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Probiotic characterization of the beneficial *Escherichia coli* CEC15 strain and role of the food matrix in probiotic activity modulation

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THÈSE DE DOCTORAT DE

L'INSTITUT AGRO RENNES ANGERS

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Par

Tales Fernando DA SILVA

« Probiotic characterization of the beneficial *Escherichia coli* CEC15 strain and role of the food matrix in probiotic activity modulation »

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Probiotic characterization of the beneficial *Escherichia coli* CEC15 strain and role of the food matrix in probiotic activity modulation

INCIPIT VITA NOVA

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Probiotic characterization of the beneficial *Escherichia coli* CEC15 strain and role of the food matrix in probiotic activity modulation

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Summarized history of medicine.

2000 BC	Polytheistic religions era	Take this herb and eat, it's good for you
1000 AD	Monotheistic religions era	That herb is bad, you'd better say this pray
1500 AD	Renaissance development era	That pray is a superstition, you'd better drink this elixir
1800 AD	Industrial revolution era	What's the use of that elixir, you'd better take this pill
1950 AD	Pharmaceutical revolution era	That pill is ineffective, you'd better take this antibiotic
2000 AD	Electronic media era	That antibiotic is chemical, you'd better eat this herb
2010 AD	Evidence-based medicine era	No study on that herb, you'd better take this probiotic

Ozen and Dinleyici, *"The history of probiotics: the untold story"*. Beneficial microbes, 2015.

"Now this is not the end. It is not even the beginning of the end.
But it is, perhaps, the end of the beginning."

Winston Churchill



ABSTRACT

Probiotics are microorganisms that, when administered in adequate quantities, are capable of exerting beneficial effects on the host. Nowadays, a variety of probiotic microorganisms, in particular bacteria, are commercialized on the market. Besides some species being considered safe to consume, such as lactic acid bacteria and bifidobacteria, the probiotic effect is considered strain-dependent and, thus, cannot be extended to all members of a recognized probiotic species. One clear example of this is the strain *Escherichia coli* Nissle 1917 (EcN), which has been recognized and commercialized as a probiotic product for over a century. *E. coli* is a species that belongs to the *Enterobacteriaceae* family: Gram-negative bacteria, rod-shaped, facultative anaerobic, commonly found in the intestine of warm-blooded organisms. This species constitutes a heterogeneous group composed of innocuous commensal strains, pathogenic strains, and probiotic strains, such as EcN and other strains contained in commercial products (Symbioflor2 and Colinfant newborn). Recently a new strain of *E. coli* revealed beneficial effects at the gut level in animals. The *E. coli* CEC15 (CEC15) strain was isolated from newborn rat's feces and was able to promote remodeling of the colonic epithelial architecture and to damages caused by TNBS-induced colitis in mice. In order to further investigate the potential of this strain as probiotic, we performed its probiogenomic analysis *in silico* and we investigated its phenotype *in vitro*, as well as its *in vivo* effect in a murine model of 5-Fluorouracyl(5-FU)-induced intestinal mucositis. CEC15 genome was sequenced, a 4.7 Mb chromosome and a 200 Kb plasmid were found. Mobile genomic elements analysis showed very few genomic islands, in comparison to EcN, with no gene related to pathogenicity and few antibiotic resistance genes. No gene responsible for antibiotic resistance was located near mobile elements. No viable prophage was identified. Genomic and *in vitro* studies revealed no hemolytic activity, yet a high tolerance towards gastrointestinal conditions. CEC15 exhibited higher adhesion to intestinal cells than EcN. Microscopic and proteomic investigations suggested that this could be due to a higher number of fimbriae proteins. When administered to healthy animals, CEC15 exerted no negative effect, yet improved the intestinal microbiota richness. When intestinal mucositis was induced, animals receiving CEC15 presented a lower weight loss and reduced damages of the intestinal structures. Perturbation of the gut microbiota by 5-FU was not prevented by CEC15 or EcN. Following these results, postbiotics fractions of CEC15 (heat-inactivated bacteria, cell-free supernatant) were tested in the same animal model. Heat-inactivated CEC15 presented almost the same preventive effects as the viable strain and prevented loss of intestinal epithelium architecture and of body weight. CEC15 cell-free supernatant, despite

showing promising results, were not comparable to CEC15 viable and heat-inactivated. All the aforementioned assays were conducted in parallel with the administration of *E. coli* EcN and of its postbiotic preparations. CEC15 outperformed EcN in all results. EcN cell-free supernatants, however exerted a promising protective effect. These results have demonstrated that CEC15 is promising as a probiotic strain. However, more pre-clinical and clinical trials are necessary to confirm its protective efficacy in humans.

Keywords: Probiotics; Probiogenomics; *Escherichia coli*; Postbiotics

Les probiotiques sont des micro-organismes qui, lorsqu'ils sont administrés en quantités adéquates, sont capables d'exercer des effets bénéfiques sur l'hôte. De nos jours, il existe une variété de micro-organismes probiotiques, en particulier des bactéries, qui sont commercialisés sur le marché. En plus de certaines espèces considérées comme sûres à consommer, telles que les bactéries lactiques et les bifidobactéries, l'effet probiotique est considéré comme dépendant de la souche. Il ne peut donc être généralisé à toutes les souches d'une espèce bactérienne probiotique reconnue. Un exemple clair en est la souche *Escherichia coli* Nissle 1917 (EcN), qui est reconnue et commercialisée en tant que produit probiotique depuis plus d'un siècle. *E. coli* est une espèce qui appartient à la famille des *Enterobacteriaceae*: bactéries à Gram négatif, en forme de bâtonnets, aérobies anaérobies facultatives, que l'on trouve couramment dans l'intestin des organismes à sang chaud. Cette espèce constitue un groupe hétérogène de souches commensales inoffensives, de souches pathogènes et de souches probiotiques, telles que EcN et deux autres contenues dans des produits commerciaux (Symbioflor2 et Colinfant newborn). Récemment, une nouvelle souche d'*E. coli* a montré des effets bénéfiques sur l'intestin dans des modèles animaux. La souche *E. coli* CEC15 (CEC15) a été isolée des selles de rats nouveau-nés. Elle prévient la destruction de l'architecture épithéliale colique et les lésions causées par la colite induite par le TNBS chez les souris. Dans le but d'étudier davantage le potentiel de cette souche en tant que probiotique, nous avons réalisé une analyse probiogénomique *in silico*, étudié son phénotype *in vitro*, ainsi que son effet *in vivo* dans un modèle murin de mucosité intestinale induite par la 5-fluorouracile (5-FU). Le génome de la CEC15 a été séquencé. Un chromosome de 4,7 Mb et un plasmide de 200 Kb ont été trouvés. L'analyse des éléments génomiques mobiles a montré un très faible nombre d'îlots génomiques, sans gène relié à une pathogénicité importante et peu de gènes de résistance aux antibiotiques. Aucun gène responsable d'antibiorésistance n'est situé à proximité d'éléments mobiles. Aucun prophage viable n'a été identifié. Les études génomiques et *in vitro* n'ont révélé aucune activité hémolytique, mais une grande tolérance vis-à-vis de conditions gastrointestinales. La souche CEC15 a montré une adhésion plus forte aux cellules intestinales que la souche EcN. Des investigations microscopiques et protéomiques suggèrent que cela pourrait être dû à un plus grand nombre de protéines de fimbriae. Lorsqu'elle est administrée à des animaux sains, la CEC15 n'a eu aucun effet négatif, et n'a pas non plus amélioré la richesse du microbiote intestinal. Lorsque la muqueuse intestinale a été induite, les animaux recevant la souche CEC15 ont présenté une perte de poids moindre et une moindre atteinte des structures intestinales. La perturbation

du microbiote sous l'effet du 5-FU, était réduite par la consommation de la souche CEC15. À la suite de ces résultats, fractions postbiotiques de la souche CEC15 ont été testées dans le même modèle animal : bactéries inactivées par la chaleur et surnageants sans cellules. Les résultats ont montré que la souche CEC15 inactivée par la chaleur présente presque les mêmes effets que la souche viable quant à la protection de l'architecture épithéliale intestinale et la perte de poids. Le surnageant sans cellules de la CEC15, bien qu'il montre des résultats prometteurs, n'était pas comparable à la CEC15 viable et inactivée par la chaleur. Tous les tests susmentionnés ont été réalisés en parallèle avec l'administration de la souche EcN et de ses préparations postbiotiques. La souche CEC15 a surpassé l'EcN dans tous les résultats. Les surnageants de la souche EcN, en revanche, ont montré des effets protecteurs prometteurs. Ces résultats ont démontré que la CEC15 est prometteuse en tant que souche probiotique. Cependant, des essais précliniques et cliniques sont nécessaires pour confirmer son efficacité protectrice chez l'homme.

Mot clés: Probiotiques; Probiogénomique; *Escherichia coli*; Postbiotiques

Probióticos são microrganismos que, quando administrados em quantidades adequadas, são capazes de exercer efeitos benéficos ao hospedeiro. Atualmente, existe uma variedade de microrganismos probióticos, principalmente bactérias, que são comercializados no mercado. Além de algumas espécies consideradas seguras para consumo, como bactérias lácticas e bifidobactérias, o efeito probiótico é considerado dependente da cepa. Portanto, não pode ser generalizado para todas as cepas de uma espécie bacteriana probiótica reconhecida. Um exemplo claro disso é a cepa *Escherichia coli* Nissle 1917 (EcN), que é reconhecida e comercializada como produto probiótico há mais de um século. *E. coli* é uma espécie que pertence à família *Enterobacteriaceae*: aeróbios Gram-negativos, em forma de bastonete, anaeróbios facultativos, comumente encontrados nos intestinos de organismos de sangue quente. Esta espécie constitui um grupo heterogêneo de cepas comensais inofensivas, cepas patogênicas e cepas probióticas, como EcN e outras duas contidas em produtos comerciais (Symbioflor2 e Colinfant newborn). Recentemente, uma nova cepa de *E. coli* demonstrou efeitos benéficos no intestino em modelos animais. A cepa de *E. coli* CEC15 (CEC15) foi isolada das fezes de ratos recém-nascidos. Previne a desestruturação da arquitetura epitelial do cólon e das lesões causadas pela colite induzida por TNBS em camundongos. Para estudar ainda mais o potencial desta cepa como probiótico, realizamos uma análise probiogenômica *in silico*, estudamos seu fenótipo *in vitro*, bem como seu efeito *in vivo* em um modelo de camundongo com mucosite intestinal induzida por 5-fluorouracil (5-FU). O genoma da CEC15 foi sequenciado. Um cromossomo de 4,7 Mb e um plasmídeo de 200 Kb foram encontrados. A análise de elementos genômicos móveis mostrou um número muito baixo de ilhas genômicas, sem genes ligados a patogenicidade significativa e poucos genes de resistência a antibióticos. Nenhum gene responsável pela resistência aos antibióticos está localizado próximo a elementos móveis. Nenhum profago viável foi identificado. Estudos genômicos e *in vitro* não revelaram atividade hemolítica, mas alta tolerância às condições gastrointestinais. A cepa CEC15 apresentou adesão mais forte às células intestinais do que a cepa EcN. Investigações microscópicas e proteômicas sugerem que isso pode ser devido a um maior número de proteínas das fímbrias. Quando administrado a animais saudáveis, a CEC15 não teve efeitos negativos, nem melhorou a riqueza da microbiota intestinal. Quando a eliminação intestinal foi induzida, os animais que receberam a cepa CEC15 apresentaram menor perda de peso e menos danos às estruturas intestinais. A perturbação da microbiota sob o efeito do 5-FU foi reduzida pelo consumo da cepa CEC15. Seguindo estes resultados,

as frações pós-bióticas da cepa CEC15 foram testadas no mesmo modelo animal: bactérias inativadas pelo calor e sobrenadantes livres de células. Os resultados mostraram que a cepa CEC15 inativada pelo calor exibe quase os mesmos efeitos que a cepa viável em termos de proteção da arquitetura epitelial intestinal e perda de peso. O sobrenadante de CEC15 livre de células, embora apresentando resultados promissores, não foi comparável ao CEC15 viável e inativada pelo calor. Todos os testes acima mencionados foram realizados em paralelo com a administração da cepa EcN e suas preparações pós-bióticas. A cepa CEC15 superou a EcN em todos os resultados. Os sobrenadantes da cepa EcN, por outro lado, mostraram efeitos protetores promissores. Estes resultados demonstraram que CEC15 é promissora como cepa probiótica. No entanto, são necessários ensaios pré-clínicos e clínicos para confirmar a sua atividades protetora em humanos.

Palavras-chave: Probióticos; Probiogenômica; *Escherichia coli*; Posbióticos

LIST OF SYMBOLS AND ABBREVIATIONS

5-FU	5-Fluorouracyl
AAD	Antibiotic-Associated Diarrhea
AD	Alzheimer's Disease
ANOVA	Analysis of variance
ARGs	Antibiotic Resistance Genes
ASD	Autism Spectrum Disorder
BAs	Biogenic amines
BDNF	Brain-derived neurotrophic factor
BGF	Bifidogenic growth stimulator
BMC	Bone marrow concentration
BMD	Bone mineral density
BMI	Body mass index
BSH	Bile salt hydrolase
CARD	Comprehensive Antibiotic Resistance Database
CBER	Center for Biologic Evaluation and research
CD	Crohn's disease
cDNA	Complementary DNA
CDS	Coding DNA sequence
CEC15	<i>Escherichia coli</i> CEC15
CEUA	Use of Animals Ethics Committee
CFA	Complete Freund's adjuvant
CFS	Cell-free supernatants
CFU	Colony-forming unit
CGE	Center for Genomic Epidemiology
CIRM-BIA	International Centre for Microbial Resources - Food-Associated Bacteria
CLSI	Clinical and Laboratory Standards Institute
CMVs	Cytoplasmic membrane vesicles
CNS	Central nervous system
COBEA	Brazilian College of Animal Experimentation
COG	Clusters of Orthologous Groups
Cox-2	Cyclooxygenase 2
CRC	Colorectal cancer
CRP	C-reactive protein
DASS	Depression, anxiety, and stress scale

DC	Dendritic cells
DM	Diabetes mellitus
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNBS	Dinitrobenzene sulfonic acid
DSS	Dextran sulfate sodium
DTPA	Diethylenetriamine penta-acetic acid
EcN	<i>Escherichia coli</i> Nissle 1917
EFSA	The European Food Safety Authority
emPAI	Exponentially Modified Protein Abundance Index
ENS	Enteric Nervous System
EOMVs	Explosive outer-membrane vesicles
EPEC	Enteropathogenic <i>E. coli</i>
ESBL	Extended-Spectrum β -Lactamase
ETEC	Enterotoxigenic <i>E. coli</i>
EUCAST	European Committee on Antimicrobial Susceptibility Testing
EVs	Extracellular vesicles
ExPEC	Extraintestinal pathogenic <i>E. coli</i>
FAO	Food and Agriculture Organization
FBS	Fetal bovine serum
FDA	Food and Drug Administration
FdUMP	Fluorodeoxyuridine monophosphate
FdUTP	Fluorodeoxyuridine triphosphate
FOS	Fructooligosaccharide
FUTP	5-fluorouridine triphosphate
GABA	Gamma aminobutyric acid
GBA	Gut-brain axis
GC-content	Guanine-cytosine content
GEIs	Genomic islands
GHQ	General health questionnaire
GIT	Gastrointestinal tract
GOS	Galactooligosaccharide
GPx	Glutathione peroxidase
GRAS	Generally Recognized as Safe
GSH	Glutathione
HE	Hematoxylin / Eosin
HIV	Human immunodeficiency virus
Hmox	Heme oxygenase
HPS	Hematein Phloxin Safran

HTAB	Hexadecyltrimethylammonium bromide
IBD	Inflammatory bowel diseases
IFN γ	Interferon-gamma
IgA	Immunoglobulin A
IL-10	Interleukin 10
IL-12	Interleukin 12
IL-17a	Interleukin 17a
IL-18	Interleukin 18
IL-1 β	Interleukin 1 beta
IL-2	Interleukin 2
IL-6	Interleukin 6
ILC	Innate lymphoid cells
IND	Investigational New Drug
ISS	Insertion sequences
KEGG	Kyoto Encyclopedia of Genes and Genomes
KO	Knock-out
LAB	Lactic acid bacteria
LB	Luria-Bertani
LBP	Live biotherapeutic products
LC/MS-MS	Liquid chromatography–mass spectrometry
LGG	<i>Lactobacillus rhamnosus</i> GG
LPS	Lipopolysaccharide
MAGs	Metagenome assembled genome
MALDI-TOF MS	Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry
MAMP	Microbial associated molecular pattern
MAPK	Mitogen-activated protein kinase
MDA	Malondialdehyde
MGEs	Mobile genetic elements
MI	Metabolic island
MIC	Minimum inhibitory concentration
MLST	Multilocus sequence typing
MOI	Multiplicity of Infection
MOS	Mannanligosaccharide
MPO	Myeloperoxidase
mRNA	Messenger ribonucleic acid
MRS	De Man–Rogosa–Sharpe
MUC2	Mucin 2
MVs	Membrane vesicles

MyD88	Myeloid differentiation primary response 88
NCBI	National Center for Biotechnology Information
NEC	Necrotizing enterocolitis
NF- κ B	Nuclear factor kappa B
NGPs	Next-generation probiotics
NK cells	Natural killer cells
NTA	Nanoparticle tracking analysis
OD	Optical density
OIMVs	Outer-inner membrane vesicles
OMVs	Outer-membrane vesicles
PAI	Pathogenicity islands
PAS	Periodic acid–Schiff
PBMC	Peripheral blood mononuclear cells
PCA	Principal components analysis
PCR	Polymerase Chain Reaction
PD	Parkinson's disease
PMSF	Phenylmethylsulphonyl fluoride
PPAR- γ	Peroxisome proliferator-activated receptor gamma
qPCR	Quantitative polymerase chain reaction
QPS	Qualified Presumption of Safety
RCT	Randomized controlled trials
RI	Resistance island
rRNA	Ribosomal ribonucleic acid
SCFA	Short-chain fatty acids
SEC	Size Exclusion Chromatography
SEM	Scanning electron microscopy
SGF	Simulated Gastric Fluid
SIF	Simulated Intestinal Fluid
SlpB	Surface layer protein B
SOD	Superoxido desmutase
SPF	Specific pathogen-free
TCA	Taurocholic acid
TDCA	Taurodeoxycholic acid
TEM	Transmission Electron microscopy
TGCA	Tauroglycocholic acid
TGF- β	Transforming growth factor beta
TJ	Tight junction
TJP1	Tight junction protein 1
TLR2	Toll-like receptor 2

TMB	3,3',5,5'-tetramethylbenzidine
TNBS	Trinitrobenzene sulfonic acid
TNF- α	Tumor Necrosis Factor alpha
tRNA	Transfer ribonucleic acid
TSMSs	Tube-shaped membranous structures
UC	Ulcerative colitis
UF	Milk ultrafiltrate
UV	Ultra-violet
WGS	Whole-genome sequencing
WHO	World Health Organization
XOS	Xylooligosaccharide
YEL	Yeast Extract Lactate
ZO-1	Zonula occludens-1
ZO-2	Zonula occludens-2

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PRESENTATION

The use of probiotics as therapy for the most variate kind of diseases has been expanding largely in the past years. The correct identification and characterization of new probiotic strains and their role on the modulation of the host is essential. New strategies using *in silico*, *in vitro*, and *in vivo* analysis have been used to better study potential probiotics and to explore key activity of the study strain in a specific disease or host. A decade ago a new strain of *Escherichia coli*, *E. coli* CEC15, has been isolated and presented beneficial effects on the intestinal epithelium structure on rats and against induced colitis in mice model. Therefore, this thesis aims to characterize the *E. coli* CEC15 strain using genomic tools and *in vitro* tests, and to evaluate its safety and modulation of the host in murine model.

In this section, the international collaboration and the funding that supported the development of this work are presented. Furthermore, the organization of the thesis is explained.

1. International collaboration

This thesis is the result of an international collaboration between Brazilian and French institutions, as part of an International Joint Laboratory that formalized an ongoing collaboration since 2000. Accordingly, this thesis was carried out under the joint supervision of:

- Dr. Vasco Ariston de Carvalho Azevedo, from the Laboratory of Cellular and Molecular Genetics (Laboratório de Genética Celular e Molecular, LGCM), in Belo Horizonte, Brazil.
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LGCM is associated to the Institute of Biological Sciences (Instituto de Ciências Biológicas, ICB) and the Postgraduation Program in Genetics (Programa de Pós-graduação em Genética, PGGen), both from the Federal University of Minas Gerais (Universidade Federal de Minas Gerais, UFMG).

STLO is associated to the French National Research Institute for Agriculture, Food and Environment (Institut National de Recherche pour l'Agriculture, l'alimentation et l'Environnement, INRAE) and the French School of Agriculture, Food, Horticultural and

Landscape Sciences (L'Institut Agro Rennes-Angers), an internal school of the National Institute of Higher Education for Agriculture, Food and the Environment.

2. Funding

This work has received financial support from INRAE (Rennes, France), L'Institut Agro Rennes-Angers (Rennes, France) the Brazilian Federal Agency for the Support and Evaluation of Graduate Education (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior, CAPES) of the Brazilian Ministry of Education, and the National Council for Scientific and Technological Development (Conselho Nacional de Desenvolvimento Científico e Tecnológico) of Brazilian Ministry of Science and Technology.

3. Organization

This thesis is divided in sections as follows:

- **General Introduction** bringing into light the most relevant topics in regards to the subject of this thesis.
- **Research question, objectives and strategy** addresses succinctly the research context, the hypothesis and the objectives.
- **Chapter 1** contains a literature review providing the theoretical background of this work, addressing the process of identification, characterization, evaluation of effects and regulations regarding probiotics and probiotic products.
- **Chapter 2** contains a literature review deepening into the theoretical background of this work addressing how probiotics, prebiotics, synbiotics, and postbiotics act in the context of 5-Fluorouracyl-induced intestinal mucositis.
- **Chapter 3** introduces an original research article in which we characterize the potential probiotic effects of the strain *E. coli* CEC15 using *in silico*, *in vitro*, and *in vivo*.
- **Chapter 4** introduces an original research about *E. coli* CEC15 and *E. coli* Nissle 1917 postbiotic (heat-inactivated bacteria and cell-free supernatant) effect in a murine model of 5-FU-induced intestinal mucositis.
- **Chapter 5** is divided into two section. The first section introduces additional studies in which we performed an comparative genomic analysis of 8 probiotic *E. coli* strains. The second section introduced results on the production and characterization of extracellular vesicles from *E. coli* CEC15 and *E. coli* Nissle 1917 strains
- **Chapter 6** introduces an original research article in which we compare the anti-colitis effect of *Propionibacterium freudenreichii* CIRM-BIA 129 fermenting whole milk, skim milk, and milk ultrafiltrate permeate in murine model of colitis induced by DSS.
- **Chapter 7** presents a general discussion and the perspectives of the work developed during this thesis.

- **Résumé étendu** presents an extended abstract written in French.
- **Thesis outputs** presents a list of the products derived from this thesis, including research articles, scientific communication and patent.
- **Annexes** presents supplemental information.
- **References** contains the list of scientific studies cited in the thesis.

GENERAL INTRODUCTION

In recent years, there has been a growing interest in the use of probiotics as a means to restore and maintain the healthy status of the host, such as maintaining a healthy gut microbiota. Probiotics are defined as “live microorganisms that, when administered in adequate amounts, confer a health benefit on the host” (Hill et al., 2014). Probiotics can be part of the composition of diverse food products, such as fermented dairy products (e.g., yogurt) and fermented vegetables (e.g., sauerkraut), as well as in dietary supplements. The concept of probiotics is not new, and its roots can be traced back to the early 20th century when Nobel laureate Elie Metchnikoff hypothesized that the consumption of fermented dairy products contributed to the longevity of Bulgarian peasants (Mercenier et al., 2003). Since then, extensive research has been conducted to explore the potential health benefits of probiotics and their mechanisms of action. The potential benefits of probiotics extend beyond gut health. Studies have shown that probiotics can have positive effects on the immune system, mental health, and skin health, among other areas. As a result, the market for probiotics has grown exponentially, with consumers seeking probiotic products to improve their overall well-being (Grumet et al., 2020).

The mostly claimed probiotic bacteria strains, which have long been studied with respect to beneficial effects, belong to the *Bifidobacterium* genera and *Lactobacillaceae* family (O’Toole et al., 2017). However, there is a growing demand for new probiotics strains. In this context, the identification of strains from strains already recognized as probiotics and gut commensal strains, as in the case of next-generation probiotics, becomes a promising strategy to fulfil this demand.

The bacterium *Escherichia coli* is well known for its pathogenicity and for also being a common member of human and animals gut microbiota, as commensal bacterium. However, a member of this specie, *E. coli* Nissle 1917 has been used as probiotic for over a century with significant effects on treating infectious intestinal diarrhea (T. M. Wassenaar, 2016) and many works showing its beneficial effects, both in humans and animal models, on diseases such as inflammatory bowel diseases (ulcerative colitis and Chron’s disease) (Schultz, 2008) and allergies (Weise et al., 2011). Other strains of *E. coli* have also shown beneficial effects in humans, in special the strains that compose the probiotic product Symbioflor2 (T. Wassenaar et al., 2014) and the colinfant newborn (Kocourková et al., 2007). A newly isolated strain of *E. coli*, CEC15, has demonstrated beneficial effects on the remodeling of the structure of intestinal tissue in rats (Tomas et al., 2015) and protected mice against ulcerative colitis induced by administration of dinitrobenzene sulfonic acid (DNBS) (Escribano-Vazquez

et al., 2019). However, more studies were made necessary to confirm and understand the beneficial effects of this strain in the host.

Phenotypical and genotypical characterization of strains are a critical step in understanding and identifying probiotics (Shokryazdan et al., 2017). One essential aspect of characterization involves taxonomically classifying probiotic strains to determine their genetic relationship with other microorganisms. Traditional methods, such as phenotypic traits, are useful for preliminary identification (FAO/WHO, 2002). However, the advent of molecular techniques, like whole-genome sequencing (WGS), has revolutionized probiotic characterization. This method enables researchers to name a probiotic strains based on the sequence of genes present in their DNA. By comparing the gene sequence with a reference database, researchers can accurately classify the probiotic strain (Ranjan et al., 2016).

Probiogenomics is an emerging field that focuses on understanding the genomic makeup of probiotic microorganisms. The genomes of probiotics hold valuable information about their metabolic capabilities and functional properties (R. D. O. Carvalho et al., 2022). By analyzing the genetic content of probiotic strains, researchers can identify specific genes responsible for probiotic functions, providing insights into their potential health benefits (Castro-López et al., 2021). Genome sequencing and bioinformatics tools play a crucial role in probiogenomics. Comparative genomics allows researchers to compare the genomes of different probiotic strains, highlighting similarities and differences in their gene content. This comparative approach aids in identifying conserved genes associated with probiotic activities and, conversely, understanding strain-specific functional characteristics (Kazou et al., 2018). Probiogenomics also contributes to optimizing probiotic formulations. By identifying essential genes involved in probiotic functionality, researchers can modify probiotic strains to enhance their efficacy (Ventura et al., 2012). Additionally, probiogenomics helps address concerns related to the genetic stability of probiotics during production and storage, ensuring that the final product maintains its intended health benefits (Ventura et al., 2009).

Another vital aspect of probiotics characterization is ensuring their safety for human consumption. Probiotics should be non-pathogenic and free from harmful traits. Extensive safety assessments, including toxicological studies and genome analysis, are conducted to verify the absence of harmful genes or virulence factors. Safety considerations are crucial, especially for vulnerable populations like infants, pregnant women, and immunocompromised individuals (Sanders et al., 2010). Furthermore, the functional properties exhibited by probiotics are paramount to their characterization. Various *in vitro* assays are conducted to assess their probiotic potential. These tests include their ability to survive the harsh conditions of the gastrointestinal tract, such as acidity and bile, and their capacity to adhere to intestinal epithelial cells (Coelho-Rocha et al., 2023). Additionally, *in vitro* studies can identify the presence of functional genes

responsible for probiotic activities, such as the production of antimicrobial compounds or the modulation of immune responses. These tests are conducted under controlled laboratory conditions, allowing researchers to simulate specific aspects of the gastrointestinal environment (de Jesus et al., 2021). One primary aspect of *in vitro* testing is assessing probiotic survival under gastric conditions. The harsh acidic environment of the stomach can be detrimental to probiotic viability. Thus, researchers subject probiotic strains to simulated gastric conditions, monitoring their survival rates to determine their resilience (Vera-Pingitore et al., 2016). Another critical parameter studied *in vitro* is probiotic adhesion to intestinal epithelial cells. Adhesion is a prerequisite for probiotics to exert their beneficial effects in the gut. Various cell culture models are used to assess probiotic adhesion, providing valuable data on their potential to colonize and interact with the gut lining (Saadat et al., 2019). Moreover, *in vitro* testing includes evaluating the antimicrobial activity of probiotics against pathogenic microorganisms. Probiotics can produce antimicrobial compounds that inhibit the growth of harmful bacteria. These antagonistic activities are crucial for maintaining gut health and preventing infections (Lindgren and Dobrogosz, 1990).

To further evaluate probiotics functions, *in vivo* models can help to simulate real diseases and probiotics' efficacy in living organisms, such as animal models and human subjects. These studies provide real-life evidence of probiotic effects on gut health and overall well-being (Rousseau et al., 2020). Animal models, including rodents and other mammalian species, are commonly used for *in vivo* probiotic testing. These models allow researchers to assess probiotic impacts on gut microbiota composition, immune responses, and other physiological parameters (Rousseau et al., 2020). Results from animal studies provide valuable insights and guide the design of human clinical trials. Human clinical trials represent the gold standard for assessing probiotic efficacy. These trials involve administering probiotics to human subjects and evaluating their effects on specific health conditions (Zawistowska-Rojek and Tyski, 2018). Randomized, double-blind, placebo-controlled trials are preferred to minimize bias and obtain reliable results. Clinical trials have demonstrated probiotics' effectiveness in various conditions, such as diarrhea, irritable bowel syndrome, and allergies (Pogačar et al., 2022).

After confirming the beneficial effect of a probiotic strain, it is necessary to establish how this strain will be administered to the host and how the matrix in which the probiotic will be contained can affect their activity. Probiotics products can be presented as suspensions, dried in the form of capsules and in the form of fermented foods preparations such as fermented milk, fermented meats, fermented vegetables, and cheeses (Ozen and Dinleyici, 2015). It has been shown before that how the probiotic is presented can affect how it modulates the host so it is important to reevaluate the probiotic activity when it is administered in a different from the one tested initially

(Mantel et al., 2023).

As probiotic research continues to evolve, it is essential to remain vigilant in critically assessing the scientific evidence behind probiotic claims. While probiotics hold immense promise, not all probiotic products on the market have been rigorously tested or proven to deliver the claimed health benefits (de Simone, 2019). Therefore, continued research, transparent reporting of study results, and collaboration between researchers, industry, and regulatory bodies are crucial to unlock the full potential of probiotics. Last, the emerging field of probiotics presents exciting opportunities to harness the power of the gut microbiota for improved health. By further understanding the complexities of the gut microbiota and the mechanisms of action of probiotics, we can unlock novel therapeutic approaches to address various health challenges (Shokryazdan et al., 2017). As probiotic research continues to progress, the future holds great promise for these beneficial microorganisms to contribute significantly to human health and well-being.

THESIS RELEVANCE, OBJECTIVES, AND STRATEGY

Changes in the way people live in developed nations, encompassing factors like diet, physical inactivity, and a growing elderly population, are contributing to a rise in chronic illnesses. A significant portion of these increasing health issues involve problems with the immune system, inflammation, and imbalances in the gut microbiota. A prime example of this trend is the increasing occurrence of inflammatory bowel diseases, which are connected to genetic predisposition, immune system irregularities, and disruptions in gut microbial balance Bouma and Strober, 2003. Furthermore, ailments such as mucositis, a severe inflammation of the gastrointestinal tract lining, affect approximately 80% of patients undergoing cancer treatments involving chemotherapy and radiopharmaceuticals R. O. Carvalho et al., 2017. Although treatments are available for conditions like inflammatory bowel diseases and mucositis, these interventions not only strain healthcare systems significantly but also offer only temporary relief between recurrences, sometimes causing unwanted side effects that further drive up treatment expenses.

Probiotic bacteria have shown the potential to alleviate symptoms of inflammatory diseases Bibiloni et al., 2005; Ghouri et al., 2014; Sood et al., 2009. However, it is well-known that this effect is strain dependent and cannot be extrapolated to other strains from the same species without profound examination of their characteristics and beneficial effects. This highlights the importance of a correct identification and characterization of a potential probiotic strain on its phenotype and also on genomic level.

The main objective of this thesis is to fully characterize the newly isolated strain *Escherichia coli* CEC15, as a potential probiotic strain evaluating aspects related to toxicity, survival to gastrointestinal tract passage, adhesion to the intestinal wall, effects on a healthy host and its microbiota and the effect of the strain in a murine model of 5-FU-induced mucositis, using the viable cell, heat-inactivated bacteria, and fermented cell-free supernatant.

To address this main objective, it has been segmented into the subsequent specific objectives:

1. Classify the strain among different *E. coli* strains;
2. Identify genes related to the pathogenicity, safety, and probiotic traits;
3. Evaluate the tolerance to acid and bile salts;
4. Determine the adhesion level to the intestinal wall *in vitro*;

5. Assess the safety of the strain in healthy mice;
6. Study the anti-inflammatory activity in the context of intestinal mucositis;
7. Study the modulation of the microbiota of healthy mice and mice with intestinal mucositis
8. Compare the CEC15 strain with the reference strain *E. coli* Nissle 1917 ;
9. Investigate the effects of postbiotic preparations from CEC15 and EcN (heat-inactivated and cell-free supernatant) on the inflammatory process;
10. Evaluate the postbiotic effect on protecting the integrity of the intestinal;
11. Analyze the modulation of barrier- and inflammation-related genes expression;
12. Compare the effects of postbiotic preparations from CEC15 and EcN strains;
13. Correlate the effects of postbiotic preparations with the effects of live strains (Probiotics vs. Postbiotics).
14. Study *E. coli* probiotic strains with genome available on NCBI;
15. Identify and analyze these different components of the strains pangenome;
16. Classify Clusters of Orthologous Genes on the pangenome;
17. Describe genomic islands present in the *E. coli* probiotic strains;
18. Determine the presence of bacteriocins and antibiotic resistance genes in the *E. coli* probiotic strains;
19. Evaluate the production of extracellular vesicles by the strains *E. coli* CEC15 and EcN;
20. Analyze physical characteristics of the produced EVs;
21. Compare the proteome among EVs and the whole cell genome of both strains;
22. Evaluate the immunomodulation control potential of EVs.
23. Evaluate the impact of growth medium on the immunomodulation of a probiotic strain effect using *Propionibacterium freudenreichii* CIRM-BIA 129 strain as test in a murine model of DSS-induced ulcerative colitis.

Objectives 1-8 are addressed on Chapter 3, which presents an original research paper using probiogenomics and *in vitro* testing to characterize the CEC15 strain and *in vivo* models to assess its safety and protective effect.

Objectives 9 - 13 are addressed on Chapter 4, which presents an original research paper where we compare the effects of heat-inactivated and cell-free supernatant against the effects of the viable bacteria (for CEC15 and Nissle 1917) in a murine model of 5-FU-induced mucositis.

Objectives 14 - 23 are addressed on Chapter 5, which shows additional results of the thesis and comprehends a comparative genomic analysis between beneficial strains of *E. coli* and on the production and characterization of extracellular vesicles by *E. coli* CEC15 and *E. coli* Nissle 1917.

Objective 23 is addressed on Chapter 6, which presents an original research paper where we compare the effects of *P. freudenreichii* CIRM-BIA 129 on a murine model of DSS-induced ulcerative colitis fermenting three different mediums: Milk permeate ultrafiltrated, Skim milk, and Whole Milk.

A general discussion of the results, the conclusions and perspectives are presented in Chapter 7.

CHAPTER 1 - LITERATURE REVIEW.

STATE OF THE ART ON PROBIOTICS AND PROBIOTIC PRODUCTS CHARACTERIZATION

In this chapter, we provide a literature review describing the process of probiotic research, from its identification to the market regulations regarding its commercialization.

We describe here the process a potential probiotic strain must undergo aiming to correctly identify the strain and to characterize its ability to survive and thrive in the gastrointestinal tract as well as identifying potential pathogenic traits associated with the strains. Bioinformatic tools, here defined as probiogenomics, aim to identify features in the genome, the transcriptome, and / or the proteome that would let to pathogenesis or to the beneficial effect attributed to the strain, and *in vitro* tests are used to further understand and characterize the results of probiogenomic analyses.

After the *in silico* and *in vitro* characterization of the strain, the potential probiotic has to be tested for its toxicity and modulation in *in vivo* studies, comprehending animal studies and clinical trials. These tests were to be performed in healthy organisms and models of specific diseases in a well defined protocol with specific dosage and administration parameters, so the results can be rigorously evaluated.

With the probiotic properly characterized we then describe the forms in which probiotics are commercialized and the legislation regarding production and quality control of ready to use probiotic products, also taking in consideration the effects that these matrix in which the probiotic products are made would have in the effect of the actual probiotic in the host.

This work was submitted to the journal "Probiotic and antimicrobial proteins" on September 22nd, 2023.

1 **FROM GENOME TO BEDSIDE: THE ROAD OF PROBIOTICS FROM CHARACTERIZATION TO**
2 **COMMERCIALIZATION**

3
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29

30 **Abstract**

31
32 This review provides a comprehensive overview of the current state of probiotic research, covering a wide range
33 of topics, including strain identification, functional characterization, preclinical and clinical evaluations,
34 mechanisms of action, therapeutic applications, manufacturing considerations, and future directions. The
35 screening process for potential probiotics involves phenotypic and genomic analysis to identify strains with health-
36 promoting properties while excluding those with any factor that could be harmful to the host. *In vitro* assays for
37 evaluating probiotic traits such as acid tolerance, bile metabolism, adhesion properties, and antimicrobial effects
38 are described. The review highlights promising findings from *in vivo* studies on probiotics mitigation of
39 inflammatory bowel diseases, chemotherapy-induced mucositis, dysbiosis, obesity, diabetes, and bone health,
40 primarily through immunomodulation and modulation of the local microbiota in human and animal models.
41 Clinical studies demonstrating beneficial modulation of metabolic diseases and human central nervous system
42 function are also presented. Manufacturing processes significantly impact the growth, viability, and properties of
43 probiotics, and the composition of the product matrix and supplementation with prebiotics or other strains can
44 modify their effects. The lack of regulatory oversight raises concerns about commercial probiotics' quality, safety,
45 and labeling accuracy, particularly for vulnerable populations. Advancements in multi-omics approaches,
46 especially probiogenomics, will provide a deeper understanding of the mechanisms behind probiotic functionality,
47 allowing for personalized and targeted probiotic therapies. However, it is crucial to simultaneously focus on
48 improving manufacturing practices, implementing quality control standards, and establishing regulatory oversight
49 to ensure the safety and efficacy of probiotic products in the face of increasing therapeutic applications.

50
51 **Keywords:** Probiotics; Probiogenomics; Probiotics Regulations; Functional characterization; Human and animal
52 models.

53
54 **Statements and Declarations**

55 **Competing Interests:** Authors declare to have no competing interests as defined by Springer, or other interests
56 that might be perceived to influence the results and/or discussion reported in this paper

57

58 **1. BACKGROUND**

59

60 For many years health-promoting bacteria, i.e., probiotic bacteria, have been widely added as live
61 components to many food preparations (e.g., in so-called functional foods). However, the precise impact of the
62 use of probiotic bacteria on the functioning of the human gastrointestinal tract (GIT) is not fully understood. The
63 elucidation of the precise mechanisms by which probiotics influence human health as well as a guaranteed
64 biosafety, are essential for the development of novel and effective probiotic products. This will have to be
65 considered in current and future research endeavors when discovering and developing the next generation of
66 probiotic bacteria[1].

67 Every bacterial strain must possess specific properties to be considered a potential probiotic. The Food
68 and Agriculture Organization (FAO) of the United Nations and the World Health Organization (WHO)[2] guide
69 the evaluation of probiotics in food. These guidelines emphasize the importance of accurately identifying potential
70 probiotic strains and conducting various *in vitro* tests to assess their functional characteristics. This is due to
71 probiotic properties being unique to each strain, influenced by specific conditions and dosage, making it unlikely
72 for two strains of the same species to possess identical probiotic attributes. However, it has been suggested that
73 for well-studied species, such as those from the *Bifidobacterium* and *Lactobacillus* genera, which are known to
74 exhibit general probiotic properties, non-strain-specific claims may be considered [3]. Nevertheless, it is crucial
75 to utilize well-defined strains exclusively in probiotic products. Numerous strain-specific criteria have been
76 developed as *in vitro* assays for the initial selection of probiotic strains. Subsequently, *in vivo* studies are conducted
77 to investigate the potential probiotic properties of the selected strains in healthy individuals [4].

78 In order to choose new probiotic strains, microbial cultures obtained from unconventional ecosystems,
79 i.e., anywhere not food related such as plants, soil, and the gut of mammals, must undergo a comprehensive
80 evaluation process, which includes *in silico* characterization, *in vitro* experiments, animal models, and clinical
81 trials [5]. For identification, the FAO/WHO guidelines recommend the utilization of the most current and valid
82 methodologies, employing a combination of phenotypic and genotypic methods for the speciation of probiotic
83 strains [2]. However, the mandatory use of molecular methods for identification is emphasized, as phenotypic
84 identifications alone are deemed insufficiently reliable [6]. Regarding *in vitro* assays, the FAO/WHO guidelines
85 list commonly employed tests for screening and characterizing potential probiotic strains. These tests evaluate
86 resistance to gastric acidity, bile salt hydrolase (BSH) activity and resistance to bile salt, adherence to mucus
87 and/or human epithelial cells, as well as antimicrobial and antagonistic activity against potentially pathogenic
88 bacteria. For instance, models simulating intestinal or systemic inflammation are employed to investigate the
89 pathogenesis of inflammation-related conditions [7].

90 Additionally, models involving high-fat diets have been developed to study metabolic disorders [8].
91 Besides, cholesterol reduction ability [9], antioxidant activity [10], or cytotoxic effects against cancer cells [11]
92 may be tested for certain probiotic strains. Furthermore, clinical trials that recruit both healthy volunteers and
93 patients are conducted, aiming to establish correlations between clinical outcomes and specific molecular changes
94 induced by probiotic supplementation [12]. These approaches collectively contribute to advancing our
95 understanding of probiotics and their therapeutic potential [13]. Nevertheless, it is worth mentioning that it is not
96 necessary for a potential probiotic strain to fulfill all these selection criteria[14].

97 This review aims to make a long trip on the road of probiotics, from the isolation and characterization of
98 strains potentially beneficial to the production and regulation of probiotics products. A briefing of the main points
99 addressed here can be found on Figure 1.

100

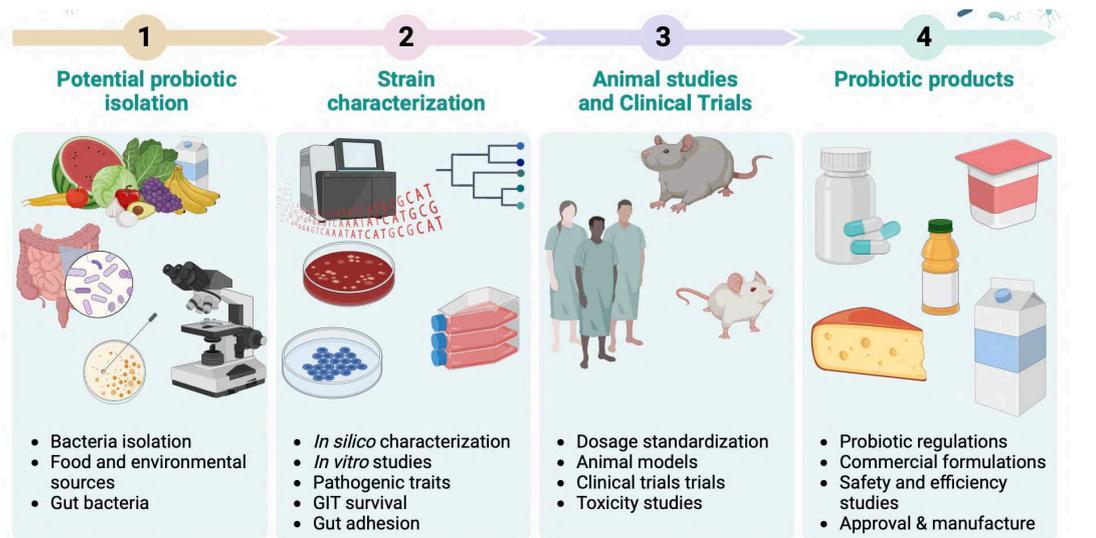


Fig 1 Main approaches used in the discovery, characterization, and production of a probiotic

2. POTENTIAL PROBIOTIC ISOLATION

Potential probiotic strains can originate from various sources such as humans, animals, plants, and the environment. Nonetheless, it is recommended that when selecting bacterial strains as probiotics for animals, they should be sourced from the animals' intestinal microflora. This approach facilitates smoother intestinal colonization and ensures a more targeted and effective application.

The gastrointestinal tract (GIT) and breast milk are dependable sources for identifying potential probiotic strains for human use [15]. Extensive research confirms that human milk plays a critical role in establishing microflora in the sterile intestines of newborns. Given the acknowledged health-promoting advantages of bacteria present in breast milk, researchers have directed attention towards isolating potential probiotic strains from this source [16–19]. Historically, the isolation of such strains has primarily encompassed traditional bacterial species like *Bifidobacterium* and *Lactobacillus*, owing to their well-established safety and efficacy in the domains of nutrition and health [20]. However, ongoing research exploring unconventional bacterial species may uncover new possibilities for these organisms to function as probiotics, thereby enhancing overall gut health. Consequently, it has been proposed that human milk harbors bacterial strains with the potential to serve as probiotic agents [21, 22]. Furthermore, research indicates that the fecal samples of adults, children, and infants also contain a significant abundance of probiotic bacteria [23]. Animal-derived food sources such as unprocessed milk [24–26] or fermented products [27, 28], as well as plant-based fermented foods [29, 30], offer ample opportunities for identifying potential probiotic strains. Numerous research studies have demonstrated that probiotic strains obtained from fermented foods can serve as a basis for creating starter cultures essential for the industrial production of fermented probiotic foods [31–33].

Independent of the source of the potential probiotic strain, the next steps involve identification to strain level, genomic and phenotypic characterization, and animal studies and clinical trials. These steps are described in the sections that follows.

3. STRAIN CHARACTERIZATION

3.1. Identification of bacterial species and strains

Probiotic strain identification relies on phenotypic and genotypic characterizations, as probiotic characteristics are predominantly strain-specific [34]. Accurate identification is paramount in probiotic research, and every potential probiotic strain should undergo correct identification using both phenotypic and genotypic methods, focusing on genotypic identification utilizing molecular techniques to identify microorganisms at the species and strain levels [2]. While phenotypic methods such as morphological evaluation and Gram staining are still employed for initial screening, computer-assisted commercial phenotypic identification systems have become outdated due to their limitations. These methods can be challenging and time-consuming, particularly for slow-growing and fastidious organisms. Additionally, identifying novel isolates using computerized numerical taxonomy does not always provide satisfactory species identification due to the reliance on characteristics observed in reference strains under optimal growth conditions, which can vary under stress [35]. Examples of

143 such systems include the Biolog microbial identification system (Biolog Inc., Hayward, CA, USA) for carbon
144 source utilization, which identifies bacteria, molds, and filamentous fungi [36], as well as the API system
145 (bioMérieux, Marcy l’Etoile, France) that identifies bacteria based on their carbohydrate fermentation patterns
146 [37].

147 In addition to phenotypic methods, Matrix-assisted laser desorption/ionization time-of-flight mass
148 spectrometry (MALDI-TOF MS) stands as a rapid, dependable, and high-capacity diagnostic tool tailored for the
149 swift identification of microorganisms. This advanced technology enables the identification of bacterial species
150 within a matter of minutes. Consistently, in the majority of these investigations, the employment of MALDI-TOF
151 MS for identification has proven to surpass conventional methods [38]. To exemplify, in a specific study involving
152 the prospective analysis of 980 routine clinical isolates through MALDI-TOF MS, a remarkable 92% of isolates
153 were accurately identified down to the species level, as opposed to 83.1% of isolates identified using conventional
154 identification methods. Notably, traditional methods exhibited a higher frequency of incorrect genus-level
155 identifications (1.6%), in contrast to the notably lower rate of 0.1% observed with MALDI-TOF MS [39]. The
156 proficiency of MALDI-TOF MS remains generally impervious to factors such as culture media, cultivation
157 conditions, or incubation durations. This inherent resilience contributes significantly to the consistent and
158 reproducible nature of bacterial identification via MALDI-TOF MS [40, 41]. This technique, however, does not
159 show high specificity at strain levels, especially for newly discovered strains, which is essential for correct
160 probiotic identification [42].

161 In contrast, genotypic identification methods have gained prominence for species identification and
162 differentiation of microbial strains. Various molecular methods, such as pulse field gel electrophoresis, sequencing
163 of rRNA genes, protein profiling, ribotyping, conventional Polymerase Chain Reaction (PCR), random
164 amplification of polymorphic DNA, and repetitive element palindromic PCR, can be employed [43]. Among the
165 various genotypic identification methods, 16S rRNA gene sequence analysis is commonly favored for microbial
166 identification due to its high accuracy and ability to determine taxonomical relationships among microbial strains
167 [44]. This method is preferred over others, such as multilocus sequence typing (MLST)[45] and metagenome-
168 assembled genomes (MAGs) [46], because it offers several advantages, including its widespread use, the
169 availability of reference databases, and its capability to provide reliable species and strain-level identification[47].
170 Other identification methods may be time-consuming, require extensive resources not readily available in many
171 laboratories, or necessitate a substantial collection of reference strains [2]. Therefore, 16S rRNA gene sequence
172 analysis has emerged as a frequently employed and reliable method for microbial identification in probiotic
173 research.

174 Despite the current whole-genome era of microbiology, there is still a pressing need for systematic and
175 standardized descriptions of bacterial genotypic variation. The need for more detailed characterization of bacterial
176 isolates has resulted in the creation of various strain-typing methods, and, in 1998, MLST was introduced as a
177 portable method using sequence data to identify clonal relationships among bacteria[45] and has become a
178 preferred technique for typing many organisms due to its ability to address the conflicting signals of vertical and
179 horizontal genetic transfer in bacterial populations, achieved by analyzing multiple ‘housekeeping’ gene loci in
180 the genome [45, 48]. Regrettably, the variability of house-keeping genes among different bacteria poses a
181 significant challenge in developing MLST schemes for anything other than closely related bacterial species. As a
182 result, it becomes necessary to have multiple MLST schemes, targeting different loci even within genera.

183 Other tools have been attempting to address this challenge by constructing MAGs, utilizing
184 advancements in sequencing technologies and computational tools. MAGs refer to groups of scaffolds with similar
185 characteristics obtained from a metagenome assembly, representing microbial genomes[49]. This process involves
186 assembling sequencing reads into scaffolds and categorizing them into candidate MAGs based on tetranucleotide
187 frequencies, abundances, complimentary marker genes [50], taxonomic alignments [51], and codon usage [52].
188 While they are commonly used to represent separate microbial taxonomic units in a sample, they are not typically
189 assumed to accurately reflect actual, genetically distinct microbial populations within that sample [53].
190 Furthermore, the precise definitions of individual, highly resolved MAGs are context-dependent and may vary
191 from one study to another.

192 Other methods have been developed to identify bacteria at the strain level from a microbial mixture using
193 long-read sequencing technology [54]. Although promising, limitations include focusing only on long reads, the
194 limited reference database, and the abundance dependency on mixture samples. This highlights the importance of
195 choosing the right tool for identifying and characterizing a potential probiotic strain, considering their
196 environment and DNA extraction method. A well-identified strain is a requirement for a probiotic to be approved
197 by the major regulatory agencies.

199 **3.2. Genomic characterization of probiotics**

200 Probiotics, as defined by the International Scientific Association for Probiotics and Prebiotics consensus
201 statement, are “live microorganisms which when administered in adequate amounts confer a health benefit on the
202

203 host"[3]. These organisms can be divided into two classes: classic probiotics, which comprises *Lactococcus* spp.,
204 the *lactobacillaceae* family., *Bifidobacterium* spp., *Bacillus* spp., Enterococci, *Weissella* spp., *Escherichia coli*
205 Nissle 1917, and *Saccharomyces* spp.; and Next-generation probiotics (NGPs), referring to a group of microbial
206 organisms that meet the conventional criteria for probiotics but have not been historically utilized for health
207 enhancement[55]. These microorganisms also align with the definition provided by the US Food and Drug
208 Administration for live biotherapeutic products (LBP), which entails being composed of live organisms, such as
209 bacteria, intended for preventing, treating, or curing human diseases or conditions, excluding vaccines[56].
210 Several microbial commensals have undergone assessment as NGPs. *Akkermansia muciniphila*, *Faecalibacterium*
211 *prausnitzii*, *Eubacterium hallii*, *Prevotella copri*, and *Bacteroides* spp. have shown significant promise. It is worth
212 noting that NGPs are genetically distant from lactic acid bacteria (LAB), which belong to the *Firmicutes* (Bacilli
213 class) or *Actinobacteria* phyla. These NGP taxa, such as *Prevotella*, *Bacteroides*, and *Akkermansia*, originate from
214 different phyla, namely *Bacteroidetes* and *Verrucomicrobia*. On the other hand, taxa like *Faecalibacterium*,
215 *Roseburia*, and *Eubacterium* are part of the *Firmicutes* phylum but belong to a different class, namely *Clostridia*.
216 This diversity in taxonomic classification highlights the wide range of potential candidates for NGPs, expanding
217 the possibilities for health promotion through probiotic interventions[55]. It becomes clear that there are numerous
218 uncharted territories to investigate regarding the variety of bacterial strains that exhibit unique and previously
219 undiscovered beneficial characteristics, and the probiogenomic is a powerful tool to tackle this issue.

220 In probiotic research, it is necessary to focus on understanding the strain-specific mechanisms of action
221 by exploring their unique genetic and metabolic characteristics. Relying solely on traditional tests to determine
222 probiotic safety and efficacy is not always reliable, making it challenging to predict their functionality besides
223 having no universally essential attributes for all probiotics, with them being able to exhibit multiple mechanisms
224 linked to a specific clinical benefit [57, 58]. These gaps in knowledge add complexity to the task of understanding
225 and forecasting the safety and functionality of probiotics. To tackle these challenges, the concept of
226 "probiogenomics" has risen as a growing field of research interest [59]. It aims to explore the diversity and
227 evolution of commensal and probiotic bacteria while uncovering the molecular basis for their beneficial activities
228 in promoting health [60]. Probiogenomics encompasses advanced techniques like genomics, transcriptomics,
229 proteomics, and metabolomics, which provide valuable resources for identifying uncharacterized strains and
230 developing predictive models for the rational selection of new probiotics [59, 61]. The public availability of
231 complete genome sequences has significantly enriched our understanding of these microorganisms' biology,
232 providing vast information on their metabolic capabilities, genetics, and phylogeny [59].

233 The genomes of newly discovered potential probiotic isolates must be fully sequenced and publicly
234 available in databases to ensure precise taxonomic identification, exploration of functional traits, and assessment
235 of safety profiles, [62]. Sequencing of probiotic strains has become more affordable and accessible. Moreover,
236 many bioinformatics tools are readily accessible to assist with genome assembly, annotation, and phylogenetic
237 analysis [63]. Phylogenetic trees can be constructed through computational analysis of complete genomes,
238 offering more accurate insights into the evolutionary relationships between strains and facilitating molecular
239 taxonomy [13].

240 Within this framework, diverse genetic events, including gene duplication, horizontal gene transfer, gene
241 decay, and chromosomal rearrangements, play a role in shaping bacterial genomes. These events contribute to the
242 genetic makeup essential for a microorganism to effectively adapt to and thrive in its specific ecological niche [1].
243 The genetic composition of the bacterial genome results from the microorganism's adaptive evolution to its
244 ecological niche [64]

245 Genomics has proven a potent approach to meeting the emerging criteria for bacteria to be recognized as
246 probiotics. The emergence of probiogenomics as a distinct field within genomics encompasses significant progress
247 in probiotic research [1]. The advancement of high-throughput sequencing technology and improvements in
248 bioinformatics have introduced new and powerful tools for conducting in-depth analyses of the evolutionary
249 patterns of bacterial strains that are of interest [13]. These advancements have facilitated in-depth investigations
250 into the biology of probiotics, their impact on cellular responses, and the validation of their health-promoting
251 properties. Genomic analyses have played a crucial role in identifying probiotic traits, such as bacteriocin
252 production or predicting acid tolerance [65], while metagenomic studies have shed light on probiotic-microbiota
253 interactions [66]. Transcriptomic, proteomic, and metabolomic platforms have enabled the examination of host-
254 microbe crosstalk and provided a comprehensive understanding of the holistic effects of probiotic
255 supplementation [67]. Additionally, recent comparative studies have strongly supported the notion that probiotic
256 actions are species-, disease-, sex-, and host-specific, underscoring the importance of targeted interventions and
257 personalized medicine [68].

258 By utilizing the genome sequences of probiotic bacteria, researchers are uncovering the mechanisms and
259 interactions involved in their activity within the host gastrointestinal tract (GIT) through the integration of
260 functional genomic techniques. In this context, three key areas of probiotic action are being emphasized: (i) the
261 ability to survive the journey through the gastrointestinal tract and adhere to the epithelial cells of the intestines;
262 (ii) engaging in competitive exclusion and displaying antimicrobial activity; (iii) modulating the immune system

263 of the host GIT; and (iv) interaction and modulation of the host intestinal microbiota (in especial by NGPs, which
264 are well-known resident of the human microbiota). These focus points provide valuable insights into the workings
265 of probiotics and their relationship with the host GIT [69].

266 Understanding microbe-host interaction is crucial for rationalizing potential probiotics targeting specific
267 health objectives. Currently, the selection of probiotic organisms is primarily based on ecological considerations
268 and phenotypic traits that ensure safety, robust manufacturing, storage stability, and survival throughout the
269 gastrointestinal tract. Empirical studies focusing on colonization, and host modulation functions (such as intestinal
270 adhesion, antimicrobial activity, and immune modulation), have contributed to a growing confidence in the
271 effectiveness of probiotics [70]. However, the intricate nature of biological interactions involving probiotics, the
272 intestinal ecosystem, and the host has made it challenging to identify specific probiotic effector molecules and
273 their functions definitively. Thus far, the probiotic effector molecules that have been identified are mainly located
274 on the bacterial surface or are secreted [71]. Nevertheless, there is an increasing body of promising evidence
275 demonstrating specific and measurable physiological effects on the host following the consumption of probiotics
276 [72].

277 Comparative genomics has significantly contributed to predicting probiotic traits and attributes [73].
278 Comparative genomics is a valuable approach utilized to explore the extent of phenotypic variation among strains
279 and identify strain-specific genes within the pangenome (the complete gene set of all strains within a monophyletic
280 group) while highlighting shared characteristics [74]. It helps uncover conserved sequences in probiotics'
281 genomes that encode essential cellular functions and regulatory elements governing their expression. Analysis of
282 clusters of orthologous genes allows for categorizing these findings into various functional categories, such as
283 transcription, metabolism, cell motility, and signal transduction, among others [75]. Furthermore, comparing the
284 genomes of newly discovered isolates with those of well-characterized probiotics can reveal functional properties
285 like adhesion to epithelial cells, auto-aggregation, stress response mechanisms, defense mechanisms (including
286 virulence factors), and antibiotic resistance [76].

288 3.3. *In silico* identification of potential pathogenic traits

289
290 The main theoretical risks associated with probiotics include the possibility of infection, adverse effects
291 caused by toxins produced by the probiotic strains or contaminants, and immunological effects [77]. Safety
292 assessments should consider factors such as the nature of the probiotic microorganism, method of administration,
293 level of exposure, health status of the recipients, and the intended physiological functions of the microbes [77].
294 Although a universal international standard for the safety evaluation of probiotics is currently lacking, in the
295 United States, the Food and Drug Administration (FDA) designates bacteria as Generally Recognized as Safe
296 (GRAS) for human consumption. Thus, probiotic blends or supplements must exclusively include strains that
297 have achieved the GRAS status [78, 79]. Furthermore, the European Food Safety Authority (EFSA) has proposed
298 the concept of "Qualified Presumption of Safety (QPS)," which may be applicable to specific groups of
299 microorganisms [80]. The determination of QPS status is based on four key considerations: (i) Taxonomy: The
300 taxonomic level or grouping for which QPS is being sought; (ii) Familiarity: The extent of knowledge available
301 regarding the proposed group of organisms to enable a decision on their safety; (iii) Pathogenicity: Whether the
302 group considered for QPS contains known pathogens, and if so, whether sufficient information exists about their
303 virulence determinants or toxigenic potential to exclude pathogenic strains; and (iv) End use: Whether viable
304 organisms from the group enter the food chain or if they are utilized in the production of other products [80].

