote : pâte pressée non cuite à croûte morgée**

Concernant cette seconde matrice, l'activité de *P. jensenii* CIRM-BIA 1774, seul ou en association avec *M. lanceolatus* UBOCC-A-109193, et de l'association *L. rhamnosus* CIRM-BIA 1952 / *M. lanceolatus* UBOCC-A-109193 a été testée. Que ce soit lors des challenge-tests ou lors des tests d'usage, tous les fermentats testés ont montré une forte activité



antifongique sans impact négatif sur les qualités organoleptiques du produit, celui issu de *P. jensenii* étant le plus actif.

*Ce travail fait l'objet d'un article à soumettre dans le journal « Food Microbiology » dans le cadre d'une édition spéciale suite au Congrès « Spoilers in Food ». L'article est présenté, ci-après, en anglais.*

1 **Development of antifungal ingredients for dairy products: from *in vitro* screening to pilot**  
2 **scale application.**

3

4 Lucille Garnier<sup>1,2</sup>, Jérôme Mounier<sup>1</sup>, Sébastien Lê<sup>3</sup>, Audrey Pawtowski<sup>1</sup>, Nicolas Pinon<sup>2</sup>,  
5 Bénédicte Camier<sup>2</sup>, Manon Chatel<sup>2</sup>, Gilles Garric<sup>2</sup>, Anne Thierry<sup>2</sup>, Emmanuel Coton<sup>1</sup> and  
6 Florence Valence<sup>2\*</sup>.

7

8 <sup>1</sup>Université de Brest, EA 3882, Laboratoire Universitaire de Biodiversité et Ecologie  
9 Microbienne, IBSAM, ESIAB, Technopôle Brest-Iroise, 29280 Plouzané, France.

10 <sup>2</sup>UMR1253 Science et Technologie du Lait et de l'OEuf, INRA, Agrocampus Ouest, 65 rue de  
11 Saint Briec, 35000 Rennes, France

12 <sup>3</sup>Applied Mathematics Department, Agrocampus Ouest, 65 rue de Saint-Briec, 35042 Rennes  
13 cedex, France

14

15 Lucille Garnier: lucille.garnier@univ-brest.fr

16 Jérôme Mounier: jerome.mounier@univ-brest.fr

17 Sébastien Lê: sebastien.le@agrocampus-ouest.fr

18 Audrey Pawtowski: audrey.pawtowski@univ-brest.fr

19 Nicolas Pinon: nicolas.pinon@inra.fr

20 Bénédicte Camier: benedicte.camier@inra.fr

21 Manon Chatel: manon.chatel@inra.fr

22 Gilles Garric: gilles.garric@inra.fr

23 Anne Thierry: anne.thierry@inra.fr

24 Emmanuel Coton: emmanuel.coton@univ-brest.fr

25

26 **\*Corresponding author:** Florence Valence :  
27 INRA - UMR 1253 Science et Technologie du Lait et de l'œuf  
29  
30 F-35042, Rennes cedex  
31 Tel: +33 (0)2 23 43 53 23  
32 Fax: +33 (0)2 23 48 53 50  
33 E-mail: [florence.valence-bertel@inra.fr](mailto:florence.valence-bertel@inra.fr)

34 Abstract

35 Biopreservation represents a complementary approach for reducing fungal spoilage in dairy  
36 products and even, in some cases, an alternative to chemical preservatives. The aim of this study  
37 was to develop antifungal fermentates derived from 2 dairy substrates using both *in vitro* and  
38 *in situ* approaches. Firstly, an *in vitro* screening of the antifungal activity of fermentates  
39 deriving from 430 lactic acid bacteria (LAB, 23 species), 70 propionibacteria (4 species) and  
40 198 fungi (87 species) was performed against four major spoilage fungi (*Penicillium commune*,  
41 *Mucor racemosus*, *Galactomyces geotrichum* and *Yarrowia lipolytica*), using a cheese-  
42 mimicking model. The most active fermentates were obtained from *Lactobacillus brevis*,  
43 *Lactobacillus buchneri*, *Lactobacillus casei/paracasei* and *Lactobacillus plantarum* among the  
44 tested LAB, *Propionibacterium jensenii* among propionibacteria, and *Mucor lanceolatus*  
45 among the tested fungi. Then, for the 11 most active fermentates, culture conditions were  
46 optimized by varying incubation time and temperature in order to enhance their antifungal  
47 activity. Finally, the antifungal activity of 3 fermentates of interest obtained from *Lactobacillus*  
48 *rhamnosus* CIRM-BIA1952, *Propionibacterium jensenii* CIRM-BIA1774 and *Mucor*  
49 *lanceolatus* UBOCC-A-109193 were evaluated in real products (sour cream and semi-hard  
50 cheese) at a pilot-scale using challenge and durability tests. In parallel, the impact of these  
51 ingredients on organoleptic properties of the product was also assessed. In semi-hard cheese,  
52 application of the selected fermentates on the cheese surface delayed the growth of spoilage  
53 molds for up to 21 days, without any effect on organoleptic properties, *P. jensenii* CIRM-  
54 BIA1774 fermentate being the most active. In sour cream, incorporation of *P. jensenii* CIRM-  
55 BIA1774 fermentate at 2 or 5% yielded a high antifungal activity but was detrimental to the  
56 product organoleptic properties. Determination of the concentration limit, compatible with  
57 product acceptability, showed that incorporation of this fermentate at 0.4% prevented fungal  
58 contamination in natural conditions but had a more limited effect against *M. racemosus* and

59 *P. commune* in challenge tests. Microbial fermentates are therefore an option of interest for the  
60 production of natural antifungal dairy ingredients and the selected fermentates are promising  
61 candidates for dairy products biopreservation.

62

63 **Keywords:** antifungal, dairy ingredient, lactic acid bacteria, propionibacteria, fungi,

## 64 **1. Introduction**

65 Fungal spoilage of dairy products is responsible for significant food waste and economic  
66 losses (Pitt and Hocking, 2009). Dairy products, despite their acidic pH and high content in  
67 organic acids, may provide a favorable environment for the development of undesirable  
68 microorganisms including fungi. Fungal spoilage is usually easy to detect due to the presence  
69 of colonies or thalli at the surface of the product. Nonetheless, fungal spoilage may also be non-  
70 visible resulting in undesirable production flavor or texture alteration via fungal metabolism  
71 (Ledenbach and Marshall, 2010), thus preventing product consumption. According to the  
72 literature, species belonging to the *Penicillium*, *Mucor*, *Cladosporium*, and *Candida*,  
73 *Galactomyces* and *Yarrowia* genera have been identified as the most important contaminating  
74 molds and yeasts, respectively (Pitt and Hocking, 2009; Garnier et al., 2017a).

75 In order to avoid or delay fungal spoilage, and hence extend product shelf life, prevention  
76 and control methods are currently used by industrials. Prevention methods applied in the dairy  
77 industry include good manufacturing and hygiene practices, implementation of Hazard  
78 Analysis Critical Control Point (HACCP) system, and use of air filtration or packaging in  
79 aseptic conditions (Garnier et al., 2017a). Control methods can involve the use of heat  
80 treatment, modified atmosphere packaging or fermentation with beneficial microorganisms  
81 (Garnier et al., 2017a; Leyva Salas et al., 2017; Nguyen Van Long et al., 2016; Sakkas et al.,  
82 2014; Bourdichon et al., 2012). In specific dairy products such as sour cream or semi-hard  
83 cheese, the addition of defined chemical preservatives with antifungal properties (e.g.

84 natamycin or organic acids and their salts) is permitted depending on the regulation in place.  
85 However, there is a strong societal demand for “preservative-free” food products since  
86 consumers are looking for more “natural” and less heavily processed foods. Therefore,  
87 industrial groups are looking for efficient solutions to replace chemical preservatives while  
88 maintaining the existing shelf-life of food products.

89 In this context, biopreservation, defined as the use of natural or added microbiota and/or their  
90 antimicrobial compounds to extend food shelf-life and increase food safety (Stiles, 1996), could  
91 represent a complementary approach to reduce fungal spoilage and when applicable, an  
92 alternative to chemical preservatives. The use of lactic acid bacteria (LAB) and propionibacteria  
93 and/or their metabolites is particularly of interest for reducing fungal spoilage in dairy products.  
94 Indeed, LAB and propionibacteria are naturally present in many foods and have a long history  
95 of safe use in fermented foods (Bourdichon et al., 2012). They produce a wide range of  
96 antifungal compounds including organic acids such as lactic, propionic, acetic, phenyllactic and  
97 fatty acids, cyclic dipeptides or proteinaceous compounds (Crowley et al., 2013, Schnürer and  
98 Magnusson, 2005, Lavermicocca et al., 2000). Fungal inhibition generally results from the  
99 additive and/or synergistic activities of several of these compounds (Varsha et al., 2016). In  
100 addition to LAB and propionibacteria, some fungi can also produce antifungal compounds such  
101 as alcohols, acids, esters, and proteinaceous compounds (Delgado et al., 2016, Leyva-Salas et  
102 al., 2017), and their utilization could be also be compatible with some dairy technologies.  
103 Commercial antifungal solutions for dairy products are currently available on the market, all of  
104 them involving LAB and/or propionibacteria in single or mixed cultures (Leyva-Salas et al.,  
105 2017; Garnier et al., 2017b). These microorganisms can be used as adjunct cultures, i.e. co-  
106 inoculated with the starter culture during product manufacturing. They can also be used to  
107 produce so-called fermentates, produced from fermentation of a dairy substrate for example  
108 and added directly to the food as an ingredient (Varsha et al., 2016; Garnier et al. 2017b).

109 While there is an increasing number of studies showing the *in vitro* antifungal activity of  
110 selected microorganisms and/or their metabolites for application in dairy products or other  
111 foods (Leyva-Salas et al., 2017), very few have dealt with the efficiency of such antifungal  
112 ingredients in real products and their potential impact on product organoleptic properties.  
113 Moreover, to our best knowledge, no work on the development of a biopreservation ingredient  
114 to replace natamycin (E235 in the European Union) for cheese surface-treatment has been  
115 reported yet in the literature.

116 The aim of this study was to develop antifungal fermentates derived from 2 dairy substrates  
117 (low-heat milk –LH- and milk permeate- UF-) for dairy food applications using both *in vitro*  
118 and *in situ* approaches.

119

## 120 **2. Materials and methods**

### 121 *2.1. Fermentate production*

122 Two different dairy substrates, UF and LH, were used to prepare fermentates. The UF substrate  
123 consisted of an ultrafiltration milk permeate (T.I.A., Bollene, France) supplemented with 10 g/l  
124 of yeast extract (Biokar Diagnosis) and 0.5 % litmus, and filtrated at 0.22 µm (VWR, Radnor,  
125 USA). For the LH substrate, 10 % reconstituted low heat skim milk powder (Triballat, Noyal,  
126 France) was supplemented with 45 % anhydrous milk fat (homogenized at 600MPa at 60°C)  
127 and 0.5 % litmus, and sterilized for 30 min at 110°C. Both substrates were then individually  
128 inoculated by 430 LAB, 70 propionibacteria and 198 fungal strains obtained from the  
129 Laboratoire Universitaire de Biodiversité et Ecologie Microbienne (LUBEM, Plouzané,  
130 France), the Université de Bretagne Occidentale Culture Collection (UBOCC, Plouzané,  
131 France) and the Centre International de Ressources Microbiennes - Bactéries d'Intérêt  
132 Alimentaire (CIRM-BIA, French National Institut for Agricultural Research, Rennes, France)  
133 culture collection (Supplementary Table S1). For LAB, LH and UF substrates were inoculated

134 (1% v/v) after 2 pre-cultures (24 h at 30°C) in de Man-Rogosa-Sharpe (MRS) broth (Difco, Le  
135 Pont de Claix, France), and incubated for 20 h at 30°C except for *Lactobacillus amylovorus*,  
136 *Lactobacillus gasseri*, *Lactobacillus acidophilus*, *Lactobacillus delbrueckii* and *Lactobacillus*  
137 *fermentum* strains which were pre-cultured and incubated at 37°C. *Lactobacillus*  
138 *sanfranciscensis* and *Lactococcus lactis* strains were pre-cultured in MRS and M17  
139 supplemented with 20 g/L maltose, respectively (Difco, Le Pont de Claix, France). Concerning  
140 propionibacteria, UF and LH media were inoculated (1% v/v) after 2 pre-cultures (48 h at 30°C)  
141 in Yeast Extract Lactate (YEL) broth (Difco, Le Pont de Claix, France), and incubated for 48 h  
142 at 30°C. For yeasts, LH and UF media were inoculated (1% v/v) after 2 pre-cultures (24 h at  
143 25°C with 120 rpm agitation speed) in Yeast Glucose Chloramphenicol (YGC) broth (Difco,  
144 Le Pont de Claix, France), and incubated for 48 h at 25°C and 120 rpm agitation speed.  
145 Concerning molds, they were first cultivated on Potato Dextrose Agar (PDA) slant for 7 d at  
146 25°C. After spore recovery with 0.01 % Tween 80 and enumeration using a Malassez cell, LH  
147 and UF media were inoculated at  $10^4$  spores/ml and incubated for 96 h at 25°C on a rotary  
148 shaker at 120 rpm. Yeast and mold fermentates were centrifuged three times (8000g at 15 min)  
149 and filtered at 0.45 µm (VWR, Radnor, USA). Fermentates were then stored at -80°C until use.  
150 For pilot scale applications, fermentates were produced in 2 to 5-L flasks. Bacterial fermentates  
151 were lyophilized (Virtis, Fisher, France, final residual water < 2 % after freeze drying under  
152 vacuum) while fungal fermentates were prepared as described above before use.

153

## 154 2.2. In vitro screening of antifungal activity

155 The antifungal activity of the obtained fermentates against *Mucor racemosus* UBOCC-A-  
156 116002, *Penicillium commune* UBOCC-A-116003, *Galactomyces geotrichum* UBOCC-A-  
157 216001 and *Yarrowia lipolytica* UBOCC-A-216006, isolated from spoiled dairy products and  
158 representative of the most common dairy product spoilers (Garnier et al., 2017b), was evaluated



159 *in vitro* using a high-throughput screening method in a cheese-mimicking model as described  
160 previously (Garnier et al., accepted).

161

### 162 2.3. Optimization of culture conditions for antifungal activity enhancement

163 Bacterial (n=12) and fungal (n=1) fermentates exhibiting the highest antifungal activity  
164 were selected for an optimization step of culture conditions. Depending on the tested  
165 microorganism, 5 time/temperature combinations were tested: 20, 48 and 72 h at 30°C as well  
166 as 48 h at 30°C followed by 24 h at either 12°C or 43°C for LAB; 48, 72 and 96 h at 30°C as  
167 well as 96 h at 30°C followed by 24 h at either 12°C or 43°C for propionibacteria and 48, 96  
168 and 168 h at 25°C as well as 96 h at 25°C followed by 48 h at either 12°C or 43°C for the  
169 filamentous fungi. Cell concentration at the end of incubation was determined on MRS and  
170 YEL agar for LAB and propionibacteria, respectively. Antifungal activities of the resulting  
171 fermentates were tested as described above against the four selected target fungal spoilers.

172

### 173 2.4. In situ antifungal activity

174

#### 175 2.4.1. Antifungal activity by fermentate surface-spraying on semi-hard cheese

176 Fermentates with antifungal activities of interest were further tested *in situ* by surface-  
177 spraying on semi-hard cheeses. Two replicates cheese trials were carried out at a pilot scale at  
178 the Dairy Platform of INRA, Rennes, on two separate weeks. Cow's milk was pasteurized at  
179 72°C for 20 s, standardised to 30 g fat/kg milk and pumped into a cylindrical jacketed, stainless  
180 steel cheese vat, with variable speed cutting and stirring (Frominox, Assat, France). Milk was  
181 added with 36 mg Ca per kg (20 mL per 100 kg milk of a 500 g /L CaCl<sub>2</sub> solution). A  
182 commercial starter comprising *Lactococcus lactis* subsp. *lactis* and *Lactococcus lactis* subsp.

183 *cremoris* (MA016, Elimeca, Thoissey, France) was resuspended in UHT semi-skimmed milk,  
184 let to rehydrate for 1 h at 20°C, and added to the cheese milk at 0.5 U/100 kg, i.e. ~106 colony-  
185 forming units (CFU)/mL). After 2 h at 12°C then 30 min pre-ripening at 33°C (pH 6.57), 0.25  
186 mL/kg of animal rennet (520 mg L<sup>-1</sup> chymosin, Carlina™145/80, Dupont Danisco, Dangé,  
187 Saint Romain, France), diluted in deionized water, was added. After coagulation (29 min), curd  
188 was cut at the size of corn grains washed with hot water, cooked for 20 min at 35 °C, and  
189 drained (pH 6.54). Curd was pre-pressed at 120 kPa for 30 min, moulded (700 g curd per mould)  
190 and pressed at 600 kPa for 30 min then 600 kPa for 90 min. The cheeses remained in the mould  
191 overnight, the temperature decreased from ~25°C to ~20°C and the pH reached 5.07 at  
192 demoulding. The composition of cheeses at this step was 50.8 % dry matter and 49 % fat in dry  
193 matter. Cheeses were salted by immersion in a sterile brine containing 33 % (m/m) NaCl, 36 mg  
194 Ca per kg, pH = 5 at 20 °C for 2 h. Cheeses were let for drying for one night at room temperature  
195 (20-25°C). Then, under a sterile laminar air flow then the external part (3 mm thickness) was  
196 cut and discarded to limit surface contamination. They were then smeared once with a mixture  
197 of the following commercial cultures: *Debaryomyces hansenii* CHOOZIT™ DH,  
198 *Kluyveromyces lactis* CHOOZIT™ KL71, and *Brevibacterium linens* CHOOZIT™ SR3  
199 (Danisco, Dangé Saint-Romain, France). After 2 h drying at room temperature under a sterile  
200 laminar air flow, 3 ml of fermentates (lyophilized ones resuspended in sterile distilled water  
201 and/or in *M. lanceolatus* supernatant), alone or in combination, were sprayed on the cheese  
202 surface. Fermentate spraying was repeated at day 3 and day 5. In-between, at day 2 after a 12 h  
203 drying period at room temperature following the first spraying, cheese surface was inoculated  
204 with 50 spores of either *P. commune* UBOCC-A-116003 or *M. racemosus* UBOCC-A 116002.  
205 Cheeses were ripened for 6 weeks in ripening chambers at 12°C and 96% relative humidity  
206 (one ripening chamber per fungal target). The ripened semi-hard cheeses had a final dry matter  
207 of 60% and pH 5.5. Fungal growth was visually evaluated every day during the 6 weeks of

208 ripening. A negative control (cheese without surface-spraying of fermentate) and a positive  
209 control (cheese-surface treated with  $1 \text{ mg/dm}^2$  in total natamycin at day 2) were also included.

210  
211

#### 2.4.2. Antifungal activity by fermentate incorporation in sour cream

212 Fermentates with antifungal activities of interest were also tested, alone or in  
213 association, by incorporation in sour cream. To do so, sour cream (final fat 33%, dry matter  
214 41%) was produced at a pilot scale from pasteurized milk (16 s at  $75^\circ\text{C}$ ) using 2.5 U/100 kg  
215 ( $10^6$  CFU/L) of commercial starter cultures MM100 (Elimeca, Thoissey, France). Sour cream  
216 (pH 4.5) was then packaged in individual 100 g jars. Various concentrations ranging from 0.1  
217 to 5 % were tested according to the selected fermentate (Table 2). The fermentates were added  
218 directly in each pot, gently mixed and stored at  $10^\circ\text{C}$  overnight before fungal target inoculation.  
219 A negative control (sour cream without fermentate) and a positive control (sour cream  
220 containing 0.08 % potassium sorbate) were also included. For challenge tests with filamentous  
221 fungi, 50 fungal spores of *P. commune* UBOCC-A-116003 or *M. racemosus* UBOCC-A 116002  
222 were inoculated on the sour cream surface followed by incubation at  $10^\circ\text{C}$  for 4 weeks. Fungal  
223 growth was visually evaluated every day. For challenge test with *Rhodotorula mucilaginosa*  
224 UBOCC-A-216004, 2 CFU/g were inoculated in the sour cream, followed by gentle mixing and  
225 incubation at  $10^\circ\text{C}$ . Yeast growth was evaluated by surface-plating after 1, 2, and 3 weeks on  
226 YGC agar incubated at  $25^\circ\text{C}$  for 2 days.

227

#### 2.4.3. Durability tests

229 Durability tests were performed to evaluate fermentate antifungal activity in a naturally  
230 contaminated environment. For semi-hard cheese, growth of spoilage fungi was evaluated on  
231 the surface of 3 different cheeses sprayed or not with selected fermentates, after ripening for 6  
232 weeks at  $12^\circ\text{C}$  in a ripening chamber. For sour cream, 100 g of sour cream with or without

233 fermentate, and a control containing 0.08 % potassium sorbate were spread out in 10 Petri  
234 dishes, exposed for 20 min to indoor air of the eating room at INRA STLO and then sealed  
235 using parafilm. Fungal spoilage at the surface was visually evaluated after 2-weeks incubation  
236 at 10°C.

237

#### 238 2.4.4. Sensory analyses

239 The impact of antifungal fermentates on the sensorial properties of cheese and cream was  
240 studied using a sorting task followed by a verbalization task. A panel of 39 and 29 untrained  
241 judges participated in the sensory evaluation of sour cream and semi-hard cheeses, respectively.  
242 Sour cream, packed in 100 g jar, was kept at 4°C for 2 weeks before sensory analysis and semi-  
243 hard cheese were tested after a 6 weeks ripening period. Products were left for 30 min at room  
244 temperature before being served and a random 3-digit code was assigned to each sample.  
245 Panelists were asked to group together the samples perceived as the most similar, taking into  
246 account the characteristics they considered as important to differentiate the products. Once  
247 groups were made, panelists had to associate specific descriptors to each group. To ensure the  
248 panelist ability to group similar samples, a random sample was tested in duplicate. Data were  
249 analyzed using a factorial approach for sorting tasks (FAST) based on multiple correspondence  
250 analysis (MCA), using the packages FactoMineR and SensoMineR implemented in the R  
251 environment, according to Cadoret et al. (2009).

252 To define the concentration of the selected fermentate at which no sensory impact could  
253 be perceived in sour cream, an acceptability test was performed with a panel of 26 untrained  
254 judges. Eight concentrations ranging from 0 to 0.7 % were tested. As for sensory tests, a random  
255 3-digit code was assigned to each sample and products were left for 30 min at room temperature  
256 before being served. Panelists were asked to indicate whether each sour cream sample was  
257 sensorily sour creams were acceptable or not for consumption.

259 **3. Results**

260 3.1. *Antifungal activity of fermentates after in vitro screening*

261 In the first part of this work, the antifungal activity of fermentates obtained by individual  
262 culture of 698 LAB, propionibacteria and fungi in LH and UF substrates (for a total of therefore  
263 1 396 fermentates) was evaluated *in vitro* against 4 fungal targets in miniaturized cheeses. The  
264 number of fermentates exhibiting antifungal activity largely varied according to the substrate  
265 used for fermentation, the tested taxon and the fungal target (Supplementary table S2).

266 LH fermentates were overall notably inhibitorier than UF fermentates with LAB being the  
267 most represented microbial group among active strains. Indeed, for LH fermentates,  
268 81 (18.9 %), 13 (3 %), and 1 (0.2 %) LAB fermentates out of the 430 tested ones, were active  
269 against *P. commune*, *M. racemosus*, and *Y. lipolytica*, respectively, while no propionibacteria  
270 of the 70 tested showed any activity. For fungal fermentates, 2 (1 %) and 6 (3 %) out of the 198  
271 tested ones were active against *M. racemosus* and *G. geotrichum*, respectively. In contrast, for  
272 UF fermentates, only fermentates from 8 LAB (1.8 % of tested LAB), 1 propionibacteria (1.4 %  
273 of tested propionibacteria) and 4 fungi (2 % of tested fungi) showed an antifungal activity, and  
274 this against only one target.

275 Among the 27 tested bacterial species, *Lactobacillus buchneri* (34 tested strains),  
276 *Lactobacillus plantarum* (115) and *Lactobacillus brevis* (8) represented the most active species  
277 with 44.1, 40.8 and 37.5 % of active LH fermentates against at least one target. Noteworthy,  
278 9 LAB LH fermentates showed an antifungal activity against both *M. racemosus* and  
279 *P. commune*, while one fermentate from a *Lactobacillus buchneri* L162 inhibited the latter 2  
280 fungi and *Y. lipolytica* as well. In contrast, in the tested conditions, only a few strains of  
281 *Lactobacillus reuteri* (n=42) and *Propionibacterium jensenii* (n=20) showed antifungal  
282 activity, with 1 (2.4 %) and 1 (5 %) active strains for LH and UF fermentates, respectively. In

283 addition, strains belonging to the *Leuconostoc mesenteroides* (n=71), *Propionibacterium*  
284 *acidipropionici* (n=17) and *Propionibacterium thoenii* (n=14) species did not show any  
285 antifungal activity whatever the substrate used.

286 As mentioned above, only a few fungal fermentates showed antifungal activities and there  
287 was no particular over represented species whatever the substrate used. Indeed, LH fermentates  
288 showing activity against *M. racemosus* and *G. geotrichum* were obtained from one isolate each  
289 of *Aureobasidium pullulans* and *Mucor lanceolatus*, and *Exophiala* sp., *Rhodotorula minuta*,  
290 *Rhodotorula mucilaginosa*, *Torulasporea desbrueckii* and *Trichoderma viride*, respectively. For  
291 UF fermentates, one isolate each of *Actinomucor elegans*, *Verticillium dahliae* and *Candida*  
292 *tenuis*, and *Trichoderma harzianum* showed activity against *P. commune* and *Y. lipolytica*,  
293 respectively.

294 Concerning the susceptibility of the fungal targets, *P. commune*, followed by *M. racemosus*  
295 were the most frequently inhibited fungi while *G. geotrichum* and *Y. lipolytica* were inhibited  
296 by a very small number of LAB or propionibacteria fermentates. Interestingly, for fungal  
297 fermentates, *M. racemosus* and *G. geotrichum* were the most sensitive targets with LH as a  
298 growth substrate whereas it was *P. commune* and *Y. lipolytica* with UF substrate.

299 After *in vitro* screening, 11 strains exhibiting the most promising antifungal activity namely,  
300 9 *Lactobacillus* strains (*L. buchneri* L151, L162, L164 and L233, *L. harbinensis* L172,  
301 *L. paracasei* L117 and L194, *L. plantarum* L244 and *L. rhamnosus* CIRM-BIA1952),  
302 *P. jensenii* CIRM-BIA1774 and *M. lanceolatus* UBOCC-A-109193 were selected for further  
303 investigation.

304

### 305 3.2. Optimization of culture conditions for antifungal activity enhancement

306 The 11 strains previously selected were inoculated and cultivated in LH and/or UF  
307 substrate with 5 different time/temperature combinations, and their antifungal activity tested

308 against the 4 selected fungal targets in miniaturized cheeses. Among the 11 selected strains and  
309 independently of the fungal targets, all showed an increased antifungal activity against at least  
310 one fungal target when culture conditions were modified (Table 1). These increases in  
311 antifungal activities were more pronounced against *P. commune*, followed by *R. mucilaginosa*  
312 and *M. racemosus*, whereas there was little improvement against *Y. lipolytica*. The increase in  
313 time before visible growth, which varied between 1 to 4 days as compared to the control  
314 depending on the tested strain, was generally higher in conditions with prolonged incubation  
315 time with the exception of *L. paracasei* L117 fermentate for which incubation for 20h at 30°C  
316 yielded the best antifungal activity. Increases in antifungal activity were also generally related  
317 to the final biomass even though this was not systematically observed. Indeed, the higher the  
318 bacterial concentrations at the end of fermentation, the higher the antifungal activities were.  
319 Finally, a final incubation step at suboptimal temperatures, e.g., 12°C or 43°C, did not improve  
320 the antifungal activity except for *L. buchneri* L233, *M. lanceolatus* UBOCC-A-109193 and  
321 *P. jensenii* CIRM-BIA1774 fermentates for which a significant increase in antifungal activity  
322 against *P. commune* and *M. racemosus*, and *M. racemosus* and *R. mucilaginosa* was observed,  
323 respectively, as compared to other conditions.

324 After this step, 3 fermentates were selected for *in situ* applications at a pilot scale,  
325 namely those of *L. rhamnosus* CIRM-BIA1952 (cultivated in LH for 48 h at 30°C) which was  
326 able to delay *P. commune* and *R. mucilaginosa* growth, *P. jensenii* CIRM-BIA1774 (cultivated  
327 in UF for 96 h at 30°C followed by 24 h at 12°C), which was able to delay the growth of the 4  
328 tested fungal targets and *M. lanceolatus* UBOCC-A-109193 (cultivated in LH for 96 h at 30°C  
329 followed by 48 h at 12°C)) which was able to delay *M. racemosus* and *P. commune* growth.

330

331 3.3. *Antifungal activity and organoleptic impact of fermentates after surface-spraying*  
332 *on semi-hard cheeses*

333 The 3 fermentates selected after the optimization step were used, alone or in association, for  
334 surface treatment of semi-hard cheeses manufactured at a pilot-scale. Their antifungal activity  
335 was compared to a negative (untreated cheese) and a positive control (cheese treated with 1  
336 mg/dm<sup>2</sup> natamycin) using challenge and durability tests. The observed times to visible growth  
337 of *M. racemosus* and *P. commune* after the challenge test are presented in Table 2. Surface-  
338 spraying of each of the tested fermentates led to an increase in time to visible growth of both  
339 fungal targets as compared to the negative control but their efficiency varied. Indeed, as  
340 compared to the negative control, time to visible growth of *M. racemosus* was increased by 3  
341 and 15 days using *L. rhamnosus* CIRM-BIA1952 associated to *M. lanceolatus* UBOCC-A-  
342 109193 and *M. lanceolatus* UBOCC-A-109193 associated to *P. jensenii* CIRM-BIA1774  
343 respectively. It and was increased by 2 and 7 days for *P. commune* with the same associations  
344 respectively. The most efficient fermentate was that of *P. jensenii* CIRM-BIA1774 which  
345 inhibited *M. racemosus* and *P. commune* for up to 21 and 14 days, respectively. No visible  
346 growth of the fungal targets was observed after natamycin application during the time of the  
347 experiment (33 days). Interestingly, we did not observe any effect of the fermentates on the  
348 smear development while that of the cheese treated with natamycin was considerably reduced  
349 (data not shown).

350 The strong antifungal activity of the fermentates after surface-treatment of semi-hard cheeses  
351 was also confirmed by the durability test results. Indeed, as shown in Figure 1, while control  
352 cheeses (untreated) were almost completely covered with mold after 6 week incubation at 10°C  
353 in a naturally-contaminated ripening chamber, those which were surface-treated with  
354 fermentates appeared less contaminated with a much smaller surface covered by mold, with the  
355 exception of one cheese replicate (out of 3) treated with *P. jensenii* fermentate.

356 To assess the impact of fermentates on cheese sensory traits, a categorization approach was  
357 used in which assessors were asked to group products in function of their sensory similarities.



358 A multiple correspondence analysis with the different cheeses assessed and their respective  
359 95% confidence ellipses is presented in Figure 2. The total variance explained by the two  
360 dimensions was 62 %, with Dim 1 and Dim 2 representing 34 and 28 % of variance,  
361 respectively. The ellipse sizes of all samples were large, especially that representing the  
362 confidence interval of the cheese treated with *P. jensenii* fermentate. Moreover, a considerable  
363 overlapping of sample confidence ellipses was observed, indicating that panelists could not  
364 clearly differentiate the samples from each other and thus suggesting that surface-spraying of  
365 cheese surfaces with fermentates had a negligible effect on cheese organoleptic  
366 characteristicscharacteristics.

367

#### 368 3.4. Antifungal activity and organoleptic impact of fermentates after incorporation in sour 369 cream

370 For this assay *L. rhamnosus* CIRM-BIA1952 and/or *P. jensenii* CIRM-BIA1774 fermentates  
371 were tested directly incorporated into the sour cream. Tested concentrations were initially 2 and  
372 5 % (m/m) of each fermentate and a mixture of both fermentates at 2.5 % (m/m) each. Once  
373 again, the *P. jensenii* CIRM-BIA1774 fermentate, alone or in combination with *L. rhamnosus*  
374 CIRM-BIA1952 fermentate, showed a high antifungal activity (similar to that of potassium  
375 sorbate) against *M. racemosus* and *P. commune* delaying their growth up to 30 days (total  
376 duration of the experiment) as compared to the negative control (Table 2). It should be noted  
377 that a more limited effect (19 days) was observed at 2 % against *P. commune*. The antifungal  
378 effect of these fermentates were also tested against *R. mucilaginosa* (Figure 3). All fermentates  
379 containing *P. jensenii* CIRM-BIA1774 totally inhibited *R. mucilaginosa* growth (counts  
380 <1CFU/g) during storage while that of *L. rhamnosus* CIRM-BIA1952 did not show any  
381 antifungal activity.

382 Durability tests confirmed the trends observed with the challenge tests. Indeed, whatever the  
383 tested concentrations, none of the samples containing *P. jensenii* CIRM-BIA1774 fermentate  
384 showed any visible fungal contamination (Figure 4). On the other hand, samples containing  
385 *L. rhamnosus* CIRM-BIA1952 fermentates at 2 and 5 % were only slightly less contaminated  
386 than control samples with 90 and 80 % of samples showing visible fungal contamination,  
387 respectively.

388 Using a similar approach to that used for semi-hard cheeses, we assessed the impact of these  
389 fermentates on sour cream sensory characteristics (Figure 5). In the multiple correspondence  
390 analysis, the total variance explained by the two dimensions was 61 %, with Dim 1 and Dim 2  
391 representing 37 and 24 % of variance, respectively. The overlapping of the confidence ellipses  
392 of the control sample and sour cream with 2 % *L. rhamnosus* CIRM-BIA1952 fermentate  
393 showed that these samples were perceived as similar by the panelists. In addition, samples  
394 containing *P. jensenii* CIRM-BIA1774 fermentate which were grouped together as well as sour  
395 creams containing 5 % *L. rhamnosus* CIRM-BIA1952 fermentate were clearly separated from  
396 the control indicating that panelists could perceive significant sensory differences with the  
397 control sour cream. Panelists described sour creams with *P. jensenii* CIRM-BIA1774  
398 fermentate as very unpleasant, strong, or acid showing a high negative impact of this fermentate  
399 on the product flavour, while sour creams with 5 % *L. rhamnosus* CIRM-BIA1952 fermentate  
400 were described as dry, pasty or burnt showing an impact on texture and flavour.

401 Based on these results, using an acceptability test, we investigated the concentration of  
402 *P. jensenii* CIRM-BIA1774 fermentate at which no organoleptic impact could be perceived in  
403 sour cream (Supplementary Figure 1). As expected, acceptability decreased with increasing  
404 concentrations of fermentate. Among the 26 panelists, 21 (80.8 %) accepted the control sour  
405 cream while only 6 (23.1 %) accepted the product with 0.7 % *P. jensenii* CIRM-BIA1774  
406 fermentate. The 21 panelists who did not reject the sour cream also accepted the sour cream

407 containing 0.1 % *P. jensenii* CIRM-BIA1774 fermentate, while 10 panelists (47.6 %) accepted  
408 that containing 0.4 % *P. jensenii* fermentate. These 2 concentrations were selected for further  
409 challenge and durability tests.

410 For challenge tests in sour creams containing 0.1 and 0.4 % *P. jensenii* CIRM-BIA1774  
411 fermentate, fungal growth delays were much shorter than those observed at 2 and 5 %. Indeed,  
412 they were 1 and 3 days, and 3 and 4 days for *M. racemosus* and *P. commune*, respectively (Table  
413 2). On the contrary, no antifungal activity was observed against *R. mucilaginosa* (Figure 3).

414 Concerning durability tests, all control sour creams were contaminated, as well as sour creams  
415 with 0.1% fermentate (Figure 6). Interestingly, as observed for sour cream samples  
416 supplemented with 0.08 % potassium sorbate (positive control), none of the samples with 0.4%  
417 fermentate showed any visible fungal contamination (Figure 6).

418

#### 419 **4. Discussion**

420 In the first part of this study, the antifungal activity of fermentates obtained from a large  
421 collection of LAB, propionibacteria and fungi was evaluated *in vitro* against 4 fungal targets,  
422 using a high-throughput screening method in a cheese-mimicking model (Garnier et al., 2017b).

423 This method allowed performing the antifungal activity screening of 1396 fermentates derived  
424 from 698 microorganisms in a reasonable time-period (1600 assays per week). Moreover,  
425 because we aimed to develop ingredients compatible with dairy technologies and because  
426 marked differences in antifungal activity can be observed between *in vitro* and *in situ* results  
427 (Leyva Salas et al., 2017), for screening, we chose to use of a cheese-mimicking medium, food  
428 structure and composition may influencing the migration of antifungal compounds, to increase  
429 the probability to select fermentates which would actually be active in real products. Indeed,  
430 our miniaturized cheeses have a physico-chemical composition and structure that better reflect  
431 those encountered by fungal spoilers in dairy products.

432 This *in vitro* screening revealed that several fermentates derived from various species,  
433 including *Lactobacillus*, *Propionibacterium*, and fungal species exhibited a significant  
434 antifungal activity. Interestingly, the proportion of active fermentates and their level of  
435 antifungal activity varied according to the dairy substrate. Indeed, LH substrate, made from low  
436 heat skimmed milk and 45 % anhydrous milk fat, allowed a better expression of antifungal  
437 activity (qualitatively and/or quantitatively) as compared to that observed for UF substrate,  
438 composed of milk ultrafiltration permeate supplemented with yeast extract. The antifungal  
439 activity was not correlated to the ability of the selected microorganisms to grow in each of these  
440 substrates. Indeed, for a dedicated strain, an assessment of the final biomass on 27 bacterial and  
441 18 fungal strains showed that similar populations were generally reached in both substrates after  
442 fermentation ( $10^7$ - $10^9$  CFU/ml for bacteria and  $10^7$  CFU/ml for yeasts), except for filamentous  
443 fungi which generally showed ~5 times more final biomass in LH than in UF (data not shown).  
444 Therefore, for most bacteria and yeasts, it is likely that the differential expression in the  
445 production of antifungal activity was related to the substrate composition, while, for  
446 filamentous fungi, both biomass level and/or substrate composition could be involved. The  
447 individual components which are responsible remain unclear. It can be underlined however that  
448 LH but not UF substrate contains milk fat and is richer in proteins, both qualitatively (presence  
449 of caseins) and quantitatively (374 g/kg total nitrogen and 190 g/kg nonprotein nitrogen in LH  
450 versus 5.7 g/kg total nitrogen and 5.5 g/kg nonprotein nitrogen in UF). Both fat and proteins  
451 are precursors of antifungal compounds such as fatty acids, peptides, organic acids such as  
452 phenyllactic acid, and volatile compounds (Hidalgo et al., 2015, Théolier et al., 2013). Similar  
453 observations were made in a recent study (Özcelik et al., 2016) showing that production of  
454 lactic, acetic, succinic, propionic, formic, and butyric acids by LAB significantly varied  
455 according to the growth medium used. Concerning the proportion of antifungal strains observed  
456 in the present study, out of 1396 tested fermentates, 17 % of LH and 1 % UF fermentates

457 showed a significant antifungal activity (growth delay or total inhibition) against at least one  
458 fungal target. These proportions are much lower than those found in previous studies. For  
459 example, Inglin et al. (2015) found that ~30 % of 504 tested lactobacilli isolates, cultivated in  
460 MRS agar, inhibited at least one of the 4 tested fungal targets. More recently, Le Lay et al.  
461 (2016) reported that ~32 % of the 270 tested LAB and 50 tested propionibacteria were active  
462 (total inhibition) against 5 fungal targets after growth in wheat flour hydrolysate agar. The lower  
463 number of active strains reported in the present study can be explained by the fact that  
464 fermentates instead of bacterial colonies (Inglin et al., 2015) or bacteria embedded in agar  
465 medium (Le Lay et al. 2016) were used, probably resulting in a more stringent selection of  
466 active isolates. In addition, the substrates used for screening were also different, for example,  
467 Inglin et al. (2015) utilized MRS medium containing acetate, which is known to potentialize  
468 antifungal activity, while in Le Lay et al. (2016) work, pH of wheat flour hydrolysate medium  
469 after LAB growth was ~3.5 (1.5 unit lower than that obtained in miniaturized cheeses),  
470 which enhances the antifungal activity of organic acids.

471 As expected from the current literature data (see Leyva-Salas et al., 2017 for a review),  
472 antifungal isolates mostly belonged to *Lactobacillus* species such as *L. brevis* (Axel et al. 2016),  
473 *L. buchneri* (Kharazian et al. 2017), *L. plantarum* (Cheong et al. 2014), *L. harbinensis*  
474 (Belguesmia et al. 2014), and *L. rhamnosus* (Fernandez et al. 2017). This can be explained by  
475 the fact that *Lactobacillus* spp. produce a large range of antifungal molecules including organic  
476 acids (Brosnan et al., 2012), hydrogen peroxide, ethanol, cyclic dipeptides, proteinaceous  
477 compounds and fatty acids (Luz et al., 2017; Crowley et al., 2013; Lavermicocca et al., 2000).  
478 The synergistic or additive action of these molecules, despite being produced at weak  
479 concentrations, could increase the antifungal activity. Interestingly, one propionibacterium and  
480 7 fungal strains also possessed antifungal activity against the selected targets. Indeed,  
481 propionibacteria produce propionic acid, as well as hydrogen peroxide, azelaic, phenyllactic

482 and hydroxyphenyllactic acids (Schwenninger et al., 2004, Fernandez et al., 2017; Le lay et al.,  
483 2016). Concerning fungi, they are known to produce antifungal compounds such as volatile  
484 compounds, killer proteins, antifungal peptides and lytic enzymes (Leyva-Salas et al., 2017,  
485 Nùñez et al., 2015, Izgu et al., 2011, Coelho et al., 2009; Weiler and Schmitt, 2003; Wheatley,  
486 2002; Schmitt et al., 1996) (see the review by Delgado et al., 2016). To our best knowledge,  
487 this is the first time that *A. pullulans*, *C. tenuis* and *M. lanceolatus* are reported for their  
488 antifungal activities. In contrast, *Trichoderma* spp. such as *T. harzianum* and *T. viride* are  
489 commonly used as biocontrol agents and are known to produce both non-volatile and volatile  
490 compounds like pyrones, butenolides, azaphylones, anthraquinones, trichothecenes, terpenoids  
491 and steroids, as well as non-ribosomal peptides, such as siderophores and peptaibols  
492 (Mukherjee et al., 2012).

493 Our results also confirmed that antifungal activities are strain- and target-dependent traits.  
494 Indeed, within each screened species, strains significantly differed in their antifungal activity  
495 as reported elsewhere (Le Lay et al., 2016, Cortés-Zavaletta et al. 2014. Russo et al., 2017). In  
496 the tested conditions, *P. commune*, followed by *M. racemosus*, were the most sensitive fungi to  
497 LH fermentate from LAB and propionibacteria, while *Y. lipolytica* and *G. geotrichum* were the  
498 most resistant. On the contrary, *G. geotrichum*, followed by *M. racemosus* were the most  
499 sensitive to LH fermentate derived from fungi. The same fungal strains as the ones used in the  
500 present study were previously characterized for their reistance to various organic acid  
501 preservatives and to natamycin Garnier et al. (2017). Interestingly, their resistance pattern  
502 followed the same pattern as that observed with LAB and propionibacteria fermentates, with  
503 *G. geotrichum* and especially *Y. lipolytica* showing the highest Minimum Inhibitory  
504 Concentrations to preservatives. Concerning LH fermentates from fungi and the relatively  
505 higher susceptibility of *G. geotrichum*, it can be hypothetized that other compounds (volatile  
506 compounds, proteinaceous compounds, fatty acids) than organic acids are involved. In future

507 studies, it would be interesting to understand which antifungal molecules are involved in the  
508 antifungal activity of the most active fermentates and to investigate their mode of action against  
509 different fungal targets.

510 Target fungal strains selected for this study were relevant since they were not only isolated  
511 from actual contaminated dairy products but also known to be representative of common dairy  
512 product contaminants (Garnier et al., 2017b, Pitt and Hocking, 2009).

513 In the next step, we assessed how culture conditions could be optimized to enhance  
514 antifungal activities. In general, the longer the incubation time and the higher the cell  
515 concentration was, the more the antifungal activity was significant. *L. paracasei* L117  
516 fermentate was an exception as its highest antifungal activity was obtained after 20 h of  
517 fermentation. This could be explained by a degradation of the antifungal compounds through  
518 time. In addition to incubation time, cold or hot thermal shock increased the antifungal activity  
519 of several strains. The reasons for this are unclear but suboptimal temperatures could trigger or  
520 increase the production of antifungal compounds. For example, suboptimal temperatures can  
521 promote the production of bacteriocins in LAB (Leal-Sánchez et al. 2002). It is worth  
522 mentioning that *L. brevis* L128 and *L. buchneri* L162 and L233, which had interesting  
523 antifungal properties (Table 1), were not kept further for *in situ* applications because they were  
524 shown to possess biogenic amines associated genes (*hdc* and *agdi* for L162 and L233, *agdi* and  
525 *tyrdc* for L128) and to produce histamine, putrescine and tyramine *in vitro* (Coton et al.  
526 submitted). The safety of the LAB strains selected for *in situ* tests, e.g. biogenic amines  
527 production and antibiotic resistance pattern were also verified in *P. jensenii* CIRM-BIA1774  
528 and *L. rhamnosus* CIRM-BIA1952, both of which were considered as safe for dairy food  
529 applications (Coton et al. submitted).

530 As previously discussed, *in situ* tests are a crucial step in the process of developing an  
531 antifungal ingredient because *in vitro* screening can lead to an overestimation of the antifungal

532 activity. Surface-spraying of antifungal fermentates on semi-hard cheese and incorporation in  
533 sour cream were performed to evaluate fermentate antifungal activity in real products against  
534 selected spoilage fungi (challenge tests) and in a naturally contaminated environment  
535 (durability test). In semi-hard cheese, surface-spraying of *P. jensenii* CIRM-BIA1774  
536 fermentate on the cheese surface was also efficient for delaying spoilage fungal growth without  
537 any impact on cheese sensory characteristics. To our best knowledge, it is the  
538 first time that the use of a fermentate is shown to efficiently replace natamycin. Further work  
539 would be necessary in an industrial context to verify its antifungal activity in different cheese  
540 types as well as to assess whether it can be produced on a large scale with an acceptable cost.  
541 In sour cream, *P. jensenii* CIRM-BIA1774 fermentate at 2 and 5 % harbored a very high  
542 antifungal activity against the 3 tested fungi and prevented fungal contaminations of natural  
543 origin for up to one month. However, at these high concentrations, it had a negative impact on  
544 sensory attributes yielding very acid sour cream with propionic odor and flavor mainly linked  
545 to high concentration of propionic acid. Nevertheless, it prevented natural contaminations at  
546 0.4 %, with a limited impact on consumer acceptance. Further work is therefore necessary to  
547 evaluate whether it could be used in this product type.

548 *P. jensenii* was previously shown to possess antifungal activities (Schwenninger and Meile,  
549 2004) and more generally, *Propionibacterium* spp. are known for their antifungal activities  
550 (Lind et al. 2007). Thus, different strains of *P. freudenreichii* are currently used to produce  
551 antifungal fermentates such as Bio Profit (Danisco Niebüll GmbH, Germany) and Microgard  
552 (Wesman Foods, Inc., OR, USA). Propionibacteria antifungal activity is due to propionic acid,  
553 well-known as an antifungal acid, but also to miscellaneous compounds including low  
554 molecular-weight compounds (Schwenninger et al. 2008). This most probably explains why,  
555 among the 70 tested propionibacteria strains which all grew in the substrate and produced  
556 propionic acid, only one had an antifungal activity of interest.



557 The use of *L. rhamnosus* CIRM-BIA1952 fermentate yielded a limited antifungal activity in  
558 sour cream and semi-hard cheese. However, it is worth mentioning that, for *in situ* challenge  
559 tests, 50 fungal spores were inoculated, which is a worst-case scenario in comparison to natural  
560 contamination, and that tested products were incubated at 10°C, which is also favorable to  
561 fungal growth. Concerning *M. lanceolatus*, this species is encountered in specific cheeses where  
562 it plays a crucial technological role (Desmasures, 2014, Hermet et al., 2014) and, to our  
563 knowledge, this is the first time that the antifungal activity of a *M. lanceolatus* fermentate is  
564 demonstrated.

565

## 566 **5. Conclusion**

567 To our best knowledge, this study is the first one in which the development of dairy-based  
568 antifungal ingredients is described from *in vitro* screening to pilot scale applications, including  
569 the evaluation of their impact on product organoleptic properties. The obtained results provided  
570 3 promising antifungal fermentates from *L. rhamnosus* CIRM-BIA1952, *P. jensenii* CIRM-  
571 BIA1774 and *M. lanceolatus* UBOCC-A-109193, which may be used in dairy foods as part of  
572 the hurdle technology to prevent fungal spoilage. From a fundamental point of view, the  
573 identification of the molecules harboring antifungal activities and their mechanism of action  
574 should be explored.

575

## 576 **Acknowledgments**

577 This work was financially supported by the Conseils Régionaux de Bretagne and Pays de la  
578 Loire,, France (grants N°13008651 and N°2014-01081, respectively) in the framework of the  
579 PROFIL interregional project under the scientific coordination of INRA (J. Leonil) and  
580 managed by the Bretagne Biotechnologies Alimentaires industrial association. We are grateful  
581 to the INRA STLO Platform ([https://www6.rennes.inra.fr/plateforme\\_lait](https://www6.rennes.inra.fr/plateforme_lait)) for providing help  
582 and support.

583  
584

585 **References**

586

587 Axel, C., Brosnan, B., Zannini, E., Furey, A., Coffey, A., Arendt, E.K., 2016. Antifungal  
588 sourdough lactic acid bacteria as biopreservation tool in quinoa and rice bread. *Int. J. Food*  
589 *Microbiol.* 239, 86-94.

590

591 Belguesmia, Y., Rabesona, H., Mounier, J., Pawtowski, A., Le Blay, G., Barbier, G., 2014.  
592 Characterization of antifungal organic acids produced by *Lactobacillus harbinensis* K. V9. 3.1  
593 Np immobilized in gellan-xanthan beads batch fermentation. *Food Control* 36, 205-211.

594

595 Bourdichon, F., Casaregola, S., Farrokh, C., Frisvad, J.C., Gerds, M.L., Hammes, W.P.,  
596 Harnett, J., Huys, G., Laulund, S., Ouwehand, A., Powell, I.B., Prajapati, J.B., Seto, Y., Ter  
597 Schure, E., Van Boven, A., Vankerckhoven, V., Zgoda, A., Tuijtelaars, S., Hansen, E.B., 2012.  
598 Food fermentations: Microorganisms with technological beneficial use. *Int. J. Food Microbiol.*  
599 154, 87–97.

600

601 Brosnan, B., Coffrey, A., Arendt, E.K., Furey, A., 2012. Rapid identification by use of the LTQ  
602 Orbitrap hybrid FT mass spectrometer, of antifungal compounds produced by lactic acid  
603 bacteria. *Anal. Bioanal. Chem.* 403, 2983-2995.

604

605 Cadoret, M., Lê, S., Pagès, J., 2009. A Factorial Approach for Sorting Task data (FAST). *Food*  
606 *Qual. Pref.* 20, 401-417.

607

608 Cheong, E.Y.L.; Sandhu, A.; Jayabalan, J.; Kieu Le, T.T.; Nhiep, N.T.; My Ho, H.T.;  
609 Zwielehner, J.; Bansal, N.; Turner, M.S., 2014. Isolation of lactic acid bacteria with antifungal  
610 activity against the common cheese spoilage mold *Penicillium commune* and their  
611 potential as biopreservatives in cheese. *Food Control* 46, 91–97.

612

613 Coelho, A.R., Tachi, M., Pagnocca, F.C., Nobrega, G.M.A., Hoffman, F.L., Harada, K.I.,  
614 Hirooka, E.Y., 2009. Purification of *Candida guilliermondii* and *Pichia ohmeri* killer toxin as  
615 an active agent against *Penicillium expansum*. *Food Addit. Contam. A Chem. Anal. Control*  
616 *Expo. Risk Assess.* 26, 73-81.

617

618 Cortés-Zavaleta, O., López-Malo, A., Hernández-Mendoza, A., García, H.S., 2014. Antifungal  
619 activity of lactobacilli and its relationship with 3-phenyllactic acid production. *Int. J. Food*  
620 *Microbiol.* 173, 30-35.

621

622 Coton, M., Lebreton, M., Leyva Salas, M., Garnier, L., Navarri, M., Pawtowski, A., Le Blay,  
623 G., Valence, F. Coton, E., Mounier, J., submitted. Safety risk assessment of potential antifungal  
624 lactic acid bacteria and propionibacteria. Submitted in *J. Dairy Sci.*

625

626 Crowley, S., Mahony, J., van Sinderen, D., 2013. Current perspectives on antifungal lactic acid  
627 bacteria as natural bio-preservatives. *Trends Food Sci. Technol.* 33, 93–109.

628

629 Delavenne, E., Cliquet, S., Trunet, C., Barbier, G., Mounier, J., Le Blay, G., 2015.  
630 Characterization of the antifungal activity of *Lactobacillus harbinensis* K.V9.3.1Np and  
631 *Lactobacillus rhamnosus* K.C8.3.1I in yogurt. *Food Microbiol.* 45, 10–17.

632

633 Delgado, J., Peromingo, B., Nunez, F., Asensio, M.A., 2016. Use of molds and their antifungal  
634 proteins for biocontrol of toxigenic molds on dry-ripened cheese and meats. *Curr. Opin. Food*  
*Sci.* 11, 40-45.

635  
636 Desmaures, N., 2014. Mold-ripened varieties, *Encycl. Food Microbiol*, p.409.  
637  
638 Fernandez, B., Vimont, A., Desfossés-Foulcault, E., Daga, M., Arora, G., Fliss, I., 2017.  
639 Antifungal activity of lactic and propionic acid bacteria and their potential as protective culture  
640 in cottage cheese. *Food Control* 78, 350-356.  
641  
642 Garnier, L., Valence, F., Mounier, J., 2017a. Diversity and control of spoilage fungi in dairy  
643 products: an update. *Microorganisms* 5, 42.  
644  
645 Garnier, L., Valence, F., Pawtowski, A., Auhustsinava-Galerie, L., Frotté, N., Deniel, F.,  
646 Coton, E., Mounier, J., 2017b. Diversity of spoilage fungi associated with various French dairy  
647 products. *Int. J. Food Microbiol.* 241, 191-197.  
648  
649 Garnier L., Leyva Salas, M., Pinon, N., Wiernasz, N., Pawtowski, A., Coton, E., Mounier, J.,  
650 Valence, F Technical note: High-throughput method for antifungal activite screening in a  
651 cheese mimicking model. *J. Dairy Sci.*, accepted.  
652  
653 Hermet, A., Mounier, J., Keravec, M., Vasseur, V., Barbier, G., Jany, J.L., 2014. Application  
654 of capillary electrophoresis single-stranded conformation polymorphism (CE-SSCP) analysis  
655 for identification of fungal communities in cheese, *Food Microbiol.* 41 82–90.  
656  
657 Hidalgo, M.E., Côrrea, A.P.F., Canales, M.M., Daroit, D.J., Brandelli, A., Risso, P., 2015.  
658 Biological and physiological properties of bovine sodium caseinate hydrolysates obtained by a  
659 bacterial protease preparation. *Food Hydrocolloids*, 43, 510-520.  
660  
661 Inglin, R.C., Stevens, M.J., Meile, L., Lacroix, C., Meile, L., 2015. High-throughput screening  
662 assays for antibacterial and antifungal activities of *Lactobacillus* species. *J. Microbiol. Meth.*  
663 114, 9-26.  
664  
665 Izgu, D.A., Kepekci, R.A., Izgu, F., 2011. Inhibition of *Penicillium digitatum* and *Penicillium*  
666 *italicum* *in vitro* and *in planta* with Panomycocin, a novel exo- $\beta$ -1,3-glucanase isolated from  
667 *Pichia anomala* NCYC 434. *Int. J. Gen. Mol. Microbiol.* 99, 85-91.  
668  
669 Katarzyna, K., Bzducha-Wrobel, A., Krasniewska, K., hlebowska-Smigiel, A., Gniewosz, M.,  
670 2017. *J. Food Prot.* 80(1), 96-103.  
671  
672 Lavermicocca, P., Valerio, F., Evidente, A., Lazzaroni, S., Corsetti, A., Gobbetti, M., 2000.  
673 Purification and characterization of novel antifungal compounds from the sourdough  
674 *Lactobacillus plantarum* Strain 21B. *Appl. Environ. Microbiol.* 66, 4084–4090.  
675  
676 Ledenbach, L.H., Marshall, R.T., 2010. Microbiological spoilage of dairy products, in: Sperber,  
677 W.H., Doyle, M.P. (Eds.), *Compendium of the Microbiological Spoilage of Foods and*  
678 *Beverages*, *Food Microbiology and Food Safety*. Springer New York, pp. 41–67.  
679  
680 Le Lay, C., Mounier, J., Vasseur, V., Weill, A., Le Blay, G., Barbier, G., Coton, E., 2016. *In*  
681 *vitro* and *in situ* screening of lactic acid bacteria and propionibacteria antifungal activities  
682 against bakery product spoilage molds. *Food Control* 60, 247-255.  
683

684 Leal-Sánchez, M.V., Jiménez-Díaz, R., Maldonado-Barragán, A., Garrido-Fernández, A.,  
685 Ruiz-Barba, J.L., 2002. Optimization of bacteriocins production by batch fermentation of  
686 *Lactobacillus plantarum* LPCO10. *App. Env. Microbiol.* 68(9), 4465-4471.

687 Leyva Salas, M., Mounier, J., Valence, F., Coton, M., Thierry, A., Coton, E., 2017. Antifungal  
688 microbial agents for food biopreservation - a review. *Microorganisms* 5(3), 37.

689 Lind, H., Sjögren, J., Gohil, S., kenne, L., Schnürer, J., Broberg, A., 2007. Antifungal  
690 compounds from cultures of dairy propionibacteria types strains. *FEMS Microbiol. Letters*  
691 271(2), 310-315.

692  
693 Luz, C., Saladina, F., Luciano, F.B., Mañes, J., Meca, G., 2017. *In vitro* antifungal activity of  
694 bioactive peptides produced by *Lactobacillus plantarum* against *Aspergillus parasiticus* and  
695 *Penicillium expansum*. *Food Sci. Technol.* 81, 128-135.

696  
697 Mukherjee, P.K., Horwitz, B.A. Kenerlet, C.M., 2011. Secondary metabolism in *Trichoderma*  
698 – a genomic perspective. *Microbiol.* 158, 35-45.

699  
700 Nguyen Van Long, N., Joly, C., Dantigny, P., 2016. Active packaging with antifungal activities.  
701 *Int. J. Food Microbiol.* 220, 73-90.

702  
703 Núñez, F., Lara, M.S., Peromingo, B., Delgado, J., Sánchez-Montero, L., Andrade, M.J., 2015.  
704 Selection and evaluation of *Debaryomyces hansenii* isolates as potential bioprotective agents  
705 against toxigenic penicillia in dry-fermented sausages. *Food Microbiol.* 46, 114-120.

706  
707 Özcelik, S., Kuley, E., Özogul, F., 2016. Formation of lactic, acetic, succinic, propionic, formic  
708 and butyric acid by lactic acid bacteria. *Food Sci. Technol.* 73, 536-542.

709  
710 Pitt, J.I., Hocking, A.D., 2009. *Fungi and food spoilage*. Springer Science & Business Media.

711  
712 Regulation (EC) No 852/2004 of the European Parliament and of the Council of 29 April 2004  
713 on the hygiene and foodstuffs.

714  
715 Russo, P., Arena, M.P., Fiocco, D., Capozzi, V., Drider, D., Spano, G., 2017. *Lactobacillus*  
716 *plantarum* with broad antifungal activity: A promising approach to increase safety and shelf-  
717 life of cereal-based products. *Int. J. Food Microbiol.* 247, 48-54.

718  
719 Sakkas, L., Moutafi, A., Moschopoulou, E., Moatsou, G., 2014. Assessment of heat treatment  
720 of various types of milk. *Food Chem.* 159, 293–301.

721  
722 Schmitt, M.J., Klavehn, P., Wang, J., Schönig, I., Tipper, D.J., 1996. Cell cycle studies on the  
723 mode of action of yeast K28 killer toxin. *Microbiol.* 142, 2655-2662.

724  
725 Schnürer, J., Magnusson, J., 2005. Antifungal lactic acid bacteria as biopreservatives. *Trends*  
726 *Food Sci. Technol.*, 16, 70–78.

727  
728 Schwenninger, S.M., Meile, L., 2004. A mixed culture of *Propionibacterium jensenii* and  
729 *Lactobacillus paracasei* subsp. *paracasei* inhibits food spoilage yeasts. *System. Appl.*  
730 *Microbiol.* 27, 229-237.

731

- 732 Schwenninger, S.M., Lacroix, C., Truttmann, S., 2008. Characterization of low-molecular-  
733 weight antiyeast metabolites produced by a food-protective *Lactobacillus-Propionibacterium*  
734 Coculture. 71, 2481-2487.  
735
- 736 Stiles, M.E., 1996. Biopreservation by lactic acid bacteria. *Antonie Leeuwenhoek* 70, 331-345.  
737
- 738 Théolier, J., Hammami, R., Labelle, P., Fliss, I., Jean, J., 2013. Isolation and identification of  
739 antimicrobial peptides derived by peptic cleavage of whey protein isolate. *J. Funct. Foods* 5,  
740 706-714.  
741
- 742 Varsha, K.K., Nampoothiri, K.M., 2016. Appraisal of lactic acid bacteria as protective cultures.  
743 *Food Control* 69, 61-64.  
744
- 745 Weiler, F., Schmitt, M. J., 2003. Zygoicin, a secreted antifungal toxin of the yeast  
746 *Zygosaccharomyces bailii*, and its effect on sensitive fungal cells. *FEMS Yeast Res.* 3, 69-76.  
747
- 748 Wheatley, R.E., 2002. The consequences of volatile organic compound mediated bacterial and  
749 fungal interactions. *Antonie van Leeuwenhoek Int. J. Gen. Microbiol.* 81, 357-364.

750 **Table 1.** Effect of culture conditions on the antifungal activity of fermentates from selected lactic acid bacteria, propionibacteria and fungi against  
 751 *M. racemosus* UBOCC-A-116002, *P. commune* UBOCC-A-116003, *R. mucilaginosa* UBOCC-A-216004 and *Y. lipolytica* UBOCC-A-216006.  
 The antifungal activity is expressed as increase in time to visible growth of the fungal target (days) as compared to a control without fermentate.

752  
 753

Fermentation substrate	Strain	Culture conditions*	Cell concentration (log <sub>10</sub> CFU/ml)	Increase in time to visible fungal growth (in days)			
				<i>M. racemosus</i>	<i>P. commune</i>	<i>R. mucilaginosa</i>	<i>Y. lipolytica</i>
Low heat milk	<i>L. buchneri</i> L151	1	5.10 <sup>7</sup>	0	0	0	0
		2	6.10 <sup>7</sup>	0	0	+1	0
		3	2.10 <sup>8</sup>	0	+1	+2	0
		4	6.10 <sup>7</sup>	0	0	+1	0
		5	7.10 <sup>7</sup>	0	0	+2	0
	<i>L. buchneri</i> L162	1	1.10 <sup>7</sup>	0	0	+1	0
		2	4.10 <sup>8</sup>	0	+1	+1	0
		3	1.10 <sup>8</sup>	0	+3	+1	0
		4	8.10 <sup>8</sup>	0	+3	+1	0
		5	1.10 <sup>9</sup>	0	+4	+2	0
	<i>L. buchneri</i> L164	1	6.10 <sup>8</sup>	0	+1	+1	0
		2	3.10 <sup>8</sup>	0	+1	+1	0
		3	2.10 <sup>8</sup>	0	0	+1	0
		4	4.10 <sup>8</sup>	0	+1	+1	0
		5	2.10 <sup>8</sup>	0	0	+1	0
<i>L. buchneri</i> L233	1	6.10 <sup>7</sup>	0	+1	0	0	
	2	7.10 <sup>6</sup>	0	+1	0	0	
	3	5.10 <sup>8</sup>	+1	+3	+1	0	
	4	2.10 <sup>8</sup>	0	+2	+1	0	
	5	4.10 <sup>8</sup>	+1	+4	+2	0	
<i>L. harbinensis</i> L172	1	2.10 <sup>8</sup>	+1	0	+1	0	
	2	7.10 <sup>7</sup>	0	0	0	0	
	3	8.10 <sup>7</sup>	0	0	+2	0	
	4	2.10 <sup>8</sup>	0	0	+1	0	
	5	1.10 <sup>8</sup>	+1	0	+1	0	
<i>L. paracasei</i> L117	1	2.10 <sup>9</sup>	+1	+4	+2	0	
	2	2.10 <sup>9</sup>	0	+1	+2	0	

		3	1.10 <sup>8</sup>	0	+1	+1	0
		4	1.10 <sup>y</sup>	0	+1	+2	0
		5	2.10 <sup>y</sup>	0	0	+1	0
	<i>L. paracasei</i> L194	1	3.10 <sup>y</sup>	0	+2	+2	0
		2	2.10 <sup>y</sup>	0	+1	+1	0
		3	3.10 <sup>8</sup>	0	+1	+1	0
		4	2.10 <sup>y</sup>	0	+1	+1	0
		5	2.10 <sup>y</sup>	0	+1	+1	0
	<i>L. plantarum</i> L244	1	7.10 <sup>8</sup>	0	+1	+1	0
		2	5.10 <sup>8</sup>	0	+1	+1	0
		3	2.10 <sup>8</sup>	0	0	+1	0
		4	6.10 <sup>8</sup>	0	+1	+1	0
		5	1.10 <sup>8</sup>	0	+1	+1	0
	<i>L. rhamnosus</i> CIRM-BIA1952	1	2.10 <sup>y</sup>	0	+2	+2	0
		2	1.10 <sup>y</sup>	0	+3	+2	0
		3	1.10 <sup>y</sup>	0	+3	+1	0
		4	1.10 <sup>y</sup>	0	+1	+1	0
		5	2.10 <sup>y</sup>	0	+1	+2	0
	<i>M. lanceolatus</i> UBOCC-A-109193	1	nd†	+1	0	nd	0
		2	nd	+2	+1	nd	0
		3	nd	+1	+1	nd	0
		4	nd	+4	+1	nd	0
		5	nd	0	+1	nd	0
UF milk permeate	<i>L. buchneri</i> L162	1	1.10 <sup>y</sup>	0	0	+1	0
		2	4.10 <sup>8</sup>	0	0	+1	0
		3	1.10 <sup>8</sup>	0	0	0	0
		4	8.10 <sup>8</sup>	0	0	0	0
		5	1.10 <sup>y</sup>	0	0	0	0
	<i>P. jensenii</i> CIRM-BIA1774	1	1.10 <sup>y</sup>	+1	+1	+1	0
		2	2.10 <sup>8</sup>	+2	+1	+1	0
		3	6.10 <sup>8</sup>	+2	+1	+2	0
		4	8.10 <sup>8</sup>	+2	+1	+2	+1
		5	8.10 <sup>8</sup>	+2	+1	+1	0

755 \*Culture conditions for lactobacilli: 1, 24h/30°C; 2, 48h/30°C; 3, 72h/30°C; 4, 48h/30°C followed by 24h/12°C; 5, 48h/30°C followed by 24h/43°C Culture conditions for  
756 *M. lanceolatus* UBOCC-A-109193: 1, 48h/25°C; 2, 96h/25°C ; 3, 168h/25°C; 4, 96h/25°C followed by 48h/12°C; 5, 96h/25°C followed by 48h/43°C. Culture conditions for  
757 *P. jensenii* CIRM-BIA1774: 1, 48h/30°C; 2, 72h/30°C; 3, 96h/30°C; 4, 96h/30°C followed by 24h/12°C; 5, 96h/30°C followed by 24h/43°C.  
758 †nd, not determined.

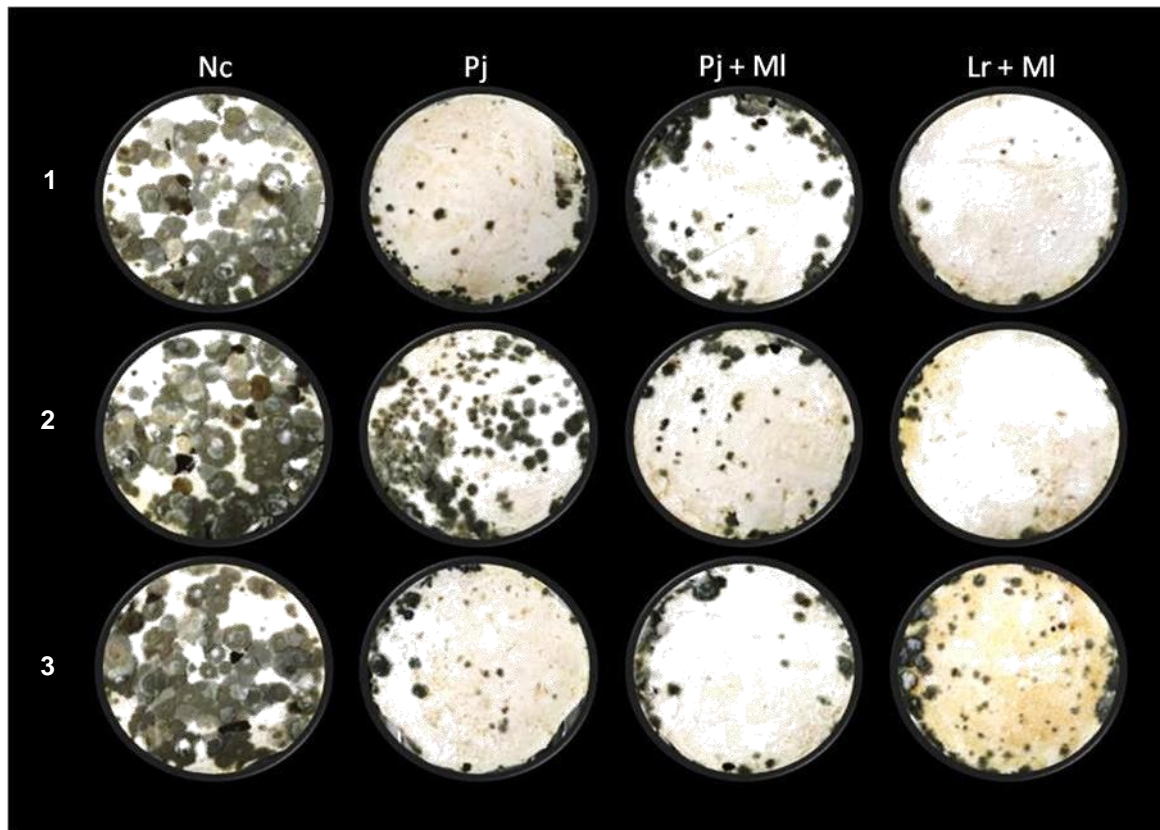


759 **Table 2.** Antifungal activity of selected fermentates against *M. racemosus* UBOCC-A-116002 and *P. commune* UBOCC-A-116003 in semi-hard  
 760 cheese and sour cream.  
 761

Product	Fermentate (fermentation substrate)	Concentration	Time before visible fungal growth (in days)	
			<i>M. racemosus</i>	<i>P. commune</i>
Semi-hard cheese	None (negative control)	-	5	7
	<i>P. jensenii</i> CIRM-BIA1774 (UF) and <i>M. lanceolatus</i> UBOCC-A-109193	0.63 g/dm <sup>2</sup> and 5 ml/dm <sup>2</sup>	20	14
	(LH) <i>P. jensenii</i> CIRM-BIA1774 (UF)	0.63 g/dm <sup>2</sup>	26	21
	<i>L. rhamnosus</i> CIRM-BIA1952 (LH) and <i>M. lanceolatus</i> UBOCC-A-109193 (LH)	1.26 g/dm <sup>2</sup> and 5 ml/dm <sup>2</sup>	8	9
	Natamycin (positive control)	1 mg/dm <sup>2</sup>	>33	>33
	Sour cream	None (negative control)		7
<i>L. rhamnosus</i> CIRM-BIA1952 (LH)		5%	7	8
<i>L. rhamnosus</i> CIRM-BIA1952 (LH)		2%	7	8
<i>P. jensenii</i> CIRM-BIA1774 (UF)		5%	30	30
<i>P. jensenii</i> CIRM-BIA1774 (UF)		2%	30	19
<i>P. jensenii</i> CIRM-BIA1774 (UF)		0.4%	10	12
<i>P. jensenii</i> CIRM-BIA1774 (UF)		0.1%	8	11
<i>L. rhamnosus</i> CIRM-BIA1952 (LH) and <i>P. jensenii</i> CIRM-BIA1774 (UF)		2.5% each	>30	>30
Potassium sorbate (positive control)		0.08%	>30	>30

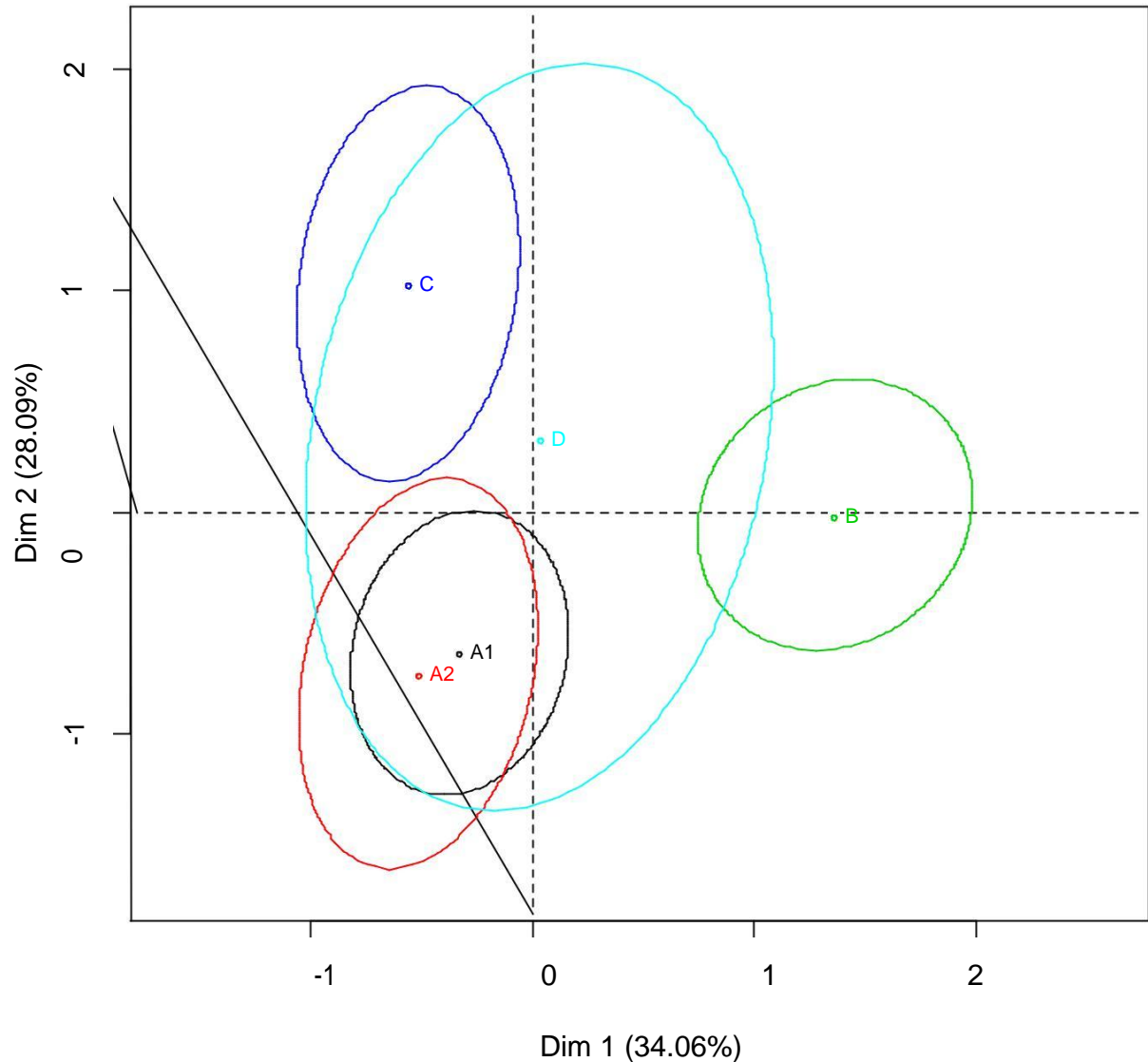
762

763



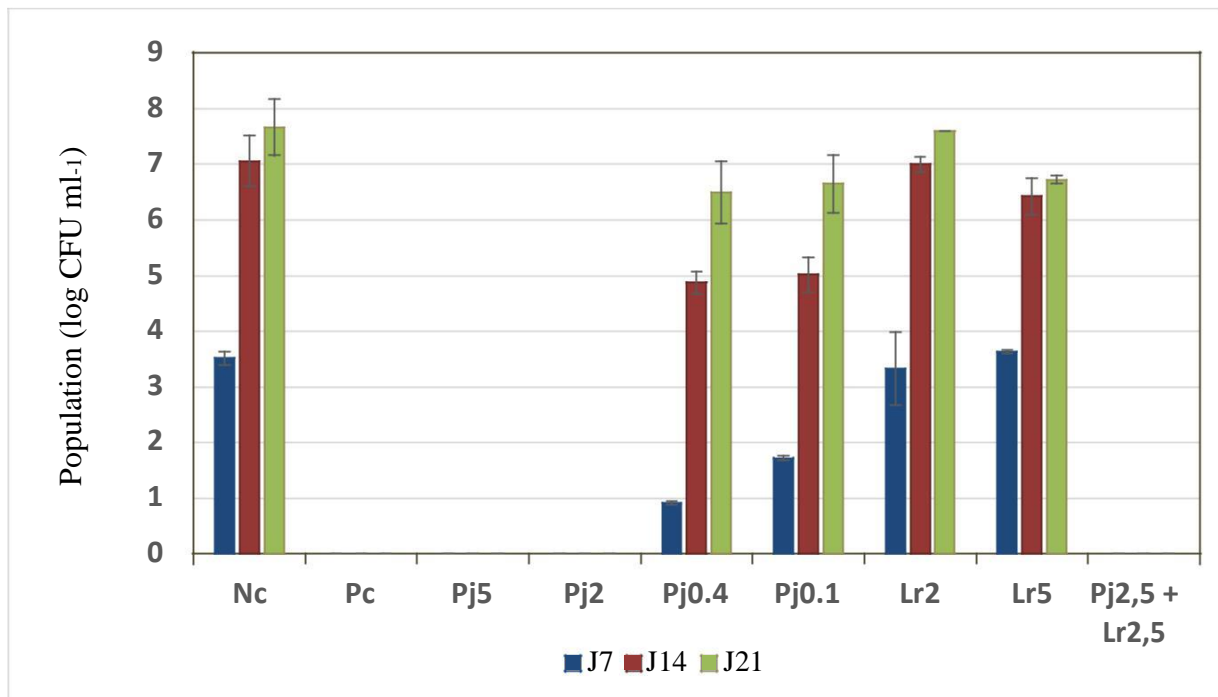
764  
765

766 **Figure 1.** Photographs showing semi-hard cheese surfaces treated or not (negative control, Nc)  
767 by surface-spraying using *P. jensenii* CIRM-BIA1774, *M. lanceolatus* CIRM-BIA1952 or  
768 *L. rhamnosus* fermentates, alone or in combination, after 6 weeks of ripening at 10°C in a  
769 naturally contaminated environment. Numbers 1 to 3 are replicates.



