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Louis Delaunay

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Par

Louis DELAUNAY

ANOPLAI – Prévalence et biodiversité d'*Anoxybacillus flavithermus*, et des autres bactéries sporulées thermophiles, associées à l'industrie des poudres laitières

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Unité de recherche : Laboratoire Universitaire de Biodiversité et d'Ecologie Microbienne

Rapporteurs avant soutenance :

Thierry BENEZECH

Directeur de recherche, INRAE Lille

Christophe NGUYEN-THE

Directeur de recherche, INRAE PACA

Composition du Jury :

Président :

Gwenaëlle LE BLAY

Professeur des Universités, UBO

Examineurs :

Thierry BENEZECH

Directeur de recherche, INRAE Lille

Christophe NGUYEN-THE

Directeur de recherche, INRAE PACA

Véronique BROUSSOLLE

Directrice de recherche, INRAE PACA

Jean-Marie PERRIER CORNET

Professeur des Universités, AgroSup Dijon

Dir. de thèse :

Ivan LEGUERINEL

Professeur des Universités, UBO

Co-dir. de thèse :

Anne-Gabrielle MATHOT

Maître de conférences, UBO

Florence POSTOLLEC

Chef de Projet, ADRIA développement

Invitée

Anne DUVIVIER

Responsable développement analytique, INGREDIA

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




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Liste des abréviations :

AW : Acid Whey

CFU: Colony Forming Unit

CIP : Cleaning In Place

cm² : centimètres carré

CP : Casein Powder

CtP : Caseinate Powder

DF : Diafiltration

DNA : Desoxyribonucleic Acid

DPA : Dipicolinic Acid

DSI : Direct Steam Injection

EPS : Exopolysaccharide

HRMSC : Heat Resistant Mesophilic Spore Count

HRTSC : Heat Resistant Thermophilic Spore Count

IF : Infant Formula

MCC : Micellar Casein Concentrate

MF : Microfiltration

mL : Millilitres

MPC : Milk Protein Concentrate

MSC : Mesophilic Spore Count

MVR : Mechanical Vapor Recompression

NDM : Non fat Dairy Milk

NEP : Nettoyage En Place

NF : Nanofiltration

PCA: Plate Count Agar

PCR : Ploymerase Chain Reaction

PP : Permeate Powder

RO : Reverse Osmosis

SMP : Skim Milk Powder

SW : Sweet Whey

TBE : Tris Borate EDTA buffer

TS : Tryptone Salt

TSA : Tryptic Soy Agar

TSB : Tryptic Soy Broth

TSC : Thermophilic Spore Count

TVR : Thermal Vapor Recompression

UF : Ultrafiltration

UFC : Unité Formant Colonies

UHT : Ultra Haute Temperature

UPGMA : Unweighted Pair Group Method with Arithmetic mean

WGS : Whole Genone Sequencing

WMP : Whole Milk Powder

WP : Whey Powder

WPC : Whey Protein Concentrat

Introduction

Les spores bactériennes sont omniprésentes dans l'environnement. Bactéries dormantes et résistantes, les spores bactériennes se disséminent dans tous les milieux, résistent aux conditions environnementales extrêmes et lorsqu'elles retrouvent des conditions favorables à leur développement elles germent, se multiplient et re-sporulent. Dans l'environnement des élevages agricoles les spores bactériennes sont présentes dans l'ensemble du biotope : la terre, la nourriture, les ensilages, l'eau et les fèces. Si les conditions de nettoyage et de désinfection des pis des vaches au moment de la traite et des équipements sont insuffisants les spores bactériennes peuvent facilement contaminer le lait cru récolté. Durant les étapes de stockage et de transport du lait avec des conditions de basses températures les spores bactériennes non psychrophiles restent dormantes et ne se développent pas. Elles résistent aux traitements thermiques de pasteurisation qui inactivent les formes végétatives des bactéries et éliminent les bactéries responsables de toxico-infections (*Listeria*, *Staphylococcus*, *Salmonella*).

Le lait est un milieu nutritif favorable au développement des micro-organismes. Les conditions environnementales observées durant les procédés de fabrication des produits laitiers vont limiter, favoriser ou sélectionner le développement de certaines espèces bactériennes en fonction de leurs caractéristiques physiologiques de croissance. Les conditions environnementales observées durant la fabrication des poudres lactées peuvent expliquer les prédominances des spores bactériennes thermophiles présentes dans les poudres commerciales alors qu'elles ne sont détectées qu'en très faibles quantités dans les laits crus. Ces bactéries sporulées thermophiles sont principalement représentées par trois espèces : *Anoxybacillus flavithermus*, *Geobacillus stearothermophilus* et *Bacillus licheniformis*.

A. flavithermus est ce que l'on peut appeler un micro-organisme émergent pour l'industrie agroalimentaire, en particulier pour les entreprises de transformation du lait en poudres lactées. Cette espèce a été initialement décrite à partir d'isolats obtenus d'échantillon récoltés dans des sources chaudes (Heinen, Lauwers, & Mulders, 1982), mais sa présence en tant qu'espèce thermophile sporulante majoritaire retrouvée dans des poudres lactées a été confirmée par plusieurs études réalisées au niveau mondial (Dettling et al., 2019; Miller, Kent, Watterson, et al., 2015; Rückert, Ronimus, & Morgan, 2004; Yuan et al., 2012). La contamination de ces

poudres par des bactéries sporulées thermophiles est déjà bien décrite, mais les études présentent généralement des analyses globales et les identifications des espèces impliquées restent rares.

Evaluer la présence d'*Anoxybacillus flavithermus* ainsi que les autres bactéries sporulées thermophiles dans les poudres de laitières est important. Les poudres laitières constituent une part non négligeable des produits de transformations du lait, elles sont faciles à conserver et à utiliser. Ces poudres laitières sont des ingrédients entrant dans la composition de nombreux produits alimentaires comme des pâtisseries, des produits de charcuterie ou des plats cuisinés. La charge microbienne de ces ingrédients laitiers peut donc potentiellement contribuer à la dégradation de la qualité de nombreux aliments finis. Aussi les qualités microbiennes exigées sont-elles souvent très contraignantes pour les industriels producteurs de ces poudres.

L'**objectif global** de cette thèse est de mieux caractériser l'espèce *Anoxybacillus flavithermus* ainsi que les autres bactéries sporulées thermophiles par rapport à l'environnement laitier et aux procédés de transformation.

Le **premier chapitre** présente un état de l'art sur les bactéries sporulées thermophiles dans le contexte de l'industrie des poudres laitières. Une description et une comparaison des caractéristiques physiologiques des trois espèces de bactéries thermophiles sporulantes majoritaires retrouvées dans les poudres de lait a été faite, ainsi que la description du fonctionnement global de la transformation du lait en poudre.

Anoxybacillus flavithermus a été identifié comme une espèce retrouvée majoritairement dans des poudres de lait de différents pays. Les poudres d'origine française analysées dans ces études sont peu nombreuses. Le **deuxième chapitre** du manuscrit a pour premier objectif de déterminer l'importance de cette espèce dans des échantillons de poudres laitières d'origine française. Cette étude a été réalisée via une analyse quantitative et une identification des bactéries sporulées (thermophiles) d'échantillons industriels de provenance diverses et connues. Le second objectif de ce chapitre a porté sur l'impact de la nature des poudres étudiées sur les prévalences observées en relation avec les procédés de fabrication utilisés. Dans les études publiées la majorité des poudres analysées étaient des poudres de lait (entier ou écrémé) et les autres types de poudre (lactosérum, protéines) ont été moins étudiés. De ce fait dans notre étude de nombreux échantillons de poudres de lactosérum, de protéines ou de caséines ont été analysés. Le troisième objectif a été d'évaluer les potentiels d'altération de souches d'espèces

sporulantes thermophiles isolées. La capacité de ces espèces bactériennes à altérer les poudres ou les aliments utilisant ces poudres lors de leur fabrication est un aspect souvent peu ou pas abordé. Nous avons apporté des éléments sur la diversité du potentiel d'altération des 3 espèces majoritaires vis-à-vis de substrats de laboratoires ou d'ingrédients retrouvés comme composants lors de formulation avec des poudres laitières. Les résultats de ces deux premiers chapitres montrent que trois espèces principales, dont *Anoxybacillus flavithermus* apparaissent comme primordiales pour la maîtrise de la qualité des poudres. Aussi, tout en restant focalisés sur l'espèce *Anoxybacillus flavithermus*, nous avons inclus dans ce chapitre et les suivants deux autres sporulés, *Geobacillus stearothermophilus* espèce thermophile ou à *Bacillus licheniformis* espèce à tendance thermophile.

Le **troisième chapitre** se focalise sur les caractéristiques (limites, taux) de croissance de ces trois espèces et la comparaison avec les conditions rencontrées (température, pH et a_w) lors des différentes étapes des procédés de transformation des poudres de lait. Cette étude a pour objectif de déterminer quelles sont les étapes à risque et quelles espèces étudiées sont les plus impactées ou favorisées par les conditions environnementales rencontrées lors des procédés de fabrication de poudre de lait.

En étudiant la prévalence des espèces, du lait cru au produit fini, ainsi que les procédés de fabrication des poudres (Chapitres 1, 2 & 3), il apparaît que le développement de biofilm, au sein des lignes de production, constitue un point crucial, surtout dans le cas des espèces strictement thermophiles qui sont initialement très peu présentes dans le lait cru. Le **chapitre 4** traite de la capacité des souches thermophiles, isolées des produits industriels, à adhérer et à former du biofilm rapidement en conditions proches des conditions industrielles, et de la capacité des espèces étudiées à former des spores au sein de ces biofilms. Pour différentes souches de l'espèce *Anoxybacillus flavithermus*, mais aussi, à titre de comparaison, pour l'espèce *Geobacillus stearothermophilus* la résistance de spores bactériennes formées en biofilm vis-à-vis des traitements alcalins et acides utilisés lors des traitements de nettoyage en place (NEP) a été évaluée dans ce chapitre.

Chapitre 1 : Poudres laitières : Etat de l'art sur les procédés et les bactéries sporulées associées

La production mondiale de poudres laitières est en augmentation constante et la qualité microbiologique de ces produits s'améliore, principalement dans les pays les plus exportateurs (Europe et Océanie). Néanmoins, la présence de bactéries sporulées thermophiles dans les poudres laitières reste très fréquente. Elles peuvent représenter une partie importante de la contamination finale des poudres laitières et sont majoritairement représentées par 3 espèces, dont deux thermophiles : *Anoxybacillus flavithermus* et *Geobacillus stearothermophilus*, et une espèce plus mésophile : *Bacillus licheniformis*.

Ces trois espèces sont quantitativement très peu importantes dans l'environnement des fermes et des lieux de la traite, mais elles peuvent être détectées dans le lait cru. Les spores, résistantes à la pasteurisation appliquée en industrie laitière, peuvent ensuite germer et se développer lors du processus de fabrication des laits en poudre.

En effet, dans l'industrie de production de poudres laitières, ces bactéries se retrouvent dans un environnement où les nutriments et les facteurs environnementaux (température, pH et activité de l'eau) sont favorables à leur croissance. En particulier, certaines étapes de pré chauffage et de concentration présentent des conditions de température et d'activité de l'eau pouvant favoriser une croissance différentielle de certaines espèces thermophiles, entraînant une sélection entre elles.

Lors de leur croissance, ces bactéries sporulées peuvent produire des biofilms, formant des niches écologiques et des environnements spécifiques favorables à la production de spores bactérienne. Le détachement de fractions de biofilms favorise la diffusion des spores dans tous les équipements et contaminent les produits finis. Les biofilms permettent également aux spores

bactériennes de mieux résister aux traitements de nettoyage et de persister dans les équipements industriels.

Une fois le produit sous forme de poudre, l'activité de l'eau est très faible et les spores restent inactives ; ceci jusqu'à ce qu'elles retrouvent un milieu hydraté, lors de la reconstitution du lait ou de l'intégration de la poudre en tant qu'ingrédient dans un autre produit alimentaire par exemple. Si les conditions de température et de pH sont favorables, les spores germent, se développent et peuvent produire des enzymes d'altération.

Ce chapitre a pour objectif de présenter et de regrouper les connaissances sur la filière des poudres laitières dans son ensemble ainsi que sur les bactéries sporulées thermophiles associées. Après une brève **introduction**, les **procédés de transformation** du lait en poudres seront décrits, permettant de visualiser les différentes étapes mises en place pour l'obtention des diverses poudres laitières. Dans la 2^{ème} partie, la **diversité et la prévalence des bactéries sporulées dans l'environnement laitier** seront abordées afin de comprendre l'origine des contaminants. Au niveau de la 3^{ème} partie, nous présenterons la **comparaison** entre les **capacités physiologiques** des bactéries sporulées thermophiles et les **caractéristiques du milieu laitier**. Puis, dans la 4^{ème} partie, l'état de l'art sur les **biofilms bactériens** formés par ces microorganismes sera présenté et nous aborderons leur **résistance face aux nettoyages**. Enfin, dans la 5^{ème} et dernière partie, les liens entre la **qualité de la poudre**, la **charge bactérienne** et l'**altération enzymatique** seront évoqués.

Ce chapitre pourra être soumis, en tant que revue bibliographique, dans un journal international portant sur la microbiologie alimentaire.

Prevalence and growth ability of
A. flavithermus, G. stearothermophilus, B. licheniformis
associated with the dairy powders industry

Prévision de soumission dans un journal international comme revue bibliographique dans International Journal of Food Microbiology (IJFM)

Abstract:

During milking steps, sporeforming bacteria present on cow's udder can enter the milking machine and be present in the raw milk. Those spores persists from the producer to the industry and resist to pasteurization steps. Thermophilic ones, known as *Geobacillus stearothermophilus*, *Anoxybacillus flavithermus* and mesophilic one: *Bacillus licheniformis*, can grow in process part (plate heat exchanger and evaporator) where temperature, pH and water activity encountered are favorable for their growth. Cell multiplication occur in biofilms adhere to stainless steel surfaces widely used in the dairy industry. This structure help bacteria to resist against cleaning in place procedure and can contaminate a new production cycle. This property can lead to a high contamination level of spore in dairy powder which can be an issue for food industries, because powder rehydration and food processing promote spore germination, enzyme production and food alteration.

1. Introduction:

To meet the needs of the world's ever-growing population, all the industries must adapt and produce ever more food materials. The dairy industry is not spared and is undergoing this production trend. In 20 years (1999/2019), the production of milk, fresh dairy products, butter, and cheese has increased by more than 152% (OECD-FAO, 2019). To facilitate the export of goods and to increase the preservation of dairy products, industries have applied concentration methods by evaporation and drying. Milk cracking separates dairy ingredients, providing dairy powders for the needs of the different industries sectors. The powder production has increased by more than 120% for skim milk powders, whey powders, and casein, and up to more than 240% for whole milk powders worldwide for a 20 years period.

After being processed into powder, milk is more easily traded on the international market where high-value dairy ingredients can be traded at over €9000 per ton for rennet casein (“Clal.it,” 2019). Some protein isolates can be traded even more expensively on the world market.

Within Europe, Germany dominates milk production, followed by France with more than 25 billion tons of cow's milk produced in 2018 (“Eurostat,” 2020). Globally, Europe is the leading producer of skimmed milk powder (1838 thousand tons) and whey powder (2101 thousand tons) for the year 2019 (Figure1). The world's leading producers of whole milk powder are Latin America, with 1544 thousand tons and Oceania, with 1526 thousand tons produced in 2019. The only two referenced casein producing continents are Europe and Oceania, with 159 and 180 thousand tons respectively (OECD-FAO, 2019).

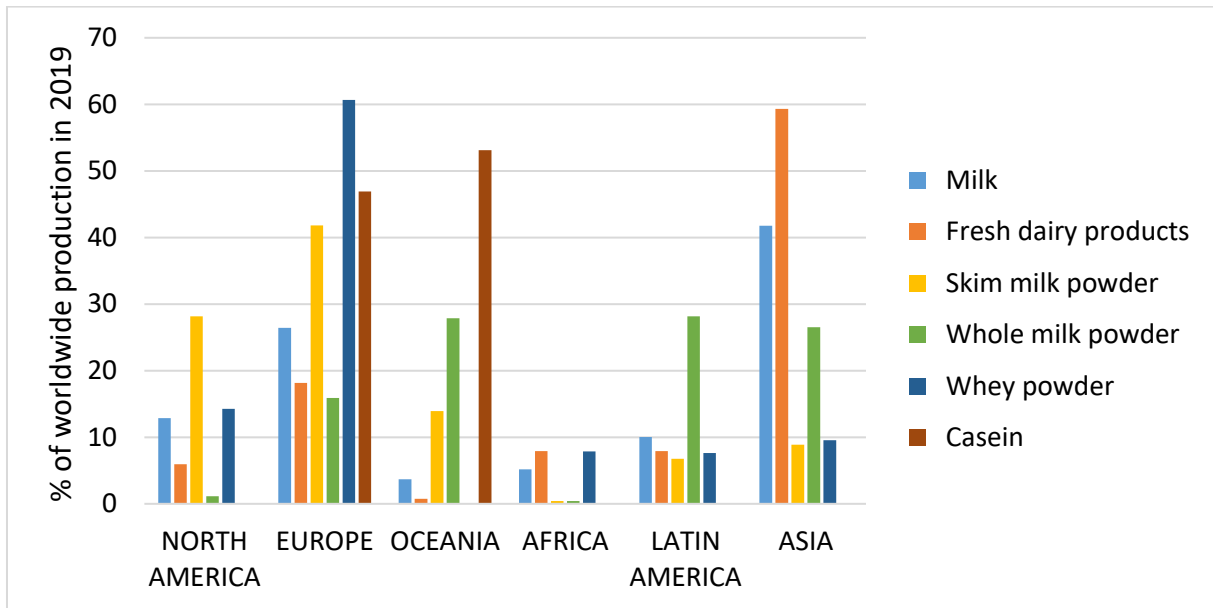


Figure 1: Percentage of the worldwide production of different kind of dairy products depending on the continent (OECD-FAO, 2019)

Dairy powder produced in Europe and Oceania are mainly traded and exported (Figure2) in Asia and Africa where the population growth need more dairy intake to feed local population (fresh dairy products are consumed preferentially but is not sufficient).

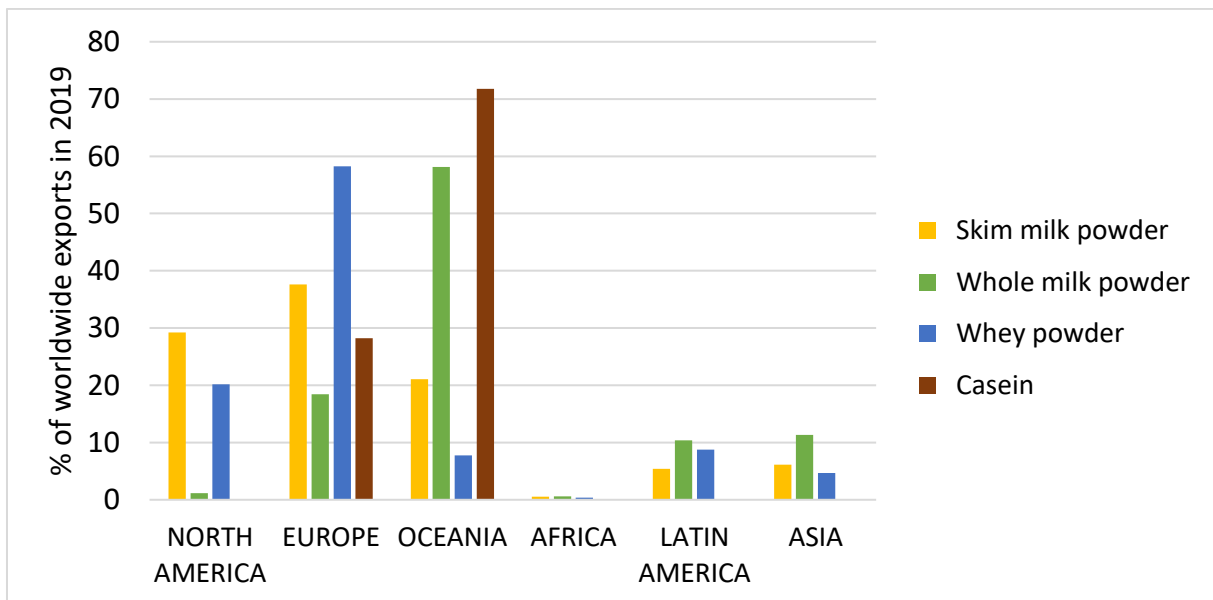


Figure 2: Percentage of the worldwide export of different kind of dairy powders depending on the continent (OECD-FAO, 2019)

Regarding the infant formula production, Europe is the main producer in the world with France, The Netherlands and Ireland at the top of producers countries (Y. Chen, 2018). On the total amount of infant formula powder produced in Europe, 60% is exported, mainly in Asia, which is a market with high demand (abolition of one child policy) and where local consumer don't trust local producers (Chi, 2017). Asian companies invest in abroad technologies and implement infant formula industries there.

To guarantee a good quality of the powders, physico-chemical, and microbiological analyses are carried out to ensure a healthy product for the consumer. The European microbiological criteria applied to dairy powders are, for milk and whey powder, a limit of 10 CFU.g⁻¹ for *Enterobacteriaceae*, and less than 100 CFU.g⁻¹ for coagulase-positive *Staphylococci*. For powdered infant formulae or for medical use, *Enterobacteriaceae* should be absent in 10g of finished product and below 500 CFU.g⁻¹ for presumptive *Bacillus cereus* (CE 1441, 2007).

However, spoilage flora, including mesophilic and thermophilic spore-forming bacteria, are not subject to any microbiological regulation. Spore requirements in dairy powders are typically linked to the final product's specifications; its processing, storage conditions, shelf life and buyer's preference. Companies purchasing dairy powders can request that the manufacturer complies with specific criteria, which may target bacterial spores (psychrophilic, mesophilic, and/or thermophilic) contained in the powdered dairy products. The U.S. Dairy Export Council, recommends a maximum of 1000 CFU.g⁻¹ of mesophilic and thermophilic spores in raw materials (SMP, NDM, WMP) (Bienvenue, 2013) for use in infant formulas, and between <500 cfu.g⁻¹ and <2000 CFU.g⁻¹, respectively, in skim milk powder and whole milk powder (McHugh, Feehily, Hill, & Cotter, 2017; Watterson, Kent, Boor, Wiedmann, & Martin, 2014).

Spore-forming bacteria found in commercially available dairy powders are present in small quantities in raw milk harvested on farms (S. I. Murphy et al., 2019). However, bacteria are found in large quantities in dairy powders (Sadiq, Li, Liu, Flint, Zhang, & He, 2016). Their high concentration in milk powders cannot be explained by the mere fact that the product is concentrated (10 time concentration from raw milk to powder). The process, therefore, favors the development of bacterial spores and the process carries out a selection of certain bacteria (Hinton, 2003).

These spore-forming bacteria can cause problems when powders are rehydrated and used by a manufacturer as ingredients in a formulation, because the development of these microorganisms degrades food matrices by secreting enzymes and acids unwanted by the manufacturer resulting in significant economic losses (Setlow & Johnson, 1999; Watterson et al., 2014; Wells-Bennik, Driehuis, & van Hijum, 2016; Y. Zhao et al., 2018). Thus, this poses a real problem in the dairy powder production industries, where spore proliferation in the process may not allow for good microbiological quality of the marketed product and where high concentrations of microbial spores reflect poor hygiene of the manufacturer (P. M. Murphy, Lynch, & Kelly, 1999; Ronimus et al., 2003).

This review is divided in five parts. To begin with, a brief description of the **dairy powders processes** is provided, in which the steps identified as problematic for thermophilic spore-forming bacteria are underlined. The second one deals with the **origin of spore forming bacteria** in this process. Then, spore forming **bacteria growth parameters** are discussed compared to the dairy industry. After that, **biofilm** formation capacities and CIP resistance are described; and finally, **powder quality** and potential risk of high-contaminated powders are depicted.

2. Process description

From the same raw material, milk, many powders can be obtained (Figure 3). The first stages of the process are common, *i.e.*: **initial storage** and **skimming**. Then, if necessary, the milk is **standardized** in fat content by re-adding cream. Milk also undergo an initial **pasteurization** step.

After these steps, the milk powders (skim or whole) are obtained by **evaporation** and **final drying**.

But, apart from skimming, milk can undergo many other **separation treatments**, especially to extract caseins, and/or **concentration (evaporation) steps** before **final drying**. Intermediate storage between operations is also often necessary.

These major operations, *i.e.*: separation treatments, membrane technology, evaporation and final drying, making it possible to obtain the various dairy powders, are described in more detail below, red steps are identified as problematic for thermophilic spore-forming bacteria.

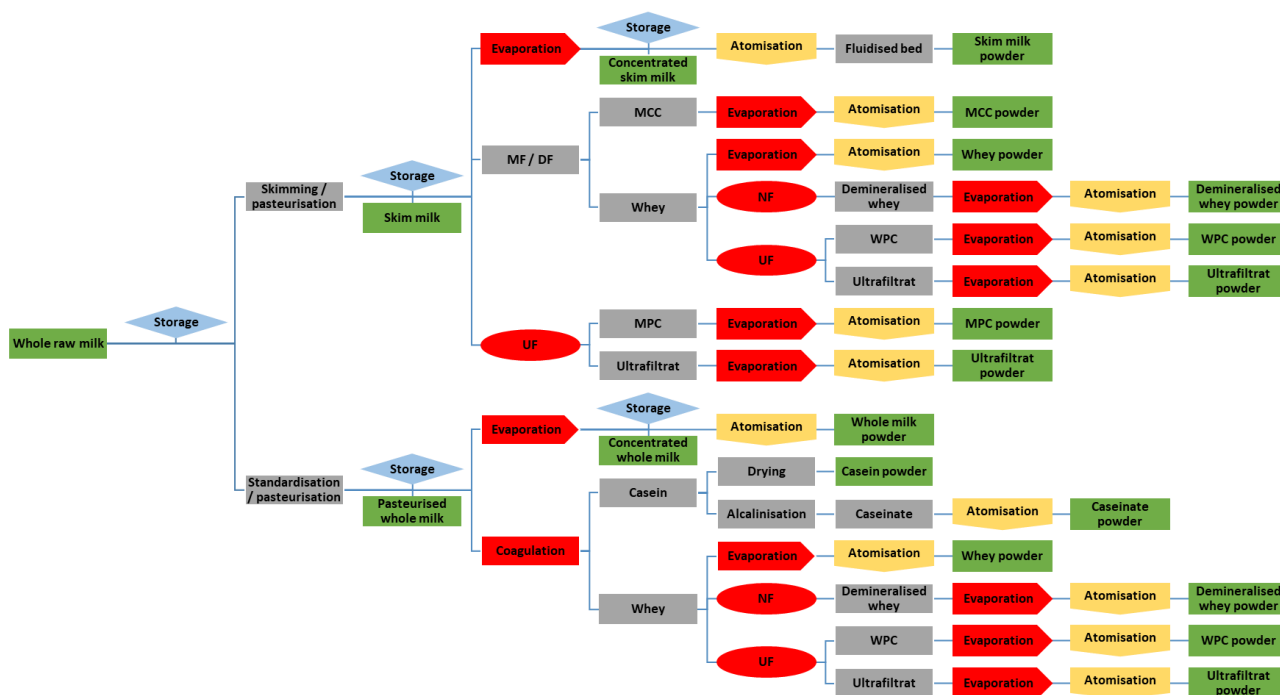


Figure 3: Process of milk cracking for the obtaining of dairy powders. In Green: products obtained from raw milk to processed powders. Red: process step favorable for thermophilic bacteria development. In Yellow: Atomization steps. In Grey: other step. DF: DiaFiltration, MCC: Micellar Casein Concentrate, MF: MicroFiltration, NF: NanoFiltration, UF: UltraFiltration, WPC: Whey Protein Concentrate. Inspired by (Schuck, 2011)

2.1. Initial and intermediate storage

The first step once the milk has been transported in the dairy industry is its cold storage until there is enough milk to start production, or until the results of analyses guaranteeing the safety of the received milk (concentration of mesophilic flora and somatic cells, antibiotic residues, fat content, protein content, freezing point...).

During the processing of milk powder, the intermediate products of production are stored in tanks whose temperature allows to preserve the techno-functional properties and to limit the proliferation of pathogenic or spoilage bacteria (4°C most of the time which can promote psychrophilic bacterial growth but inhibit thermophilic bacteria growth).

2.2. Skimming

The milk can then be skimmed in a skimming machine, which separates the cream and fat from the raw milk by centrifugation. This step can also eliminate impurities present in the milk (straw, hair, somatic cells, leukocytes, red blood cells, bacteria, etc.). Once the skimming stage has been carried out, cream can be added to the skimmed milk to be standardized at 35g.L⁻¹ of fat (whole milk).

2.3. Pasteurization

During the processing of raw milk into milk powder, a pasteurization step (72°C during 15sec) ensures the safety of production by destroying vegetative bacteria potentially pathogenic to the consumer and also reduces the proteolytic activity of enzymes naturally present in the milk (Schuck, 2011). But this weak heat treatment has quite no effect on the destruction of bacterial spores.

2.4. Other separation treatments (apart from skimming and membrane technology)

The milk can then undergo various treatments: the proteins in the milk can be coagulated by adding acid (at pH 4.6) or rennet to obtain a casein clot (solid phase) and whey (liquid phase). From this casein clot, caseinates can be extracted by alkalization, and the whey can be evaporated or dried directly to obtain a whey powder or undergo stages of filtration and separation of compounds (cracking) to isolate the proteins present. A large number of dairy powders can be obtained by different processes (Figure 3).

2.5. Heating steps

Before the evaporation stage, and high temperature processes, the milk could be preheated on the one hand by heat exchanger (indirect heating: Figure 4) and on the other hand by steam injection (direct heating). During this stage, the cold milk is injected into a heat exchanger heated by a heat-transfer liquid or steam that will transfer its calories to the milk, which will heat up as it passes through the exchanger.

The temperatures used in the exchangers are between 50 °C and 65 °C corresponding to temperatures of thermophilic spore forming bacteria. Scott et al., (2007) observed strong growth during this warming stage and possible spore formation in biofilms. These temperatures observed in plate heat exchangers allow thermophilic bacteria to grow rapidly, sporulate and

adhere to surface to form biofilms. When biofilm become mature, a part of it can detach and cause cross contamination by vegetative cells or thermoresistant spores.

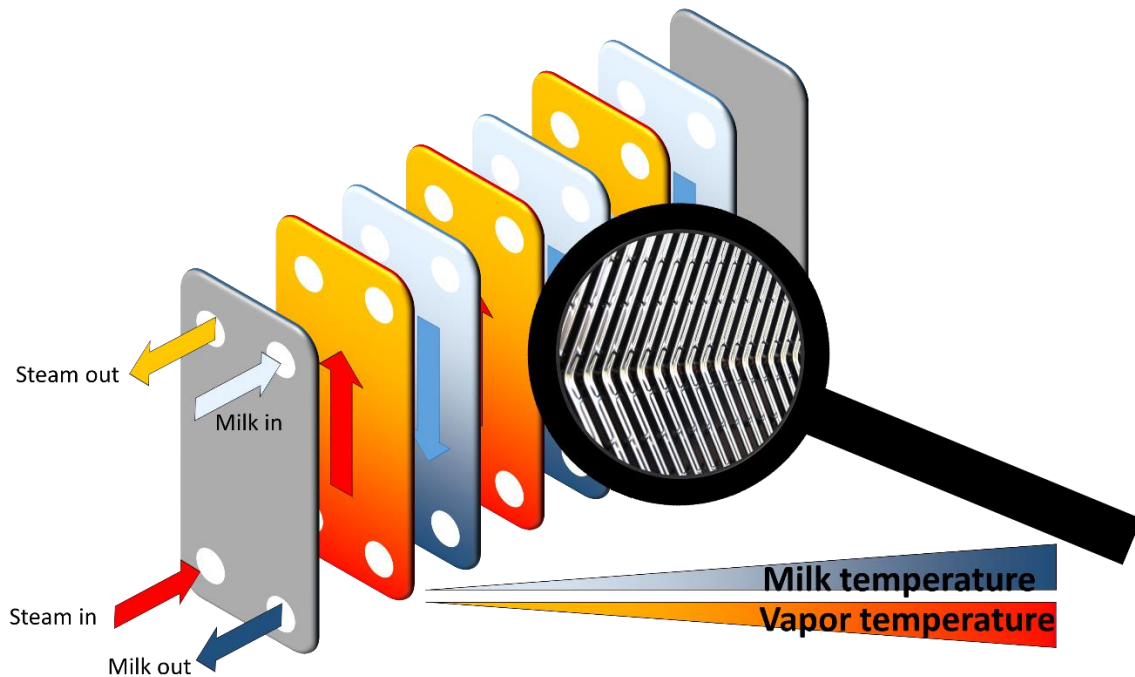


Figure 4: Plate heat exchanger principle. Inspired by (Kakaç, Liu, & Pramuanjaroenkij, 2012)

In addition, the heating steps in plate heat exchangers for pasteurization or for preheating steps promote fouling of surfaces after a few hours of use. This fouling is due to high temperatures that lead to the denaturation of serum proteins and the precipitation of mineral compounds (Bansal & Chen, 2005) and deposits on stainless steel surfaces retaining microorganisms. According to Flint et al., (2001), *G. stearotherophilus* spores attach 100 more in the presence of fouling deposits on stainless steel surfaces.

2.6. Membrane technology

Membrane technologies (Figure 5) are separation processes widely used in the dairy industry. They allow the separation of microorganisms by microfiltration, proteins by ultra-filtration, and mineral carbohydrates and salts by nanofiltration or reverse osmosis (P. Kumar et al., 2013). During the manufacture of dairy powders, the use of membrane techniques allows cold pasteurization of milk or concentration of milk or whey proteins before drying by reducing lactose concentrations (Saxena, Tripathi, Kumar, & Shahi, 2009).

However, the use of membranes is limited by clogging effect which reduces porosity and efficiency. This fouling is due to the accumulation of particles but especially bacteria forming biofilms with a production of exopolysaccharide (EPS) matrix (Anand, Singh, Avadhanula, & Marka, 2014). Although many works have described these biofilm developments in membranes (Anand & Singh, 2013; Anand et al., 2014; Julien Chamberland, Lessard, Doyen, Labrie, & Pouliot, 2017), few works mention the presence of thermophilic or facultative thermophilic bacteria in dairy filtration membranes. *Anoxybacillus* has been detected in used spiral ultrafiltration membranes but mainly in nanofiltration membranes filtering whey permeates (J. Chamberland, Lessard, Doyen, Labrie, & Pouliot, 2017). These authors explain this majority presence (93%) by its resistance to hot alkaline sanitation treatments. This resistance to strong alkaline conditions can be related to the maximum growth pH of this bacterium which is pH 10 (Ellis & Magnuson, 2012; D. Jiang et al., 2015; Wang et al., 2014). On the other hand, Bogler et al., (2019) observed the high capacity of *Anoxybacillus sp.* to form biofilms reducing permeate flows in reverse osmosis membranes. For other species, Chamberland et al., (2017) observed a low prevalence of *Bacillus* bacteria in all membrane types studied and did not identify bacteria of *Geobacillus* genus.

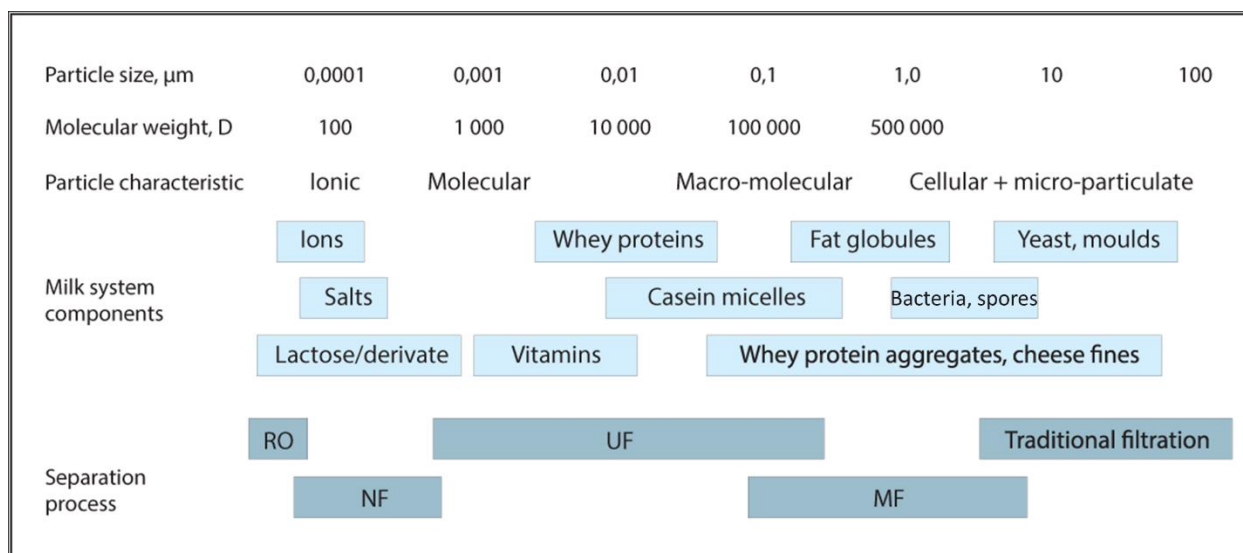


Figure 5: Use of membrane technology in dairy industry. MF: MicroFiltration, NF: NanoFiltration, RO: Reverse Osmosis, UF: UltraFiltration. Based on (Bylund, 2003)

2.7. Evaporation

The evaporation stage itself allows the removal of water from the milk before drying by limiting energy consumption. Evaporation of water is carried out under partial vacuum (from 160 to 320 mbar (Tetrapack, 2019)) in evaporators by exchanging the latent heat of evaporation between the steam and the product through the stainless steel tube wall. The milk flows as a film on the inner surface of the tubes and the water evaporate during the falling time. To increase the evaporation capacity and reduce specific energy consumption, evaporators increase the effects, cascade system recycling the vapors and increase the heat exchange surfaces up to 10000m² per unit (Decloux & Rémond, 2009). The boiling temperature varies from 70°C at the first effect to 45°C at the last effect depending on the number of effects in falling-flow evaporators in dairy industries. The residence time in the evaporator is between 20 and 120 seconds for multiple-effect systems (Goff, 2019) and the milk solids content increase from 9-13% to 40-50%.

In evaporators (Figure 6), the environmental temperature conditions encountered and the large exchange surfaces are favorable to fouling by whey serum proteins, mineral salts (and fats) as well as to the formation of biofilms by thermophilic bacteria. Besides, the configuration and dimensions of the falling-flow evaporators (22 meters high) make cleaning difficult, limiting the effectiveness of the alkaline and acidic treatments used in cleaning-in-place procedures. In each new effect the temperature decreases and the dry matter content increases. Madoumier et al., (2015) present the temperature and dry matter changes in a 4-effect evaporator. The number of effect could be more or less depending on the temperature encountered in evaporator, vacuum and product to concentrate. The dry matter content increases from 12.5% to 20.5% at 66°C in the first effect, then to 25.2% at 61°C in the second effect, then to 32.8% at 56°C in the third effect, and then to 48% at 46°C in the fourth effect. These temperature and dry extract changes correspond to the data presented by Scott et al., (2007) for a falling film evaporator. As the dry extract values increase with the number of effects, the water activities (a_w) will decrease approaching or becoming lower than the minimum growth values of the thermophilic spore forming species. As a result, biofilm formation or bacterial sporulation will be very limited in the latter effects or will not be able to occur as observed by Scott et al. (2007).

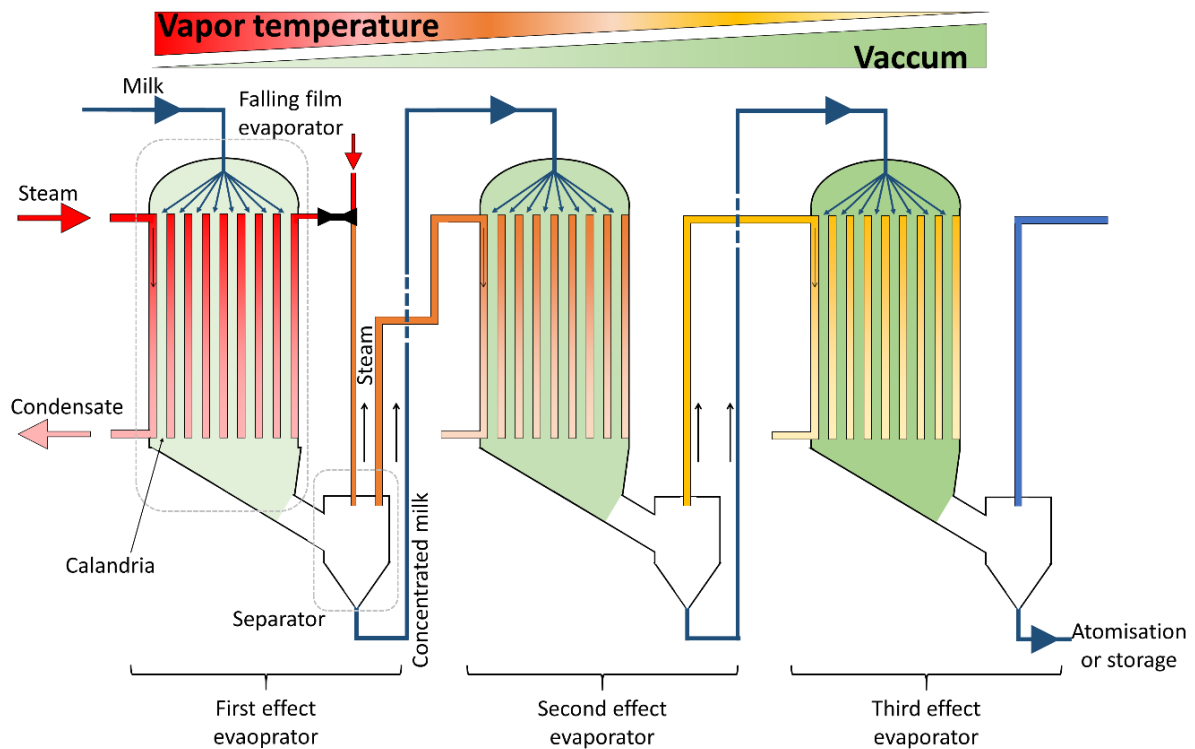


Figure 6: Multiple effect evaporator for dairy fluid concentration. Inspired by (Písecký, 2012)

2.8. Drying and atomization

Concentrated dairy fluids can be transformed into powder when their dry matter concentration is high enough, using an atomization tower (Figure 7). Concentrated dairy solutions are heated before entering the atomization tower, then passing through a pressurized nozzle and the fluid is dispersed into 10^{11} microdroplets of $50\mu\text{m}$ diameter (1L representing 120m^2 of surface area after atomization). This fluid is introduced under pressure into a heated and ventilated chamber at $160/230^\circ\text{C}$ depending on the product. The droplet (thanks to an equilibrium of the humidity) loses its water and dries quickly. The milk stays in the atomization chamber for a very short time and the internal temperature of the droplets does not exceed $70/80^\circ\text{C}$. At the end of the tower, the powder is recovered on a fluidized bed and cooled quickly with dry air. During this stage, the fine particles can be recovered to be re-injected during the atomization of the concentrated milk at the top of the tower. The powder is then packaged for storage.