305 Probiotic strains, including *Lactobacillus*, *Lactococcus*, *Bifidobacterium*, and *Streptococcus* species,
306 have a long-standing record of safe use and are generally recognized as safe (GRAS) [81]. Evidence suggests that
307 the consumption of probiotic lactobacilli does not pose a greater risk of infection compared to commensal strains,
308 and it is highly unlikely to associate lactobacilli consumption with a risk of death [15]. Instances of infection
309 caused by lactobacilli and bifidobacteria are exceedingly rare and estimated to account for only 0.05% to 0.4% of
310 cases of infective endocarditis and bacteremia [23]. As for NGPs, a comprehensive and extensive research on the
311 safety and tolerability is imperative and must be conducted through both animal and human trials. Currently,
312 human trials are lacking for the majority of candidate NGPs, and when they are conducted, they are primarily
313 exploratory in nature, featuring small sample sizes and excluding sensitive populations such as frailty subjects,
314 the elderly, or children [55].

315 While most species and genera of LAB are considered safe, certain strains may present concerns, such
316 as those known to produce biogenic amines from protein sources. Additionally, the transmission of antibiotic
317 resistance genes among different bacterial strains is a health concern, and the FAO/WHO guidelines recommend
318 assessing the antibiotic resistance/susceptibility pattern of each probiotic strain [2].

319 Although some studies have attempted to identify virulence factors for lactobacilli, such approaches are
320 more applicable to known pathogens and may be inherently flawed when applied to normal commensals such as
321 lactobacilli or bifidobacteria [15]. In general, safety studies encompassing taxonomy clarification, *in vitro* tests,
322 *in vivo* tests, human trials, and genome sequencing are necessary to establish the safety profile of bacteria [23]

323 Before considering their potential application, selected strain candidates must undergo characterization
324 based on various safety traits. These criteria can be found in multiple published works, reports, and
325 recommendations provided by diverse committees, organizations, and expert/advisory groups [82, 83]. One
326 crucial aspect of evaluating probiotic safety involves a thorough examination of intrinsic properties, such as
327 pathogenicity/virulence, toxin production, and antibiotic resistance. This assessment has become obligatory due
328 to several studies suggesting that probiotics may have adverse effects on the health of the host, primarily resulting
329 in allergic and infectious diseases, harmful metabolic activities, and infectivity [84]. These disorders have been
330 associated with a decrease in immune system stimulation and subsequent imbalance between Th1 and Th2
331 responses [85]. Therefore, ensuring probiotic safety can be achieved through individual or integrated
332 probiogenomics approaches.

334 3.3.1. Virulence factors

335
336 Virulence factors play a crucial role in microbial pathogenesis and encompass a variety of components,
337 such as enzymes, toxins, secreted effectors, and cell-associated products. A comprehensive safety assessment of
338 probiotics must include an evaluation of their potential to express virulence or toxin genes that could lead to
339 disease. This assessment typically involves two main aspects: determining whether the probiotic strain belongs to
340 a species known for virulence or toxigenicity and investigating the presence of virulence or toxin genes within the
341 microbe's genome.

342 Defining the virulence of a microbe at the molecular genetic level is challenging, as it greatly depends
343 on the dynamic host-microbe relationship. Generally, factors contributing to colonization, invasion, and evasion
344 of host immune-related elements are considered critical genetic foundations of virulence [58]. Other factors may
345 enable a microbe to thrive in a specific host environment and contribute to virulence by complementing the
346 harmful effects of toxins and other directly acting agents associated with virulence [86].

347 It is important to note that individual "virulence" factors do not act in isolation; Instead, their coordinated
348 expression and underlying genetic foundation collectively contribute to disease potential [58]. A prime example
349 is the species of *Escherichia coli*, which includes both commensal strains and pathogenic strains causing disease.
350 This example highlights the complexity of host-microbe interactions and the role of designated "virulence factors"
351 in pathogenicity. Determining pathogenicity requires a systems biology approach and a deeper understanding of
352 the mechanisms underlying the beneficial relationship between probiotics and the host [87].

353 High-resolution information on core genome relationships and accessory genome elements can further
354 enhance our understanding of the genetic content of concern. Recent developments in this field have prompted
355 the EFSA to update its QPS list of microorganisms to address these changes [88].

356 Abriouel et al. [89] conducted an *in silico* evaluation of the safety of *Weissella confusa* LBAE C39-2 and
357 *Weissella cibaria* KACC 11862 strains by analyzing their whole-genome sequences. The analysis revealed that
358 *W. confusa* LBAE C39-2 possesses four virulence factors associated with genes encoding collagen adhesion,
359 hemolysin, and mucus-binding proteins. On the other hand, *W. cibaria* KACC 11862 was found to contain two
360 virulence factors coding for hemolysins. Although these factors have been linked to invasion and infectivity
361 events, their specific role in the virulence of *Weissella* species remains unknown, thus requiring further studies to
362 confirm their expression.

363 Similarly, Li et al. [90] assessed the safety of the potentially probiotic strain *Enterococcus durans*
364 KLDS6.0930, which was isolated from a traditional fermented cream. Whole-genome sequencing and analysis
365 were employed to examine virulence-related genes. The authors identified 45 putative virulence factors
366 predominantly related to cell surface molecules, primarily involved in host or surface adhesion and promotion of
367 biofilm formation. These factors have been recognized as important elements in the initiation of infections.
368 However, it is worth noting that an additional analysis using PathogenFinder [91], a web-based server that predicts
369 bacterial pathogenicity through the analysis of user-provided proteome, genome, or raw reads, predicted *E. durans*
370 KLDS6.0930 as a non-pathogenic bacterium.

371 In a related study, the genome sequences of *Bifidobacterium bifidum* BGN4 and *Bifidobacterium longum*
372 BORI, obtained from fecal samples of healthy breast-fed infants, were compared with the genome sequences of
373 four significant human pathogens: *E. coli*, *Enterococcus*, *Listeria*, and *Staphylococcus aureus* using
374 VirulenceFinder tool[92], a part of a freely accessible web-based platform for analyzing whole-genome
375 sequencing (WGS) data, provided by the Center for Genomic Epidemiology (CGE) at
376 <http://www.genomicepidemiology.org/>. No virulence-associated genes related to offensive traits, such as Shiga
377 toxin, exoenzymes, or genes involved in immune evasion or alteration, were detected. Consequently, the authors
378 proposed that these strains could have the GRAS status[93].

380 3.3.2. Prophages and integrases

381

382 Unwanted genetic traits such as those related to disease-causing factors and resistance to antimicrobial
383 agents are frequently connected to mobile genetic elements (MGEs) that can be acquired during the process of
384 adaptive evolution. Therefore, it is essential to examine the mobilome of a probiotic strain, which includes
385 components like phages, plasmids, genomic islands (GEIs), transposons, and insertion sequences (Iss). This
386 examination is crucial for evaluating the strain's safety and for determining whether its health-promoting benefits
387 are innate characteristics or acquired attribute' [94, 95]. Prophages are segments of bacteriophage genomes
388 inserted and integrated into bacterial chromosomes. They are commonly found in probiotic genomes; however,
389 their presence requires further investigation due to their association with a higher likelihood of encoding virulence
390 factors and promoting genetic variability [96].

391 In a study by Liu, Wang, et al. [97], the complete genome of *L. plantarum* 5-2, isolated from fermented
392 soybeans, was sequenced using pyrosequencing to explore the potential presence of prophage elements. The
393 analysis revealed that the genome of strain 5-2 harbored four prophage regions, three of which were intact, and
394 one was incomplete. The first intact prophage region exhibited similarity to *Lactobacillus* phage Sha1 (44.1 kb,
395 referred to as region 1), while the other two regions shared similarity with *Lactobacillus* phage phi1e (42.3 kb
396 and 45.6 kb, referred to as region 2 and region 3). Conversely, the incomplete prophage region 4 showed
397 resemblance to *Sphingomonas* phage PAU (14.1 kb). Within the three intact prophage elements, three integrases
398 were identified. These integrases are known to be associated with phage morphogenesis, including the packaging,
399 head, tail gene clusters, and lysis cassette. Furthermore, the presence of intact prophage regions suggests their
400 recent acquisition into the bacterial genome, while the incomplete prophage region implies strong selection by the
401 bacteria leading to prophage inactivation. Sequences belonging to prophages are recognized for their lack of
402 stability in terms of maintaining the integrity of the genome. The presence of complete and functional prophage
403 regions indicated high risk of gene transfer from the strain to others present in the host, needing further
404 investigation on the content of these sequences. The genetic content of prophages must be extensively characterized
405 to guarantee the absence of any pathogenic genes that could lead to a pathogenic trait, avoiding the transfer to
406 commensal or even already pathogenic strains. As a result, it is preferable to not have intact prophages in order to
407 uphold genome stability and to guarantee the appropriateness of probiotic bacteria for use in industrial contexts
408 [98].

409 Similarly, Abriouel et al. [99] conducted a comprehensive analysis of the complete genome sequence of
410 *L. pentosus* MP-10, a bacterium isolated from the brine of naturally fermented olives, to identify prophage DNA
411 elements. The genome of MP-10 was found to harbor five temperate phage regions: two regions were determined
412 to be intact (region 2 and region 5), two regions were uncertain (region 1 and region 4), and one region was
413 incomplete (region 3). The intact prophage regions were identified as *Lactobacillus* phage Sha1 (39.2 kb) and
414 *Oenococcus* phage phi 9805 (51.7 kb). The questionable regions showed similarity to *Streptococcus pyogenes*
415 phage 315.2 (15.4 kb) and *Listeria* phage B025 (20.9 kb). The incomplete region was associated with
416 *Lactobacillus* phage Sha1 (26.7 kb). Furthermore, each prophage region contained integrases, specifically,
417 PP_00601 (region 2), PP_02352 (region 5), PP_00037 (region 1), PP_01319, and PP_01321 (region 3), which are
418 responsible for encoding proteins. Based on these findings, the authors recommended further investigations into
419 the application of *L. pentosus* MP-10.

420 More recently, Tarrah et al. [100] conducted a study to explore potential safety characteristics,
421 particularly the presence of prophages, in *L. paracasei* DTA93, which was isolated from fecal samples of healthy
422 infants. The analysis involved complete genome sequencing using Illumina paired-end sequencing technology.
423 The data revealed the presence of only three incomplete prophage regions in the genome of DTA93. These regions
424 were identified in contig 12 (from 47.8 to 66.8 kb) for region 1, contig 28 (from 2.1 to 24.3 kb) for region 2, and
425 contig 47 (from 2.1 to 24.3 kb) for region 3. The authors suggested that these prophage remnants did not contain
426 sufficient genes to be considered as complete functional phages.

427 Pei and colleagues [101] evaluated 1,472 genomes of Lactobacilli strains, from 16 different species,
428 presenting a wide variety of prophages. The distribution of referred prophages is uneven, with a wide variety of
429 genome characteristics and distinct clusters linked to host species, allowing to better understand the genetic
430 diversity of prophages in Lactobacilli. Additionally, antibiotic resistance genes (ARGs) were found in prophages
431 from 10% of the studied strains. The study also showed that most of the intact identified prophages were able to
432 be induced producing successfully temperate phages [102]. The transfer of the genes and, consequently, the
433 phenotype related to the gene, were not tested. The findings of this study could be of interest to a variety of
434 biotechnological and clinical fields that require a more comprehensive safety assessment and functional
435 understanding of Lactobacilli species.

436 3.3.3. Antibiotic resistance

437 The global health concern of antibiotic resistance is rapidly spreading. As mentioned above, genes
438 culminating to the resistance to antibiotics are commonly found inside of prophage and other MGEs. Probiotic
439 bacteria that possess inherent resistance to antibiotics are generally considered safe, as they pose minimal risk of
440
441

442 transferring drug-resistant genes to more harmful species. However, in probiotic strains where resistance genes
443 are acquired, primarily carried on MGEs, like plasmids, transposons, prophages, and integrons, there is a
444 significant potential for horizontal transmission, presenting a serious safety concern [103].

445 Given that certain probiotics may serve as reservoirs for antibiotic-resistant genes, it is reasonable to
446 speculate that genetic transfer of antibiotic resistance to opportunistic bacteria could occur. Therefore, it is
447 important to investigate the mechanisms of resistance, the genetic nature of resistance traits, and the elements
448 contributing to resistance dissemination [84]. The evaluation of antibiotic resistance in novel probiotic strains
449 involves both phenotypic assessment through determining minimal inhibitory concentrations [104], and genotypic
450 analysis using PCR-based techniques and sequencing [105, 106]. DNA microarrays and whole-genome
451 sequencing (WGS) platforms can also be employed to identify and locate previously unidentified antibiotic
452 resistance genes [106, 107].

453 EFSA has established a protocol for evaluating antibiotic resistance, which includes assessing phenotypic
454 resistance based on MIC (minimum inhibitory concentration) values above pre-established thresholds and
455 searching the genome for the presence of antibiotic resistance genes [62, 108]. According to EFSA's guidance,
456 any functional antibiotic resistance above the threshold should be further characterized as intrinsic or
457 transmissible, and the genetic basis of resistance should be identified and explained. The updated EFSA guidance
458 from 2018 emphasizes the need for both phenotypic (MIC) and genotypic analysis of antimicrobial resistance,
459 without limiting the analysis to resistance above a specific threshold or cutoff value [62]. This aligns with the
460 safety requirements of several countries worldwide.

461 Understanding the genetic nature of antibiotic resistance is crucial for assessing the risk of potential
462 spread, given the transferability of resistance genes among bacteria. It is well-known that live microbes can harbor
463 antibiotic resistance genes, and horizontal gene transfer in the human gut has been demonstrated [109]. Strains
464 with intrinsic resistance to antibiotics, which are commonly found within strains from the same species, poses a
465 low risk of horizontal spread and are considered safe to use by regulatory agencies [62]. Acquired resistance,
466 which is not typically present in a species, is considered a higher concern. However, chromosomal mutations
467 leading to acquired resistance are associated with a low potential for horizontal spread [108]. According to EFSA
468 guidance and global regulatory requirements, the genetic nature of clinically relevant antibiotic resistance genes
469 must be explained. Strains carrying transmissible resistance genes flanked by genetic elements known to mediate
470 horizontal transfer must be addressed and explained, as per EFSA guidance. Additionally, the risk of transfer
471 potential should be assessed. Strains for which the nature of resistance cannot be explained should be avoided [62,
472 108].

473 474 **3.3.4. Biogenic amines production**

475
476 Biogenic amines (BAs) are organic nitrogen compounds formed mainly by decarboxylation of amino
477 acids by microorganisms and are associated with toxicity concerns, particularly in relation to their microbial
478 production in certain fermented foods like certain cheeses [110]. They are considered undesirable metabolic
479 products of bacteria, due to their adverse effects and potential toxicological risks. While BAs are important for
480 various biological functions such as neurotransmitters, excessive levels can lead to toxic effects [111]. Some
481 commonly studied BAs include monoamines (e.g., histamine, tyramine), diamines (e.g., cadaverine, putrescine),
482 and polyamines (e.g., spermidine, spermine). Histamine and tyramine are particularly concerning due to their
483 potential toxicities. Therefore, probiogenomics studies can provide insights into whether probiotic strains harbor
484 genes or operons responsible for the biosynthesis of Bas. To assess the potential for additional BA production by
485 administered probiotic strains, the presence of genes encoding BAs production can be determined using WGS or
486 targeted PCR amplification. Phenotypic confirmation of biogenic amine production by bacterial species can be
487 analyzed using decarboxylase screening media and high-performance liquid chromatography [112].

488 Li et al. [113] investigated the production of Bas by *Lactobacillus helveticus* KLDS1.8701, isolated from
489 Chinese traditional fermented dairy products, using WGS and LC/MS-MS analysis. The genome of *L. helveticus*
490 KLDS1.8701 contained pseudogenes related to the conversion of arginine into putrescine (HUO_RS05500,
491 arginine deiminase; HUO_RS05505, ornithine carbamoyltransferase; HUO_RS05510, carbamate kinase) and the
492 conversion of ornithine into putrescine (HUO_RS06750, ornithine decarboxylase). Additionally, the strain
493 harbored a cluster for spermidine/putrescine ABC transporter (HUO_RS08405 to HUO_RS08420). Despite the
494 presence of these genes, LC/MS-MS analysis revealed the absence of BAs production by *L. helveticus*
495 KLDS1.8701, indicating that the pathway may be incomplete and, therefore, non-functional.

496 The potential production of BAs by *Bifidobacterium animalis* subsp. *Lactis* AD011, isolated from an
497 infant fecal sample, was recently investigated using a metabolomics approach through LC/MS-MS analysis [114,
498 115]. Since the presence of BAs can serve as a quality indicator for fermented probiotic products, the authors
499 focused on identifying four main Bas, namely putrescine, cadaverine, histamine, and tyramine, in two different
500 growth media (MRS and whole milk) after 15 hours of bacterial cultivation. The results demonstrated the absence
501 of detectable Bas ($\mu\text{g/mL}$) production in both supernatants, suggesting that, regarding the production of BAs,

502 AD011 is likely safe and acceptable for human consumption as a probiotic. Other pathogenicity traits were still
503 to be evaluated to fully confirm strain safety.

504

505 3.3.5. Mucin degradation

506

507 Mucin, a protein found in mucus, plays a crucial role in the structure of mucosal surfaces in the digestive
508 tract and serves as a protective barrier against microbial and chemical invasion [116]. More than 40 bacterial
509 genes involved in mucin metabolism have been identified [117]. WGS can be utilized to identify bacterial genes
510 associated with mucin degradation, and the activity of these genes can be assessed *in vitro* by evaluating the ability
511 of bacterial strains to grow in the presence of mucin as the sole carbon source in liquid or agar media [118].

512 The gut microbiota naturally degrades mucus to utilize it as a carbon source. For example, *Akkermansia*
513 *muciniphila* feeds on mucins and converts them into short-chain fatty acids (SCFAs), which serve as an essential
514 energy source for the cells lining the gastrointestinal tract. However, a disruption in the balance between mucin-
515 degrading bacteria and other bacteria has been suggested to contribute to disease and infection in humans [119,
516 120]. It's important to note that mucin degradation is tightly regulated within gut microbes and influenced by the
517 availability of dietary polysaccharides [121]. Assessing the mucin degradation capability of probiotic strains is
518 part of a comprehensive safety analysis since if there is an excessive increase on mucin-degrading bacteria would
519 lead to destruction of the intestinal protective barrier allowing pathogenic and opportunistic bacteria to cause
520 infections. However, the determination of the associated risk requires a systems biology approach and a deeper
521 understanding of the mechanisms underlying the beneficial relationship between probiotics and the host.
522 Therefore, the number of mucin-degrading genes present in the genome of a probiotic species should be
523 considered as part of a holistic assessment of the strain's safety, rather than being solely regarded as a virulence
524 characteristic.

525

526 3.4. *In vitro* characterization of probiotic candidates

527

528 Numerous health advantages associated with the consumption of these microorganisms have been
529 elucidated through clinical investigations. These benefits encompass a range of outcomes, such as the mitigation
530 of diarrhea's duration and occurrence, relief from lactose intolerance symptoms, decreased likelihood of
531 pathogenic infections, immune system stimulation, and regulation of the inflammatory response [122]. The GIT
532 serves as a primary site where probiotic microorganisms exert their effects. The underlying concept behind
533 incorporating probiotics into food and supplements to harness their potential health benefits relies on the
534 microorganisms' capacity to withstand the transit through the GIT, ensuring their adequate quantity of live
535 probiotic bacteria reach either the small or large intestine and interact with, adhere to, and colonize the host.
536 However, probiotic microorganisms encounter various challenges during their passage through the GIT, including
537 the highly acidic environment of the stomach (pH 1.5–4.0), bile salts, and digestive enzymes [123].

538 FAO and WHO have established guidelines for probiotics in food. These guidelines propose several
539 criteria for the selection of probiotics, including resistance to adverse conditions in the human body, ability to
540 adhere to epithelial cells, antimicrobial activity, and safety assessment [3]. Evaluating the stress tolerance
541 characteristics of novel strains under gastrointestinal conditions is crucial to ensure their functionality and expand
542 the range of microbial species and products available as probiotics.

543

544 3.4.1. Acid tolerance and survival in artificial gastric juice

545

546 To be considered as a probiotic, bacterial strains must possess several crucial characteristics, including
547 maintaining their viability and activity throughout production, product storage, and passage through the GIT
548 [124]. The GIT poses a stressful condition for probiotics, beginning with the stomach. The transit time in the
549 stomach can vary from less than 1 hour to 3 to 4 hours, influenced by factors such as individual differences, diet,
550 and other variables [125]. During this time, the pH in the stomach can reach extremely low levels, around 1.5,
551 while the concentration of bile in the upper intestinal tract can be unpredictable and vary [126]. *In vitro* simulated
552 stomach survival methods are commonly employed as initial steps in evaluating new probiotic strains. These
553 methods involve subjecting the strains to incubation in an acidified medium (such as MRS broth/buffer/peptone;
554 pH 2.0–3.0 for 1–4 hours) or simulated gastric juices (pH 2.0–3.0 in the presence of pepsin) [127, 128].
555 Additionally, more complex gastrointestinal models are utilized to simulate various aspects of gastrointestinal
556 transit and provide a more comprehensive evaluation of probiotic survival. *In vitro* tests assessing acid and bile
557 tolerance often serve as a predictive measure of a strain's ability to survive in the host's body environment.
558 However, it should be noted that acid and bile tolerance primarily pertain to the oral administration of probiotics
559 and may not be relevant for other applications [14].

560 The resistance to gastrointestinal conditions varies among different genera and species. Lactobacilli are
561 generally more resistant, while bifidobacteria tend to be more sensitive to low pH, resulting in lower survival rates

562 at pH 2 and pH 3.0 [5]. In a study on Serpa cheese, a total of 116 LAB strains were isolated to investigate their
563 probiotic properties. None of the LAB isolates survived at pH 2.5, but all of them survived at pH 3.0. Additionally,
564 20 isolates demonstrated survival at an intermediate pH of 2.75 after a 2-hour exposure [129].

565 Lee et al. [130] conducted a study to examine the acid tolerance of LAB, exposing them to a pH of 2.5
566 for 1 hour. Among the LAB tested, only *L. reuteri* and *L. gasseri* exhibited tolerance to acidic conditions. In a
567 study by Song et al. [131], the functional properties of LAB isolated from various sources were screened. Except
568 for *L. acidophilus* M23, the tested *Lactobacillus* strains demonstrated tolerance to pH 2.5 for 2 hours in the
569 presence of pepsin. It has been reported that bifidobacteria are more susceptible to a pH of 2.0 in an HCl acidified
570 medium, when compared to a medium acidified by a mixture of pepsin and HCl, which better simulates the
571 composition of gastric juices. Furthermore, it has been suggested that pepsin may protect bifidobacterial cells
572 during exposure to low pH by reducing their hyperpolarization, which is associated with H⁺-ATPase activity
573 [132].

574 Finally, dynamic models of human digestion have been used to evaluate the survival of probiotic bacteria
575 during transit, and to determine the impact of the food matrix, or of the probiotic encapsulation, during simulated
576 digestion. Rabah et al [133] tested the survival of *Propionibacterium freudenreichii* CIRM-BIA 129 through the
577 GIT using *in vitro* models of static [134] and dynamic [135] digestion with three different matrices, milk
578 ultrafiltrate, milk, and a mono-strain 'Swiss-type' cheese. During static digestion, all three matrices were able to
579 be recovered at viability of around 40% after gastric phase, when later submitted to intestinal phase, only the
580 cheese matrix was able to maintain the same viability while milk and milk ultrafiltrate reduced to 20%. When
581 submitted to the dynamic digestion cheese matrix was able to maintain viability up to 60% while milk ultrafiltrate
582 had CFU reduced up to 3 logs after 2h of gastric phase. After the intestinal phase, both matrices maintained the
583 viability they had after the end of gastric phase. In addition. This study [133] did evaluate the integrity of the SlpB
584 (surface layer protein B), a anti-inflammatory protein found on the surface of some *P. freudenreichii* strains,
585 during the digestion using both digestion models. It was found that SlpB was completely degraded during static
586 gastric phase on milk and milk ultrafiltrate matrices while it was protected by the cheese matrix. As for the
587 dynamic digestion, SlpB was found up to 80 minutes of digestion (mid gastric phase) on milk ultrafiltrate matrix
588 and to 150 min (30 min at intestinal phase). This demonstrates the importance of the probiotic matrix not only on
589 the probiotic viability but also on their immunomodulatory function as we have seen here with the preservation
590 of SlpB protein.

591 3.4.2. Bile tolerance and metabolism

592 Passage of probiotics through the small intestine can pose challenges as they encounter bile acids, bile
593 salts, and pancreatic enzymes, which can significantly reduce viability. Unlike the stomach, the small intestine's
594 neutral pH range of 6.1 to 7.8 does not inhibit probiotic survival [136, 137]. Bile acids play a crucial role in lipid
595 digestion in the small intestine and impact the microbial ecosystem in the small and large intestines [138]. The
596 liver produces bile acids to aid lipid digestion, while bile salts are secreted into the duodenum [138]. These
597 components comprise more than 50% of the organic composition of bile. Furthermore, bile acids act as digestive
598 surfactants, facilitating the emulsification of lipids for easier absorption [139]. Bile acids have also been shown
599 to possess antimicrobial properties against various bacterial species [140].

600 Conjugated bile salts, conjugated ionically with either taurine or glycine by hepatic enzymes, have been
601 observed to inhibit the growth of both Gram-negative and Gram-positive bacteria (e.g., *E. coli* and *Klebsiella* and
602 *Enterococcus* genera) [141]. However, Gram-positive bacteria tend to be more susceptible to the inhibitory effects
603 of bile salts, when compared to Gram-negative bacteria [142]. Some probiotic strains possess the capability to
604 produce bile salt hydrolase (BSH), which allows them to hydrolyze bile salts. This ability is believed to confer
605 resistance to the presence of conjugated bile salts and serves as a defense mechanism against their toxic effects.
606 The hydrolysis of glycine- and taurine-conjugated bile salts by BSH leads to the release of the corresponding
607 amino acids and deconjugated bile acids [143]. The BSH activity of probiotic bacteria has been considered a
608 significant criterion in the selection of potential probiotic strains due to its association with various mechanisms
609 that contribute to the reduction of plasma cholesterol levels in the host [144]. Deconjugated bile salts, being less
610 soluble than their conjugated counterparts, are more likely to be excreted in the feces. Consequently, the synthesis
611 of new bile salts from cholesterol in the liver is reduced, resulting in decreased plasma cholesterol levels.
612 Furthermore, deconjugated bile salts can precipitate with cholesterol, reducing its solubility and promoting its
613 excretion via feces. It should be noted, however, that while BSH activity contributes to cholesterol reduction,
614 excessive deconjugation of bile salts may have negative implications in the human intestine. This is because other
615 intestinal bacteria can convert primary bile acids into secondary bile acids, which have been shown to exhibit
616 mutagenic and tumor-promoting properties in animal models. Thus, excessive deconjugation of bile salts may
617 pose potential harm to the human host [145].

620 In a study by Song et al. [131], the bile salt tolerance of 10 *Lactobacillus* strains was assessed. The results
621 revealed that *Lactobacillus sp.* JNU 8829, *L. casei* MB3, *L. sakei* MA9, *L. sakei* CH8, and *L. acidophilus* M23

622 exhibited high tolerance to bile acid. The assessment was performed using MRS broth supplemented with 0.3%
623 oxgall and incubated for 24 hours at 37°C. In a study conducted by Bin Masalam et al. [146], it was found that
624 the most bile-tolerant strains were predominantly enterococci, including *E. faecium* ZiNb3, *E. faecium* Rashad3,
625 and *E. faecium* SMBM3. Other species such as *L. casei* BgShn3, *L. casei* Dwan5, *L. casei* MSJ1, *L. plantarum*
626 EyLan2, *L. acidophilus* Musallam2, *L. paracasei* NMBM1, *S. equinus* Salam7, *L. garvieae* Emad4, *L. garvieae*
627 ZSJ5, and *W. confusa* SYary1 also demonstrated bile tolerance. The assessment was performed using MRS broth
628 supplemented with 0.5% wt/vol bile and incubated for 4 hours.

629 BSH enzymes have been found in various bacterial genera, including *Enterococcus*, *Listeria*,
630 *Lactobacillus*, *Bifidobacterium*, *Clostridium*, and *Bacteroides* [147, 148]. Generally, most bifidobacteria and
631 lactobacilli strains in the GIT possess BSH enzymes [147]. It has been observed that *Lactobacillus* strains
632 originating from the gut can deconjugate both glyco- and tauro-conjugated bile acids, while *Lactobacillus* strains
633 isolated from fermented milk products exhibit a greater capability to deconjugate glyco-conjugated bile acids
634 [139]. BSH activity serves multiple purposes, including the utilization of liberated amino acids as nutrients
635 (carbon and nitrogen sources) and the reduction of bile salt toxicity. Additionally, it may play a role in
636 incorporating cholesterol into the cell wall [148]. Some lactobacilli strains have been reported to reduce
637 cholesterol levels through mechanisms such as cholesterol binding to their cells, which could potentially lead to
638 lower serum cholesterol levels *in vivo* [149].

639 In a study by Saravanan et al. [150], 10 strains isolated from traditional fermented foods belonging to the
640 genera *Leuconostoc* spp., *Weissella* spp., *Pediococcus* spp., *Lactococcus* spp., and *Bacillus* spp. All exhibited
641 strong BSH activity when tested on MRS agar containing 0.5% taurodeoxycholic acid (TDCA) sodium salt at
642 30°C for 72 hours. However, Kumari et al. [151], which tested 20 different lactobacilli strains isolated from
643 fermented foods and beverages, found that none of them exhibited BSH activity. Awasti et al. [152] evaluated
644 BSH activity in 12 bifidobacteria isolates obtained from human sources using MRS medium supplemented with
645 0.5% sodium salts of taurocholic acid (TCA), tauroglycocholic acid (TGCA), and TDCA at 37°C for 48 hours.
646 Out of the 12 isolates, 5 showed high levels of BSH activity, 8 exhibited moderate activity, while one strain
647 (NBIF-1) did not demonstrate BSH activity on TCA and TDCA.

648 These findings highlight the variability in BSH activity among different bacterial species and even within
649 strains of the same species, indicating the influence of strain-specific factors and environmental adaptation.

650

651 **3.4.3. Adherence to the host's intestinal wall**

652

653 The capacity to adhere to intestinal epithelial cells is critical for successfully colonizing probiotic strains
654 and is often regarded as a prerequisite for colonization. Consequently, it represents one of the primary criteria for
655 selecting a probiotic microorganism [153]. The colonization of the human intestinal microbiota begins at birth
656 and continues throughout life. However, the composition of the intestinal microbiota can change over the host's
657 lifetime, and probiotics have the potential to influence these changes. Although probiotics do not permanently
658 colonize the host's intestine, they can have a transient colonization effect [154]. The adhesion of probiotic strains
659 is an essential factor in their colonization ability, and this adhesion can be associated with changes in the intestinal
660 microbiota. For instance, the decline in bifidobacteria in elderly individuals may be attributed to reduced adhesion
661 of bifidobacteria to intestinal mucus, which correlates with age [155].

662 Several mechanisms have been proposed to explain bacterial adhesion to the intestinal mucosa. One
663 possible mechanism is hydrophobic interactions between the probiotic candidates and the intestine's surface [156].
664 The presence of mucin-binding proteins in the bacterial cell envelope enhances adhesion capability by binding to
665 the mucin proteins of the mucus layer on the gastrointestinal epithelia [157]. Pili, which are hair-like appendages,
666 can also contribute to bacterial attachment to the mucosal surface of the intestine. Certain bacteria, such as
667 bifidobacteria, can employ pili to facilitate adhesion [158]. Fibronectin-binding and surface-layer proteins are
668 additional surface proteins embedded in the bacterial cell wall that promote bacterial adherence to the intestinal
669 mucosa [159]. Producing extracellular polysaccharides by probiotic candidates has also been associated with
670 adhesion to the intestinal surface [160].

671 The *in vitro* adhesion test is commonly used to evaluate the ability of a probiotic candidate to attach to
672 human epithelial cell lines such as Caco-2, HT-29, and fetal I-407 [161]. This test examines the capability of the
673 probiotic candidate to attach to epithelial cell lines (notably to mucin) [161, 162]. However, the *in vitro* adhesion
674 test has several drawbacks. One primary concern is the reproducibility of the test. The conditions under which the
675 adherence of the probiotic candidate is assessed in the epithelial cell lines deviate from the natural conditions in
676 the host intestine. Important factors such as other microorganisms, digestive enzymes like pancreatin, and physical
677 contractions are absent in the *in vitro* test [161, 162].

678

679 **3.4.4. Production of antimicrobials and pathogens antagonists**

680

681 The evaluation of antimicrobial activity against pathogens is a crucial factor in the selection of potential
682 probiotic strains. While the production of antimicrobial compounds is a primary mechanism for this activity, there
683 are additional mechanisms involved. These include competition between probiotic and pathogenic strains for
684 nutrients, the attachment of probiotics to epithelial cells, and the stimulation of the immune system. These
685 combined mechanisms contribute to the overall antimicrobial efficacy exhibited by probiotic strains [163].

686 Antimicrobial substances produced by LAB can be classified into two main groups: non-bacteriocin
687 antimicrobial substances and bacteriocins [164]. The non-bacteriocin antimicrobial metabolites encompass a
688 variety of compounds such as organic acids (e.g., lactic acid, acetic acid), hydrogen peroxide, diacetyl,
689 acetaldehyde, acetoin, carbon dioxide, reuterin, reutericyclin, antifungal cyclic dipeptides, phenyllactic acid, 4-
690 hydroxyphenyllactic acid, and 3-hydroxy fatty acids. Among them, organic acids, particularly lactic and acetic
691 acids, are the most important and extensively studied. LAB play a significant role in modulating the intestinal
692 environment by producing organic acids, which leads to a decrease in pH and favors the colonization by beneficial
693 microorganisms, while reducing the population of pathogens [165]. Heterofermentative LAB species possess
694 flavoprotein oxidase enzymes that catalyze the reduction of oxygen, resulting in the production of hydrogen
695 peroxide. The antimicrobial activity of hydrogen peroxide is attributed to its oxidative impact on bacterial cells,
696 leading to the disruption of essential molecular structures of cell proteins [166].

697 The second group of antimicrobial compounds produced by LAB consists of bacteriocins, which are
698 peptides or proteins synthesized within the ribosomes of certain bacterial strains. Bacteriocins exhibit
699 antimicrobial activity against other bacteria, while the cells producing them are immune to their own bacteriocins
700 [167]. To be classified as bacteriocins, these antimicrobial peptides must meet the criteria of being modified or
701 unmodified peptide antimicrobials produced by bacteria, accompanied by a dedicated immunity system that
702 protects the producer cells [168]. The investigation of LAB for their bacteriocin-like inhibitory activity has gained
703 significant attention in recent studies [23].

704 Studies as the ones mentioned above regarding acid and bile tolerance, adhesion to intestinal cells, and
705 production of antimicrobial compounds are essential to determine the potential of a probiotics to survive and
706 compete with the native microbiota and pathogens/pathobionts.

708 3.4.5. Probiotics modulation of gene expression *in vitro*

709 *In vitro* studies are also useful to understand how probiotics modulates different cell types on the host.
710 Immortalized cells and fresh isolated cells have been widely used to evaluate modulation of the expression of key
711 genes related to barrier function, signaling, inflammation, differentiation, and chemoattraction of immune cells.
712 This helps to predict the effect that could be obtained in a complex organism. As most probiotics aims to treat
713 intestinal diseases, intestinal epithelial cells, such as Caco-2 and HT-29 (human colorectal adenocarcinoma), and
714 PBMCs (peripheral blood mononuclear cells), such as lymphocytes, monocytes, natural killer cells (NK cells) or
715 dendritic cells) are the most used cell lines in *in vitro* studies.

716 One of the well-recognized effects of probiotics is their ability to induce a shift from Th2 to Th1 cells,
717 leading to a reduction in allergic reactions. When human peripheral blood lymphocytes and PBMCs are exposed
718 to lactic acid bacteria (LAB), they exhibit an increase in the production of interferon-gamma (IFN-g) by T and
719 natural killer (NK) cells [169, 170]. These findings align with *in vitro* experiments that demonstrate lactobacilli
720 present in fermented foods strongly stimulate the production of pro-IFN-g cytokines like IL-12 and IL-18 by both
721 human and murine leukocytes [171, 172]. This capacity to steer the immune response towards a Th1 profile could
722 prove beneficial in conditions characterized by Th2-driven inflammation, including atopic disorders and other
723 Th2-associated inflammatory diseases.

724 Certain probiotic strains can exert varying effects on NK cells. For example, *L. rhamnosus* GG and *L.*
725 *reuteri* DSM 17938 hinder the activation of T cells and NK cells, as well as the release of IFN-gamma from
726 PBMCs stimulated with *Staphylococcus aureus* [173]. The intricate interplay among probiotics, dendritic cells
727 (DC), and NK cells emphasizes how distinct strains can uniquely shape the immune system and inflammatory
728 responses, potentially yielding advantageous outcomes by balancing NK and DC interactions [174]. An emerging
729 strain, *L. reuteri* LMG P-27481, discovered and studied by Sagheddu et al. [175], demonstrates a remarkable
730 ability to prompt significant secretion of IL-10 when exposed to immature human DCs. In comparison to other *L.*
731 *reuteri* strains, it manifests a more pronounced anti-inflammatory impact. *In vitro* co-culture experiments reveal
732 that *L. reuteri* LMG P-27481 effectively curbs the growth of *E. coli*, *Salmonella*, and Rotavirus, with the unique
733 ability to hinder *Clostridium difficile* growth. Notably, its genetic makeup enables it to metabolize lactose, proving
734 especially valuable in scenarios involving diarrhea [175].

735 Luerce et al. [176], through a colitis-recurrence model conducted on Caco-2 cells, demonstrated the
736 capability of *L. lactis* NCDO 2118 to reduce the secretion of IL-8 triggered by IL-1beta. Similarly, *B. animalis*
737 subsp. *lactis* and *L. acidophilus* have also exhibited the ability to decrease IL-8 production, suppress the
738 expressions of pro-inflammatory agents, and enhance TLR2 expression in an *in vitro* model. This anti-
739

740 inflammatory effect is achieved by influencing the TLR2-mediated NF- κ B and mitogen-activated protein kinase
741 (MAPK) signaling pathways within inflamed intestinal epithelial cells [177].

742 Certain Lactobacillus species have shown the ability to mitigate barrier disruptions by upregulating tight
743 junction (TJ) proteins. For instance, *L. acidophilus* and *L. plantarum* have been shown to increase the expression
744 of the occludin protein *in vivo* and *in vitro* models, respectively [178, 179]. Moreover, *L. plantarum* triggers the
745 relocalization of ZO-1 and occludin to the apical region of cells by stimulating Toll-like receptor 2 (TLR2) [180,
746 181]. It's important to note, however, that while coincubation of Caco-2 cells with *L. plantarum* leads to increased
747 transcription of genes related to the disassembly of tight junctions and occludin degradation, the elevated occludin
748 expression and apical localization might be a defensive response prompted by initial bacterial-induced degradation
749 of the TJ structures, rather than a means of maintaining them [178].

750 In certain cases, the realization of beneficial effects from probiotics hinges on the prior disruption of TJ
751 homeostasis. *E. coli* Nissle 1917 incubation of T84 human intestinal epithelial cells does not significantly alter
752 intestinal barrier function [182]. However, when T84 cells are co-incubated with enteropathogenic *E. coli* (EPEC)
753 causing barrier disruptions, supplementing with *E. coli* Nissle 1917 restores barrier integrity to levels similar to
754 those in control cells. Consequently, *E. coli* Nissle 1917's beneficial effects on the intestinal barrier might manifest
755 post-infection, possibly without requiring preemptive supplementation, which can be challenging from a clinical
756 standpoint. Additionally, alongside reduced barrier permeability, *E. coli* Nissle 1917 supplementation leads to
757 heightened expression of ZO-2 and its robust relocalization to the tight junctions [182]. While these initial findings
758 do not establish definitive causation, they suggest that *E. coli* Nissle 1917's positive impact on intestinal barrier
759 function might be influenced by the bacterium's ability to regulate ZO-2 expression and localization.

760 Mucins play a crucial role in maintaining the protective function of the intestinal barrier. Probiotic
761 bacteria exhibited diverse impacts on mucin gene and protein expression. Furthermore, the effects of probiotic
762 treatments on mucin gene expression varied. When Caco-2:HT29-MTX (90:10) co-cultures were incubated with
763 *L. rhamnosus* HN001, increased levels of all mucin mRNA were observed, with a significant increase in
764 MUC5AC mRNA compared to untreated samples [183]. This contrasted with a study by Mack et al. [184], where
765 *L. plantarum* 299v increased the expression of MUC2 and MUC3 genes in HT29 cells. This difference might arise
766 from the co-culture conditions in this study versus the monoculture of mostly undifferentiated HT29 cells in the
767 previous one.

768 Although important for the identifying mechanism of actions of probiotics and predict the effects in the
769 host, *in vitro* studies lack the complexity of a tissue, an organ, and an organism. To fulfil the gaps left from *in*
770 *vitro* studies and to continue the tests on probiotic effects, *in vivo* studies involving animal models and, later,
771 clinical trials in humans are widely used.

773 4. ANIMAL STUDIES AND CLINICAL TRIALS

774
775 The seek for proof of efficacy *in vivo* while exploring the potential of probiotics has resulted in the
776 development of various biological models, with varying levels of complexity. These models encompass a wide
777 range, from simple multicellular organisms, such as worms and invertebrates, to advanced knock-out (KO) models
778 in rodents, and even clinical trials involving different populations of humans [162]. Hence, although the ultimate
779 evaluation of probiotic functionality should ideally be conducted directly in the target population, such as the
780 general population or a specific subgroup with a particular condition [185], the initial selection of strains to be
781 included in these costly clinical trials may require the use of suitable *in vivo* models. Rodent models, especially
782 mice and rats, serve as cost-effective and publicly acceptable screening tools, but they still fall short of
783 representing human physiology. Therefore, it is necessary to develop more relevant experimental models for
784 evaluating probiotic functionality. These models should allow for the study of various dynamic states and address
785 specific diseases with multifactorial origins. It is important to note that the accuracy of results obtained from
786 animal models are not always the same as the results in humans and can sometimes present challenges. As recently
787 stated, there can be inflammatory findings and difficulties in extrapolating results from one species to another
788 [186, 187]. The use of animals to predict human response to drugs, chemicals, or foods (including probiotics)
789 remains a contentious issue. While some advocate for a ban on animal experimentation due to a perceived lack of
790 scientific evidence for human predictivity [188], the relevance of animal disease models, such as mice, for
791 studying human conditions, has been positively evaluated [189].

792 The effective utilization of rodent models for probiotic research will rely on rigorous standardization,
793 including the composition of the microbiota. It is crucial to always consider the relevance to the human situation
794 since many bacterial species that are commensal in humans can be pathogenic in mice, and vice versa [190, 191].
795 Despite these potential drawbacks, rats and mice will inevitably continue to be used as models to address
796 numerous research questions related to probiotics. This includes evaluating immune and metabolic
797 responsiveness, regulatory processes, neuro-endocrinological and nutritional aspects, all of which play important
798 roles in the complex relationships between the microbiota and the host. Additionally, small animals allow for the
799 mimicking of specific diseases with genetically modified specimens (conditional and tissue-specific knock-

800 in/knock-out mutants) or specific chemicals (e.g., TNBS to induce intestinal inflammation) and infectious
801 challenges. Manipulating the microbiota also enables the investigation of the role of specific microorganisms in
802 these models [162].

803 The regulations permit the use of animals for scientific purposes but require strict adherence to restrictive
804 conditions. To enhance the welfare of animals in research, the application of the 3Rs serves as an ethical guide.
805 3R means replacement (the use of alternative methods such as *in vitro*, *in silico*, *ex vivo*, and the use of less sentient
806 species, i.e., invertebrates), reduction (to utilize the least number possible of animals without interfering with the
807 statistics), and refinement (experimental protocols curation aiming to minimize stress and suffering on the
808 animals)- proposed originally by Russel and Burch in 1959 [192, 193]. The implementation of the 3Rs principle
809 contributes to enhancing the well-being of animals utilized in scientific research through various means. It tackles
810 multiple issues related to animal use in science, prioritizes the welfare of individual animals, incorporates new
811 knowledge and insights, strikes a balance between scientific requirements and animal welfare, and fosters
812 collaboration among diverse stakeholders concerned about the welfare of animals used in scientific studies [194].
813 Numerous Replacement technologies offer notable benefits, including enhanced consistency and accuracy, rapid
814 results, and lower costs, when compared to using animals, besides certain studies being simply impractical to
815 conduct in animal models due to limitations in throughput or the necessity of human-relevant tissues[192]. By
816 employing stringent criteria in experimental design to ensure reproducibility, Reduction also leads to improved
817 scientific outcomes, and diligently applying the principles of Refinement helps minimize stress as a scientific
818 variable, ensuring a more refined and reliable research environment [192].

819 However, despite the efforts to find alternative methods, the current state of knowledge does not yet
820 allow these methods to address all scientific questions in biology and medical research comprehensively.
821 Therefore, alternative methods often serve as complementary approaches to *in vivo* methods, rather than complete
822 replacements, as they may not fully substitute for the complexity of biological processes and physiological
823 interactions observed in living organisms.

824

825 4.1. Animal toxicity studies

826

827 When the history of use for a particular probiotic strain is unknown or insufficient, authoritative guidance
828 suggests conducting additional safety studies, including animal studies. However, there is limited specific
829 guidance on the design and conduct of animal toxicity studies for probiotics. Unlike chemicals, a standard non-
830 clinical toxicology testing paradigm may not be applicable to probiotics due to their unique nature, and it may
831 provide limited information [77, 195]. Therefore, specific requirements for probiotic testing should be carefully
832 evaluated on a case-by-case basis.

833 It may be reasonable to conduct tests in animal models for certain probiotic strains. For example, if there
834 is insufficient historical use data available for a particular strain or species, or in the case of “novel” or next-
835 generation probiotics, animal toxicity assessment may be necessary before proceeding to testing in humans. Even
836 when candidate probiotic strains are human commensal microbes, they are still considered “not-self” and cannot
837 be assumed to be harmless.

838 Rousseau et al. [196] provided an overview of *in vitro*, *ex vivo*, and *in vivo* non-clinical models that they
839 considered relevant for microbiome research on various products, including probiotics. While these models hold
840 promise for microbiome research purposes, most of them are not currently suitable for assessing standard
841 toxicology endpoints.

842 There are indeed notable differences between rodents and humans that should be considered when
843 evaluating potential probiotic strains. Factors such as differences in mucus growth rate and dietary patterns
844 between rodents (herbivores) and humans (omnivores) can impact the relevance of animal models to human
845 outcomes. In this respect, pigs constitute preclinical animal models with a higher similarity, when compared to
846 rodents, in terms of physiology, digestive and associated metabolic processes, nutritional requirements, and
847 intestinal microbiota [197]. It is important to acknowledge that no animal model can fully represent humans [58].

848 However, when toxicity studies are deemed necessary for probiotics, it is common to rely on standard
849 rodent models, such as rats or mice. Rodents have a long history of use in toxicology studies and provide a wealth
850 of data on toxicity findings that are specific to rodents and do not have a direct correlation to humans (lack clinical
851 relevance). This historical data can be informative in assessing the safety of probiotics [82].

852 Once an appropriate rodent model is chosen, the next question is related to the duration of dosing required
853 to support further safety testing in human clinical trials. Interestingly, a review of traditional oral repeated dose
854 animal toxicity studies with probiotics, as reported in the scientific literature, has not revealed any adverse effects
855 regardless of the duration of dosing. This includes acute (single dose), repeated dose (e.g., 14-28 days), sub-
856 chronic (e.g., 28-90 days), and even chronic (12+ months) studies conducted at high doses of the probiotic under
857 investigation [82, 198].

858 For example, studies have been conducted where rats were dosed with a proprietary preparation of
859 *Bacillus coagulans* for 90 days at high doses with no observed toxicity. Similarly, a 12-month study in rats with

860 *Clostridium butyricum* and a sub-chronic rodent study with a probiotic product containing various strains showed
861 no toxicity [199].

862 The duration of dosing in toxicity studies with probiotics is connected to the consideration of relevant
863 toxicity endpoints. When assessing the safety of live microorganisms, such as probiotics, specific endpoints
864 related to in vivo administration should be evaluated. One important endpoint is translocation, which refers to the
865 passage of live microbes from the gastrointestinal tract to other sites within the body. To assess translocation,
866 various organs such as lymph nodes, spleen, liver, bloodstream, or other tissues are collected at necropsy,
867 homogenized, and plated for enumeration of bacterial colonies [200]. Genetic methods can be used for confirming
868 the presence of specific bacterial strains. Translocation of microbes to other organs is a concern because it could
869 potentially lead to infection in the host, such as bacteremia or septicemia.

870 Therefore, when studying the translocation potential of probiotics, it is more relevant to use healthy
871 animals in the research [201]. Several in vivo studies have been performed to assess the translocation potential of
872 various probiotic strains in healthy mice or rats, typically lasting for four weeks. This timeframe is considered
873 sufficient to observe potential translocation and infectivity [202].

874 Shorter-term studies, such as repeated dose studies, may provide sufficient assurance of safety to proceed
875 with clinical studies in healthy humans. These initial human studies should be carefully designed to collect safety
876 endpoint data and ensure appropriate monitoring of potential adverse effects [199]. It is important to note that the
877 safety assessment of probiotics should be tailored to the characteristics of the strain and the intended use, and a
878 case-by-case approach is recommended.

879 Besides toxicity studies, animal models and clinical trials have been extensively used to identify the
880 effects of potential probiotics in disease models and healthy individuals. These effects attributed to probiotics
881 found on these studies are resumed in Figure 2 and are described in more details in the following sections.

882

883 4.2. Probiotics modulation of intestinal diseases *in vivo*

884

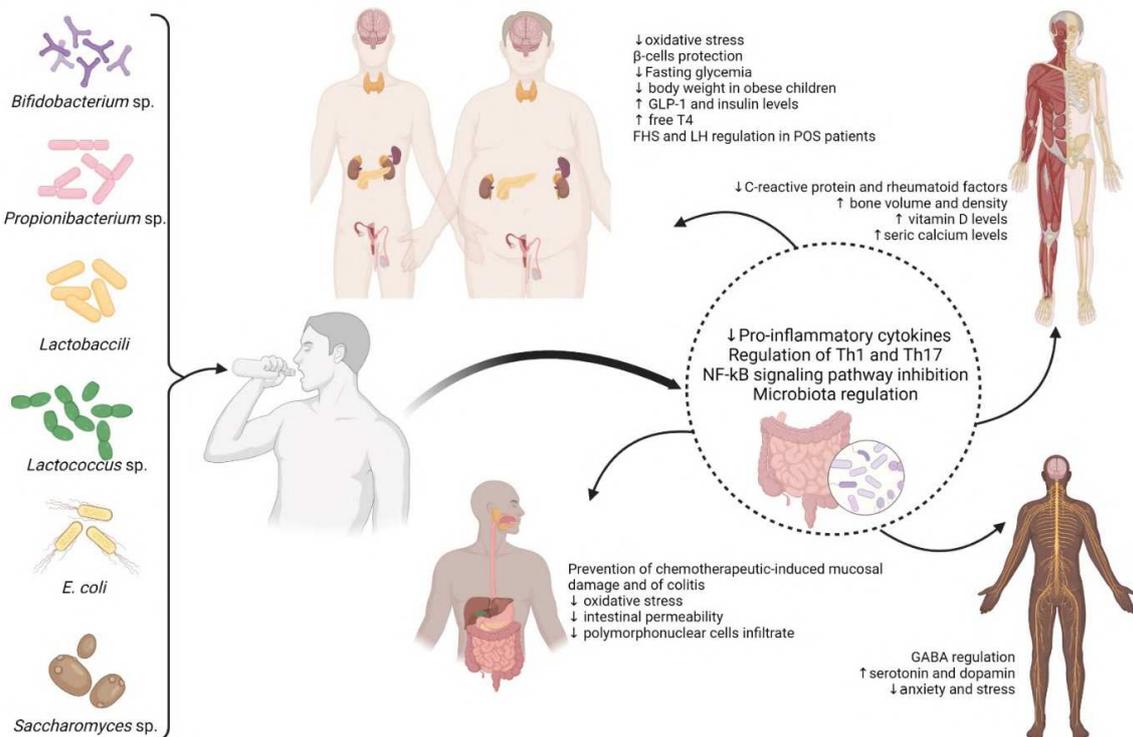
885 The commensal microbiota has constant interaction with the GIT, which under normal conditions,
886 maintains the mucosal immune homeostasis and promotes benefits to the host [203–205]. However, when
887 intestinal microbial balance is disturbed, the intestinal microbiota might have a role on the establishment and / or
888 development of chronic inflammatory diseases, such as inflammatory bowel diseases (IBDs) and intestinal
889 mucositis [206, 207].

890 The IBDs are described as multifactorial disorders that involve chronic inflammation of the GIT. IBS
891 include the ulcerative colitis (UC), which affects mainly the mucosa and submucosa of the colon, as well as
892 Crohn's disease (CD), that causes a multifocal transmural granulomatous inflammation along the lining of GIT
893 [208].

894 The UC mainly affects adults aged 30-40 years, with no sex predominance. The CD has a bimodal
895 distribution between ages 15 to 30 and 40 to 60 years old. The highest incidence and prevalence of UC are seen
896 in Northern Europe and North America, while CD is most seen in the western developed world in North America,
897 Northern Europe, and New Zealand [209–211]

898 The IBD associated disorders are commonly characterized by blood in the stool, diarrhea, tenesmus,
899 fatigue, fevers, increased frequency of bowel movements, abdominal pain, and weight loss. The diagnosis is based
900 on clinical information, physical examination, and endoscopic and histological investigations [212, 213]. The
901 main risk factors associated with these diseases' etiology are family history, genetic susceptibility, environmental
902 factors (e.g., cigarette smoking, stress), intestinal dysbiosis, and altered immune response [209–211].

903 A loss of tolerance towards enteric commensal bacteria and an exacerbated Toll-like or NOD-like
904 receptors' expression levels lead to an activated innate (macrophage, neutrophil) and acquired (T and B cell)
905 immune response in IBD patients. These immune cells increase the production of different cytokines and
906 chemokines involved in Th1, Th2, and Th17 responses, such as pro-inflammatory markers Tumor Necrosis
907 Factor-alpha (TNF- α), Interferon-gamma (IFN- γ), Interleukins (IL-1 β , IL-18, IL-6, IL-17, IL-23), Chemokine (c-
908 c motif) ligand (CCL2, CCL4, CCL5), chemokine (C-X-C ligand) motif (CXCL8, CXCL10) [214, 215]. All these
909 immunological factors are associated with CD and UC development. In this way, to suppress the inflammatory
910 responses, current treatments for IBDs have been done with corticosteroids, aminosalicylates, monoclonal
911 antibodies anti-TNF-a (e.g., infliximab and adalimumab), antibiotics, and surgery. Moreover, the choice of the
912 therapeutic approach is based on the extent and severity of the disease, inducing and maintaining clinical remission
913 [213].



914

915 **Fig 2** Main effects of probiotics in the inflammatory process of diverse systems

916

917

918 4.2.1. Probiotics as Alternative Therapeutic Approach for Treatment of IBD

918

919

920 Knowing that dysbiosis also plays a key role in the pathogenesis of IBDs, the modulation of the patient
921 microbiota, via the administration of probiotic bacteria, has been proposed as a promising therapeutic approach
922 for the treatment of these diseases due to selected probiotic strains anti-inflammatory effects on GIT and the
923 microbiota restoration/regulation [216]. A strain-dependent positive effect of probiotic consumption in IBD
924 treatment has been extensively proven in animal models and clinical trials, although most studies are still
925 conducted in animal models, especially colitis induced by either DSS (Dextran Sodium Sulfate) 2% and TNBS

926

927 (2,4,6-trinitrobenzene sulfonic acid) in rats and mice.
928 The positive effect of probiotic administration has been tested in clinical trials. The commercial probiotic
929 preparation VSL#3 (including *L. plantarum*, *L. delbrueckii*, *L. casei*, *L. acidophilus*, *B. longum*, *B. breve*, *B.*
930 *infantis*, and *S. salivarius*) was able to prevent CD recurrence after surgery by reducing mucosal inflammatory
931 cytokine levels (IL-8 and IL-1 β) and improving IBD questionnaire score [217]. In another study, this commercial
932 probiotic formulation could also induce remission in patients with UC [218]. Meta-analysis of randomized
933 controlled clinical trials further confirmed the strong evidence of VSL#3 efficacy in the context of IBD, with 8
934 conclusive clinical trials [219].

933

934 The beneficial effects of *L. Rhamnosus* GG administration was also reported, which improved the gut
935 barrier function and clinical status in children with mildly to moderately active Crohn's disease [220]. Promising
936 results were also found for the *L. delbrueckii* and *L. fermentum*, which consumption was associated with intestinal
937 inflammation reduction in patients with UC. This included a decrease in colonic concentration of IL-6, in
938 expression of TNF- α and NF- κ B p65, in leukocyte recruitment, and in colonic MPO (myeloperoxidase)
939 activity[221].

939

940 A review analyzed 18 studies on the effectiveness of probiotics, prebiotics, and symbiotics in inducing
941 or maintaining remission of UC in adults and children and concluded that probiotics appear to be beneficial in
942 achieving remission in patients with UC [222]. One of the reviewed studies demonstrated changes in the
943 composition of the intestinal microbiota, the more significant number of Bifidobacteria on the mucosal surface of
944 patients fed with a formula containing *B. longum* and the prebiotic Synergy1® (inulin and oligofructose) [222].

944

945 As for the studies conducted in animal models, pre-clinical trial showed that oral administration of
946 *Lactobacillus delbrueckii* subsp. *Lactis* CNRZ327 (2.5×10^{10} colony-forming units (CFU)/mL) [223] or of
947 *Propionibacterium freudenreichii* CIRM-BIA 129 (2×10^9 CFU/mL) [224] were able to attenuate DSS and TNBS

946

947 induced colitis in mice, respectively. These probiotic bacteria showed anti-inflammatory, as evidenced by a
948 reduction of oxidative stress markers (Cyclooxygenase 2 (Cox-2) and heme oxygenase (Hmox)), and neutrophil
949 inflammatory infiltrate (MPO assay) [224]. These probiotics both modulated the balance between Th1, Th2, Th17
950 and Treg cells[223] and prevented epithelial architecture damage [223, 224]. The same results were observed in
951 rats for *Propionibacterium freudenreichii* KCTC 1063 strain, according to Ma et al.[225]. They administrated this
952 probiotic strain (10^8 CFU/rat/day) for 22 days, and it showed an improvement of DSS (5%)-induced colitis (last
953 8 days) in rats by stimulating MUC2 protein expression and down-regulating the pro-inflammatory cytokines
954 TNF- α , IL-6, and IL-1 β [225].

955 Jang and colleagues [226] showed that administration of *Bifidobacterium longum* LC67 (2×10^9
956 CFU/mL) reduced the severity of TNBS-induced colitis in mice by preventing myeloperoxidase activity,
957 inhibiting NF- κ B activation, restoring Th17/Treg balance and gut microbiota composition by restoring the
958 Proteobacteria to Bacteroidetes ratio.

959 Finally, the above-mentioned VSL#3 mix of 8 probiotic strains, which was effective against IBD in
960 clinical trials, also afforded protection against DSS-induced colitis and TNBS-induced colitis in rats and mice.
961 Dai et al., [227] shows that VSL#3, in rats, fosters anti-inflammatory characteristics through a lowered disease
962 activity index and reduced MPO activity. Furthermore, the administration of VSL#3 over a span of 7 days led to
963 a decline in iNOS, COX-2, NF- κ B, TNF- α , IL-6, and p-Akt levels, accompanied by an elevation in IL-10
964 expression within the colonic tissue. Simultaneously, this resulted in reduced TNF- α and IL-6 levels, while IL-10
965 serum levels exhibited an increase. As for TNBS induced colitis, VSL#3 treatment prevented weight loss and
966 mitigated colon shortening. Additionally, VSL#3 treatment demonstrated the ability to reduce damage to intestinal
967 epithelial cells and the infiltration of inflammatory cells within the lamina propria and submucosa. Moreover,
968 VSL#3 restored the levels of HMGB1, a proinflammatory mediator crucial in experimental colitis, as well as
969 F4/80+ levels, a pan-marker indicating macrophages within the lamina propria mononuclear cells of mice with
970 TNBS-induced colitis. The notable reduction in the expression of tight junction proteins ZO-1, occludin, and
971 claudin-1 in TNBS-exposed mice, caused by TNBS administration, was ameliorated through VSL#3 treatment
972 [228].

973 The aforementioned results highlight the potential effects of certain probiotic strains on IBD, opening
974 pathways for their use as therapeutics for humans in the future.

975 4.2.2. Probiotics as Alternative Therapeutic Approach for Treatment of intestinal mucositis

976 Intestinal mucositis is a cytotoxic effect resulting from non-selective antineoplastic drugs (e.g., 5-
977 Fluorouracil, Oxaliplatin, Methotrexate, and Irinotecan) that, besides destroying neoplastic cells, promote damage
978 to healthy cells [229]. This inflammatory disorder is mainly characterized by inflammation-related signaling
979 pathways. These include NF- κ B and mitogen-activated protein kinase (MAPKs) activation, and consequently,
980 pro-inflammatory cytokines and chemokines production (e.g., TNF- α , IL-6, IL-22, CXCL1, CXCL5), loss of the
981 intestinal epithelial barrier, damage to the crypts and villus shortening, mucus-producing goblet cells reduction,
982 inflammatory cells infiltration in the lamina propria (e.g., macrophages, neutrophils and eosinophils), microbiota
983 composition alteration, tight junctions disruption, increasing intestinal permeability, and predisposition to
984 infections by pathogenic microorganisms[230, 231].