770

771 **Figure 2.** Impact of cheese surface treatment with antifungal fermentates on sensorial  
 772 properties of semi-hard cheese determined using a categorization approach: representation of  
 773 the cheeses and their respective 95 % confidence ellipses in the plane defined by Dim 1 and  
 774 Dim 2 of a multiple correspondence analysis. A1 and A2, control cheeses (without fermentate);  
 775 B, cheese surface-treated with a combination of  $4.8 \text{ mg/dm}^2$  *L. rhamnosus* CIRM-BIA1952 and  
 776  $10 \text{ ml/dm}^2$  *M. lanceolatus* UBOCC-A-109193 fermentates; C, cheese surface-treated with a  
 777 combination of  $2.4 \text{ mg/dm}^2$  *P. jensenii* CIRM-BIA1774 and  $10 \text{ ml/dm}^2$  *M. lanceolatus*  
 778 UBOCC-A-109193 fermentates and D, cheese surface-treated with  $2.4 \text{ mg/dm}^2$  *P. jensenii*  
 779 CIRM-BIA1774 fermentate.



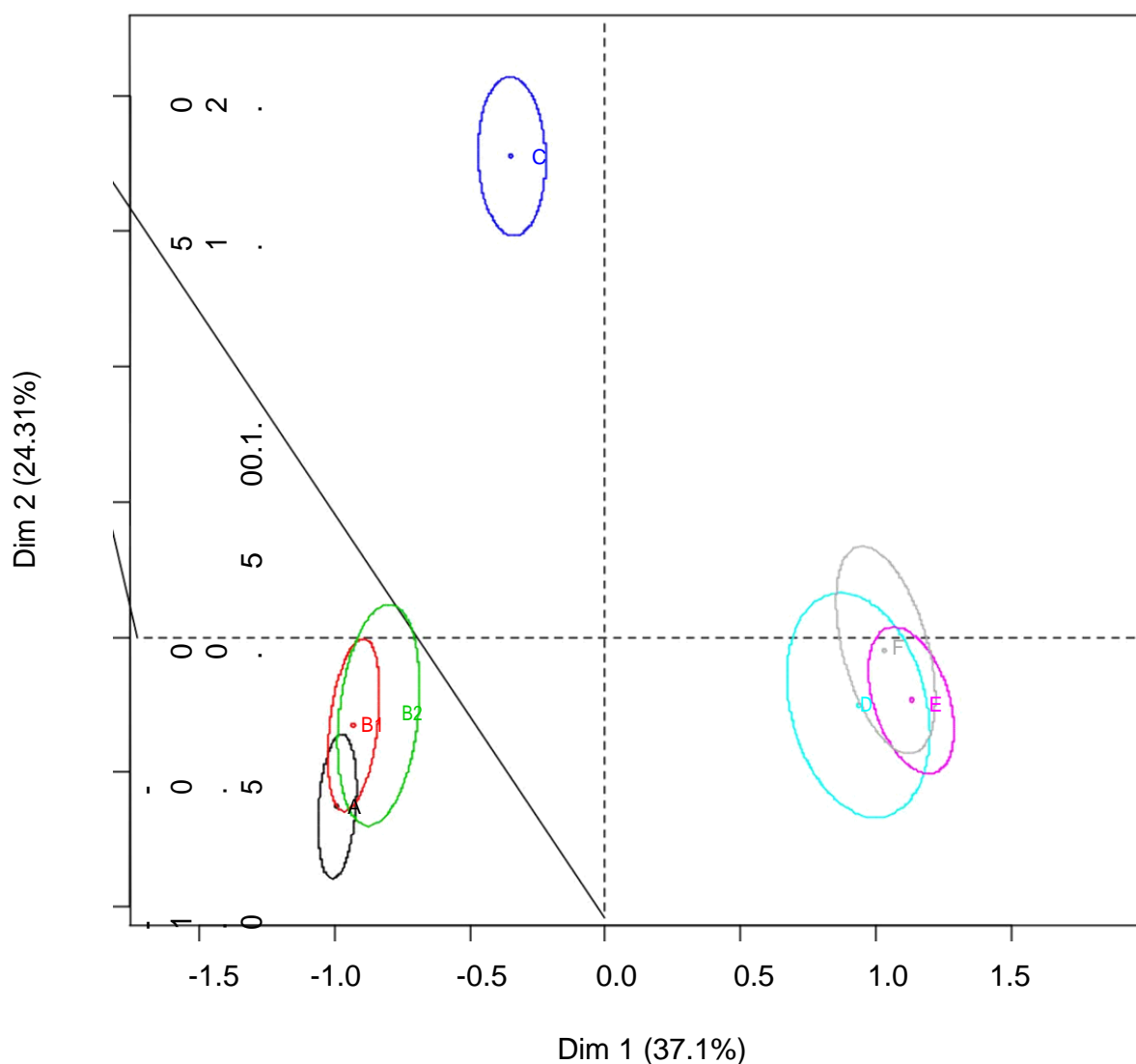
780

781

782 **Figure 3.** Growth of *R. mucilaginosa* UBOCC-A-216004 in sour cream after incorporation or  
 783 not of *P. jensenii* CIRM-BIA1774 and/or *L. rhamnosus* CIRM-BIA1952 fermentates. Nc,  
 784 negative control (sour cream without fermentate); Pc, positive control (sour cream with 0.08 %  
 785 potassium sorbate); Pj2, *P. jensenii* fermentate at 2 % m/m; Pj5, *P. jensenii* fermentate at 5 %  
 786 m/m, Lr2, *L. rhamnosus* fermentate at 2 % m/m; Lr5, *L. rhamnosus* fermentate at 5 % m/m;  
 787 Pj2.5+Lr2.5, mixture of *P. jensenii* and *L. rhamnosus* fermentates at 2.5 % m/m each.



789 **Figure 4.** Photographs showing sour cream surfaces in which fermentates of *P. jensenii* CIRM-  
790 BIA1774 (2 % and 5%), *L. rhamnosus* CIRM-BIA1952 (2 and 5 %) and mixture of *P. jensenii*  
791 and *L. rhamnosus* at 2.5 % each were incorporated. Negative controls corresponded to sour  
792 cream samples without fermentate. Samples were stored for 2 weeks at 4°C, naturally  
793 contaminated in an indoor environment (20 min) and stored again for 2 weeks at 10°C. Nc,  
794 positive control; Pj 2%, *P. jensenii* fermentate at 2% m/m; Pj 5 %, *P. jensenii* fermentate at 5 %  
795 m/m, Lr 2 %, *L. rhamnosus* fermentate at 2% m/m; Lr 5 %, *L. rhamnosus* fermentate at 5 %  
796 m/m; Pj 2.5 %+Lr 2.5 %, mixture of *P. jensenii* and *L. rhamnosus* fermentates at 2.5 % m/m  
797 each. Numbers 1 to 10 are replicates. Scores correspond to the number of contaminated cream  
798 / total number of cream.  
799



800

801 **Figure 5.** Impact of the incorporation of antifungal fermentates on sensorial traits of sour cream  
 802 determined using a categorization approach: representation of the sour creams and their  
 803 respective 95 % confidence ellipses in the plane defined by Dim 1 and Dim 2 of a multiple  
 804 correspondence analysis. A, control sour cream (without fermentate); B1 and B2, sour cream  
 805 with 2 % *L. rhamnosus* CIRM-BIA1952 fermentate, C, sour cream with 5 % *L. rhamnosus*  
 806 CIRM-BIA1952 fermentate; D, sour cream with 2 % *P. jensenii* CIRM-BIA1774 fermentate,  
 807 E, sour cream with 5 % of *P. jensenii* CIRM-BIA1774 fermentate; F, sour cream with 2.5 %  
 808 *L. rhamnosus* CIRM-BIA1952 and 2.5 % of *P. jensenii* CIRM-BIA1774 fermentates.



809

810

**Figure 6.** Photographs showing sour cream surfaces in which *P. jensenii* CIRM-BIA1774 fermentates were incorporated at 0.1 and 0.4 %. Negative

811

control (Nc) correspond to sour cream samples without fermentate and positive control to sour cream samples containing 0.08 % potassium sorbate.

812

Samples were stored for 2 weeks at 4°C, naturally contaminated in an indoor environment and stored again for 2 weeks at 10°C. Numbers 1 to 10

813

are replicates. Scores correspond to number of contaminated sour cream/ total number of sour cream.

814



<b>Species</b>	<b>Strain</b>	<b>Species</b>	<b>Strain</b>
<i>Lactobacillus acidophilus</i>	CIRM-BIA443	<i>Lactobacillus casei/paracasei</i>	L101
<i>Lactobacillus acidophilus</i>	CIRM-BIA445	<i>Lactobacillus casei/paracasei</i>	L102
<i>Lactobacillus acidophilus</i>	CIRM-BIA448	<i>Lactobacillus casei/paracasei</i>	L103
<i>Lactobacillus acidophilus</i>	CIRM-BIA1674	<i>Lactobacillus casei/paracasei</i>	L106
<i>Lactobacillus amylovorus</i>	CIRM-BIA669	<i>Lactobacillus casei/paracasei</i>	L107
<i>Lactobacillus brevis</i>	L128	<i>Lactobacillus casei/paracasei</i>	L108
<i>Lactobacillus brevis</i>	L30	<i>Lactobacillus casei/paracasei</i>	L109
<i>Lactobacillus brevis</i>	L31	<i>Lactobacillus casei/paracasei</i>	L110
<i>Lactobacillus brevis</i>	CIRM-BIA608	<i>Lactobacillus casei/paracasei</i>	L112
<i>Lactobacillus brevis</i>	CIRM-BIA932	<i>Lactobacillus casei/paracasei</i>	L117
<i>Lactobacillus brevis</i>	CIRM-BIA933	<i>Lactobacillus casei/paracasei</i>	L118
<i>Lactobacillus brevis</i>	CIRM-BIA1523	<i>Lactobacillus casei/paracasei</i>	L119
<i>Lactobacillus brevis</i>	CIRM-BIA1531	<i>Lactobacillus casei/paracasei</i>	L120
<i>Lactobacillus buchneri</i>	L111	<i>Lactobacillus casei/paracasei</i>	L121
<i>Lactobacillus buchneri</i>	L137	<i>Lactobacillus casei/paracasei</i>	L122
<i>Lactobacillus buchneri</i>	L147	<i>Lactobacillus casei/paracasei</i>	L124
<i>Lactobacillus buchneri</i>	L149	<i>Lactobacillus casei/paracasei</i>	L127
<i>Lactobacillus buchneri</i>	L151	<i>Lactobacillus casei/paracasei</i>	L129
<i>Lactobacillus buchneri</i>	L162	<i>Lactobacillus casei/paracasei</i>	L13
<i>Lactobacillus buchneri</i>	L164	<i>Lactobacillus casei/paracasei</i>	L130
<i>Lactobacillus buchneri</i>	L169	<i>Lactobacillus casei/paracasei</i>	L136
<i>Lactobacillus buchneri</i>	L170	<i>Lactobacillus casei/paracasei</i>	L138
<i>Lactobacillus buchneri</i>	L192	<i>Lactobacillus casei/paracasei</i>	L139
<i>Lactobacillus buchneri</i>	L203	<i>Lactobacillus casei/paracasei</i>	L140
<i>Lactobacillus buchneri</i>	L212	<i>Lactobacillus casei/paracasei</i>	L142
<i>Lactobacillus buchneri</i>	L217	<i>Lactobacillus casei/paracasei</i>	L143
<i>Lactobacillus buchneri</i>	L233	<i>Lactobacillus casei/paracasei</i>	L144
<i>Lactobacillus buchneri</i>	L239	<i>Lactobacillus casei/paracasei</i>	L148
<i>Lactobacillus buchneri</i>	L243	<i>Lactobacillus casei/paracasei</i>	L150
<i>Lactobacillus buchneri</i>	L245	<i>Lactobacillus casei/paracasei</i>	L157
<i>Lactobacillus buchneri</i>	L25	<i>Lactobacillus casei/paracasei</i>	L158
<i>Lactobacillus buchneri</i>	L265	<i>Lactobacillus casei/paracasei</i>	L159
<i>Lactobacillus buchneri</i>	L271	<i>Lactobacillus casei/paracasei</i>	L16
<i>Lactobacillus buchneri</i>	L273	<i>Lactobacillus casei/paracasei</i>	L161
<i>Lactobacillus buchneri</i>	L277	<i>Lactobacillus casei/paracasei</i>	L179
<i>Lactobacillus buchneri</i>	L278	<i>Lactobacillus casei/paracasei</i>	L184
<i>Lactobacillus buchneri</i>	L290	<i>Lactobacillus casei/paracasei</i>	L186
<i>Lactobacillus buchneri</i>	L56	<i>Lactobacillus casei/paracasei</i>	L188
<i>Lactobacillus buchneri</i>	L84	<i>Lactobacillus casei/paracasei</i>	L19
<i>Lactobacillus buchneri</i>	L90	<i>Lactobacillus casei/paracasei</i>	L194
<i>Lactobacillus buchneri</i>	L99	<i>Lactobacillus casei/paracasei</i>	L196
<i>Lactobacillus buchneri</i>	CIRM-BIA659	<i>Lactobacillus casei/paracasei</i>	L199
<i>Lactobacillus buchneri</i>	CIRM-BIA1324	<i>Lactobacillus casei/paracasei</i>	L208
<i>Lactobacillus buchneri</i>	CIRM-BIA1514	<i>Lactobacillus casei/paracasei</i>	L209
<i>Lactobacillus buchneri</i>	CIRM-BIA1515	<i>Lactobacillus casei/paracasei</i>	L211
<i>Lactobacillus buchneri</i>	CIRM-BIA1516	<i>Lactobacillus casei/paracasei</i>	L215

<i>Lactobacillus buchneri</i>	CIRM-BIA1525	<i>Lactobacillus casei/paracasei</i>	L220
<i>Lactobacillus casei/paracasei</i>	L1	<i>Lactobacillus casei/paracasei</i>	L222
<i>Lactobacillus casei/paracasei</i>	L100	<i>Lactobacillus casei/paracasei</i>	L224
<i>Lactobacillus casei/paracasei</i>	L225	<i>Lactobacillus casei/paracasei</i>	L69
<i>Lactobacillus casei/paracasei</i>	L226	<i>Lactobacillus casei/paracasei</i>	L7
<i>Lactobacillus casei/paracasei</i>	L227	<i>Lactobacillus casei/paracasei</i>	L72
<i>Lactobacillus casei/paracasei</i>	L228	<i>Lactobacillus casei/paracasei</i>	L73
<i>Lactobacillus casei/paracasei</i>	L229	<i>Lactobacillus casei/paracasei</i>	L74
<i>Lactobacillus casei/paracasei</i>	L23	<i>Lactobacillus casei/paracasei</i>	L75
<i>Lactobacillus casei/paracasei</i>	L230	<i>Lactobacillus casei/paracasei</i>	L82
<i>Lactobacillus casei/paracasei</i>	L231	<i>Lactobacillus casei/paracasei</i>	L85
<i>Lactobacillus casei/paracasei</i>	L232	<i>Lactobacillus casei/paracasei</i>	L86
<i>Lactobacillus casei/paracasei</i>	L234	<i>Lactobacillus casei/paracasei</i>	L93
<i>Lactobacillus casei/paracasei</i>	L237	<i>Lactobacillus casei/paracasei</i>	L95
<i>Lactobacillus casei/paracasei</i>	L24	<i>Lactobacillus delbrueckii</i>	L160
<i>Lactobacillus casei/paracasei</i>	L240	<i>Lactobacillus delbrueckii</i>	L218
<i>Lactobacillus casei/paracasei</i>	L242	<i>Lactobacillus farciminis</i>	CIRM-BIA1634
<i>Lactobacillus casei/paracasei</i>	L247	<i>Lactobacillus fermentum</i>	CIRM-BIA42
<i>Lactobacillus casei/paracasei</i>	L249	<i>Lactobacillus fermentum</i>	CIRM-BIA666
<i>Lactobacillus casei/paracasei</i>	L250	<i>Lactobacillus gasseri</i>	CIRM-BIA665
<i>Lactobacillus casei/paracasei</i>	L252	<i>Lactobacillus gasseri</i>	CIRM-BIA1437
<i>Lactobacillus casei/paracasei</i>	L253	<i>Lactobacillus gasseri</i>	CIRM-BIA1438
<i>Lactobacillus casei/paracasei</i>	L257	<i>Lactobacillus gasseri</i>	CIRM-BIA1447
<i>Lactobacillus casei/paracasei</i>	L261	<i>Lactobacillus harbinensis</i>	L172
<i>Lactobacillus casei/paracasei</i>	L263	<i>Lactobacillus pentosus</i>	CIRM-BIA660
<i>Lactobacillus casei/paracasei</i>	L264	<i>Lactobacillus pentosus</i>	CIRM-BIA853
<i>Lactobacillus casei/paracasei</i>	L266	<i>Lactobacillus pentosus</i>	CIRM-BIA854
<i>Lactobacillus casei/paracasei</i>	L267	<i>Lactobacillus pentosus</i>	CIRM-BIA1490
<i>Lactobacillus casei/paracasei</i>	L269	<i>Lactobacillus perolens/harbinensis</i>	L191
<i>Lactobacillus casei/paracasei</i>	L27	<i>Lactobacillus perolens/harbinensis</i>	L244
<i>Lactobacillus casei/paracasei</i>	L270	<i>Lactobacillus perolens/harbinensis</i>	L283
<i>Lactobacillus casei/paracasei</i>	L274	<i>Lactobacillus plantarum</i>	L10
<i>Lactobacillus casei/paracasei</i>	L276	<i>Lactobacillus plantarum</i>	L11
<i>Lactobacillus casei/paracasei</i>	L279	<i>Lactobacillus plantarum</i>	L12
<i>Lactobacillus casei/paracasei</i>	L281	<i>Lactobacillus plantarum</i>	L123
<i>Lactobacillus casei/paracasei</i>	L284	<i>Lactobacillus plantarum</i>	L125
<i>Lactobacillus casei/paracasei</i>	L287	<i>Lactobacillus plantarum</i>	L14
<i>Lactobacillus casei/paracasei</i>	L288	<i>Lactobacillus plantarum</i>	L145
<i>Lactobacillus casei/paracasei</i>	L289	<i>Lactobacillus plantarum</i>	L146
<i>Lactobacillus casei/paracasei</i>	L3	<i>Lactobacillus plantarum</i>	L15
<i>Lactobacillus casei/paracasei</i>	L32	<i>Lactobacillus plantarum</i>	L152
<i>Lactobacillus casei/paracasei</i>	L37	<i>Lactobacillus plantarum</i>	L155
<i>Lactobacillus casei/paracasei</i>	L38	<i>Lactobacillus plantarum</i>	L156
<i>Lactobacillus casei/paracasei</i>	L40	<i>Lactobacillus plantarum</i>	L163
<i>Lactobacillus casei/paracasei</i>	L47	<i>Lactobacillus plantarum</i>	L167
<i>Lactobacillus casei/paracasei</i>	L51	<i>Lactobacillus plantarum</i>	L17
<i>Lactobacillus casei/paracasei</i>	L52	<i>Lactobacillus plantarum</i>	L171
<i>Lactobacillus casei/paracasei</i>	L57	<i>Lactobacillus plantarum</i>	L173
<i>Lactobacillus casei/paracasei</i>	L58	<i>Lactobacillus plantarum</i>	L174
<i>Lactobacillus casei/paracasei</i>	L59	<i>Lactobacillus plantarum</i>	L175
<i>Lactobacillus casei/paracasei</i>	L61	<i>Lactobacillus plantarum</i>	L176

<i>Lactobacillus casei/paracasei</i>	L62	<i>Lactobacillus plantarum</i>	L18
<i>Lactobacillus casei/paracasei</i>	L68	<i>Lactobacillus plantarum</i>	L180
<i>Lactobacillus plantarum</i>	L181	<i>Lactobacillus plantarum</i>	L43
<i>Lactobacillus plantarum</i>	L182	<i>Lactobacillus plantarum</i>	L45
<i>Lactobacillus plantarum</i>	L183	<i>Lactobacillus plantarum</i>	L46
<i>Lactobacillus plantarum</i>	L193	<i>Lactobacillus plantarum</i>	L48
<i>Lactobacillus plantarum</i>	L195	<i>Lactobacillus plantarum</i>	L49
<i>Lactobacillus plantarum</i>	L198	<i>Lactobacillus plantarum</i>	L5
<i>Lactobacillus plantarum</i>	L20	<i>Lactobacillus plantarum</i>	L50
<i>Lactobacillus plantarum</i>	L200	<i>Lactobacillus plantarum</i>	L53
<i>Lactobacillus plantarum</i>	L201	<i>Lactobacillus plantarum</i>	L53
<i>Lactobacillus plantarum</i>	L202	<i>Lactobacillus plantarum</i>	L54
<i>Lactobacillus plantarum</i>	L204	<i>Lactobacillus plantarum</i>	L6
<i>Lactobacillus plantarum</i>	L205	<i>Lactobacillus plantarum</i>	L6
<i>Lactobacillus plantarum</i>	L206	<i>Lactobacillus plantarum</i>	L63
<i>Lactobacillus plantarum</i>	L207	<i>Lactobacillus plantarum</i>	L64
<i>Lactobacillus plantarum</i>	L21	<i>Lactobacillus plantarum</i>	L65
<i>Lactobacillus plantarum</i>	L210	<i>Lactobacillus plantarum</i>	L65
<i>Lactobacillus plantarum</i>	L216	<i>Lactobacillus plantarum</i>	L66
<i>Lactobacillus plantarum</i>	L219	<i>Lactobacillus plantarum</i>	L70
<i>Lactobacillus plantarum</i>	L22	<i>Lactobacillus plantarum</i>	L71
<i>Lactobacillus plantarum</i>	L221	<i>Lactobacillus plantarum</i>	L76
<i>Lactobacillus plantarum</i>	L241	<i>Lactobacillus plantarum</i>	L77
<i>Lactobacillus plantarum</i>	L246	<i>Lactobacillus plantarum</i>	L8
<i>Lactobacillus plantarum</i>	L248	<i>Lactobacillus plantarum</i>	L87
<i>Lactobacillus plantarum</i>	L251	<i>Lactobacillus plantarum</i>	L88
<i>Lactobacillus plantarum</i>	L254	<i>Lactobacillus plantarum</i>	L88
<i>Lactobacillus plantarum</i>	L255	<i>Lactobacillus plantarum</i>	L89
<i>Lactobacillus plantarum</i>	L256	<i>Lactobacillus plantarum</i>	L9
<i>Lactobacillus plantarum</i>	L258	<i>Lactobacillus plantarum</i>	L96
<i>Lactobacillus plantarum</i>	L259	<i>Lactobacillus plantarum</i>	L98
<i>Lactobacillus plantarum</i>	L26	<i>Lactobacillus plantarum</i>	CIRM-BIA466
<i>Lactobacillus plantarum</i>	L260	<i>Lactobacillus plantarum</i>	CIRM-BIA652
<i>Lactobacillus plantarum</i>	L268	<i>Lactobacillus plantarum</i>	CIRM-BIA845
<i>Lactobacillus plantarum</i>	L272	<i>Lactobacillus plantarum</i>	CIRM-BIA850
<i>Lactobacillus plantarum</i>	L28	<i>Lactobacillus plantarum</i>	CIRM-BIA855
<i>Lactobacillus plantarum</i>	L282	<i>Lactobacillus plantarum</i>	CIRM-BIA1105
<i>Lactobacillus plantarum</i>	L285	<i>Lactobacillus plantarum</i>	CIRM-BIA1106
<i>Lactobacillus plantarum</i>	L286	<i>Lactobacillus plantarum</i>	CIRM-BIA1108
<i>Lactobacillus plantarum</i>	L29	<i>Lactobacillus plantarum</i>	CIRM-BIA1109
<i>Lactobacillus plantarum</i>	L291	<i>Lactobacillus plantarum</i>	CIRM-BIA1110
<i>Lactobacillus plantarum</i>	L292	<i>Lactobacillus plantarum</i>	CIRM-BIA1111
<i>Lactobacillus plantarum</i>	L293	<i>Lactobacillus plantarum</i>	CIRM-BIA1419
<i>Lactobacillus plantarum</i>	L294	<i>Lactobacillus plantarum</i>	CIRM-BIA1420
<i>Lactobacillus plantarum</i>	L298	<i>Lactobacillus plantarum</i>	CIRM-BIA1520
<i>Lactobacillus plantarum</i>	L299	<i>Lactobacillus reuteri</i>	L154
<i>Lactobacillus plantarum</i>	L35	<i>Lactobacillus reuteri</i>	L165
<i>Lactobacillus plantarum</i>	L36	<i>Lactobacillus reuteri</i>	L177
<i>Lactobacillus plantarum</i>	L39	<i>Lactobacillus reuteri</i>	L178
<i>Lactobacillus plantarum</i>	L4	<i>Lactobacillus reuteri</i>	L185
<i>Lactobacillus plantarum</i>	L41	<i>Lactobacillus reuteri</i>	L187

<i>Lactobacillus plantarum</i>	L41	<i>Lactobacillus reuteri</i>	L189
<i>Lactobacillus reuteri</i>	L190	<i>Lactococcus lactis</i>	L168
<i>Lactobacillus reuteri</i>	L197	<i>Lactococcus lactis</i>	L2
<i>Lactobacillus reuteri</i>	L213	<i>Leuconostoc citreum</i>	CIRM-BIA742
<i>Lactobacillus reuteri</i>	L214	<i>Leuconostoc citreum</i>	CIRM-BIA852
<i>Lactobacillus reuteri</i>	L223	<i>Leuconostoc citreum</i>	CIRM-BIA1453
<i>Lactobacillus reuteri</i>	L235	<i>Leuconostoc citreum</i>	CIRM-BIA1454
<i>Lactobacillus reuteri</i>	L236	<i>Leuconostoc citreum</i>	CIRM-BIA1455
<i>Lactobacillus reuteri</i>	L238	<i>Leuconostoc citreum</i>	CIRM-BIA1456
<i>Lactobacillus reuteri</i>	L262	<i>Leuconostoc citreum</i>	CIRM-BIA1457
<i>Lactobacillus reuteri</i>	L275	<i>Leuconostoc mesenteroides</i>	L104
<i>Lactobacillus reuteri</i>	L280	<i>Leuconostoc mesenteroides</i>	L113
<i>Lactobacillus reuteri</i>	L295	<i>Leuconostoc mesenteroides</i>	L114
<i>Lactobacillus reuteri</i>	L296	<i>Leuconostoc mesenteroides</i>	L115
<i>Lactobacillus reuteri</i>	L297	<i>Leuconostoc mesenteroides</i>	L116
<i>Lactobacillus reuteri</i>	L33	<i>Leuconostoc mesenteroides</i>	L126
<i>Lactobacillus reuteri</i>	L44	<i>Leuconostoc mesenteroides</i>	L131
<i>Lactobacillus reuteri</i>	L55	<i>Leuconostoc mesenteroides</i>	L132
<i>Lactobacillus reuteri</i>	L60	<i>Leuconostoc mesenteroides</i>	L133
<i>Lactobacillus reuteri</i>	L67	<i>Leuconostoc mesenteroides</i>	L134
<i>Lactobacillus reuteri</i>	L78	<i>Leuconostoc mesenteroides</i>	L135
<i>Lactobacillus reuteri</i>	L79	<i>Leuconostoc mesenteroides</i>	L141
<i>Lactobacillus reuteri</i>	L80	<i>Leuconostoc mesenteroides</i>	CIRM-BIA694
<i>Lactobacillus reuteri</i>	L81	<i>Leuconostoc mesenteroides</i>	CIRM-BIA740
<i>Lactobacillus reuteri</i>	L83	<i>Leuconostoc mesenteroides</i>	CIRM-BIA847
<i>Lactobacillus reuteri</i>	L91	<i>Leuconostoc mesenteroides</i>	CIRM-BIA1146
<i>Lactobacillus reuteri</i>	L92	<i>Leuconostoc mesenteroides</i>	CIRM-BIA1147
<i>Lactobacillus reuteri</i>	L94	<i>Leuconostoc mesenteroides</i>	CIRM-BIA1153
<i>Lactobacillus reuteri</i>	L97	<i>Leuconostoc mesenteroides</i>	CIRM-BIA1154
<i>Lactobacillus reuteri</i>	CIRM-BIA522	<i>Leuconostoc mesenteroides</i>	CIRM-BIA1155
<i>Lactobacillus reuteri</i>	CIRM-BIA696	<i>Leuconostoc mesenteroides</i>	CIRM-BIA1156
<i>Lactobacillus reuteri</i>	CIRM-BIA911	<i>Leuconostoc mesenteroides</i>	CIRM-BIA1159
<i>Lactobacillus reuteri</i>	CIRM-BIA912	<i>Leuconostoc mesenteroides</i>	CIRM-BIA1176
<i>Lactobacillus reuteri</i>	CIRM-BIA929	<i>Leuconostoc mesenteroides</i>	CIRM-BIA1185
<i>Lactobacillus reuteri</i>	CIRM-BIA1439	<i>Leuconostoc mesenteroides</i>	CIRM-BIA1187
<i>Lactobacillus reuteri</i>	CIRM-BIA1534	<i>Leuconostoc mesenteroides</i>	CIRM-BIA1191
<i>Lactobacillus rhamnosus</i>	CIRM-BIA607	<i>Leuconostoc mesenteroides</i>	CIRM-BIA1192
<i>Lactobacillus rhamnosus</i>	CIRM-BIA909	<i>Leuconostoc mesenteroides</i>	CIRM-BIA1193
<i>Lactobacillus rhamnosus</i>	CIRM-BIA910	<i>Leuconostoc mesenteroides</i>	CIRM-BIA1194
<i>Lactobacillus rhamnosus</i>	CIRM-BIA913	<i>Leuconostoc mesenteroides</i>	CIRM-BIA1228
<i>Lactobacillus rhamnosus</i>	CIRM-BIA930	<i>Leuconostoc mesenteroides</i>	CIRM-BIA1229
<i>Lactobacillus rhamnosus</i>	CIRM-BIA1107	<i>Leuconostoc mesenteroides</i>	CIRM-BIA1230
<i>Lactobacillus rhamnosus</i>	CIRM-BIA1112	<i>Leuconostoc mesenteroides</i>	CIRM-BIA1231
<i>Lactobacillus rhamnosus</i>	CIRM-BIA1113	<i>Leuconostoc mesenteroides</i>	CIRM-BIA1232
<i>Lactobacillus rhamnosus</i>	CIRM-BIA1436	<i>Leuconostoc mesenteroides</i>	CIRM-BIA1233
<i>Lactobacillus sakei</i>	CIRM-BIA467	<i>Leuconostoc mesenteroides</i>	CIRM-BIA1234
<i>Lactobacillus sakei</i>	CIRM-BIA892	<i>Leuconostoc mesenteroides</i>	CIRM-BIA1235
<i>Lactobacillus sakei</i>	CIRM-BIA1559	<i>Leuconostoc mesenteroides</i>	CIRM-BIA1236
<i>Lactobacillus sanfranciscensis</i>	CIRM-BIA1357	<i>Leuconostoc mesenteroides</i>	CIRM-BIA1237
<i>Lactobacillus zeae</i>	CIRM-BIA524	<i>Leuconostoc mesenteroides</i>	CIRM-BIA1238
<i>Lactobacillus zeae</i>	CIRM-BIA525	<i>Leuconostoc mesenteroides</i>	CIRM-BIA1239

<i>Leuconostoc mesenteroides</i>	CIRM-BIA1242	<i>Propionibacterium freudenreichii</i>	CIRM-BIA12
<i>Leuconostoc mesenteroides</i>	CIRM-BIA1244	<i>Propionibacterium freudenreichii</i>	CIRM-BIA41
<i>Leuconostoc mesenteroides</i>	CIRM-BIA1245	<i>Propionibacterium freudenreichii</i>	CIRM-BIA118
<i>Leuconostoc mesenteroides</i>	CIRM-BIA1246	<i>Propionibacterium freudenreichii</i>	CIRM-BIA1324
<i>Leuconostoc mesenteroides</i>	CIRM-BIA1249	<i>Propionibacterium freudenreichii</i>	CIRM-BIA141
<i>Leuconostoc mesenteroides</i>	CIRM-BIA1250	<i>Propionibacterium freudenreichii</i>	CIRM-BIA508
<i>Leuconostoc mesenteroides</i>	CIRM-BIA1251	<i>Propionibacterium freudenreichii</i>	CIRM-BIA509
<i>Leuconostoc mesenteroides</i>	CIRM-BIA1252	<i>Propionibacterium freudenreichii</i>	CIRM-BIA510
<i>Leuconostoc mesenteroides</i>	CIRM-BIA1259	<i>Propionibacterium freudenreichii</i>	CIRM-BIA512
<i>Leuconostoc mesenteroides</i>	CIRM-BIA1260	<i>Propionibacterium freudenreichii</i>	CIRM-BIA514
<i>Leuconostoc mesenteroides</i>	CIRM-BIA1261	<i>Propionibacterium freudenreichii</i>	CIRM-BIA515
<i>Leuconostoc mesenteroides</i>	CIRM-BIA1262	<i>Propionibacterium freudenreichii</i>	CIRM-BIA527
<i>Leuconostoc mesenteroides</i>	CIRM-BIA1264	<i>Propionibacterium freudenreichii</i>	CIRM-BIA683
<i>Leuconostoc mesenteroides</i>	CIRM-BIA1265	<i>Propionibacterium freudenreichii</i>	CIRM-BIA686
<i>Leuconostoc mesenteroides</i>	CIRM-BIA1266	<i>Propionibacterium freudenreichii</i>	CIRM-BIA687
<i>Leuconostoc mesenteroides</i>	CIRM-BIA1267	<i>Propionibacterium freudenreichii</i>	CIRM-BIA688
<i>Leuconostoc mesenteroides</i>	CIRM-BIA1268	<i>Propionibacterium freudenreichii</i>	CIRM-BIA690
<i>Leuconostoc mesenteroides</i>	CIRM-BIA1269	<i>Propionibacterium freudenreichii</i>	CIRM-BIA692
<i>Leuconostoc mesenteroides</i>	CIRM-BIA1270	<i>Propionibacterium jensenii</i>	CIRM-BIA39
<i>Leuconostoc mesenteroides</i>	CIRM-BIA1271	<i>Propionibacterium jensenii</i>	CIRM-BIA455
<i>Leuconostoc mesenteroides</i>	CIRM-BIA1272	<i>Propionibacterium jensenii</i>	CIRM-BIA1347
<i>Leuconostoc mesenteroides</i>	CIRM-BIA1302	<i>Propionibacterium jensenii</i>	CIRM-BIA1431
<i>Leuconostoc mesenteroides</i>	CIRM-BIA1304	<i>Propionibacterium jensenii</i>	CIRM-BIA1432
<i>Leuconostoc mesenteroides</i>	CIRM-BIA1305	<i>Propionibacterium jensenii</i>	CIRM-BIA1494
<i>Leuconostoc mesenteroides</i>	CIRM-BIA1314	<i>Propionibacterium jensenii</i>	CIRM-BIA1505
<i>Leuconostoc mesenteroides</i>	CIRM-BIA1315	<i>Propionibacterium jensenii</i>	CIRM-BIA1507
<i>Leuconostoc mesenteroides</i>	CIRM-BIA1317	<i>Propionibacterium jensenii</i>	CIRM-BIA1658
<i>Leuconostoc mesenteroides</i>	CIRM-BIA1318	<i>Propionibacterium jensenii</i>	CIRM-BIA1660
<i>Leuconostoc mesenteroides</i>	CIRM-BIA1320	<i>Propionibacterium jensenii</i>	CIRM-BIA1661
<i>Leuconostoc mesenteroides</i>	CIRM-BIA1326	<i>Propionibacterium jensenii</i>	CIRM-BIA1774
<i>Pediococcus pentosaceus</i>	L42	<i>Propionibacterium jensenii</i>	CIRM-BIA1775
<i>Pediococcus pentosaceus</i>	CIRM-BIA1701	<i>Propionibacterium jensenii</i>	CIRM-BIA1777
<i>Propionibacterium acidipropionici</i>	CIRM-BIA64	<i>Propionibacterium jensenii</i>	CIRM-BIA1778
<i>Propionibacterium acidipropionici</i>	CIRM-BIA609	<i>Propionibacterium jensenii</i>	CIRM-BIA1779
<i>Propionibacterium acidipropionici</i>	CIRM-BIA928	<i>Propionibacterium jensenii</i>	CIRM-BIA1781
<i>Propionibacterium acidipropionici</i>	CIRM-BIA1384	<i>Propionibacterium jensenii</i>	CIRM-BIA1782
<i>Propionibacterium acidipropionici</i>	CIRM-BIA1424	<i>Propionibacterium jensenii</i>	CIRM-BIA1785
<i>Propionibacterium acidipropionici</i>	CIRM-BIA1425	<i>Propionibacterium jensenii</i>	CIRM-BIA1794
<i>Propionibacterium acidipropionici</i>	CIRM-BIA1427	<i>Propionibacterium thoenii</i>	CIRM-BIA40
<i>Propionibacterium acidipropionici</i>	CIRM-BIA1535	<i>Propionibacterium thoenii</i>	CIRM-BIA1329
<i>Propionibacterium acidipropionici</i>	CIRM-BIA1652	<i>Propionibacterium thoenii</i>	CIRM-BIA1423
<i>Propionibacterium acidipropionici</i>	CIRM-BIA1656	<i>Propionibacterium thoenii</i>	CIRM-BIA1429
<i>Propionibacterium acidipropionici</i>	CIRM-BIA1664	<i>Propionibacterium thoenii</i>	CIRM-BIA1433
<i>Propionibacterium acidipropionici</i>	CIRM-BIA1762	<i>Propionibacterium thoenii</i>	CIRM-BIA1435
<i>Propionibacterium acidipropionici</i>	CIRM-BIA1763	<i>Propionibacterium thoenii</i>	CIRM-BIA1500
<i>Propionibacterium acidipropionici</i>	CIRM-BIA1764	<i>Propionibacterium thoenii</i>	CIRM-BIA1663
<i>Propionibacterium acidipropionici</i>	CIRM-BIA1765	<i>Propionibacterium thoenii</i>	CIRM-BIA1786
<i>Propionibacterium acidipropionici</i>	CIRM-BIA1769	<i>Propionibacterium thoenii</i>	CIRM-BIA1788
<i>Propionibacterium acidipropionici</i>	CIRM-BIA1770	<i>Propionibacterium thoenii</i>	CIRM-BIA1789
<i>Propionibacterium freudenreichii</i>	CIRM-BIA10	<i>Propionibacterium thoenii</i>	CIRM-BIA1790
<i>Propionibacterium thoenii</i>	CIRM-BIA1791	<i>Gliocladium deliquescens</i>	UBOCC-A-101174

<i>Propionibacterium thoenii</i>	CIRM-BIA1793	<i>Gliocladium roseum</i>	UBOCC-A-101175
<i>Actinomucor elegans</i>	UBOCC-A-101333	<i>Gliocladium</i> sp.	UBOCC-A-102025
<i>Actinomucor elegans</i>	UBOCC-A-102005	<i>Gliocladium virens</i>	UBOCC-A-101176
<i>Actinomucor elegans</i>	UBOCC-A-106035	<i>Gliomastix murorum</i>	UBOCC-A-101178
<i>Aureobasidium pullulans</i>	UBOCC-A-101091	<i>Gliomastix murorum</i>	UBOCC-A-101179
<i>Aureobasidium pullulans</i>	UBOCC-A-101092	<i>Lodderomyces elongisporus</i>	UBOCC-A-214062
<i>Aureobasidium pullulans</i>	UBOCC-A-108047	<i>Meyerozyma guilliermondii</i>	L1lev7
<i>Aureobasidium pullulans</i>	UBOCC-A-108048	<i>Meyerozyma guilliermondii</i>	L2lev17
<i>Aureobasidium pullulans</i>	UBOCC-A-108056	<i>Meyerozyma guilliermondii</i>	UBOCC-A-214105
<i>Aureobasidium pullulans</i>	UBOCC-A-108057	<i>Meyerozyma guilliermondii</i>	UBOCC-A-208004
<i>Aureobasidium pullulans</i>	UBOCC-A-111234	<i>Mucor bruneogriseus</i>	UBOCC-A-109204
<i>Candida catenulata</i>	L1lev3	<i>Mucor bruneogriseus</i>	UBOCC-A-109205
<i>Candida davisiana</i>	UBOCC-A-214090	<i>Mucor bruneogriseus</i>	UBOCC-A-109206
<i>Candida haemulonis</i>	UBOCC-A-214059	<i>Mucor bruneogriseus</i>	UBOCC-A-109207
<i>Candida methapsilosis</i>	L1lev6	<i>Mucor bruneogriseus</i>	UBOCC-A-109208
<i>Candida parapsilosis</i>	UBOCC-A-214083	<i>Mucor bruneogriseus</i>	UBOCC-A-109209
<i>Candida parapsilosis</i>	L1lev5	<i>Mucor bruneogriseus</i>	UBOCC-A-109210
<i>Candida parapsilosis</i>	UBOCC-A-214060	<i>Mucor fragilis</i>	UBOCC-A-109196
<i>Candida pseudoglaebosa</i>	UBOCC-A-214089	<i>Mucor fragilis</i>	UBOCC-A-109199
<i>Candida sorbophila</i>	UBOCC-A-214111	<i>Mucor fragilis</i>	UBOCC-A-113030
<i>Candida tenuis</i>	UBOCC-A-214064	<i>Mucor fuscus</i>	UBOCC-A-101355
<i>Candida zeylanoides</i>	L2lev6	<i>Mucor fuscus</i>	UBOCC-A-109194
<i>Candida zeylanoides</i>	UBOCC-A-214114	<i>Mucor fuscus</i>	UBOCC-A-109195
<i>Chaetomium dolichotrichum</i>	UBOCC-A-101010	<i>Mucor fuscus</i>	UBOCC-A-109202
<i>Chaetomium funicola</i>	UBOCC-A-101011	<i>Mucor fuscus</i>	UBOCC-A-110148
<i>Chaetomium globosum</i>	UBOCC-A-101012	<i>Mucor griseocyanus</i>	UBOCC-A-102010
<i>Cleistothelobolus nipigonensis</i>	UBOCC-A-214079	<i>Mucor hiemalis</i>	UBOCC-A-101359
<i>Cryptococcoccus curvatus</i>	UBOCC-A-214084	<i>Mucor hiemalis</i>	UBOCC-A-101360
<i>Cryptococcoccus curvatus</i>	UBOCC-A-214094	<i>Mucor hiemalis</i>	UBOCC-A-102003
<i>Cryptococcoccus albidus</i>	UBOCC-A-214081	<i>Mucor hiemalis</i>	UBOCC-A-109197
<i>Cryptococcoccus diffluens</i>	UBOCC-A-214070	<i>Mucor hiemalis</i>	UBOCC-A-111119
<i>Cryptococcus magnus</i>	UBOCC-A-214092	<i>Mucor hiemalis</i>	UBOCC-A-112185
<i>Cryptococcus uzbekistanensis</i>	UBOCC-A-214131	<i>Mucor lanceolatus</i>	UBOCC-A-109193
<i>Cystofilobasidium infirmominiatum</i>	UBOCC-A-214077	<i>Mucor mucedo</i>	UBOCC-A-101361
<i>Debaryomyces hansenii</i>	L1lev4	<i>Mucor mucedo</i>	UBOCC-A-101362
<i>Debaryomyces hansenii</i>	UBOCC-A-214074	<i>Mucor plumbeus</i>	UBOCC-A-109203
<i>Exophiala castellanii</i>	UBOCC-A-214056	<i>Mucor plumbeus</i>	UBOCC-A-111125
<i>Exophiala dermatitidis</i>	UBOCC-A-214076	<i>Mucor plumbeus</i>	UBOCC-A-111126
<i>Exophiala</i> sp.	UBOCC-A-101133	<i>Mucor plumbeus</i>	UBOCC-A-111128
<i>Exophiala</i> sp.	UBOCC-A-108008	<i>Mucor plumbeus</i>	UBOCC-A-111129
<i>Exophiala</i> sp.	UBOCC-A-108012	<i>Mucor plumbeus</i>	UBOCC-A-111132
<i>Exophiala</i> sp.	UBOCC-A-108017	<i>Mucor racemosus</i>	UBOCC-A-101366
<i>Exophiala</i> sp.	UBOCC-A-108021	<i>Mucor racemosus</i>	UBOCC-A-102017
<i>Exophiala</i> sp.	UBOCC-A-108022	<i>Mucor racemosus</i>	UBOCC-A-109186
<i>Exophiala</i> sp.	UBOCC-A-108025	<i>Mucor racemosus</i>	UBOCC-A-109211
<i>Exophiala</i> sp.	UBOCC-A-108026	<i>Mucor racemosus</i>	UBOCC-A-109212
<i>Exophiala</i> sp.	UBOCC-A-108035	<i>Mucor racemosus</i>	UBOCC-A-109213
<i>Exophiala</i> sp.	UBOCC-A-108059	<i>Mucor</i> sp.	UBOCC-A-110121
<i>Exophiala xenobiotica</i>	UBOCC-A-214088	<i>Mucor</i> sp.	UBOCC-A-112143
<i>Phoma betae</i>	UBOCC-A-111053	<i>Schizosaccharomyces pombe</i>	UBOCC-A-201052
<i>Phoma glomerata</i>	UBOCC-A-101141	<i>Schizosaccharomyces pombe</i>	UBOCC-A-201053

<i>Phoma</i> sp.	UBOCC-A-102026	<i>Sporobolomyces roseus</i>	UBOCC-A-214093
<i>Phoma</i> sp.	UBOCC-A-109091	<i>Sporobolomyces roseus</i>	UBOCC-A-214078
<i>Phoma valerianellae</i>	UBOCC-A-101242	<i>Torulasporea delbrueckii</i>	L2lev7
<i>Phoma valerianellae</i>	UBOCC-A-101243	<i>Torulasporea delbrueckii</i>	L2lev3
<i>Phoma valerianellae</i>	UBOCC-A-101244	<i>Torulasporea delbrueckii</i>	L1lev2
<i>Pichia anomala</i>	UBOCC-A-201001	<i>Torulasporea delbrueckii</i>	UBOCC-A-214128
<i>Pichia anomala</i>	UBOCC-A-205011	<i>Trichoderma harzianum</i>	UBOCC-A-101288
<i>Pichia anomala</i>	UBOCC-A-212007	<i>Trichoderma harzianum</i>	UBOCC-A-101289
<i>Pichia anomala</i>	UBOCC-A-212008	<i>Trichoderma harzianum</i>	UBOCC-A-111253
<i>Pichia anomala</i>	UBOCC-A-212011	<i>Trichoderma harzianum</i>	UBOCC-A-111254
<i>Pichia burtonii</i>	UBOCC-A-212009	<i>Trichoderma koningii</i>	UBOCC-A-101290
<i>Pichia fermentans</i>	UBOCC-A-201032	<i>Trichoderma viride</i>	UBOCC-A-101292
<i>Pichia fermentans</i>	UBOCC-A-201033	<i>Trichoderma viride</i>	UBOCC-A-111251
<i>Pichia membranifaciens</i>	UBOCC-A-214071	<i>Trichoderma viride</i>	UBOCC-A-111252
<i>Pichia norvegensis</i>	UBOCC-A-201034	<i>Trichoderma viride</i>	UBOCC-A-112152
<i>Pseudozyma</i> sp.	UBOCC-A-214138	<i>Trichosporon asahii</i>	UBOCC-A-214080
<i>Rhizomucor pusillus</i>	UBOCC-A-101365	<i>Trichosporon coremiiforme</i>	UBOCC-A-214058
<i>Rhizomucor pusillus</i>	UBOCC-A-111202	<i>Trichosporon dulciturum</i>	UBOCC-A-214085
<i>Rhodotorula laryngis</i>	UBOCC-A-214057	<i>Trichosporon montevidense</i>	UBOCC-A-214068
<i>Rhodotorula minuta</i>	UBOCC-A-214082	<i>Verticillium albo-atrum</i>	UBOCC-A-101303
<i>Rhodotorula mucilaginosa</i>	UBOCC-A-214069	<i>Verticillium albo-atrum</i>	UBOCC-A-101304
<i>Rhodotorula mucilaginosa</i>	UBOCC-A-214075	<i>Verticillium albo-atrum</i>	UBOCC-A-101305
<i>Rhodotorula mucilaginosa</i>	UBOCC-A-214091	<i>Verticillium albo-atrum</i>	UBOCC-A-101306
<i>Rhodotorula mucilaginosa</i>	UBOCC-A-214102	<i>Verticillium albo-atrum</i>	UBOCC-A-101307
<i>Rhodotorula slooffiae</i>	UBOCC-A-214103	<i>Verticillium dahliae</i>	UBOCC-A-101308
<i>Rhodotorula slooffiae</i>	UBOCC-A-214073	<i>Verticillium dahliae</i>	UBOCC-A-101309
<i>Rhodotorula vanillica</i>	UBOCC-A-214104	<i>Verticillium dahliae</i>	UBOCC-A-101310
<i>Saccharomyces bailii</i>	UBOCC-A-201004	<i>Verticillium dahliae</i>	UBOCC-A-101311
<i>Saccharomyces bailii</i>	UBOCC-A-201035	<i>Verticillium dahliae</i>	UBOCC-A-101312
<i>Saccharomyces bailii</i>	UBOCC-A-205008	<i>Verticillium dahliae</i>	UBOCC-A-101313
<i>Saccharomyces carlsbergensis</i>	UBOCC-A-201036	<i>Verticillium dahliae</i>	UBOCC-A-101314
<i>Saccharomyces carlsbergensis</i>	UBOCC-A-201038	<i>Verticillium dahliae</i>	UBOCC-A-101315
<i>Saccharomyces carlsbergensis</i>	UBOCC-A-205034	<i>Verticillium dahliae</i>	UBOCC-A-101316
<i>Saccharomyces cerevisiae</i>	UBOCC-A-214061	<i>Verticillium dahliae</i>	UBOCC-A-101317
<i>Saccharomyces cerevisiae</i>	UBOCC-A-201006	<i>Verticillium dahliae</i>	UBOCC-A-101318
<i>Saccharomyces cerevisiae</i>	UBOCC-A-201039	<i>Verticillium intertextum</i>	UBOCC-A-101319
<i>Saccharomyces cerevisiae</i>	UBOCC-A-201040	<i>Verticillium lecanii</i>	UBOCC-A-101320
<i>Saccharomyces cerevisiae</i>	UBOCC-A-201041	<i>Verticillium lecanii</i>	UBOCC-A-108019
<i>Saccharomyces cerevisiae</i>	UBOCC-A-205009	<i>Verticillium lecanii</i>	UBOCC-A-108023
<i>Saccharomyces cerevisiae</i>	UBOCC-A-201042	<i>Verticillium nigrescens</i>	UBOCC-A-101322
<i>Saccharomyces kluyveri</i>	UBOCC-A-201045	<i>Verticillium nonalfalfae</i>	UBOCC-A-112135
<i>Saccharomyces ludwigii</i>	UBOCC-A-205010	<i>Verticillium tricorpus</i>	UBOCC-A-101323
<i>Saccharomyces rouxii</i>	UBOCC-A-201046	<i>Wickerhamomyces anomalus</i>	UBOCC-A-214106
<i>Saccharomyces uvarum</i>	UBOCC-A-201047	<i>Zygosaccharomyces bailii</i>	UBOCC-A-201062
<i>Saccharomyces uvarum</i>	UBOCC-A-201048	<i>Zygosaccharomyces mellis</i>	UBOCC-A-201063
<i>Saccharomyces uvarum</i>	UBOCC-A-201049	<i>Zygosaccharomyces rouxii</i>	UBOCC-A-201064
<i>Saccharomyces uvarum</i>	UBOCC-A-201050	<i>Zygosaccharomyces rouxii</i>	UBOCC-A-209001
<i>Saccharomyces uvarum</i>	UBOCC-A-201051	<i>Zygosaccharomyces</i> sp.	UBOCC-A-202008

**Supplementary Table S2.** Antifungal activity of fermentates from lactic acid bacteria, propionibacteria and fungi grown in low heat milk (LH) and ultrafiltration milk permeate (UF) against *M. racemosus* UBOCC-A-116002, *P. commune* UBOCC-A-116003, *G. geotrichum* UBOCC-A-216001 and *Y. lipolytica* UBOCC-A-216006 after *in vitro* screening in miniaturized cheeses.

Taxon	Number of isolates	Number of active* fermentates obtained on LH					Number of active fermentates obtained on UF				
		<i>M. racemosus</i>	<i>P. commune</i>	<i>G. geotrichum</i>	<i>Y. lipolytica</i>	More than one target (%)	<i>M. racemosus</i>	<i>P. commune</i>	<i>G. geotrichum</i>	<i>Y. lipolytica</i>	More than one target (%)
<i>Lactobacillus acidophilus</i>	4	0	0	0	0	0 (0)	0	0	0	0	0 (0)
<i>Lactobacillus amylovorus</i>	1	0	0	0	0	0 (0)	0	0	0	0	0 (0)
<i>Lactobacillus brevis</i>	8	1	3	0	0	1 (12.5)	0	0	0	0	0 (0)
<i>Lactobacillus buchneri</i>	34	3	15	0	1	3 (8.8)	0	3	0	0	0 (0)
<i>Lactobacillus casei/paracasei</i>	112	4	12	0	0	2 (1.8)	0	0	0	0	0 (0)
<i>Lactobacillus delbrueckii</i>	2	0	0	0	0	0 (0)	0	0	0	0	0 (0)
<i>Lactobacillus farciminius</i>	1	0	0	0	0	0 (0)	0	0	0	0	0 (0)
<i>Lactobacillus fermentum</i>	2	0	0	0	0	0 (0)	0	0	0	0	0 (0)
<i>Lactobacillus gasserii</i>	4	0	0	0	0	0 (0)	0	0	0	0	0 (0)
<i>Lactobacillus harbinensis</i>	1	1	0	0	0	0 (0)	0	0	0	0	0 (0)
<i>Lactobacillus pentosus</i>	4	0	0	0	0	0 (0)	0	0	0	0	0 (0)
<i>Lactobacillus</i>	3	1	0	0	0	0 (0)	0	0	0	0	0 (0)
<i>Lactobacillus plantarum</i>	115	3	47	0	0	3 (2.6)	0	0	0	0	0 (0)
<i>Lactobacillus reuteri</i>	42	0	1	0	0	0 (0)	0	0	0	0	0 (0)
<i>Lactobacillus rhamnosus</i>	9	0	3	0	0	0 (0)	0	0	0	0	0 (0)
<i>Lactobacillus sakei</i>	3	0	0	0	0	0 (0)	0	0	0	1	0 (0)
<i>Lactobacillus sanfranciscensis</i>	1	0	0	0	0	0 (0)	0	0	0	0	0 (0)
<i>Lactobacillus zeae</i>	2	0	0	0	0	0 (0)	0	0	0	1	0 (0)
<i>Lactococcus lactis</i>	2	0	0	0	0	0 (0)	0	0	0	2	0 (0)
<i>Leuconostoc citreum</i>	7	0	0	0	0	0 (0)	0	1	0	0	0 (0)
<i>Leuconostoc mesenteroides</i>	71	0	0	0	0	0 (0)	0	0	0	0	0 (0)
<i>Pediococcus pentosaceus</i>	2	0	0	0	0	0 (0)	0	0	0	0	0 (0)
<i>Propionibacterium</i>	17	0	0	0	0	0 (0)	0	0	0	0	0 (0)
<i>Propionibacterium freudenreichii</i>	19	0	0	0	0	0 (0)	0	0	0	0	0 (0)
<i>Propionibacterium jensenii</i>	20	0	0	0	0	0 (0)	1	0	0	0	0 (0)
<i>Propionibacterium thoenii</i>	14	0	0	0	0	0 (0)	0	0	0	0	0 (0)
<i>Actinomucor elegans</i>	3	0	0	0	0	0 (0)	0	1	0	0	0 (0)
<i>Aureobasidium pullulans</i>	7	1	0	0	0	0 (0)	0	0	0	0	0 (0)
<i>Candida catenulata</i>	1	0	0	0	0	0 (0)	0	0	0	0	0 (0)
<i>Candida davisiana</i>	1	0	0	0	0	0 (0)	0	0	0	0	0 (0)
<i>Candida haemulonis</i>	1	0	0	0	0	0 (0)	0	0	0	0	0 (0)
<i>Candida methapsilosis</i>	1	0	0	0	0	0 (0)	0	0	0	0	0 (0)
<i>Candida parapsilosis</i>	3	0	0	0	0	0 (0)	0	0	0	0	0 (0)
<i>Candida pseudoglebosa</i>	1	0	0	0	0	0 (0)	0	0	0	0	0 (0)
<i>Candida sorbophila</i>	1	0	0	0	0	0 (0)	0	0	0	0	0 (0)
<i>Candida tenuis</i>	1	0	0	0	0	0 (0)	0	0	0	1	0 (0)
<i>Candida zeylanoides</i>	2	0	0	0	0	0 (0)	0	0	0	0	0 (0)
<i>Chaetomium dolichotrichum</i>	1	0	0	0	0	0 (0)	0	0	0	0	0 (0)

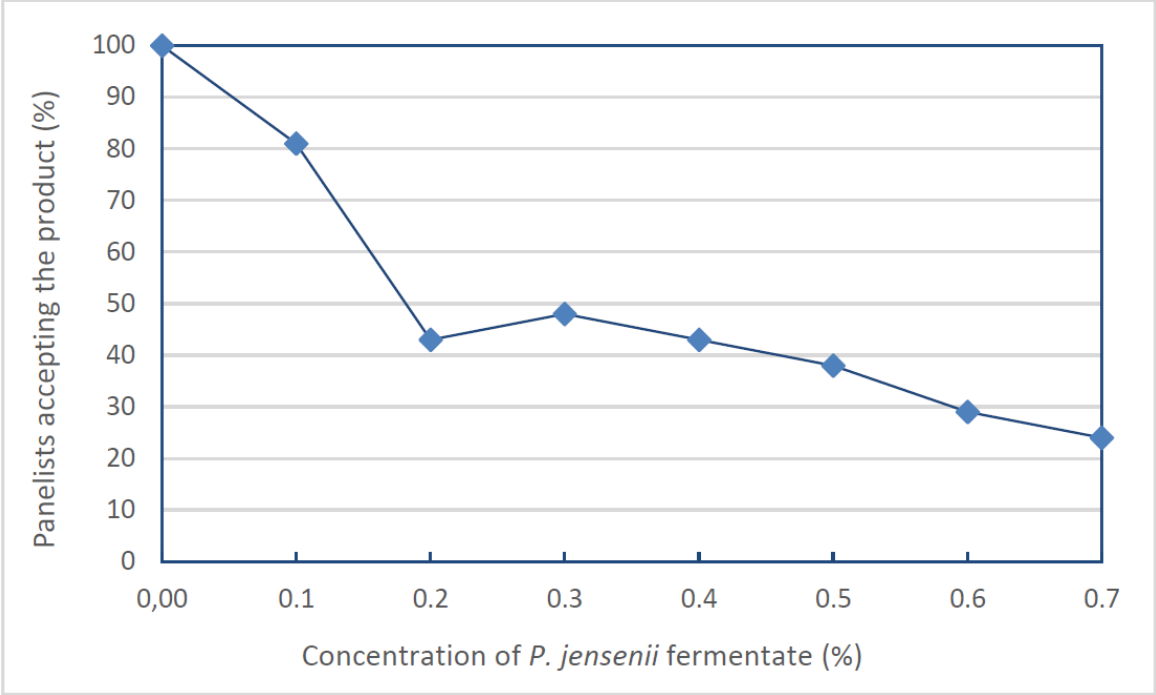


<i>Chaetomium funicola</i>	1	0	0	0	0	0 (0)	0	0	0	0	0 (0)
<i>Chaetomium globosum</i>	1	0	0	0	0	0 (0)	0	0	0	0	0 (0)
<i>Cleistothelobolus nipigonensis</i>	1	0	0	0	0	0 (0)	0	0	0	0	0 (0)
<i>Cryptococcus albidus</i>	1	0	0	0	0	0 (0)	0	0	0	0	0 (0)
<i>Cryptococcus curvatus</i>	2	0	0	0	0	0 (0)	0	0	0	0	0 (0)
<i>Cryptococcus diffluens</i>	1	0	0	0	0	0 (0)	0	0	0	0	0 (0)
<i>Cryptococcus magnus</i>	1	0	0	0	0	0 (0)	0	0	0	0	0 (0)
<i>Cryptococcus uzbekistanensis</i>	1	0	0	0	0	0 (0)	0	0	0	0	0 (0)
<i>Cystofilobasidium</i>	1	0	0	0	0	0 (0)	0	0	0	0	0 (0)
<i>Debaryomyces hansenii</i>	2	0	0	0	0	0 (0)	0	0	0	0	0 (0)
<i>Exophiala castellanii</i>	1	0	0	0	0	0 (0)	0	0	0	0	0 (0)
<i>Exophiala dermatidis</i>	1	0	0	0	0	0 (0)	0	0	0	0	0 (0)
<i>Exophiala xenobiotica</i>	1	0	0	0	0	0 (0)	0	0	0	0	0 (0)
<i>Exophiala sp.</i>	10	0	0	2	0	0 (0)	0	0	0	0	0 (0)
<i>Gliocladium deliquescens</i>	1	0	0	0	0	0 (0)	0	0	0	0	0 (0)
<i>Gliocladium roseum</i>	1	0	0	0	0	0 (0)	0	0	0	0	0 (0)
<i>Gliocladium virens</i>	1	0	0	0	0	0 (0)	0	0	0	0	0 (0)
<i>Gliocladium sp.</i>	1	0	0	0	0	0 (0)	0	0	0	0	0 (0)
<i>Gliomastix murorum</i>	2	0	0	0	0	0 (0)	0	0	0	0	0 (0)
<i>Lodderomyces elongisporus</i>	1	0	0	0	0	0 (0)	0	0	0	0	0 (0)
<i>Meyerozyma guilliermondii</i>	4	0	0	0	0	0 (0)	0	0	0	0	0 (0)
<i>Mucor brunneogriseus</i>	7	0	0	0	0	0 (0)	0	0	0	0	0 (0)
<i>Mucor fragilis</i>	3	0	0	0	0	0 (0)	0	0	0	0	0 (0)
<i>Mucor fuscus</i>	5	0	0	0	0	0 (0)	0	0	0	0	0 (0)
<i>Mucor griseocyanus</i>	1	0	0	0	0	0 (0)	0	0	0	0	0 (0)
<i>Mucor hiemalis</i>	6	0	0	0	0	0 (0)	0	0	0	0	0 (0)
<i>Mucor lanceolatus</i>	1	1	0	0	0	0 (0)	0	0	0	0	0 (0)
<i>Mucor mucedo</i>	2	0	0	0	0	0 (0)	0	0	0	0	0 (0)
<i>Mucor plumbeus</i>	6	0	0	0	0	0 (0)	0	0	0	0	0 (0)
<i>Mucor racemosus</i>	6	0	0	0	0	0 (0)	0	0	0	0	0 (0)
<i>Mucor sp.</i>	2	0	0	0	0	0 (0)	0	0	0	0	0 (0)
<i>Phoma betae</i>	1	0	0	0	0	0 (0)	0	0	0	0	0 (0)
<i>Phoma glomerata</i>	1	0	0	0	0	0 (0)	0	0	0	0	0 (0)
<i>Phoma valerianellae</i>	3	0	0	0	0	0 (0)	0	0	0	0	0 (0)
<i>Phoma sp.</i>	2	0	0	0	0	0 (0)	0	0	0	0	0 (0)
<i>Pichia anomala</i>	5	0	0	0	0	0 (0)	0	0	0	0	0 (0)
<i>Pichia burtonii</i>	1	0	0	0	0	0 (0)	0	0	0	0	0 (0)
<i>Pichia fermentans</i>	2	0	0	0	0	0 (0)	0	0	0	0	0 (0)
<i>Pichia membranifaciens</i>	1	0	0	0	0	0 (0)	0	0	0	0	0 (0)
<i>Pichia norvegensis</i>	1	0	0	0	0	0 (0)	0	0	0	0	0 (0)
<i>Pseudozyma sp.</i>	1	0	0	0	0	0 (0)	0	0	0	0	0 (0)
<i>Rhizomucor pusillus</i>	2	0	0	0	0	0 (0)	0	0	0	0	0 (0)
<i>Rhodotorula laryngis</i>	1	0	0	0	0	0 (0)	0	0	0	0	0 (0)
<i>Rhodotorula minuta</i>	1	0	0	1	0	0 (0)	0	0	0	0	0 (0)
<i>Rhodotorula mucilaginosa</i>	4	0	0	1	0	0 (0)	0	0	0	0	0 (0)
<i>Rhodotorula slooffiae</i>	2	0	0	0	0	0 (0)	0	0	0	0	0 (0)
<i>Rhodotorula vanillica</i>	1	0	0	0	0	0 (0)	0	0	0	0	0 (0)
<i>Saccharomyces carlsbergensis</i>	3	0	0	0	0	0 (0)	0	0	0	0	0 (0)
<i>Saccharomyces cerevisiae</i>	7	0	0	0	0	0 (0)	0	0	0	0	0 (0)

<i>Saccharomyces kluyveri</i>	1	0	0	0	0	0 (0)	0	0	0	0	0 (0)
<i>Saccharomyces ludwigii</i>	1	0	0	0	0	0 (0)	0	0	0	0	0 (0)
<i>Saccharomyces uvarum</i>	5	0	0	0	0	0 (0)	0	0	0	0	0 (0)
<i>Schizosaccharomyces pombe</i>	2	0	0	0	0	0 (0)	0	0	0	0	0 (0)
<i>Sporobomyces roseus</i>	2	0	0	0	0	0 (0)	0	0	0	0	0 (0)
<i>Torulasporea delbrueckii</i>	4	0	0	1	0	0 (0)	0	0	0	0	0 (0)
<i>Trichoderma harzianum</i>	4	0	0	0	0	0 (0)	0	0	0	1	0 (0)
<i>Trichoderma koningii</i>	1	0	0	0	0	0 (0)	0	0	0	0	0 (0)
<i>Trichoderma viride</i>	4	0	0	1	0	0 (0)	0	0	0	0	0 (0)
<i>Trichosporon asahii</i>	1	0	0	0	0	0 (0)	0	0	0	0	0 (0)
<i>Trichosporon coremiiforme</i>	1	0	0	0	0	0 (0)	0	0	0	0	0 (0)
<i>Trichosporon dulcitum</i>	1	0	0	0	0	0 (0)	0	0	0	0	0 (0)
<i>Trichosporon montevidense</i>	1	0	0	0	0	0 (0)	0	0	0	0	0 (0)
<i>Verticillium albo-atrum</i>	5	0	0	0	0	0 (0)	0	0	0	0	0 (0)
<i>Verticillium dahliae</i>	11	0	0	0	0	0 (0)	0	1	0	0	0 (0)
<i>Verticillium intertextum</i>	1	0	0	0	0	0 (0)	0	0	0	0	0 (0)
<i>Verticillium lecanii</i>	3	0	0	0	0	0 (0)	0	0	0	0	0 (0)
<i>Verticillium nigrescens</i>	1	0	0	0	0	0 (0)	0	0	0	0	0 (0)
<i>Verticillium nonalfalfae</i>	1	0	0	0	0	0 (0)	0	0	0	0	0 (0)
<i>Verticillium tricorpus</i>	1	0	0	0	0	0 (0)	0	0	0	0	0 (0)
<i>Wickerhamomyces anomalus</i>	1	0	0	0	0	0 (0)	0	0	0	0	0 (0)
<i>Zygosaccharomyces bailii</i>	4	0	0	0	0	0 (0)	0	0	0	0	0 (0)
<i>Zygosaccharomyces mellis</i>	1	0	0	0	0	0 (0)	0	0	0	0	0 (0)
<i>Zygosaccharomyces rouxii</i>	3	0	0	0	0	0 (0)	0	0	0	0	0 (0)
<i>Zygosaccharomyces sp.</i>	1	0	0	0	0	0 (0)	0	0	0	0	0 (0)
<b>Total bacteria</b>	<b>500</b>	<b>13</b>	<b>81</b>	<b>0</b>	<b>1</b>	<b>9 (1.8)</b>	<b>1</b>	<b>4</b>	<b>0</b>	<b>4</b>	<b>0 (0)</b>
<b>Total fungi</b>	<b>198</b>	<b>2</b>	<b>0</b>	<b>6</b>	<b>0</b>	<b>0 (0)</b>	<b>0</b>	<b>2</b>	<b>0</b>	<b>2</b>	<b>0 (0)</b>
<b>Total</b>	<b>698</b>	<b>15</b>	<b>81</b>	<b>6</b>	<b>1</b>	<b>9 (1.3)</b>	<b>1</b>	<b>6</b>	<b>0</b>	<b>6</b>	<b>0 (0)</b>

\* Delayed growth or total inhibition of the fungal target in comparison with the negative control.

**Supplementary figure 1.** Acceptability test for sour cream inoculated with increased concentrations of *P. jensenii* CIRM-BIA1774 fermentate ranging from 0.1 to 0.7 %. Results corresponding to the 21 of 26 panelists accepting the the control (sour cream without fermentate addition).



## **Données complémentaires**

### **1. Evaluation du spectre d'action**

*Suite à l'étape de criblage in vitro des fermentats pour leur activité antifongique, 22 fermentats bactériens ont été retenus afin d'étudier leur spectre d'activité.*

#### **Matériels et méthodes**

##### **Fermentats bactériens**

Les fermentats sélectionnés sont issus des espèces *L. brevis* (n=1), *L. buchneri* (n=6), *L. casei* (n=6), *L. harbinensis* (n=2), *L. plantarum* (n=4), *L. rhamnosus* (n=2) et *P. jensenii* (n=1). Ils ont été obtenus selon le protocole décrit dans l'article ci-avant. Après 2 pré-cultures de 24 ou 48h à 30°C dans un milieu Man-Rogosa-Sharpe (MRS) ou Yeast Extract Lactate (YEL) pour les bactéries lactiques et propioniques respectivement, les différentes souches ont été inoculées dans les 2 milieux laitiers, à savoir le lait low heat complétement en matière grasse (LH) et le perméat d'ultrafiltration de lait complétement en extrait de levure (UF), dont les compositions sont données dans le chapitre 2, puis incubées 20h ou 48h à 30°C pour les bactéries lactiques et propioniques respectivement. Les fermentats obtenus ont ensuite été conservés à -80°C jusqu'à utilisation.