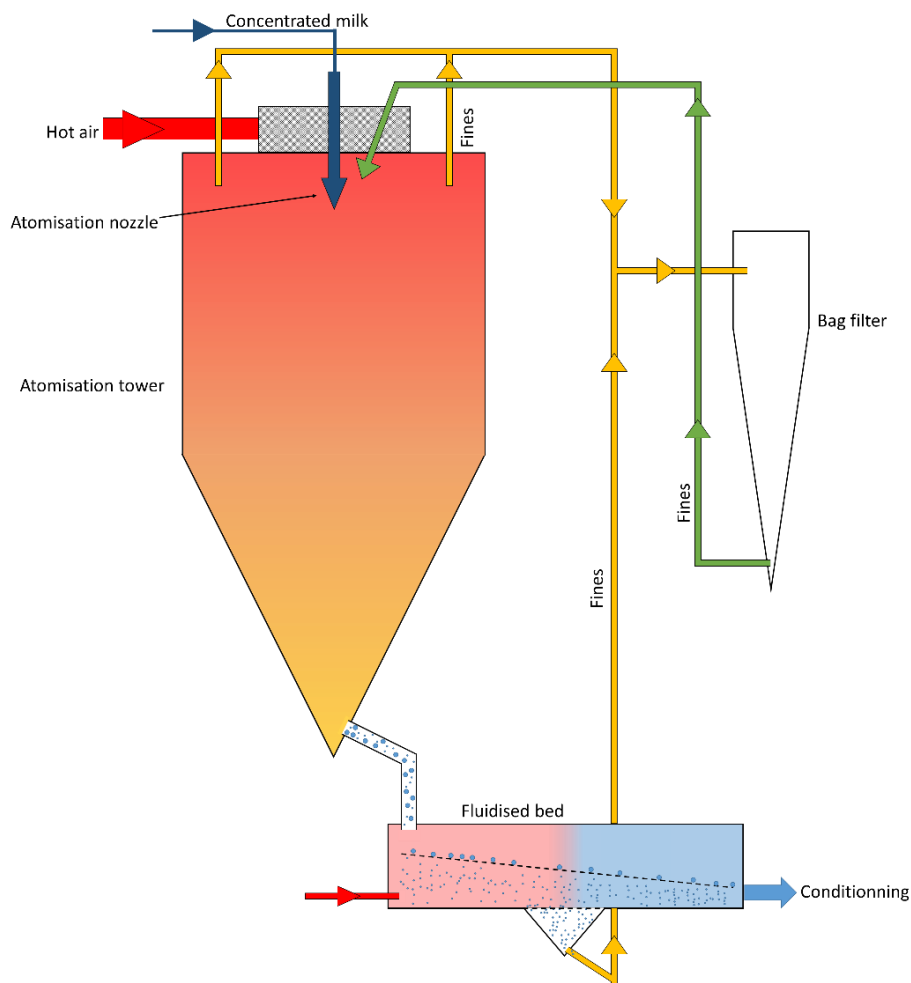


Figure 7: Atomization tower for dairy powder production. Inspired by (Písecký, 2012)

The drying step is very fast, the low water activities do not allow the growth of the microorganisms and the spray treatment only slightly affects the survival of the microorganisms in vegetative form and does not affect the survival of the bacterial spores. The atomization step does not inactivate the microorganisms and does not allow their growth but their concentration per gram of product increases by drying the product (from 50% Total solid to 95%).

3. Prevalence and origin of sporeforming bacteria in the dairy powders processes

3.1. Overall prevalence in raw milk and dairy powders

The range (minimum and maximum observed) of mesophilic and thermophilic spores in publications dealing with prevalence in raw milk and dairy powders are reported in Figure 8.

In most international studies conducted on the presence of bacteria in raw milk, the quantity of mesophilic spores rarely exceeds $1.3 \log \text{ MesophilicSporeCount.ml}^{-1}$ except for some studies where raw milk was contaminated with more than $3.5 \log \text{ MSC.g}^{-1}$ (McGuiggan, McCleery, Hannan, & Gilmour, 2002; Scheldeman, Pil, Herman, De Vos, & Heyndrickx, 2005), and thermophilic spores are rarely present and represent less than $1.12 \log \text{ TSC.mL}^{-1}$ (Buehner, Anand, & Garcia, 2014; N. H. Martin, Kent, Evanowski, Zuber Hrobuchak, & Wiedmann, 2019; Martinez, Stratton, & Bianchini, 2017; McGuiggan et al., 2002; S. I. Murphy et al., 2019; Scheldeman et al., 2005).

But this amount of bacteria increases during the process and can reach more than 3.5 logs MSC.g^{-1} on milk powder (Reginensi et al., 2011; Sadiq, Li, Liu, Flint, Zhang, & He, 2016) and more than $4 \log \text{ TSC.g}^{-1}$ or even $5 \log \text{ TSC.g}^{-1}$ (Cho et al., 2018; Kent, Chauhan, Boor, Wiedmann, & Martin, 2016; Reginensi et al., 2011; Rückert et al., 2004; Sadiq, Li, Liu, Flint, Zhang, & He, 2016; Watterson et al., 2014; Yuan et al., 2012).

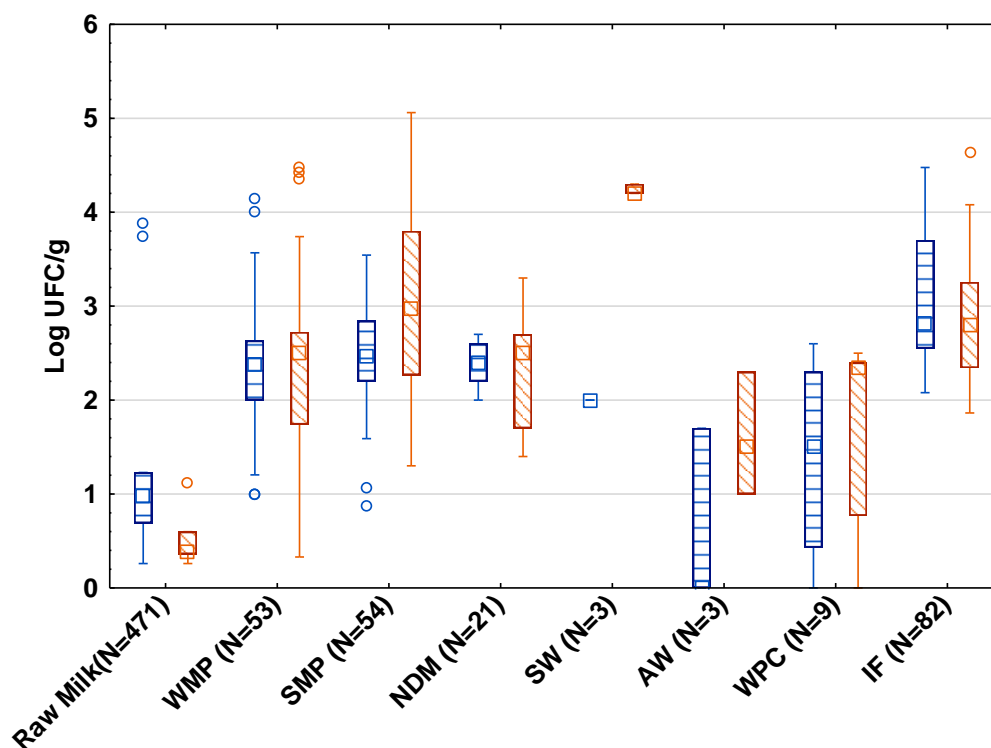


Figure 8: Mesophilic and thermophilic spore enumeration in dairy powders. WMP : Whole Milk Powder; SMP : Skim Milk Powder; NDM : Non-fat Dairy Milk; SW : Sweet Whey; AW : Acid Whey; WPC : Whole Protein Concentrate ; IF : Infant Formula (Abd, Abdula'Al, & Abood, 2014; Buehner et al., 2014; Cho et al., 2018; Kent et al., 2016; N. H. Martin et al., 2019; Martinez et al., 2017; McGuiggan et al., 2002; S. I. Murphy et al., 2019; Reginensi et al., 2011; Ronimus, Rueckert, & Morgan, 2006; Rückert et al., 2004; Sadiq, Li, Liu, Flint, Zhang, & He, 2016; Scheldeman et al., 2005; Watterson et al., 2014; Yuan et al., 2012).

In most studies on powders from all over the world, more thermophilic than mesophilic spores are often found. Depending on process applied, mesophilic and thermophilic spore formers concentration vary.

In whole milk powder (WMP), mesophilic and thermophilic spore counts are quite equal, *i.e.* from 1 to 4.14 log MSC.g⁻¹ (Kent et al., 2016; Sadiq, Li, Liu, Flint, Zhang, & He, 2016) and from 0.33 to 4.47 logTSC.g⁻¹ (Cho et al., 2018; Reginensi et al., 2011).

In skim milk powder (SMP), mesophilic spore count is a little lower, with 0.87 (Cho et al., 2018) to 3.54 log MSC.g⁻¹ (Sadiq, Li, Liu, Flint, Zhang, & He, 2016), while the thermophilic ones can be as high as 1.30 (Rückert et al., 2004) to 5.06 log TSC.g⁻¹ (Cho et al., 2018).

Whey powders (whether sweet or acid) have not been studied much (only 3 data available for each) as have protein concentrate powders (WPC, 9 data), so results on these types of powders should be taken with caution. For Acid whey (AW) and protein concentrate powders (WPC), mesophilic spores counts are lower and vary from <1 log MSC.g⁻¹ (Watterson et al., 2014) to 2.6 log MSC.g⁻¹ (Watterson et al., 2014), and thermophilic ones from <1 log TSC.g⁻¹ (Watterson et al., 2014) to 2.5 log TSC.g⁻¹ (Watterson et al., 2014).

Infant formulas (IF), which are assembly products have been mainly studied in a work carried out on Chinese products by Sadiq et al., (2016) and had mesophilic spore counts ranging from 2.07 log MSC.g⁻¹ up to 4.47 log MSC.g⁻¹, and concentrations of thermophilic spores in can range from 2 log TSC.g⁻¹ to 4.64 log TSC.g⁻¹ (Yuan et al., 2012).

During milk powder production, water removal (by evaporator and spray drying) concentrate milk compounds as lactose, milk proteins, and salts by a factor of 10. Sporeforming bacteria present in pasteurized milk will also be concentrated by this process. But the high concentration of bacteria found in powder cannot be explain only by this concentration factor. That seems that mesophilic and thermophilic sporeforming bacteria find during the milk powder process some steps allowing their growth and proliferation.

3.2. Nature of the spore forming bacteria contaminant in dairy powders

An attempt to identify more precisely thermophilic and/or mesophilic isolates from these products has been made by some authors. When available the data were summarized in Figure 9.

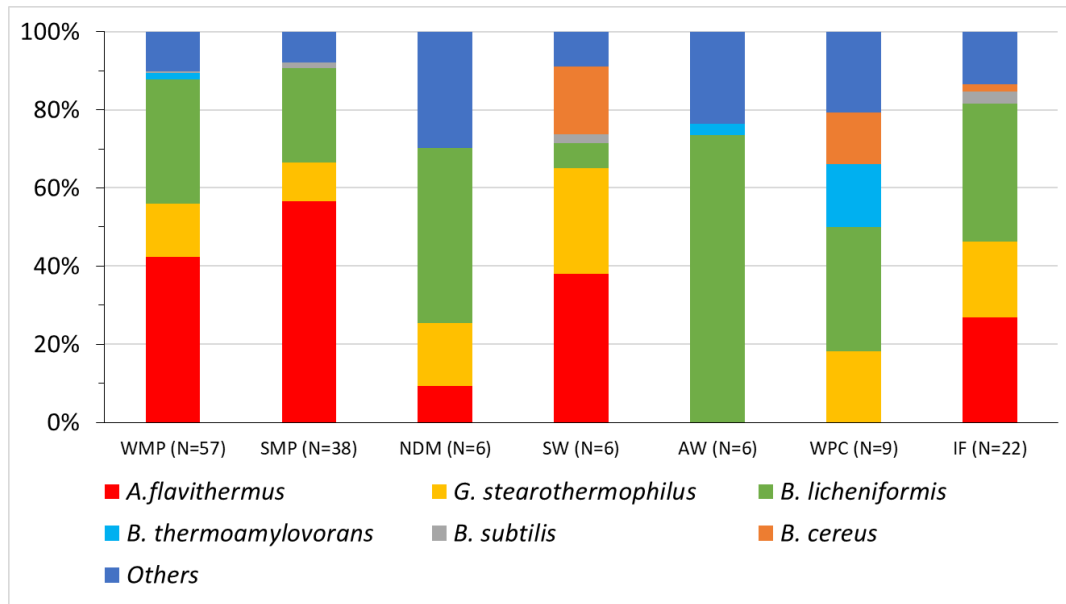


Figure 9: Percentage of the main contaminants in dairy powders. WMP: Whole Milk Powder; SMP: Skim Milk Powder; NDM: Non-fat Dairy Milk; SW: Sweet Whey; AW: Acid Whey; WPC: Whole Protein Concentrate; IF: Infant Formula. Others: different species found at less than 6%. Data from (Chauhan et al., 2013; Dettling et al., 2019; Miller, Kent, Watterson, et al., 2015; Reginensi et al., 2011; Ronimus et al., 2006; Rückert et al., 2004; Sadiq, Li, Liu, Flint, Zhang, & He, 2016; Yuan et al., 2012).

In the vast majority of dairy powders (WMP, NDM, SMP and IF), the 3 most common species are *A. flavithermus*, *G. stearothermophilus* and *B. licheniformis* (Ronimus et al., 2006; Rückert et al., 2004; Sadiq, Li, Liu, Flint, Zhang, & He, 2016; Yuan et al., 2012). Except for acid whey powder (AW), where *B. licheniformis* represent more than 70% of the isolates, and no *A. flavithermus* or *G. stearothermophilus* were found. For sweet whey powder (SW), *B. licheniformis* is less represented as compared to *B. cereus*. For whey protein concentrate (WPC) *B. thermoamylovorans* and *B. cereus* are found instead of *A. flavithermus* (Miller, Kent, Watterson, et al., 2015). *B. thermoamylovorans* could be an emerging spore forming bacteria in the dairy industry (S. H. Flint, Gonzaga, Good, & Palmer, 2017).

Other genera/species have been identified in a minority and/or less systematic manner, such as *Aneurinibacillus thermoaerophilus*, *Thermoactinomyces vulgaris*, *Brevibacillus aydinogluensis*, *B. coagulans*, *B. subtilis* group, *B. cereus* group, and *B. pumilus* group (Chauhan et al., 2013; Dettling et al., 2019; Miller, Kent, Watterson, et al., 2015; Reginensi et al., 2011; Ronimus et al., 2006; Rückert et al., 2004; Sadiq, Li, Liu, Flint, Zhang, & He, 2016; Yuan et al., 2012).

The industrial processes used for WMP, NDM and SMP are quite similar, using centrifugation, evaporator and spray dryer. For AW and SW, some treatments can be added as acidic and basic solution addition, compare to WPC, which usually pass through filtration membrane. Although these different process steps may select different species, few articles, if any, have specifically addressed this aspect.

Finally, since infant formula are composed of a mix of dairy powders, contamination can be related to those of their constituents.

3.3. Global levels of spore forming bacteria in the farm and dairy cows environment

Knowing the origin of contaminants can help to choose the right levers in order to limit their level. A first hypothesis is that spore-forming bacteria found in dairy powders could originate from the raw material and be linked to the level of contamination of the dairy primary environment.

In fact, spore-forming bacteria, due to their ubiquity, are found in soil, silage, feed and cow feces (Heyndrickx, 2011; Scheldeman et al., 2005; Te Giffel, Wagendorp, Herrewegh, & Driehuis, 2002; Vaerewijck et al., 2001; Wu, Walker, Vanselow, Chao, & Chin, 2007). For example, mesophilic spores are present at 5.68 log MSC.g⁻¹ in soil samples (N. H. Martin et al., 2019), 6.22 log MSC.g⁻¹ in corn silage (Buehner et al., 2014), 5.99 log MSC.g⁻¹ in bedding (S. I. Murphy et al., 2019). Although present in smaller quantities (about 1 log), thermophilic spores can reach 4.61 log TSC.g⁻¹ in soil (N. H. Martin et al., 2019); 5.59 log TSC.g⁻¹ in corn silage (Buehner et al., 2014) and 5.06 log TSC.g⁻¹ in an organic litter (S. I. Murphy et al., 2019).

Silage is a rather favorable environment for anaerobic germs (Zucali et al., 2015). However, aerobic mesophilic and thermophilic spores are also found (Scheldeman et al., 2005).

Since the feed of dairy cows is likely to contain spores and since spores are resistant to many conditions, it seems logical to find them in cattle faeces. Indeed, the rumen microbiota contains potentially spore-forming bacteria (*Ruminococcus bromii*) and spore-forming genes of the genera *Clostridium* and *Bacillus* but its composition has not been studied extensively and aerobic spore-forming bacteria are probably present as a minority (K. M. Singh et al., 2014). However, the high presence of spores in the farm environment can lead to contamination of

udders and thus contamination of raw milk if the teat cleaning and disinfection process of milking equipment and parlor is not well followed (Christiansson, Bertilsson, & Svensson, 1999).

Different studies looking at the amounts of spores present in raw milk have found the presence of Mesophilic Spore Count with a wide range from 0.26 log MSC.ml⁻¹ (S. I. Murphy et al., 2019) to more than 5.5 log MSC.ml⁻¹(Abd et al., 2014). For Thermophilic Spores Count (TSC), the concentration in raw milk is estimated from less than 0.26 log TSC.ml⁻¹(S. I. Murphy et al., 2019) to 2.31 log TSC.g⁻¹ (Abd et al., 2014) figure 8.

The presence of spore-forming bacteria in raw milk has been linked to various parameters. Thus, the size of the herd (the larger it is), the elimination of the first milk sprays and dry massage of the udder before milking reduce the quantity of mesophilic and thermophilic spores (Miller, Kent, Boor, Martin, & Wiedmann, 2015). The type of bedding also modulates the spores contamination levels: sawdust and sand bedding seem to decrease the part of mesophilic spores, while straw bedding seems to decrease the part of thermophilic spores (Miller, Kent, Boor, et al., 2015; S. I. Murphy et al., 2019).

However, spore-forming bacteria constitute a small proportion of the total microbiome of raw milk. Thus Kable et al., (2016) indicate that the genus *Bacillus* corresponds to 0.51%, while *Clostridium* corresponds to 7.47% of the microbiota.

The spores are a minority in raw milk and in small quantities, but due to selection pressures applied by the processing (pasteurization, high process temperature, product formulation), this flora becomes the majority and in high concentration in intermediate and finished products (Alcaraz et al., 2008; Guinebretière et al., 2008; Postollec et al., 2010).

Overall, spores do exist in the environment and can contaminate the raw material. However, at least two scenarios seem possible to explain the levels reached for some species in the final product.

3.4. Hypotheses on the probable contamination routes

The question of the origin of the contamination is not a simple one and the answers are multiple. Data from studies that identified isolates from plate counts of various environmental samples are summarized in Figure 10.

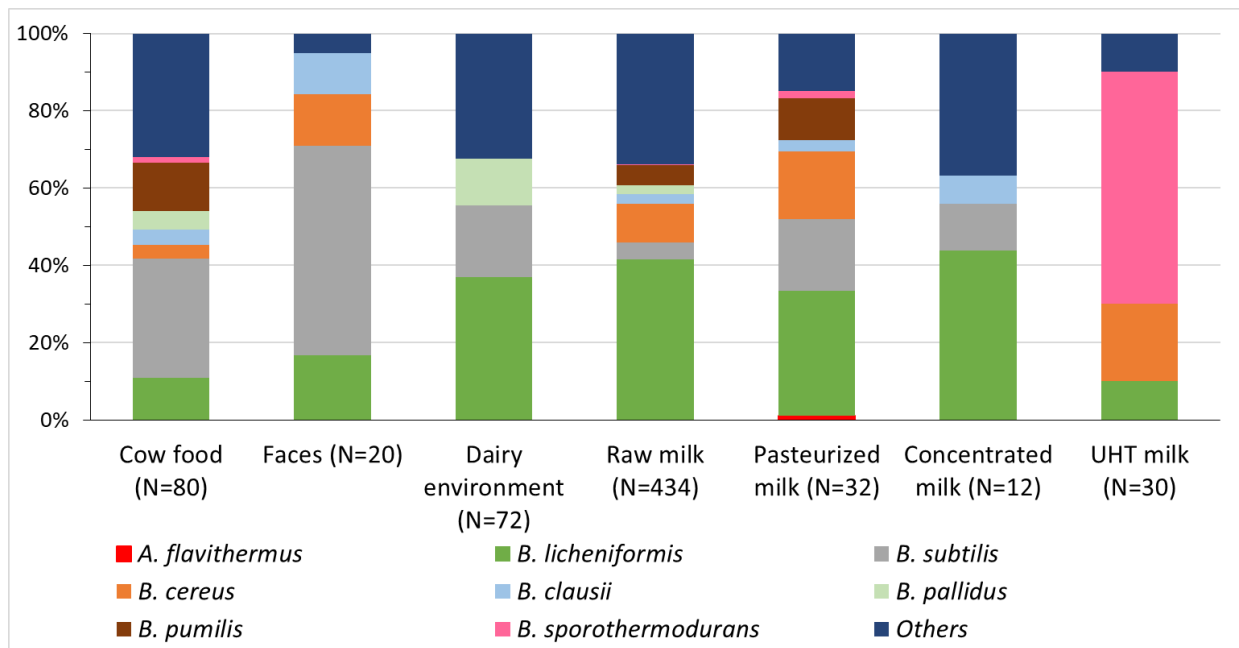


Figure 10: Percentage of the major spore-forming contaminants in dairy environment: Cow food; Faces; Dairy environment; and in milk: Raw, Concentrated; Pasteurized and UHT. Data from (Gupta & Brightwell, 2017; Lücking, Stoeckel, Atamer, Hinrichs, & Ehling-Schulz, 2013; Ronimus et al., 2006; Scheldeman et al., 2005; Vaerewijck et al., 2001; Wu et al., 2007)

In the case of *B. licheniformis* (and some other mesophilic species such as the *B. subtilis* group) their presence in raw milk has been confirmed many times. In fact, even if the quantity of spores found varies between the different studies, the species of aerobic mesophilic spore-forming bacteria identified in raw milk remain similar, with the majority being *B. licheniformis*; *B. cereus* and *B. subtilis* (Aouadhi, Maaroufi, & Mejri, 2014; Chauhan et al., 2013; J. H. Martin, 1974; Martinez et al., 2017; Miller, Kent, Watterson, et al., 2015; Scheldeman et al., 2005) (Figure 10).

This is due to the presence of these species in many parts of the environment. Thus, the aerobic mesophilic and thermophilic spores found in silage and cow food have been identified as *B. subtilis* (30.30%), *B. pumilis* (12.19%), *B. licheniformis* (10.78%), *B. pallidus* (4.90%), *B. clausii* (3.90%), *B. cereus* (3.41%) or other species or genera (e.g. *B. farraginis*, *Geobacillus* spp., *Brevibacillus*, *Aneurinibacillus*, *Paenibacillus*, *B. flexus*, *B. oleronius*, *Virgibacillus*...) for 34.52% (Scheldeman et al., 2005; Vaerewijck et al., 2001; Wu et al., 2007). Similarly, in bovine faeces, the mesophilic spores found belong to *B. subtilis* (54.17%), *B. licheniformis* (16.67%), *B. cereus* (13.33%), *B. clausii* (10.83%), and other species (e.g. *B. coagulans*, *Bacillus* spp. or *B. sphaericus*) (Wu et al., 2007).

When raw milk is heat treated, a selection of heat resistant bacteria is done by the process, although it doesn't change that much the repartition of the spore forming contaminants in pasteurized milk, it can lead to a clear predominance of highly heat resistant species such as *B. sporothermodurans* in UHT milk (Aouadhi et al., 2014).

Several studies shown that seasons have an impact (number and diversity) on the microbiote of raw milk and therefore on heat treated milk (Buehner et al., 2014; Crielly, Logan, & Anderton, 1994; Kable et al., 2016). Even if variations have been observed, species present are the same: *B. licheniformis*, *B. subtilis*, *B. sonorensis*, *B. cereus*. This variation is certainly due to milk composition and physiochemical properties variations between the seasons (S. Li, Ye, & Singh, 2019).

Thus, *Bacillus licheniformis* can be brought in dairy industries by raw milk in significant quantities.

The picture is quite different as far as the thermophilic species are concerned. As previously mentioned, overall, the nature of thermophilic spores from the farm environment and even from raw milk has been relatively less investigated than for mesophilic ones.

Few studies have documented the proportion of thermophilic spore species in raw milk. Only the study by Coorevits et al., (2008) identified *B. licheniformis*, *U. thermosphaericus*, *Bacillus sp.* and *B. thermoamylovorans* as the major thermotolerant spore forming species in raw milk. The proportion of these different species varies according to the type of milk analyzed in this study, with *B. licheniformis* accounting for up to 89.29% of the total isolates identified. This confirms the data provided by the studies on the mesophilic spore forming bacteria.

Some species are rarely present in raw (or mild heat-treated) milk such as *Anoxybacillus flavithermus* which is only found at about 1.6% in pasteurized milk (Chauhan et al., 2013). However, as demonstrated by Kable et al., (2019), even if *Anoxybacillus* is only present at 0.8 to 1.6% in raw milk, it can reach 40% of the total isolates in some samples collected during the summer.

Geobacillus stearothermophilus is not found in raw milk (Buehner et al., 2014; Chauhan et al., 2013) or at a level of 0.2% (Miller, Kent, Watterson, et al., 2015) or 0.7% (J. H. Martin, 1974).

Other *Geobacillus* species could be present at low levels as *G. pallidus* (Witthuhn, Lücking, Atamer, Ehling-Schulz, & Hinrichs, 2011) or *G. thermoleovorans* (Lücking et al., 2013).

It thus appears that, with few exceptions (summer milk loaded with thermophilic species), the spores of thermophilic species are brought in very small or even undetectable quantities by the raw milk. It is therefore accepted and logical to think that the industrial processes, in line with the characteristics of the strains, are the origin of the significant contamination found in finished products.

4. Main species characteristics and adaptation to dairy environments

Milk, as well as whey, are rich media, composed of water, proteins, carbohydrates, lipids and mineral salts that allow the growth of many germs even demanding one. When these liquids are heated (during the process) to optimal temperatures, they allow the growth (biofilm formation and sporulation) of the species present. Through selection pressures, limits and growth rates, some species may be favored or on the contrary disappear. Lactose utilization, effect of ions concentration, growth limits for pH, aw and temperature, have been identified as potentially important factors and will be presented in this paragraph.

4.1. Effect of lactose on bacterial growth

The main carbohydrate source in milk is lactose and could select species that are able to use it. Depending on the bacterial genome and capacity to degrade and use lactose, some species can grow faster due to this factor and can be mentioned as a selection pressure.

Some studies have been done comparing some type strains (mostly originated from hot spring or wastewater), and dairy isolates, identifying lactose utilization genes in dairy isolates but not in types strains.

The non-dairy *A. flavithermus* strain WK1 is not able to use lactose (Y. Zhao et al., 2018), compare to dairy one (Burgess, 2016; Tasara, Morach, Klumpp, & Stephan, 2017). This is also the case of *G. stearothermophilus* isolated from dairy environments, which differ from wild or types strains (59 strains tested). Dairy strains have a putative *lac* operon not found in the genomes of the other strains, and are able to use lactose (Burgess, Flint, Lindsay, Cox, & Biggs, 2017; X. Y. Zhao, 2014). Selection of these strains could occur if they enter the dairy plants. Why and how lactose utilization is earned or lost is not quite clear. Some industrially originated

A. flavithermus strains (strain 52-1A for instance) indeed possess a plasmid but lactose operon is not located on this one (Tasara et al., 2017).

On another hand Zain et al., (2016) managed to isolate some beta-galactosidase negative *B. licheniformis* strains from Chinese dairy powder; lactose fermentation analysis was done on 33 *B. licheniformis* dairy isolates and they all also gave negative results (Zain, Bennett, & Flint, 2017). These results support the fact that lactose negative strains could persist in the dairy environment and can develop by degrading protein presents in liquid whey via the production of large amount of protease. In addition, most of *B. licheniformis* isolates in this last study were able to form biofilm in 1% WPC80 without lactose or minerals on stainless steel (Zain et al., 2017).

Synergy between dairy strains have also been observed as a growth mechanism: *G. thermoglucosidans* TNO-09.020 only grows in the presence of lactose positive *A. flavithermus* TNO-09.006 strains because of its lack of lactose permease or hydrolysis equipment (Yu Zhao et al., 2013). Co-cultures between lactose positive and negative strains certainly occur in dairies and in biofilm formed, promoting lactose negative bacteria fast growth.

Different studies carried out on *A. flavithermus*, *G. stearothermophilus* and *B. licheniformis* show that these three species are adapted to the nutritional conditions encountered in dairies where they grow and sporulate easily. *B. licheniformis* in raw milk, for example, shows a growth rate of 0.16 h^{-1} at 20°C (De Jonghe et al., 2010). The maximum specific growth rate of *G. stearothermophilus* in evaporated milk stored at 62°C was $2.083 \pm 0.288 \text{ h}^{-1}$ (mean \pm st.dev.) which was almost identical to that observed in TSB ($2.068 \pm 0.036 \text{ h}^{-1}$) (Kakagianni, Gougouli, & Koutsoumanis, 2016). For *A. flavithermus*, the growth rate is similar to *Geobacillus* one with 1.83 h^{-1} at 55°C with a sporulation rate of 0.9 h^{-1} in UHT skim milk medium at laboratory scale (100mL) (Graf, Hehnke, Neuwirth, & Hinrichs, 2021).

All three species have also proteolytic and lipolytic activities. Thus, Sadiq et al., (2016) indicate that almost all isolates of these species, derived from Chinese dairy powders, possess these enzymatic activities. Variations in intensity are visible between isolates but *B. licheniformis* appears to be more effective for both. More information on the properties of enzymes produced by thermophilic spore forming bacteria are developed in part 6.1.

4.2. Ion effect on strains and species behavior

Milk composition can vary depending on the farm, cow's diet, season (S. Li et al., 2019), modifying protein, carbohydrates, fat and salt proportion.

Salts and ions encountered in milk are known to have an effect on bacterial sporulation and biofilm gene regulation. For example, calcium is present as high as 1 to 1.2g.kg⁻¹ (Gaucheron, 2005) in milk; it is accumulated in the prespore of *Bacillus subtilis* (Jonhstone, Ellar, & Appleton, 1980; Alfredo Palop, Sala, & Condón, 1999) and allows the activation of the sporulation genes but also the genes for the formation of biofilm by vegetative bacteria (Oomes et al., 2009). Calcium is also widely used in sporulation media of *Geobacillus* studied by Seale et al., (2008) and *Anoxybacillus flavithermus* (Burgess, Brooks, Rakonjac, Walker, & Flint, 2009; Rönner, Husmark, & Henriksson, 1990; Scott, 2005). This high concentration in milk can then promote *Anoxybacillus*, *Geobacillus* and *Bacillus* sporulation and biofilm formation.

According to a study by Somerton et al., (2013), the addition of Ca²⁺ and Mg²⁺ ions seems to have a positive effect on the growth of *A. flavithermus* E16; DSM2641; *Geobacillus sp.* F75 and *G. thermoleovorans* DSM5366, in a medium composed of casein digestate. Sinnelä et al., (2019) shown the same effect on *B. licheniformis* with calcium and magnesium. These ions, present in milk, stimulate the production of exopolysaccharides, surface proteins and increase the adhesion of bacteria to surfaces (Patrauchan, Sarkisova, Sauer, & Franklin, 2005; Ben Somerton et al., 2013; Song & Leff, 2006; Turakhia & Characklis, 1989). According to a study by Somerton et al., (2015), high Na⁺ and low Ca²⁺ and Mg²⁺ ions have a negative impact on biofilm formation in *Geobacillus spp.* but do not appear to impact biofilm formation in *Anoxybacillus flavithermus*.

The presence of ions in the medium have also an impact on spore thermoresistance as demonstrated by Cazemier et al., (2001), where the heat resistance of spores of *B. subtilis* AdHC1, A163; and of *B. licheniformis* VBBBa and AS 1-1813 formed in a medium supplemented with Ca²⁺, Mg²⁺, Fe²⁺, K⁺ and Mn²⁺ are more heat resistant than spores formed in a medium supplemented only with Mn²⁺.

4.3. pH effect on the growth ability

During the various stages of milk processing, the pH value varies only slightly during concentration or filtration. It is only during the addition of soda for casein precipitation and the addition of acid for caseinate recovery that the pH is strongly impacted (from 6.7 to 4.6), which can also affect the survival of thermophilic spore-forming bacteria. Sweet whey is distinguished from acid whey by its pH. . Sanitation treatments also lead to extreme pH values.

The limits or cardinal values of pH are fewly referenced in the bibliography, however the data collected allow us to conclude that the three species are neutrophilic with a slight alkaliphile tendency (Figure 11) (Ellis & Magnuson, 2012; D. Jiang et al., 2015; Kakagianni et al., 2016; Trunet et al., 2015; Wang et al., 2014). As mentioned, if caseins are not precipitated and the pH value not modified, all the three species can grow easily if the other parameters ($T^{\circ}C$ and a_w) are still optimum.

It therefore seems implausible that *A. flavithermus* or *G. stearothermophilus* grow in acid whey, only *B. licheniformis* has a limit value close to (and lower than) that of the product and seems likely to grow there, which is in accordance with Figure 9.

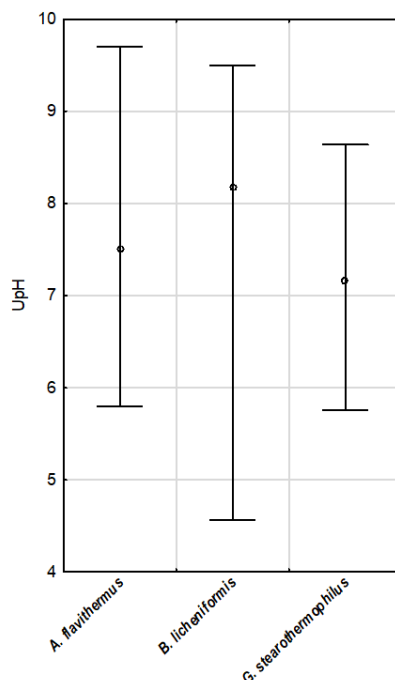


Figure 11: Average pH growth limits obtained for 5 strains of *A. flavithermus*, 2 strain of *B. licheniformis* and 3 strain of *G. stearothermophilus*. data from : (Chiş et al., 2013; Ellis & Magnuson, 2012; D. Jiang et al., 2015; Kakagianni et al., 2016; Lauwers & Heinen, 1983; Llarch, Logan, Castellví, Prieto, & Guinea, 1997; Mtimet, 2015; Trunet et al., 2015; Wang et al., 2014). Whiskers: min and max pH growth; o: optimal pH

4.4. Effect of water activity on growth

Water play a very huge role in life and also in bacterial growth, and is needed for every chemical reaction encountered in organisms. In an environment, water can be found as available (free water) or not (bound water). Only the free water part can be used by organisms for their growth and is determined as water activity (a_w) of a medium or availability of water at a determined temperature. The bound water is not available for organism growth because that part is linked to molecules known as salt or sugar. If the a_w is equal to 1, all the water of the media is free water and available for bacterial growth; but if a_w is close to 0, the less water is available for growth.

Concerning the optimal a_w , there is few data available. However, *A. flavithermus* can grow with a maximum NaCl concentration of 3% ($a_w=0.983$) (Ellis & Magnuson, 2012; D. Jiang et al., 2015; Wang et al., 2014); *G. stearothermophilus* can grow in the presence of 3% (Durand, 2014) or 5% salt ($a_w=0.970$)(Nazina et al., 2001) whereas *B. licheniformis* have an $a_{w\ min}$ between 0.904 and 0.920 depending on the suppressor and strains studied (Baril, Coroller, Couvert, El Jabri, et al., 2012; Torres, Martínez, Pandey, & Castro, 2009).

As shown, few data are available for water activity effect on those three bacteria and determination of cardinal parameters have to be done.

4.5. Effect of temperature

During the manufacture of dairy powders, the microbial ecology of the product evolves throughout the process from raw milk to the different final products according to the contamination (of initial product and of process parts); as well as the evolution of the environmental factors associated with the different processes and unit operations applied.

For *A. flavithermus*, the growth temperature range is from 30 to 75°C, (Chiş et al., 2013; Ellis & Magnuson, 2012; Heinen et al., 1982; D. Jiang et al., 2015; Wang et al., 2014) with an average optimum temperature of 60°C. These temperatures are close to those observed in *G. stearothermophilus*, a thermophilic bacterium that can grow from 33.76 to 74°C with a mean optimum species temperature of 65°C (Kakagianni et al., 2016; Rosso, Lobry, & Flandrois, 1993; Warth, Scientific, & Ryde, 1978) (data obtained with modeling). *B. licheniformis* species is classified as mesophilic but behaves similarly to thermophilic bacteria as it can grow between

11.3 and 58°C with a mean optimal growth temperature of 49.51°C (Llarch et al., 1997; Trunet et al., 2015; Warth et al., 1978).

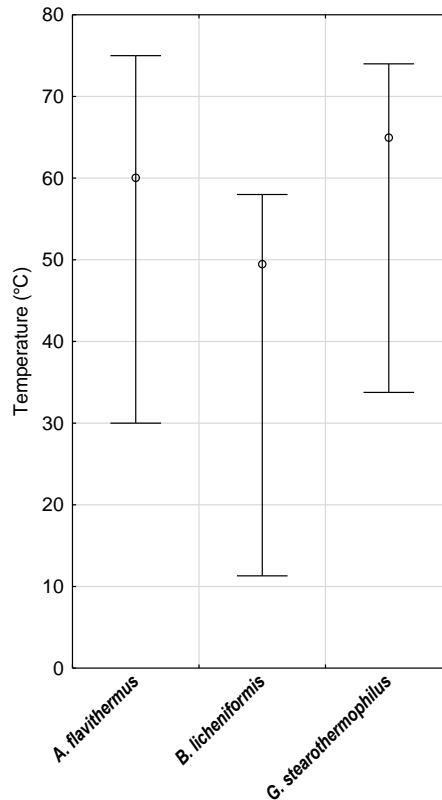


Figure 12: Temperature growth limits of *A. flavithermus*; *B. licheniformis* and *G. stearothermophilus* according to (Ellis & Magnuson, 2012; Heinen et al., 1982; D. Jiang et al., 2015; Kakagianni et al., 2016; Torres et al., 2009; Trunet et al., 2015; Wang et al., 2014; Warth et al., 1978). Whiskers: min and max temperature; ○: optimal temperature

Cardinal growth values of *A. flavithermus*, *G. stearothermophilus* and *B. licheniformis* seems quite well adapted for growing in dairy powder process as well as the composition of milk which is not bounding. All of this characteristic will lead to a bacterial growth, biofilm formation and milk powder contamination. To limit this high concentration of spore in dairy powders, industrial producers use cleaning in place procedure to clean the process.

5. Biofilms of thermophilic spore forming bacteria and CIP

5.1. Biofilms

A biofilm (Figure 13) is defined as a sessile microbial community characterized by adherence to a solid biotic or abiotic surface and by the production of a matrix surrounding

actively growing bacterial cells comprising extracellular polysaccharides (EPS), proteins and DNA (Bjarnsholt et al., 2009; Costerton, Lewandowski, Caldwell, Korber, & Lappin-Scott, 1995; Flemming & Wingender, 2010; Sadiq et al., 2017; Wingender, Strathmann, Rode, Leis, & Flemming, 2001) according to Marchand et al., (2012).

Briefly the first stage of biofilm formation occurs when bacteria (or spore) adhere to surfaces (Mittelman, 1998). This step is achieved by interactions between cells and surfaces that are of different types: electrostatic charges, weak bonds, as well as the hydrophobicity of the bacteria (Marchand et al., 2012). During this step, surface proteins play a very important role in the adhesion of even dead cells (Parker, Flint, Palmer, & Brooks, 2001). During the second phase of biofilm formation, planktonic bacteria bind to the surface through fibrin, pili, flagella, and secrete EPS (Kokare, Chakraborty, Khopade, & Mahadik, 2009). This step firmly adhere cells to surface and result to an irreversible attachment. The third phase consist on a development of the biofilm structure. The fourth phase is the maturation of biofilm. The fifth and final phase consists of biofilm dissemination in the environment or food matrix (Marchand et al., 2012; Petrova & Sauer, 2016) (Figure 13).

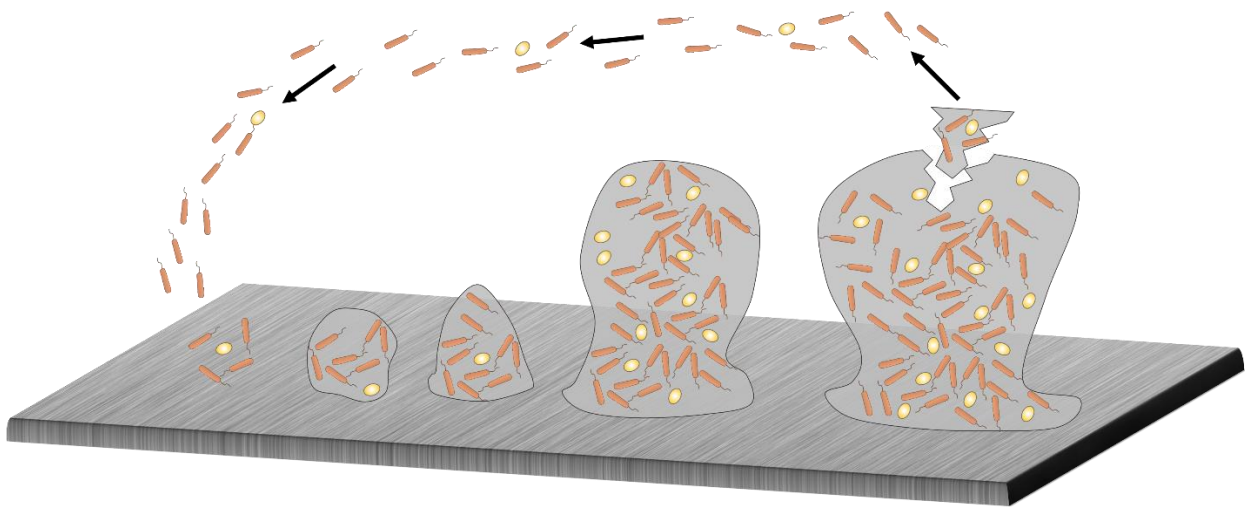


Figure 13: Schematic representation of biofilm formation inspired by (Stoodley, Sauer, Davies, & Costerton, 2002)

Biofilms are present in all food processes and especially in the dairy industry. It concerns particularly thermophilic spore forming species: *A. flavithermus*, *G. stearothermophilus* and *B. licheniformis*, which ability to grow on stainless steel has been demonstrated (Burgess et al., 2009; S. H. Flint, Palmer, et al., 2001; Sadiq et al., 2017; Seale et al., 2008).

Concerning the first step of biofilm formation, *i.e.* adhesion, the capacity of thermophilic species has been measured in optimal conditions (TSB at 55°C) by Yuan et al., (2018). They show that vegetative cells of *G. stearothermophilus* have a higher ability to adhere to stainless steel coupons (grade 304) in 30 minutes, counting on average 2.92log CFU.cm⁻² of stainless steel; in contrast to *A. flavithermus* which adheres only at 1.55log CFU.cm⁻² and *B. licheniformis* at 1.06log CFU.cm⁻².