985 This is a relevant gastrointestinal inflammatory condition in humans, a severe clinical issue, and
986 alteration of the intestinal microbiota has a relevant role in the progression of this inflammatory condition[207].
987 The modulation of the digestive microbiota through oral administration of probiotic bacteria has thus been
988 proposed as a therapeutic alternative for the intestinal mucositis treatment. In this context, several studies have
989 indeed demonstrated the strain-dependent beneficial effects of probiotic bacteria on the prevention of
990 chemotherapy-induced intestinal mucositis [232–234]

991 *Bifidobacterium infantis* (10^9 CFU/day) ameliorated the mucosal damage induced in a synergic colorectal
992 cancer model, in rats, with 5-Fluorouracil (5-FU) (75 mg/kg/3 days) and oxaliplatin (8 mg/kg/3 days). It improved
993 intestinal mucositis by increasing the CD4+, CD25+, Foxp3+, Tregs cells and decreasing Th1 and Th17 cells
994 response [235]. Positive effects were also reported for *B. bifidum* G9-1 (10^7 - 10^9 CFU), which reduced the
995 intestinal damage induced by 5-FU (50 mg/kg/9 days) in mice, via regulation of the intestinal microbiota
996 (increasing Bacteroidetes and decreasing Firmicutes abundance), and reduction of inflammatory infiltrate and pro-
997 inflammatory IL-1 β and TNF- α cytokines levels [236].

1000 Promising results were also reported using different *Lactobacillus* strains. Epithelial damage induced by
1001 5-FU (450 mg/kg) in mice was attenuated by the consumption of *L. acidophilus*, which improved the intestinal
1002 mucositis via inhibition of the NF- κ B signaling pathway and reduced the levels of pro-inflammatory cytokines
1003 and chemokines, such as TNF- α , IL-1 β , and C-X-C motif chemokine ligand 1 (CXCL-1) [237]. *L. casei*, triggered
1004 similar protective effect against intestinal damage caused by chemotherapy association with 5-FU (30 mg/Kg),
1005 Leucovorin (10mg/kg) and Oxaliplatin (1mg/Kg) per 5 days-induced intestinal mucositis in a syngeneic colorectal
1006 cancer model. This was related to the downregulation of pro-inflammatory cytokines (IL-6, TNF- α , IL-1 β , IFN-

1007 γ) due to NF- κ B inhibition and microbiota regulation (decreasing Firmicutes and increasing Bacteroidetes
1008 abundance) [238]

1009 Probiotic formulations containing different bacteria strains and fermented products also have
1010 demonstrated effectiveness in mitigating intestinal damages induced by 5-FU chemotherapy. A probiotic
1011 formulation containing *L. acidophilus*, *L. paracasei*, *L. rhamnosus*, and *B. lactis* prevented epithelial damage in
1012 mice induced by 5-FU (450 mg/kg), and it increased the villus/crypt ratio, glutathione (GSH) levels, reduced
1013 neutrophils infiltrate, and malondialdehyde (MDA) and pro-inflammatory cytokines (TNF- α and IL-6) levels in
1014 the duodenum, jejunum, and ileum [239]. Another study also showed that administration of the commercial DM#1
1015 (*L. acidophilus* DM8302, *L. casei* DM8121, *B. breve* DM8310, and *S. thermophilus* DM8309) ameliorated the
1016 intestinal damage of mice treated with 5-FU (30 mg/kg/5 days) by decreasing intestinal permeability, neutrophil
1017 infiltration, and pro-inflammatory cytokines (TNF- α , IL-4, IL-6) [240].

1018 Fermented milk formulations composed of *P. freudenreichii* CIRM-BIA138 and/or *L. paracasei* BL23
1019 supplemented with whey protein (30%) [233], and *L. delbrueckii* CIDCA 133 (7.5×10^7 CFU) [241] were able to
1020 prevent the intestinal mucosa damage induced by 5-FU (300 mg/kg). These studies observed the prevention of
1021 villus shortening and goblet cell degeneration and polymorphonuclear cells infiltration reduction [233, 241].

1022 Probiotic yeasts can also be highlighted in intestinal mucositis treatment. Post-treatment with
1023 *Saccharomyces cerevisiae* UFMG A-905 (10^9 CFU/mL) was able to protect mice against irinotecan (75 mg/kg)-
1024 induced intestinal mucositis, reducing the weight loss, villus shortening, intestinal permeability, oxidative stress,
1025 and prevented goblet cells degeneration [242]. Promising results were also reported for *Saccharomyces boulardii*
1026 (10^9 CFU/kg/3 days), which prevented 5-FU (450 mg/kg)-induced intestinal mucositis, controlling TLR2, TLR4,
1027 MyD88, reducing NF- κ B, ERK1/2, phospho-p38, phospho-JNK, and pro-inflammatory markers TNF- α , IL-1 β ,
1028 and CXCL-1 [243].

1029 Therefore, all these findings show that oral administration of probiotic microorganisms modulates the
1030 inflammatory response in chemotherapeutic agents-induced mucositis, revealing a promising therapeutic strategy
1031 in treating this intestinal inflammatory disorder.

1032

1033 4.2.3. Intestinal dysbiosis resolution and microbiota modulation by probiotics

1034

1035 The human intestinal microbiota is known to fulfil diverse functions in the host, including intestinal
1036 development, homeostasis, and protection against pathogenic bacteria. Moreover, various studies have indicated
1037 that the intestinal microbiota dysbiosis contributes to the development of metabolic diseases, including obesity,
1038 diabetes, and intestinal diseases such as antibiotic-associated diarrhea (AAD), IBDs, and colorectal cancer (CRC)
1039 [12, 244–246]. Modulating the microbiota by probiotics supplementation seems a promising way to treat and
1040 prevent diverse conditions.

1041 In dysbiosis, probiotics can restore intestinal biodiversity, bringing it back to normal [247]. The
1042 overgrowth of the Proteobacteria and/or reduction of Bacteroidetes are observed conditions of dysbiosis related
1043 to aberrant diet habitats. Abnormal increase in Proteobacteria levels can lead to energetic imbalance between
1044 different bacteria species and growing suppression of other bacteria species. The proliferation of some species of
1045 Proteobacteria also may cause illness development [248]. In celiac disease, studies suggested that patients with
1046 gastrointestinal symptoms present different microbiota compositions, higher abundance of Proteobacteria phylum,
1047 and lower abundance of Bacteroidetes and Firmicutes compared to control groups [245, 249]. These microbiota
1048 alterations may have pathogenic implications, promoting persistent gastrointestinal symptoms [249].

1049 The mechanisms by which probiotics alter the intestinal microbiota are diverse. They may stimulate
1050 mucin production by intestinal goblet cells, induce antimicrobial peptides production, improve stability of cell
1051 junctions, increase the release of IgA by activated B cells, and inhibit the growth of pathogens or promote their
1052 elimination through the production of antimicrobial molecules, such as short-chain fatty acids, bacteriocins, and
1053 microcin [250]. These mechanisms maintain homeostasis of the microbiota, prevent adhesion and proliferation of
1054 potentially pathogenic microorganisms, and promote health in the host.

1055 He et al., [251] performed a clinical trial with 276 previously untreated patients infected with
1056 *Helicobacter pylori*, which were divided into two groups through random assignment. One group (n=140)
1057 received a 14-day esomeprazole, bismuth, amoxicillin, and furazolidone, supplemented with probiotics
1058 (*Bifidobacterium* tetravaccine tablets), while the other group (n=136) received a placebo for 28 days. The
1059 occurrence of gastrointestinal adverse events was notably lower in the probiotics group compared to the placebo
1060 group (23.6% vs 37.7%, p=0.016). Immediate eradication of *H. pylori* led to considerable disruptions in the gut
1061 microbiota, with Proteobacteria replacing commensal Firmicutes and Bacteroidetes. However, this alteration
1062 gradually normalized after two weeks. The reduction of gut Bacteroidetes induced by eradication drugs was
1063 counteracted by the addition of probiotics. The gastric microbiota fully restored itself as *H. pylori* decreased and
1064 other taxa flourished. Importantly, individuals treated with probiotics exhibited more stable fluctuations in gastric
1065 microbiota compared to those who received a placebo.

1066 On another trial [252], fifty patients receiving hemodialysis were enrolled and randomized, receiving
1067 either probiotics (2.2×10^9 CFU *B. longum* NQ1501, 0.53×10^9 CFU *L. acidophilus* YIT2004, and 1.1×10^9 CFU
1068 *E. faecalis* YIT0072) or placebo for 6 months. In comparison to the placebo group, the administration of probiotics
1069 did not result in a significant alteration of species diversity within the fecal microbiome. However, probiotics did
1070 play a role in restoring the community composition, and this effect was particularly notable in non-diabetic
1071 hemodialysis patients ($P = 0.007$ as indicated by Adonis analysis). Specifically, based on the findings from linear
1072 discriminate analysis effect size, the introduction of probiotics led to an increase in the proportions of the
1073 Bacteroidaceae and Enterococcaceae families, while reducing the presence of Ruminococcaceae,
1074 Halomonadaceae, Peptostreptococcaceae, Clostridiales Family XIII. Incertae Sedis, and Erysipelotrichaceae
1075 families in non-diabetic hemodialysis patients.

1076 *B. longum* BB536 and *L. rhamnosus* HN001, in combination with vitamin B6, were administered to a
1077 group of 23 lactose intolerant individuals who continued to experience symptoms despite adhering to a lactose-
1078 free diet [253]. This administration took place over a period of 30 days. In comparison to the placebo, the intake
1079 of probiotics and vitamin B6 resulted in a significant reduction in bloating ($p = 0.028$) and an improvement in
1080 constipation ($p = 0.045$). The composition of the fecal microbiome varied between the treatment group and the
1081 placebo. The treatment led to the enrichment of several genera associated with lactose digestion, including
1082 Bifidobacterium. Additionally, there were changes in the relative abundance of certain compounds, such as an
1083 increase in acetic acid, 2-methyl-propanoic acid, nonenal, and indolizine 3-methyl, and a decrease in phenol.

1084 These findings underscore the importance of specific probiotics, and sometimes adjuvants, in alleviating
1085 symptoms and addressing gut dysbiosis in individuals with dysbiosis and persistent functional gastrointestinal
1086 symptoms.

1087

1088 4.3. Probiotics in metabolic diseases

1089

1090 Metabolic syndrome refers to a set of metabolic disorders characterized by dyslipidemia, hyperglycemia,
1091 insulin resistance, oxidative stress, inflammation, hypertension, and neurodegeneration. These disorders are
1092 associated with various metabolic diseases like obesity, diabetes mellitus, non-alcoholic fatty liver disease
1093 (NAFLD), and osteoarthritis [254]. It is common for multiple metabolic diseases to coexist, where obesity
1094 increases the risk of type 2 diabetes mellitus, and excessive body weight contributes to the development of
1095 NAFLD [255]. Recent studies have highlighted the significant role of imbalanced gut microbiota in metabolic
1096 diseases [256]. Therefore, modulating the gut microbiota has emerged as a promising approach to address this
1097 situation [257].

1098 The approach to maintaining a healthy balance of gut microbiota is by using probiotics. Another approach
1099 involves the use of prebiotics, non-digestible food ingredients that selectively stimulate the growth and activity of
1100 specific bacteria in the digestive tract [258]. Experimental studies and clinical trials have shown promising effects
1101 of probiotics in alleviating conditions such as obesity, type 2 diabetes mellitus, and other metabolic diseases in
1102 many cases [259].

1103 Consumption of probiotic kefir was reported to improve serum apolipoprotein A1 in metabolic syndrome
1104 patients [260]. Consumption of *L. plantarum* [261], *L. acidophilus* and some Bifidobacterium species (*B. bifidum*,
1105 *B. lactis*, and *B. longum*) [262] led to a reduction in blood sugar and cholesterol. More precisely, consumption of
1106 *L. Plantarum* during 90 days led to reduction in LDL cholesterol, blood glucose, and homocysteine levels in
1107 postmenopausal women [263].

1108

1109 4.3.1. Diabetes Mellitus

1110

1111 Probiotics can be a strategy capable of attenuating hyperglycemia, improving the function of pancreatic
1112 β cells [264], insulin secretion [265], insulin resistance [266], regulating lipid and lipoprotein metabolism [267],
1113 modulating oxidative stress and inflammatory processes, improving the body weight [268], and preventing micro
1114 and macrovascular complications [269]. Experimental studies suggest a possible beneficial effect of the use of
1115 probiotics in the prevention and treatment of diabetes mellitus (DM), a metabolic syndrome of multiple origin,
1116 resulting from the lack of insulin and/or the inability of insulin to adequately exert its effects, characterizing
1117 permanently high blood sugar levels (hyperglycemia), by modulation of the intestinal microbiota and immune
1118 response [270, 271].

1119 *L. acidophilus* and *L. casei* reduced oxidative stress and exhibited an anti-diabetic effect in animal models
1120 of diabetes [272, 273]. *Lactobacillus* is the bacterial genus most used in DM studies because it can improve
1121 hyperglycemia in the short and long term, reducing fasting and post-meal plasma glucose, glycated hemoglobin,
1122 serum insulin concentration, and insulin resistance [274].

1123 Matsuzaki et al. [275] reported that the ingestion of *L. casei* by alloxan-treated Balb/c mice inhibited the
1124 disappearance of pancreatic β -cells. In another study, researchers observed that autoimmune destruction of
1125 pancreatic β -cells was also inhibited by the oral administration of *L. casei* in non-obese diabetic mice. The

1126 administration of *Lactobacillus* GG significantly delayed elevated glucose intolerance and hyperglycemia during
1127 the development of streptozotocin-induced diabetes in rats [276].

1128 Oral administration of dahi, (a fermented milk product from India, containing *L. acidophilus* and *L. casei*)
1129 delayed the progression of streptozotocin-induced diabetes in rats. The results suggested that the supplementation
1130 of probiotic cultures increased effectiveness in suppressing chemically induced diabetes through insulin depletion.
1131 In addition, the product was able to prevent diabetic dyslipidemia, inhibiting lipid peroxidation and nitrite
1132 formation [272].

1133 Supplementation with *L. acidophilus* NCFM was evaluated in healthy or insulin-sensitive individuals.
1134 According to Andreasen et al. [277], after treatment, insulin sensitivity was preserved in the probiotic group,
1135 decreased in the placebo group, and the inflammatory markers and systemic inflammatory response (TNF, IL-6,
1136 IL1ra, and C-reactive protein) were not affected in either group.

1137 Probiotics, over 13 randomized clinical trials involving 840 subjects with type 2 DM improved glucose
1138 metabolism with a potentially more significant effect when the duration of intervention was higher than 8 weeks
1139 in a meta-analysis study and patients with type 2 diabetes mellitus [278]. These studies performed administration
1140 of (1) yogurt containing *L. acidophilus* La5, *B. lactis* Bb12 for 6 weeks, (2) capsule with *L. acidophilus*, *L. casei*,
1141 *L. rhamnosus*, *L. bulgaricus*, *B. breve*, *B. longum*, *S. thermophilus* for 8 weeks, (3) capsule with *L. acidophilus*,
1142 *L. bulgaricus*, *L. bifidum*, *L. casei* for 6 weeks, (4) Synbiotic food containing *L. sporogenes* and inulin for 6 weeks,
1143 (5) Synbiotic food containing *L. sporogenes*, inulin, and beta-carotene for 6 weeks, (6) Synbiotic bread with *L.*
1144 *sporogenes* and inulin for 8 weeks, (7) Probiotic soy milk with *L. plantarum* A7 for 8 weeks, (8) Capsule containing
1145 *L. acidophilus*, *L. casei*, *B. bifidum* for 12 weeks, (9) Fermented milk with *L. acidophilus* La5, *B. animalis* subsp.
1146 *lactis* Bb12 for 6 weeks, (10) Probiotic capsule containing *L. acidophilus*, *B. bifidum*, *L. reuteri*, *L. fermentum*, for
1147 12 weeks, (11) Probiotic honey with *B. coagulans* T4 for 12 weeks, (12) Probiotic capsule with *L. acidophilus*, *B.*
1148 *bifidum*, *L. casei*, *L. fermentum* for 12 weeks, and (13) Probiotic capsule with *L. acidophilus*, *B. bifidum*, *L. casei*,
1149 *L. fermentum* for 12 weeks.

1150 *Lactobacillus gasseri* BNR17 from human breast milk significantly reduced fasting and postprandial
1151 glycemia and HbA1c (glycated hemoglobin) in a murine model of type 2 DM [279]. *L. rhamnosus* CCFM0528
1152 and *L. casei* CCFM 0412 also reduced fasting glucose, postprandial glycemia and HbA1c, and increased serum
1153 insulin levels and hepatic glycogen after 13 weeks of intervention in diabetic rats, showing an improvement in
1154 glucose tolerance [280, 281]. Another study with diabetic rats using soy milk fermented with *L. rhamnosus* CRL
1155 981 demonstrated a significant reduction in fasting glycemia [282].

1156 The use of shubat (also known as chal), a Turkic beverage of fermented camel milk, sparkling white with
1157 a sour flavor, and a mixture of LAB strains (*L. plantarum*, *L. helveticus*, *L. harbinensis*, *L. hilgardii*, *L. rhamnosus*,
1158 *L. mucosae*, *L. par*, *L. paracasei* subsp. *tolerans*, *L. pentosus*, and *Lactococcus lactis*) and yeasts (*Kluyveromyce*
1159 *smarxianus*, *Pichia membranifaciens*, *Candida ethanolica*, and *Issatchenkia orientalis*), promoted a reduction of
1160 fasting blood glucose and HbA1c and increased in serum levels of C-peptide and GLP -1[283].

1161 In studies with gestational DM, probiotics led to significant reductions in fasting glucose and insulin
1162 resistance and a tendency to increase insulin receptors sensitivity, as reported before [284].

1163 4.3.2. Obesity

1164 There is evidence supporting the connection between gut bacteria and obesity in infancy and adults where
1165 alterations in bacterial strains colonizing the human intestines have been suggested to potentially contribute to the
1166 development of obesity [285]. The etiology of obesity includes several genetic, metabolic, inflammatory
1167 mechanisms, and dysbiosis [286, 287]. Microbial changes in the human gut can be considered a factor involved
1168 in obesity development in humans [288]. The modulation of the bacterial strains in the digestive tract may help to
1169 reshape the metabolic profile in the obese human host [289].

1170 *Lactobacillus* species (for example, *L. casei* strain Shirota (LAB13), *L. gasseri*, *L. rhamnosus*, and *L.*
1171 *plantarum*) and *Bifidobacterium* species (for example, *B. infantis*, *B. longum*, and *B. breve* B3) were used with
1172 success in well-established animal models of obesity due to their safety (i.e., low levels of pathogenicity traits and
1173 of antibiotic resistance genes) [290]. These treatments led to decreased weight gain and fat accumulation, when
1174 compared to the placebo group [291]. However, experimental studies differ in the duration of treatment (ranging
1175 from 4 weeks to 6 months) and administration of the daily dose of probiotics, which lead to greater or lesser effects
1176 on body weight or fat mass [292].

1177 On the other hand, some studies using different species of *Lactobacillus* and *Bifidobacterium* have failed
1178 to demonstrate the beneficial effects of therapy with probiotics in obese animals. Different strains of *L. plantarum*,
1179 *L. acidophilus* NDC13, *L. gasseri* SBT2025, *L. casei* shirota 4,159,029, and *L. coryniformis* CECT57 showed
1180 no significant effect on the weight of obese rats [292]. Bubnov et al. [293] reported that a combination of *B.*
1181 *animalis* VKB and *B. animalis* VKL did not have significant anti-obesity effects, although both probiotics
1182 administered alone reduced body weight in female BALB/C mice fed with high-fat diet. These studies suggest
1183 potential interactions between food ingredients and certain probiotic strains [290].

1186 Looking at the role of probiotics on obesity in pregnant women, it was found that one month before
1187 delivery and child up to 6 months old were supplemented with *L. rhamnosus* GG demonstrated less weight gain
1188 up to 4 years old [291]. Regarding the administration to subjects from different age groups, the supplementation
1189 with *L. salivialis* Is-33 or with VSL#3® in obese adolescents were not able to reduce body weight, waist
1190 circumference, and visceral fat. However, VSL#3® showed a beneficial effect on body mass index (BMI), liver
1191 fat index, insulin resistance, and GLP-1 levels in obese children treated with probiotics [294, 295]. In addition,
1192 Sanchis-Chordà et al. [296] showed that body weight was significantly reduced after *Bifidobacterium*
1193 *pseudocatenulatum* CECT 7765 administration in obese children with insulin resistance.

1194 Studies have shown that *L. curvatus* HY7601 and *L. plantarum* KY1032 [297], *L. acidophilus* LA-14, *L.*
1195 *casei* LC-11, *L. lactis* LL-23, *B. bifidum* BB-06, and *B. lactis* BL-4 [298], and *Pediococcus pentosaceus* LP28
1196 [299] strains led to a significant reduction in body weight, BMI, waist circumference, and fat mass in overweight
1197 human subjects. The administration of *L. rhamnosus* CGMCC1.3724 and a restricted-calorie diet caused
1198 significantly more significant weight loss in obese women than men [300]. The same results were not observed
1199 in a study with only obese women using supplementation of different doses of Ecologic® (*B. bifidum* W23, *B.*
1200 *lactis* W51, *B. lactis* W52, *L. acidophilus* W37, *L. brevis* W63, *L. casei* W56, *L. salivarius* W24, *L. lactis* W19,
1201 and *L. lactis* W58) [301].

1202 These results have shown commonly used probiotics as adjuvant on the treatment of diabetes and on
1203 weight loss processes beside their already known effects on inflammation and immunomodulation. It highlights
1204 the importance of testing the effects of potential probiotics in different disease models.
1205

1206 4.4. Probiotics and inflammation of the bone-muscular system

1207

1208 It is well known that the crosstalk between intestinal microbiota and host cells plays a critical role in
1209 regulating many critical biological processes. However, the link between allying intestinal microbiota and bone
1210 health is still being elucidated. Current research suggests a complex relationship that demands further
1211 investigation to establish the exact mechanisms by which these microorganisms may modulate bone health [302].
1212 In this context, the term “Osteomicrobiology” was introduced by [303] to refer to the research field on the role of
1213 microbiota in bone health and disease.

1214 It has been shown that the imbalance in the communities of intestinal microorganisms (dysbiosis) directly
1215 contributes to the development of several bone-inflammatory diseases and bone loss in general [304]. Sjogren et
1216 al. [305] showed that female germ-free mice presented an increase in bone mass associated with a low number of
1217 osteoclasts in the trabecular bone. When colonizing Germ-Free females with intestinal microbiota of healthy
1218 animals, normalization in bone mass parameters, osteoclasts, and bone marrow immune status was observed,
1219 revealing the physiological importance of intestinal microbiota to the maintenance of bone mass [305]. Thus,
1220 treatment with probiotic microorganisms can beneficially modulate the microbiota to improve general bone health,
1221 which several studies have corroborated.

1222 The prominent bone inflammatory condition is osteoporosis, whose risk factors are highly associated
1223 with women's aging process and menopause [306]. This disease affects more than 200 million people worldwide,
1224 and it is characterized by a reduction in bone mass, resulting in deterioration of bone microarchitecture due to a
1225 combination of causes: decreased absorption of calcium by the intestine, inactivation of vitamin D, osteoblasts
1226 lifetime and sex hormones [307] Such factors resulted in an increase in bone fragility and a higher occurrence of
1227 fractures, which is a global concern due to the increasing aging population [307]. Current therapies for the
1228 prevention and treatment of osteoporosis comprise calcium and D vitamin supplementation. For high-risk patients,
1229 however, antiresorptive drugs are more often prescribed. Nevertheless, it has side effects, including
1230 gastrointestinal irritation, osteonecrosis of the jaw, and atypical subtrochanteric femoral fractures [308]. In this
1231 context, many studies revealed the role of probiotics as novel therapies in preventing and controlling
1232 postmenopausal osteoporosis models like the administration of *L. plantarum* A41 and *L. fermentum* SRK414 in
1233 ovariectomized rats [309], soymilk-honey fermented with *L. casei* subsp. *casei* R-68 and soymilk-honey
1234 fermented with *L. plantarum* 1 R 1.3.2 administered in menopausal women [310], *L. paracasei* DSM 13434, *L.*
1235 *plantarum* DSM 15312 and *L. plantarum* DSM 15313 in postmenopausal women [311], *B. subtilis* C-3102 in
1236 postmenopausal women [312], *L. reuteri* 6475 in postmenopausal women [313], and GeriLact® (*L. casei*, *B.*
1237 *longum*, *L. acidophilus*, *L. rhamnosus*, *L. bulgaricus*, *B. breve*, and *S. thermophilus*) in postmenopausal women
1238 [314].

1239 Probiotic *B. clausii* (Enterogermina®) was consumed orally as suspension of 200 µL (10⁹ CFU/mL
1240 daily) in drinking water for six weeks in female BALB/c mice after ovariectomy (which simulates postmenopausal
1241 osteoporosis conditions). In this study, treated animals showed a lower rate of bone resorption, increased bone
1242 volume, trabecular density, and bone mineral density, and reduced pro-inflammatory cytokines, proving *B. clausii*
1243 as an excellent therapeutic candidate [315]. Another report showed that *L. reuteri*, *L. casei*, and *B. coagulans*
1244 significantly increased serum vitamin D concentrations in ovariectomized rats [316]. Additionally, *L. acidophilus*,
1245 *L. casei*, and *Bifidobacterium* sp. significantly increased serum calcium compared to non-treated groups. *L.*

1246 *acidophilus* and *L. casei* indicated the most beneficial effects on bone mineral density (BMD). Regarding bone
1247 marrow concentration (BMC) and bone area, *L. acidophilus*, *L. reuteri*, and *L. casei* showed the most significant
1248 enhancement [317]. A recent meta-analysis study with 497 postmenopausal women showed that daily
1249 supplementation with probiotics for 24 weeks to 12 months was associated with a decrease in bone turnover marks
1250 (BTM) compared to the placebo group. BMD loss at the lumbar spine was significantly lower in the probiotic
1251 group, while hips did not have a significant BDM difference [318].

1252 Another clinically meaningful bone inflammatory condition is rheumatoid arthritis, a chronic
1253 autoimmune disease that damages bones and cartilage, leading to severe joint pain, disability, and premature death
1254 if not adequately treated [319]. The current therapies are focused on non-steroidal anti-inflammatory drugs,
1255 glucocorticoids, and disease-modifying antirheumatic drugs (methotrexate). However, even with improvement in
1256 arthritis treatment, the frequency and degree of responses are restricted, and some patients do not reach the
1257 treatment targets as clinical remission [320]. Due to these challenges, probiotic therapies are also pointed out as
1258 possible adjuvant or alternative therapies [321].

1259 *L. casei* ATCC 334 was reported to promote an anti-inflammatory effect on collagen-induced arthritis
1260 (CIA) in female Wistar rats due to COX-2 and NF- κ B downregulation [322]. Likewise, it was found to inhibit the
1261 increase of inflammatory markers like erythrocyte sedimentation rate (ESR), serum C-reactive protein (CRP),
1262 serum rheumatoid factor (RF), and serum TNF- α [323]. Also, *L. plantarum* showed to be effective in exerting
1263 anti-arthritic activity in model of Complete Freund's adjuvant (CFA)-induced arthritis in female Wistar rats.

1264 A meta-analysis involving 361 patients showed that pro-inflammatory cytokine IL-6 was lower in those
1265 who received probiotics than placebo. However, there was no improvement between probiotics and placebo
1266 groups in disease activity scores [324]. Hence, there are signs of the beneficial effect of probiotics in treating
1267 human arthritis. However, more randomized controlled trials (RCTs) are still needed.

1268 Regarding bone loss, studies showed that pretreatment with *L. casei* for 8 weeks before a surgical process
1269 for administration of CoCrMo to the cranium of C57BL/36 mice (which promote rapid osteolysis), followed by
1270 two more weeks of treatment, was able to reduce 40% in bone porosity and in osteoclasts formation, as well as
1271 activated M1 and M2 macrophages with an anti-inflammatory profile [325]. This same microorganism was tested
1272 in elderly patients who had suffered a fracture in the distal region of the radius. Those patients received daily
1273 supplementation with *L. casei* Shirota for six months and were evaluated monthly according to pain level and
1274 limb function recovery. Results showed a significant reduction in pain level during the first 4 months of treatment
1275 and greater flexibility and strength in the wrist than in the placebo group, accelerating the fracture healing process
1276 [326].

1277 In addition, *L. reuteri*, known for its immunomodulatory potential, improved bone health in female
1278 BALB/c mice submitted to a surgical incision on the back three times a week with probiotic administration. The
1279 probiotic consumption led to an increase in bone volume, trabecular density, and a reduction in pro-inflammatory
1280 cytokines [327].

1281 Several studies correlate improvement in the bone condition and probiotic supplementation. However,
1282 mechanisms involving such benefits and signaling pathways in this complex interaction remain poorly studied.
1283 Therefore, metabolomics and proteomics studies and high-quality randomized controlled trials are essential tools
1284 for further clarification of this complex intestine-bone interaction.

1285

1286 4.5. Probiotics and the central nervous system

1287

1288 Psychobiotics refer to a specific group of probiotics that influence functions and behaviors of the central
1289 nervous system (CNS) through the gut-brain-axis (GBA). This communication occurs via various pathways,
1290 including immune, humoral, neural, and metabolic pathways. The application of psychobiotics, in both animal
1291 models and clinical trials, not only improves gastrointestinal function but also exhibits potential antidepressant
1292 and anxiolytic effects, opening a new frontier in neuroscience research[328].

1293 Probiotics modulate the intestinal microbiota, increasing the diversity of microorganisms and the
1294 composition of beneficial bacteria, modulating the CNS via direct and indirect mechanisms [329]. Psychobiotics
1295 are able to modulate, in humans and animal models, important neurotransmitters and proteins, such as gamma-
1296 aminobutyric acid (GABA), serotonin, glutamate, tryptophan metabolism, and brain-derived neurotrophic factor
1297 (BDNF) [330]. These substances play crucial roles in controlling neural excitatory-inhibitory balance, mood,
1298 cognitive functions, learning, and memory processes [331–333]. These microbiologically synthesized
1299 neurotransmitters can cross the intestinal mucosa, acting indirectly on the enteric nervous system (ENS) [334,
1300 335]. Much of the research on psychobiotics is conducted through animal studies, where stress is induced, and
1301 behavioral tests are performed on rodents to assess motivation, anxiety, and depression[336].

1302 Certain strains of *Lactobacillus* spp. and *Bifidobacterium* spp., like *L. brevis*, *B. dentium*, and *L.*
1303 *plantarum*, have been found to produce GABA and serotonin, as well as *Lactococcus lactis* strains [337–340].
1304 Additionally, specific *Lactobacillus* strains, such as *L. plantarum* and *L. odontolyticus*, can produce
1305 acetylcholine[341]. Recent studies have also demonstrated that microbes can regulate serotonin synthesis in the

1306 gut[328]. *L. rhamnosus* JB-1 has demonstrated the potential to reduce anxiety and depression. Its intake results in
1307 specific changes in GABA receptor expression within different brain regions and leads to a decrease in plasma
1308 corticosterone levels[342]. According to Liu et al., [343] *L. plantarum* PS128 activity in CNS functions in mice,
1309 increased locomotor activity, decreased anxiety, depression, corticosteroid levels, and increased serotonin levels,
1310 with a dose of 10^9 CFU in 28-day treatment. Studies with strains of *B. breve* and *L. fermentum* had an anxiolytic
1311 effect, reducing the anxiety behavior [344]. Similarly, the administration of a single strain, *B. longum* NCC3001,
1312 has shown effectiveness in treating anxiety. This strain also upregulates the expression of BDNF in the
1313 hippocampus[345]. Apart from promising results from animal studies, several research studies have also shown
1314 positive effects of probiotics on mental health in humans. In one study, healthy volunteers who received *B. longum*
1315 1714 for a duration of 4 weeks experienced reduced stress levels and improved memory[346]. Furthermore, a
1316 randomized, double-blind, placebo-controlled trial investigated the effects of probiotic yogurt (containing *L.*
1317 *acidophilus* LA5 and *B. lactis* BB12) and probiotic capsules (comprising *L. casei*, *L. acidophilus*, *L. rhamnosus*,
1318 *L. bulgaricus*, *B. breve*, *B. longum*, and *S. thermophiles*) on petrochemical workers[347]. The participants who
1319 consumed both probiotic yogurt and probiotic capsules demonstrated improvements in mental health parameters,
1320 as assessed by the depression, anxiety, and stress scale (DASS) and the general health questionnaire (GHQ) [347].

1321 Alzheimer's disease (AD) is a chronic neurodegenerative disorder that is characterized by cognitive and
1322 memory impairments. However, the evidence regarding the effects of probiotics in ameliorating cognitive
1323 disorders, including AD, is currently limited[328]. A study focused on examining the impact of multiple probiotic
1324 strains, namely *L. acidophilus*, *L. fermentum*, *B. lactis*, and *B. longum*, on an animal model of AD. After the
1325 probiotic intervention, there was an increase in the total counts of *Bifidobacterium* spp. and *Lactobacillus* spp. in
1326 the stool, while Coliform counts decreased[348]. Moreover, the study found that the probiotic supplementation
1327 led to improvements in learning and memory deficits in AD rats compared to the control rats. Additionally, the
1328 Alzheimer-probiotics group showed reductions in the number of amyloid plaques, as well as decreased
1329 inflammation and oxidative stress, suggesting potential therapeutic benefits of probiotics in mitigating certain
1330 aspects of Alzheimer's disease [348]. In a study conducted by Mehrabadi and Sadr[349], it was demonstrated that
1331 treatment with probiotic strains *L. reuteri*, *L. rhamnosus*, and *B. infantis* at a dose of 10 billion CFU per day for a
1332 duration of 10 weeks showed beneficial effects in rat models of AD. The probiotic treatment was found to be
1333 effective in reducing inflammation and oxidative stress in these animal models of AD.

1334 Parkinson's disease (PD) is a neuropsychiatric disorder that affects around two percent of the elderly
1335 population and, among the various nonmotor symptoms experienced by patients with PD, constipation is a
1336 common issue[350]. In a randomized controlled study focusing on inflammation, insulin, and lipid-related genes
1337 in peripheral blood mononuclear cells (PBMCs) from individuals with Parkinson's disease (PD), a 12-week
1338 intervention with a probiotic supplement resulted in significant changes in gene expression. The subjects with PD
1339 who received the probiotic supplement showed a downregulation of interleukin-1 (IL-1), IL-8, and tumor necrosis
1340 factor alpha (TNF- α) expression, while there was an upregulation of transforming growth factor beta (TGF- β) and
1341 peroxisome proliferator-activated receptor gamma (PPAR- γ) compared to the placebo control group[351]. In a
1342 study conducted by Hsieh et al.[352], it was reported that the consumption of a probiotic mixture containing *B.*
1343 *bifidum*, *B. longum*, *L. rhamnosus* GG, *L. lactis* subsp. *lactis*, and *L. plantarum* LP28 at a dose of 10 billion CFU
1344 per day for 16 weeks provided effective protection for dopamine-releasing neurons. This protection subsequently
1345 led to a reduction in the deterioration of motor dysfunctions in MitoPark PD mice. In another clinical study, the
1346 effects of fermented milk containing 6.5×10^9 of *L. casei* Shirota were assessed in PD patients over a period of 5
1347 weeks. The study reported that the fermented milk consumption resulted in reduced bloating, decreased
1348 constipation, and less abdominal pain in PD patients[353].

1349 Autism Spectrum Disorder (ASD) is a neurodevelopmental disorder characterized by challenges in social
1350 communication and interactions in various settings. These difficulties are often accompanied by repetitive and
1351 restricted patterns of behaviors, interests, and activities[354]. In a study conducted by Shaaban et al.[355], the
1352 beneficial effects of probiotics on behavioral and gastrointestinal manifestations of ASD were reported. Autistic
1353 children were treated with probiotic strains containing *L. acidophilus*, *L. rhamnosus*, and *B. longum* for a period
1354 of 3 months. The treatment resulted in an increase in the population of Bifidobacteria and Lactobacilli levels in
1355 the gut. Additionally, the children showed weight reduction and improvement in gastrointestinal symptoms,
1356 indicating potential positive impacts of probiotics in managing symptoms related to ASD.

1357 The balance on the intestine-brain axis can be maintained by metabolites derived from probiotic bacteria,
1358 which aid in the production of neurotransmitters and the maturation of the nervous system. Probiotic bacteria
1359 produce digestion and fermentation metabolites of nutritional components that affect the brain process and
1360 immune responses [356] therefore, probiotics are crucial to maintaining the balance on the brain-gut axis [357].
1361 These findings highlight the potential of specific probiotic strains in positively influencing mental health and brain
1362 function.

1363
1364
1365

5. PROBIOTIC PRODUCTS

1366 Probiotics are not only utilized in the management of various health conditions but are aggressively
1367 marketed to consumers to enhance or preserve health, largely fueled by media coverage. Bacteria with alleged
1368 probiotic properties are now readily available in the form of dairy products, juices, capsules, drops, powders, and
1369 functional foods [358, 359]. To cater to consumer convenience, probiotics have also been dehydrated and
1370 formulated as food supplements. The dehydration process aims to improve the shelf life of the bacterial strains.
1371 In fact, probiotic supplement products require refrigeration to preserve the viability of the bacteria, with a
1372 minimum count of 10^7 colony-forming units per gram (CFU/g) [360]. Recent advancements in technology have
1373 led to the development of dehydrated probiotics that exhibit shelf stability at room temperature. These
1374 formulations maintain a high concentration of viable cells, possess resistance to acidic conditions, and demonstrate
1375 resilience against bile in the small intestinal tract. However, there is currently a lack of information regarding the
1376 survival of commercial probiotic supplements when exposed to the low pH environment of the GIT and the bile
1377 conditions of the small intestinal tract [361].

1378 These food supplements may contain numerous strains of bacteria, with the most commonly available
1379 strains belonging to the *Lactobacillus* and *Bifidobacterium* species. However, other strains like *E. coli* Nissle
1380 1917, certain strains of *Enterococcus*, and yeasts such as *S. boulardii* are also utilized as probiotics [362].
1381 Additionally, many probiotic products may contain a combination of multiple species. These multi-strain products
1382 offer the advantage of providing a broader range of health benefits compared to products with only one strain
1383 [363]. Additionally, the term multispecies is used to describe products that contain strains from multiple genera,
1384 indicating a broader range of bacterial diversity within the product [364]. Ensuring the viability of probiotic
1385 species is crucial when selecting strains for use, as they need to survive in the food product or capsule and
1386 throughout the digestive system while maintaining their original health-promoting effects [365].

1387 An important factor to consider in the manufacturing of probiotic products is the presence of dead
1388 bacteria. Dead bacteria are inevitable in probiotic products and can originate from various stages of manufacturing,
1389 such as harvesting, lyophilization (freeze-drying), and degradation processes [366]. These dead bacterial bodies
1390 accompany the live bacteria from the early stages of manufacturing and cannot be eliminated from the final
1391 product. To ensure that the advertised number of live bacteria is maintained, manufacturers often "overfill" each
1392 sachet or capsule with an excess of bacteria, considering the inevitable loss of viability during storage. This
1393 practice compensates for the expected number of dead bacteria in the product [367].

1394 If bacteria produced under specific manufacturing conditions have reduced viability consumers end up
1395 ingesting a greater number of bacteria (both live and dead) compared to what is reported on the label for each
1396 dose. Current regulations for labeling probiotic products require informing consumers about the number of live
1397 bacteria expressed as CFU per dose [366]. However, this information does not consider the number of dead
1398 bacteria. Consequently, the CFU information provided does not accurately inform consumers about the "total
1399 number" of bacteria they are ingesting [367]. As a result, health professionals are unaware of this "hidden content"
1400 and the true potency of the product they are administering to patients. In individuals with dysreactive immune
1401 disorders, whether the bacteria are viable or dead, complete or fragmented, "good" bacteria can potentially be
1402 harmful if present in high numbers that can impact the balance between anti- and pro-inflammatory cytokines, as
1403 well as other cellular functions [367–369].

1404 1405 **5.1. Role of probiotic matrix on the probiotic effect**

1406 One additional factor that contributes to the variability observed in the results of clinical studies, apart
1407 from variations in study populations, selection criteria, and study design, is the utilization of different production
1408 conditions, growth media, drying conditions, or cryoprotectants for the same bacterial strain, as well as the
1409 combination of a successful probiotic with other bacteria or strains [370].

1410 For instance, research has demonstrated that the adhesion properties of the *L. rhamnosus* GG strain
1411 (ATCC 53103) are contingent upon the composition of the growth media and the number of starter culture
1412 transfers [371]. Furthermore, when *L. rhamnosus* GG was combined with *L. rhamnosus* LC705, *Bifidobacterium*
1413 *breve* Bb99, and *Propionibacterium freudenreichii* ssp. *shermanii*, no significant clinical or immunological effects
1414 were observed [372]. Likewise, as early as 1983, it was found that the clinical outcome of *L. acidophilus* varied
1415 depending on the specific production lot [373].

1416 Various factors, including fermentation, matrix composition, cell harvesting, spray-drying, freeze-
1417 drying, as well as storage conditions such as temperature, humidity, and pH, play significant roles in determining
1418 the viability, growth, and survival of the microorganisms. Ultimately, these factors can influence the outcomes of
1419 research studies and clinical trials involving probiotics [370, 374, 375].

1420 *L. delbrueckii* CIDCA 133, for example, showed different results in an animal model of 5-FU-induced
1421 mucositis when administered with fermented milk[241] or fermented MRS medium[376]. CIDCA133 fermented
1422 milk at a dosage of 10^7 CFU was effective in protecting the intestinal mucosa from damage caused by 5-FU, better
1423 than the fermented MRS administration. Fermented milk presented better results at reducing intestinal neutrophils
1424 infiltrate, had a better protection on weight loss, and protected the intestinal epithelial architecture, including
1425

1426 preservation of villus and crypts in a more effective manner. Although both treatments were able to reduce the
1427 effects promoted by the 5-FU administration, these experiments made clear the role of the matrix on probiotic
1428 activity.

1429 Mantel et al, [377] tested the effect of *P. freudenreichii* CIRM-BIA129 on a DSS-induced colitis model
1430 in mice with 3 different fermented matrices, milk ultrafiltration permeate, skim milk and whole milk. The work
1431 showed the increase in protein and fat in the fermented matrix positively influenced the anti-inflammatory effect
1432 of *P. freudenreichii*, with the fermented whole milk obtaining the best results. This shows that the production of
1433 fermented functional foods should consider not only bacterial fermentation but also the various components of
1434 the matrix and the structure of the food. This highlights the importance of considering the interactions between
1435 the microorganisms and the food matrix, as well as the overall food structure, in order to develop effective and
1436 beneficial fermented functional products.

1437

1438 5.2. Regulation and safety of probiotics products

1439

1440 When it comes to the use of probiotics as a treatment, it is important to consider safety due to the potential
1441 consumption of a substantial quantity of bacteria. Safety considerations encompass two aspects. Firstly, it involves
1442 determining the adverse effect profile of specific preparations, both mono- and multi-strain, to assess the safety
1443 of the products themselves. Secondly, it entails ensuring that marketed probiotic preparations adhere to rigorous
1444 quality standards. This ensures that the correct strains of bacteria are present in the product and that it is free from
1445 any contamination[87]. These measures are in place to safeguard the well-being of individuals using probiotic
1446 treatments.

1447 The relatively unregulated nature of the probiotic market allows for the transfer of claims from tested
1448 products to others that may have notable differences in formulation or manufacturing processes. This practice
1449 gives rise to numerous problems and questions. Furthermore, when probiotic formulations are utilized to manage
1450 significant conditions like IBD or disorders associated with immunosuppression, such as human
1451 immunodeficiency virus (e.g. HIV), the lack of strict regulation can have severe consequences for patients [366].

1452 It is worth noting that most commercially available probiotics are derived from fermented foods with a
1453 long history of safe consumption or from microbes that naturally colonize healthy individuals [362]. EFSA
1454 considers all common probiotic species safe for the general population [378]. However, this definition does not
1455 offer specific guidance for the increasing use of probiotics in individuals with medical conditions. It is important
1456 to mention that EFSA is cautious in accepting the term "probiotic," although it is tolerated by health authorities
1457 in certain countries like Italy. In the United States, FDA evaluates and classifies probiotics individually, but many
1458 have been classified as safe for use in food products [379]. Regulations concerning NGPs remain inadequate and
1459 vary among different countries. In Europe, any microorganisms that have not been used in foods prior to 1997
1460 must undergo a thorough evaluation by the EFSA[56] before they can be approved for the market, whether they
1461 are intended as novel foods or drugs [380]. As mentioned before, the FDA Center for Biologic Evaluation and
1462 Research (CBER) has defined NGPs as LBPs. This category presents a promising opportunity for novel
1463 microorganisms extracted from the microbiota, although they require meticulous characterization of any
1464 microorganism falling under this category, akin to the standards demanded for vaccines[381]. Although the
1465 pathway for human research on LBPs is well-defined, there are currently no known examples that have completed
1466 this process and the Investigational New Drug (IND) process must be followed[381]. It is worth noting that in the
1467 past, the FDA classified almost all probiotic research as drug research.

1468 Most clinical trials investigating probiotics have not raised significant safety concerns [77]. However, a
1469 few isolated cases of serious adverse effects have been documented independently of the formulation, dosage, and
1470 daily intake. These adverse effects include instances of bacterial sepsis associated with lactobacilli-containing
1471 probiotic supplements and the death of a preterm infant due to gastrointestinal mucormycosis, a severe and rare
1472 fungal infection resulting from a category of molds known as mucormycetes, which was linked to mold
1473 contamination in a probiotic supplement [87, 382]. Furthermore, in patients with predicted severe acute
1474 pancreatitis, treatment with a multispecies probiotic preparation was associated with an elevated risk of mortality
1475 [383]. Therefore, a thorough safety evaluation is necessary prior to the use of probiotics in vulnerable populations,
1476 including individuals with compromised intestinal mucosa or immune dysregulation, as seen in patients with IBD,
1477 liver diseases, HIV, and other conditions [77]. Safety concerns become even more crucial when dealing with a
1478 small number of products that contain high concentrations of probiotic bacteria, ranging from 450 to 900 billion
1479 bacteria per dose. The yeast *S. boulardii*, a natural yeast found in some probiotic formulations, has been associated
1480 with fungemia in critically ill patients and immunocompromised individuals [384, 385].

1481 The accuracy of labeling for commercial probiotic products currently on the market is not always reliable.
1482 It has been observed that some microorganisms claimed to be present in these products may be absent or their
1483 quantities may be lower than what is stated on the label. In a study conducted by Weese et al. in 2002, deficiencies
1484 were identified in the labels of numerous Canadian commercial probiotics intended for oral consumption.
1485 Specifically, 43% of the analyzed products had improperly identified bacteria, and 25% of the products had

1486 misspelled content [386]. Similarly, Toscano et al. conducted a quality assessment of the main probiotic products
1487 available in the Italian market in 2011 [387] and obtained similar results to the study by Weese. In the Italian
1488 study, it was observed that 42% of the analyzed products did not contain the declared number of bacteria for at
1489 least one of the labeled strains. Additionally, 17% of the products showed no viable microorganisms, and 8% were
1490 contaminated with *E. faecium* [387]. The presence of an undisclosed microorganism, which may potentially
1491 possess pathogenic traits, poses a significant risk to the host's health. These studies underscore the necessity for
1492 specific legislation that mandates accurate identification and characterization of probiotic strains in commercial
1493 products, as well as thorough testing of all products available on the market.

1494 Ensuring the stability of strain characteristics in the final product is essential to produce functional
1495 probiotic foods. This becomes particularly important when treating young infants who have compromised gut
1496 barrier function, abnormal gut microbiota, and increased sensitivity to dietary substances [370]. It is increasingly
1497 recognized that the existing regulatory approach is insufficient and can give rise to issues related to quality, safety,
1498 and the validity of claims in commercial probiotic products used in medical contexts, including products used in
1499 vulnerable populations. There is a regulatory void that needs to be addressed to ensure appropriate oversight and
1500 regulation of probiotic products.

1501

1502 6. FINAL CONSIDERATIONS AND FUTURE PERSPECTIVES

1503

1504 This review explores the current state of probiotic research and development, from initial characterization
1505 of potential probiotic strains to clinical applications and commercial product considerations. The process of
1506 identifying and screening new probiotic candidates involves a combination of traditional phenotypic assays and
1507 advanced genomic analysis. Genomic analysis provides valuable insights into the genetic factors related to
1508 functionality and safety, including the absence of pathogenicity factors and antibiotic resistance genes. Indeed,
1509 the main criteria for selecting probiotic microorganisms in many studies include their tolerance to acid and bile,
1510 as well as their adhesion ability, among others. However, the variation in experimental conditions (*in vitro*), such
1511 as the types of bile, adhesion test methods, medium composition, pH, and duration of the tests, hinders the overall
1512 comparison of results. Standardization of testing methods and conditions, and support from genomic data, are
1513 essential for meaningful comparisons and reliable conclusions regarding probiotic characteristics. This allows for
1514 better understanding of the potential benefits and functionality of probiotic strains and facilitates the selection of
1515 appropriate candidates for further research and application.. These tests serve as predictive measures of a strain's
1516 ability to survive the journey through the gastrointestinal tract and exert beneficial effects. While animal models
1517 are useful for preliminary screening, they have limitations in accurately replicating human physiology and clinical
1518 outcomes. Therefore, well-designed human trials are crucial to demonstrate the health benefits of probiotic strains
1519 in a disease-specific and often strain-specific manner. Probiotics have shown promising results, in both animal
1520 models and clinical trials, in mitigating various conditions, including infectious diarrhea, inflammatory bowel
1521 disease, mucositis, metabolic disorders, musculoskeletal inflammation, and even psychiatric conditions.

1522 However, the translation of probiotics from the laboratory to the market encounters several challenges.
1523 Manufacturing processes significantly impact bacterial growth, viability, and functional properties of probiotic
1524 products. The composition of the product matrix and supplementation with prebiotics or other bacterial strains
1525 can further modify the effects of the primary probiotic strains. Currently, there is a lack of regulatory oversight in
1526 the probiotics market, leading to issues related to product quality, safety, and label accuracy. Improvement in
1527 manufacturing practices, labeling requirements, and regulation is necessary to ensure consumer safety and
1528 confidence, particularly for vulnerable populations relying on probiotic products.

1529 Looking ahead, advances in probiogenomics and multi-omics approaches will expand mechanistic
1530 knowledge and allow for predictive modeling to rationally select novel probiotics suited for specific health goals.
1531 Relevant *in vitro* and animal models that better represent human intestinal conditions will improve clinical
1532 predictability. Elucidating the bioactive molecules derived from probiotics and their impact on cellular signaling
1533 is key to developing "postbiotic" therapies beyond live cells. With greater personalization on the horizon,
1534 combinations tailored to an individual's microbiome, genetics and health status may provide greater benefits
1535 compared to broad-spectrum probiotic products currently dominating the market. Overall, exciting innovations in
1536 probiotic research and application hold promise for revolutionizing therapeutic approaches for diverse conditions
1537 and improving public health. However, these require parallel efforts to improve quality standards, manufacturing
1538 practices, and regulatory oversight of probiotic products to ensure safety and efficacy.

1539

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1547
1548 **Competing interests**
1549

1550 Authors declare to have no competing interests as defined by Springer, or other interests that might be perceived
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1552

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CHAPTER 2 - LITERATURE REVIEW.

THE USE OF PROBIOTIC, PREBIOTIC, SYNBIOTIC AND POSTBIOTICS IN THE CONTEXT OF INTESTINAL MUCOSITIS

Here, in this chapter, we describe an important gastrointestinal disease, the intestinal mucositis, which affects individuals under chemotherapeutic treatment for diverse types of cancers using, in the case of this work, the anti-metabolic drug 5-Fluorouracyl (5-FU). Intestinal mucositis is a predictable disease, as it is caused as a collateral effect of the use of a drug, and, for this instance, we are able to use strategies beforehand that would protect the mucosa and inhibiting major damage and associated symptoms. On another note, 5-FU-induced intestinal mucositis is easily reproducible in animal models what makes it a good disease to evaluate probiotic beneficial effects.

As there is no pharmaceutical approved treatment for mucositis in the market, patients are normally treated with anti-inflammatory drugs to alleviate the symptoms, and here is where the probiotics enter. Probiotics have been tested extensively in animal models of 5-FU-induced intestinal mucositis. They have presented protective effect of the intestinal epithelium architecture and are well-known for their anti-inflammatory and immunomodulatory activities, which are applicable to the model in question. Their derivatives, inactivated microorganisms and cell-free supernatant, here describes as paraprobiotic (newly renamed to postbiotics), have shown much similar results to the viable bacteria and a promising alternative for patients for each the administration of viable bacteria is too risky. Prebiotics, which are fibers that improve the effects of beneficial effects of the indigenous microbiota, and synbiotics, which are a mix of probiotics and prebiotics administered together, are also the focus of studies targeting the treatment and prevention of 5-FU-induces mucositis.

Therefore, this chapter describes the pathology of intestinal mucositis induced by 5-FU administration and the mechanisms behind the treatment using probiotics, prebiotics, synbiotics and postbiotics in animal models and clinical trials.

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Probiotics, Prebiotics, Synbiotics, and Paraprobiotics as a Therapeutic Alternative for Intestinal Mucositis

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Intestinal mucositis, a cytotoxic side effect of the antineoplastic drug 5-fluorouracil (5-FU), is characterized by ulceration, inflammation, diarrhea, and intense abdominal pain, making it an important issue for clinical medicine. Given the seriousness of the problem, therapeutic alternatives have been sought as a means to ameliorate, prevent, and treat this condition. Among the alternatives available to address this side effect of treatment with 5-FU, the most promising has been the use of probiotics, prebiotics, synbiotics, and paraprobiotics. This review addresses the administration of these “biotics” as a therapeutic alternative for intestinal mucositis caused by 5-FU. It describes the effects and benefits related to their use as well as their potential for patient care.

Keywords: lactic acid bacteria, chemotherapy, intestinal inflammation, treatment, mucositis

INTRODUCTION

Cancer is a disease characterized by uncontrolled proliferation of cells with cellular differentiation properties, having the capacity to invade tissues and organs and spread to other regions of the body, causing metastases (World Health Organization [WHO], 2018). This disease is the second leading cause of death globally, according to the World Health Organization, accounting for an estimated 9.6 million deaths in 2018; lung (1.76 million deaths), colorectal (862,000 deaths), stomach (783,000 deaths), liver (782,000 deaths), and breast cancer (627,000 deaths) are the most common types and have the highest mortality rates (World Health Organization [WHO], 2018).

Despite the high incidence and mortality rates, when identified early, cancer is a potentially curable and treatable disease. Treatment may be done through surgery, chemotherapy, radiotherapy, or bone marrow transplantation, depending on the type of cancer, degree of tumor aggressiveness, as well as the patient's physical and immunological status. It is often necessary to combine more than one type of treatment to achieve satisfactory results (World Health Organization [WHO], 2018).

Antineoplastic chemotherapy consists of the use of drugs that destroy cancer cells, inhibit their growth, and prevent their spread by targeting DNA or critical processes involved in cell division (Guichard et al., 2017; Shields, 2017). The traditional chemotherapeutics are classified according to their mechanisms of action, including antimetabolites, microtubule-targeting agents, topoisomerases, and antibiotics (Shields, 2017). The therapeutic arsenal mostly used in the

treatment of neoplasms include oxaliplatin, irinotecan, capecitabine, cisplatin, methotrexate, 5-fluorouracil (5-FU), and FOLFIRI (an association of 5-fluorouracil, irinotecan, and leucovorin), among others (Nussbaumer et al., 2011; Cassidy and Syed, 2017; Guichard et al., 2017).

The medication 5-FU is highlighted among the chemotherapeutic alternatives and has been mainly used in the treatment of advanced types of cancer, such as colorectal cancer, as well as malignant head and neck cancer, breast, stomach, and some skin cancers (Longley et al., 2003; Martins and Wagner, 2013; Cassidy and Syed, 2017; Guichard et al., 2017). This drug is an analog of uracil and thymine (Figure 1), which is metabolized in the liver, producing many metabolites. One of them binds to and inhibits the enzyme thymidylate synthase and, consequently, ends up interfering with DNA synthesis and cell division (see the *Mechanism of Action of 5-FU* section). On the other hand, this drug can act by the incorporation of its metabolites into the DNA and/or RNA of these cells (Sonis, 2004), which impedes their normal functioning and induces apoptosis (Longley et al., 2003; Miura et al., 2010).

However, 5-FU's non-specific mechanism of action results in side effects such as nausea, cardiotoxicity, leukopenia, alopecia, myelosuppression, diarrhea, and oral and intestinal mucositis (Duncan and Grant, 2003; Soveri et al., 2014; Thomas et al., 2016; Cinausero et al., 2017). Intestinal mucositis is the most prevalent side effect of 5-FU therapy (50–80% of reported cases) and one of the main limiting factors for continuing treatment (Kim et al., 2015).

Mucositis is an inflammation of the gastrointestinal tract (GIT), with symptoms that include diarrhea, abdominal pain, bleeding, fatigue, malnutrition, electrolyte imbalance, and infections, causing complications that may be life threatening (Sonis, 2004; Toucheffeu et al., 2014; Kim S. et al., 2018). The cytotoxic effects of 5-FU in the GIT cells are a severe problem for oncological therapeutics, as they decrease the patient's ability to tolerate treatment, affecting the quality of life, directly influencing the success of therapy (Jamali et al., 2018).

Within this context, therapeutic alternatives have been sought as a means to prevent or ameliorate intestinal mucositis. Among these alternatives, the most promising are the use of probiotics [*“live microorganisms which when administered in adequate amounts confer a health benefit on the host”* (FAO/WHO, 2001)], prebiotics [*“a substrate that is selectively utilized by host microorganisms conferring a health benefit”* (Gibson et al., 2017)], synbiotics [*“a mixture of probiotics and implantation of live microbial dietary supplements in the GIT, by selectively stimulating the growth and/or activating the metabolism of one or a limited number of health-promoting bacteria, and thus improving host welfare”* (Gibson and Roberfroid, 1995)], paraprobiotics, and postbiotics, which can be defined as non-viable microorganisms, cell fractions or cell metabolites, bacteriocins, organic acids, and enzymes (Rad et al., 2020).

In this review, we address the evidence for the suitability of probiotics, prebiotics, synbiotics, and paraprobiotics as a therapeutic alternative for intestinal mucositis caused by the antineoplastic drug 5-FU.

MECHANISM OF ACTION OF 5-FU

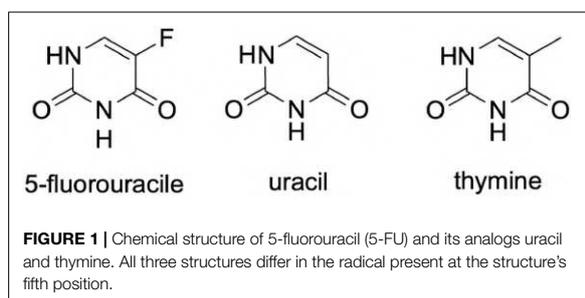
The drug 5-FU is an antimetabolite analogous to uracil, which differs by the substitution of a hydrogen atom with fluorine at the fifth position of the uracil molecule. Developed in the 1950s and introduced in cancer therapy to inhibit cell division and proliferation of cancer cells, this substance is among the class of antineoplastic drugs with a vast spectrum of action in oncological practice, being widely used for the treatment of a variety of tumors (Thomas et al., 2016; Kato et al., 2017).

To control the abnormal proliferation of cancer cells, 5-FU enters into the cells through facilitated transport, which is the same mechanism involved in its intracellular conversion into active metabolites [fluorodeoxyuridine monophosphate (FdUMP), fluorodeoxyuridine triphosphate (FdUTP), and 5-fluorouridine triphosphate (FUTP)]. These metabolites may exhibit three different mechanisms of action: (1) FdUMP inhibits the activity of the enzyme thymidylate synthase causing an imbalance in the pool of nucleotides, consequently decreasing the concentration of the deoxynucleotides dTTP and dATP, essential for DNA repair; (2) FdUTP binds to the DNA structure, inhibiting its synthesis, blocking cell division; and (3) FUTP can be incorporated into RNA, damaging it, leading to functional loss and cell death (Longley et al., 2003; Zhang et al., 2008; Miura et al., 2010; Figure 2).

Clinical evidence of patients undergoing oncologic therapy with 5-FU shows that the effects of this chemotherapy vary among users. From 20 to 40% of the patients treated with the standard dose of this drug (10–15 mg/kg body weight, for 3–4 days intravenously) develop some degree of mucositis, and about 80–100% of the patients treated with high doses (350–500 mg/kg body weight) develop GIT problems (Crombie and Longo, 2016; Cinausero et al., 2017).