##### **Cibles fongiques**

En plus des 4 cibles utilisées pour le criblage, à savoir *M. racemosus* UBOCC-A-116002, *P. commune* UBOCC-A-116003, *G. geotrichum* UBOCC-A-216001 et *Y. lipolytica* UBOCC-A-216006, 7 autres cibles fongiques (*P. bialowiezense* 5f22, *C. pseudolongus* 1f7b, *C. parapsilosis* UBOCC-A-216002, *Meyerozyma guilliermondii* UBOCC-A-216003, *R. mucilaginosa* UBOCC-A-216004, *T. asahii* UBOCC-A-216005, et *D. pinodella* UBOCC-A-116004) également isolées de produits laitiers contaminés, ont été sélectionnées selon leur occurrence et leur positionnement taxonomique. Les spores ou cellules de ces champignons ont été obtenues comme décrit précédemment par Delavenne *et al.* (2012), mis à part que les levures ont été cultivées dans un milieu Potato Dextrose Broth (PDB) au lieu d'un milieu Yeast ExtractMalt Agar (YEMA).

## Evaluation de l'activité antifongique des fermentats

L'activité antifongique des fermentats obtenus a été évaluée *in vitro* en utilisant la méthode de criblage haut-débit dans un modèle fromage, comme décrit dans l'article méthodologique (voir chapitre 2 de la partie résultats).

### Résultats

Sur les 22 fermentats sélectionnés, tous ont montré une activité antifongique contre au moins 1 des 11 cibles fongiques (**Tableau 4**). Des observations similaires à celle du criblage *in vitro* découlent de cet essai. En effet, une fois encore, l'impact du substrat de fermentation sur l'activité antifongique est important puisque 81,2% des fermentats issus de la fermentation du milieu LH sont actifs contre au moins 2 cibles fongiques alors que seulement 27,3% des fermentats issus de la fermentation du milieu UF le sont. Concernant les fermentats LH les plus actifs, ils dérivent des espèces *L. brevis*, *L. buchneri*, *L. harbinensis* et *P. jensenii* avec 100% des souches testées actives contre au moins 2 cibles fongiques (**Tableau 4**). De même, les fermentats UF les plus actifs dérivent de *L. brevis* et *P. jensenii* avec, ici encore, 100% des souches actives contre au moins 2 cibles (**Tableau 4**).

Ces résultats montrent également les différences de sensibilité entre les cibles fongiques confirmant ainsi le caractère cible-dépendant de l'activité antifongique des fermentats, ce qui sous entend la production de composés antifongiques différents selon les microorganismes et selon les substrats de fermentation. En effet, *P. bialowiezense* 5f22 est la cible la plus sensible, peu importe le substrat de fermentation. En revanche, *P. commune* UBOCC-A-116003 est très sensible aux fermentats LH mais n'est inhibé par aucun fermentat UF (**Tableau 4**). Au contraire, 4 fermentats UF sont capables d'inhiber *G. geotrichum* UBOCC-A-216001 alors qu'un seul fermentat LH est actif contre cette même cible (**Tableau 4**).

*Cette étape d'évaluation du spectre d'action a, non seulement permis de valider l'activité antifongique des fermentats sélectionnés mais elle a également participé à la sélection de 12 fermentats bactériens pour l'optimisation de l'activité antifongique présentée dans l'article qui précède.*

**Tableau 4 – Activité antifongique d’une sélection de 22 fermentats issus de bactéries lactiques et propioniques contre 11 cibles fongiques après un test *in vitro* sur le modèle fromage.**

Espèce	Nombre de souche testées	Nombre de fermentats LH actifs*											Nombre de fermentats UF actifs											Nombre de fermentats actifs contre plusieurs cibles (%)	
		<i>M. racemosus</i>	<i>P. commune</i>	<i>G. geotrichum</i>	<i>Y. lipolytica</i>	<i>P. bialowiezense</i>	<i>C. pseudolongus</i>	<i>T. asahii</i>	<i>C. parapsilosis</i>	<i>M. guilliermondii</i>	<i>R. mucilaginososa</i>	<i>D. pinodella</i>	<i>M. racemosus</i>	<i>P. commune</i>	<i>G. geotrichum</i>	<i>Y. lipolytica</i>	<i>P. bialowiezense</i>	<i>C. pseudolongus</i>	<i>T. asahii</i>	<i>C. parapsilosis</i>	<i>M. guilliermondii</i>	<i>R. mucilaginososa</i>	<i>D. pinodella</i>		
<i>L. brevis</i>	1	0	0	0	0	1	0	0	0	0	0	1	1 (100%)	1	0	0	0	1	0	1	0	0	1	1	1 (100%)
<i>L. buchneri</i>	6	2	6	0	0	6	0	1	0	0	4	6	6 (100%)	0	0	0	0	3	0	1	0	0	0	4	3 (50%)
<i>L. casei</i>	6	0	5	0	0	6	0	1	0	0	2	2	5 (83,3%)	0	0	0	0	2	0	0	0	0	0	0	0
<i>L. harbinensis</i>	2	0	0	0	0	2	0	2	0	0	1	2	2 (100%)	0	0	1	0	0	0	0	0	0	0	0	0
<i>L. plantarum</i>	4	0	0	0	0	1	0	1	0	0	0	0	2 (50%)	0	0	1	0	0	0	0	0	0	0	0	0
<i>L. rhamnosus</i>	2	0	1	1	0	1	0	1	0	0	1	0	1 (50%)	0	0	1	0	0	0	0	0	0	0	0	0
<i>P. jensenii</i>	1	1	0	0	0	1	0	0	0	0	0	0	1 (100%)	1	0	1	0	1	0	0	0	0	0	0	1 (100%)
<b>Totaux</b>	<b>22</b>	<b>3</b>	<b>12</b>	<b>1</b>	<b>0</b>	<b>18</b>	<b>0</b>	<b>6</b>	<b>0</b>	<b>0</b>	<b>8</b>	<b>11</b>	<b>18 (81,2%)</b>	<b>2</b>	<b>0</b>	<b>4</b>	<b>0</b>	<b>7</b>	<b>0</b>	<b>2</b>	<b>0</b>	<b>0</b>	<b>1</b>	<b>5</b>	<b>6 (27,3%)</b>

\* retard de croissance ou inhibition totale de la cible fongique par rapport au contrôle positif.

## **2. Etape intermédiaire au changement d'échelle**

Suite à l'optimisation de l'activité antifongique de 11 fermentats actifs, 7 fermentats bactériens et fongiques, issus des espèces *L. buchneri* (n=2), *L. paracasei* (n=1), *L. harbinensis* qui a été en co-cultivé avec *S. thermophilus* (n=1), *L. rhamnosus* (n=1), *P. jensenii* (n=1) et *M. lanceolatus* (n=1) ont été sélectionnés pour des tests sur produits réels afin de pouvoir sélectionner les 3 fermentats les plus performants à tester, par la suite, à l'échelle pilote. Ces tests complémentaires nous ont aussi permis de définir les modalités d'application des fermentats pour une utilisation en produits "réels". Pour ce faire nous avons évalué par le biais de challenge-tests l'activité antifongique de ces fermentats sur 2 produits issus du commerce : un fromage blanc, dans lequel le fermentat lyophilisé était directement incorporé dans la masse, et un fromage à pâte pressée à croûte morgée où le fermentat était pulvérisé en surface.

### **2.1. Challenge-test sur fromage blanc issu du commerce**

Sur les 7 fermentats sélectionnés après optimisation, 4 ont été testés dans le fromage blanc. Ces 4 fermentats, issus des souches *L. paracasei* L117, *L. rhamnosus* CIRM-BIA 1952, *P. jensenii* CIRM-BIA 1774 et de la co-culture de *L. harbinensis* L172 avec *S. salviarius* subsp. *thermophilus* CIRM-BIA 16, ont été testés seuls et/ou en association contre 3 cibles fongiques, à savoir *M. racemosus* UBOCC-A-116002, *P. commune* UBOCC-A-116003 et *R. mucilaginosa* UBOCC-A-216004.

### **Matériels et méthodes**

#### **Obtention des fermentats**

Après 2 pré-cultures de 24 ou 48h dans un milieu Mann-Rogosa-Sharpe (MRS) ou Yeast Extract Lactose (YEL) pour les souches de bactéries lactiques et propioniques, les milieux laitiers sélectionnés suite à l'optimisation (lait low heat pour les bactéries lactiques et le perméat d'ultrafiltration pour la bactérie propionique) ont été inoculés (à 1% pour les souches pures et 0,1%/0,01% pour la co-culture *L. harbinensis* L172 / *S. thermophilus* CIRM-BIA 16) et incubés dans les conditions optimales pour l'expression de l'activité antifongique de chaque souche, à savoir : 20h à 30°C pour *L. paracasei* L117, 48h à 30°C pour *L. rhamnosus* CIRM-BIA 1952 et 96h à 30°C puis 24h à 12°C pour *P. jensenii* CIRM-BIA 1774. En ce qui

concerne la co-culture, elle a été incubée d'abord 6h à 40°C puis 30h à 30°C. Les fermentats ont ensuite été lyophilisés puis conservés sous vide à 4°C jusqu'à utilisation.

### **Cibles fongiques**

Les challenge-tests ont été réalisés contre 3 cibles fongiques : *M. racemosus* UBOCC-A-116002, *P. commune* UBOCC-A-116003 et *R. mucilaginosa* UBOCC-A-216004. Les spores et cellules ont été obtenues comme indiqué précédemment.

### **Challenge-tests**

Les 4 fermentats ont été testés seuls, à 1 et 2% dans le fromage blanc ainsi que les trois associations de fermentats suivantes : *P. jensenii* CIRM-BIA1774 / *L. rhamnosus* CIRM-BIA 1952, *P. jensenii* CIRM-BIA1774 / *L. paracasei* L117, et *L. rhamnosus* CIRM-BIA 1952 / *L. paracasei* L117. Pour chaque association, les fermentats ont été ajoutés à 2% (m/m) dans le fromage blanc, soit 1% de chaque fermentat. Après homogénéisation, 50 spores d'une des 2 cibles fongiques, *M. racemosus* UBOCC-A-116002 ou *P. commune* UBOCC-A-116003 ont été déposées à la surface du fromage blanc et la lecture des résultats a été faite visuellement et quotidiennement pendant l'incubation à 10°C. En ce qui concerne *R. mucilaginosa* UBOCC-A-216004, environ 2 unités formant colonie (UFC)/g ont été inoculées dans le fromage. Après homogénéisation, les fromages blancs ont été incubés à 10°C et un dénombrement a été réalisé après 1, 2 et 3 semaines.

### **Résultats**

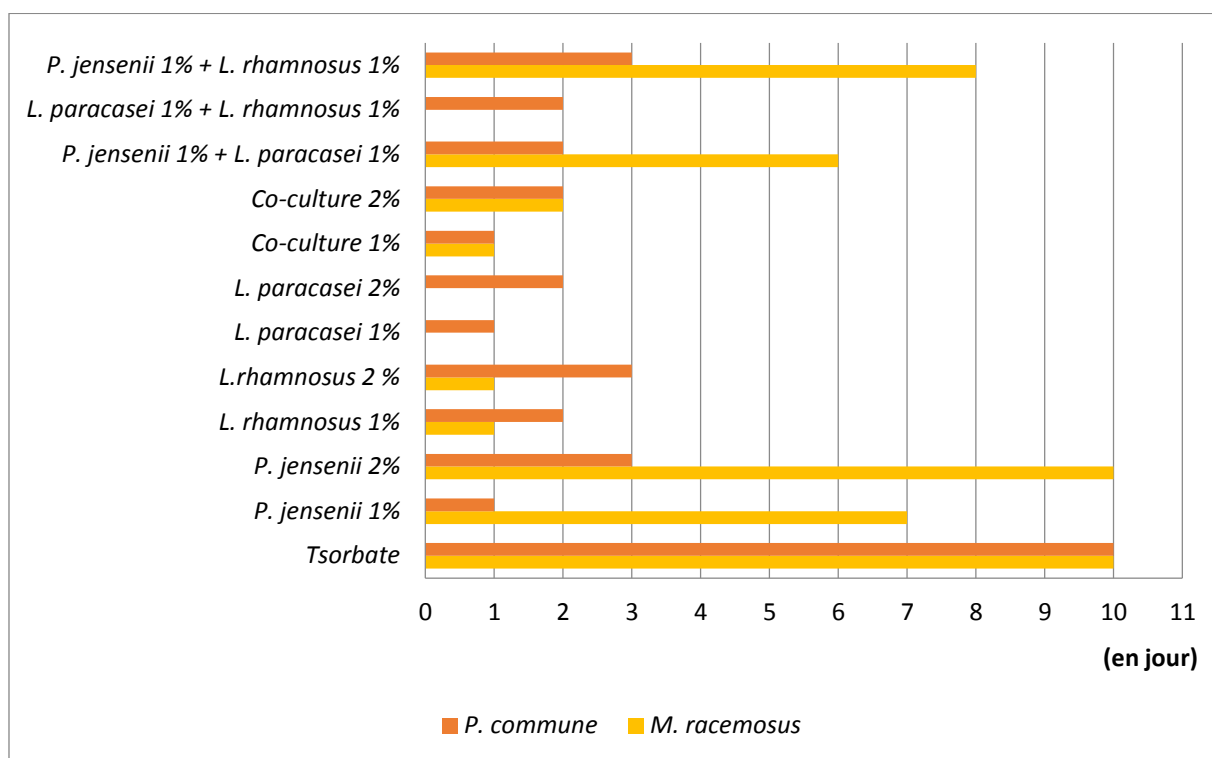
De manière synthétique, ces résultats ont montré un effet dose important, en effet, à 1%, l'efficacité des fermentats est très faible alors qu'à 2%, des retards de croissance de plusieurs jours sont observés. En effet, à 2%, le fermentat de *L. rhamnosus* CIRM-BIA 1952 permet un retard de croissance de 2 à 4 jours de *P. commune* UBOCC-A-116003 et le fermentat de *P. jensenii* CIRM-BIA 1774 retarde de 10 jours la croissance de *M. racemosus* UBOCC-A-116002 (**Figure 10**) et permet de réduire de 2 log<sub>10</sub> la concentration des levures après 14 jours de stockage à 10°C (**Figure 11**).

L'association des fermentats *L. rhamnosus* CIRM-BIA 1952 à 1% et *P. jensenii* CIRM-BIA 1774 à 1% montre également des résultats intéressants puisque cette association permet un retard de 7 jours pour *M. racemosus* UBOCC-A-116002 et 3 jours pour *P. commune*

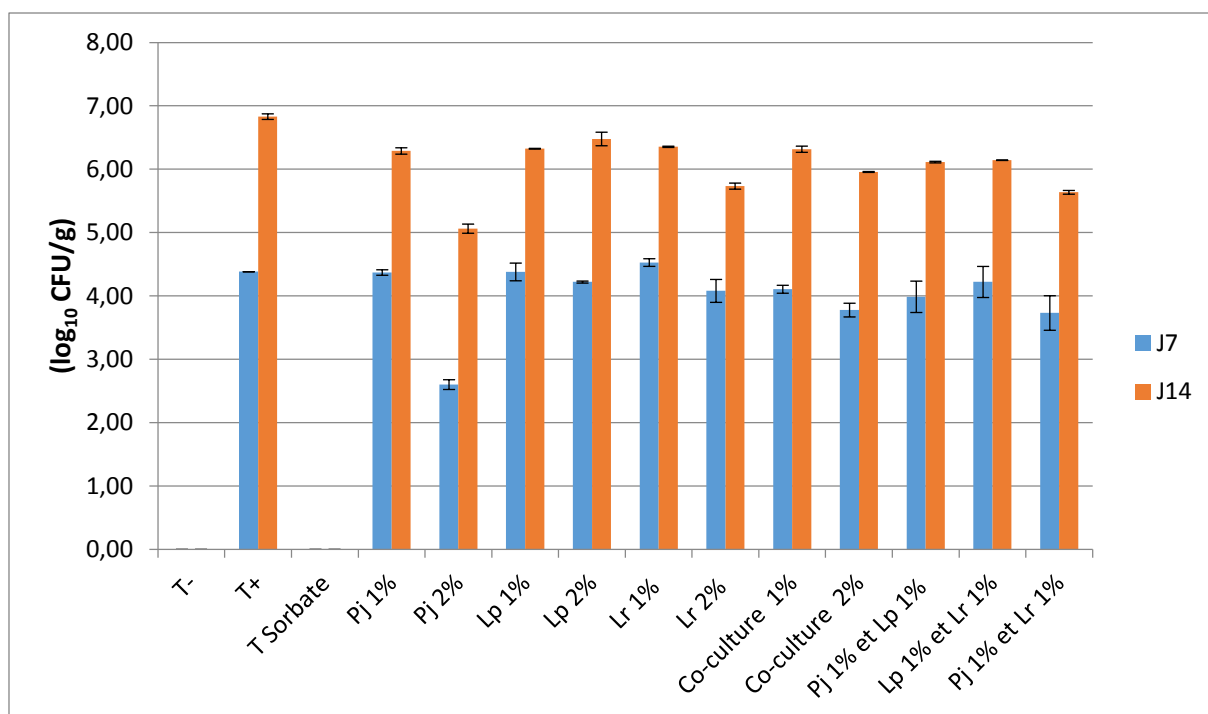


UBOCC-A-116003 soit une augmentation de 1 et 2 jours respectivement par rapport au fermentat *P. jensenii* CIR-BIA 1774 employé seul à 1% (**Figure 10**). Ces 2 fermentats, ainsi que leur association seront testés à l'échelle pilote dans des fromages blancs et des crèmes fraîches.

Ces résultats confirment donc l'activité antifongique des fermentats sélectionnés dans un produit réel. De plus, tous les fermentats testés ont montré une activité contre au moins 1 des 3 cibles fongiques validant ainsi la méthode de criblage mise au point. Enfin, ces résultats montrent également que les conditions d'applications choisies, à savoir l'incorporation du fermentat lyophilisé à 1 ou 2 % directement dans le fromage blanc sont adaptées à ce type de produit et pourront donc être appliquées pour le reste de l'étude.



**Figure 10. Retard de croissance (en jour) de *P. commune* UBOCC-A-116003 et *M. racemosus* UBOCC-A-116002, par rapport aux témoins respectifs, à la surface des fromages blancs pendant une incubation à 10°C. Tsorbate : Témoin sorbate à 0,08% ; Co-culture : *L. harbinensis* L172 en co-culture avec *S. thermophilus* CIRM-BIA 16; les pourcentages indiqués correspondent aux concentrations (en %) de fermentats ajoutés dans le produit.**



**Figure 11. Dénombrement de *R. mucilaginosa* UBOCC-A-216004 après 7 et 14 jours d'incubation à 10°C (en log<sub>10</sub> CFU/g de fromage). T- : Contrôle négatif correspondant à la crème fraîche non inoculée ; T+ : Témoin positif correspondant à la concentration cellulaire de *R. mucilaginosa* UBOCC-A-216004 dans la crème fraîche ne contenant pas de fermentat ; T Sorbate : Témoin sorbate de potassium à 0,08% ; Pj : *P. jensenii* CIRM-BIA 1774 ; Lp : *L. paracasei* L117 ; Lr : *L. rhamnosus* CIRM-BIA 1952 ; Co-culture : *L. harbinensis* L172 en co-culture avec *S. thermophilus* CIRM-BIA 16 ; les pourcentages indiqués correspondent aux concentrations (en %) de fermentats ajoutés dans le produit.**

## 2.2. Challenge-test sur fromage à pâte pressée à croûte morgée

L'activité antifongique des 7 fermentats sélectionnés suite à l'optimisation a été évaluée contre *P. commune* UBOCC-A-116003 et *M. racemosus* UBOCC-A-116002 lors de challenge-tests sur un fromage à pâte pressée à croûte morgée.

### Matériels et méthodes

#### Obtention des fermentats

Les fermentats issus de *L. rhamnosus* CIRM-BIA 1952, *P. jensenii* CIRM-BIA 1774, *L. paracasei* L117 et de la co-culture *L. harbinensis* L172 avec *S. thermophilus* CIRM-BIA 16 ont été obtenus comme décrit dans la partie précédente. Les 2 autres fermentats bactériens ont été obtenus après 2 pré-cultures de 24h à 30°C des souches *L. buchneri* L162 et L233 dans un milieu MRS puis le milieu lait low heat a ensuite été inoculé à 1% et incubé 48h à 30°C puis 24h à 43°C. Ces fermentats ont également été lyophilisés et conservés sous vide à 4°C jusqu'à utilisation ; En ce qui concerne le fermentat fongique, il a été obtenu après culture de *M. lanceolatus* UBOCC-A-109193 ( $10^4$  spores/ml), dans le milieu lait low heat, pendant 96h à 25°C puis 48h à 12°C. Ce fermentat a ensuite été centrifugé (3 fois 10min à 8000rpm) puis filtré (0,45µm) afin de récupérer un surnageant ne contenant aucune spore fongique. Ce surnageant a été conservé à -80°C jusqu'à utilisation.

Les associations de fermentats bactériens sont obtenues en resuspendant (1X) les fermentats lyophilisés dans 10 mL d'eau préalablement stérilisée. Concernant les associations de fermentats bactériens avec le fermentat fongique, les fermentats lyophilisés ont été resuspendus (1X) dans 10mL de surnageant de *M. lanceolatus* UBOCC-A-109193.

#### Cibles fongiques

Les challenge-tests sur fromages à pâte pressée à croûte morgée ont été réalisés contre 2 cibles fongiques : *M. racemosus* UBOCC-A-116002, *P. commune* UBOCC-A-116003. Les spores ont été obtenues comme indiqué précédemment.

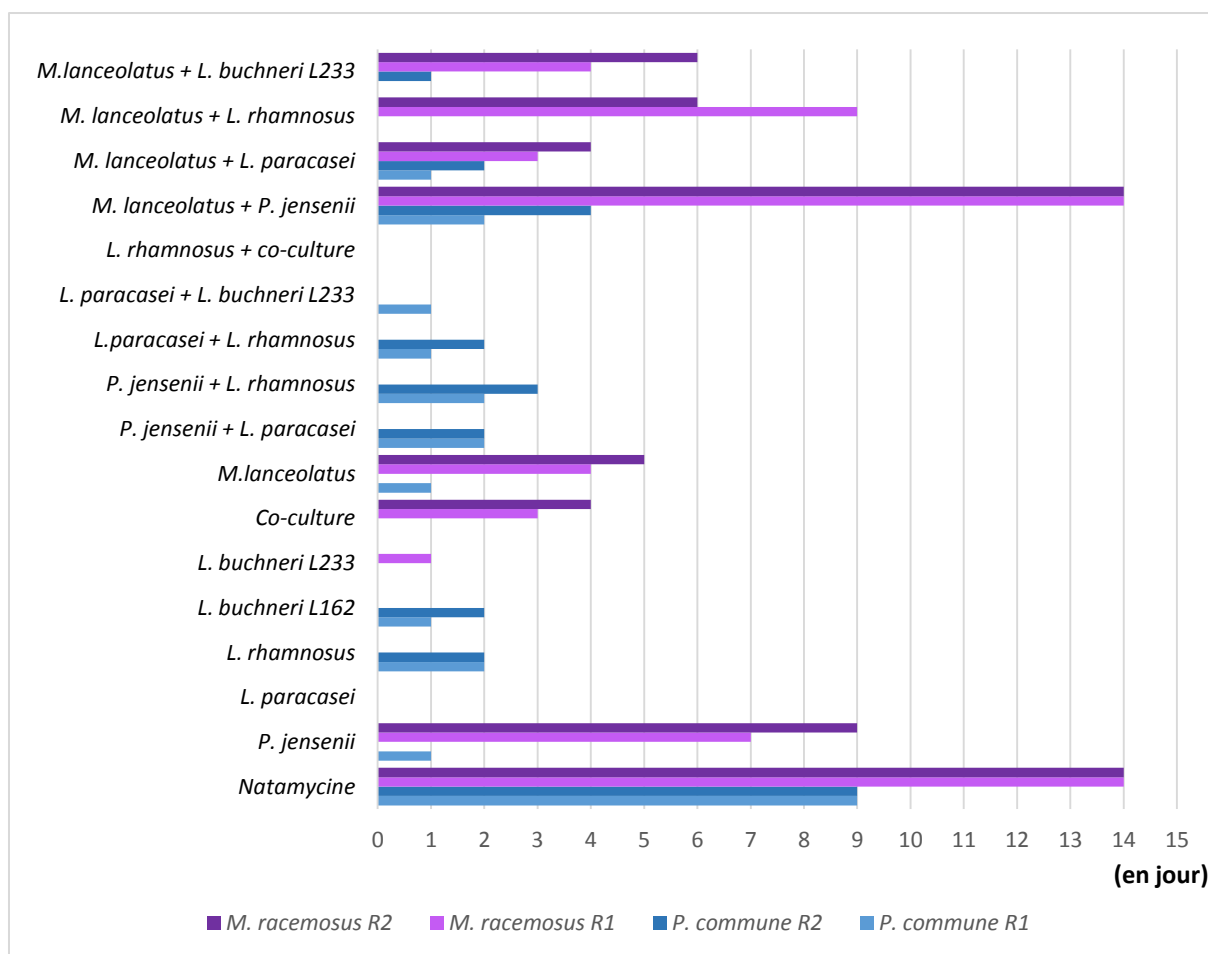
#### Challenge-tests

Les 7 fermentats ont été testés seuls ou en association (**Figure 12**), par pulvérisation après resuspension des lyophilisats dans de l'eau ou dans du surnageant de *M. lanceolatus* UBOCC-

A-109193. Après écroutage des fromages et application de la morge (voir article précédent pour les détails de la méthode), 2 modalités de pulvérisation ont été testées : 1 dose (environ 5mL/dm<sup>2</sup>) à été appliquée par pulvérisation à J0 (jour d'application de la morge) ou 3 doses ont été appliquées à J0, J2 et J7. Les cibles fongiques (*P. commune* UBOCC-A-116003 et *M. racemosus* UBOCC-A-116002) ont été inoculées en surface (50 spores) à J1 puis la lecture des résultats a été faite visuellement et quotidiennement jusqu'à croissance des cibles fongiques.

### **Résultats**

Comme observé lors des essais sur fromage blanc, la dose de fermentat appliquée est déterminante pour l'activité antifongique. En effet, avec une seule pulvérisation, aucun effet n'a été observé (résultats non présentés). En revanche, avec 3 pulvérisations, des retards de croissance de plusieurs jours ont pu être observés (**Figure 12**). Sur ce type de produits, le fermentat *P. jensenii* CIRM-BIA 1774 retarde de 8 jours la croissance de *M. racemosus* UBOCC-A-116002, l'association des fermentats *P. jensenii* CIRM-BIA 1774 et *M. lanceolatus* UBOCC-A-109193 retarde de 14 jours la croissance de *M. racemosus* UBOCC-A-116002 et de 3 jours celle de *P. commune* UBOCC-A-116003 tandis que l'association des fermentats *L. rhamnosus* CIRM-BIA 1952 et *M. lanceolatus* UBOCC-A-109193 montre également une activité intéressante contre *P. commune* UBOCC-A-116003 puisqu'un retard de 3 à 4 jours a été observé (**Figure 12**).



**Figure 12. Retard de croissance (en jour) des cibles *P. commune* UBOCC-A-116003 et *M. racemosus* UBOCC-A-116002, par rapport aux témoins respectifs, à la surface des pâtes pressées à croûte morgée pendant une incubation à 12°C et 95% d'humidité relative. R1 et R2 correspondant aux 2 réplicats techniques réalisés pour chaque fermentat testé.**

Sur 16 fermentats ou associations de fermentats testés sur fromage à pâte pressée, 14 ont montré une activité contre au moins une des deux cibles fongiques validant, comme pour le fromage blanc, la méthode de criblage utilisée. De plus, cette méthode nous a permis de choisir les conditions d'application des fermentats. Ainsi 3 doses à J0, J2 et J7 (J0 étant le jour d'application de la morge) sont nécessaires pour avoir une activité antifongique sur les fromages à pâte pressée à croûte morgée.

***En conclusion de cette Etude ~ Développement d'ingrédients laitiers antifongiques : du criblage in vitro aux applications à l'échelle pilote :***

*A partir de données de criblage in vitro, nous avons pu valider l'activité antifongique de 3 fermentats, utilisés seuls ou en association en conditions réelles sur 3 produits laitiers de technologies différentes : le fromage frais, la crème fraîche et un fromage à pâte pressée à croûte morgée. En réalisant des tests organoleptiques, nous avons également pu définir un cadre d'utilisation de ces fermentats en fonction des produits considérés. Les industriels devront donc maintenant s'appropriier les différents fermentats, adapter et optimiser leur fabrication (possibilité par exemple de le produire en plus grand volume par séchage par atomisation) mais aussi les modes d'application en fonction des produits et des technologies visés. D'un point de vue fondamental, il est maintenant nécessaire d'identifier les molécules mises en jeu dans cette activité et de comprendre les mécanismes d'action liés à ces fermentats. C'est l'objet du chapitre 4 qui suit.*



## **Etude 4**

~

# **Identification des composés antifongiques et des mécanismes d'action**





En produisant une grande variété de composés antifongiques, les bactéries lactiques, propioniques et les champignons jouent un rôle actif dans la conservation des produits laitiers fermentés. En effet, lors des travaux précédents, des fermentats issus de ces microorganismes ont montré leur activité contre différentes cibles fongiques.

Ainsi, l'activité antifongique de 3 fermentats, dérivant (i) de la fermentation d'un perméat d'ultrafiltration de lait complété en extrait de levure par *P. jensenii* CIRM-BIA 1774, (ii) de la fermentation d'un lait reconstitué complété en matière grasse par *L. rhamnosus* CIRM-BIA 1952 et (iii) de la fermentation d'un lait reconstitué complété en matière grasse par *M. lanceolatus* UBOCC-A-109193, a été caractérisée via l'identification des molécules antifongiques.

### **Principaux résultats**

L'utilisation de différentes méthodes d'analyse (HPLC, GC, MS), décrites dans la partie « matériels et méthodes » de l'article suivant, a permis l'identification de 56, 53 et 43 composés dans les fermentats dérivant de *L. rhamnosus* CIRM-BIA 1952, *P. jensenii* CIRM-BIA 1774 et *M. lanceolatus* UBOCC-A-109193, respectivement.

#### Fermentat de *P. jensenii*

Parmi les 61 composés identifiés, des acides organiques ont été identifiés, notamment les acides propionique, citrique et acétique qui sont présents en concentrations importantes. Au sein des acides organiques, on retrouve aussi quelques acides gras libres tels que les acides palmitiques et steariques. De plus, des composés volatiles, tels que le 3-méthyl-butanal et le butyl-propionate, ont également été détectés.

#### Fermentat de *L. rhamnosus*

Ce fermentat, avec 63 composés, est celui qui présente la plus grande diversité de composés (potentiellement) antifongiques. Les principaux composés détectés sont l'acide lactique, l'acide succinique, l'acide acétique, l'acide palmitique, l'acide stéarique, l'acide hexanoïque et l'acétoïne. De plus, un peptide, issu de l'hydrolyse de la caséine  $\alpha$ -S2 a été identifié et son activité antifongique contre *M. racemosus* UBOCC-A-109155 et *R. mucilaginosa* UBOCC-A-216004 a été démontrée.

### Fermentat de *M. lanceolatus*

Ce fermentat, seul, montre la diversité de composés antifongiques la moins importante (41 composés) mais présente une forte concentration en acide butyrique, connu pour son activité antifongique. De plus, des molécules telles que le diacétyl, l'éthyl butanoate, le 3-méthyl-1-butanol et l'acide myristique ont été également été détectés.

Finalement, parmi ces 3 fermentats, celui dérivant de *P. jensenii* CIRM-BIA 1774 montrait l'activité antifongique la plus intéressante et il a donc été sélectionné pour étudier son impact sur 2 cibles fongiques : *M. racemosus* UBOCC-A-109155 et *R. mucilaginosa* UBOCC-A-216004. Concernant *M. racemosus*, aucun impact n'a été observé sur la germination à 1 et 2%. En revanche, à 5%, le temps et la capacité de germination sont significativement impactés. De plus, des changements morphologiques ont été observés sur les spores en germination. Quand à la croissance radiale de *M. racemosus*, elle décroît significativement avec l'augmentation de la concentration en fermentat. La croissance de *R. mucilaginosa* a également été affectée par ce fermentat. En effet, à 1%, le temps de latence est légèrement augmenté, à 2% le temps de latence et le taux de croissance sont affectés et à 5%, le fermentat inhibe complètement la croissance de *R. mucilaginosa*. Cependant, aucun changement morphologique des cellules n'a été observé.

Actuellement, une étude transcriptomique est en cours de réalisation afin de comprendre l'impact du fermentat à un niveau moléculaire.

*Ce travail fait l'objet d'un article scientifique rédigé en anglais et présenté ci-après.*

1 **Antifungal activity of fermented dairy ingredients: identification of antifungal**  
2 **compounds and effects on fungal growth.**  
3

4 Lucille Garnier<sup>1,2</sup>, Marine Penland<sup>1</sup>, Anne Thierry<sup>2</sup>, Marie-Bernadette Maillard<sup>2</sup>, Julien  
5 Jardin<sup>2</sup>, Marcia Leyva-Salas<sup>1,2</sup>, Emmanuel Coton<sup>1</sup>, Florence Valence<sup>2</sup> and Jérôme Mounier<sup>1\*</sup>.

6  
7 <sup>1</sup> Université de Brest, EA 3882, Laboratoire Universitaire de Biodiversité et Ecologie  
8 Microbienne, IBSAM, ESIAB, Technopôle Brest-Iroise, 29280 Plouzané, France.

9 <sup>2</sup> STLO, Agrocampus Ouest, INRA, 35000 Rennes, France

10

11 Lucille Garnier : [lucille.garnier@univ-brest.fr](mailto:lucille.garnier@univ-brest.fr)

12 Marine Penland: [marine.penland@etudiant.univ-brest.fr](mailto:marine.penland@etudiant.univ-brest.fr)

13 Florence Valence : [florence.valence-bertel@inra.fr](mailto:florence.valence-bertel@inra.fr)

14 Anne Thierry: [anne.thierry@rennes.inra.fr](mailto:anne.thierry@rennes.inra.fr)

15 Marie-Bernadette Maillard: [marie-bernadette.maillard@inra.fr](mailto:marie-bernadette.maillard@inra.fr)

16 Julien Jardin: [julien.jardin@inra.fr](mailto:julien.jardin@inra.fr)

17 Marcia. Leyva Salas : [marcia.leyva-salas@univ-brest.fr](mailto:marcia.leyva-salas@univ-brest.fr)

18 Emmanuel Coton : [emmanuel.coton@univ-brest.fr](mailto:emmanuel.coton@univ-brest.fr)

19

20 **\*Corresponding author** : Jérôme Mounier

21 Laboratoire Universitaire de Biodiversité et Ecologie Microbienne,

22 Parvis Blaise-Pascal, Technopôle Brest-Iroise

23 29280 Plouzané, France

24 Tel : +33 (0)2.90.91.51.00

25 Fax : +33 (0)2.90.91.51.01

26 E-mail: [Jerome.mounier@univ-brest.fr](mailto:Jerome.mounier@univ-brest.fr)

27

28 **Keywords: fermentate, antifungal, organic acids, fatty acids, volatile compounds, action**

29 **mechanisms**

30 **Abstract**

31 **Fungi are commonly responsible for dairy product spoilage and leads to substantial**  
32 **economic losses for the dairy industry as well as consumers' dissatisfaction. Lactic acid**  
33 **bacteria, propionibacteria and fungi can play an active role in preservation of fermented**  
34 **dairy product, being able to produce a large range of antifungal metabolites. In a**  
35 **previous study (Garnier et al., submitted, "étude 3") an extensive screening performed**  
36 **both, *in vitro* and *in situ*, allowed the selection of 3 dairy fermentates exhibiting an**  
37 **antifungal activity and derived from *Propionibacterium jensenii* CIRM-BIA 1774,**  
38 ***Lactobacillus rhamnosus* CIRM-BIA 1952 and *Mucor lanceolatus* UBOCC-A-109193**  
39 **respectively. Using different analytical methods (high performance liquid phase**  
40 **chromatography, head-space trap gas chromatography combined or not with mass**  
41 **spectrometry), 64 compounds with known antifungal activity were detected in**  
42 ***L. rhamnosus* fermentate, 61 in *P. jensenii* fermentate and 51 in *M. lanceolatus* one. Most**  
43 **abundant antifungal compounds in *P. jensenii*, *L. rhamnosus* and *M. lanceolatus* derived**  
44 **fermentates were propionic and acetic acids, lactic and acetic acids, and butyric acid,**  
45 **respectively. Many other compounds with a known antifungal activity (weak acids, free**  
46 **fatty acids, volatile copounds) were also evidenced but were present at detected at lower**  
47 **levels. In addition, an untargeted approach using by nano LC-MS/MS allwed to unravel**  
48 **in *L. rhamnosus* derived fermentate a peptide of 9-amino acids derived from the  $\alpha$  s2-**  
49 **casein pepa4c177, identified, which inhibited *in vitro* *M. racemosus* and *R. mucilaginosa*.**  
50 **The effect of the *P. jensenii* fermentate on the growth of the latter 2 fungi was further**  
51 **investigated *in vitro*. When exposed at 2 and 5% of *P. jensenii* fermentate, significant**  
52 **growth delays were observed on both fungi as well as a fungistatic activity on *R.***  
53 ***mucilaginosa* along with morphological changes in *M. racemosus* germlings. This study**  
54 **provides new insights on the molecules supporting antifungal activity and action**

55 **mechanism of food-grade microorganism fermentates which could be used as antifungal**  
56 **ingredients in the dairy industry.**

57

58

## 59 1. Introduction

60 Fungi are responsible for the spoilage of dairy products. Indeed, due to their intrinsic  
61 characteristics (e.g., acidic pH and intermediate water activity, nutrient composition), dairy  
62 products constitutes a favorable environment for growth of a large diversity of yeasts and  
63 molds (Rohm et al., 1992). Fungal spoilage leads food and substantial economic losses and  
64 can also impact the brand image of commercial products in the eye of the consumer (Pitt and  
65 Hocking, 2009). The most common fungi involved in spoilage of dairy products belong to  
66 *Penicillium*, *Mucor* and *Cladosporium* and *Candida*, *Kluyveromyces* and *Yarrowia* for mould  
67 and yeast genera, respectively (Pitt and Hocking, 2009; Garnier et al., 2017).

68 Control of fungal spoilers in the dairy industry is of a great importance. Today, different  
69 preventive and control methods, i.e., the so called “hurdle technologies”, are implemented to  
70 prevent contamination or recontamination during product manufacturing and/or inhibit and/or  
71 slow down growth of fungal spoilers. These methods includes for example application of  
72 good manufacturing and hygiene practices, implementation of hazard analysis and critical  
73 control points (HACCP) system, air filtration and packaging in aseptic conditions, heat  
74 treatment, refrigeration and salting/brining. In addition, chemical preservatives can be used  
75 and extend dairy product shelf-life with respect of the relevant legislation (Garnier et al.,  
76 2017). However, regarding chemical preservatives, consumers are more and more requesting  
77 preservatives-free products and therefore “natural” preservation alternatives, such as  
78 biopreservation, are increasingly used (Leyva Salas et al. 2017).

79 Lactic acid bacteria (LAB), propionibacteria and fungi are good candidates for  
80 biopreservation as they have a long history of safe use in human consumption. LAB are used  
81 in various dairy products such as yogurt, kefir or cheese (Corrieu and Luquet, 2008). As a  
82 consequence, some LAB and propionibacteria species are used as bioprotective cultures, or  
83 antifungal ingredients, in dairy products. Commercial antifungal solutions such as Holdbac

84 (DuPont Danisco, Dangé, Saint Romain, France), FRESHQ (CHR Hansen, Hovedstaden,  
85 Danemark) and Natamax (DuPont Danisco, Dangé, Saint Romain, France ) protective cultures  
86 containing LAB species and/or propionibacteria, as well as antifungal ingredient such as  
87 MicroGARD®, derived from Propionibacteria species, are currently available on the market  
88 (Leyva Salas et al. 2017). Concerning fungi biocontrol agents, they are mostly used in fruit  
89 preparation or fermented meat (Leyva Salas et al., 2017). Regarding LAB and  
90 propionibacteria, they are able to produce a large spectrum of compounds such as organic  
91 acids, fatty acids (Sjogren et al., 2003), cyclic dipeptides (Strom et al., 2002) and  
92 proteinaceous compounds (Rizello et al., 2011) which may be involved in antifungal activity,  
93 acting in synergy (Crowley et al., 2013; Leyva Salas et al., 2017). Concerning fungi, studies  
94 also showed their capacity to produce antifungal compounds such as killer-toxins, organic  
95 acids or peptides (Acosta et al., 2009 ; Coda et al., 2013 ; Ignatova et al., 2015 ; Nally et al.,  
96 2015 ; Leyva-Salas et al., 2017).

97 Antifungal agents can be classified in 4 groups based on their targets: (i) inhibitors of  
98 ergosterol biosynthesis, (ii) fungal membrane disruptors, (iii) inhibitors of cell wall synthesis,  
99 (iv) inhibitors of sphingolipids biosynthesis, (v) nucleic acid synthesis inhibitors, (vi) protein  
100 biosynthesis inhibitors and (vi) microtubules biosynthesis inhibitors (Campoy and Adrio,  
101 2017). In the context of dairy product biopreservation, only few studies have dealt with the  
102 action mechanisms of bioprotective cultures (Crowley et al., 2013). In a recent study of  
103 Mieszkin et al. (2017), the action mechanisms of *Lactobacillus harbinensis* K.V9.3.1.Np  
104 against *Yarrowia lipolytica* in fermented milk was studied. The fungistatic effect of this  
105 bioprotective culture involved different organic acids which led to intracellular pH decrease  
106 and membrane depolarization.

107 In a recent study (Garnier et al., to be submitted, “étude 3”), 3 fermented dairy ingredients  
108 with antifungal activity were selected after an *in vitro* screening and their efficiency was



109 validated in real dairy products, *i.e.*, after incorporation in sour cream and surface-spraying on  
110 semi-hard cheese. Thus, the aim of the present study was to investigate which compounds  
111 were responsible for the observed antifungal activity. For that purpose, different analytical  
112 methods *i.e.*, conventional LC and GC, GC-MS, LC-QToF and nano-LC-MS/MS, were  
113 applied, to detect and quantify antifungal metabolites, many of which previously known for  
114 their antifungal activity, including organic acids and fatty acids (targeted approach), and  
115 volatile compounds and peptides (non-targeted approach). In addition, we studied the effect of  
116 different concentrations of the most active fermentate obtained from *Propionibacterium*  
117 *jensenii* CIRM-BIA 1774 on the growth and morphology of *M. racemosus* and *R.*  
118 *mucilaginosa*.

119

## 120 2. Material and methods

### 121 2.1.Preparation of fermentates

122 Fermentates with antifungal activity were selected from a previous study (Garnier et al., to be  
123 submitted, “étude 3”). They were obtained from the fermentation of 2 dairy media : a  
124 reconstituted 10% low heat milk supplemented with 45% anhydrous milk fat (for  
125 *Lactobacillus rhamnosus* CIRM-BIA 1952 and *Mucor lanceolatus* UBOCC-A-109119  
126 strains) and an ultrafiltration permeate supplemented with 1% yeast extract (for  
127 *Propionibacterium jensenii* CIRM-BIA 1774 strain). They were kept as a lyophilisate for *P.*  
128 *jensenii* CIRM-BIA 1774 and *L. rhamnosus* CIRM-BIA 1952 and as a 0.45 µm filtered  
129 culture supernatant for *Mucor lanceolatus* UBOCC-A-109119, as described previously  
130 (Garnier et al. to be submitted, “étude 3”). Three biological replicates of each fermentate were  
131 analyzed.

132

### 133 2.2.Fungal strains and culture conditions

134 The impact of *P. jensenii* CIRM-BIA 1774 fermentate on fungal growth was studied on *R.*  
135 *mucilaginosa* UBOCC-A-216004 and *M. racemosus* UBOCC-A-109155 obtained from the  
136 Université de Bretagne Occidentale Culture Collection (UBOCC, Plouzané, France) and were  
137 both isolated from spoiled dairy products (Garnier et al. 2017). For germination and radial  
138 growth studies, *M. racemosus* spores were harvested in Tween 80 (0.01%, v/v) from Potato  
139 Dextrose Agar (PDA) incubated for 5 days at 25 °C and the suspension was used to inoculate  
140 Roux flasks (Easy Flask 225 cm<sup>2</sup> Nunc – Thermo Fisher Scientific, Denmark) filled with  
141 PDA. After incubation for 15 days at 25 °C, spores were harvested by centrifugation at 7000 g  
142 at 4 °C for 15 min. The pellet was resuspended in water with glycerol (15%, v/v) and the  
143 concentration was adjusted to 5.10<sup>7</sup> spores/mL before aliquot storage at -80 °C.  
144 *R. mucilaginosa* was streaked on PDA incubated for 72 h at 25°C and one colony was  
145 suspended in Potato Dextrose Broth (PDB). After incubation for 24 h at 25 °C under shaking  
146 at 150 rpm, 1% (v/v) of this culture was used to inoculate fresh PDB which was incubated in  
147 the same conditions. Then, the entire culture was centrifuged at 7000 g at 4 °C for 15 min, the  
148 pellet resuspended in water supplemented with glycerol (15%, v/v) and the concentration  
149 adjusted at 2.10<sup>8</sup> cells/mL. Aliquots were stored at -80 °C until use.

150

## 151 **2.3. Identification and quantification of potential antifungal compounds**

### 152 **2.3.1. Antifungal compounds identification by LC-QToF**

#### 153 **2.3.1.1. Standard preparation**

154 Thirty-one compounds with known antifungal activity were searched as described previously  
155 (Brosnan et al. 2012, 2014; Le Lay et al., 2016,) except that 5 additional molecules were  
156 added, *i.e.*, 4 -di-tert-butylphenol (D), mevalonolactone (X), N-acetyl-D-glucosamine (Y),  
157 phenyl acetate (Z) and ricinoleic acid (Ab) (**Supplementary Table S1**). Standards were  
158 prepared by mixing all compounds at different concentrations. Individual stock solutions at 5

159 mg/mL were prepared in water or acetonitrile and mixed together to obtain a standard mix at  
160 100 ppm in H<sub>2</sub>O/ACN (90/10, v/v). A matrix-matched calibration curve was built by diluting  
161 the standard mix in a blank extract of acidified semi-skimmed milk (adjusted to pH 5 with  
162 lactic acid) at the following concentrations: 1 ppm, 5 ppm, 10 ppm, 30 ppm and 50 ppm.  
163 Standards were kept at -20 °C in amber vials.

164

#### 165 **2.3.1.2. Sample preparation**

166 Compound extraction from lyophilized fermentates and supernatant were performed as  
167 described by Brosnan et al., (2014) with slight modifications. First, 10 mL of lyophilized  
168 cultures resuspended in sterile water (1:10, w/v) or supernatant were mixed with 4 g of  
169 MgSO<sub>4</sub>, 1 g of NaCl and 10 mL ethyl acetate supplemented with 1 % of formic acid. The  
170 mixture was then vigorously shaken and centrifuged for 10 min at 8500 g at 4 °C. The organic  
171 phase was transferred into a dSPE tube (dispersive Solid Phase Extraction, Agilent  
172 technologies), vortexed and centrifuged for 10 min at 2600 g. Liquid phase was then  
173 recovered in a 15 mL-tube, mixed with 100 µL of DMSO and evaporated under nitrogen gas.  
174 The remaining 100 µL were reconstituted with 900 µl of H<sub>2</sub>O/Acetonitrile (90/10, v/v),  
175 filtered at 0.22 µm and stored in amber vials at -20 °C until analysis.

176

#### 177 **2.3.1.3. LC-QToF analysis and method validation**

178 Detection and quantification were performed on a 1260 Infinity binary HPLC and a 6530  
179 Accurate Mass LC-QToF LC/MS (Agilent Technologies) as described by Le Lay et al.,  
180 (2016). Compounds separation was done on a Zorbax Extend-C18 column (201 x 150 mm, 5  
181 µ) equipped with a pre-column (2.1 x 12.5 mm, 5 µ) (Agilent technologies) and the mass  
182 spectrometer operated in negative electrospray ionization. Analysis were performed under the

183 conditions described by Le Lay et al. (2016) except that injected volumes were 10 and 50  $\mu\text{L}$ .  
184 Standards and samples were injected in triplicate.  
185 To validate the method, extraction recovery was determined on acidified semi-skimmed milk  
186 spiked with 10 ppm of each compound. Standard mix at 10 ppm in blank extract was injected  
187 3 times a day for 5 consecutive days to assess the intra- and inter-day. Moreover, for each  
188 compound, the limit of Detection (LOD) and limit of quantification (LOQ) were determined  
189 based on the standard deviation of the analyte response and the standard deviation slope (ICH  
190 Harmonized Tripartite Guideline, 2005)

191

192

### 2.3.2. Antifungal organics acids quantification by HPLC

193 Nine mL of culture supernatant or 3 g of lyophilized fermentates dissolved in sterile water  
194 (1:2) were centrifuged for 30 min at 10 000 g. The liquid phase was half diluted in 5 mM  
195 sulfuric acid, vigorously mixed and stored overnight at  $-20^{\circ}\text{C}$ . After thawing, samples were  
196 once again centrifuged at 10 000 g for 30 min, the upper phase recovered and filtered on 0.45  
197  $\mu\text{m}$  PTFE membrane. Analysis was performed on a HPLC Dionex system (Sunnydale, CA,  
198 USA) equipped with a Aminex HPX-87H column (Biorad, Hercules, CA, USA) with 5 mM  
199  $\text{H}_2\text{SO}_4$  as the mobile phase, an UV and refractometer detectors (210 nm). Identification and  
200 quantification were achieved using standard solutions of the following organic acids at  
201 concentrations ranging from 0 to 1 mg/mL: acetic acid, benzoic acid, citric acid, lactic acid,  
202 propionic acid, succinic acid and 2-pyrrolidone-5-carboxylic acid. Standards and samples  
203 were injected in triplicate.

204

205

### 2.3.3. Free fatty acid identification by gas chromatography

206

#### 2.3.3.1. Standard preparation

207 Identification and quantification of free fatty acids, adapted from Jong and Badings (1990),  
208 was performed with different external standards of concentrations ranging from 5 to 500 µg/g  
209 including acetic, propionic, butyric, valeric, isovaleric, caproic, enanthic, caprylic, pelargonic,  
210 capric, undecyclic, lauric, myristic, palmitic, margaric, stearic, oleic, linoleic and  
211 octadecadienoic acids. An internal standard, to take into account the extraction yield, was  
212 used (0.5 mg/g of valeric acids (C5:0), tridecanoic acid (C13:0) and margaric acid (C17:0)  
213 diluted in 30 mL of heptan).

214

#### 215 **2.3.3.2.** Fatty acid extraction

216 Free fatty acid extraction was performed with 1 g of reconstituted (1X) lyophilized fermentate  
217 or 1 ml of culture supernatant, 3g of anhydrous sodium sulfate (Na<sub>2</sub>SO<sub>4</sub>) and mixture was  
218 ground. Then 0.3 mL of hydrogen sulfur (H<sub>2</sub>SO<sub>4</sub> at 2.5M), 1 mL of internal standards and 15  
219 mL of ether/heptan (1:1) were added before centrifugation (3 min at 100 g). Organic layer  
220 was recovered with 100 mL of Na<sub>2</sub>SO<sub>4</sub> to capture residual water. Pellet was resuspended in 15  
221 mL of ether/heptan (1:1) and was centrifuged (3min at 100 g). This step was repeated 3 times  
222 by pooling the organic layer. Free fatty acid purification was performed with a Manifold, with  
223 a SPE 500 mg column (Phenomenex, UK) conditioned with 10 mL heptan. The organic layer  
224 was transferred onto the column and 10 mL heptan/2-propanol (3:2) were added for neutral  
225 lipids removal. Free fatty acids were eluted with 5 mL ether diethyl with 2% formic acid and  
226 transferred in an amber vial.

227

#### 228 **2.3.3.3.** Analysis of fatty acids by GC

229 Fatty acid annalysis was performed by gas chromatography (Varian CP-3800) equipped with  
230 a flame ionization detector (FID) and a capillary column (BP21 25m x 0.53 mm, layer 0.5

231  $\mu\text{m}$ , JW Scientific, Folsom, USA). Vector gas was dihydrogen at 9.7 mL/min. A temperature  
232 gradient was applied from 65°C to 240°C with a ratio of 10°C/min then 10 min at 240°C  
233 using direct injection.

234 Quantification was performed using standard calibration curves with the Star Varian (version  
235 5.3) software. A correction of possible free fatty acids losses during extraction was performed  
236 with internal standards using the following calculation:

237

$$238 \text{ Free fatty acid } (\mu\text{g}) = \frac{([\text{Calculated concentration of free fatty acid}] * [\text{Standard Y } (\mu\text{g})])}{[\text{Calculated concentration of standard Y}]} \text{ (Equation1)}$$

239

## 240 2.3.4. Volatile compounds identification by GC-MS

### 241 2.3.4.1. Preparation of standards for GC-MS analysis

242 Standard compounds were used to generate standard curves and to check the response of the  
243 HS-trap GC-MS system during the samples analysis. Two solutions of standard compounds  
244 were prepared: one with neutral volatiles and another with short-chain fatty acids. Neutral  
245 standard compounds included four esters (ethyl acetate, ethyl propanoate, ethyl butanoate and  
246 ethyl hexanoate), two aldehydes (3-methylbutanal and benzaldehyde), one ketone (2-  
247 heptanone), 2,3-butanedione, dimethyl disulfide at concentrations ranging from 5 to 1200  
248 ng/g and 3-methylbutanol at concentrations ranging from 260 to 50,000 ng/g. For short-chain  
249 acids, the standard mix 46975-U (Supelco, Sigma-Aldrich, Saint-Louis, USA) containing  
250 acetic, propanoic, butanoic, 2-methylpropanoic, pentanoic, 3-methylbutanoic, hexanoic, 4-  
251 methylpentanoic and heptanoic acids at 10mM each was used to prepare standard solutions at  
252 concentrations ranging from 20 to 1000 ng/g. The pH of these standard acid solutions was  
253 adjusted to  $6.35 \pm 0.15$  with NaOH.

254

### 255 2.3.4.2. Extraction of volatile compound using headspace trap

256 Extraction of volatile compounds was performed using a Perkin Elmer Turbomatrix HS-40  
257 trap automatic headspace sampler with trap enrichment. The principle of HS-trap method has  
258 been previously described in detail (Barani et al., 2006; Schulz et al., 2007), aliquots (2.5 g)  
259 of fermentates were placed in 22 mL PerkinElmer vials with polytetrafluorethylene  
260 (PTFE)/silicone septa and the used conditions were as described in Pogačić et al. (2015).

261

#### 262 2.3.4.3. Analysis of volatile compounds using GC-MS

263 Volatiles were analyzed using a Clarus 680 gas chromatograph coupled to a Clarus 600T  
264 quadrupole mass spectrometer (PerkinElmer, Courtaboeuf, France as described in Pogačić et  
265 al. (2015). All samples were analyzed in the same GC-MS run. Standards were regularly  
266 injected to verify the absence of instrumental drift of the GC-MS system and blank samples  
267 (boiled deionized water) were also injected to check the absence of carry-over.

268 Volatile compounds were identified as described in Pogačić et al. (2015), comparing their  
269 retention index and mass spectral data from the NIST 2008 Mass Spectral Library (Scientific  
270 Instrument Services, Ringoes, NJ, USA) with those of authentic standards purchased from  
271 Sigma Aldrich (St. Quentin Fallavier, France).

272

#### 273 2.3.4.4. Data processing

274 As described by Pogačić et al. (2015), the PerkinElmer Turbomass software, version  
275 5.4.2.1617, was used to perform data pre-processing. After conversion of the GC-MS raw  
276 data files to netCDF format with Data Bridge (Perkin Elmer, Waltham, Massachusetts, USA),  
277 raw data were converted to time- and mass-aligned chromatographic peaks areas using the  
278 open source XCMS package implemented with the R statistical language (Smith et al., 2006).

279

#### 280 2.3.5. Identification of potential antifungal peptides

281 **2.3.5.1. Identification of peptides by nano-LC-MS/MS**

282 Mass spectrometry (MS) analysis was adapted from that described by Nyemb et al. (2016),  
283 using a nanoRSLC Dionex U3000 system fitted to a Q Exactive mass spectrometer (Thermo  
284 Scientific, San Jose, USA) equipped with a nanoelectrospray ion source. Briefly, samples  
285 were first concentrated using a  $\mu$ -precolumn pepMap100 (C18 column, 300  $\mu$ m i.d.  $\times$  5 mm  
286 length, 5  $\mu$ m particle size, 100 Å pore size; Dionex, Amsterdam, The Netherlands), before  
287 peptide separation was performed on a PepMap RSLC column (C18 column, 75  $\mu$ m i.d.  $\times$  150  
288 mm length, 3  $\mu$ m particle size, 100 Å pore size; Dionex); column temperature was maintained  
289 at 35 °C along peptide separation that was performed at a flow rate of 0.3  $\mu$ L  $\cdot$  min<sup>-1</sup> using  
290 solvent A (2% (v/v) acetonitrile, 0.08% (v/v) formic acid and 0.01% (v/v) TFA in deionized  
291 water) and solvent B (95% (v/v) acetonitrile, 0.08% (v/v) formic acid and 0.01% (v/v) TFA in  
292 deionized water). Elution gradient was as follows: a first rise from 5 to 35% solvent B over 35  
293 min, followed by a second rise from 35 to 85% solvent B over 2 min. Eluted peptides were  
294 directly electrosprayed into the mass spectrometer operating in positive ion mode with a  
295 voltage of 1.8 kV. The mass spectra were recorded in full MS mode using the m/z range 250–  
296 2000. The resolution of the mass analyzer for a m/z of 200 a.m.u (atomic mass unit) was set to  
297 70,000 in the acquisition method. For each scan, the ten most intense ions were selected for  
298 fragmentation. MS/MS spectra were recorded with a resolution of 17,500 at m/z of 200 a.m.u  
299 and the parent ion was subsequently excluded from MS/MS fragmentation for 15 s. The  
300 instrument was externally calibrated according to the supplier's instructions. Peptides were  
301 identified from the MS/MS spectra using X!Tandem pipeline software (Langella et al., 2017)  
302 The peptide identification database was an in-house database composed of major milk and  
303 egg proteins derived from [www.uniprot.org](http://www.uniprot.org) (207 proteins in total). Database search  
304 parameters were specified as follows: a non-specific enzyme cleavage was selected; a 0.05 Da  
305 mass error was allowed for fragment ions while 10 ppm mass error was allowed for parent



306 ions. The phosphorylation of serine and threonine were selected as variable modifications as  
307 well as the oxidation of methionine residues. For each peptide identified, a minimum score  
308 corresponding to an e-value below 0.05 was considered to be a prerequisite for valid peptide  
309 identification. The peptide false discovery rate was calculated by the software to be less than  
310 0.5% using these parameters. Bioinformatic analysis to identify potential antifungal peptides  
311 Peptides (n=1236) described as possessing an antifungal activity were selected from the  
312 following database: ADP (Wang and Wang, 2004), BIOPEP (Minkiewicz et al., 2008),  
313 CAMP (Wadhwa et al., 2014), EROP (Zamyatnin et al., 2006), MilkAMP (Théolier et al.,  
314 2013) and PeptideDB. Then a blast between these peptides and those identified by nano-  
315 LCMS/MS in our 3 fermentates and in the 2 dairy substrates was performed to obtain a list of  
316 61 peptides found in fermentates and/or in dairy substrates. After elimination of peptides  
317 present in the non-fermented dairy substrates, a short list of 16 potential antifungal peptides  
318 was obtained.

319

#### 320 2.3.5.2. Antifungal assay of peptides isolated from lyophilized cultures and 321 supernatant

322 Sixteen peptides potentially with antifungal activity (**Table 5**) and previously identified by  
323 nano-LC-QToF in the lyophilized cultures and supernatant were neosynthesized (Biomatik,  
324 Ontario, Canada). Their individual antifungal activity against *M. racemosus* UBOCC-A-  
325 109155 and *R. mucilaginosa* UBOCC-A-216004 was assessed using an agar well diffusion  
326 method. Briefly, the targeted fungi were included at a final concentration of  $1.10^4$  spores or  
327 cells per ml in PDA medium containing 1% agar and the mix poured into 90 mm Petri dishes.  
328 Once solidified, six 9-mm-wells were cut per plate and filled with 100  $\mu$ L of the peptide  
329 solutions prepared as follows. Each of the tested peptides was dissolved in sterile distilled  
330 water following manufacturer's guidelines to obtain a 5 mg/mL stock solution and diluted to

331 2, 1, 0.5 and 0.25 mg/mL. Agar plates were then incubated at 25 °C and the potential  
332 antifungal activity evaluated daily for 7 days. If applicable, diameters of inhibition zones were  
333 measured.

334

335 2.4. Impact of *Propionibacterium jensenii* CIRM 1774 lyophilized culture on fungal  
336 growth

337 2.4.1. Effect on *Mucor racemosus* germination

338 The impact of *P. jensenii* lyophilized fermentate on *M. racemosus* germination was studied.  
339 Four different concentrations were tested: 1, 2, 5 and 0 %. The appropriate amount of  
340 lyophilized fermentate was dissolved in PDB adjusted to pH 5.0 with sodium hydroxide 0.1  
341 M and then filter-sterilized at 0.45 µm. Spores prepared as described above were inoculated at  
342  $1.10^6$  spores/mL in PDB and incubated at 25°C under shaking at 120 rpm. Germination  
343 kinetic was followed by determining on a hemocytometer, the percentage of germinated  
344 spores, expressed as the number of germinated spores on the total number of counted spores.  
345 A spore was considered germinated when the length of the germ tube was equal or superior to  
346 the swollen spore diameter. Three replicates were made per tested concentration. For  
347 comparing germination kinetics, germination parameters were determined by adjusting  
348 experimental data using the asymmetric model (Dantigny et al., 2011)) which expresses  
349 percentage of germinated spores ( $P(t)$ ) as a function of time ( $t$ ) (**Equation 1**) :

350 
$$P(t) = P_{max} \times \left( 1 - \frac{1}{1 + \left(\frac{t}{\tau}\right)^d} \right) \text{ (Equation 2)}$$

351 where  $P_{max}$  is the highest percentage of germinated spores,  $\tau$ , the median germination time, is  
352 the time when P equals half of Pmax and  $d$  a shape parameter. The model fitting was  
353 performed using the MATLAB curve fitting tool by minimizing the sum of squares of the  
354 residuals (*lsqcurvefit function*, Matlab 2014 The Mathworks Inc., USA).

355

#### 2.4.2. Effect on radial growth of *M.racemosus*

356 The effects of 0, 1, 2 and 5% of *P. jensenii* fermentate on *M. racemosus* radial growth were  
357 also investigated. PDA was obtained by mixing 2X agar (30 g/L) and 2X PDB containing  
358 lyophilized fermentates prepared as described above and poured into Petri dishes.  
359 Approximately 50 spores were inoculated in the center of agar medium followed by  
360 incubation at 25 °C. Radial growth was determined daily by measuring two perpendicular  
361 diameters until the thallus diameter reached 85 mm or for up to 10 days when no increase in  
362 thallus diameter was observed. Four replicates were prepared per tested concentration.  
363 Experimental data were fitted with the MATLAB curve fitting tool using Buchanan equation  
364 (**Equation 2**) adapted for a two-phase fungal growth (Buchanan, 1993):

$$365 \quad r(t) = \begin{cases} r_0, & t \leq \lambda \\ r_0 + \mu(t - \lambda), & t > \lambda \end{cases} \text{ (Equation 3)}$$

366 where  $r(t)$  and  $r_0$  are the radius at time  $t$  (days) and  $t_0$ , respectively,  $\mu$  is the growth rate in  
367  $\text{mm}\cdot\text{d}^{-1}$  and  $\lambda$  is the lag time defined as the time from which the thallus diameter is larger than  
368  $2r_0$ .

369

370

#### 2.4.3. Effect on growth kinetics of *R. mucilaginosa*

371 Yeast growth was monitored in PDB containing 0, 1, 2 and 5 % *P. jensenii* lyophilized  
372 fermentate. First, a pre-culture of *R. mucilaginosa* was grown in PDB for 24 h at 25 °C under  
373 shaking at 120 rpm. Then, PDB supplemented with *P. jensenii* fermentate was inoculated with  
374  $1.10^6$  cells/mL from the pre-culture and incubated at 25 °C under shaking at 120 rpm. Culture  
375 samples were taken every one or two hours and cell concentration determined on a  
376 hemocytometer until stationary phase was reached. Three replicates were made for each tested  
377 concentration. Experimental data for each condition were fitted with the Matlab curve fitting  
378 tool using the logistic model (**Equation 3**) (Zwietering et al., 1990):

379 
$$\ln\left(\frac{N}{N_0}\right) = \frac{A}{\left(1+e^{\left(\frac{A\mu m}{A}(\lambda-t)+2\right)}\right)} \text{ (Equation 4)}$$

380 where  $N$  and  $N_0$  are the cell concentrations at time  $t$  and  $t_0$ , respectively,  $\lambda$  is the lag time and  
381  $A$ , the value  $N/N_0$  when the stationary phase is reached. Yeast populations were also  
382 determined on PDA medium after 0 and 24 h of incubation.

383

### 384 3. Results

#### 385 3.1. Identification and quantification of potential antifungal compounds

##### 386 3.1.1. Organic and fatty acids quantification by HPLC and LC-QToF

387 Regarding LC-QToF method validation, extraction recovery was satisfying for 23 compounds  
388 out the 31 tested compounds with recovery percentages between 70 and 120 % (data not  
389 shown). Eight compounds (i.e., cytidine, 2-deoxycytidine, N-acetylglucosamine, glucuronic  
390 acid, decanoic acid, ricinoleic acid, hydroxymyristic acid and succinic acid) could not be  
391 accurately detected and quantified. Standard deviation between 0 and 3 ppm were obtained  
392 after repeated injections of 10 ppm standard mixture over 5 days, thus, repeatability was  
393 considered acceptable.

394 Based on HPLC and LC-QToF analysis, 15, 19 and 13 compounds were detected in the  
395 *Propionibacterium jensenii* CIRM-BIA 1774, *Lactobacillus rhamnosus* CIRM-BIA 1952 and  
396 *Mucor lanceolatus* UBOCC-A-109119 fermentates, respectively. Eleven molecules were  
397 systematically detected in all 3 fermentates (**Table** ). Concerning bacterial fermentates, 3  
398 molecules (propionic, citric and acetic acids), were quantified at concentrations above 10  
399 mg/g in *P. jensenii* culture while only 2 (lactic and citric acids) were present above this level  
400 in *L. rhamnosus* fermentate. In addition, 15 molecules were detected at concentrations  
401 comprised between LOQ and 1 mg/g in *L. rhamnosus* fermentate against 10 in *P. jensenii*  
402 fermentate. Other molecules could not be quantified as they were present below their LOQ.

403 Concerning *M. lanceolatus*, concentrations of the 13 detected compounds never exceeded  
404 1 mg/g except for citric acid. Lactic acid was present at high concentration ( $86.018 \pm 3.05$  mg/g  
405 fermentate) in *L. rhamnosus* fermentate but was not detected in *P. jensenii* or *M. lanceolatus*  
406 fermentates. As expected, propionic acid was only present in the *P. jensenii* lyophilisate at  
407  $59.94 \pm 21.28$  mg/g. Acetic acid concentrations were  $16.893 \pm 5.676$  mg/g in *P. jensenii*  
408 culture,  $2.57 \pm 0.62$  mg/g – approximately 6-fold less- in *L. rhamnosus* culture and  $0.104 \pm$   
409  $0.002$  mg/g in *M. lanceolatus* fermentate. For several compounds including 2-pyrrolidone-5-  
410 carboxylic acid, hydroxyphenyllactic and phenyllactic acid, (S)-(-)-2-Hydroxyisocaproic acid  
411 and mevalonolactone the highest concentrations were also observed in *P. jensenii* fermentate.

412

### 413 3.1.2. Identification of volatile compounds by head-space trap GC-MS

414 An untargeted approach was used to search for potential antifungal volatile compounds  
415 present in the different fermentates using a head-space trap coupled with gas chromatography.  
416 Results, presented in relative abundance, showed that 36 volatile compounds were detected in  
417 the different fermentates. Among them, 7 (2-n-butyl furan, 2-pentyl furan, hexanal, heptanal,  
418 octanal, nonanal and 2-undecanone) were detected with relative abundance inferior to  $1.10^8$   
419 and acetate and propionate were detected with relative abundance superior to  $1.10^9$  in the 3  
420 fermentates (**Table 3**). In *P. jensenii* CIRM-BIA 1774, *L. rhamnosus* CIRM-BIA 1952 and *M.*  
421 *lanceolatus* UBOCC-A-119109 fermentates, 18, 15 and 16 volatile compounds, respectively,  
422 were detected with relative abundance superior to  $1.10^8$ , including acetate and propionate.  
423 Concerning *P. jensenii* CIRM-BIA 1774, propionate ( $6.26.10^{10}$ ) and acetate ( $4.69.10^{10}$ ) were  
424 the main volatile compounds, followed by 3-methyl butanal, butyl propanoate, butyl acetate,  
425 ethyl propanoate, hexanoate, 2-methyl propanoate and propyl propanoate, all showing relative  
426 abundance superior to  $1.10^9$  (**Table 3**). Likewise, propanoate ( $1.86.10^{10}$ ) and acetate  
427 ( $1.21.10^{10}$ ) were the main compounds identified from *L. rhamnosus* CIRM-BIA 1952 but only

428 3 other compounds (acetoin, hexanoate and 2 furanmethanol) showed relative abundance  
429 superior to  $1.10^9$  (**Table 3**). On the contrary, *M. lanceolatus* had a different profil, ethyl  
430 butyrate ( $2.51.10^9$ ) was the main volatile compounds, followed by propanoate ( $2.37.10^9$ ), 3-  
431 methyl, 1-butanol ( $2.36.10^9$ ), acetate ( $1.80.10^9$ ) and diacetyl ( $1.36.10^9$ ) (**Table 3**).

432

### 433 3.1.3. Identification and quantification of free fatty acids by GC

434 Potential antifungal free fatty acids were searched for and quantified using gas  
435 chromatography. Nineteen free fatty acids were detected in the different fermentates (**Table**  
436 **4**). Among them, 13 compounds (acetic, propionic, butyric, caproic, nonanoic, capric, lauric,  
437 myristic, pentadecanoic, palmitic, palmitoleic, stearic and oleic acids) were found  
438 systemically in the 3 fermentates at different concentration (**Table 4**). One and 3 compounds  
439 were only found in *L. rhamnosus* and *P. jensenii* fermentate respectively but concentrations  
440 were extremely low ( $< 2 \mu\text{g/g}$ ) and standard deviation significant. Among the 13 common  
441 compounds, 7 (caproic, nonanoic, capric, lauric, pentadecanoic, palmitoleic and oleic acids)  
442 showed concentrations inferior to  $10 \mu\text{g/g}$ . Among the 6 common compounds left,  
443 quantitative differences existed between fermentates (**Table 4**). Indeed, propionic  
444 ( $1027.0 \pm 270.79 \mu\text{g/g}$ ) and acetic ( $507.2 \pm 84.04 \mu\text{g/g}$ ), acids were the main compounds of *P.*  
445 *jensenii* CIRM-BIA 1774 fermentate and butyric acid was the principal compound of *M.*  
446 *lanceolatus* UBOCC-A-109193 fermentate with a concentration of  $444.7 \pm 0.94 \mu\text{g/g}$ .  
447 Nonetheless, for acetic and propionic acids, standard deviation were very high and differed  
448 significantly from HPLC results. Concerning *L. rhamnosus* CIRM-BIA 1952 fermentate,  
449 concentrations of the 6 common compounds were all inferior to  $100 \mu\text{g/g}$ . For this fermentate,  
450 the higher concentrations were found for acetic ( $58.1 \pm 13.7 \mu\text{g/g}$ ) and palmitic ( $49.9 \pm 5.60$   
451  $\mu\text{g/g}$ ) acids (**Table 4**).

452

#### 453 3.1.4. Identification of potential antifungal peptides

454 An untargeted approach was performed to search for potential antifungal peptides present in  
455 the different fermentates. Regarding the nano-LC-MS/MS analysis, a total of 1040 peptides  
456 were identified in the 3 tested fermentates. Among these 1040 peptides, 22, 253 and 853 were  
457 identified from *P. jensenii* CIRM-BIA 1774, *L. rhamnosus* CIRM-BIA 1952 and  
458 *M. lanceolatus* UBOCC-A-103193 fermentates, respectively. In addition, *P. jensenii* and *L.*  
459 *rhamnosus* fermentates respectively shared 3 and 85 peptides with *M. lanceolatus* fermentate.  
460 There was not any common peptide in *P. jensenii* and *L. rhamnosus* fermentates. After a blast  
461 search against a list of 1236 known antifungal peptides, 16 peptides, with E-values ranging  
462 from  $1.6 \times 10^{-2}$  to  $2 \times 10^{-6}$ , identities between 47 and 100% and relative abundances between  
463  $1.7 \times 10^{-2}$  and  $8.9 \times 10^{-8}$ , corresponding to  $\alpha$  s2 and  $\kappa$ -casein hydrolysates were neosynthesized  
464 for *in vitro* antifungal assays (**Table 1**).

465

#### 466 3.1.5. Antifungal activity of identified peptides

467 Out of the 16 peptides evaluated for their activity by the agar diffusion method against *M.*  
468 *racemosus* and *R. mucilaginosa*, only one, pepa4c177 which was detected in *L. rhamnosus*  
469 fermentate, showed a significant antifungal activity (Figure 1). This: peptide, contained 9  
470 amino acids and was derived from  $\alpha$  s2-casein f(165-203) (Table 1). This peptide inhibited  
471 both *M. racemosus* and *R. mucilaginosa* at the highest tested concentrations (2.5 and 5 mg/ml)  
472 while only *M. racemosus* was inhibited at 1 mg/mL (**Figure**). After 7 days at 25°C, the  
473 peptide had remained active against the two tested fungi, with inhibition zones of 2 and 3 mm  
474 in diameter around wells containing 2 and 5 mg/mL, respectively.