Those differences can be explained by hydrophobicity of bacteria or spores; but this effect is not very clear in the literature. According to some authors, the hydrophobicity of the cells would be strongly related to the ability to adhere (Yuan et al., 2018), and extracellular proteins are the major actors allowing the adhesion of vegetative bacteria and spores. On the contrary, some authors claimed that, even if spores are more hydrophobic than vegetative cells, there is no clear relationship between hydrophobicity and adhesion capacity and surface polysaccharides do not seem to have an impact on these interactions (Parkar et al., 2001). The adhesion ability could be explained by the presence of extracellular proteins allowing the adhesion of vegetative bacteria and spores (Parkar et al., 2001).

Concerning the biofilm growth and maturation, data are scarce, but it can be noticed that, after 18h of culture in flow skim milk at 55°C, *A. flavithermus* B12-C^m can form a biofilm of 30µm thickness (Parkar, Flint, & Brooks, 2003) and 109µm after 4 days of culture for *Anoxybacillus* sp. at 25°C versus 87µm for *P. aeruginosa* (Bogler et al., 2019). For *B. licheniformis* TT33 originated from hot spring, it can produce a 33 µm thick biofilm within 72h at 37°C (Suthar, Hingurao, Desai, & Nerurkar, 2009). No data have been found for *G. stearothermophilus* biofilm thickness.

The industrial environment is quite important for this biofilm aspect. As previously mentioned (see 4.2) ions concentration can influence the biofilm formation; it is also possible, in whole milk powder production, that fat contained in milk create a small layer on stainless steel avoiding cells and spores adhesion (as it has been tested on *B. cereus* by Ribeiro et al., 2019). Biofilm formation is promoted at the air/liquid interface in all three species originating from dairy environments (Ostrov, Paz, & Shemesh, 2019; Yu Zhao et al., 2013) this could modulate its appearance, even if dairy process lines are mostly exempt of air and bubbles,

Considering the genetic aspects of this behavior, dairy originated strains forming biofilm present, for *B. licheniformis*, 68 genes that are absent in nonbiofilm producer strains, not originated from a dairy environment (type strain) (WGS comparison, Ostrov et al., 2019). These genes are involved in the synthesis of flagellar proteins (role in adhesion); sporulation and germination proteins (increasing resistance to CIP); regulator of genes involved in the production of extracellular matrix (in particular γ -poly-DL-glutamic acid). No genetic studies on *A. flavithermus* and *G. stearothermophilus* have been done regarding biofilms genes. But those two species have the necessary genes to effect quorum sensing by activation of the LuxS; Pfs and RbsB (AI-2 synthesis and receptor), according to Kaur et al., (2018).

An interesting point is that, because of competency genes present in these three species, horizontal transfer could appear in biofilm leading in a gene gain allowing a better resistance or a new alteration activity. *A. flavithermus* from dairy sources (WK1 and TNO-09.006) has competency genes (ComX, ComP and ComA) controlled by Quorum Sensing and similar to those observed in *Geobacillus spp.* and *Bacillus spp.* (Goh et al., 2014). These horizontal gene transfer could explain an adaptation of strains to dairy processes by the exchange of genes within biofilm.

5.2. Sporulation within biofilm

Spore is a dormant state of the cell and sporulation is a defense mechanism that allows the bacteria to resist multiple physical or chemical effects. This transformation is induced by unfavorable conditions such as lack of nutrients (amino acids, carbon source or minerals) or overpopulation (Lazazzera, 2000). Sporulation (Figure 14) is a long and costly process for the cell. It can be divided into 7 steps involving specific genes and sigma factors. The whole processes leads to the formation of a mature spore consisting of an exosporium, a coat, separated from the cortex by an inner membrane and finally the core of the spore.

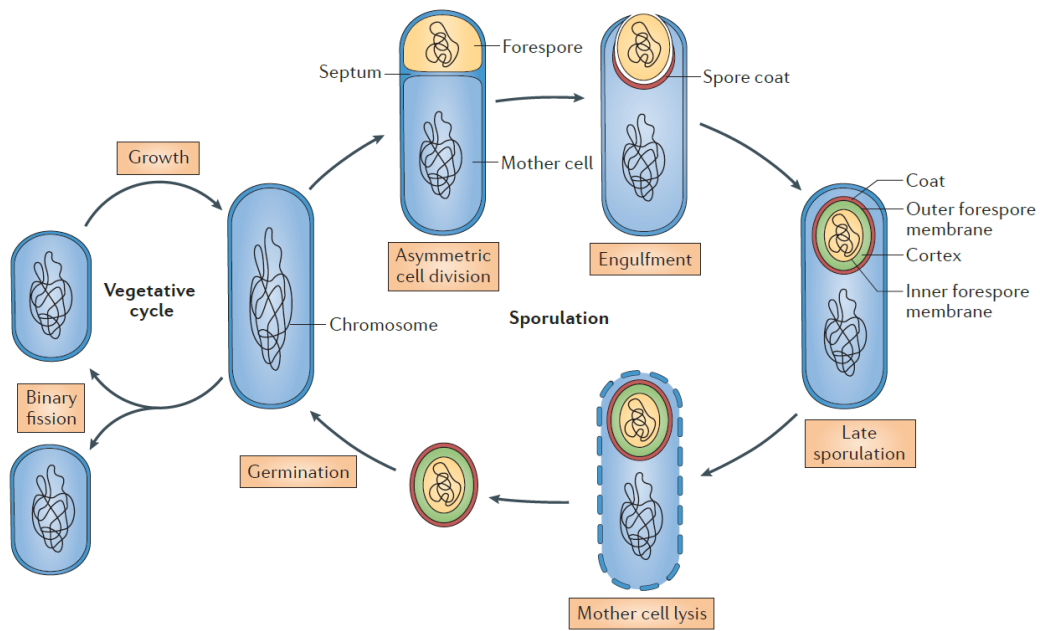


Figure 14 : Description of sporulation cycle (Mckenney, Driks, & Eichenberger, 2013)

The properties of the spore are due to the low water content of the spore core, the high concentration of divalent cations in the spore core and the presence of SASP-like proteins (Small, Acid-soluble Spore Proteins) that limit DNA damage (Moeller, Setlow, Reitz, & Nicholson, 2009; Nicholson, Munakata, Horneck, Melosh, & Setlow, 2000). When the spore's environment becomes favorable for its growth, it can then germinate and grow back into a vegetative bacteria (Setlow, 2014b).

Sporulation mechanism has been widely described for *Bacillus subtilis* and appears to be similar in other spore-forming bacterial species and genera (Setlow, 2014a). The metabolic activity of the spores is strongly reduced (Segev, Smith, & Ben-Yehuda, 2012) due to a strong decrease of a_w in the spore, and the presence of DPA (Dipicolinic acid) and Ca-DPA (Gerhardt, Scherrer, Black, & Halvorson, 1990).

Concerning the thermophilic species, it is noteworthy that sporulation of *A. flavithermus* occurs rapidly: 8h to 11h (Burgess et al., 2009 ; Scott et al., 2007), in industrial like conditions (flow cell with pasteurized milk heated to 55°C) and is simultaneous with biofilm formation. As mentioned by Burgess et al., (2009), temperature have an effect on sporulation and biofilm formation by *A. flavithermus* : sporulation can only occur when temperature is higher than 48°C but biofilm formation is not impacted at 48°C, 55°C or 60°C. In some conditions, 50% of the biofilm can be form of spores (Burgess et al., 2009). *G. stearothermophilus*, seems a little

slower, but, after 16h of growth in microplate on stainless steel coupon, spore can represent more than 70% of total cells (Burgess, 2016). This quick sporulation gives a fast resistance to stress and cleaning. On the contrary, in other species, such as *B. subtilis*, spores are only produced when the biofilm is "mature" (Hamon & Lazazzera, 2001; Lindsay, Brözel, & von Holy, 2005). No data have been found on sporulation kinetics of *B. licheniformis*.

Within biofilms recovered from whole milk powder plant, biofilms can reached more than 7 log CFU.g⁻¹ of fouled material, containing more than 95% of spores. The majority of this fouling was composed of *A. flavithermus* and *Geobacillus spp* (Scott et al., 2007).

5.3. CIP procedures

In order to minimize the presence of thermophilic spore-forming bacteria in the manufactured product and to avoid any fouling that could promote bacterial growth, the dairy industries are implementing Cleaning In Place (CIP) procedures, allowing the cleaning of the stages of the manufacturing process without dismantling it. Table 1 lists some CIP procedures referenced in the bibliography. Most often these cleaning procedures are divided into 4 steps.

Firstly a rinse with water is performed to remove milk from the various pipes and machines, followed by a passage of a sodium hydroxide solution more or less concentrated (0.5 to 2% hot from 65 to 80°C for a specified time, 10 to 30min). This second step is intended to remove organic residues present on surfaces, to remove biofilms present and to have also a saponification effect against lipids (Thomas & Sathian, 2014). To remove the soda residues, a new rinse with water is carried out before circulating a nitric acid solution (0.5 to 1.5% from 50 to 65°C for a defined time), allowing the removal of inorganic residues (salts, ions ...) and especially to have a bactericidal effect. A final rinsing is carried out to guarantee the absence of cleaning solution in the different parts of the process. Some CIP protocols are complemented by rinsing with additives, usually enzymes, for better cleaning or disinfection (Table 1).

Table 1 : Different CIP procedures described in bibliography (P. Bremer, Fillery, & McQuillan, 2006; Burgess, Lindsay, & Flint, 2010; Faille et al., 2001; S. H. Flint, Bremer, & Brooks, 1997; Malek, 2019; Marchand et al., 2012; P. M. Murphy et al., 1999; Ribeiro et al., 2019; Scott, 2005; Scott et al., 2007; R. P. Singh & Zorrilla, 2011; Tetrapack, 2019; Wedel et al., 2020; Wirtanen, Husmark, & Mattila-Sandholm, 1996). RT : Room Temperature

Principal author	1 st Rinse	1 st Wash	2 nd Rinse	2 nd Wash	3 rd Rinse
Bremer		1% NaOH 65°C 10min	5min RT water	1% nitric acid 65°C 10min	5min RT water
Burgess	water hot	1.5% NaOH 75°C 30min	hot water	0.5% nitric acid 70°C 20min	hot water
Faille	water 5min 0.5m.s ⁻¹	1% Galor 50°C 10min 1m.s ⁻¹	water 5min 0.5m.s ⁻¹		
Flint	water RT	1.5% NaOH 70-80°C	water	0.5% nitric acid 60°C	Bleach 200ppm 10 -20°C
Malek	water RT	2% NaOH 80°C 10min	water	1% nitric acid 70°C 5 min	water
Marchand	water	1% NaOH 65°C 10min	water	1% nitric acid 65°C 10min	water
Murphy		2% NaOH 75°C 30min	water	1.5% nitric acid 65°C 30min	water
Ribeiro	Pre rinse 1min RT water	Anionic tensioactive detergent 7min 40°C	RT water 1min	Sanitizer (sodium hypochlorite) 10min RT	
Ribeiro	Pre rinse 1min RT water	Anionic tensioactive detergent 7min 40°C	RT water 1min	Acid detergent 10min 40°C	Rinse 1 min RT + Sanitizer sodium hypo 10min RT
Scott (1)	hot water	1.5% NaOH 75°C 30min	hot water	0.5% nitric acid 70°C 20min	hot water
Scott	water	1.6% NaOH	water	0.9% nitric acid	water
Singh	hot water 10min	alkaline detergent 0.5- 1.5% 30min 75°C	hot water 5min	nitric acid 0.5-1.5% 20min 70°C	RT water 8min
Tetrapack	hot water 10min	0.5-1.5% NaOH 75°C 30min	hot water 5min	0.5-1% nitric acid 70°C 20min	RT water
Wedel	water 50°C 5min	alkaline detergent 1% 70°C 20min	water	1% acid detergent 65°C 25min	water
Wirtanen	RT water ->75°C 5min	1% NaOH 75°C 7min	RT water -> 70°C 15min	0.8% nitric acid 70°C 4min	RT water 5min

5.4. Bacterial effect of CIP agents on thermophilic spore forming bacteria

First of all, the presence of biofilms limits the bactericidal effectiveness of cleaning because bacteria and spores are protected by the extracellular matrices of the biofilm, reducing their inactivation (Watkinson, 2009).

The effectiveness of treatments applied during CIP on strains of *A. flavithermus*, *G. stearothermophilus* and *B. licheniformis* have been presented and quantified by different authors and referenced in the Table 2 below using different protocols and CIP solutions.

For Sodium hydroxide solutions at 0.5% at 65°C during 10min of contact with spore suspension have a major effect on *G. stearothermophilus* with a reduction of 1.43log of spores.mL⁻¹ compared to *A. flavithermus*, which spores are reduced of only 0.33 log spores.mL⁻¹. If the concentration of soda was increased, from 0.5 to 2%, the reduction between strains is lower for *A. flavithermus* with a 2.75log of spores.mL⁻¹ reduction versus 3.97 log of spores.mL⁻¹ inactivation for *G. stearothermophilus* (Wedel, Wenning, Dettling, Scherer, & Hinrichs, 2019). Results obtained from *G. stearothermophilus* in other studies are lower with a reduction of a reduction of 2log of spores.mL⁻¹ on *G. stearothermophilus* D4 spore suspension at 1% of NaOH at 65°C during 10min (Seale, Flint, James Mcquillan, & Bremer, 2011), and 2.6log of CFU.cm⁻² on stainless steel fouled surface with a 2% NaOH treatment (Hinton, Trinh, Brooks, & Manderson, 2002). CIP resistance of *B. licheniformis* have also been tested in Zou & Liu (2018) study and report a 2.5log CFU.mL⁻¹ reduction after a treatment of 2% at 80°C during 20min on biofilms formed in synthetic media.

For acid treatments, data are not numerous but *A. flavithermus* and *G. stearothermophilus* spore resistance seems equal with 5 and 5.4log of spores.mL⁻¹ reduction after a treatment with 0.5% HNO₃ at 65°C during 10min (Wedel et al., 2019). Only one study (Zou & Liu, 2018) refer treatments applied on *B. licheniformis* biofilms formed in synthetic media with an inactivation of 3.5 log CFU.mL⁻¹ after a 1.5% HNO₃ treatment at 80°C during 20min.

The addition of enzymes in the alkaline solution or disinfectants in acid solutions have a low impact in *A. flavithermus* biofilm removal and increase the inactivation by 0.3 log CFU.g⁻¹ on fouling material (Wedel et al., 2020).

Table 2 : Data obtained for CIP resistance of *A. flavithermus*, *G. stearothermophilus* and *B. licheniformis* species depending on strains, material to treat, CIP parameters and log inactivation. RT: Room temperature; TSB: Tryptic Soy Broth.

Species	Strains	Treated cells	CIP product	Concentration	Temperature	Time of treatment	Log inactivation	References
<i>A. flavithermus</i>	Avg. of 6 strains	Spore suspension	NaOH	0.5%	65°C	10min	0.33 log spores.mL ⁻¹	(Wedel et al., 2019)
	Avg. of 6 strains	Spore suspension	NaOH	1%	65°C	10min	0.61 log spores.mL ⁻¹	
	1958	Fouling skim milk	Alkaline	1%	RT	1min	1 log CFU.g ⁻¹	(Wedel et al., 2020)
	1958	Fouling skim milk	Alkaline / enzymatic	1%	RT	1min	1.3 log CFU.g ⁻¹	
	Avg. of 6 strains	Spore suspension	NaOH	2%	65°C	10min	2.75 log spores.mL ⁻¹	(Wedel et al., 2019)
	Avg. of 2 strains	Spore suspension	HNO ₃	0.5%	65°C	10min	5 log spores.mL ⁻¹	
	1958	Fouling skim milk	Acid cleaning	1%	60°C	5min	1.4 log CFU.g ⁻¹	(Wedel et al., 2020)
	1958	Fouling skim milk	Acid disinfection	1% + 0.1%	60°C	5min	1.7 log CFU.g ⁻¹	
<i>G. stearothermophilus</i>	Avg. of 5 strains	Spore suspension	NaOH	0.5%	65°C	10min	1.43 log spores.mL ⁻¹	(Wedel et al., 2019)
	Avg. of 5 strains	Spore suspension	NaOH	1%	65°C	10min	2.79 log spores.mL ⁻¹	
	Avg. of 5 strains	Spore suspension	NaOH	2%	65°C	10min	3.97 log spores.mL ⁻¹	
	B12-Cm	Fouled surface	NaOH	2%	65°C	10min	2.6 log CFU.cm ⁻²	(Hinton et al., 2002)
	G8742	Spore suspension	HNO ₃	0.5%	65°C	10min	5.4 log spores.mL ⁻¹	(Wedel et al., 2019)
<i>Geobacillus sp.</i>	D4	Spore suspension	NaOH	1%	65°C	10min	2 log spores.mL ⁻¹	(Seale et al., 2011)
<i>B. licheniformis</i>	/	Biofilms (TSB)	NaOH	2%	80°C	20min	2.5 log CFU.mL ⁻¹	(Zou & Liu, 2018)
	/		HNO ₃	1.5%	80°C	20min	3.5 log CFU.mL ⁻¹	

5.5. Effect of CIP on *in situ* biofilms

The analysis of pure strains allows to understand their resistance to CIP treatment, however this does not fully reflect industrial reality where biofilms are mostly multi-species. This is why various studies have been conducted on this subject.

After reconstitution of a milk powder, Bremer et al., (2006) have passed in stainless steel tubes, a flow of milk for 18 hours at 55 ° C, mimicking industrial conditions with indigenous bacteria. The effects of different cleaning treatments were evaluated, using caustic soda solutions (1% 65°C for 10min), nitric acid (1% 65°C 10min), commercial solutions (soda, disinfectants, foaming agents). Addition of a chelating and wetting agent enhance destruction of the adhered bacteria (destruction of 3 log.cm²) by soda treatment followed by of nitric acid (destruction of 2 log.cm²). The best removal of biofilm is achieved after contact of the biofilm with a soda solution containing a wetting and chelating agent and then, after washing, with a nitric acid solution containing surfactants (destruction of 3.8 log.cm²).

The vicious cycle caused by incomplete cleaning is illustrated in Figure15.

A CIP must therefore eliminate as much as possible the bacteria and spores present in a biofilm, without neglecting the elimination of residual milk matter that can promote from 10 to 100 times the adhesion of cells to stainless steel (S. H. Flint, Palmer, et al., 2001) and also of the adhered dead cells that can have the same consequence (Hinton et al., 2002).

Even if the cost of fouling and CIPs have been evaluated at more than 80% of the production costs due to the loss of process efficiency, energy and quality (Van Asselt & De Jong, 2005), it is very important to change CIP solutions as often as possible because the presence of dairy compounds in soda solutions decreasing the sporicidal effect but does not have an impact on the effect of nitric acid solutions (Wedel et al., 2019). In addition, an incomplete CIP treatment leaving some of the biofilm on the stainless steel could allow a spread of 2.9 log CFU.m² in 1 hour to a treated milk during a new production (Wedel et al., 2020).

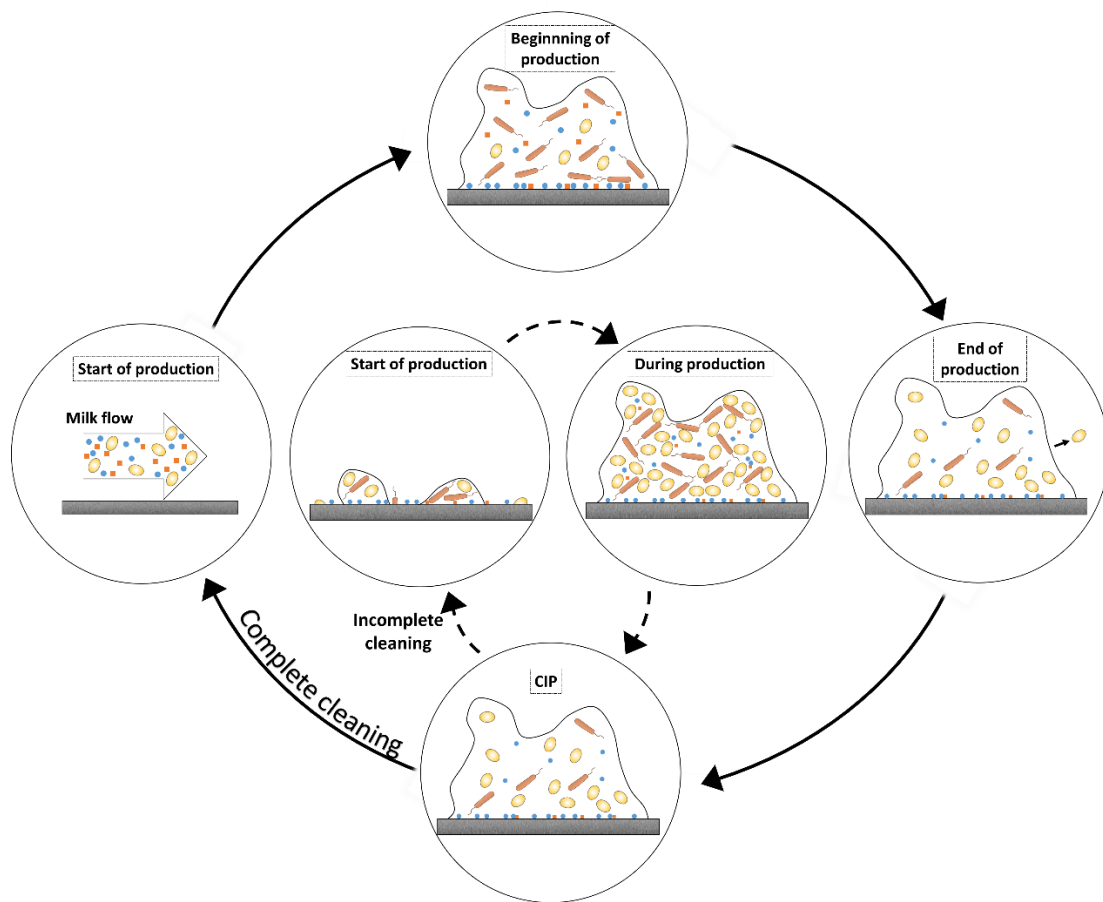


Figure 15: Biofilm formation and proliferation on stainless steel in dairy industry. Inspired from (Wedel et al., 2020). — : vegetative bacteria; ● : bacterial spores; ● : milk proteins; ■ : milk salt

6. Powder quality and use

Powders are highly dehydrated products and this property ensures their stability, particularly at the microbiological level. Only Enzymes such as lipases can alter powders during storage (Bracco et al., 2020). However, powders of dairy origin are also ingredients for many food products. Those usually have a higher water content and therefore the presence of spores or enzymes can cause their spoilage. The growth limits, already presented (see 4.3 to 4.5), of the different species can predict the risk involved. Other parameters must be taken into account such as the heat resistance of the spores (for sterilized products) or the limits of the enzymes produced by these species and which can be found in these products

6.1. Heat resistance of spores

The heat resistance of bacterial spores varies depending on the species. As Warth et al., (1978) noticed, there is a relationship between the heat resistance of bacterial spores and the cardinal

values of growth temperature. So thermophilic species are known to produce extremely heat-resistant spores.

However, this characteristic does not intervene directly at the level of the powder manufacturing processes since the heat treatments undergone by the product remain limited. But, many products, including canned foods, use dairy powders as ingredients and these can be a source of highly heat-resistant spores. Thus cases of instability at 55 ° C could be attributed to them. This aspect remains scarcely documented. In canned food spoilage, *G. stearothermophilus* is often incriminated (flat sour) and the contamination could originate from powdered milk and dehydrated ingredients (Postollec et al., 2012; Rueckert, Ronimus, & Morgan, 2005a). Lastly, *Anoxybacillus flavithermus* was identified exclusively from spoiled fish and vegetable soups (André, Zuber, & Remize, 2013).

Heat resistance of bacterial spores is highly variable according to species and strains but also according to the environmental conditions of sporulation, heat treatment and recovery parameters (Baril, Coroller, Couvert, Leguérinel, et al., 2012; Condon, Bayarte, & Sala, 1992; Iciek, Papiewska, & Molska, 2006; Igura, Kamimura, Islam, Shimoda, & Hayakawa, 2003; Mafart, Couvert, & Leguérinel, 2002; Trunet et al., 2015; Warda, Tempelaars, Abee, & Nierop Groot, 2016). However, in order to compare thermoresistances between species, the numerous bibliographic data allow to determine thermoresistance ranges at a given processing temperature (DT_{tref} parameter values) as well as a range of thermo-sensitivity (zT parameter values).

Even if the thermoresistance of a strain depends on its cellular history, this capacity seems mainly related to genetic factors, such as the presence of spoVA and spoVA2mob operons in some *Bacillus* species. For *Bacillus amyloliquefaciens* and *B. licheniformis*, the higher the number of operons in the genome, the higher the thermoresistance will be (Erwin M Berendsen, Boekhorst, Kuipers, & Wells-Bennik, 2016). This operon also impacts the resistance of strains to high pressures (Z. Li, Schottroff, Simpson, & Gänzle, 2019).

In the figure below (Figure 16), the thermoresistance of spores of the three species is presented according to two parameters. The D_{100°C}, which is the decimal reduction time of spores at 100°C, expressed in minutes, corresponding to the time of treatment to destroy 1 log of spores

at 100°C. And the parameter z_T which corresponds to the temperature to vary the D value by a factor 10.

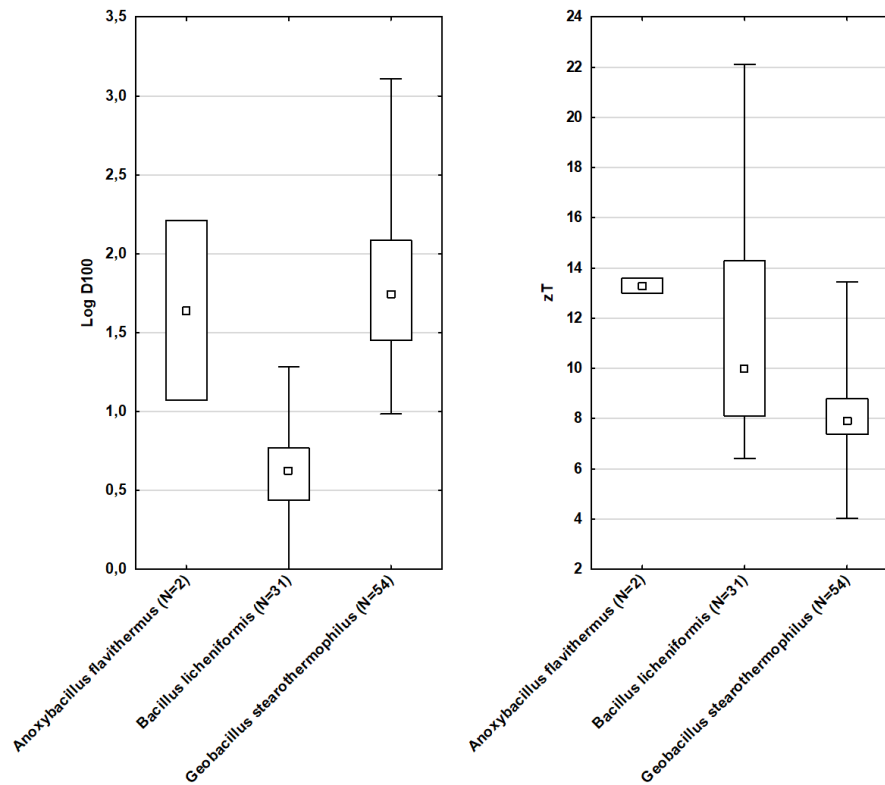


Figure 16: Thermoresistance parameters (z_T and $\text{Log}D_{100}$) of spore of three species : *Anoxybacillus flavithermus*, *Bacillus licheniformis* and *Geobacillus stearothermophilus* (Anderson & Friesen, 1974; André, LeBa, & Zuber, 2009; André et al., 2013; Erwin Mathijs Berendsen, 2016; Couvert & Mafart, 2002; Davies, 1975; Davies, Underwood, Perkin, & Burton, 1977; Feeherry, Munsey, & Rowley, 1987; Fernandez, Ocio, Sanchez, & Martinez, 1994; Haas, Behnsilian, & Schubert, 1996; Janštová & Lukášová, 2001; Le Jean, Abraham, Debray, Candau, & Piar, 1994; Leguérinel, Couvert, & Mafart, 2007; Leuschner, O'callaghan, & Arendt, 1998; Lopez, Martinez, Gonzalez, Martin, & Bernardo, 1998; López, Mazas, González, González, & Bernardo, 1996; Mikolajcik, 1970; Montville & Sapers, 1981; Nakayama, Yano, Kobayashi, Ishikawa, & Sakai, 1996; Ocio, Fernández, Rodrigo, & Martínez, 1996; A. Palop, Raso, Pagán, Condón, & Sala, 1996; Periago, Leontidis, Fernández, Rodrigo, & Martínez, 1998; Raso, Palop, Bayarte, Condón, & Sala, 1995; Rodrigo, Rodrigo, Fernández, Rodrigo, & Martínez, 1999; Rodriguez, Cousin, & Nelson, 1993; Sadiq, Li, Liu, Flint, Zhang, Yuan, et al., 2016; Tanaka, Ito, & Kamikado, 2012; Tejedor, Rodrigo, & Martínez, 2001; Wallace, Nordsiden, Wolf, Thompson, & Zottola, 1978; Wescolt, Fairchild, & Foegeding, 1995; Yu Zhao et al., 2013)

The data present on the thermoresistance (Figure 16) of *A. flavithermus* spores are few (N=2). D_{100} values range from 11.8 to 162.8min with a median value of 87.3min. The z_T values are very close between the two studies, with a minimum z_T of 13.0°C and a maximum z_T of 13.6°C. For *G. stearothermophilus* spores (N=54), D_{100} values range from 9.6 to 1282.6min with a median value of 55.4min. z_T values range from 4.02 to 13.45 with a median value of

7.9°C. The data obtained on spores of *B. licheniformis* strains are slightly variable. D100 values (N=31) range from 0 to 19.2 with a median value of 4.2min, while zT values vary from 6.4 to 22.1°C with a median value of 10°C.

Data obtained for *B. licheniformis* show that the thermoresistance of those spores is significantly lower than those of *A. flavithermus* and *G. stearothermophilus* and therefore less likely to cause non-stability in products heat-treated at high temperatures. A high variability of the zT parameter appears within this species. Thermoresistance data of *G. stearothermophilus* are highly variable, which may be related to the diversity of spore origin studied.

6.2. Spoilage activity

The spoilage enzymes produced by thermophilic spore-forming bacteria can have a significant effect in the use and reconstitution of dairy powders in industry. They can come from spore re-growth during rehydration or they can be pre-formed and persist in powders and be reactivated during rehydration.

In the search for enzyme activity on specific agar media, De Jonghe et al., (2010) evaluated the enzymatic activities for *A. flavithermus* species which were identified as lipolysis and β -galactosidase, present in more than 50% of the isolates tested (N=3). These authors also determined the ability of *G. stearothermophilus* species to have proteolysis, lipolysis and β -galactosidase activities on 20-49% of the isolates tested (N=21). For *B. licheniformis* species (N=54) all of them have β -galactosidase, lipolysis, and proteolysis activity. These results are comparable with those obtained by Lücking et al., (2013).

In addition, enzyme production is generally evaluated only in single species culture. However, in industrial processes, species are present in microbial communities. Teh et al., (2012, 2011) demonstrated that the production of spoilage enzymes is increased when dairy strains form biofilms in coculture VS monoculture. Results correlated with those of Abate et al., (1999) on *Bacillus amyloliquefaciens* and *Zymomonas mobilis* biofilms.

Although native enzyme can be present and incriminated in powders spoilage, bacterial enzymes can participate to this phenomena. Thomas et al, 2004 published a review about the aging of powders, including that linked to enzymatic activities. They indicated that after drying,

enzymes are less solvated, and molecular mobility is decreased. Compared to milk, enzymatic activities are lower in milk powders. The enzymatic activities that are the most important in milk powders are lipolysis and proteolysis. Lipolysis is observed even in a restricted water environment, such as in food powders. Celestino et al., (1997) have studied lipase activity and the content in free fatty acids in whole milk powders. Lipase activity was found to be stable throughout eight months of storage. Free fatty acids were constantly liberated from triglycerides by lipases and accumulated during powder storage. This is detrimental to milk powder quality, since free fatty acids readily oxidize and bring off-flavors. Celestino et al., (1997) noticed that the storage of whole milk powders did not significantly affect the proteolytic activity. Non-protein nitrogen content, which reflects protein degradation, increased during the storage of milk powders. Proteolytic activity also was observed in reconstituted milk. Thus, proteinases remain stable in milk powder upon storage. This is detrimental to the quality of reconstituted milk, as some peptides are known to be responsible for bitter flavors.

6.3. Heat resistance of enzymes

In the dairy environment or during the production of dry milks, mesophilic or thermophilic sporeforming *Bacillus* species release exo-cellular enzymes that can alter the quality of dry milks. Produced by the species *A. flavithermus*, *G. stearothermophilus* or *B. licheniformis*, these enzymes have been isolated purified and their characteristics quantified (see Table 3).

The β -galactosidase hydrolyze lactose into glucose and β -galactose, sources of carbon allowing the development of microorganisms in milk, products or dairy environments. As previously mentioned, the majority of strains of *B. licheniformis*, *G. stearothermophilus* and all strains of *A. flavithermus* isolated from milk powder by Sadiq et al., (2016) show an activity of β - galactosidase. These enzymes produced by these three species appear thermostable (W. Chen et al., 2008; Rani, Sharma, & Dev, 2019). It should be noted that the optimal pH of β -galactosidases activity is close to the milk pH of 6.7 (W. Chen et al., 2009; Goodman & Pederson, 1976; Juajun et al., 2011; Tran et al., 1998).

The proteolytic activities of strains of *A. flavithermus*, *G. stearothermophilus* or *B. licheniformis* isolated from dry milk powder were detected by Sadiq et al., (2016). The proteolytic activities of the enzymes produced by these microorganisms are diverse. Proteases

produced by the species *B. licheniformis*, *G. stearothermophilus* isolated from dairy products or milk powder production lines are thermostable with decimal reduction times at 70°C ranging from 51.6 minutes to 132.6 minutes (L. Chen, Coolbear, & Daniel, 2004; L. Chen, Daniel, & Coolbear, 2003; Chopra & Mathur, 1984). It should be noted that no published studies have quantified proteolytic activities produced by *A. flavithermus*.

The lipolytic activities of strains of *A. flavithermus*, *G. stearothermophilus* or *B. licheniformis* isolated from dry milk were detected and quantified by Sadiq et al., (2016). The lipolytic activities of the enzymes produced by these microorganisms isolated from dairy products or milk powder production lines are more active on short-chain fatty acid esters (L. Chen et al., 2004). Lipases produced by the species *B. licheniformis*, *G. stearothermophilus* appear more thermostable than the proteases produced by these same strains, but all can resist to pasteurization treatment: 72°C 2min (L. Chen et al., 2004).

Because of their high optimal temperature, enzymes produced by species *A. flavithermus*, *G. stearothermophilus* or *B. licheniformis* isolated from dairy powders or dairy environments are not inactivated by the temperatures encountered during the pasteurization, evaporation and drying steps carried out during the manufacturing processes of dairy powders. These enzymes can still be found active in dry milk. For the enzymes produced by these three species the optimal temperatures of activity of β galactosidases and lipases (median value of 50°C and 60°C) are lower than for proteases (median value of 70°C). The optimal pH values for the activity of proteases and lipases are alkaline (median 9 and 8.5). Besides, the optimal pH values for the activity of β galactosidases (median 6.75) are close to the pH of milk. During storage of dry milk powders, the activities of β galactosidase, proteases and lipases will be strongly reduced because the environmental conditions of temperature pH and low water content encountered are far from the optimal conditions of activity of these enzymes. However, their residual activities during long storage periods could lead to an alteration of the dry milk (L. Chen et al., 2003). Enzymes could also be fully re activated after dairy milk reconstitution with water during other food production.

Table 3: Compilation of data on the optimal parameters (T°C and pH) for an enzymatic activity of lipase protease and β -galactosidase on three species : *A. flavithermus*, *B. licheniformis* and *G. stearothermophilus*. *N/C : Native or Cloned

Enzyme	Species	Strain	N/C*	T°C opt (min/max)	pH opt (min/max)	Reference
β -galactosidase	<i>A. flavithermus</i>	PW10	N	60 (4/80)	9 (3/11)	(Rani et al., 2019)
		DSM13	C	50 (4/80)	6.5 (3/11)	(Juajun et al., 2011)
	<i>B. licheniformis</i>	ATCC 9800	C	45 (4/80)	5.8 (3/11)	(Trân et al., 1998)
		PS7	N	60 (4/80)	7 (3/11)	(Rani et al., 2019)
	<i>G. stearothermophilus</i>	ATCC8005	C	70 (30/80)	7 (5.5/8)	(W. Chen et al., 2008)
		AT 7	N	65 (30/80)	6.2 (5.5/8)	(Goodman & Pederson, 1976)
Protease	<i>B. licheniformis</i>	USC13	N	37	7.5	(Ageitos, Vallejo, Sestelo, Poza, & Villa, 2007)
		MIR29	N	60	12	(Ferrero, Castro, Abate, Baigori, & Siñeriz, 1996)
		VSG1	N	45	9	(Sangeetha, Geetha, & Arulpandi, 2010)
		NH1	N	70	10	(Hmidet et al., 2009)
		NH1	N	70	10.5	(Hadj-Ali et al., 2007)
		MP1	N	70	10	(Jellouli et al., 2011)
	<i>G. stearothermophilus</i>	WF146	N	80	8	(Tang, Zhou, Chen, Dai, & Peng, 2000)
		RM-67	N	70	8	(Chopra & Mathur, 1984)
		F1	N	85	9	(Rahman et al., 1994)
		TLS33 (Prot. S)	N	70	8.5	(Sookkheo, Sinchaikul, Phutrakul, & Chen, 2000)
		TLS33 (Prot. N)	N	85	7.5	(Sookkheo et al., 2000)
		TLS33 (Prot. B)	N	90	7	(Sookkheo et al., 2000)
		C	65	7.5	(M. Zhang, Zhao, Du, Lu, & Gao, 2008)	
Lipase	<i>A. flavithermus</i>	HBB 134	N	50	9	(Bakir & Metin, 2016)
		H1	N	55	10	(Khyami-Horani, 1996)
	<i>B. licheniformis</i>	VSG1	N	55	9	(Sangeetha et al., 2010)
		RSP-09	C	40	10	(Madan & Mishra, 2009)
			C	60	9	(G. Kaur et al., 2016)
			N	45	9	(Annamalai, Elayaraja, Vijayalakshmi, & Balasubramanian, 2011)
	<i>G. stearothermophilus</i>	AB-1	N	35	7	(Abada, 2008)
		5	N	60	7	(Berekaa, Zaghoul, Abdel-Fattah, Saeed, & Sifour, 2009)
		MC7	N	75	7.5/9	(Kambourova, Kirilova, Mandeva, & Derekova, 2003)
		JC	C	55	9	(Y. Jiang, Zhou, & Chen, 2010)
L1		C	62.5	9.5	(Kim, Park, Lee, & Oh, 1998)	
	P1	C	55	8.5	(Sinchaikul, Sookkheo, Phutrakul, Pan, & Chen, 2001)	

7. Conclusion

Dairy powders are widely produced and marketed around the world. They therefore constitute an important part of the activity of dairies. The processes implemented involve very large stainless steel structures, representing huge surfaces, potentially colonizable by microorganisms. These surfaces are maintained at fairly high temperatures in a stream of pasteurized milk or whey. These conditions are favorable mainly for three species of thermophilic spore-forming bacteria: *Anoxybacillus flavithermus*, *Geobacillus stearothermophilus* and *Bacillus licheniformis*, although other species can occasionally be found. Prevalence studies show that the latter, probably because of its lower growing temperatures, may originate in raw milk through contamination of the production environment. On the contrary the two others, clearly thermophilic, are nearly absent in the raw material but found a very favorable habitat in the industrial environment. Indeed, these microorganisms adhere to stainless steel and sporulate rapidly in a biofilm. Their elimination by the cleaning-in-place operation therefore constitutes a real challenge. In this sense, they are rightly considered as indicators of the cleanliness of the facilities. They also constitute a real problem because of their potential to spoil powders and products using dairy powders as ingredients. Acidification, proteolysis and lipolysis have been attributed to them. In addition, the significant heat resistance of their spores and enzymes constitutes a threat for canned products using these dairy ingredients. Although the danger is clearly identified and manufacturers are on the lookout for information to control them, knowledge of their diversity and the diversity of their behavior remains incomplete. This is particularly true for *Anoxybacillus flavithermus* which has been described relatively recently. Their non-pathogenic nature has also put them in the background until now. But it is important to fill these gaps because they constitute a real economic problem and can even be an obstacle to innovation.

8. Conclusion du Chapitre 1

Ce chapitre bibliographique a permis de faire le point sur l'état de l'art concernant les microorganismes associés aux installations industrielles et aux poudres laitières.

Au niveau mondial, la prédominance et l'origine des 3 espèces majeures : *Anoxybacillus flavithermus*, *Geobacillus stearothermophilus* et *Bacillus licheniformis* ne font aucun doute. Mais les poudres françaises sont très peu représentées dans les études réalisées et il semblait important de valider sur des échantillons issus d'entreprises françaises ces conclusions, ainsi que de récupérer des souches industrielles permettant par la suite d'étudier la diversité des comportements. En effet, comme indiqué dans la conclusion de cette revue, ces microorganismes non pathogènes restent moins étudiés (tout particulièrement *Anoxybacillus flavithermus*) que leur « collègue sporulé » pathogène mésophile *Bacillus cereus*.

C'est pourquoi le Chapitre 2, visera dans un premier temps à évaluer, à partir d'échantillons de diverses poudres laitières française, la concentration en bactéries sporulées mésophiles et thermophiles et la diversité d'espèces de ces contaminants. Une première analyse de la diversité phénotypique, portant sur le potentiel altérant des isolats récoltés, y sera également proposée.

Chapitre 2 : Prévalence, diversité génotypique et potentiel d'altération des contaminants sporulés thermophiles dans les poudres lactières françaises

La contamination des poudres lactières par les bactéries sporulées thermophiles est étudiée dans de nombreux pays et a permis d'identifier *Anoxybacillus flavithermus*, *Geobacillus stearothermophilus* et *Bacillus licheniformis* comme étant les plus présentes dans différents types de poudres analysées (Dettling et al., 2019; Miller, Kent, Boor, et al., 2015; Rueckert, Ronimus, & Morgan, 2005b). Cependant la contamination des poudres lactières par les bactéries sporulées thermophiles au niveau français a été très peu étudié (Rückert et al., 2004) et les études réalisées ont porté principalement sur les poudres de lait entier ou écrémé, et très peu sur les autres poudres lactières comme les poudres de lactosérum ou les poudres de protéines lactières.

Toutes ces poudres lactières ont des propriétés fonctionnelles et organoleptiques intéressantes (agent de texture : émulsifiant, épaississant/gélifiant ; exhausteur de goût, agent brunisseur...) et sont fortement utilisées comme ingrédients en industrie agroalimentaire (fabrication de sauces, de produits de boulangerie, viennoiserie et pâtisserie, de produits laitiers ou encore dans la transformation de viande). Elles sont omniprésentes dans le domaine alimentaire et représentent la 5^{ème} famille d'ingrédient les plus utilisés dans les formulations (derrière le sel, le sucre, l'eau, les matières grasses, selon OpenFoodFacts).