EFFECTS OF 5-FU ON THE GASTROINTESTINAL TRACT

In addition to having a digestive and nutrient absorption role, the GIT mucosa acts as a physical and immunological barrier, having the ability to defend the body against potentially harmful agents that can trigger inflammatory responses in the intestine (Salvo Romero et al., 2015; König et al., 2016). The intestinal barrier is categorized according to the various levels of protection, as well as the location and nature of its cellular and extracellular components (Vancamelbeke and Vermeire, 2017). These include mainly the mucus layer associated with the commensal microbiota of the gut, antimicrobial peptide and immunoglobulin A (IgA) secretion, the monolayer of specialized epithelial cells (enterocytes, Paneth cells, goblet cells, stem cells, and enteroendocrine cells), and the lamina propria, a specialized connective tissue in which innate and adaptive immune cells reside, such as T cells, B cells, dendritic cells (DCs), macrophages, neutrophils, eosinophils, and the newly discovered innate lymphoid cells (ILCs) (Vancamelbeke and Vermeire, 2017).

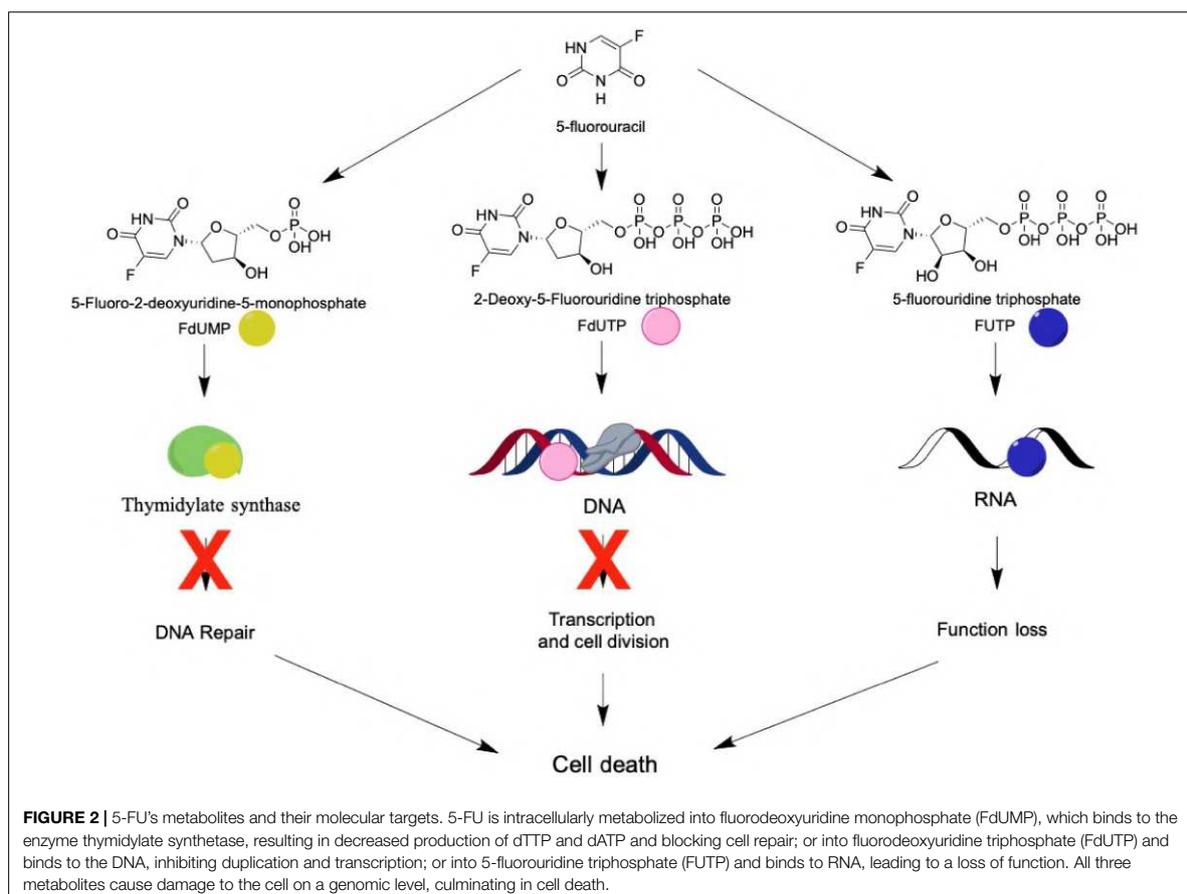


Although the intestinal barrier plays an essential role in the body's homeostasis, it is susceptible to 5-FU oncologic therapy (Yu, 2013). The intestinal mucositis caused by 5-FU mainly affects the small intestine (duodenum, jejunum, and ileum), characterized by inflammation, loss of intestinal structure and functionality, villous atrophy, goblet and Paneth cell degeneration, reduction in mucin secretion, increased intestinal permeability, cell death, polymorphonuclear cell infiltration, and increased production of proinflammatory cytokines, such

as interleukin-1 β (IL-1 β), IL-6, and tumor necrosis factor- α (TNF- α), mucosal tissue exposed to infection, and alteration of the intestinal microbiota composition (Chang et al., 2012; Lee, 2014).

The pathology of mucositis can be divided into five phases (initiation, response to primary damage, signal amplification, ulceration, and healing) (Sonis, 2004; **Figure 3**). The *initiation phase* occurs when the intestinal mucosa is exposed to 5-FU, which promotes DNA/RNA damage, either because it binds directly to these biomolecules or through the oxidative stress caused by reactive oxygen species (ROS) production. These factors induce tissue damage (Sonis, 2004; Villa and Sonis, 2015; Cereda et al., 2018), which activates several signal transduction pathways, such as nuclear factor κ B (NF- κ B) pathway signaling. This situation leads to the induction of various inflammatory mediators, such as IL-8, TNF- α , cyclooxygenase-2 enzyme (COX-2), IL-6, and IL-1 β , among others, that are responsible for mucosal toxicity (Sonis, 2004; Cinausero et al., 2017).

The recruitment of these proinflammatory cytokines acts indirectly on *signal amplification* (amplification phase) via a positive feedback mechanism, activating pathways that increase



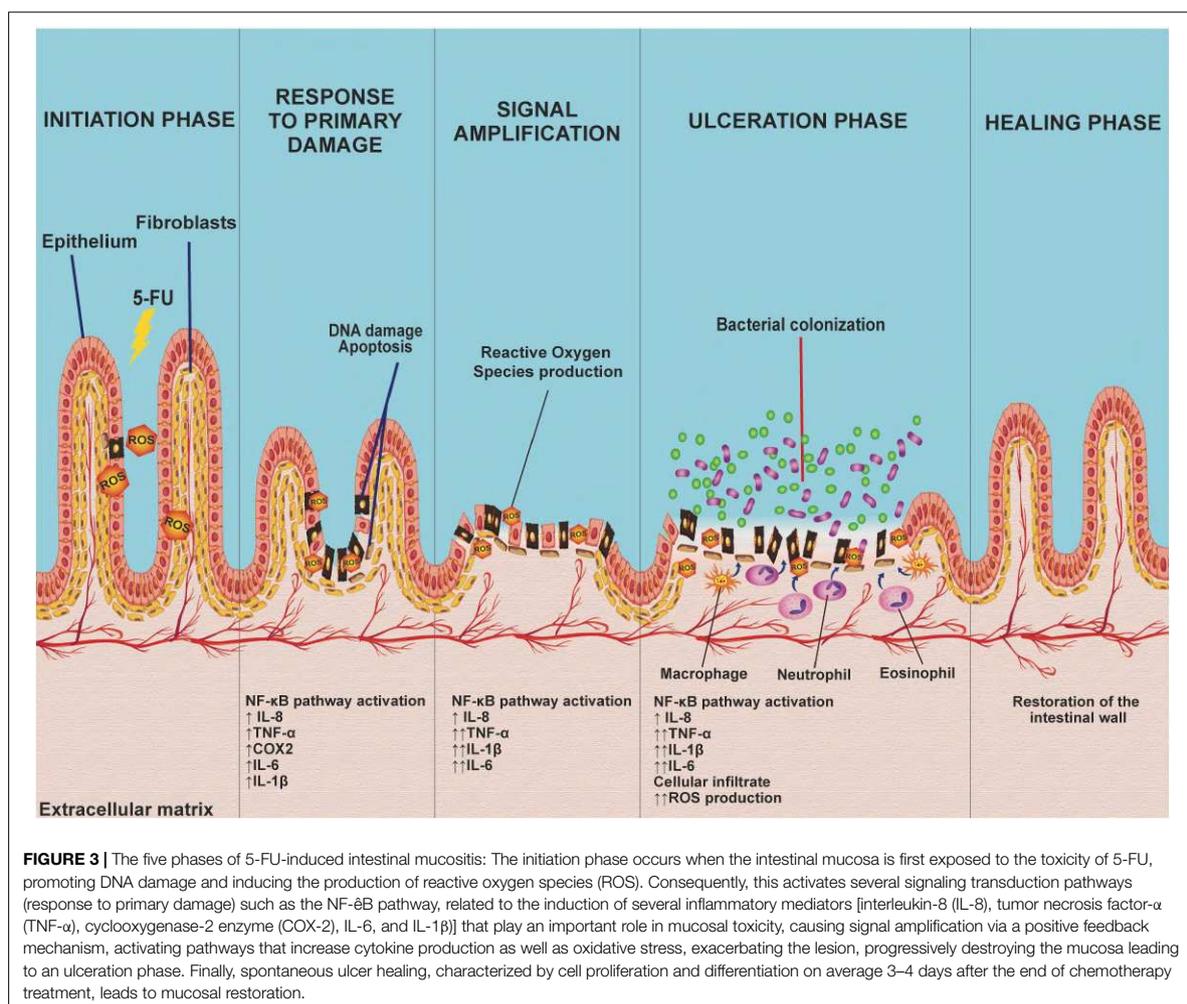


FIGURE 3 | The five phases of 5-FU-induced intestinal mucositis: The initiation phase occurs when the intestinal mucosa is first exposed to the toxicity of 5-FU, promoting DNA damage and inducing the production of reactive oxygen species (ROS). Consequently, this activates several signaling transduction pathways (response to primary damage) such as the NF-κB pathway, related to the induction of several inflammatory mediators [interleukin-8 (IL-8), tumor necrosis factor-α (TNF-α), cyclooxygenase-2 enzyme (COX-2), IL-6, and IL-1β] that play an important role in mucosal toxicity, causing signal amplification via a positive feedback mechanism, activating pathways that increase cytokine production as well as oxidative stress, exacerbating the lesion, progressively destroying the mucosa leading to an ulceration phase. Finally, spontaneous ulcer healing, characterized by cell proliferation and differentiation on average 3–4 days after the end of chemotherapy treatment, leads to mucosal restoration.

proinflammatory cytokine production (TNF-α, IL-1β, and IL-6), as well as oxidative stress. The increase in the production of these factors initiates a cascade of reactions that leads to the activation of matrix metalloproteinases, resulting in tissue damage or an increase in TNF-α production, exacerbating the initial lesion (Sonis, 2004).

The progressive destruction of the mucosa culminates in an *ulceration phase*, which occurs when loss of integrity and function of the epithelium occurs. At this stage, there are symptomatic lesions that, apart from being prone to pathogenic bacterial colonization, stimulate the activation and infiltration of defense cells, including macrophages, neutrophils, and eosinophils, in the intestinal mucosa. These cells increase the production of oxidant compounds, resulting in an increase in the depth of intestinal ulcers, consequently increasing bacterial translocation (Villa and Sonis, 2015; Cinausero et al., 2017; Cereda et al., 2018).

Finally, the *healing phase* is characterized by cell proliferation and differentiation. This phase occurs, on average, 3–4 days after

the last chemotherapy treatment, leading to restoration of the mucosa (Sonis, 2004; Villa and Sonis, 2015).

EFFECTS OF 5-FU ON INTESTINAL MICROBIOTA

In addition to causing structural damage to the intestinal epithelium, the mucositis caused by chemotherapeutic agents has a crucial influence on the intestinal microbiota (van Vliet et al., 2010). The GIT has a complex ecological population, constituted by more than a thousand different species of microorganisms, though their distribution varies along the GIT (Mowat and Agace, 2014; Rajlić-Stojanović and de Vos, 2014); low concentrations and bacterial diversity (up to 10³ CFU/ml) are found in the upper GIT (stomach, duodenum, jejunum, and proximal ileum) (Walter and Ley, 2011). A larger number of bacteria (10⁹–10¹² CFU/ml) reside

in the lower compartments of the GIT (distal ileum and colon), which constitutes, to date, the habitat with the highest known microbial density (Mowat and Agace, 2014; Jandhyala et al., 2015; Thursby and Juge, 2017). Due to the low oxygen tension in the colon, the most prevalent bacterial groups consist of anaerobic species, such as *Clostridia*, *Enterobacteria*, *Enterococcus*, *Bacteroides*, *Bifidobacteria*, *Fusobacteria*, *Lactobacilli*, *Peptococci*, *Peptostreptococci*, *Prevotellaceae*, *Roseburia*, *Ruminococci*, and *Verrucomicrobia* (Simon and Gorbach, 1982; Bäckhed et al., 2005; Mowat and Agace, 2014).

The intestinal microbiota acts through several mechanisms to maintain the homeostasis of the organism, living in mutualism with the host, benefiting from the nutrient-rich environment offered by the organism and, in exchange, performing innumerable beneficial functions, including elimination of pathogens, production of vitamins and short-chain fatty acids (SCFA), as well as modulation of the enteric and systemic immune systems (Lane et al., 2017; Thursby and Juge, 2017). However, when this mutualism becomes unbalanced, the intestinal microbiota can contribute to the onset of infectious diseases, chronic inflammation, and autoimmune diseases (de Oliveira et al., 2017).

The commensal microbiota, such as *Bifidobacterium infantis* and *Bacteroides thetaiotaomicron*, have been shown to decrease NF- κ B activation, decreasing levels of endotoxins and of plasma proinflammatory cytokines (Stringer et al., 2009). Studies have demonstrated that treatment with 5-FU alters the relative abundance of several genera of the intestinal microbiota, such as *Clostridium*, *Lactobacillus*, *Enterococcus*, *Bacteroides*, *Staphylococcus*, *Streptococcus*, and *Escherichia* (Stringer et al., 2009). Thus, disrupted homeostasis of the intestinal microbiota can affect the mucosal immune system due to an imbalance between the production of pro- and anti-inflammatory mediators, resulting in intestinal inflammation (Autenrieth and Baumgart, 2017; Holleran et al., 2017).

Given the possibility that intestinal mucositis is closely related to intestinal microbiota dysbiosis (von Bültzingslöwen et al., 2003; Yu, 2018), probiotic microorganisms have been presented as an alternative treatment due to their beneficial properties in the GIT. Given these characteristics, several studies have shown that probiotics can be an effective therapeutic alternative for the reduction of antineoplastic-induced intestinal mucositis.

PROBIOTICS

In order, to be considered a probiotic and be able to exert health benefits for the host, microorganisms must have some specific attributes, such as being capable of remaining viable during transport and storage, and tolerating the low pH of the gastric lumen and the action of bile, and pancreatic and intestinal secretions. Many probiotics are able to colonize the GIT and stimulate the immune system (Wang M. et al., 2016; Mokoena, 2017). Furthermore, resistance to antibiotics in probiotic strains should be analyzed in order to assess their safety, as well as the level and the source of this resistance (Zhang et al., 2018). Intrinsic resistance is unlikely to spread horizontally between bacteria (Mathur and Singh, 2005), while acquired

resistance could be transferred to other organisms, including pathogens, representing a potential risk to the health of the host (van Reenen and Dicks, 2011). The most well-studied and characterized probiotics belong to the lactic acid bacteria (LAB) group. However, other microorganisms also present probiotic properties, such as some *Saccharomyces* spp., and bacteria of the genera *Bifidobacterium* and *Faecalibacterium* (Pot et al., 2013; Bastos et al., 2016; Chang et al., 2019).

LAB mainly include the genera *Lactobacillus*, *Leuconostoc*, *Lactococcus*, *Pediococcus*, and *Streptococcus*, among others, and constitute a group of Gram-positive microorganisms, anaerobic or aerotolerant, non-spore forming, resistant to low pH, and able to produce lactic acid as the final product of the fermentation of carbohydrates (Wang Y. et al., 2016; Mokoena, 2017; Plavec and Berlec, 2019). Furthermore, these bacteria have been used for a long time in several industrial processes for the production of fermented foods, such as cheese, yogurts, etc. (Socol et al., 2010), and they frequently present probiotic properties. Additionally, these organisms have been explored for protein heterology production and as live delivery systems for gene and biotherapeutic vaccines, with potential applications for the treatment and prevention of various pathological conditions, in both human and veterinary medicine (Carvalho et al., 2017; Gomes-Santos et al., 2017; LeCureux and Dean, 2018; Kuczkowska et al., 2019).

MECHANISMS OF ACTION OF PROBIOTICS

Studies have shown that benefits for human health are attributed to consumption of probiotics, mainly for GIT diseases (Fedorak et al., 2015; Acurcio et al., 2017), though also for other diseases, including osteoporosis (Collins et al., 2018), cancer (Zaharuddin et al., 2019), obesity and type 2 diabetes (Saez-Lara et al., 2015; Wang et al., 2017; Hsieh et al., 2018), depression (Wallace et al., 2020), and atopic dermatitis (Rather et al., 2016). In this context, the main mechanisms of action described for these microorganisms in the host include: (i) colonization and regulation of a dysbiotic intestinal microbiota (Shi et al., 2017); (ii) protection of the epithelial barrier by maintaining tight junction integrity (Blackwood et al., 2017); (iii) induction of mucin production (Aliakbarpour et al., 2012) and B-cell-secreting IgA, which are important defense mechanisms necessary to maintain epithelial integrity and to protect the intestine from the external environment; (iv) increasing adherence to the intestinal mucosa and inhibiting of concomitant pathogen adherence based on competition for available nutrients and sites of mucosal adhesion (Collado et al., 2010; Monteagudo-Mera et al., 2019); (v) competitive exclusion of pathogenic microorganisms, such as *Staphylococcus aureus* and *Salmonella typhimurium* (Halder et al., 2017; Plaza-Díaz et al., 2017); (vi) production of antimicrobial substances such as acetic and lactic acids, and bacteriocins, which have strong inhibitory effects against Gram-negative bacteria and have been considered as the main antimicrobial compounds produced by probiotics against pathogens (Alakomi et al., 2000; De Keersmaecker et al., 2006; Makras et al., 2006; Bermudez-Brito et al., 2012; Mokoena, 2017;

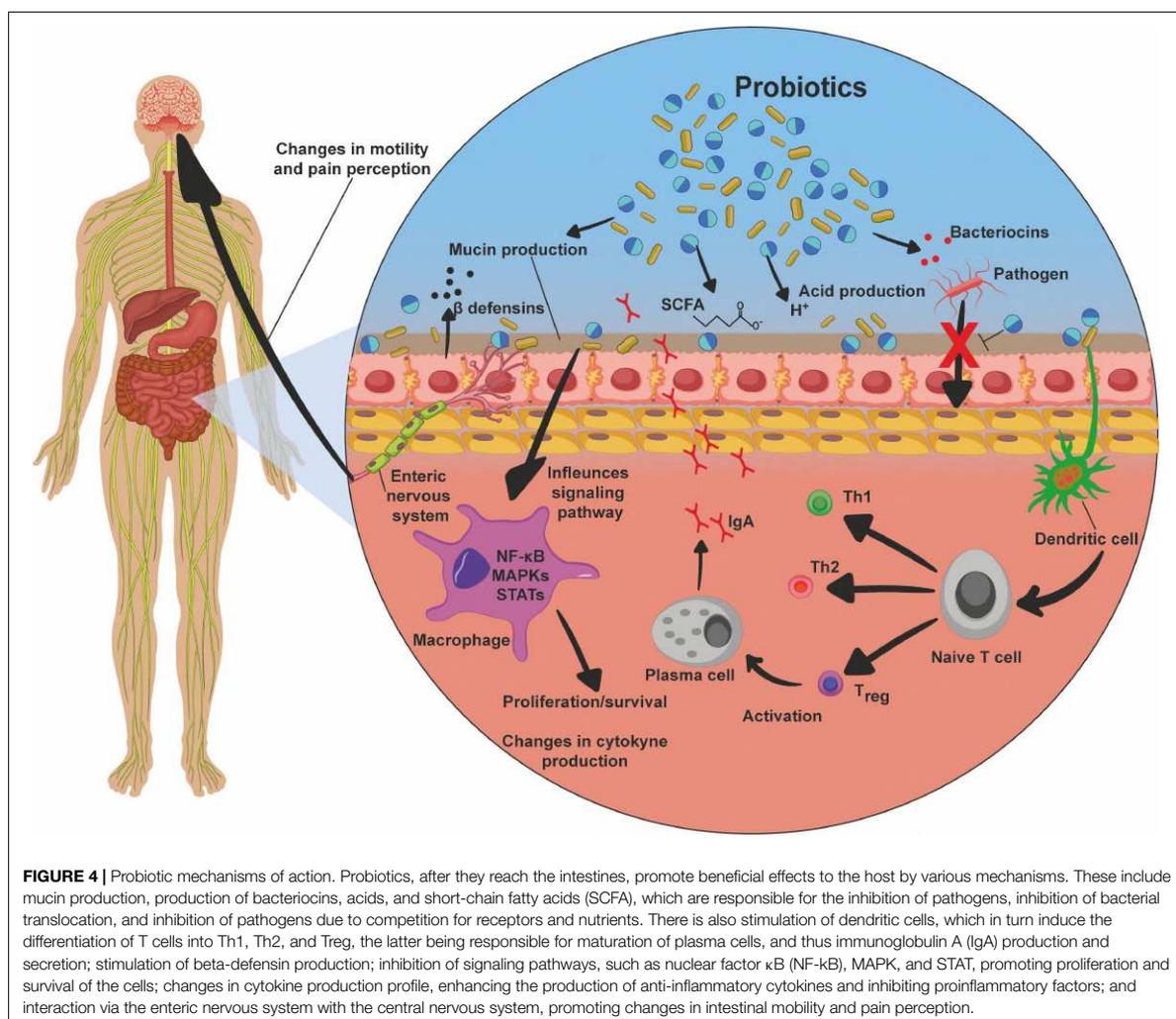
Gaspar et al., 2018; Castilho et al., 2019); (vii) production and secretion of metabolites of SCFAs with anti-inflammatory properties, such as acetate, propionate, and butyrate, which exert beneficial effects on intestinal and immune cells, being important compounds for cell proliferation, cell differentiation, and gene expression, and they are signaling molecules of immunological pathways; butyrate is the primary energy source of colonocytes, and it has an epithelial barrier function; SCFAs can also induce expression of the anti-inflammatory cytokine IL-10, inhibiting inflammatory responses (Parada Venegas et al., 2019); (viii) inhibition of the activation of the NF- κ B signaling pathway (Kaci et al., 2011; Gao et al., 2015); (ix) interaction with the gut-brain axis via the production of metabolites such as γ -aminobutyric acid (GABA) (Kim N. et al., 2018); and (x) modulation of the host's innate and/or adaptive immune system responses through interaction with epithelial cells, dendritic cells, monocytes, macrophages, and lymphocytes (Azad et al., 2018).

In addition, probiotics can act by inducing host autophagy to attenuate oxidative stress-induced intestine injury (Wu et al., 2019; **Figure 4**).

Thus, due to the numerous possible pathways in which probiotics could be involved, their study as therapeutics of various diseases, especially those related to the GIT, is of particular importance.

EFFECTS OF PROBIOTICS ON INTESTINAL MUCOSITIS

The proposed mechanisms of action for the beneficial effects of probiotic microorganisms in diseases affecting the GIT are diverse, heterogeneous, strain specific, and depend on the quantity of probiotics used (Plaza-Diaz et al., 2017). Since the immunomodulatory and anti-inflammatory effects



reported for LAB, as well as other probiotics, are strain dependent, it is necessary to identify and characterize species and strains with probiotic potential and investigate their effects on different targets or diseases (Plaza-Díaz et al., 2017). **Table 1**

presents the main findings for the effects of probiotics on intestinal mucositis.

In this context, studies conducted *in vitro* using Caco-2 cells (Fang et al., 2014) and *in vivo* with rats and mice

TABLE 1 | Effects of probiotics, prebiotics, synbiotics, and paraprobiotics in intestinal mucositis.

	Effects of intestinal mucositis	References
Probiotics strain		
<i>Lactobacillus acidophilus</i>	Inhibited nuclear factor κB (NF-κβ) (NF-κβ) pathway signaling Regulated levels of the proinflammatory cytokines [tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and the C-X-C motif chemokine ligand (CXCL)] Reversed gastrointestinal dysmotility, increased gastric emptying and intestinal transit	Justino et al., 2015
<i>Lactobacillus acidophilus</i> A4	Stimulated the overexpression of mucin genes (MUC2 and MUC5AC) Reduced myeloperoxidase (MPO) activity Reduced expression of proinflammatory cytokine IL-1β	Oh et al., 2017
<i>Lactobacillus casei</i> variety <i>rhamnosus</i> (Lcr35)	Reduced production of proinflammatory cytokines (TNF-α, IL-1β, IFN-γ, IL-10, and IL-6). Attenuated the loss of goblet cells, decreased Firmicutes and increased Bacteroidetes Reduced the frequency of diarrhea Restored villus/crypt ratio	Yeung et al., 2015; Chang et al., 2018
<i>Bifidobacterium bifidum</i> G9-1	Attenuated histopathological alteration, with decrease cell infiltrate in crypts Regulated intestinal microbiota (decrease Firmicutes and increase Bacteroidetes abundance) Reduced the concentrations of proinflammatory cytokines TNF-α and IL-1β and MPO activity Reduced diarrhea and interrupt weight loss	Kato et al., 2017
<i>Bifidobacterium infantis</i>	Improved the histologic parameters, ameliorating mucosal damage Reduced Th1 and Th17 cells, and increased CD4 + CD25 + Foxp3 + Tregs response	Mi et al., 2017
Association: (<i>B. breve</i> , <i>L. acidophilus</i> , <i>L. casei</i> , and <i>Streptococcus thermophilus</i>)	Reduced neutrophil infiltration, proinflammatory cytokines (TNF-α, IL-4, and IL-6), and intestinal permeability Restored of the intestinal epithelium architecture	Tang et al., 2017
Association: (<i>L. acidophilus</i> , <i>L. paracasei</i> , <i>L. rhamnosus</i> , and <i>B. lactis</i>)	Prevented epithelial injury in intestinal mucositis, with an increase in the villus/crypt ratio Reduced the malondialdehyde (MDA), MPO, TNF-α, and IL-6 levels Increased glutathione (GHS) levels in the duodenum and jejunum sections	Quaresma et al., 2019
Association: Whey protein isolate, to skim milk fermented by <i>L. casei</i> and <i>Propionibacterium freudenteichii</i>	Ameliorated histological scores and prevented villus shortening Reduced weight loss and degeneration of goblet cells	Cordeiro et al., 2018
<i>Lactobacillus delbrueckii</i> subsp. <i>lactis</i> CIDCA 133	Prevented body mass loss Inhibited length reduction of the intestine caused by 5-fluorouracil (5-FU) Restored histopathological damage Reduced inflammatory parameters: neutrophil, eosinophil, leukocyte infiltrate reduction, and immunoglobulin A (IgA) secretion Reduced intestinal permeability	De Jesus et al., 2019
<i>Saccharomyces boulardii</i>	Reduced cells apoptosis and inflammatory factors (nitrite concentration, neutrophil infiltrate TNF-α, IL-1β cytokines, and CXCL-1 chemokine) Improved the intestinal functions such as gastric emptying, gastrointestinal transit, absorption, and intestinal permeability Modulated the expression of TLR2, TLR4, MyD88, NF-κB extracellular signal, regulated kinase 1/2 (ERK1/2), phospho-p38 MAPK, phospho-c-Jun N-terminal kinase (phospho-JNK) in jejunum/ileum and in Caco2 cells	Justino et al., 2015, 2020
Prebiotics		
Fructooligosaccharide (FOS)	Reduced MPO activity in jejunum section Decreased inflammatory infiltrate and preserved intestinal epithelium Attenuated weight loss and increased catalase levels	Smith et al., 2008; Galdino et al., 2018
Synbiotics		
Simbioflora®	Attenuated weight loss Improved histology of the intestinal mucosa and preserved epithelial architecture Reduced eosinophil infiltrate Decreased intestinal permeability Increased the production of extracellular factors, such as SCFA (acetate and butyrate)	Trindade et al., 2018
Paraprobiotics		
<i>L. rhamnosus</i> inactivated by heat	Prevented the expression of monocyte chemoattractant protein 1 (MPC-1) Regulated the expression of TNF-α, IL-12	Fang et al., 2014

have demonstrated strain-dependent effects of probiotics for the prevention/treatment of experimental mucositis induced by 5-FU, proving to be an effective therapeutic alternative for the treatment of this disease. Thus, they could be used in parallel with chemotherapy to promote the attenuation of gastrointestinal toxicity caused by cancer drugs, which is promising for improving the quality of life of patients undergoing chemotherapy treatment (Mi et al., 2017; Chang et al., 2018).

Lactobacillus acidophilus can decrease intestinal damage caused by 5-FU (applied at a dose of 450 mg/kg) by inhibiting the signaling of the NF- κ B pathway, reducing levels of proinflammatory cytokines, such as TNF- α , IL-1 β , and the C-X-C motif chemokine ligand 1 (CXCL-1); reversion in gastrointestinal dysmotility and increased gastric emptying and intestinal transit were observed (Justino et al., 2015). This probiotic was able to reduce inflammation and normalize bowel function in mice (Justino et al., 2015). Additionally, Oh et al. (2017) demonstrated that *L. acidophilus* A4 decreased the severity of intestinal mucositis induced by 5-FU (150 mg/kg) by stimulating overexpression of mucin genes (MUC2 and MUC5AC), reducing myeloperoxidase (MPO) activity, and inhibiting expression of proinflammatory cytokines, such as IL-1 β , in mice (Oh et al., 2017).

Lactobacillus casei variety *rhamnosus* (Lcr35, Antibiohilus®, France) reduced the production of proinflammatory cytokines (TNF- α , IL-1 β , IFN- γ , and IL-6), attenuated the loss of goblet cells, reduced the frequency of diarrhea, and restored the villus/crypt ratio, demonstrating an anti-inflammatory effect on tissue damage caused in the intestinal mucosa by administering 5-FU (30 mg/kg) for 5 days (Yeung et al., 2015). The protective effect of Lcr35 (1×10^7 CFU) was also demonstrated in a colorectal cancer model; Balb/c mice were treated with a chemotherapeutic association called FOLFOX (30 mg/kg of 5-FU; 10 mg/kg of leucovorin, and 1 mg/kg of oxaliplatin) during 5 days (Chang et al., 2018). Lcr35 treatment was able to attenuate intestinal mucosa damage through regulation of the expression of proinflammatory cytokines (IL-1 β , IL-6, TNF- α , and IL-10) induced by FOLFOX in the jejunum segment and also affected the gut microbiota composition, decreasing *Firmicutes* and increasing *Bacteroidetes* abundance (Chang et al., 2018). Thus, Lcr35 is a promising therapeutic strategy for the prevention or management of chemotherapy-induced intestinal mucositis (Chang et al., 2018).

A component in the intestinal microbiota, *Bifidobacterium bifidum* G9-1 (BBG9-1), has been widely used as a treatment for diarrhea and constipation, as well as for intestinal mucositis induced by 5-FU (50 mg/kg/6 days) (Kato et al., 2017). This probiotic can reduce diarrhea and interrupt weight loss, as well as being able to attenuate villus shortening and goblet cell degeneration. It can decrease inflammatory infiltrate in crypt cells, reduce MPO activity, reduce TNF- α and IL-1 β levels, and also regulate the intestinal microbiota (decrease *Firmicutes* and increase *Bacteroidetes* abundance), demonstrating its ability to reduce the severity of 5-FU-induced intestinal mucositis (Kato et al., 2017).

Mi et al. (2017) demonstrated that *B. infantis* (1×10^9 CFU/11 days) administration, in a synergic colorectal

cancer treatment model with 5-FU (75 mg/kg/3 days) and oxaliplatin (8 mg/kg/3 days), was able to reduce the deleterious effects to the intestinal mucosa induced by chemotherapy. This probiotic improved the histology parameters, ameliorating the mucosal damage by decreasing Th1 and Th17 cells, and increasing the CD4⁺ CD25⁺ Foxp3⁺ Tregs response (Mi et al., 2017).

A combination of probiotic strains also demonstrated effectiveness in the reduction of intestinal damage induced by 5-FU chemotherapy. DM#1 mixture (*B. breve* DM8310, *L. acidophilus* DM8302, *L. casei* DM8121, and *Streptococcus thermophilus* DM8309) administration improved the restoration of the epithelial architecture, reduced neutrophil infiltration, reduced proinflammatory cytokines (TNF- α , IL-4, IL-6), and decreased intestinal permeability in mice treated with 5-FU (30 mg/kg/5 days) (Tang et al., 2017). Another study using a probiotic mix (*L. acidophilus*, *L. paracasei*, *L. rhamnosus*, and *B. lactis*) showed that the mixture was able to prevent epithelial injury in intestinal mucositis induced by 5-FU (450 mg/kg), with an increase in the villus/crypt ratio and reduced malondialdehyde (MDA), MPO, TNF- α , and IL-6 levels in all small intestinal segments (duodenum, jejunum, and ileum) (Quaresma et al., 2019). In addition, administration of the probiotic mix resulted in an increase in glutathione (GSH) levels in the duodenum and jejunum sections and attenuated the delay in gastric emptying (Quaresma et al., 2019).

The therapeutic effects of probiotics also have been demonstrated for fermented products, which can be consumed by cancer patients. Milk fermented by *Lactobacillus delbrueckii* CIDCA 133 (7.5×10^7 CFU) attenuated the damage caused to the intestinal mucosa by 5-FU (300 mg/kg), both in the recovery of the architecture of the epithelium, including prevention of goblet cell degeneration, and reduction of the polymorphonuclear cell infiltrate, with reduced IgA secretion and intestinal permeability (De Jesus et al., 2019).

A mulberry leaf extract fermented by *L. acidophilus* A4 strain stimulated overexpression of mucin genes (MUC2 and MUC5AC), promoted reduction of MPO, inhibited expression of proinflammatory cytokines, such as IL-1 β , and reduced the loss of intestinal barrier function generated by 5-FU (150 mg/kg) administration (Oh et al., 2017).

The role of whey protein isolate (WPI) added to skim milk fermented by *Lactobacillus casei* BL23 (*L. casei* BL23) or by *Propionibacterium freudenreichii* CIRM-BIA138 (*P. freudenreichii* 138) was studied in a 5-FU-induced mucositis mouse model (Cordeiro et al., 2018). Milk fermented by both bacteria was sufficient to reduce weight loss, reduce histological scores, and prevent villus shortening and degeneration of goblet cells. WPI addition to fermented milk improved the effects of these probiotics, compared to when they were administered alone (Cordeiro et al., 2018).

In addition to bacteria, yeasts can also have a beneficial effect on gastrointestinal mucositis. In this context, Porto et al. (2019) showed the effect of *Saccharomyces cerevisiae* UFMG A-905 alone or after enrichment with selenium, for intestinal mucositis treatment. This probiotic composition was able to preserve intestinal architecture and reduce nitrite concentration,

lipid peroxidation, intestinal permeability, and inflammatory parameters, protecting mice against pathological consequences caused by 5-FU administration (Porto et al., 2019).

The probiotic, thermophilic, non-pathogenic yeast, *Saccharomyces boulardii*, was also tested for intestinal mucositis treatment; the histopathological changes caused by 5-FU were significantly reduced, including cell apoptosis and inflammatory parameters (nitrite concentration, neutrophil infiltrate, TNF- α and IL-1 β cytokines, and CXCL-1 chemokine). This probiotic organism also improved the intestinal functions, such as gastric emptying, gastrointestinal transit, absorption, and intestinal permeability (Justino et al., 2015).

The effects of *S. boulardii* were evaluated by *in vitro* (Caco-2 cells treated with 1 mM 5-FU/24 h) and *in vivo* assays [Swiss mice treated with *S. boulardii* (1×10^9 CFU/kg/3 days), mucositis induction by 5-FU (450 mg/kg)] (Justino et al., 2020). *S. boulardii* was able to modulate TLR2, TLR4, MyD88, NF- κ B, ERK1/2, phospho-p38, phospho-JNK, TNF- α , IL-1 β , and CXCL-1 expression, in these two different experimental models.

Based on the above studies, probiotics could be an effective therapeutic alternative for attenuating, preventing, and treating 5-FU-induced intestinal mucositis, although clinical studies will be required to test their safeness and usefulness for treatment.

PREBIOTICS, SYNBIOTICS, PARAPROBIOTICS, AND POSTBIOTICS

The use of probiotics to treat intestinal mucositis is widely reported; however, research has also demonstrated the importance of fiber consumption to improve their benefit for the intestinal microbiota. These fibers are used by the microbiota organisms during the fermentation process, resulting in the production of various compounds, such as SCFAs, which are able to modulate the function of immune cells in the intestine, showing mainly anti-inflammatory effects (Tan et al., 2014; Luu and Visekruna, 2019).

To classify dietary fibers as prebiotic, it is necessary to satisfy six basic criteria: (i) they must be resistant to gastric acidity, hydrolysis by mammalian enzymes, and gastrointestinal absorption, (ii) they should not be digested in the upper gastrointestinal tract, (iii) they should be fermented in the colon by beneficial bacteria, (iv) they should be beneficial to the host's health, (v) they should stimulate the growth of probiotics, and (vi) they should withstand food processing conditions while remaining unchanged (Wang, 2009; Markowiak and Ślizewska, 2017; Cerdó et al., 2019).

Prebiotics may be added to food or may be obtained through consumption of natural products, such as fruit, vegetables, cereals, and other edible plant products in which carbohydrate availability is high (Markowiak and Ślizewska, 2017). A wide variety of compounds have the potential to be classified as prebiotics. Most are non-digestible oligosaccharides extracted from plants, including fructooligosaccharide (FOS) (L'homme et al., 2003), galactooligosaccharide (GOS) (Ziegler et al., 2007), mannanoligosaccharide (MOS), and xylooligosaccharide

(XOS) (Playne and Crittenden, 2002), oligofructose, and inulin (Roberfroid, 2007).

Prebiotic compounds stimulate growth, activating metabolism and promoting protection of bacteria that are beneficial to the host organism (e.g., saccharolytic bacteria, *Bifidobacterium*, and *Lactobacillus*). Prebiotic fermentation by indigenous microbiota can modulate the composition and the function of these microorganisms (Gibson and Roberfroid, 1995; Slavin, 2013; Davani-Davari et al., 2019). Furthermore, prebiotic fermentation can benefit the host through production of some compounds, such as SCFAs and lactic acid, produced by *Bifidobacterium* and *Lactobacillus* spp., which cause a reduction in the intestinal pH, inhibiting the development of gastrointestinal pathogens (Gibson and Wang, 1994; Bovee-Oudenhoven et al., 2003; Amani Denj et al., 2015). Prebiotics are also able to exert beneficial effects via mucin production by providing fermentable compounds that contribute to a lower incidence of bacterial translocation (Satchithanandam et al., 1990; Schley and Field, 2002).

Another mechanism proposed for prebiotics is their interaction with carbohydrate receptors (mannose, fucose and C-type lectin receptors, and galectins) on immune cells [phagocytes, natural killer (NK) cells, DCs]. The production of metabolites (e.g., folate and riboflavin, vitamins, and SCFAs) during their fermentation by gut microbiota showcases antimicrobial activity and maintains a healthy gut barrier (Hosono et al., 2003; Roller et al., 2004; Furusawa et al., 2013; Comstock et al., 2014; Levit et al., 2018; Enam and Mansell, 2019).

As prebiotics stimulate probiotic action, the synbiotic concept was created to overcome difficulties faced by probiotics in the GIT, demonstrating that this association (prebiotics + probiotics) intensifies their individual beneficial effects (Markowiak and Ślizewska, 2017).

Information on prebiotic stimulation of known probiotic strains leads to the choice of the ideal microorganism-substrate synbiotic pairs; the consumption of appropriately selected probiotics and prebiotics can increase the beneficial effects of each. Synbiotics have beneficial synergistic effects, greater than those observed for individual administration of prebiotics and probiotics (Geier et al., 2006).

The main criteria for synbiotic formulation should be a selection of appropriate probiotic and prebiotic pairs; the prebiotic should selectively stimulate the growth of probiotic microorganisms, having a beneficial effect on health, with no or limited stimulation of other microorganisms. The main probiotic species and prebiotics used in synbiotic formulations include, respectively, *Lactobacillus* spp., *Bifidobacteria* spp., *S. boulardii*, and *B. coagulans*, and FOS, GOS, and XOS. The health benefits from the administration of synbiotics to humans include: (i) increased levels of *lactobacilli* and *bifidobacteria* and balanced gut microbiota, (ii) improvement of immunomodulating ability, (iii) prevention of bacterial translocation; and (iv) improvement of liver function and reduction of incidence of nosocomial infections in surgical patients (Pandey et al., 2015; Markowiak and Ślizewska, 2017). Evidence shows that physical and chemical changes in the colon and intestinal microbiota caused by synbiotic

consumption, such as increased production of SCFAs and an increase in antitumor or antimutagenic compounds, can provide protection against rectal colon cancer, as they result in an improved immune response due to changes in the microbiota (Machado et al., 2014).

The studies listed above show the advantages of using live organisms; however, despite the fact that probiotics have proven benefits for the health of the host, current research emphasizes that the living organisms are not necessary for probiotic action; their different components, such as carbohydrates, proteins, lipids, vitamins, organic acids, cell wall components, and other complex molecules, generated after cell death, also have health benefits (Cuevas-González et al., 2020). The administration of non-viable organisms and their secreted products can present advantages in safety, reducing the possibility of infection and microbial translocation, which have been reported after the administration of probiotics to immunocompromised individuals (Aguilar-Toalá et al., 2018; Cuevas-González et al., 2020).

In this context, the terms “paraprobiotics” and “postbiotics” have been defined to refer to inactivated organisms and their metabolites. The difference between them is that paraprobiotics, also known as “non-viable probiotics” refer to inactivated cells, while postbiotics refer to soluble factors, which can be products (or metabolic byproducts) secreted by viable bacteria or released after their lysis (Cuevas-González et al., 2020). It is already possible to find products on the market that contain inactivated bacteria (e.g., Lactéol Fort® from PUMC Pharmaceutical Co., Ltd. and Fermenti Lattici Tindalizati® from Frau, AF United Spa) (Taverniti and Guglielmetti, 2011).

Microorganisms can be inactivated through ultrasound (Ojha et al., 2016), high temperatures (Chuang et al., 2007), UV radiation (Lopez et al., 2008), and other options. However, it is necessary to evaluate some details to choose the best inactivation method, as well as to evaluate the effects on microbial structure and components (Ananta and Knorr, 2009; Taverniti and Guglielmetti, 2011).

The mechanism of action of paraprobiotics is not yet fully understood, but it is known that they are capable of acting in immunomodulation (Adams, 2010). *L. rhamnosus* GG (LGG), inactivated by UV radiation (Lopez et al., 2008) or heat killed (Li et al., 2009), has shown interesting results. UV-inactivated LGG is as effective as living LGG in downregulating the IL-8 response in Caco-2 cells; IL-8 is a proinflammatory chemokine released by intestinal cells (Lopez et al., 2008). Heat-killed LGG was tested in an infant rat model with LPS-induced inflammation and both live and inactivated strains administered enterally (10^8 CFU/kg); both were able to decrease proinflammatory mediators induced by LPS and to positively regulate anti-inflammatory mediators in the liver, plasma, and lung (Li et al., 2009).

The strains *L. acidophilus* A2, *L. gasseri* A5, and *L. salivarius* A6 inactivated by heat, in an *in vitro* experiment, were both able (at 10^5 CFU/ml) to stimulate splenocyte and dendritic cell proliferation and production of IL-10, IL-12-p70, and IFN- γ . Likewise, *L. salivarius* was able to activate splenocytes and dendritic cells in mice to induce T cells toward a Th1 immune response. It was concluded that heat-inactivated bacteria can

play an important role in modulating the immune response (Chuang et al., 2007).

A comparison was made of the *in vitro* potential of viable *L. rhamnosus*, the same bacteria inactivated by heat and the culture supernatant, for inducing the synthesis of cytokines by macrophages. Viable and heat-inactivated *L. rhamnosus* were able to induce the production of TNF- α , IL-6, and IL-10, demonstrating a capability to exert an immunoregulatory effect on macrophages (Jorjão et al., 2015).

Postbiotics is another term that emerged after it was found that not only live probiotic bacteria are capable of promoting health benefits. Postbiotics comprise all products obtained from the metabolic processes of live bacteria or released after bacterial lysis, with biological benefits for the host (Tsilingiri and Rescigno, 2013). These products include cell surface proteins (surface-layer proteins), cell-free supernatants (CFS), cell lysates, bacteriocins, enzymes such as glutathione peroxidase (GPx) and superoxide dismutase (SOD), peptides, teichoic acids, exopolysaccharides, B-group vitamins, secreted polysaccharides, organic acids (lactate), and SCFAs (acetate, propionate, and butyrate) (Tsilingiri and Rescigno, 2013).

Postbiotic mechanisms of action have not been fully elucidated; nonetheless, there is evidence that they promote antioxidant (Xu et al., 2011; Xing et al., 2015) and antiproliferative effects (Escamilla et al., 2012; Chuah et al., 2019), stimulating antipathogenic, immunomodulatory, and anti-inflammatory properties (Wang et al., 2018; Gao et al., 2019).

PREBIOTICS, SYNBIOTICS, AND PARAPROBIOTICS IN INTESTINAL MUCOSITIS

A few studies describe the action of prebiotics (Figure 5A), synbiotics (Figure 5B), and paraprobiotics (Figure 5C) on intestinal mucositis. Table 1 presents the main findings of their effects in intestinal mucositis. FOS supplement (3 and 6%) was administered to evaluate the effect on 5-FU (150 mg/kg)-induced intestinal mucositis in a murine model (Smith et al., 2008; Galdino et al., 2018). FOS was able to reduce MPO activity in a jejunum section. This was the only parameter that showed a significant reduction (Smith et al., 2008). In addition, beneficial effects of FOS (6%) administration in an experimental model of intestinal mucositis induced by 5-FU (300 mg/kg) were observed (Galdino et al., 2018). There was a decrease in inflammatory infiltrate, partial preservation of the intestinal epithelium, attenuation in body weight loss, and increased catalase levels, showing that supplementation with FOS could be an important adjuvant for the prevention and treatment of intestinal mucositis (Galdino et al., 2018).

Regarding the effects of synbiotics on intestinal mucositis, a commercial product called Simbioflora®, which is a synbiotic compound composed of 5.5 g of FOS plus four probiotic strains, *L. paracasei*, *L. rhamnosus*, *L. acidophilus*, and *B. lactis*, was evaluated (Trindade et al., 2018). This synbiotic was able to attenuate weight loss, decrease intestinal permeability,

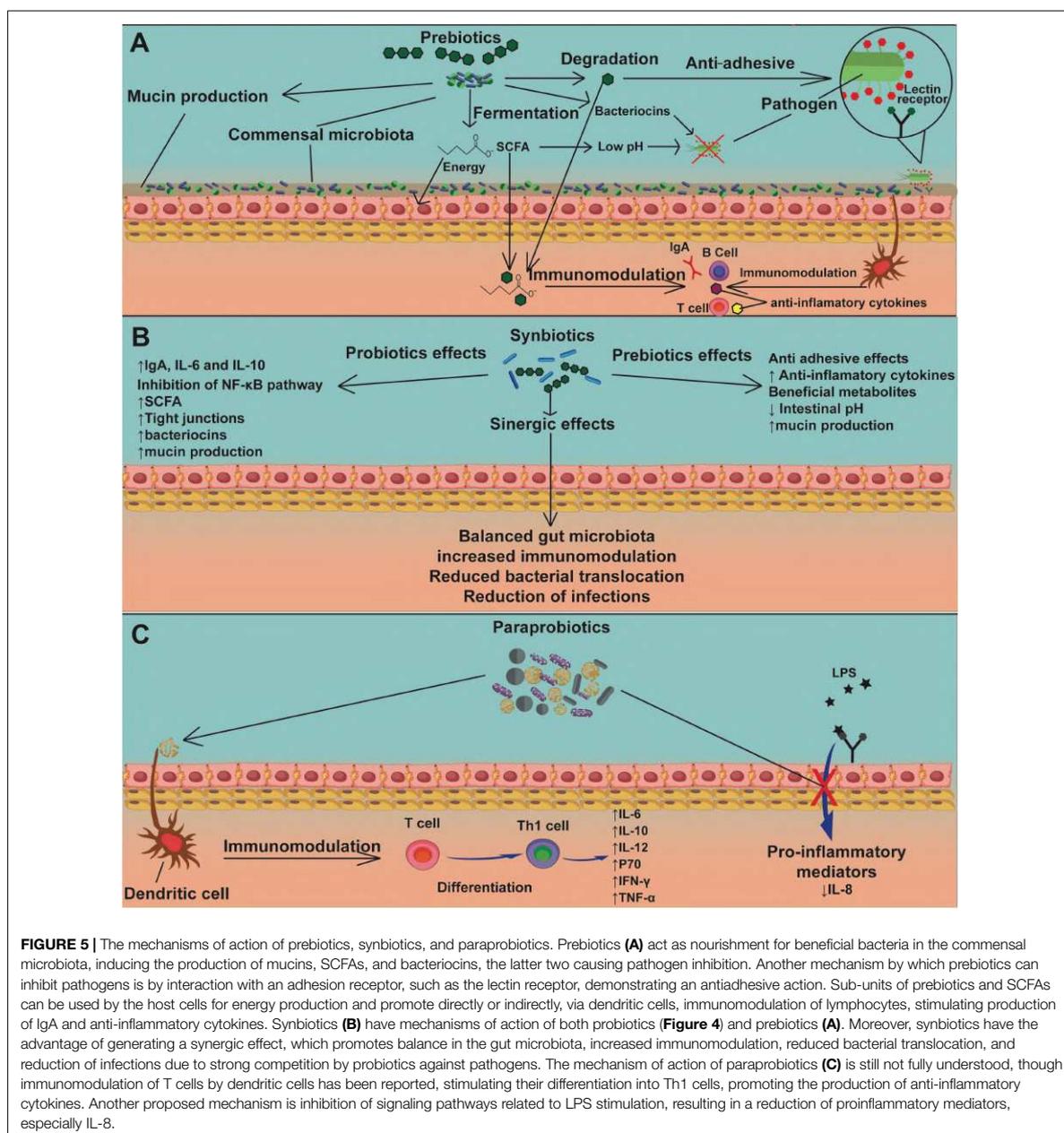


FIGURE 5 | The mechanisms of action of prebiotics, synbiotics, and paraprobiotics. Prebiotics (**A**) act as nourishment for beneficial bacteria in the commensal microbiota, inducing the production of mucins, SCFAs, and bacteriocins, the latter two causing pathogen inhibition. Another mechanism by which prebiotics can inhibit pathogens is by interaction with an adhesion receptor, such as the lectin receptor, demonstrating an antiadhesive action. Sub-units of prebiotics and SCFAs can be used by the host cells for energy production and promote directly or indirectly, via dendritic cells, immunomodulation of lymphocytes, stimulating production of IgA and anti-inflammatory cytokines. Synbiotics (**B**) have mechanisms of action of both probiotics (**Figure 4**) and prebiotics (**A**). Moreover, synbiotics have the advantage of generating a synergic effect, which promotes balance in the gut microbiota, increased immunomodulation, reduced bacterial translocation, and reduction of infections due to strong competition by probiotics against pathogens. The mechanism of action of paraprobiotics (**C**) is still not fully understood, though immunomodulation of T cells by dendritic cells has been reported, stimulating their differentiation into Th1 cells, promoting the production of anti-inflammatory cytokines. Another proposed mechanism is inhibition of signaling pathways related to LPS stimulation, resulting in a reduction of proinflammatory mediators, especially IL-8.

reduce eosinophil infiltrate, and also improve the histology of the intestinal mucosa, with preservation of the epithelial architecture, when compared to the administration of the isolated prebiotics (Trindade et al., 2018). In addition, it was found that this synbiotic increases the production of extracellular factors, such as SCFAs (acetate and butyrate), which could contribute to the observed immunomodulating activity (Trindade et al., 2018).

The effects of paraprobiotics on mucositis were demonstrated by Fang et al. (2014). To examine the immunomodulatory properties of *L. rhamnosus*, the bacteria were inactivated by heat and evaluated in an *in vitro* model of intestinal mucositis using Caco-2 cells (Fang et al., 2014). This revealed that heat does not affect the cell integrity of this bacterial species, maintaining its rod-shaped structure intact, considerably reducing the expression of monocyte

chemoattractant protein 1 (MCP-1), and regulating the expression of TNF- α and IL-12. The same results were obtained with live bacteria, revealing that this bacterial species conserved intact probiotic properties after heat inactivation, making it a promising candidate for further studies (Fang et al., 2014).

In a study of the postbiotic effect on 5-FU-induced intestinal mucositis, Prisciandaro et al. (2011) found that *Escherichia coli* Nissle 1917 (EcN) supernatant partially protected the mouse intestine from 5-FU damage (150 mg/kg) (Prisciandaro et al., 2011). It was observed that this postbiotic was able to help avoid histological damage (villus height and crypt depth) and prevented a decrease in acidic mucin-producing goblet cells. Another study showed that oral butyrate supplementation (9 mM) was able to reduce the damage to the intestinal mucosa caused by this antineoplastic agent (200 mg/kg). Reduction in histological damage, ulceration, and amelioration in intestinal permeability were observed. The gene expression of the *tight junction* protein ZO-1 (zonulin) was increased, and proinflammatory cytokines, such as TNF- α and IL-6, were reduced (Ferreira et al., 2012).

The supernatant of mulberry leaf extract fermented by *L. acidophilus* A4 was able to reduce gene expression of proinflammatory cytokines IL-1 β and myeloperoxidase (MPO), and stimulate overexpression of mucin genes (MUC2 and MUC5AC), thus reducing the severity of intestinal mucositis induced by 5-FU (150 mg/kg) (Oh et al., 2017). Additionally, *Lactobacillus plantarum* supernatant inhibited the expression of the specific markers CD44, CD133, CD166, and ALDH1 of 5-FU-resistant colorectal cancer cells (CRC) (HT-29 and HCT116) (An and Ha, 2016). The combination therapy of this postbiotic and 5-FU induced an anticancer mechanism by inactivating the Wnt/ β -catenin signaling of chemoresistant CRC cells and led to cell death by inducing caspase-3 activity. These results suggest that probiotic secretory substances can regulate cell proliferation in colorectal cancer and may be a therapeutic alternative for treating chemoresistant colorectal cancer (An and Ha, 2016).

To date, there have been few rigorous investigations examining the effect of prebiotics on 5-FU-induced intestinal mucositis. Knowing its potential in the intestinal mucosa, their supplementation with probiotics may be an attractive therapeutic alternative to ameliorate symptoms caused by mucositis, as well as other diseases involving the GIT.

Despite the significant impact of mucositis and advances in research to understand this pathology, existing therapies are mainly limited to clinical management of symptoms, aiming at electrolyte replacement, oral rehydration, and the use of adjuvant agents, such as loperamide octreotide, sucralfate enemas, sulfasalazine, and hyperbaric oxygen, to reduce fluid loss and decrease intestinal motility and diarrhea associated with mucositis, which are important debilitating symptoms (Van Sebille et al., 2015; Ribeiro et al., 2016). Given that it is necessary to find more effective therapeutic alternatives to combat intestinal mucositis, the “biotics” are strong candidates.

FINAL CONSIDERATIONS

The antineoplastic drug 5-FU is an essential and useful option for cancer treatment; however, its side effects, especially mucositis, can complicate treatment continuity and may lead to death. Effective measures to combat these symptoms, improving the quality of life of cancer patients, are crucially needed.

The probiotics have been investigated in various studies because of their beneficial properties for the GIT, including attenuation of dysbiosis. Several probiotic bacteria studied in intestinal mucositis murine models were able to attenuate and prevent intestinal histological damage, and also decrease weight loss and proinflammatory cytokine secretions, proving to be quite efficient in ameliorating the side effects to the intestine caused by 5-FU.

Though they can improve the health of the host, administration of viable microorganisms to immunosuppressed individuals still leads to controversial clinical findings. Paraprobiotics could be an effective alternative to address this concern, since microbial cells are dead or inactivated, thus avoiding risks associated with their administration to immunocompromised individuals.

Prebiotics are also described in the literature for their regulatory ability, acting to modify the commensal microbiota to a beneficial state. However, there are a few studies evaluating their potential for helping avoid intestinal mucositis. The existing studies demonstrate that prebiotics, when associated with a probiotic, are more efficient than when they are used separately, attenuating the symptoms of mucositis and improving to almost normal status the histology of the GIT.

Therefore, probiotics, prebiotics, synbiotics, paraprobiotics, and postbiotics may be useful alternatives for the treatment of intestinal mucositis induced by 5-FU. However, further studies are needed to elucidate all of the mechanisms of action of these bacteria and prebiotics to evolve into human clinical trials.

AUTHOR CONTRIBUTIONS

VB, TS, LT, LJ, FB, and NC-R wrote the original draft of the manuscript. VA, MD, and PM-A reviewed and revised the manuscript, obtained funding, and supervised the project. All authors contributed to the article and approved the submitted version.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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CHAPTER 3 - ORIGINAL PAPER.

PROBIOGENOMICS OF *Escherichia coli* CEC15

This chapter is composed of an original research paper on the probiogenomics of the *Escherichia coli* CEC15 strain and its effects on a murine model of intestinal mucositis induced by 5-FU administration.

E. coli CEC15 is a newly isolated strain which presented remodeling effects on the intestinal architecture of rats and protective effects in a mice model of ulcerative colitis. Based on these results and the potential this strain could carry, in this paper we carried out a probiogenomic analysis of this strain searching in its genome, the proteome, and its phenotype factors that could lead to its beneficial effect to the host's.

Theses analyses looked up for genes and proteins that are related to antibiotic resistance, production of toxins, presence of mobile elements in the genome, factors to survive to adhere and colonize the gastrointestinal tract. These findings were confirmed using *in vitro* tests. Furthermore, the safety of the strain was tested in healthy animals by administration of high dosages for long periods without causing any significant alteration in the animal. And finally, to further evaluate the protective effects of the strain, *E. coli* CEC15 was tested in a murine model of 5-FU-induced mucositis, which included the analysis of microbiota remodeling by the treatment.

In addition, all these tests were performed in parallel with the *E. coli* Nissle 1917 strain, a reference probiotic strain among *E. coli* specie.

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RESEARCH

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Comprehensive probiogenomics analysis of the commensal *Escherichia coli* CEC15 as a potential probiotic strain

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Abstract

Background Probiotics have gained attention for their potential maintaining gut and immune homeostasis. They have been found to confer protection against pathogen colonization, possess immunomodulatory effects, enhance gut barrier functionality, and mitigate inflammation. However, a thorough understanding of the unique mechanisms of effects triggered by individual strains is necessary to optimize their therapeutic efficacy. Probiogenomics, involving high-throughput techniques, can help identify uncharacterized strains and aid in the rational selection of new probiotics. This study evaluates the potential of the *Escherichia coli* CEC15 strain as a probiotic through *in silico*, *in vitro*, and *in vivo* analyses, comparing it to the well-known probiotic reference *E. coli* Nissle 1917. Genomic analysis was conducted to identify traits with potential beneficial activity and to assess the safety of each strain (genomic islands, bacteriocin production, antibiotic resistance, production of proteins involved in host homeostasis, and proteins with adhesive properties). *In vitro* studies assessed survival in gastrointestinal simulated conditions and adhesion to cultured human intestinal cells. Safety was evaluated in BALB/c mice, monitoring the impact of *E. coli* consumption on clinical signs, intestinal architecture, intestinal permeability, and fecal microbiota. Additionally, the protective effects of both strains were assessed in a murine model of 5-FU-induced colitis.

Results CEC15 mitigates inflammation, reinforces intestinal barrier, and modulates intestinal microbiota. *In silico* analysis revealed fewer pathogenicity-related traits in CEC15, when compared to Nissle 1917, with fewer toxin-associated genes and no gene suggesting the production of colibactin (a genotoxic agent). Most predicted antibiotic-resistance genes were neither associated with actual resistance, nor with transposable elements. The genome of CEC15 strain encodes proteins related to stress tolerance and to adhesion, in line with its better survival during digestion and higher adhesion to intestinal cells, when compared to Nissle 1917. Moreover, CEC15 exhibited beneficial effects on mice and their intestinal microbiota, both in healthy animals and against 5FU-induced intestinal colitis.

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Conclusions These findings suggest that the CEC15 strain holds promise as a probiotic, as it could modulate the intestinal microbiota, providing immunomodulatory and anti-inflammatory effects, and reinforcing the intestinal barrier. These findings may have implications for the treatment of gastrointestinal disorders, particularly some forms of diarrhea.

Keywords Probiotics, Probiogenomics, *Escherichia coli* CEC15, Genomics, *Escherichia coli* Nissle 1917, Mucositis, Immunomodulation, Gastrointestinal tract

Background

Probiotics are commonly used to mitigate the severity of certain illnesses, such as diarrhea caused by antibiotics, childhood diarrhea, ulcerative colitis, pouchitis, and eczema associated with cow's milk allergy [1]. Probiotics are "live microorganisms that when administered in adequate amounts confer health benefits on the host" [2], and it is important to note that each probiotic strain has specific effects, and the success of one strain does not guarantee the success of another. The genetic differences between probiotic bacteria can be greater than the differences between humans and goldfish [3]. While some characteristics, like safety status, are common among probiotic species, mechanisms for probiotic activity are less common and only present in certain strains (strain-dependent effect) [4–6]. For example, strains of *Enterococcus faecium* can be beneficial as a probiotic, while other strains of the same species can also be pathogens that cause problems due to antibiotic resistance [1]. The most common probiotics belongs to the lactic acid bacteria (LAB) group, the genera *Bifidobacterium* and *Protonibacterium*, and the yeast *Saccharomyces* [7]. There is one Gram-negative bacterium, which has been considered as a probiotic due to its protective effect against enteropathogenic bacteria, the *Escherichia coli* Nissle 1917 strain [8].