475

#### 476 3.2. Impact of *Propionibacterium jensenii* CIRM-BIA 1774 fermentate on fungal growth

477 *P. jensenii* CIRM-BIA 1774 fermentate antifungal activity against *M. racemosus* and  
478 *R. mucilaginosa* was further investigated. Regarding *M. racemosus*, the impact on both  
479 germination and radial growth was studied. Growth parameters were calculated from the  
480 experimental data at different concentrations and compared using the LSD test of Fisher ( $p$   
481  $<0.05$ ) (**Table 5**). Concentrations of 1 and 2 % of fermentate had no significant impact on  
482 neither germination time ( $\tau$ ) nor maximal germination percentage ( $P_{max}$ ) as compared to the  
483 control condition although a slight increase in lag time was observed. In contrast, in the  
484 presence of 5 % fermentate,  $P_{max}$  significantly decreased to 34.54 % and median germination  
485 time was increased to more than 20 hours, i.e. 3 times that of the control condition.  
486 Morphological changes of germinating spores were observed when exposed to the antifungal  
487 culture. During early germination, spores were oval-shaped and germ tubes appeared larger  
488 and right-angled while spores from the control condition were round with thin germ tubes. In  
489 a more advanced germination stage, especially with 2 and 5 % fermentate, a large amount of  
490 germlings had large vacuoles and the germ tube extremities showed signs of lysis (**Figure 2**).  
491 It is noteworthy that, at the highest tested concentration, spore germination did not occur in all  
492 replicates. Indeed, spores remained dormant for half of them even after prolonged incubation  
493 and the variability between replicates was more pronounced (**Figure 3**). Spores were taken  
494 from non-germinated replicates after a 24 h incubation period and inoculated in control  
495 medium in the same incubation conditions. These spores were able to germinate again with a  
496 similar morphotype than control spores. Such results may indicate that a 5% fermentate  
497 concentration was closed to the minimum inhibition concentration and that *P. jensenii* CIRM-  
498 BIA 1774 fermentate exhibited mainly a fungistatic activity despite signs of lysis were  
499 observed. Experiments on the radial growth led to similar conclusions as no differences in lag  
500 time were observed at 1 and 2 %. However, radial growth rates significantly decreased at  
501 these concentrations. Indeed,  $\mu$  was divided by 2 from  $12.44 \pm 0.47$  mm/day at 0% to  $6.49 \pm$



502 0.1 mm/day and  $5.48 \pm 0.72$  mm/day at 1 and 2%, respectively. At 5%, growth was  
503 considerably delayed as a thallus was only visible after incubation for 12 days and a 85-mm  
504 diameter was reached after 29 days with an average radial growth rate of 0.78 mm/day when  
505 only 5 days were necessary to reach this diameter at 0%. Once again, a high variability could  
506 be observed between replicates at this concentration as complete growth (85-mm diameter)  
507 was only reached, with different kinetics, for half of the replicates with different kinetics  
508 (Figure 4).

509 *R. mucilaginosa* growth was also affected by *P. jensenii* CIRM-BIA 1774 fermentate.  
510 Exposition at 1% only had a moderate impact on the yeast growth since lag time increased  
511 from  $5.85 \pm 0.47$  h to  $7.27 \pm 0.71$  h ( $p > 0.05$ ) and growth rate decreased from  $0.53 \pm 0.02$   
512 hours to  $0.42 \pm 0.03$  (Table 6). At 2 %, both lag time and growth rate significantly increased  
513 and decreased, respectively. Biological responses at this concentration were heterogeneous  
514 with two distinct kinetics (**Figure 5**). The observed variability mostly affected lag time as  
515 growth rate estimated by the curve slope were similar. A 5% fermentate concentration  
516 completely inhibited *R. mucilaginosa* growth. No budding nor lysing yeast cells could be  
517 observed during the 7-days follow-up. Thus, to assess the physiological state of yeast cells,  
518 they were plated on conventional PDA after 0 and 24 h incubation and no significant  
519 difference ( $<0.5 \log_{10}$ ) was observed between populations estimated microscopically  
520 enumerated and that enumerated on plates, suggesting a fungistatic effect.

521

#### 522 **4. Discussion**

523 In this study, 3 fermentates obtained from *L. rhamnosus*, *P. jensenii* and *M. lanceolatus* that  
524 were previously shown to possess promising antifungal activities in real dairy products  
525 (Garnier et al. to be submitted), were studied to identify the potential antifungal compounds  
526 involved in their activity. Because antifungal activities of microorganisms can result from a

527 large panel of active molecules belonging to various chemical families (Crowley et al., 2013,  
528 Leyva-Salas et al., 2017), multiple extraction and analysis methods allowing to identify them,  
529 were combined, using targeted and untargeted approaches. As expected, these approaches  
530 allowed us highlighting a large variety of organic and fatty acids, volatile compounds and one  
531 antifungal peptide thus providing new insights in the understanding of the molecules  
532 supporting antifungal activities of microorganisms used for bioprotection. Organic acids  
533 include numerous compounds such as fatty acids, hydroxylated and cyclic derivatives which,  
534 for many of them, exhibit antifungal activities. As expected, lactic and acetic acids for *L.*  
535 *rhamnosus* and propionic and acetic acids for *P. jensenii* were the most abundant fermentation  
536 products. Their respective and combined antifungal activities are well-known and have been  
537 reported many times in the literature (Bian et al., 2016; Gerez et al., 2010; Lind et al., 2007).  
538 At pH 5 in PDA, minimum inhibitory concentrations (MIC) of lactic, acetic and propionic  
539 acids against *R. mucilaginosa* UBOCC-A-216004 and *M. racemosus* UBOCC-A-109155  
540 were 2, 0.1 and 0.05, and 2, 0.2 and 0.05 M, respectively (data not shown). It thus indicates  
541 that lactic acid *per se*, as reported elsewhere on several mold species (Dagnas et al. 2015),  
542 brought a limited contribution to the antifungal activity of *L. rhamnosus* fermentate. The high  
543 MIC of lactic acid at this pH can be explained by the weak acid theory which states that the  
544 antifungal activity of weak acids depends on both the matrix pH and the acid pKa  
545 (corresponding to the pH at which 50% of the acid is in its dissociated and undissociated  
546 forms). Indeed, the more the acid is under its undissociated form, the more it can passively  
547 diffuse through the membrane and the more it is active (Stratford et al., 2009). Therefore, at  
548 pH 5.0, lactic acid, with a pKa of 3.9, is unlikely to be responsible for the observed antifungal  
549 activity, even if it is present at high concentration in *L. rhamnosus* fermentate. Noteworthy,  
550 Dagnas et al. (2015) showed that in combination with acetic acid, both acted in synergy. In  
551 contrast to lactic acid, acetic acid has a higher pKa (pKa =4.75) as well as propionic acid (pKa

552 =4.87) which indicate that they significantly contributed to the observed antifungal activity of  
553 the fermentates. For example, when *P. jensenii* fermentate was added at 5% in PDA, its final  
554 concentration was ~3 g/L, which is closed to its MIC (3.7 g/L). Organic acid concentration in  
555 *M. lanceolatus* fermentate were substantially inferior to those in the two other fermentates  
556 except for citric acid which was found in substantial amount in all the fermentates but is also  
557 naturally present in milk. Although the antifungal activity of this acid has been reported, its  
558 efficiency is limited compared to other organics acids such as acetate and benzoate (Hassan et  
559 al., 2015; Shokri, 2011) .However, it also acts as a precursor of other identified compounds  
560 such as diacetyl which is well known for its antifungal activity as discussed below. .

561 *P. jensenii* fermentate, and to a smaller extent *L. rhamnosus* fermentate, contained numerous  
562 hydroxylated compounds such as S)-(-)-2-Hydroxyisocaproic acid, 3-(4-  
563 Hydroxyphenyl)propionic acid and DL-Hydroxyphenyllactic acid. The hydroxyl group  
564 improves acid bioactivity by enhancing viscosity and reactivity as compared to their non-  
565 hydroxylated counterpart and by providing an easier partition into the membrane (Pohl et al.,  
566 2011). Honoré et al. (2016) showed that these compounds mostly contributed to the antifungal  
567 activity of *Lactobacillus paracasei* DGCC 2132. It has also been reported that decanoic acid  
568 derived hydroxylated acids inhibited *R. mucilaginosa* growth at concentrations as low as 10  
569 µg/mL (Sjögren et al., 2003). This compound could therefore contribute to the antifungal  
570 effect of *P. jensenii* fermentate. Phenyllactic acid which was quantified at concentrations of  
571 0.491 mg/g in *P. jensenii* fermentate is also a quite well known antifungal compounds  
572 (Lavermicocca et al. 2003).

573 Free fatty acids were also searched for and quantified. Concerning *L. rhamnosus* and *P.*  
574 *jensenii* fermentates, concentrations were low (<0.06 mg/g) for all identified free fatty acids  
575 with palmitic and stearic acids being the most prevalent. Low concentrations were also  
576 observed in *M. lanceolatus* fermentate except for butyric acid which had a concentration of

577 0.44 mg/ml. Butyric acid is known for its antifungal activity (Pohl et al., 2011) and could play  
578 a significant role in the antifungal activity of *M. lanceolatus* fermentate. Its presence in high  
579 concentration likely results from *M. lanceolatus* lipolytic activity.

580 Because volatile compounds may possess antifungal activity as recently shown by Aunbjerg  
581 et al. (2015) for diacetyl, they were also analyzed in the different fermentates. Even though an  
582 absolute quantification was not possible, it gave us an overview of the most prevalent  
583 compounds as well as their chemical nature. Diacetyl was present in the 3 fermentates with  
584 the highest prevalence in *L. rhamnosus* and *M. racemosus* fermentates. This compound was  
585 recently shown to play a non-negligible role in the antifungal activity of *L. paracasei* DGCC  
586 2132 against two strains of *Penicillium* spp. Similarly, ethyl acetate which was found in all  
587 fermentates was found to be the main compound responsible of the antifungal activity of  
588 *Wickerhamomyces anomalus* in sourdough bread (Coda et al. 2011). The fermentate derived  
589 from *M. lanceolatus* also contained high relative abundances of ethyl-butanoate and 3-methyl  
590 1-butanol. Ethyl-butanoate, also known as ethyl butyrate, has a fruity smell and occurs  
591 naturally in many fruits (Schieberle et al., 2009). It is a flavour agent showing a flavour  
592 profile of burnt, cocoa, floral and malt (FAO/WHO, 2017). Likewise, 3-methyl 1-butanol is  
593 also a flavouring agent with apple, butter, cheese, pineapple and strawberry flavours  
594 (FAO/WHO, 2017). To our best knowledge, their antifungal activity is not known.  
595 Consequently, it could be of interest to test their potential antifungal activity as well as that of  
596 other abundant volatile compounds and then, if active, to quantify them accurately.

597 Peptides can also be involved in the antifungal activity of biocontrol agents (Leyva-Salas et  
598 al., 2017), therefore, an untargeted approach was used to identify such compounds. Overall,  
599 the sequence of more than 1000 peptides contained in these 3 fermentates was determined by  
600 nano-LC-MS/MS and 16 of them identified as short (9 to 25 aa) casein fragments stood out  
601 when sequences were compared to antimicrobial peptide databases. It is likely that they

602 derived from the proteolytic activities of the microorganisms since they were absent in the  
603 different control media. To confirm their antifungal potential, these 16 peptides were  
604 chemically synthesized and one basic peptide pepa4c177 (RLNFLKKIS) from  $\alpha_{s2}$ -casein  
605 f(165-203) and identified in *L. rhamnosus* CIRM-BIA 1952 fermentate showed a clear *in*  
606 *vitro* antifungal activity against *R. mucilaginosa* and *M. racemosus* at 2 and 1 mg/ml  
607 ,respectively, for at least seven days. The C-terminal part of the fragment is undoubtedly  
608 essential for its antifungal effect since the peptides pepa4b61, pepa4c101 and pepa4b79  
609 whose sequences partially overlap the pepa4c177 turned out to be inactive. Previous work  
610 outlined the antimicrobial potential of casein hydrolysates from ovine and bovine milk  
611 (McCann et al., 2006; Recio and Visser, 1999; Zucht et al., 1995). However, experiments  
612 were based on controlled hydrolysis of caseins and the fragments only tested for their  
613 antibacterial activity. The peptide described in this report were generated by pepsin hydrolysis  
614 of ovine  $\alpha_{s2}$ -casein and successfully inhibited bacterial growth of *Escherichia coli*,  
615 *Staphylococcus* and *Listeria* spp. (López-Expósito *et al.*, 2006) with a bactericidal activity  
616 against *E. coli* at 2 mg/ml (López-Expósito *et al.*, 2007). It is worth noting that the natural  
617 antifungal bioactivity of the peptide identified in the present study could be underestimated  
618 since its conformation may be different from that encountered in the fermentate. In addition, it  
619 would be necessary to quantify pepa4c177 in the fermentates to define whether it takes part or  
620 not in the observed *in vitro* (present work) and *in situ* antifungal activity. It is noteworthy that  
621 this antifungal peptide is not de novo synthesized by *L. rhamnosus* but results from its  
622 proteolytic activities. In most studies on antifungal LAB, cyclic dipeptides are the main  
623 peptides related to their antifungal activity. Their production by *Lactobacillus* species have  
624 been reported (Magnusson *et al.*, 2003; Ström *et al.*, 2002). A MIC of 20 mg/mL was  
625 determined for *L. plantarum* cyclo(L-Phe-L-Pro) against *Aspergillus fumigatus* and  
626 *Penicillium roqueforti* (Ström *et al.*, 2002) which is 10 times higher than that observed for

627 pepa4c177 whereas cyclo(L-Pro-D-Leu) produced by a *Bacillus cereus* strain had a MIC of 8  
628  $\mu\text{g/mL}$  against *Aspergillus flavus*. It would also be interesting to further investigated the  
629 action spectrum of pepa4c177 peptide which was evidenced for the first time in the present  
630 study Overall, more than fifty compounds were identified in the 3 fermentates and it is  
631 reasonable to assume that they were not equally involved in the antifungal activity observed  
632 against *R. mucilaginosa* and *M. racemosus*. Moreover, synergistic and/or additive effects  
633 between compounds have been reported and make it more complex to understand their  
634 mechanism of action (Crowley et al., 2013; Suomalainen and Mäyrä-Makinen, 1999). To  
635 clarify this aspect, the determination of MIC of each identified organic acids as well as their  
636 MIC in mixture against the two fungi should be conducted.

637 In the second part of this study, the effect of *P. jensenii* CIRM-BIA 1774 fermentate on  
638 growth of *R.mucilaginosa* and *M. racemosus* was further investigated. Based on growth  
639 parameters comparison, *R. mucilaginosa* growth was significantly impacted at 2% with an  
640 enhanced lag time and a fungistatic effect was observed at 5% whereas, for *M. racemosus*,  
641 growth was significantly delayed at the different tested concentration without any total growth  
642 inhibition. Comparison of growth parameters showed that *P. jensenii* fermentate mainly  
643 affected *M. racemosus* radial growth rather than its germination capacity except at 5% for  
644 which a significant decrease in Pmax and increase in median germination time were observed.  
645 Le Lay et al. (2016) also observed such phenomena after studying the effect of a fermentate  
646 from *Leuconostoc citreum* on *Eurotium repens* germination. A high variability was also  
647 observed between biological replicates for the 5% concentration as growth and germination  
648 did not occur in all biological replicates suggesting that CMI was close to that concentration.  
649 However, despite germination did not occur, spores were still viable indicating that external  
650 conditions sensed by spores prevented triggering initiation of the germination process. Such  
651 behavior was also observed for *R. mucilaginosa* in PDB with 5% fermentate as growth did not

652 occur at all but cells were still viable. A similar observation was made by Mieskin et al.  
653 (2017) on *Yarrowia lipolytica* grown into yogurt cell-free supernatant of *L. harbinensis*  
654 K.V9.3.1.Np, except that a loss of cultivability was also evidenced for this bioprotective  
655 culture.

656 Interestingly, radial growth curve of *M. racemosus* in the presence of 5% fermentate differed  
657 with the others, which resulted in a lack of fit with the two-phase growth model used in the  
658 present study. Indeed, instead of two phases, three successive phases were seen – a lag phase,  
659 a first growth phase with a limited growth rate and a second growth phase with an increased  
660 growth rate. Such results may suggest that *M. racemosus* was able to cope with the high stress  
661 caused by *P. jensenii* fermentate after a second phase of adaptation starting on day 20.  
662 Another hypothesis consistent with this late start might imply that the antifungal effect of the  
663 fermentate decreased over time during incubation. Whether this phenomenon would be  
664 independent of the fungi through a decrease in volatile compound concentrations during  
665 incubation, i.e. propionate, or whether *M. racemosus* actively degraded antifungal compounds  
666 or adapted to such stress is yet to be determined. To our knowledge, such a three-phase  
667 growth behavior has not been reported in other studies even though degradation of weak acids  
668 by fungi has been previously shown in other mold species such *A. niger* (Plumridge et al.  
669 2008). These aspects are worth further investigation to better apprehend the adaptation  
670 mechanisms of this fungus to antagonistic molecules. Paradoxically, significant  
671 morphological changes were noticed in germinating spores but not for mycelium in the  
672 presence of fermentate as compared to the control condition. Exposition to the fermentate  
673 provoked germ tube damages leading to cell death for some germlings and might therefore  
674 partially explain the prolonged lag time observed at 5% of fermentate. Similar phenomena  
675 have been reported in an *Aspergillus fumigatus* strain exposed to a  $\beta$ -glucanase synthesis  
676 inhibitor, which are an essential component of the fungal cell wall (Bowman et al., 2002). The

677 phenomenon observed here could also be amplified by the physiological state of the fungi.  
678 Indeed, germlings are more vulnerable at the germ tube apex as the cell wall is thinner and  
679 likely allows an increased diffusion of antifungal molecules. Other morphological changes  
680 frequently described in this context mainly consist of swollen hyphae or deformed yeast cells  
681 (Ström et al., 2005). In contrast to *M. lanceolatus*, *R. mucilaginosa* cells exhibited here no  
682 visible damage. However, these observations were performed under phase-contrast  
683 microscopy and it would be interesting to investigate for ultrastructural changes using  
684 scanning electron microscopy or transmission electron microscopy as described previously  
685 (Mieszkin et al., 2017). The results reported in this study provide some response elements for  
686 a better understanding of the action mechanism of *P. jensenii* fermentate. Further work will be  
687 necessary to identify precisely which are the main molecules supporting antifungal activities  
688 of the studied fermentates and how these molecules act and affect fungal cell physiology at a  
689 cellular level.

690

691

692

693

#### 694 **Acknowledgements**

695 We are grateful to Regional Council of Brittany (grant no. 13008651) and Pays de la Loire  
696 (grant no. 2014-07081) for the financial support and INRA for scientific coordination (J.  
697 Leonil) through the interregional project PROFIL, managed by BBA industrial association.

698



699 **References**

- 700  
701 Acosta, R., Rodríguez-Martín, A., Martín, A., Núñez, Asensio, M. A., 2009. Selection of  
702 antifungal protein-producing molds from dry-cured meat products. *Int. J. Food Microbiol.*  
703 135, 39-46.  
704  
705 Aunbjerg, S. D., Honoré, A. H., Marcussen, J., Ebrahimi, P., Vogensen, F. K., Benfeldt, C.,  
706 Skov, T. and Knøchel, S. (2015). Contribution of volatiles to the antifungal effect of  
707 *Lactobacillus paracasei* in defined medium and yogurt. *International Journal of Food*  
708 *Microbiology* 194, 46–53.
- 709 Axel, C., Brosnan, B., Zannini, E., Furey, A., Coffey, A., Arendt, E.K., 2016. Antifungal  
710 sourdough lactic acid bacteria as biopreservation tool in quinoa and rice bread. *Int. J. Food*  
711 *Microbiol.* 239, 86-94.  
712
- 713 Bian, X., Muhammad, Z., Evivie, S. E., Luo, G.-W., Xu, M. and Huo, G.-C. (2016).  
714 Screening of antifungal potentials of *Lactobacillus helveticus* KLDS 1.8701 against spoilage  
715 microorganism and their effects on physicochemical properties and shelf life of fermented  
716 soybean milk during preservation. *Food Control* 66, 183–189.
- 717 Biede, S.L., Hammond, E.G., 1979. Swiss cheese flavor: II. Organoleptic analysis 1, 2. *J.*  
718 *Dairy Sci.* 62, 238–248.  
719
- 720 Bowman, J. C., Hicks, P. S., Kurtz, M. B., Rosen, H., Schmatz, D. M., Liberator, P. A. and  
721 Douglas, C. M. (2002). The Antifungal Echinocandin Caspofungin Acetate Kills Growing  
722 Cells of *Aspergillus fumigatus* In Vitro. *Antimicrob. Agents Chemother.* 46, 3001–3012.
- 723 Brosnan, B., Coffey, A., Arendt, E. K. and Furey, A. (2014). The QuEChERS approach in a  
724 novel application for the identification of antifungal compounds produced by lactic acid  
725 bacteria cultures. *Talanta* 129, 364–373.
- 726 Buchanan, R. L. (1993). Predictive food microbiology. *Trends in Food Science Technology* 4,  
727 6–11.  
728
- 729 Caplice, E., Fitzgerald, G., 1999. Food fermentations: role of microorganisms in food  
730 production and preservation. *Int. J. Food Microbiol.* 50, 131–149.  
731
- 732 Campoy S., Adrio, J.L., (2017). Antifungals. *Biochem. Pharmacol.* 133, 86-96.  
733
- 734 Chamba, J.-F., Irlinger, F. (2004). Secondary and adjunct cultures. In *Cheese: Chemistry,*  
735 *Physics and Microbiology* (Fox, P. F., McSweeney, P. L. H., Cogan, T. M., Guinee, T. P.,  
736 Eds.), Vol. 2, pp. 191-206.  
737
- 738 Coda, R., Cassone, A., Rizzello, C.G., Nionelli, L., Cardinali, G., Gobbetti, M., (2011).  
739 Antifungal activity of *Wickerhamomyces anomalus* and *Lactobacillus plantarum* during  
740 sourdough fermentation: identification of novel compounds and long-term effect during  
741 storage of wheat bread. *Appl. Environ. Microbiol.* 77, 3484–92.

- 742 Coda, R., Rizello, C.G., Di Cagno, R., Trani, A., Cardinali, G., Gobbetti, M., 2013.  
743 Antifungal activity of *Meyerozyma guilliermondii*: Identification of active compounds  
744 synthesized during dough fermentation and their effect on long-term storage of wheat bread.  
745 Food Microbiol. 33, 243-251.  
746
- 747 Coloretti, F., Carri, S., Armaforte, E., Chiavari, C., Grazia, L. and Zambonelli, C. (2007).  
748 Antifungal activity of lactobacilli isolated from salami. *FEMS Microbiology Letters* 271, 245–  
749 250.
- 750 Corrieu, G., Luquet, F.-M., 2008. Bactéries lactiques: de la génétique aux ferments. Ed;  
751 Lavoisier/TEC DOC, Paris, France.
- 752 Cortés-Zavaleta, O., López-Malo, A., Hernández-Mendoza, A. and García, H. S. (2014).  
753 Antifungal activity of lactobacilli and its relationship with 3-phenyllactic acid production.  
754 *International Journal of Food Microbiology* 173, 30–35.
- 755 Crowley, S., Mahony, J. and van Sinderen, D. (2013). Current perspectives on antifungal  
756 lactic acid bacteria as natural bio-preservatives. *Trends in Food Science Technology* 33, 93–  
757 109.
- 758 Dantigny, P., Nanguy, S. P.-M., Judet-Correia, D. and Bensoussan, M. (2011). A new model  
759 for germination of fungi. *Int. J. Food Microbiol.* 146, 176–181.
- 760 FAO/WHO, 2017. Food additive evaluations.  
761
- 762 Garnier, L., Mounier J., Lê, S., Pawtowski, A., Pinon, N., Camier, B., Chatel, M., Garric G.,  
763 Coton, E., Valence, F., Development of antifungal ingredients for dairy products: from *in*  
764 *vitro* screening to pilot scale application. Unpublished work  
765
- 766 Garnier, Valence, F., L., Mounier J., 2017. Diversity and control of spoilage fungi in dairy  
767 products: un update. *Microorganisms* 5, 42.  
768
- 769 Gerez, C. I., Carbajo, M. s., Rollán, G., Torres Leal, G. and Font de Valdez, G. (2010).  
770 Inhibition of Citrus Fungal Pathogens by Using Lactic Acid Bacteria. *Journal of Food*  
771 *Science* 75, M354–M359.
- 772 Gripon, J. C. (1999). Mould-ripened cheeses. In *Cheese: Chemistry, physics and microbiology*  
773 (Fox, P.F., Ed.), Vol. 2, pp. 111–136.  
774
- 775 Hassan, R., El-Kadi, S., Sand, M., (2015). Effect of some organic acids on some fungal  
776 growth and their toxins production. *Int. J. Adv. Biol.* 2(1), 1-11.  
777
- 778 Honoré, A. H., Aunbjerg, S. D., Ebrahimi, P., Thorsen, M., Benfeldt, C., Knøchel, S. and  
779 Skov, T. (2016). Metabolic footprinting for investigation of antifungal properties of  
780 *Lactobacillus paracasei*. *Anal Bioanal Chem* 408, 83–96.
- 781 ICH, 2005. Q2 (R1), “Validation of analytical procedures: text and methodology”, ICH  
782 Harmonised Tripartite Guideline. International Conference on Harmonisation of Technical  
783 Requirements for Registration of Pharmaceuticals for Human Use, Chicago, USA, 2005.  
784

785 Ignatova, L.V., Brazhnnikova, Y.V., berzhanova, R.Z., Mukasheval, T.D. 2015. Plant growth-  
786 promoting and antifungal activity of yeasts from dark chesnut soil. *Microbial Research*, 175,  
787 78-83.  
788

789 Jong C. and Badings H.T. (1990). Determination of free fatty acids in milk and cheese  
790 procedures for extraction, clean up, and capillary gas chromatographic analysis. *J High Res*  
791 *Chromatog*, 13: 94-98.  
792

793 Langella O., Valot B., Balliau T., Blein-Nicolas M., Bonhomme L., and Zivy M. (2017),  
794 X!TandemPipeline: A Tool to Manage Sequence Redundancy for Protein Inference and  
795 Phosphosite Identification, *Journal of Proteome Research*, 16 (2), 494–503).

796 Le Lay, C., Mounier, J., Vasseur, V., Weill, A., Le Blay, G., Barbier, G. and Coton, E. (2016).  
797 In vitro and in situ screening of lactic acid bacteria and propionibacteria antifungal activities  
798 against bakery product spoilage molds. *Food Control* 60, 247–255.

799 Leyva Salas, M., Mounier, J., Valence, F., Coton, M., Thierry, A., Coton, E., 2017.  
800 Antifungal microbial agents for food biopreservation - a review. *Microorganisms* 5(3), 37.

801 Lind, H., Jonsson, H. and Schnürer, J. (2005). Antifungal effect of dairy propionibacteria—  
802 contribution of organic acids. *International Journal of Food Microbiology* 98, 157–165.

803 Lind, H., Sjögren, J., Gohil, S., Kenne, L., Schnürer, J. and Broberg, A. (2007). Antifungal  
804 compounds from cultures of dairy propionibacteria type strains. *FEMS Microbiology Letters*  
805 271, 310–315.

806 López-Expósito, I., Gómez-Ruiz, J. Á., Amigo, L. and Recio, I. (2006). Identification of  
807 antibacterial peptides from ovine  $\alpha$ 2-casein. *International Dairy Journal* 16, 1072–1080.

808 López-Expósito, I., Quirós, A., Amigo, L. and Recio, I. (2007). Casein hydrolysates as a  
809 source of antimicrobial, antioxidant and antihypertensive peptides. *Lait* 87, 241–249.

810 Magnusson, J., Ström, K., Roos, S., Sjögren, J., Schnürer, J., 2003. Broad and complex  
811 antifungal activity among environmental isolates of lactic acid bacteria. *FEMS Microbiol.*  
812 *Lett.* 219, 129–135.  
813

814 Mantere-Alhonen, S., Makinen, E., 1987. A new type of sour milk with propionibacteria.  
815 *Meijeritieteellinen Aikakauskirja* 45, 49–61.  
816

817 McCann, K. B., Shiell, B. J., Michalski, W. P., Lee, A., Wan, J., Roginski, H. and Coventry,  
818 M. J. (2006). Isolation and characterisation of a novel antibacterial peptide from bovine  $\alpha$ S1-  
819 casein. *International Dairy Journal* 16, 316–323.

820 Miezkin, S., Hymery, N., Debaets, S., Coton, E., Le Blay, E., Valence, F., Mounier, J.,  
821 (2017). Action mechanisms involved in the bioprotective effect of *Lactobacillus harbinensis*  
822 K.V9.3.1.Np against *Yarrowia lipolytica* in fermented milk. *Int. J. Food Microbiol.* 248, 47-  
823 55.

824 Minkiewicz, P. Dziuba, J., Iwaniak, A., Dziuba, M., Darewicz, M., (2008). BIOPEP database  
825 and other programs for processing bioactive peptide sequences. *J. AOAC Int.* 91, 965-980.  
826

827 Nally, M.C., Pesce, V.M., Maturano, Y.P., Rodriguez Assaf, L.A., Toro, M.E., Castellanos de  
828 Figueroa, L.I., Vazquez, F., 2015. Antifungal modes of action of *Saccharomyces* and other  
829 biocontrol yeasts against fungi isolated from sour and grey rots. *Int. J. Food Microbiol.* 204,  
830 91-100

831 Nyemb-Diop, K., Causeur, D., Jardin, J., Briard-Bion, V., Guérin-Dubiard, C., Rutherford,  
832 S.M., Dupont, D., Nau, F., (2016). Investigating the impact of egg white gel structure on  
833 peptde kinetics profil during in vitro digestion. *Food Res. Int.* 88, 302-309.  
834

835 Pesce, V.M., Nally, M.C., Carrizo, G.P., Rojo, C., Pérez, B.A., Toro, M.E., Castellanos de  
836 Figueroa, L.I., Vazquez, F. 2017. Antifungal activity of native yeasts from different  
837 microenvironments against *Colletotrichum gloeosporioides* on ripe olice fruits. *Biol. Control*,  
838 *In Press*.  
839

840 Pitt, J.I., Hocking, A.D., 2009. *Fungi and Food Spoilage*. Springer Science Business Media,  
841 Berlin, Germany.

842 Plumridge, A., Startford, M., Kenneth, C.L., Archer, D.B., 2008. The weak-acid  
843 preservative sorbic acid is decarboxylated and detoxified by a phenylacrylic acid  
844 decarboxylase, PadA1, in the spoilage mold *Aspergillus niger*. *Appl. Env. Microbiol.* 74(2),  
845 550-552.  
846

847 Pogacic, T., Maillars, M-B., Leclerc, A., Hervé, C., Chuat, V., Yee, A.L., Valence, F. (2015).  
848 A methodological approach to screen diverse cheese-related bacteria for their ability to  
849 produce aroma compounds. *Food Microbiol*, 46: 145-153.  
850

851 Pohl, C. H., Kock J. L. F. and Thibane, V. S. 2011. Antifungal free fatty acids: A Review, p  
852 61–71 *In Méndez-Vilas A, editor. (ed), Science against microbial pathogens: communicating*  
853 *current research and technological advances. Formatex Research Center, Badajoz, Spain*  
854

855 Recio, I. and Visser, S. (1999). Identification of two distinct antibacterial domains within the  
856 sequence of bovine  $\alpha$ 2-casein. *Biochimica et Biophysica Acta (BBA) - General Subjects*  
857 1428, 314–326.

858 Rizzello, C.G., Cassone, A., Coda, R., Gobbetti, M., 2011. Antifungal activity of sourdough  
859 fermented wheat germ used as an ingredient for bread making. *Food Chem.* 127, 952–959.  
860

861 Schieberle, H.-D. Belitz, W. Grosch, P. (2009). *Food chemistry* (4th rev. and extended ed.).  
862 Springer, Berlin, Germany.

863 Schnürer, J. and Magnusson, J. (2005). Antifungal lactic acid bacteria as biopreservatives.  
864 *Trends in Food Science Technology* 16, 70–78.

865 Schwenninger, S. M. and Meile, L. (2004). A mixed culture of *Propionibacterium jensenii*  
866 and *Lactobacillus paracasei* subsp. *paracasei* inhibits food spoilage yeasts. *Syst. Appl.*  
867 *Microbiol.* 27, 229–237.

868 Shokri, H., (2011). Evaluation of inhibitory effects of citric and tartaric acids and their  
869 combination on the growth of *Trichophyton mentagrophytes*, *Aspergillus fumigatus*, *Candida*  
870 *albicans* and *Malassezia furfur*. *Comp. Clinic. Pathol.* 20(5), 543-545.

871 Sjögren, J., Magnusson, J., Broberg, A., Schnürer, J. and Kenne, L. (2003). Antifungal 3-  
872 Hydroxy Fatty Acids from *Lactobacillus plantarum* MiLAB 14. *Appl Environ Microbiol* 69,  
873 7554–7557.

874 Smith, C.A. Want, E.J., O'Maille, G., Abagyan, R., Siuzdak, G., 2006. XCMS: processing  
875 mass spectrometry data for metabolite profiling using nonlinear peak alignment, matching,  
876 and identification. *Anal. Chem.*, 78 (2006), pp. 779-787

877 Stratford, M., Plumridge, A., Nebe-von-Caron, G. and Archer, D. B. (2009). Inhibition of  
878 spoilage mould conidia by acetic acid and sorbic acid involves different modes of action,  
879 requiring modification of the classical weak-acid theory. *International Journal of Food*  
880 *Microbiology* 136, 37–43.

881 Ström, K., Sjögren, J., Broberg, A., Schnürer, J., 2002. *Lactobacillus plantarum* MiLAB 393  
882 produces the antifungal cyclic dipeptides cyclo (L-Phe-L-pro) and cyclo (L-Phetrans-4-OH-L-  
883 pro) and 3-phenyllactic acid. *Appl. Environ. Microbiol.* 68, 4322–4327.

884  
885 Ström, K., Schnürer, J. and Melin, P. (2005). Co-cultivation of antifungal *Lactobacillus*  
886 *plantarum* MiLAB 393 and *Aspergillus nidulans*, evaluation of effects on fungal growth and  
887 protein expression. *FEMS Microbiol. Lett.* 246, 119–124.

888 Suomalainen, T. H. and Mäyrä-Mäkinen, A. M. (1999). Propionic acid bacteria as protective  
889 cultures in fermented milks and breads. *ResearchGate* 79, 165–174.

890 Théolier, J., Fliss, I., Jean, J., Hammami, R. (2014). MilkAMP: a comprehensive database of  
891 antimicrobial peptides of dairy origin. *Dairy Sci. Technol.* 94(2), 181-193.

892 Wagh, F.H., Gopi, L., Barai, R.S., Ramteke, P., Nizami, B., Idicula-Thomas, S., (2014).  
893 CAMP: Collection of sequences and structures of antimicrobial peptides. *Nucleic acids Res.*  
894 42, 1154-1158.

895 Wang, Z., Wang, G., (2004). ADP: the antimicrobial peptide databe. *Nucleic acids res.* 32,  
896 590-592.

897 Zamyatnin, A.A., Borchikov, A.S., Vladimirov, M.G., Voronina, O.L. (2006). The EROP-  
898 Moscow oligopeptide database. *Nucleic acids res.* 34, 261-266.

899 Zucht, H.-D., Raida, M., Adermann, K., Mägert, H.-J. and Forssmann, W.-G. (1995).  
900 Casocidin-I: a casein- $\alpha$ 2 derived peptide exhibits antibacterial activity. *FEBS Letters* 372,  
901 185–188.

902 Zwietering, M. H., Jongenburger, I., Rombouts, F. M. and van 't Riet, K. (1990). Modeling of  
903 the Bacterial Growth Curve. *Appl Environ Microbiol* 56, 1875–1881.

904  
905  
906

907  
908

**Table 1.** Peptides identified in the bacterial and fungal fermentate used for the antifungal activity test

<b>Peptide</b>	<b>Amino acid sequence</b>	<b>Length</b>	<b>Charge</b>	<b>Protein</b>	<b>Fermentate</b>
<b>pepa5c165</b>	YYQQKPVALINNQFLPYPPYYAKPAA	25	2	$\kappa$ -casein f(43-97)	<i>L. rhamnosus</i>
<b>pepa5c104</b>	QQKPVALINNQFLPYPPYYAKPA	22	1	$\kappa$ -casein f(43-97)	<i>L. rhamnosus</i>
<b>pepa5b48</b>	LINNQFLPYPPYYAKPA	16	1	$\kappa$ -casein f(43-97)	<i>P. jensenii</i>
<b>pepa4c101</b>	KTKLTETEEKNRLNFLK	16	2	$\alpha$ s2-casein f(165-203)	<i>P. jensenii</i>
<b>pepa4b79</b>	KLTEEEKNRLNFL	13	0	$\alpha$ s2-casein f(165-203)	<i>P. jensenii</i>
<b>pepa5b99</b>	QKPVALINNQFLPYPPYYAKPA	21	1	$\kappa$ -casein f(43-97)	<i>L. rhamnosus</i>
<b>pepa4c190</b>	STEVFTKTKTKLTEEEKNR	18	1	$\alpha$ s2-casein f(165-203)	<i>M. lanceolatus</i>
<b>pepa4b98</b>	KTKLTETEEKNRL	12	1	$\alpha$ s2-casein f(165-203)	<i>M. lanceolatus</i>
<b>pepa4c197</b>	TKKTKLTETEEKNR	13	2	$\alpha$ s2-casein f(165-203)	<i>M. lanceolatus</i>
<b>pepa5b83</b>	PYYAKPAAVRSPAQILQWQVL	21	2	$\kappa$ -casein f(43-97)	<i>L. rhamnosus</i>
<b>pepa4b97</b>	KTKLTETEEKNR	11	1	$\alpha$ s2-casein f(165-203)	<i>M. lanceolatus</i>
<b>pepa4b61</b>	LTEEEKNRLNF	11	-1	$\alpha$ s2-casein f(165-203)	<i>M. lanceolatus</i>
<b>pepa4c177</b>	RLNFLKKIS	9	3	$\alpha$ s2-casein f(165-203)	<i>L. rhamnosus</i>
<b>pepac192</b>	TKLTETEEKNR	10	0	$\alpha$ s2-casein f(165-203)	<i>M. lanceolatus</i>
<b>pepa4c223</b>	YLKTVYQHQ	9	2	$\alpha$ s2-casein f(165-203)	<i>L. rhamnosus</i>
<b>pepa4b78</b>	KLTEEEKNR	9	0	$\alpha$ s2-casein f(165-203)	<i>M. lanceolatus</i>

909

910 **Table 2. Comparison of the antifungal compounds identified in the fermentates by HPLC and LC-QToF**

Compound	<i>P. jensenii</i> CIRM-BIA 1774 (mg/g)	<i>L. rhamnosus</i> CIRM-BIA 1952 (mg/g)	<i>M. lanceolatus</i> UBOCC-A-109193 (mg/g)
Lactic acid	-	<b>86.018 ± 3.05</b>	-
Propionic acid	<b>59.94 ± 21.28</b>	0.999 ± 0.89	-
Acetic acid	<b>16.893 ± 5.676</b>	2.57 ± 0.62	0.104 ± 0.002
Benzoic acid (HPLC)	-	<b>0.09 ± 0.047</b>	-
Benzoic acid	0.004 ± 0.001	<b>0.118 ± 0.008*</b>	0.0171 ± 0.0002
Citric acid	<b>32.477 ± 5.437</b>	14.541 ± 1.271	2.514 ± 0.030
Succinic acid	4.85 ± 2.78	<b>5.221 ± 0.541</b>	0.099 ± 0.024
2-pyrrolidone-5-carboxylic acid	<b>2.23 ± 0.33</b>	0.18 ± 0.087	0.0011 ± 0
(S)-(-)-2-Hydroxyisocaproic acid	<b>0.389 ± 0.012*</b>	0.164 ± 0.006*	0.0007 ± 0.0001
2-Hydroxydodecanoic acid	+	+	+
3-(4-Hydroxyphenyl)propionic acid	<b>0.468 ± 0.024*</b>	0.013 ± 0.0004	-
3-Hydroxydecanoic acid	+	+	+
Azelaic acid	-	+	<b>0.0005 ± 0.0001</b>
DL-Hydroxylauric acid	-	+	-
DL-Hydroxyphenyllactic acid	<b>0.312 ± 0.0125*</b>	0.011 ± 0.001	-
Hydrocinnamic acid	+	+	+
Mevalonolactone	<b>0.302 ± 0.024*</b>	0.151 ± 0.007*	+
Phenyllactic acid	<b>0.491 ± 0.024*</b>	0.012 ± 0.001	0.0012 ± 0.0001
Salicylic acid	0.0005 ± 0	0.0006 ± 0.0001	+

911 Results are expressed as the mean of three replicates ± standard deviation. Legend: (\*) Estimated concentration; (+) value between LOD and  
912 LOQ; (-) molecule not detected or at concentration < LOD. Bold values correspond to the highest concentration among the three analyzed  
913 fermentates (**bold characters correspond to the highest observed values**).

914 **Table 3. Relative abundance of potential antifungal volatiles in fermentates derived**  
 915 **from *M. Lanceolatus* UBOCC-A-109193, *P. jensenii* CIRM-BIA 1774 and *L. rhamnosus***  
 916 **CIRM-BIA 1952 (bold characters correspond to the highest observed values).**

Volatiles	Fermentate derived from <i>L.</i> <i>rhamnosus</i>	Fermentate derived from <i>M.</i> <i>lanceolatus</i>	Fermentate derived from <i>P.</i> <i>jensenii</i>
Ethyl acetate	1,08.10 <sup>7</sup>	1,55.10 <sup>7</sup>	<b>3,93.10<sup>8</sup></b>
Butyl acetate	6,46.10 <sup>7</sup>	2,08.10 <sup>7</sup>	<b>1,84.10<sup>9</sup></b>
Butanol 2-methyl-acetate	1,37.10 <sup>7</sup>	5,29.10 <sup>6</sup>	<b>8,71.10<sup>8</sup></b>
Ethyl propanoate	2,66.10 <sup>6</sup>	2,47.10 <sup>6</sup>	<b>1,52.10<sup>9</sup></b>
2-methyl propanoate	1,30.10 <sup>7</sup>	5,81.10 <sup>7</sup>	<b>1,37.10<sup>9</sup></b>
Butyl propanoate	9,36.10 <sup>6</sup>	4,68.10 <sup>6</sup>	<b>3,40.10<sup>9</sup></b>
Propyl propanoate	1,20.10 <sup>6</sup>	5,58.10 <sup>5</sup>	<b>1,00.10<sup>9</sup></b>
2-Pentanol propanoate	7,16.10 <sup>6</sup>	3,28.10 <sup>6</sup>	<b>5,75.10<sup>8</sup></b>
3-methyl butanal	5,53.10 <sup>8</sup>	3,99.10 <sup>8</sup>	<b>3,53.10<sup>9</sup></b>
2-ethyl furan	1,66.10 <sup>7</sup>	<b>2,61.10<sup>8</sup></b>	4,39.10 <sup>6</sup>
2-n-butyl furan	1,59.10 <sup>5</sup>	<b>1,72.10<sup>7</sup></b>	3,64.10 <sup>4</sup>
2-pentyl furan	3,37.10 <sup>7</sup>	<b>6,26.10<sup>7</sup></b>	1,42.10 <sup>6</sup>
2-Furanmethanol	2,75.10 <sup>8</sup>	<b>1,21.10<sup>9</sup></b>	1,98.10 <sup>7</sup>
Butyrate	<b>7,39.10<sup>8</sup></b>	2,09.10 <sup>8</sup>	4,44.10 <sup>8</sup>
Ethyl butyrate	<b>2,51.10<sup>9</sup></b>	2,81.10 <sup>7</sup>	1,69.10 <sup>7</sup>
3-methyl butyrate	4,11.10 <sup>7</sup>	1,15.10 <sup>7</sup>	<b>2,67.10<sup>8</sup></b>
3-methyl, 1-butanol	<b>2,36.10<sup>9</sup></b>	5,83.10 <sup>6</sup>	3,69.10 <sup>7</sup>
2-pentanone	<b>5,92.10<sup>8</sup></b>	2,45.10 <sup>8</sup>	3,87.10 <sup>7</sup>
2,3-pentanedione	<b>1,11.10<sup>8</sup></b>	2,88.10 <sup>6</sup>	3,15.10 <sup>6</sup>
Diacetyl	<b>1,36.10<sup>9</sup></b>	7,45.10 <sup>8</sup>	1,07.10 <sup>8</sup>
Styrene	<b>1,65.10<sup>8</sup></b>	2,26.10 <sup>7</sup>	1,06.10 <sup>6</sup>
Acetoin	2,93.10 <sup>8</sup>	<b>1,61.10<sup>9</sup></b>	1,56.10 <sup>8</sup>
Benzaldehyde	<b>1,42.10<sup>8</sup></b>	4,57.10 <sup>7</sup>	1,28.10 <sup>8</sup>
4-methyl benzaldehyde	<b>1,36.10<sup>8</sup></b>	2,67.10 <sup>7</sup>	6,45.10 <sup>7</sup>
Hexanoate	4,22.10 <sup>8</sup>	<b>1,24.10<sup>9</sup></b>	1,46.10 <sup>9</sup>
Ethyl hexanoate	<b>7,37.10<sup>8</sup></b>	8,98.10 <sup>5</sup>	2,67.10 <sup>5</sup>
Hexanal	1,65.10 <sup>7</sup>	2,71.10 <sup>7</sup>	<b>3,43.10<sup>7</sup></b>
Heptanoate	8,70.10 <sup>6</sup>	<b>1,09.10<sup>8</sup></b>	4,19.10 <sup>7</sup>
Heptanal	3,79.10 <sup>7</sup>	<b>8,37.10<sup>7</sup></b>	2,33.10 <sup>7</sup>
Octanoate	4,77.10 <sup>7</sup>	1,84.10 <sup>8</sup>	<b>5,48.10<sup>8</sup></b>
Octanal	3,51.10 <sup>5</sup>	<b>3,15.10<sup>6</sup></b>	1,85.10 <sup>5</sup>
2-Nonanone	2,32.10 <sup>7</sup>	<b>4,78.10<sup>8</sup></b>	6,29.10 <sup>5</sup>
Nonanal	3,08.10 <sup>6</sup>	<b>5,89.10<sup>6</sup></b>	1,48.10 <sup>6</sup>
2-Undecanone	4,26.10 <sup>6</sup>	<b>8,17.10<sup>7</sup></b>	5,71.10 <sup>6</sup>



918 **Table 4. Quantification of potential antifungal free fatty acids by GC/MS in fermentates**  
 919 **derived from *M. Lanceolatus* UBOCC-A-109193, *P. jensenii* CIRM-BIA 1774 and *L.***  
 920 ***rhamnosus* CIRM-BIA 1952. (bold characters correspond to the highest observed values)**

Free fatty acids		Fermentate derived from <i>M. lanceolatus</i> (µg/mL)	Fermentate derived from <i>P. jensenii</i> (µg/g)	Fermentate derived from <i>L. rhamnosus</i> (µg/g)
C2 : 0	Acetic acid	47.1±1.31	<b>507.2±84.04</b>	58.1±13.70
C3 : 0	Propionic acid	14.2±0.59	<b>1027.0±270.79</b>	22.6±25.40
C4 : 0	Butyric acid	<b>444.7±0.94</b>	8.7±2.44	6.6±0.70
C6 : 0	Caproic acid	<b>6.3±0.22</b>	1.2±0.51	1.4±0.40
C7 : 0	Heptanoic acid	0.0	0.0	<b>0.1±0.20</b>
C8 : 0	Caprylic acid	0.0	0.8±0.75	<b>1.4±0.10</b>
C9 : 0	Nonanoic acid	1.4±0.09	1.1±0.09	<b>1.8±0.30</b>
C10 : 0	Capric acid	<b>4.5±0.25</b>	1.6±0.50	3.0±0.30
C12 : 0	Lauric acid	3.1±0.01	2.7±0.17	<b>5.0±0.20</b>
C13 : 0	Tridecanoic acid	0.0	<b>1.5±0.20</b>	0.3±0.30
C14 : 0	Myristic acid	<b>11.0±6.69</b>	2.8±1.05	9.7±0.80
C15 : 0	Pentadecanoic acid	<b>4.1±2.78</b>	1.8±0.67	1.1±0.10
C16 : 0	Palmitic acid	31.9±15.94	21.0±5.65	<b>49.9±5.60</b>
C16 : 1	Palmitoleic acid	<b>6.6±4.85</b>	0.9±0.89	1.6±1.00
C18 : 0	Stearic acid	8.5±3.13	13.2±5.02	<b>32.0±3.40</b>
C18 : 1	Oleic acid	5.8±2.52	0.6±1.00	<b>8.8±1.30</b>
C18 : 2	Linoleic acid	0.0	<b>0.3±0.46</b>	0.0
C19 : 0	Nonadecanoic acid	0.0	<b>0.9±1.48</b>	0.0
C20 : 0	Arachidic acid	0.0	<b>1.7±2.88</b>	0.0

921

922 **Table 5. Germination and growth parameters of *Mucor racemosus* UBOCC-A-109155**  
 923 **exposed to *Propionibacterium jensenii* CIRM-BIA 1774 fermentate**

Concentration	0%	1%	2%	5%
<i>P</i> max (%)	73.27 ± 1.5 <sup>a</sup>	73.8 ± 1.96 <sup>a</sup>	84.05 ± 2.57 <sup>a</sup>	34.54 ± 8.25 <sup>b</sup>
$\tau$ (h)	6.43 ± 0.09 <sup>a</sup>	7.3 ± 0.18 <sup>a</sup>	8.33 ± 0.11 <sup>a</sup>	20.7 ± 2.46 <sup>b</sup>
<i>d</i> (SU)	20 ± 0 <sup>a</sup>	14.38 ± 2.81 <sup>b</sup>	12.16 ± 0.61 <sup>b</sup>	5.46 ± 2.91 <sup>c</sup>
$\lambda$ (d)	0.86 ± 0.04 <sup>a</sup>	0.59 ± 0.03 <sup>a</sup>	0.79 ± 0.24 <sup>a</sup>	9.62 ± 3.09 <sup>b</sup>
$\mu$ (mm.d <sup>-1</sup> )	12.44 ± 0.47 <sup>a</sup>	6.49 ± 0.1 <sup>b</sup>	5.48 ± 0.72 <sup>c</sup>	0.78 ± 1.47 <sup>d</sup>
<i>Diameter at 7 days</i> (mm)	85 ± 0 <sup>a</sup>	81.67 ± 1.04 <sup>a</sup>	67.63 ± 7.81 <sup>b</sup>	< 4 <sup>c</sup>

924 Values are expressed as mean ± standard deviation of the replicates. Means with different  
 925 letters within a row are significantly different according to Fisher's LSD test (p < 0.05).  
 926

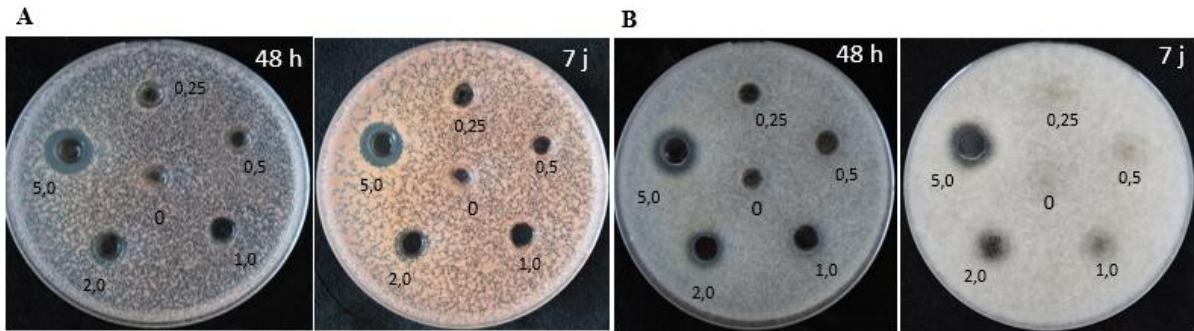
927

928 **Table 6. Growth parameters of *Rhodotorula mucilaginosa* UBOCC-A-216004 cultivated**  
 929 **in PDB (pH 5) at different concentrations of *Propionibacterium jensenii* CIRM-BIA 1774**  
 930 **fermentate.**

Concentration	0%	1%	2%	5%
$\mu$ (h <sup>-1</sup> )	0.53 ± 0.02a	0.42 ± 0.03b	0.37 ± 0.02c	-
$\lambda$ (h)	5.85 ± 0.47a	7.27 ± 0.71a	40.24 ± 6b	-
A	5.99 ± 0.06a	6.76 ± 0.27b	7.61 ± 0.24c	-

931 Values are expressed as mean ± standard deviation of the replicates. Means with different  
 932 letters within a row are significantly different according to a Fisher's LSD test (p <0.05)  
 933  
 934

935



936

937

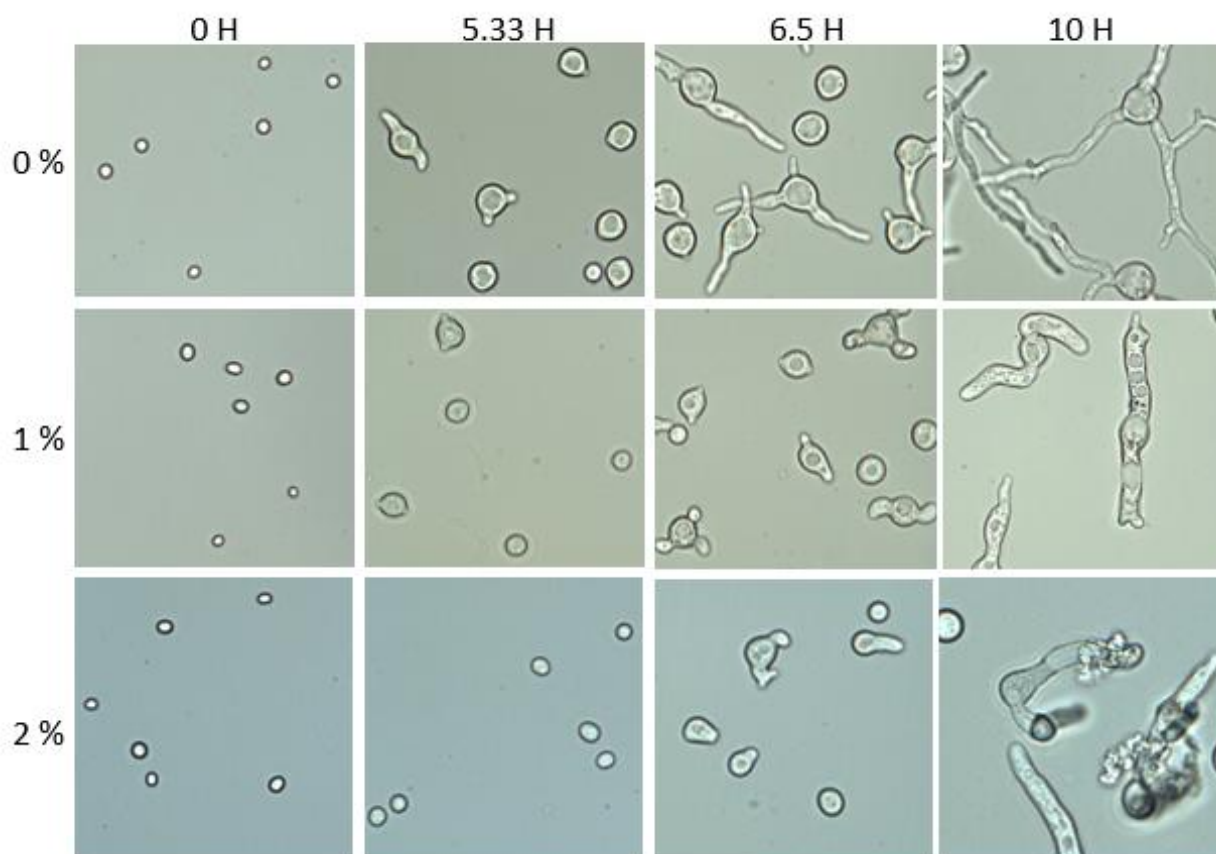
938

939

940

941

**Figure 1. Antifungal activity of peptide pepa4c177 on *Rhodotorula mucilaginosa* (A) and *Mucor racemosus* (B) grown on PDA 1% agar (final concentration:  $1.10^4$  spores or cells/mL) at 25 °C for 7 days. Five concentrations were tested 0,25 ; 0,5 ; 1,0 ; 2,0 and 5 mg/mL. Central wells were filled with sterile water as negative control.**



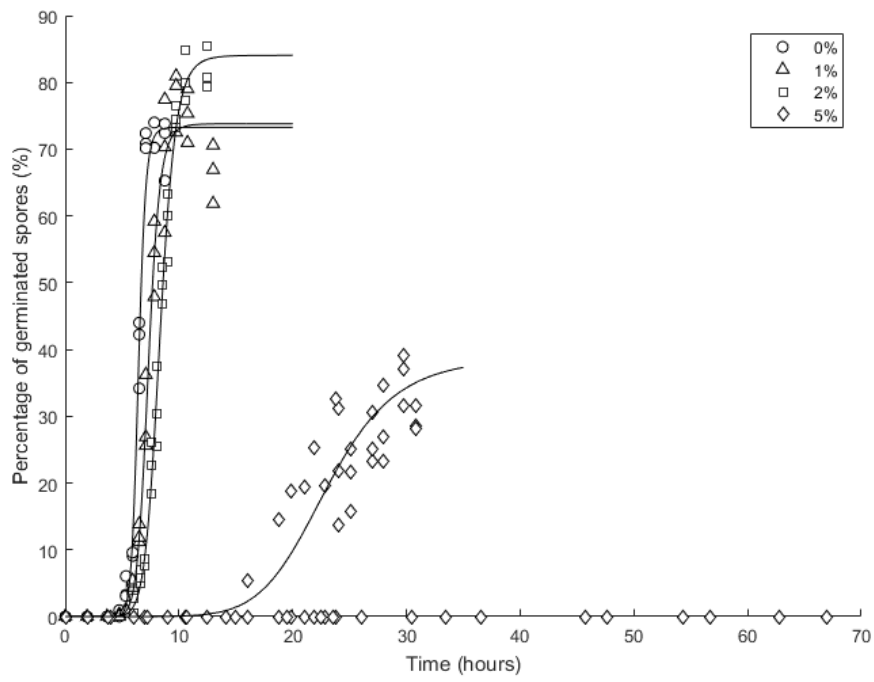
942

943 **Figure 2. *Mucor racemosus* UBOCC-A-109155 germlings after cultivation at different**  
 944 **concentrations of *Propionibacterium jensenii* CIRM1774 fermentate.**

945

946

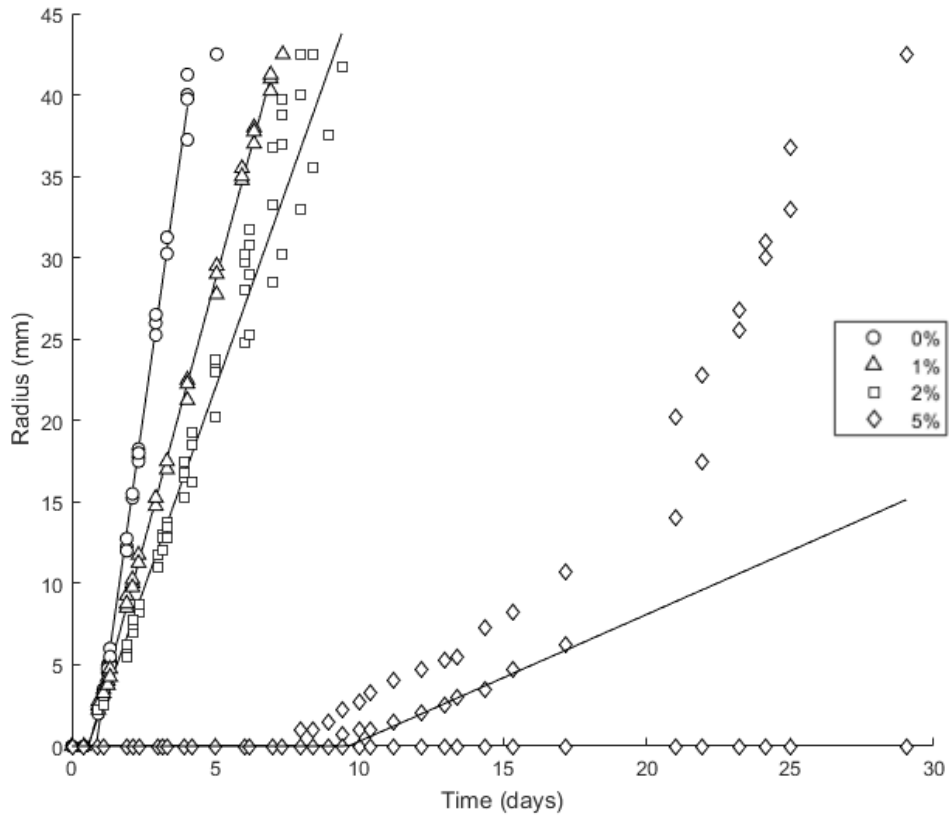
947



948 **Figure 3. Germination kinetics of *Mucor racemosus* UBOCC-A-109155 exposed to**  
949 **growing concentrations of *Propionibacterium jensenii* CIRM-BIA 1774 lyophilized**  
950 **culture.**

951

952



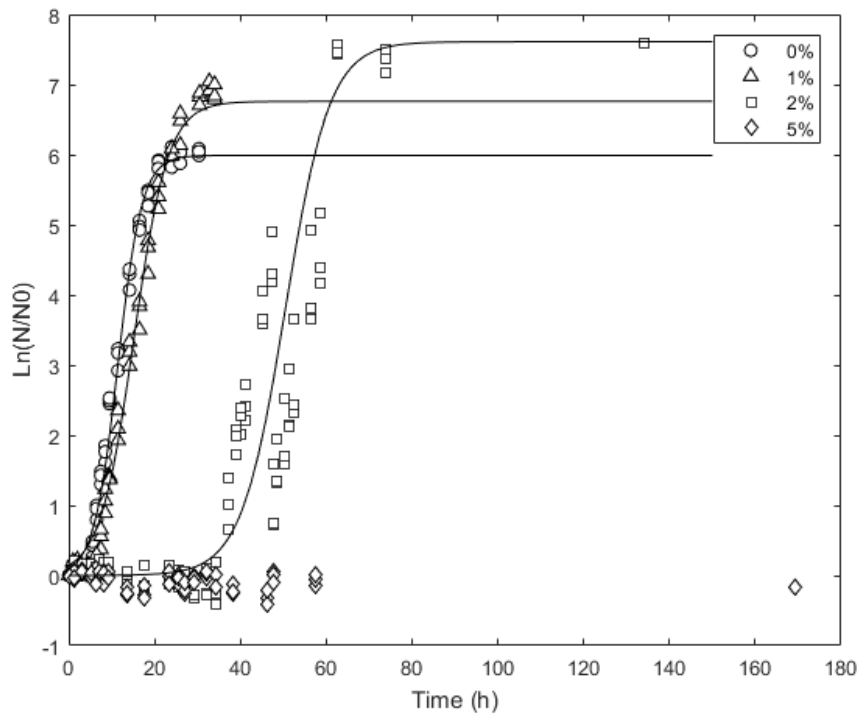
953

954 **Figure 4. Radial growth of *Mucor racemosus* UBOCC-A-109155 exposed to different**  
 955 **concentrations of *Propionibacterium jensenii* CIRM-BIA 1774 fermentate.**

956

957

958  
959



**Figure 5. Growth kinetics of *Rhodotorula mucilaginosa* UBOCC-A-216004 exposed at growing concentrations of *Propionibacterium jensenii* CIRM-BIA 1774 fermentate**

960



961 **Supplementary data**962 **Table S 1. Antifungal compounds searched for using LC-QToF analysis**

	<b>Antifungal compounds</b>	<b>(M-H)- theoric</b>	<b>(M-H)- found</b>	<b>Supplementary qualifier ions</b>
A	2- Deoxycytidine	226.0833	226.0829	
B	1,2-Dihydroxybenzene	109.0295	109.0297	
C	3,4-Dihydroxyhydrocinnamic acid	181.0506	181.0503	
D	4 -di-tert-butylphenol	205.1598	205.1591	
E	(S)-(-)-2-Hydroxyisocaproic acid	131.0714	131.0713	
F	2-Hydroxydodecanoic acid	215.1653	215.1649	
G	3-(4-hydroxy-3-methoxyphenyl) propionic acid	195.0663	195.0660	
H	3-(4-Hydroxyphenyl)propionic acid	165.0557	165.0554	
I	3-Hydroxydecanoic acid	187.1340	187.1334	
J	4-Hydroxybenzoic acid	137.0244	137.0243	
K	Azelaic acid	187.0976	187.0971	
L	Benzoic acid	121.0295	121.0294	
M	Caffeic acid	179.0350	179.0346	
N	$\rho$ - Coumaric acid	163.0401	163.0398	
O	Cytidine	242.0782	242.0779	
P	Decanoic acid	171.1391	171.1386	
Q	D-Glucuronic acid	193.0354	193.0350	
R	DL- $\beta$ -Hydroxylauric acid	215.1653	215.1649	
S	DL- $\beta$ -Hydroxymyristic acid	243.1966	243.1960	
T	DL-Hydroxyphenyllactic acid	181.0506	181.0503	112.9856 223.0139
U	Ferulic acid	193.0506	193.0503	
V	Hydrocinnamic acid	149.0608	149.0606	
W	Methylcinnamic acid	161.0608	161.0604	
X	Melanovolactone	129.0557	129.0556	
Y	N-Acetyl-D-glucosamine	220.0827	220.0821	
Z	Phenyl acetate	135.0452	135.0450	196.0785
Aa	Phenyllactic acid	165.0551	165.0554	
Ab	Ricinoleic acid	297.2435	297.2429	
Ac	Salicylic acid	137.0244	137.0242	
Ad	Succinic acid	117.0193	117.0193	147.0650
Ae	Vanillic acid	167.0350	167.0345	

963

964

En conclusion de cette Etude 4 ~ Identification des composés antifongiques :

*Grâce aux différentes méthodes d'identification mises en place, 64, 61 et 51 molécules de nature différente (acides organiques, acides gras, composés volatils et peptides) potentiellement impliquées dans l'activité antifongique des fermentats dérivant de P. jensenii CIRM-BIA 1744, L. rhamnosus CIRM-BIA 1952 et M. lanceolatus UBOCC-A-109193 respectivement, ont pu être identifiées. Néanmoins, leur implication respective dans l'activité antifongique des différents fermentats reste encore à définir.*

*De plus l'effet fongistatique de P. jensenii CIRM-BIA 1774 sur les cibles fongiques R. mucilaginosa UBOCC-A-216004 et M. racemosus UBOCC-A-109155 et son impact sur la physiologie des spores de M. racemosus ont pu être démontrés, donnant ainsi des éléments de réponse quant aux mécanismes d'action de ce fermentat.*



# **Discussion, conclusions et** **perspectives**



Pour lutter contre les altérations fongiques des produits laitiers, de nouvelles pistes de biopréservation, utilisant des substrats laitiers fermentés par des bactéries lactiques, propioniques et des champignons utilisés en tant qu'ingrédients, ont été étudiées au travers de cette thèse qui s'est faite dans le cadre d'une collaboration étroite entre partenaires académiques et partenaires industriels.

La première partie de cette thèse (**Etude 1 de la partie « Résultats »**) avait pour objectif de faire un état des lieux de la diversité des contaminants fongiques rencontrée dans les produits laitiers et leur environnement par le biais d'une collecte de produits laitiers contaminés (n=53), de boîtes d'ambiance (n=59) et d'isolats fongiques (n=63) d'origine variée fournis par les différents partenaires industriels. Cette étude nous a permis de mettre en évidence la grande diversité de contaminants fongiques des produits laitiers et leur environnement. En effet, 105 isolats dont 64 champignons filamenteux et 41 levures ont été isolés de produits laitiers contaminés. Ces isolats appartenaient à 19 genres différents, les plus fréquemment trouvés étant *Penicillium*, *Cladosporium*, *Mucor* et *Dydimella* pour les champignons filamenteux et *Candida*, *Trichosporon*, *Meyerozyma*, *Yarrowia* et *Kluyveromyces* pour les levures. Parmi ces genres, les espèces les plus retrouvées étaient *P. bialowiezense*, *P. commune*, *P. solitum* et *T. asahii*, *M. guilliermondi*, *C. inconspicua* et *Y. lipolytica* pour les champignons filamenteux et les levures respectivement. À l'exception de *P. bialowiezense*, ces espèces sont couramment retrouvées en tant que contaminants des produits laitiers (Pitt and Hocking, 2009). Cependant, *P. brevicompactum*, considéré comme un contaminant commun des produits laitiers et très proches génétiquement de *P. bialowiezense* aurait pu être confondu dans de précédentes études (Samson *et al.*, 2002). Cette proximité génétique pourrait expliquer l'absence de *P. bialowiezense* dans les différentes études sur la diversité des contaminants fongiques. Il est également important de noter que l'échantillonnage des produits contaminés n'était pas homogène. En effet, certains produits, tels que le beurre, la crème fraîche ou le lait pasteurisé, n'étaient représentés que par 1 ou 2 échantillons. Cette hétérogénéité est liée à la collecte des échantillons qui s'est faite par opportunité par les partenaires industriels en fonction des productions en cours au moment de l'échantillonnage. L'idéal aurait donc été d'avoir un même nombre d'échantillon pour chaque typologie de produit étudiée. Cependant, cet échantillonnage nous a tout de même permis d'avoir une bonne idée de la diversité des contaminants fongiques rencontrée dans les produits laitiers contaminés en France.

L'environnement laitier a également été étudié et a permis l'identification de 70 isolats dont 65 champignons filamenteux et 5 levures. La prédominance des champignons filamenteux était attendue puisque leurs spores sont facilement disséminées par l'air. Ces isolats appartenaient notamment aux genres *Penicillium* et *Cladosporium* qui sont également les genres les plus retrouvés dans les produits laitiers contaminés. Comme Kure *et al.* (2004) l'ont démontré, les résultats montraient donc que l'environnement était la source primaire de contamination des produits laitiers par les champignons filamenteux. Néanmoins, les partenaires industriels fournissant les échantillons d'ambiance, pour des raisons de confidentialité, n'étaient pas toujours en mesure de communiquer les zones spécifiques où avaient été prélevés ces échantillons d'ambiance, nous n'avons pas pu aller plus loin dans l'étude des sources et des flux de contaminations fongiques des produits laitiers. Il aurait été très intéressant de collecter des échantillons d'ambiance en parallèle d'échantillons issus des équipements et des surfaces, pour chaque pièce de chaque usine afin de pouvoir étudier la diversité fongique associée à l'environnement de chaque partenaire industriel. Ce type d'étude a été réalisé par Bokulich *et al.* (2013) qui ont étudié, grâce à des techniques de séquençage haut-débit, l'écosystème microbien de 2 usines fabriquant du fromage de manière artisanale. Ils ont ainsi pu montrer l'importance de la diversité microbienne de l'environnement dans l'établissement des communautés microbiennes des fromages. Ce type d'étude pourrait donc permettre, à terme, d'identifier les points critiques de contaminations pour chaque partenaire industriel.

Cette première étape nous a permis de faire un état des lieux de la diversité fongique rencontrée dans les produits laitiers et leur environnement mais elle nous a également permis de disposer d'une collection de référence de contaminants fongiques des produits laitiers. Au sein de cette collection, nous avons pu sélectionner 10 espèces (*C. halotolerans* UBOCC-A-116001, *G. geotrichum* UBOCC-A-216001, *M. racemosus* UBOCC-A-116002, *P. commune* UBOCC-A-116003, *D. pinodella* UBOCC-A-116004, *C. parapsilosis* UBOCC-A-216002, *M. guilliermondii* UBOCC-A-2016003, *R. mucilaginosa* UBOCC-A-216004, *T. asahii* UBOCC-A-216005 et *Y. lipolytica* UBOCC-A-216006) représentatives de la diversité des contaminations fongiques dans les produits laitiers. Ces 10 souches ont servi à l'étude de la résistance aux conservateurs chimiques couramment utilisés dans l'industrie laitière soit le benzoate de sodium (E 211), le sorbate de potassium (E 202), le propionate de calcium (E 282) et la natamycine (E 235). Cette étude a notamment révélé la forte résistance de *Y. lipolytica* à ces conservateurs, en prenant en compte les concentrations maximum autorisées dans le marché européen. En effet, *Y. lipolytica* a montré des concentrations minimales

inhibitrices supérieures à 3 g/l pour le propionate de calcium, le benzoate de sodium et à 1 mg/dm<sup>2</sup> pour la natamycine, toutes 3 étant supérieures aux concentrations maximales autorisées par la Regulation (CE) No 1129/2011. De plus, *Y. lipolytica* à une CMI de 1 g/l pour le sorbate de potassium dont les concentrations maximales autorisées sur le marché en Europe sont comprises entre 0,3 et 2 g/l. Ces résultats confirment une résistance naturelle des champignons aux conservateurs. En effet, des mécanismes de résistances ont déjà été démontrés par le passé. Ainsi, Ullah *et al.* (2013) ont montré que l'adaptation de *S. cerevisiae* au sorbate provenait de la diminution de son entrée (par diffusion) dans la cellule et que cette diminution pourrait être due à la modification de la structure ou de la composition de la membrane plasmique. Néanmoins, cette étude a été réalisée sur une seule souche par espèce et il serait donc intéressant d'étudier le comportement, vis-à-vis du développement de résistance, de plusieurs souches, d'origines différentes (différents produits laitiers ou autres produits alimentaires, traités ou non avec ces conservateurs) au sein d'une même espèce afin d'évaluer le caractère souche-dépendant de cette résistance et de déterminer si une adaptation à la présence d'un conservateur pourrait expliquer l'apparition de ces résistances. En effet, une étude récente de Streekstra *et al.* (2016) a montré que *T. asahii* était capable de développer une tolérance à la natamycine. En effet, lors de repiquages successifs à des concentrations croissantes de natamycine, *T. asahii* était capable de se développer en présence d'une concentration 225% supérieure à sa concentration minimale inhibitrice initiale. Ces résultats montrent par conséquent, l'importance de trouver des solutions alternatives à l'utilisation des conservateurs pour lutter contre les contaminations fongiques.

Au delà de cette notion de diversité et de résistance, cette première étape nous a également permis de sélectionner, sur la base de l'occurrence et de la représentation taxonomique des espèces, mais aussi en accord avec les partenaires industriels et la littérature, 4 isolats fongiques comme cibles représentatives pour la suite de l'étude, à savoir, *G. geotrichum* UBOCC-A-216001, *M. racemosus* UBOCC-A-116002, *P. commune* UBOCC-A-116003 et *Y. lipolytica* UBOCC-A-216006.

La deuxième partie de la thèse consistait, sur la base d'un criblage *in vitro*, à rechercher des microorganismes présentant une activité antifongique en matrice laitière (**Etudes 2 et 3 de la partie « Résultats »**). Pour cela, une sélection de 698 microorganismes a été réalisée au sein des collections de l'UBOCC, du LUBEM et du CIRM-BIA. Cette sélection était constituée de bactéries lactiques, propioniques et de champignons qui sont de bons candidats à la biopréservation. En effet, les bactéries lactiques et propioniques sont naturellement présentes



dans les produits laitiers (Pfeiler and Klaenhammer, 2007), elles ont un long historique d'utilisation sans risque dans l'alimentation humaine et sont connues pour produire une grande variété de composés antifongiques tels que des acides organiques, des acides gras ou des peptides (Crowley *et al.*, 2013 ; Leyva Salas *et al.*, 2017). De même, les champignons sont largement utilisés en tant que flores technologiques dans les produits laitiers et sont également connus pour produire des composés antifongiques tels que des molécules volatiles et des composés de nature protéique (Leyva Salas *et al.*, 2017). Ainsi, 430 bactéries lactiques appartenant aux genres *Lactobacillus*, *Lactococcus*, *Leuconostoc* et *Pediococcus*, 70 bactéries propioniques et 198 champignons dont 99 levures et 99 champignons filamenteux ont été sélectionnés pour être criblés dans le cadre de cette thèse.