Lorsque ces poudres sont réhydratées, et si les conditions sont favorables à leurs croissances, les spores peuvent germer, se développer et retrouver une activité métabolique pouvant altérer

la qualité et sécurité des aliments. Dans le cas des espèces sporulées thermophiles étudiées dans ce manuscrit, il n'y a pas d'impact sanitaire associé à ces contaminations mais c'est la production d'enzymes non désirées par l'industriel qui peut fortement impacter les qualités techno-fonctionnelles des ingrédients ou les qualités organoleptiques du produit fini, notamment par une acidification de la matrice. Cependant il est difficile de décrire les différents types d'altération produite à la suite du développement de ces contaminants car cela dépend grandement de la composition de l'aliment. Dans le cadre industriel, les contaminations en spores thermophiles sont habituellement suivies en tant qu'indicateurs d'hygiène des lignes de production et la distinction des espèces présentes n'est pas réalisée sauf en cas d'identification de la flore persistante aux procédures de sanitation ou altération majeure du produit fini produit à partir de ces poudres.

L'objectif de ce deuxième chapitre est d'évaluer le niveau de contamination en spores mésophiles et thermophiles des poudres laitières produites en France. Ainsi des échantillons de poudres laitières ont été collectés auprès de différents industriels de manière volontaire. En plus des poudres de lait entier ou écrémé de lait de vache, des poudres de lait de jument, de brebis et de chèvre, plus atypiques ont été collectées ainsi que des poudres de caséines, caséinates, protéines laitières et poudres infantiles. Cette phase de collecte et d'analyse des échantillons a permis la constitution et caractérisation d'une banque d'isolats bactériens.

Ce chapitre comporte la **description des méthodes** utilisées pour : la récolte des échantillons, le dénombrement des sporulés des poudres laitières (dont une courte étude bibliographique), l'étude de la prévalence des bactéries sporulées dans les poudres laitières, ainsi que la constitution de la souchothèque et sa caractérisation moléculaire et phénotypique. Les résultats de prévalence et biodiversité des spores thermophiles ayant été publiés sous forme **d'une « short communication »**, présentée telle quelle dans ce chapitre, ils sont complétés par une **partie résultats complémentaires**. Celle-ci comprend des points sur la prévalence des spores mésophiles hautement thermorésistantes, la biodiversité moléculaire rencontrée ainsi que le « potentiel d'altération » (caractérisation des capacités enzymatiques) de souches choisies au sein des trois espèces majoritaires.

1. Méthodologie

1.1. Collecte des poudres laitières

Afin d'évaluer la contamination en spores des poudres laitières françaises, une campagne d'échantillonnage a été effectuée en demandant des poudres aux entreprises productrices en France. Les échantillons ont donc été obtenus au bon vouloir de chaque entreprise, sans plan d'analyse préalable. Ce dernier point rendant l'analyse statistique des résultats délicate, mais présente l'intérêt d'étudier une grande diversité de types de poudres laitières. Ainsi cette collecte a permis l'obtention de poudres laitières plus atypiques issues de filières plus courtes comme celle de la production de poudres de lait de jument (WMPm), de chèvre (WMPg) ou de brebis (WMPs). De même des poudres de lactosérum (WP), protéines laitières (MPC), caséines (CP), caséinates (CtP), protéines de lactosérum (WPC) et perméat (PP) ont été étudiées en plus des poudres de lait entier (WMP) et écrémé (SMP) de vache et poudres infantiles (IF) plus fréquemment reportées dans les études bibliographiques. Un total de 19 sites industriels de taille variable ont répondu favorablement à cet appel à collecte et 61 poudres ont été récupérées et stockées à 4°C jusqu'à l'étape de dénombrement et la constitution de la souchothèque. La Figure 17 indique le nombre et type de poudre étudiés.

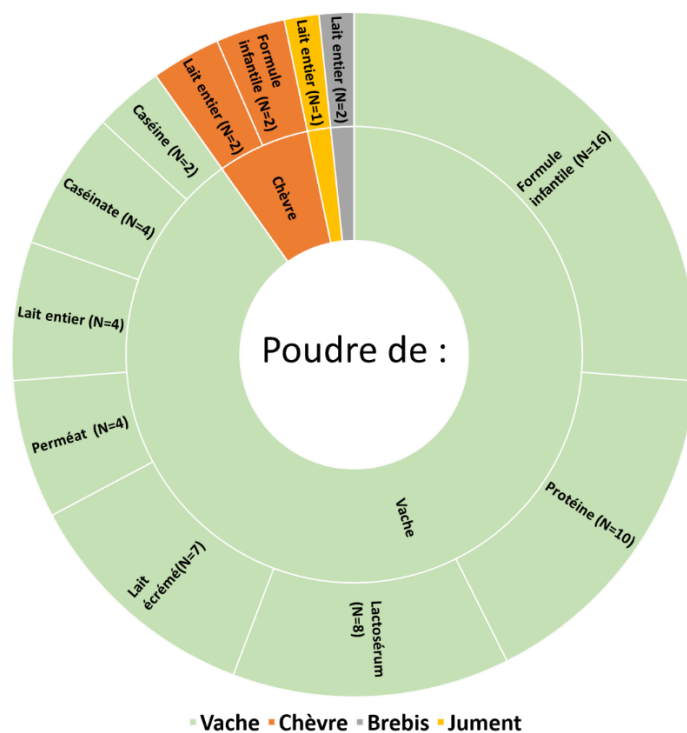


Figure 17: Récapitulatif des poudres obtenues après demande auprès d'industriels français.

1.2. Dénombrement des bactéries sporulées contaminant les poudres laitières

1.2.1. Choix méthodologiques

Après analyse de la bibliographie sur les méthodes utilisées pour le dénombrement des matrices issues de la ferme ou de laiterie, de multiples protocoles de dénombrement des spores par méthode culturale sont utilisés.

Pour le dénombrement des spores et la destruction des formes végétatives, le traitement thermique appliqué est de 80°C pendant un temps variable, *e.g.* 10, 12, 15, 20 ou 30min en fonction des publications (Abd et al., 2014; Buehner et al., 2014; McGuiggan et al., 2002). Pour évaluer la part des spores hautement thermorésistantes, les publications citent des traitements de 100°C ou 106°C pendant 30min (Kent et al., 2016; N. H. Martin et al., 2019). Comme les conditions de traitements thermiques, les milieux de dénombrement et les conditions d'incubation varient. Les milieux gélosés utilisés peuvent être du Plate Count Agar (PCA) (Cho et al., 2018) additionné ou non de lait en poudre (Scheldeman et al., 2005), du Brain Heart Infusion agar (S. I. Murphy et al., 2019; Watterson et al., 2014), du Tryptic Soy Agar (TSA) (Rückert et al., 2004; Wu et al., 2007) additionné ou non d'amidon (Ronimus et al., 2006; Sadiq, Li, Liu, Flint, Zhang, & He, 2016) ou encore du Milk starch agar (Rajput et al., 2009). De plus les conditions d'incubation varient de 30 à 37°C pendant 24 à 72h (Reginensi et al., 2011; Scheldeman et al., 2005) pour les mésophiles avec une incubation à 55°C pendant 16 à 48h pour les thermophiles (Rückert et al., 2004; Yuan et al., 2012).

Tous ces paramètres étant variables d'une étude à l'autre, nous nous sommes donc appuyés sur les normes en vigueur pour la reconstitution des poudres (ISO 6887-5 :2010), pour les traitements thermiques appliqués pour le dénombrement des spores (80°C 10min, NF V08-250, 2010) et spores hautement thermorésistantes (106°C 30min : ISO/TS 27265, 2009) ainsi que pour l'incubation des boîtes de dénombrements à 37°C et 55°C, respectivement pour les flores mésophiles et thermophiles, pendant 24h et 48h (NF V08-602, 2011).

1.2.2. Protocoles retenus

Les dénombrements de spores ont été réalisés après reconstitution des poudres avec un diluant adapté. Le choix du diluant a été fait selon la norme ISO 6887-5 : Préparation des échantillons, de la suspension mère et des dilutions décimales en vue de l'examen microbiologique — Partie

5 : Règles spécifiques pour la préparation du lait et des produits laitiers ». La liste des diluants utilisés en fonction des types de poudres est indiquée dans le Tableau 4. Les diluants utilisés dans cette étude sont le peptone sel, ainsi que l'hydrogenophosphate de potassium sans antimousse.

Table 4 : Liste des diluants à utiliser pour réhydrater les poudres laitières selon la norme ISO 6887-5. Les symboles « ✓ » indique les diluants possibles selon les types de poudre et le terme « NA » indique que le diluant n'est pas applicable pour la poudre correspondante.

Type de poudre	10g de prise d'essai 90mL de diluant	Diluant						
		Peptone sel pH=7	Solution de Ringer pH=6,9	Eau peptonée	Tampon phosphate	Eau peptonée tamponnée	Hydrogenophosphate de potassium pH= 7,5	Solution citrate de sodium
Poudre de lait		✓	✓	✓	✓	✓	NA	NA
Poudre de lait Roller Dried		✓	✓	✓	✓	✓	✓ pH= 7,5	✓
Poudre de lactosérum doux		✓	✓	✓	✓	✓	NA	NA
Poudre de lactosérum acide		NA	NA	NA	NA	NA	✓ pH=8,4	NA
Poudre de babeurre		✓	✓	✓	✓	✓	NA	NA
Poudre de lactose		✓	✓	✓	✓	✓	NA	NA
Poudre de caséine		NA	NA	NA	NA	NA	✓ + antimousse pH=8,4	NA
Poudre de caséinate		✓	NA	NA	NA	NA	✓ pH= 7,5	✓
Poudre infantile		✓ +/- α amylase	NA	NA	NA	NA	NA	NA

La solution laitière est ensuite séparée en deux flacons, l'un subissant un traitement thermique de 80°C 10min (NF V08-250, 2010 : Traitements thermiques préalables au dénombrement ou à la recherche de spores bactériennes) et l'autre subissant un traitement thermique de 106°C 30min (ISO/TS 27265, 2009 : Dénombrement des spores spécialement thermorésistantes des bactéries thermophiles pour les laits secs), comme présenté dans la figure 18. L'utilisation de ces deux traitements couplés à l'incubation des boîtes de gélose à 37 et 55°C permettent, respectivement le dénombrement de spores mésophiles (MSC), de spores mésophiles hautement thermorésistantes (HRMSC) et de spores thermophiles (TSC), spores thermophiles hautement thermorésistantes (HRTSC).

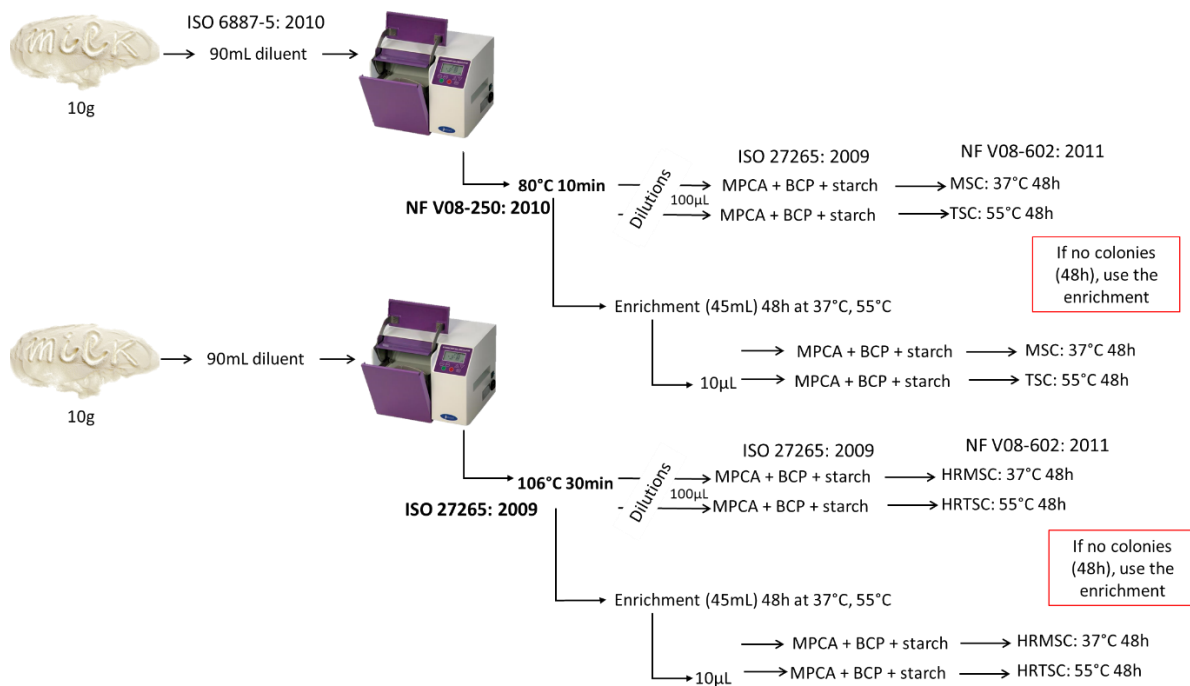


Figure 18: Protocole utilisé pour les dénombrements des spores mésophiles (MSC), thermophiles (TSC), mésophiles hautement thermorésistantes (HRMSC) et thermophiles hautement thermorésistantes (HRTSC)

La montée en température des solutions a été menée dans un bain marie au glycérol dans des flacons fermés hermétiquement et permettant une montée rapide en température avec un suivi de température réalisé dans des flacons témoins. A la fin du traitement, la solution laitière est rapidement refroidie dans un bain d'eau froide, des dilutions en série sont réalisées, puis les dénombrements sont effectués en inclusion (1mL) pour la solution laitière non diluée et en surface pour les dilutions (100µL) sur milieu de dénombrement classique (PCA) supplémenté en lait ainsi qu'en pourpre de bromocrésol et en amidon, comme précisé dans la norme ISO/TS 27265 :2009.

Les dénombrements sont réalisés après incubation des boîtes pendant 24h et 48h à 37°C et 55°C (NF V08-602, 2011). Ces deux modes d'inoculation (surface et inclusion) et cette double lecture permettent le dénombrement des colonies avant envahissement des boîtes de Petri. De plus, un enrichissement des échantillons a été réalisé en parallèle afin de distinguer une contamination inférieure au seuil de détection ($10\text{UFC}\cdot\text{g}^{-1}$) ou une absence de flore recouvrable dans les conditions testées à l'issue du traitement thermique appliqué. Les résultats des niveaux de contamination des spores thermophiles apparaîtront tels que publiés dans le journal à comité

de lecture « International Dairy Journal » (Delaunay et al., 2021) alors que les résultats des contaminations en spores mésophiles hautement thermorésistantes apparaîtront séparément, tout comme ceux de la biodiversité génétique et phénotypique qui seront détaillés et analysés de manière globale.

1.3. Constitution de la banque d'isolat

Après dénombrement des poudres laitières collectées, seuls les isolats issus du dénombrement des spores thermophiles ont été récupérés. La sélection des isolats a été réalisée en fonction du nombre et des différents aspects morphologiques de colonies observés pour chaque boîte de dénombrements aux dilutions comptables. Par exemple, sur une boîte de dénombrement contenant entre 30 et 300 colonies, l'aspect de toutes les colonies est évalué pour ré isoler toutes les colonies ayant un aspect unique au moins une fois. Puis, si plus de 20 colonies ont un aspect identique, alors une nouvelle colonie est isolée. Enfin si plus de 50 colonies ont un aspect identique, alors deux colonies sont isolées et si plus de 80 colonies ont un aspect identique, alors trois colonies sont isolées. Ainsi, sur plus de 3336 colonies analysées, 14 aspects morphologiques principaux (taille des colonies, couleur, aspect, couleur) ont été décrits, permettant d'évaluer la diversité à 87% après calcul du coefficient de Simpson (Hunter & Gaston, 1988). Même si plusieurs espèces peuvent présenter un aspect de colonies similaire, cette sélection a permis de conserver la proportion des différents aspects morphologiques observés. Toutes les 313 souches de la collection ont été purifiées et sont conservées dans un mélange de TSB (50%) et de glycérol à 40% (50%) et stockées à - 80°C.

Cette sélection de souches a par ailleurs été analysée par typage M13 afin de déterminer la diversité génétique présente dans les poudres laitières testées.

Les souches sélectionnées ont ainsi été caractérisées pour leur activité enzymatique (ce chapitre), leur capacité de croissance (chapitre3), leur capacité à former des biofilms et résister aux traitements de sanitation (chapitre4).

1.4. Diversité génétique de la collection

Chacun des isolats composant la souchothèque a été préalablement purifié et l'analyse de typage moléculaire après une nuit de culture, extraction de l'ADN total, amplification de régions ciblées, acquisition d'empreinte moléculaire après migration sur gel et analyse biostatistique permettant la génération de dendrogramme ou regroupement des empreintes a été mené.

1.4.1. Extraction de l'ADN et amplification par M13 PCR

Pour chacune des 313 souches de la collection d'étude, une culture de nuit à 55°C en bouillon TSB a été réalisée. Un volume de 1mL de culture a été centrifugé. La lyse et l'extraction de l'ADN ont été réalisés sur le culot bactérien après ajout de 180µL de tampon de lyse (Tris 1M pH=8 : 2% ; EDTA 0.5M pH8 : 0.4%, Triton 100x : 1.2 %, lysozyme : 2%) et ajout de 1.12% de mutanolysine (Sigma-Aldrich). Les différentes étapes permettant l'extraction ont ensuite été réalisées automatiquement par le QIAcube (QiaGen). Le volume d'élution final a été de 2x50µL. Les ADN extraits ont été calibrés à 10ng.µL⁻¹ grâce au Nanodrop 2000 (ThermoFischer).

Les amplifications ont été effectuées dans un volume final de 50µL, comprenant 1µM d'amorce M13 consensus : 5'-GAGGGTGGCGGCTCT-3' (Vassart et al., 1987); 0.3mM de dNTP ; 3.5mM de MgCl₂ ; 1U de Taq Polymerase ; dilué dans de l'incubation mix 10X Mg free (New England Biolabs); et 100ng d'ADN étaloné. L'amplification a été réalisée par une première étape de dénaturation à 94°C pendant 6 min, puis par 40 cycles avec une dénaturation à 94°C pendant 1 min, puis une hybridation à 45°C pendant 2min puis une amplification à 72°C pendant 2 min. Une extension finale de 10min à 72°C a été appliquée. Un contrôle négatif a été amplifié comprenant le mix de réaction sans ADN. Un contrôle positif composé de l'ADN de la souche *Bacillus weihenstephanensis* KBAB4 a été aussi amplifié et a permis de s'assurer de la conformité et répétabilité des profils obtenus.

Les amplicons ont été alors déposés sur un gel d'agarose (1%) pour migration en électrophorèse dans du TBE 1X (Sigma-Aldrich). L'agent intercalant utilisé dans le gel a été le Bromure d'éthidium (BET)(Sigma-Aldrich), et a été ajouté à une concentration de 0,002% avant de couler les gels. Après dépôt des échantillons, des témoins (positifs et négatifs) et des marqueurs

de taille (DNA Molecular Weight Marker VI) (Sigma-Aldrich), la migration a été réalisée pendant 4h à 100V.

1.4.2. Numérisation des gels et analyse biostatistique pour la détermination de la diversité

Les gels ont ensuite été visualisés et numérisés sous une lampe UV Transluminator 2000 à 302nm couplé à un analyseur d'image : Gel Doc™ (Bio-Rad).

Les photos des gels ont été analysées grâce au logiciel BioNumerics® (version 7.6.3.) et les clusters de différenciation ont été réalisés grâce au coefficient de Person et la transformation de ces coefficients en distance selon la méthode UPGMA(Unweighted Paired Group Method with Arithmetic mean) pour la réalisation du dendrogramme. Les regroupements d'empreintes ou clusters ont été réalisés en appliquant le pourcentage de diversité de 87% calculé lors de la récupération des isolats sur la base des aspects morphologiques observés sur boîte de dénombrement. En fonction des empreintes obtenues, le dendrogramme a classé 302 souches dans 27 clusters différents avec 11 souches individuelles

1.4.3. Séquençage 16S pour identification des isolats représentatifs des clusters

Après l'obtention du dendrogramme, une sélection d'un ou plusieurs isolats par cluster ainsi que les 11 souches n'appartenant à aucun cluster ont été sélectionnées. Cette sélection de 61 souches a permis l'identification des isolats représentatifs de la diversité obtenue après séquençage de l'ADNr 16S et comparaison des séquences aux banques de données internationales. Ainsi, pour chaque isolats sélectionné, les extraits d'ADN ont été utilisés pour amplifier spécifiquement par PCR de la région 16S de l'ADNr ciblées par les amorces W18 (5'-GGAGTTTGATCMTGGCTCAG-3') et W02 (5'-GNTACCTTGTTAACGACTT-3') décrites par Delbès et al., (2000). Les amplifications ont été réalisées dans un volume final de 72.5µL, comprenant 0.5µM d'amorce sens ; 0.5µM d'amorce antisens ; 200mM de mix dNTP ; une solution d'incubation (MgCl₂ :3.5mM) à 1X ; 0.725U de Taq DNA polymérase ; et 50ng d'ADN. L'amplification a été réalisée par une première étape de dénaturation à 98°C pendant 1min 30sec, puis par 35 cycles avec une dénaturation à 98°C pendant 30 secondes, puis une hybridation à 57°C pendant 30sec puis une amplification à 72°C pendant 1 min 30sec. Une extension finale de 5min à 72°C a été appliquée. Le séquençage de ces séquences a été réalisé par Biofidal (Lyon ; France).

Les séquences nucléotidiques obtenues après séquençage ont été comparées avec des banques de données internationales (NCBI, DDBJ, EBI, RDP et LeBIBI), et l'identification des souches est déterminée grâce à la comparaison des résultats de Blast réalisé sur ces banques. L'utilisation de ces différentes bases de données sont équivalente. A noter que l'identification à l'espèce est fonction de la richesse des bases de données et de la taxonomie actuelle.

Tableau 5 : Table récapitulative des isolats obtenus en fonction du type de poudre analysé et des séquençages effectués

	Total	Séquençage
WMP	49	10
SMP	59	10
MPC / WPC	67	13
WP	44	13
CP	8	1
CtP	2	1
PP	1	1
IF	83	12
Total	313	61
Séquençage	61	

1.5. Etude des capacités enzymatiques des souches

Avant chaque manipulation, les souches ont été d'abord repiquées sur une gélose TSA puis dans un bouillon TSB et incubées pendant une nuit à 55°C sous agitation.

La capacité d'altération a été menée en ciblant des carbohydrates et ingrédients retrouvés dans les denrées alimentaires. La sélection de ces composées a été réalisées en fonction des évaluations déjà reportées par différentes études et donnant des résultats de discrimination intéressants.

1.5.1. Choix des souches

De plus, des caractérisations phénotypiques ont portées sur une sélection de 62 souches. Cette sélection a été réalisé afin d'optimiser la diversité rencontrée en fonction du type de poudre et de l'appartenance aux groupes M13. Plus spécifiquement cette sélection contient 32 souches d'*Anoxybacillus flavithermus* (31 souches isolées de poudre laitière + DSM2641), 15 souches de *Geobacillus stearothermophilus* (14souches isolées de poudre laitière + DSM22) et 15

souches de *Bacillus licheniformis* (11 souches isolées de poudre laitière + 2 souches d'origine laitières (banque d'isolats de l'ADRIA) + 1 souche d'origine alimentaire : NVH1032 (Madslie, Olsen, Granum, & Blatny, 2012) + DSM13). Ainsi les caractérisations phénotypiques des souches isolées à partir des poudres laitières analysées dans cette étude ont été comparées à celles des souches type. A noter que les souches types de la collection DSM ont été isolées de source d'eau chaude pour la souche d'*A. flavithermus*, de conserve altérée pour la souche de *G. stearothermophilus* et d'origine inconnue pour la souche de *B. licheniformis*.

Tableau 6: Liste des souches utilisées pour l'évaluation de la diversité phénotypique

	WMP	SMP	WP	MPC	WPC	CtP	CP	PP	IF	Hors poudres	Souches Types	Total
<i>A. flavithermus</i>	3	6	7	1	5	/	/	1	8	/	1	32
<i>G. stearothermophilus</i>	3	4	4	/	/	/	1	/	2	/	1	15
<i>B. licheniformis</i>	3	2	2	/	1	/	1	/	2	3	1	15
Total	9	12	13	1	6	0	2	1	12	3	3	62

Les souches ont été sélectionnées en fonction de leur groupe d'appartenance au cluster M13, de la distance calculée par la classification UPGMA, ainsi que par la poudre dont elles ont été isolées.

1.5.2. Utilisation de carbohydrates

La capacité des souches à utiliser une source de carbohydrate a été réalisé grâce au milieu API 50CHB (Biomérieux, France), milieu synthétique sans source de carbone normalement utilisé pour identifier les bactéries lactiques. Ce milieu a été ajouté dans les puits d'une microplaque 96 puits contenant déjà une solution de sucre stérilisée par filtration et déshydratée (24h sous hotte à flux laminaire) afin que la concentration finale en sucre soit de 1% dans le puits. Les sources de carbohydrates ont été sélectionnées afin d'obtenir une diversité d'utilisation chez *A. flavithermus*, *G. stearothermophilus* et *B. licheniformis* selon les résultats obtenus par Burgess et al., (2016), Flint et al., (2001) et Ronimus et al., (2003).

Les différentes sources de carbohydrates sont les suivantes : D(+)-Glucose; D(-)-Fructose; D(+)-Galactose; D(+)-Maltose; D-Lactose; Saccharose; D(+)-Trehalose; D(+)-Melezitose;

L(+)-Arabinose; D(-)-Ribose; D(+)-xylose; D(+)-Mannose; L(+)-Rhamnose; Mannitol; D(-)-Sorbitol; Inuline; D(+)-Raffinose; D-Cellobiose et Salicine.

Toutes les souches cultivées sur gélose TSA (24h 55°C) ont été resuspendues dans 3mL de milieu API50CHB et réparti (150µL) dans les puits des microplaques 96 puits.

Un témoin négatif de croissance est réalisé en remplaçant la source de carbohydrate par de l'eau et un témoin de contamination est également réalisé en utilisant du API50CHB stérile avec une source de carbohydrate, mais sans ajout de bactéries. Les plaques 96 puits ont été incubées en chambre humide à 55°C pour les souches d'*Anoxybacillus flavithermus* et de *Geobacillus stearothermophilus*, et à 30°C pour les souches de *Bacillus licheniformis*. Une lecture visuelle (virage de l'indicateur coloré du rouge au jaune) a été réalisée après 6 ; 16 ; 24 et 48h.

1.5.3. Utilisation d'ingrédients

En plus des sources de carbohydrates, une sélection de 7 ingrédients a été réalisées pour évaluer un potentiel d'altération des souches laitières en fonction de leur association aux poudres laitières et ingrédients laitiers selon la base de donnée Open Food Facts (Gigandet, 2020). Ainsi, une recherche de l'activité caséinase, lecithinase, tributyrase, gélatinase, amylase, pectinase, cellulase sont menées sur des milieux gélosés. A noter que la dégradation des sucres plus complexes tels que la cellulose, pectine et amidon ont été réalisés sur gélose car trop complexe à mettre en place en microplaque.

La recherche de l'activité caséinase a été effectuée sur un milieu composé d'agar 1.5% autoclavé et de lait écrémé UHT ajouté post autoclave à hauteur de 10% final (Vos et al., 2011). La capacité à pousser sur milieu contenant de la lécithine a été menée sur milieu TSA additionné de jaune d'oeuf recueilli stérilement et ajouté à hauteur de 1.5% final (Mohammad, Al Daghistani, Jaouani, Abdel-Latif, & Kennes, 2017). La recherche de l'activité tributyrase a été réalisée sur milieu composé d'extrait de viande 0.3%, de peptone 0.5%, de tributyrine 1%, et d'agar 2%, pH=7.2+/-0.2 (Deshmukh, 2007). La recherche de la gélatinase a été effectuée sur un milieu gélosé composé d'extrait de viande 0.4%, d'extrait de levure 0.1%, de gélatine 2%, et d'agar 2%, pH=7.2+/-0.2 (Smith & Goodner, 1958). La recherche de la gélatinase a été révélée par ajout d'une solution saturée de sulfate d'ammonium (réaction de Stone 1935). La recherche de l'amylase a été effectuée sur un milieu composé d'amidon 1%, d'extrait de levure 0.2%, de peptone 0.5%, de MgSO4 0.05%, de NaCl 0.05%, de CaCl2 0.015% et d'agar 2% ;

pH=7.2±0.2 (Santos & Martins, 2003). La recherche de l'activité pectinase a été réalisée sur milieu composé de NaNO₃ 0.1%, KCl 0.1%, K₂HPO₄ 0.1%, MgSO₄ 0.05%, d'extrait de levure 0.05%, de pectine 1%, et d'agar 2% (Mohandas et al., 2018). Le pH a été ajusté après dissolution à chaud de la pectine et avant ajout de l'agar à pH=11.4. La recherche de l'activité cellulase a été effectuée sur milieu composé de carbométhylcellulose 1%, d'extrait de levure 0.5%, de peptone 0.5%, et d'agar 1.5%, pH=7.2±0.2 (Hazarika et al., 2019). Les recherches enzymatiques de l'amylase, de la pectinase et de la cellulase ont été révélées après ajout d'une solution d'iode (lugol). Afin d'exploiter les résultats, une mesure a été réalisée au bout de 48 et 96h après avoir mesuré la taille de la colonie et la taille du halo observé.

Les souches ont été repiquées dans 5mL de milieu TSB et après une croissance d'une nuit à 55°C sous agitation pour *A. flavithermus* et *G. stearothermophilus*, et à 30°C pour *B. licheniformis*, un dépôt de 5µL est réalisé en surface des boîtes pour chacune des souches. Les boîtes ont été incubées aux mêmes températures que celles réalisées pour la croissance, à savoir 30°C pour *B. licheniformis* et 55°C pour les deux espèces thermophiles.

1.5.4. Analyse statistique des résultats

Les résultats obtenus pour l'analyse de la phénotypie sont renseignés en tant que données binaires (activité détectée : 1 ; ou absence d'activité enzymatique :0), et les souches sont classées en grâce à une Classification ascendante hiérarchique effectué avec le logiciel Statistica version 13.5.0.17.

2. Short Communication: Occurrence and diversity of thermophilic sporeformers in French dairy powders

Delaunay, L., Cozien, E., Gehannin, P., Mouhali, N., Mace, S., Postollec, F., Leguerinel, I., Mathot, A.-G. (2020). Occurrence and diversity of thermophilic sporeformers in French dairy powders. *International Dairy Journal*, 104889. <https://doi.org/10.1016/j.idairyj.2020.104889>

Abstract

This study reports the prevalence of sporeforming bacteria and contamination levels of a variety of powdered dairy products from cows, ewes, goats and mares' milk produced in France. The concentration of mesophilic spores, thermophilic spores and highly heat-resistant thermophilic spores was assessed in 61 dairy powders. Thermophilic spore concentration was highly variable between powders, likely due to the different manufacturing processes used for transforming milk into dairy powders. The different stages of processing, particularly heat treatment close to 55°C had a strong impact on selection of thermophilic bacteria contaminating dairy powders. For some products, thermophilic spore counts were as high as 5.89 log TSC.g⁻¹. Of the 313 thermophilic isolates selected, 93.3% belonged to the species *Geobacillus stearothermophilus* (43.5%), *Bacillus licheniformis* (30.0%) and *Anoxybacillus flavithermus* (19.8%). These results confirm the presence of the three majority species reported worldwide.

2.1. Introduction

The manufacture of powdered products is an important part of the dairy industry because of their long shelf life and ease of use. France is one of the leading producers and processors of milk in Europe. Although non-pathogenic, spore-forming bacteria and more specifically thermophilic aerobic species have become an important parameter for monitoring production hygiene in this type of product. Three species dominate this group of bacteria. They include *Geobacillus stearothermophilus*, *Anoxybacillus flavithermus* and *Bacillus licheniformis* (Rückert, Ronimus, & Morgan, 2004).

In the dairy industry, the provenance of bacterial spores is highly diverse as spores originate from soil (Heyndrickx, 2011), water (Christiansson et al., 1999) or silage (Te Giffel et al., 2002). The spores can be consumed by cows, resist transit and end up in the faeces, from which they can contaminate the milk, through dirty teats and thus entering milking equipment (Miller, Kent, Boor, et al., 2015). Contamination can also occur during the transport of milk from the farm to the dairy industry (Huck, Sonnen, & Boor, 2008). All these sources of contamination contribute to explaining the presence of bacterial spores in raw milk (Huck, Hammond, Murphy, Woodcock, & Boor, 2007). Concentrations of mesophilic bacterial spores can be as high as $3.88 \log \text{ mL}^{-1}$ in raw milk (McGuiggan et al., 2002). More specifically, thermophilic spores have been found at concentrations ranging from 0.26 to $2.41 \log \text{ spore mL}^{-1}$ in raw milk (Buehner et al., 2014; S. I. Murphy et al., 2019). Those thermophilic spores are mainly represented by *Bacillus licheniformis* but also slightly by *Geobacillus stearothermophilus* or *Anoxybacillus flavithermus* (from not detected to minus than 2% (Chauhan et al., 2013)).

In addition to milk contamination and the concentration effect by processing, it has been shown that spore-forming (especially thermophilic) bacteria can multiply during processes and thereby persist in industrial sites as a biofilm (Scott et al., 2007).

After pasteurisation, which has no effect on spores, the manufacturing processes for dairy powders differ according to the raw material and desired end product.

Milk powders (whole or skimmed) and whey powders are obtained by concentration in evaporators followed by atomization, while milk protein or whey protein powders need an ultrafiltration before concentration and atomization (see McHugh, Feehily, Hill, & Cotter, 2017 for nice scheme). In addition, other casein precipitation processes such as acid, alkali or rennet

can be used. These different processes lead to different temperatures, a_w , pH conditions and durations that will select and influence the development of microorganisms. Cleaning in place operations can also affect contamination levels.

Infant formulas are another category of powder products of dairy origin, which can be manufactured either by dry formulation (mixing of powders) or wet formulation (mixing of liquid ingredients), concentrated and atomised (Happe & Gambelli, 2015).

Studies on the prevalence of mesophilic or thermophilic spore-forming bacteria have mainly focused on Whole Milk Powders (WMP) and Skimmed Milk Powders (SMP) (Dettling et al., 2019; Miller et al., 2015; Reginensi et al., 2011; Rückert, Ronimus, & Morgan, 2004; Sadiq et al., 2016). However, few other studies have focused on whey powders, whey protein powders and even infant formula powders (Miller, Kent, Watterson, et al., 2015; Sadiq, Li, Liu, Flint, Zhang, & He, 2016; Yuan et al., 2012; Zain et al., 2017).

The objective of this study was to evaluate the contamination levels and prevalence of sporeforming bacteria in French dairy powders targeting specific products of interest for dairy powder production from cows, ewes, goats and mares' milk.

2.2. Material and methods

2.2.1. Sampling and spore enumeration

A total of 61 French dairy powders was collected from 19 industrial plants on a voluntary base. Powders were derived from cows, ewes, goats and mares' milk. Analysed samples were composed of whole milk powder (WMP), skimmed milk powder (SMP), whey powder (WP), whey protein concentrate (WPC), milk protein concentrate (MPC), casein powder (CP), caseinate powder (CtP), permeate powder (PP) and infant formula (IF).

Analyses were performed according to ISO 6887-5, 2010 for milk solutions reconstitution; to NF V08-250 2010, for Mesophilic Spore Counts (MSC) and Thermophilic Spore Counts (TSC), and according to ISO standard ISO/TS 27265:2009 (106°C for 30 min) for highly Heat-Resistant Thermophilic Spore Count (HRTSC).

All bacterial spores were enumerated on agar according to ISO/TS 27265, 2009. Counts were performed in duplicate for each powder. The enumeration data were processed with Statistica version 13.5.0.17.

2.2.2. Isolate recovery

Thermophilic spore-forming bacterial counts were used to select isolates for further identification. Based on the colony morphology diversity, a proportional number of isolates were selected and isolated twice on TSA (Biokar Diagnostics) + potato starch 0.2% (Panreac). Selected isolates were stored in a 20% sterile glycerol solution at -80°C.

2.2.3. DNA extraction and isolate identification

Procedures used for DNA extraction, M13 PCR amplification, gel migration, clustering fingerprints, isolates selection for 16S rDNA sequencing, and identification, are similar to Ronimus et al., (2003) and are fully explicated in Supplementary material.

2.3. Results & discussion

Milk powders analysed in this study can be classified into four major groups. This included powders processed with evaporation and atomisation (WMP; SMP and WP) which have to be re-divided because of their protein composition (into WMP and SMP on the one hand and WP on the other) ; powders processed with filtration step (MPC and WPC); powders made of reconstitution (IF). And, finally, we arbitrarily grouped in “others” the remaining samples (CP, CtP, PP) which did not fit into these categories and for which there are few samples analysed. Enumerations and diversity between those four groups is different (Table 7).

Table 7: Median of enumeration of Mesophilic (MSC), Thermophilic (TSC) and Highly Heat Resistant Thermophilic Spores (HRTSC) in the four types of dairy powders studied and diversity of TSC isolated. WMP: Whole Milk Powder; SMP: Skimmed Milk Powder; WP: Whey Powder; WPC: Whey Protein Concentrate; MPC: Milk Protein Concentrate; IF: Infant Formula; Others (Permeate Powder; Casein Powder; Caseinate Powder). SE: Standard Error. NA: Not Applicable

Categories of powder	WMP / SMP	WP	WPC / MPC	IF	Others	Total	
N samples (N=61)	15	8	10	18	10	61	
Median MSC / SE	2.60 / 0.46	1.57 / 0.56	2.37 / 0.68	1.51 / 0.67	NA	NA	
Median TSC / SE	4.02 / 1.47	1.94 / 0.80	2.78 / 0.53	1.60 / 0.83	NA	NA	
Median HRTSC / SE	1.81 / 1.27	1.24 / 0.26	0 / 0.48	0.94 / 0.98	NA	NA	
ANOVA Grouping TSC	a	a/b	a/b	b	NA	NA	Simpson Index (1/D)
Total TSC isolates	108	44	67	83	11	313	
<i>A. flavithermus</i> (%)	17%	29%	22%	18%	NA	19.8%	4.17
<i>B. licheniformis</i> (%)	25%	14%	63%	17%	NA	30.0%	1.72
<i>G. stearothermophilus</i> (%)	50%	52%	9%	59%	NA	43.5%	2.22
Others (%)	8%	5%	6%	6%	NA	6.7%	
Simpson index (1/D)	2.94	2.78	2.27	2.50	NA		

The high concentrations of thermophilic spores in WMP or SMP (Figure 19) from cow may be explained by the temperature conditions during manufacturing processes, which were

favourable for the development of thermophilic bacteria and the formation of biofilms (Yu Zhao et al., 2013). Indeed, in plate heat exchangers used for pasteurization, preheaters used before evaporation, and evaporators, temperatures classically range from 45 to 70°C (Goff, 2019). In WMP and SMP, *G. stearothermophilus* seems to be predominant, followed by *B. licheniformis* and *A. flavithermus*. International studies demonstrate that *B. licheniformis* is the main contaminant in some WMP (Rückert et al., 2004; Sadiq, Li, Liu, Flint, Zhang, & He, 2016) and SMP (Sadiq, Li, Liu, Flint, Zhang, & He, 2016). However, other studies demonstrate that *A. flavithermus* is also the main contaminant of some WMP (Dettling et al., 2019) and SMP (Dettling et al., 2019; Ronimus et al., 2003). Results obtained in our study are in agreement with worldwide one where *G. stearothermophilus* reaches more than 22% of identified isolates (Sadiq, Li, Liu, Flint, Zhang, & He, 2016). The contamination level of WMP from ewe and goat milk seems less important (avg 1.68log TSC.g⁻¹) and could be linked to the size of the industry, in addition, no thermophilic spores were quantified in mare milk probably because of the process used (lyophilisation).

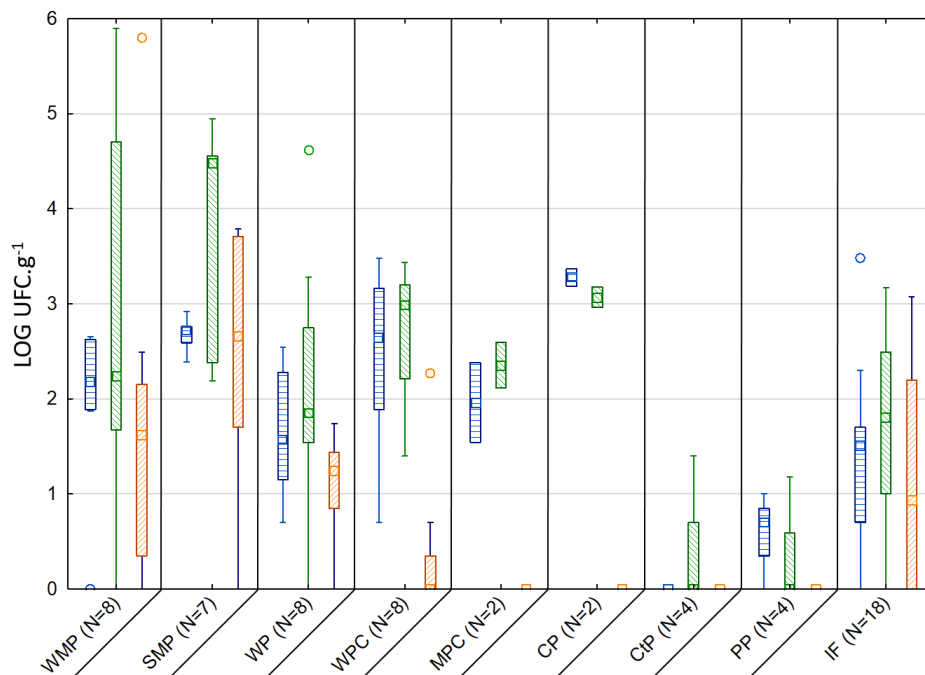







Figure 19: Enumeration of mesophilic spores (80°C 10 min, incubation 37°C: ) , thermophilic spores (80°C 10 min, incubation 55°C: ) and heat resistant thermophilic spores (106°C 30 min, incubation 55°C: ) in milk powders. WMP: Whole Milk Powder; SMP: Skimmed Milk Powder; CP: Casein Powder; CtP: Caseinate Powder; MPC: Milk Protein Concentrate; WP: Whey Powder; WPC: Whey Protein Concentrate; PP: Permeate Powder; IF: Infant Formula; : Median; Box: 25^e percentiles; Whisker: non-outlier min and max (coefficient 1); : Outlier values (coefficient 1.5).

Whey can be concentrated and evaporated directly, or fractioned by filtration, giving different types of dairy powders, protein powders or permeate powders (Snappe, Lepoudere, & Sredzinski, 2010). The high contamination of WP can be explained by evaporation steps like WMP and SMP, and filtration (for some of them), increasing the bacterial population rate. The spore diversity is quite similar to WMP and SMP, where *G. stearothermophilus* is predominant (52%), but *A. flavithermus* is more present in WP (29%), which is in relation with the study from Miller et al., (Miller, Kent, Watterson, et al., 2015) who found, in acid WP, that those two sporeformers represent 27.1% and 38% respectively.

Concentration of thermophilic spores of proteins obtained from whey (WPC) or milk (MPC) is consistent with the work of Kent et al., (2016). Bacterial spores are retained by the filtration membrane into the retentate (Julien Chamberland et al., 2017). Besides predominance of *B. licheniformis* in this type of powder was explained by Zain et al., (2017) by the development upstream of ultrafiltration processes in tanks or plate exchangers, where whey protein and lactose concentrations and temperatures are favourable for its development.