The *E. coli* Nissle 1917 strain (hereafter referred to as EcN) was first isolated in 1915 from feces of a soldier by German army physician Alfred Nissle [9, 10]. This strain presented good antagonistic effects against the bacteria that were causing a diarrhea outbreak at the moment, i.e. *Salmonella enterica* serovar Paratyphi, *Shigella dysenteriae*, *S. flexneri*, *Proteus vulgaris* and *P. mirabilis* [10]. A preparation containing EcN (Mutaflor) was administered to the sick soldiers and was able to restore the healthy state on them [11]. Over a century later, this strain is still being used worldwide to treat intestinal infectious diseases [10, 12, 13] and its probiotic activities have been the subject of intensive research [14–20]. However, complete genome sequencing of EcN [21, 22], as well as the advance of the genomic era evidenced that this strain has genes responsible to produce colibactin, a genotoxic secondary metabolite produced by some enterobacteria, that creates interstrand crosslinks in DNA,

which could lead to the development and the progression of colorectal cancer [23, 24]. Furthermore, the beneficial effect of this strain is linked to the presence of colibactin in a way that the knock-out of genes in the referred cluster inhibits greatly the anti-inflammatory effect of the strain on a DSS-induced colitis rat's model [25, 26]. This has raised concerns regarding the safe use of this strain.

The general ways in which probiotic microorganisms improve human health can be grouped into several categories, such as enhancing the intestinal barrier [27], regulating the immune system [28], and combating harmful pathogens through antimicrobial production [29] or competition for binding sites in the mucus barrier [30]. Although there is some supporting evidence for these claims, the specific molecular processes responsible for these activities are still largely unknown [31].

To select new probiotic strains, microbial cultures from unconventional ecosystems need to undergo a thorough evaluation process, including *in vitro* experiments, animal models, and clinical trials [32]. However, the traditional tests are not always reliable indicators of probiotic safety and efficacy, making it difficult to predict their functionality. Additionally, there are no specific attributes that are essential to all probiotics, and probiotics may exert more than one mechanism associated with a given clinical benefit [33]. These knowledge gaps complicate the efforts to understand and predict the safety and functionality of probiotics. To address these issues, the concept of "probiogenomics" has emerged as a growing area of research interest [34]. Probiogenomics involves high-throughput techniques, such as genomics, transcriptomics, proteomics, and metabolomics, which can provide a useful resource for revealing uncharacterized strains and allow for the design of predictive models for the rational selection of new probiotics [34, 35].

A new strain of *E. coli* with beneficial properties was recently isolated from suckling rodents' feces [36]. The *E. coli* CEC15 has demonstrated barrier reinforcement effect in the colonic epithelium and anti-inflammatory related immunomodulation on germ-free and conventional mice affected by TNBS-induced colitis and in IL10^{-/-} mice [37]. These effects suggest a promising effect of the CEC15 strain in the treatment of intestinal inflammatory diseases.

The aim of this work was to make a thorough evaluation of the CEC15 strain through *in silico*, *in vitro*, and *in vivo* analyses on its potential as a probiotic strain, comparing it to the well-known probiotic EcN reference strain. Their genomic composition and their potential for immunomodulation, barrier reinforcement, anti-inflammatory effect, and ability to modulate the intestinal microbiota are the focus of this work.

Results

General features of the *E. coli* CEC15 genome

The complete genome of the *E. coli* CEC15 strain consisted of a circular chromosome of length 4,780,804 bp, with a GC content of 50.66%, and a plasmid of length 200,825 bp with a GC content of 50.7%. The genome annotation showed a total of 4,505 CDS for the chromosome, with 4248 predicted as proteins, 152 being hypothetical proteins, 22 corresponding to rRNA, and 83 to tRNA, while the plasmid presented 213 CDS, from which 40 are hypothetical proteins.

The CEC15 genome was compared with that of the probiotic *E. coli* strain Nissle 1917 (EcN). CEC15 has a slightly smaller genome when compared to EcN (5.05 Mb) presenting 220 fewer CDS (4,725 CDS on the EcN chromosome). On the other hand, CEC15 harbors a larger plasmid in size and number of CDS than the EcN plasmids pMUT1 (3,173 bp with 6 CDS) and pMUT2 (5,514 bp with 8 CDS). CEC15 was classified as *E. coli* serotype O180:H14, while EcN has the serotype O6:H1.

A phylogenomic tree was constructed with the two studied strains and representative *E. coli* isolates of phylogroups A, B1, B2, C, D, E, including strains from the commercial probiotic product Symbioflor2, and using 1,000 single-copies genes common to all strains (Fig. 1). The CEC15 and EcN strains were scattered throughout the phylogenetic tree. EcN clustered with *E. coli* S88 and 536, two virulent strains belonging to the B2 phylogroup, while CEC15 was found closely related to the strains IAI1 and 55989, a commensal and a pathogenic enteroaggregative strain, respectively, which belong to the *E. coli* phylogroup B1. This analysis showed the high heterogeneity among *E. coli* strains with phylogroups composed of pathogens, commensal, and probiotics. Moreover, it indicated that an association between phylogroup clusters of *E. coli* strains and probiotic properties could not be found.

CEC15 genome presented fewer genomic islands and mobile elements than EcN

Prediction analysis revealed the presence of 25 genomic islands (Additional file 1) corresponding to 5 metabolic islands (MI), 14 pathogenicity islands (PAI), and 6 prophage regions (Additional file 2) in the CEC15

genome (Fig. 2A). MI presented lengths ranging from 6 to 18 kb and contained 6 to 23 genes coding for proteins involved notably in the utilization of propanediol, fructose, and mannose (e.g., propanediol utilization (*pdu*) gene cluster, numerous components of PTS sugar transporters). The PAI sizes were larger, with the higher size at 67.8 kb for PAI 2 and the smallest at 7.8 kb for PAI 3. In addition to metabolic functions, PAI2 notably contained genes coding for bacteria competition-related proteins such as colicin immunity domain-containing protein, contact-dependent growth inhibition system immunity protein, and toxin-antitoxin system toxin CbtA family protein. The PAI 1 (48.2 kb) is composed, mainly, of type II secretion system genes, while the PAI 11 (37.1 kb) contains genes from type VI secretion system. The PAI 10 (37.4 kb) contains most genes related to flagella production and assembly, followed by PAI 13 (923.1 kb) that contains genes for fimbriae production. Among the prophage regions found, 3 were predicted to be intact: regions 2 (36 kb), 4 (48.7 kb), and 5 (30.1 kb) that belong to the viral families *Myoviridae*, *Podoviridae*, and *Siphoviridae*, respectively. Note that some PAI and prophage regions overlapped. Prophage region 1 can be found inside PAI 2 while phage region 2 merges with PAI 3 and 4, and phage region 3 merges with PAI 5 almost completely. The large phage region 4 contains the PAI 7 and 8. Those PAI that were found inside prophage regions are mostly composed of transposase genes.

The EcN genome contained more genomic islands than CEC15 with 10 MI, 22 PAI, 1 resistance island (RI), and 6 prophage regions (Fig. 2B) (Additional files 3 and 4). The EcN MI ranged from 6.3 kb to 27 kb and contained mainly genes related to the transport and metabolism of a variety of carbohydrates. The EcN 6.7 kb resistance island is composed of 7 genes, notably one coding for the SMR family multidrug efflux protein EmrE that confers resistance to a wide range of toxic compounds [38]. As for the PAI, besides the large number of islands found, they also have a wide array of sizes ranging from 5.6 kb to 135.6 kb. Many of these PAI contain genes of type II and VI secretion systems, a variety of transposases (IS66, ISL3, ISL100, ISL3, IS21, and IS3), adhesion proteins, iron-binding proteins, and genes encoding proteins associated with antibiotic resistance. An important PAI to be mentioned is the EcN PAI 9 (54.7 kb in size), which contains the biosynthetic gene cluster that produces colibactin, a secondary metabolite that induces DNA double-strand breaks leading to genotoxic effects. None of those genes are found on the CEC15 genome. Of the 6 prophage regions on the EcN genome, 2 were intact (phage region 3 [52.8 kb] and 4 [39.9 kb]), both *Siphoviridae* and these prophage regions merge with genomic islands. The prophage region 3 contains 2 PAI (PAI 11

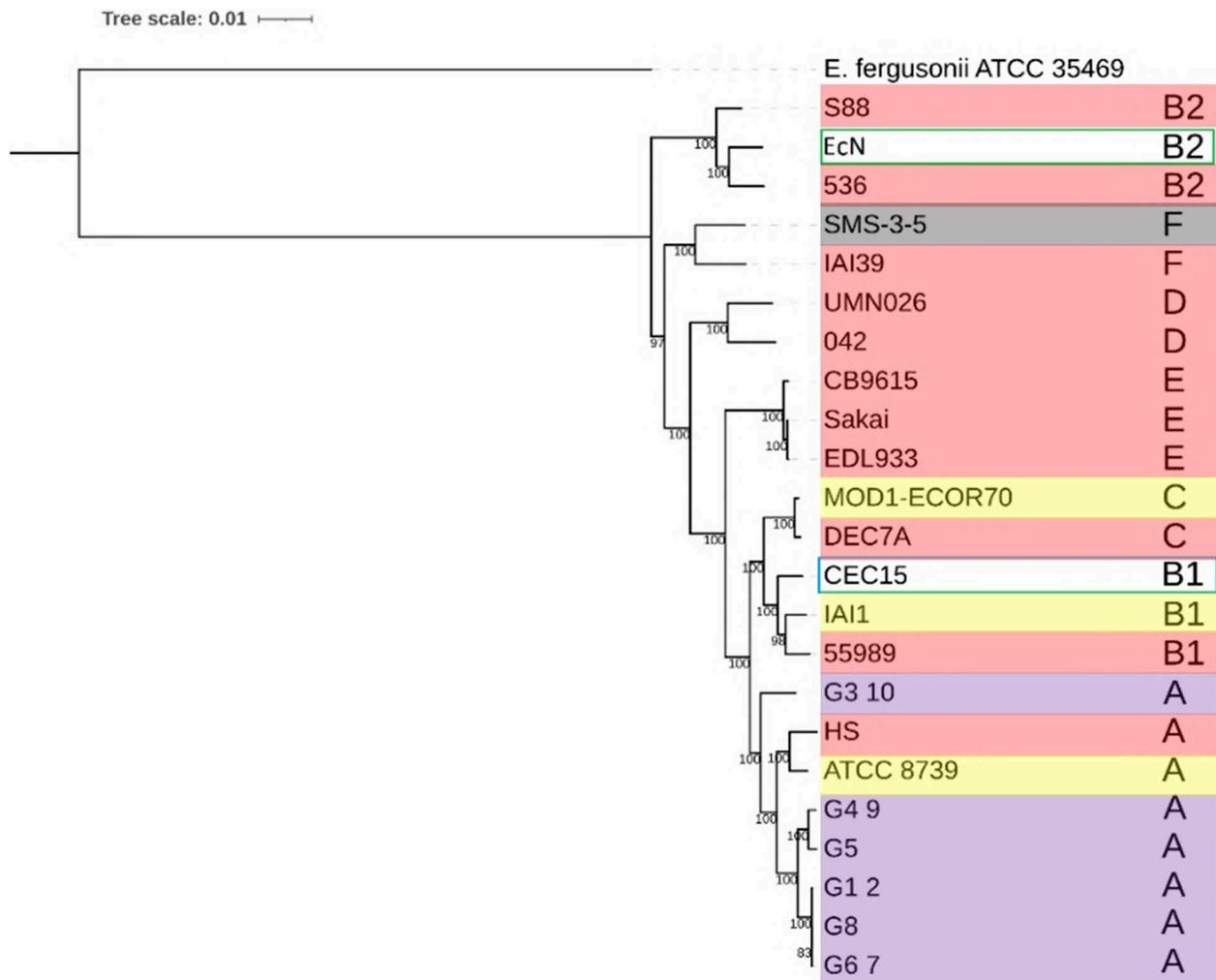


Fig. 1 Phylogenomic tree of *Escherichia coli* strains. The phylogenomic analysis was based on 1,000 single-copies genes shared among all the strains. CEC15 and EcN strains are highlighted by a blue and a green box, respectively. Strains highlighted in red are pathogenic strains, while highlights in yellow and gray indicates commensal and environmental strains, respectively. The strains in purple are from the commercial probiotic Synbioflor2

and PAI 12), while prophage region 4 contains partially the PAI 14 and the whole PAI 15. As for the incomplete prophage regions, prophage region 1 is located completely inside PAI 4, while prophage regions 5 and 6 have some degree of overlapping with PAI 19 and 21, respectively. The prophage region 2 has no overlapping with any PAI. Those PAI contained or overlapping with prophage regions are mainly composed by iron-binding genes, transposases, and metal transport systems.

Analysis of transposable elements, insertion sequences (IS), by the ISSaga tool [39] found 21 complete transposase genes in the CEC15 genome (Additional file 5), from which 9 are present in genomic islands (PAI 2, PAI 7, PAI 11, and PAI 13). EcN has over twice more of transposases genes (48) than CEC15 (Additional file 6),

from which 38 were found on PAI (PAI 4, PAI 8, PAI 9, PAI 16, PAI 18, PAI 19, PAI 20, PAI 21, and PAI 22). The CEC15 transposases were characterized in four families (IS3, ISAs1, ISNCY, and ISS66), the IS66 being the most abundant, and, for EcN, into 11 families (IS1, IS110, IS200, IS21, IS3, IS30, IS4, IS630, IS66, ISL3, and ISNCY), IS3 being the most abundant. The majority of IS in the CEC15 genome surrounds sugar metabolism-related genes with 4 IS from the ISS66 family enclosing a phosphotransferase system (PTS) sugar transport cluster, and 2 IS nearby phage regions. The EcN's IS are, in their majority, surrounding transport-related genes, in addition to four important gene clusters (sialic acid catabolizing gene cluster, flagellar hook-associated protein cluster, salmochelin biosynthesis cluster, and ferric citrate ABC

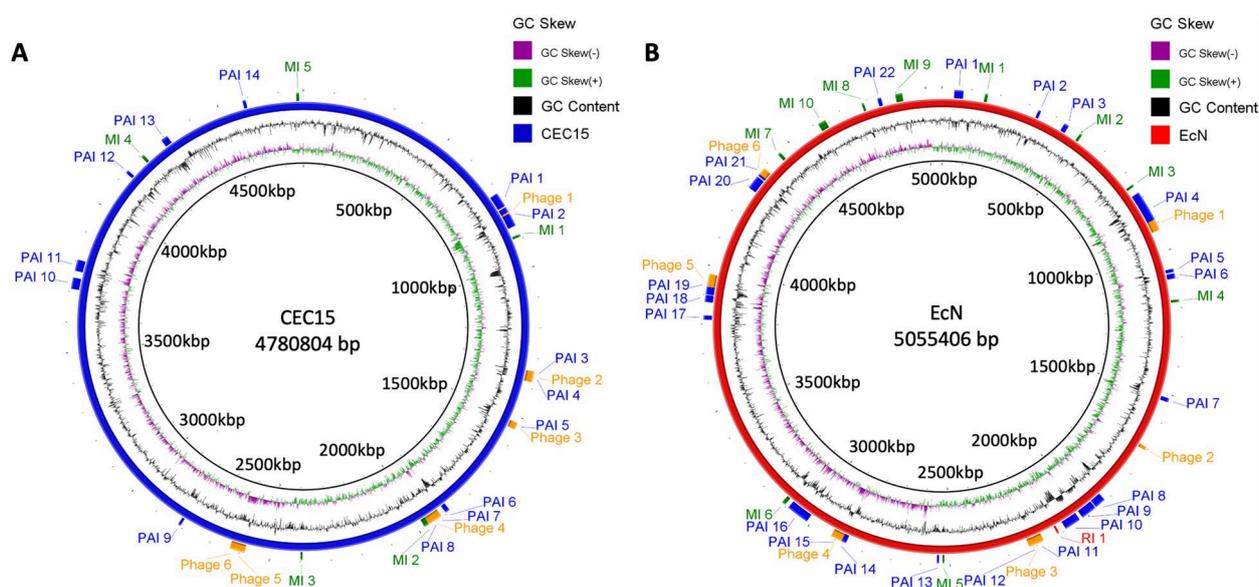


Fig. 2 Schematic circular representation of CEC15 (A) and EcN (B) genomic islands. Pathogenicity Island (PAI), Metabolic Island (MI), Resistance Island (RI), and Prophage regions were found on the genome. Figure generated by BRIG software. Circles, from the inside-out, indicate chromosome size (black circle), the GC skew positive (green) and negative (purple), the GC content (in black indicating higher content outwards and lower content inwards), and the chromosome (blue in figure A for CEC15 and red in figure B for EcN) with the location of PAI (blue), MI (green), RI (red), and prophage regions (orange)

cluster), a few antibiotic resistance genes, and type II and IV toxin/anti-toxin genes. A more detailed superposition of genomic features (PAI, MI, RI, prophages, IS, and antibiotic related genes) of CEC15 and EcN can be found on Additional files 7 and 8, respectively.

Most antibiotic resistance genes present did not translate to resistance phenotype in CEC15

Forty-five genes coding for proteins potentially related to antibiotic resistance were found in the CEC15 genome by aligning against the Comprehensive Antibiotic Resistance Database (CARD) [40] (Additional file 9). These genes are classified into three resistance mechanisms: antibiotic efflux ($n = 37$), antibiotic target alteration/protection ($n = 5$), and antibiotic inactivation ($n = 3$). The antibiotic classes comprised by these genes are mostly fluoroquinolones, β -lactams, macrolides, glycopeptides, and aminoglycosides. The EcN genome, similarly, presented 44 genes potentially related to antimicrobial resistance (Additional file 10), most of them coding for antibiotic efflux mechanisms ($n = 38$). Four EcN genes were related to antibiotic target alteration/protection, and 2 for antibiotic inactivation. These genes promote resistance to different classes of antibiotics, including aminoglycosides, β -lactams, tetracyclines, fluoroquinolones, macrolides, and glycopeptides.

The distance between antibiotic resistance-related genes and IS is important to evaluate the possibility of

genetic transfer to other strains. CEC15 has 12 genes that are < 30 kb distance from an IS gene (*mdtM*, *pmrF*, *evgS*, *evgA*, *emrK*, *emrY*, *eptA*, *mdtE*, *ugd*, *mdtF*, *gadW*, and *gadX*), while EcN has only 6 within the same criteria (*mdtM*, *bacA*, *pmrF*, *ugd*, *cpxA*, and *tolC*) (Additional file 9 and 10, respectively), which are related to resistance to fluoroquinolones, tetracycline, and polymyxin in CEC15 and fluoroquinolones, lincosamides, bacitracin, polymyxin, aminoglycosides, penam, and tetracycline in EcN.

Both strains were submitted to antibiotic susceptibility testing using the disc-diffusion method with antibiotics from nine different classes (Table 1). CEC15 and EcN strains showed susceptibility to most antibiotics but were resistant to erythromycin. The strain EcN showed additional resistance to kanamycin, according to Clinical and Laboratory Standards Institute (CLSI) standards, and to gentamicin, tobramycin, and fosfomycin, according to European Committee on Antimicrobial Susceptibility Testing (EUCAST) standards. Both strains showed intermediate resistance to streptomycin, ampicillin, and ciprofloxacin. Note that the beta-lactamase coding gene *ampC* was found on both strains.

Despite the high number of genes related to resistance to fluoroquinolones and tetracycline (19 and 11 genes, respectively in both strains), CEC15 and EcN showed sensitivity to all antibiotics tested from these classes.

Table 1 The antibiotic sensibility of *E. coli* strains (disc-diffusion method)

Antibiotic class	Antibiotic (CODE/ μ g)	CEC15			EcN		
		halo (mm)	CLSI result	EUCAST result	halo (mm)	CLSI result	EUCAST result
Penicillin	Ampicillin (AMP/10)	15	I	S	16	I	S
	Oxacillin (OXA/5)	0	R	R	0	R	R
Quinolones	Ciprofloxacin (CIP/5)	25	I	S	24	I	ATU
	Chloramphenicol (CHL/30)	25	S	S	26	S	S
	Norfloxacin (NXN/10)	25	S	S	24	S	S
	Nalidixic acid (NAL/30)	19	S	n.d.	20	S	n.d.
Macrolides	Erythromycin (ERY/15)	13	R	n.d.	10	R	n.d.
Aminoglycosides	Gentamicin (GMI/15)	20	S	S	15	S	R
	Kanamycin (KMN/30)	18	S	n.d.	13	R	n.d.
	Streptomycin (SMN/10)	12	I	n.d.	12	I	n.d.
	Tobramycin (TMN/10)	19	S	S	14	I	R
Tetracyclines	Tetracycline (TET/30)	19	S	S	20	S	S
Lincosamides	Lincomycin (LCN/15)	0	R	R	0	R	R
	Clindamycin (CMN/2)	0	R	R	0	R	R
Phosphonic antibiotics	Fosfomicin (FSF/50)	30	S	S	23	S	R

S susceptible, R resistant, I intermediate, ATU area of technical uncertainty, n.d not described

CEC15 and EcN did not present hemolytic activity

Four hemolysis related genes were found in both CEC15 and EcN genome: genes coding for a ShlB/FhaC/HecB family hemolysin secretion/activation protein, a hemolysin III family protein, a hemolysin family protein, and hemolysin HlyE. Moreover, EcN presented the hemolysin expression modulator *hha* gene. The ShlB/FhaC/HecB family hemolysin secretion/activation protein-encoding gene is found inside PAI for both strains (PAI 2 and 18 for CEC15 and EcN, respectively), and *hha* gene is found in PAI 4 for EcN, remaining genes are found elsewhere in the chromosome. The hemolytic activity of strains CEC15 and EcN was therefore evaluated on sheep-blood agar, with the two *S. aureus* strains Bk and IT2 as a control for α - and β -hemolysis, respectively. Complete hemolysis was observed for strain IT2 (Fig. 3, spot 1) with a yellow halo corresponding to a β -hemolytic activity, whereas strain Bk only resulted in partial degradation of erythrocytes leading to a greenish halo, which is characteristic of α -hemolytic activity (Fig. 3, spot 2). No halo was observed for strains CEC15 and EcN showing their inability to degrade erythrocytes (Fig. 3, spots 3 and 4 respectively).

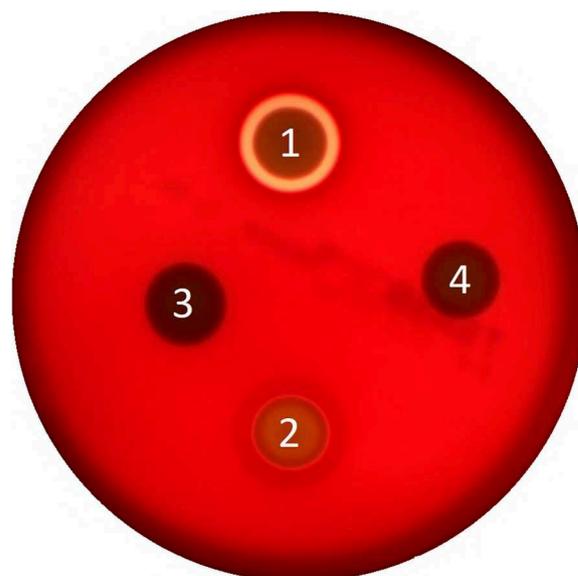


Fig. 3 Hemolytic activity assay of *E. coli* strains. Strains *Staphylococcus aureus* IT2 (1), *S. aureus* Bk (2), CEC15 (3), and EcN (4) were spotted on sheep-blood agar and incubated overnight, the presence of a halo was observed for the two control strains (1 and 2) but not for the tested strains in this study (3 and 4)

Metabolic profile of CEC15 revealed exclusive pathways for GABA and sugar production, amino acid metabolism, and xenobiotics degradation

The different number of MI between the two strains prompted us to examine their metabolic abilities. As expected, these strains share most metabolic pathways

(KEGG [Kyoto Encyclopedia of Genes and Genomes] modules) identified by the BlastKOALA [41] analysis. The strains share, in total, 100 complete metabolic modules, while 5 modules, found exclusively in the CEC15 genome, are involved in polyamine biosynthesis (synthesis of gamma-aminobutyric acid, GABA, from

putrescine), aromatic amino acid metabolism (homoprotocatechuate degradation), polyketide sugar unit biosynthesis (dTDP-L-rhamnose biosynthesis), and two aromatics (xenobiotics) degradation modules (phenylacetate degradation and trans-cinnamate degradation). No exclusive modules were found on EcN (Additional file 11).

Both strains have the machinery necessary to produce 6 from the 8 essential amino acids (lysine, threonine, isoleucine, methionine, phenylalanine, and tryptophan) and other 7 non-essential amino acids (arginine, cysteine, histidine, proline, serine, tyrosine, and glutamate), and cofactors and vitamins, especially from the B group (pantothenate, biotin, pyridoxal-p, and riboflavin). The predicted gene repertoires of complete pathways for sugar utilization in the CEC15 and EcN genomes allow the metabolism of galactose, fructose, xylulose, ribulose, ribose, erythrose, lactose, ascorbate, glycogen, and starch as primary carbon source. Another gene class with an important role on the carbohydrate metabolism is the group of PTS sugar transport systems, which are present in large amount in both genomes (59 and 64 genes for CEC15 and EcN, respectively), allowing the entry of

sugars into the cell to be metabolized. The genome of both strains also comprises genes involved in two terpenoids biosynthesis, C5 isoprenoid and C10-C20 isoprenoid (Additional file 11).

CEC15 demonstrated high fitness on simulated human digestion

Twenty-five genes related to acid tolerance were found, 23 shared among both strains and 2 exclusives of CEC15 (peroxide/acid resistance protein YodD and YceO family protein) (Table 2). The highly associated acid resistance genes from the glutamate decarboxylase family (GAD family) [42] and the acid stress response sigma factor RpoS [43] were found in the genome of both strains, which could indicate a high survival rate for both in the gastric environment.

To evaluate this hypothesis, the viability of the two strains was assessed in gastrointestinal conditions using a simulated human digestion protocol. Both strains underwent a considerable loss of viability, just after the pH was adjusted to 3, with a survival rate of 73.7% (\pm 0.08, $p=0.016$) for CEC15 and 37.71% (\pm 0.15%, $p<0.0001$) for EcN (Fig. 4, T1). After 120 min of incubation at pH

Table 2 Acid-resistance proteins found on the genome of CEC15 and EcN

Locus tag	Gene	Product	
CEC15_000207	EcN_000211	<i>gadA</i>	glutamate decarboxylase
CEC15_000208	EcN_000212	<i>gadX</i>	acid resistance transcriptional activator GadX
CEC15_000213	EcN_000217	<i>gadE</i>	acid resistance transcriptional activator GadE
CEC15_000215	EcN_000219	<i>hdeA</i>	acid-activated periplasmic chaperone HdeA
CEC15_000216	EcN_000220	<i>hdeB</i>	acid-activated periplasmic chaperone HdeB
CEC15_000474	EcN_000513	<i>yhcN</i>	peroxide/acid stress response protein YhcN
CEC15_001489	EcN_001445	<i>oxc</i>	oxalyl-CoA decarboxylase
CEC15_001491	EcN_001447	<i>yfdE</i>	CoA:oxalate CoA-transferase
CEC15_001977		<i>yodD</i>	peroxide/acid resistance protein YodD
CEC15_002333	EcN_002372	<i>asr</i>	acid resistance repetitive basic protein Asr
CEC15_002338	EcN_002377	<i>clcB</i>	voltage-gated ClC-type chloride channel ClcB
CEC15_002404	EcN_002437	<i>ydeO</i>	acid stress response transcriptional regulator YdeO
CEC15_002411	EcN_002444	<i>gadB</i>	glutamate decarboxylase
CEC15_002412	EcN_002445	<i>gadC</i>	acid resistance gamma-aminobutyrate antiporter GadC
CEC15_002520	EcN_002529	<i>ldhA</i>	D-lactate dehydrogenase
CEC15_002734	EcN_002690	<i>ychM</i>	C4-dicarboxylic acid transporter DauA
CEC15_002784	EcN_002738	<i>ariR</i>	biofilm/acid-resistance regulator AriR
CEC15_002800	EcN_002809	<i>phoQ</i>	two-component system sensor histidine kinase PhoQ
CEC15_002870		<i>yceO</i>	YceO family protein
CEC15_002912	EcN_003035	<i>ymdF</i>	general stress protein
CEC15_003598	EcN_003686	<i>yagU</i>	YagU family protein
CEC15_003764	EcN_003863	<i>clcA</i>	H(+)/Cl(-) exchange transporter ClcA
CEC15_004198	EcN_004368	<i>adiC</i>	arginine/agmatine antiporter
CEC15_004546	EcN_004770	<i>ilvD</i>	dihydroxy-acid dehydratase
CEC15_001098	EcN_001127	<i>rpoS</i>	RNA polymerase sigma factor RpoS

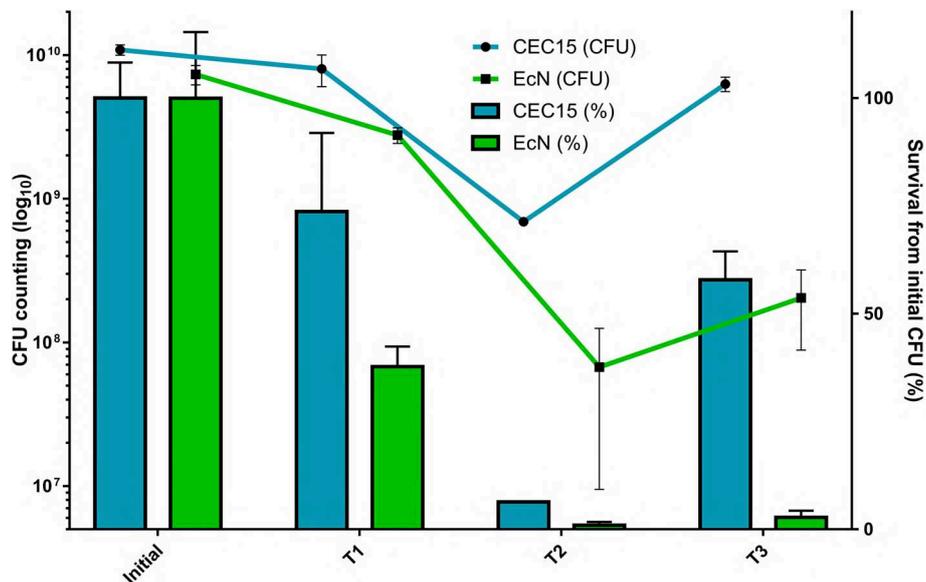


Fig. 4 Bacterial survival in the simulated human digestive tract. Both strains were submitted to an artificial digestion process and, at each step, aliquots were collected to estimate the quantity of viable bacteria. CFU counting was made before the experiment begins (Initial), at the start of gastric phase (pH adjusted to 3 - T1), at the end of gastric phase and beginning of intestinal phase (120 min in pH 3 - T2), and at the end of intestinal phase (pH restored to 7 and 120 min incubation - T3). The lines represent the CFU count in each step of the digestion processes while the bars represent the viability in percentage relative to the initial CFU. Data are expressed as mean and standard deviation of three independent experiments

3 and in the presence of pepsin, simulating the gastric environment, 6.3% ($\pm 0.001\%$, $p < 0.0001$) of the initial concentration of CEC15 were still viable, against 0.91% ($\pm 0.01\%$, $p < 0.0001$) for EcN (Fig. 4, T2). After changing to the intestinal environment (pH 7, pancreatin, and bile salts) and incubating for another 120 min, the CEC15 strain presented a considerable recovery of colony forming units (CFU), restoring its viability to 57.85% ($\pm 0.07\%$, $p = 0.0004$) of the initial concentration, while EcN CFU was maintained at 2.77% ($\pm 0.02\%$, $p < 0.0001$) (Fig. 4, T3), which represents no significant difference with the previous phase (EcN T2 vs T3, $p = 0.9939$) (Fig. 4). These results indicate that the CEC15 strain is likely more fit to survive the stress promoted by the gastrointestinal tract environment, being more able to thrive in those conditions than the EcN strain.

High adhesion rate of CEC15 can be associated to the presence of fimbriae and pili

According to the SPAAN software [44], 84 genes of CEC15 and 89 of EcN, six from each are duplicated genes (Additional file 12) were predicted with a high probability profile (score > 0.8) to code for adhesins. A total of 33 genes were found exclusively on CEC15 genome, against 32 on EcN. From these exclusive gene products, CEC15 presents 13 fimbriae proteins, 2 flagella proteins, 9 transport proteins, and 4 phage related

proteins. EcN, on the other hand, possess 7 fimbriae proteins, 5 transport proteins, and 3 phage related proteins. Among the predicted CEC15 adhesin genes, 29 are related to fimbriae/pili proteins (34%), 18 to porins/transporters (21%), and 8 to flagella proteins (10%). A similar categorical distribution of adhesins was observed for EcN: 30% of fimbriae/pili ($n = 27$), 13% of porins/transporters ($n = 15$), and 7% of flagella ($n = 6$). The 5 highest-scored genes on CEC15 are related to contact-dependent inhibition toxin CdiA, type 1 fimbria D-mannose specific adhesin FimH, lateral flagellin LafA, exopolysaccharide production protein YjbE, and type 1 fimbrial major subunit FimA, while for EcN we found contact-dependent inhibition effector tRNA nuclease, type 1 fimbria D-mannose specific adhesin FimH, phase-variable autotransporter adhesin UpaE, DUF823 domain-containing adhesin, and F1C fimbria minor subunit FocG.

The presence of surface appendages like fimbriae/pili and flagella was confirmed by electron microscopy in both strains. The scanning electron microscopy (SEM) and transmission electron microscopy (TEM) images (Fig. 5A-F) suggest that the CEC15 strain expresses more fimbriae/pili (white arrows) on its surface than the EcN strain. To study the expression of these proteins on the surface of both strains, a mechanical shearing of overnight still culture was performed. The extracted proteins

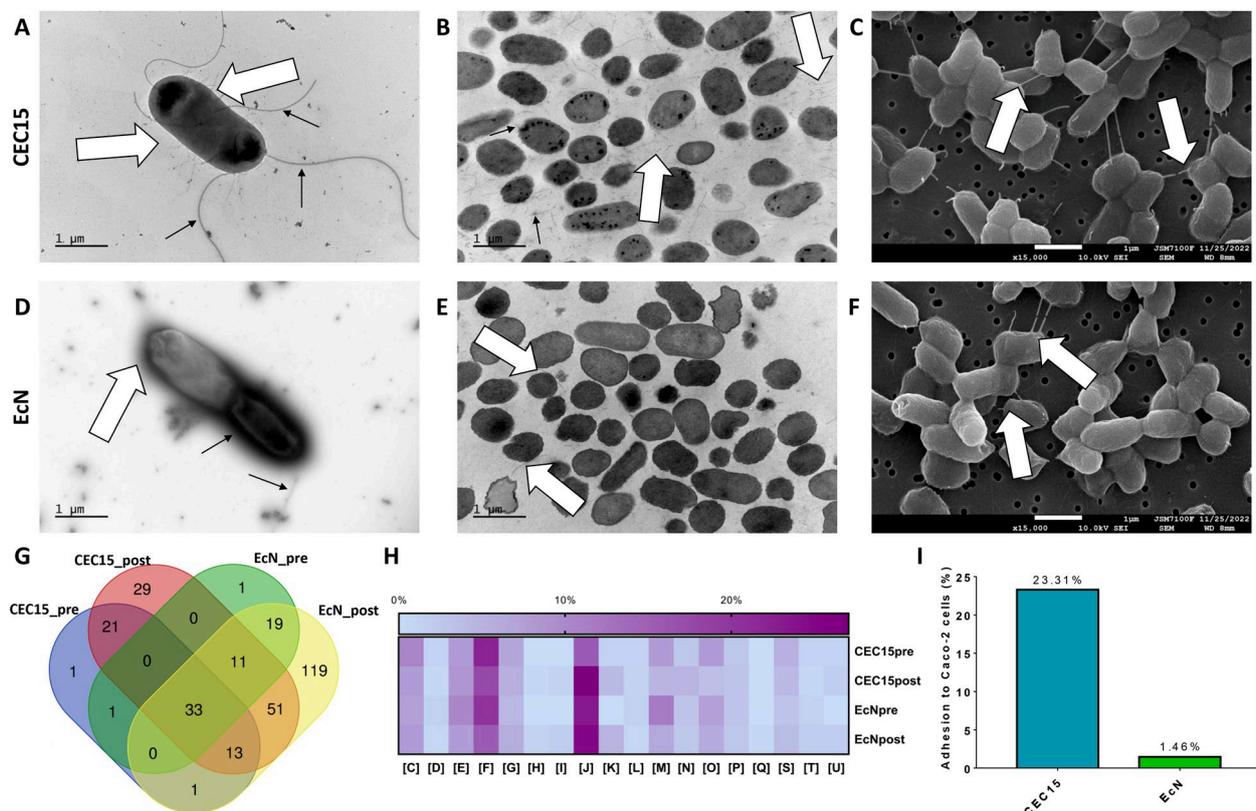


Fig. 5 Adhesive profile of CEC15 and EcN strains. The presence of fimbriae/pili and flagella in CEC15 and EcN strains could be confirmed by Transmission electron microscopy of fresh (**A** and **D**) and fixated (**B** and **E**), and by scanning electron microscopy (SEM) of fixated samples (**C** and **F**) of CEC15 and EcN, respectively. A significant quantity of proteins were found on sheared samples of both strains, about half of them being shared between strains (**G**). The heatmap (**H**) present the percentage of COG-classified proteins presented in each condition, according to the code: [C] Energy production and conversion; [D] Cell cycle control, cell division, chromosome partitioning; [E] Amino Acid metabolism and transport; [F] Nucleotide metabolism and transport; [G] Carbohydrate metabolism and transport; [H] Coenzyme metabolism; [I] Lipid metabolism; [J] Translation; [K] Transcription; [L] Replication and repair; [M] Cell wall/membrane/envelop biogenesis; [N] Cell motility; [O] Post-translational modification, protein turnover, chaperone functions; [P] Inorganic ion transport and metabolism; [Q] Secondary metabolites biosynthesis, transport and catabolism; [S] Function Unknown; [T] Signal Transduction; [U] Intracellular trafficking and secretion. The effectiveness of these adhesins were tested by adhesion assay on Caco-2 cells (**I**) were CEC15 presented a better adhesive profile (23.31%) than EcN (1.46%). White arrows indicate the presence of fimbriae/pili. Black arrows indicate flagella. Scale in all pictures is equivalent to 1 μ m and the pictures were taken on amplification of 30,000 for TEM and 15,000 for SEM

(Additional file 13) were digested by in-gel trypsinolysis and identified through MS/MS mass spectrometry.

A variety of proteins were found following shearing of the bacteria. The identification of proteins proceeded with samples pre-shearing (only resuspended in phosphate-buffered saline [PBS] buffer) and post-shearing (resuspended in PBS and then blended, 5 min, max speed). Regarding pre-shearing samples, a total of 70 and 65 proteins were detected for CEC15 and EcN, respectively, 34 of those shared among both strains, while after shearing, the quantity of proteins detected increased to 158 on CEC15 and to 247 for EcN, with 108 being shared (Fig. 5G) (Additional file 14). A total of 50 proteins were exclusive to CEC15, among those, 1 (autotransporter

outer membrane beta-barrel domain-containing protein) were exclusive to pre-shearing CEC15, 29 on post-shearing CEC15 (notably flagellar hook protein FlgE, flagellar filament capping protein FliD, flagellar hook-associated protein FlgL, type 1 fimbria chaperone FimC, type 1 fimbria D-mannose specific adhesin FimH, and type 1 fimbria minor subunit FimG), and 20 shared on pre- and post-shearing (notably flagellin FliC). EcN, on the other hand, presented 139 exclusive proteins, 1 (peptidoglycan-associated lipoprotein Pal) on pre-shearing sample, 119 on post-shearing samples (notably flagellar hook protein FlgE, autotransporter adhesin Ag43, Ag43/Cah family autotransporter adhesin, flagellar hook-associated protein FlgK, F1C fimbrial major subunit FocA, F1C fimbrial

protein subunit FocH), and 19 shared on pre- and post-shearing (notably FliC/FljB family flagellin). Based on the emPAL, we can infer that flagellin FliC is the main protein on CEC15 samples, FliC/FljB family flagellin on EcN pre-shearing, and F1C fimbrial major subunit FocA on EcN post-shearing (Supplementary Table S12).

The identified proteins were categorized according to their COG (Clusters of Orthologous Genes) classes. All samples had a high prevalence of translation proteins [J] (15.58% and 27.05% for CEC15 and 23.18% and 24.62% for EcN, pre- and post-shearing respectively) and Nucleotide metabolism and transport [F] (22.07% and 17.64% for CEC15 and 20.28% and 15.53% for EcN, pre- and post-shearing respectively), what could indicate cell lysis, as they are represented mainly by ribosomal proteins and enzymes. An important COG class for adhesion proteins is the Cell wall/membrane/envelop biogenesis [M] category, and it represented 7.79% and 4.70% for CEC15, and 11.59% and 6.43% for EcN (pre- and post-shearing, respectively) (Fig. 5H).

Based on the previous results, the adhesion of strains CEC15 and EcN to the human Caco-2 intestinal epithelial cell line was investigated. CEC15 exhibited the highest adhesion ability (~23%) on Caco-2 cells when compared to EcN strain (~1.5%). (Fig. 5I).

More bacteriocins clusters were detected on EcN than CEC15

Three gene clusters related to the synthesis of bacteriocins were found in EcN genome: a cluster coding for genes involved in the synthesis of bottromycin, an inhibitor of protein synthesis that blocks aminoacyl-tRNA binding; a cluster coding for microcin production and transport, a channel-forming bacteriocin active against enterobacteria; and a cluster coding for colicin-E9 production and transport, a polypeptide toxin with endonuclease activity against *E. coli* strains and closed-related bacteria. The EcN genome presented all three gene clusters, while only the bottromycin-encoding cluster was found in the CEC15 genome (Additional file 15).

CEC15 promoted modulation of immune- and barrier-related gene expression in Caco-2 cells

The ability of both strains to modulate the expression of intestinal epithelial cell genes coding for key factors of immunoregulation and epithelial integrity was evaluated. For this purpose, Caco-2 cell monolayers were incubated with the bacterial supernatants or with heat inactivated bacterial cells of both strains and the expression of Caco-2 genes was evaluated after 24 h of treatment. CEC15 strain appeared to be more immunomodulatory than EcN. Indeed, CEC15 supernatant and/or inactivated CEC15 cells increased the expression

of 6 genes, (*Il1b*, *Il8*, *Mcp1*, *Nfkb1a*, *Tnf*, and *Muc2*), while EcN only modulated 5 genes, increasing the expression of *Il8*, *Mcp1*, *Tnf*, and *Ptgs2*, and reducing the expression of *Ocln* among those tested. The remaining genes were not altered by any of the treatments (Additional file 16). Indeed, among the 6 barrier-related genes tested, only heat treated CEC15 at multiplicity of infection (MOI) of 100:1 induced the expression of *Muc2*, while only heat treated EcN at MOI of 100:1 lowered the expression of *Ocln*. In addition, *Ptgs2* expression, which in the colonic environment is highly associated with the promotion of colorectal carcinoma, was only induced by EcN (Fig. 6).

CEC15 showed better intestinal protection against 5-FU-induced mucositis than EcN

We evaluated the impact of a high-dosage daily administration of strains CEC15 and EcN, and of their anti-inflammatory and protective effects, in the context of 5-FU-induced intestinal mucositis in a BALB/c mice model.

Both strains were administered, as a daily dose of 10^{10} CFU, via gavage, for 12 consecutive days, to healthy animals and to animals with 5-fluorouracil (5-FU)-induced mucositis. During the experimental period, no significant difference in body weight and in food and water intake was found between groups of healthy animals that received either PBS (control group; NC), CEC15 or EcN. The induction of mucositis led to a weight loss of about 3.5 g per animal. Consumption of CEC15 or of EcN did not totally overcome mucositis-related weight loss in animals. However, consumption of the CEC15 strain (CEC15-MUC) was able to partially prevent this weight loss, when compared to the MUC group (Fig. 7A). 5-FU-induced mucositis drastically increased intestinal permeability, as indicated by the increased blood counts of DTPA-⁹⁹Tc by almost 2-fold, in comparison to the NC group. However, both strains prevented this increase in permeability. Moreover, in the absence of 5-FU, they reduced permeability to levels below that of the NC group (Fig. 7B). The neutrophilic infiltration, as indicated by the intestinal myeloperoxidase (MPO) activity, was increased in mucositis animals. Among the tested strains, only CEC15 reduce MPO activity down to a level close to that of healthy animals (Fig. 7C).

The structure of the ileal epithelium can be observed on the HE-stained tissue sections in Fig. 7D. The structural damages caused by 5-FU-induced mucositis were evaluated by histopathological scoring of such sections (Fig. 7E). The analysis showed an extensive damage of the ileal epithelial structure caused by the administration of 5-FU. This damage, however, was attenuated by the administration of CEC15 (Fig. 7E). In line with the observed alterations of the ileal mucosa structure,

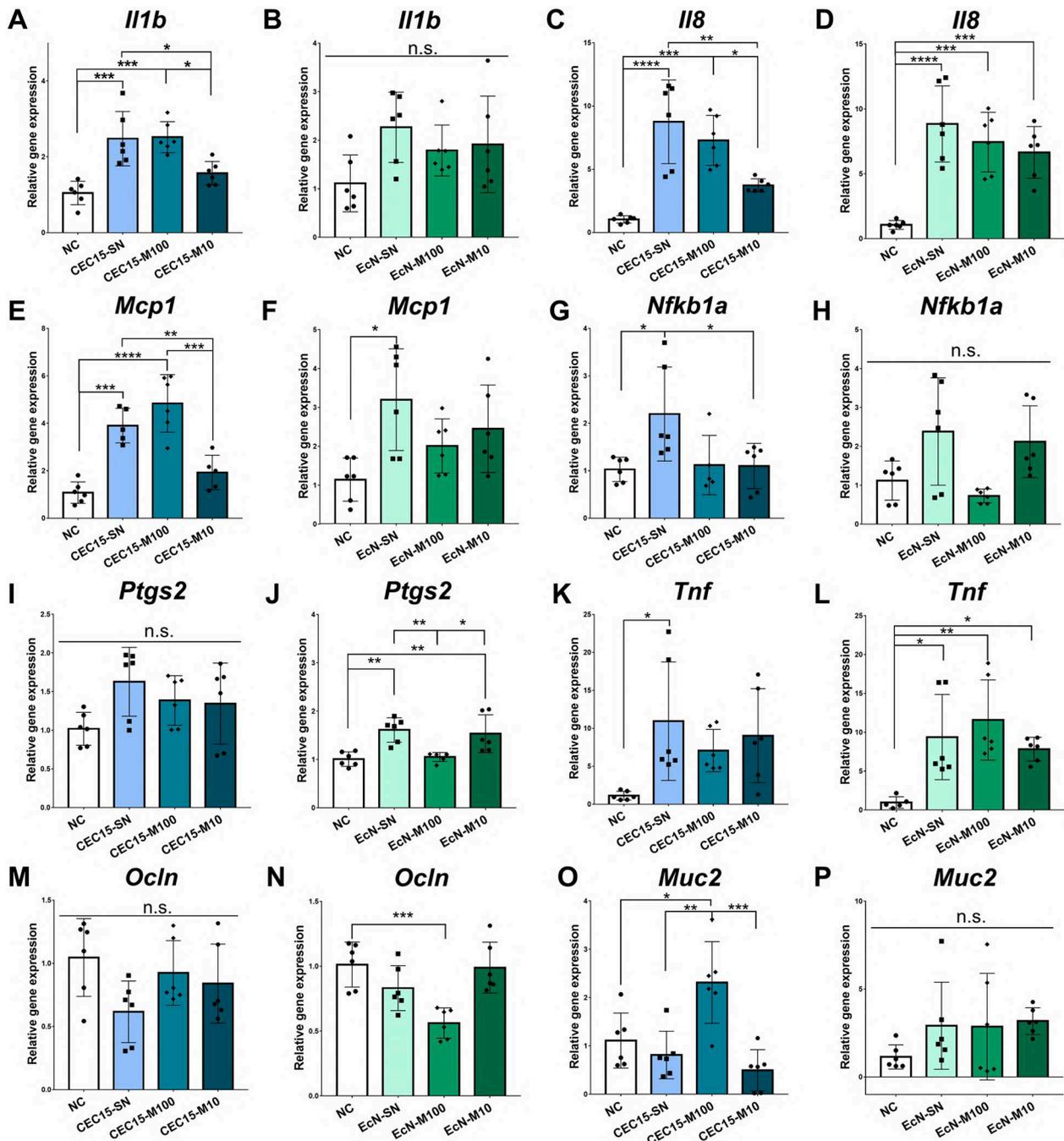


Fig. 6 Modulation of immunoregulatory and barrier-related genes expression in Caco-2 cells. The relative gene expression of genes related to immunomodulation and intestinal barrier (*Il1b* (A and B), *Il8* (C and D), *Mcp1* (E and F), *Nfkb1a* (G and H), *Ptgs2* (I and J), *Tnf* (K and L), *Ocln* (M and N), and *Muc2* (O and P)) on CEC15- and EcN-treated cells, respectively, were evaluated with the *Gapdh*, *B2m*, and *Hprt1* genes as reference ($2^{-\Delta\Delta ct}$). Statistical analyses were performed by One-way ANOVA with Tukey's post-test on GraphPad Prism 7.0. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$. NC: negative control; CEC15-SN: CEC15 supernatant; CEC15-M100: CEC15 treatment at MOI 100; CEC15-M10: CEC15 treatment at MOI 10; EcN-SN: EcN supernatant; EcN-M100: EcN treatment at MOI 100; EcN-M10: EcN15 treatment at MOI 10

infiltration of inflammatory neutrophils was quantified by monitoring the MPO activity. This infiltration was prevented by consumption of CEC15 (Fig. 7C). 5-FU

administration also affected the height of villi and the crypts depth (Fig. 7F-G). The treatments with CEC15 and EcN were able to reduce the damage on the villi height

(Fig. 7F), but only the CEC15 treatment was able to prevent reduction of crypts depth (Fig. 7G).

CEC15 led to increased microbial diversity in healthy animals and reduced dysbiosis in 5-FU-induced mucositis.

We evaluated how the *E. coli* strains modulated the gut microbiota, both in a healthy context, and in the context of 5-FU-induced mucositis. In that aim, feces collected on the last day of the animal experiment above mentioned were analyzed by 16S rRNA amplicon sequencing. The alpha diversity of the groups, represented by the Shannon index, showed statistical difference between the healthy and the mucositis groups (Fig. 8A). CEC15 presented a higher diversity than NC but showed no difference from EcN. 5-FU administration caused a loss of diversity in the intestinal microbiota, demonstrated by the difference between the NC and MUC groups. Interestingly, the treatment with CEC15 (CEC/MUC group) partially protected against loss of diversity. The analysis of the relative abundance in the groups at the phylum level (Fig. 8B) showed no significant difference on abundance in the healthy groups and an imbalance of the microbiota with increasing in abundance of *Bacteroidota* and *Proteobacteria* member in the MUC group samples compared to the NC. Although both CEC15 and EcN treatments reduced the abundance of *Proteobacteria* in the 5-FU groups, the impact of CEC15 was more prominent. The heatmap (Fig. 8C) shows the diversity on the microbiota in the genus level among samples and treatment groups, the genera with high abundance are *Parabacteroides*, *Clostridia*, *Lachnospiraceae*, and *Eubacterium*.

The twelve genera with the highest abundance were compared individually between groups (Fig. 9). Three genera belonging to the *Firmicutes* phylum did not present any significant difference among groups (*Clostridia* UCG-014, panel A; *Oscillibacter*, panel B; *Blautia*, panel C). *Escherichia* (panel D) is the only genus that present significant difference between NC and MUC groups ($p=0.0356$), no difference was seen among the healthy groups. *Escherichia* abundance was the same between

NC and the CEC/MUC and EcN/MUC groups however, these two groups presented different abundance among themselves (CEC/MUC vs EcN/MUC, $p=0.0388$). Regarding *Parabacteroides* (panel E), CEC15/MUC and EcN/MUC showed a higher abundance in relation to NC ($p=0.0006$), while only EcN/MUC presented a higher abundance than EcN ($p=0.0007$), and MUC ($p=0.0373$). *Lachnospiraceae* (panel F) and *Colidextribacter* (panel G) presented similar pattern, with EcN showing difference from NC ($p=0.0142$; $p=0.0052$), MUC ($p=0.0079$; $p=0.0025$), and EcN/MUC ($p=0.0113$; $p=0.0044$). As for *Lactobacillus* (panel H), CEC15 presented a higher abundance than EcN ($p=0.0174$) and MUC ($p=0.0025$), in the *Eubacterium* genus (panel I) CEC15 presented difference with NC ($p=0.0408$) and MUC ($p=0.0352$). The *Butyrivococcus* genus (panel J) was increased in CEC and EcN, presenting difference to NC ($p=0.0004$; $p=0.0232$), MUC ($p=0.0006$; $p=0.0190$), and the treatments, CEC/MUC ($p=0.0060$ against CEC15), and EcN/MUC ($p=0.0005$; $p=0.0249$). The *Clostridia* vadinBB60 (panel K) group was modulated positively with the treatment with CEC15 on mucositis animals (CEC/MUC vs MUC, $p=0.0029$), which was not seen for the treatment with EcN. Finally, the *Anaeroplasma* genus (panel L) presented no difference among the healthy groups and the MUC group, in the CEC/MUC group, however, the number of OTUs was highly increase ($p<0.0001$ against all groups), the same was not observed for EcN treatment. These results showed the capability of CEC15 and EcN to modulate the gut microbiota composition, including some important genera in healthy animals and in the context of an inflammatory gastrointestinal disease.

Discussion

Although there are numerous probiotic strains available in the market, there is still a need for new strains with new or enhanced beneficial properties. It is crucial to note that such properties are specific to each strain, and as such, it is of utmost importance to thoroughly identify and characterize any potential new probiotic strain

(See figure on next page.)

Fig. 7 Clinical and histopathological aspects of *E. coli* strains' administration. Mice ($n = 8$) were administered either sterile PBS, CEC15 (10^{10} CFU/mice/day) or EcN (10^{10} CFU/mice/day) for 12 days with administration of 5-FU (300 mg/kg) or PBS on the day 10. The results above show the weight variation (day 10-13) (A) and the morphological characteristics, such as intestinal permeability (B) and tissue neutrophilic infiltration (C). The structural damage caused by the 5-FU administration and the partial protection promoted by CEC15, as well as the unmodified morphology on the control groups and of the EcN treatment after mucositis induction can be observed on the slides (D), dyed with hematoxylin and eosin (Magnification of 20X). The histopathologic inflammatory scoring based on villous atrophy, rupture of the surface enterocyte borders, depletion of calyceal cells, loss of crypt architecture, destruction of crypt cells, abscess formation in the crypts, infiltration of lymphocytes and polymorphonuclear cells, dilation of capillaries and lymphatic vessels, and thickening with edema formation in the submucosa and external muscle layers. Histological features were scored on a scale of 0 (average) to 3 (max damage), and points were summed for each animal accordingly (E), villus height (F), and the depth of the crypts (G) were measured from these slides. Statistical analyses were performed by One-way ANOVA with Tukey's post-test on GraphPad Prism 7.0. * $p<0.05$; ** $p<0.01$; *** $p<0.001$; **** $p<0.0001$. NC: negative control; CEC15: healthy CEC15-treated; EcN: healthy EcN-treated; MUC: mucositis control; CEC15/MUC: mucositis CEC15-treated; EcN/MUC: mucositis EcN-treated

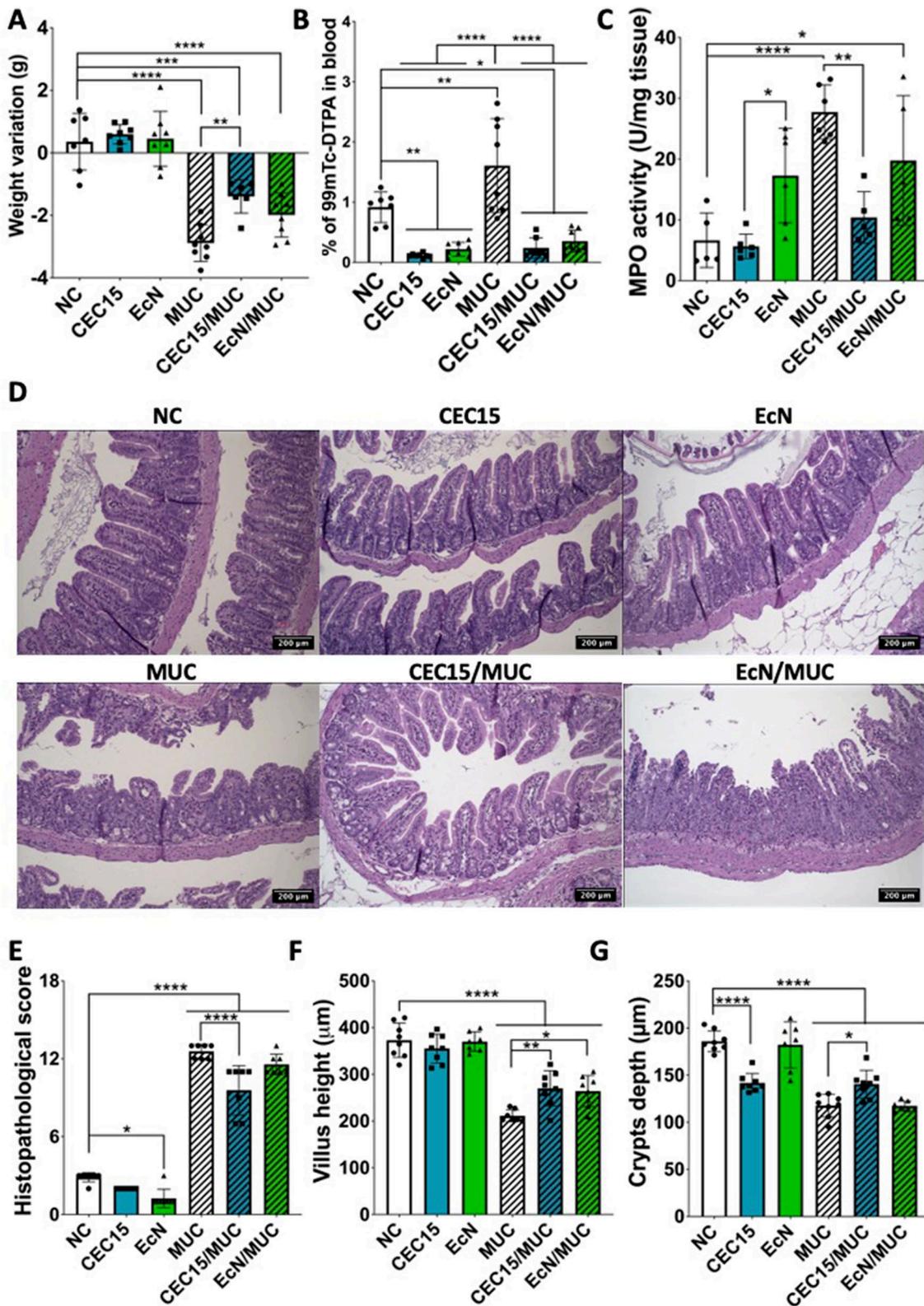


Fig. 7 (See legend on previous page.)