Cette thèse s'étant focalisée sur la production d'ingrédients laitiers antifongiques, il était nécessaire de sélectionner un ou plusieurs substrats de fermentation, compatibles avec les produits laitiers et permettant la croissance des microorganismes sélectionnés. Ainsi, 2 substrats laitiers ont été sélectionnés à savoir un perméat d'ultrafiltration de lait (UF) supplémenté en extrait de levure pour permettre la croissance des microorganismes et un lait low heat reconstitué (LH) supplémenté en matière grasse, qui est potentiellement un précurseur dans la synthèse de composés antifongiques tels que les acides gras. Afin de valider ces 2 substrats laitiers, la croissance de 26 et 18 espèces représentatives de la sélection, appartenant à 8 et 15 genres de bactéries et de champignons respectivement, a été testée. La croissance de 100% des espèces fongiques et de 86% des espèces bactériennes testées dans les 2 substrats a été validée, validant de ce fait l'utilisation de ces 2 substrats de fermentation (données préliminaires à la thèse non présentées). Par conséquent, nous avons à cribler l'activité de 1396 fermentats/ingrédients, issus de la fermentation des substrats UF et LH par les 598 microorganismes sélectionnés, contre les 4 cibles fongiques sélectionnées précédemment (*G. geotrichum* UBOCC-A-216001, *M. racemosus* UBOCC-A-116002, *P. commune* UBOCC-A-116003 et *Y. lipolytica* UBOCC-A-216005). Afin de diminuer le nombre de faux-positifs ou -négatifs, dus notamment aux erreurs de manipulation, il était important de réaliser ces tests en duplicat. Ainsi, nous devons réaliser, au total, plus de 11000 essais nécessitant par conséquent la mise au point d'une méthode de criblage haut-débit (Chapitre 2 de la partie « Résultats »). De plus, des études récentes ont montré l'impact du milieu de criblage sur l'expression de l'activité antifongique. En effet, une perte significative de l'activité antifongique était souvent observée entre les études *in vitro* et *in situ* (Delavenne *et al.* 2014 ; Le Lay *et al.*, 2016). Travailler directement avec un produit laitier aurait donc été l'idéal mais représentait beaucoup trop de travail pour cette étape de criblage. Travailler dans

une matrice la plus proche possible des produits laitiers afin de faciliter, par la suite, le passage du criblage *in vitro* aux études *in situ*, nous paraissait donc être une bonne alternative. Nous avons donc développé un milieu mimant le fromage, constitué d'un rétentat d'ultrafiltration, emprésuré et ensemencé avec un ferment commercial acidifiant (concentration finale en matière grasse de 6% et pH de 4,5) et distribué en plaque de 24 puits. Les produits laitiers étant usuellement conservés à des températures réfrigérées, nous avons décidé d'incuber nos plaques, après inoculation avec les cibles fongiques, à 12°C, une température proche de celle des réfrigérateurs des consommateurs (environ 8°C) mais qui permet une croissance relativement rapide des champignons, qui sont, pour la plupart, psychrotrophes (Gougouli *et al.*, 2011). Il était cependant nécessaire de valider la croissance des 4 cibles fongiques avant de commencer le criblage afin de valider la matrice et les conditions d'incubation. De plus, cette validation nous a permis de définir les jours adéquats de lecture des résultats pour chaque cible fongique. En effet, selon la cible considérée, 5 à 8 jours d'incubation étaient nécessaires avant de voir apparaître un thalle ou une colonie sur la matrice. Finalement, cette méthode nous permettait de tester environ 200 fermentats par semaine. De plus, de par la nature de la matrice, il était également possible d'utiliser cette méthode de criblage pour d'autres applications, notamment pour le criblage de cultures bioprotectrices, application qui fait l'objet d'une seconde thèse dans le cadre du projet PROFIL. Une fois tous les paramètres validés, cette méthode nous a permis de tester, *in vitro*, le potentiel antifongique des 1396 fermentats en moins de 10 semaines.

Les résultats de ce criblage, présentés dans l'**étude 3 de la partie « Résultats »**, ont montré que le nombre de fermentats présentant une activité antifongique variait largement selon (i) le substrat de fermentation utilisé, (ii) la souche sélectionnée et (iii) la cible fongique considérée. En effet, les fermentats issus du milieu LH montraient des activités antifongiques plus intéressantes que ceux issus du milieu UF, excepté contre *Y. lipolytica*, qui était préférentiellement inhibée par les fermentats issus du milieu UF. Cela sous-entendait donc que, selon le substrat de fermentation utilisé, c'est-à-dire selon la composition du milieu de culture, une même souche était capable de produire des composés antifongiques différents. En effet, la matière grasse et les caséines présentes dans le milieu LH pourraient être des précurseurs d'acides gras ou de peptides antifongiques (Hidalgo *et al.*, 2015 ; Theolier *et al.*, 2013). Ces résultats confirmaient donc les études de Katarzyna *et al.* (2017) et Stiles *et al.* (2002) qui ont montré l'impact du substrat de fermentation sur l'expression de l'activité antifongique de microorganismes.

Indépendamment du substrat de fermentation, les souches les plus actives appartenaient principalement au genre *Lactobacillus* tels que *L. brevis*, *L. buchneri*, *L. plantarum* et *L. rhamnosus*. Comme discuté dans l'étude 3 de la partie « Résultats », toutes ces espèces sont connues pour produire des composés antifongiques variés, tels que des acides organiques ou des peptides (Leyva Salas *et al.*, 2017 ; Luz *et al.*, 2017) agissant de manière additive ou synergique. De plus, une souche de *P. jensenii* et 12 souches fongiques appartenant aux genres *Actinomucor*, *Aureobasidium*, *Candida*, *Exophiala*, *Mucor*, *Rhodotorula*, *Torulaspora*, *Trichoderma* et *Verticillium* ont également montré des activités antifongiques intéressantes. Ces résultats ne sont pas surprenants dans la mesure où les bactéries propioniques sont connues pour produire de l'acide propionique, du peroxyde d'hydrogène ou de l'acide phényllactique possédant une activité antifongique (Leyva Salas *et al.*, 2017 ; Fernandez *et al.*, 2017 ; Le Lay *et al.*, 2016). De même, les champignons produisent des composés antifongiques tels que des composés volatils, des enzymes lytiques ou des peptides (Leyva Salas *et al.*, 2017 ; Delgado *et al.*, 2016). Ensemble, et en accord avec de récentes études (Russo *et al.*, 2017 ; Le Lay *et al.*, 2016 ; Cortés-Zavaletta *et al.*, 2014), ces résultats soulignent le caractère souche-dépendant de l'activité antifongique puisqu'au sein d'une même espèce, les souches testées montrent des niveaux variables d'activité antifongique.

Les résultats du criblage ont également permis d'identifier des degrés variables de sensibilité des cibles fongiques. En effet, *G. geotrichum* UBOCC-A-216001 et *Y. lipolytica* UBOCC-A-216005 sont les souches les plus difficiles à inhiber alors que *P. commune* UBOCC-A-116003 est la souche la plus sensible aux activités des fermentats, indépendamment du substrat de fermentation ou des microorganismes considérés. Ces observations ont donc apporté la confirmation du caractère souche-dépendant de l'activité antifongique (Fernandez *et al.*, 2017 ; Le Lay *et al.*, 2016 ; Inglin *et al.*, 2015 ; Delavenne *et al.*, 2014). Ces résultats établissent également l'importance de bien définir le spectre d'activité des fermentats les plus efficaces lors du criblage, en évaluant une variété plus large et surtout représentative des contaminants fongiques des produits laitiers. C'est ainsi qu'ont été sélectionnées les cibles fongiques utilisées pour l'étude du spectre d'activité des fermentats, à savoir les 4 cibles utilisées pour le criblage et *C. parapsilosis* UBOCC-A-216002, *C. pseudolongus* 1f7b, *D. pinodella* UBOCC-A-116004, *M. guilliermondii* UBOCC-A-216003, *P. bialowiezense* 5f22, *R. mucilaginosus* UBOCC-A-216004 et *T. asahii* UBOCC-A-216005. Indépendamment du substrat, les souches les plus actives appartenaient aux espèces *L. brevis* (n=1), *L. buchneri* (n=6), *L. casei* (n=6), *L. harbinensis* (n=2), *L. plantarum* (n=4), *L. rhamnosus* (n=2) et *P. jensenii* (n=1) et leur spectre d'activité a donc été évalué pour les 2 substrats (UF et LH).

Par manque de temps, les fermentats issus de champignons n'ont malheureusement pas pu être testés.

Les résultats ont montré que certaines souches, telles que *L. rhamnosus* CIRM-BIA 1952, *L. harbinensis* L172, *L. buchneri* L164 et L169 étaient capables de produire des fermentats actifs contre 5 cibles fongiques différentes à partir du seul milieu LH. De même, les souches *L. brevis* L128 et *P. jensenii* CIRM-BIA 1774 ont produit des fermentats actifs contre 7 et 6 cibles respectivement, à partir du milieu UF. Au contraire, les souches *L. buchneri* L164 et *L. casei* L109 n'étaient capables d'inhiber qu'une seule cible à partir des milieux UF et LH respectivement. Encore une fois, ces résultats démontraient (i) l'impact du substrat de fermentation sur l'activité antifongique, (ii) le caractère souche-dépendant et (iii) cible-dépendant de cette activité et nous a permis de définir le spectre d'activité des fermentats testés. Enfin, ces résultats nous ont permis de faire une sélection de fermentats bactériens pertinents pour la suite de l'étude, à savoir les fermentats LH dérivant des souches *L. buchneri* L151, L162, L164 et L233, *L. harbinensis* L172, *L. paracsei* L117 et L194, *L. plantarum* L1244 et *L. rhamnosus* CIRM-BIA 1952 et les fermentats UF dérivant de *L. buchneri* L162 et *P. jensenii* CIRM-BIA 1774. A ces fermentats issus de bactéries, un fermentat fongique issu de la fermentation du milieu LH par *M. lanceolatus* UBOCC-A-109193 a été ajouté. Cette étude a été menée sur une seule souche de chaque espèce cible et il aurait pu être intéressant de tester plusieurs souches cibles, là encore, afin de savoir dans quelle mesure la sensibilité des cibles fongiques est, elle aussi, souche dépendante.

Il faut également noter que, pour obtenir les différents fermentats, chaque espèce de microorganismes a été cultivée dans les conditions optimales de croissance or il est maintenant avéré que les conditions de cultures peuvent avoir un impact sur l'activité antifongique des microorganismes (Delavenne *et al.*, 2015 ; Le lay *et al.*, 2016). Nous nous sommes donc demandé si l'activité antifongique des fermentats sélectionnés pouvait être optimisée en variant les conditions de fermentation (temps et température d'incubation) de chaque microorganisme (chapitre 3 de la partie « Résultats »). Les résultats obtenus ont montré qu'il n'y avait pas de règle générale pour optimiser l'activité antifongique des fermentats. Dans certains cas, le temps d'incubation pouvait augmenter ou diminuer l'activité antifongique, avec ou sans corrélation avec la concentration cellulaire. Dans d'autres cas, c'est la température qui avait un effet sur l'activité antifongique des fermentats. En effet, pour certains microorganismes, la production de composés antifongiques semble être plus importante en conditions de stress (choc thermique chaud ou froid). Ainsi, si les mécanismes liés à l'augmentation de l'activité antifongique des fermentats n'ont pas pu être expliqués

grâce à ces essais, ils ont néanmoins abouti à la sélection de 7 fermentats, à savoir *L. buchneri* L162 et L233, *L. harbinensis* L172, *L. paracasei* L117, *L. rhamnosus* CIRM-BIA 1952, *P. jensenii* CIRM-BIA 1774 et *M. lanceolatus* UBOCC-A-109193, et de leurs conditions optimales de culture pour des tests *in situ*.

L'évaluation *in situ*, sur produits issus du commerce, de l'activité antifongique des fermentats sélectionnés a ensuite été réalisée (**étude 3 de la partie « Résultats »**). Selon le type de produit laitier considéré, deux approches différentes ont été choisies pour l'utilisation de conservateurs. D'une part, l'intégration des substances actives directement dans le produit lors du processus de fabrication (ex : fromage blanc, crème fraîche,...) et d'autre part, l'application en surface par pulvérisation lors du processus de maturation des fromages (ex : fromages à pâte pressée non cuite). Par conséquent, afin de se rapprocher des conditions industrielles et d'évaluer la faisabilité d'utilisation de nos fermentats, ces deux approches ont été testées, lors d'une étape intermédiaire, dans 2 produits issus du commerce : un fromage blanc et un fromage à pâte pressée à croûte morgée. En effet, pour valider notre méthode (concentrations, mode et nombre d'application,...) et nous assurer que l'activité observée *in vitro* était globalement conservée *in situ* avant de passer à l'échelle pilote, il était nécessaire de tester préalablement nos fermentats. L'incorporation des fermentats dans la masse a été testée dans un fromage blanc, inoculé par *M. racemosus* UBOCC-A-116002 et *P. commune* UBOCC-A-116003 à la surface et par *R. mucilaginosa* UBOCC-A-216004 dans la masse, et la pulvérisation a été testée sur un fromage à pâte pressée non cuite à croûte morgée contaminé en surface par *M. racemosus* UBOCC-A-116002 et *P. commune* UBOCC-A-116003 (données supplémentaires – chapitre 3 de la partie « Résultats »).

L'incorporation dans la masse, à 1 et 2%, de 3 fermentats issus de *L. paracasei* L117 (LH), *L. rhamnosus* CIRM-BIA 1952 (LH) et *P. jensenii* CIRM-BIA 1774 (UF), seuls ou en couple et sur 1 fermentat issu de la co-culture (LH) de *L. harbinensis* L172 et *S. thermophilus* CIRM-BIA 16. Cette association résulte de données issues d'une étude antérieure, réalisée au LUBEM, où la co-culture entre *L. harbinensis* et *S. thermophilus* avait montré des résultats intéressants (Delavenne et al., 2014). Les résultats obtenus ont montré que tous les fermentats présentaient une activité contre au moins 1 des cibles fongiques dans le fromage blanc et que cette activité augmentait avec la concentration de fermentat incorporée.

Pour l'application par pulvérisation, avec 1 ou 3 pulvérisations, les 7 fermentats ou associations de fermentats cités ci-dessus ont également été testés. En plus, les fermentats LH issus de *L. buchneri* L233, L162, *M. lanceolatus* UBOCC-A-109193 ont été testés seuls et les associations de *M. lanceolatus* UBOCC-A-109193 (LH) avec les fermentats *L. buchneri* L233

(LH), *L. rhamnosus* CIRM-BIA 1952 (LH), *L. paracasei* L117 (LH), *P. jensenii* CIRM-BIA 1774 (UF) et les associations des fermentats LH *L. paracasei* L117 avec *L. buchneri* L233 et *L. rhamnosus* CIRM-BIA 1952 avec la co-culture (LH) *L. harbinensis* L172 - *S. thermophilus* CIRM-BIA 16, ont également été testés. Les résultats ont montré un effet dose important. En effet, avec une seule pulvérisation, aucun effet antifongique n'a été observé alors qu'avec 3 pulvérisations, 81,3% des fermentats (13/16) présentaient une activité contre au moins une des cibles fongiques.

Pris dans leur ensemble, ces résultats montrent que le choix de réaliser le criblage sur une matrice proche des produits laitiers était important puisque 100% et 81% fermentats, testés dans le fromage blanc et sur le fromage à pâte pressée à croûte morgée respectivement, se sont révélés être actifs contre au moins une cible fongique. Enfin, cette étape intermédiaire nous a permis de valider les conditions d'utilisation. Elle nous a également permis de sélectionner 2 fermentats issus de *L. rhamnosus* CIRM-BIA 1952 (LH) et *P. jensenii* CIRM-BIA 1774 (UF), seuls et en association, pour l'incorporation dans une matrice type fromage blanc, et 1 fermentat issu de *P. jensenii* CIRM-BIA 1774 (UF) ainsi que 2 associations (*M. lanceolatus* UBOCC-A-109193 [LH] associé à *P. jensenii* CIRM-BIA 1774 [UF] ou à *L. rhamnosus* CIRM-BIA 1952 [LH]) pour la pulvérisation sur une matrice type fromage à pâte pressée à croûte morgée, pour la validation à l'échelle pilote.

Cette validation a été réalisée en testant les fermentats cités précédemment dans le cadre d'une production de crème fraîche et de fromage à pâte pressée à croûte morgée à l'échelle pilote afin de valider définitivement l'efficacité, mais aussi la faisabilité et l'acceptabilité des fermentats en condition de production industrielle. Cette étape a été réalisée par la mise en place de challenge-tests et de tests d'usage, pour des contaminations présentes naturellement dans l'environnement, complétés par une analyse sensorielle. Les 6 fermentats ou association de fermentats sélectionnés se sont révélés actifs contre au moins une cible dans les deux matrices lactiques. En effet, dans la crème fraîche et dans le cadre des challenge-tests, la présence du fermentat *P. jensenii* CIRM-BIA 1774, à 5%, inhibe totalement la croissance de *M. racemosus* UBOCC-A-116002, *P. commune* UBOCC-A-116003 et *R. mucilaginosa* UBOCC-A-216004 pendant 3 semaines, soit les mêmes résultats que ceux obtenus avec le sorbate de potassium. De même, aucune contamination d'origine environnementale n'a été observée lors des tests d'usage. Concernant *L. rhamnosus* CIRM-BIA 1952, aucun retard significatif de croissance n'a été observé lors des challenge-tests. En revanche, il permet une diminution importante du niveau de contamination naturelle. En ce qui concerne les essais sur fromage à pâte pressée, encore une fois, tous les fermentats testés se sont révélés actifs

lors des challenge-tests et des tests d'usage. Il faut cependant noter que, dans le cas des fromages à pâte pressée, un seul type de pulvérisation (3 pulvérisations à J+1, J+3 et J+6 après morgeage) a été testé sans étape d'optimisation. Il serait donc intéressant de pouvoir tester différentes méthodes d'application (nombre d'applications, temps entre 2 pulvérisations) afin d'optimiser l'activité antifongique des fermentats. De plus, il est important de souligner que les fermentats testés n'ont pas montré d'impact négatif sur le développement de la morge des fromages. Une application industrielle peut donc tout à fait être envisagée pour ce type de fermentats avec, qui plus est, une optimisation possible en fonction des technologies considérées.

Au delà de l'aspect antifongique, il nous a fallu vérifier que nos fermentats n'avaient pas d'impact sur les qualités organoleptiques des crèmes fraîches et des fromages à pâte pressée à croûte morgée. Pour ce faire, nous avons réalisé des tests sensoriels en suivant la méthodologie mise en place par Cadoret *et al.* (2009) (**étude 3 de la partie « Résultats »**). En ce qui concerne les fromages à pâte pressée à croûte morgée, aucun des fermentats testés n'a montré d'impact sur les qualités organoleptiques du produit. Cela peut entre autre s'expliquer par le fait que le fermentat est appliqué à la surface du produit, sur la croûte du fromage que certains panélistes n'avaient pas consommé. Ces résultats montrent également que le fermentat ne diffuse pas suffisamment dans le fromage pour être détecté par les consommateurs. Ces travaux confirment donc que ces fermentats, dans ces conditions, sont une solution envisageable pour une application en industrie laitière.

En revanche, en ce qui concerne la crème fraîche, la présence du fermentat *P. jensenii*, à 2 et 5%, et du fermentat *L. rhamnosus*, à 5%, entraîne un fort impact négatif sur le goût et l'odeur et sur la texture du produit respectivement. A 2%, les crèmes contenant le fermentat *L. rhamnosus* sont confondues avec les crèmes fraîches témoins, ce qui signifie qu'à cette concentration, le fermentat *L. rhamnosus* n'a pas d'impact sur les qualités organoleptiques de la crème fraîche mais montre en revanche une activité antifongique faible lors des challenge-tests. Cependant, les résultats des tests d'usage sont très encourageants puisque l'activité de ce fermentat sur des contaminants de l'environnement est notable et significative. Ce fermentat peut donc être envisagé comme solution de bio-préservation, éventuellement en association avec d'autres méthodes ou d'autres fermentats afin de garantir la conservation de certains produits laitiers. Concernant les fermentats issus de *P. jensenii*, malgré une activité antifongique aussi efficace que celle des conservateurs, une utilisation, dans ces conditions, n'était pas envisageable à cause de son impact très négatif sur les qualités organoleptiques du produit. Des tests supplémentaires ont donc été réalisés dans le cadre de cette étude afin de

trouver le seuil à partir duquel l'impact organoleptique devenait acceptable, tout en conservant une activité antifongique intéressante.

Pour ce faire, une seconde analyse sensorielle a donc été mise en place afin de déterminer le seuil d'acceptabilité des fermentats *P. jensenii* CIRM-BIA 1774 dans les crèmes fraîches (Chapitre 3 de la partie « Résultats »). Les résultats ont montré qu'incorporé à la crème fraîche à raison de 0,1%, plus de 90% des consommateurs ne détectaient plus le fermentat et qu'à 0,4%, près de la moitié des panélistes ne le détectaient pas non plus. Ces 2 concentrations ont donc été retenues afin d'évaluer l'activité antifongique du fermentat *P. jensenii* CIRM-BIA 1774 à ces concentrations. Pour ce faire, des challenge-tests, sur les 3 cibles fongiques précédemment utilisées, et des tests d'usage ont été effectués. Lors des challenge-tests, l'activité antifongique des fermentats était plus faible pour ces 2 concentrations mais des retards de croissance de 1 à 4 jours ont tout de même été observés. En revanche, les tests d'usage ont permis de montrer que le fermentat de *P. jensenii* CIRM-BIA 1774 à 0,4% diminuait de manière significative le niveau de contamination d'origine naturelle. Par conséquent, ce type de solution pourrait être envisagée en complément d'autres moyens de conservation mais ne peut pas être utilisée seule comme le laissait présager les premiers tests. En ce qui concerne les différences observées entre les challenge-tests et les tests d'usage, elles pourraient s'expliquer par les différences de mode et de taux d'inoculation des contaminants fongiques mais aussi des espèces en présence. En effet, dans le cas des challenge-tests, 50 spores étaient déposées en surface (ou 2 UFC/g incorporés dans la masse pour les levures) ce qui représente un niveau de contamination extrêmement élevé et très supérieur aux contaminations "naturelles" de produit usuellement observées (une dizaine de spores ou cellule sur ou dans le produit tout au plus). On pourrait donc supposer que l'activité antifongique des fermentats observée lors des tests d'usage nous donne une meilleure idée de son activité potentielle en conditions réelles que celle observée lors des challenge-tests.

Enfin, pour qu'un ingrédient/fermentat puisse être utilisé dans le commerce, il ne doit pas présenter de risque pour la santé humaine. En effet, l'innocuité des souches sélectionnées doit être garantie pour une utilisation en industrie agroalimentaire. Ainsi, comme indiqué par l'approche QPS, la résistance acquise aux antibiotiques et la production d'amines biogènes ont été testées pour chaque microorganisme sélectionné. Ces travaux ont été réalisés par Mégane Lebreton, stagiaire de M2, sous la responsabilité de Monika Coton. Les résultats obtenus font partie d'un article soumis dans International Journal of Microbiology intitulé « *Safety assessment of potential antifungal lactic acid bacteria and propionibacteria* », présenté en annexe 1.



Ensemble, ces résultats sont une preuve de concept pour les partenaires industriels utilisateurs qui doivent maintenant s'approprier ces fermentats. En effet, le processus de fabrication de ces fermentats pourrait être optimisé en utilisant par exemple un séchage par atomisation plutôt qu'une lyophilisation pour les fermentats bactériens ou encore en utilisant des méthodes de filtration mécanique plutôt que manuelle pour le surnageant de champignon. D'autre part, les partenaires industriels vont également devoir adapter l'application de ces fermentats aux processus de fabrication des différents produits laitiers. Enfin, le coût de production de ces fermentats est également un point important pour les partenaires industriels, or, le fait d'avoir travaillé en collaboration, pour le choix des matrices laitières à utiliser comme substrat de fermentation, mais aussi pour le choix des souches et des cibles, nous a permis de prendre en compte cet élément et d'anticiper le coût d'une production industrielle.

Enfin, la 4<sup>ème</sup> partie de cette thèse consistait à identifier les molécules impliquées dans l'activité antifongique des fermentats et à en comprendre les mécanismes d'action (**Etude 4 de la partie « Résultats »**). Différentes méthodes ont donc été mises en place afin d'identifier les molécules potentiellement antifongiques, notamment les acides organiques (HPLC, LC-Q-ToF) dont les acides gras libres (GC), les composés volatils (Head-space trap GC/MS [Chromatographie en phase gazeuse couplée à la spectrométrie de masse]) et les peptides (nano LC-MS/MS). Au total, 64, 61 et 51 molécules, connues pour leur activité antifongique, ont été identifiées, par HPLC et LC-Q-ToF, dans les fermentats *M. lanceolatus* UBOCC-A-109193, *P. jensenii* CIRM-BIA 1774 et *L. rhamnosus* CIRM-BIA 1952 respectivement. Parmi ces molécules, on retrouve notamment les acides lactique et succinique à 86,0 mg/g et 5,2 mg/g respectivement, dans le fermentat *L. rhamnosus* CIRM-BIA 1952, les acides propionique, citrique et acétique à 59,9 mg/g, 32,5 mg/g et 16,9 mg/g respectivement, dans le fermentat *P. jensenii* CIRM-BIA 1774 et l'acide citrique à 2,5 mg/g dans le fermentat *M. lanceolatus* UBOCC-A-109193. A cela s'ajoutent d'autres molécules en plus faibles quantités et pouvant agir de manière additive ou synergique notamment les acides 2-hydroxyisocaproïque, 3-(4-hydroxyphényl)propionique, hydroxyphényllactique et phényllactique et le mévalonolactone dont la somme des concentrations étaient de 1,96 mg/g chez *P. jensenii* CIRM-BIA 1774.

De plus, 50, 47 et 46 molécules (acides gras libres et composés volatils) potentiellement antifongiques ont été identifiées par GC et GC-MS dans ces 3 mêmes fermentats. Parmi ces molécules on retrouvait notamment des acides tels que les acides propionique (1 mg/g) et acétique (0,5 mg/g) dans le fermentat *P. jensenii* CIRM-BIA 1774 et les acides butyrique (0,4

mg/g) et palmitique (50 µg/g) identifiés dans les fermentats *M. lanceolatus* UBOCC-A-109193 et *L. rhamnosus* CIRM-BIA 1952 respectivement. Concernant les composés volatils, le diacétyl ou l'éthyl acetate peuvent également avoir une activité antifongique (Aunsbjerg *et al.*, 2015 ; Coda *et al.*, 2011). Ces composés ont été identifiés en utilisant une méthode semi-quantitative donnant leur abondance relative. Le diacétyl était présent dans les 3 fermentats étudiés à des abondances relatives de  $1,07 \cdot 10^8$ ,  $1,36 \cdot 10^9$  et  $7,45 \cdot 10^8$  pour *P. jensenii* CIRM-BIA 1774, *L. rhamnosus* CIRM-BIA 1952 et *M. lanceolatus* UBOCC-A-109193 respectivement. De même, l'éthyl acétate a été retrouvé dans les 3 fermentats et pourrait également jouer un rôle dans leur activité antifongique. D'autres composés volatils ont été identifiés grâce à cette méthode. Par exemple l'éthyl butyrate et le 3-méthyl 1-butanol, 2 agents aromatiques dont l'activité antifongique n'est pas connue, ont également été identifiés dans les fermentats. Il serait donc intéressant de pouvoir quantifier ces différents composés pour ensuite pouvoir tester leur activité antifongique et ainsi avoir une idée de leur rôle respectif dans l'activité des fermentats. Finalement, bien que la nature de ces composés diffère peu entre les fermentats, les concentrations quant à elles, sont bien différentes et pourraient en partie expliquer les différents niveaux d'activité antifongique entre les fermentats.

Comme discuté dans la revue de littérature de Leyva Salas *et al.* (2017), certains peptides sont aussi connus pour avoir une activité antifongique. Grâce à une approche non ciblée utilisant une méthode de nano LC-MS/MS, 22, 253 et 853 peptides ont été identifiés dans les fermentats *P. jensenii* CIRM-BIA 1774, *L. rhamnosus* CIRM-BIA 1952 et *M. lanceolatus* UBOCC-A-109193, respectivement. Sur la base d'une comparaison avec les peptides antifongiques référencés dans les bases de données et de leur abondance relative, 3, 6 et 7 peptides issus respectivement de ces 3 mêmes fermentats et non présents dans les milieux LH et UF non fermentés, ont été sélectionnés et néosynthétisés afin de tester leur activité antifongique *in vitro* contre le champignon filamenteux *M. racemosus* UBOCC-A-109155 et la levure *R. mucilaginosa* UBOCC-A-216004. Parmi ces 16 peptides, un seul a montré une activité antifongique. Il s'agit du peptide pepa4c177 issu du fermentat *L. rhamnosus* CIRM-BIA 1952, composé de 9 acides aminés (RLNFLKKIS), correspondant à un hydrolysate de la caséine  $\alpha$ -s2 et actif contre les 2 cibles fongiques à 2 et 5 mg/ml. Il est important de noter que cette méthode ne nous a pas permis de rechercher les dipeptides cyclique. Or, les bactéries lactiques sont connues pour en produire et leur activité antifongique a été démontrée (Crowley *et al.*, 2013). Par exemple, le dipeptide cyclique cyclo(L-Phe-L-Pro), produit par *L. plantarum*

est actif contre *P. roquefortii* avec une CMI de 20 mg/ml (Ström *et al.*, 2002). Il serait donc intéressant de pouvoir rechercher ces dipeptides cycliques dans les différents fermentats.

Ensemble, ces résultats montrent que, comme le suggère Crowley *et al.* (2013), l'activité antifongique des fermentats repose certainement sur l'action additive ou synergique de ces nombreux composés. Cependant, d'autres analyses et essais sont nécessaires afin de déterminer le rôle de chacun des composés dans l'activité antifongique de chaque fermentat et des études sont en cours afin de comprendre les mécanismes d'action liés à ces fermentats. Cette étude nous a donc permis d'identifier les différentes molécules antifongiques ou potentiellement antifongiques dans les différents fermentats mais ne nous a pas permis d'évaluer leur rôle respectif dans l'activité des fermentats. Pour clarifier cet aspect, il serait intéressant de déterminer les CMI pour les molécules identifiées contre les différentes cibles fongiques utilisées dans les études précédentes. De plus, il serait également intéressant de tester des combinaisons de différentes molécules, aux concentrations identifiées, afin d'avoir une idée de leur part dans l'activité antifongique des fermentats.

Enfin, la compréhension des mécanismes d'action liés aux fermentats est également importante. Pour ce faire, l'étude de l'impact du fermentat *P. jensenii* (montrant les activités antifongiques les plus importantes) sur la germination et la croissance radiale de *M. racemosus* UBOCC-A-109155 et sur la croissance de *R. mucilaginosa* UBOCC-A-216004 a été mise en place (**Etude 4 de la partie « Résultats »**). Différentes concentrations (0, 2 et 5%) du fermentat dérivant de *P. jensenii* CIRM-BIA 1774 ont été appliquées et la croissance des deux champignons a été affectée à différentes intensités. Concernant *M. racemosus* UBOCC-A-109155, à 2 et 5%, sa croissance était ralentie mais aucune inhibition complète n'a été observée. Ces résultats montraient que le fermentat *P. jensenii* CIRM-BIA 1774 semble affecter la croissance radiale de *M. racemosus* UBOCC-A-109155 plutôt que sa germination. Cependant, des changements morphologiques significatifs ont été observés sur les spores en germination, notamment au niveau des tubes germinatifs, et non sur le mycélium. Dans la littérature, il a été montré que l'application d'acide phényllactique (présent à 0,5 mg/g dans le fermentat *P. jensenii* CIRM-BIA 1774) à des concentrations sub-létales menait à la diminution du nombre de spores germées et de la longueur du tube germinatif (Coloretti *et al.*, 2007). Ainsi, l'acide phényllactique pourrait avoir un rôle dans l'activité antifongique du fermentat *P. jensenii* CIRM-BIA 1774 contre *M. racemosus* UBOCC-A-116002 (utilisé lors du criblage) et UBOCC-A-109155 (utilisé pour cette étude car son génome est séquencé). Concernant *R. mucilaginosa* UBOCC-A-216004, son temps de latence était rallongé dès une concentration de 2% et un effet fongistatique était observé à 5%.

Contrairement à *M. racemosus* UBOCC-A-109155, les cellules de *R. mucilaginosa* UBOCC-A-216004 ne présentaient aucun changement morphologique visible au microscope à contraste de phase à une concentration de 2% du fermentat. Quant à l'inhibition totale à 5%, elle peut être expliquée par le fait qu'à cette concentration de fermentat, la concentration en propionate est estimée à environ 3g/L, or selon Lind *et al.* (2005), la concentration minimale inhibitrice du propionate pour *R. mucilaginosa* est de 1,85 g/L à pH 5 montrant ainsi que le propionate est la principale molécule agissant contre cette levure. Cependant, il ne faut pas négliger la présence d'acide phenyllactique et d'acide acétique dont l'activité antifongique contre *R. mucilaginosa* a déjà été démontrée (Cortés-Zavaleta *et al.*, 2014; Schwenninger and Meile, 2004). Ces résultats apportent donc quelques éléments de réponse afin de mieux comprendre les mécanismes d'action du fermentat *P. jensenii* CIRM-BIA 1774 mais des approches complémentaires, telles que la transcriptomique, sont actuellement mises en place afin d'identifier l'impact du fermentat, au niveau moléculaire, sur *M. racemosus* UBOCC-A-109155 et *R. mucilaginosa* UBOCC-A-216004. En effet ce type de méthode, déjà mise en place par Muñoz *et al.* (2013) et Wang (2017) qui ont étudié respectivement la réponse transcriptionnelle de cibles fongiques à un peptide antifongique et à des composés antifongiques dérivés de plantes, nous permettrait d'avoir une vision globale des changements dans le profil d'expression des gènes de *M. racemosus* UBOCC-A-109155 en réponse à une exposition à différentes concentrations du fermentat *P. jensenii* CIRM-BIA 1774.

Finalement, l'objectif finalisé de cette thèse était de développer des ingrédients antifongiques 100% laitiers grâce à l'action de microorganismes sur des fractions laitières. Les résultats ont montré que le fermentat UF dérivant de *P. jensenii* CIRM-BIA 1774 présentait l'activité antifongique la plus intéressante. En effet, il a montré de fortes activités antifongiques *in vitro* et *in situ* contre *P. commune*, *M. racemosus* et *R. mucilaginosa*. Les essais en produits réels ont montré que ce fermentat était compatible avec différentes typologies de produits laitiers (crème fraîche et fromage à pâte pressée à croûte morgée). Enfin, bien que les autres fermentats sélectionnés, ou leurs associations, aient montré une activité antifongique moins importante lors des challenge-tests, leur activité lors des tests d'usage était intéressante et ils pourraient donc être utilisés en complément d'autres méthodes.

Enfin, d'un point de vue fondamental, les résultats de l'**étude 1** nous ont permis de montrer que la diversité des contaminants fongiques des produits laitiers et de leur environnement est très importante, que l'environnement est la source principale de ces contaminations et que la résistance aux conservateurs est une problématique réelle et actuelle. Les résultats des **études**

**2 et 3** ont montré que des souches de bactéries lactiques, propioniques et fongiques étaient capables de générer des ingrédients antifongiques à partir de la fermentation d'un substrat laitier et que cette activité est souche-dépendante, cible-dépendante et substrat-dépendante. Ces résultats ont également montré que le fait de cribler les fermentats dans des conditions proches de leurs conditions d'utilisation finales permettait de faciliter le changement d'échelle et ensemble, ces résultats ont permis d'obtenir de nouvelles solutions de biopréservation. Enfin, les résultats de l'**étude 4** ont permis d'identifier les principales molécules antifongiques et potentiellement antifongiques présentes dans les fermentats retenus, à savoir *P. jensenii* CIRM-BIA 1774, *L. rhamnosus* CIRM-BIA 1952 et *M. lanceolatus* UBOCC-A-109193. De plus, l'étude du mécanisme d'action du fermentat *P. jensenii* CIRM-BIA 1774 nous a permis de montrer l'effet fongistatique du fermentat sur *R. mucilaginosa* et *M. racemosus* ainsi que son impact sur la germination des spores de *M. racemosus*. Finalement, cette étude est, à notre connaissance, la première qui présente toutes les étapes (de l'étude de la diversité des contaminants fongiques des produits laitiers puis du criblage *in vitro* aux applications à l'échelle pilote pour enfin s'intéresser aux molécules antifongiques et aux mécanismes d'action mis en jeu) du développement d'un ingrédient laitier antifongique. Pour terminer, cette étude ouvre de nombreuses questions concernant notamment les mécanismes de résistance des champignons aux différents conservateurs, sur la souche-dépendance de la sensibilité aux fermentats, sur le rôle des différentes molécules identifiées dans les fermentats ainsi que sur les mécanismes d'action de ces fermentats.

*D'un point de vue personnel, ce travail collaboratif entre partenaires académiques et industriels a été très enrichissant. En effet, cette thèse m'a permis d'acquérir une expérience en recherche appliquée et fondamentale tout en ayant une vision globale des contraintes liées aux applications industrielles, telles que des questions de coûts de production, de sécurité sanitaire et de réglementation. Ce partenariat m'a également permis de présenter mes travaux à différents publics: scientifiques spécialistes et non-spécialistes et industriels mais aussi politiques ou totalement néophytes me donnant ainsi les clés pour une communication réussie, peu importe le public. Enfin, la complémentarité des partenaires et l'ensemble des personnes impliquées m'ont permis de mener et de terminer ce projet dans les temps tant du point de vue fondamental que de l'applicabilité industrielle.*

# **Références**

# **bibliographiques**



## ~ A ~

---

- Aarestrup, F. M., 2005. Veterinary drug usage and antimicrobial resistance in bacteria of animal origin. *Basic. Clinical. pharmacol. toxicol.*, 96, 271-281.
- Acosta, R., Rodríguez-Martín, A., Martín, A., Núñez, Asensio, M. A., 2009. Selection of antifungal protein-producing molds from dry-cured meat products. *Int. J. Food Microbiol.* 135, 39-46.
- Ainsworth, G.C., 1973. Introduction and keys to higher taxa, in: Ainsworth, G.C., Sparrow, F.K., Sussman, A.S. (Eds.), *The Fungi: An advanced treatise*. Academic Press, London, UK, pp. 1-7
- Alexopoulos, C.J., Mims, C.W., Blackwell, M., 1996. *Introductory mycology*. John Wiley and Sons, New York, USA, p. 869.
- Almena-Aliste, M., Mietton, B., 2014. Cheese classification, characterization and categorization: a global perspective, in: Donnelly, C.W. (Ed.), *Cheese and Microbes*. ASM Press, Washington, USA, pp. 39-71.
- Ammor, M. S., Mayo, B., 2007. Selection criteria for lactic acid bacteria to be used as functional starter cultures in dry sausage production: An update. *Meat Science*, 76, 138-146.
- Ampuero, S., Bosset, J., 2003. The electronic nose applied to dairy products: a review. *Sensors and Actuators B: Chemical* 94, 1-12.
- Anderson, I. C., Cairney, J. W. G., 2004. Diversity and ecology of soil fungal communities: increased understanding through the application of molecular techniques. *Env. Microbiol.* 6(8), 769-779.
- Andrighetto, C., De Dea, P., Lombardi, A., Neviani, E., Rossetti, L., Giraffa, G., 1998. Molecular identification and cluster analysis of homofermentative thermophilic lactobacilli isolated from dairy products. *Research microbiol.* 149(9), 631-643.
- Avise, J.C., Ball, R.M.J., 1990. Principles of genealogical concordance in species concepts and biological taxonomy. *Oxf. Surv. Evol. Biol.* 7, 45-67.
- Axelsson, L., 2004. Lactic acid bacteria: Classification and physiology. In Salminen, S., Wright, A.V., Ouwehand, A. (Eds.), *Lactic acid bacteria: microbiology and functional aspects*, 3e Ed. Marcel Dekker, Inc, New-York, USA, 1-66.

## ~ B ~

---

- Baldauf, S.L., 2008. An overview of the phylogeny and diversity of eukaryotes. *J. Syst. Evol.* 46, 263-273.
- Baldauf, S.L., Doolittle, W.F., 1997. Origin and evolution of the slime molds (Mycetozoa). *Proc. Natl. Acad. Sci.* 94, 12007-12012.
- Banjara, N.; Suhr, M.J.; Hallen-Adams, H.E., 2015. Diversity of yeast and mold species from a variety of cheese types. *Curr. Microbiol.*, 70, 792-800.
- Barlowska, J., Sz wajkowska, M., Litwińczuk, Z., Król, J., 2011. Nutritional value and technological suitability of milk from various animal species used for dairy production. *Comp. Rev. Food Sci. and Food Safety*, 10(6), 291-302.
- Bass, D., Richards, T.A., 2011. Three reasons to re-evaluate fungal diversity “on Earth and in the ocean.” *Fungal Biol. Rev.* 25, 159-164.
- Béal, C., and Sodini, I., 2003. Fabrication des yaourts et des laits fermentés. *Techniques de l'ingénieur, Bioprocédés F6 315*, 1-16.



- Bengtson, S., Rasmussen, B., Ivarsson, M., Muhling, J., Broman, C., Marone, F., Stampanoni, M., Bekker, A., 2017. Fungus-like mycelia fossils in 2.4-billion-year-old vesicular basalt. *Nat. Ecol. Evol.* 1,
- Beimforde, C., Feldberg, K., Nylinder, S., Rikkinen, J., Tuovila, H., Dörfelt, H., Gube, M., Jackson, D.J., Reitner, J., Seyfullah, L.J., Schmidt, A.R., 2014. Estimating the Phanerozoic history of the Ascomycota lineages : Combining fossil and molecular data. *Molecul. Phylogen. Evol.* 78, 386-398.
- Beresford, T.P., Fitzsimons, N.A., Brennan, N.L., Cogan, T.M., 2001. Recent advances in cheese microbiology. *Int. Dairy J.* 11(4-7), 259-274.
- Bernardeau, M., Guguen, M. and Vernoux, J.P., 2006. Beneficial lactobacilli in food and feed: long-term use, biodiversity and proposals for specific and realistic safety assessments. *FEMS Microbiol. Rev.* 30, 487–513.
- Berthier, F., Beuviel, E., Dasen, A., Grappin, R., 2001. Origin and diversity of mesophilic lactobacilli in Comté cheese, as revealed by PCR with repetitive and species-specific primers. *Int. Dairy J.* 11(4), 293–305.
- Besl, H., Bresinsky, A., 1997. Chemosystematics of *Suillaceae* and *Gomphidiaceae* (suborder *Suillineae*). *Plant Syst. Evol.* 206, 223–242.
- Blackwell, M., Hibbett, D.S., Taylor, J.W., Spatafora, J.W., 2006. Research coordination networks: a phylogeny for kingdom Fungi (Deep Hypha). *Mycolog.* 98(6), 829-837.
- Bokulich, N.A., Mills, D.A., 2013. Facility-specific “House” microbiome drives microbial landscape of artisan cheesemaking plants. *App. Env. Microbiol.* 79(17):5214.
- Bourdichon, F., Casaregola, S., Farrokh, C., Frisvad, J. C., Gerds, M. L., Hammes, W. P., Harnett, J., Huys, G., Laulund, S., Ouwehand, A., Powell, I.B., Prajapati, J.B., Seto, Y., Schure, E.T., Van Boven, A., Vankerckhoven, V., Zgodá, A., Tuijelaars, S., Bech Hansen, E., 2012. Food fermentations: Microorganisms with technological beneficial use. *Int. J. Food Microbiol.* 154, 87–97.
- Boutrou, R., Guéguen, M., 2005. Interests in *Geotrichum candidum* for cheese technology. *Int. J. Food Microbiol.* 102(1), 1-20.
- Brillet, A., Pilet, M. F., Prevost, H., Cardinal, M., Leroi, F., 2005. Effect of inoculation of *Carnobacterium divergens* V41, a biopreservative strain against *Listeria monocytogenes* risk, on the microbiological, chemical and sensory quality of cold-smoked salmon. *Int. J. Food Microbiol.* 104, 309-324.
- Brul, S. and Coote, P., 1999. Preservative agents in foods: Mode of action and microbial resistance mechanisms. *Int. J. Food Microbiol.* 50, 1–17.

~ C ~

- 
- Cadoret, M., Lê, S., Pagès, J., 2009. A Factorial Approach for Sorting Task data (FAST). *Food Qual. Pref.* 20(6), 401-417.
- Carreira, A., Ferreira, L. M., Loureiro, V., 2001. Brown pigments produced by *Yarrowia lipolytica* result from extracellular accumulation of homogentisic acid. *App. Env. Microbiol.* 67(8), 3463–3468.
- Carbone, I., Kohn, L.M., 1999. A method for designing primer sets for speciation studies in filamentous ascomycetes. *Mycolog.* 91, 553.
- Cavalier-Smith, T., 1998. A revised six-kingdom system of life. *Biol. Rev.* 73, 203–266.
- Cerning, J., Gripon, J. C., Lamberet, G., Lenoir, J. 1987. Biochemical activities of *Penicillium* used in cheese making. *Le Lait*, 67, 3–39.

- Chamba, J.-F., Irlinger, F., 2004. Secondary and adjunct cultures. In Cheese: Chemistry, Physics and Microbiology (Fox, P. F., McSweeney, P. L. H., Cogan, T. M., Guinee, T. P., Eds.), Vol. 2, pp. 191-206.
- Chamba, J. F., Perreard, É. 2002. Contribution of propionic acid bacteria to lipolysis of Emmental cheese. *Le Lait*, 82, 33-44.
- Chandi, G. K., Gill, B. S. 2011. Production and characterization of microbial carotenoids as an alternative to synthetic colors: a review. *Int. J. Food Properties*, 14(3), 503-513.
- Chávez, R., Fierro F., García-Rico, R. O., Laich, F. 2011. Mold-fermented foods: *Penicillium* spp. as ripening agents in the elaboration of cheese and meat products. In *Mycofactories* (Leitã, A. L., Ed.), pp. 73-98.
- CNIEL, 2017. L'économie laitière en chiffres, Edition 2017, CNIEL.
- Cocolin, L., Ercolini, D., 2015. Zooming into food-associated microbial consortia: a "cultural" evolution. *Curr. Opinion Food Sci.* 2, 43-50.
- Coda, R., Rizello, C.G., Di Cagno, R., Trani, A., Cardinali, G., Gobbetti, M., 2013. Antifungal activity of *Meyerozyma guilliermondii*: Identification of active compounds synthesized during dough fermentation and their effect on long-term storage of wheat bread. *Food Microbiol.* 33, 243-251.
- CODEX Alimentarius Commission, 2003. CODEX standard for fermented milks. Codex Stan 243-2003.
- Disponible sur : [www.codexalimentarius.net/download/standards/400/CXS\\_243e.pdf](http://www.codexalimentarius.net/download/standards/400/CXS_243e.pdf)
- Couret, V., Gueguen, M., Vernoux, J. P., 2004. *In vitro* screening of potential probiotic activities of selected lactobacilli isolated from unpasteurized milk products for incorporation into soft cheese. *J. Dairy Research*, 71(04), 451-460.
- Collins, M., Rodrigues, U., Ash, C., Aguirre, M., Farrow, J., Martinez-Murcia, A., Phillips, B., Williams, A., Wallbanks, S., 1991. Phylogenetic analysis of the genus *Lactobacillus* and related lactic acid bacteria as determined by reverse transcriptase sequencing of 16S rRNA. *FEMS Microbiol. Letters* 77, 5-12.
- Collins, Y.F., McSweeney, P.L.H., Wilkinson, M.G., 2003. Lipolysis and free fatty acid catabolism in cheese: a review of current knowledge. *Int. Dairy J.* 13, 841-866.
- Collins, Y. F., McSweeney, P. L. H., Wilkinson, M. G., 2004. Lipolysis and catabolism of fatty acids in cheese. In *Cheese: Chemistry, Physics and Microbiology* (Fox, P. F., McSweeney, P. L.H., Cogan, T. M., Guinee, T. P., Eds.), Vol. 1, pp. 373-389. Academic Press.
- Coloretti, F., Carri, S., Armaforte, E., Chiavari, C., Grazia, L., Zambonelli, C., 2007. Antifungal activity of lactobacilli isolated from salami. *FEMS Microbiol. Letters* 271, 245-250.
- Corrieu, G., Luquet, F.-M., 2008. Bactéries lactiques, De la génétique aux ferments, Ed. TecDoc Lavoisier, Paris, France.
- Cortés-Zavaleta, O., López-Malo, A., Hernández-Mendoza, A. and García, H. S. 2014. Antifungal activity of lactobacilli and its relationship with 3-phenyllactic acid production. *Int. J. Food Microbiol.* 173, 30-35.
- Coton, M., Delbés-Paus, C., Irlinger, F., Desmasures, N., Le Fleche, A., Stahl, V., Montel, M.C., Coton, E., 2012. Diversity and assessment of potential risk factors of Gram-negative isolates associated with French cheeses. *Food Microbiol.*, 29(1), 88-98.
- Cracraft, J., 1983. Species concepts and speciation analysis, in: Johnston, R.F. (Ed.), *Current Ornithology*. Springer US, pp. 159-187.
- Crowley, S., Mahony, J., van Sinderen, D., 2013. Current perspectives on antifungal lactic acid bacteria as natural bio-preservatives. *Trends Food Sci. Technol.* 33(2), 93-109.

## ~ D ~

---

- Dasen, A., Berthier, F., Grappin, R., Williams, A. G., Banks, J., 2003. Genotypic and phenotypic characterization of the dynamics of the lactic acid bacterial population of adjunct containing Cheddar cheese manufactured from raw and microfiltered pasteurized milk. *J. App. Microbiol.*, 94(4), 595–607.
- Davidson, M.P., 2001. Chemical preservatives and natural antimicrobial compounds. In Doyle, M.P., Beuchat, L.R., Montville, T.J. (Eds.), *Food microbiology: fundamentals and frontiers*, ASM press, Washington, 593-627.
- De Vuyst, L. and Degeest, B., 1999. Heteropolysaccharides from lactic acid bacteria. *FEMS Microbiol. Rev.* 23, 153–177.
- Delavenne, E., Cliquet, S., Trunet, C., Barbier, G., Mounier, J., Le Blay, G., 2015. Characterization of the antifungal activity of *Lactobacillus harbinensis* K.V9.3.1Np and *Lactobacillus rhamnosus* K.C8.31I in yogurt. *Food Microbiol.* 45, 10-17.
- Delavenne, E., Mounier, J., Asmani, K., Jany, J. L., Barbier, G., Le Blay, G. 2011. Fungal diversity in cow, goat and ewe milk. *Int. J. Food Microbiol.*, 151(2), 247–251.
- Delavenne, E., Mounier, J., Deniel, F., Barbier, G., Le Blay, G., 2012. Biodiversity of antifungal lactic acid bacteria isolated from raw milk samples from cow, ewe and goat over one-year period. *Int. J. Food Microbiol.* 155, 185–190.
- Desmazeaud, M., 1983. L'état des connaissances en matière de nutrition des bactéries lactiques. *Le lait* 63, 267–316.
- Devle, H., Vetti, I., Naess-Andresen, C. F., Rukke, E. O., Vegarud, G., Ekeberg, D., 2012. A comparative study of fatty acid profiles in ruminant and non-ruminant milk. *European J. of Lipid Sci. and Technol.*, 114(9), 1036–1043
- Doleyres, Y., 2003. Production en continu de ferments lactiques probiotiques par la technologie des cellules immobilisées. Thèse, Université de Laval, Faculté des Sciences de l'Agriculture et de l'Alimentation, Département des Sciences des aliments de de nutrition, Quebec.
- Drouault, S., Corthier, G., 2001. Effets des bactéries lactiques ingérées avec des laits fermentés sur la santé. *Vet. Research*, 32, 101-117.
- Dubeuf, J.-P., Boyazoglu, J., 2009. An international panorama of goat selection and breeds. *Livest. Sci., Special Issue: Animal Genetic Resources* 120, 225-231.
- Dugat-Bony, E., Garnier, L., Denonfoux, J., Ferreira, S., Sarthou, A.-S., Bonnarme, P., Irlinger, F., 2016. Highlighting the microbial diversity of 12 French cheese varieties. *Int. J. Food Microbiol.* 238, 265-273.
- Dunne, J., Evershed, R.P., Salque, M., Cramp, L., Bruni, S., Ryan, K., Biagetti, S., Di Lernia, S., 2012. First dairying in green Saharan Africa in the first millennium bc. *Nature* 486 (7403), 390-394.

## ~ E ~

---

- EFSA, 2009. Scientific opinion of the panel on biological hazards on the maintenance of the list of QPS microorganisms intentionally added to food or feed. *EFSA Journal*, 712, 1-93.
- Eliskases-Lechner, F., Ginzinger, W., 1995. The bacterial flora of surface-ripened cheeses with special regard to coryneforms. *Le Lait*, 75(6), 571–584.
- Ercolini, D., 2004. PCR-DGGE fingerprinting: novel strategies for detection of microbes in food. *J. Microbiol. Methods*, 56(3), 297–314.

- Ercolini, D., 2013. High-throughput sequencing and metagenomics: moving forward in the culture-independent analysis of food microbial ecology. *Appl. Environ. Microbiol.* 79, 3148-3155.
- Erwin, D.C., Ribeiro, O.K., 1996. Phytophthora diseases worldwide. *Am. Phytopathol. Soc.*
- Evans, T. N., Seviour, R. J., 2012. Estimating biodiversity of fungi in activated sludge communities using culture-independent methods. *Microbial Ecol.*, 23(4), 773-786.

~ F ~

---

- Farnworth, E.R. (Ted), 2003. Handbook of fermented functional foods. CRC Press.
- Felis, G.E. and Dellaglio, F., 2007. Taxonomy of lactobacilli and bifidobacteria. *Intestinal Microbiol.* 8, 44–61.
- Fernandez, B., Vimont, A., Desfossés-Foulcault, E., Daga, M., Arora, G., Fliss, I., 2017. Antifungal activity of lactic and propionic acid bacteria and their potential as protective culture in cottage cheese. *Food Control* 78, 350-356.
- Findik, O., Andiç, S., 2017. Some chemical and microbiological properties of the butter and the butter oil produced from the same raw material. *Food Sci. Technol.* 86, 233-239.
- Fitzsimmons, N. A., Cogan, T. M., Condon, S., Beresford, T., 1999. Phenotypic and genotypic characterization of non-starter lactic acid bacteria in mature cheddar cheese. *App. Env. Microbiol.*, 65(8), 3418–3426
- Fleet, G.H., 1990. Yeasts in dairy products. *J. Appl. Bacteriol.* 68, 199–211.
- Fox, P. F., Kelly, A. L., 2006. Indigenous enzymes in milk: Overview and historical aspects— Part 1. *Int. Dairy J.*, 16(6), 500–516.
- Fox, P.F., McSweeney, P.L.H., 2004. Cheese: an overview, in: Fox, P.F., McSweeney, P.L.H., Cogan, T.M., Guinee, T.P. (Eds.), *Cheese: Chemistry, Physics and Microbiology*. Elsevier Academic Press, London, pp. 1–18.
- Fox, P. F., Wallace, J. M., 1997. Formation of Misvor Compounds in Cheese. In *Advances in Applied Microbiology* (Neidleman, S. L., Laskin, A.I., Eds.), Vol. 45, pp. 17-85. Academic Press.
- Freitas, R., Chuat, V., Madec, M.N., Nero, L.A., Thierry, A., Valence, F., de Carvalho, A.F., 2015. Biodiversity of dairy *Propionibacterium* isolated from dairy farms in Minas Gerais, Brazil. *Int. J. Food Microbiol.* 203, 70-77.
- Frisvad, J.C., Filtenborg, O., 1990. Secondary metabolites as consistent criteria in *Penicillium* taxonomy and a synoptic key to *Penicillium* subgenus *Penicillium*, in: Samson, R.A., Pitt, J.I. (Eds.), *Modern Concepts in Penicillium and Aspergillus Classification*, NATO ASI Series. Springer US, pp. 373–384.
- Frisvad, J.C., Filtenborg, O., 1983. Classification of terverticillate penicillia based on profiles of mycotoxins and other secondary metabolites. *Appl. Environ. Microbiol.* 46, 1301–1310.
- Frisvad, J.C., Samson, R.A., 2004. Polyphasic taxonomy of *Penicillium* subgenus *Penicillium* - A guide to identification of food and air-borne terverticillate Penicillia and their mycotoxins. *Stud. Mycol.* 49, 1–174.
- Frohlich-Wyder, M. T., Bachmann, H. P., 2004. Cheeses with propionic acid fermentation. In *Cheese: Chemistry, Physics and Microbiology* (Fox, P. F., McSweeney, P. L. H., Cogan, T. M., Guinee, T. P., Eds.), Vol. 2, pp. 191-206.

~ G ~

---

- Gänzle, M.G., 2015. Lactic metabolism revisited: metabolism of lactic acid bacteria in food fermentations and food spoilage. *Curr. Opin Food Sci.* 2, 106-117.
- Gardini, F., Martuscelli, M., Crudele, M. A., Paparella, A., Suzzi, G., 2002. Use of *Staphylococcus xylosum* as a starter culture in dried sausages: effect on the biogenic amine content. *Meat Sci.*, 61, 275-283.
- Geisen, R., 2000. *P. nalgiovensis* carries a gene which is homologous to the *paf* gene of *P. chrysogenum* which codes for an antifungal peptide. *Int. J., Food Microbiol.* 62, 95-101.
- Geiser, D.M., Jiménez-Gasco, M. del M., Kang, S., Makalowska, I., Veeraghavan, N., Ward, T.J., Zhang, N., Kulda, G.A., O'Donnell, K., 2004. FUSARIUM-ID v. 1.0: A DNA sequence database for identifying *Fusarium*, in: Mulè, G., Bailey, J.A., Cooke, B.M., Logrieco, A. (Eds.), *Molecular Diversity and PCR-Detection of Toxicogenic Fusarium Species and Ochratoxigenic Fungi*. Springer Netherlands, pp. 473–479.
- Giannino, M. L., Marzotto, M., Dellaglio, F., Feligini, M., 2009. Study of microbial diversity in raw milk and fresh curd used for Fontina cheese production by culture-independent methods. *Int. J. Food Microbiol.*, 130(3), 188–195
- Glass, N.L., Donaldson, G.C., 1995. Development of primer sets designed for use with the PCR to amplify conserved genes from filamentous ascomycetes. *Appl. Environ. Microbiol.* 61, 1323–1330.
- Goudéranche, H., Camier-Caudron, B., Gassi, J.-Y., Schuck, P., 2002. Procédés de transformation fromagère (partie 3). *Techniques de l'Ingénieur*.
- Goudéranche, H., Camier-Caudron, B., Gassi, J.-Y., Schuck, P., 2001a. Procédés de transformation fromagère (partie 1). *Techniques de l'Ingénieur*.
- Goudéranche, H., Camier-Caudron, B., Gassi, J.-Y., Schuck, P., 2001b. Procédés de transformation fromagère (partie 2). *Techniques de l'Ingénieur*.
- Gougouli, M., Kalantzi, K., Beletsiotis, E., Koutsoumanis, K.P., 2011. Development and application of predictive models for fungal growth as tools to improve quality control in yoghurt production. *Food Microbiol.* 28, 1453-1462.
- Gould, G.W., 1996. Methods for preservation and extension of shelf life. *Int. J. Food Microbiol.* 33, 51–64.
- Gripon, J. C., 1999. Mould-ripened cheeses. In *Cheese: Chemistry, physics and microbiology* (Fox, P.F., Ed.), Vol. 2, pp. 111–136.
- Grube, M., Gaya, E., Kauserud, H., Smith, A.M., Avery, S.V., Fernstad, S.J., Muggia, L., Martin, M.D., Eivindsen, T., Køljalg, U., Bendiksby, M., 2017. The next generation fungal diversity researcher. *Fungal Biol. Rev.* 31, 124-130.

## ~ H ~

- 
- Hawksworth, D.L., 1991. The fungal dimension of biodiversity: magnitude, significance, and conservation. *Mycol. Res.* 95, 641–655
- Hawksworth, D.L., Rossman, A.Y., 1997. Where are all the undescribed fungi? *Phytopathology* 87, 888–891
- Head, I. M., Saunders, J. R., Pickup, R. W., 1998. Microbial evolution, diversity, and ecology: a decade of ribosomal RNA analysis of uncultivated microorganisms. *Microbial Ecol.*, 35(1), 1–21
- Hermet, A., Méheust, D., Mounier, J., Barbier, G., Jany, J.-L., 2012. Molecular systematics in the genus *Mucor* with special regards to species encountered in cheese. *Fungal Biol.* 116, 692-705.
- Hey, J., 2006. On the failure of modern species concepts. *Trends Ecol. Evol.* 21, 447–450.

- Hibbett, D.S., Binder, M., Bischoff, J.F., Blackwell, M., Cannon, P.F., Eriksson, O.E., Huhndorf, S., James, T., Kirk, P.M., Lücking, R., Thorsten Lumbsch, H., Lutzoni, F., Matheny, P.B., McLaughlin, D.J., Powell, M.J., Redhead, S., Schoch, C.L., Spatafora, J.W., Stalpers, J.A., Vilgalys, R., Aime, M.C., Aptroot, A., Bauer, R., Begerow, D., Benny, G.L., Castlebury, L.A., Crous, P.W., Dai, Y.-C., Gams, W., Geiser, D.M., Griffith, G.W., Gueidan, C., Hawksworth, D.L., Hestmark, G., Hosaka, K., Humber, R.A., Hyde, K.D., Ironside, J.E., Kõljalg, U., Kurtzman, C.P., Larsson, K.-H., Lichtwardt, R., Longcore, J., Miądlikowska, J., Miller, A., Moncalvo, J.-M., Mozley- Standridge, S., Oberwinkler, F., Parmasto, E., Reeb, V., Rogers, J.D., Roux, C., Ryvarden, L., Sampaio, J.P., Schüßler, A., Sugiyama, J., Thorn, R.G., Tibell, L., Untereiner, W.A., Walker, C., Wang, Z., Weir, A., Weiss, M., White, M.M., Winka, K., Yao, Y.-J., Zhang, N., 2007. A higher-level phylogenetic classification of the Fungi. *Mycol. Res.* 111, 509–547.
- Hidalgo, M.E., Côrrea, A.P.F., Canales, M.M., Daroit, D.J., Brandelli, A., Risso, P., 2015. Biological and physiological properties of bovine sodium caseinate hydrolysates obtained by a bacterial protease preparation. *Food Hydrocolloids*, 43, 510-520.
- Hoffmann, K., Voigt, K., Kirk, P. M., 2011. *Mortierellomycotina* subphyl. nov., based on multi-gene genealogies. *Mycotaxon*. 115(1), 353–363.
- Hoier, E., Janzen, T., Henriksen, C. M., Rattray, F., Brockmann, E., Johansen, E., Law, B. A., 1999. The production, application and action of lactic cheese starter cultures. *Technology of cheesemaking*, 99–131.
- Holzappel, W., Geisen, R. and Schillinger, U., 1995. Biological preservation of foods with reference to protective cultures, bacteriocins and food-grade enzymes. *Int. J. Food Microbiol.* 24, 343–362.
- Hong, S.-B., Go, S.-J., Shin, H.-D., Frisvad, J.C., Samson, R.A., 2005. Polyphasic taxonomy of *Aspergillus fumigatus* and related species. *Mycologia* 97, 1316–1329.
- Houbraken, J., Frisvad, J.C., Samson, R.A., 2010. Sex in *Penicillium* series *Roqueforti*. *IMA Fungus* 1, 171–180.
- Hugenholtz, P., Goebel, B. M., Pace, N. R., 1998. Impact of culture-independent studies on the emerging phylogenetic view of bacterial diversity. *J. Bacteriol.*, 180(18), 4765–4774.

~ I ~

---

- Ignatova, L.V., Brazhnikova, Y.V., Berzhanova, R.Z., Mukasheva, T.D., 2015. Plant growth-promoting and antifungal activity of yeasts from dark chesnut soil. *Microbiol. Research* 175, 78-83.
- Inglis, R.C., Stevens, M.J., Meile, L., Lacroix, C., Meile, L., 2015. High-throughput screening assays for antibacterial and antifungal activities of *Lactobacillus* species. *J. Microbiol. Meth.* 114, 9-26.
- Irlinger, F., Mounier, J., 2009. Microbial interactions in cheese: implications for cheese quality and safety. *Current Opinion in Biotechnology*, 20(2), 142-148.
- Ishikawa, M., Kodama, K., Yasuda, H., Okamoto-Kainuma, A., Koizumi, K., Yamasato, K., 2007. Presence of halophilic and alkaliphilic lactic acid bacteria in various cheeses. *App. Microbiol.*, 44(3), 308–313.

~ J ~

---

- James, T. Y., Kauff, F., Schoch, C. L., Matheny, P. B., Hofstetter, V., Cox, C., Celio, G., Gueidan, C., Fraker, E., Miadlikowska, J., Lumbsh, T.H., Rauhut, A., Reeb, V., Arnold, A.E., Wynns, A.A., Stajich, J., Hosaka, K., Sung, G., Jonhson, D., o'Rourke, B., Crockett, M., Binder, M., Curtis, J.M., Slot, J., Wang, Z., Wilson, A., Schüßler, A., Longcore, J.E., O'Donnell, K., Mozley-Standridge, S., Porter, D., Letcher, P.M., Powell, M.J., Taylor, J.W., White, M.M., Griffith, G.W., Davies, D.R., Humber, R.A., Morton, J.B., Sugiyama, J., Rossman, A., Rogers, J.D., Kohlmeyer, B., Spotts, R.A., Serdani, M., Crous, P.W., Hughes, K.W., Matsuura, K., Langer, E., Langer, G., Untereiner, W., Lücking, R., Büdel, B., Geiser, D.M., Aptroot, A., Diederich, P., Scmitt, I., Schultz, M., Yahr, R., Hibett, D., Lutzoni, F., McLaughlin, D.J., Spatafora, J.W., Vilgalys, R., 2006. Reconstructing the early evolution of Fungi using a six-gene phylogeny. *Nature*, 443(7113), 818–822
- James, T.Y., Letcher, P.M., Longcore, J.E., Mozley-Standridge, S.E., Porter, D., Powell, M.J., Griffith, G.W., Vilgalys, R., 2006. A molecular phylogeny of the flagellated fungi (*Chytridiomycota*) and description of a new phylum (*Blastocladiomycota*). *Mycolog.* 98, 860–871.
- Jandal, J. M., 1996. Comparative aspects of goat and sheep milk. *Small Ruminant Research*, 22(2),177-185.
- Johnson, M., Law, B. A., 1999. The origins, development and basic operations of cheesemaking technology. *Technol. Cheesemaking*, 1–32.

~ K ~

---

- Katarzyna, K., Bzducha-Wrobel, A., Krasniewska, K., Hlebowska-Smigiel, A., Gniewosz, M., 2017. Effect of magnesium acetate on the antimold activity of *Lactobacillus*. *J. Food Prot.* 80(1), 96-103.
- Kirk, P.M., Cannon, P.F., Minter, D.W., Stalpers, J.A., 2008. *Dictionary of the Fungi*, 10th ed. CAB International, Wallingford
- Kure, C., 2001. Mould contaminants on Jarlsberg and Norvegia cheese blocks from four factories. *Int. J. Food Microbiol.* 70, 21–27.
- Kure, C.F., Skaar, I., 2000. Mould growth on the Norwegian semi-hard cheeses Norvegia and Jarlsberg. *Int. J. Food Microbiol.* 62, 133–137.
- Kure, C.F., Skaar, I., Brendehaug, J., 2004. Mould contamination in production of semi-hard cheese. *Int. J. Food Microbiol.* 93, 41–49.
- Kurtzman, C.P., Robnett, C.J., 1997. Identification of clinically important ascomycetous yeasts based on nucleotide divergence in the 5' end of the large-subunit (26S) ribosomal DNA gene. *J. Clin. Microbiol.* 35, 1216–1223.
- Kwon-Chung, K.J., 2012. Taxonomy of fungi causing mucormycosis and entomophthoromycosis (Zygomycosis) and nomenclature of the disease: molecular mycologic perspectives. *Clin. Infect. Dis.* 54, S8–S15
- Kwon-Chung, K.J., Sugui, J.A., 2009. Sexual reproduction in *Aspergillus* species of medical or economical importance: why so fastidious? *Trends Microbiol.* 17, 481–487.
- Irlinger, F., Mounier, J., 2009. Microbial interactions in cheese: implications for cheese quality and safety. *Curr. Opin. Biotechnol.*, 20(2), 142-148.

~ L ~

---

- LaPointe, G., 2009. La production d'exopolysaccharides. In Drider, D., Prevost, H. (Eds.), *Bactéries lactiques: Physiologie, Métabolisme, Génomique et Applications industrielles, Economica*, 73-98.
- Le Lay, C., Mounier, J., Vasseur, V., Weill, A., Le Blay, G., Barbier, G. and Coton, E., 2016. *In vitro* and *in situ* screening of lactic acid bacteria and propionibacteria antifungal activities against bakery product spoilage molds. *Food Control* 60, 247–255.
- Ledenbach, L.H., Marshall, R.T., 2010. Microbiological spoilage of dairy products. In: Sperber, W.H., Doyle, M.P. (Eds.), *Compendium of the Microbiological Spoilage of Foods and Beverages, Food Microbiology and Food Safety*. Springer, New York, pp. 41–67.
- Leyva Salas, M., Mounier, J., Valence, F., Coton, M., Thierry, A., Coton, E., 2017. Antifungal microbial agents for food biopreservation - a review. *Microorganisms* 5(3), 37.
- Lind, H., Jonsson, H., Schnürer J., 2005. Antifungal effect of dairy propionibacteria contribution of organic acids. *Int. J. Food Microbiol.*, 98, 157-65.
- Liu, J., Sui, Y., Wisniewski, M., Droby, S., Liu, Y., 2013. Review: Utilization of antagonistic yeasts to manage postharvest fungal diseases of fruit. *Int. J. Food Microbiol.* 167, 153-160.
- Liu, S.Q., Tsao, M., 2009. Biocontrol of dairy moulds by antagonistic dairy yeast *Debaryomyces hansenii* in yoghurt and cheese at elevated temperatures.
- Loubière, P. and Coccagn-Bousquet, M., 2009. Devenir du carbone. In Drider, D., Prévost, H. (Eds.), *Bactéries Lactiques: Physiologie, Métabolisme, Génomique et Applications industrielles, Economica*, 29-50.
- Lucey, J.A., 2004. Formation, structural properties and rheology of acid-coagulated milk gels. In Fox, P.F., McSweeney, P.L.H., Cogan, T.M., Guinee, T.P. (Eds.), *Cheese: Chemistry, Physics and Microbiology*, 3e Ed., Elsevier Academic Press, London 1, 105–122.
- Luz, C., Saladina, F., Luciano, F.B., Mañes, J., Meca, G., 2017. In vitro antifungal activity of bioactive peptides produced by *Lactobacillus plantarum* against *Aspergillus parasiticus* and *Penicillium expansum*. *Food Sci. Technol.* 81, 128-135.

## ~ M ~

- 
- Mannu, L., Comunian, R., Francesca Scintu, M., 2000. Mesophilic lactobacilli in Fiore Sardo cheese: PCR-identification and evolution during cheese ripening. *Int. Dairy J.*, 10(5), 383–389
- Margulis, L., Schwartz, K.V., 1998. *Five kingdoms: an illustrated guide to the phyla of life on earth*, 3rd ed. W H Freeman and Company, New-York.
- Martin, G.W., 1960. The systematic position of the Myxomycetes. *Mycologia* 52, 119.
- Mayoral, M.B., Martin, R., Sanz, A., Hernandez, P.E., Gonzalez, I. and Garcia, T., 2005. Detection of *Kluyveromyces marxianus* and other spoilage yeasts in yoghurt using a PCR-culture technique. *Int. J. Food Microbiol.* 105, 27–34.
- Mayr, E., 1940. Speciation phenomena in birds. *Am. Nat.* 74, 249–278.
- McCann, D., Barrett, A., Cooper, A., Crumpler, D., Dalen, L., Grimshaw, K., Kitchin, E., Lok, K., Porteous, L., Prince, E., Sonuga-Barke, E., Warner, J.O., Stevenson, J., 2007. Food additives and hyperactive behaviour in 3-year-old and 8/9-year-old children in the community: a randomised, double-blinded, placebo-controlled trial. *The Lancet* 370, 1560–1567.



- McSweeney, P.L.H., Ottogalli, G., Fox, P.F., 2017. Diversity of cheese varieties: an overview, in: Fox, P.F., McSweeney, P.L.H., Cogan, T.M., Guinee, T.P. (Eds.), *Cheese: Chemistry, Physics and Microbiology*. Elsevier Academic Press, London, pp. 1–22.
- Molimard, P., Spinnler, H.E., 1996. Review : Compounds involved in the flavor of surface mold-ripened cheeses: origins and properties. *J. Dairy Sci.* 79, 169-184.
- Monnet, V., Atlan, D., Béal, C., Champomier-Vergès, M.C., Chapot-Chartier, M.P., Chouayekh, H., Cocaign-Bousquet, M., Deghorain, M., Gaudu, P., Gilbert, C., Guéden, E., Guillouard, I., Goffin, P., Guzzo, J., Hols, P., Juillard, V., Ladero, V., Lindley, N., Lortal, S., Loubière, P., Maguin, E. Monnet, C., Rul, F., Tourdot-Maréchal, P., Yvon, M., 2008. *Métabolisme et ingénierie métabolique*. In Corrieu, G., Luquet, F.-M. (Eds.), *Bactéries lactiques, De la génétique aux ferments*, TecDoc Lavoisier, Paris, France, 271–509.
- Montel, M.C., Buchin, S., Mallet, A., Delbes-Paus, C., Vuitton, D.A., Desmasures, N., Berthier, F., 2014. Traditional cheeses: Rich and diverse microbiota with associated benefits. *Int. J. Food Microbiol.* 177, 136-154.
- Mounier, J., Gelsomino, R., Goerges, S., Vancanneyt, M., Vandemeulebroecke, K., Hoste, B., Scherer, S., Swings, J., Fitzgerald, G.F., Cogan, T.M., 2005. Surface Microflora of Four Smear-Ripened Cheeses. *App. Env. Microbiol.*, 71(11), 6489-6500.
- Mounier, J., Monnet, C., Jacques, N., Antoinette, A., Irlinger, F., 2009. Assessment of the microbial diversity at the surface of Livarot cheese using culture-dependent and independent approaches. *Int. J. Food Microbiol.*, 133(1), 31–37.
- Muñoz, A., Gandía, M., Harries, E., Carmona, L., Read, N.D., Marcos, J.F., 2013. Understanding the mechanism of action of cell-penetrating antifungal peptides using the rationally designed hexapeptide PAF26 as a model. *Fungal Biol. Rev.* 26(4), 146-155.

~ N ~

- 
- Nally, M.C., Pesce, V.M., Maturano, Y.P., Rodriguez Assaf, L.A., Toro, M.E., Castellanos de Figueroa, L.I., Vazquez, F., 2015. Antifungal modes of action of *Saccharomyces* and other biocontrol yeasts against fungi isolated from sour and grey rots. *Int. J. Food Microbiol.* 204, 91-100.

~ O ~

- 
- Orla-Jensen, S., 1919. *The lactic acid bacteria*. Høst and Son, Copenhagen, Denmark.