For casein powder (CP), the high amount of thermophilic bacteria is due to use of rennet. There was no modification of pH value and bacteria were concentrated in the protein part. Moreover, during casein powder production, rennet casein is washed with hot water (45/60°C) which may promote thermophilic bacteria growth. The low concentration of bacterial spores in CtP may be explained by the combination of acid and alkaline treatments as well as the absence of heat treatment in plate heat exchangers or evaporation before drying. To our knowledge, no comparison data for CP or CtP are available in the bibliography. Permeate powders originate from the liquid part obtained by ultrafiltration of milk or whey (bacteria and proteins are retained in the retentate). This observation explains the absence or very limited level of 1.18 log TSC g⁻¹ in one of the four permeate powder samples analysed. No correlation between diversity and process can be done due to the lack of isolates obtained from those types of powder.

Infant powders are produced by blending various milk ingredients (SMP, lactose and WPC ...) from the fractionation of milk constituents to approximate the composition of human breast milk. It is, therefore, difficult to explain the variability in spore concentrations observed by the impact of the manufacturing process. This difference in composition may explain the high

presence of *G. stearothermophilus*, followed by *A. flavithermus* and *B. licheniformis*. Those results are not in accordance with studies of Yuan et al., (2012) which demonstrate that *A. flavithermus* is the most prevalent contaminant with more than 40.4% of isolates obtained. But Sadiq et al., (Sadiq, Li, Liu, Flint, Zhang, & He, 2016) demonstrate that *B. licheniformis* was the most predominant isolate with more than 47.2%. Those two studies shown that *G. stearothermophilus* was present at a level of 21% and 17.1% respectively. The contamination level of thermophilic spores in goat and ewe formula (avg 1.72 log TSC.g⁻¹) seems equal to IF from cow milk, but mesophilic concentration is higher (avg 2.51log MSC.g⁻¹ vs 1.06log MSC.g⁻¹ in cow's IF).

Regarding enumeration of TSC present in the four groups, ANOVA analysis (Table 7) can classify them in two groups (a and b) resulting in a high contamination for WMP and SMP compared to IF (group b). WP, WPC and MPC are part of groups a and b due to of their median TSC contamination.

The high concentration of HRTSC (Figure 19 & Table 7) in WMP and SMP seems to be related to the presence of *G. stearothermophilus* and *A. flavithermus*, unlike to MPC and WPC mainly contaminated by *B. licheniformis* resulting of low amount of HRTSC.

Besides the three major species, other species also isolated from the dairy environment or ingredients such as *Aneurinibacillus thermoaerophilus*, *Bacillus smithii*, *Brevibacillus brevis*, *Bacillus thermoamylovorans*, *Bacillus coagulans* and *Brevibacillus bortelensis* (Lücking, Stoeckel, Atamer, Hinrichs, & Ehling-Schulz, 2013; Miller et al., 2015; Ronimus, Rueckert, & Morgan, 2006; Scheldeman, Pil, Herman, De Vos, & Heyndrickx, 2005; Yuan et al., 2012) or never isolated from dairy environment such as *Bacillus ginsengihumi* and *Paenibacillus naphthalenovorans* accounted for 6.71% of all the isolates in our study.

Calculated Simpson Index (1/D) (Simpson, 1949) on powder type didn't highlight any difference between them, but the strain diversity reflected by the M13 PCR footprint shows that diversity of *A. flavithermus* (1/D=4.17) is higher (Table 7) comparing *G. stearothermophilus* (1/D=2.22) and *B. licheniformis* (1/D=1.72).

2.4. Conclusion

The presence and concentration of mesophilic and thermophilic spore-forming bacteria in dairy powders varies according to the type of powder analysed. At each stage of milk or whey

processing, the environmental conditions such as – temperature, pH or composition – may or may not promote their proliferation, biofilm formation and sporulation in the raw material or residual biofilms. Furthermore, concentration processes, including evaporation, filtration or precipitation, influence the concentrations of bacterial spores in dairy powders. Thermophilic spore forming bacteria are used as a hygiene indicator if the cleaning in place procedure is not well respected. Due to their resistance to processes and cleaning products, it is important to monitor these thermophilic spores in order to limit their proliferation and potential impact in products using the reconstituted powders. Future experiments will be conducted on the phenotypic diversity of thermophilic spores, particularly their capacity to form biofilms and their resistance to different cleaning treatments.

Declarations of interest

None.

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Remarque : le “Supplementary material” soumis avec la « Short communication » présentée ci-dessus, n’a pas été repris dans ce manuscrit car le détail des méthodes a été décrit dans la partie méthodologie.

3. Résultats complémentaires

3.1. Niveau de contamination en spores mésophiles hautement thermorésistantes (HRMSC) des poudres lactières produite en France

En plus des résultats présentés dans la publication, le dénombrement des spores mésophiles hautement thermorésistantes (HRMSC) a également été réalisé. La figure 20 présente les résultats des dénombrements de la flore mésophile (MSC) comparés avec ceux de la flore hautement thermorésistante (HRMSC).

La concentration de spores mésophiles varie de $< 1 \text{ MSC.g}^{-1}$ à $3.48 \text{ log MSC.g}^{-1}$ pour les échantillons les plus contaminés avec une médiane de $1.70 \text{ log MSC.g}^{-1}$. Pour ce qui est de la flore HRMSC cette concentration varie de 0 HRMSC.g^{-1} à $2.97 \text{ log HRMSC.g}^{-1}$ avec une médiane à 0 HRMSC.g^{-1} pour l'ensemble des échantillons. L'absence de spores HRMSC dans certains échantillons est confirmé par l'absence de croissance après enrichissement.

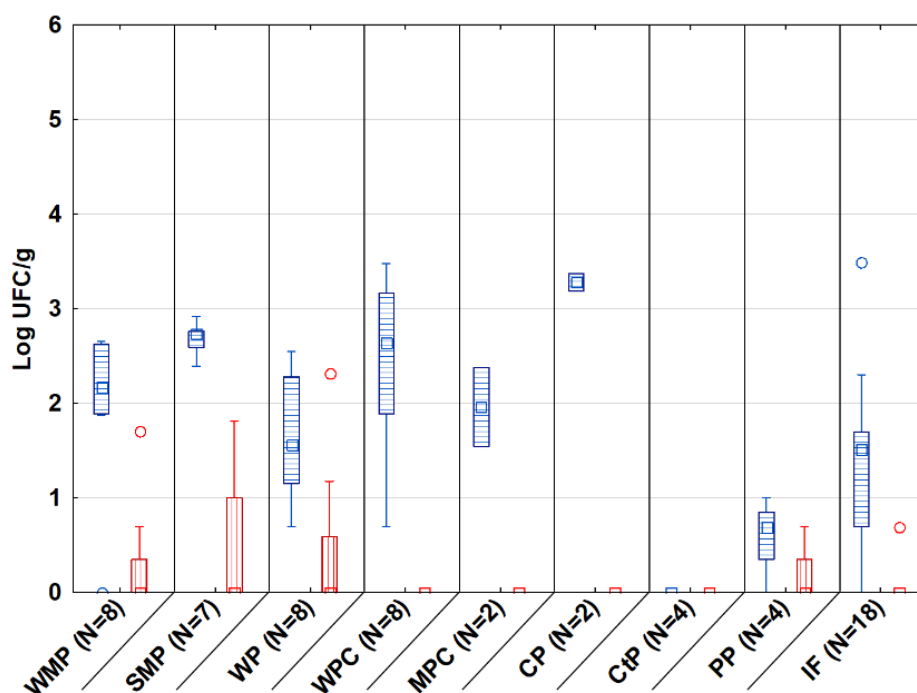




Figure 20: Dénombrement des spores mésophiles ($80^{\circ}\text{C } 10\text{min}$: ) et des spores mésophiles hautement thermorésistantes ($106^{\circ}\text{C } 30\text{min}$: ) dans des poudres lactières. WMP : Whole Milk Powder ; SMP : Skimmed Milk Powder ; WP : Whey Powder ; WPC : Whey Protein Concentrate ; MPC : Milk Protein Concentrate ; CP : Casein Powder ; CtP : Caséinate Powder ; PP : Permeat Powder ; IF : Infant Formula. \square : médiane ; boîte : 25^e percentiles ; moustache : min et max non aberrants (coefficient 1) ; \circ : valeurs aberrantes (coefficient 1.5).

Les résultats obtenus pour le dénombrement des HRMSC sont comparables avec ceux obtenus par Kent et al., (2016). La part des spores hautement thermorésistantes mésophiles (HRMSC)

dans les poudres laitières est très faible comparée aux MSC, TSC ou aux HRTSC. Cette différence de concentration en spores résistant à un traitement thermique de 106°C-30min peut s'expliquer par le fait que les spores mésophiles dénombrées sont majoritairement représentées par le genre *Bacillus*, qui produit des spores moins thermorésistantes que celle des espèces thermophiles représentées par *A. flavithermus* et *G. stearothermophilus*.

3.2. Biodiversité génétique des spores thermophiles isolées de poudres laitières

Les résultats de typage M13, utilisés pour évaluer la diversité des isolats obtenus et classer nos souches en clusters afin de les identifier, sont ici détaillés davantage. La figure 21 présente le dendrogramme obtenu après analyse biostatistique des empreintes moléculaires obtenues pour les 313 isolats de la collection. Les souches identifiées par séquençage de l'ADNr 16S sont présentés avec une flèche rouge dans le dendrogramme.

Ce dendrogramme met en évidence 3 espèces majoritaires dans les spores isolées des poudres laitières après incubation des boîtes de dénombrement à 55°C, à savoir *A. flavithermus*, *G. stearothermophilus*, et *B. licheniformis*. Ainsi le dendrogramme présente 5 sous-groupes d'*Anoxybacillus flavithermus* avec des sous-groupes composés de 4 à 22 isolats, 5 sous-groupes de *G. stearothermophilus* avec des sous-groupes composés de 2 à 101 isolats, et 7 sous-groupes de *B. licheniformis*, avec des sous-groupes composés de 2 à 61 isolats. Ces résultats illustrent bien la biodiversité estimée par le coefficient de Simpson (1/D indiqué en tableau 7 de la « short communication »). En effet plus le coefficient 1/D est grand, plus la diversité de la population l'est également. Ainsi une forte diversité génétique est obtenue pour l'espèce d'*A. flavithermus* (1/D de 4.17) avec la présence de 5 sous-groupes sans groupe majoritaire. Pour l'espèce *B. licheniformis* (1/D de 2.22), 7 sous-groupes sont obtenus avec la présence d'un groupe majoritaire de 61 souches représentant 65% de l'espèce (groupe M13, n°3). De même l'espèce *G. stearothermophilus* (1/D de 1.72) avec la présence de 5 sous-groupes dont un groupe majoritaire de 101 souches représentant 74% de l'espèce (groupe M13, n°19)

Cette diversité décrite dans de nombreuses études internationales est bien retrouvée dans cette étude, sauf sur les protéines laitières, *i.e.* poudre de caseinate (CtP) et perméat (PP) pour lesquelles de très faibles contaminations de spores thermophiles sont observées. Ainsi 1 échantillon de CtP sur les 4 analysés a permis la récupération d'un isolat de *G. stearothermophilus* d'un groupe minoritaire (groupe M13, n°17). Ce même échantillon

contenant également 1 isolat d'*Aneurinibacillus thermoaerophilus* (groupe M13, n°11). De même échantillon de PP sur les 4 analysés a permis la récupération d'un isolat d'*A. flavithermus* appartenant au groupe majoritaire (groupe M13, n°15).

En marge de ces espèces majoritaires, d'autres espèces telles que *Aneurinibacillus thermoaerophilus*, *Bacillus smithii*, *Brevibacillus brevis*, *Bacillus thermoamylovorans*, *Bacillus coagulans* et *Brevibacillus bortelensis* sont retrouvées et ont également été décrites comme pouvant contaminer l'environnement laitier. A noter que 2 espèces thermotrophes telles que *B. ginsengihumi* décrite à partir de sol de culture du ginseng (Ten et al., 2006) ou *Paenibacillus naphthalenovorans* décrite à partir de rhizosphère de plant de marais salés présentant une forte activité de dégradation de naphtaline (Daane et al., 2002) n'ont jamais été décrites comme pouvant contaminer l'environnement agroalimentaire et notamment laitier. Ces autres espèces minoritaires sont retrouvées ponctuellement sur 3 échantillons sur 8 de WMP, sur 3/7 échantillons de SMP, 1/8 échantillons de WP, 3/8 échantillons de WPC et 3/8 échantillons d'IF. A noter que seuls 3/8 échantillons d'IF ne présentent pas d'isolat recouvrable. Ces espèces minoritaires sont retrouvées ponctuellement chez plusieurs industriels. En prenant en compte l'information du producteur de poudre il peut être émis l'hypothèse d'une plus grande variété de production chez l'industriel A entrainerait de multiples pressions de sélection, pouvant favoriser la diversité microbienne. Cependant ce point ne peut être confirmé par l'absence de plan d'échantillonnage conséquent au niveau de la production des poudres laitières françaises.

A noter que les poudres de lait de jument n'ont pas permis l'isolement de souches thermotrophes ni thermophiles. Cet échantillon de poudre de lait WMP est d'ailleurs le seul à partir duquel aucune spore n'a été récupérée (l'échantillon ne présentait aucune spore après enrichissement). De même les contaminations des poudres de lait de brebis et chèvre sont faibles ($2 \log.mL^{-1}$) et permettent le recouvrement d'un isolat de *G. stearothermophilus*, de 5 *B. licheniformis*, et d'un isolat de *Brevibacillus brevis*. Malgré le nombre d'échantillons limités, aucun isolat d'*A. flavithermus* n'a été retrouvé dans ces poudres de lait de chèvre ou brebis.

Un regroupement par famille de poudre permet de mettre en évidence une prévalence d'*Anoxybacillus flavithermus* de l'ordre de 20% dans les WP, WPC/MPC, IF (Table 7). Une prévalence de l'ordre de 52% et 59% de *G. stearothermophilus*, respectivement dans les WP et IF. Ainsi qu'une prévalence de 63% de *B. licheniformis* dans les protéines laitières WPC/MPC.

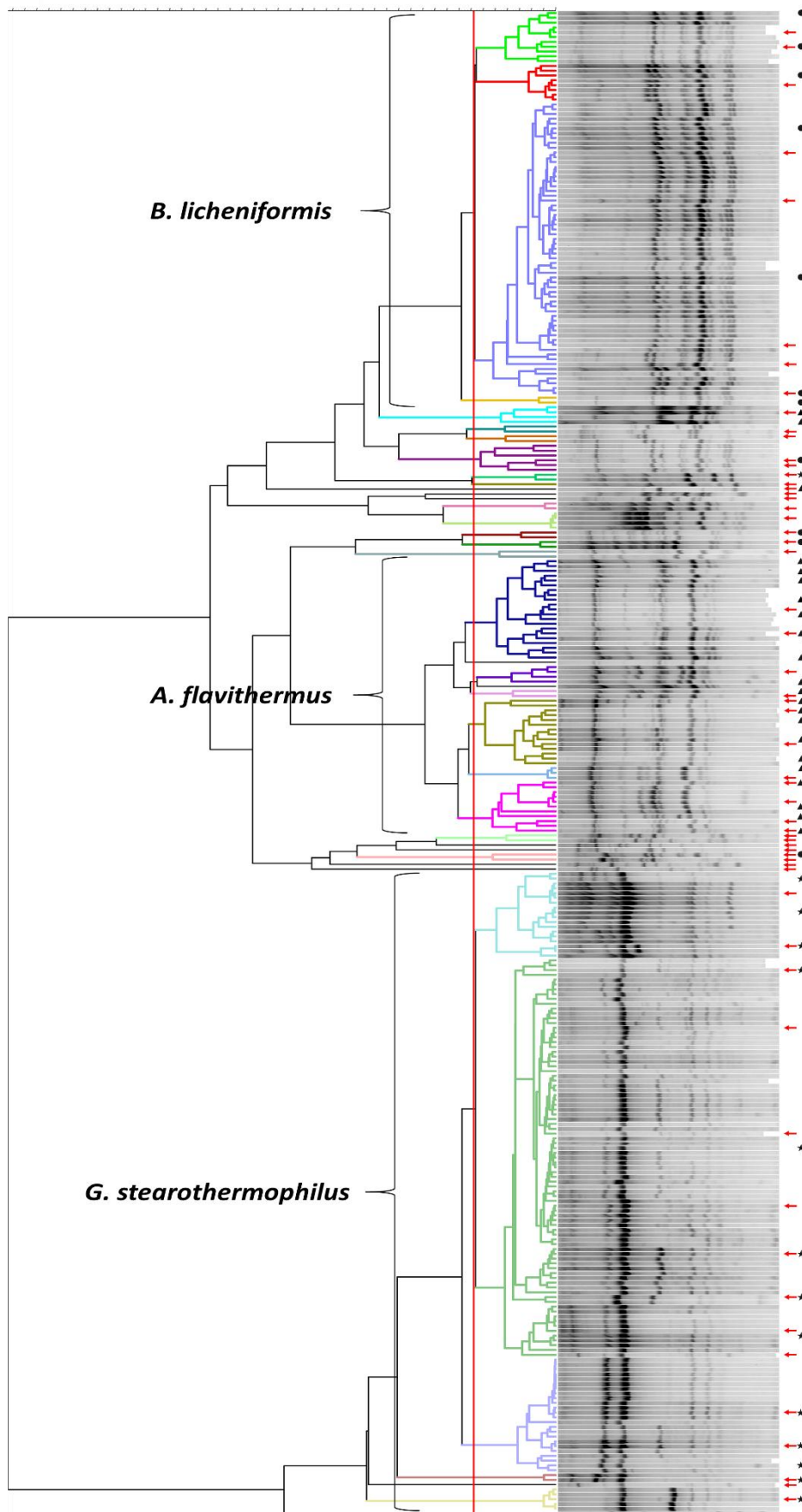


Figure 21: Dendrogramme obtenu après analyse des profils M13 PCR pour les 313 isolats thermophiles. — : seuil de similarité de 87%. ← : souches séquencées permettant d'identifier les espèces associées aux clusters. ● : Souches de *B. licheniformis* ; ▲ : d'*A. flavithermus* ; et ★ : *G. stearothermophilus* utilisées pour l'étude de la diversité phénotypique et la formation de biofilms (Chapitre 4).

3.3. Potentiel d'altération de la sélection de souches de bactéries sporulées isolées des poudres laitières

La capacité d'altération des souches d'origine alimentaire a été évaluée sur la capacité des souches à utiliser diverses sources de carbohydrates et ingrédients retrouvés associés aux poudres laitières dans la production de diverses denrées alimentaires.

3.3.1. Capacité à dégrader des carbohydrates

L'analyse de la capacité des souches à utiliser une sélection de carbohydrates comme unique source de carbone a permis de séparer les espèces et de créer des groupes inter-espèces. Même si des variations entre les souches d'une même espèce peuvent être observées, une tendance dominante est observée pour toutes les souches testées dans une même espèce. Le tableau 8 présente les tendances observées par espèces pour les carbohydrates testés. Les résultats pour le D(+)-Glucose ; D(-)-Fructose ; D(+)-Maltose ; Saccharose sont positifs pour toutes les souches testées. Ces résultats sont confirmés par les témoins (positifs et négatifs) ainsi que par l'absence de virage de l'indicateur coloré de pH dans les puits stériles.

Tableau 8: Pourcentage de résultats positifs en fonction de l'espèce testée et du sucre ajouté au milieu API50CHB

	Number of tested strains	D(+)-Glucose	D(-)-Fructose	D(+)-Maltose	Saccharose	D(+)-Galactose	D(-)-Lactose	Melezitose	D(+)-Raffinose	D(+)-Trehalose	D(+)-Mannose	D(-)-Salicine	D(-)-Ribose	D(-)-Cellobiose	Mannitol	L(+)-Arabinose	D(+)-Xylose	D(-)-Sorbitol	L(+)-Rhamnose
<i>A. flavithermus</i>	32	100	100	100	100	100	96.9	71.9	25.0	84.4	40.6	6.3	0	6.3	3.1	0	0	0	0
<i>G. stearothermophilus</i>	15	100	100	100	100	86.7	93.3	93.3	86.7	53.3	100	93.3	60.0	53.3	13.3	6.7	6.7	6.7	0
<i>B. licheniformis</i>	15	100	100	100	100	6.7	0	0	0	100	100	100	100	100	100	100	93.3	93.3	6.7

Pour *B. licheniformis*, toutes les souches testées sont négatives pour la dégradation du D(-)-Lactose, D(+)-Raffinose, Melezitose, et seule la souche type est positive pour la dégradation du D(+)-Galactose. La seule souche positive pour la dégradation du L(+)-Rhamnose est une souche isolée de poudre de lactosérum.

Pour *A. flavithermus* et *G. stearothermophilus*, toutes les souches sauvages sont positives pour le D(-)-Lactose contrairement aux souche type. Pour *A. flavithermus*, c'est le contraire, en ce qui concerne la dégradation du mannitol, seule la souche type peut utiliser cet hydrate de carbone.

Pour *G. stearothermophilus*, toutes les souches isolées de poudres laitières sont également positives sur la dégradation de la D(-)Salicine contrairement à la souche type. Pour ces deux espèces thermophiles, l'incapacité à dégrader le D(-)Lactose par les souches types a déjà été mentionné dans la bibliographie (Burgess, 2016; Y. Zhao et al., 2018) et suggèrent une adaptation des souches d'*A. flavithermus* et de *G. stearothermophilus* à l'environnement laitier. De même, les résultats obtenus pour l'absence de dégradation du D(-)Lactose par toutes les souches sauvages de *B. licheniformis* ont déjà été démontrés (Sadiq, Li, Liu, Flint, Zhang, Yuan, et al., 2016; Zain et al., 2017) et expliqué par l'absence de β -Galactosidase. Cependant cela n'empêche pas les souches de *B. licheniformis* de dégrader les protéines présentes dans le lait et de se développer en mélange avec d'autres espèces pouvant dégrader le lactose en glucose. Ce mécanisme symbiotique a en effet déjà été décrit entre une souche positive au lactose : *A. flavithermus* et une négative au lactose *G. thermoglucosidans* (Y. Zhao et al., 2018).

3.3.2. Capacité à dégrader une sélection d'ingrédients spécifiques

La dégradation bactérienne d'ingrédients utilisés en complément de poudres laitières pour la formulation de nombreuses denrées alimentaires peut également permettre d'évaluer le potentiel d'altération des bactéries sporulées retrouvées (tableau 9) L'utilisation de ces ingrédients permet d'évaluer les activités lipolytiques (dégradation bactérienne de la tributyrine et lécithine), protéolytiques (dégradation de la caséine et gélatine) ainsi que la capacité à dégrader des sucres complexes (cellulose, pectine, amidon).

Tableau 9: Pourcentage des résultats positifs en fonction des espèces testées et des ingrédients et sucres complexes.

	Number of tested strains	Cellulose	Casein	Pectine	Gelatine	Starch	Lecithine	Tyributyryne
<i>A. flavithermus</i>	32	100.0	93.8	50.0	65.6	3.1	3.1	0.0
<i>G. stearothermophilus</i>	15	100.0	100.0	66.7	26.7	13.3	6.7	0.0
<i>B. licheniformis</i>	15	100.0	100.0	100.0	93.3	66.7	100.0	100.0

Sur les 62 souches testées, toutes ont été positives au test de dégradation de la cellulose. Seules deux souches ont été négatives pour la dégradation de la caséine, la souche type *A. flavithermus*, et une souche sauvage isolée de formulation infantile (AF112). Pour les autres ingrédients testés, les résultats sont très variables mais *B. licheniformis* semble avoir un pouvoir d'altération plus important que les deux autres espèces avec une grande majorité des souches testées étant capable de dégrader les ingrédients testés. Ces résultats présentent la tendance inverse de ceux publiés par Lücking et al., (2013), qui observait une très forte activité lipolytique (envers la

tributyryne et la lécithine) et n'observait pas d'activité protéolytique (dégradation de la caséine) chez *A. flavithermus*. L'activité lipolytique n'a pas été détectée dans notre étude chez *G. stearotherophilus* contrairement à ce qu'observait Lücking et al., (2013) qui évalue cette activité sur 15 à 49% des 21 isolats testés. Pour *B. licheniformis*, la part des souches positives pour la recherche de l'activité protéolytique et lipolytique est comparable avec les données bibliographiques (De Jonghe et al., 2010; Lücking et al., 2013). Les différences entre notre étude et les résultats obtenus dans la bibliographie peuvent s'expliquer par l'origine des souches (divers aliments dans la bibliographie), et la composition des milieux utilisés.

3.3.3. Capacités d'altération des bactéries sporulées thermophiles isolées de poudres laitières

L'ensemble des résultats d'activité enzymatique obtenus sur les souches peuvent être compilés dans la figure 22 présentant un dendrogramme réalisé en utilisant une matrice d'agrégation et une représentation UPGMA.

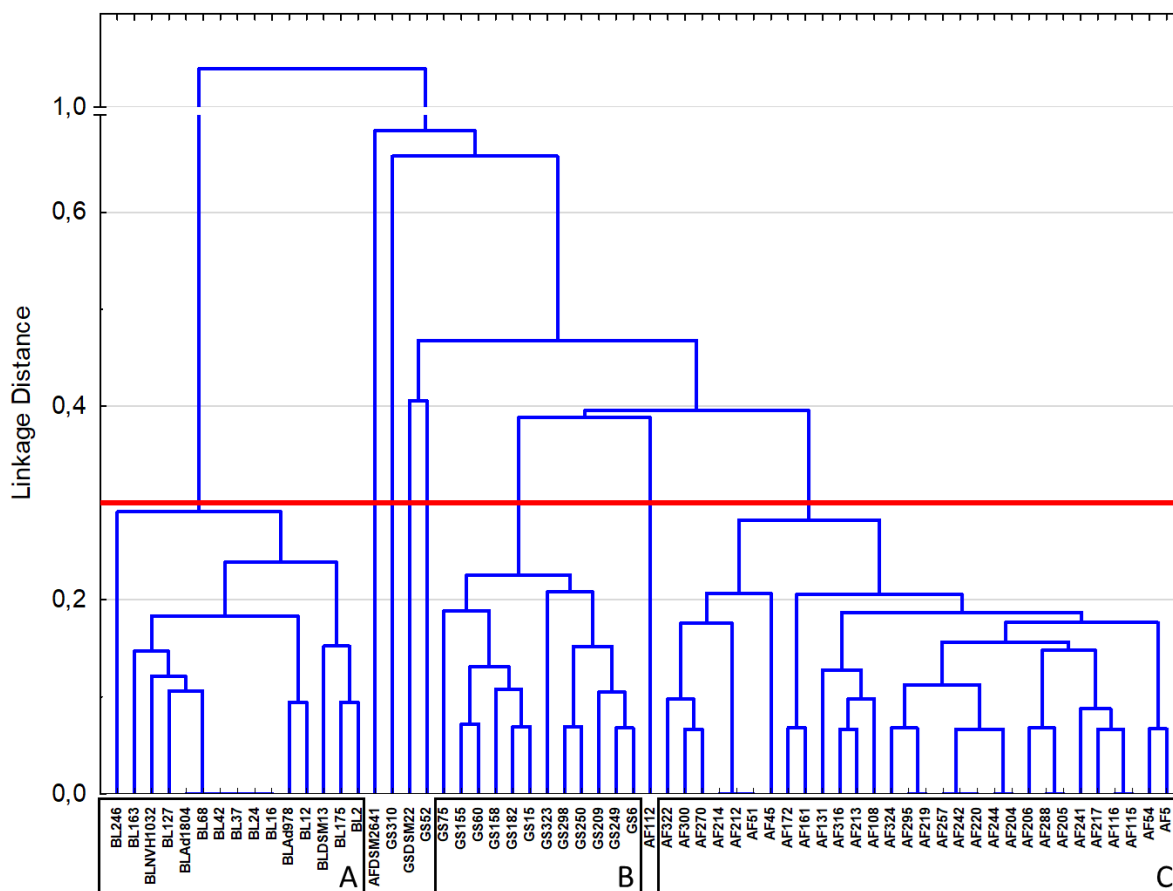


Figure 22: Dendrogramme des réponses phénotypiques de toutes les souches testées sur la capacité à utiliser les glucides et les ingrédients. BL : *B. licheniformis*, GS : *G. stearotherophilus*, AF : *A. flavithermus*, — : seuil de

similarité. Dendrogramme utilisant la matrice de distance d'agrégation UPGMA utilisant le coefficient de Pearson. Les souches types se distinguent par leur numérotation BL DSM 13, GS DSM22, AF DSM2641

Les souches de l'espèce *B. licheniformis* sont toutes classées ensemble dans le groupe A, et les groupes B et C ne regroupent que *G. stearothermophilus* et *A. flavithermus* respectivement. La souche type *A. flavithermus* DSM2641 ainsi que la souche type de *G. stearothermophilus* DSM22 se retrouvent séparées des groupes contenant les autres souches d'*A. flavithermus* (groupe C) et *G. stearothermophilus* (groupe B) notamment compte tenu de leur incapacité à utiliser le lactose. Ainsi la souche sauvage AF112 d'*A. flavithermus* semble plus proche de *Geobacillus* en raison de sa capacité à dégrader la salicine et le cellobiose, et de son incapacité à dégrader la caséine. L'empreinte M13 de cette souche se classe également en individuel. Les souches sauvages de *G. stearothermophilus* GS52 et GS310 semblent éloignées des autres souches sauvages de cette espèce à cause de leurs activités Mannitol, Arabinose, Xylose et lécithinase. De même les empreintes de ces souches n'appartiennent pas au groupe M13 majoritaire de *G. stearothermophilus*. Il pourrait être intéressant de valider l'identification de ces souches atypiques par des méthodes complémentaires. Pour l'espèce *B. licheniformis* l'ensemble des souches isolées de poudres laitières et la souche type sont regroupées au sein du groupe A.

4. Conclusion du Chapitre 2

Le Chapitre 2 présente l'approche méthodologique et les résultats obtenus pour le dénombrement des spores thermophiles, la collecte des souches isolées de poudres laitières ainsi que leur diversité génotypique et phénotypique. La prévalence et les niveaux de contamination en spores montrent une faible prévalence des spores mésophiles et une forte prévalence des spores thermophiles. La caractérisation de la diversité génétique de cette collection d'étude permet de mettre en évidence, comme déjà présenté dans les études internationales, une prédominance de trois espèces : *Geobacillus stearothermophilus*, *Bacillus licheniformis* et *Anoxybacillus flavithermus*. Certaines peuvent apparaître plus ou moins liées au procédé utilisé comme *B. licheniformis*, fortement représenté parmi les isolats issus de procédés comprenant une étape de filtration.

La diversité intra spécifique d'*A. flavithermus* permet de mettre en évidence 5 sous-groupes d'empreintes génotypiques avec une plus forte diversité de souches que celle des deux autres espèces. L'espèce *B. licheniformis* présente 7 sous-groupes avec un groupe majoritaire représentant 65% des souches de *B. licheniformis* testées (Groupe M13, n°3). De même, l'espèce *G. stearothermophilus* présente 5 sous-groupes avec un groupe majoritaire représentant 74% des souches de *G. stearothermophilus* testées (Groupe M13, n°19). Les groupes obtenus pour la diversité génétique sont éclatés dès l'instant que les regroupements se font sur une diversité phénotypique basée sur les capacités des souches à dégrader des sucres et ingrédients retrouvés dans les denrées alimentaires.

Ainsi toutes les souches testées (toutes espèces confondues) sont positives pour l'activité caséinase et cellulase, ce qui peut être lié à une sélection ou adaptation des souches à l'environnement laitier (pour l'activité caséinase). De même toutes les souches d'*A. flavithermus* et de *G. stearothermophilus* isolées de poudres laitières présentent la capacité de dégrader spécifiquement le lactose du lait contrairement aux souches de *B. licheniformis* testées. En prenant en considération les différents éléments obtenus, le panel d'activités enzymatiques de *B. licheniformis* semble être le plus diversifié, cette espèce étant capable de dégrader tous les ingrédients testés sauf quelques isolats négatifs pour l'amylase. Bien que *B. licheniformis* ne semble pas être l'espèce majoritaire dans les poudres WMP, SMP et WP, sa forte prévalence dans les poudres de concentré protéique peut poser des problèmes lors de

l'utilisation de ces poudres comme ingrédients (7000 produits alimentaires selon OpenFoodFacts).

Les trois espèces majoritaires retrouvées dans ce chapitre sont également décrites dans les études bibliographiques sur le sujet. Cependant la proportion des espèces en fonction du type de poudre analysé varie. Cela indique donc un effet du processus de fabrication sur la sélection de souches ou espèces.

Cette prévalence et diversité peut-elle vraiment s'expliquer par des variations de procédés technologiques, favorisant la croissance d'espèces ?

Quels sont les leviers pouvant permettre la sélection de certaines espèces ou souches ?

Ce sont des questions qui découlent de cette première approche et auxquelles nous tenterons d'apporter des réponses dans le Chapitre

Chapitre 3 : Identification des étapes du procédé de transformation du lait en poudre favorisant le développement des bactéries sporulées thermophiles

Les bactéries sporulées thermophiles sont présentes en très faible concentration dans le lait cru de 1 à 2.06 log UFC.ml⁻¹ (Buehner et al., 2014) mais sont retrouvées en grande quantité dans les poudres laitières après production. Au cours de sa transformation, le lait est concentré (facteur 10), mais cela ne suffit pas à expliquer une telle contamination des poudres. Il y a donc, au cours du process, un ou des étapes favorables au développement des bactéries sporulées thermophiles. Cependant, le temps de résidence au long des installations est relativement court ne permettant pas de justifier d'un tel degré de contamination. Il est donc fortement probable que les bactéries sporulées thermophiles forment une niche écologique sur les surfaces de certaines parties de l'installation et s'y développent (S. Flint et al., 2020; Teh, Flint, Brooks, & Knight, 2015; Yu Zhao et al., 2013).

Dans ce chapitre, les conditions rencontrées au cours de procédés de transformation du lait en poudres seront comparées avec les valeurs cardinales des bactéries et les zones critiques permettant la croissance des bactéries sporulées thermophiles seront exposées. Les conditions industrielles ont été tirées de la bibliographie et une partie expérimentale, en particulier pour relier extrait sec et activité de l'eau, a été réalisée. En ce qui concerne les limites et capacités de croissance des différentes espèces, les données bibliographiques récoltées ont été complétées par la détermination expérimentale (limites de pH, a_w et taux de croissances) pour 5 souches de chacune des 3 espèces étudiées. Ce chapitre est présenté sous forme d'un article qui sera soumis prochainement pour publication.

Dairy powder industry: risk area associated to thermophilic sporeforming bacteria using growth limits

En relecture pour soumission à International Journal of Food Microbiology (IJFM).

Abstract:

Anoxybacillus flavithermus, *Geobacillus stearothermophilus* and *Bacillus licheniformis* represent the main contaminants encountered in dairy powders. These thermophilic sporeforming bacteria, rarely detected in raw milk, persist and develop during the manufacturing process of milk powders. Moreover, as spores, these species resist and are concentrated in powders during processes. The objective of this study was to determine the steps of dairy powder processes that are favorable to the growth of such contaminants.

A total of 5 strains were selected for each three species as natural contaminant of dairy pipelines to determine minimal and maximal values enabling growth for temperature, pH and a_w . These growth limits were associated with the environmental conditions encountered at each step of the manufacturing of whole milk, skim milk and milk protein concentrate powders. The growth ability range appear wider for *B. licheniformis* (pH over 4 units and $a_{w \min} > 0.881$) than for *A. flavithermus* and *G. stearothermophilus* which show equivalent and narrower range (pH over 2 units and $a_{w \min} > 0.978$ and > 0.966 , respectively). During the early stages of manufacturing, *i.e.* pre-treatment, pasteurization, standardization and pre-heating prior to concentration, the encountered physico-chemical conditions are suitable for the development and growth of *A. flavithermus*, *G. stearothermophilus* and *B. licheniformis*. While during the evaporation step, conditions appears to be most suitable for *G. stearothermophilus* growth. High temperatures appear as a limiting factor for the growth of *B. licheniformis* and low water activity strongly limits the development of *A. flavithermus*.

1. Introduction

Dairy powders are worldwide produced and used (whole milk, skim milk and whey powders represented in 2019, more than 13.335 thousand tons worldwide). They can be easily traded and exported because of their low moisture content giving a long shelf life.

The contamination of dairy powders by thermophilic bacterial spores has been described by many authors (Ronimus et al., 2006; Scott et al., 2007). *Geobacillus stearothermophilus*, *Anoxybacillus flavithermus*, and *Bacillus licheniformis* are the three thermophilic major species, showing the highest prevalence in dairy powders (Burgess et al., 2010; Ronimus et al., 2003; Sadiq, Li, Liu, Flint, Zhang, & He, 2016).

Levels of spores in dairy powders is mainly monitored by clients who want the lowest concentration of thermophilic spores in finished product (reaching 3.7 log TSC.g⁻¹ for milk powders used in infant formula, or 4 log TSC.g⁻¹ for SMP and WMP (Bienvenue, 2013)). Indeed, strains of these thermophilic spore forming bacteria species are able to synthesize enzymes, which affect physicochemical properties of dairy powder and lead to an undesirable texture or taste (Lücking et al., 2013).

Even though these species are ubiquitous, thermophilic spore are found at relatively low levels in raw milk; from 0.29 to 2.23 log spores.ml⁻¹ (N. H. Martin et al., 2019; P. M. Murphy et al., 1999). While concentrations as high as 4.7 log of thermophilic spores.g⁻¹ in milk powders (Rückert et al., 2004) or 6.9 log of thermophilic spore.g⁻¹ in foulant of milk separator (Scott et al., 2007) have been observed. This may be explained by favorable environmental conditions encountered during processes in the production lines (temperature, pH, water activity (a_w)) as well as the composition of the milk or milk protein concentrates that support germination, vegetative growth and increasing the contaminations. Even if residence times at each stage of the manufacturing process vary from around a few seconds to a few minutes, high contamination can be explained by the capacity of those species to adhere to surface and produce biofilms, causing a permanent and persistent contamination of the production lines (Brooks & Flint, 2008). Indeed time in between each cleaning in place procedures (CIP) may range from 7 to 20 hours depending on the process, promoting bacteria growth. It is, therefore, interesting to question the operations of the process that allow the development of these

different species by crossing the data on the conditions encountered (pH, Temperature and a_w) and on the growth and survival boundaries of these bacteria.

The dairy processes for transforming powdered milk are fairly well described, as well as the temperature conditions encountered in each phase (Bylund, 2003; Písecký, 2012). Although the initial pH of milk (whole or skimmed) or protein concentrates is generally constant and known, modifications can occur during the processing steps. Thus, Singh (2007) observed an acidification during the concentration phase, but this has not been characterized according to the dry extract reached. In addition, concentration of dairy components in scientific papers is usually expressed as a percentage of dry matter or "total solids", which is difficult to compare to a_w limit for bacteria.

For these thermophilic bacteria, growth limits with respect to temperature are easily found in the literature. Boundaries concerning pH values are less frequent. Likewise, some $a_{w\ min}$ values exist but are generally determined by adding salt, which does not correspond to dairy conditions, for which it is the effect of the concentration process which modifies the a_w . Indeed, NaCl has itself a significant inhibitory effect. In addition, these values were determined on strains of various origins, but mainly non-dairy ones: hot springs for *Anoxybacillus flavithermus* or compost for *Geobacillus stearothermophilus*. The *Bacillus licheniformis* strains studied so far mainly come from diverse food samples.

It is therefore clear that there is a lack of data to completely link these different aspects. Therefore, the objective of this work is twofold. It involves finding the data and filling in the gaps, then analyzing the overall pattern to identify the most risky stages for each of the species studied during the manufacturing processes of milk powders or protein concentrates.

In this study, on the one hand, temperature, pH, a_w growth limits values of dairy strains from these species have been determined experimentally to complete those obtained from literature data. On the other hand, pH and a_w of whole milk, skimmed milk and milk protein concentrates at different dry matters have been determined to propose a modeling and equations linking these parameters.

Finally, combining these bacterial growth limit values and environmental conditions encountered during manufacturing processes allow to identify steps of the dairy powder process that support growth of thermophilic spore forming bacteria.

2. Materials and methods

2.1. Bacterial strains

A total of five strains is selected for each species, *i.e.* *G. stearothermophilus* (GS52, GS209, GS250, GS310, DSM22); *A. flavithermus* (AF5, AF45, AF108, AF161, DSM2641) and *B. licheniformis* (BL16, NVH1032, Ad978, Ad1804, DSM13). Table 10 reports the strain selection composed of one reference strain (DSM collection) and four industrial strains previously isolated from dairy powders or processed environments (Delaunay et al., 2021; Madslie et al., 2012). All bacteria were inoculated in TSB and incubated at 55°C for *G. stearothermophilus* and *A. flavithermus*, and 37°C for *B. licheniformis*, during 16h before experiments.

2.2. Determination of pH growth limits

Seven pH were studied, ranging from 3.65 to 7. Slant TSA surfaces were made by mixing pH adjusted (HCl), double concentrated, and filtrated TSB with sterile double concentrated agar, in tubes, and were inoculated with bacterial suspension. Growth limits were determined by the presence or absence of colonies on the TSA surface after 7 days incubation at 37°C for *B. licheniformis* or 55°C for *G. stearothermophilus* and *A. flavithermus*. The pH values were measured in sterile control agars. Tests were made in triplicate.

2.3. Determination of a_w growth limits

Ten water activity levels were tested on slant TSA ranging from 0.995 to 0.898, by adding glycerol at concentrations determined from Marcolli & Peter (2005). The media were autoclaved and the water activity were measured using an a_w meter (FA-st1 GBX; France Scientific Instrument). All agar slants were inoculated and incubated as described previously for pH (see 1.2.).

2.4. Determination of growth rate

A total volume of 250mL of UHT skim milk was inoculated by 2mL of an overnight culture of bacteria in TSB at 55°C; and placed in 55°C water bath under agitation. Every 30 minutes, agar

plates were made in triplicate by pouring 1mL of milk (or dilutions in tryptone salt) in 15mL of TSA with a double layer of agar during 8h. A final sampling was made and enumerated after 24h of growth. After 24h of incubation at 55°C; total bacteria were enumerated on agar plates, growth curves were adjusted using Rosso model (Rosso et al., 1993) and growth rate was determined.

2.5. Determination of the relation between the total solid content, pH and water activity of dairy fluids

Relationships between water activity and dry matter of whole milk, skim milk and milk protein concentrate were obtained by measuring the dry matter and water activities of samples at different concentration levels. Concentrations of whole and skim milk (UHT milk from local store) and milk protein concentrate (from local industry) were performed using a rotavapor (Laborota 4000 efficient; Heidolph Instruments GmbH & Co. KG, Germany) at 50 °C under vacuum (200hPa). The dry extract measurements were performed by weight difference before and after a night at 100°C and water activities were measured using an a_w meter (FA-st1 GBX; France Scientific Instrument). pH values of milk or protein concentrate samples at different concentration levels were determined using a pH-meter (PHM210, MeterLab, Radiometer, Copenhagen). Polynomial equation parameters values describing relations between a_w and %TS were obtained using solve function (Excel Microsoft 2016).