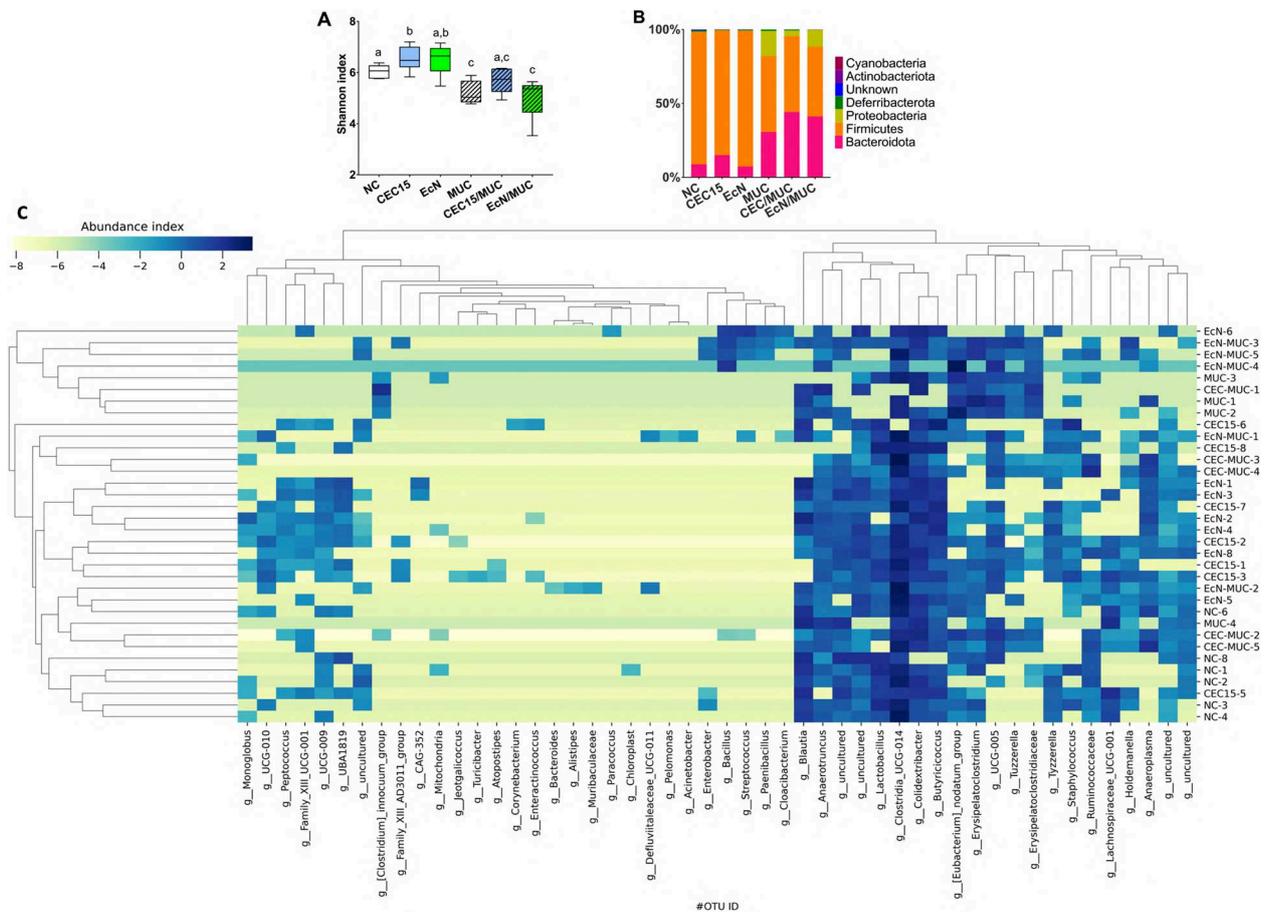


Fig. 8 Alpha diversity and abundance of OTU of intestinal bacteria. **A** Alpha diversity shown by Shannon index estimated for each group: NC: negative control ($n = 6$); CEC15: healthy CEC15-treated ($n = 7$); EcN: healthy EcN-treated ($n = 7$); MUC: mucositis control ($n = 4$); CEC/MUC: mucositis CEC15-treated ($n = 5$); EcN/MUC: mucositis EcN-treated ($n = 5$). **B** Relative abundance of intestinal microbiota at the phylum level among the groups. **C** Heatmap analysis of the bacterial genus distribution among the 34 samples based on hierarchical clustering. One-way ANOVA and Bonferroni multiple comparisons test. Different letters indicate significant differences, p value < 0.05

to determine the most beneficial ones [45]. The commensal *Escherichia coli* strain CEC15 has shown promising protective properties in a chronic colitis mouse model [37]. In the present study, CEC15 was assessed for other properties relevant to a probiotic bacterium, using a probiogenomics approach combined with *in vitro* and *in vivo* analyses. We tested its safety, its antibiotic resistance, the presence of pathogenic characteristics, tolerance towards gastrointestinal conditions, adhesion to intestinal cells, immunomodulatory properties, and protective effects in a 5-FU-induced intestinal mucositis mice model. Among the relevant features we identified for a probiotic, we can mention the absence of hemolytic activity, the presence of genes associated with antioxidant properties (e.g., biosynthesis of terpenoids) and ability to modulate the inflammatory process. While some beneficial properties are shared by both strains, others, which are of great value for a probiotic,

are specific to the CEC15 strain. In this study, we conducted a comprehensive comparison of CEC15, focusing on its probiotic attributes, with the well-established *E. coli* Nissle 1917, a probiotic strain with a notable history of application across various contexts. This comparative analysis aimed to gauge the relative efficacy of these two probiotics in conferring health benefits. The insights garnered from this comparison shed light on the underlying mechanisms that drive the beneficial properties of CEC15, underscoring the significance of such comparative investigations. While our current study primarily focused on assessing the specific characteristics of CEC15 that contribute to its probiotic properties, it is worth noting that there is room for further exploration in this field, involving comparing CEC15 with other *E. coli* strains, including commensal strains and those with established probiotic activities. Such studies could significantly enhance our understanding of the probiotic

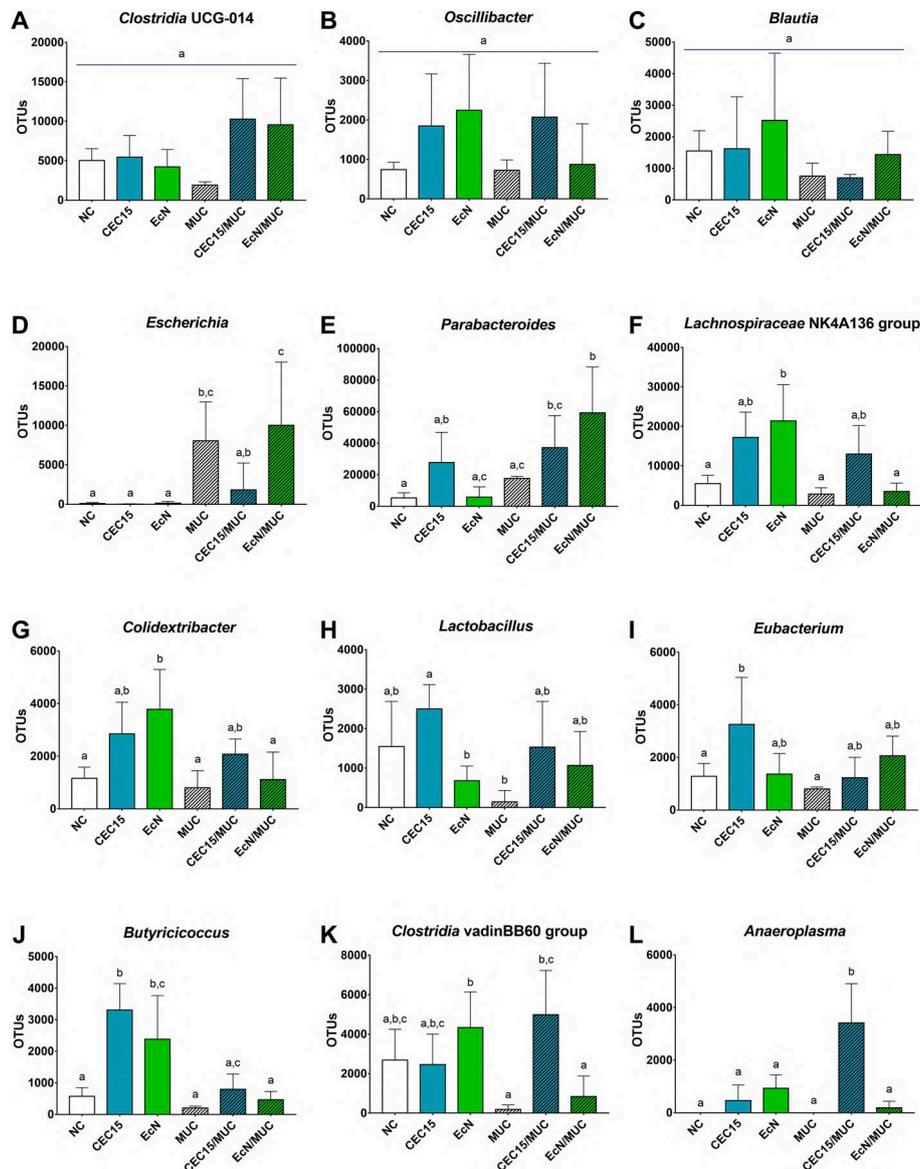


Fig. 9 Relative abundance of the main genus of fecal bacteria. **A–L** abundances of genus in the feces of mice in different groups. Data are expressed in absolute OTU reads (\pm S.E.M.). Different letters indicate statistical significance ($P < 0.05$; one-way ANOVA and Bonferroni multiple comparisons test). NC: negative control ($n = 6$); CEC15: healthy CEC15-treated ($n = 7$); EcN: healthy EcN-treated ($n = 7$); MUC: mucositis control ($n = 4$); CEC/MUC: mucositis CEC15-treated ($n = 5$); EcN/MUC: mucositis EcN-treated ($n = 5$)

mechanisms at play. Additionally, investigating CEC15 in comparison to pathogenic strains holds the potential to elucidate the critical distinctions between fitness and pathogenicity within the *E. coli* strains. Although these avenues of analysis hold substantial importance, it's essential to clarify that they serve as prospects for future research, rather than the primary focus of our current study, which aimed to comprehensively evaluate the probiotic properties of CEC15. Below, we delve into what we consider the most pertinent aspects of these properties.

The first, which can be highlighted, is a genomic one. *E. coli*, a versatile bacterial species presents in the intestinal tract of many vertebrates, as well as in the external environment, is characterized by a great genetic, genomic, and phenotypic diversity among the strains it encompasses [46]. The *E. coli* species, which includes commensal and pathogenic strains, is divided into seven phylogroups, including four major phylogroups A, B1, B2, and D [47–49]. Whole genome phylogenetic analysis classified the two probiotic strains CEC15 and EcN

into separate phylogroups. CEC15 clusters within the phylogroup B1, and EcN within B2. Among the *E. coli* phylogroups, the phylogroup B2 is the one most often associated with infections, especially urinary tract-related, and sepsis, followed by phylogroups A and D [50, 51], while members of the phylogroup B1 are more widely related to intestinal commensal bacteria of healthy animals [52]. In line with this result, CEC15 belongs to the O180:H14 serotype, which is mostly associated with non-pathogenic strains [53, 54], while the EcN serotype is O6:H1, a serotype often associated with pathogenic strains, especially enterotoxigenic *E. coli* (ETEC) and extraintestinal pathogenic *E. coli* (ExPEC) [55–57]. Although belonging to a phylogroup/serotype is not a safety indicator, it is nevertheless reassuring to note that CEC15 is phylogenetically close to commensal strains. Another important feature highly related to the phylogroup B2 is the presence of the *pks* island, allowing production of the genotoxic compound colibactin [58]. Auvray et al [59] isolated 785 *E. coli* strains from healthy bovines ($n = 418$), healthy humans ($n = 278$), and human sepsis ($n = 89$). Among those, 3%, 22%, and 39%, respectively, presented the *pks* island. On total, 42% of strains from the phylogroup B2 presented the *pks* island, while it was present in only 2% of strains from the phylogroup B1, from which none were isolated from human sepsis [59]. Interestingly, the CEC15 strain is devoid of *pks* island as well as of genes involved in colibactin synthesis. The *pks* island that was located on a chromosomal pathogenicity island 9 of strain EcN is present in various members of the *Enterobacteriaceae* family, particularly in *E. coli* and *Klebsiella pneumoniae* strains isolated from different sources, such as intestinal microbiota [60, 61], septicemia [62, 63], newborn meningitis [64], and urinary tract infections [65, 66]. These bacteria that produce colibactin are known to cause DNA damage and chromosomal instability in eukaryotic cells, leading to the senescence of epithelial cells and apoptosis of immune cells. Although many studies link the production of colibactin to the beneficial effect of the EcN strain, notably its anti-inflammatory effect [25, 26, 67–69], the absence of the *pks* gene cluster in strain CEC15 is an unambiguously advantageous feature exhibited by this promising probiotic.

Undesirable genetic traits such as virulence factors and antimicrobial resistances are often related to mobile genetic elements (MGEs) that can be acquired throughout adaptive evolution. The characterization of the mobilome of a probiotic strain, including phages, plasmids, genomic islands (GEIs), transposons, and insertion sequences (ISs), is therefore pivotal to evaluate its safety and to determine if its health-promoting benefits are acquired or intrinsic traits [70, 71]. Although GEIs were initially established in pathogenic bacteria, the

comparison of DNA sequences from different microorganisms, including an increasing number of complete genome sequence of commensal and probiotic bacteria, has shown that regions with characteristics of GEIs can also be found in many non-pathogenic bacteria [72]. CEC15 is no exception. However, when compared to strain EcN, CEC15 presents a lower number of transposases and GEIs, including pathogenicity islands (PAI), metabolic islands (MI), and resistance islands (RI). Sequence analysis showed that, in general, a significant proportion of the gene clusters found in GEIs code for functions that aid in the survival and propagation of the strains. Hence, these genes may confer a selective advantage to microorganisms carrying the islands, when exposed to stress, *in vivo* conditions, or to antibacterial substances, by enhancing microbial transmission, survival, or colonization within a niche [73]. The lower GEIs content does not confer a disadvantage for CEC15 as the number of proteins linked to adaptation and survival on CEC15 genome is close to what is found on EcN genome.

Typically, the CEC15 PAI contain genes related to bacteria-bacteria competition, type II and IV secretion systems and the production of flagella and pili, while EcN PAI are composed mainly by type II and VI secretion systems, a wide variety of transposases, adhesion related genes and the *pks* gene cluster. In the context of a pathogen, all these features would represent a better chance for this pathogenic organism to begin a disease process. On the other hand, here in the context of two beneficial bacteria, these features could allow CEC15 and EcN to compete against pathogenic bacteria and to colonize the environment, leading to better chances to beneficially modulate the host response [74].

In the process of assessing the safety of strains, in addition to virulence factors, particular attention is given to the presence of antibiotic resistance determinants and their potential mobility [75]. Here, a total of 44 and 45 potential antimicrobial resistance (AMR) related genes were found on the EcN and CEC15 genome, respectively. These include genes coding for potential resistance to fluoroquinolones, β -lactams, macrolides, glycopeptides, and aminoglycosides. Antibiotic susceptibility testing (disc-diffusion method) was performed to confirm AMR gene prediction. This analysis has shown that besides having the larger number of AMR genes associated to fluoroquinolones and tetracycline, both strains were sensitive to the tested antibiotics from these antibiotic classes. Both strains displayed resistance to two lincosamides, lincomycin and clindamycin, and the macrolide erythromycin. This result corroborates genomic data with the presence of efflux pump genes such as *acrAB-tolC*, *emrAB*, *mdfA*, *emrE*, *acrE*, and *emrB*. In addition, both strains were resistant to oxacillin. However, neither

strain presented the *bla* gene, associated with Extended-Spectrum β -Lactamase (ESBL). These enzymes can break down penicillin, cephalosporins (excluding cephamycin), and monobactams, but are not effective against carbapenems [76]. The *ampC* gene, on the other hand, was found on both strains and seems to be the responsible for Oxacillin resistance. Unlike ESBL, AmpC β -lactamase does not cause β -lactam resistance in wild strains [77, 78]. Finally, EcN was found resistant to kanamycin, while CEC15 was sensitive. Even if the presence of AMR genes is far from being wanted, AMR genes are detected into the genome of many commensal, food, and probiotic bacteria [79–84]. The presence of AMR genes is likely not a safety issue but can become when there is a risk of resistance transfer to other bacteria, notably to the human microbiota [75]. It has been proposed that if an AMR gene is found within 31 kb of an IS/transposon, it should be considered associated with the MGE, implying that it has the potential to be mobilized [85]. Among the AMR genes identified, few are at transposable distance from an IS in the genome of EcN ($n = 6$) and CEC15 ($n = 12$). Moreover, most of them are related to classes of antibiotics for which strains EcN and CEC15 are sensitive (fluoroquinolones and tetracycline). For genes likely involved in lincosamide and erythromycin resistance, only the EcN *tolC* and *ermE* genes could be transferable as close to an IS and within a GEI, respectively. This shows that, besides presenting a high number of AMR genes (44 in EcN and 45 in CEC15) a very low number is considered transferable, yet these genes are not the principal resistance gene related to a specific antibiotic class and, on CEC15, did not produce the phenotype.

Among the phenotypic different features identified between the two strains, the highest ability of strain CEC15 to tolerate acid and bile and to adhere to intestinal epithelial cells, two properties related to the survival and to the colonization of the human gastrointestinal tract (GIT), may be of great interest for a probiotic [45]. Indeed, *E. coli* has an impressive capability to endure low acidity levels and has various molecular mechanisms that facilitate this survival. The corresponding machinery can be expressed constantly, usually during a stationary phase, or triggered by different growth conditions [86]. We showed that strain CEC15 was more tolerant than strain EcN towards simulated gastrointestinal conditions and exhibited the highest survival rate during the intestinal phase. In the model here used, we applied a brutal change of pH from the initial to the gastric phase (pH 7 to pH 3) and then from the gastric to the intestinal phase (pH 3 to pH 7) whereas, *in vivo*, the pH would be much higher at the beginning of the gastric phase and then decrease slowly because of acidic secretions and gastric emptying. Therefore, the viability obtained with the INFOGEST

model is probably underestimated. Similar differences in stress tolerance among *E. coli* strains were already reported in simulated human digestive environment [87]. Notably it has been shown that differences in acid resistance of strains were a consequence of their glutamate decarboxylase activity [86, 87]. Genomic comparison revealed that the genetic potentials associated to acid-resistance, including the decarboxylation of glutamate (*gadA/B*, *gadC*, *gadE*, *gadX*), were almost identical between CEC15 and EcN. Future work will be needed to determine whether the level of acid resistance of strains is linked to the production of the GAD system, its activity, or other mechanisms.

In addition to the survival under gastrointestinal conditions, mucosal adhesion is also a critical step for the establishment of probiotic strains in the gut, which is commonly viewed as a necessary requirement [88]. Numerous bacterial factors have been shown to be involved in adhesion to host surfaces [89]. Among the molecules involved in *E. coli* adhesion, flagella and pili/fimbriae are key actors during the initial attachment to surfaces [90]. As mentioned before, some genes associated to PAI are not exclusively associated to pathogenic traits. For instance, the possession of genes responsible for producing pili, which are frequently found inside PAI, gives the bacterium an advantageous position in various environments. Clusters of genes coding for pili were found in PAI 1 (type II secretion system for pseudopilin *gsp*) and PAI 13 (outer membrane usher protein *pef*) of CEC15 and were found on PAI 2 (fimbrial usher protein), PAI 3 (type II secretion system for pseudopilin *gsp*), PAI 4 (type II secretion system for pseudopilin *gsp*), and PAI 16 (Fimbrial S/F1C cluster) of EcN. From the 84 adhesins found on CEC15 genome, 34% were fimbriae/pili proteins while on EcN these proteins corresponded to 30% (of 89 proteins). Fimbriae/pili-like surface appendages are clearly seen on CEC15 electron microscopy images, by contrast with EcN images. As for the proteins detected on the surface of the strains, 14% of CEC15 exclusive proteins were fimbriae proteins while for EcN exclusive proteins they correspond to 5%.

The type 1 fimbriae of *E. coli*, especially the *fim* fimbriae gene cluster found exclusively on CEC15, have been demonstrated to facilitate the process of adhesion to epithelial host cells and contribute to the colonization of the intestinal tract [91]. Nonetheless, it is known that fimbriae/pili are hard to detect through proteomic analysis due to their structure and, consequently, resistance to proteolysis, especially by trypsin [92–94], which could be by-passed with the western-blot analysis of the different fimbriae types. The most frequent proteins detected belong to the F1C fimbriae family, associated to biofilm formation and intestinal strains by commensal strains, in

special the EcN strain [95, 96]. According to Kleta et al., [95] F1C fimbriae, with H1 flagella also playing a role as bridges between EcN cells, as can be observed on the SEM images on Fig. 5, is the main protein responsible for adhesion capacities and the inhibitory effect against enteropathogenic *E. coli* (EPEC). The presence of these appendage-like proteins seen on the electron micrography and the detected proteins (notably type 1 fimbria chaperone FimC, type 1 fimbria D-mannose specific adhesin FimH, and type 1 fimbria minor subunit FimG and flagellin FliC) could correlate with the high adhesion of CEC15 to Caco-2 cells when compared to EcN. While the adhesion ability of probiotics to the host does not guarantee a health benefit, this interaction could lead to transient or permanent colonization, which may enhance their effects and hinder pathogen growth through competitive exclusion and bacterial antagonism mechanisms [97, 98]. Both a high survival rate, which could lead to many viable bacterial cells in the GIT, and a strong ability to attach to intestinal cells can be key factors enabling CEC15 to exert its probiotic activities *in vivo* and confer health benefits.

Bacterial components and metabolites of CEC15 and EcN were compared regarding their potential to modulate Caco-2 cells genetic expression. CEC15 and ECN modulated the gene expression of key factors for immunoregulation and epithelial integrity. In the conditions here tested, supernatant and inactivated bacteria, were able to promote some degree of modulation in most of the genes tested, notably the increased expression of Interleukin 8 (*Il8*). *Il8* has multiple effects on neutrophils, including their recruitment, activation of their granule release, induction of superoxide generation, and enhancement of adhesion molecule expression [99, 100]. It has been shown before that EcN is able to increase expression of *Il8* in different human intestinal epithelial cell lines, including Caco-2 cells, and that this increase is related mainly to EcN's flagella [101], its capsule (K5) [102], and other unknown factors [103]. Both strains present similar *Il8* fold increase when stimulating with supernatant and inactivated cells at MOI 100 (~8-fold), where, yet, at low MOI only EcN was able to stimulate increased expression.

The Interleukin 1 β (*Il1b*) gene expression was increased under CEC15 supernatant and inactivated bacteria at MOI 100 stimulation while no modulation was observed for the EcN strain. In healthy condition, the production of *Il1b* acts on the production of monocytes/macrophages, mediating innate immunity training, and promotes mucus secretion, induces proliferation and surface coagulability in barrier cells [104], essential activities to promote protection against pathogens.

CEC15 was able to modulate alone a few genes involved in host defense. The mucin 2 gene (*Muc2*) was stimulated by co-incubation with CEC15 at an MOI of 100. On the other hand, the expression of the Occludin gene (*Ocln*) was slightly reduced by stimulation with EcN co-incubation at MOI 100. Mucins are a crucial component of the intestinal barrier that protects against pathogens, and they form a major part of the intestinal mucous gel layer [105]. Our findings are consistent with those reported for other probiotic bacteria, such as *L. acidophilus* [106], *L. plantarum* [107], and *Lactobacillus* GG [108]. In addition, rats treated with the VSL#3 probiotic formula have been shown to exhibit an increase in colonic mucin secretion [109]. This agreement suggests that increased expression of *Muc2* may be a protective mechanism allowing probiotics to enhance intestinal barrier function and prevent pathogen colonization. In addition to its ability to antagonize pathogens, increased mucin production has been shown to enhance intestinal barrier function and provide protection against aggressions from luminal content or environmental matter [110].

The chemokine Monocyte chemoattractant protein-1 gene (*Mcp1*) was induced by all conditions of CEC15 while only EcN supernatant had similar effect. *Mcp1* is crucial in the regulation of septic shock as it facilitates the production of reactive oxygen species and various cytokines, which are vital components of the immune response against bacterial infections that can cause septic shock by attracting monocytes and other immune cells to the site of infection. [111]. At the same time, the expression of Tumor necrosis factor (*Tnf*) was induced by ECN in all conditions but only by CEC15 supernatant. In various inflammatory disorders, including Crohn's disease (CD), TNF-alpha is known to play a crucial role in intestinal inflammation and induce increase in the permeability of intestinal epithelial tight junctions (TJ). This increase in permeability can exacerbate the inflammatory response in the gut [112]. *Tnf* increased expression could be related to the decrease on expression of *Ocln* mentioned above. The Prostaglandin-endoperoxide synthase 2 (*Ptgs2*) was slightly stimulated by EcN, yet not by CEC15, which has been associated with the development of colorectal cancer [113, 114]. In summary, this result shows a more protective profile regarding CEC15 effects on increased expression of barrier genes and modulation of the immune system by increasing *Il1b* and *Mcp1* while EcN co-incubation led to increased pro-inflammatory genes.

Finally, *in vivo* studies were carried out to confirm the safety and effectiveness of CEC15 as a probiotic strain. For that, we administrate daily both strains to healthy mice, at high dosage (10^{10} CFU/mice/day) and evaluated their

effects on the host and its intestinal microbiota after 13 days. To assess and compare their health effects, CEC15 and EcN strains were also tested in a mice 5-FU mucositis model. 5-FU is widely used as chemotherapeutic agent for the treatment of different types of cancer [115], it targets rapidly dividing and proliferating cells, effectively eradicating cancerous cells, and impeding their multiplication and division. Unfortunately, this process also affects healthy cells, especially those with a high proliferative rate, resulting in unwanted and detrimental side effects such as the intestinal mucositis [116, 117]. Therefore, chemotherapy impacts various aspects of the intestinal barrier, such as the mucus layer, epithelium, neuroendocrine feedback signaling, immune system, and gut vascular barrier [115]. These effects can lead to heightened immunological responses, increased intestinal permeability to toxins, and potentially facilitate the movement of bacteria from the gut into adjacent organs or the systemic circulation [118]. Given the critical role of the microbiota in sustaining a healthy gastrointestinal mucosa, a highly promising avenue for mitigating the adverse effects of 5-FU-induced mucositis could involve the utilization of probiotics, which hold significant potential in this regard [119]. In recent years, the significance of probiotics and their derivatives for the treatment of mucositis has been increasingly recognized [115]. This is underscored by the inclusion of probiotics in the guidelines of the Multinational Association of Supportive Care in Cancer and International Society for Oral Oncology (MASCC/ISOO) regarding mucositis management. The guidelines, which distill the most credible scientific evidence into practical clinical recommendations, indicate that probiotics containing *Lactobacillus* spp. could offer advantages in averting diarrhea induced by radiotherapy or chemotherapy in patients with pelvic malignancies [120]. Furthermore, in addition to the abovementioned, the reason for selecting this model is its high severity, which would enable the observation of notable protective effects promoted by any potential probiotic strains [7, 121, 122] and the fact that it is a well-established model [123–127]. Furthermore, it is crucial to emphasize the absence of studies assessing the impact of both EcN and CEC15 on the 5-FU-induced intestinal mucositis model, apart from two studies evaluating the effects of EcN supernatant [128, 129].

In healthy animals, with the criteria used, no detrimental effects were associated with the consumption of the two strains. We only observed a small reduction on crypt depth following the CEC15 administration, as previously observed when conventional and gnotobiotic rats colonized by CEC15 were compared [36]. Both strains were able to improve intestinal barrier and epithelial integrity as have been reported before [36, 37, 130–132]. Likewise, no significant variation in microbiota composition,

richness and diversity was observed following probiotic administration, even though the microbiota of CEC15-treated mice seemed closer to that of control mice than those treated with strain EcN. As expected, 5-FU administration led to a consistent inflammatory process in the ileum, which was characterized by excessive weight loss, increase in intestinal permeability, neutrophils infiltration, and an accentuated destruction of ileal epithelial structure, as it has been reported by many studies before [123, 124, 127]. No strain was able to prevent weight loss, a result that is not surprising given the aggressive nature of the 5-FU therapy. Nevertheless, CEC15, yet not EcN, partially prevented the weight loss, and such protection is known to depend on the probiotic strain, as has been observed with many other probiotics [125, 127, 133–138]. While both strains prevented both the increase of intestinal permeability and the decrease of villus height, CEC15 intervention specifically and significantly reduced the histological score that reflects the architectural damages of tissues caused by 5FU treatment. It further prevented decrease of crypt depth and increase of MPO activity, a biomarker of inflammation and oxidative stress.

The 5-FU administration also resulted in an imbalance of the intestinal microbiota, as evidenced by a decreased abundance of *Firmicutes*, yet increased abundance of *Bacteroidota* and *Proteobacteria* in mice. Metagenomics studies conducted in both experimental animal models and patients with intestinal inflammatory diseases have reported conflicting results, with some studies showing a decrease in the *Firmicutes* phylum [139, 140]. Interestingly, CEC15 administration showed a large reduction on *Proteobacteria* restoring with no alteration in the *Firmicutes/Bacteroidota* ratio. As for EcN there was only a slight restoration with even an increase on *Bacteroidota*. Moreover, the treatment with CEC15 in 5-FU-induced mucositis promoted a degree of protection showing no difference to the NC group in relation to the diversity of the samples. Both *Firmicutes* and *Bacteroidota* phyla have been negatively correlated with mortality and DAI score [141], suggesting that restoring their abundance may have played an important role in the protection of the intestinal architecture. Altogether, our results suggest that, as EcN, CEC15 is also a strain to be safely administrated in healthy conditions.

In the context of this work, the *Parabacteroides* genus, which saw an increase in levels with EcN treatment, has garnered attention for its association with host health. Recent reports indicate its decrease in conditions such as inflammatory bowel disease and obesity, underscoring its significance. Furthermore, *Parabacteroides* exhibits physiological traits related to carbohydrate metabolism and the production of short-chain fatty acids [142]. Notably,

Parabacteroides levels have risen in the EcN/MUC group in comparison to the control groups.

Conversely, several members of the *Eubacterium* genus are known for their butyrate production, playing pivotal roles in maintaining energy balance, regulating colonic motility, modulating the immune system, and suppressing inflammation within the gut [143]. *Eubacterium* species are also involved in transformations of bile acids and cholesterol, contributing to their regulation. Dysbiosis and altered representation of *Eubacterium* species have been linked to various human diseases [143]. Surprisingly, *Eubacterium* levels remained unaffected by 5-FU administration, and an increase was observed after treatment of healthy animals with CEC15, a phenomenon not observed with EcN. Additionally, the *Eubacterium* group, alongside the *Lachnospiraceae* NK4A136 group, are prolific producers of butyrate, a key compound involved in regulating gut inflammatory processes and immune system development [144]. Furthermore, the butyrate producing *Butyricoccus* has displayed its ability to prevent necrotic enteritis and reduce pathogen abundance in the cecum and ileum [145]. The treatments with CEC15 and EcN led to increased levels of *Butyricoccus* compared to the control and mucositis-treated groups. Also, working in conjunction with *Lachnospiraceae*, *Lactobacillus* serves as a crucial group of bacteria responsible for synthesizing short-chain fatty acids [146]. Notably, CEC15 exhibited higher levels of *Lactobacillus* members than the EcN and MUC groups.

Colidextribacter demonstrates a significant correlation with inflammation-related serum metabolites from gut microbes, suggesting its potential role in producing inflammatory metabolites [147]. Interestingly, this genus was only elevated by EcN administration. Research has pointed to the *Clostridia* vadinBB60 group's inverse correlation with obesity, dyslipidemia, and insulin resistance in a mouse model [148–151]. Its reduction has also been associated with elevated trimethylamine N-oxide levels and an increased risk of thrombosis [148–151]. Notably, CEC15 treatment positively modulated this group. Furthermore, it has been reported that *Anaeroplasm* is strongly associated with intestinal IgA and TGF- β secretion, playing a key role in regulating intestinal inflammation [152]. In this study, only the CEC/MUC group exhibited positive modulation of the *Anaeroplasm* genus.

Conclusions

All things considered, the commensal *E. coli* CEC15 has a potential as a probiotic strain, due to its ability to modulate the intestinal microbiota, provide protective and anti-inflammatory effects, and reinforce the intestinal barrier. The modulation of the microbiota, in especial

by CEC15, has led to positive modulation of SCFA and anti-inflammatory-related genera. The study suggests that the CEC15 strain is effective against an inflammation model of 5FU-induced intestinal mucositis, which could translate to a treatment for patients under similar conditions. However, further research is needed to evaluate the safety and effectiveness of the CEC15 strain in humans.

Methods

In silico analysis

Strain, growth, and DNA extraction

Two *Escherichia coli* strains were used in this work. We previously isolated the primo-colonizing *E. coli* CEC15 (CEC15) strain from freshly pooled fecal samples of 15-day-old suckling rodents [36]. The probiotic *E. coli* Nissle 1917 (EcN) strain was kindly given by professor Flaviano Martins from Federal University of Minas Gerais, Brazil. For DNA extraction, CEC15 was grown on Luria-Bertani (LB) medium (1% peptone, 0.5% yeast extract, and 0.5% NaCl) for 24 h at 37 °C under shaking conditions (150 rpm). Colony Forming Units (CFUs) were enumerated by serial dilutions in peptone water prior to spreading on top of solid LB medium added with agar. DNA was extracted using Wizard Genomic DNA Purification Kit (Promega, Wisconsin, EUA), according to the manufacturer's instructions. DNA was quantified using the nanodrop 2000 spectrophotometer (ThermoFisher, Massachusetts, EUA) and proceeded to sequencing.

Genome sequencing, assembly, annotation, and phylogenomic analysis

DNA sequencing was performed using the Illumina HiSeq platform, with a pair-end library of 2x151 bp and an insert size of 450 bp (Göttingen, Germany), and by the PacBio platform. The analysis of the quality of the reads was performed using the software FastQC (FastQC: a quality control tool for high throughput sequence data. Available online at: <http://www.bioinformatics.babraham.ac.uk/projects/fastqc>). Data from Illumina sequencing generated a Phred value of 39 for 6280224*2; thus, trimming was unnecessary. Sequenced from PacBio presented 122,634 reads and, after the Phred value was adjusted to 24, a new file was generated with 12238 reads with a range size of 500-24781 bp.

The assembly was performed *ab initio* with the SPAdes software (v. 3.15.3 [Python version: 3.5.2]) [153] with a hybrid assembly approach from the two sequencing platforms' results. A scaffold assembly was reached with 4,772,817 bp (four gaps) forming a chromosome, one contig of 201,163 bp representing the plasmid, and 26 contigs of 7588 bp also belonging to the chromosome; however, these 26 contigs were excluded from the analysis by an

assembly quality filter for being recognized as an artifact of low sequencing coverage and without significant similarity by BLAST analysis to any *E. coli* genome deposited on NCBI database (April 6th, 2022). MOB-suite software [154] was used to classify contigs from the chromosome and the plasmid. The chromosome scaffold had its origin fixed at the *dnaA* gene, with a total of 5 gaps, and the plasmid had its origin fixed at the *repB* gene. The remaining gaps were closed using the software GFinisher (v. 1.4) [155] based on contig assembled by the software EDENA (v.3.131028) [156]. In the end, we have a chromosome with 4,780,804 bp, with sequence coverage of 383.49-fold and GC content of 50.78%, and a plasmid of 200,825 bp, with sequencing coverage of 604.24-fold and GC content of 47.25%. The software CLC Genomics Workbench (v. 22) was used for the final mapping of reads resulting in 99.71% of reads mapped. The sequence was deposited on NCBI under the access numbers CP133657.1 (chromosome) and CP133658.1 (pCEC15).

The genome of CEC15 and EcN strains (NCBI access: CP058217.1 [chromosome], CP058218.1 [pMUT1], and CP058219.1 [pMUT2]) used in this study were automatically annotated by the Prokaryotic Genome Annotation Pipeline (PGAP-NCBI) [157–159]. Functional annotation was performed with EggNOG-mapper [160, 161]. The orthology between the two genomes was analyzed by the OrthoFinder tool [162].

Twenty-two *E. coli* strains were subjected to phylogenomic analysis. We added 20 strains representing the *E. coli* phylogroups A, B1, B2, C, D, E, and F, to the CEC15 and EcN strains. The phylogenomic tree was constructed with the phylogenomic tree tool from PATRIC (<https://www.patric.cbrc.org/app/PhylogeneticTree>) by the codon tree method. In this method, the orthologous genes were identified via annotation of Protein Global Families (PGFams) of PATRIC [163]. The sequences of protein were aligned by MUSCLE software [164], and the corresponding codon sequences were concatenated. The phylogenomic inference was realized via the RAxML program [165] with support values estimated by 100 fast bootstrapping runs [166]. The tree was visualized and edited with the tool iTOL (v.6.53) (<https://itol.embl.de/>).

Genomic islands prediction, transposases, and insertion elements

Prediction of Metabolic (MI), Resistance (RI), and Pathogenicity (PAI) islands in CEC15 and EcN strains was performed with the software GIPSY (Genomic Island Prediction Software, v.1.1.3) [167], using *Escherichia coli* O157:H7 str. Sakai genome (NC_002695) as a reference. Phage islands were predicted utilizing PHASTER tool (PHAge Search Tool Enhanced Release) [168, 169]. Visualization of the genomic island's map was performed with BRIG software (BLAST Ring Image Generator, v.

0.95) [170]. The annotation of insertion elements was done using the tool ISSaga (Insertion Sequence Semi-Automatic Genome Annotation) (<http://issaga.biotoul.fr/>) [39]. The serotyping of CEC15 was identified based on genes for specific O-antigen (O typing) and flagellin genes (H typing) with the SerotypeFinder 2.0 web tool hosted by the Center for Genomic Epidemiology (CGE) (www.genomicepidemiology.org). Data was curated manually and tabulated.

Antibiotic resistance genes

The identification of genes related to the resistance of antibiotic compounds in the genome of the CEC15 and EcN strains was performed by alignment to CARD (Comprehensive Antibiotic Resistance Database) [40], using the ABRicate (<https://github.com/tseemann/abricate>) software.

Bacteriocins, adhesin, stress response-related genes predictions, and metabolic profiling

Bacteriocins-coding genes were predicted with BAGEL4 (<http://bagel4.molgenrug.nl/>) [171]. The presence of adhesin proteins in the genomes of CEC15 and EcN was analyzed by SPAAN software (score>0.8) [44]. The identification of genes related to stress response (acid and osmolarity) was curated manually based on the protein function described on the UniProt database. Metabolic profiling was performed using the BlastKOALA tool (<https://www.kegg.jp/blastkoala>) [41].

In vitro assays

Survival under simulated gastrointestinal conditions

CEC15 and EcN strains were grown on LB medium for 16 h at 37 °C under shaking conditions, the cultures were then diluted 100-fold in Simulated Gastric Fluid (SGF) (KCl 6.9 mM, K₂HPO₄ 0.9 mM, NaHCO₃ 25 mM, NaCl 47.2 mM, MgCl₂ 0.1 mM, (NH₄)₂CO₃ 0.5 mM, and CaCl₂ 0.15mM, pH 3) and submitted to the INFOGEST *in vitro* simulation of gastrointestinal food digestion [172] with some modifications. In brief, diluted cultures were centrifuged at 5000 x g for 10 min at 4 °C, the supernatant was removed, and the pellet was washed twice with sterile PBS prior to centrifugation. The washed pellet was then resuspended in 10 mL of SGF, at this point, an aliquot of 500 µL was collected for CFU counting (T1). To simulate the digestion, 200 U/mL of porcine pepsin (Sigma-Aldrich, cat. no. P7012) were added. SGF was added to a final volume of 20 mL, and the tubes were incubated in a water bath at 37 °C with agitation at 60 rpm for 2 h. After the incubation period, another 500 µL aliquot was collected for CFU counting (T2), and the samples passed to the intestinal phase where 20 mL of Simulated Intestinal Fluid (SIF) (KCl 6.8 mM, K₂HPO₄ 0.8 mM, NaHCO₃ 85 mM, NaCl 38.4 mM, MgCl₂ 0.33 mM, and CaCl₂ 0.6 mM,

pH 7) was added. The pH of the solution was adjusted to 7 using 1N NaOH and, to simulate the intestinal environment, 10 mM of bile salts (Sigma-Aldrich, cat. no. B3883) and pancreatin (equivalent to trypsin activity of 100 U/mL) (Sigma-Aldrich, cat. no. P7545) were added. The tubes were again incubated, as previously, for 2 h, and a final aliquot was collected for CFU counting (T3). CFU quantification was performed on LB agar plates, incubated at 37 °C overnight before manually counting colonies. The results were expressed in % of survival to the initial CFU. The experiment was done in triplicate.

Antibiotic susceptibility

The susceptibility towards antimicrobials was performed using the Kirby-Bauer method (disk diffusion). For that, 250 µL of overnight culture (CEC15 and EcN) on LB medium were placed in a Mueller-Hinton agar plate and spread evenly with the aid of a sterile swab, the plate was left open to dry for about 10 min, and four antibiotic disks were placed in each plate. The plates were then incubated at 37 °C for 20 h, and the halo was measured with a millimetric ruler. The following classes, and their respective antibiotics (BIO-RAD, France), were tested: Penicilins: Ampicillin (AMP, 10 µg), and Oxacillin (OXA, 5 µg); Quinolones: Ciprofloxacin (CIP, 5µg), Chloramphenicol (CHL, 30µg), Norfloxacin (NXN, 10µg), and Nalidixic acid (NAL, 30µg); Macrolides: Erythromycin (ERY, 15 µg); Aminoglycosides: Gentamicin (GMI, 15 µg), Kanamycin (KNM, 30 µg), Streptomycin (SMN, 10 µg) and Tobramycin (TMN 10µg); Tetracyclines: Tetracycline (TET, 30 µg); Lincosamides: Lincomycin (LCN, 15 µg) and Clindamycin (CMN, 2µg); Phosphonic antibiotics: Fosfomicin (FSF, 50 µg); Glycopeptides: Vancomycin (VAN, 30 µg); and Ansamycin antibiotics: Rifampicin (RAM, 30µg). The results were analyzed according to Clinical and Laboratory Standards Institute (CLSI) and the European Committee on Antimicrobial Susceptibility Testing (EUCAST) standards for *Enterobacteriaceae*, when available, and expressed as susceptible, intermediate, resistant, and Area of Technical Uncertainty (ATU).

Hemolytic activity assay

For this assay, bacteria (CEC15 and EcN) were grown on LB medium overnight, and 10 µL of each culture were spotted in blood agar, supplemented with defibrinized sheep blood (5%), and incubated overnight at 37 °C. The strains *Staphylococcus aureus* BK and IT2 were used as α - and β-hemolytic strain control, respectively. The *S. aureus* was grown in BHI broth at 37 °C and 150 rpm overnight and 10 µL was spotted on the plate as described for *E. coli* strains. The results are expressed as α-hemolysis (presence of a greenish halo around the

bacteria), β-hemolysis (presence of a clear halo), and γ-hemolysis (no halo).

Adhesion assay in human colon carcinoma (Caco-2) cells

The human Caco-2 colon adenocarcinoma cell line (ATCC-HTB-37) was cultured in DMEM high glucose (DMEM-HG) medium supplemented with 10% (v/v) fetal bovine serum (FBS), 100 U/mL penicillin and 100 µg/mL streptomycin (P0781, Sigma-Aldrich). The cells were seeded in a 75 cm² flask at a density of 1x10⁴ cells/cm² and incubated at 37 °C and 5% CO₂ until reached 80% confluence. The cells were washed twice with phosphate-buffered saline (PBS) and detached with trypsin 0.25% for 5 min at 37 °C. Live cells were counted using the TC20 Automated Cell Counter (BIO-RAD) with trypan blue staining. A 12-well plate was prepared by seeding 7x10⁴ cells/well, and it was kept at 37 °C and 5% CO₂ for 21 days until differentiation. The culture medium was changed every 2 to 3 days for flasks and plates.

After 21 days of differentiation, the adhesion assay proceeded. For this, an overnight culture of *E. coli* (CEC15 and EcN) was diluted to 1% in fresh LB broth and incubated until reaching an optical density (OD_{600nm}) of 0.5 (≈ 2 x10⁸ CFU/mL). One mL sample of bacterial cultures was centrifuged at 6000 x g for 10 min, and the pellet resuspended in 1 mL of DMEM-HG medium without FBS, penicillin and streptomycin. One aliquot was collected to calculate the initial CFU. The Caco-2 monolayers were washed with PBS and incubated with DMEM-HG medium containing 2 x10⁸ bacterial cells corresponding to a multiplicity of infection of 100 bacteria for each Caco-2 cell (MOI 100). After 2 h of incubation, the monolayers were then washed extensively three times with PBS to remove unattached bacteria. Caco-2 cells and adherent bacteria were then detached by the addition of 0.5 mL of trypsin (0.25%) and incubated for 5-7 min. Trypsin was neutralized by adding 0.5 mL of DMEM-HG with FBS. The cell suspension was then centrifuged at 6000 x g for 10 min at 4 °C, and the pellet was resuspended in 1 mL of Triton 0.1% in water to detach bacteria from Caco-2 cells. Serial dilutions of the cells suspension were plated on LB agar and incubated overnight for counting of viable bacteria. Adhesion experiments were performed in triplicates and expressed as % of adhered bacteria to Caco-2 cells in relation to the initial bacterial CFU added.

Scanning and Transmission Electron Microscopy (SEM and TEM)

For scanning electron microscopy (SEM) observations, 16-hours-old CEC15 and EcN cultures in LB were filtrated through 0.22 µm pore size nitrocellulose filter

membrane, which were then cut into small pieces and placed into a fixation solution (2.5% glutaraldehyde, 100 mM sodium cacodylate). After 24 h, the filter pieces were transferred to a fresh solution of 0.25% glutaraldehyde and 100 mM sodium cacodylate. For SEM observations, the filters were removed from fixating solution, washed with fresh solution (0.25% glutaraldehyde and 100mM sodium cacodylate), dehydrated with ethanol (10, 25, 50, 75, 95, and finally 100%), CO₂ dried, and coated with gold. The filter membranes were examined and photographed with a JEOL JSM-7100F scanning electron microscope, operating at 10 kV.

For transmission electron microscopy (TEM) observations, 16-hours-old CEC15 and EcN cultures were centrifugated (5,000 x g, 5 min), and the bacterial pellets were resuspended in the above fixation solution. After 24 h, the fixation solution was removed, and the bacterial pellets resuspended in 0.25% glutaraldehyde and 100 mM sodium cacodylate. The pellets were post-fixed with 1% osmium tetroxide containing 1.5% potassium cyanoferrate and 2% uranyl acetate in water before gradual dehydration in ethanol (30% to 100%) and embedding in Epon resin. Thin sections (70 nm) were collected on 200-mesh copper grids and counterstained with lead citrate before the examination. Fresh non-fixated samples were also examined by TEM, where a glow-discharged formvar-coated copper EM grid was placed on a drop of bacterial culture for 1 min, blotted with a filter paper, placed on a drop of 2% uranyl acetate for 1 min, blotted again, and air dried. All samples (fixed and fresh) were analyzed with JEOL 1400 transmission electron microscope (JEOL Ltd.) operating at 120 kV.

Shearing of fimbriae proteins

Overnight still-grown cultures (37 °C and no agitation) were centrifugated at 10000 x g for 10 min and the harvested cells were resuspended in PBS at 1/100 the initial culture volume. Fimbriae proteins were sheared using a waring blender at maximum speed for 5 min, two aliquots were collected, before and after shearing, and centrifuged at 10000 x g for 30 min to remove cells and debris. The resulting supernatant was collected, the protein content was quantified and resolved on precast NuPAGE Bis-Tris gradient gels (4-12%, ThermoFisher Scientific) for profile verification.

Proteomic analysis

Three independent replicates of shearing-derived proteins (10 µg each) were separated on 12% home-made SDS-PAGE minigels (Miniprotean II, Bio-Rad) and stained with Coomassie-blue (BIO-RAD, France). In-gel trypsin digestion was performed as described before [173]. Peptides were identified by mass spectrometry as

described elsewhere [174], followed by protein identification (maximum e-value of 0.05) from the MS/MS spectra with the X!TandemPipeline software [175]. The peptides were searched against the genome sequences of the two strains described above with parameters as described before [176]. A minimum of 3 peptides per protein was necessary for the validation of the identification and a protein was only considered present when it was identified in at least two of the three replicates. The relative quantification of proteins was obtained by the Exponentially Modified Protein Abundance Index (emPAI) [177]. Proteins were categorized into Clusters of Orthologous Groups (COG).

Modulation of Caco-2 cells

Sixteen-hours-old CEC15 and EcN cultures in LB were diluted 10 and 100-fold and inactivated by heating at 60 °C for 1 hour. Inactivated cultures were centrifuged (5000 x g, 10 min) and the bacterial pellets were resuspended in 1 mL of DMEM-HG with FBS and antibiotics. The bacterial culture supernatants were prepared as follows. 1 mL of 16-hours-old CEC15 or EcN culture was centrifuged as described above, and the supernatant was filtered (0.22 µm pore diameter). Caco-2 cells were prepared as described above. For this assay, 6-well plates were prepared by seeding 1x10⁵ cells/well and incubated at 37 °C and 5% CO₂ for 21 days until differentiation. The media was changed every 2 to 3 days. On the day of the assay, the medium was removed, and cells were washed twice with sterile PBS. The PBS was then replaced by DMEM (control), DMEM containing inactivated bacteria at MOI 10 and MOI 100, DMEM + EVs at the concentration of 1x10⁹ and 1x10¹⁰ EVs/mL, and DMEM with bacterial culture supernatant (final dilution of 100-fold). The plate was incubated for 24 h at 37 °C and 5% CO₂. After incubation the supernatant was removed, and the cells were washed with PBS to remove the media and bacteria. The assay was performed in three independent experiments.

RT-qPCR assay

Total RNA was extracted using the RNeasy mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The cDNA was prepared from 1 µg of RNA using the qScript cDNA Synthesis Kit (Quantabio - Beverly, MA, EUA). The qPCR analysis was performed using the iQ SYBR Green Supermix (BIO-RAD - Hercules, California, EUA) according to the manufacturer for a final volume of 20 µL and run in the CFX96 Real-Time system Thermal cycler (BIO-RAD - Hercules, California, EUA) with the following program: 95 °C for 3 min, 40 cycles of 95 °C for 10 s and 60 °C for 30 s, followed by a melting curve 55 °C – 95 °C increasing 0.5 °C per cycle.

Data were analyzed by the $2^{-\Delta\Delta CT}$ method for the reference genes (*GAPDH*, *b2m*, and *Hprt1*). The list of primers used can be found in Additional file 18.

In vivo assays

Experimental design

Male BALB/c mice, 4-5 weeks old with specific pathogen-free (SPF) status were obtained from the “Biotério central” of the Federal University of Minas Gerais (UFMG). Mice were randomly divided into 6 groups (8 animals per group) and kept in a microisolator ($n = 4$ each) with a 12 h light/dark cycle, temperature of $25\text{ }^{\circ}\text{C} \pm 2$, and sterile filtered water and standard chow food *ad libitum*. The experiment was conducted in agreement with the Brazilian College of Animal Experimentation (COBEA) and approved by the Use of Animals Ethics Committee from UFMG (CEUA – UFMG) under the protocol 67/2021.

For 12 days, mice were gavaged with 300 μL of sterile PBS (negative control group [NC] and mucositis group [MUC]), of *E. coli* CEC15 (1×10^{10} CFU) (CEC15 control group [CEC15] and CEC15 treatment group [CEC15-MUC]), or *E. coli* Nissle 1917 (1×10^{10} CFU) (EcN control group [EcN] and EcN treatment group [EcN-MUC]). On the 10th day of experiments, the animals from the groups MUC, CEC15-MUC, and EcN-MUC received an intraperitoneal injection of 5-fluorouracil (5-FU, 300 mg/kg) to induce intestinal mucositis, while the other groups received injection of sterile PBS. On the last day of experimentation, to evaluate the intestinal permeability, all mice received by gavage 100 μL of a solution containing 18.5 MBq of diethylenetriamine penta-acetic acid labeled with technetium-99m ($^{99\text{m}}\text{Tc}$ -DTPA) showing radiochemical purity of 99.4% performed by chromatography on Wattman paper. After 4 h, all mice were euthanized by anesthetic deepening (300 mg/mL of ketamine and 30 mg/mL of xylazine) (Ceva, São Paulo, Brazil), the blood was collected for permeability assay, and the ileum was collected for the remaining analyses. Water and food consumption, as well as animal weight, were evaluated daily for the duration of the experiment.

Permeability analysis

The blood was weighed and placed in appropriate tubes to determine radioactivity levels using an automated gamma counter (PerkinElmer Wallac Wizard 1470–020 Gamma Counter; PerkinElmer Inc., Waltham, EUA). The results are presented as the percentage of the radiation dose, which was calculated by the % dose per gram of $^{99\text{m}}\text{Tc}$ -DTPA in blood following the equation:

$$\% \text{dose/g} = \frac{\text{cpm in gram of blood}}{\text{cpm of standard}} \times 100$$

where cpm = counts (of radioactivity) per minute.

Histopathological analysis

A section of approximately 4 cm of ileum was opened, washed with PBS to remove fecal matter, rolled up, and fixated with a 10% formalin solution. Later, tissue was embedded in paraffin, and sections of 4 μm were placed in microscope slides and stained with hematoxylin and Eosin (HE).

From each animal, 10 pictures from different tissue sections were collected using a BX41 optical microscope (Olympus, Tokyo, Japan) (20x). The pictures were blindly scored according to the system previously described by Howarth et al. [178], and the villus height and crypt depth (20 per animal) were measured with the assistance of the Image-J software (v. 1.51j.8 – NIH, Bethesda, MD, USA).

Neutrophilic infiltration assay

Neutrophilic infiltration was evaluated by detecting the myeloperoxidase enzyme activity (MPO assay) as described elsewhere [125]. Briefly, 50 mg of ileum were homogenized by maceration, centrifugated, and lysed by hypotonic solution, followed by three cycles of freezing in liquid nitrogen. After the last thawing samples were centrifugated and the supernatant was used for MPO assay (colorimetric). The assay absorbance was read at 450 nm and the results were expressed as MPO arbitrary units/ mg of tissue.

16S rRNA amplicon metagenome analysis

Total DNA was extracted from fresh pooled feces of mice collected on the day of the euthanasia. An average of 50 mg of feces was used and the DNA extraction was performed with the QIAamp DNA stool Mini kit (QIAGEN) following the manufacture’s instruction. Library preparation and sequencing were performed as described before [124].

The FASTQ files underwent quality filtering, involving the removal of truncated and low-quality reads (those with a Phred score < 20), which was carried out using Trimmomatic [179]. Subsequently, the forward and reverse paired reads were merged to form contigs. These sequences were then subjected to a series of processing steps, which included dereplication, sorting by abundance, removal of singletons, and filtering for chimeric sequences using mothur [180]. Following this preprocessing, the sequences were clustered into Operational Taxonomic Units (OTU) at a 97% similarity threshold and taxonomically assigned using QIIME2 [181], with the taxonomic assignments being based on a 97% sequence similarity to the SILVA database [182].

Statistical analysis

All *in vitro* experiments were done in triplicate while the *in vivo* experiments were performed with a technical

duplicate. The results are presented as the mean \pm the standard deviation. The *in vitro* and *in vivo* analysis were submitted to ANOVA test followed by the post-test of *Tukey*. The data of relative abundance of OTU were analyzed using ANOVA followed by Bonferroni multiple comparison test. The graphics were plotted on GraphPad Prism 7.0 where a *p*-value under 0.05 was statistically significant.

Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1186/s12866-023-03112-4>.

Additional file 1: Supplementary Table S1. Genomic Islands identified in the *Escherichia coli* strain CEC15 genome by Gipsy software.

Additional file 2: Supplementary Table S2. Prophages regions identified in the *Escherichia coli* strain CEC15 genome by PHASTER tool.

Additional file 3: Supplementary Table S3. Genomic Islands identified in the *Escherichia coli* strain Nissle 1917 genome by Gipsy software

Additional file 4: Supplementary Table S4. Prophages regions identified in the *Escherichia coli* strain Nissle1917 genome by PHASTER tool.

Additional file 5: Supplementary Table S5. Putative insertion sequence family predicted in *Escherichia coli* CEC15 genome using ISSaga tool.

Additional file 6: Supplementary Table S6. Putative insertion sequence family predicted in *Escherichia coli* EcN genome using ISSaga tool.

Additional file 7: Supplementary Figure S1. Schematic circular representation of CEC15 genomic features.

Additional file 8: Supplementary Figure S2. Schematic circular representation of EcN genomic features.

Additional file 9: Supplementary Table S7. Genes involved in resistance to antibiotic mechanisms in *Escherichia coli* CEC15.

Additional file 10: Supplementary Table S8. Genes involved in resistance to antibiotic mechanisms in *Escherichia coli* Nissle 1917.

Additional file 11: Supplementary Table S9. Metabolic profiling

Additional file 12: Supplementary Table S10. Proteins of *Escherichia coli* CEC15 and EcN with high adhesin profile probability

Additional file 13: Supplementary figure S3. Pre- and post-shearing protein profile of CEC15 and EcN strains.

Additional file 14: Supplementary Table S11. Proteins identification of pre-shearing and post-shearing samples of *E. coli* CEC15 and *E. coli* Nissle 1917

Additional file 15: Supplementary figure S4. Bacteriocins-encoding genes present in the genome of CEC15 and EcN strains.

Additional file 16: Supplementary figure S5. Relative gene expression of Caco-2 cells is unaltered by treated with either CEC15 or EcN strains

Additional file 17: Supplementary table S12. Number of OTUs per sample at the genus level.

Additional file 18: Supplementary Table S13. Primers used in this study

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Authors' contributions

Conceptualization, TdS, VA, GJ and EG; methodology, TdS, FA, EG, GJ, DD and VA; formal analysis, TdS, RG, TS, MA, AF, MV, LdJ, LP, ND, OM, MFC, JJ, AB, VC, SF, RP, RC, BB, EF, EG, CC, PL and FA; resources, YLL, VA, GJ and EG; writing-original draft preparation, TdS and EG; writing-review and editing, TdS, GJ, VA, and EG; supervision, GJ, VA and EG; funding acquisition, VA, SF, VC, GJ and EG. All authors have read and agreed to the published version of the manuscript.

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Availability of data and materials

The datasets generated and/or analyzed during the current study are available in the figshare repository, <http://doi.org/10.6084/m9.figshare.23657679>. The genome sequence of CEC15 was deposited on NCBI under the access numbers CP133657.1 (chromosome) and CP133658.1 (plasmid - pCEC15). Other data supporting the conclusions of this article are included in this published article [and its supplementary information files].

Declarations

Ethics approval and consent to participate

All procedures adhere to the ARRIVE guidelines (<https://arriveguidelines.org>), which govern the reporting of animal experiments, and were conducted in accordance with appropriate guidelines and regulations. The animal experiment was conducted in agreement with the 'Brazilian College of Animal Experimentation' (COBEA) and approved by the 'Use of Animals Ethics Committee' from Federal University of Minas Gerais - Brazil (CEUA - UFMG) under the protocol number 67/2021.

Consent for publication

Not applicable

Competing interests

The authors declare no competing interests.

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CHAPTER 4 - ORIGINAL PAPER.

POSTBIOTIC ACTIVITY OF *E. coli* CEC15 ON 5-FU-INDUCED MUCOSITIS

This chapter comprehends a original research paper on the effects of postbiotic versions of *E. coli* CEC15 and Nissle 1917 on a murine model of 5-FU induced mucositis.

Postbiotics, as non viable bacteria, are considered safer to use as there is no to minimal chance of infections and other negative effects caused by the administration of live bacteria, especially in immunocompromised individuals, children, and the elderly. Postbiotics comprises a vast number of factor which includes non-viable but intact bacteria (inactivated by heat, UV, or ultrasound), fragments of bacterial wall, secreted metabolites, cytoplasmic factors, and other by-factors. They are easy to produce and more stable than the viable bacteria for storage purposes. The main question to be asked is if the postbiotic will present the same or similar beneficial effect of the viable strain.

To this end, this paper makes a comparison between viable bacteria and their postbiotics (of *E. coli* CEC15 and Nissle 1917) in a murine model of mucositis induces by the administration of 5-FU. This paper shows that the effects of viable CEC15 is transferred to its postbiotics formulations, the same not occurring for *E. coli* Nissle 1917 strain as one of the postbiotic formulations performs better than the viable and the inactivated bacteria.

This comes to show that, when well characterized and tested, postbiotics can be a viable substitute for probiotics in the treatment of diverse diseases.

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1 Postbiotic effect of *Escherichia coli* CEC15 and *Escherichia coli* Nissle 1917 on a
2 murine model of 5-FU-induced intestinal mucositis

3
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34
35 **ABSTRACT**

37 Postbiotics are preparations of inanimate microorganisms and/or their components that confers a health benefit on the
38 host. The *Escherichia coli* strains Nissle 1917 (EcN) and CEC15 have shown beneficial effects, in their live form, in
39 murine models of intestinal inflammation, such as colitis and mucositis. However, the use of live bacteria, in special
40 by immunocompromised individuals, had raised concerns. Based on this, the present study evaluated the effects as
41 postbiotic of heat-inactivated, and cell-free supernatant preparations of EcN and CEC15 in attenuating 5-fluorouracil
42 (5-FU)-induced intestinal mucositis in mice and compared them with the probiotic effects of the live preparations.
43 BALB/c mice were fed daily, by gavage, with 10^{10} CFU of live or inactivated bacteria, or with 300 μ L of cell-free
44 supernatant for 12 days. On the 10th day, all animals, except for the control group, received an intraperitoneal injection
45 of 5-FU (300 mg/kg). After 72 h of 5-FU administration, animals were euthanized, and the ileum and blood were
46 collected for analysis. Treatments with live and heat-inactivated CEC15 mitigated weight loss, preserved intestinal
47 length, reduced histological damage, maintained goblet cells, decreased neutrophil infiltration, and modulated
48 expression of inflammatory and barrier genes, when compared to 5-FU mucositis controls. EcN showed more limited
49 effects. CEC15 upregulated mRNA expression of the mucin *MUC2* and tight junction protein *TJPI*. Overall, CEC15
50 demonstrated protective effects against 5-FU-induced mucositis, whatever the way it was administered: live bacteria,
51 heat-inactivated bacteria, or cell-free supernatant. This suggests CEC15 mediates a protective response via secreted
52 metabolites and does not require viability.