~ P ~

- 
- Panchal, B.R., Truong, T., Prakash, S., Bansal, N., Bhandari, B., ARC Dairy Innovation Hub, 2017. Effect of fat globule size on the churnability of dairy cream. *Food Research Int.* 99, 229-238.
- Papp, T., Nagy, G., Csernetics, A., Szekeres, A., Vágvölgyi, C., 2009. Beta-carotene production by mucoralean fungi. *Ann Faculty Engg Hunedoara*, 4, 173–176.
- Pfeiler, E.A. and Klaenhammer, T.R. The genomics of lactic acid bacteria. *Trends Microbiol.* 15, 546–553.

- Pesce, V.M., Nally, M.C., Carrizo, G.P., Rojo, C., Pérez, B.A., Toro, M.E., Castellanos de Figueroa, L.I., Vazquez, F., 2017. Antifungal activity of native yeasts from different microenvironments against *Colletotrichum gloeosporioides* on ripe olive fruits. *Biol. Control*, *In Press*.
- Pfeiler, E.A., Klaenhammer, T.R., 2007. The genomics of lactic acid bacteria. *Trends Microbiol.* 15, 546-553.
- Pitt, J.I., 1979. The genus *Penicillium* and its teleomorphic states *Eupenicillium* and *Talaromyces*. Academic Press, London.
- Pitt, J.I., Hocking, A.D., 2009. *Fungi and food spoilage*, 3rd Ed. Springer Science and Business Media, New York
- Pot, B., 2008. The taxonomy of lactic acid bacteria. In Corrieu, G., Luquet, F.-M. (Eds.), *Bactéries lactiques, De la génétique aux ferments*, TecDoc Lavoisier, Paris, 1–152
- Privat, K. and Thonart, P., 2011. Action des cultures protectrices : cas des germes lactiques sur la flore alimentaire indésirable. *Biotechnol. Agron. Society Env.* 15,339–348.
- Punt, P.J., an Beizen, N., Conesa, A., Albers, A., Mangnus, J., van Hondel, C., 2002. Filamentous fungi as cell factories for heterologous protein production. *Trends Biotechnol.* 20, 200-206.

~ Q ~

---

- Quigley, L., O'Sullivan, O., Stanton, C., Beresford, T.P., Ross, R.P., Fitzgerald, G.F., Cotter, P.D., 2013. The complex microbiota of raw milk. *FEMS Microbiol. Rev.* 37, 664–698

~ R ~

---

- Regulation EC 1333/2008 of the European Parliament and of the Council of 16 december 2008 on food additives.
- Reynolds, D.R., 1993. The fungal holomorph: an overview, in: Reynolds, D.R., Taylor, J.W. (Eds.), *The Mitotic, Meiotic and Pleomorphic Speciation in Fungal Systematics*. CABI, Wallingford, pp. 15–25.
- Richards, T.A., Jones, M.D.M., Leonard, G., Bass, D., 2012. Marine fungi: their ecology and molecular diversity. *Annu. Rev. Mar. Sci.* 4, 495–522. doi:10.1146/annurev-marine-120710-100802
- Rodgers, S., 2008. Novel applications of live bacteria in food services: probiotics and protective cultures. *Trends Food Sci. Technol.* 19, 188–197.
- Ropars, J., Cruaud, C., Lacoste, S., Dupont, J., 2012. A taxonomic and ecological overview of cheese fungi. *Int. J. Food Microbiol.* 155, 199–210.
- Ruse, M., 1969. Definitions of species in biology. *Br. J. Philos. Sci.* 20, 97–119.

~ S ~

---

- Salque, M., Bogucki, P.I., Pyzel, J., Sobkowiak-Tabaka, I., Grygiel, R., Szmyt, M., Evershed, R.P., 2013. Earliest evidence for cheese making in the sixth millennium BC in northern Europe. *Nature* 493, 522–525.

- Salvetti, E., Torriani, S., Felis, G.E., 2012. The genus *Lactobacillus*: A taxonomic update. *Probiotics Antimicro. Prot.* 4, 217-226.
- Samson, R.A., Seifert, K.A., Kuijpers, A.F.A., Houbraken, J.A.M.P., Frisvad, J.C., 2004. Phylogenetic analysis of *Penicillium* subgenus *Penicillium* using partial  $\beta$ -tubulin sequences. *Stud. Mycol.* 49, 175-200.
- Samson, R., Frisvad, J., Hoekstra, E., Filtenborg, O., 2002. Introduction to food and airborne fungi. Revised 6th edition. Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands 389 pp. Distributed in the United States by ASM Press., in: *Mycopathologia*.pp. 609-609.
- Sandine, W.E., Elliker, P.R., 1970. Microbiologically induced flavors and fermented foods. Flavor in fermented dairy products. *J. Agric. Food Chem.* 18, 557-562.
- Schmidt, J. L., Rouger, C., Diez, M., Lenoir, J., 1993. Activités biochimiques de levures du genre *Candida* isolées de fromages de chèvre. *MAN Microbiologie, aliments, nutrition*, 11(2), 165-182.
- Schnürer, J. and Magnusson, J., 2005. Antifungal lactic acid bacteria as biopreservatives. *Trends Food Sci. Technol.* 16, 70-78.
- Schoch, C.L., Seifert, K.A., Huhndorf, S., Robert, V., Spouge, J.L., Levesque, C.A., Chen, W., Bolchacova, E., Voigt, K., Crous, P.W., Miller, A.N., Wingfield, M.J., Aime, M.C., An, K.-D., Bai, F.-Y., Barreto, R.W., Begerow, D., Bergeron, M.-J., Blackwell, M., Boekhout, T., Bogale, M., Boonyuen, N., Burgaz, A.R., Buyck, B., Cai, L., Cai, Q., Cardinali, G., Chaverri, P., Coppins, B.J., Crespo, A., Cubas, P., Cummings, C., Damm, U., Beer, Z.W. de, Hoog, G.S. de, Del-Prado, R., Dentinger, B., Diéguez-Uribeondo, J., Divakar, P.K., Douglas, B., Dueñas, M., Duong, T.A., Eberhardt, U., Edwards, J.E., Elshahed, M.S., Fliegerova, K., Furtado, M., García, M.A., Ge, Z.-W., Griffith, G.W., Griffiths, K., Groenewald, J.Z., Groenewald, M., Grube, M., Gryzenhout, M., Guo, L.-D., Hagen, F., Hambleton, S., Hamelin, R.C., Hansen, K., Harrold, P., Heller, G., Herrera, C., Hirayama, K., Hirooka, Y., Ho, H.-M., Hoffmann, K., Hofstetter, V., Högnabba, F., Hollingsworth, P.M., Hong, S.-B., Hosaka, K., Houbraken, J., Hughes, K., Huhtinen, S., Hyde, K.D., James, T., Johnson, E.M., Johnson, J.E., Johnston, P.R., Jones, E.B.G., Kelly, L.J., Kirk, P.M., Knapp, D.G., Kõljalg, U., Kovács, G.M., Kurtzman, C.P., Landvik, S., Leavitt, S.D., Liggenstoffer, A.S., Liimatainen, K., Lombard, L., Luangsa-ard, J.J., Lumbsch, H.T., Maganti, H., Maharachchikumbura, S.S.N., Martin, M.P., May, T.W., McTaggart, A.R., Methven, A.S., Meyer, W., Moncalvo, J.-M., Mongkolsamrit, S., Nagy, L.G., Nilsson, R.H., Niskanen, T., Nyilasi, I., Okada, G., Okane, I., Olariaga, I., Otte, J., Papp, T., Park, D., Petkovits, T., Pino-Bodas, R., Quaedvlieg, W., Raja, H.A., Redecker, D., Rintoul, T.L., Ruibal, C., Sarmiento-Ramírez, J.M., Schmitt, I., Schüßler, A., Shearer, C., Sotome, K., Stefani, F.O.P., Stenroos, S., Stielow, B., Stockinger, H., Suetrong, S., Suh, S.-O., Sung, G.-H., Suzuki, M., Tanaka, K., Tedersoo, L., Telleria, M.T., Tretter, E., Untereiner, W.A., Urbina, H., Vágvölgyi, C., Vialle, A., Vu, T.D., Walther, G., Wang, Q.-M., Wang, Y., Weir, B.S., Weiß, M., White, M.M., Xu, J., Yahr, R., Yang, Z.L., Yurkov, A., Zamora, J.-C., Zhang, N., Zhuang, W.-Y., Schindel, D., 2012. Nuclear ribosomal internal transcribed spacer (ITS) region as a universal DNA barcode marker for Fungi. *Proc.Natl. Acad. Sci. USA*, 109, 6241-6246.
- Schoch, C.L., Sung, G.-H., López-Giráldez, F., Townsend, J.P., Miadlikowska, J., Hofstetter, V., Robbertse, B., Matheny, P.B., Kauff, F., Wang, Z., Gueidan, C., Andrie, R.M., Trippe, K., Ciuffetti, L.M., Wynns, A., Fraker, E., Hodkinson, B.P., Bonito, G., Groenewald, J.Z., Arzanlou, M., Hoog, G.S. de, Crous, P.W., Hewitt, D., Pfister, D.H., Peterson, K., Gryzenhout, M., Wingfield, M.J., Aptroot, A., Suh, S.-O., Blackwell, M., Hillis, D.M., Griffith, G.W., Castlebury, L.A., Rossman, A.Y., Lumbsch, H.T., Lücking, R., Büdel, B., Rauhut, A., Diederich, P., Ertz, D., Geiser, D.M., Hosaka, K., Inderbitzin, P., Kohlmeyer, J., Volkmann-Kohlmeyer, B., Mostert, L., O'Donnell, K., Sipman, H., Rogers, J.D.,

- Shoemaker, R.A., Sugiyama, J., Summerbell, R.C., Untereiner, W., Johnston, P.R., Stenroos, S., Zuccaro, A., Dyer, P.S., Crittenden, P.D., Cole, M.S., Hansen, K., Trappe, J.M., Yahr, R., Lutzoni, F., Spatafora, J.W., 2009. The *Ascomycota* tree of life: a phylum-wide phylogeny clarifies the origin and evolution of fundamental reproductive and ecological traits. *Syst. Biol.* 58, 224–239.
- Schwenninger, S.M., Meile, L., 2004. A mixed culture of *Propionibacterium jensenii* and *Lactobacillus paracasei* subs. *paracasei* inhibits food spoilage yeasts. *Syst. Appl. Microbiol.* 27, 229–237.
- Smiddy, M. A., Huppertz, T., Van Ruth, S. M., 2012. Triacylglycerol and melting profiles of milk fat from several species. *Int. Dairy J.*, 24(2), 64–69.
- Smit, G., Smit, B.A. and Engels, W.J.M., 2005. Flavour formation by lactic acid bacteria and biochemical flavour profiling of cheese products. *FEMS Microbiol. Rev.* 29, 591–610.
- Smith, J. and Hong-Shum, L., 2003. Food additives data book. Blackwell Science, Great Britain.
- Sørensen, L. M., Gori, K., Petersen, M. A., Jespersen, L., Arneborg, N., 2011. Flavour compound production by *Yarrowia lipolytica*, *Saccharomyces cerevisiae* and *Debaryomyces hansenii* in a cheese-surface model. *Int. Dairy J.*, 21(12), 970-978.
- Spatafora, J.W., Chang, Y., Benny, G.L., Lazarus, K., Smith, K.E., Berbee, M.L., Bonito, G., Corradi, N., Grigoriev, I., Gryganskyi, A., James, T.Y., O'Donnell, K., Roberson, R.W., Taylor, T.N., Uehling, J., Vilgalys, R., White, M.M., Stajich, J.E., 2016. A phylum-level phylogenetic classification of zygomycete fungi based on genome-scale data. *Mycologia* 108, 1028-1046.
- Spinnler, H. E., Gripon, J. C., 2004. Surface mould-ripened cheeses. In *Cheese: Chemistry, Physics and Microbiology* (Fox, P. F., McSweeney, P. L. H., Cogan, T. M., Guinee, T. P., Eds.), Vol. 2, pp. 157-174. Academic Press.
- Stiles, J., Penkar, S., Plocková, M., Chumchalová, J., Bullerman, L.B., 2002. Antifungal activity of sodium acetate and *Lactobacillus rhamnosus*. *J. Food Prot.* 65(7), 1188-1191.
- Stiles, M.E. and Holzapfel, W.H. 1996. Lactic acid bacteria of foods and their current taxonomy. *Int. J. Food Microbiol.* 36, 1–29.
- Stratford, M.; Nebe-von-Caron, G., Steels, H., Novodvorska, M., Ueckert, J., Archer, D.B., 2013. Weak-acid preservatives: pH and proton movements in the yeast *Saccharomyces cerevisiae*. *Int. J. Food Microbiol.* 161, 164–171.
- Streekstra, H., Verkennis, A.E.E., Jacobs, R., Dekker, A., Stark, J., Dijksterhuis, J., 2016. Fungal strains and the development of tolerance against natamycin. *Int. J. Food Microbiol.* 238, 15-22.
- Ström, K., Sjögren, J., Broberg, A., Schnürer, J., 2002. *Lactobacillus plantarum* MiLAB 393 produces the antifungal cyclic dipeptides cyclo (L-Phe-L-pro) and cyclo (L-Phetrans-4-OH-L-pro) and 3-phenyllactic acid. *Appl. Environ. Microbiol.* 68, 4322–4327.
- Swindell, S.R., Benson, K.H., Griffin, H.G., Renault, P., Ehrlich, S. and Gasson, M.J., 1996. Genetic manipulation of the pathway for diacetyl metabolism in *Lactococcus lactis*. *App. Env. Microbiol.* 62, 2641–2643.

## ~ T ~

- 
- Tailliez, P., 2001. Mini-revue: les bactéries lactiques, ces êtres vivants apparus il y a près de 3 milliards d'années. *Le Lait* 81, 1–11.
- Tailliez, P., 2004. Les lactobacilles: propriétés, habitats, rôle physiologique et intérêt en santé humaine. *Antibiotiques* 6, 35–41.
- Taniwaki, M.H., Hocking, A.D., Pitt, J.I., Fleet, G.H., 2001. Growth of fungi and mycotoxin production on cheese under modified atmospheres. *Int. J. Food Microbiol.* 68, 125-133.

- Théolier, J., Hammami, R., Labelle, P., Fliss, I., Jean, J., 2013. Isolation and identification of antimicrobial peptides derived by peptic cleavage of whey protein isolate. *J. Funct. Foods* 5(2), 706-714.
- Thierry, A., Valence, F., Deutsch, S.-M. Even, S., Falentin, H., Le Loir, Y., Jan, G., Gagnaire, V., 2015. Strain-to-strain differences within lactic and propionic acid bacteria species strongly impact the properties of cheese – a review. *Dairy Sci. Technol.* 95, 895-918.
- Toomey, N., Bolton, D. and Fanning, S., 2010. Characterisation and transferability of antibiotic resistance genes from lactic acid bacteria isolated from Irish pork and beef abattoirs. *Research Microbiol.* 161, 127–135.
- Tungjaroenchai, W., White, C., Holmes, W. and Drake, M., 2004. Influence of adjunct cultures on volatile free fatty acids in reduced-fat Edam cheeses. *J. Dairy Sci.* 87, 3224–3234.

---

~ U ~

- 
- Ullah, A., Chandrasekaran, G., Brul, S., Smits, G.J., 2013. Yeast adaptation to weak acids prevents futile energy expenditure. *Front. Microbiol.* 4, 142, 1-10.
- Upadhyay, V. K., McSweeney, P. L. H., Magboul, A. A. A., Fox, P. F., 2004. Proteolysis in cheese during ripening. In *Cheese: Chemistry, Physics and Microbiology* (Fox, P. F., McSweeney, P. L. H., Cogan, T. M., Guinee, T. P., Eds.), Vol. 1, pp. 391-443.

---

~ V ~

- 
- Van den Tempel, T., Jakobsen, M., 2000. The technological characteristics of *Debaryomyces hansenii* and *Yarrowia lipolytica* and their potential as starter cultures for production of Danablu. *Int. Dairy J.*, 10(4), 263–270.
- Vacheyrou, M.; Normand, A.C.; Guyot, P.; Cassagne, C.; Piarrou, R.; Bouton, Y., 2011. Cultivable microbial communities in raw cow milk and potential transfers from stables of sixteen French farms. *Int. J. Food Microbiol.* 146, 253–262.
- Velisek, J., Cejpek, K., 2011. Pigments of higher fungi: a review. *Czech J. Food Sci.* 29, 87-102.
- Vigne, J.D., Helmer, D., 2007. Was milk a "secondary product" in the Old World Neolithisation process? Its role in the domestication of cattle, sheep and goats. *Anthropozoologica*, 42(2), 9-40.
- Viljoen, B.C., 2001. The interaction between yeasts and bacteria in dairy environments. *Int. J. Food Microbiol.* 69, 37–44.

---

~ W ~

- 
- Wang, S.Y., Chen, H.C., Liu, J.R., Lin, Y.C., Chen, M.J., 2008. Identification of yeasts and evaluation of their distribution in taiwanese kefir and viili starters. *J. Dairy Sci.* 91, 3798–3805.
- Wang, Z., 2017. Plant-derived antifungal compounds trigger a common transcriptional response. *Infection, Genetics and Evolution* 54, 1-3.

- Ward, D. M., Weller, R., Bateson, M. M., 1990. 16S rRNA sequences reveal uncultured inhabitants of a well-studied thermal community. *FEMS Microbiol. Letters*, 75(2), 105–115.
- Ward, D. M., Bateson, M. M., Weller, R., Ruff-Roberts, A. L., 1992. Ribosomal RNA analysis of microorganisms as they occur in nature. *Adv. in Microbial Ecol.*, 12, 219–286.
- Wessels, S., Axelsson, L., Bech Hansen, E., De Vuyst, L., Laulund, S., Lahteenmaki, L., Lindgren, S., Mollet, B., Salminen, S. and von Wright, A., 2004. The lactic acid bacteria, the food chain, and their regulation. *Trends Food Sci. Technol.* 15, 498–505.
- Whittaker, R., 1969. New concepts of kingdoms of organisms. *Science* 163, 150–160.
- Wolfe, B.E., Button, J.E., Santarelli, M., Dutton, R.J., 2014. Cheese rind communities provide tractable systems for *in situ* and *in vitro* studies of microbial diversity. *Cell*, 158, 422–433.
- Wood H.G., 1981. Metabolic cycles in the fermentation by propionic acid bacteria. *Curr. Top. Cell. Regul.* 18, 255-287
- Wyder, M.-T., Amrein, R., Winkler, H., Häni, J.-P., 2000. La morge, un écosystème complexe. Groupe de discussion des fromagers « Gruyère et fromages à pâte mi-dure ». Station fédérale de recherche laitière, Liebefeld, Switzerland.

~ Y ~

- 
- Yang, Z.L., 2011. Molecular techniques revolutionize knowledge of Basidiomycota evolution. *Fungal Divers.* 50, 47–58.



# **Valorisation du travail de** **thèse**





## **Communications orales :**

**Garnier L.**, Valence F., Pinon N., Pawtowski A., Galerne L., Deniel F., Frotté N., Coton E., Mounier J., Production d'ingrédients laitiers fonctionnalisés par des microorganismes producteurs de composés antifongiques. Assemblée Générale du Pôle Agronomique Ouest, 15 mai 2015, Nantes, France.

**Garnier L.**, Valence F., Pawtowski A., Pinon N., Galerne L., Frotté N., Coton E., Mounier J., Production d'ingrédients laitiers fonctionnalisés par des microorganismes producteurs de composés antifongiques. Journée des doctorants du Laboratoire Universitaire de Biodiversité et d'Écologie Microbienne, 4 juillet 2015, Quimper, France.

**Garnier L.**, Valence F., Pinon N., Pawtowski A., Galerne L., Deniel F., Frotté N., Coton E., Mounier J., Production d'ingrédients laitiers fonctionnalisés par des microorganismes producteurs de composés antifongiques. Assemblée Générale de l'Association Bba, 19 mai 2016, Nantes, France.

**Garnier L.**, Production d'ingrédients laitiers fonctionnalisés par des microorganismes producteurs de composés antifongiques. Finale régionale du concours Ma thèse en 180 secondes, Bretagne, 7 avril 2016, Brest, France.

**Garnier L.**, Production d'ingrédients laitiers fonctionnalisés par des microorganismes producteurs de composés antifongiques. Finale inter-régionale du concours Ma thèse en 180 secondes, Bretagne-Pays de la Loire, 29 avril 2016, Rennes, France.

**Garnier L.**, Valence, F., Pawtowski, A., Pinon, N., Camier, B., Coton, M., Coton, E., Mounier, J., Development of antifungal ingredients for dairy products: from *in vitro* screening to pilot scale applications. Spoilers in Food, 28-30 June 2017, Quimper, France.

*Prize of the most transferable results to fight spoilage in the food industry décerné par Saupiquet*



Support pour les présentations aux différentes étapes du concours « Ma thèse en 180 secondes »





Section: How to fight food spoilers?

Development of antifungal ingredients for dairy products: from *in vitro* screening to pilot-scale applications  
**Authors :** Lucille Garnier, Florence Valence, Manon Chatel, Nicolas Pinon, Audrey Pawtowski, Bénédicte Camier, Monika Coton, Franck Deniel, Marielle Harel-Oger, Gilles Garric, Emmanuel Coton and Jérôme Mounier.

**Key words:** antifungal activity, screening, bacteria, molds, dairy products

**Introduction**

In dairy products, bioprotective cultures and their metabolites represent an interesting alternative to chemical preservatives. The aim of this study was to develop dairy ingredients with antifungal properties, active against a large diversity of spoilage fungi and to test their efficiency and organoleptic impact in real products.

**Materials and methods**

First, the biodiversity of spoilage fungi in a large variety of dairy products was investigated using molecular methods in order to select the most appropriate fungal targets. Then, the antifungal activity of fermentates obtained from 698 lactic acid bacteria, propionibacteria and fungal strains were screened *in vitro* using a novel high-throughput method in a cheese mimicking model. After optimizing fermentation conditions to improve antifungal activity, the best fermentates were tested at a pilot scale in uncooked pressed cheese (UPC) and sour cream to evaluate their antifungal activity using challenge and durability tests and their impact on organoleptic properties using sensorial analysis.

**Results**

The *in vitro* screening allowed selecting antifungal ingredients that were also active *in situ*. Durability tests revealed that a *Lactobacillus rhamnosus* fermentate had a promising activity in sour cream. For UPC, a *Propionibacterium jensenii*-based ingredient could be used instead of natamycin delaying *Mucor racemosus* and *Penicillium commune* for up to 16 days.

**Conclusion**

These approaches led to the development of new antifungal dairy-based ingredients that might replace natamycin in UPC.

## Communications affichées :

**Garnier L.**, Valence F., Pawtowski A., Pinon N., Galerne L., Frotté N., Coton E., Mounier J. Production d'ingrédients laitiers fonctionnalisés par des microorganismes producteurs de composés antifongiques. 5<sup>ième</sup> Journée des doctorants de l'Ecole Doctorale Santé, Information – Communications, Mathématiques, Matière, 24 septembre 2015, Plouzané, France.

**Garnier L.**, Valence F., Pawtowski A., Galerne L., Deniel F., Frotté N., Coton E., Mounier J. Diversity of spoilage fungi in dairy products and environment, and their resistance to chemical preservatives. 3<sup>rd</sup> International conference on microbial diversity, the challenge of complexity, 27-30 october 2015, Perugia, Italy.

2<sup>nd</sup> prix du poster décerné par *International Committee on Food Microbiology and Hygiene*

**Garnier L.**, Mounier J., Pawtowski A., Pinon N., Frotté N., Coton E., Valence F. High-throughput screening for antifungal activities of bacterial and fungal isolates in a cheese-like medium. 3<sup>rd</sup> International conference on microbial diversity, the challenge of complexity, 27-30 october 2015, Perugia, Italy.

**Garnier L.**, Valence F., Pawtowski A., Pinon N., Galerne L., Frotté N., Camier B., Coton E., Mounier J. Production d'ingrédients laitiers fonctionnalisés par des microorganismes producteurs de composés antifongiques. Colloque SFR ScInBioS IBSAM, 7 juin 2016, UFR Sciences, UBO, Brest, France.

Prix du poster décerné par l'ED SICMA et IBSAM

**Garnier L.**, Valence F., Pawtowski A., Galerne L., Deniel F., Frotté N., Coton E., Mounier J. Diversity of spoilage fungi in dairy products and environment, and their resistance to chemical preservatives. FoodMicro, 19-22 July 2016, Dublin, Ireland.

**Garnier L.**, Mounier J., Pawtowski A., Pinon N., Frotté N., Coton E., Valence F. New high-throughput screening method for antifungal activities in a cheese-like medium. FoodMicro, 19-22 July 2016, Dublin, Ireland.

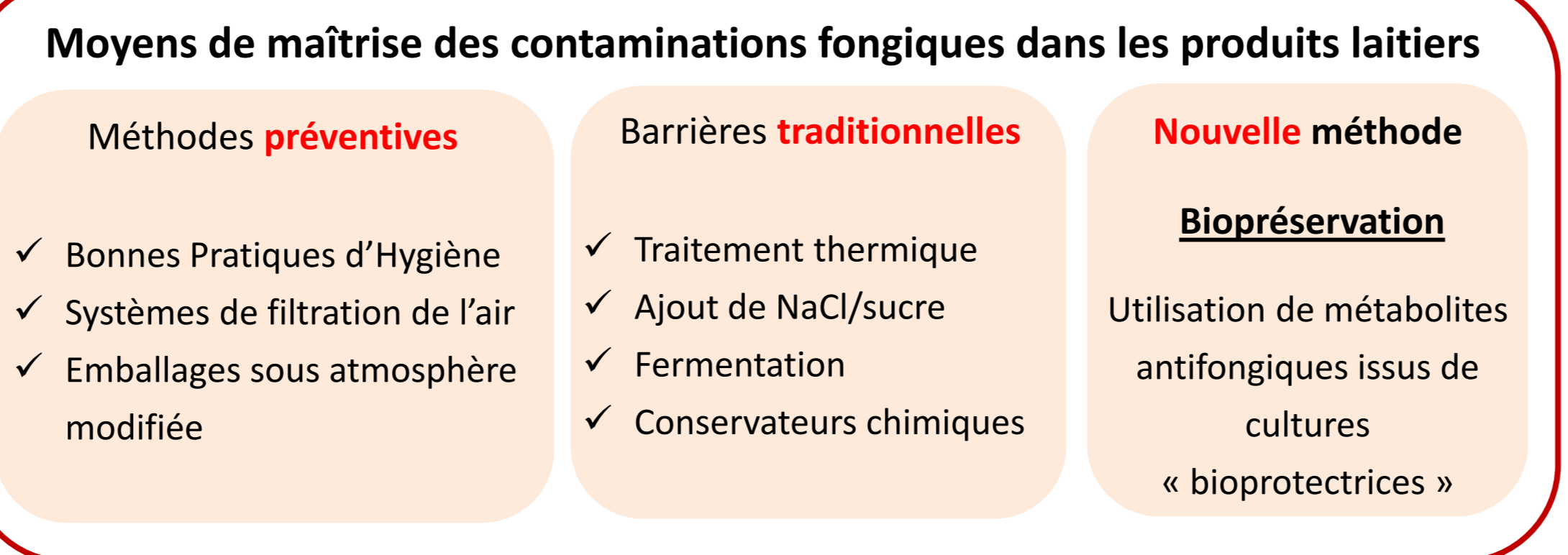
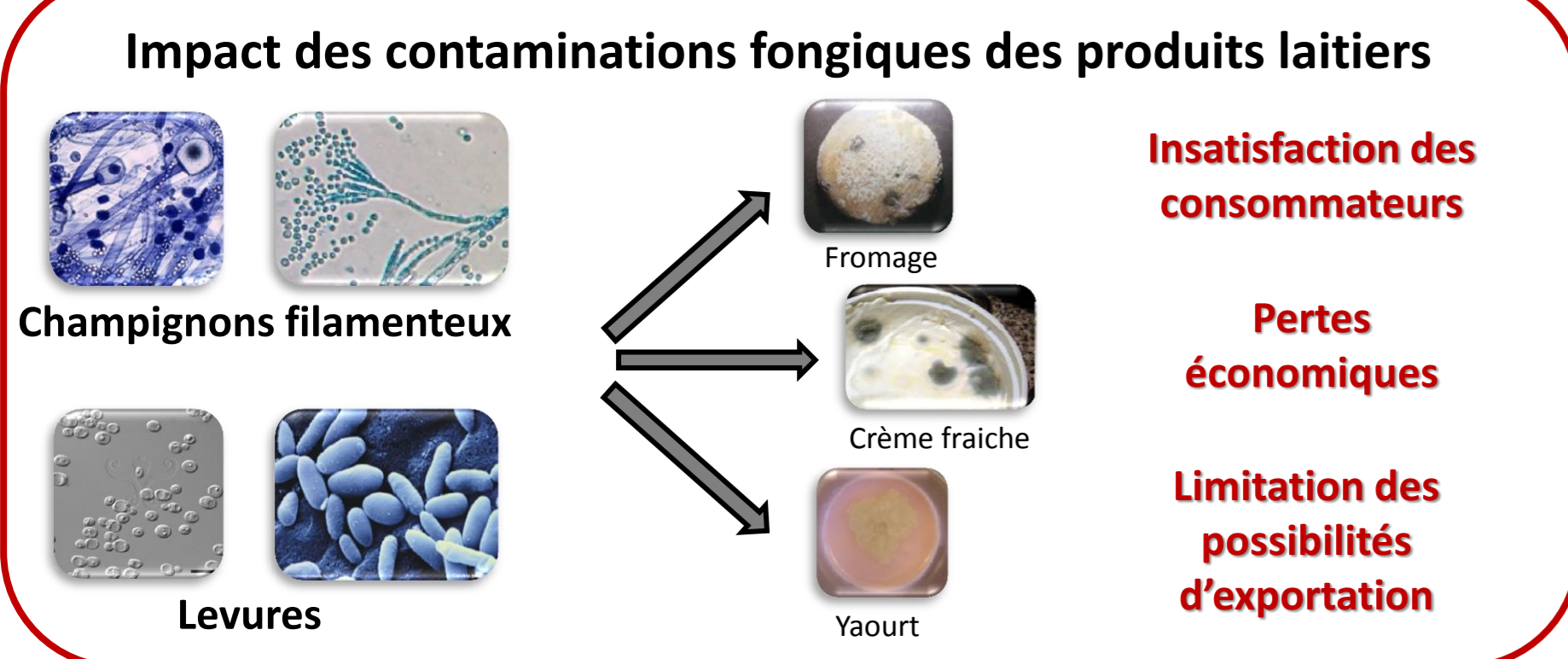


L. Garnier<sup>1</sup>, F. Valence<sup>2</sup>, A. Pawtowski<sup>1</sup>, N. Pinon<sup>2</sup>, F. Deniel<sup>1</sup>, L. Galerne<sup>1</sup>, N. Frotté<sup>1</sup>, E. Coton<sup>1</sup> et J. Mounier<sup>1</sup>

<sup>1</sup> Université de Brest, EA 3882, Laboratoire Universitaire de Biodiversité et Ecologie Microbienne, ESIAB, Technopôle Brest-Iroise, 29280 Plouzané, France.  
<sup>2</sup> INRA, UMR1253 Science et Technologie du Lait et de l'Œuf, F-35042 Rennes, France.

Contact: [lucille.garnier@univ-brest.fr](mailto:lucille.garnier@univ-brest.fr)  
[www.researchgate.net/profile/Garnier\\_Lucille](http://www.researchgate.net/profile/Garnier_Lucille)

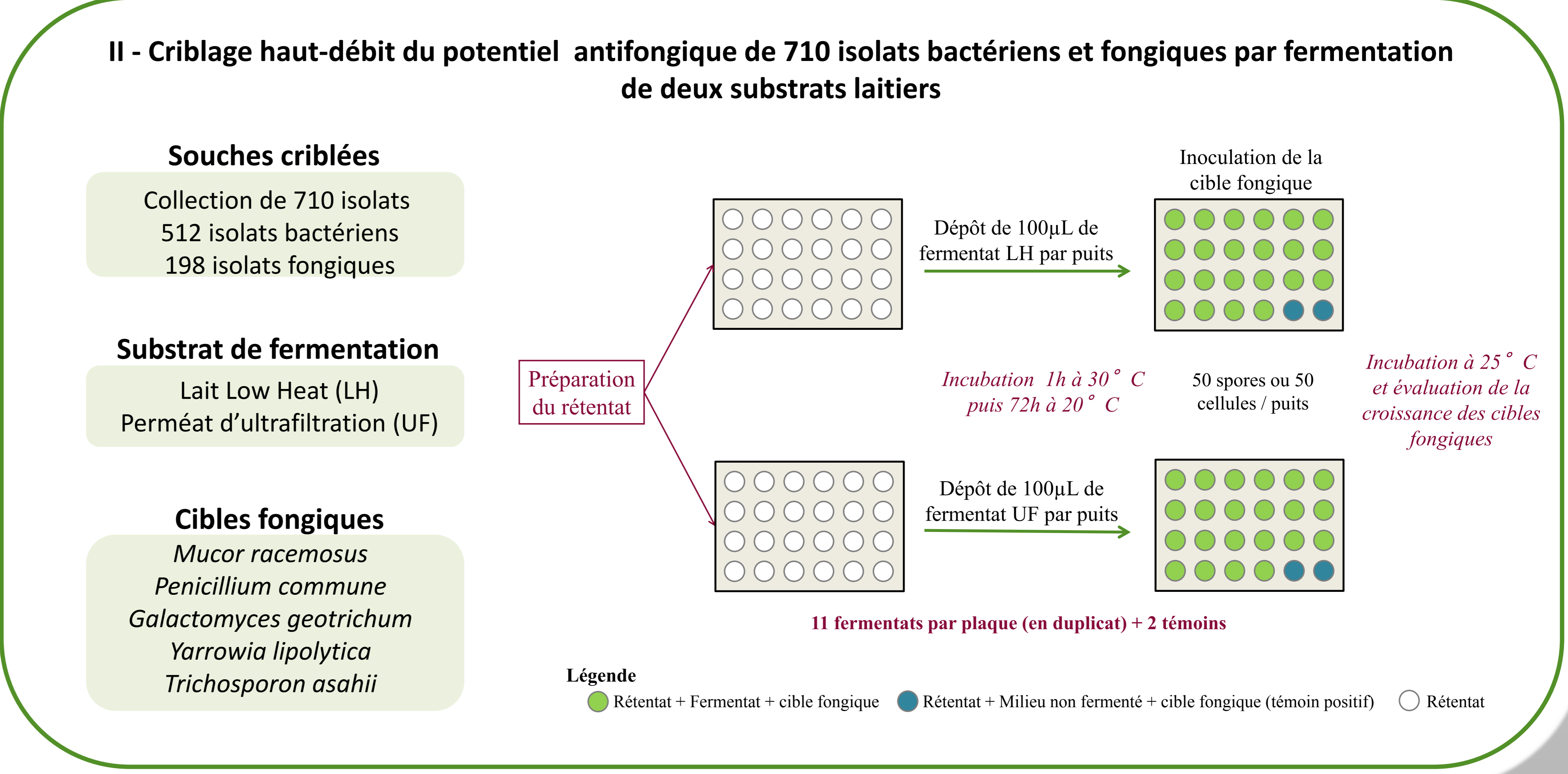
## INTRODUCTION



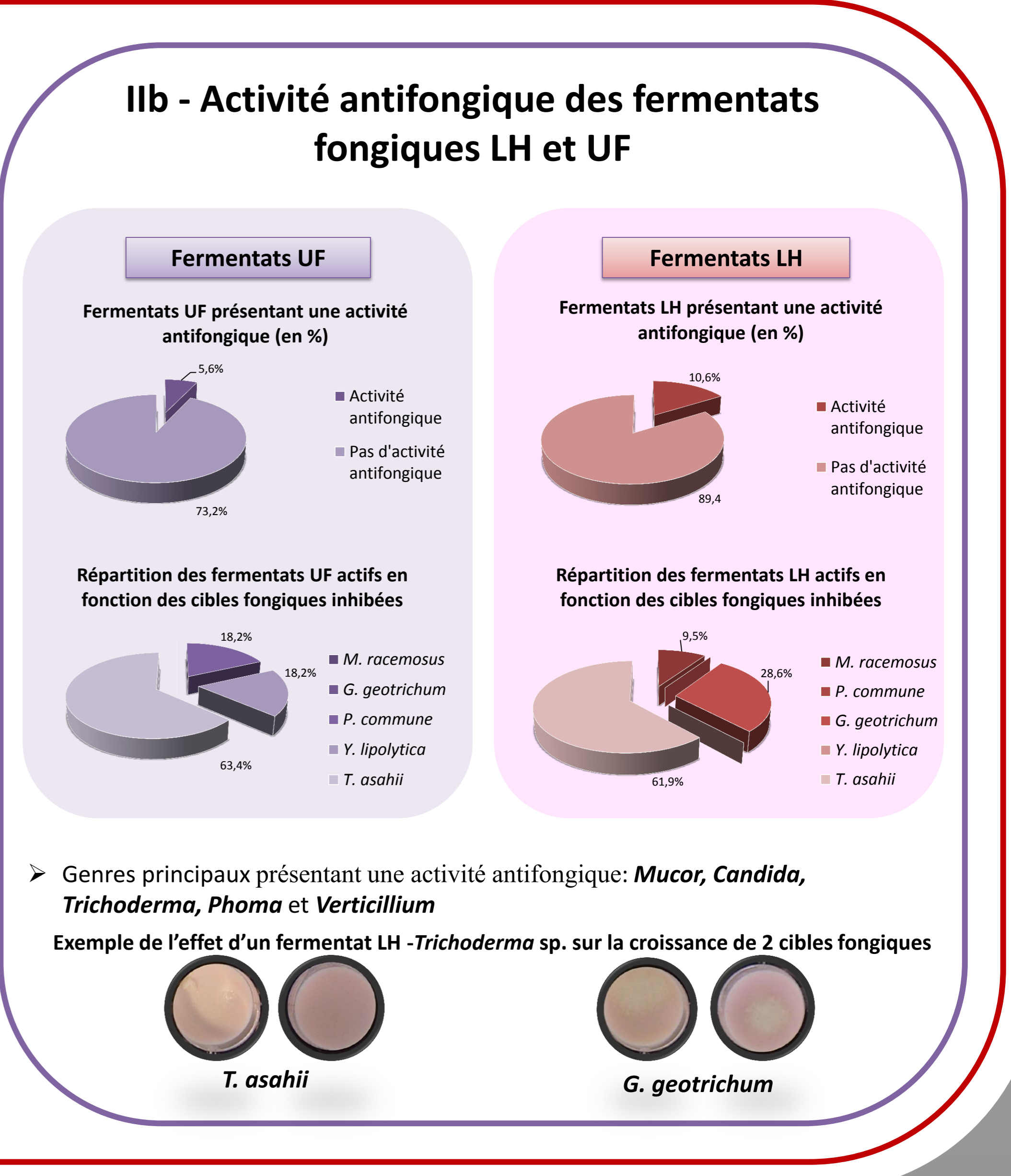
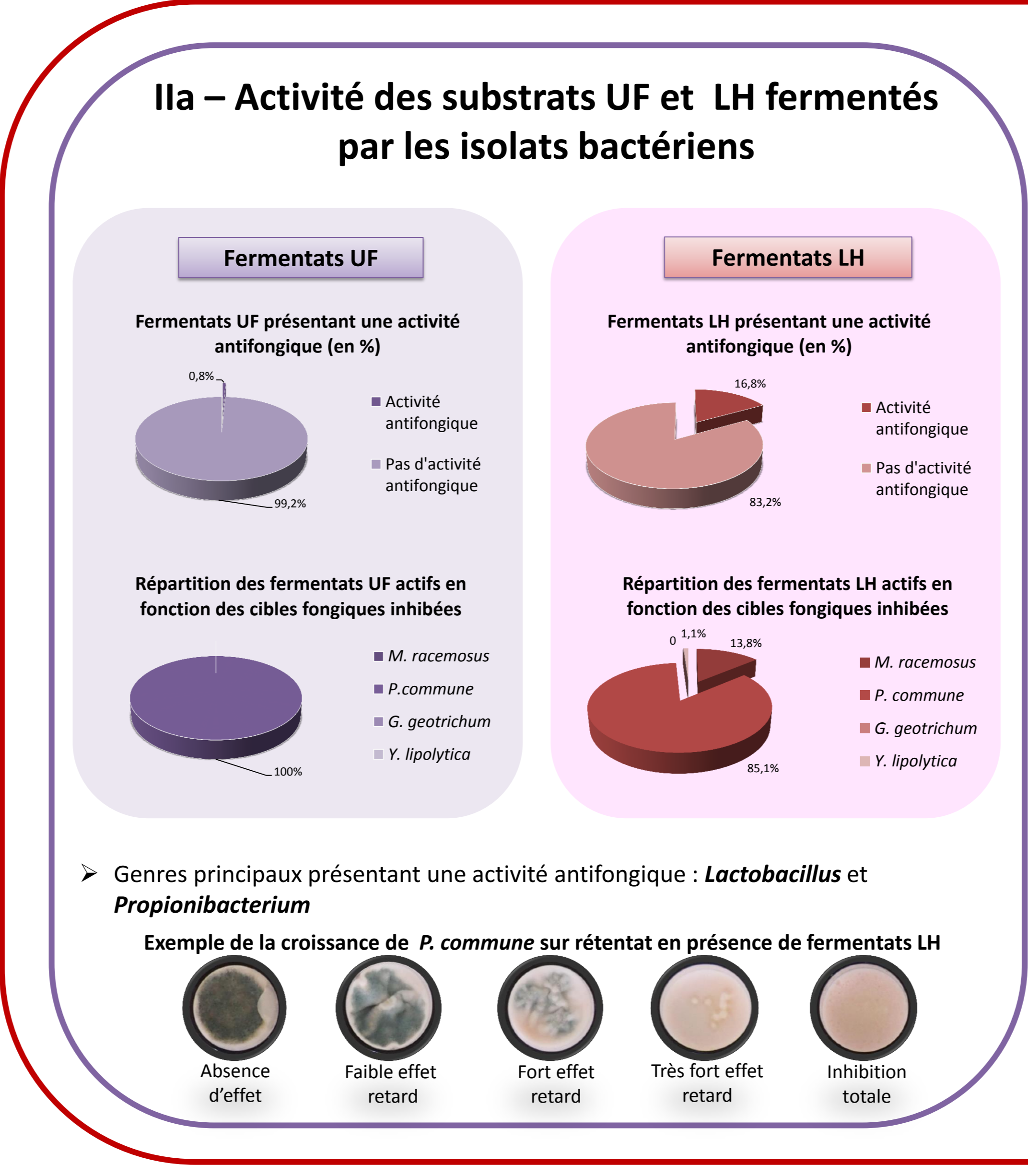
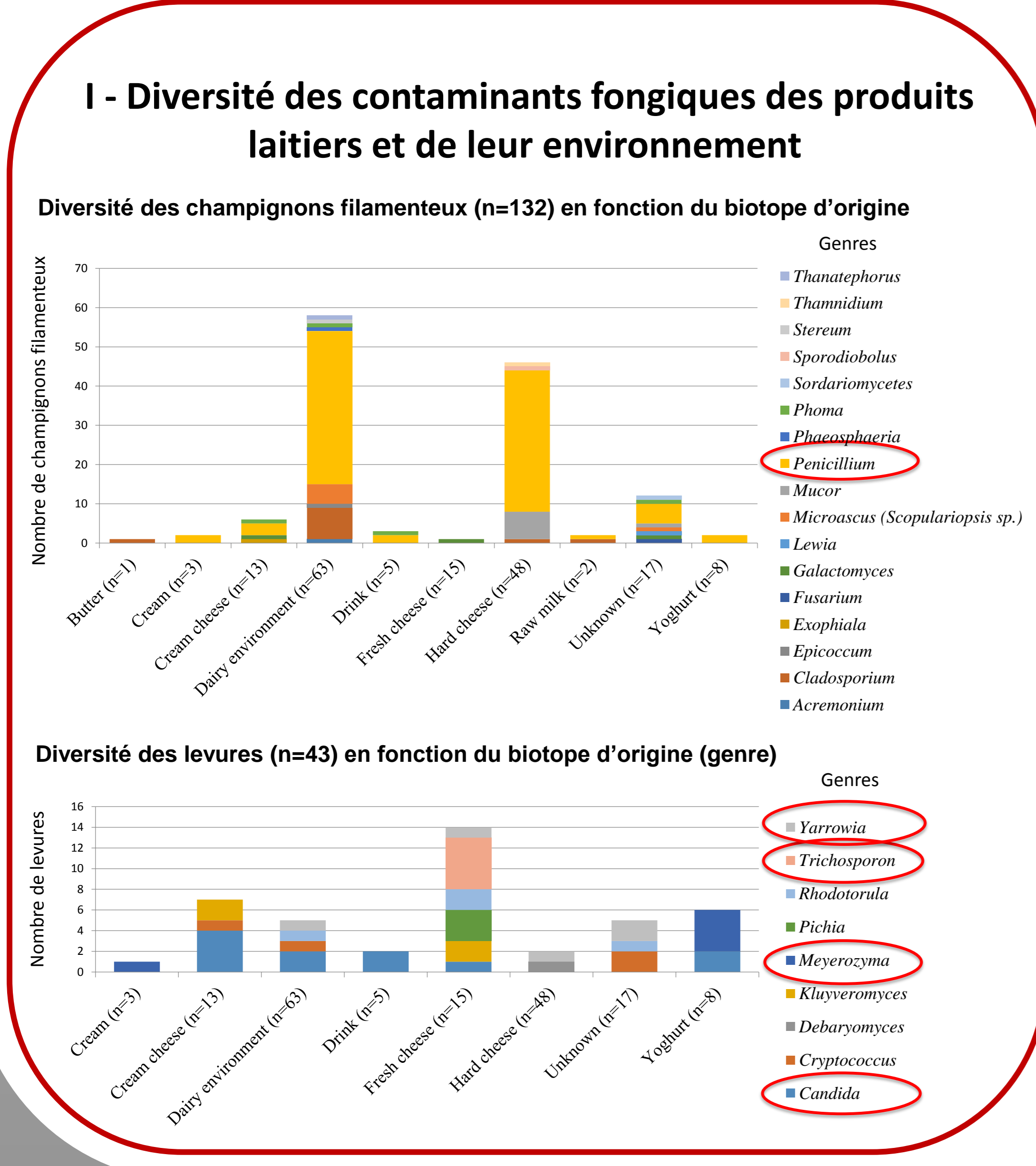
## OBJECTIFS

- Quelle est la diversité des contaminants fongiques des produits et de l'environnement laitiers?
- Quels sont les microorganismes présentant une activité antifongique dans un milieu laitier?

## MATÉRIEL ET MÉTHODES



## RÉSULTATS



## CONCLUSION

L'étude de la diversité des contaminants fongiques des produits laitiers et de leur environnement a permis la mise en place d'une collection de 175 isolats composée de **43 levures** et **132 champignons filamenteux**. Au sein de cette collection, **5 cibles fongiques représentatives** de cette diversité et issues de produits contaminés ont été utilisées lors des criblages: *M. racemosus*, *P. commune*, *G. geotrichum*, *Y. lipolytica* et *T. asahii*. Le criblage de l'activité antifongique de la collection a permis la **sélection de 22 isolats bactériens** appartenant aux espèces *Lactobacillus buchneri*, *Lactobacillus casei*, *Lactobacillus brevis*, *Lactobacillus plantarum*, *Lactobacillus rhamnosus*, *Lactobacillus lactobacillus* et *Propionibacterium jensenii*. Chacune de ces souches est capable de retarder de un à plusieurs jours la croissance de 2 à 4 cibles fongiques. Il a également permis la **sélection de 3 isolats fongiques**, appartenant aux genres *Mucor*, *Torulasporea* et *Trichoderma*, capables de retarder fortement la croissance de 1 ou 2 cibles fongiques chacun. De plus, il est important de noter l'**impact du substrat de fermentation** sur l'activité antifongique des différents isolats.

## PERSPECTIVES

L'optimisation de l'activité antifongique des souches d'intérêt est actuellement en cours pour 13 fermentats. Par la suite, des **tests sur produits réels à l'échelle pilote** sont prévus, de même que l'**identification des composés et des mécanismes** impliqués dans l'activité antifongique de souches bactériennes et fongiques. Ainsi, la finalité du projet est d'obtenir un ingrédient antifongique et 100% laitier.



# Diversity of spoilage fungi in dairy products and environment, and their resistance to chemical preservatives

L. Garnier<sup>a,b</sup>, F. Valence<sup>b</sup>, A. Pawtowski<sup>a</sup>, L. Galerne<sup>a</sup>, F. Deniel<sup>a</sup>, N. Frotté<sup>a</sup>, E. Coton<sup>a</sup>, and J. Mounier<sup>a</sup>

<sup>a</sup> Université de Brest, EA 3882, Laboratoire Universitaire de Biodiversité et Ecologie Microbienne, ESIAB, Technopôle Brest-Iroise, 29280 Plouzané, France.

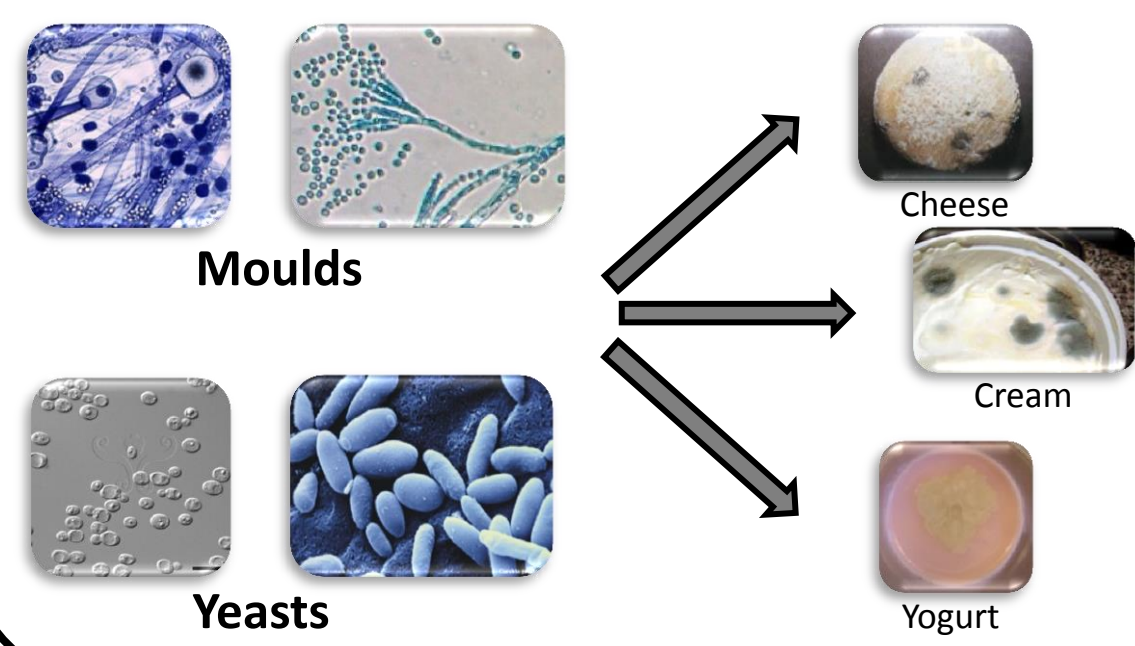
<sup>b</sup> INRA, UMR1253 Science et Technologie du Lait et de l'Œuf, F-35042 Rennes, France.

Contact: [lucille.garnier@univ-brest.fr](mailto:lucille.garnier@univ-brest.fr)

[https://www.researchgate.net/profile/Garnier\\_Lucille](https://www.researchgate.net/profile/Garnier_Lucille)

## INTRODUCTION

### Impact of fungal contaminations in dairy products



**Substantial economic losses**  
**Consumer Dissatisfaction**  
**Negative brand image**  
**Limitation of export possibilities**

### Control of fungal contaminations

#### Preventive methods

- ✓ Good manufacturing practices
- ✓ Good hygiene practice
- ✓ Air filtration systems

#### Hurdle technologies

- ✓ Heat treatment
- ✓ Cold storage
- ✓ Addition of NaCl/sugar
- ✓ Fermentation
- ✓ Modified atmosphere packaging
- ✓ **Chemical preservatives**

## SCIENTIFIC QUESTIONS

I

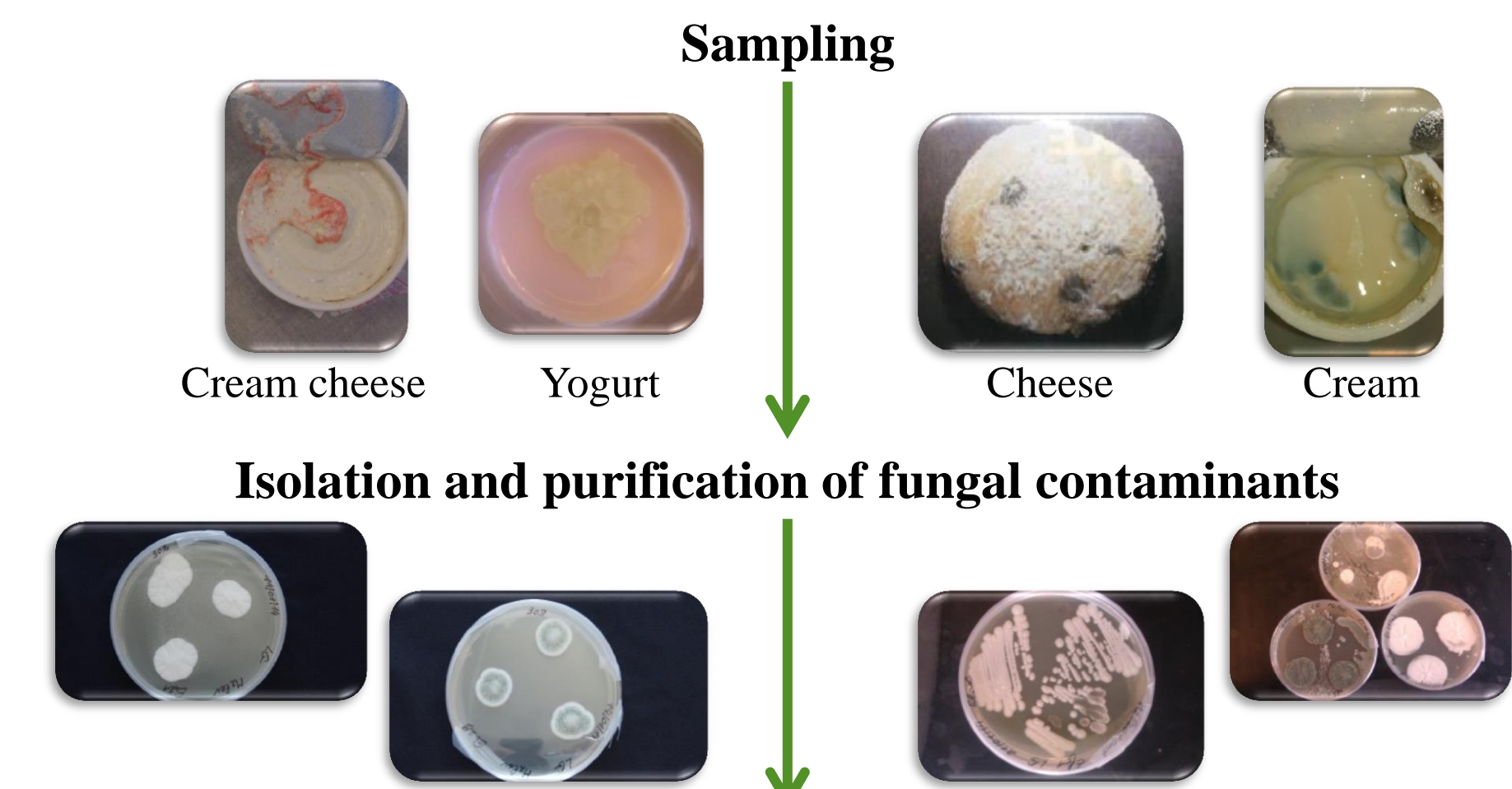
What is the diversity of fungal contaminants present in the dairy environment and spoiled products?

II

What is the resistance of spoilage fungi to chemical preservatives?

## MATERIALS AND METHODS

### I - Characterization of the diversity of fungal contaminants in dairy products and their environment



### Identification of fungal contaminants by sequencing

- ❖ D1-D2 domain of the 26S rRNA gene → **Yeasts**
- ❖ ITS region → **Moulds**
- ❖ Partial  $\beta$ -tubulin → **Moulds**
- ❖ EF1- $\alpha$  → **Moulds**

### II - Determination of the resistance to chemical preservatives

#### Fungal targets

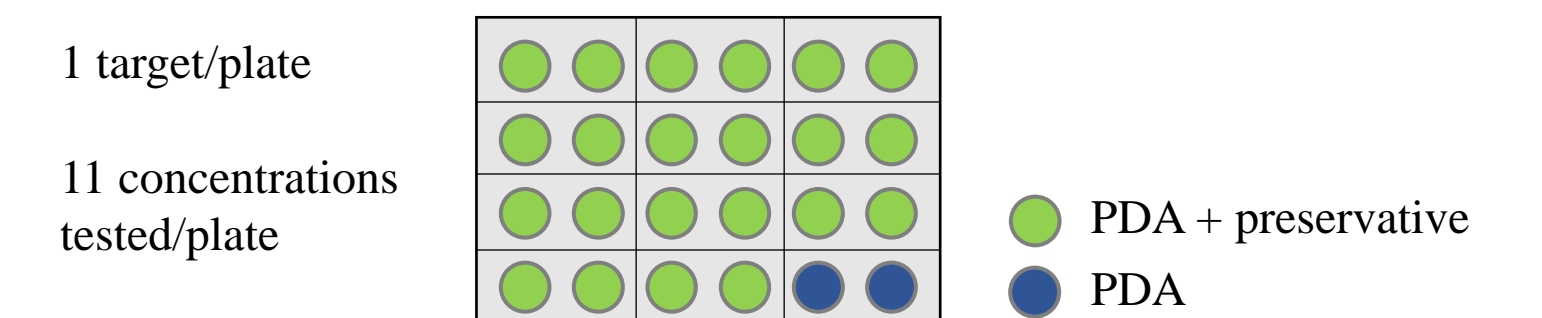
**Moulds**  
*Cladosporium halotolerans*  
*Galactomyces geotrichum*  
*Mucor racemosus*  
*Penicillium commune*  
*Phoma pinodella*

**Yeasts**  
*Candida parapsilosis*  
*Meyerozyma guilliermondii*  
*Rhodotorula mucilaginosa*  
*Trichosporon asahii*  
*Yarrowia lipolytica*

#### Chemical preservatives

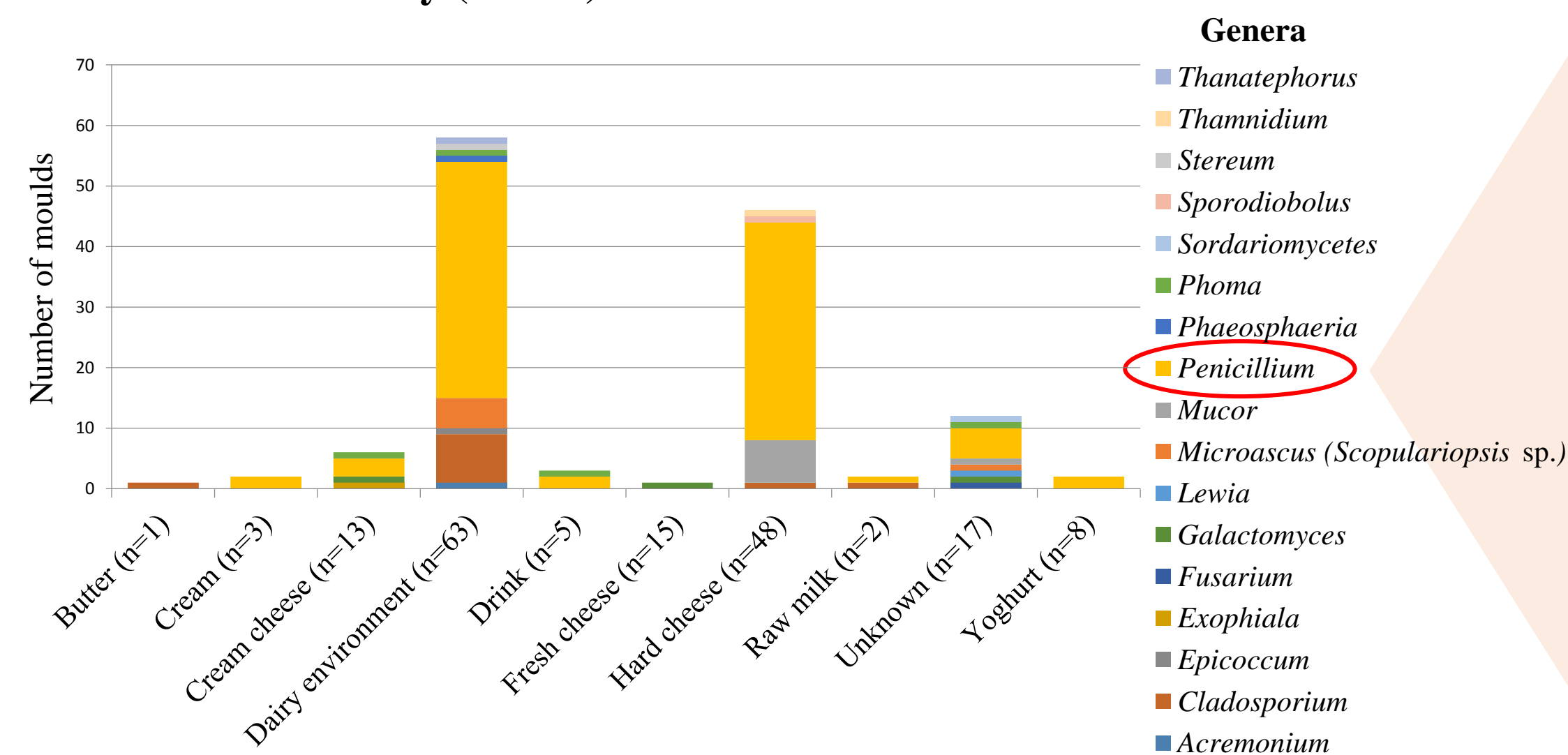
Calcium propionate } Tested concentrations (pH 5)  
Potassium sorbate } 0.04 0.08; 0.1; 0.15; 0.3; 0.5; 1; 1.5; 2  
Sodium benzoate } 2.5; 3 g.l<sup>-1</sup>

Natamycin (Pimaricin) } Tested concentrations (pH 5)  
(surface treatment) } 0.01; 0.02; 0.04; 0.06; 0.08; 0.1; 0.12;  
0.14; 0.16; 0.18; 0.2 g.l<sup>-1</sup>

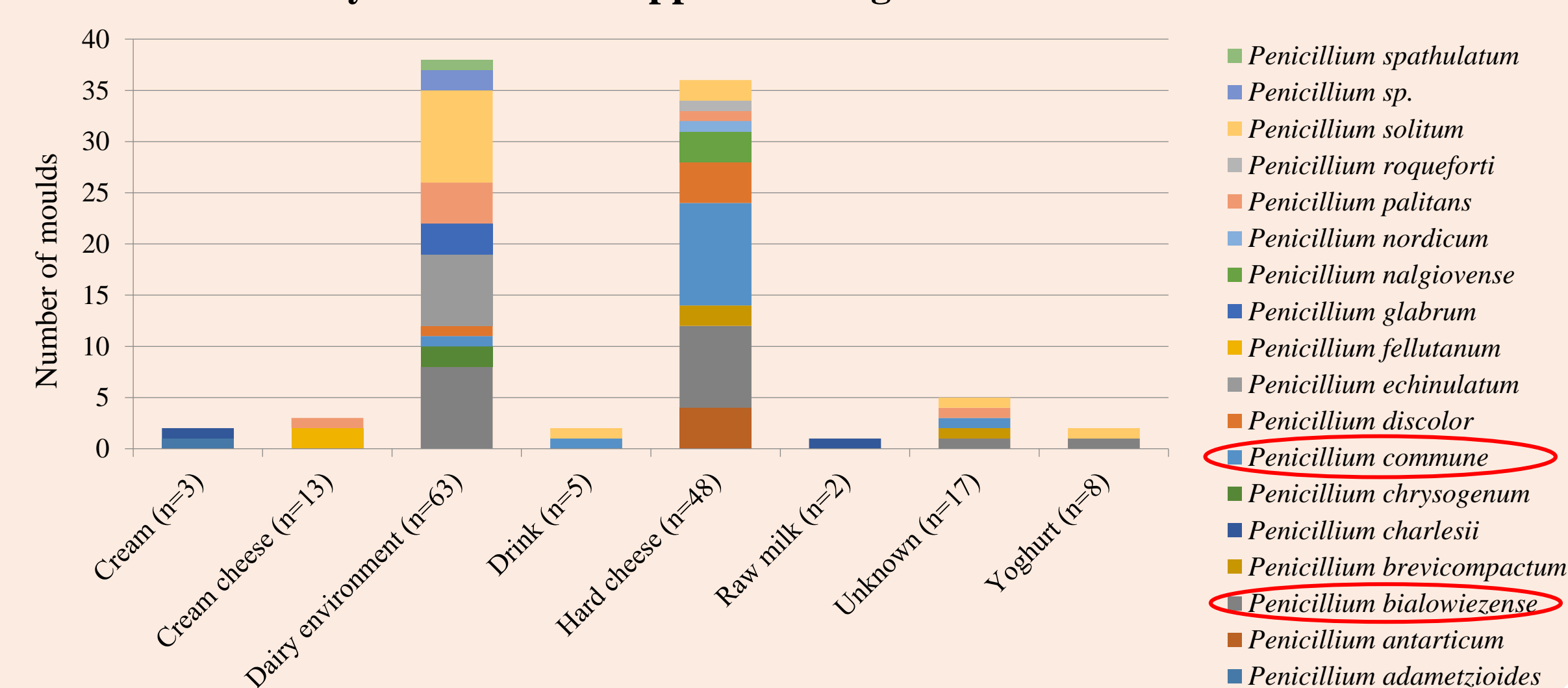


## RESULTS

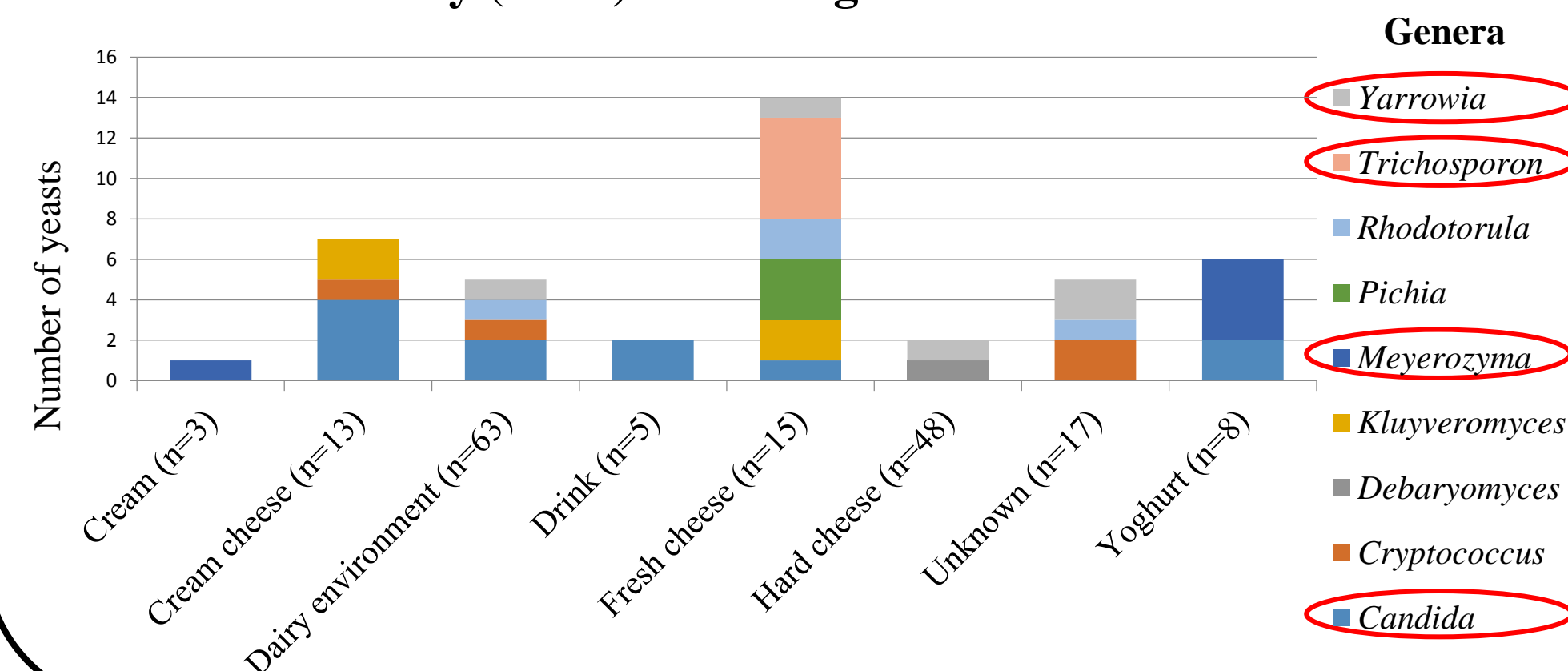
### Ia - Mould diversity (n=132) in relation to their isolation source



### Ib - Diversity of *Penicillium* spp. according to their isolation source



### Ic - Yeast diversity (n=43) according to their isolation source



### II - Resistance of yeasts and moulds to chemical preservatives

	Minimal inhibitory concentrations			
	Calcium propionate (g.l <sup>-1</sup> )	Potassium sorbate (g.l <sup>-1</sup> )	Sodium benzoate (g.l <sup>-1</sup> )	Natamycin (g.l <sup>-1</sup> )
<i>Cladosporium halotolerans</i>	2	0.1	0.3	0.04
<i>Galactomyces geotrichum</i>	> 3*	1	1	0.1
<i>Mucor racemosus</i>	> 3*	0.5	1	0.1
<i>Penicillium commune</i>	> 3*	0.3	2	0.06
<i>Phoma pinodella</i>	3*	0.15	0.5	0.1
<i>Candida parapsilosis</i>	> 3*	1	2	0.1
<i>Meyerozyma guilliermondii</i>	> 3*	0.5	1	0.08
<i>Rhodotorula mucilaginosa</i>	> 3*	0.15	1	0.1
<i>Trichosporon asahii</i>	> 3*	0.5	1	0.1
<i>Yarrowia lipolytica</i>	> 3*	1	> 3*	> 0.2*

\* Highest tested concentrations

## CONCLUSION

A **high diversity** was found among fungal isolates. Indeed, **25 genera representing 67 species** were identified out of 175 isolates, with ***Penicillium* being the most prevalent** (51.4% of total species), were ***Cladosporium*, *Mucor* and *Microascus***. At the species level, ***Penicillium commune* and *Penicillium bialowiezense* were the most prevalent** filamentous fungi, representing 10.2% and 7.4% of fungal isolates, respectively. Concerning yeast, ***Candida*, *Meyerozyma*, *Trichosporon* and *Yarrowia* are the most prevalent**. Moreover, fungal species such as ***Penicillium antarcticum* and *Penicillium salamii* were identified for the first time in dairy products**. In addition, numerous species were both identified in spoiled products and after air-sampling suggesting that **dairy environment acts as a primary source of contamination**. Concerning chemical preservatives, **natamycin and potassium sorbate were the most efficient** followed by sodium benzoate and calcium propionate. Finally, several species, such as ***Penicillium commune* and *Yarrowia lipolytica* presented high minimal inhibitory concentrations for calcium propionate, sodium benzoate and natamycin**. In conclusion, this study provided useful information on the occurrence of fungal contaminants in dairy products and should help to develop adequate strategies for combating fungal spoilage.

# High-throughput screening for antifungal activities of bacterial and fungal isolates in a cheese-like medium

L. Garnier<sup>a,b</sup>, J. Mounier<sup>a</sup>, A. Pawtowski<sup>a</sup>, N. Pinon<sup>b</sup>, N. Frotté<sup>a</sup>, E. Coton<sup>a</sup> and F. Valence<sup>b</sup>

<sup>a</sup> Université de Brest, EA 3882, Laboratoire Universitaire de Biodiversité et Ecologie Microbienne, ESIAB, Technopôle Brest-Iroise, 29280 Plouzané, France.

<sup>b</sup> INRA, UMR1253 Science et Technologie du Lait et de l'Œuf, F-35042 Rennes, France.

## INTRODUCTION

**Fungal spoilage** is an important cause of food losses in the dairy industry. In this context, the use of **bioprotective cultures** can be an alternative or a complementary approach to be considered. Lactic acid bacteria (LAB) and propionibacteria, as well as fungi, can exhibit antifungal activities. Nevertheless, antifungal activities of LAB, propionibacteria and fungi are **highly strain-dependent** and **vary according to the growth medium**. This activity is based on **multifactorial mechanisms** and related to the production of antifungal compounds such as organic acids, fatty acids, proteinaceous compounds, cyclic dipeptides or reuterin. In this context, it is necessary to develop high-throughput screening methods to test a large number of strains in order to find the most efficient ones.

## AIM

To develop a high-throughput screening method allowing the rapid detection of antifungal activities in a cheese-like model in order to elaborate a new antifungal dairy ingredient.

## DESCRIPTION OF THE SCREENING METHOD DEVELOPPED

703 screened isolates...

505 bacterial isolates (25 species belonging to 5 genera)  
198 fungal isolates (81 species belonging to 29 genera)

... cultivated in 2 dairy media ...

- ❖ 10%-reconstituted low heat skim milk supplemented with 45% anhydrous milk fat (LH)
- ❖ 6-fold concentrated milk ultrafiltration (0,2 µm) permeate and complemented with 10g/l yeast extract and a pH indicator (UF)

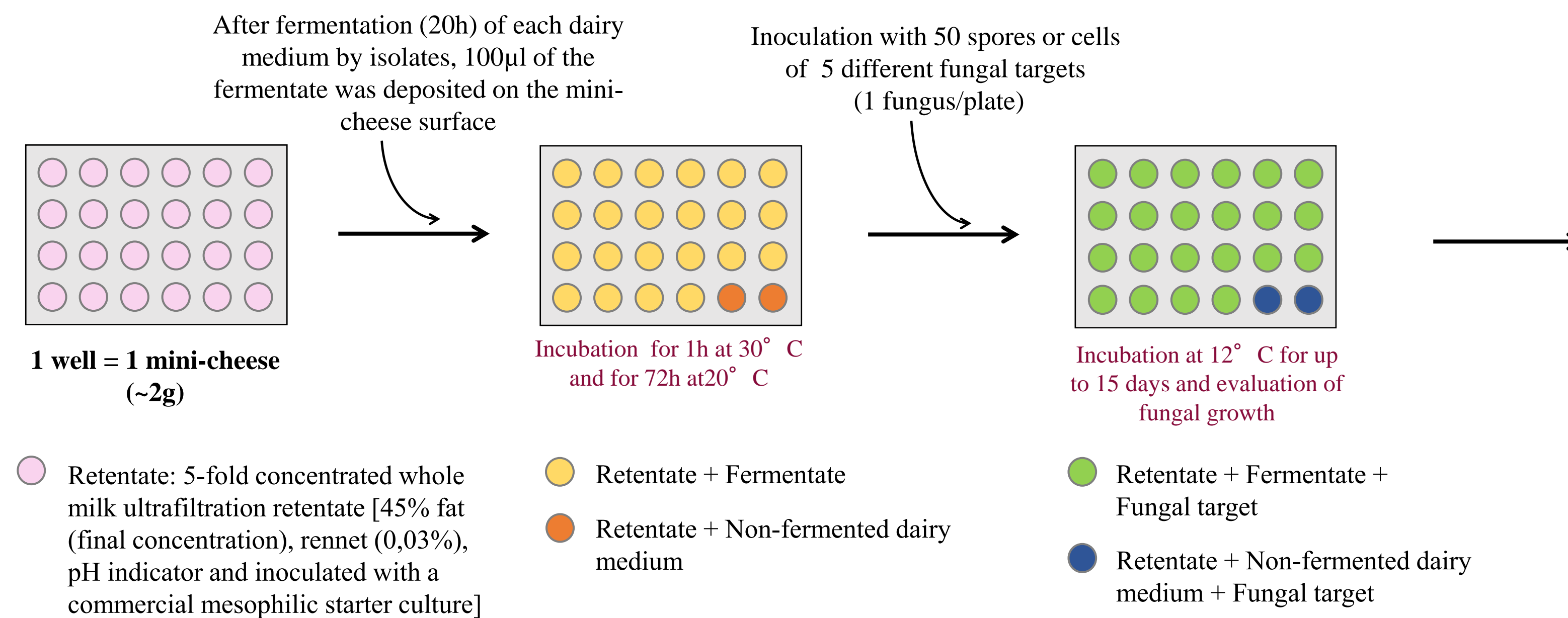
...and tested against 4 fungal targets, representative of the diversity of spoilage fungi in dairy products\*,...