3. Results and discussion

3.1. Environmental growth limits of *A. flavithermus*, *G. stearothermophilus*, and *B. licheniformis*

3.1.1. pH growth limits

The minimal pH values allowing growth for the five strains for each of the three species studied *Anoxybacillus flavithermus*; *Geobacillus stearothermophilus* and *Bacillus licheniformis* are presented in Table 10 and compared with data obtained from the literature. For *Anoxybacillus flavithermus*, the pH_{min} is ranging between pH 5.3 and pH 6.3 for the different strains tested. This range is similar to those obtained for strains isolated from hot springs (pH ranging from 5.5 to 6, using a growth no growth method) (Chiş et al., 2013; Dai et al., 2011; Ellis &

Magnuson, 2012; Heinen et al., 1982; D. Jiang et al., 2015). For *Geobacillus stearothermophilus*, its pH_{\min} is ranging from 3.9 to 5.3. These data are consistent with the pH_{\min} of 5.27 determined by Mtimet et al., (2015) for ATCC 12980 (data obtained using Rosso model adjustment (Rosso et al., 1993)). Among 20 strains of *G. stearothermophilus* isolated from spoiled canned food, Durand et al., (2015) observed growth for a minimal pH below pH 5 for 6 strains and below 5.5 for the others. For *Bacillus licheniformis* pH_{\min} range between 3.9 and 5. These minimum pH values for growth appear homogeneous between strains of the same species and consistent with those determined by Rodriguez, Cousin, & Nelson (1992) and Baril et al., (2012). The strains of *B. licheniformis* can grow at lower pH than the strains of *A. flavithermus*, *G. stearothermophilus*.

3.1.2. Water activity growth limits

The development of strains of *A. flavithermus* are strongly affected by the decrease in water activity (Table 10). A water activity of 0.984 inhibits the development of strains AF5 and AF161. The other dairy strains and the type strain DSM2641 from hot springs are inhibited at a water activity of 0.978. Strains of *A. flavithermus* show relatively high $a_{w \min}$ values promoting growth ranging from 0.995 to 0.984. These values are consistent with the minimum values corresponding to the maximum NaCl concentrations of 2.5% to 3% w/v for strains isolated from geothermal water (Dai et al., 2011; Ellis & Magnuson, 2012; Heinen et al., 1982; D. Jiang et al., 2015).

G. stearothermophilus strains have lower minimum water activities allowing growth, some strains have $a_{w \min}$ at 0.978, while others have $a_{w \min}$ at 0.966 as determined by Putri et al., (2017), respectively for a strain isolated from dairy product and by Durand et al., (2015) for a strain isolated from spoiled canned food.

As compared to the two other thermophilic species, strains of *B. licheniformis* have lower $a_{w \min}$, *i.e.* 0.918 for the Ad1804 strain and 0.898 for the other four strains tested. Strains of *B. licheniformis* isolated from dairy powder can grow at a_w values between 0.909 and 0.881. These values are slightly lower than those determined by Yakimov et al., (1995), Torres et al., (2009) and Baril et al., (2012) which are between 0.904 and 0.920. The growth ability of *B. licheniformis* at water activities close to 0.9 allows this specie to grow in concentrated dairy products.

Comparisons made with data from this study and collected data seems to be comparable and showing no effect of the depressor origin at the tested concentration (glycerol, salt or concentrated milk).

3.1.3. Determination of growth rate

Growth rate was determined for all tested strains and data are presented in table 10. For all wild *A. flavithermus* strains, μ was ranging from 1.49 to 3.65 h⁻¹, reaching a total of bacteria from 7.59 to 8.41 log UFC.ml⁻¹ after 24h. The *A. flavithermus* type strain can't develop in milk and can't grow, because of the lack of lactose degradation enzyme (Ellis & Magnuson, 2012). For wild strains of *G. stearothermophilus*, results ranging from 1.21 to 1.8h⁻¹ and reach from 5.23 to 7.68 log UFC.ml⁻¹. For the type strain, result is similar to *A. flavithermus* one, with an absence of grow due to a lack of lactose degradation gene (Burgess et al., 2017). For *B. licheniformis*, all tested strains can grow, with a growth rate ranging from 0.35 to 1.88h⁻¹ and reaching 2.52 to 8.4 log CFU.ml⁻¹. By comparing species tested, *A. flavithermus* is the bacteria with the fastest growth speed (median of 2.78h⁻¹), followed by *G. stearothermophilus* (median of 1.60h⁻¹) and by *B. licheniformis* with a median of 0.96h⁻¹ at 55°C in skim milk.

Table 10: Comparison of growth abilities obtained from this study and literature for *A. flavithermus*, *G. stearothermophilus* and *B. licheniformis*

Species	Strain	Origin	pH _{min}	a _{w min}	T _{min} °C	T _{opt} °C	T _{max} °C	μ (h ⁻¹)	References	
<i>A. flavithermus</i>	AF5	Skim milk powder	5.82>pH _{min} >6.31	0.978<a _{w min} <0.984				2.26	This study	
	AF45	Milk protein powder	5.34> pH _{min} >5.82	0.978< a _{w min} <0.984				1.49		
	AF108	Milk protein powder	5.34> pH _{min} >5.82	0.978< a _{w min} <0.984				3.65		
	AF161	Whey powder	5.34> pH _{min} >5.82	0.984< a _{w min} <0.995				3.29		
	DSM2641	Hot spring	5.82> pH _{min} >6.31	0.978< a _{w min} <0.984				ND		
	DSM2641	Hot spring	5> pH _{min} >6	0.983< a _{w min} <0.986 (NaCl 2.5 or 3%)	30	60	72			(Heinen et al., 1982)
	CM	Milk powder plant			34	48-58	67			(Scott, 2005)
	TWXYL3	Hot spring	6**	0.983 (NaCl 3% w/w)	35	65	75			(Ellis & Magnuson, 2012)
	T1	Hot spring	5> pH _{min} >5.5		37	60	70			(Chiş et al., 2013)
	E13T	Hot spring	5.5**	0.977< a _{w min} <0.981 (NaCl 3.5 or 4% w/v)	30	60	66			(Dai et al., 2011)
TCC9-4	Hot spring	6**	0.970< a _{w min} <0.983 (NaCl 3 or 5% w/w)	30	60	68		(D. Jiang et al., 2015)		
1958	Milk or whey product						1.83 (55°C)	(Graf et al., 2021)		
<i>G. stearothermophilus</i>	GS52	Whole milk powder	3.91> pH _{min} >4.42	0.978< a _{w min} <0.984				1.21	This study	
	GS209	Whey powder	4.42> pH _{min} >5.01	0.978< a _{w min} <0.984				1.54		
	GS250	Skim milk powder	4.42> pH _{min} >5.01	0.966< a _{w min} <0.978				ND		
	GS310	Infantile formula	5.01> pH _{min} >5.34	0.966< a _{w min} <0.978				1.8		
	DSM22	Canned food	4.42> pH _{min} >5.01	0.966<a _{w min} <0.978				ND		
	DSM 22	Spoiled canned food	5> pH _{min} >5.5	0.966< a _{w min} <0.971 (NaCl 3 or 4% w/v)						(Durand, 2014)
	W14	Milk processing plant		0.983 (concentrated milk)	40	58-65	73			(Putri, 2017)
	B631	Canned Cabbage				65	74			(Warth et al., 1978)
	308	Compost			30.80	57.20	65.50			(Rosso et al., 1993)
	238	Compost			33.60	64.80	71.80			(Rosso et al., 1993)
ATCC 12980	Unkown	5.27*		38.52	57.59	68.02		(Mtimet, 2015)		
ATCC 7953	Unkown			33.76	61.82	68.14	2.068 +/- 0.036	(Kakagianni et al., 2016)		
Donk 1920	Unkown	6**	0.967< a _{w min} <0.970 (NaCl 5 or 5.5% w/v)	30-45		70-75		(Nazina et al., 2001)		
<i>B. licheniformis</i>	BL16	Dairy powder	3.91> pH _{min} >4.42	0.881< a _{w min} <0.898				1.88	This study	
	NVH1032	canned food	3.91> pH _{min} >4.42	0.881< a _{w min} <0.898				ND		
	Ad978	Dairy product	4.42> pH _{min} >5.01	0.881< a _{w min} <0.898				1.59		
	Ad1804	Dairy procesing plant	4.42> pH _{min} >5.01	0.909< a _{w min} <0.918				ND		
	DSM13	Unknow	3.91> pH _{min} >4.42	0.881< a _{w min} <0.898				0.96		
	AD978	Dairy powder	4.9*	0.920 (glycerol)	20	50.90	55			(Baril, Coroller, Couvert, El Jabri, et al., 2012)
	B691	Unheated milk				48	56			(Warth et al., 1978)
	64-83-46	Acidified onions	4.2> pH _{min} >4.4		15		60			(Rodriguez et al., 1992)
	BAS50	oil storage cavern	5.4**	a _{w min} <0.918 (NaCl 13% w/v)	25		55			(Yakimov et al., 1995)
	S-86	Soil		0.904 (NaCl 15% w/v)		50	55			(Torres et al., 2009)
R-27356	raw milk						0.16 (20°C)	(De Jonghe et al., 2010)		

*Model adjustment; ** One data available

3.1.4. Growth characteristics of strains

For the three strains tested, minimal pH, minimal a_w require for bacterial growth and maximal growth rate at 55°C in skim milk was achieved. The temperature growth limit for strains of thermophilic *G. stearothermophilus*, *A. flavithermus* and thermotolerant *B. licheniformis* are well documented and mentioned in the Table 11. For all tested strains, there was no difference in term of cardinal parameters comparing wild strains and type strains.

Table 11: Summarize of growth cardinals used for the comparison with process line

	<i>A. flavithermus</i>	<i>G. stearothermophilus</i>	<i>B. licheniformis</i>
T°C min	30	30,8	15
T°C opt	60	64	50
T°C max	75	74	60
a_w min	>0.978	>0.966	>0.881
pH min	>5.34	>3.91	>3.91

As mentioned, *B. licheniformis* is thermotolerant, meaning that its cardinal growth temperature are between mesophilic and thermophilic ones, with an optimum at 50°C. For *A. flavithermus* and *G. stearothermophilus*, they are both thermophilic with an optimum at 60 and 62°C respectively. Considering pH behavior, it appears that strains of *G. stearothermophilus* and *B. licheniformis* can grow at lower pH than strains of *A. flavithermus* (Table 11).

For minimal a_w , data reported in the literature ($a_w=f([NaCl])$) and results obtained ($a_w=f[glycerol]$) are similar or close (regarding NaCl and glycerol results). *B. licheniformis* can develop at lowest a_w values followed by *G. stearothermophilus* and *A. flavithermus*, indicating that it can develop in high concentrated milk parts.

3.2. Determination of the relationship a_w versus total solid content (TS%) as well as pH versus %TS of milk concentrates

The relationships between total solids contents and water activity of whole milk, skim milk and milk protein concentrate are shown in Figure 23.

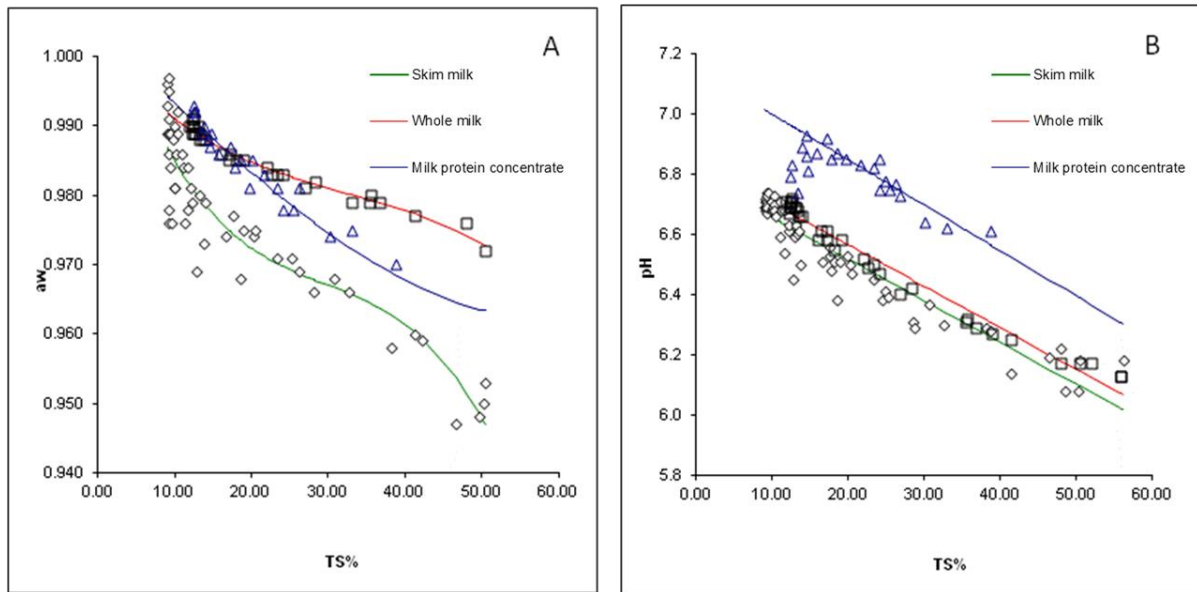


Figure 23: Relationship between water activity versus total solid content (%) (A) and relationship between pH versus total solid content (%) (B). These relationship were determined experimentally for concentrated whole milk; skim milk and milk proteins concentrate.

During concentration, for the same dry matter values, the water activity of concentrated skim milk is lower than the water activity of whole milk and milk protein concentrate obtained by ultrafiltration. Lactose is the most water-depressing compound in condensed milk compared to triglycerides in fat and protein. The water activity of the different concentrated milks can be related to the concentration of lactose. Its concentration in the total solid content is higher in skim milk (53% TS) than in whole milk (37% TS). Concerning the 80% protein concentrates obtained by ultrafiltration (MPC 80), part of the lactose is eliminated in the filtrate reducing its concentration to 6% TS. Empirical polynomial equations (Equation 1-3) have been developed.

Whole Milk: Equation 1

$$a_w = 1.00 - 1.40 \times 10^{-3} * (TS\%) + 3.37 \times 10^{-5} \times (TS\%)^2 - 3.49 \times 10^{-7} \times (TS\%)^3$$

Skim Milk: Equation 2

$$a_w = 1.01 - 3.74 \times 10^{-3} * (TS\%) + 1.12 \times 10^{-4} \times (TS\%)^2 - 1.26 \times 10^{-6} \times (TS\%)^3$$

Milk Protein Concentrate: Equation 3

$$a_w = 1.00 - 1.08 \times 10^{-3} * (TS\%) + 8.42 \times 10^{-8} \times (TS\%)^2 - 1.05 \times 10^{-7} \times (TS\%)^3$$

They have then been applied to estimate the water activities of the concentrates of skim milk, whole milk and MPC, using bibliographic data of the total solids contents, determined at the different steps of the manufacturing process of dairy powders (see figure 24).

As previously observed by Singh (2007), we report an acidification of milk during concentration steps (figure 23 B). The pH decreasing is similar for the three dairy solutions evaluated (WM, SM and MPC). The initial pH values of whole and skim milk is close to 6.7 pH, compare to 6.9 pH for protein concentrate. The slope is the same for the three representations. At 40 %TS, pH values of concentrated whole and skim milk are close to 6.2 pH compare to 6.5 pH for protein concentrate. The decrease of pH values of milk solutions during concentration is due to the concentration of lactic acid present in raw milk (0.10 to 0.19% of lactic acid (Fabro et al., 2006; Goff, 2020)).

3.3. Process description

The manufacturing processes for dairy powders are described in a few publications, articles or books. Data for temperature, pH, TS% and correlated a_w , were recorded at each stage of the major processes, *i.e.*: whole milk (WMP), skimmed milk (SMP) and milk protein (MPC). Description of all parameters (temperature, pH, TS% and correlated a_w) at each step was made for three major products: whole milk powder (WMP), skim milk powder (SMP) and milk protein concentrate (MPC) production. When available, time between CIP procedures are also reported. All Data are presented in the figure 24.

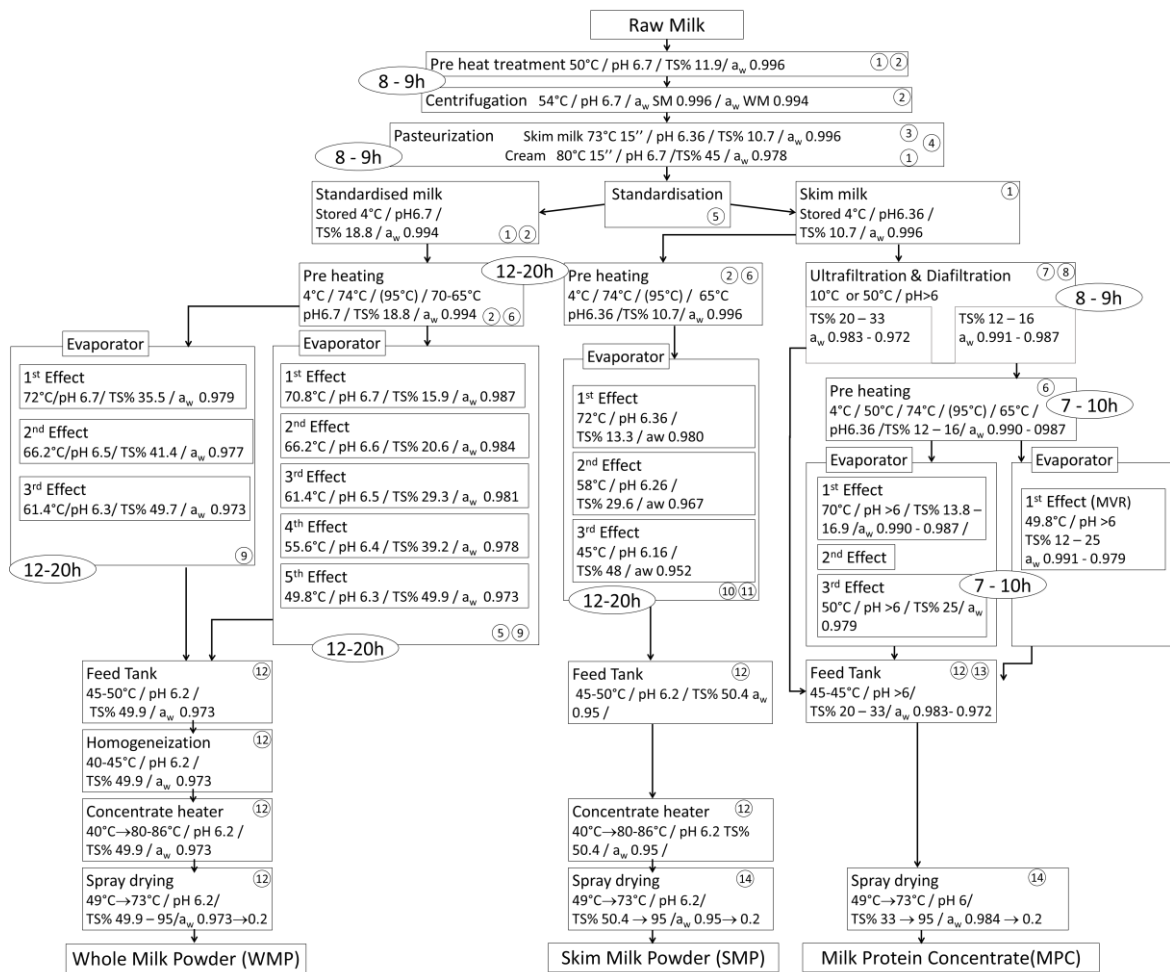


Figure 24: Flow chart for whole milk powder (WMP), skim milk powder (SMP) and milk protein concentrate powder (MPC) production. For each process step, the environmental physicochemical conditions encountered by the bacterial contaminants are indicated with associated values of temperature, pH, a_w and total solid content (TS%). Time between two CIP procedures, which is compatible with bacterial multiplication, are mentioned in ovals. References (1), Chirife & Fontan (1982); (2), Scott (2005); (3), Munir et al., (2014); (4), Fernandez-Salgero et al., (1993); (5), Singh (2007); (6), Refstrup (2000); (7), Getler et al., (1997); (8), Meena et al., (2017); (9), Zhang et al., (2018); (10), Karimi et al., (2007); (11), Murphy et al., (1999); (12), Pisecký (2012); (13), Augustin et al., (2012); (14), Schuck et al., (2008).

3.4. Growth capacity of *A. flavithermus*, *G. stearothermophilus* and *B. licheniformis* species in dairy powder processes.

The first observation is that, in these processes, the pH of the fluid remains above six in all stages, so minimal pH values of strains are never reached in dairy powder processes and will not be discussed. Therefore, this factor is not a limiting factor for the growth of the thermophilic bacteria studied and will not be discussed. Considering that, growth boundaries, temperature

(4-95°C) and water activity (0.2-0.996) are the main factors that will impact the growth, for these thermophilic bacteria, during the different steps of the dairy powder process.

Extreme values, obtained for strains of the different species were used, in order to take into account the worst scenario. Those are indicated in table 11. By combining Figure 24 and Table 11, growth capacities of *A. flavithermus*, *G. stearothermophilus*, and *B. licheniformis* can be determined in milk powder process and are represented in Table 12.

The mutual steps are present in the three process. Their parameters allow bacterial growth for the three species, except for *B. licheniformis* in pasteurization step. For milk preheating and centrifugation, the temperature is ranging between 50°C and 54°C, (Schuck, 2011; Scott, 2005) with a given a_w of 0.994 for whole milk (Chirife & Fontan, 1982). These environmental conditions clearly allow the growth and development of *A. flavithermus*, *G. stearothermophilus* and *B. licheniformis*. During the pasteurization step, the temperature of 73°C of skim milk (Munir et al., 2014) is close to the maximum growth temperatures of *A. flavithermus* and *G. stearothermophilus*. This temperature strongly limits but allow the growth of these microorganisms. However, they are not sufficient to thermally reduce the concentrations of vegetative forms and spores of these two species or the spores of *B. licheniformis* (D_{70} of vegetative cells of *A. flavithermus* (N=4) ranging from 0.7 to 3.33min, and 0.67min for *G. stearothermophilus* (Reich et al., 2017)).

For whole milk or skim milk, the preheating stage increases the temperature from 4°C to 40°C and then from 40°C to 70°C (Atkins, Walmsley, Walmsley, Fodor, & Neale, 2012; Karimi et al., 2007; Refstrup, 2000; Scott, 2005). During this step, the standardized milk (pH 6.7, a_w 0.994 - 0.996) and these temperature conditions (40°C-70°C) are highly favorable for the development of *A. flavithermus*, *G. stearothermophilus* and *B. licheniformis* (Table 12). Refstrup (2000) observed an increase in the concentration of thermophilic bacteria from 3 to more than 6 log cells.g⁻¹ of solid skimmed milk after 14 hours of heating. To limit the growth of thermophilic cells, Refstrup (2000) and Písecký (2012) presented the use of direct steam injection (DSI) heating at the end of preheating bringing the milk to a temperature of 80-95°C.

Table 12: Growth potential of *A. flavithermus*, *G. stearothermophilus* and *B. licheniformis* in dairy powder process. ✓: part of the process promoting bacterial growth.

		<i>A. flavithermus</i>	<i>G. stearothermophilus</i>	<i>B. licheniformis</i>
Mutual steps	Pre heating	✓	✓	✓
	Centrifugation	✓	✓	✓
	Pasteurization	✓	✓	
	Storage 4°C			
WMP	Pre heating	✓	✓	✓
	Evapo 3 effects E1	✓	✓	
	Evapo 3 effects E2		✓	
	Evapo 3 effects E3		✓	
	Evapo 5 effects E1	✓	✓	
	Evapo 5 effects E2	✓	✓	
	Evapo 5 effects E3	✓	✓	
	Evapo 5 effects E4	✓	✓	✓
	Evapo 5 effects E5		✓	✓
	Feed Tank		✓	✓
	Homogeneization		✓	✓
Concentr. Heater		✓	✓	
SMP	Pre heating	✓	✓	✓
	Evapo 3 effects E1	✓	✓	
	Evapo 3 effects E2		✓	✓
	Evapo 3 effects E3			✓
	Feed tank			✓
	Concentr. Heater			✓
MPC	UF et DF	✓	✓	✓
	UF et DF	✓	✓	✓
	Pre heating	✓	✓	✓
	Evapo 3 effects E1	✓	✓	
	Evapo 3 effects E3	✓	✓	✓
	Evapo 1 effect E1	✓	✓	✓
	Feed tank	✓	✓	✓
Mutual step	Spray drying			

However, the DSI treatment does not inactivate previously formed bacterial spores. These spores can originate in biofilms formed in the preheaters which can allow sporulation. Indeed, the time between two successive CIP, ranges from 12h to 20h, allowing biofilm formation. The length for growth and sporulation of thermophilic bacteria can be estimated at 9 hours in dairy industry preheaters (Scott et al., 2007). Other methods can be used to limit the development of biofilms of thermophilic bacteria during this step. Duplex preheating systems can be used to reduce the time between two cleanings of the preheating section or an increase of the cycle time up to 20 hours to limit the risk of development of sporulating thermophilic bacteria (Pisecký, 2012). However, the preheating step appears crucial for the entire process.

Evaporators concentrate the milk under vacuum, lowering its boiling temperature. For whole milk and skim milk, processed in multiple-effect systems the temperature is ranging from 70°C to 72°C in the first effect and from 45 to 62°C in the last effect depending on the number of effects and the type of evaporator (Karimi et al., 2007; Y. Zhang et al., 2018). Milk solids contents increase from 12.5% to 50% for whole milk (Y. Zhang et al., 2018) and from 8.5% to 45% for skim milk (Karimi et al., 2007; P. M. Murphy et al., 1999). For milk protein concentrates, the concentration rate per evaporator before drying is ranging from 12-16% to 25% (Industrial data). The vapor from the evaporation of water from the milk is recycled to the multiple-effect evaporators with a reduction in pressure and boiling temperature or in the same effect with multiple passes by thermal vapor recompression (TVR unit) or mechanical vapor recompression (MVR unit). MVR evaporators can be used with multiple passes with a boiling temperature of 50°C. The temperatures observed in the first effect of evaporators are lower or equal to the maximum growth temperatures of the thermophilic species *A. flavithermus* (66°C-75°C) and *G. stearothermophilus* (65.5°C-75°C) allowing their growth. The thermotolerant specie, *B. licheniformis* (Tmax 55°C-58°C), could grow in the MVR evaporators used for milk protein concentration (50°C) or in the last effect of multiple effect TVR evaporators where the temperature is less or equal to 58°C.

However the evolution of water activity in the evaporators could limit the development of these species. During evaporation, each effect eliminates water that increases the dry extract. Zhang et al., (2018) and Karimi et al., (2007) reported the evolution of the dry extracts at the level of each effect of the evaporators , respectively for the concentration of whole and skim milk. The mathematical equations determined in our study to determine a_w from dry extracts enables the identification of steps allowing growth of thermophilic bacteria for the process of whole milk, skimmed milk and liquid milk protein concentrates (Figure 24). During the concentration stage the decrease in water activity strongly limits the development capacity of *A. flavithermus* ($a_{w \text{ min}}$ 0.986-0.979), but, it appears that *A. flavithermus* can grow during the concentration of whole milk in three effects (TS% 29.3, a_w 0.981), in skim milk evaporator in the first effect (TS% 13.3, a_w 0.980) and in all the effects of MPC concentration.

The minimal a_w value for *G. stearothermophilus* growth ($a_{w \text{ min}}$ 0.979-0.968) allows this bacterium to develop, in all evaporator effects at whole milk and milk protein concentration but

not in skim milk concentration steps where a_w is lower than minimal required for growth (TS% 48, a_w 0.952).

For *B. licheniformis*, the range of a_w allowing growth is rather large ($a_{w \text{ min}}$ 0.902-0.887). This factor therefore does not appear to be a limiting factor during the concentration. This bacteria can grow in all effects as soon as the temperature allows it. *B. licheniformis* spores present in milk could, in the last effects of the evaporators, adhere to the stainless steel walls and form biofilms (Parkar et al., 2001; Yuan et al., 2018). This allow *B. licheniformis* to grow when the temperature is lower than T_{max} 56°C.

For powders of protein concentrates obtained from skimmed milk, an ultrafiltration step (UF) is carried out, with or without an additional diafiltration step (DF) to increase protein concentrations. During this step caseins and serum proteins are concentrated in the retentate part; lactose and calcium are eliminated in the filtrate. Bacteria and spores are retained with the proteins by the ultrafiltration and diafiltration membranes and remain in the retentate. The temperatures used in this step can vary from 10°C to 50°C (Meena et al., 2017). At 10°C no growth of thermophilic bacteria is possible but the low temperatures increase the viscosity of the liquids limiting the permeate flow rates, the protein concentration rate and the productivity. On the other hand, at 50°C the permeate flow rate is increased, but this temperature allows the growth of thermophilic bacteria. During this step the total solid content increases from 15 to 33% (Getler et al., 1997; Meena et al., 2017) which decreases the water activity (from 0.988 to 0.973). These water activity values do not limit the growth *G. stearothermophilus* and *B. licheniformis* but inhibit the growth of *A. flavithermus* with a_w lower than 0.978. These data are in accordance with Chamberland et al., (2019); where after 10h at 50°C using ultrafiltration membrane, process conditions lead to a selection of thermophilic bacteria and resulted in a high contamination of concentrated milk. At the end of this step, UF and DF concentrates, with high total solids concentrations (TS 25%), will be directly dried in a spray drying tower. Concentrates obtained only by UF have a lower total solids concentrations, and may be concentrated in an evaporator to increase the concentration up to 31% before spray drying (Augustin et al., 2012).

Prior to atomization, milk concentrates are stored in feed tanks at 40-45°C, then pumped, homogenized for whole milk and heated to 80°C by direct injection or tubular heat exchangers

before dispersion in the drying tower (Písecký, 2012). Although the temperatures during storage are favorable for the development of thermophilic bacteria. But water activity encounter restrict some bacterial growth. For WMP, water activity reached can only promote *G. stearothermophilus* and *B. licheniformis* growth (TS% 49.9 , a_w 0.973). For SMP, only *B. licheniformis* can growth in feed tank (TS% 50.4 , a_w 0.950). Whereas, for MPC feed tank, all the three species can develop and/or sporulate (TS% 20-33 , a_w 0.983 – 0.972).

During spray drying the condensed milk is dispersed as a spray in a stream of hot air from 180°C to 250°C. During a treatment time of about 30 seconds (Verdurmen & de Jong, 2003) the temperature at the surface of the droplets varies depending on the water activity of the concentrate, the temperature and the humidity of the incoming air. The temperature evolution at the droplet surface can be determined using the Mollier-Ramzine enthalpic diagram of the humid air. According to data published by Schuck et al., (2008), it is possible to determine the evolution of the surface temperature of a droplet between the inlet and outlet of the drying tower. At the beginning of the drying process, a droplet of solution with a water activity of 0.95 in air at 230°C with a humidity of 1g of water.kg⁻¹ of dry air has a surface temperature of 49°C. At the end of the drying process the milk powder with a water activity of 0.28 in air at 86°C with a humidity of 27g of water.kg⁻¹ of dry air at a temperature close to 73°C. These temperatures are in the range of the growth temperatures of the thermophilic spore-forming bacteria studied. However, the very low activity of the water, totally inhibits their growth or the germination of their bacterial spores. The heat treatment in the drying tower does not inhibit the growth of thermophilic microorganisms present in the milk but the conditions of water activity do not allow their multiplication.

4. Conclusion

The presence of thermophilic bacterial spores *A. flavithermus*, *G. stearothermophilus*, *B. licheniformis* in milk powders is related to the environmental conditions observed in the various stages of the manufacturing process and to the ability of these bacterial species to develop. Milk is a nutritive medium favorable to the development of strains of these species isolated in food products or processes and its pH changes only slightly during the manufacturing process of milk powders. The main factors that limit the development of these thermophilic

bacteria are temperature and water activity. Temperatures higher than 55°C in the last evaporator effects inhibit the growth of *B. licheniformis*. Decreases in water activity affect the growth of *A. flavithermus*, have a small impact on the growth of *G. stearothermophilus* but do not inhibit the growth of *B. licheniformis* prior to the drying step. *G. stearothermophilus* is the thermophilic species most likely to grow during the milk powder manufacturing process, the growth of *A. flavithermus* is limited by the low water activities observed in the last evaporator effects and subsequent steps. Temperatures observed in all stages of milk powder manufacturing before drying are insufficient to inactivate the vegetative forms of these thermophilic bacterial species and spray drying does not inactivate bacterial spores

The association of bacterial growth ranges (temperature, pH and water activity) and the conditions encountered during the processes, make the risk estimation of development of these bacteria during the manufacturing processes of food products possible.

Declarations of interest

None.

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5. Conclusion du Chapitre 3

En fonction des conditions rencontrées, les bactéries sporulées peuvent se développer au cours du procédé de transformation du lait en poudre lactières. Les paramètres limitants pour leur croissance sont la température et l'activité de l'eau. *G. stearothermophilus* et *A. flavithermus*, deux espèces thermophiles peuvent se multiplier dans les sections chaudes du process, mais la croissance de cette dernière va être inhibée par la diminution rapide de l'activité de l'eau au fur et à mesure que le pourcentage de matière sèche augmente dans le produit (dans les évaporateurs). Quant à *B. licheniformis*, sa croissance va être inhibée quand les températures rencontrées excèdent sa température maximale de croissance, mais est très peu impactée par les diminutions d' a_w .

Ces différentes capacités de croissance peuvent expliquer les variations de diversité expliquées dans le chapitre 2 de cette thèse, démontrant une forte présence de *G. stearothermophilus* dans les poudres de lait écrémé ou entier, et des poudres de lactosérum où les températures élevées et les a_w faibles favorisent la croissance de *G. stearothermophilus* au détriment d'*A. flavithermus*. La croissance de *B. licheniformis* est rendu possible dans le procédé d'obtention des poudres de concentré protéiques car les séparations sur membrane se font à des températures plus faibles que celles rencontrées en évaporateur. Il devient alors compétitif par rapport aux espèces plus thermophiles. Comme le montre la figure 25, à 55°C les cellules de *B. licheniformis* arrivent à se développer mais la température est très proche de leur température maximale de croissance, d'où la vitesse de croissance faible obtenus pour cette espèce sur lors de nos essais. *G. stearothermophilus* (Figure 25) et *A. flavithermus* (pas de données disponibles) ont la même tendance thermophile et se situent proche de leurs températures optimales de croissance à 55°C, et atteignent une vitesse de croissance maximale.

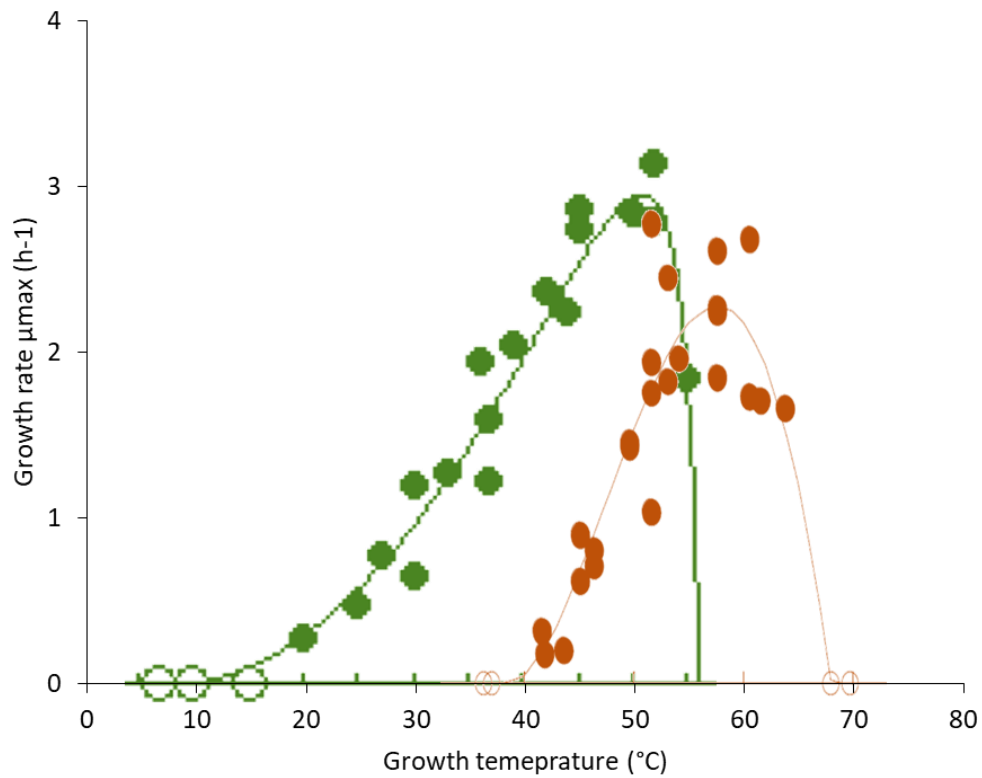


Figure 25: Vitesse spécifique de croissance de *B. licheniformis* (●) et *G. stearothermophilus* (●) en fonction de la température. Données issues de thèses réalisées au sein du LUBEM (Baril, 2011; Mtimet, 2015)

Afin de limiter la présence de ces bactéries dans le procédé de fabrication des poudres lactières, il est nécessaire de savoir comment contrôler leur développement et les éliminer le plus facilement sans trop impacter le rendement de production, via l'application de cycles de nettoyage en place.

Chapitre 4 : Comparaison de la formation de biofilm, et de leur résistance aux agents de NEP, chez les principales espèces laitières de bactéries sporulées thermophiles

En raison de leurs capacités de croissance adaptées au processus de transformation du lait en poudres laitières, les bactéries sporulées thermophiles peuvent s'y développer, notamment dans les parties du procédé de fabrication où les températures sont favorables et où la diminution de l'activité de l'eau ne devient pas limitante.

Cependant, la concentration initiale dans le lait cru et le temps de séjour dans les différentes étapes du procédé de fabrication ne suffisent pas à expliquer les fortes contaminations par les spores thermophiles dans les poudres de lait commercialisées. L'augmentation de la concentration de spores bactériennes observée ne peut s'expliquer que par la formation de biofilms sur les surfaces des équipements. Les biofilms sont des niches écologiques permettant aux bactéries thermophiles de se multiplier suffisamment et de re-sporuler.

Il apparaît donc intéressant de s'interroger sur les capacités des espèces thermophiles *A. flavithermus*, *G. stearothermophilus* et *B. licheniformis* à former des biofilms sur des surfaces en inox rencontrées en industrie.

De plus, dans l'industrie laitière les nettoyages des installations sont réalisés par des nettoyages en place (NEP). Les traitements de nettoyages usuellement appliqués sont alternativement des rinçages et l'utilisation de détergents alcalin (Hydroxyde de sodium) et acide (Acide Nitrique).

Il est donc important de traiter aussi ce deuxième volet concernant la persistance sur les surfaces et l'on peut se poser la question de l'efficacité des étapes de nettoyages alcalins et acides sur l'élimination de biofilms formés (spores) par les espèces thermophiles étudiées.

Dans le chapitre 4 les études réalisées ont eu pour objectif d'apporter des réponses à ces deux questionnements. Les travaux réalisés chez *A. flavithermus* et *G. stearothermophilus* sont présentés dans une proposition d'article ci-après tandis que les résultats obtenus pour *B. licheniformis* seront présentés brièvement en fin de ce chapitre, dans une partie « résultats complémentaires ».

Biofilm and sporulation ability of dairy powder sporeformers and their resistance against cleaning in place procedures

Article en cours de relecture pour être publié dans International Dairy Journal ou Food Micro

Abstract :

Thermophilic sporeforming bacteria are highly prevalent in dairy powder industries and can easily contaminate process. Those bacteria mainly represented by *Anoxybacillus flavithermus* and *Geobacillus stearothermophilus* are able to resist cleaning in place procedure through biofilm production. This study compares the ability of species to form biofilms by different techniques and their resistance toward parameters modifications (temperature, sodium hydroxide and nitric acid concentration, and duration of treatments) during cleaning procedures. It appears that in submerged conditions during 6h of growth, *A. flavithermus* can produce more biofilms compared to *Geobacillus* and can be composed of more than 79% of spores. Those structures lead to a better resistance against weak sodium hydroxide treatments and no differences between species can be observed using nitric acid solutions.

1. Introduction

The thermophilic bacterial spores *Anoxybacillus flavithermus* *Geobacillus stearothermophilus* are species frequently found in milk powders (P. M. Murphy et al., 1999; Rückert et al., 2004; Sadiq, Li, Liu, Flint, Zhang, & He, 2016; Schwartzberg, 1989; Scott et al., 2007). This high prevalence is related to favorable conditions allowing their development during the manufacturing process of dairy powders. Milk is a rich bacterial growth medium and the physiological growth characteristics of these species of thermophilic spore-forming bacteria allow them to develop, particularly in parts where temperatures are favorable and where the decrease in water activity does not become limiting.

However, the initial concentration of spores in the raw milk and the residence time in the various stages of the manufacturing process are not sufficient to explain the contamination by thermophilic spore forming bacteria spores in commercial milk powders. The increase of bacterial spores concentration can be linked to the biofilms formation on the surfaces of the equipment allowing thermophilic bacteria to grow and multiply sufficiently and sporulate.

From dairy biofilms obtained on plant surfaces, the aerobic thermophilic spore-forming species *A. flavithermus* *G. stearothermophilus* have been isolated and identified in numerous studies (S. Flint et al., 2020; Gopal et al., 2015; Langeveld, Montfort Quasig, Weerkamp, Waalewijn, & Wever, 1995; Sadiq et al., 2017; Yu Zhao et al., 2013). The biofilm formation capacities of these three species were studied under laboratory conditions, on different media, in different surface and for several strains per species (Burgess et al., 2009; S. H. Flint, Palmer, et al., 2001; Ostrov, Paz, et al., 2019; Seale et al., 2008). Sadiq et al., (2017) showed that for different strains isolated from dairy industry equipment (*A. flavithermus*, *G. stearothermophilus* and *B. licheniformis*), biofilm formation is highly impacted by the surface (polystyrene and stainless steel), media (Tryptic soy broth or milk) and temperature (37°C or 55°C).

To remove bacterial biofilm, and food residues, industrials use specific cleaning procedures to respect their material shelf life and environment. Industrials use Cleaning In Place (CIP) procedures to wash their process line without dismantling or opening. In the dairy powder industry plate heat exchanger, evaporators and pipes are contaminated with thermophilic spore formers. Because of industrial equipment size (more than 10 000m² of surface for an

evaporator), and shape (curved pipes, corners, dead ends, valves) it is hard to clean and remove all the bacterial contaminations.

The first step of cleaning is a water rinsing of the installations to remove milk deposits present on the surface, followed by a circulation of an alkaline solution (0.5/2% at 60 to 80°C during 10 to 30min). The purpose of this step is to remove the remaining proteins as well as to saponify the persistent fats in the system (Watkinson, 2009). A water rinse is then performed to remove alkaline residues, and acid solutions (0.5/1% at 60 or 70°C for 10 to 30 min) is injected into the process to remove persistent mineral salts (P. J. Bremer & Seale, 2010; Tetrapack, 2019; Thomas & Sathian, 2014). At the same concentration, nitric acid has a higher sporicidal activity than soda (Wedel et al., 2019).

Dairy biofilms are constituted by bacteria and bacterial spores in a matrix composed of Exopolysaccharides (EPS), external DNA, microbial proteins, and milk residues (Hinton et al., 2002; Wedel et al., 2020). This matrix protect vegetative cells and spores and lead to a better resistance to CIP compared to planktonic cells (Watkinson, 2009). The presence of spore embedded in biofilm increase the resistance, persistence and dissemination of biofilm in industrials or natural environment.