53

54 Keywords: *Escherichia coli*; Postbiotics; mucositis; 5-Fluorouracyl; CEC15; Nissle 1917

55

56 STATEMENTS AND DECLARATIONS

57

58 **Ethics approval and consent to participate:** The experiment was conducted in agreement with the Brazilian College
59 of Animal Experimentation (COBEA) and approved by the “Use of Animals Ethics Committee” from UFMG (CEUA
60 – UFMG) under the protocol 210/2022.

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73

74 **1. INTRODUCTION**

75

76 Probiotics are live microorganisms that, when administered in adequate amounts, can potentially bring health
77 benefits to the host [1]. Lactic acid bacteria (LAB), such as members of the *Lactobacillaceae* family, *Lactococcus*
78 spp., and *Streptococcus* spp., represent most probiotics found on the market today, together with *Bifidobacterium* spp.,
79 *Propionibacterium* spp., and the yeast *Saccharomyces* spp. Most of these organisms are found naturally in the human
80 gastrointestinal tract. Nonetheless, some Gram-negative bacteria have also been identified with beneficial effects on
81 the host [2].

82 One of the most studied Gram-negative probiotic bacteria is *Escherichia coli* Nissle 1917 (EcN). EcN is
83 considered safe and effective and has been approved by the FDA for treating acute diarrhea in children. Studies have
84 shown that EcN can produce various bioactive compounds that may have beneficial effects on human health. For
85 example, EcN can produce bacteriocins, small peptides with antimicrobial activity against many pathogenic bacteria
86 [3]. In addition, EcN can produce short-chain fatty acids, such as acetic acid, that promote a stimulatory effect on
87 intestinal motility [4], through the modulation of the gut microbiota, by colonizing the human gastrointestinal tract
88 (GIT), compete with other bacteria, and interact with host cells [5–7], and by modulating the diversity of the gut
89 microbiota by reducing the number of pathogenic bacteria, which may lead to improved gut function and overall health
90 [8–14].

91 Although EcN is generally considered safe, some concerns exist regarding its use in immunocompromised
92 patients. These concerns relate to characteristics suggested by EcN genome sequence and which may negatively affect
93 the health of these individuals, such as the toxin called colibactin, a potential genotoxic agent that can lead to DNA
94 damage in intestinal cells and increase the risk of colorectal cancer [15, 16]. Finally, some studies have suggested that
95 EcN may increase the production of pro-inflammatory cytokines in patients with inflammatory bowel disease (IBD).
96 Although the production of pro-inflammatory cytokines is a normal immune system response to infection, this
97 response can be exacerbated in IBD patients, leading to damage to intestinal tissue [17]. Therefore, there are concerns
98 regarding the use of EcN in IBD patients.

99 In 2013, *E. coli* CEC15 (CEC15) was isolated from the feces of a newborn rat during a study that sought to
100 identify bacteria with probiotic potential [18]. This study showed that early colonization by CEC15 triggered an
101 adaptive response in the colonic epithelium of the mice, leading to the remodeling of the epithelial tissue, thickening
102 of the mucus layer, and affecting ion and water transport. Colonization by *E. coli* CEC15 had a long-term effect on
103 the colonic epithelium, leading to a shift in intestinal homeostasis toward a new equilibrium state [18].
104 Since then, new studies have sought to characterize this strain by evaluating its beneficial potential *in vivo* in murine
105 models of intestinal inflammation. One work using gnotobiotic mice evaluated the effects of CEC15 colonization of
106 the gastrointestinal tract, in comparison with the commercial EcN strain. CEC15 colonization increased expression of
107 genes related to immunity and to defense against pathogens, mainly due to an increased production of mucin, a protein
108 that coats the intestinal epithelium and helps preventing pathogen entry, evidencing the potential of this strain in
109 modulating gut defense mechanisms [19]. More recently we evaluated and compared the protective effect of both
110 strains, EcN and CEC15, in a model of intestinal mucositis induced by the drug 5-Fluorouracil (5-FU) in mice [20].

111 This model presents high severity in the inflammatory process and allows a better evaluation of probiotics' anti-
112 inflammatory effects [21]. We demonstrated the preventive beneficial issue of CEC15 in this acute inflammatory
113 model [20].

114 Recent research emphasizes the effect of various bacterial components, suggesting that live microorganisms
115 are not required for beneficial action [22]. Furthermore, the use of killed microorganisms (also named paraprobiotics
116 or ghost probiotics) and of their secreted products (also named metabiotics) may offer safety advantages, reducing the
117 risk of infection and microbial translocation observed in immunocompromised individuals after probiotic
118 administration [22, 23]. Therefore, an alternative to the possible unwanted effects caused by probiotics is the use of
119 postbiotics [24]. A postbiotic is a preparation of inanimate microorganisms and/or their components that confers a
120 health benefit on the host. [25]. Because they are inactivated or inert components, postbiotics are considered safer than
121 probiotics given the concerns related to the use of live microorganisms in specific patient populations, including
122 immunosuppressed individuals [26]. These preparations can significantly reduce the risk of microbial translocation
123 and infections, are more stable than probiotics, and have a lower risk of associated antimicrobial resistance.

124 Some of the mechanisms associated with the efficacy of postbiotics include epithelial barrier enhancement,
125 immunomodulatory effects, and antimicrobial effects [27–29]. Currently, postbiotics are applied as a promising
126 treatment strategy for health conditions, especially in gastrointestinal disorders [30]. Therefore, their application
127 would be an efficient complement to probiotics and a driving force for developing a comprehensive health industry.
128 Here we evaluated the potential beneficial role of postbiotic preparations from *E. coli* CEC15 and Nissle 1917 strains
129 in the context of an acute intestinal inflammation, an *in vivo* intestinal mucositis induced by the cancer chemotherapy
130 drug 5-FU. For that, we compared the effect of live bacteria (as control), heat-inactivated bacteria, and cell-free
131 supernatant of both strains on the intestinal barrier and on inflammatory markers.

132

133 **2. MATERIALS AND METHODS**

134

135 **2.1. Animals**

136

137 Male BALB/c mice aged 6 weeks (n=8/group), acquired from the Central Animal Facility of the Federal
138 University of Minas Gerais (UFMG), were used. During the experiments, the mice were kept in mini-isolators in the
139 animal house of the Department of Genetics, Ecology and Evolution of the Institute of Biological Sciences (ICB),
140 UFMG, fed with standard mouse chow, water *ad libitum*, and a 12-hour light cycle. The experimental protocols used
141 in this study were performed according to the guidelines approved by the Ethics Committee on Animal Use (CEUA)
142 - UFMG (Protocol: 210/2022).

143

144 **2.2. Bacterial cultures and sample preparation**

145

146 For this experiment, both *E. coli* strains (Nissle 1917 and CEC15) were grown in LB medium with shaking
147 (150 rpm) for 18 h at 37 °C. To collect the supernatant, 30 mL of culture was centrifuged at 6000 x g for 15 min. The

148 supernatant was subsequently filtered using a 0.22 μm syringe filter and aliquoted into 300 μL samples, kept at -80
149 $^{\circ}\text{C}$ until use for the inactivated bacteria. The overnight culture was diluted to an optical density of 0.6 ($\lambda=600\text{ nm}$)
150 (OD_{600}), equivalent to 2×10^8 CFU/mL, and centrifuged at $6000 \times g$ for 15 minutes. The supernatant was discarded,
151 the pellet was washed three times with sterile PBS, and finally resuspended in 3 mL of PBS. The wash was then
152 incubated at $65\text{ }^{\circ}\text{C}$ for 1 hour to inactivate the bacteria, and after incubation, 300 μL samples were aliquoted. The
153 inactivated bacteria and supernatant were seeded on LB agar and incubated for 48 hours at $37\text{ }^{\circ}\text{C}$ to confirm sterility.
154 The live bacteria doses were prepared daily throughout the experiment. For that, overnight culture was diluted to an OD_{600} of
155 0.2, to a final volume of 30 mL, and grown until reach an OD_{600} of 0.6 and centrifuged at $6000 \times g$ for 15 minutes. The
156 supernatant was discarded, the pellet was washed three times with sterile PBS, and resuspended in 3 mL of PBS. The
157 doses were aliquoted into microtubes in 300 μL volumes.

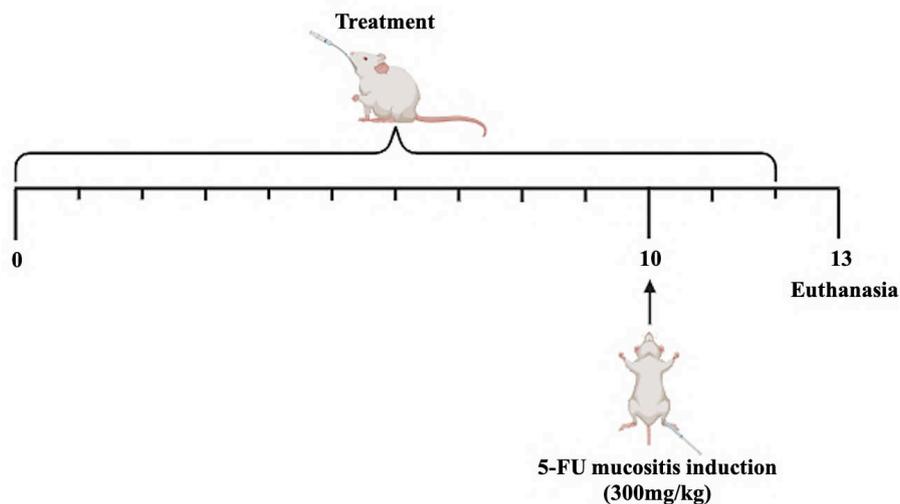
158

159 2.3. Experimental model and animal groups

160

161 The animals were randomly divided into eight experimental groups as described below: Ct- (negative
162 control), MUC (induction of mucositis by 5-FU without treatment), EcN (treated with live EcN and induction of
163 mucositis by 5-FU), EcNi (treated with heat-inactivated EcN and induction of mucositis by 5-FU), EcNs (treated with
164 supernatant of EcN culture and induction of mucositis by 5-FU), CEC (treated with live CEC15 and induction of
165 mucositis by 5-FU), CECi (treated with heat-inactivated CEC15 and induction of mucositis by 5-FU), CECs (treated
166 with culture supernatant of CEC15 and induction of mucositis by 5-FU). Over 12 days, all animals received daily by
167 gavage 300 μL of live, inactivated bacteria or supernatant according to the corresponding group, except for the Ct-
168 and MUC-groups that received PBS. The experimental design is shown in Figure 1.

169



170

171

172 **Fig 1** Experimental design. The experimental groups received doses of live, heat-inactivated *E. coli* Nissle 1917 or
173 CEC15 or cell-free culture supernatant for 12 days. On the 10th day of experimentation, the animals received 300
174 mg/kg of 5-FU (or PBS) intraperitoneally and were euthanized after 72 hours.

175 The induction of intestinal mucositis in mice was based on the model previously described by Carvalho and
176 colleagues (Carvalho et al., 2017). The animals received an intraperitoneal injection containing 300 mg/kg of 5-FU in
177 a single dose for induction of inflammation 10 days after the beginning of the experimental period. Sterile PBS
178 administered by the same route was used as a control. After 72 hours after the induction of mucositis, the animals were
179 euthanized by deep anesthesia with ketamine + xylazine (270 and 90 mg/kg, respectively) to perform the analyses.

180

181 **2.4. Food/water consumption and weight change**

182

183 The feed was weighed on a semi-analytical scale during all the days of the experiment, and the water volume
184 was measured daily with the aid of a volumetric measuring cylinder to evaluate the food and water consumption. The
185 results were presented in two periods: before the induction of the disease (days 0-10) and after the induction of
186 inflammation (days 11-13), to verify if the proposed treatments can improve the clinical picture of low food and water
187 consumption caused by intestinal mucositis. The animals were weighed on semi-analytical scales every day of the
188 experiment to assess weight variation in grams (weight gain or loss before and after the induction of mucositis) in the
189 animals.

190

191 **2.5. Evaluation of intestinal permeability**

192

193 To evaluate the intestinal permeability of the animals, on the 13th experimental day, all the mice received by
194 gavage 100 μ L of a solution containing 18.5 MBq of diethylenetriaminepentaacetic acid radiolabeled with technetium-
195 99m (^{99m}Tc -DTPA) with a radiochemical purity of 99.4% performed by chromatography on Wattman paper. After 4
196 hours, all mice were euthanized, and blood was collected by axillary puncture, weighed, and placed in appropriate
197 tubes for radioactivity determination. The levels of radioactivity in the blood were determined using an automated
198 gamma counter (PerkinElmer Wallac Wizard 1470-020 Gamma Counter; PerkinElmer Inc.) The results are presented
199 as the percentage of radiation dose, which was calculated using the following equation:

200

$$\%DTPA/g = \frac{\text{cpm blood treatment}}{\text{cpm blood control}} \times 100$$

201

Where cpm represents counts of radioactivity per minute.

202

203 **2.6. Histological analysis**

204

205 After the euthanasia procedure, samples of the ileum from the animals in each group were collected for
206 histological analysis. The intestines were cut longitudinally, stretched with the serosal layer in contact with the filter
207 paper, and opened by the anti-mesenteric edge, removing all its contents without damage to the mucosa. The organs
208 were then placed on a flat surface, and they were rolled in a spiral with the mucosa facing inward to form rolls from
209 the distal to the proximal portion. Subsequently, the rolls were fixed by immersion in a 10% buffered formalin solution,
210 where they were kept until processed. The material was processed for paraffin embedding, and 4 μ m sections of each
211 sample were stained with hematoxylin/eosin (HE), and periodic acid–Schiff (PAS). The pathologist examined

212 microscopic slides, and a histopathological grading system evaluated mucosal and muscular lesions. Photographic
213 images were obtained using an Olympus BX43 microscope and a Q-Color 5 digital camera captured with Q-Capture
214 Pro 7 software (QImaging, BC, Canada).

215

216 *2.6.1. Histological score*

217

218 Histological score analyses were performed using the histological sections described above. The score
219 proposed by Howarth and collaborators was used [31] to evaluate the association of mucositis with histological
220 changes of the ileal mucosa so that the microscopic findings evaluated were villous atrophy, rupture of the surface
221 enterocyte borders, depletion of calyceal cells, loss of crypt architecture, destruction of crypt cells, abscess formation in
222 the crypts, infiltration of lymphocytes and polymorphonuclear cells, dilation of capillaries and lymphatic vessels, and
223 thickening with edema formation in the submucosa and external muscle layers. Each histological variable was scored
224 from 0 (average) to 3 (maximum damage), and based on the findings, the points were added for each animal. For
225 morphometric evaluation of the intestinal epithelium structure, 20 villi and 20 crypts were measured per animal. The
226 measurements were performed with the help of ImageJ software on images from random regions of the histological
227 section.

228

229 *2.6.2. Goblet cells counting*

230

231 Additionally, the quantification of goblet cells per crypt was established using histology images. Five images
232 were captured from random ileum sections for each animal. Goblet cell counting was conducted for the entire image
233 and presented as the goblet cell count per field.

234

235 **2.7. Myeloperoxidase (MPO) enzyme activity**

236

237 Myeloperoxidase enzyme activity was measured to determine the rate of neutrophil recruitment into the small
238 intestine. Fragments of the distal ileum of the animals were collected, weighed, and stored at -80 °C. After thawing, the
239 tissue was homogenized with ice-cold buffer (Buffer 1) (NaCl 0.1 M; Na₂EDTA 0.015 M, pH 4.7) (ratio: 1.9 mL/100
240 mg tissue) and centrifuged at 4 °C for 15 min at 10,000 rpm. Immediately afterward, the supernatant was discarded,
241 and the precipitate was submitted to hypotonic lysis by adding ice-cold 0.2% NaCl and 1.6% NaCl supplemented with
242 5% glucose (ratio: 1.5 mL/100 mg of tissue) to break up red blood cells. The samples were homogenized and
243 centrifuged again at 4 °C for 15 min at 10,000 rpm. The supernatant was discarded, and the precipitate resuspended
244 with buffer (Buffer 2) (NaH₂PO₄ 0.05 M; Hexadecyltrimethylammonium bromide (HTAB) 0.5%, pH 5.4) (ratio: 1.9
245 mL/100 mg tissue) and homogenized again at room temperature. Successively, 1 mL aliquots of the suspension were
246 transferred to 1.5 mL microtubes, frozen in liquid nitrogen, and thawed in water at room temperature alternately three
247 times. Subsequently, the samples were centrifuged at 4 °C for 15 minutes at 10,000 rpm, and the supernatant was
248 collected for the enzymatic assay. In a 96-well plate (Nunc-Immuno, MaxiSorp), 25 µL of the collected supernatant

249 was added along with 25 μ L of the 1.6 mM TMB substrate (3,3',5,5'-tetramethylbenzidine) (Sigma-Aldrich®),
 250 previously diluted in dimethyl sulfoxide (DMSO). Subsequently, the samples were incubated at 37 °C for 5 min, and
 251 then 100 μ L of 0.002 % hydrogen peroxide was added to each well and again incubated at 37 °C for 5 min. Soon after,
 252 100 μ L of 1 M H₂SO₄, whose function is to stop the reaction, was added. After this step, the absorbance was measured
 253 by spectrophotometry at a wavelength of 450 nm using a microplate reader (Bio-Rad model 450, Bio-Rad Laboratories,
 254 Hercules, CA, USA), and the results were plotted as enzyme units per milligram of tissue (U/mg).

255

256 2.8. Gene expression analysis of inflammatory and barrier markers

257

258 Quantitative PCR was carried out with the PowerUp™ SYBR® Green Master Mix (Thermo Fisher) on the ABI
 259 PRISM 7900HT Sequence Detection System (Applied Biosystems™) under the following steps: 95 °C for 10 min, and
 260 40 cycles of 95 °C for 15 s, and 60 °C for 1 min. The modulation of gene expression was performed for genes coding for
 261 the nuclear factor-kappa B (*NFKB1*), interleukin 1 beta (*IL1B*) and 17 alfa (*IL17A*), mucin 2 (*MUC2*), occludin (*OCLN*),
 262 tight junction protein 1 (*TJPI*), and claudin 1 (*CLDN1*). Gene expression results were analyzed following the $2^{-\Delta\Delta CT}$
 263 method using glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) and eukaryotic elongation factor 2 (*EEF2*) genes as
 264 endogenous references (Table 1).

265

Table 1. Sequence of primers used in this study

Gene	Primer sequence	Reference
<i>Gapdh</i> *	F: TCACCACCATGGAGAAGGC R: GCTAAGCAGTTGGTGGTGCA	[32]
<i>Eef2</i> *	F: TGTCAGTCATCGCCCATGTG R: CATCCTTGCGAGTGTCAGTGA	[33]
<i>Nfkb1</i>	F: GTGGAGGCATGTTCCGGTAGTG R: TCTTGGCACAATCTTTAGGGC	[34]
<i>Il1b</i>	F: CAACCAACAAGTGATATTCTCCATG R: GATCCACACTCTCCAGCTGCA	[35]
<i>Il17a</i>	F: GCTCCAGAAGGCCCTCAGA R: AGCTTCCCTCCGCATTGA	[36]
<i>Muc2</i>	F: GATGGCACCTACCTCGTTGT R: GTCCTGGCACTTGTTGGAAT	[37]
<i>Ocln</i>	F: ACTCCTCCAATGGCAAAGTG R: CCCCACCTGTCTGTAGTCT	[38]
<i>Tjp1</i>	F: CCACCTCTGTCCAGCTCTTC R: CACCGGAGTGATGGTTTTCT	[39]
<i>Cldn1</i>	F: TCCTTGCTGAATCTGAACA R: AGCCATCCACATCTTCTG	[40]

* Reference genes

266

267 2.9. Statistical Analysis

268

269 The test for the normality of the data was performed using the Shapiro-Wilk test. Data were evaluated using
270 one-way ANOVA followed by Tukey's post-hoc test (for parametric data) or Kruskal-Wallis test and further tested by
271 Dunn's test (for non-parametric data). The Mann-Whitney test was used to analyze food and liquid intake before and
272 after induction of inflammation by 5-FU. Spearman's test was used for correlation analysis. All data were performed
273 using GraphPad Prism 8.0 software (GraphPad Software, San Diego, CA, United States), p-value < 0.05. Results are
274 presented as mean \pm standard deviation.

275

276 3. RESULTS

277

278 3.1. *Escherichia coli* CEC15, as well as its inactivated form limited weight loss during mucositis

279

280 During the 10 days of treatment administration, there was no change in food and fluid intake, but a notable
281 weight gain in the Ct- (negative control – healthy animals) (1.2 ± 0.5 g), MUC (mucositis control – healthy until day
282 10) (0.8 ± 0.6 g), CEC (Live CEC15 treatment) (1.1 ± 0.2 g), CECi (Heat inactivated CEC15 treatment) (1.2 ± 0.7
283 g), and CECs (CEC15 cell-free supernatant treatment) (1.9 ± 0.5 g) treated groups was observed (Figure 2A). No
284 increase in weight gain was observed in the groups treated with EcN (Live EcN treatment) (0 ± 1.5 g) and its
285 derivatives EcNi (Heat inactivated EcN treatment) (0.5 ± 1 g) and EcNs (EcN cell-free supernatant treatment) ($0.4 \pm$
286 1.6 g) (Figure 2B). The treatment with 5-FU drastically decreased feed and water consumption (data not shown),
287 which consequently led to a significant weight loss ($-2.7 \text{ g} \pm 0.5$, $p < 0.0001$), while healthy animals (Ct- group) gained
288 weight ($1.0 \text{ g} \pm 0.09$) (Figure 2a and 2b). Treatment with EcN ($-2.1 \text{ g} \pm 0.71$, $p = 0.4208$), EcNi ($-2.0 \text{ g} \pm 0.64$,
289 $p = 0.2238$), and CECs ($-2.1 \text{ g} \pm 0.44$, $p = 0.2692$) could not prevent animal weight loss. In contrast, animals treated with
290 CEC ($-1.6 \text{ g} \pm 0.49$, $p = 0.0035$), CECi ($-1.5 \text{ g} \pm 0.34$, $p = 0.0004$), and EcNs ($-1.9 \text{ g} \pm 0.54$, $p = 0.0462$) showed limited
291 weight loss, when compared to the MUC (Intestinal mucositis control – no treatment) group (Figure 2c). However,
292 there was no significant difference when the different treatments were compared.

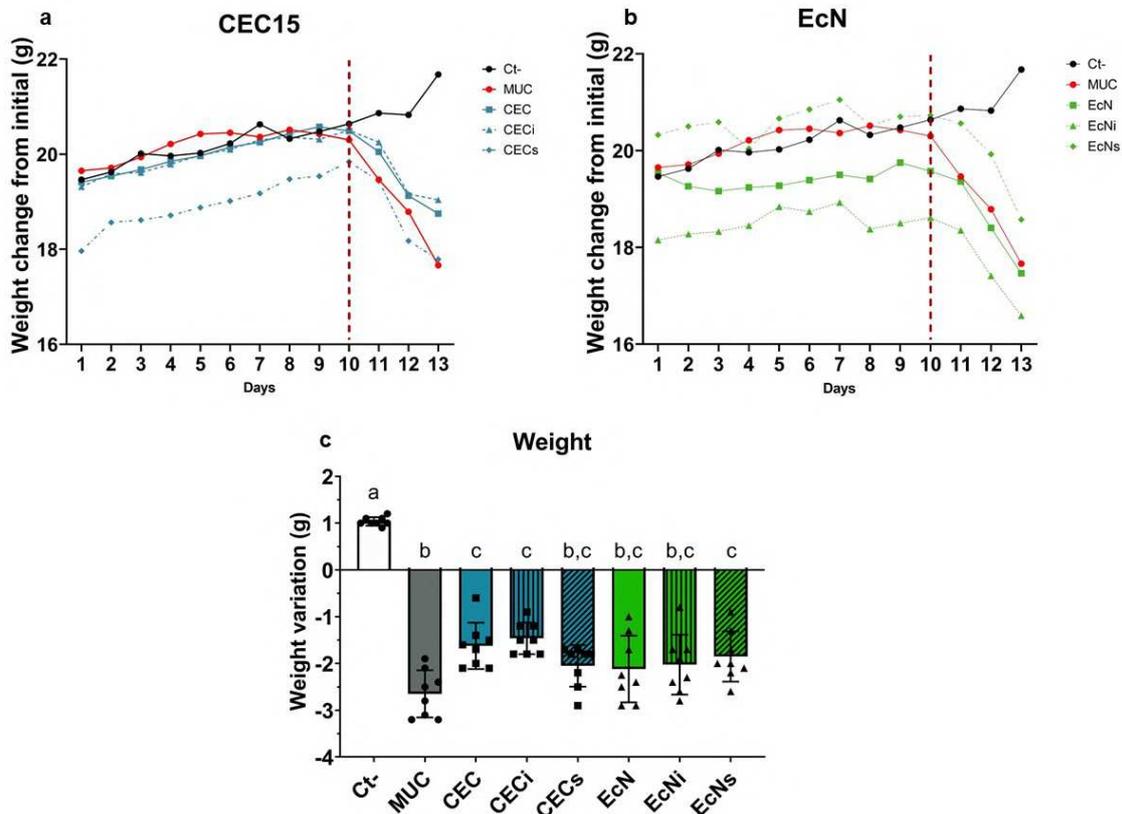
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294 3.2. *Escherichia coli* CEC15, as well as its postbiotic fractions, limited intestinal architecture damages

295

296 The effects of CEC15, EcN, and their derivatives on the intestinal structure were also evaluated at the macroscopic
297 and microscopic levels. The protective effect of the treatments on the intestinal mucosa can also be observed in the
298 histological images (Figure 3a), where we can see great destruction of the epithelial architecture of the ileum in the
299 MUC group and its preservation, more pronounced, in the groups treated with CEC15 and its derivatives. The
300 induction of mucositis led to a relevant shortening on small intestine length (MUC: $29.8 \text{ cm} \pm 2.03$, $p < 0.0001$)
301 compared with the control group ($36.7 \text{ cm} \pm 2.03$) with an average loss of 6.9 cm. The treatments with CEC (34.5 cm

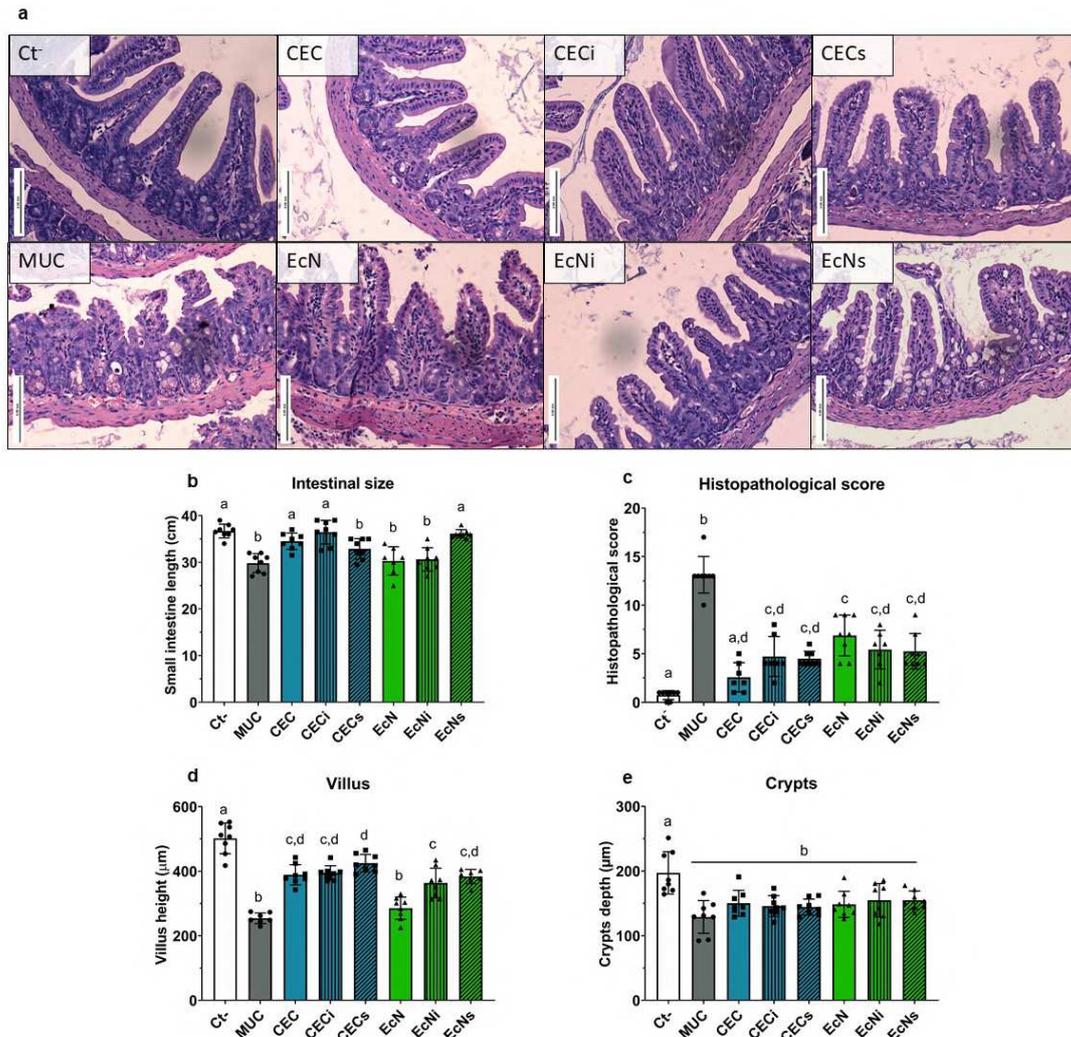
302 ± 1.75 , $p=0.0012$), CECi ($36.4 \text{ cm} \pm 2.54$, $p<0.0001$), and EcNs ($36.1 \text{ cm} \pm 0.88$, $p<0.0001$) maintained intestinal
 303 length (Figure 3b), preventing thus the shortening of the small intestine promoted by 5-FU administration. In contrast,
 304 EcN ($30.3 \text{ cm} \pm 3.04$, $p=0.9999$), EcNi ($30.6 \text{ cm} \pm 2.5$, $p=0.9943$), and CECs ($32.9 \text{ cm} \pm 2.18$, $p=0.0973$) did not help
 305 prevent small intestine shortening.



306 **Fig 2** Treatment and 5-FU-induced weight modulation. (a) CEC15 groups weight variation during 10 days of treatment
 307 and 3 days after 5-FU-induction of mucositis. (b) EcN groups weight variation during 10 days of treatment and 3 days
 308 after 5-FU-induction of mucositis. The dotted red line indicated the date of mucositis induction. (c) Weight change
 309 after mucositis induction by administering 5-FU. Statistical analysis was performed by 2-way ANOVA followed by
 310 a post-test of Tukey. Different letters indicated significant differences.
 311

312 All treatments reduced the damage caused by 5-FU in the intestinal mucosa, as can be observed by the
 313 histological score, which was preserved in all treatments (Figure 3c). Healthy animals presented a very low score (Ct-
 314 , 0.8 ± 0.46), which was increased by 5- FU administration (MUC, 13.1 ± 1.89 , $p<0.0001$). However, only the group
 315 treated with live CEC15 (CEC, 3.3 ± 2.38 , $p<0.0001$ to MUC) did not show significant differences compared with the
 316 healthy group. Furthermore, apart from the EcN group (6.9 ± 2.1 , $p<0.0001$ to MUC), the other treatments did not
 317 show difference between them (EcNi, 5.4 ± 1.99 ; EcNs, 5.3 ± 1.83 ; CECi, 4.7 ± 2.06 ; CECs, 4.5 ± 0.76 , $p<0.0001$ for
 318 all to MUC group). 5-FU treatment induced a considerable reduction in villus height (Figure 3d) (MUC, $254.3 \mu\text{m} \pm$
 319 16.56 , $p<0.0001$) and crypt depth (Figure 3E) (MUC, $128.9 \mu\text{m} \pm 25.33$, $p<0.0001$) when compared to healthy animals
 320 (Ct-, $501.8 \mu\text{m} \pm 47.5$ and $197.1 \mu\text{m} \pm 32.74$, respectively). Although no treatment prevented villus shortening, all

321 treatments (CEC, 389.0 $\mu\text{m} \pm 22.15$; CECi, 394.4 $\mu\text{m} \pm 22.54$; CECs, 425.1 $\mu\text{m} \pm 27.11$; EcNi, 363.6 $\mu\text{m} \pm 45.81$;
 322 EcNs, 383.4 $\mu\text{m} \pm 22.15$, $p < 0.0001$ for all), except for the EcN group (285.2 $\mu\text{m} \pm 34.45$, $p = 0.6164$), showed a
 323 reduction in shortening, with this protection being more pronounced in groups containing CEC15. Regarding the depth
 324 of the crypts, no group could prevent or reduce the reduction of the crypts (Figure 3e) (CEC, 150.2 $\mu\text{m} \pm 20.04$,
 325 $p = 0.5176$; CECi, 145.8 $\mu\text{m} \pm 16.06$, $p = 0.7764$; CECs, 144.2 $\mu\text{m} \pm 12.38$, $p = 0.8509$; EcN, 148.3 $\mu\text{m} \pm 20.19$, $p = 0.6304$;
 326 EcNi, 154.8 $\mu\text{m} \pm 25.80$, $p = 0.2726$; EcN, 154.9 $\mu\text{m} \pm 14.16$, $p = 0.2681$).



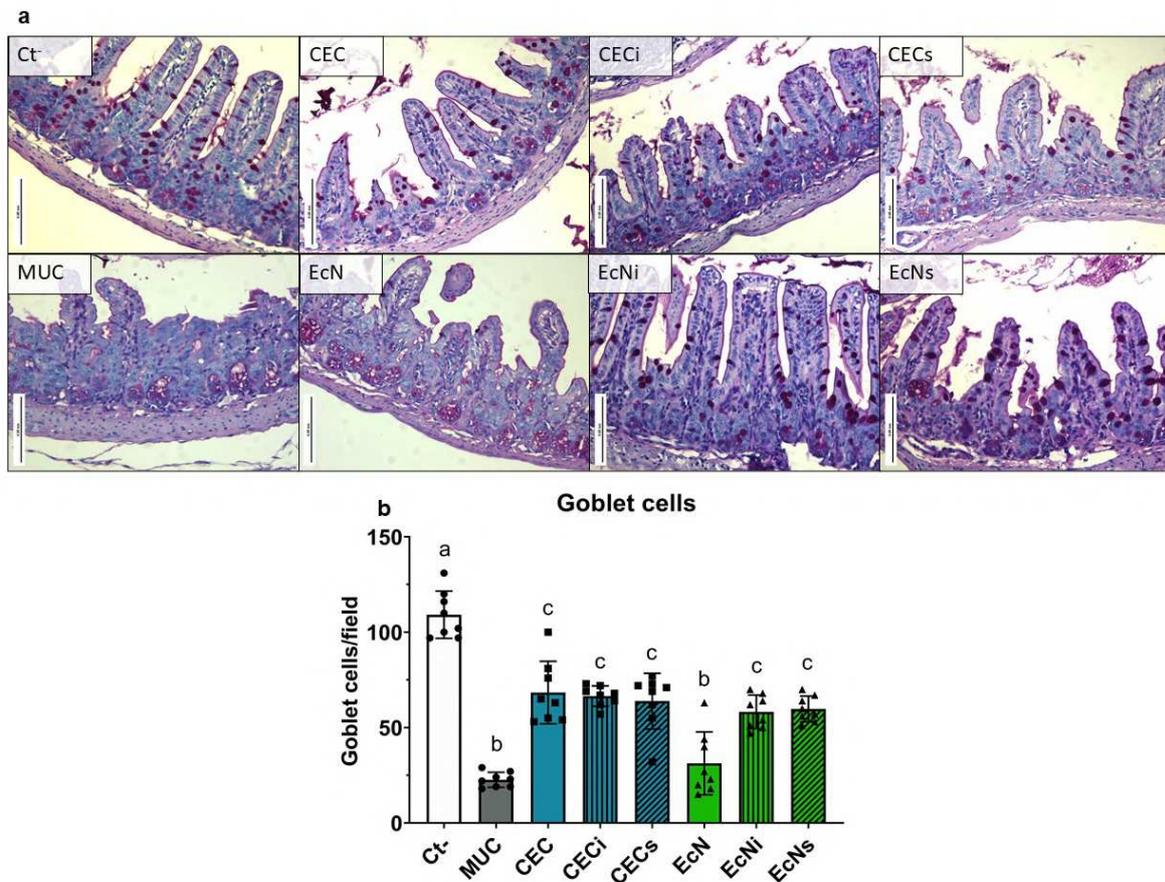
327
 328 **Fig 3** Modulation of macro and microscopic aspects of 5-FU-induced intestinal mucositis. (a) Histology of the iliac
 329 portion of the intestine stained in HE. (b) Modulation of intestinal length. (c) Histological score of the iliac portion.
 330 (d) Height of intestinal villus. (e) Depth of intestinal crypts. Villus height and crypt depth measurements were
 331 performed using ImageJ 1.51j.8 software (NIH, Bethesda, MD, United States). Statistical analysis was performed by
 332 2-way ANOVA followed by a post-test of Tukey. Different letters indicated significant differences.

333

334 **3.3. *Escherichia coli* CEC15, as well as its postbiotic fractions, restored goblet cells counts**

335

336 The variation in the number of goblet cells was evaluated in PAS staining slides (Figure 4a). Significant
 337 reduction on goblet cells' number was also observed in the 5- FU-treated (MUC: 22.6 ± 3.96 , $p < 0.0001$) ileum-
 338 inflamed mice compared to the Ct- group (109.1 ± 12.38). Goblet cell loss was minimized in mice treated with CEC15
 339 (CEC, 68.4 ± 16.39 , $p < 0.0001$) and its derivatives (CECi, 66.5 ± 5.32 , $p < 0.0001$; CECs, 63.9 ± 14.62 , $p < 0.0001$).
 340 Similar results were observed in groups treated with the postbiotic forms of EcN (EcNi, 58.3 ± 8.83 , $p < 0.0001$; EcNs,
 341 59.8 ± 6.76 , $p < 0.0001$), while the treatment with live EcN (31.3 ± 16.47 , $p = 0.8103$) was unable to alleviate this loss
 342 (Figure 4b).



343

344 **Fig 4** Goblet cell counting. (a) Histology of the iliac portion of the intestine stained in PAS. (b) Goblet cell count per
 345 field. Statistical analysis was performed by 2-way ANOVA followed by a post-test of Tukey. Different letters
 346 indicated significant differences.

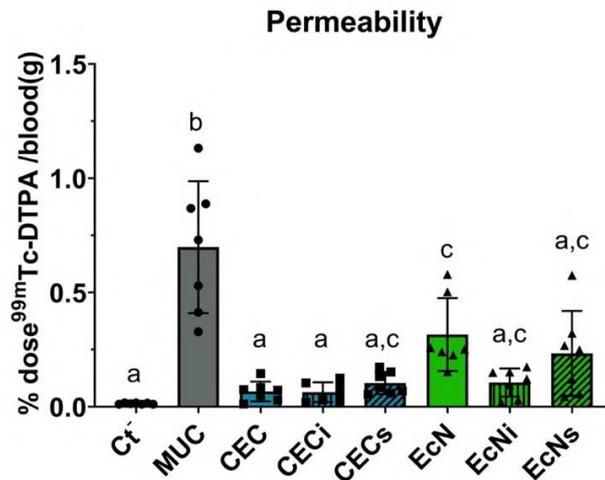
347

348 3.4. *Escherichia coli* CEC15, as well as its postbiotic fractions, limited gut permeability increase

349

350 The 5-FU administration triggered an increased intestinal permeability (Ct-, $0.014\% \pm 0.003$; MUC, 0.698%
 351 ± 0.289 , $p < 0.0001$). Although all tested treatments reduced the permeability caused by 5-FU (CEC, $0.067\% \pm 0.043$,
 352 $p < 0.0001$; CECi, $0.064\% \pm 0.043$, $p < 0.0001$; CECs, $0.103\% \pm 0.048$, $p < 0.0001$; EcN, $0.316\% \pm 0.159$, $p = 0.0001$;

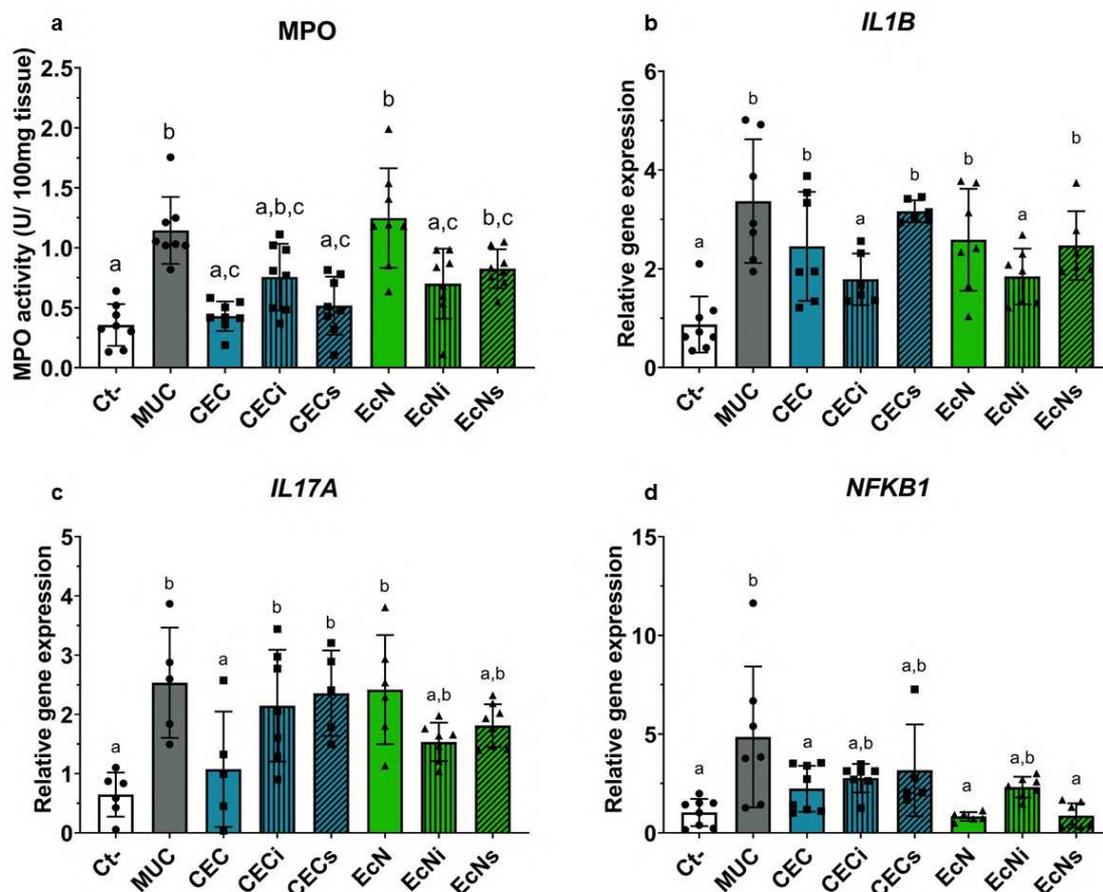
353 EcNi, $0.106\% \pm 0.062$, $p < 0.0001$; EcNs, $0.233\% \pm 0.185$, $p < 0.0001$), the EcN group was the only one that showed a
 354 significant difference with groups Ct- ($p = 0.0038$), CEC ($p = 0.0297$), and CECi ($p = 0.0266$) (Figure 5). Consumption
 355 of CEC and of its postbiotic fractions led to permeability values close to that of the control healthy group. Consumption
 356 of EcN only partially limited (5-FU-induced permeability increase).



357
 358 **Fig 5** Intestinal permeability. Serum ^{99m}Tc-DTPA dosage. Statistical analysis was performed by 2-way ANOVA
 359 followed by a post-test of Tukey. Different letters indicated significant differences.

360
 361 **3.5. *Escherichia coli* CEC15, as well as its postbiotic fractions, limited induction of gut inflammation**

362
 363 Neutrophil infiltrate, which is an important marker of the inflammatory process, was evaluated by monitoring
 364 myeloperoxidase enzyme activity (Figure 6a). Myeloperoxidase activity was highly increased by 5-FU administration
 365 (Ct-, 0.36 ± 0.17 ; MUC, 1.14 ± 0.28 , $p < 0.0001$) and was only reduced in the groups treated with CEC (0.43 ± 0.12 ,
 366 $p < 0.0001$), CECs (0.52 ± 0.24 , $p = 0.0003$), and EcNi (0.70 ± 0.29 , $p = 0.0250$), while the other groups did not differ
 367 from the MUC group (EcN, 1.25 ± 0.41 , $p = 0.9928$; EcNs, 0.82 ± 0.16 , $p = 0.2373$; CECi, 0.76 ± 0.28 , $p = 0.0748$)
 368 (Figure 6a). 5-FU administration also induced the expression of the pro-inflammatory related genes *Il1b* ($p < 0.0001$),
 369 *Il17a* ($p = 0.0021$), and *Nfkb1* ($p = 0.0006$) compared to the control group (Ct-). Modulation of these markers was also
 370 observed after treatment with both strains and their postbiotic derivatives. We noticed a reduction of *Il1b* mRNA
 371 transcription only in group CECi ($p = 0.0246$) and EcNi ($p = 0.0239$) (Figure 6b), while *Nfkb1* transcription was reduced
 372 in groups CEC ($p = 0.0471$), EcN ($p = 0.0009$) and EcNs ($p = 0.0006$) (Figure 6d). Finally, a decrease of *Il17a* expression
 373 ($p = 0.0477$) was only detected in the CEC group when compared to to MUC group (Figure 6c). Among the tested
 374 fractions, inactivated CECi and EcNi were the most efficient at reducing *Il1b* induction, while live CEC was the most
 375 efficient regarding *Il17a* expression.

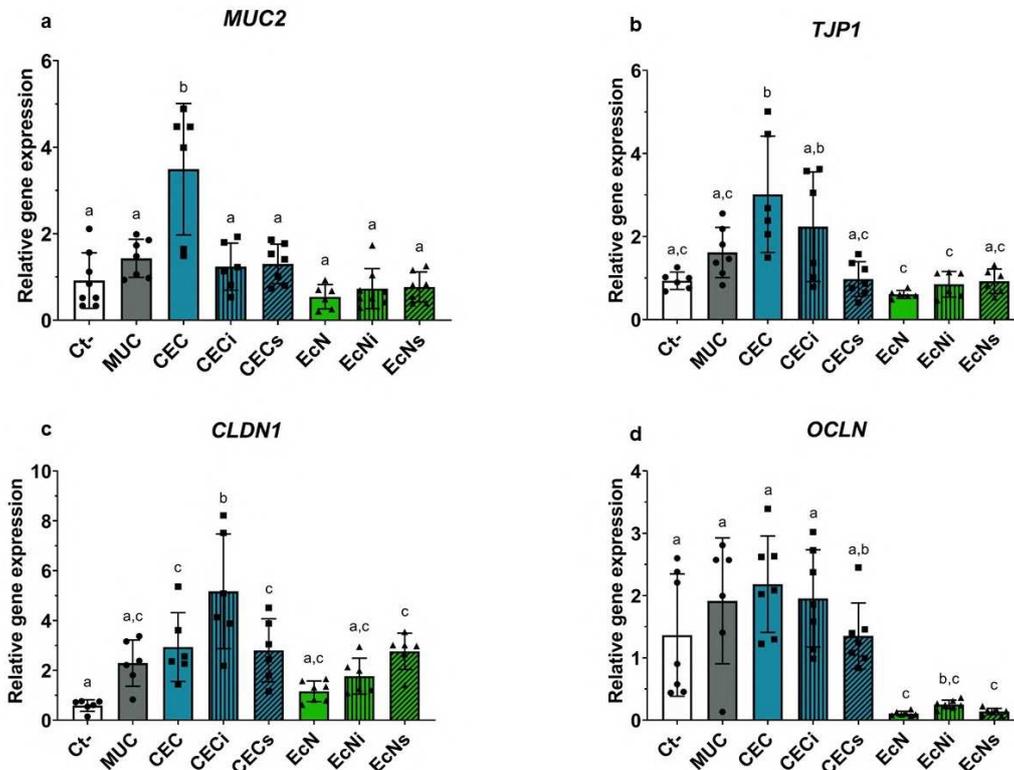


376
 377 **Fig 6** Modulation of inflammation by regulation of MPO activity and pro-inflammatory gene expression by CEC15,
 378 EcN, and their derivatives. MPO activity (a) and relative expression level of (b) *Il1b*, (c) *Il17a*, and (d) *Nfkb1* genes
 379 in all treated groups versus the Ct- control group. Statistical analysis was performed by 2-way ANOVA followed by
 380 a post-test of Tukey. Different letters indicated significant differences.

381
 382 **3.6. *Escherichia coli* CEC15, as well as its postbiotic fractions, modulated expression of proteins involved in gut**
 383 **barrier function**

384
 385 The expression of intestinal barrier genes (*Muc2*, *Tjp1*, *Cldn1*, and *Ocln*), which are closely related to
 386 intestinal mucosa integrity, was also evaluated. No significant variation in the expression of these genes was observed
 387 between the control group (Ct-) and the MUC group (Figure 7). Despite of this, among the treatments, there was a
 388 higher transcription of *Muc2* gene in the CEC group compared to the groups MUC ($p < 0.0001$), EcN ($p < 0.0001$), EcNi
 389 ($p < 0.0001$) and EcNs ($p < 0.0001$) (figure 7a). The *Tjp1* gene was also overexpressed in the CEC group compared with
 390 the groups MUC ($p = 0.0294$), EcN ($p < 0.0001$), EcNi ($p = 0.0002$) and EcNs ($p = 0.0004$). Although there was no
 391 difference between CECi and MUC groups, the CECi treatment enhanced the *Tjp1* expression when compared to EcN
 392 ($p = 0.0095$) and EcNi ($p = 0.0433$) groups (figure 7b). The *Cldn1* gene transcript was enhanced in the CECi group when

393 compared to the MUC ($p= 0.0023$), EcN ($p<0.0001$), EcNi ($p=0.0002$) and EcNs ($p=0.0176$) groups (Figure 7c).
 394 Finally, while all treatments with EcN and its derivatives downregulated *Ocln* expression, no difference in its
 395 transcription was observed between the Ct-, MUC, CEC, CECi, and CECs groups (Figure 7d).



396
 397 **Fig 7** Modulation of barrier gene expression by CEC15, EcN and their derivatives. Relative expression level of (a)
 398 *Muc2*, (b) *Tjpl*, (c) *Cldn1*, and (d) *Ocln* genes in all treated groups versus the Ct- (control group). Statistical analysis
 399 was performed by 2-way ANOVA followed by a post-test of Tukey. Different letters indicated significant differences.

400
 401 **4. DISCUSSION**

402
 403 Probiotics are considered essential tools in modulating inflammatory and infectious intestinal diseases, as
 404 well as a promising therapeutic alternative to alleviate symptoms of 5-FU induced mucositis [24]. Nonetheless,
 405 questions have been raised related to the safety of administering live bacteria in immunocompromised individuals and
 406 there is a constant search for solutions to this issue. The number of studies regarding the use of postbiotics, inactivated
 407 microorganisms and/or their metabolites, as an alternative to probiotics in these cases have risen with promising results
 408 [24, 41–43].

409 In this study, we have evaluated the postbiotic activity of formulations, heat-inactivated bacteria, and cell-
 410 free supernatant, derived from the *E. coli* strains Nissle 1917 and CEC15, in a murine model of 5-FU-induced intestinal
 411 mucositis. Beneficial activity of the two probiotic strains has been already established *in vivo* in various mouse models
 412 of inflammation [11, 14, 19, 44–46]. In particular, we previously compared the beneficial activity of both strains on

413 5-FU-induced intestinal mucositis in mice and showed the better protective effect of the CEC15 strain [20]. Here we
414 confirmed these results, notably in showing the specific effect of CEC15, compared to EcN, in the prevention of
415 animal weight loss, villous height reduction, goblet cell depletion, neutrophil infiltrate, intestinal permeability
416 increases, and inflammatory related genetic markers.

417 Mucositis is a global inflammation in the GIT, giving rise to various symptoms such as bleeding, diarrhea,
418 abdominal pain, fatigue, malnutrition, electrolyte imbalance, and infections. These symptoms can cause life-
419 threatening complications of cancer chemotherapy [47–49]. The cytotoxic effects of 5-FU on epithelial cells of the
420 GIT represent a major challenge for cancer treatment, as they impair the patient's ability to tolerate therapy,
421 consequently reducing the quality of life and influencing treatment success [50]. The inflammation caused by 5-FU,
422 which extends throughout the whole GIT, is highly associated with weight loss and reduced fluid and food intake.
423 This weight loss can occur either by reduced consumption or by decreased ability to absorb nutrients [48]. As expected,
424 the induction of intestinal mucositis with 5-FU reduced significantly the ingesta of food and liquid and, therefore,
425 induced a considerable weight loss on mice. Here, none of the treatments was able to maintain food and liquid intake
426 in face of 5-FU administration. This inability to attenuate the decrease of food and liquid intake has been reported for
427 other probiotic species in this animal model, like *Lactobacillus delbrueckii* [51, 52], *Lactiplantibacillus plantarum*
428 (formerly *Lactobacillus plantarum*) [21], and *Lacticaseibacillus rhamnosus* (formerly *Lactobacillus rhamnosus*) [42].
429 Among the four postbiotic formulations tested, only two, heat-inactivated CEC15 (CECi) and EcN cell-free
430 supernatant (EcNs), were able to attenuate weight loss. Similar results were found after administration of heat-
431 inactivated *L. delbrueckii* CIDCA133 were the weight loss reduced from 10% to 6.5% from the initial weight [41],
432 and with administration of heat-inactivated *L. rhamnosus* CGMCC1.3724, reducing weight loss from 2.4 g to 1.7 g
433 [42].

434 Looking further on the effects of 5-FU administration, changes in the architecture of the intestinal epithelium
435 are one of the commonly seen in the inflammatory process promoted in the intestinal mucositis [48]. We observed
436 that all postbiotic treatments could reduce the damage associated with the intestinal mucosa by maintaining the
437 integrity of the brush border of enterocytes and reducing the reactivity of the submucosa. The structural damage caused
438 by the administration of 5-FU led to changes in the height of intestinal villi, the deepening of the crypts, and the
439 reduction of goblet cells, whose main function is the production of mucin, a key component of the protective intestinal
440 mucus layer. These are parameters for assessing the severity of the damage associated with mucositis and are also
441 associated with the signs mentioned above [31, 53]. As expected, we could see a reduction on villus height and crypts
442 depth, and a depletion on the number of goblet cells after 5-FU administration. All postbiotic formulations derived
443 from both strains were able to mitigate the reduction of villus height and depletion of goblet cells. As reported before,
444 the effectiveness of live CEC15 was confirmed as well as the absence of protection from live EcN facing 5-FU
445 administration, the results here presented consolidate the CEC15 effect, as well as the effect of its postbiotic
446 preparations in the protection of intestinal epithelium structure, in special the inactivated CEC15. As for EcN
447 preparations, cell-free supernatant presented promising results, as it has already been demonstrated before [53] with
448 the heat-inactivated form showing some level of protection in a lower extent.

449 The damage caused to the intestinal mucosa by 5-FU is associated with increased intestinal permeability.
450 This last is associated with an increased risk of infection and transit of complex molecules, including toxins, through
451 the epithelium and promotes intestinal bleeding and fluid and nutrient loss. All treatments were found to prevent
452 increased intestinal permeability. Nevertheless, the permeability result is partially in accordance with the results on
453 gene expression modulation of barrier related genes. The groups with the lowest protection against increased
454 permeability (EcN and EcNs, also EcNi and CECs to a lesser extent) are also the ones with reduced expression of *Tjp1*
455 and *Ocln* genes, highly associated with the integrity of intestinal barrier. CEC15 groups, in special the CEC and CECi
456 ones, present the best results in both permeability and gene expression of barrier related genes with an over expression
457 of *Muc2* and *Tjp1* on the CEC group and of *Cldn1* on the CECi group. These results express the important role of
458 CEC15, live or inactivated, in the maintenance of intestinal barrier integrity, by elevating the expression of tight
459 junction proteins and the production of mucus, maintaining barrier integrity, and reducing the transit of toxic
460 compounds and microorganisms. Previous research has highlighted the enhancement of mucins and tight junction
461 proteins following treatment with heat-inactivated probiotics and/or their respective supernatants. An illustrative
462 example is the supernatant of mulberry leaf extract fermented by *L. acidophilus*, whose consumption induced
463 upregulation of *Muc2* and *Muc5ac* gene expression in 5-FU-inflamed mice [54]. Additionally, the EcN-derived
464 supernatant was found to mitigate the epithelial barrier disruption induced by enteropathogenic *E. coli*, in Caco-2
465 cells, by elevating the gene expression of tight junction proteins such as *Tjp1*, *Cldn14*, and *Cldn2* [55].

466 All preparations of both strains promoted a protective effect on the intestinal mucosa, reducing the damage
467 associated with mucositis. The infiltration of neutrophils in tissue is associated with inflammation as these cells are
468 recruited in response to tissue damage [56]. The enzyme myeloperoxidase is produced exclusively by neutrophils and
469 serves as an indirect measure of the number of cells present in the tissue [52]. MPO enzyme activity in injured tissue
470 was attenuated by all treatments with the CEC15-derived preparations, in contrast to what is observed with those
471 derived from the EcN strain. Similar results were seen after administration of postbiotics preparations of *L. delbrueckii*
472 CIDCA133 [41]. The lower levels of MPO indicates that the CEC15 preparations are more efficient than the EcN in
473 limiting neutrophil recruitment into the intestinal epithelium.

474 Mucositis is associated with altered expression of pro- and anti-inflammatory cytokine genes. These changes
475 generate greater activation of inflammatory pathways such as NFkB and TNF, loosening of the intestinal barrier, and
476 weakening the protection of the epithelium through the modulation of genes such as *Il1b*, *Il17a*, *Nfkb1*, among others
477 [24]. Here we have seen an elevation of the expression of these three genes after administration of 5-FU, we have also
478 observed a protection against *Il1b* by treatment with CECi and EcNi, and of *Nfkb* after treatment with EcNs in addition
479 to live CEC15 and EcN. Expression of *Il17a* was only reduced by administration of live CEC15. Other groups, like
480 EcNi and EcNs, have shown a tendency for protection (no difference to the Ct- group), however they did not present
481 statistical difference from the MUC group. The increase on *Il1b* expression has been correlated to increase on
482 permeability by degradation of the occludin mRNA *in vitro* and in a murine model [57] and it seems to be the case on
483 treatments with EcN and its derivatives. The *Il17a* gene is known to be overexpressed in intestinal inflamed tissue [58],
484 its overexpression, however, it is not the sole responsible for the inflammatory process and there have been studies

485 showing *Il17* as an anti-inflammatory cytokine, depending on the co-expression of other cytokines such as *Il10* and
486 *Inf-γ* [59–62].

487 It's important to emphasize that not all probiotics demonstrated anti-inflammatory effects in the context of 5-
488 FU-induced epithelial damage [53, 63]. Despite sharing similar probiotic characteristics [64], the mechanisms
489 involved in mitigating 5-FU-induced inflammation may vary among strains or species. This variability could be
490 attributed to factors such as the probiotic dosage, the type of antineoplastic agents, the specific experimental protocols
491 used to induce mucositis, and the form in which beneficial microorganisms are administered, extending to their
492 postbiotic forms (i.e., live, inactivated, or their secreted products) as we have demonstrated in this study [24, 65–67].

493

494 5. CONCLUSION

495

496 The results so far demonstrate that the *E. coli* CEC15 strain can protect the intestinal epithelium from the
497 deleterious effects of 5-FU, maintaining its integrity besides modulating the inflammatory response. The study shed
498 light on the protective role of CEC15 in preserving intestinal barrier integrity, promoting the upregulation of tight
499 junction proteins and of mucin. Additionally, the observed reduction in neutrophil infiltration and modulation of pro-
500 and anti-inflammatory cytokine genes confirms the anti-inflammatory potential of this probiotic. This protective effect
501 of CEC15 may be mediated by bacterial structures and/or secreted metabolites considering that postbiotic preparations
502 of the strain promoted beneficial effects like those promoted by live bacteria. Such postbiotic fractions may be used
503 with less risk to patients. By contrast, despite being a recognized probiotic bacterium and having proven efficacy in
504 several models of inflammatory bowel disease, EcN demonstrated here no protective effects in the model of intestinal
505 mucositis used here, confirming that probiotic effects may be highly strain dependent. Overall, this study contributes
506 to the growing understanding of probiotics and postbiotics as potential therapeutic strategies in mitigating mucositis-
507 associated intestinal damage. Future research should further explore and refine these approaches to optimize their
508 therapeutic benefits.

509

510 DECLARATIONS

511

512 **Ethics approval and consent to participate:** The experiment was conducted in agreement with the Brazilian College
513 of Animal Experimentation (COBEA) and approved by the “Use of Animals Ethics Committee” from UFMG (CEUA
514 – UFMG) under the protocol 210/2022.

515

516 **Availability of data and materials:** Raw data can become available upon reasonable request to the corresponding
517 author.

518

519 **Competing interests:** The authors declare no competing interests.

520

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524
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526 TdS, TG, KV, EF; resources, SF, VC and VA; writing-original draft preparation, RG and TdS; writing-review and
527 editing, RG, TdS, GJ, YIL, CC, PL, JC, EG and VA; supervision, GJ, EG and VA; funding acquisition, GJ, EG and
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532
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CHAPTER 5 - ADDITIONAL RESULTS

This chapter contains additional results on the work with *E. coli* CEC15. These results are presented here in two sections:

- A comparative genomics study using *E. coli* strains that have shown any beneficial effect on the host (i.e. EcN and the six strains that compose the Symbioflor2 probiotic product).
- The production and characterization of extracellular vesicles from CEC15 and EcN.