*Mucor racemosus*  
*Penicillium commune*  
*Galactomyces geotrichum*  
*Yarrowia lipolytica*

(\*See poster entitled "Diversity of spoilage fungi in dairy products and environment, and their resistance to chemical preservatives")

...in duplicate.

**11248 cultures performed**



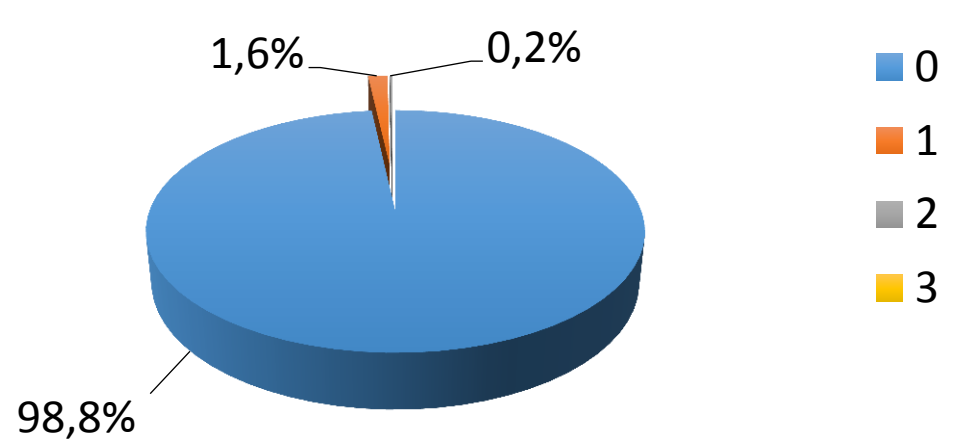
- Retentate: 5-fold concentrated whole milk ultrafiltration retentate [45% fat (final concentration), rennet (0,03%), pH indicator and inoculated with a commercial mesophilic starter culture]
- Retentate + Fermentate
- Retentate + Non-fermented dairy medium
- Retentate + Fermentate + Fungal target
- Retentate + Non-fermented dairy medium + Fungal target

Visual appearance					
Antifungal effect	No effect	Weak effect	High effect	Very high effect	Total inhibition
Antifungal score	0	1	2	2	3

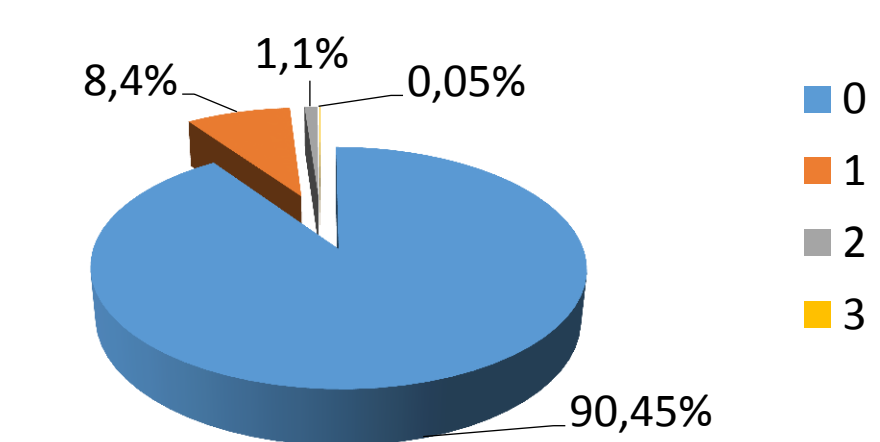
Example of the effect of LH medium fermented by different *Lactobacillus* spp. on the growth of *P. commune*

## RESULTS

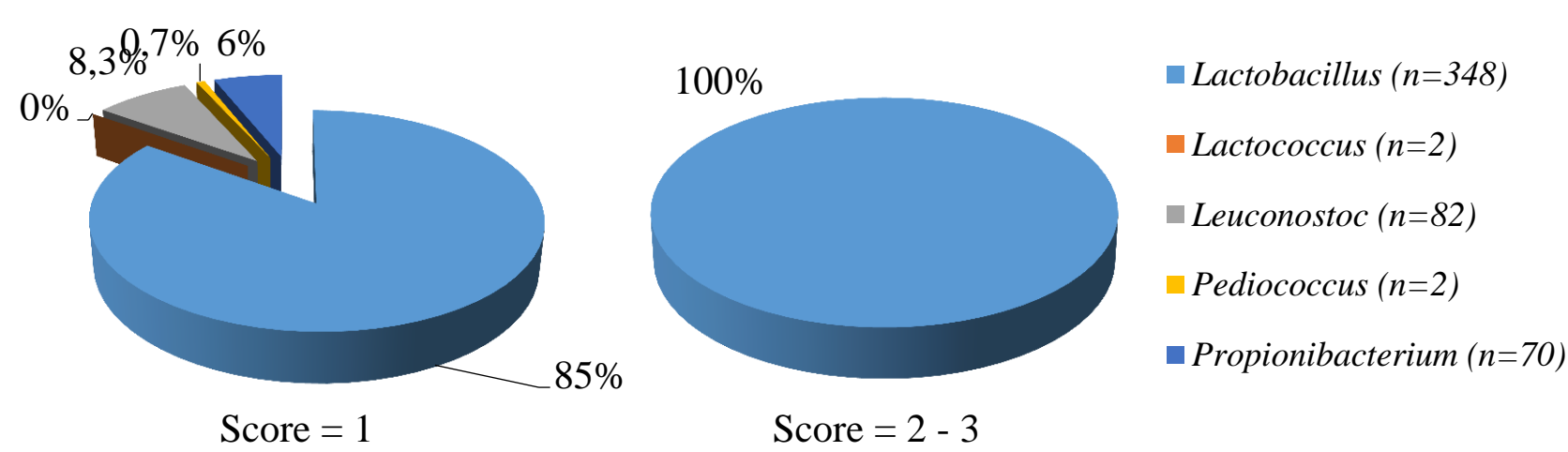
Distribution of antifungal scores of UF fermentates (%)



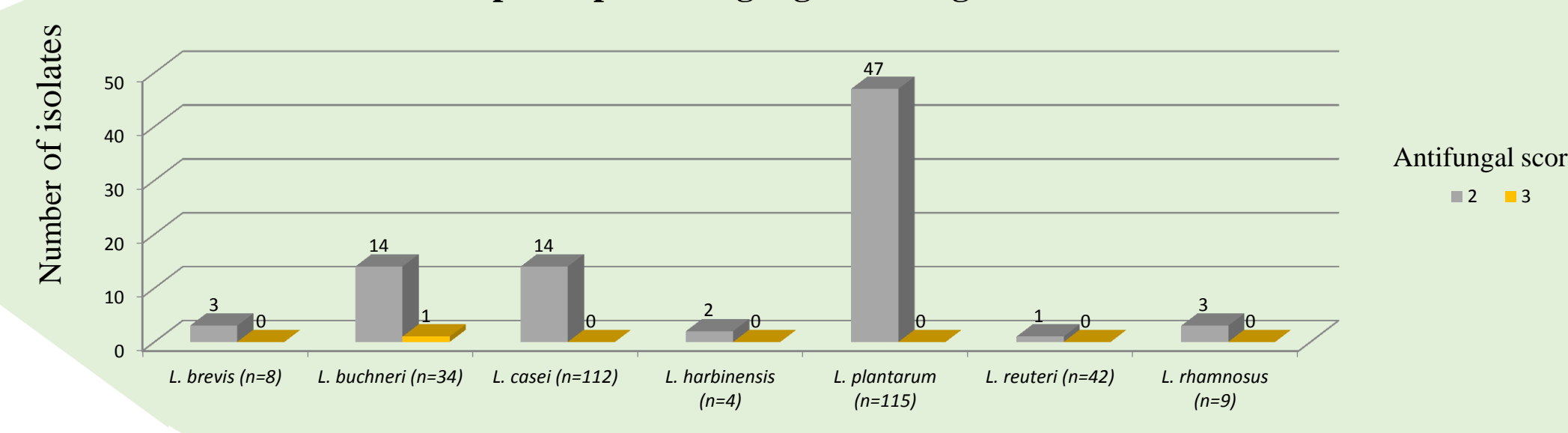
Distribution of antifungal scores of LH fermentates (%)



Distribution of the different genera presenting weak (score = 1) and high (score = 2 or 3) antifungal activities



*Lactobacillus* species presenting high antifungal activities after LH fermentation



Antifungal scores attributed to different isolates of *Lactobacillus buchneri* against the 4 fungal targets

	<i>M. racemosus</i>	<i>P. commune</i>	<i>G. geotrichum</i>	<i>Y. lipolytica</i>
<i>Lactobacillus buchneri</i> L164	2	2	0	3
<i>Lactobacillus buchneri</i> L162	2	2	1	0
<i>Lactobacillus buchneri</i> L111	1	2	1	0
<i>Lactobacillus buchneri</i> L151	2	2	0	0
<i>Lactobacillus buchneri</i> L233	1	2	0	0
<i>Lactobacillus buchneri</i> L169	1	2	0	0

## CONCLUSION

This high-throughput screening for antifungal activity allowed the testing of **1400 cultures per week** on average and confirmed the **impact of the growth medium** used on the expression of antifungal activities by bacteria or fungi. Indeed, 4 and 48 bacteria, and, 11 and 21 fungi, inhibited at least one fungal target after cultivation in UF and LH, respectively. This screening showed that antifungal activity was **strain-dependent** and **varied as a function of the tested fungal target**. Among the 4 tested fungal targets, *P. commune* was the most frequently inhibited fungus, followed by *M. racemosus* while only few isolates were able to inhibit *G. geotrichum* or *Y. lipolytica*. This method was validated for 2 ingredients compatible with dairy technologies and should facilitate the scale-up of promising isolates. Finally, this new miniaturized high-throughput screening method opens new possibilities to screen microorganisms for their antifungal activities in dairy media.

L. Garnier<sup>1</sup>, F. Valence<sup>2</sup>, A. Pawtowski<sup>1</sup>, N. Pinon<sup>2</sup>, F. Deniel<sup>1</sup>, L. Galerne<sup>1</sup>, N. Frotté<sup>1</sup>, B. Camier<sup>2</sup>, E. Coton<sup>1</sup> et J. Mounier<sup>1</sup>

<sup>1</sup> Université de Brest, EA 3882, Laboratoire Universitaire de Biodiversité et Ecologie Microbienne, ESIAB, Technopôle Brest-Iroise, 29280 Plouzané, France.  
<sup>2</sup> INRA, UMR1253 Science et Technologie du Lait et de l'CEuf, F-35042 Rennes, France.

Contact: [lucille.garnier@univ-brest.fr](mailto:lucille.garnier@univ-brest.fr)  
[www.researchgate.net/profile/Garnier\\_Lucille](http://www.researchgate.net/profile/Garnier_Lucille)

## INTRODUCTION

**Impact des contaminations fongiques des produits laitiers**

Champignons filamenteux  
Levures

Insatisfaction des consommateurs  
Pertes économiques  
Limitation des possibilités d'exportation

**Moyens de maîtrise des contaminations fongiques dans les produits laitiers**

Méthodes préventives	Barrières traditionnelles	Nouvelle méthode
<ul style="list-style-type: none"> <li>Bonnes Pratiques d'Hygiène</li> <li>Systèmes de filtration de l'air</li> <li>Emballages sous atmosphère modifiée</li> </ul>	<ul style="list-style-type: none"> <li>Traitement thermique</li> <li>Ajout de NaCl/sucre</li> <li>Fermentation</li> <li>Conservateurs chimiques</li> </ul>	<p><b>Biopréservation</b></p> <p>Utilisation de métabolites antifongiques issus de cultures « bioprotectrices »</p>

## QUESTIONS SCIENTIFIQUES

- I  
Quelle est la diversité des contaminants fongiques des produits et de l'environnement laitiers?
- II  
Quels sont les microorganismes présentant une activité antifongique dans un milieu laitier?
- III  
Parmi ces microorganismes, lesquels présentent une activité dans un produit réel?

## MATÉRIEL ET MÉTHODES

**I - Caractérisation de la diversité des contaminants fongiques présents dans les produits laitiers et leur environnement**

Collecte des échantillons  
Crème fraîche, Yaourt, Fromage, Crème

Isolement et purification des contaminants

Identification des contaminants par séquençage

- Domaine D1-D2 du gène codant l'ARNr 28S → **Levures**
- Région ITS → **Champignons filamenteux**
- Portion du gène codant la β-tubuline
- EF1-α

**II - Criblage haut-débit du potentiel antifongique de 703 isolats bactériens et fongiques par fermentation de deux substrats laitiers**

703 isolats criblés...  
505 isolats bactériens  
198 isolats fongiques

... cultivés dans 2 fractions laitères...  
❖ LH: Lait Low Heat reconstitué à 10% + émulsion de MGLA à 45% (30g MG/l final)  
❖ UF: Perméat d'ultrafiltration 6X (stérilisé par filtration 0.22µm) complétement à 10g/l d'extrait de levure + pourpre de bromocrésol

...et testés contre 4 cibles fongiques, représentatives de la diversité des contaminants fongiques des produits laitiers,...

...en duplicats.  
**11360 cultures**

*Mucor racemosus*  
*Penicillium commune*  
*Galactomyces geotrichum*  
*Yarrowia lipolytica*

Après fermentation (20h) des fractions laitères par les isolats, 100µl du milieu fermenté (Fermentat) ont été ajoutés à la surface de chaque mini fromage

Inoculation avec 50 spores ou cellules de 4 cibles fongiques (1 cible/plaque)

Évaluation de la croissance fongique et score attribué à l'activité antifongique des fermentats

Aspect visuel				
Effet antifongique	Aucun	Faible	Fort	Inhibition totale
Score	0	1	2	3

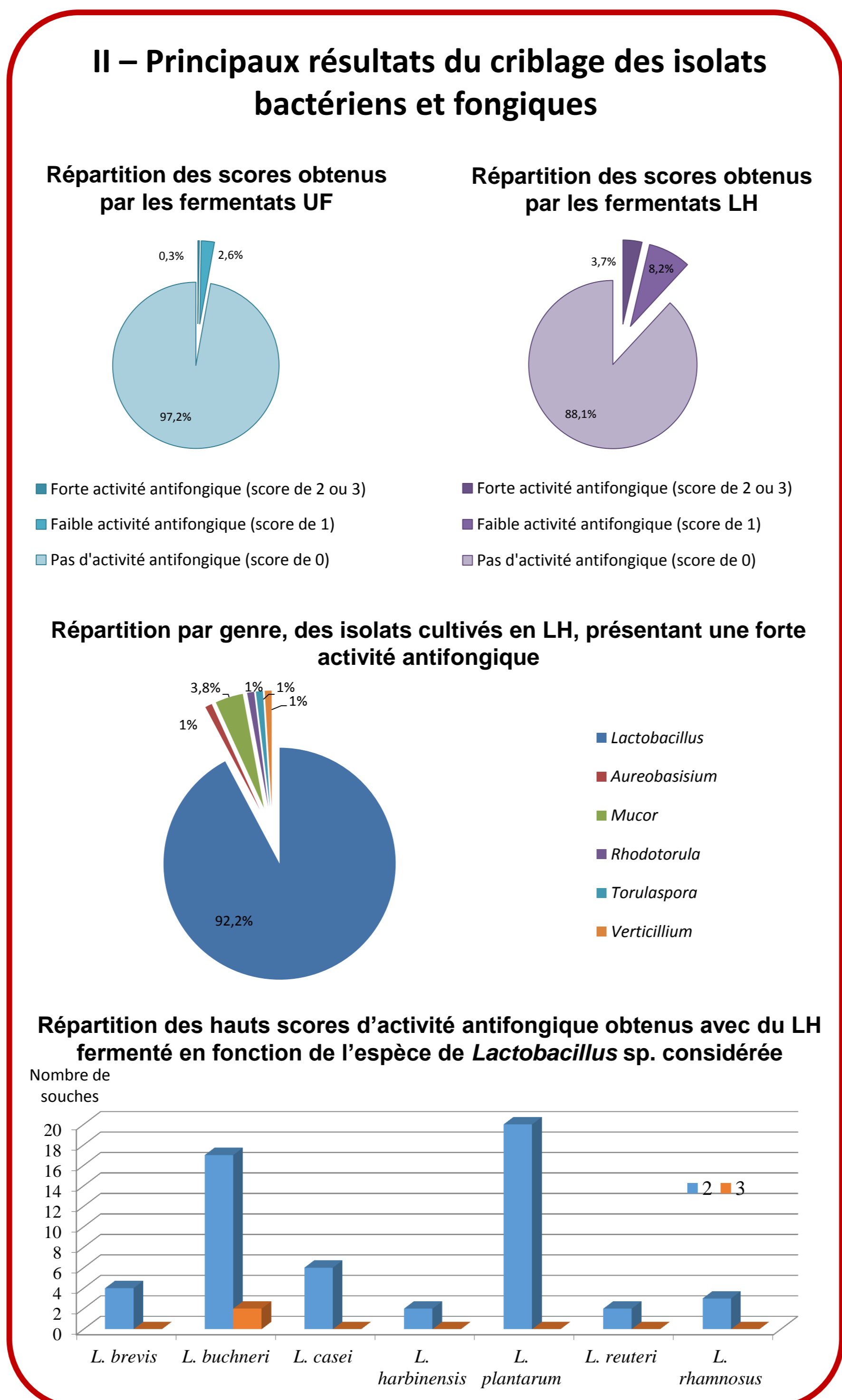
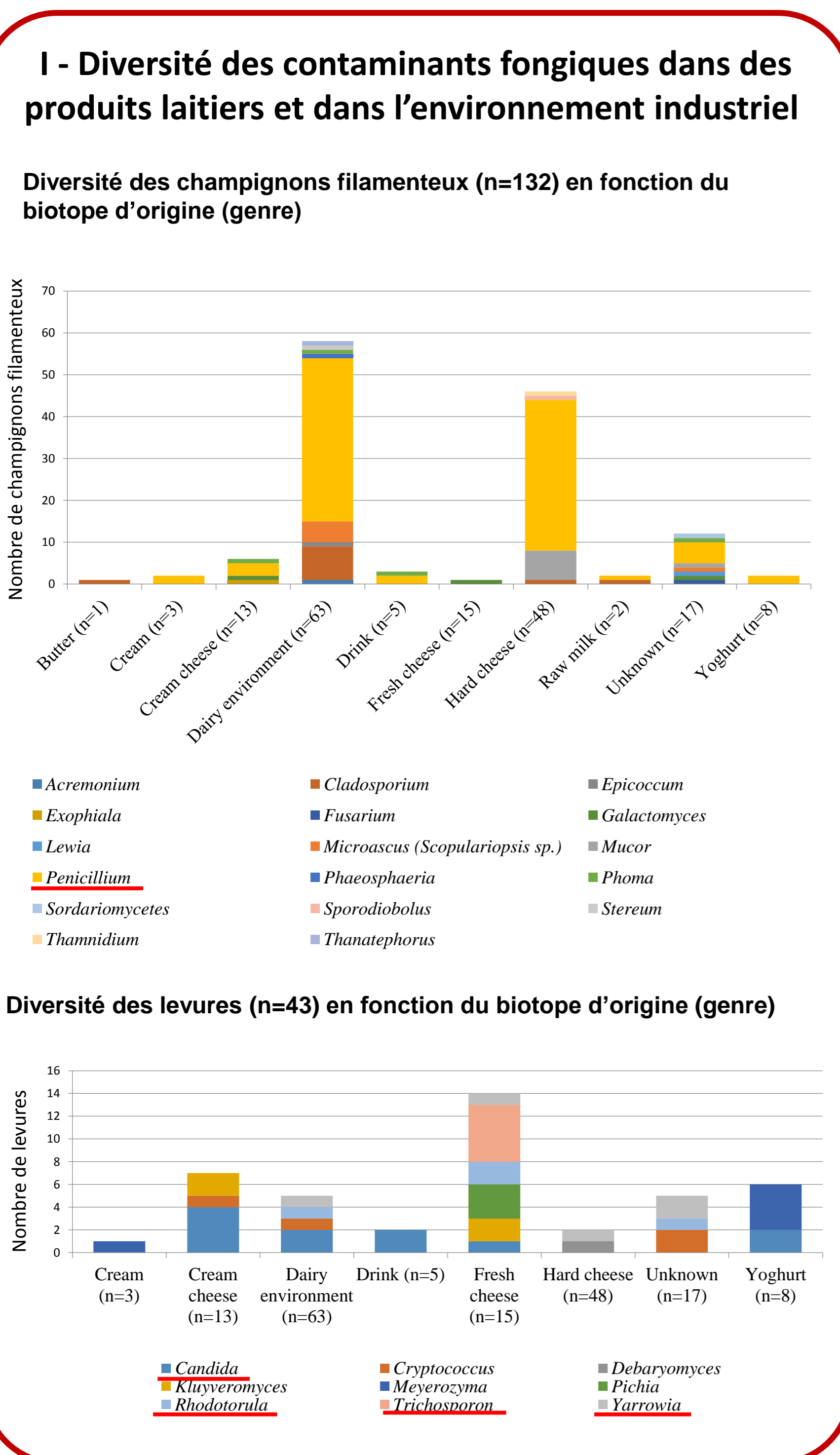
Exemple de l'effet du milieu LH fermenté par *Lactobacillus* sp. sur la croissance de *P. commune*

**III - Caractérisation de l'activité antifongique de 2 fermentats après validation et optimisation de leur activité antifongique - exemple d'une contamination par *P. commune***

Fromage blanc → Fermentat lyophilisé 1 et 2% → Inoculation avec *P. commune* (50 spores en surface) → Incubation à 10 ° C pendant 14 jours → Lecture et photo tous les jours à partir de 9 jours et comparaison avec les témoins

Témoin négatif (T-) = (ni fermentat ni cible)  
Témoin positif (T+) = (cible sans fermentat)  
Témoin avec un conservateur chimique (TS) = (Sorbate à 0,08%)

## RÉSULTATS



**III - Effet antifongique de LH fermenté par *L. paracasei* L117 ou *L. rhamnosus* CIRM1952 sur le développement de *P. commune* à la surface d'un fromage blanc**

❖ Retard de croissance de 1 jour observé pour LH - *L. paracasei* à 1%

❖ Retard de croissance de 1 jour observé et physiologie de *P. commune* affectée par LH - *L. paracasei* à 2%

❖ Retard de croissance de 3 jours observés pour LH - *L. rhamnosus* à 1%

❖ Retard de croissance de 4 jours observés pour LH - *L. rhamnosus* à 2%

## CONCLUSIONS

L'étude de la diversité des contaminants fongiques des produits laitiers et de leur environnement a permis la mise en place d'une collection de 175 isolats composée de **43 levures** et **132 champignons filamenteux**. Au sein de cette collection, **4 cibles fongiques représentatives** de cette diversité et issues de produits contaminés ont été utilisées lors des criblages: *M. racemosus*, *P. commune*, *G. geotrichum* et *Y. lipolytica*. Le criblage de l'activité antifongique de la collection a permis la **sélection de 22 isolats bactériens** appartenant aux genres *Lactobacillus* et *Propionibacterium*. Chacune de ces souches est capable de retarder la croissance de 2 à 4 cibles fongiques. Il a également permis la **sélection de 11 isolats fongiques**, appartenant aux genres *Aureobasidium*, *Candida*, *Exophiala*, *Mucor*, *Phoma*, *Pichia*, *Torulaspota* et *Trichoderma*, capables de retarder la croissance de 1 ou 2 cibles fongiques chacun. De plus, il est important de noter l'**impact du substrat de fermentation** sur l'activité antifongique des différents isolats. L'**optimisation de l'activité antifongique** des souches d'intérêt a été réalisée et a permis la **sélection de 4 souches bactériennes** et **1 souche fongique** pour la réalisation de challenge-tests sur produits réels. L'**activité antifongique de certains ingrédients fermentés a été validée dans des produits réels** et les conditions d'application de l'ingrédient antifongique dans ces produits ont été définies.

## PERSPECTIVES

Des challenge-tests à l'échelle pilote vont être réalisés prochainement et l'identification des composés et des mécanismes impliqués dans l'activité antifongique des ingrédients obtenus est en cours. Ainsi, la finalité du projet est **d'obtenir un ingrédient antifongique et 100% laitier**.

L. Garnier<sup>1,2</sup>, F. Valence<sup>2</sup>, A. Pawtowski<sup>1</sup>, F. Deniel<sup>1</sup>, L. Galerne<sup>1</sup>, N. Frotté<sup>1</sup>, E. Coton<sup>1</sup> and J. Mounier<sup>1</sup>

<sup>1</sup> Université de Brest, EA 3882, Laboratoire Universitaire de Biodiversité et Ecologie Microbienne, ESIAB, Technopôle Brest-Iroise, 29280 Plouzané, France.

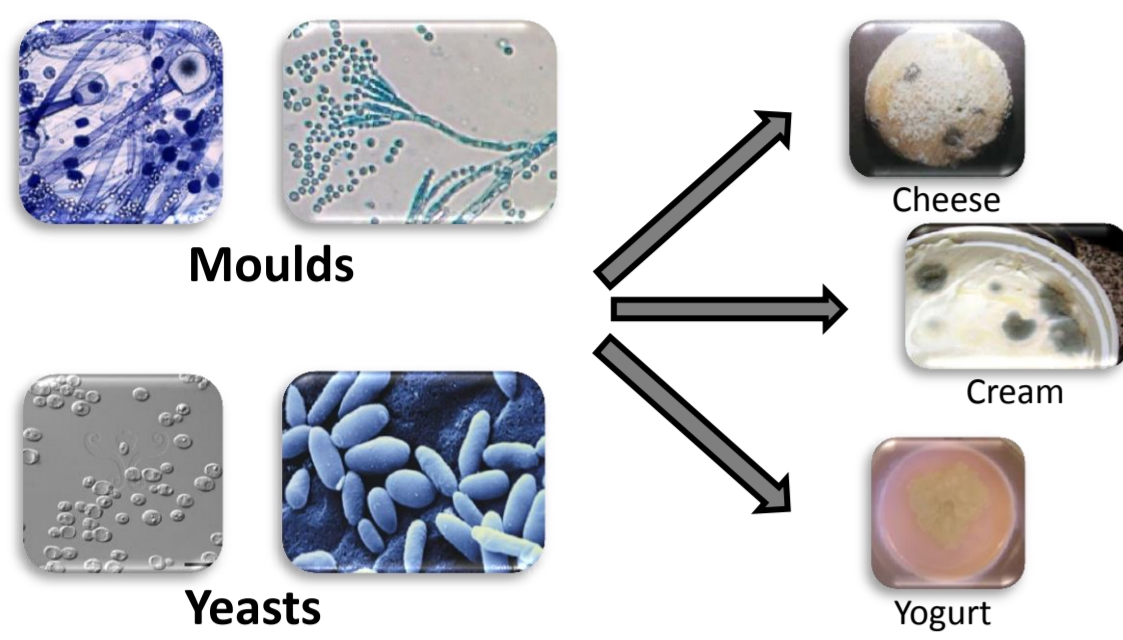
<sup>2</sup> INRA, UMR1253 Science et Technologie du Lait et de l'Œuf, F-35042 Rennes, France.

Contact: [lucille.garnier@univ-brest.fr](mailto:lucille.garnier@univ-brest.fr)

[www.researchgate.net/profile/Garnier\\_Lucille](http://www.researchgate.net/profile/Garnier_Lucille)

## INTRODUCTION

### Impact of fungal contaminations in dairy products



**Substantial economic losses**  
**Consumer Dissatisfaction**  
**Negative brand image**  
**Limitation of export possibilities**

### Control of fungal contaminations

#### Preventive methods

- ✓ Good manufacturing practices
- ✓ Good hygiene practice
- ✓ Air filtration systems

#### Hurdle technologies

- ✓ Heat treatment
- ✓ Cold storage
- ✓ Addition of NaCl/sugar
- ✓ Fermentation
- ✓ Modified atmosphere packaging
- ✓ **Chemical preservatives**

## SCIENTIFIC QUESTIONS

### I

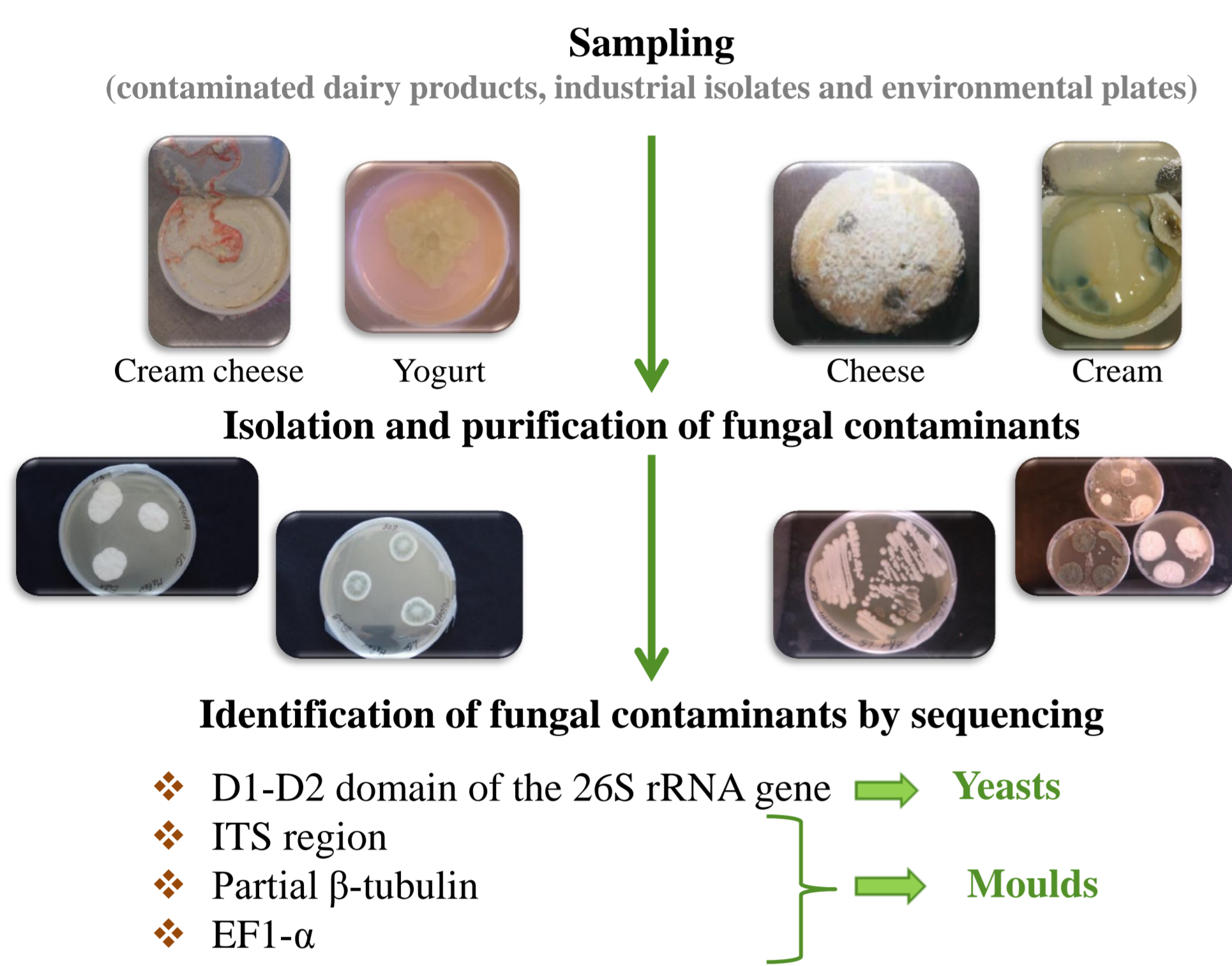
What is the diversity of fungal contaminants present in the dairy environment and spoiled products?

### II

What is the resistance of spoilage fungi to chemical preservatives?

## MATERIALS AND METHODS

### I - Characterization of the diversity of fungal contaminants in dairy products and their environment



### II - Determination of the resistance to chemical preservatives

#### Fungal targets

##### Moulds

*Cladosporium halotolerans*  
*Galactomyces geotrichum*  
*Mucor racemosus*  
*Penicillium commune*  
*Phoma pinodella*

##### Yeasts

*Candida parapsilosis*  
*Meyerozyma guilliermondii*  
*Rhodotorula mucilaginosa*  
*Trichosporon asahii*  
*Yarrowia lipolytica*

#### Chemical preservatives

Calcium propionate  
Potassium sorbate  
Sodium benzoate

**Tested concentrations (pH 5)**  
0.04 0.08; 0.1; 0.15; 0.3; 0.5; 1; 1.5; 2 2.5; 3 g.l<sup>-1</sup>

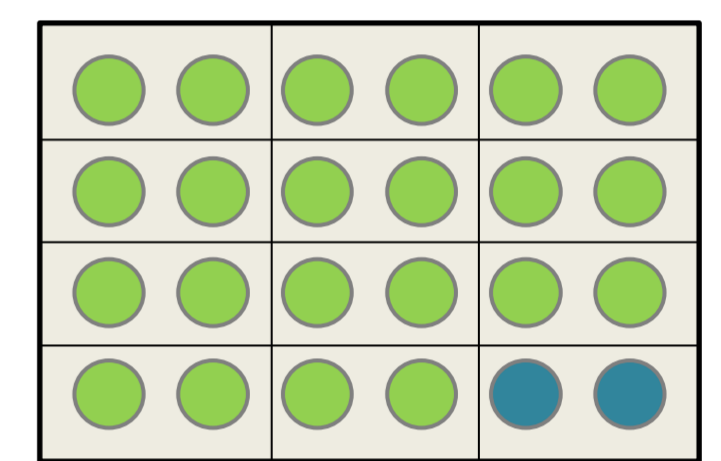
Natamycin (Pimaricin)  
(surface treatment)

**Tested concentrations (pH 5)**  
0.01; 0.02; 0.04; 0.06; 0.08; 0.1; 0.12; 0.14; 0.16; 0.18; 0.2 g.l<sup>-1</sup>

1 target/plate  
(inoculation with 50 spores or cells in each well)

11 concentrations tested/by plate

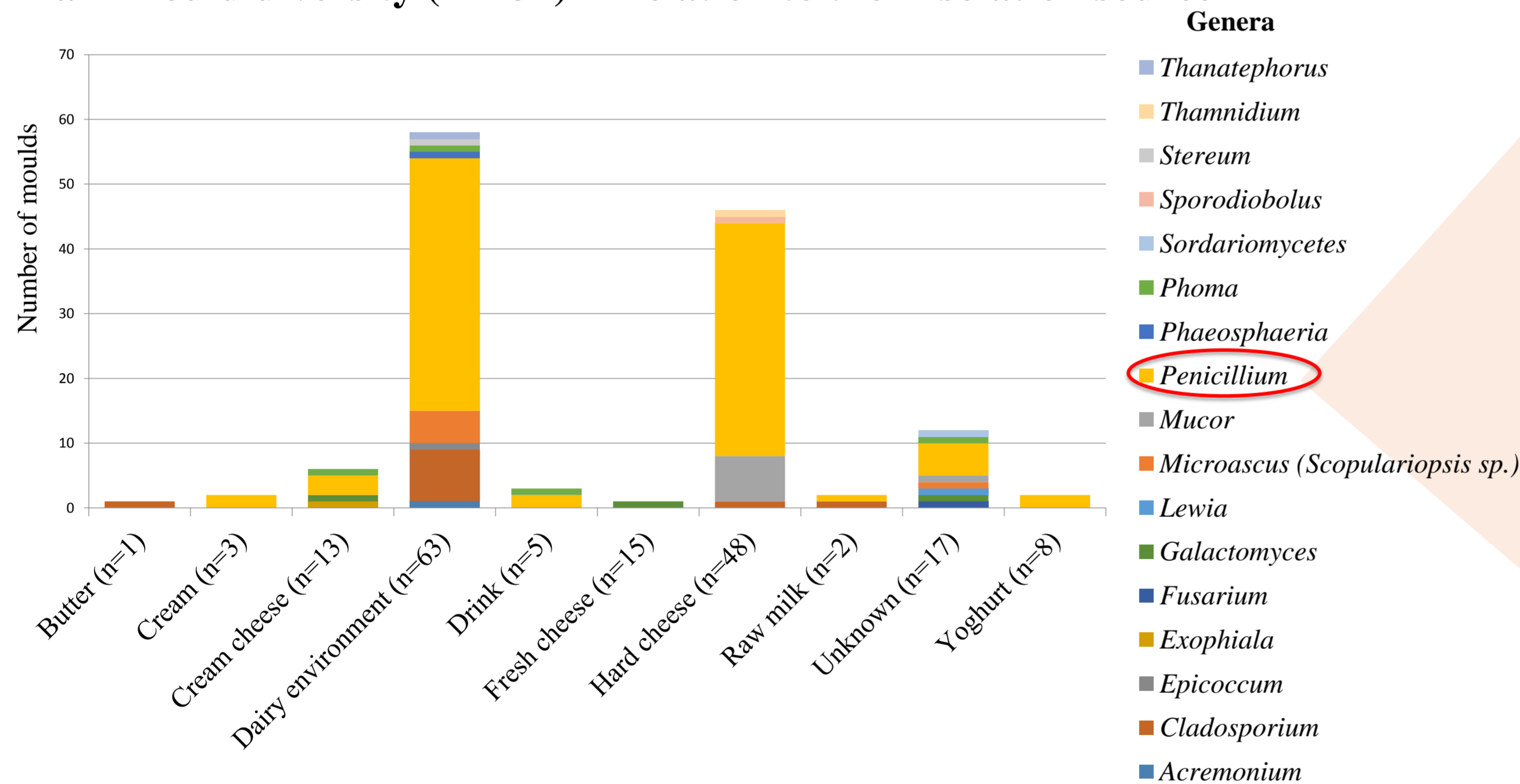
PDA: Potato Dextrose Agar  
(Defined medium used for fungal culture)



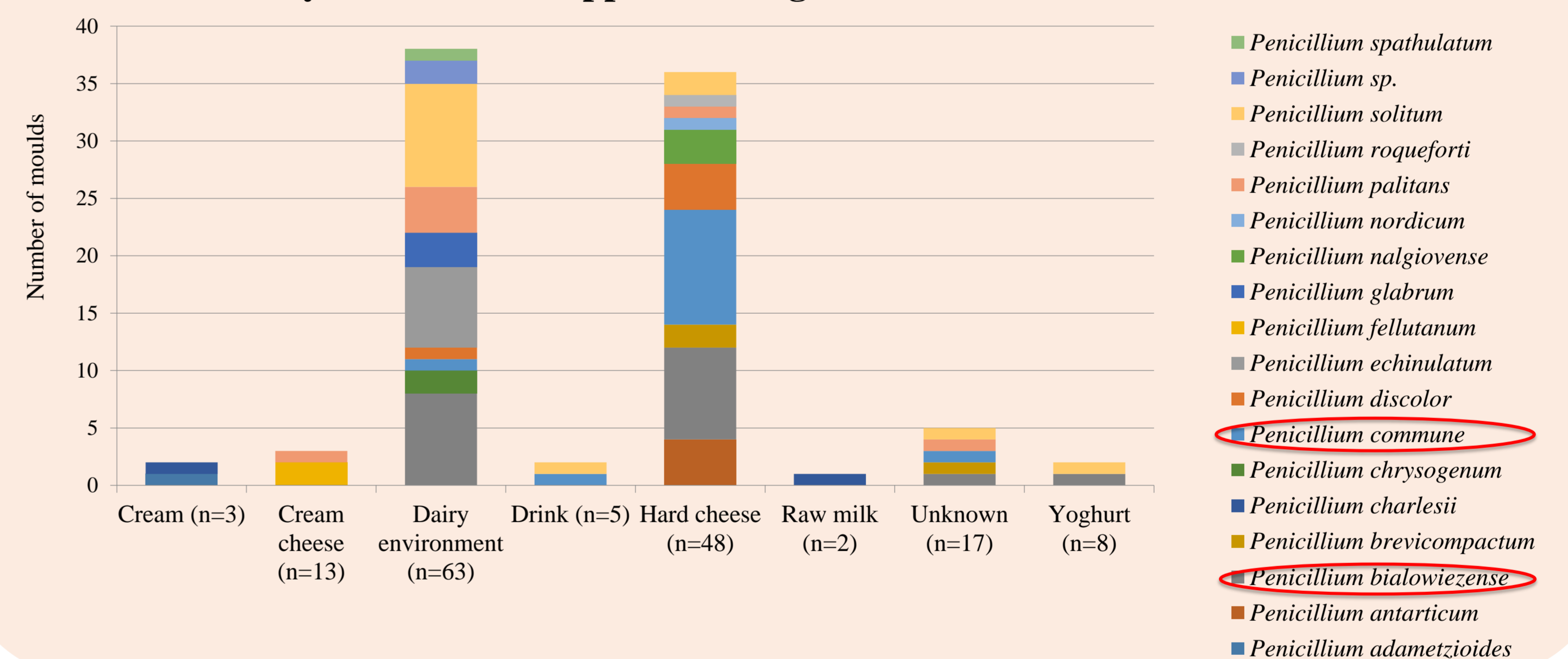
● PDA + preservative  
● PDA

## RESULTS

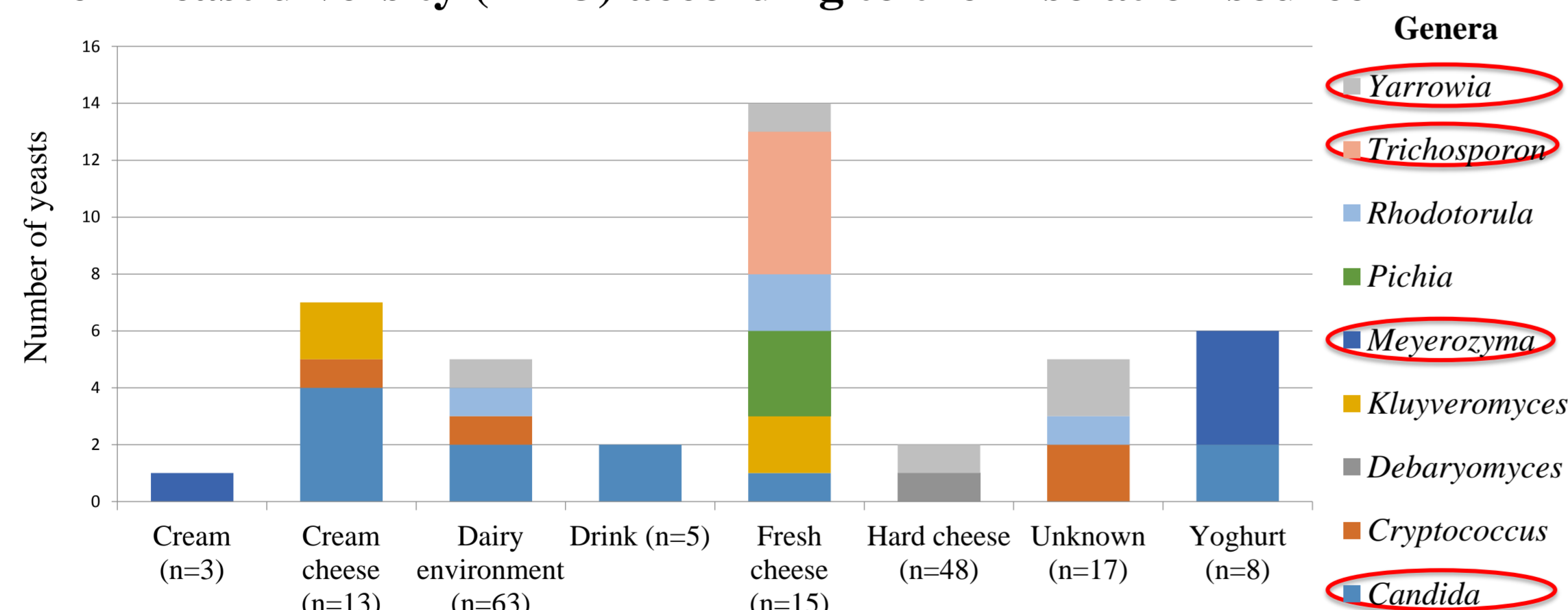
### Ia - Mould diversity (n=132) in relation to their isolation source



### Ib - Diversity of *Penicillium* spp. according to their isolation source



### Ic - Yeast diversity (n=43) according to their isolation source



### II - Resistance of yeasts and moulds to chemical preservatives

	Minimal inhibitory concentrations			
	Calcium propionate (g.l <sup>-1</sup> )	Potassium sorbate (g.l <sup>-1</sup> )	Sodium benzoate (g.l <sup>-1</sup> )	Natamycin (g.l <sup>-1</sup> )
<i>Cladosporium halotolerans</i>	2	0.1	0.3	0.04
<i>Galactomyces geotrichum</i>	> 3*	1	1	0.1
<i>Mucor racemosus</i>	> 3*	0.5	1	0.1
<i>Penicillium commune</i>	> 3*	0.3	2	0.06
<i>Phoma pinodella</i>	3*	0.15	0.5	0.1
<i>Candida parapsilosis</i>	> 3*	1	2	0.1
<i>Meyerozyma guilliermondii</i>	> 3*	0.5	1	0.08
<i>Rhodotorula mucilaginosa</i>	> 3*	0.15	1	0.1
<i>Trichosporon asahii</i>	> 3*	0.5	1	0.1
<i>Yarrowia lipolytica</i>	> 3*	1	> 3*	> 0.2*

\* Highest tested concentrations, corresponding to the highest concentrations authorized in dairy products in France

## CONCLUSION

A **high diversity** was found among fungal isolates. Indeed, **25 genera representing 67 species** were identified out of 175 isolates, with ***Penicillium* being the most prevalent** (51.4% of total species), were ***Cladosporium*, *Mucor* and *Microascus***. At the species level, ***Penicillium commune* and *Penicillium bialowiezense* were the most prevalent** filamentous fungi, representing 10.2% and 7.4% of fungal isolates, respectively. Concerning yeast, ***Candida*, *Meyerozyma*, *Trichosporon* and *Yarrowia* are the most prevalent**. Moreover, fungal species such as ***Penicillium antarcticum* and *Penicillium salamii* were identified for the first time in dairy products**. In addition, numerous species were both identified in spoiled products and after air-sampling suggesting that **dairy environment acts as a primary source of contamination**. Concerning chemical preservatives, **natamycin and potassium sorbate were the most efficient** followed by sodium benzoate and calcium propionate. Finally, several species, such as ***Penicillium commune* and *Yarrowia lipolytica* presented high minimal inhibitory concentrations for calcium propionate, sodium benzoate and natamycin**. In conclusion, this study provided useful information on the occurrence of fungal contaminants in dairy products and should help to develop adequate strategies for combating fungal spoilage.

L. Garnier<sup>1,2</sup>, J. Mounier<sup>1</sup>, A. Pawtowski<sup>1</sup>, N. Pinon<sup>2</sup>, N. Frotté<sup>1</sup>, E. Coton<sup>1</sup> and F. Valence<sup>2</sup>

<sup>1</sup> Université de Brest, EA 3882, Laboratoire Universitaire de Biodiversité et Ecologie Microbienne, ESIAB, Technopôle Brest-Iroise, 29280 Plouzané, France.

<sup>2</sup> INRA, UMR1253 Science et Technologie du Lait et de l'Œuf, F-35042 Rennes, France.

Contact: [lucille.garnier@univ-brest.fr](mailto:lucille.garnier@univ-brest.fr)

[www.researchgate.net/profile/Garnier\\_Lucille](http://www.researchgate.net/profile/Garnier_Lucille)

## INTRODUCTION

**Fungal spoilage** cause significant food losses in the dairy industry. In this context, biopreservatives (**bioprotective cultures or fermentates**) can be an alternative or a complementary approach to traditional methods. Lactic acid bacteria (LAB) and propionibacteria, as well as fungi, can exhibit antifungal activities. These activities are based on **multifactorial mechanisms** and related to the production of antifungal compounds such as organic acids, fatty acids, proteinaceous compounds, cyclic dipeptides or reuterin. Nevertheless, antifungal activities of LAB, propionibacteria and fungi are **highly strain-dependent** and **vary according to the growth medium**. In order to find the most active antifungal biopreservatives for further industrial applications, it is thus necessary to develop high-throughput screening methods that allow testing large strains number in medium as close as possible as those where they are intended to be used.

## AIM

The objective of this study was to develop a new high-throughput screening method allowing the rapid detection of fermentate antifungal activities in a cheese-like model.

## DESCRIPTION OF THE SCREENING METHOD DEVELOPPED – APPLICATION FOR BIOPROTECTIVE CULTURES

### 703 screened isolates...

505 bacterial isolates  
(25 species belonging to 5 genera)  
  
198 fungal isolates  
(81 species belonging to 29 genera)

### ... cultivated in 2 dairy media (« fermentates ») ...

- ❖ 10%-reconstituted low heat skim milk supplemented with 45% anhydrous milk fat (LH)
- ❖ 6-fold concentrated milk ultrafiltration (0,2 µm) permeate and complemented with 10g/l yeast extract and a pH indicator (UF)

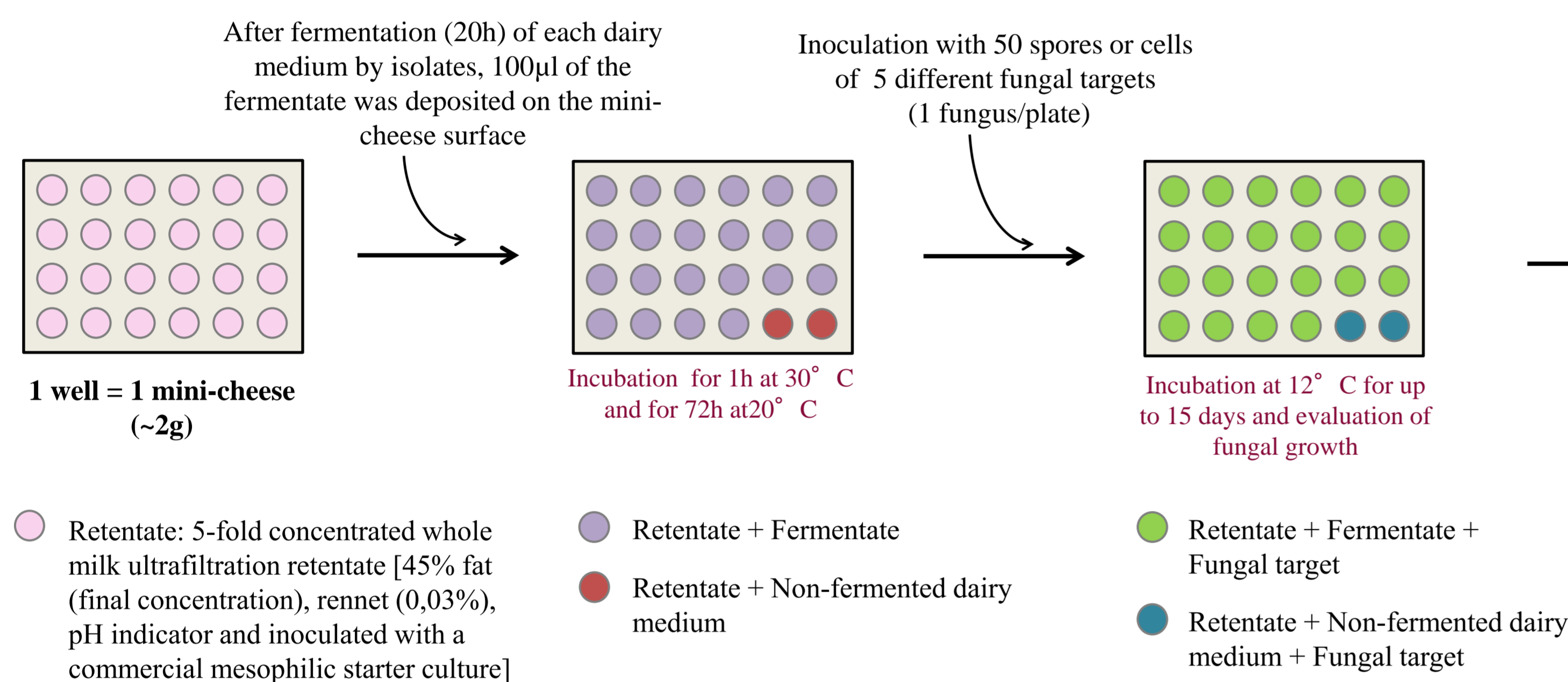
### ...and tested against 4 fungal targets, representative of the diversity of spoilage fungi in dairy products\*,...

*Mucor racemosus*  
*Penicillium commune*  
*Galactomyces geotrichum*  
*Yarrowia lipolytica*

(\*See poster entitled "Diversity of spoilage fungi in dairy products and environment, and their resistance to chemical preservatives")

### ...in duplicate.

**11 248**  
**cultures**  
**performed**  
**in 8 weeks**



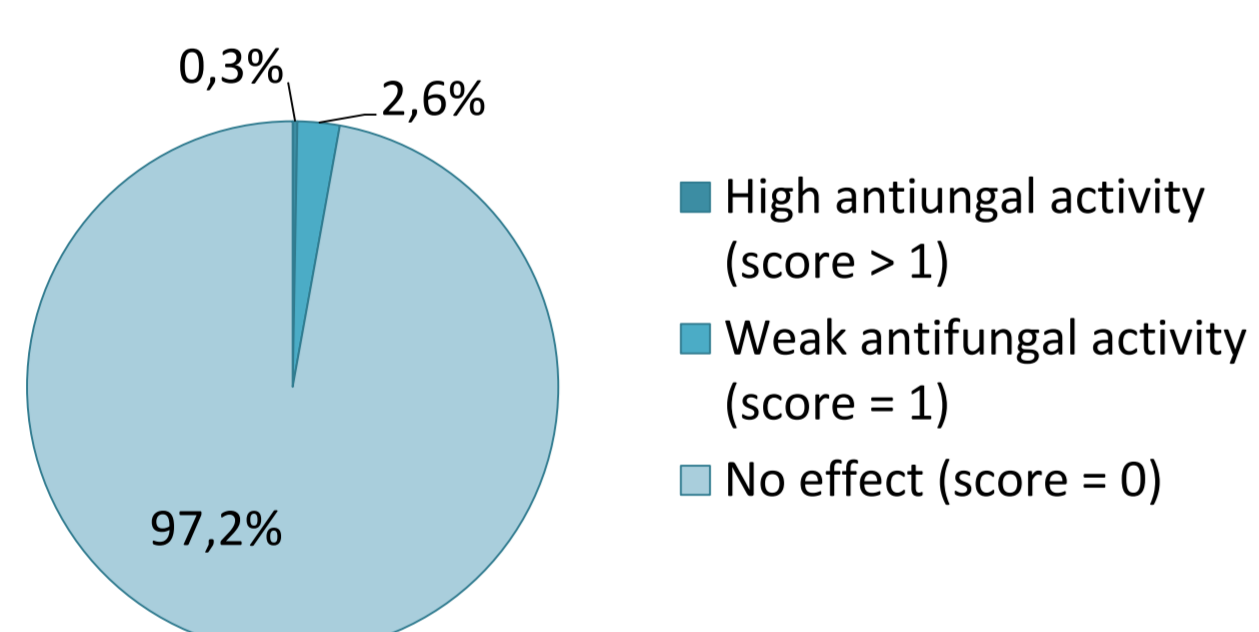
### Evaluation of fungal growth to determine antifungal activities

Visual appearance				
Antifungal effect	No effect	Weak effect	High effect	Total inhibition
Antifungal score	0	1	2	3

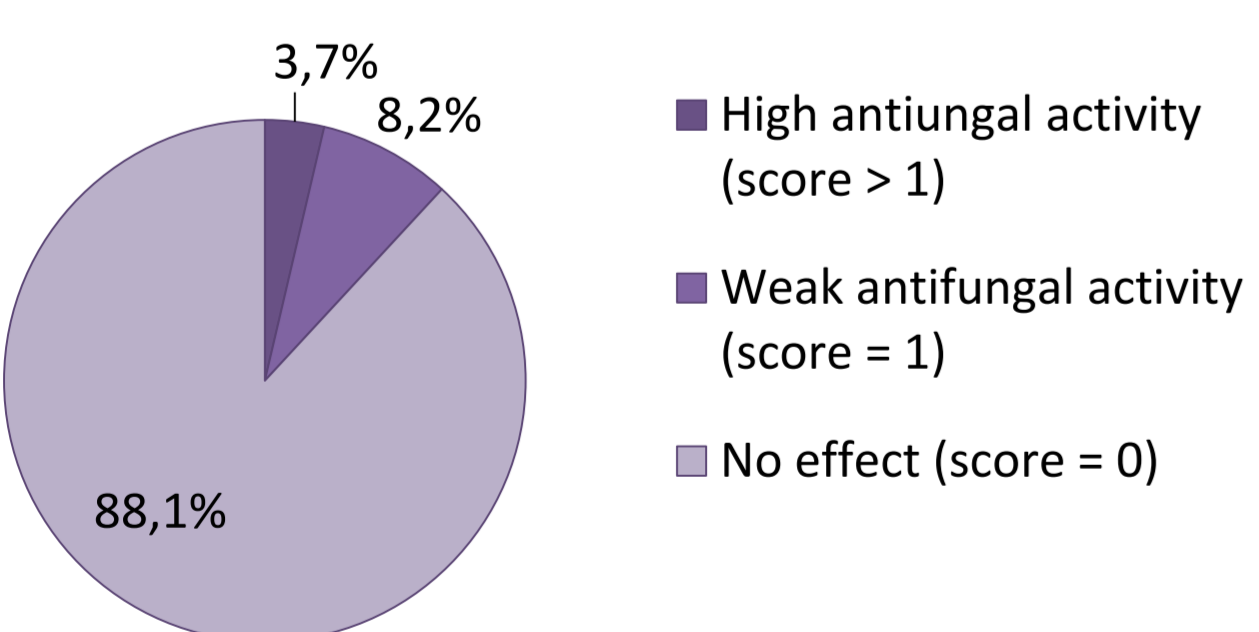
Example of the effect of LH medium fermented by different *Lactobacillus* spp. on the growth of *P. commune*

## RESULTS FOR BIOPROTECTIVE CULTURES

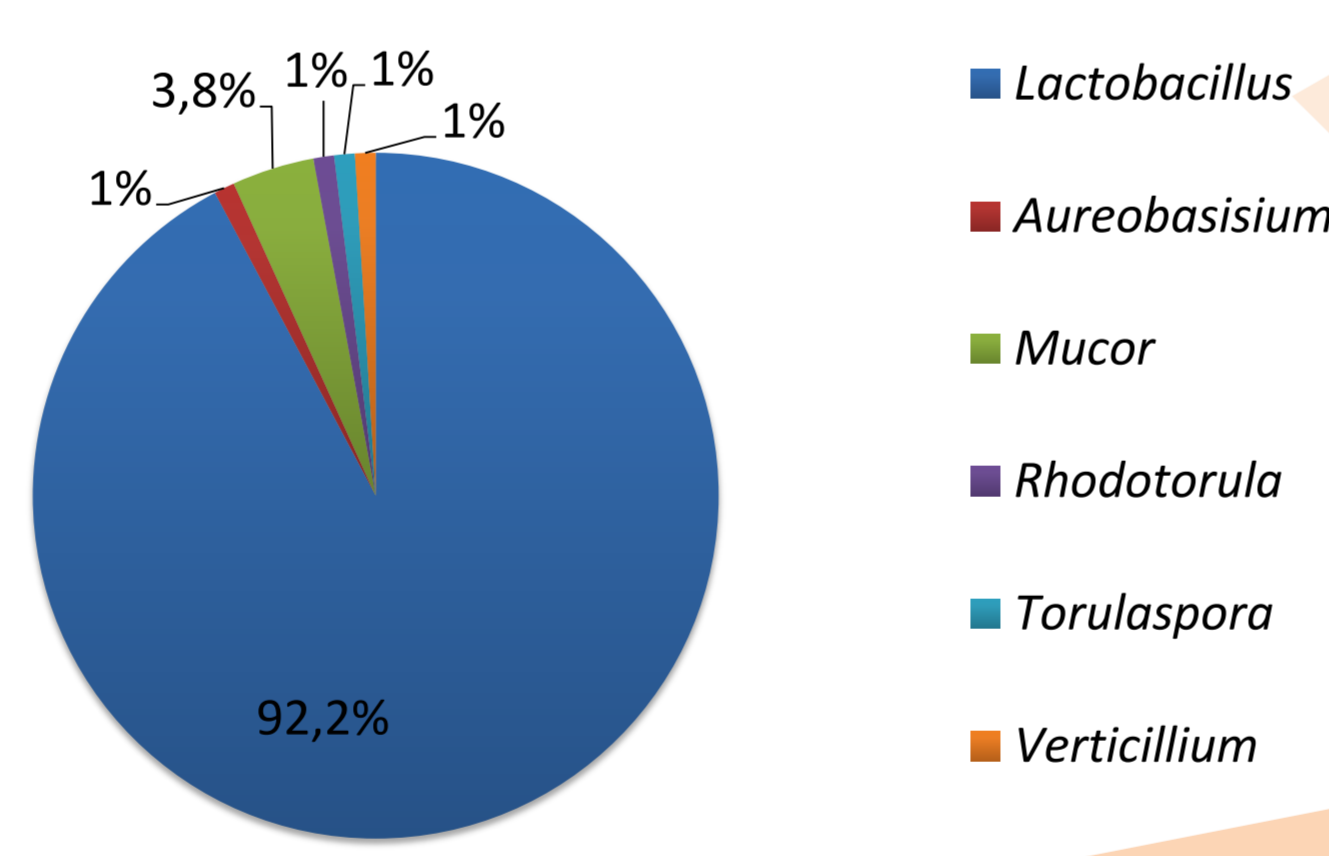
### Distribution of antifungal activities of UF fermentates (%)



### Distribution of antifungal activities of LH fermentates (%)



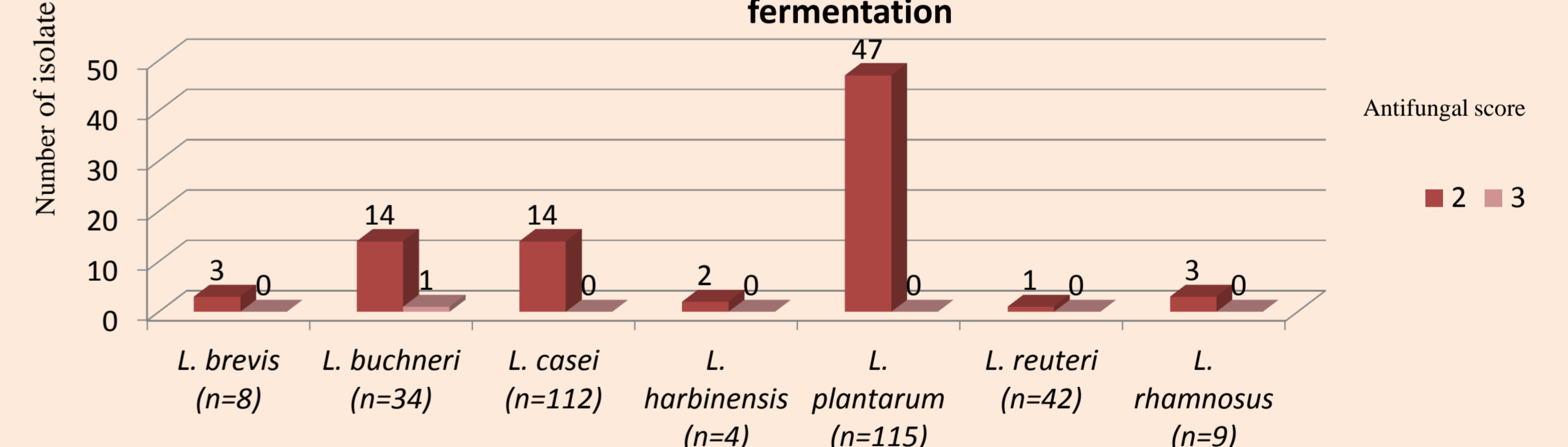
### Distribution of the different genera presenting high (score>1) antifungal activities in LH fermentates



Antifungal scores attributed to different isolates of *Lactobacillus buchneri* against the 4 fungal targets

	<i>M. racemosus</i>	<i>P. commune</i>	<i>G. geotrichum</i>	<i>Y. lipolytica</i>
<i>Lactobacillus buchneri</i> L164	2	2	0	3
<i>Lactobacillus buchneri</i> L162	2	2	1	0
<i>Lactobacillus buchneri</i> L111	1	2	1	0
<i>Lactobacillus buchneri</i> L151	2	2	0	0
<i>Lactobacillus buchneri</i> L233	1	2	0	0
<i>Lactobacillus buchneri</i> L169	1	2	0	0

### *Lactobacillus* species presenting high antifungal activities (score>1) after LH fermentation



## CONCLUSION

This high-throughput antifungal activity screening method allowed the testing of **1400 cultures per week** and confirmed the **impact of the growth medium** used on the antifungal activities expression of bacteria or fungi. Indeed, 4 and 48 bacteria, principally *Lactobacillus* sp., and, 11 and 21 fungi, inhibited at least one fungal target after cultivation in UF and LH, respectively. This screening showed that antifungal activity was **strain-dependent** and **varied as a function of the tested fungal target**. Among the 4 tested fungal targets, ***P. commune* was the most frequently inhibited fungus**, followed by *M. racemosus* while only few isolates were able to inhibit *G. geotrichum* and *Y. lipolytica*. The most effective fermentates were those obtained with *L. buchneri*, *L. rhamnosus* and *L. plantarum*. This **method was applied to produce 2 antifungal ingredients compatible with dairy technologies**. The activities in real products of the most promising ones are currently under evaluation. This new high-throughput screening method may be used for other types of antifungal products. As examples, it may be used to test:

- ✓ antifungal bioprotective cultures as **adjunct cultures** simultaneously with the mesophilic starter cultures
- ✓ the antifungal activity of **essential oils or other antifungal compounds**
- ✓ the resistance of fungi to **chemical preservatives** in a cheese-like medium (determination of minimal inhibitor concentration)

# Annexes

**Annexe 1:** Coton, M., Lebreton, M., Leyva-Salas, M., Garnier, L., Navarri, M., Pawtowski, A., Le Blay, G., Valence, F., Coton, E., Mounier, J. « Safety risk assessment of potential antifungal lactic acid bacteria and propionibacteria ». *Article soumis à International Journal of Food Microbiology*.

1 *Safety risk assessment of potential antifungal lactic acid bacteria and*  
2 *propionibacteria*

3 Monika Coton<sup>a\*</sup>, Mégane Lebreton<sup>a</sup>, Marcia Leyvas Salas<sup>a,b</sup>, Lucille Garnier<sup>a,b</sup>, Marion  
4 Navarri<sup>a</sup>, Audrey Pawtowski<sup>a</sup>, Gwenaëlle Le Blay<sup>c</sup>, Florence Valence<sup>b</sup>, Emmanuel Coton<sup>a</sup>,  
5 Jérôme Mounier<sup>a</sup>

6  
7 <sup>a</sup>*Université de Brest, EA 3882, Laboratoire Universitaire de Biodiversité et Ecologie*  
8 *Microbienne, IBSAM, ESIAB, Technopôle Brest-Iroise, 29280 Plouzané, France*

9 <sup>b</sup>*UMR1253 Science et Technologie du Lait et de l'Œuf, INRA, Agrocampus Ouest, 65 rue de*  
10 *Saint Briec, 35000 Rennes, France*

11 <sup>c</sup>*Université de Brest, UMR 6539 CNRS/UBO/IRD/Ifremer, Laboratoire des Sciences de*  
12 *l'Environnement Marin, IUEM, Technopôle Brest-Iroise, 29280 Plouzané, France*

13

14 \* **Correspondance to: Dr. Monika COTON**

15 **Laboratoire Universitaire de Biodiversité et Ecologie Microbienne EA3882**

16 **Technopôle Brest Iroise**

17 **29280 Plouzané, France**

18 **E-mail: [monika.coton@univ-brest.fr](mailto:monika.coton@univ-brest.fr)**

19 **Tel: +33 2 99 91 51 55**

20 **Fax: +33 2 90 91 51 01**

21

22 **Running title: LAB and propionibacteria safety assessment**

23

24 **Abstract**

25 Lactic acid and propionibacteria are among the main microbial groups currently studied for  
26 their potential use as alternatives to chemical additives in foods. To consider them for use as  
27 bioprotective cultures, not only does their antimicrobial efficiency in a food matrix need to be  
28 ensured, but their safety should also be assessed. In this study, 21 lactic acid bacteria and 1  
29 propionibacterial strain exhibiting antifungal properties of interest were screened for two  
30 major risk factors, biogenic amine production and antibiotic resistances. Low risk profiles  
31 were observed for 15 strains (9 different lactic acid bacteria and 1 propionibacteria species)  
32 thereby highlighting their potential for use as bioprotective cultures in the future. Some  
33 exceptions were found as both tested *L. buchneri* strains were identified as histamine-  
34 producers, while 1 *L. brevis*, 2 *L. rhamnosus* and 1 *Lc. mesenteroides* strains were identified  
35 as tyramine-producers. Moreover, these strains were also shown to be resistant to up to 5  
36 antibiotics, including potentially acquired resistances, and therefore excluded for use as  
37 bioprotective cultures. This safety assessment approach is proposed as a model procedure for  
38 low risk lactic acid bacteria (non-enterococci) and propionibacteria safety evaluation.

39

40 **Keywords:** bioprotective cultures, lactic acid bacteria, propionibacteria, safety assessment

41



## 42 **1. Introduction**

43 Continual improvement of food quality and safety is an on-going food industry  
44 challenge associated with both public authorities' policies and guidelines, and consumer  
45 demands worldwide. Actual trends correspond to promoting better health via enhanced  
46 nutritional value foods (i.e. reduced fat, salt and sugar), disease prevention (related to  
47 nutritional value but also by preventing the presence of pathogens, toxic compounds or  
48 antimicrobial resistances – pesticides and other chemical compounds), but also provision of  
49 foods with pleasurable sensory characteristics. More specifically, recent societal demands for  
50 the food industry correspond to convenient, fresh (i.e. locally produced), less heavily-  
51 processed, all natural or preservative-free healthy foods as shown by the “Clean Label” trend.  
52 From a microbiological point of view, there is a clear growing interest for natural alternatives  
53 for food preservation. In this context, while for several years inhibition of pathogens was the  
54 main target, inhibition of spoilage microorganisms during storage has a growing interest as  
55 they are responsible, not only for important economic losses at the industrial and consumer  
56 level but also contribute to major food losses and food waste, which are very important issues  
57 worldwide. In this context, biopreservation applications are currently in demand.

58 Food preservation has been used by man since the Neolithic era to avoid loss of  
59 otherwise perishable foodstuffs (Bourdichon et al., 2012; Prajapati and Nair, 2003). Among  
60 the preservation methods, microbial fermentations have been used for their key role in both  
61 food processing and preservation. The antimicrobial potential of several microorganisms,  
62 combined with the need for alternative preservation methods, has led to the biopreservation  
63 concept, i.e. the selection and use of bioprotective cultures, (Leyva Salas et al., 2017).  
64 Bioprotective cultures are an interesting alternative to traditional preservatives as they can be  
65 directly used (or indirectly for the compounds they produce) to inhibit or delay growth of  
66 spoilage and pathogenic microorganisms in a given food or feed product, and increase product

67 shelf life (Stiles, 1996). However, before the direct use of otherwise efficient bioprotective  
68 cultures (or their metabolites) by the food industry, several aspects must be considered: i)  
69 microbial safety and regulatory considerations (e.g. Generally Recognized as Safe (GRAS) or  
70 Qualified Presumption of Safety (QPS) status in the US or EU, respectively) and, ii) microbial  
71 stability and sensorial impact in a given product (i.e. microbial cell stabilisation after  
72 lyophilisation or active dry powder preparation, high viability, maintaining bioactivity in the  
73 product during use, limited sensorial impact unless desirable) (Melin et al., 2007). Among  
74 these aspects, evaluation of bioprotective culture safety is an essential criterion to ensure that  
75 a given culture does not pose any risks to human health during food processing and  
76 conservation. Indeed, these cultures or their products will be intentionally added by the user at  
77 different stages of food processing into the food chain. According to the considered type of  
78 microorganism (Gram positive or negative bacteria, yeasts), different safety criteria should be  
79 assessed and may include the current body of knowledge (i.e. history of use, intended end  
80 use), definition of the taxonomic unit (i.e. well-defined taxonomy and species identity),  
81 possible safety concerns including pathogenicity (e.g. adhesion to intestinal epithelial cells,  
82 virulence factors, etc.), production of toxic or undesirable compounds (e.g. toxins, virulence  
83 factors, biogenic amines, allergens, etc.) and antimicrobial resistance patterns (intrinsic versus  
84 acquired antibiotic resistances) (Bourdichon et al., 2012; EFSA, 2016; Gasser, 1994; Miceli et  
85 al., 2011; Mogensen et al., 2002; Vankerckhoven et al., 2008). This type of safety assessment  
86 has been well documented by the European Food Safety Agency and has led to the QPS status  
87 of many microorganisms, either directly or indirectly (e.g. as a food additive or ingredient)  
88 added into the food chain including many bacterial and yeast species (EFSA, 2013, 2016).  
89 This is also used by the American Food and Drug Administration for the definition of the  
90 GRAS Status.