Because of their resistance, biofilms may persist, allowing spore-forming bacteria to re-contaminate the transformation process surfaces and product (S. H. Flint et al., 1997; Hinton et al., 2002; Ostrov, Paz, et al., 2019; Ribeiro et al., 2019).

The aim of this study was to qualify the ability or capacity of thermophilic spore forming bacteria *A. flavithermus* *G. stearothermophilus* to form biofilm on stainless steel in dairy conditions, to quantify them by microscopic method and cell concentration. The resistance of these biofilms during CIP procedures alkaline and acid step were quantified. For alkaline and acid cleaning step the efficiency of detergent concentration, temperature and time contact were evaluated and analyzed.

2. Materials and methods

2.1. Bacterial strains

A total of 313 strains of sporeforming bacteria has been isolated from dairy powders and characterized with molecular typing (Delaunay et al., 2021). A selection of 45 strains of

A. flavithermus and *G. stearothermophilus* species was done to represent encountered biodiversity, taking into account type of dairy powder, origin of the powder. The type strains of each species, *A. flavithermus* DSM2641 and *G. stearothermophilus* DSM22, have also been studied. These 47 selected strains were used for biofilm formation ability and resistance against CIP.

2.2. Inocula preparation

All strains were cultured on TSA for 24 hours, 5mL of TSB were inoculated, incubated for 6 hours at 55°C and then 100µL were transferred to 50ml of TSB broth and incubated at 55°C with agitation for 16 hours. The cultures were centrifuged at 6000 rpm for 10 min at 4°C; the pellets were rinsed with 10 mL of sterile TS and suspended in 1 mL of TS. The optical density (measured at 600nm) of the suspensions was used to inoculate UHT commercial skim milk at a 5 log of CFU.mL⁻¹ concentration.

2.3. Screening of biofilm formation

2.3.1. Biofilm on submerged coupons

This method evaluates the ability of strains to form biofilms on a totally submerged surface. Each sterile stainless steel coupon (grade 304, 1cm²) was placed in a 6cm Petri dish and covered with 10mL of inoculated milk. Petri dishes were incubated for 45 minutes at 55°C without shaking. The milk was removed and replaced by sterile UHT skimmed milk and the Petri dishes were incubated back at 55°C, shaken (50rpm 3mm orbital shaker) for 6 hours. After incubation, the contents was removed, the coupons were washed twice with 10mL of sterile water to remove as much milk residue and planktonic bacteria as possible. The coupons were covered with acridine orange (0.01% in 0.22µm filtered water) and left in the dark for 15 minutes. The excess acridine orange was then removed by two rinses with 1mL of filtered water. Each coupon was covered with a glass slide and observed with a x100 lens under oil immersion. A series of 10 pictures were taken at random per coupon and images obtained were analysed using a script on ImageJ (Schneider, Rasband, & Eliceiri, 2012) to calculate a percentage of biofilm coverage by microscopic field. Each measure per strain was carried out on two separate coupons and each strain was analysed twice from different inoculum.

2.3.2. Screening of spore formation in biofilms in 96 wells stainless steel plates

In order to evaluate the ability of strains to form spores inside biofilm on stainless steel, a fast quantification of spores and vegetative bacteria was carried out on a 96-well stainless steel microplates (custom made, food grade stainless steel, T2C, Quimper, France) in UHT skimmed milk. A volume of 200 μ L of inoculated milk was distributed in each well. Then the plate was incubated at 55°C for 45min without stirring, allowing the planktonic bacteria to adhere to the surfaces. Then the milk was removed and replaced by sterile skimmed milk already at temperature. The plates were incubated for 6 hours at 55°C with agitation (100rpm, 5cm orbital shaker). Then liquid milk was removed and wells are rinsed with 250 μ L of sterile water. Biofilm was detached by sonication (10min, 40% of 40KHz) (Konrat et al., 2016) in 250 μ L of TS solution. Preliminary tests for biofilm removal using ultrasonic bath were done and protocol was validated (data not shown). Total cells and spores (10min at 80°C) were enumerate using the microcolonies counts (Konrat et al., 2016) on square Petri dishes in TSA supplemented with potato starch, after 16h at 55°C. Depending on the dimensions of the well, the volume contained and the agitation applied, the surface covered by the liquid during handling was estimated at 1.52 cm². All the results were brought back to CFU.cm⁻² taking this surface into account. The amount of vegetative cells are calculated by subtracting the spore concentration on the total cell concentration per cm square.

2.4. Alkaline and acid effect on biofilm removal

Seven strains of *Anoxybacillus flavithermus* and three strains of *Geobacillus stearothermophilus* able to form a biofilm presenting more than 4log of spores.cm⁻² were selected. Biofilm were developed in milk on stainless steel plates for 6 hours. After the first water wash, caustic soda (0.25, 0.5 or 1% (v/v) at 60, 70 or 80°C) or nitric acid (0.05, 0.1 or 0.7% (v/v) at 60, 65, 70°C) were added for treatment duration 10, 20 or 30min. Then alkaline or acid solutions (250 μ L) were eliminated and 250 μ L of water were added for rinsing. Remaining biofilm was detached in tryptone salt solution by sonication and enumerated (drop plate method) as described previously. Negative control was done using the same protocol with TS solution replacing alkaline or acid solution. The effect of the treatment was assessed by comparing enumeration made from testing wells and negative control. So both release of

biofilm and cells inactivation by the treatment were taken into account. All conditions was assessed on technical triplicate.

2.5. Data analysis

Data quantifying the decrease of cell concentration (per cm²) of *A. flavithermus* and *G. stearothermophilus* biofilms were analyzed by variance analysis. These analysis assess the impact of the factors studied (NaOH or HNO₃ concentrations, temperature and contact time) on the biofilm remove. Statistical analyses (ANOVA) and graphics have been done using Statistica software (13.5.0.17).

3. Results

3.1. Screening of biofilm formation

3.1.1. Screening of biofilm formation on submerged stainless steel

For the two species *i.e.* *A. flavithermus* and *G. stearothermophilus*, the capabilities of 47 strains to produce biofilm within 6h, on stainless steel coupon, are presented in the Table 13. *Anoxybacillus flavithermus* is the species that forms the most biofilms according to the strains tested. Out of the 32 strains tested (including the DSM2641 type strain), 62.5% form a biofilm allowing an average microscopic field coverage of more than 35%. This is more than the results obtained for *Geobacillus stearothermophilus*. From 15 strains tested a single strain of *G. stearothermophilus* (6.7%) formed biofilm. However, this strain present a high coverage percentage (75%).

Table 13: Biofilm formation obtained on 47 strains on two major species found in dairy powder after 6h of growth at 55°C using acridine orange staining. * see figure 25 for an example of 2D/3D biofilm observation.

	Number of strains	% of tested strains			% coverage max
		Showing a biofilm coverage >5%	2D biofilm*	3D biofilm*	
<i>Anoxybacillus flavithermus</i>	32	62.50%	25%	37.50%	35
<i>Geobacillus stearothermophilus</i>	15	6.70%	/	6.70%	75

Within thermophilic sporeforming bacteria, two types of biofilm could be observed as two dimension or three dimension biofilms. On the 32 strains of *A. flavithermus* tested, 25% of them

could form two-dimensional biofilm (Figure 26.B), compared to 37.5% of strains, which could form three-dimensional biofilm in 6h of growth (Figure 26, A). The only one biofilm formed by a *G. stearotherophilus* strain was characterized as a three-dimensional one (Figure 26, C).

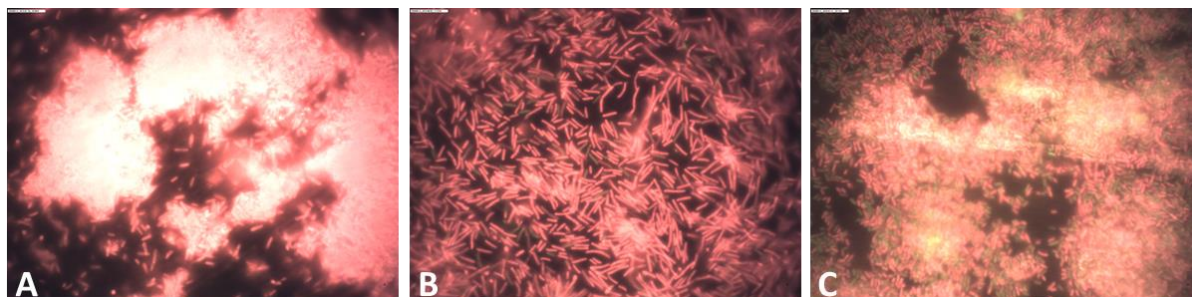


Figure 26: Microscopic observation of formed biofilm after acridine staining for ; A : *Anoxybacillus flavithermus* AF288 (forming a 3D biofilm) ; B : AF5 (forming a 2D biofilm) and C : for *Geobacillus stearotherophilus* GS30 (forming a 3D biofilm) in tested conditions

3.1.2. Screening of spore formation in biofilms

For the 32 strains of *A. flavithermus* and the 15 strains of *G. stearotherophilus*, the Figure 27 shows the concentrations of vegetative cells and spores produced in the biofilms in the stainless steel microplates.

Most of *A. flavithermus* strains can form biofilm in those conditions composed for more than 5log vegetative cells.cm⁻² for 90.7% of them, which is quite similar to *G. stearotherophilus*, with 80% of the 15 strains tested. Regarding spores, *A. flavithermus* biofilms could be composed of more than 4 log of spores.cm⁻² for 71.9% of them and only 20% of them for *G. stearotherophilus*. As shown, the quantity of spores in *A. flavithermus* biofilm is higher than *G. stearotherophilus* ones, with 79.8% and 16.3% of maximum sporulation ratio respectively.

On the 32 *A. flavithermus* strains tested, 9 of them are above the 20% sporulation ratio (Red line figure 27), versus no one for *G. stearotherophilus*. For this specie, 8 strains (53%) are above the 0.2% sporulation ratio line (green line Figure 27), similar to 17 strains (53%) for *A. flavithermus*, between 20% and 0.2% sporulation ratio.

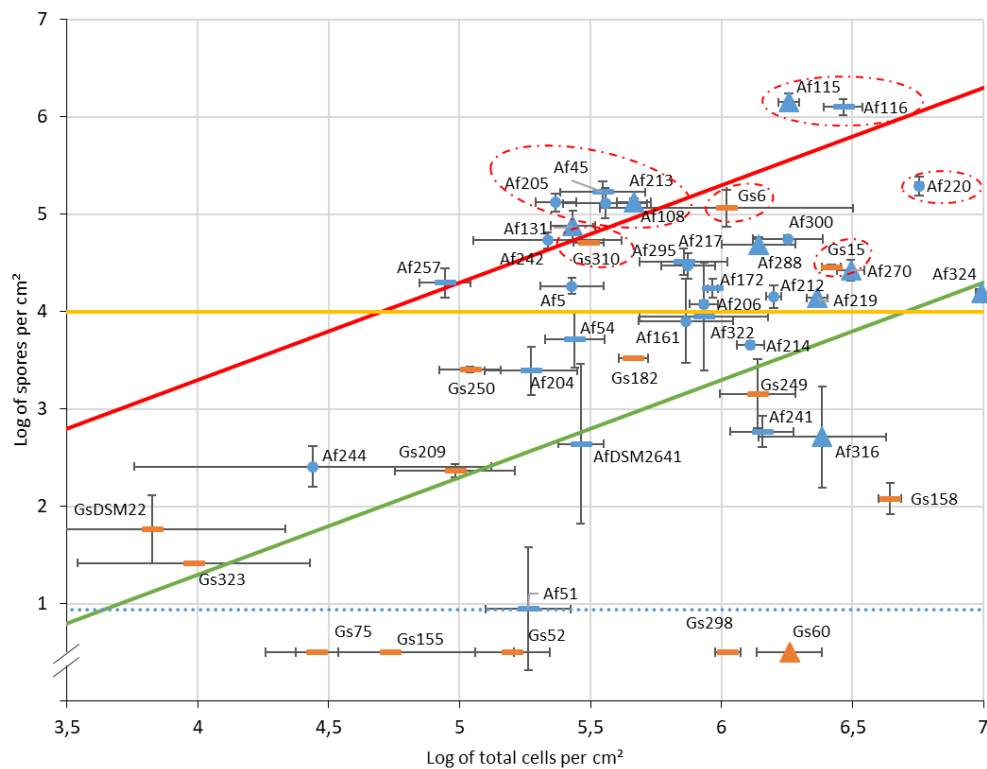


Figure 27: Correlation between log of spores per cm^2 and total cells per cm^2 present in biofilms formed by *A. flavithermus* (blue) and *G. stearothersophilus* (orange), in stainless steel microplate after 6h of growth at 55°C in milk. Results from coupon technique were combined as: non biofilm former bacteria (—), 2D biofilm (●) and 3D biofilm (▲). Yellow line: arbitrary limit of 4 log of spores. cm^{-2} ; Red line (—): 20% of sporulation ratio; Green line (—): 0.2% of sporulation ratio. Blue dotted line (—): Detection limit of the method (0.94log.cm^{-2}). Spores from strains under detection limit (Gs75, 155, 52, 298 and 60) were not able to be enumerated in tested conditions. One strain Af112 neither form vegetative cells nor spore using microplate technique.

The yellow line (Figure 27) represent an arbitrary level of 4log of spores. cm^{-2} . As already mentioned, many strains of *A. flavithermus* biofilms are composed of more of 4log of spore. cm^{-2} compare to *G. stearothersophilus*. This specie then could be an issue for total removal of bacterial biofilm by the industry.

3.2. CIP effect, Alkaline and acid effect on biofilm removal

For alkaline treatment, as shown, for two strains, in the Figure 28, the inactivation kinetics are biphasic. An inactivation of 2-3log CFU. cm^{-2} is observed at the beginning of the treatment between 0 and 10 minutes, then the decrease of the population is less important between 10, 20 and 30 minutes. For acidic treatment, the inactivation kinetics appear log linear (Figure 26). Similar patterns were observed for all strains (data not shown).

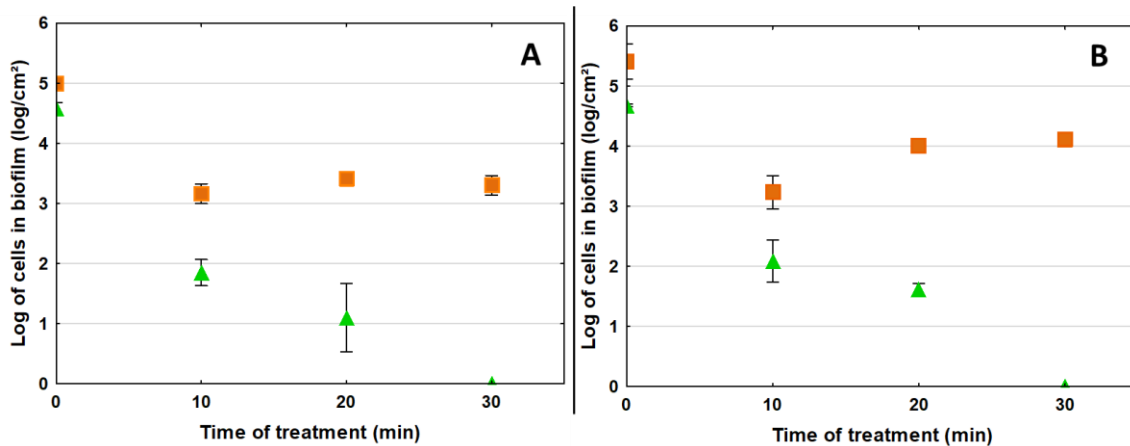


Figure 28: Kinetic of inactivation of *A. flavithermus* AF45 (A) and *G. stearothermophilus* GS6 (B), against 0.25% NaOH 70°C (■), and 0.1% HNO₃ at 65°C (▲) depending on the time of treatment (min).

For all strains of each species, the impact of the studied factors on biofilm removal was globally evaluated by analysis of variance. Indeed, the effects of the concentration, temperature and contact time were assessed for alkaline and acidic treatments for *A. flavithermus* (7 strains) and *G. stearothermophilus* (3 strains) (Table 14 and Figure 29).

Table 14: Significant effects of three parameters on the destruction of biofilms formed by 7 strains of *A. flavithermus* and 3 strains of *G. stearothermophilus*. NS : Non significant (p -value $>0,05$), * : p -value $\leq 0,05$, ** : p -value $\leq 0,01$, *** : p -value $\leq 0,001$, **** : p -value $\leq 0,0001$

	NaOH treatment		Nitric acid treatment	
	<i>A. flavithermus</i>	<i>G. stearothermophilus</i>	<i>A. flavithermus</i>	<i>G. stearothermophilus</i>
Concentration	* (P-value=0.01854)	**** (P-value=0.00000)	**** (P-value=0.00000)	*** (P-value=0.00053)
Temperature	* (P-value=0.01353)	*** (P-value=0.00023)	**** (P-value=0.00000)	NS (P-value=0.57928)
Treatment duration	NS (P-value=0.12692)	NS (P-value=0.44855)	**** (P-value=0.00000)	**** (P-value=0.00000)

Alkaline treatments inactivate bacterial spores. NaOH concentration has a significant effect on the inactivation of spores of both species studied. As the concentration increases, the removal of bacteria increase. The decrease is significantly (p -value= 0.0000) more marked for *G. stearothermophilus* than for *A. flavithermus*. The effect of temperature is globally significant but is variable according to the species and strains studied. The increase of temperature from

60°C to 80°C, increases or reduces the efficiency of the soda treatment. Increases were observed for 4 strains of *A. flavithermus* and decreases for the 3 strains of *G. stearothermophilus* studied. Nitric acid treatments inactivate bacterial spores in biofilms. Both studied species are affected significantly and identically to the treatment time, concentration and temperature factors. Increasing the concentration of nitric acid increases the inactivation of spores in biofilms for both species studied. For *A. flavithermus* strains, the increase in temperature increases the inactivation effects of nitric acid treatments but this effect is variable depending on the strains studied.

The impacts of each factor on biofilms decrease are presented in the Figure 29.

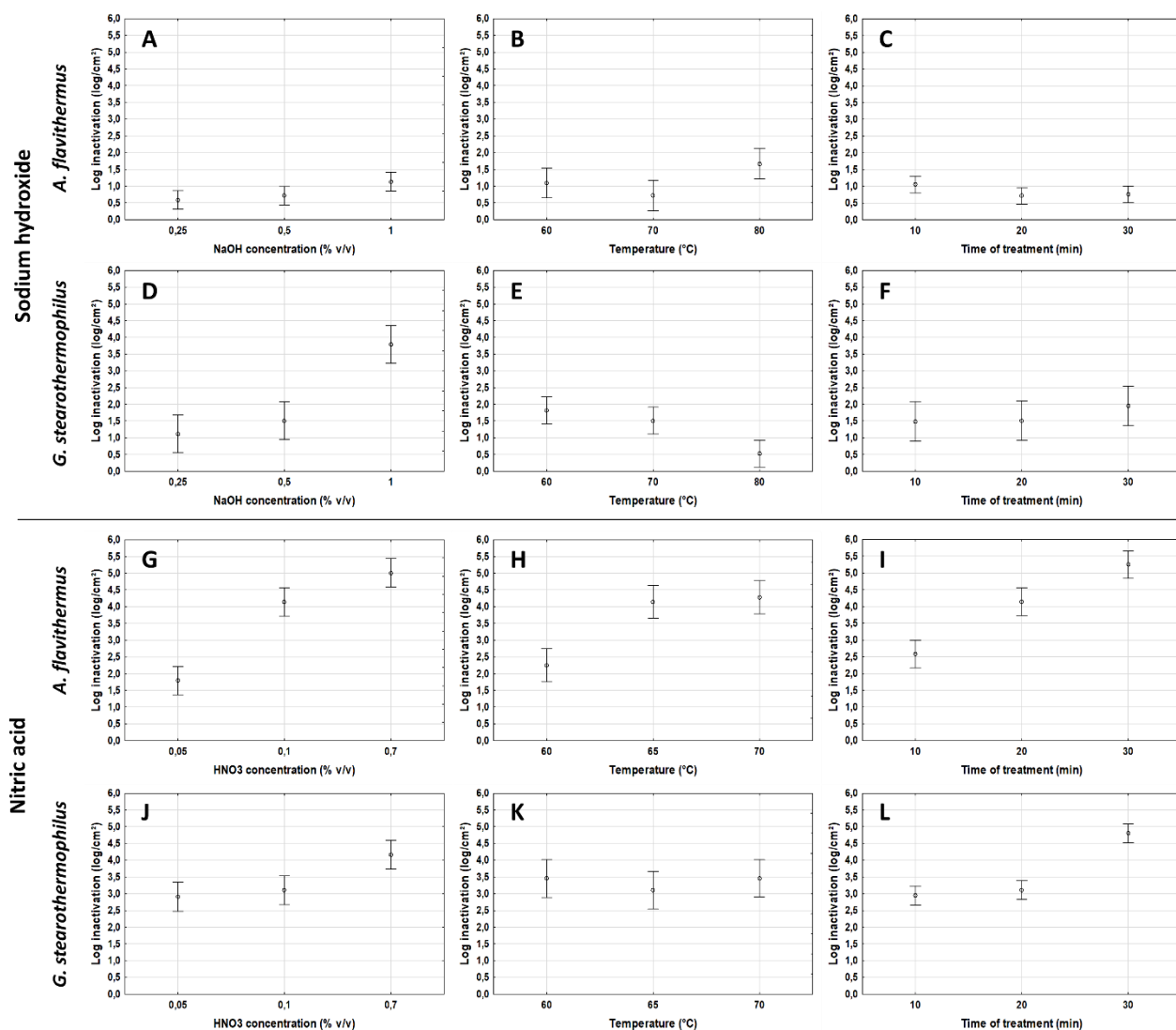


Figure 29: Log inactivation of *A. flavithermus* or *G. stearothermophilus* spores.cm⁻², depending of the treatment applied, using caustic soda (upper part) and nitric acid (bottom part), regarding different parameters: solution concentration (A; D; G and J); Temperature (B; E; H and K), and duration of the treatment (C; F; I and L). ○ : least square means, whisker : 95% of confidence intervals.

4. Discussion

A. flavithermus and *G. stearothermophilus* are two thermophilic spore-forming species that can grow in biofilms on stainless steel surfaces of dairy equipment (Burgess et al., 2009; Scott et al., 2007).

A. flavithermus shows a great ability to form biofilms in 6 hours at 55°C on stainless steel coupons immersed in agitated and not renewed skim milk. Indeed 60% of the 32 strains studied formed biofilms observable by microscopy. This rapid formation of biofilms of this species was observed by Burgess et al., (2009) in dynamic conditions at the same temperature. This rapidity can be explained by the incubation temperature close to the optimal growth temperature for this species (60°) (Ellis & Magnuson, 2012; Heinen et al., 1982).

G. stearothermophilus also has the ability to form biofilms on stainless steel surfaces (M. Kumar, Flint, Palmer, Chanapha, & Hall, 2021; Seale et al., 2008). However, at 55°C the ability of this species to form biofilms appears limited, only one strain out of 15 strains studied was able to develop biofilms on stainless steel coupons immersed in milk. This lack of biofilm formation observed may be related to the incubation temperature far from the optimal growth temperatures (58°C-65°C) which would delay biofilm formation. As observed by Kumar et al., (2021) the biofilm onset time for this species is longer at 55°C than at 60°C or 65°C. These biofilm times are also dependent on the conditions studied.

The use of stainless steel microplates also allows on the one hand to quantify the formation of biofilms on surfaces close to the surfaces of industrial equipment and on the other hand to easily carry out screening to quantify the formation and the effects of the treatments of detachment of biofilms by the use of alkaline or acid detergents.

Compared to biofilms on immersed coupons, in these microplates more strains of *A. flavithermus* and *G. stearothermophilus* developed biofilms with concentrations of 4 log bacteria.cm⁻² to 7 log bacteria.cm⁻². This difference can be explained by biofilm developments at liquid-air interfaces (Yu Zhao et al., 2013). Moreover *G. stearothermophilus* is a strictly aerobic bacteria unlike *A. flavithermus* which is able to develop anaerobically. The availability of O₂ in the coupon system could be limiting.

Bacterial spore formation is concomitant with bacterial growth (Gauvry et al., 2019; M. Kumar et al., 2021) However, the ratio between the number of vegetative cells and spores formed at 6

hours of incubation varies among the strains studied. Using those protocols, *A. flavithermus* appears to be the best spore-forming bacterium suited to rapidly make biofilms in milk industry (in the tested conditions) in milk at 55°C and could rapidly (in 6h) contaminate specific parts of the process using these temperatures. This specie could therefore be pioneer in biofilm formation allowing other sporulated bacteria to adhere and form a stronger and multi species biofilm. Its rapid formation of biofilm and spores production (up to more than 87% of total biofilm bacteria) could also enable it to resist CIPs used even in short manufacturing processes (less than 10h) as already demonstrated by Burgess et al., (2009).

Evaluations of the effectiveness of cleaning treatments with alkaline and nitric acid solution usually used in CIP were studied by different authors on bacteria or bacterial spores suspensions or on biofilms formed on immersed stainless steel coupons. (P. Bremer et al., 2006; Parkar, Flint, & Brooks, 2004; Wedel et al., 2019). The use of stainless steel microplates to study biofilm formation and cleaning treatment efficiencies presents several interests. On the one hand, it allows a rapid screening by performing multiple tests per plate by studying the capacity of biofilm formation by numerous strains or bacterial species on stainless steel surfaces. On the other hand, it allows the study of the effects of different factors affecting the effectiveness of alkaline and acid treatments on the removal and inactivation of bacteria and spores within biofilms with rinsing steps.

Alkaline inactivation studies of biofilms obtained on stainless steel coupons immersed in flow or in stainless steel micro-plates gave similar results. Analyses performed on *A. flavithermus* by Wedel et al., (2020) evaluated the resistance of biofilms formed on flow-through coupons to a 1% NaOH treatment at 60°C for 1 min, allowing the removal of 1.6 log.cm². These data are comparable to our results of treatment with 0.5% NaOH at 70°C for 10min, with eliminations of 0.10 log.cm² to 2.01 log.cm² depending on the *A. flavithermus* strains.

The kinetics of bacterial inactivation by soda are biphasic as observed by Bénézech et al., (2018) and Wedel et al., (2019). In a first phase the bacterial spores are rapidly inactivated then in the second phase the inactivation rate decreases where the time factor (10, 20 and 30 minutes) does not appear significant for the removal of *A. flavithermus* and *G. stearothermophilus* biofilms.

The NaOH concentration effect on the removal and inactivation of biofilm bacteria is significant for both species in our study. An increase from 0.5% to 1% NaOH at 70°C for 20

minutes leads to a decrease from 0.02 log.cm^{-2} to 0.86 log.cm^{-2} according to *A. flavithermus* strains and from 1.10 log.cm^{-2} to 4 log.cm^{-2} according to *G. stearothermophilus* strains. For these two species Wedel et al., (2019) observed similar results, on planktonic spores with 10 min treatments at 65°C . An increase in soda concentration from 0.5% to 1% increases the removal by 0 log.mL^{-1} to 0.75 log.mL^{-1} depending on the *A. flavithermus* strains studied and by 0.39 to 1.94 log.mL^{-1} depending on the *G. stearothermophilus* strains studied. Bacteria elimination and inactivation seems to be more important in biofilms than in planktonic conditions on spore. The elimination of spores in biofilms depends on both the biofilm remove and their inactivation by NaOH, whereas in planktonic conditions only the effect of inactivation by NaOH present an effect against bacterial spores.

Few papers have quantified the effect of temperature on the removal and inactivation of bacteria or spores in biofilms by NaOH. For the two species studied this factor is significant, increasing temperature decreases the effectiveness of the treatment on biofilms of the three strains of *G. stearothermophilus* studied.

Nitric acid in CIP procedures is used to remove mineral deposits, but also acts to loosen biofilms and inactivate spores (P. J. Bremer & Seale, 2010).

The treatment time has a significant effect on spore inactivation by HNO_3 . The kinetics of spore inactivation of *A. flavithermus* and *G. stearothermophilus* strains by nitric acid treatments are log linear in Figure 28. Decimal reduction time values D can be calculated. At 65°C with a nitric acid concentration of 0.1% for *A. flavithermus* the $D_{65^{\circ}\text{C}}$ values vary from 3.82 min to 7.83 min depending on the strain and from 5.95 min to 6.59 min depending on the strain of *G. stearothermophilus* inactivated in biofilms. In planktonic conditions, for *G. stearothermophilus* G8742, Wedel et al., (2019), had determined a $D_{60^{\circ}\text{C}}$ value of 3.7 and 0.58 min for nitric acid concentrations of 0.5% and 1% respectively. Based on the data from Wedel et al., (2019) it can be calculated and estimated that the D value for *G. stearothermophilus* G8742 would be 1.2 minutes at 65°C and 0.1% nitric acid. This data comparison would confirm the protective effect of biofilms for bacterial spore against the bactericidal effect of nitric acid

The effect of the concentration is significantly impacting on the elimination, when the concentration increases, the level of surviving spores decreases. *G. stearothermophilus* strains

are more sensitive to low concentration treatments than *A. flavithermus* strains. The increase in the inactivation efficiency of bacterial spores in biofilm as a function of nitric acid concentration appears to be greater between concentrations of 0.05% and 1% than between 0.1% and 0.7%. This observation is different from Wedel et al., (2019) for *G. stearothermophilus* strains. In planktonic condition a log linear relationship appears between the time to first decimal reduction and the concentrations.

The effect of temperature on nitric acid treatment is significant for *A. flavithermus* strains, with increasing temperature leading to better removal and inactivation of bacterial spores in biofilms. This effect is in agreement with the observations of Wedel et al., (2019).

5. Conclusion

Anoxybacillus flavithermus seems to be the most suitable and the most adapted bacteria to form biofilm rapidly on stainless steel surfaces compare to *Geobacillus stearothermophilus* at 55°C in skimmed milk medium on stainless steel. Its biofilms can be composed of more than 75% of spores and can have a better resistance to weak treatments compare to *G. stearothermophilus*. At high CIP conditions, both thermophilic bacteria have the same behavior and cannot survive high parameters.

6. Etude complémentaire : Formation de biofilms chez *Bacillus licheniformis*.

L'évaluation des capacités à former un biofilm rapidement sur de l'inox immergé dans du lait à 55°C a également été réalisée sur des souches de *Bacillus licheniformis* d'origine laitière (N=14) ou non (N=1 ; souche type DSM13).

Sur les coupons en inox ; aucune des souches de *B. licheniformis* testées n'a formé du biofilm (couverture >5%) en 6h dans les conditions établies. Cette faible capacité est corrélée avec les résultats obtenus dans l'étude de Zain et al., (2017), qui, après isolement de souches de protéines de lactosérum et formation de biofilm sur de l'inox pendant 24h, obtiennent 36% de souches productrices de biofilm à 37°C, contre 9% à 55°C.

Afin de compléter nos résultats, la capacité de 3 souches de *B. licheniformis* à former un biofilm sur la surface d'une microplaque en inox (interface air/liquide) a également été évalué. Aucune des souches testées n'a pu former un biofilm de plus de 3 log de bactéries végétatives.cm⁻² et ne contenait pas de spores.

Comme cité précédemment l'effet de la température sur la formation de biofilm est un paramètre clé, favorisant ou non l'adhésion et la croissance des bactéries. De plus, il semble que *B. licheniformis* ai besoin de plus de temps pour former un biofilm (24 ou 48h) comparé aux espèces thermophiles comme *A. flavithermus* et *G. stearothermophilus*.

7. Conclusion du Chapitre 4

La formation de biofilm chez les bactéries sporulées thermophiles en industrie laitières est un moyen utilisé pour résister plus facilement aux traitements de nettoyage usuels, et permet également une dissémination rapide des bactéries dans le système. Les résultats présentés dans ce chapitre permettent de mettre en avant *Anoxybacillus flavithermus* comme étant l'espèce la plus adaptée pour former rapidement du biofilm dans les conditions testées, en seulement 6h de croissance à 55°C. Les différentes souches de *Geobacillus stearothermophilus* et de *Bacillus licheniformis* n'ont pas permis de former un biofilm assez conséquent sur les coupons en inox immergés (sauf une seule souche de *Geobacillus*).

Cependant, après utilisation d'une seconde méthode, la majorité des souches d'*A. flavithermus* et de *G. stearothermophilus* semblent former, à l'interface air/liquide, un biofilm, dont la part des spores peut représenter plus de 75% de la population totale. Celui-ci s'est révélé assez important pour mesurer l'effet des solutions de nettoyage et mimer les NEP rencontrés en industrie.

Selon les résultats obtenus, il semble que les biofilms formés par *A. flavithermus* soient plus résistants aux faibles concentrations de soude et d'acide, comparés à ceux de *G. stearothermophilus*. Cependant, les deux espèces ne résistent pas aux forts traitements appliqués et utilisés lors de ces travaux. L'ensemble des résultats peut être synthétisé en trois dimensions comme présenté dans la figure ci-dessous (Figure 30).

Cette représentation permet de visualiser une meilleure résistance des souches d'*A. flavithermus* à des fortes concentrations en soude à des temps de contact courts, alors que *G. stearothermophilus* est plus impacté (inactivation et élimination de plus de 3.5log.cm⁻² à 1% en soude pendant 10min de traitement). Pour les données de résistance aux traitements acides, les données sont plus complexes à analyser. Pour le traitement à l'acide nitrique, il semble que *G. stearothermophilus* soit plus sensible aux basses concentrations lors de traitement courts permettant la destruction et l'élimination de 2.5log de spores.cm⁻² (Figure 30 D) contre 1.8 log pour *A. flavithermus* (figure 30 B). Dès que le temps est allongé ou que la concentration en acide est augmentée, les réponses pour ces deux espèces sont identiques avec une destruction et une élimination de 4log de spores.cm⁻². A forte concentration et avec une durée maximale de 30min, il semble que *A. flavithermus* soit plus sensible que *G. stearothermophilus*, ce qui n'est

pas le cas mais est dû à la représentation (prolongation de la surface de réponse dans des conditions maximales non testées dans le plan d'expérience).

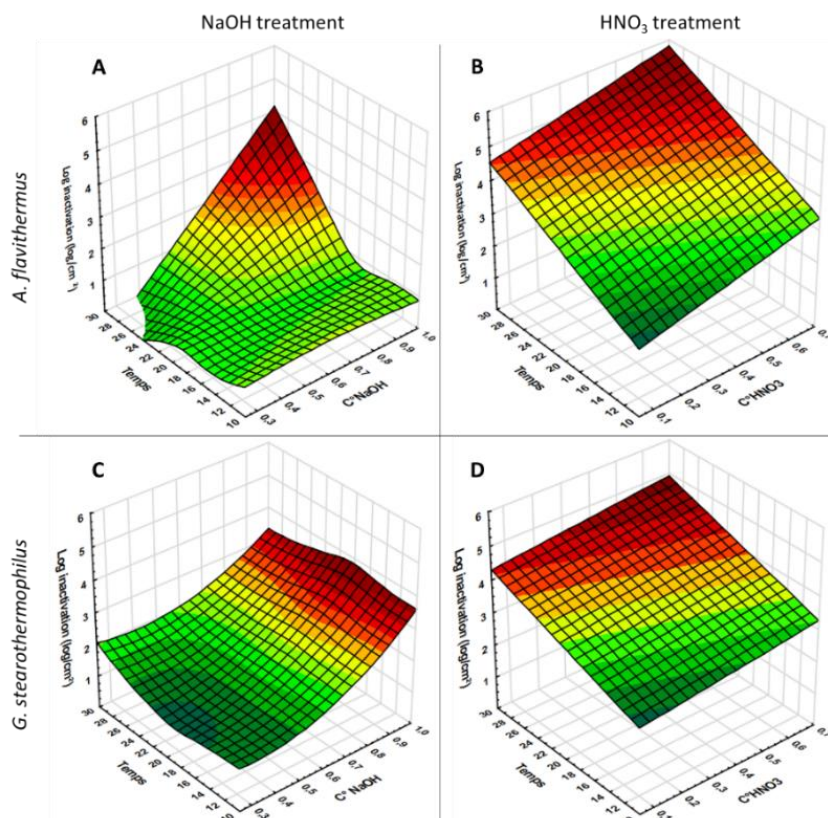


Figure 30: Modélisation polynomiale de la surface de réponse des traitements à la soude caustique à 70°C (A & C) et à l'acide nitrique à 65°C (B & D) sur les biofilms de *A. flavithermus* (A & B) et *G. stearothermophilus* (C & D). Le temps de contact est représenté sur les axes X, la concentration de la solution, sur les axes Y et l'inactivation des spores ($\log.cm^{-2}$) sur les axes Z. En vert : faible destruction des spores ; en rouge : forte destruction des spores.

Afin de mimer au mieux les conditions rencontrées en industrie de transformation du lait en poudre, il serait intéressant d'effectuer ces différentes manipulations sur du lait reconstitué à partir de poudre, afin d'obtenir un lait contaminé avec des bactéries sauvages, en conservant la diversité et la proportion des espèces. Il serait également intéressant d'évaluer la capacité à former des biofilms chez *B. licheniformis* sur des périodes d'incubation plus longues et avec des températures plus basses, ou avec d'autres espèces, en flux (effet des forces de cisaillement, renouvellement de la matrice, interaction entre espèces).

Conclusion

La majorité des procédés de transformation de produits alimentaires présentent des conditions favorables à la croissance de bactéries d'altération, pouvant affecter les qualités des aliments, ou pathogènes, pouvant entraîner des toxiinfections chez le consommateur. Pour y remédier, les industriels mettent en place des plans de contrôle des zones critiques (HACCP), permettant de maîtriser la contamination et la prolifération des microorganismes non désirés. C'est le cas lors de la transformation du lait cru en poudres laitières.

Lors de la traite, et si les conditions de nettoyage des pis et de l'environnement sont bien maîtrisés, la quantité de spores thermophiles dans le lait cru est très faible ne dépassant que très rarement les 10 spores.mL⁻¹. La présence de la flore thermophile dans le lait cru est très peu recherchée par les industriels et aucune norme française ni européenne ne légifère sur leur présence. Une fois le lait dépoté en laiterie et sa qualité validée (mesure du point de congélation, présence d'antibiotique, mesure du nombre de cellules somatiques ...), celui-ci subit une pasteurisation garantissant une bonne qualité microbiologique, puis est stocké à 4°C. Cette pasteurisation inactive la majorité des bactéries pathogènes végétatives mais est inefficace contre les bactéries thermophiles et les spores. La transformation du lait en poudre utilise de multiples étapes : chauffage, concentration, séparation par ultrafiltration séchage en fonction du type de produit. Certaines étapes (comme les échangeurs à plaques utilisés pour augmenter rapidement la température du lait ou les évaporateurs permettant de réduire la quantité d'eau libre contenue dans le lait) permettent la croissance de bactéries thermophiles, la formation de biofilm et leur sporulation. Ces développements bactériens et la formation de biofilms peuvent entraîner de fortes contaminations des poudres laitières par ces spores thermophiles. L'utilisation de ces poudres contaminées comme additifs dans la confection d'aliments peuvent être à l'origine d'altérations rapides.

Dans nos études, nous avons évalué les concentrations de spores bactériennes de différents échantillons de poudres laitières produites en France. Les niveaux de concentrations sont apparus très hétérogènes selon le produit étudié. Les poudres laitières peuvent être exemptes de spores dans 5 grammes d'échantillon mais les concentrations peuvent atteindre 5.89 log de spore thermophile par gramme de poudre. Cette forte disparité semble être reliée au type de poudre fabriqué et aux procédés mis en place pour leur obtention. Certaines étapes de fabrication présentant des températures proches de 50 ou 60°C vont favoriser le développement des bactéries thermophiles,

Suite à cette étude de dénombrement, une banque d'isolat bactérien a été réalisée. Les souches isolées ont été sélectionnées en fonction de l'aspect macroscopique des colonies obtenues pour chaque dénombrement de poudre laitière dénombrée. Une identification par une approche génomique (M13 PCR) de ces 313 isolats a permis de démontrer une forte diversité des espèces et également une spécificité de la diversité en fonction des types de poudre analysés : poudre de lait entier, écrémé, poudre de lactosérum, poudre de concentré de protéines, caséines, caséinates, formules infantiles, et de leurs origines : lait de vache, brebis, chèvre et jument. Pour l'ensemble des poudres laitières étudiées l'identification des isolats a permis de démontrer l'appartenance de 43.5% des isolats à l'espèce *Geobacillus stearothermophilus*, 30.0% appartenant à *Bacillus licheniformis* et 19.8% appartenant à *Anoxybacillus flavithermus*. Ces premiers résultats sont en accord avec les études internationales décrivant la présence majoritaire de ces trois espèces dans les poudres laitières. La diversité, évaluée grâce à l'index de Simpson, n'a pas révélé de différence entre la nature des poudres laitières étudiées, mais entre les espèces. En effet, la diversité des empreintes M13 obtenues sur les isolats d'*A. flavithermus* est plus importante que pour les deux autres espèces *G. stearothermophilus* et *B. licheniformis*. Plus la diversité intra espèce est faible, plus les isolats obtenus (de cette espèce) risquent d'être identiques (clones). Pour *A. flavithermus* la diversité importante caractérise une grande variété de souches isolées.

Une fois produites, les poudres laitières sont destinées à être commercialisées pour les particuliers, mais également aux industriels qui utilisent des poudres laitières comme ingrédient de fabrication. La forte contamination des poudres laitières par des bactéries sporulées

thermophiles peut engendrer des problèmes d'altération. Lors de son utilisation, la réhydratation d'une poudre laitière et des conditions de température, de pH et d' a_w favorables peuvent permettre la germination des spores et la croissance des bactéries ainsi que la synthèse d'enzymes responsables d'altérations des aliments.

En se basant sur des études bibliographique visant à évaluer la diversité des souches de *G. stearothermophilus* (Burgess, Flint, & Lindsay, 2014), et des informations du site OpenFoodFacts (Gigandet, 2020), une liste de produits d'intérêt permettant d'évaluer la diversité phénotypique et la capacité des trois espèces bactériennes à dégrader certains ingrédients a été retenue.

Les capacités des souches des trois espèces à dégrader 19 sources de carbohydrates et 7 ingrédients utilisés en compléments de poudres laitières lors de la formulation d'aliments transformés ont été évaluées. L'analyse des différents comportements bactériens vis-à-vis des substrats testés a permis de classer les bactéries en groupes, de différencier les espèces entre elles et de mettre en avant une variabilité intra espèce. *B. licheniformis* ayant les activités enzymatiques les plus diverses, cette espèce présente la plus grande capacité d'altération de produits alimentaires. Lors de la formulation d'aliment transformés, les poudres laitières utilisées par les industriels sont généralement contaminées avec ces trois espèces et un effet « cocktail » des activités enzymatiques augmentent les risques d'altération.

Pour évaluer les capacités de croissance de *A. flavithermus*, *G. stearothermophilus* et *B. licheniformis* lors des procédés de fabrication des poudres de lait, les conditions environnementales rencontrées dans les procédés de transformation du lait en poudre ont été confrontés aux valeurs les paramètres de croissance des trois espèces.

Les conditions de température pH et a_w à chaque étape du procédé de fabrication de laits secs ont été définis à partir de données bibliographiques.

Les températures cardinales de croissances de ces trois espèces sont bien documentées et ont été déterminées à partir de données bibliographiques. Les valeurs limites de pH et a_w de croissance ont dû être déterminées expérimentalement pour 5 souches de chacune des espèces. En complément, les taux de croissance ont été évalués en milieu laitier.