91           The main bacterial groups currently used for food biopreservation correspond to lactic  
92 acid bacteria (LAB) followed by propionic acid bacteria (PAB) and to a lesser extent some  
93 staphylococci or *Brevibacterium* species (Elsser-Gravesen and Elsser-Gravesen, 2014).  
94 Among the first two main microbial groups, many LAB and PAB already have a long history  
95 of safe use in foods (in particular, many *Lactobacillus*, *Lactococcus* and *Leuconostoc* species  
96 and *Streptococcus thermophilus*) and agricultural applications, and many species/strains have  
97 already been granted the QPS or GRAS status. Moreover, LAB and/or PAB members (i.e.  
98 lactobacilli, lactococci, propionibacteria) can be considered to be ingested at relatively high  
99 concentrations, even on a daily basis, by many healthy consumers consuming fermented foods  
100 around the world and no documented ill-effects have been described to the best of our  
101 knowledge. This is due to the fact that most food related LAB (excluding any strains  
102 belonging to *Enterococcus* species as they have not been recommended for the QPS list in the  
103 EU due to their potentially associated risk factors related to virulence or multidrug-resistances  
104 (EFSA, 2009) and PAB species have been described not to exhibit many safety concerns (i.e.  
105 no known virulence factors, no toxins produced, limited antimicrobial resistances). This  
106 finding was reinforced in a recent study by (Bennedsen et al., 2011) who screened 28 LAB  
107 genomes *in silico* (including 16 strains representing 8 *Lactobacillus* species, 3 *Lactococcus*  
108 *lactis* strains and 3 *Streptococcus thermophilus* strains) for antimicrobial resistance genes and  
109 virulence factors and showed that no risk factors were associated with these species and  
110 strains.

111           Despite the clearly low risks associated with many LAB (in particular, strains  
112 belonging to *Lactobacillus*, *Lactococcus* and *Streptococcus* species) and PAB (i.e.  
113 *Propionibacterium freudenreichii*, *Propionibacterium acidipropionici*, *Propionibacterium*  
114 *jensenii* and *Propionibacterium thoenii* (Meile et al., 2007; Thierry et al., 2011), efficient  
115 safety assessment is still required. Based on current knowledge, candidate LAB and PAB

116 strains should be verified for biogenic amines (BA) production due to their potential  
117 physiological effects on the consumer if ingested in high quantities from food, especially as  
118 BA production was also described as a strain-dependent trait in many LAB species (Coton,  
119 Mulder, et al., 2010; Coton, Romano, et al., 2010; Ladero et al., 2015; Lucas et al., 2008;  
120 Lucas et al., 2005). Antimicrobial resistance profiles are also required, in particular, to ensure  
121 that no acquired antibiotic resistances are harboured by candidate strains, which could lead to  
122 future dissemination of unwanted genetic content among microorganisms. Therefore, a  
123 thorough and adapted safety assessment needs to be carried out on any novel LAB and PAB  
124 bioprotective culture. Moreover, with the constant evolution in taxonomy and the description  
125 of novel species, it is possible to encounter newly described species therefore apparently  
126 lacking a wide body of knowledge, but that have actually been used or present for long  
127 periods of time in foods, and therefore not likely to pose any risk to human health. Such  
128 criteria should also be considered during safety risk assessment.

129 In this study, we propose a model safety assessment approach for food and/or feed  
130 related LAB (excluding enterococci species) and PAB species risk assessment, which  
131 includes biogenic amine production and antibiotic resistance pattern evaluation. We applied  
132 this methodology to various LAB and PAB species strains pre-selected for their antifungal  
133 activity in dairy products and their potential use in food industry applications.

134

## 135 **2. Materials and Methods**

136

### 137 *2.1 Bacterial strains and culture conditions*

138 In this study, 22 lactic acid bacteria (LAB) with interesting antifungal activities  
139 (Delavenne et al., 2015; Delavenne et al., 2013; Mieszkin et al., 2017), including 19  
140 *Lactobacillus* species, 1 *Lactococcus*, 1 *Leuconostoc* and 1 *Streptococcus*, and 1 propionic

141 acid bacteria (PAB) belonging to the *Propionibacterium jensenii* species were evaluated for  
142 different safety criteria. In addition, 4 lactobacilli, 1 streptococci and 1 propionibacteria were  
143 used as biogenic amine positive controls or reference strains for antibiotic resistance testing.  
144 Isolates were originally isolated from different milk-related sources or came from  
145 international culture collections. Table 1 provides a detailed list of these strains and their  
146 characteristics.

147 Lactobacilli pure cultures were grown on De Man, Rogosa and Sharpe (MRS) plates  
148 (Biokar, France) and incubated at 30°C for 48h under anaerobic conditions, except for  
149 *Lactobacillus* L30a that was incubated at 37°C under anaerobic conditions. *Streptococcus*  
150 *thermophilus* was cultured on M17 medium (bioMérieux, France) and incubated at 37°C for  
151 24h, while PAB were grown on yeast extract lactate medium (YEL; composition as follows:  
152 21.4 g/L sodium lactate, 10 g/L tryptone, 10 g/L yeast extract, 1 ml of a 328 g/l  
153 K<sub>2</sub>HPO<sub>4</sub>·3H<sub>2</sub>O and 56 g/L MnSO<sub>4</sub>·H<sub>2</sub>O solution) and incubated at 30°C for 4 to 5 days under  
154 anaerobic conditions as described by (Malik et al., 1968). All strains were cryoconserved for  
155 long term storage in 15% glycerol (v/v) at -80°C.

156

## 157 2.2 Biogenic amine determination

### 158 2.2.1 Culture conditions

159 LAB strains were grown in MRS broth supplemented or not with 2.5 g/L tyrosine  
160 (Sigma, France), 2.5 g/L L-histidine monohydrochloride (Sigma-Aldrich, France), 2.5 g/L L-  
161 ornithine monohydrochloride (Sigma, France) and 1 g/L agmatine sulfate (Sigma-Aldrich,  
162 France) and adjusted to pH 5.3 before autoclaving. Cultures were incubated at 30°C (except  
163 for *Lactobacillus* 30a at 37°C) for 72h in static conditions. For *Propionibacterium jensenii*  
164 and *Streptococcus thermophilus* strains, MRS-based medium was replaced by YEL and M17,  
165 respectively, supplemented accordingly and incubated at 30°C and 43°C. Three consecutive

166 cultures in amino acid enriched media were performed before HPLC analysis. Each strain was  
167 also enumerated on the appropriate medium (as described above) to ensure similar growth in  
168 all biological triplicates.

169

### 170 *2.3.2. Biogenic amine quantification by HPLC*

171 Biogenic amine quantification was performed using an Agilent 1200 HPLC system  
172 (Agilent Technology, France) according to the method developed by (Duflos et al., 1999)  
173 with minor modifications. Briefly, prefiltered samples were injected into a LUNA 3  $\mu\text{m}$   
174 C18 150 mm X 4.5 mm column (Phenomenex, France). The column was maintained at 30°C  
175 and the flow rate set to 1 ml/min using as mobile phase: solvent A HPLC grade water and  
176 solvent B acetonitrile (Carlo Erba Reagents, France). Solvent B was set to 40%, followed by a  
177 4 step gradient: 40-55% for 15 min, then 55-80% for 15 min before maintaining at 80% for 7  
178 min with a final increase to 90% for 13 min and 10 min post-time. This method can  
179 simultaneously detect and quantify 11 biogenic amines, namely methylamine, ethylamine,  
180 tryptamine, 2-phenyl ethylamine, isoamylamine, cadaverine, putrescine, histamine, tyramine,  
181 spermidine and spermine. All standards were obtained from Sigma-Aldrich (France). For  
182 biogenic amine quantification, a 6 point calibration curve was used by combining all 11  
183 biogenic amine standard solutions at concentrations ranging from 50 mg/L (standard 1) to 500  
184 mg/L (standard 6). Linearity for all standards was confirmed to have  $R^2$  values  $>0.99$ . Before  
185 analysis, 2 ml of bacterial culture were centrifuged to pellet cells, and the supernatant was  
186 transferred to a new Eppendorf tube and stored at -20°C until use. One hundred  $\mu\text{L}$  of each  
187 supernatant were filtered using 0.45  $\mu\text{m}$  acetate membrane-filter before dansyl-chloride  
188 derivatization of biogenic amines as described by (Duflos et al., 1999) with a modification.  
189 Samples were incubated for 30 min at -20°C after adding 500  $\mu\text{l}$  toluene. Biogenic amines  
190 concentrations were determined by HPLC as described above.

191

### 192 2.3.3. Biogenic amine gene detection by PCR

193 DNA was extracted from bacterial cultures grown to stationary phase using 1 ml of culture  
194 with the NucleoSpin Tissue Kit (Macherey Nagel, France) according to the manufacturer's  
195 instructions. Purified DNA samples were stored at -20°C. The molecular method,  
196 simultaneously detecting 4 major BA associated genes (*hdc*, *agdi*, *odc* and *tyrdc*) and a PCR  
197 internal control (targeting the 16S rRNA gene), was used as described by (Coton, Romano, et  
198 al., 2010). Simplex PCR reactions were also performed and targeted each gene individually  
199 for positive reaction confirmation and gene sequencing. PCR reactions were carried out in a  
200 50 µl final volume using 0.2 µM primers, 0.2 mM dNTPs, 1X buffer (Promega), 1.5 mM  
201 MgCl<sub>2</sub> (Promega), 1.25 U GoTaq DNA polymerase (Promega) and 50 to 100 ng DNA. For  
202 simplex PCR, the amplification program varied from that described by (Coton, Romano, et  
203 al., 2010) and was as followed: for *hdc*, 95°C for 5 min, 35 cycles of 95°C for 30 sec, 48°C  
204 for 30 sec, 72°C for 45 sec with a final extension at 72°C for 5 min; for *tyrdc*, 95°C for 45  
205 sec, 35 cycles of 95°C for 45 sec, 50°C for 45 sec, 72°C for 1 min with a final extension at  
206 72°C for 5 min; for *odc/agdi*, 95°C for 5 min, 35 cycles of 95°C for 1 min, 52°C for 1 min,  
207 72°C for 1.5 min with a final extension at 72°C for 5 min. PCR products were analyzed using  
208 0.8% (w/v) agarose gels (Promega) in 1x TBE buffer at 130 V for 50 min then visualized  
209 using GelRed staining.

210

### 211 2.3.4. Gene sequencing and sequence analysis

212 PCR fragments were sequenced by Eurofins MWG (Abersberg, Germany).  
213 Alignments were performed using DNA Baser or Bionumerics software (Applied Maths,  
214 Belgium) and sequence similarity was determined using BLAST in the NCBI GenBank  
215 database.

216

217 *2.4 Antibiotic susceptibility tests*

218

219 *2.2.1 Determination of antibiotic susceptibility*

220 Minimum inhibitory concentration (MIC) determination for a wide panel of antibiotics  
221 was conducted according to the ISO 10932 IDF 223 International Standard (2010-06-15)  
222 applicable to bifidobacteria and non-enterococcal lactic acid bacteria related to milk and milk  
223 products. To do so, 13 antibiotics were selected and corresponded to ampicillin (AMP) for  
224 beta-lactam antibiotics; gentamicin (GEN), kanamycin (KAN), neomycin (NEO),  
225 streptomycin (STR) for aminoglycosides; tetracycline (TET), oxytetracycline (OXY) for  
226 cyclins; erythromycin (ERY) for macrolides, clindamycin (CLI) for lincosamides;  
227 chloramphenicol (CHL) for phenicols; trimethoprim (TMP) for diaminopyrimidines;  
228 vancomycin (VAN) for glycopeptides and rifampicin (RIF) for rifamycins. The antibiotics  
229 mentioned above were available as salts or ready to use solutions (Sigma-Aldrich, USA). For  
230 each one, a stock solution was prepared in water (GEN, KAN, STR, ERY, CLI, CHL, AMP,  
231 NEO, VAN and OXY), DMSO (TRI) or a water / ethanol mixture (60/40 v/v) (TET and RIF)  
232 and then filtered through 0.2µm cellulose acetate filters. Then, two-fold dilutions were made  
233 in sterile water to obtain the concentration range indicated by the ISO standard for each  
234 antibiotic.

235 For MIC determinations, 50 µl aliquots of each antibiotic solution were added into  
236 sterile 96-well plates according to the ISO standard. After preparation, plates were stored at -  
237 20°C until use.

238

239 *2.2.1 Culture conditions and antibiotic resistance pattern tests*



240 LAB were first inoculated on MRS plates, PAB on YEL plates and streptococci on  
241 M17-sucrose plates and incubated in anaerobic conditions as follows: 48 h at 37°C for LAB  
242 (except *L. brevis* and *L. plantarum*), 24 h at 37°C for streptococci, 96 h at 30°C for PAB and  
243 48 h at 28°C for *L. brevis* and *L. plantarum* as required by the ISO standard. Briefly, 1 Mac  
244 Farland ( $OD_{625nm}=0.16-0.2$ ) suspensions were prepared for each strain in physiological water  
245 (0.85% w/v NaCl) and diluted 500-fold in the required test medium (see below) then  
246 inoculated into 96 well plates. Each LAB strain was cultured in LSM 2X medium composed  
247 of 90% (v/v) 2X Iso-Sensitest medium (Oxoid, UK) and 10% (v/v) 2X MRS medium. For  
248 streptococci, strains were cultured in IST-lactose 2X medium containing 90% (v/v) 2X Iso-  
249 Sensitest medium and 10% (v/v) lactose solution (20 g/L). For PAB, the ISO standard did not  
250 indicate any specification therefore an IST-YEL 2X medium containing 90% (v/v) 2X Iso-  
251 Sensitest medium and 10% (v/v) 2X YEL medium was used.

252 For each plate, the reference strains for each microbial group specified by the ISO  
253 standard (namely *L. plantarum* LMG6907, *L. paracasei* LMG13552 and *S. thermophilus*  
254 LMG18311 and listed in Table 1) and negative controls were included. However, this method  
255 did not include any information on PAB antimicrobial susceptibility testing, therefore we  
256 included the control strain, *Propionibacterium jensenii* CIRM-BIA 39<sup>T</sup> (equivalent to  
257 DSMZ20535) based on recently published data on this strain (Darilmaz and Beyatli, 2012).  
258 These control strains were included in each series (systematically performed in biological  
259 triplicates for all studied strains) for proper validation of all results by comparing the obtained  
260 results to those provided in the ISO method or that were published. Positive controls  
261 contained 50µl of 500-fold diluted culture suspensions (for each strain included) and 50 µl of  
262 sterile solvent (without added antibiotic solution), while negative controls contained 50 µl  
263 sterile water and 50 µl non-inoculated sterile medium. All plates were incubated for 48 h at  
264 37°C for LAB except *L. brevis* (30°C), *L. plantarum* (28°C) and *L. harbinensis* (30°C)

265 strains, while PAB plates were incubated at 30°C for 72 h. MIC values were then determined  
266 and corresponded to the ATB concentration in the first well where no growth was visible.  
267 Plate readings were carried out by an automatic plate reader set to 620nm (Lecteur Multiskan  
268 FC, Thermo Scientific).

269

### 270 **3. Results**

#### 271 *3.1 In vitro biogenic amine determination*

272 The selection of bioprotective cultures lacking decarboxylase or deiminase activities  
273 was the first step during this safety evaluation. In this study, 22 LAB and PAB strains (along  
274 with control strains) were analyzed for the presence of 4 major BA associated genes, *hdc*,  
275 *tyrdc*, *odc* and *agdi*, involved in histamine, tyramine and putrescine (*odc* and *agdi*)  
276 production, respectively, by multiplex and/or simplex PCR. Moreover, BA were  
277 simultaneously quantified in liquid decarboxylase medium cultures (i.e. MP or optimal  
278 growth medium supplemented with amino acids) for each strain by HPLC.

279 *P. jensenii* CIRM-BIA 1774 did not harbor any of the target genes according the  
280 results obtained using the different primers in both multiplex and simplex PCR results (Table  
281 2). This finding was further validated by HPLC analyses as no BA were detected in the tested  
282 decarboxylase medium culture supernatants (< detection limit, DL) (Table 3). Complementary  
283 to these analyses, an *in silico* search was also performed using 20 available  
284 *Propionibacterium freundrichii* genomes (only PAB species with available genome  
285 sequences; H. Falentin, INRA STLO) using known and available decarboxylase genes and  
286 entire clusters (i.e. corresponding to *hdc*, *tyrdc*, *odc*, *agdi* as well as *ldc* involved in  
287 cadaverine production) as almost no literature data exists to date on BA production in PAB. A  
288 standard BLAST strategy was applied to search for both nucleotide and protein level

289 similarities. No positive BLAST results were obtained for any sequence, therefore this species  
290 was also considered not to harbor genes involved in BA production.

291 For LAB, 15 of the tested strains did not harbor any of the target BA genes and  
292 corresponded to *L. casei* L142, *L. coryniformis* K.V9.3.10, *L. harbinensis* (L172 and  
293 K.V9.3.1NP), *L. paracasei* L117, *L. plantarum* (L11, L14, L17 and L244), *L. rhamnosus*  
294 (CIRM-BIA 1112, CIRM-BIA 1113 and CIRM-BIA 1952), *L. zea* M.C7.5.2C, *L. lactis*  
295 CIRM 47, and *S. thermophilus* CIRM-BIA 16 (Table 2). On the other hand, *tyrdc* and *agdi*  
296 genes were simultaneously detected by PCR in *L. brevis* L128, while only *tyrdc* was  
297 amplified for the 3 tested *L. rhamnosus* strains (CIRM-BIA 574, CIRM-BIA 578 and  
298 M.C8.4.1B) (Table 2). Concerning the 2 *L. buchneri* strains (L162 and L233), simultaneous  
299 detection of both *hdc* and *agdi* genes was observed (Table 2). In each case, all PCR products  
300 were sequenced to ensure accurate BA gene identifications. *L. brevis* L128 *tyrdc* and *agdi*  
301 amplification products were 100% and 99% identical to those of *L. brevis* ATCC367  
302 (Genbank accession n°CP000416.1). This clearly confirmed the identity of this gene, while in  
303 *L. rhamnosus* (CIRM-BIA 574, CIRM-BIA 578) and *L. mesenteroides* (M.C8.4.1B) strains,  
304 *tyrdc* fragments were 97% and 98% identical with the same sequence in Genbank, again  
305 confirming valid gene identification. For *L. buchneri* strains (L162 and L233), *hdc* gene  
306 products were 100% identical to *L. parabuchneri* DSM5987 *hdc* sequence (Genbank  
307 accession n°LN877765.1), confirming the presence of these genes, while *agdi* amplification  
308 products showed 75% homology with the one described in *L. brevis* ATCC367.

309 To determine whether strains produced BA *in vitro* and to confirm gene detection  
310 results by PCR, HPLC determinations were also carried out on MP- and/or MRS-based  
311 culture supernatants. The same results were obtained using this method on all LAB strains as  
312 no BA (among the 11 targeted BA for HPLC analyses) were detected in culture supernatants  
313 among the same 15 BA-gene negative strains using multiplex PCR. Among positive BA-gene

314 results, *L. brevis* L128 simultaneously produced tyramine and putrescine in both amino acid  
315 supplemented media (either MRS or MP based media) (Table 3), thereby confirming the  
316 obtained PCR data. For both tyramine and putrescine, slightly higher concentrations were  
317 observed in MP medium ( $641 \pm 7$  mg/L and  $292 \pm 6$  mg/L, respectively) versus MRS medium  
318 ( $574 \pm 50$  mg/L and  $200 \pm 12$  mg/L, respectively). Two *L. rhamnosus* and 1 *L. mesenteroides*  
319 strains were also confirmed to be tyramine producers with concentrations reaching close to  
320 410 mg/L (CIRM-BIA 578), 700 mg/L (CIRM-BIA 574) and 580 mg/L (M.C8.4.1B),  
321 respectively, in MRS based medium (Table 3). Finally, *L. buchneri* L162 and L233 both  
322 produced tyramine in MRS-based medium supernatants at concentrations reaching  $723 \pm 39$   
323 mg/L and  $743 \pm 33$  mg/L histamine, respectively.

324

### 325 3.2. Antimicrobial susceptibility testing

326 Antimicrobial susceptibility testing was the second step during this safety evaluation  
327 of the 22 different LAB and PAB strains. To do so, the standardized ISO 10932:2010 method  
328 (ISO, 2010) was used to determine MIC values for 13 antibiotics belonging to 8 families.  
329 MIC values obtained for the studied strains were compared to the cut-off values published by  
330 EFSA to determine whether a given strain was resistant to an antibiotic (EFSA, 2012). All  
331 antibiotic susceptibility testing results are given in Table 4.

332 All lactobacilli strains were resistant to vancomycin (a glycopeptide), while both  
333 *S. thermophilus* CIRM-BIA 16 and *P. jensenii* CIRM-BIA 1774 were sensitive to this  
334 antibiotic. Many strains were also resistant to kanamycin (an aminoglycoside) and included  
335 *L. plantarum* L11, L14 and L17, *L. coryniformis* K.V9.3.1O, *L. harbinensis* K.V9.3.1Np,  
336 *L. paracasei* L117, *L. rhamnosus* CIRM-BIA 578 and *L. zae* M.C7.5.2C. Moreover,  
337 *L. coryniformis* K.V9.3.1O, *L. harbinensis* K.V9.3.1Np and *Ln. mesenteroides* M.C8.4.1B

338 were weakly resistant to kanamycin (biological triplicates around the cut-off values); all other  
339 strains showed what we considered to be intermediate values, therefore below resistant cut-off  
340 values or when these values were not known. Four strains were resistant to tetracycline (*L.*  
341 *buchneri* L162 and L233, *L. zeae* M.C7.5.2C and to a lesser extent *L. harbinensis*  
342 K.V9.3.1Np), while all others presented intermediate profiles. Two *L. rhamnosus* strains  
343 (CIRM-BIA 1113 and CIRM-BIA 1952) were also resistant to trimethoprim, a  
344 diaminopyrimidin antibiotic, while all other strains were either sensitive (*L. paracasei* L117,  
345 *L. harbinensis* L172 and *P. jensenii* CIRM-BIA 1774) or gave an intermediate result  
346 (*L. plantarum* L11, L14, L17 and L244, *L. casei* L142, *L. coryniformis* K.V9.3.10, *L. brevis*  
347 L128, *L. buchneri* L162 et L233, *L. rhamnosus* CIRM-BIA 1112, CIRM-BIA 574 and CIRM-  
348 BIA 578, *L. zeae* M.C7.5.2C, *L. harbinensis* K.V9.3.1Np, *L. lactis* CIRM-BIA 47, *L.*  
349 *mesenteroides* M.C8.4.1B and *S. thermophilus* CIRM-BIA 16). Many strains were also found  
350 to be resistant to the lincosamide, clindamycin, and included *L. plantarum* L11, L14 and L17,  
351 *L. brevis* L128 and *L. rhamnosus* CIRM-BIA 1112. On the other hand, *L. plantarum* L244, *L.*  
352 *buchneri* L162 and L233 and *S. thermophilus* CIRM-BIA-16 were all sensitive to this  
353 antibiotic, while the others gave intermediate MIC values. Six strains were also resistance to  
354 chloramphenicol (*L. buchneri* L233, *L. casei* L142, *L. rhamnosus* CIRM BIA 1112 and  
355 CIRM-BIA 1952, *L. zeae* M.C7.5.2C, *S. thermophilus* CIRM-BIA 16). Finally, one *L.*  
356 *rhamnosus* strain (CIRM-BIA-1112) was resistant to multiple antibiotics namely  
357 streptomycin, erythromycin, clindamycin, chloramphenicol and, as already mentioned,  
358 vancomycin.

359 On the other hand, *L. buchneri* L162 and L233, *L. harbinensis* L172, *L. rhamnosus*  
360 CIRM-BIA-1113 and CIRM-BIA-1952, *S. thermophilus* CIRM-BIA 16 and *P. jensenii*  
361 CIRM-BIA-1774 were highly sensitive to rifampicin. *L. buchneri* L162 and L233 strains were  
362 also sensitive to gentamicin, neomycin, clindamycin and rifampicin. *P. jensenii* CIRM-

363 BIA 1774 was sensitive to trimethoprim, vancomycin and, as already mentioned, rifampicin  
364 and intermediate MIC values were obtained for all other ATB tested (no resistances were  
365 identified). *S. thermophilus* CIRM-BIA-16 was also sensitive to 4 ATBs, intermediate for 8  
366 ATBs and resistant to only 1 ATB, chloramphenicol.

367 Overall, most of the tested strains were resistant to either 1 or 2 ATBs, including  
368 vancomycin (except for *P. jensenii* CIRM-BIA 1774 and *S. thermophilus* CIRM-BIA 16).  
369 Only 8 strains were resistant to 3 or more ATBs and corresponded to *L. buchneri* L233,  
370 *L. plantarum* L11, L14 and L17, *L. rhamnosus* CIRM-BIA 1112 and CIRM-BIA-1952, *L.*  
371 *zeae* M.C7.5.2C. *L. harbinensis* K.V9.3.1Np showed 2 ATB MIC values only weakly in the  
372 cut-off value zone therefore this strain could be considered to have only 1 notable resistance  
373 to vancomycin. It is worth underlining that intermediate MIC values were observed for most  
374 ATBs and strains and all results are provided in Table 4.

375

#### 376 **4. Discussion**

377 The selection of bioprotective cultures for use in the food chain is of growing interest  
378 to avoid food or feed spoilage and extend product shelf life. This can have a major impact on  
379 food quality for the consumer but also economically at the industrial and consumer levels by  
380 reducing food loss and waste. In this context, prior to bioprotective culture use, safety  
381 assessment of selected strains is necessary. Among potential bioprotective cultures, and as  
382 mentioned previously, non-enterococcal LAB and PAB species are of particular interest due  
383 to their described antimicrobial properties against spoilage fungi in *in vitro* and/or *in situ*  
384 studies (Aunbjerg et al., 2015; Delavenne et al., 2015; Delavenne et al., 2013; Le Lay,  
385 Coton, et al., 2016; Le Lay, Mounier, et al., 2016; Schwenninger and Meile, 2004). According  
386 to current knowledge on these bacterial groups, we have considered that safety assessment  
387 requires two major steps once taxonomy at both the species and strain levels has been well-

388 established: i) determining whether a strain is capable or not of producing biogenic amines  
389 (due to their potential physiological effects on the consumer) and, ii) determining antibiotic  
390 susceptibility patterns and acquired antibiotic resistances. Indeed, the presence of acquired  
391 resistances associated with mobile elements can contribute to antibiotic resistances  
392 dissemination that is a major worldwide public health concern. This proposed 2-step safety  
393 evaluation is reinforced by the fact that many non-enterococcal LAB and PAB species, to be  
394 used as potential bioprotective cultures, already have a long history of safe use in the food  
395 chain and/or already have GRAS or QPS status, and/or are well defined taxonomically. In this  
396 study, this approach was used to assess a collection of 21 LAB and 1 PAB strains that were  
397 previously shown to have antifungal activities both *in vitro* and *in situ* (in yogurt, fresh  
398 cream,...) (Delavenne et al., 2015; Delavenne et al., 2013; Mieszkin et al., 2017).

399         Biogenic amines are nitrogenous basic compounds that are mainly formed by the  
400 decarboxylation of free amino acids (Shalaby, 1996; Spano et al., 2010; Ten Brink et al.,  
401 1990). This is particularly the case in fermented foods when BA-producing bacteria,  
402 harbouring at least a decarboxylase or deiminase enzyme and BA/amino acid transporter, are  
403 present and environmental conditions are favourable (Martin and Coton, 2017). Bioprotective  
404 cultures lacking decarboxylase or deiminase activities, the key enzymes involved in BA  
405 production in LAB, should be selected to avoid BA production in foods. This study evaluated  
406 a panel of 22 strains for their potential to produce BA by using molecular tools (to detect  
407 decarboxylase and/or deiminase enzymes) followed by HPLC analysis to quantify BA  
408 production *in vitro*. The molecular method (using multiplex PCR) detected the 4 major genes  
409 involved in the biosynthesis of 3 of the most relevant BA related to human health in foods,  
410 namely tyramine, histamine and putrescine (Coton, Romano, et al., 2010). The target genes  
411 correspond to *hdc* involved in histamine production, *tyrdc* involved in tyramine production,  
412 and *odc* and *agdi* that can both lead to putrescine production; although cadaverine is also of

413 major interest in food, no molecular tools to detect the *ldc* gene are currently available for  
414 LAB and was therefore not targeted. This method was complementary to standard HPLC  
415 analysis of culture supernatants and, in particular, can detect strains harbouring the different  
416 genes, even if production is not observed in the studied conditions.

417 Six strains belonging to *L. brevis* (n=1), *L. buchneri* (n=2), *L. mesenteroides* (n=1) and  
418 *L. rhamnosus* (n=2) species were identified as BA-producers *in vitro*. *L. brevis* L128  
419 produced quantifiable amounts of both tyramine and putrescine in the tested conditions and  
420 both the *tyrdc* and *agdi* genes were detected thus well correlated. This species has already  
421 been well described as a tyramine and putrescine producer and the biosynthesis clusters  
422 involved in their production have been documented (Coton and Coton, 2009; Coton, Mulder,  
423 et al., 2010; Lucas et al., 2007). Moreover, *L. brevis* strains from different fermented foods  
424 such as dry cured sausages or wine, have already been described to produce high levels of  
425 tyramine (from ~380 to >1220 mg.L<sup>-1</sup>) and putrescine (~420 mg.L<sup>-1</sup>) *in vitro* (Bover-Cid et  
426 al., 2001; Coton, Romano, et al., 2010). Two *L. rhamnosus* strains (CIRM-BIA-574 and  
427 CIRM-BIA-578) were identified as *tyrdc*<sup>+</sup> and tyramine producers *in vitro*; to our knowledge,  
428 this is the first description of this species as a strong tyramine producer. Interestingly, *tyrdc*  
429 sequence homologies only showed 97% identities with the well-known *L. brevis tyrdc* gene  
430 sequences. In the future, further studies could be carried out to determine cluster organization  
431 and gene synteny, as differences have already been described among known tyramine-  
432 producing bacteria, in particular between the *L. brevis* operon structure and  
433 *Sporolactobacillus* sp. (Coton et al., 2011; Lucas et al., 2003; Lucas et al., 2007). *L.*  
434 *mesenteroides* (M.C8.4.1B) was also identified a tyramine producing strain and the *tyrdc* gene  
435 was detected in this strain. Strains belonging to this species that were isolated from different  
436 fermented foods or beverages (Coton, Romano, et al., 2010) have already been described in  
437 the literature as tyramine producers (Gonzalez de Llano et al., 1998). Finally, 2 *L. buchneri*



438 strains, L162 and L233, were identified as histamine producers and the *hdc* gene was also  
439 detected and confirmed by PCR. Interestingly, while the *agdi* gene was also detected, no  
440 putrescine was detected in culture supernatants. To our knowledge, this is the first time that  
441 these two genes are simultaneously described in *L. buchneri*. This species is a well-known  
442 histamine producer and the biosynthesis pathway was previously described (Martín et al.,  
443 2005). More recently, (Elsanhoty and Ramadan, 2016) identified a *L. buchneri* strain with a  
444 putative *agdi* gene (however, no *agdi* sequence data was made available), but not with a *hdc*  
445 gene. Complementary to our findings, we also performed an *in silico* search using the *L.*  
446 *buchneri* CD034 genome sequence (accession n°CP003043.1) (Heinl et al., 2012), which  
447 allowed us to detect this gene therefore suggesting that this species may be a potential  
448 putrescine producer. Further studies will be required to elucidate the *agdi* cluster in this  
449 species and determine whether it is functional. Overall, only few strains were identified as  
450 BA-producers in this study including *L. brevis*, *L. mesenteroides* and *L. buchneri* strains that  
451 have all been previously described to produce the corresponding biogenic amines.  
452 Interestingly, 2 strains belonging to *L. rhamnosus* were shown to be tyramine producers for  
453 the first time, suggesting that they likely obtained this trait via horizontal gene transfer. BA  
454 production is well known to be a strain-dependent trait (Coton and Coton, 2009; Coton,  
455 Mulder, et al., 2010; Ladero et al., 2012; Ladero et al., 2015; Lucas et al., 2008; Lucas et al.,  
456 2005; Priyadarshani and Rakshit, 2011) and horizontal gene transfer events have already been  
457 described in many LAB species (Coton and Coton, 2009; Coton, Mulder, et al., 2010; Coton  
458 et al., 2011; Ladero et al., 2012; Lucas et al., 2005). Concerning PAB, this study did not  
459 detect any BA related genes or BA production using different molecular and biochemical  
460 approaches, thus suggesting that PAB can be considered as low risk for BA production to  
461 date. It is important to note that the molecular tools were originally designed based on LAB  
462 species sequences, therefore it cannot be concluded whether they will be functional on other

463 genera (i.e. PAB); however, the *in silico* search performed in this study on the available PAB  
464 genomes clearly confirmed that no BA related genes were present. To date, BA screening  
465 among PAB strains has not been largely explored, although this risk factor should be included  
466 during any safety evaluation procedure. Finally, concerning the BA risk, even if BA are not  
467 produced *in vitro* and/or *in situ* in the actual target food matrix by a given strain, the presence  
468 of BA associated genes should incite to reject the positive isolate from food applications.

469         The second step of the safety evaluation was to determine antibiotic resistance patterns  
470 (via MIC determinations) using a panel of 13 ATBs belonging to different families and that  
471 are commonly used in scientific papers and recommended by the ISO or EFSA guidelines.  
472 The clinically important ATB, vancomycin, was included in the study and all tested bacteria  
473 were resistant except *S. thermophilus* CIRM-BIA 16 and *P. jensenii* CIRM-BIA 1774 that  
474 were both sensitive. Lactobacilli are already well-known to be naturally resistant to  
475 vancomycin and this resistance has already been observed among yogurt related *S.*  
476 *thermophilus* isolates (Aslim and Beyatli, 2004; Bernardeau et al., 2008) as well as a  
477 *P. jensenii* isolate from Emmental cheese (Campaniello et al., 2015). High natural resistance  
478 to trimethoprim have also been described among some LAB species (Danielsen and Wind,  
479 2003; Mathur and Singh, 2005) and two out of five *L. rhamnosus* strains (CIRM-BIA 1113  
480 and CIRM-BIA 1952) were resistant to trimethoprim. All other strains were either sensitive or  
481 values were below the defined resistance level to this ATB. Multiple strains were also  
482 resistant to clindamycin and included some *L. plantarum*, *L. brevis* and *L. rhamnosus* strains,  
483 which tends to suggest an acquired resistance to this ATB. The same was observed for  
484 kanamycin resistance for 9 isolates belonging to *L. plantarum*, *L. coryniformis*, *L.*  
485 *harbinensis*, *L. paracasei*, *L. rhamnosus*, *L. zae* and *L. mesenteroides*. Kanamycin resistance  
486 has already been described among lactobacilli, including *L. plantarum* (Danielsen and Wind,  
487 2003). More surprisingly, 1 of the tested *L. rhamnosus* strains (CIRM-BIA 1112) was

488 multiresistant to 5 ATBs, namely, streptomycin, erythromycin, clindamycin, chloramphenicol  
489 and vancomycin. This finding has never been described in literature data, although (Korhonen  
490 et al., 2010) already identified 3 *L. rhamnosus* strains possessing 3 ATB resistances  
491 (clindamycin, erythromycin and streptomycin).

492 On the other hand, many strains were highly sensitive to rifampicin (no growth in the  
493 presence of this ATB), they belonged to *L. buchneri*, *L. harbinensis*, *L. rhamnosus*,  
494 *S. thermophilus* and *P. jensenii* species. MIC values obtained for the two *L. rhamnosus* strains  
495 were found to be in accordance with previous findings on other strains belonging to this  
496 species (Danielsen and Wind, 2003). Some strains also showed multiple sensitivities, such as  
497 *L. buchneri* (strains L162 and L233) to gentamicin, clindamycin, neomycin and rifampicine.  
498 This finding is in accordance with the fact that many *Lactobacillus* spp. are described as  
499 sensitive to ATB inhibiting protein synthesis (i.e. macrolids, glycopeptides and lincosamides)  
500 (Ammor et al., 2007). MIC values obtained for both *L. buchneri* strains were also very close  
501 to those previously described for other strains by (Korhonen, 2010). This was also observed  
502 for *P. jensenii* CIRM-BIA 1774 that was sensitive to vancomycin, trimethoprim and  
503 rifampicin. Sensitivities to trimethoprim (Campaniello et al., 2015) and rifampicin (Darilmaz  
504 and Beyatli, 2012) have already been documented for this species. Moreover, *P. jensenii*  
505 CIRM-BIA 1774 was not resistant to any of the tested ATBs and a previous study suggested  
506 that many *Propionibacterium* spp. strains of milk origin have not acquired ATB resistances  
507 (Ammor et al., 2007). Finally, most MIC values determined in this study for *L. plantarum*, *L.*  
508 *paracasei* and *L. rhamnosus* strains were well correlated to previously published data  
509 (Danielsen and Wind, 2003). Other data obtained on the different LAB and PAB species  
510 included in this work will provide new insight on antimicrobial susceptibility patterns.

511 The safety assessment procedure proposed in this study identified the majority of LAB  
512 and PAB strains belonging to 13 different species as non-BA-producers and with low

513 antibiotic resistance patterns. The only exceptions were for *L. rhamnosus* strains that were  
514 either observed to produce high levels of tyramine *in vitro* (CIRM-BIA 574 and CIRM-  
515 BIA 578), a biogenic amine with potential negative effects on human health if ingested in  
516 high quantities from foods, or were multiresistant to 5 (CIRM-BIA 1112), 3 (CIRM-  
517 BIA 1952) or 2 (CIRM-BIA 1113) ATBs, including a natural resistance to vancomycin.  
518 Another exception was also identified for 2 *L. buchneri*, 1 *L. brevis* and 1 *L. mesenteroides*  
519 strain that were identified as either histamine or tyramine producers *in vitro*, and displayed  
520 between 2 and 3 ATB resistances. The obtained results excluded these strains for future food  
521 applications due to the fact that BA biosynthesis genes were harbored and BA production was  
522 also observed *in vitro* and multiple resistances to different ATB were identified and likely  
523 correspond to acquired resistances that could pose a putative risk related to gene  
524 dissemination. In this context, further studies should be carried out to determine the genetic  
525 element that led to these resistance profiles.

526 In conclusion, the proposed safety assessment procedure was an efficient model to  
527 select low risk non-enterococcal LAB and PAB candidate bioprotective cultures for future use  
528 in food applications. Overall, the following strains did not present risks associated to BA  
529 production and ATB resistance patterns and can therefore be of great interest for industrial  
530 use as antifungal cultures: *L. casei* L142, *L. coryniformis* K.V9.3.1O, *L. harbinensis* L172 and  
531 K.V9.3.1Np, *L. paracasei* L117, *L. plantarum* L11, L14, L17 and L244, *L. rhamnosus* CIRM-  
532 BIA 1113 and CIRM-BIA 1952, *L. zeae* M.C7.5.2C, *L. lactis* CIRM-BIA 47, *S. thermophilus*  
533 CIRM-BIA 16, *P. jensenii* CIRM-BIA 1774.

534

### 535 **Acknowledgments**

536 This work was partly supported by the French National Agency for Research (ANR) program  
537 FUNGINIB (ANR-09-ALIA-005-01). We are also grateful to the Regional Council of

538 Brittany (grant no. 13008651) and Pays de la Loire (grant no. 2014-07081) for their financial  
539 support and INRA for scientific coordination (J. Leonil) through the interregional project  
540 PROFIL, managed by BBA industrial association.

541

542

## References

- 543  
544  
545  
546 Ammor, M.S., Flórez, A.B., Mayo, B., 2007. Antibiotic resistance in non-enterococcal lactic  
547 acid bacteria and bifidobacteria. *Food Microbiol* 24, 559-570.  
548  
549 Aslim, B., Beyatli, Y., 2004. Antibiotic resistance and plasmid DNA contents of  
550 *Streptococcus thermophilus* strains isolated from Turkish yogurts. *Turkish Journal of*  
551 *Veterinary and Animal Sciences* 28, 257-263.  
552  
553 Aunbjerg, S.D.H., A.H., Marcussen, J., Ebrahimi, P., Vogensen, F.K., Benfeldt, C., Skov, T.,  
554 Knøchel, S., 2015. Contribution of volatiles to the antifungal effect of *Lactobacillus*  
555 *paracasei* in defined medium and yogurt. *Int J Food Microbiol* 194, 46-53.  
556  
557 Bennedsen, M., Stuer-Lauridsen, B., Danielsen, M., Johansen, E., 2011. Screening for  
558 Antimicrobial Resistance Genes and Virulence Factors via Genome Sequencing. *Appl*  
559 *Environ Microbiol* 77, 2785-2787.  
560  
561 Bernardeau, M., Vernoux, J.P., Henri-Dubernet, S., Gueguen, M., 2008. Safety assessment of  
562 dairy microorganisms: the *Lactobacillus* genus. *Int J Food Microbiol* 126, 278-285.  
563  
564 Bourdichon, F., Casaregola, S., Farrokh, C., Frisvad, J.C., Gerds, M.L., Hammes, W.P.,  
565 Harntt, J., Huys, G., Laulund, S., Ouwehand, A., Powell, I.B., Prajapati, J.B., Seto, Y., Ter  
566 Schure, E., Van Boven, A., Vankerckoven, V., Zgoda, A., Tuijelaars, S., Bech Hansen, E.,  
567 2012. Food fermentations: microorganisms with technological beneficial use. *Int J Food*  
568 *Microbiol* 154, 87-97.  
569  
570 Bover-Cid, S., Izquierdo-Pulido, M., Vidal-Carou, M.C., 2001. Effect of the interaction  
571 between a low tyramine-producing *Lactobacillus* and proteolytic staphylococci on biogenic  
572 amine production during ripening and storage of dry sausages. *Int J Food Microbiol* 65, 113-  
573 123.  
574  
575 Campaniello, D., Bevilacqua, A., Sinigaglia, M., Altieri, C., 2015. Screening of  
576 *Propionibacterium* spp. for potential probiotic properties. *Anaerobe* 34, 169-173.  
577  
578 Coton, E., Coton, M., 2009. Evidence of horizontal transfer as origin of strain to strain  
579 variation of the tyramine production trait in *Lactobacillus brevis*. *Food Microbiol* 26, 52-57.  
580  
581 Coton, E., Mulder, N.L., Coton, M., Pochet, S., Trip, H., Lolkema, J.S., 2010. Origin of the  
582 Putrescine-Producing Ability of the Coagulase-Negative Bacterium *Staphylococcus*  
583 *epidermidis* 2015B. *Appl Environ Microbiol* 76, 5570-5576.  
584  
585 Coton, M., Fernández, M., Trip, H., Ladero, V., Mulder, N.L., Lolkema, J.S., Alvarez, M.A.,  
586 Coton, E., 2011. Characterization of the tyramine-producing pathway in *Sporolactobacillus*  
587 sp. P3J. *Microbiol* 157, 1841-1849.  
588  
589 Coton, M., Romano, A., Spano, G., Ziegler, K., Vetrana, C., Desmarias, C., Lonvaud-Funel,  
590 A., Lucas, P., Coton, E., 2010. Occurrence of biogenic amine-forming lactic acid bacteria in  
591 wine and cider. *Food Microbiol* 27, 1078-1085.

592 Danielsen, M., Wind, A., 2003. Susceptibility of *Lactobacillus* spp. to antimicrobial agents.  
593 Int J Food Microbiol 82, 1-11.  
594

595 Darilmaz, D.O., Beyatli, Y., 2012. Acid-bile, antibiotic resistance and inhibitory properties of  
596 propionibacteria isolated from Turkish traditional home-made cheeses. Anaerobe 18, 122-  
597 127.  
598

599 Delavenne, E., Cliquet, S., Trunet, C., Barbier, G., Mounier, J., Le Blay, G., 2015.  
600 Characterization of the antifungal activity of *Lactobacillus harbinensis* K.V9.3.1Np and  
601 *Lactobacillus rhamnosus* K.C8.3.1I in yogurt. Food Microbiol 45, 10-17.  
602

603 Delavenne, E., Ismail, R., Pawtowski, A., Mounier, J., Barbier, G., Le Blay, G., 2013.  
604 Assessment of lactobacilli strains as yogurt bioprotective cultures. Food Control 30, 206-213.  
605

606 Duflos, G., Dervin, C., Malle, P., Bouquelet, S., 1999. Use of biogenic amines to evaluate  
607 spoilage in plaice (*Pleuronectes platessa*) and whiting (*Merlangus merlangus*). J AOAC Int  
608 86, 1357-1363.  
609

610 EFSA, 2012. Scientific Opinion. Guidance on the assessment of bacterial susceptibility to  
611 antimicrobials of human and veterinary importance. The EFSA Journal 10, 1-10.  
612

613 EFSA, 2013. Scientific Opinion on the maintenance of the list of QPS biological agents  
614 intentionally added to food and feed (2013 update). The EFSA Journal 11, 3449-3555.  
615

616 EFSA, 2016. Update of the list of QPS-recommended biological agents intentionally added to  
617 food or feed as notified to EFSA 4: suitability of taxonomic units notified to EFSA until  
618 March 2016. EFSA Journal 14, 4522.  
619

620 Elsanhoty, R.M., Ramadan, M.F., 2016. Genetic screening of biogenic amines production  
621 capacity from some lactic acid bacteria strains. Food Control 68, 220-228.  
622

623 Elsser-Gravesen, D., Elsser-Gravesen, A., 2014. Biopreservatives. Adv Biochem Eng  
624 Biotechnol 143, 29-49.  
625

626 Gasser, F., 1994. Safety of lactic acid bacteria and their occurrence in human clinical  
627 infections. Bulletin de l'Institut Pasteur 94, 45-67.  
628

629 Gonzalez de Llano, D., Cuesta, P., Rodriguez, A., 1998. Biogenic amine production by wild  
630 lactococcal and leuconostoc strains. Lett Appl Microbiol 26, 270-274.  
631

632 Heintl, S., Wibberg, D., Eikmeyer, F., Szczepanowski, R., Blom, J., Linke, B., Goesmann, A.,  
633 Grabherr, R., Schwab, H., Pühler, A., Schlüter, A., 2012. Insights into the completely  
634 annotated genome of *Lactobacillus buchneri* CD034, a strain isolated from stable grass silage.  
635 J Biotechnol 161, 153-166.  
636

637 ISO, N., 2010. ISO 10932:2010 (IDF 223:2010) Lait et produits laitiers – Détermination de la  
638 concentration minimale inhibitrice (CMI) d'antibiotiques applicable aux bifidobacteria et  
639 bactéries lactiques non-entérocoques.  
640

641 Korhonen, J., 2010. Antibiotic resistance of lactic acid bacteria. Kopijyvä.

642 Korhonen, J.M., Van Hoek, A.H.A.M., Saarela, M., Huys, G., Mayrhofer, S., A., V.W., 2010.  
643 Antimicrobial susceptibility of *Lactobacillus rhamnosus*. Beneficial Microbes 1, 75-80.  
644  
645 Ladero, V., Fernández, M., Calles-Enríquez, M., Sánchez-Llana, E., Cañedo, E., Martín,  
646 M.C., Alvarez, M.A., 2012. Is the production of the biogenic amines tyramine and putrescine  
647 a species-level trait in enterococci? Food Microbiol 30, 132-138.  
648  
649 Ladero, V., Martín, M.C., Redruello, B., Mayo, B., Belen Florez, A., Fernandez, M., Alvarez,  
650 M., 2015. Genetic and functional analysis of biogenic amine production capacity among  
651 starter and non-starter lactic acid bacteria isolated from artisanal cheeses. Eur Food Research  
652 Technol 241, 377-383.  
653  
654 Le Lay, C., Coton, E., Le Blay, G., Chobert, J.M., Haertlé, T., Choiset, Y., Nguyen Van Long,  
655 N., Meslet-Cladière, L., Mounier, J., 2016. Identification and Quantification of Antifungal  
656 Compounds Produced by Lactic Acid Bacteria and Propionibacteria Int J Food Microbiol 239,  
657 79-85.  
658  
659 Le Lay, C., Mounier, J., Vasseur, V., Weill, A., Le Blay, G., Barbier, G., Coton, E., 2016. *In*  
660 *vitro* and *in situ* screening of lactic acid bacteria and propionibacteria antifungal activities  
661 against bakery product spoilage molds. Food Control 60, 247-255.  
662  
663 Leyva Salas, M., Mounier, J., Valence, F., Coton, M., Thierry, A., Coton, E., 2017.  
664 Antifungal Microbial Agents for Food Biopreservation-A Review. Microorganisms 5, E37.  
665 Lucas, P., Claisse, O., Lonvaud-Funel, A., 2008. High frequency of histamine-producing  
666 bacteria in enological environment and instability of the phenotype. Appl Environ Microbiol  
667 74, 811-817.  
668  
669 Lucas, P., Landete, J., Coton, M., Coton, E., Lonvaud-Funel, A., 2003. The tyrosine  
670 decarboxylase operon of *Lactobacillus brevis* IOEB 9809: characterization and conservation  
671 in tyramine-producing bacteria. FEMS Microbiol Lett 229, 65-71.  
672  
673 Lucas, P.M., Blancato, V.S., Claisse, O., Magni, C., Lolkema, J.S., Lonvaud-Funel, A., 2007.  
674 Agmatine deiminase pathway genes in *Lactobacillus brevis* are linked to the tyrosine  
675 decarboxylation operon in a putative acid resistance locus. Microbiology 153, 2221-2230.  
676  
677 Lucas, P.M., Wolken, W.A., Claisse, O., Lolkema, J.S., Lonvaud-Funel, A., 2005. Histamine-  
678 producing pathway encoded on an unstable plasmid in *Lactobacillus hilgardii* 0006. Appl  
679 Environ Microbiol 71, 1417-1424.  
680  
681 Malik, A.C., Reinbold, G.W., Vedamuthu, E.R., 1968. An evaluation of the taxonomy of  
682 Propionibacterium. Canadian J Microbiol 14, 1185-1191.  
683  
684 Martin, J.F., Coton, M., 2017. Blue Cheese: Microbiota and Fungal Metabolites, Fermented  
685 Foods in Health and Disease Prevention. Elsevier, pp. 275-303.  
686  
687 Martín, M.C., Fernández, M., Linares, D.M., Alvarez, M.A., 2005. Sequencing,  
688 characterization and transcriptional analysis of the histidine decarboxylase operon of  
689 *Lactobacillus buchneri*. Microbiol 151, 1219-1228.  
690



691 Mathur, S., Singh, R., 2005. Antibiotic resistance in food lactic acid bacteria-a review. Int J  
692 Microbiol 105, 281-295.  
693  
694 Meile, L., Le Blay, G., Thierry, A., 2007. Safety Assessment of Dairy Microorganisms:  
695 *Propionibacterium* and *Bifidobacterium* Int J Food Microbiol 126, 316-320.  
696  
697 Melin, P., Sundh, I., Hakansson, S., Schnurer, J., 2007. Biological preservation of plant  
698 derived animal feed with antifungal microorganisms: safety and formulation aspects.  
699 Biotechnology Letters 29, 1147-1154.  
700  
701 Miceli, M.H., Diaz, J.A., Lee, S.A., 2011. Emerging oppportunistic yeast infections. The  
702 Lancet Infectious Diseases 11, 142-151.  
703  
704 Mieszkin, S., Hymery, N., Debaets, S., Coton, E., Le Blay, G., Valence, F., Mounier, J., 2017.  
705 Action mechanisms involved in the bioprotective effect of *Lactobacillus harbinensis*  
706 K.V9.3.1.Np against *Yarrowia lipolytica* in fermented milk. Int J Food Microbiol 248, 47-55.  
707  
708 Mogensen, G., Salminen, S., O'Brien, J., Ouwehand, A., Holzapfel, W.H., Shortt, C., Fonden,  
709 R., Miller, G.D., Donohue, D., Playne, M., Crittenden, R., Salvadori, B., Zink, R., 2002.  
710 Inventory of microorganisms with a documented history of use in food. Bulletin of IDF 377,  
711 10-19.  
712  
713 Prajapati, J.B., Nair, B.M., 2003. The history of fermented foods. CRC Press, Boca Raton.  
714 Priyadarshani, W.M.D., Rakshit, S.K., 2011. Screening selected strains of probiotic lactic acid  
715 bacteria for their ability to produce biogenic amines (histamine and tyramine). Int J Food Sci  
716 Technol 46, 2062-2069.  
717  
718 Schwenninger, S.M., Meile, L., 2004. A mixed culture of *Propionibacterium jensenii* and  
719 *Lactobacillus paracasei* subsp. *paracasei* inhibits food spoilage yeasts. Syst Appl Microbiol  
720 27, 229-237.  
721  
722 Shalaby, A.R., 1996. Significance of biogenic amines in food safety and human health. Food  
723 Research International 29, 675-690.  
724  
725 Spano, G., Russo, P., Lonvaud-Funel, A., Lucas, P., Alexandre, H., Grandvalet, C., Coton, E.,  
726 Coton, M., Barnavon, L., Bach, B., Rattray, F., Bunte, A., Magni, A., Ladero, V., Alvarez,  
727 M., Fernández, M., Lopez, P., Palencia, P.F., Corbi, A., Trip, H., Lolkema, J.S., 2010.  
728 Biogenic amines in fermented foods. Eur J Clin Nutr 64, 95-100.  
729  
730 Stiles, M.E., 1996. Biopreservation by lactic acid bacteria. . Ant Van Leeuwenhoek 70, 331-  
731 345.  
732  
733 Ten Brink, B., Damink, C., Joosten, H.M.L.J., Huis In't Veld, J.H.J., 1990. Occurence and  
734 formation of biologically active amines in foods. Int J Food Microbiol 11, 73-84.  
735  
736 Thierry, A., Deutsch, S.M., Falentin, H., Dalmaso, M., Cousin, F.J., Jan, G., 2011. New  
737 insights into physiology and metabolism of *Propionibacterium freudenreichii*. Int J Food  
738 Microbiol 149, 19-27.  
739

740 Vankerckhoven, V., Huys, G., Vancanneyt, M., Vael, C., Klare, I., Romond, M.B., Entenza,  
741 J.M., Moreillon, P., Wind, R.D., Knol, J., Wiertz, E., Pot, B., Vaughan, E.E., Kahlmeter, G.,  
742 Goossens, H., 2008. Biosafety assessment of probiotics used for human consumption:  
743 recommendations from the EU-PROSAFE project. Trends Food Sci. Tech. 19, 102-114.

744

745

746

747

748

749

750

751 **Table 1. Species, sources and strain characteristics for lactobacilli and propionibacteria**  
 752 **strains used in this study.**

Species	Strain number	Biotope	Strain characteristics	Culture conditions
<i>L. brevis</i>	IOEB 9809	France-Wine	BA control (TyrDC+, AgDI+)	MRS – 30°C – Anaerobic
<i>L. saerimneri</i>	ATCC 33222	Horse stomach	BA control (HDC+, ODC+, LDC+)	MRS – 37°C – Anaerobic
<i>L. plantarum</i>	LMG 6907	Sour kraut	Antibiotic testing control	MRS – 28°C – Anaerobic
<i>L. paracasei</i>	LMG 13552	Emmental	Antibiotic testing control	MRS – 37°C – Anaerobic
<i>S. thermophilus</i>	LMG 18311	Yogurt	Antibiotic testing control	M17 – 37°C – Anaerobic
<i>P. jensenii</i>	CIRM-BIA 39 <sup>T</sup>	Buttermilk	Antibiotic testing control	YEL – 30°C – Anaerobic
<i>L. buchneri</i>	L162	2009-France-Raw goat milk	this study	MRS – 30°C – Anaerobic
<i>L. buchneri</i>	L233	2009-France-Raw goat milk	this study	MRS – 30°C – Anaerobic
<i>L. brevis</i>	L128	2009-France-Raw sheep milk	this study	MRS – 30°C – Anaerobic
<i>L. casei</i>	L142	2009-France-Raw goat milk	this study	MRS – 30°C – Anaerobic
<i>L. coryniformis</i>	K.V9.3.10	2009-France-Raw cow milk	this study	MRS – 30°C – Anaerobic
<i>L. harbinensis</i>	L172	2009-France-Raw cow milk	this study	MRS – 30°C – Anaerobic
<i>L. harbinensis</i>	K.V9.3.1NP	2009-France-Raw cow milk	this study	MRS – 30°C – Anaerobic
<i>L. paracasei</i>	L117	2009-France-Raw cow milk	this study	MRS – 30°C – Anaerobic
<i>L. plantarum</i>	L11	2009-France-Raw cow milk	this study	MRS – 30°C – Anaerobic
<i>L. plantarum</i>	L14	2009-France-Raw goat milk	this study	MRS – 30°C – Anaerobic
<i>L. plantarum</i>	L17	2009-France-Raw goat milk	this study	MRS – 30°C – Anaerobic
<i>L. plantarum</i>	L244	2009-France-Raw cow milk	this study	MRS – 30°C – Anaerobic
<i>L. rhamnosus</i>	CIRM-BIA 1112	Fermented milk	this study	MRS – 30°C – Anaerobic
<i>L. rhamnosus</i>	CIRM-BIA 1113	Unknown	this study	MRS – 30°C – Anaerobic
<i>L. rhamnosus</i>	CIRM-BIA 1952	Unknown	this study	MRS – 30°C – Anaerobic
<i>L. rhamnosus</i>	CIRM-BIA 574	Raw milk cheese	this study	MRS – 30°C – Anaerobic
<i>L. rhamnosus</i>	CIRM-BIA 578	Raw milk cheese	this study	MRS – 30°C – Anaerobic
<i>L. zeae</i>	M.C7.5.2C	2009-France-Raw goat milk	this study	MRS – 30°C – Anaerobic
<i>L. lactis</i> subsp. <i>lactis</i>	CIRM-BIA 47	Diacetyl producing starter	this study	M17 – 30°C – Anaérobic
<i>Ln. mesenteroides</i>	M.C8.4.1B	2009-France-Raw goat milk	this study	MRS – 30°C – Anaerobie
<i>P. jensenii</i>	CIRM-BIA 1774	Raw cow milk	this study	YEL – 30°C – Anaerobie
<i>S. thermophilus</i>	CIRM-BIA 16	Buttermilk	this study	M17 – 37°C – Anaerobie

753

754

755 **Table 2. Multiplex PCR to detect the four main genes (*tyrdc*, *hdc*, *odc* and *agdi*) involved**  
 756 **in tyramine, histamine and putrescine production. Results are provided as absent (-) or**  
 757 **present (+).**

758

759

760

761

762

763

764

765

766

767

768

769

770

771

772

773

774

775

776

777

778

779

780

781

782

783

Species and strain code	<i>agdi</i>	<i>tyrdc</i>	<i>hdc</i>	<i>odc</i>
<i>L. buchneri</i> L162	+	-	+	-
<i>L. buchneri</i> L233	+	-	+	-
<i>L. brevis</i> L128	+	+	-	-
<i>L. casei</i> L142	-	-	-	-
<i>L. coryniformis</i> K.V9.3.10	-	-	-	-
<i>L. harbinensis</i> L172	-	-	-	-
<i>L. harbinensis</i> K.V9.3.1NP	-	-	-	-
<i>L. paracasei</i> L117	-	-	-	-
<i>L. plantarum</i> L11	-	-	-	-
<i>L. plantarum</i> L14	-	-	-	-
<i>L. plantarum</i> L17	-	-	-	-
<i>L. plantarum</i> L244	-	-	-	-
<i>L. rhamnosus</i> CIRM-BIA 1112	-	-	-	-
<i>L. rhamnosus</i> CIRM-BIA 1113	-	-	-	-
<i>L. rhamnosus</i> CIRM-BIA 1952	-	-	-	-
<i>L. rhamnosus</i> CIRM-BIA 574	-	+	-	-
<i>L. rhamnosus</i> CIRM-BIA 578	-	+	-	-
<i>L. zeae</i> M.C7.5.2C	-	-	-	-
<i>L. lactis</i> CIRM-BIA 47	-	-	-	-
<i>L. mesenteroides</i> M.C8.4.1B	-	+	-	-
<i>S. thermophilus</i> CIRM-BIA 16	-	-	-	-
<i>P. jensenii</i> CIRM-BIA 1774	-	-	-	-

784 **Table 3. *In vitro* production of biogenic amines by some antifungal LAB strains as**  
785 **determined by HPLC. Cultures were carried out in optimal culture medium (MRS)**  
786 **and/or MP decarboxylase medium. BA quantifications are expressed in mg/L and ND**  
787 **signifies BA was not detected. BAs not mentioned in this table were not detected at**  
788 **quantifiable amounts in any samples.**

Species name and strain code	Cadaverine	Histamine	Putrescine	Tyramine
<i>L. saerimneri</i> ATCC 33222 ( <i>hdc+</i> , <i>odc+</i> , <i>ldc+</i> )	493 ± 14 (MRS)	887 ± 62 (MRS)	601 ± 13 (MRS)	ND
<i>L. brevis</i> IOEB 9809 ( <i>tdc+</i> , <i>agdi+</i> )	ND	ND	223 ± 18 (MRS)	768 ± 80 (MRS)
<i>L. brevis</i> (L128)	ND	ND	200 ± 12 (MRS) 292 ± 6 (MPTa)	574 ± 50 (MRS) 641 ± 7 (MPTa)
<i>L. buchneri</i> (L162)	ND	743 ± 33 (MRS) 855 ± 13 (MPH)	ND	ND
<i>L. buchneri</i> (L233)	ND	723 ± 39 (MRS) 972 ± 47 (MPH)	ND	ND
<i>L. mesenteroides</i> (M.C8.4.B1)	ND	ND	72 ± 12 (MRS) 41 (MPTa)	580 (MRS) 413 (MPTa)
<i>L. rhamnosus</i> (CIRM-BIA 574)	ND	ND	ND	702 ± 39 (MRS)
<i>L. rhamnosus</i> (CIRM-BIA 578)	ND	ND	ND	410 (MRS)

789

**Table 4. Antibiotic susceptibility testing for 21 LAB and 1 PAB strains with 13 antibiotics. Values in tables correspond to <sup>a</sup>minimal inhibitory concentrations (mg/L) and <sup>b</sup>microbiological cut-off values (mg/L) according to EFSA, 2012. Abbreviations: n.r.: not required; nd: not determined.**

Species	ATB with action on the cell wall		ATB inhibiting protein synthesis									ATB inhibiting nucleic acid synthesis	
	Ampicillin	Vancomycin	Chloramphenicol	Clindamycin	Erythromycin	Gentamicin	Kanamycin	Neomycin	Streptomycin	Tetracycline	Oxytetracycline	Trimethoprim	Rifampicin
<i>L. brevis</i> L128	0.5 (2)	>128 (n.r.)	4 (4)	16 (1)	0.5 (1)	1 (16)	32 (32)	[2 – 4] (nd)	16 (64)	[8 – 16] (8)	16 (nd)	[0.12 – 1] (nd)	0.25 (nd)
<i>L. buchneri</i> L162	1 (2)	>128 (n.r.)	4 (4)	0.03 (1)	0.5 (1)	0.5 (16)	[8 – 16] (32)	0.5 (nd)	4 (64)	16 (8)	[16 – 32] (nd)	2 (nd)	[0.12 – 0.25] (nd)
<i>L. buchneri</i> L233	1 (2)	>128 (n.r.)	[8 – 16] (4)	0.03 (1)	0.5 (1)	0.5 (16)	[8 – 16] (32)	0.5 (nd)	[4 – 8] (64)	16 (8)	[16 – 32] (nd)	2 (nd)	[0.12 – 0.25] (nd)
<i>L. casei</i> L142	0.5 (4)	>128 (n.r.)	8 (4)	[0.06 – 0.12] (1)	0.5 (1)	4 (32)	[32 – 64] (64)	8 (nd)	16 (64)	1 (4)	1 (nd)	[1 – 2] (nd)	[0.25 – 0.5] (nd)
<i>L. coryniformis</i> K.V9.3.1O	[0.5 – 1] (4)	>128 (n.r.)	[0.5 – 1] (4)	[0.06 – 0.12] (1)	[0.5 – 1] (1)	[1 – 8] (16)	[64 – 128] (64)	4 (nd)	[8 – 16] (64)	[1 – 2] (8)	[2 – 4] (nd)	0.125 (nd)	[0.5 – 1] (nd)
<i>L. harbinensis</i> K.V9.3.1Np	[0.25 – 0.5] (4)	>128 (n.r.)	[1 – 2] (4)	[0.25 – 2] (1)	[0.25 – 1] (1)	[4 – 8] (16)	[32 – 128] (64)	[4 – 16] (nd)	[32 – 64] (64)	[8 – 16] (8)	[16 – 8] (nd)	[1 – 4] (nd)	[0.5 – 2] (nd)
<i>L. harbinensis</i> L172	0.5 (4)	>128 (n.r.)	[2 – 4] (4)	1 (1)	0.5 (1)	2 (16)	32 (64)	8 (nd)	4 (64)	0.25 (8)	0.25 (nd)	0.12 (nd)	0.12 (nd)
<i>L. lactis</i> CIRM-BIA 47	[1 – 4] (2)	>128 (4)	1 (8)	[1 – 2] (1)	0.5 (1)	[1 – 4] (32)	[16 – 64] (64)	[2 – 8] (nd)	[8 – 32] (32)	[8 – 16] (4)	[8 – 16] (nd)	[0.125 – 2] (nd)	[0.5 – 1] (nd)
<i>L. mesenteroides</i> M.C8.4.1B	[1 – 4] (2)	>128 (n.r.)	2 (4)	0.032 (1)	[0.5 – 1] (1)	[2 – 8] (16)	[32 – 64] (32)	[16 – 8] (nd)	[8 – 32] (64)	[0.5 – 1] (8)	[0.5 – 2] (nd)	[8 – 32] (nd)	[0.5 – 2] (nd)
<i>L. paracasei</i> L117	0.25 (4)	>128 (n.r.)	4 (4)	[0.12 – 0.25] (1)	0.5 (1)	4 (32)	128 (64)	[8 – 16] (nd)	32 (64)	1 (4)	1 (nd)	0.12 (nd)	0.25 (nd)
<i>L. plantarum</i> L11	0.12 <sup>a</sup> (2) <sup>b</sup>	>128 (n.r.)	4 (8)	[2 – 4] (2)	1 (1)	8 (16)	128 (64)	[8 – 16] (nd)	64 (n.r.)	[8 – 16] (32)	8 (nd)	[0.5 – 1] (nd)	2 (nd)
<i>L. plantarum</i> L14	0.12 (2)	>128 (n.r.)	4 (8)	[2 – 4] (2)	1 (1)	8 (16)	128 (64)	[8 – 16] (nd)	64 (n.r.)	[8 – 16] (32)	8 (nd)	0.5 (nd)	2 (nd)
<i>L. plantarum</i> L17	0.12 (2)	>128 (n.r.)	4 (8)	[2 – 4] (2)	1 (1)	8 (16)	128 (64)	[8 – 16] (nd)	64 (n.r.)	[8 – 16] (32)	[8 – 16] (nd)	[0.5 – 1] (nd)	2 (nd)
<i>L. plantarum</i> L244	[0.12 – 0.25] (2)	>128 (n.r.)	4 (8)	0.03 (2)	[0.5 – 1] (1)	2 (16)	64 (64)	4 (nd)	32 (n.r.)	8 (32)	8 (nd)	[0.5 – 2] (nd)	[1 – 2] (nd)
<i>L. rhamnosus</i> CIRM-BIA 1112	0.5 (4)	>128 (n.r.)	8 (4)	>16 (1)	>8 (1)	[2 – 4] (16)	32 (64)	[8 – 16] (nd)	>256 (32)	1 (8)	1 (nd)	[8 – 16] (nd)	0.25 (nd)
<i>L. rhamnosus</i> CIRM-BIA 1113	[0.25 – 0.5] (4)	>128 (n.r.)	4 (4)	[0.5 – 1] (1)	[0.5 – 1] (1)	2 (16)	32 (64)	8 (nd)	8 (32)	[0.5 – 1] (8)	[0.5 – 1] (nd)	[64 – >64] (nd)	[0.12 – 0.25] (nd)
<i>L. rhamnosus</i> CIRM-BIA 1952	0.5 (4)	>128 (n.r.)	[4 – 8] (4)	0.5 (1)	0.5 (1)	2 (16)	[32 – 64] (64)	[4 – 8] (nd)	8 (32)	[0.5 – 1] (8)	[0.5 – 1] (nd)	>64 (nd)	0.12 (nd)
<i>L. rhamnosus</i> CIRM-BIA 574	[0.063 – 2] (4)	>128 (n.r.)	[0.25 – 1] (4)	[0.125 – 0.5] (1)	[0.063 – 2] (1)	[4 – 8] (16)	[32 – 64] (64)	[1 – 32] (nd)	[4 – 32] (32)	[0.25 – 0.5] (8)	[0.125 – 0.5] (nd)	[2 – 8] (nd)	[0.125 – 1] (nd)
<i>L. rhamnosus</i> CIRM-BIA 578	[2 – 4] (4)	>128 (n.r.)	1 (4)	[0.25 – 0.5] (1)	[0.25 – 0.5] (1)	[4 – 8] (16)	[64 – 128] (64)	[16 – 64] (nd)	[4 – 8] (32)	[0.25 – 0.5] (8)	[0.25 – 0.5] (nd)	[2 – 8] (nd)	[0.25 – 1] (nd)
<i>L. zeae</i> M.C7.5.2C	[0.25 – 1] (4)	>128 (n.r.)	[2 – 4] (4)	[0.25 – 1] (1)	[0.5 – 1] (1)	4 (16)	[64 – 256] (64)	8 (nd)	[32 – 64] (64)	[8 – 16] (8)	[8 – 16] (nd)	[1 – 4] (nd)	[2 – 4] (nd)
<i>P. jensenii</i> CIRM-BIA 1774	0.5 (2)	0.25 (4)	0.5 (2)	[0.12 – 0.25] (0.25)	0.5 (0.5)	4 (64)	[16 – 32] (64)	[1 – 2] (nd)	2 (64)	[0.25 – 0.5] (2)	0.5 (nd)	0.12 (nd)	0.12 (nd)
<i>S. thermophilus</i> CIRM-BIA 16	0.03 (2)	0.25 (4)	[4 – 16] (4)	0.03 (2)	0.5 (2)	[2 – 4] (32)	32 (64)	8 (nd)	[4 – 16] (64)	0.25 (4)	0.25 (nd)	32 (nd)	0.12 (nd)







## Production d'ingrédients laitiers fonctionnalisés par des microorganismes producteurs de composés antifongiques

Dans les produits laitiers, les cultures bioprotectrices et leurs métabolites représentent une alternative d'intérêt aux conservateurs chimiques pour lutter contre les contaminations fongiques. L'objectif de cette thèse était de développer des ingrédients antifongiques, issus de la fermentation d'un substrat laitier par différents microorganismes, bactéries ou champignons, utilisables dans des produits laitiers variés. Pour ce faire, nous avons dans un premier temps caractérisé la diversité des contaminants fongiques des produits laitiers et de leur environnement afin de sélectionner les cibles fongiques les plus pertinentes pour le reste de notre étude. Ensuite, nous avons criblé, *in vitro*, l'activité antifongique de fermentats issus de la fermentation de 2 substrats laitiers par 698 souches de bactéries lactiques, propioniques et de champignons grâce à une nouvelle méthode de criblage haut-débit, dans une matrice mimant le fromage. Après optimisation des conditions de fermentation pour améliorer l'activité antifongique de ces fermentats, les plus actifs ont été testés à l'échelle du laboratoire sur des fromages blancs et des fromages à pâte pressée à croûte morgée (PPCM) avant d'être testés à l'échelle pilote dans des crèmes fraîches et des fromages à PPCM. Cette étape nous a permis (i) de valider l'activité antifongique des fermentats en produits réels en mettant en place des challenge-tests et des tests d'usage et (ii) d'évaluer leur impact sur les qualités organoleptiques des produits grâce à des analyses sensorielles. Les molécules impliquées dans l'activité antifongique ont ensuite été identifiées grâce à différentes méthodes (chromatographie en phase gazeuse ou liquide couplée ou non à la spectrométrie de masse) et l'impact de ces composés sur la croissance des cibles fongiques a été évalué. Le criblage *in vitro* a permis la sélection de 3 ingrédients antifongiques qui se sont tous révélés actifs *in situ*. Les tests de durabilité ont révélé que le fermentat issu de *Lactobacillus rhamnosus* CIRM-BIA 1952 avait une activité prometteuse dans les crèmes fraîches. Pour le fromage à PPCM, le fermentat issu de *Propionibacterium jensenii* CIRM-BIA 1774, qui retarde jusqu'à 16 jours la croissance de *Mucor racemosus* et *Penicillium commune* pourrait être utilisé à la place de la natamycine. La caractérisation des composés antifongiques dans les fermentats a mis en évidence certains acides organiques, des acides gras, des composés volatils et un peptide qui, ensemble, jouent très probablement un rôle dans l'activité antifongique de ces fermentats. Nous avons enfin montré l'effet fungistatique du fermentat le plus actif sur *Rhodotorula mucilaginosa* UBOCC-A-216004 et *Mucor racemosus* UBOCC-A-109155 ainsi que son impact sur la physiologie des spores de *M. racemosus*. L'ensemble de ces résultats devrait donc conduire au développement de nouveaux ingrédients laitiers antifongiques qui pourraient remplacer avantageusement les conservateurs dans les produits laitiers, ils apportent en outre des éléments d'information sur les molécules et les mécanismes mis en œuvre dans l'inhibition antifongique.

**Mots clés : ingrédients laitiers, antifongiques, bioprotection, mécanismes d'action**

## Production of dairy-based ingredients through microorganisms producing antifungal compounds

In dairy products, bioprotective cultures and their metabolites represent an interesting alternative to chemical preservatives. The aim of this PhD thesis was to develop antifungal dairy ingredients, derived from the fermentation of dairy substrates by different microorganisms, bacteria and fungi, which could be used in various dairy products. To do so, we first studied the diversity of spoilage fungi in a large variety of dairy products and their environment in order to select the most appropriate fungal targets. Then, we screened the *in vitro* antifungal activity of fermentates obtained from the fermentation of 2 dairy substrates by 698 lactic acid bacteria, propionibacteria and fungal strains using a novel high-throughput method in a cheese mimicking model. After optimizing fermentation conditions to enhance antifungal activity, the most active fermentates were tested at a lab scale in semi-hard and fresh cheese before evaluation at a pilot scale in semi-hard cheese and sour cream. This step allowed us (i) to validate the fermentate antifungal activities in real products using challenge- and durability-tests and (ii) to evaluate their impact on the products' organoleptic properties using sensorial analysis. Antifungal molecules were then identified using different analytical methods (high performance liquid chromatography, gas chromatography coupled or not with mass spectrometry) and impact of these compounds on fungal growth was studied. The *in vitro* screening allowed selecting 3 antifungal ingredients that were also active *in situ*. Durability tests revealed that *Lactobacillus rhamnosus* CIRM-BIA 1952 fermentate had a promising activity in sour cream. In semi-hard cheese, *Propionibacterium jensenii* CIRM-BIA 1774 fermentate, which delayed *Mucor racemosus* and *Penicillium commune* growth for up to 16 days, could be used instead of natamycin. Antifungal compounds present in fermentates consisted of organic acids, free fatty acids, volatile compounds and peptides which altogether might play a role in the antifungal activity of these fermentates. Finally, the fungistatic effect of *P. jensenii* CIRM-BIA 1774 fermentate against *R. mucilaginosa* UBOCC-A-216004 and *M. racemosus* UBOCC-A-109155 was demonstrated, as well as its impact on the physiology of *M. racemosus* spores. Together, these results should lead to the development of new antifungal dairy ingredients which could replace preservatives in dairy products. Finally, these results gave us new information concerning antifungal molecules and their action mechanisms.

**Keywords: dairy-based ingredients, antifungal, biopreservation, action mechanisms**