Le pH n'évoluant que faiblement durant le procédé de fabrication du lait en poudre ce facteur n'affecte pas la croissance des espèces étudiées durant le procédé de fabrication des poudres. Selon les données obtenues et celles recueillies dans la bibliographie, les températures observées dans certaines étapes de fabrication de poudre de lait sont favorables au développement des espèces thermophiles : *A. flavithermus* et *G. stearothermophilus*. Cependant la diminution de l'activité de l'eau affecte rapidement la croissance de ces espèces sporulantes thermophiles. L' a_w limite de croissance de l'espèce *A. flavithermus* est élevée (>0.978), et dans les premiers effets des évaporateurs l'augmentation de l'extrait sec et la diminution de l' a_w inhérente suffisent à inhiber la croissance de cette espèce. Ayant une a_w limite de croissance inférieure à celle de *A. flavithermus*, *G. stearothermophilus* n'est inhibé que dans les derniers effets des évaporateurs lorsque l' a_w diminue fortement. Pour l'espèce *B. licheniformis*, la température va être le facteur limitant sur la capacité de croissance dans les évaporateurs (espèce mésophile à tendance thermophile), l' a_w du lait concentré dans les évaporateurs reste supérieur à la valeur de l'activité de l'eau minimale de croissance de cette espèce. Dans les poudres de lait, les activités de l'eau inhibent la germination des spores bactériennes et leur développement, les spores bactériennes peuvent y rester dormantes durant de longues périodes.

Les capacités de croissance des espèces étudiées dans le procédé de fabrication des poudres de lait ne suffisent pas seulement à expliquer la présence de ces trois espèces retrouvées en majorité dans les poudres laitières. Les temps de séjours du lait dans les équipements ne sont pas suffisants pour permettre leur développement. Ces espèces bactériennes ne peuvent très probablement se développer dans les procédés de fabrication qu'en formant des biofilms sur les équipements.

La capacité de 62 isolats issus de ces trois espèces à former des biofilms sur de l'inox (surface très majoritairement utilisé en industrie alimentaire et laitière) a été évaluée. Il s'est avéré qu'en milieu clos et après une incubation de 6h à 55°C, plus de 62% de 32 souches d'*A. flavithermus* peuvent former un biofilm (cellules adhérees 2D ou 3D) recouvrant plus de 5% d'un champ microscopique, contrairement à *G. stearothermophilus* qui n'a donné qu'une seule souche positive sur les 15 souches testées dans ces conditions. Les tests menés sur 15

souches de *B. licheniformis* n'ont pas donné de résultats positifs. Cette absence de formation de biofilm peut s'expliquer par la température d'incubation de 55°C proche ou supérieure à la température de croissance de cette espèce ainsi qu'un temps d'incubation trop court. Cette première approche sur des coupons en inox immergés met en évidence une capacité d'adhésion rapide et d'une formation de biofilm par *A. flavithermus*. Une deuxième étude complémentaire a quantifié et dénombré les cellules végétatives et les spores en biofilm (dans une microplaque en inox favorisant la formation de biofilm à la surface air/liquide). Il s'est avéré qu'en 6h de croissance 90% des souches d'*A. flavithermus* testées peuvent former un biofilm composé de plus de 5log de cellules végétatives.cm⁻², et contenant plus de 4 log de spores.cm⁻² pour 70% d'entre eux. Pour *G. stearothermophilus*, 80% des souches testées forment un biofilm composé de plus de 5 log de cellules végétatives.cm⁻² et composés pour 20% d'entre eux de plus de 4 log de spores.cm⁻². Les résultats obtenus sur *B. licheniformis* ne permettent pas une formation de biofilm (>3log de cellules végétatives.cm⁻², et très peu de spores formées) dans les conditions testées.

L'élimination des biofilms des surfaces des installations laitières doit être réalisée régulièrement avec une fréquence de 9 à 12 heures. Les industriels appliquent un nettoyage en place (NEP), permettant de faire circuler des fluides détergents ou désinfectants à des températures et pendant des durées définies. Ce nettoyage a pour but d'éliminer les résidus laitiers (protéines adhérentes, matières grasses, ions ...), ainsi que de détruire les biofilms adhérents pour éviter des re-contaminations des surfaces en aval. L'efficacité des traitements alcalin et acide sur l'inactivation des spores d'espèces thermophiles dans les biofilms a été étudiée. Des mesures de l'efficacité des solutions alcalines de soude et d'acide nitrique à différentes concentrations, à différentes températures et pendant des temps de contact définis ont été menées. Un effet significatif de la concentration en soude et de la température sur la destruction des biofilms d'*A. flavithermus* a été observé alors que seule la concentration en soude impacte significativement la destruction des spores dans les biofilms de *G. stearothermophilus*.

Pour le traitement à l'acide nitrique, la destruction et l'élimination des biofilms est plus importante comparée aux résultats avec la soude, comme déjà démontré dans d'autres études internationales. Les cinétiques d'inactivation apparaissent log linéaires. La survie des cellules d'*A. flavithermus* en biofilm est significativement impactée par la concentration en acide

nitrique et par la température. Pour *G. stearothermophilus*, la survie de ses cellules en biofilm est significativement impactée par la concentration en acide.

L'appartenance à des groupes génotypiques (M13 PCR), comparée avec la capacité des isolats à former des biofilms, des spores au sein de ces biofilms et à résister à des traitements chimiques, n'a pas permis de définir des liens entre ces profils phénotypiques et les groupes génotypiques. La même constatation peut être faite en ce qui concerne le potentiel d'altération.

Perspectives

Afin d'évaluer la diversité génotypique des contaminants des poudres, une démarche similaire à celle de Ronimus et al., (2003) a été choisie. Elle consiste, pour rappel, à utiliser des colonies issues des dénombrement et à une réaliser une amplification par M13 PCR, un regroupement des profils d'électrophorèse par classification ascendante hiérarchique puis une identification, via le séquençage de l'ADNr 16S d'isolats sélectionnés. D'autres méthodes auraient pu (ou pourront) compléter cette démarche et/ou affiner l'identification. On peut citer l'amplification par RAPD PCR (Rückert et al., 2004; Sadiq, Li, Liu, Flint, Zhang, Yuan, et al., 2016) ou par PFGE qui permettent, après migration sur gel, l'obtention d'un profil type par espèce voire même des différences intra espèces comme déjà observé par Ronimus et al., (2003). Le séquençage de gènes de ménage (CcpA, Cpn60, FtsY, GroEL, GyrB, PycA, RecN, RpoD, Spo0A) (Colak, Inan, Karaoglu, Canakci, & Belduz, 2012; Durak, Fromm, Huck, Zadoks, & Boor, 2006) peut également permettre d'évaluer cette diversité en ciblant spécifiquement des gènes conservés chez ces espèces et ainsi obtenir une différenciation des bactéries sporulées. Une autre approche, valable uniquement si les espèces ciblées ont été au préalable identifiées serait d'utiliser pour le dénombrement à partir de nouveaux échantillons de poudre une méthode non culturale (PCR en temps réel) ciblée. En effet, celle-ci, pourrait permettre d'évaluer plus rapidement le taux de contamination des poudres laitières analysées, mais aussi de connaître spécifiquement la prévalence des trois espèces en utilisant des amorces spécifiques. Une première analyse de potentielles amorces utilisables en qPCR avait été faite en début de thèse, mais la mise en place d'une telle technique demande une mise au point du protocole qui n'a pas été jugée prioritaire à ce moment-là. Depuis, de nouvelles publications sont parues, dont une portant sur l'évaluation de la contamination en bactéries sporulées thermophiles (*A. flavithermus*, *G. stearothermophilus*) de poudres laitières par méthode qPCR (Ohkubo, Uchida, Motoshima, & Katano, 2019). Les amorces proposées, ainsi que l'une de celles proposées pour *B. licheniformis* par Almeida et al., (2018), sont étudiées actuellement au LUBEM, pour les valider sur la diversité des isolats industriels obtenus (stage de Master 2 en cours). Il faudra ensuite valider l'extraction sur les spores et matrices envisagées. Si cette méthode s'avère utilisable, elle pourrait permettre de quantifier rapidement la diversité des trois espèces dans des échantillons de poudres, ou d'autres matrices comme le biofilm, mais

également faciliter l'identification des isolats laitiers de ces trois espèces. Par contre, l'évaluation de la diversité intraspécifique ou encore la récupération des isolats pour des études phénotypiques complémentaires n'est pas réalisable par ces techniques moléculaires.

Une caractérisation d'isolats choisis d'*Anoxybacillus flavithermus* par WGS avait été envisagée dans le projet de thèse initial. Cependant, l'apport que pourrait avoir la génomique comparative à ce stade n'apparaît pas évident. En effet, les différences de caractères phénotypiques mises en évidence ne semblent pas s'y prêter pour l'instant. Les isolats laitiers, apparaissent homogènes par rapport à des caractères importants dans ce milieu : utilisation du lactose et galactose, hydrolyse de la caséine, absence d'activité visible sur la tributyrine. La caractérisation de la capacité à former du biofilm et la vitesse de sporulation sont probablement multifactorielles et l'identification d'isolats réellement différents est malaisée.

Les méthodes de screening utilisant des microplaques en inox ont permis d'évaluer les capacités des espèces sporulantes thermophiles à former des biofilms et les efficacités des nettoyages alcalins et acides des nettoyages en place pour éliminer ou inactiver les spores sur des espèces bactériennes très présentes dans les procédés de fabrication des poudres laitières. Il apparaît intéressant de poursuivre cette étude en validant des résultats à une échelle pilote réduite utilisable en laboratoire (Stage de Master 2 en cours). L'objectif serait de valider la formation de biofilms des espèces sporulantes thermophiles sur des surfaces en inox (tubes en inox) avec un renouvellement en continu du milieu. Le milieu utilisé peut être du lait du lactosérum, du lait concentré ou des extraits protéiques modifié pour simuler différentes étapes du process de fabrication des poudres laitières. Cette méthode refléterait les conditions environnementales et physicochimiques rencontrés en industrie. Une évaluation du temps de génération des bactéries et des spores en biofilm pourra être effectuée, ainsi que la vitesse de formation du biofilm, mono ou multi espèces. Dans ce pilote à l'échelle du laboratoire l'étude de la résistance de ces biofilms face aux traitements de nettoyage peut être envisagée, afin de les optimiser pour éliminer les espèces rencontrées et minimiser les effluents industriels. De plus, pour mieux refléter les conditions industrielles, des solutions de nettoyage déjà encrassées et contaminées pourraient être utilisées, mimant la recirculation des solutions de NEP.

La maîtrise de l'altération des aliments pour limiter le gaspillage alimentaire est un sujet d'actualité. Dans ce travail de thèse nous avons décrit la biodiversité des spores thermophiles présentes dans les poudres de lait et les activités enzymatiques associées à ces espèces sur les principaux différents substrats. Afin de mieux appréhender les conséquences de ces activités enzymatiques, ces résultats pourraient être validés dans des matrices alimentaires. Ces validations permettraient d'évaluer la capacité d'altération des bactéries sporulées thermophiles en quantifiant les évaluations de critères physicochimiques et organoleptiques de ces matrices. Par exemple le suivi de l'altération de lait reconstitué en évaluant les cinétiques d'évolution de la concentration en lactose, protéines totales, protéines du lactosérum, acidification et le pH en lien avec l'évolution des populations bactériennes. Une analyse de l'altération sur une flore multi espèces pourrait également être menée afin d'évaluer l'effet symbiotique des altérations, comme cela a été réalisé par Zhao et al., (2018) associant *A. flavithermus* et *G. thermoglucosidans*.

Ces validations pourraient être complétées par des suivis de la dégradation d'aliments formulés utilisant des poudres laitières. Dans de multiples formulations alimentaires, les poudres sont introduites comme ingrédients. Les nouvelles conditions environnementales température, pH ou a_w peuvent favoriser la germination et la synthèse d'enzymes d'altération par les bactéries sporulées. Des aliments modèles pourraient être formulés en utilisant des poudres laitières plus ou moins contaminées et les évolutions des caractéristiques physico chimiques organoleptiques ou techno fonctionnelles de ces produits pourraient être évaluées.

A notre connaissance il n'existe pas de travaux scientifiques ayant présenté et quantifié les risques d'altération dues aux espèces bactériennes sporulées et leurs activités enzymatiques en lien avec les formulations utilisant les poudres laitières

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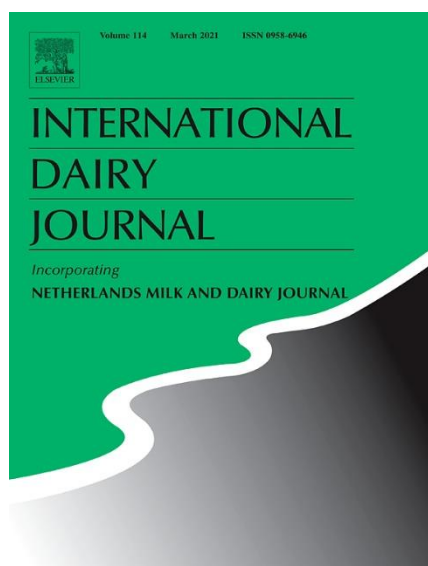
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Valorisations de la thèse



Les résultats du chapitre 2 ont fait l'objet d'une publication dans un journal scientifique à comité de lecture. Cet article a été publié sous la forme d'une short communication dans le journal : International Dairy Journal en septembre 2020 (<https://doi.org/10.1016/j.idairyj.2020.104889>) :

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Short communication

Occurrence and diversity of thermophilic sporeformers in French dairy powders



Louis Delaunay ^a, Emeline Cozien ^b, Pierre Gehannin ^b, Nassim Mouhali ^b, Sabrina Mace ^b, Florence Postollec ^b, Ivan Leguerinel ^a, Anne-Gabrielle Mathot ^{a,*}

^a Université de Brest, EA3882, Laboratoire Universitaire de Biodiversité et Ecologie Microbienne, UMT 19.03 ALTER'IX, IBSAM, 6 Rue de l'Université, 29334, Quimper, France

^b ADRIA Food Technology Institute, UMT 19.03 ALTER'IX, Z.A. de Creac'h Gwen, 29196, Quimper Cedex, France

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ABSTRACT

This study reports the prevalence of sporeforming bacteria and contamination levels of a variety of powdered dairy products from cows', ewes', goats' and mares' milk produced in France. The concentration of mesophilic spores, thermophilic spores and highly heat-resistant thermophilic spores was assessed in 61 dairy powders. Thermophilic spore concentration was highly variable between powders, likely due to the different manufacturing processes used for transforming milk into dairy powders. The different stages of processing, particularly heat treatment close to 55 °C had a strong impact on selection of thermophilic bacteria contaminating dairy powders. For some products, thermophilic spore counts were as high as 5.89 log TSC g⁻¹. Of the 313 thermophilic isolates selected, 93.3% belonged to the species *Geobacillus stearothermophilus* (43.5%), *Bacillus licheniformis* (30.0%) and *Anoxybacillus flavithermus* (19.8%). These results confirm the presence of the three majority species reported worldwide.

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Un article portant sur le chapitre 3 et une revue bibliographique sont prévues pour être publiées d'ici mi 2021.

Les résultats obtenus dans le chapitre 2 ont également fait l'objet d'un poster lors des journées scientifiques de l'école doctorale EGAAL en juillet 2019.

Journées scientifiques de l'ED EGAAL



Prévalence et biodiversité des espèces sporulées thermophiles associées à l'industrie des poudres lactières en France- ANOPLAI

Louis DELAUNAY^{1*}, Sabrina MACE², Emeline COZIEN², Florence POSTOLLEC², Ivan LEGUERINEL¹, Anne-Gabrielle MATHOT¹

1 – LUBEM, Université de Bretagne Occidentale - UMT ACTIA19.03 ALTER'IX, 6 rue de l'université, 29334 Quimper, France

2 – ADRIA Food Technology Institute - UMT ACTIA19.03 ALTER'IX, 20 Avenue de la Plage des Gueux, 29000 Quimper

Mots clés : Spores thermophiles, Poudres lactières, Alimentation

Résumé :

Lors de la fabrication des poudres lactières, la pasteurisation est la seule étape thermique permettant d'inactiver les microorganismes mais elle n'affecte que peu les spores bactériennes présentes dans le lait cru. Ces spores bactériennes peuvent se former dans des biofilms se développant dans les usines, en particulier sur les échangeurs thermiques et dans les concentrateurs sous vide. Les conditions qui y sont rencontrées (température, aw) sont optimales pour la germination et la croissance des spores thermophiles. Ces bactéries sporulées se retrouvant ensuite dans différents produits alimentaires, utilisant les poudres de lait dans leurs formulations, pourront altérer le produit final lors de sa conservation.

L'objectif de ce projet est de détecter, de quantifier, d'identifier et de caractériser les spores thermophiles dans les poudres laitières françaises permettant d'avoir une vision globale de leurs prévalences.

Un total de 62 échantillons de poudres laitières françaises d'origines et de natures diverses ont été traitées thermiquement afin d'éliminer les bactéries végétatives; à 80°C pendant 10min et à 106°C pendant 30min. Pour chaque échantillon, la proportion de bactéries mésophiles et thermophiles a été établie selon la norme ISO/TS 27265:2009. Ainsi 313 bactéries ont pu être isolées et identifiées après extraction de leur ADN et amplification par méthode M13 RAPD. Les amplicons sont séparés sur gel d'électrophorèse, les empreintes sont analysées grâce au logiciel BioNumerics, des regroupements ont été formés par l'étude des coefficients de similarité sur une analyse Pearson et un cluster a été réalisé par UPGMA. L'assignation des clusters à une espèce a été réalisée par séquençage du gène de l'ADNr 16S d'isolats représentatifs.

Les dénombrements font apparaître que plus une poudre laitière a subi d'étapes de transformation, moins la contamination apparaît importante. En utilisant l'analyse UPGMA, les 313 isolats ont été séparés en 23 groupes. L'identification des espèces par séquençage 16S a mis en évidence la diversité des spores. Trois espèces présentent de fortes prévalences *Geobacillus stearothermophilus* 43 %, *Bacillus licheniformis* 30 % et *Anoxybacillus flavithermus* 20 %. D'autres espèces sont détectées plus rarement.

Ces résultats montrent la prévalence de trois espèces dans les poudres laitières françaises, résultats en accord avec les travaux publiés mondialement sur ce sujet. Les isolats de ces espèces seront caractérisés par une approche phénotypique: activités enzymatiques, thermorésistance, capacité à croître et former des biofilms, afin d'appréhender les risques d'altération liés à ces espèces bactériennes. La fonctionnalité des poudres sera également analysée afin d'évaluer l'impact de ces contaminations sur la qualité et la stabilité des poudres.

Poster présenté :

Prévalence et biodiversité d'*Anoxybacillus flavithermus* associé à l'industrie des poudres laitières

Louis DELAUNAY^a, Sabrina MACE^b, Emeline COZIEN^b, Florence POSTOLLEC^c, Ivan LEGUERINEL^c, Anne-Gabrielle MATHOT^d

^a Université de Brest, EA3882, Laboratoire Universitaire de Biodiversité et Ecologie Microbienne, UMT14.01 SPORE-RISK, IBSAM, 6 rue de l'Université, 29334, Quimper, France

^b ADRIA Développement, UMT14.01 SPORE-RISK, Z.A. de Creac'h Gwen, F-29196, Quimper Cedex, France

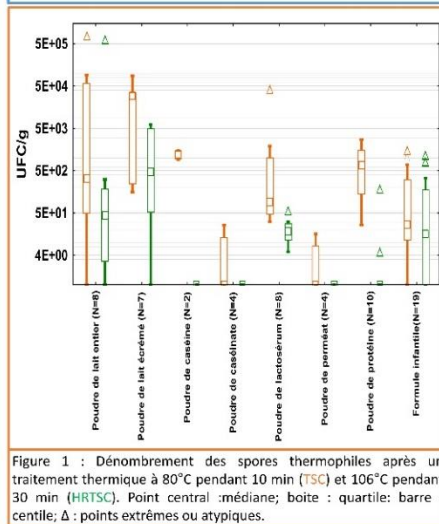
Contexte et objectifs :

Lors de la fabrication des poudres laitières, la pasteurisation est la seule étape thermique permettant de détruire les microorganismes végétatifs et potentiellement pathogènes. Cependant, les spores bactériennes présentes dans le lait cru persistent. Celles-ci peuvent germer, se développer, former des biofilms sur les surfaces en inox (spécifiquement dans les échangeurs thermiques et dans les évaporateurs). De plus, les conditions rencontrées (température, a_w) sont optimales pour la germination et la croissance des spores thermophiles. Ces bactéries sporulées pourront altérer le produit final lors de sa réhydratation ou de sa conservation. L'objectif de cette étude a été d'évaluer les prévalences des bactéries sporulées thermophiles dans les poudres laitières d'origines françaises.

Matériels et Méthodes :

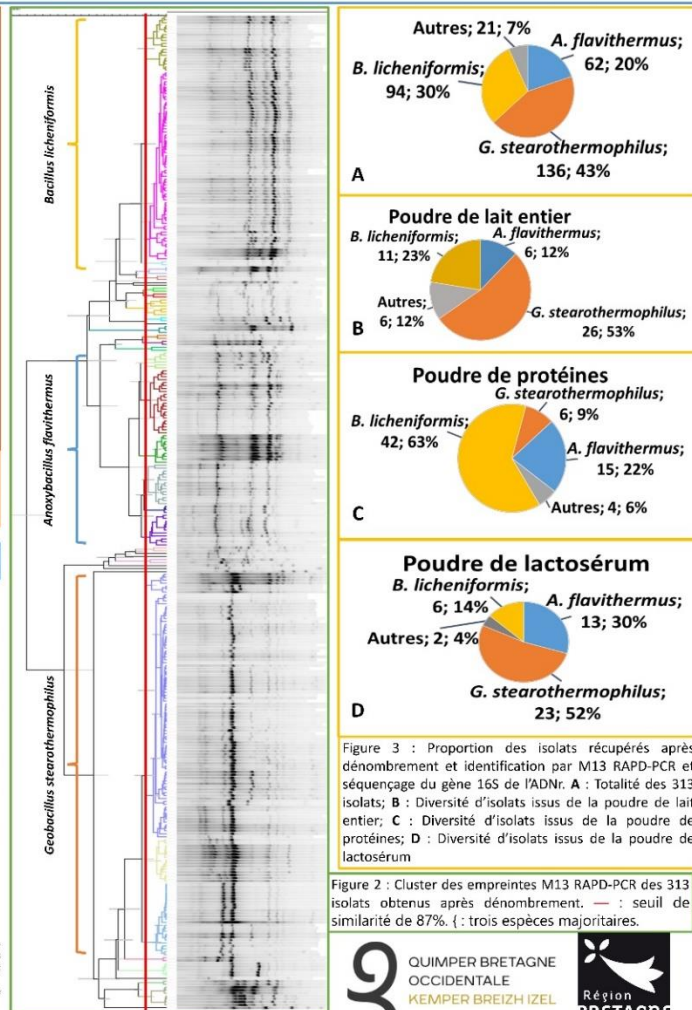
- Récupération des échantillons (N=62)
- Poudres de différents mammifères : vache, chèvre, brebis, jument
- Différents types de poudres (N=8) : Poudre de lait entier, de lait écrémé, de caséine, de caséinate, de lactosérum, de perméat, de protéines et formules infantiles
- Dénombrement des spores thermophiles : traitement thermique du lait reconstitué à 80°C 10 min et 106°C 30 min
- Récupération d'isolats (N=313)
- Différenciation des isolats par M13 RAPD-PCR
- Séquençage du gène 16S des souches représentatives des clusters
- Identification des espèces majoritaires

Résultats :



Conclusions

- Les dénombrements démontrent que plus un produit est processé, plus la quantité de spores thermophiles retrouvées est faible.
- L'analyse M13 RAPD-PCR est assez discriminante pour pouvoir évaluer la diversité des isolats obtenus
- Espèces majoritaires : *Geobacillus stearothermophilus*, *Bacillus licheniformis* et *Anoxybacillus flavithermus* : en accord avec la bibliographie (Rückert *et al.*, 2004; Postollec *et al.*, 2012; Sadiq *et al.*, 2018)
- Diversité spécifique par type de poudres: sélection du process



Postollec, F., Mathot, A.G., Bernard, M., Divanac, M.L., Pavan, S., and Schier, D. (2012) Tracking spore-forming bacteria in food: From natural biodiversity to selection by processes. *Int J Food Microbiol* 158: 1-8
Rückert, A., Ronimus, R.S., and Morgan, H.W. (2004) A RAPD-based survey of thermophilic bacilli in milk powders from different countries. *Int J Food Microbiol* 96: 203-212
Sadiq, F.A., Flint, S., and He, G. (2018) Microbiota of milk powders and the heat resistance and sporage potential of aerobic spore-forming bacteria. *Int Dairy J* 85: 159-168



Les résultats du chapitre 2 ont également été présentés lors du congrès international IAFP US, organisé à Louisville aux Etats-Unis en Juillet 2019, par un membre de l'UMT : Sabrina Macé.

Le résumé proposé a été le suivant :

Prevalence and characterization of thermophilic sporeformers in French dairy powders

Louis DELAUNAY^a; Sabrina MACE^b; Emeline COZIEN^b ; Florence POSTOLLEC^b; Ivan LEGUERINEL^a; Anne-Gabrielle MATHOT^a

^a Université de Brest, EA3882, Laboratoire Universitaire de Biodiversité et Ecologie Microbienne, UMT14.01 SPORE-RISK, IBSAM, 6 rue de l'Université, 29334, Quimper, France

^b ADRIA Développement, UMT14.01 SPORE-RISK, Z.A. de Creac'h Gwen, F-29196, Quimper Cedex, France

Introduction:

In the process of dairy powders, pasteurization is the only heat treatment applied for the destruction of microorganisms. However, bacterial spores present in raw milk remain alive. Biofilm could also be formed in factories, especially in heat exchangers and vacuum concentrators. The temperature conditions in those promote the growth of thermophilic species. These spores forming bacteria can alter the quality during the process or when the final product is re-hydrated for use.

Purpose:

The aim of this project is to detect, to identify and to characterize thermophilic spores in French dairy powders.

Methods:

Sixty-two dairy powders samples were treated at 80°C/10min and 106°C/ 30min. For each sample, total thermophilic spores population was enumerated (or isolated after enrichment) on MPCA+BCP+PS (ISO/TS 27265:2009) and 313 bacteria were isolated. DNA of all of these bacterial isolates was extracted and amplified with a M13-RAPD PCR method. PCR products were separated by gel electrophoresis. Fingerprints have been analysed using Bionumerics

Software (version 7.6) and clusters were formed with UPGMA analysis. The 16S RNA gene was sequence for 61 DNA samples regarding their clusters.

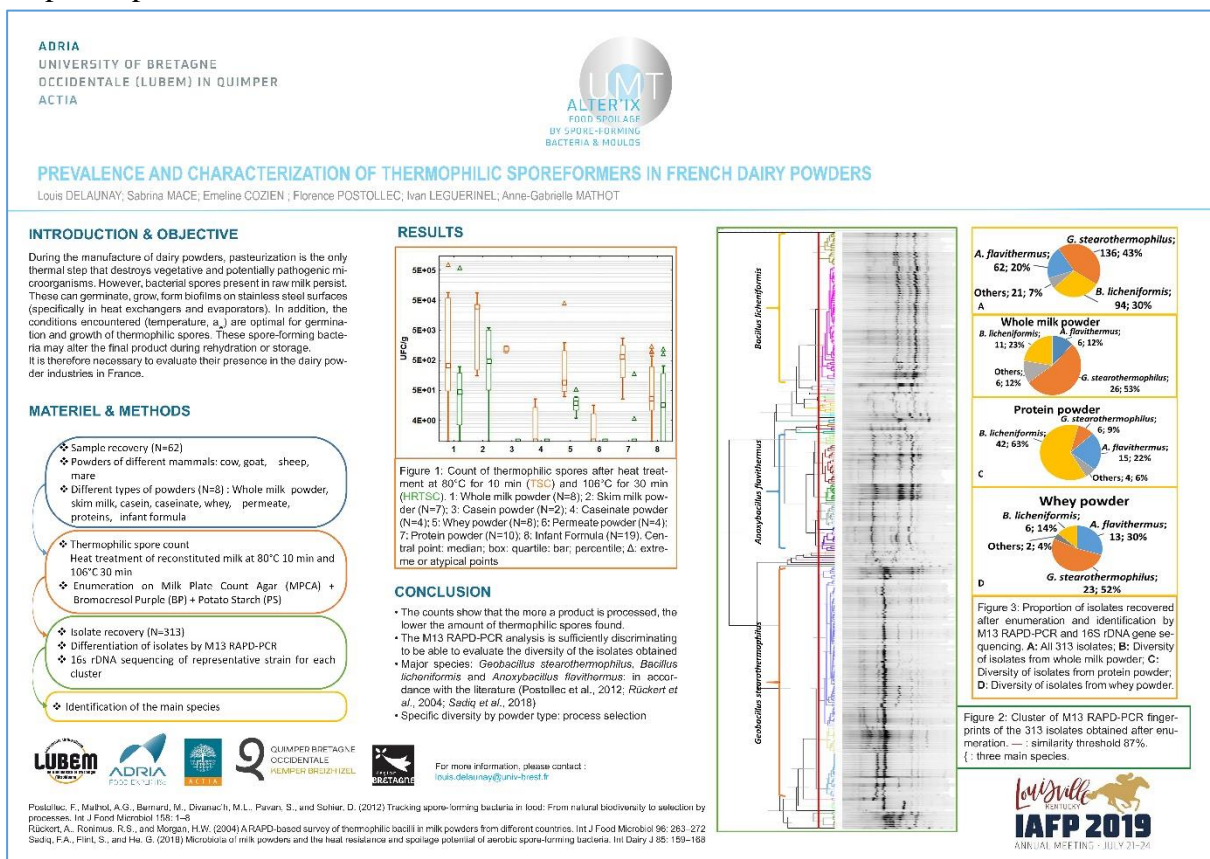
Results:

Enumeration results shows that higher a powder is processed, lower the contamination will be. Using UPGMA analysis, the 313 fingerprints obtained were separated into 23 clusters. Species identification of the isolates belonging to these clusters highlighted the spore diversity : 43% were identify as *Geobacillus stearothermophilus* species, 33% from *Bacillus* (29% *B. licheniformis*, 1% *B. thermoamylovorans*, 1% *B. coagulans*, 1% *B. ginsenghumi*, 1% *B. smithii*), 20% from *Anoxybacillus flavithermus*, 1% *Aneurinibacillus thermoaerophilus*, 1% *Brevibacillus group*, 1% *Paenibacillus naphthalenovorans*, 1% of undetermined bacteria.

Significance:

Those results show the prevalence of some genus in milk powder. They are correlated with worldwide studies. Bacterial isolates will be characterized by a phenotypic approach (enzymatic activities, heat resistance, ability to form biofilms ...).

Le poster présenté était le suivant :



Les résultats du chapitre 4 ; ont fait l'objet d'une présentation sous forme d'un poster dans un congrès international en septembre 2020, Biofilms 9, 2020, Karlsruhe, Allemagne.

Le résumé proposé a été le suivant :

Screening of fast biofilm formation on stainless steel by thermophilic sporeformers originated from dairy powder and their resistance against CIP

Louis DELAUNAY^a; Florence POSTOLLEC^b; Ivan LEGUERINEL^a; Anne-Gabrielle MATHOT^a

^a Université de Brest, EA3882, Laboratoire Universitaire de Biodiversité et Ecologie Microbienne, UMT14.01 SPORE-RISK, IBSAM, 6 rue de l'Université, 29334, Quimper, France

^b ADRIA Développement, UMT14.01 SPORE-RISK, Z.A. de Creac'h Gwen, F-29196, Quimper Cedex, France

Introduction:

Thermophilic sporeformers are present in raw milk at very low concentration and resist to pasteurisation applicated to destruct vegetative and pathogenic cells. Those spores can adhere to stainless steel due to their hydrophobicity and can form biofilms. Early stage biofilms are important because it can increase the matrix and the adhesion of other cells. Because of those biofilms, the three main species: *Geobacillus stearothermophilus*, *Anoxybacillus flavithermus* and *Bacillus licheniformis* can resists to Cleaning In Place (CIP) procedure, and contaminate a new process.

Material and Methods:

Early stage adhesion was conducted on stainless steel submerged by milk inoculated with a fresh culture of bacteria (*G. stearothermophilus* (N=15), *A. flavithermus* (N=32) and *B. licheniformis* (N=15)) for 6h of growth at 55°C under agitation. The ability of sporeformers to form biofilms under those conditions were measured by image analysis after a fluorescent coloration (acridine orange) and random photography. A coverage percentage was calculated by ImageJ ; and a positive threshold was set up at 5% of covering.

The efficiency of CIP procedures were obtained after a caustic soda and nitric acid treatment during different duration and temperature of treatment. Tested biofilms were formed in milk during 12h at 55°C, in stainless steel microplates (96 wells) on the same species (3 strains for each) under agitation. Surviving spores were enumerated by the microcolony method.

Results:

Early stage adhesion shows that 62.5 % (N=20) of *A. flavithermus* strains can form biofilm within 6h, whereas only 6.7% (N=1) of *G. stearothermophilus* and 0% (N=0) of *B. licheniformis* biofilm in 6h at 55°C on submerged stainless steel. However, the maximum covering % on *A. flavithermus* was 35%; while on the only biofilm forming strain of *G. stearothermophilus*, this percentage reach 75%. Image analysis also shows biofilm structure from 2D to 3D.

The presence and the resistance of spores to chemical cleaning was highly variable within strains. Nitric acid appears to be more effective than caustic soda against biofilms formed by vegetative cells and spores from these strains.

Significance:

Those results shows that strong biofilms are mainly composed of spore and are very resistant to CIP used in dairy industries. That is why a better understanding of control methods can lead to a finer and suitability use of cleaning products.


Le diaporama présenté pendant cet échange en distanciel a été le suivant :

ADRIA UNIVERSITY OF BRETAGNE OCCIDENTALE (LUBEM) IN QUIMPER ACTIA | louis.delaunay@univ-brest.fr | **KIT** Karlsruhe Institute of Technology | biofilms 9.1 - Biofilm control

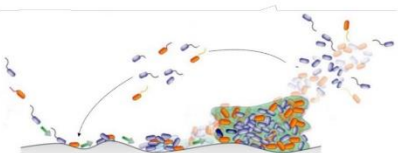
biofilms 9

Screening of fast biofilm formation on stainless steel by thermophilic sporeformers originated from dairy powder and their resistance against CIP


Louis Delaunay, Florence Postollec, Ivan Leguerinel, and Anne-Gabrielle Mathot






Raw milk is contaminated with few thermophilic sporeformers




They grow in process and can adhere to stainless steel to form biofilm




Conditions encountered in the process are optimal and biofilms can resist CIP procedures



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biofilms 9


Screening of fast biofilm formation in 6h on stainless steel coupons (1cm x 1cm)

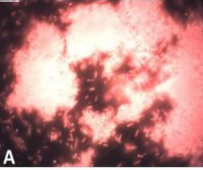
3 species isolated from dairy powders : *Anoxybacillus flavithermus*, *Geobacillus stearothermophilus* and *Bacillus licheniformis*

- Inoculation of UHT skimmed milk, incubation 55°C 6h in rotary shaker with submerged coupons
- Washing steps with tempered water, coloration with Acridine Orange and microscopy.

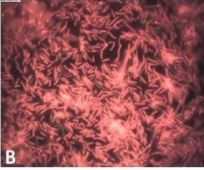
	Number of strains	Positive results (coverage >5%)	% positive	Type of biofilm		% coverage max.
				2D	3D	
<i>A. flavithermus</i>	32	20	62,5	8	12	35
<i>G. stearothermophilus</i>	15	1	6,7	/	1	75
<i>B. licheniformis</i>	15	0	0	/	/	0

→ ***A. flavithermus* is a fast biofilm former in tested conditions and could be the pioneer strain allowing others strains to form biofilm**

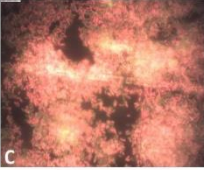




A







B




C

A: *A. flavithermus* strain 288; B: *A. flavithermus* strain 5; C: *G. stearothermophilus* strain 60



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Biofilm resistance against CIP procedures used in dairy powders industries

7 strains of *A. flavithermus*
3 strains of *G. stearothermophilus*
No biofilm formation: *B. licheniformis*

- Inoculation of UHT skimmed milk, incubation 55°C 6h in rotary shaker
- CIP procedures (washing with water, NaOH or HNO₃, washing with water)
- Counting survivals with micro colony method (5µL) after US (10min 16kHz)



	NaOH			HNO ₃		
	T°C	[%]	Duration (min)	T°C	[%]	Duration (min)
NaOH	60	0,25	10	60	0,05	10
	70	0,5	20	65	0,1	20
	80	1	30	70	0,7	30

Results :

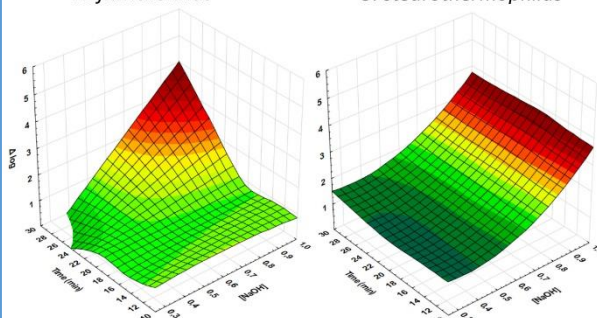
CIP	Specie	Parameters	P value
NaOH	<i>A. flavithermus</i>	T°C	0,348
		[%]	0
		Duration	0,019
	<i>G. stearothermophilus</i>	T°C	0,144
		[%]	0
		Duration	0,959
HNO ₃	<i>A. flavithermus</i>	T°C	0,606
		[%]	0,138
		Duration	0,015
	<i>G. stearothermophilus</i>	T°C	0
		[%]	0
		Duration	0

Biofilm resistance against CIP procedures used in dairy powders industries

NaOH at 70°C

A. flavithermus

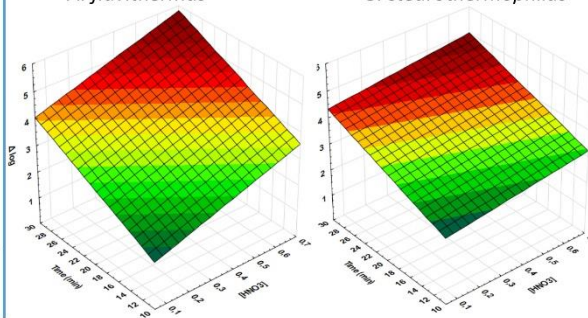
G. stearothermophilus



HNO₃ at 65°C

A. flavithermus

G. stearothermophilus



Biofilms formed by *A. flavithermus* are more resistant than *G. stearothermophilus* ones against CIP
Need to validate those results using flow cell biofilm protocol and with multi species biofilms.

En raison de la pandémie mondiale, et pour limiter la diffusion du virus lors de congrès, de multiples évènements ont été annulés.



Des résumés ont été proposés pour European Spore Conférence 2020 prévu à Naples, FoodMicro 2020 prévu à Athènes, IAFP EU 2020 prévu à Munich ; mais tous les trois ont été annulés. Les différents résumés proposés avaient été acceptés pour European Spore Conference et IAFP EU. Une demande de bourse de mobilité avait également été demandée auprès de l'école doctorale EGAAL.



J'ai également pu assister à BSPIT 2018 à Paris en mars 2018 et à European Spore Conference à Londres en avril 2018.

Titre : ANOPLAI – Prévalence et biodiversité d'*Anoxybacillus flavithermus*, et des autres bactéries sporulées thermophiles, associées à l'industrie des poudres lactières

Mots clés : Spores thermophiles, Biofilm, Diversité, Poudres lactières, Nettoyage en place.

Résumé : Les bactéries sporulées thermophiles présentes dans l'environnement de la ferme, se retrouvent en faibles concentrations dans le lait cru. Elles résistent aux traitements de pasteurisation appliqués en industrie et peuvent se développer au cours des procédés de fabrication des poudres lactières pour s'y retrouver en fortes concentrations. Afin d'évaluer la contamination des poudres lactières produites en France, un dénombrement et une évaluation de la diversité des espèces des spores thermophiles mené sur 61 échantillons recueillis auprès d'industriels a permis de mettre en évidence une grande diversité de contaminants sporulés. Il apparaît que *A. flavithermus*, *G. stearothermophilus* et *B. licheniformis* sont les espèces bactériennes sporulées thermophiles les plus présentes dans les poudres lactières. Ces prévalences dépendent du type de poudre lactière étudiées. Une diversité phénotypique est observée lors de l'analyse des activités enzymatiques avec un potentiel d'altération observée pour *A. flavithermus*, *G. stearothermophilus* et plus particulièrement pour *B. licheniformis*.

Il en résulte que les poudres contaminées utilisées comme ingrédients dans les formulations alimentaires sont de possibles sources d'altération. Une analyse détaillée du procédé de fabrication des poudres lactières, associée aux capacités de croissance des bactéries sporulées thermophiles a permis d'évaluer les étapes du procédé de fabrication des poudres susceptibles de favoriser le développement des espèces étudiées, de la formation de biofilms et de spores. Enfin les estimations d'une part de la capacité de formations de biofilms par *A. flavithermus* et par *G. stearothermophilus* et d'autre part de leurs résistances aux traitements de nettoyage en place ont révélé que l'espèce *A. flavithermus* est la plus résistante aux traitements alcalins alors que les résistances aux traitements acides semblent similaires pour les deux espèces. Les résultats obtenus au cours cette thèse ont permis d'évaluer les capacités de croissance des bactéries sporulées thermophiles dans les industries des poudres lactières et d'appréhender des leviers permettant de mieux maîtriser leurs développements et leurs éliminations.

Title : ANOPLAI – Prevalence and biodiversity of *Anoxybacillus flavithermus*, and other thermophilic spore forming bacteria, associated with the dairy powder industry

Keywords : Thermophilic spores, Biofilm, Diversity, Dairy powders, Cleaning in place

Abstract: Thermophilic spore forming bacteria present in the farm environment are found at low concentrations in raw milk. They are resistant to pasteurization treatments applied in industry and can develop during the manufacturing process of dairy powders and be found in high concentrations. In order to evaluate the contamination of dairy powders produced in France, an enumeration and an evaluation of the species diversity of thermophilic spores was carried out on 61 samples collected from industrials allowed to highlight a great diversity of spore contaminants. It appears that *A. flavithermus*, *G. stearothermophilus* and *B. licheniformis* are the most present thermophilic spore-forming bacterial species in dairy powders. These prevalence depend on the type of dairy powder studied. A phenotypic diversity regarding the enzymatic activity has shown with a spoilage potential for *A. flavithermus*, *G. stearothermophilus* and more particularly for *B. licheniformis*.

As a result, contaminated powders used as ingredients in food formulations are possible sources of spoilage. A detailed analysis of the manufacturing process of dairy powders, associated with the growth capacities of thermophilic spore-forming bacteria, allowed to evaluate the steps of the manufacturing process of the powders favouring the development, the formation of biofilms and spores of the studied species. Finally, the estimations of the capacity of biofilm formation by *A. flavithermus* and *G. stearothermophilus* on the one hand and of their resistance to the cleaning treatments on the other hand revealed that the *A. flavithermus* species is the most resistant to the alkaline treatments whereas the resistances to the acid treatments seem to be similar for both species. The results obtained during this thesis have allowed to evaluate the growth capacities of thermophilic spore forming bacteria in the dairy powder industry and to understand the levers allowing to better control their development and their elimination.