These additional results help us to understand how the beneficial *E. coli* strain are genetically related and also how can the extracellular vesicles produced by the two studied strains can exert effects in the host independent of the presence and activity of the viable strain.

COMPARATIVE GENOMICS OF *Escherichia coli* PROBIOTIC STRAINS

1. INTRODUCTION

Probiotics, as defined by the Food and Agriculture Organization of the United Nations/World Health Organization (FAO/WHO), are living microorganisms that, when administered in sufficient quantities, confer health benefits to the host (Hill et al., 2014). Probiotic microorganisms display diverse characteristics, encompassing their origin, taxonomy, optimal dosage, and specific health advantages, all contingent on the particular strain being employed. Within the category of Gram-negative microorganisms possessing probiotic properties, *Escherichia coli* strain Nissle 1917 (EcN) stands out as one of the most intensively researched bacterial strains. For over a century, EcN has served as the active pharmaceutical ingredient in a licensed medicinal product available in Germany and several other countries (Sonnenborn, 2016). Over the past few decades, novel probiotic activities unique to this versatile *E. coli* strain have been identified.

Selecting *E. coli* as a probiotic may seem logical due to its assumed widespread presence in the gut. However, the actual occurrence of *E. coli* in the human gut, its abundance, and its significance as a "major player" in that environment are subjects of interest (T. M. Wassenaar, 2016). In his book on the normal human microbiota, Tannock notes that *E. coli* is typically located in the ileum, which is the last part of the small intestine, as well as in the colon. However, it tends not to outnumber other more prevalent species (Tannock, 1994). Caugant and colleagues describe a coexistence of transient and persistent *E. coli* clones in the gut, with rapid changes in the genetic composition of the *E. coli* population. Unfortunately, specific quantitative data regarding *E. coli* abundance are not provided (Caugant et al., 1981).

E. coli bacteria serve as the foundation for at least three probiotic products available in the market, known by their commercial names: Mutaflor (EcN), Symbioflor 2 (6 strains, G1/2, G3/10, G4/9, G5, G6/7, and G8), and Colinfant (A0 34/86) (T. M. Wassenaar, 2016). These products have been employed in numerous scientific studies aimed at uncovering their potential beneficial impacts on human health. Besides these strains, *E. coli* CEC15, the focus of this thesis, is a commensal bacteria isolated from newborn rat's feces (Tomas et al., 2015) and has shown good results on the protection of intestinal diseases, especially colitis (Escribano-Vazquez et al., 2019) and intestinal mucositis (this thesis), as proven in animal models.

Given the advancements in high-throughput sequencing technology and comparative genomics approaches, numerous studies have centered on investigating the genomes of probiotic bacteria, such as *Limosilactobacillus reuteri* (Zheng et al., 2015;

Jatuponwiphat et al., 2019), *Bifidobacterium longum* (da Silva et al., 2021), *Pediococcus acidilactici* (Z. Li et al., 2021), *Enterococcus faecium* (Ghattargi et al., 2018), *Lactococcus lactis* (Oliveira et al., 2017), among others. These earlier research efforts have provided valuable insights into genome characteristics, phylogenomic relationships, and functional genes related to niche adaptation and probiotic activity across various strains. Consequently, researchers have gained a more profound understanding of the genetic underpinnings of probiotic strains functional roles. Nonetheless, besides EcN, the genome of probiotic *E. coli* strains have been poorly characterized, and its distinct functional properties are yet to be explored. Thus, the goal of this work is to perform a comparative genomic analysis on the 8 *E. coli* strains genomes available (EcN, CEC15, and the 6 strains from Symbioflor2) aiming to find genomic characteristics that could lead to their beneficial effects.

2. MATERIAL AND METHODS

2.1. Phylogenomic analysis

For phylogenomic analysis, we added 15 strains representing the *E. coli* phylogroups A, B1, B2, C, D, E, and F, plus eight probiotic strains. The phylogenomic tree was constructed with the phylogenomic tree tool from PATRIC by the codon tree method, which is based on the codon sequence of one thousand genes shared among all genomes and of a single copy. In this method, the orthologous genes were identified via annotation of Protein Global Families (PGFams) of PATRIC (Davis et al., 2016). The sequences of protein were aligned by MUSCLE software (Edgar, 2004) and the corresponding codon sequences were concatenated. The phylogenomic inference was realized via the RAxML program (Stamatakis, 2014) with support values estimated by 100 fast bootstrapping runs (Stamatakis et al., 2008). The tree was visualized and edited with the tool iTOL (v.6.53) (<https://itol.embl.de/>).

2.2. Pangenomics

The pangenome analysis was carried out via Roary software (v13.13.0) (Page et al., 2015) with a minimum of 70% identity on BLASTp. Eight *E. coli* strains genome, available publicly on the NCBI database, were used in this comparison (Table 1). Orthologous clusters were analyzed from annotation using OrthoVenn3 platform (Xu et al., 2019). Functional analyses of the pangenome was performed using the BPGA tool (Chaudhari et al., 2016) to find the KEGG pathway distribution and Clusters of Orthologous Groups of proteins (COGs).

2.3. Genomic islands prediction

Prediction of Metabolic (MI), Resistance (RI), and Pathogenicity (PAI) islands in *E. coli* CEC15 strain, Nissle 1917 strain and Symbioflor 2 strains was performed with

software GIPSy (Genomic Island Prediction Software, v.1.1.3) (Soares et al., 2016), using *E. coli* O157:H7 str. Sakai (*E. coli* O157) (NC_002695) genome as a reference. Visualization of the genomic island's map was performed with software BRIG (BLAST Ring Image Generator, v. 0.95) (Alikhan et al., 2011). Data were curated manually

2.4. Bacteriocins and adhesin predictions

Genes coding bacteriocins were predicted with BAGEL4 (van Heel et al., 2018). Their distribution among the genomes was visualized through a heat map of presence and absence. The presence of adhesins proteins in the genomes of the strains was analyzed by SPAAN software (score>0.8) (Sachdeva et al., 2005), and later, the sub-cellular localization of these proteins was predicted using CELLO2GO (Yu et al., 2014).

2.5. Antibiotic resistance genes in the genome of *E. coli* probiotic strains

The identification of genes related to the resistance of antibiotic compounds in the genome of the CEC15 strain was performed by alignment to the CARD (Comprehensive Antibiotic Resistance Database) (Alcock et al., 2019), with coverage $\geq 90\%$ as selection criteria, and e-value $< e^{-2}$ for the identification of antibiotic resistance genes in potential.

3. RESULTS AND DISCUSSION

3.1. Features of the *E. coli* probiotic strains genome

A general comparison of genomic features from the 8 strains can be found in table 1 and 2. As for genome conservation, most strains show linearity between the gene clusters with a large inversion on the G5 strain genome (Figure 1).

Table 1. Genome features of the 8 complete genomes of *Escherichia coli* used in the present study

Strain	GC%	Size (Mb)	Prokka annotation				Plasmids	Accession Number
			CDS	tRNA	rRNA	tmRNA		
CEC15	50.7	4.78	4,422	86	22	1	1	CP133657.1
EcN	50.6	5.05	4,612	88	22	1	2	CP058217.1
G1/2	50.9	5.28	5,055	96	22	1	-	CP060083.1
G3/10	50.9	4.95	4,694	89	22	1	7	CP060075.1
G4/9	50.7	4.67	4,281	86	22	1	1	CP060073.1
G5	50.9	4.84	4,586	88	22	1	7	CP060065.1
G6/7	50.9	5.32	5,081	98	22	1	3	CP060061.1
G8	50.9	5.29	5,064	98	22	1	3	CP060057.1

3.2. Phylogenomic analysis

The phylogenomic analysis tree (Figure 2) was based on 1000 single-copies genes shared among all the strains. It shows the *E. coli* CEC15 strain forming a clade

Table 2. Genomic characterization of the studied strains plasmids.

Strain	Plasmid	GC%	CDS	Plasmid size (pb)	Accession Number
CEC15	pCEC	48.96	203	200,825	CP133658.1
Nissle 1917	pMUT1	46.9	5	3,173	CP058218.1
	pMUT2	46.01	7	5,514	CP058219.1
G1/2	-	-	-	-	-
G3/10	pSYM1	44.61	58	50,572	CP060077.1
	unnamed1	46.01	2	1,887	CP060080.1
	pSYM2	46.01	3	4,197	CP060078.1
	unnamed2	46.03	1	1,240	CP060082.1
	pSYM3	46.01	2	1,934	CP060079.1
	pSYM4	51.53	1	1,304	CP060081.1
	pSYM5/6	50.8	106	100,783	CP060076.1
G4/9	pSYM4	51.53	1	1,304	CP060074.1
	unnamed1	51.31	138	148,192	CP060074.1
	unnamed2	50.22	51	35,863	CP060067.1
G5	pSYM3	51.19	3	1,934	CP060072.1
	pSYM7	52.92	6	4,461	CP060069.1
	pSYM8	33.45	2	2,350	CP060071.1
	pSYM9	60.53	14	12,706	CP060068.1
	pSYM11	49.1	7	3,214	CP060070.1
G6/7	unnamed1	47.88	131	99,817	CP060062.1
	pSYM10	51.06	1	1,549	CP060064.1
	pSYM12	48.67	9	7,136	CP060063.1
G8	unnamed1	47.88	131	99,817	CP060058.1
	pSYM10	51.06	1	1,549	CP060060.1
	pSYM12	48.67	10	7,136	CP060059.1

with the strains *E. coli* IA1 and *E. coli* 55989, a commensal and a pathogenic enteroaggregative strain, respectively, which belong to the *E. coli* phylogroup B1. All strains from the probiotic Symbioflor 2 (*E. coli* G1/2, G3/10, G4/9, G5, G6/7, and G8) are closely related to the strain *E. coli* ATCC 8739, a multiple antibiotic resistance strain, and the pathogenic *E. coli* HS, forming the *E. coli* phylogroup A. The *E. coli* Nissle 1917 strain was classified together with the strains *E. coli* S88 and *E. coli* 536, both virulent strains belonging to the B2 phylogroup. This shows a high heterogeneity among *E. coli* strains with phylogroups composed of pathogens, commensal, and beneficial bacteria, and the beneficial strains being situated in phylogenomic distant groups.

3.3. Pangenome results

The 8 *E. coli* strains demonstrated an open genome pattern, revealing a substantial number of new genes each time a new genome was incorporated into the set of genomes under examination. The pangenome of probiotic *E. coli* strains has 3336 genes in the core genome, 2533 genes in the cloud (i.e. unique genes), and 2447 genes in the shell (i.e. shared between 2 to 7 strains) (Figure 3A). The clustering of strains based on the core genome can be seen on the pan genome matrix (Figure 3B). *E. coli* CEC 15 presents 346 exclusive genes in its chromosome, 238 (68%) of those are hypothetical proteins, we can also highlights 8 transposases genes, mainly from IS3 and IS66 families, a cluster from propanediol utilization, and 8 proteins for the

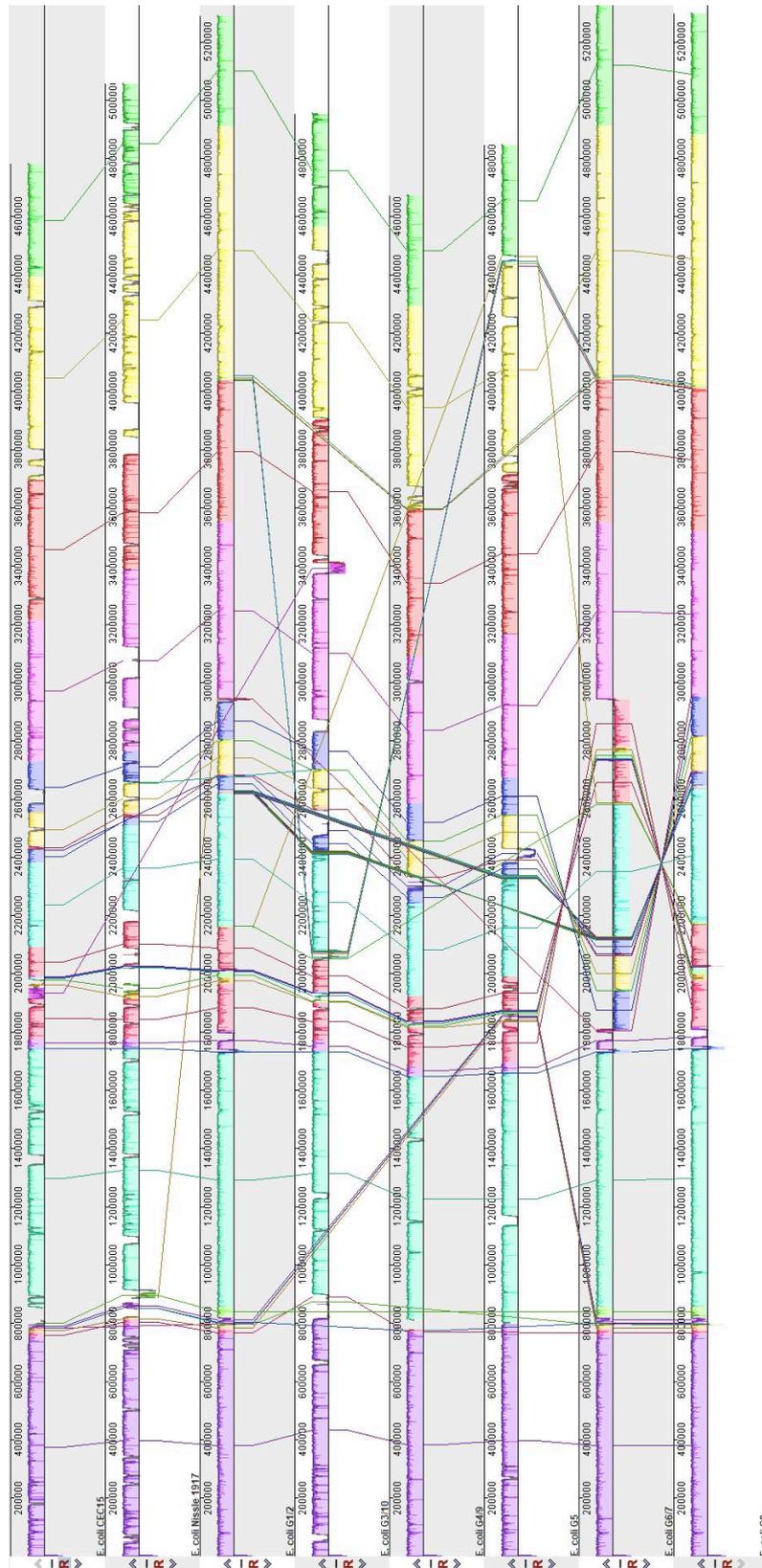


Figure 1: Multiple sequence alignment of the whole genome from the 8 strains of *E. coli* showing the conserved structure and synteny among the gene clusters.

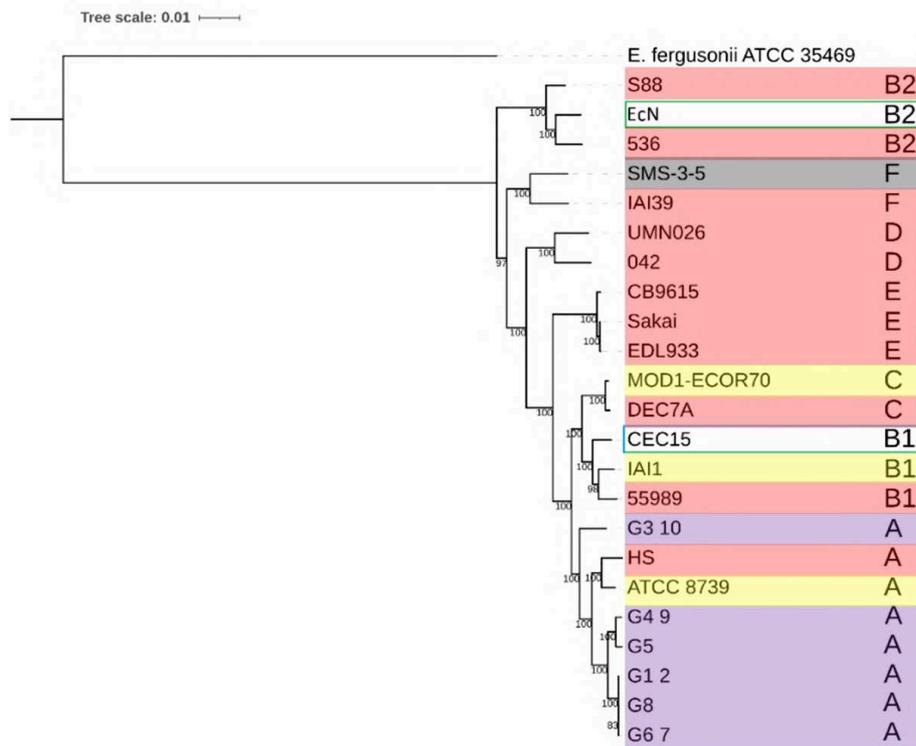


Figure 2: Phylogenomics of *E. coli* probiotic strains. *Escherichia coli* strains are grouped into 7 phylogroups (A, B1, B2, C, D, E, and F). *E. coli* CEC15 (CEC15) and Nissle 1917 (EcN) are in white with green box, strains from Symbioflor 2 (G1/2, G3/10, G4/9, G5, G6/7, and G8) are highlighted in purple. Yellow boxes represents commensal strains, red boxes indicate pathogenic strains, and the gray box indicate an environmental strain.

production of fimbriae. EcN presented 717 exclusive genes, 376 (52%) of hypothetical proteins, a cluster for colicin production (6), a cluster of HTH-type transcription regulation (9), 38 (5%) transposase genes, mainly from IS3 (19) and ISL3 (5) families, the cluster of 20 genes for colibactin production, 16 genes for fimbriae production, 4 multidrug resistance genes, PTS system for fructose, galactitol, and sorbose metabolism, and 4 genes for vitamin B12 use. *E. coli* G1/2 has 69 exclusive proteins, 71% of those being hypothetical proteins, 8 transposases, mainly from IS21 family. 667 genes were found exclusively on *E. coli* G3/10, 420 (63%) of hypothetical proteins, 56 transposase genes from families IS30, IS66, IS6, IS3, IS1, and InsAB mainly, a phage-like integrase cluster (KpLE2 family, 5 genes), the Rac (21) and Qin (5) prophage clusters, a cluster of Type IV secretion system (4), and a variety of transporter proteins. *E. coli* 4/9 has 141 exclusive genes, 68 (48%) being hypothetical proteins, 30 of transposase genes, mainly IS150 and IS3 families, and three CPS-53 (KpLE1) prophage genes. The *E. coli* G5 presented 483 exclusive genes, with 310 (64%) being hypothetical proteins, the highest number of transposases genes (63, 13%), mainly from families IS1, IS110, IS3, and IS5, four prophage clusters from the families CP4-44, e14, KpLED2, and Rac, Type IV secretion system proteins, and a variety of transport related proteins. The *E.*

coli G6/7 present the least number of exclusive genes (47), with 38 (80%) being hypothetical proteins, one prophage (e14) gene, 4 IS21 transposase genes, 2 transcription regulators, one tRNA-Ala gene, and one vitamin B12 transporter. And least, *E. coli* G8 with 63 exclusive genes, 52 (82%) hypothetical proteins, one CP4-6 and one Qin prophage genes, 7 transposase genes, majority from IS21 family, one outer membrane protein PagN, and a tRNA-Lys gene. In the *E. coli* genome, 3,267 orthogroups existing in the 8 genomes constituted the core gene pool (core genome), We noticed that orthogroups present only in one genome (Singletons) could also be seen as species-specific and they vary widely among the strains (Table3).

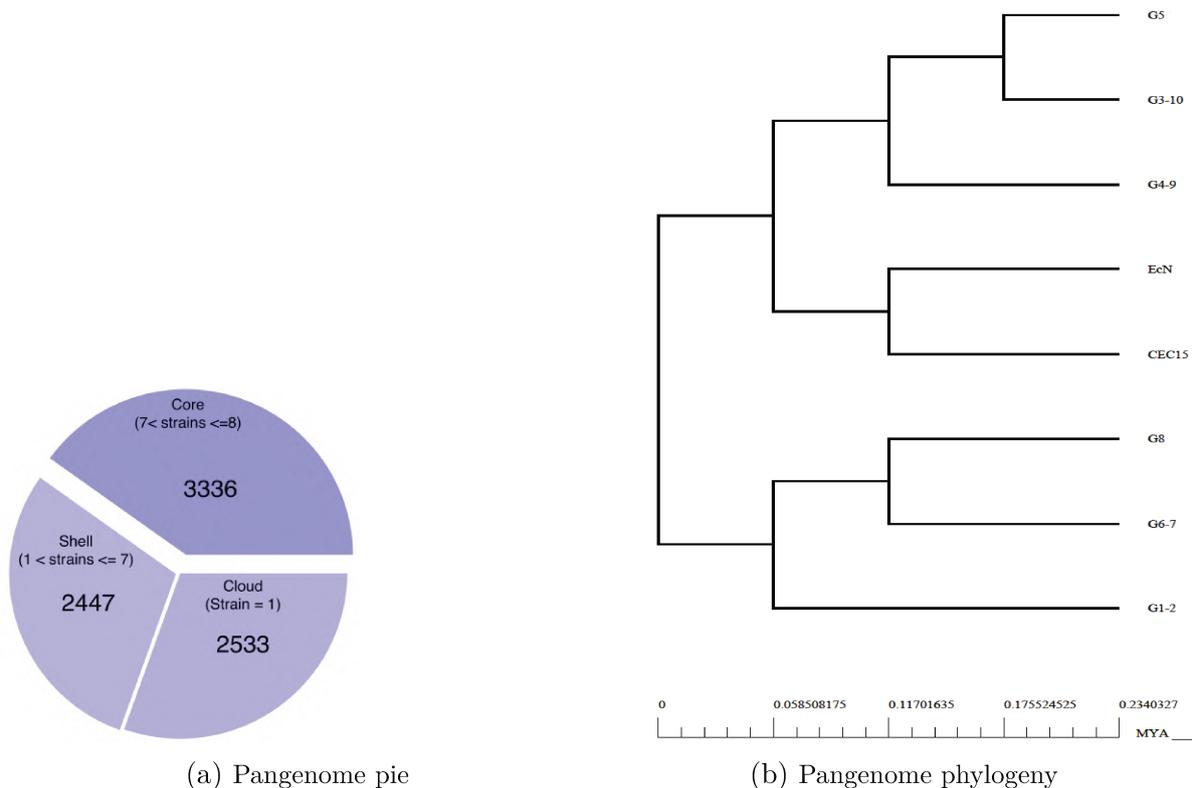


Figure 3: Pangenome analysis of *E. coli* probiotic strains

While the functions of genes aren't always clearly understood, many proteins have been categorized into functional groups based on their similarity to already characterized proteins. We analyzed the primary COG groups for the genomes under investigation and initially compared the genes within their core and pan-genomes (Figure 5). As expected, the majority of genes are related to metabolism (Figure 5A), followed by information storage and processing, and poorly characterized proteins. From metabolism genes, the majority are core genes, followed by accessory and unique genes, the same does not occur for the other classes as information storage and processing genes are more present in the accessory genome, followed by unique and core, and cellular processing and signaling together with poorly characterized genes

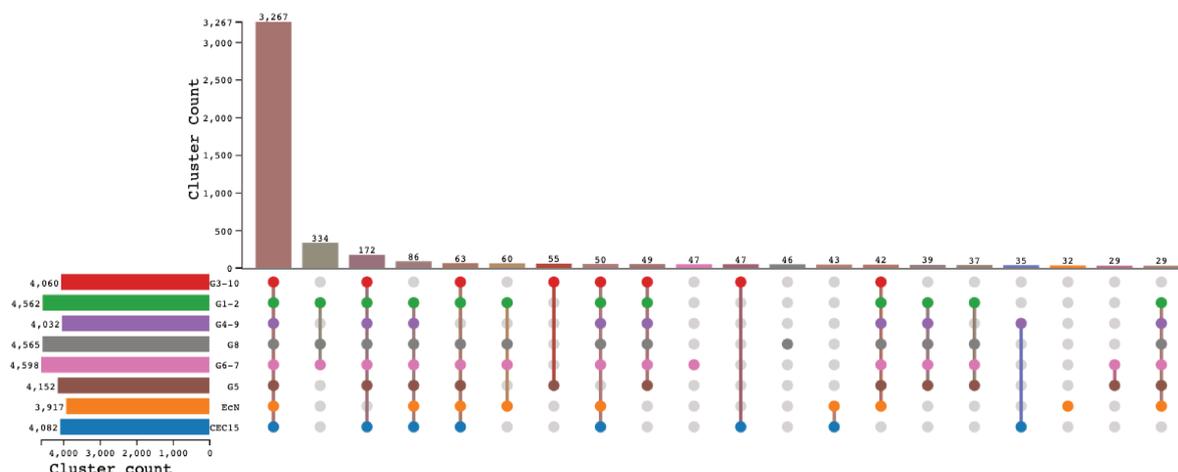


Figure 4: Orthogroups clustering of the genomes

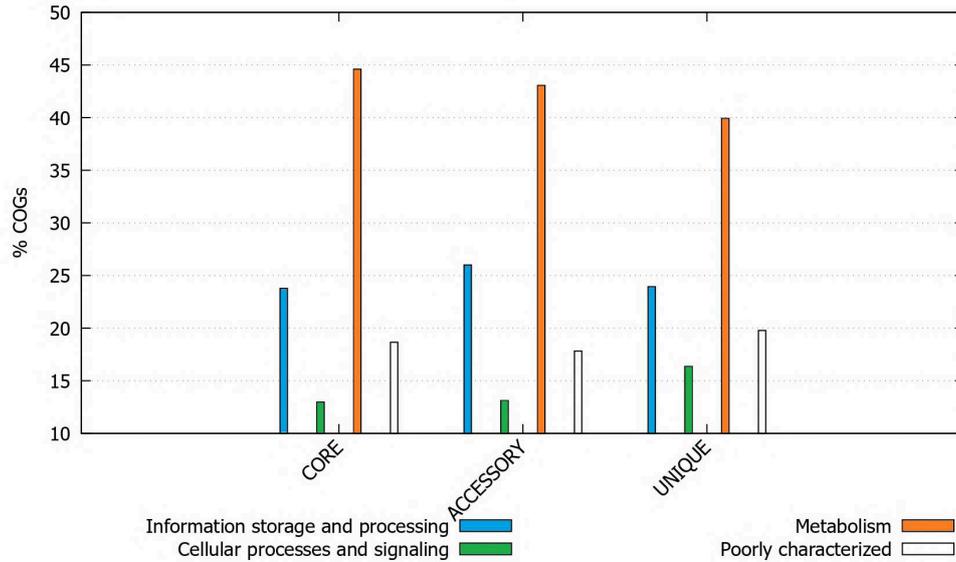
with the majority being found on the unique genome. Taking a more detailed look at COG distribution we see that the majority of metabolism category belongs to carbohydrate [G] and amino acid [E] metabolism and transport, while information storage and signaling is dominated by transcription genes [K], and cellular processing and signaling is dominated by [M] Cell wall/membrane/envelop biogenesis. There seem to have no great difference between core, accessory, and unique genome in each category.

As expected, when we analyzed the KEEG distributions the majority of genes were found related to metabolism, with most being present in the core genome. On another hand, the remaining categories (Cellular processes, Environmental information processing, Human diseases, and organismal systems) were mostly represented by accessory or unique genomes. Detailed analysis showed that cell motility, infectious diseases, replication and repair, and signal transduction were mainly represented by unique genes, and only carbohydrate metabolism, energy metabolism, metabolism of co-factor and vitamins, and translation are majorly represented by core genes. Accessory genome dominates the categories of amino acid metabolism, membrane transport, overview, and xenobiotics biodegradation and metabolism.

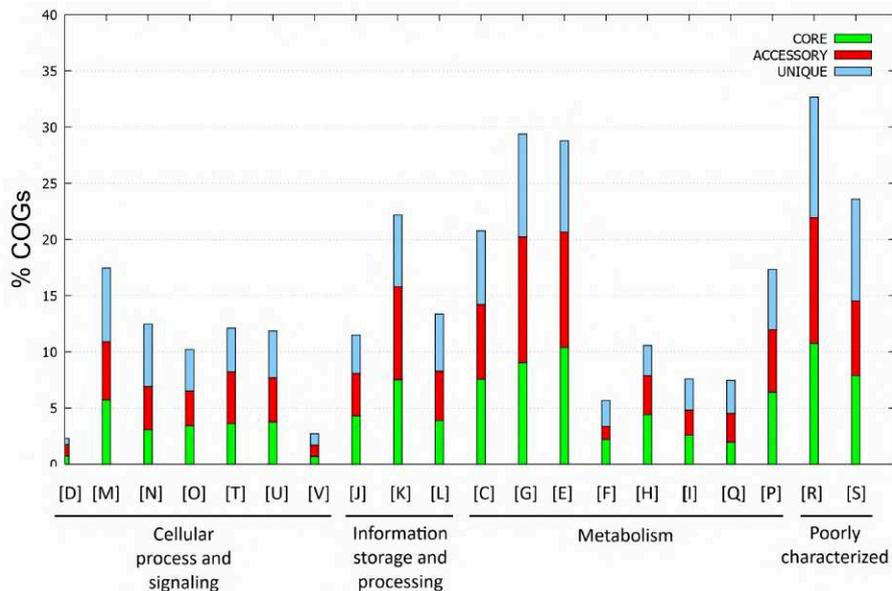
Table 3. Protein clusters organization on probiotic *E. coli* genomes

Strain	Proteins	Clusters	Singletons
CEC15	4398	4089	157
EcN	4519	3924	216
G1/2	4826	4563	16
G3/10	4521	4065	224
G4/9	4179	4037	35
G5	4425	4160	112
G6/7	4892	4599	10
G8	4851	4565	9

3.4. Genomic Islands Analysis



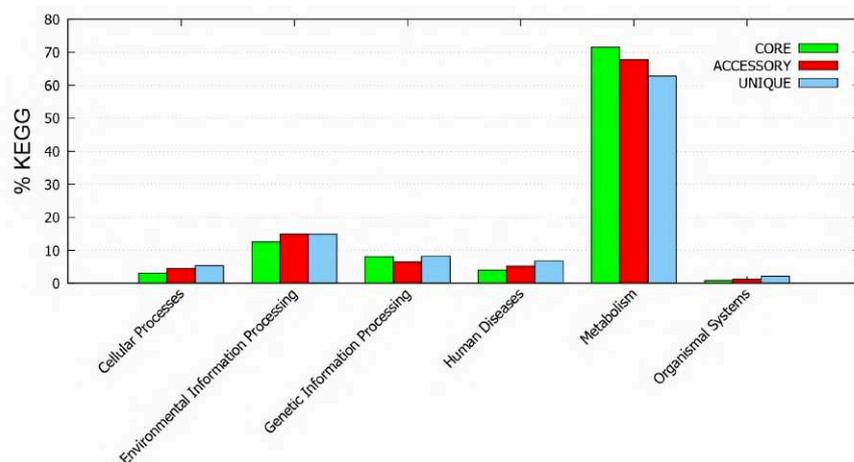
(a) COG distribution by category



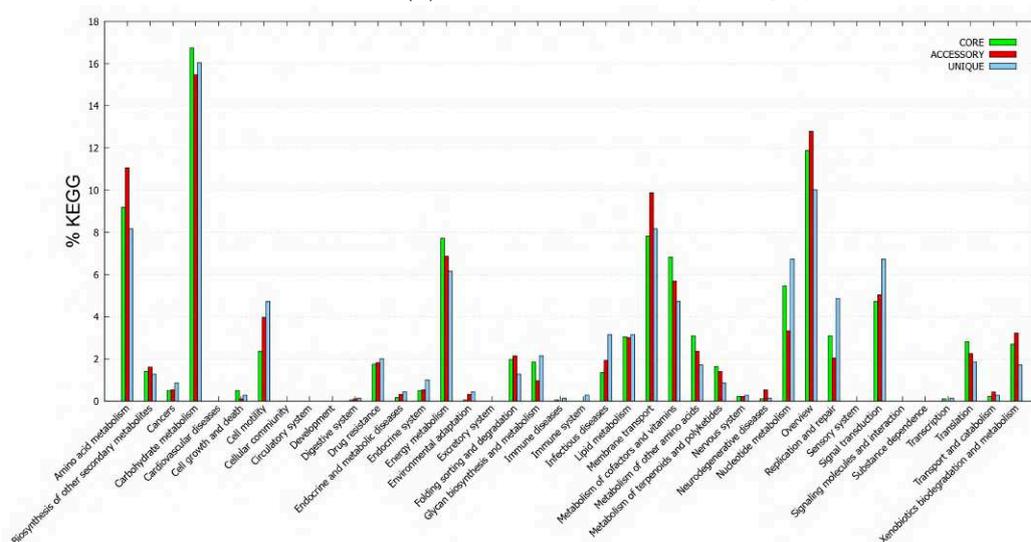
(b) Detailed COG distribution

Figure 5: COG distribution among the Pan genome of *E. coli* probiotic strains. [C] Energy production and conversion; [D] Cell cycle control, cell division, chromosome partitioning; [E] Amino Acid metabolism and transport; [F] Nucleotide metabolism and transport; [G] Carbohydrate metabolism and transport; [H] Coenzyme metabolism; [I] Lipid metabolism; [J] Translation; [K] Transcription; [L] Replication and repair; [M] Cell wall/membrane/envelop biogenesis; [N] Cell motility; [O] Post-translational modification, protein turnover, chaperone functions; [P] Inorganic ion transport and metabolism; [Q] Secondary metabolites biosynthesis, transport and catabolism; [R] General Functional Prediction only; [S] Function Unknown; [T] Signal Transduction; [U] Intracellular trafficking and secretion.

Prediction analysis of the CEC15 strain revealed the presence of 19 genomic islands (GEIs) (Figure 7 / Table 5). Five metabolic islands (MI) were found, with genetic content related to the utilization of substrates such as propanediol, fructose, and mannose (PduU, PduB, DGL, KDPG aldolase, L-rhamnulokinase e KdgT) (Supplementary



(a) KEEG distribution by category



(b) Detailed KEEG distribution

Figure 6: KEEG distribution among the Pan genome of *E. coli* probiotic strains

File S1). Pathogenicity islands (PAI) presented genes from the transposase families IS3, IS4, and IS66, recombinase XerC, flagella-related genes (FlhS, FlgG, and FlgF), and proteins from the secretion system II (Supplementary File S1).

The EcN strain presented 33 GEIs (Table 6). From the PAIs present, PAI 9 deserves attention for containing the full cluster for colibactin production and a few transposases from IS3 family, PAI 4, the largest of this strain, contains a wide variety of genes including metabolic pathways for carbohydrate assimilation, type II secretion systems, adhesins such as Ag43 and fimbriae, genes for the biosynthesis of K5 polysaccharide, and diverse transposases. PAIs 8, 11, and 12 are mostly composed by prophage related proteins. PAI 16 contains a wide variety of genes, including transposases, fimbriae and other adhesins, and antimicrobial genes such as microcins and salmochelin/enterobactin, and type IV toxins systems. The MI of EcN con-

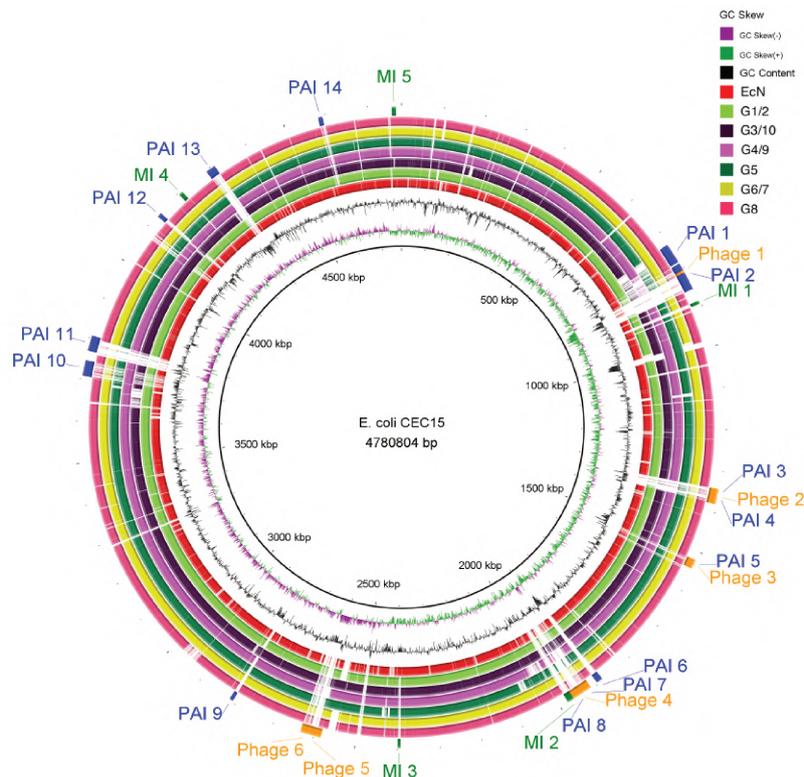


Figure 7: Schematic circular representation of *E. coli* CEC15 genomic islands compared to *E. coli* Nissle 1917 and the Symbioflor2 strains. Pathogenicity Islands (PAI), and Metabolic Islands (MI) were found on the genome. Each ring from the circle represents one genome, differentiated by colors. Figure generated by BRIG software.

tain transporters and enzymes for the metabolism of different carbohydrates, like fructose, tagatose, glucose, and riboses. The RI contain the multidrug efflux protein EmrE (Supplementary File S1).

The strain G1/2 presents 26 GEI and the second highest number of PAIs (25) (Table 7). The majority of small PAI are composed of transposases and hypothetical proteins (PAI 4, 6, 7, 10, 11, 13, 15, 16, 17, 18, 19, 20, and RI 1). From the remaining islands, PAI 24 contains 2 genes for hemolysin production, PAI 23 contains a cluster for lactate utilization, PAI 21 has a cluster for iron transport, PAI 1 is mainly composed of type II secretion system genes (Supplementary File S1).

Eighteen genomic islands were detected in the chromosome of G3/10 strain (Table 8), 15 PAI, 2 MI, and 1 RI. No key gene was found of either MI identified. RI 1 contains a cluster that promotes resistance to copper (Cu). PAIs 2, 4, 8, 10, 14, and 15 are mainly composed of transposases and hypothetical proteins, PAI 13 contain genes that allows the transport and metabolism of aldols, PAI 5 contains genes for lactate metabolism and PAI 6 and 7 for metabolism of carbohydrates such as glucose, galactiol, ribulose, and hexulose. Finally, PAI 3 is composed of genes from type II secretion systems (Supplementary File S1).

Strain G4/9 presents the lowest number of GEIs from the analyzed strains (16) (Table 9). The MI 1 of G4/9 contain an epoxidase cluster, linked to the biosynthesis of sterols while MI 2 contains genes for beta-glucoside assimilation. As for the PAIs, PAI 2, 5, 6, 8, 9, 10, 11, and 14 are mainly composed of hypothetical proteins and transposases. PAI 1 contain type II secretion system proteins, PAI 3 contains the arginine/agmatine system, responsible for acid resistance, and iron assimilation mechanism-related proteins. PAI 12 is mostly composed of flagella-production proteins (Supplementary File S1).

G5 strain presents 22 GEI and the highest number of RI (3) (Table 10). Although no key gene is found on RI 2 and 3, RI 1 contains the copper resistance cluster mentioned on G3/10 strain. The MI 1 contains similar genes, related to beta-glucoside metabolism, found on strain G4/9. As for the PAIs, PAI 2, 4, 5, 6, 7, 10, and 11 are, mostly composed of transposons and hypothetical proteins. PAI 1, as in many strains before, contain type II secretion system proteins, while PAI 8 has toxins and cold-shock proteins, and PAI 12 contains genes for assimilation of propionate (Supplementary File S1).

The G6/7 strain (Table 11) contains 27 GEIs, 23 PAI, 3 MI, and 1 RI. MIs and RI has no key gene beside a few permease proteins. PAI 3, 5, 7, 8, 11, 12, 15, 16, 17, and 18 are filled with transposases and hypothetical proteins. Again, PAI 1 is composed of type II secretion system proteins, as is PAI 2 with extra genes related to permeases, fatty acid utilization, and N-acetylneuroaminic acid utilization. A few cold-shock genes are scattered around PAI 4, 5, and 6 (Supplementary File S1).

Finally, the G8 strain contains 28 GEIs and the highest number of PAIs (26) (Table 12) (Table 12). No key genes were found on G8 MIs. PAIs 5, 6, 7, 9, 10, 12, 13, 14, 15, 16, 17, 18, 19, 20, and 21 contain, mostly, hypothetical genes and transposases. Cold-shock proteins can be found on PAI 11 and 12, PAI 1 and 2 contain genes very similar genes as PAI 1 and 2 of G6/7. PAI 22 contains genes for iron assimilation (Supplementary File S1).

Table 4. Presence of mobile genetic elements in the genome of the studied strains

Strain	Pathogenicity Islands	Metabolic Islands	Resistance islands	Phages
CEC	14	5	0	6
EcN	22	10	1	6
G1/2	25	0	1	18
G3/10	15	2	1	17
G4/9	14	2	0	7
G5	18	1	3	10
G6/7	23	3	1	18
G8	26	3	0	18

3.5. Bacteriocins and adhesins

The eight probiotics *E. coli* strains showed different distributions in bacteriocins-

Table 5. Number and size of genomic islands in CEC15 genome

Strain	Genomic Islands						Total GEI
	PAI	Size (Kb)	MI	Size (Kb)	RI	Size (Kb)	
CEC15	PAI 1	48.1					
	PAI 2	67.8	MI 1	7.6			
	PAI 3	7.8					
	PAI 4	20.0					
	PAI 5	11.8	MI 2	18.1			
	PAI 6	14.9					
	PAI 7	10.1					
	PAI 8	24.3	MI 3	6.2	-	-	19
	PAI 9	8.4					
	PAI 10	37.4					
	PAI 11	37.1	MI 4	9.8			
	PAI 12	10.2					
	PAI 13	23.1	MI 5				
	PAI 14	10.3		9.4			

PAI= Pathogenicity islands; MI= Metabolic islands; RI= Resistance islands; GEI = Genomic islands

Table 6. Number and size of genomic islands in EcN genome

Strain	Genomic Islands						Total GEI
	PAI	Size (Kb)	MI	Size (Kb)	RI	Size (Kb)	
EcN	PAI 1	30.8					
	PAI 2	9.3	MI 1	10.1			
	PAI 3	16.8					
	PAI 4	135.5	MI 2	8.6			
	PAI 5	10.4					
	PAI 6	17.3	MI 3	7.7			
	PAI 7	13.5					
	PAI 8	37.8	MI 4	8.1			
	PAI 9	54.6					
	PAI 10	56.3	MI 5	6.2			
	PAI 11	20.9			RI 1	6.7	33
	PAI 12	10.8	MI 6	12.8			
	PAI 13	6.5					
	PAI 14	18.3	MI 7	10.5			
	PAI 15	5.6					
	PAI 16	78.1	MI 8	7.7			
	PAI 17	14.2					
	PAI 18	23.8	MI 9	23.5			
	PAI 19	59.2					
	PAI 20	47.1					
	PAI 21	27.6	MI 10	27.2			
	PAI 22	12.6					

PAI= Pathogenicity islands; MI= Metabolic islands; RI= Resistance islands; GEI = Genomic islands

encoding genes. Among these bacteria, the EcN strain presented a greater diversity of bacteriocin genes followed by the strains G1/2, G6/7, and G8. A single bacteriocin was identified in the CEC15 genome: the bottromycin, which appears to be conserved in *E. coli* strains with probiotic profiles (Figure 8).

As for adhesins, CEC15 presented 78 potential adhesins, EcN presented 89, G1/2 and G3/10 presented 69 each, G4/9 presented 64, G5 presented 62, G6/7 pre-

Table 7. Number and size of genomic islands in G1/2 genome

Strain	PAI	Size (Kb)	Genomic Islands		RI	Size (Kb)	Total GEI	
			MI	Size (Kb)				
G1/2	PAI 1	16.8						
	PAI 2	11.4						
	PAI 3	33.5						
	PAI 4	8.7						
	PAI 5	36.9						
	PAI 6	13.3						
	PAI 7	7.7						
	PAI 8	20.5						
	PAI 9	56.0						
	PAI 10	9.0						
	PAI 11	12.9						
	PAI 12	22.6						
	PAI 13	11.6		-	-	RI 1	7.3	26
	PAI 14	7.3						
	PAI 15	46.5						
	PAI 16	13.2						
	PAI 17	13.2						
	PAI 18	9.6						
	PAI 19	12.8						
	PAI 20	9.4						
	PAI 21	62.0						
	PAI 22	9.8						
	PAI 23	25.2						
	PAI 24	13.2						
	PAI 25	83.4						

PAI= Pathogenicity islands; MI= Metabolic islands; RI= Resistance islands; GEI = Genomic islands

Table 8. Number and size of genomic islands in G3/10 genome

Strain	PAI	Size (Kb)	Genomic Islands		RI	Size (Kb)	Total GEI	
			MI	Size (Kb)				
G3/10	PAI 1	48.1						
	PAI 2	67.8						
	PAI 3	7.8						
	PAI 4	20.0						
	PAI 5	11.8		MI 1	10.5			
	PAI 6	14.9						
	PAI 7	10.1						
	PAI 8	24.3				RI 1	28.5	18
	PAI 9	8.4						
	PAI 10	37.4						
	PAI 11	37.1						
	PAI 12	10.2		MI 2	8.9			
	PAI 13	23.1						
	PAI 14	10.3						
	PAI 15	10.3						

PAI= Pathogenicity islands; MI= Metabolic islands; RI= Resistance islands; GEI = Genomic islands

sented 71, and G8 presented 68. In general, among Symbioflor2 strains, the difference in the number of adhesins were not relevant as they present a similar quantity. the same cannot be said about CEC15 and EcN, with considerably more adhesin-like proteins. Fimbriae proteins are the most common adhesin found with 27 in CEC15, 22 in EcN,

Table 9. Number and size of genomic islands in G4/9 genome

Strain	PAI	Size (Kb)	Genomic Islands		RI	Size (Kb)	Total GEI
			MI	Size (Kb)			
G4/9	PAI 1	16.8					
	PAI 2	6.4					
	PAI 3	62.0					
	PAI 4	13.9	MI 1	20.6			
	PAI 5	7.6					
	PAI 6	22.5					
	PAI 7	27.5					
	PAI 8	13.7			-	-	16
	PAI 9	10.2					
	PAI 10	7.0					
	PAI 11	20.6	MI 2	9.4			
	PAI 12	38.2					
	PAI 13	23.6					
	PAI 14	10.7					

PAI= Pathogenicity islands; MI= Metabolic islands; RI= Resistance islands; GEI = Genomic islands

Table 10. Number and size of genomic islands in G5 genome

Strain	PAI	Size (Kb)	Genomic Islands		RI	Size (Kb)	Total GEI
			MI	Size (Kb)			
G5	PAI 1	16.8					
	PAI 2	27.7					
	PAI 3	16.2					
	PAI 4	5.5			RI 1	33.5	
	PAI 5	9.1					
	PAI 6	7.5					
	PAI 7	35.5					
	PAI 8	20.3					
	PAI 9	13.4					
	PAI 10	11.9	MI 1	9.4	RI 2	8.4	22
	PAI 11	6.8					
	PAI 12	51.1					
	PAI 13	32.9					
	PAI 14	9.3					
	PAI 15	27.2					
	PAI 16	5.3			RI 3	6.7	
	PAI 17	6.3					
	PAI 18	20.3					

PAI= Pathogenicity islands; MI= Metabolic islands; RI= Resistance islands; GEI = Genomic islands

14 in G1/2, 15 in G3/10, 19 in G4/9, 14 in G5, 14 in G6/7, and 14 in G8. The common adhesin Ag43 appeared in all genomes but G3/10 and G4/9 (Supplementary material S2).

3.6. Antibiotic resistance genes

Genomic analysis of the antibiotic resistance genes presence in the genome of the 8 studied strains shows a wide variety in the number of genes and mechanism of action among the strains. Table 13 shows a summary of the number of genes for each resistance mechanism in each strain. In all strains, the highest number of genes cor-

Table 11. Number and size of genomic islands in G6/7 genome

Strain	Genomic Islands						Total GEI	
	PAI	Size (Kb)	MI	Size (Kb)	RI	Size (Kb)		
G6/7	PAI 1	16.8						
	PAI 2	92.5						
	PAI 3	18.1						
	PAI 4	58.8						
	PAI 5	11.7		MI 1	12.3			
	PAI 6	22.6						
	PAI 7	12.9						
	PAI 8	9.0						
	PAI 9	17.7						
	PAI 10	10.0						
	PAI 11	32.9						
	PAI 12	20.5		MI 2	8.7	RI 1	7.4	27
	PAI 13	42.3						
	PAI 14	13.2						
	PAI 15	18.6						
	PAI 16	9.6						
	PAI 17	12.8						
	PAI 18	9.4						
	PAI 19	62.0		MI 3	9.4			
	PAI 20	25.2						
	PAI 21	14.5						
	PAI 22	83.7						
	PAI 23	0.6						

PAI= Pathogenicity islands; MI= Metabolic islands; RI= Resistance islands; GEI = Genomic islands

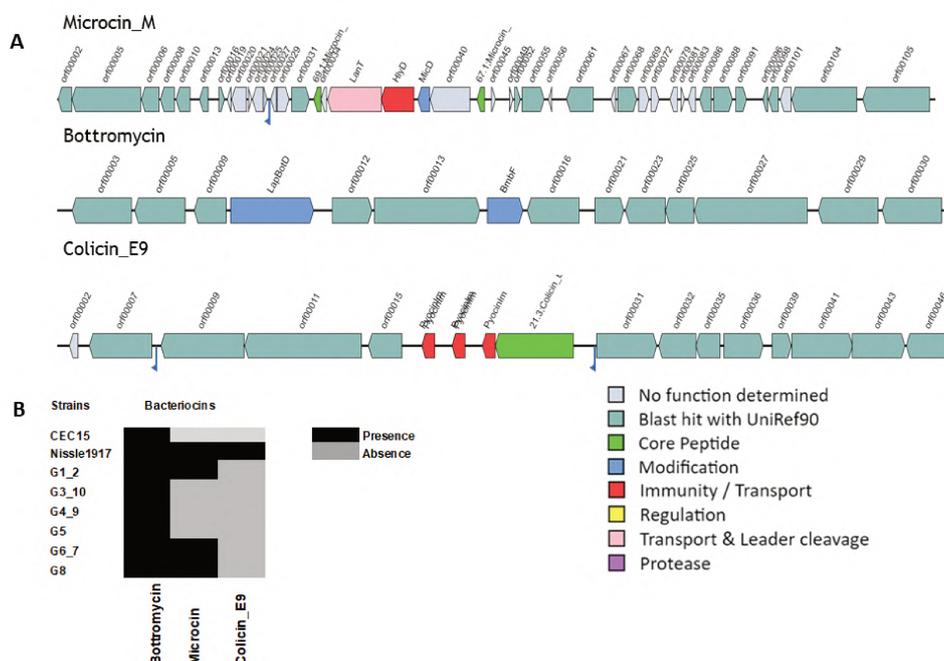


Figure 8: Detection of three bacteriocins gene clusters in the genome of the eight *E. coli* strains studied. (A) Schematic cluster structure representation of the three bacteriocins identified. (B) Heat-map of presence/absence of the three bacteriocins in the genome of the studied strains

respond to antibiotic efflux mechanism, followed by antibiotic target alteration. A total

Table 12. Number and size of genomic islands in G8 genome

Strain	PAI	Size (Kb)	Genomic Islands		RI	Size (Kb)	Total GEI
			MI	Size (Kb)			
G8	PAI 1	16.8					
	PAI 2	16.8					
	PAI 3	115.8					
	PAI 4	33.6					
	PAI 5	54.0					
	PAI 6	20.5					
	PAI 7	1.0		MI 1	12.3		
	PAI 8	16.0					
	PAI 9	32.8					
	PAI 10	12.9					
	PAI 11	22.6					
	PAI 12	11.7					
	PAI 13	7.4					28
	PAI 14	18.5					
	PAI 15	17.5					
	PAI 16	13.2					
	PAI 17	18.6					
	PAI 18	9.6					
	PAI 19	7.4					
	PAI 20	12.8		MI 2	9.8		
	PAI 21	9.4					
	PAI 22	61.9					
	PAI 23	25.2					
	PAI 24	13.2					
	PAI 25	83.7					
	PAI 26	35.4					

PAI= Pathogenicity islands; MI= Metabolic islands; RI= Resistance islands; GEI = Genomic islands

of 6 genes are present in all strains (HTH-type transcriptional regulator GadW; HTH-type transcriptional regulator GadX; Multidrug resistance protein MdtE; Multidrug resistance protein MdtF; and Inner membrane metabolite transport protein YhjE), all of them associated with resistance by antibiotic efflux, the first four promoting multidrug resistance to fluoroquinolones, macrolides, and penams, and the last one promoting resistance to fosfomycin. Another 11 genes were shared between all strains but CEC15 (cAMP-activated global transcriptional regulator CRP; Cell division ATP-binding protein FtsE; Dihydropteroate synthase; HTH-type transcriptional regulator DmlR; HTH-type transcriptional regulator ZntR; Lipopolysaccharide export system ATP-binding protein LptB; Multidrug export protein AcrE; Multidrug export protein AcrF; putative ABC transporter ATP-binding protein YheS; Sensor protein QseC; and Transcriptional regulatory protein QseB), being the Dihydropteroate synthase associated with antibiotic target replacement, the putative ABC transporter ATP-binding protein YheS with antibiotic target protection, and the remaining with antibiotic efflux.

On another note, EcN has in its genome, besides the mentioned above, 24 genes from multidrug resistance family while G4/9 presents 18 from the same families.

Three were found on G1/2 and five on G8. The remaining strains did not present any more genes of these families besides the mentioned in the paragraph above. EcN and G4/9 presents also 2 genes exclusively related to resistance to macrolides (Macrolide export ATP-binding/permease protein MacB; Macrolide export protein MacA), EcN also present 3 genes related to exclusive resistance to fosfomycin (Fosfomycin resistance protein AbaF; Fosmidomycin resistance protein; Fosfomycin resistance protein AbaF; Fosmidomycin resistance protein) while only the first two genes are found on G4/9. EcN and G4/9 also presents one gene for the production of the β -lactamase enzyme, responsible for resistance to β -lactam antibiotics. None of the remaining strains presents these genes (Supplementary material S3).

Table 13. Summary of antibiotic resistance genes separated by mechanism of action

Strain	AE	RP	AI	ATA	ATP	ATR	Total genes
CEC15	10	2	-	3	-	-	15
EcN	108	8	5	17	4	1	143
G1/2	24	-	3	3	2	1	33
G3/10	35 (1)	1	-	9	5	1	51
G4/9	102	7	6	19	3	1	138
G5	21	1 (1)	1 (1)	4	2	1	30
G6/7	19 (1)	-	-	4	2	1	26
G8	31 (1)	1	2	7	2	1	44

AE= antibiotic efflux; RP= reduced permeability; AI= antibiotic inactivation; ATA= antibiotic target alteration; ATP= antibiotic target protection; ATR= antibiotic target replacement; (n)= number of these genes that were found in plasmids

4. FINAL REMARKS

In conclusion, the extensive genomic analysis of eight distinct *E. coli* strains has unveiled compelling insights into the genetic diversity and evolutionary relationships within this bacterial species. The phylogenomic analysis reveals a clear demarcation between probiotic strains and their pathogenic or commensal counterparts, indicating a notable divergence among *E. coli* strains. This phylogenomic heterogeneity reflects the evolutionary pathways these strains have taken, potentially influenced by their specific ecological niches and adaptations. Furthermore, the identification of exclusive genes associated with antibiotic resistance and metabolic pathways within these strains underscores the vast genetic repertoire inherent to *E. coli*. The presence of unique genes linked to antibiotic resistance is of particular interest, as it highlights the potential implications for bacterial fitness and response to selective pressures. These genetic variations might provide a competitive advantage, aiding survival in diverse environments, including those challenged by antibiotics.

This study lays a strong foundation for future research endeavors aimed at elucidating the functional implications of the identified genomic features. Understanding the roles and mechanisms of these unique genes in antibiotic resistance, metabolic processes, and other biological functions is critical for harnessing this knowledge in

medical, biotechnological, and ecological applications. Moreover, the findings invite exploration into the potential applications of these probiotic strains in various biological contexts, including human health and environmental remediation. Ultimately, this genomic exploration enriches our understanding of *E. coli*'s genetic landscape and sets the stage for further in-depth investigations.

Data availability

The data supporting this chapter can be found on:
<http://doi.org/10.6084/m9.figshare.24188553>.

PRODUCTION AND CHARACTERIZATION OF *Escherichia coli* CEC15 and Nissle 1917 EXTRACELLULAR VESICLES

1. INTRODUCTION

Most studies on probiotics aim at better understanding of the mechanisms behind the beneficial effects associated with these organisms (Ogles et al., 2003). These mechanisms include the production of antimicrobial substances (Castilho et al., 2019), such as bacteriocins, modulation of the intestinal microbiota (Y. Shi et al., 2017), maintenance of the intestinal epithelial barrier (Blackwood et al., 2017), increase in intestinal epithelial barrier (Blackwood et al., 2017), mucin production and defense mechanisms (Aliakbarpour et al., 2012), inhibition of nuclear factor- κ B (NF- κ B) pathways (Kaci et al., 2011), production of short-chain fatty acids (SCFA), which are an essential source of energy for enterocytes and critical molecules for signaling pathways for the maintenance of intestinal health, and immunomodulation (Azad et al., 2018). Most of these beneficial effects are directly linked to humoral modulation of the inflammatory process, modulation of non-specific cellular immunity, and protection of immune barriers. Studies have demonstrated the action of bacteria such as members of the Lactobacillaceae family, *Lactococcus* spp., *Bifidobacterium* spp., *Propionibacterium* spp., *Escherichia coli* Nissle 1917, and the yeast *Saccharomyces* spp. on dendritic cells and regulatory T lymphocytes that are able to control inflammation in the gastrointestinal tract and other systems in the host (Liu et al., 2018; Meijerink and Wells, 2010; Smits et al., 2005). Recently, the role of extracellular vesicles produced by some of these strains have shown effects in the prevention and/or regulation of the inflammatory process.

Several living organisms secrete extracellular vesicles (EVs) into the environment, including bacteria, archaea, and eukaryotes. These are spherical and nanometric lipid bilayers that mediate intercellular communication (Deatherage and Cookson, 2012; Nieuwland and Sturk, 2010; Tkach and Théry, 2016). EVs can transport biologically active molecules, including proteins, lipids, metabolites, and nucleic acids from donor cells to distant recipient cells, contributing to important physiological and pathological processes. Bacterial EVs play several roles in interactions with other bacteria and with the host, through direct vesicle-mediated activation of target cells, or through the transfer of functional vesicular content to recipient cells. Therefore, they can participate in numerous processes, depending on the species of bacteria, including pathogenesis, elimination of competing bacteria, microbiota homeostasis, bacterial survival, gene transfer, and host cell modulation (Brown et al., 2015; Haurat et al., 2015; J. H. Kim et al., 2015). In terms of nomenclature, Gram-negative bacteria are linked to the generation of various types of vesicles, including outer-membrane vesicles

(OMVs), explosive outer-membrane vesicles (EOMVs), outer-inner membrane vesicles (OIMVs), and tube-shaped membranous structures (TSMSs). On the other hand, Gram-positive bacteria are associated with cytoplasmic membrane vesicles (CMVs or MVs) and TSMSs (Nagakubo et al., 2020; Toyofuku et al., 2019). Nevertheless, it's important to note that the all-encompassing term "extracellular vesicles" or "EVs" encompasses all these various vesicle types (Théry et al., 2018). When it comes to the mechanisms underlying their formation, research involving different bacterial species has primarily focused on OMVs and MVs. These studies have indicated that while there are complex genetic and environmental factors at play, the formation of EVs is primarily influenced by factors that impact membrane stability and cell wall permeability (Briaud and Carroll, 2020; Volgers et al., 2018).

Studies involving the mechanisms of interaction between EVs and hosts have become an attractive area, with rapid development and great potential for clinical applications. However, there are few studies available for Gram-positive and Gram-negative bacteria, particularly in relation to bacteria with probiotic potential (Brown et al., 2015; Haurat et al., 2015; J. H. Kim et al., 2015). Currently, EVs derived from *Lactobacilli* species Hao et al., 2021; Tong et al., 2021, *Bifidobacterium* spp. Nishiyama et al., 2020, and *Propionibacterium freudenreichii* CIRM-BIA 129 have been studied (de Rezende Rodvalho et al., 2020). EVs isolated from these strains have been shown to reproduce the immunoregulatory effects similar to the live parental bacterium. In addition, EVs were found to be produced by the *E. coli* Nissle 1917 strain with activity on the modulation of immune response in intestinal epithelial cells (Cañas et al., 2018), modulate the intestinal microbiome and metabolome (J. Shi et al., 2023), enhancing immunomodulation and antimicrobial activity in macrophages (Hu et al., 2020), regulate intestinal barrier dysfunction (Alvarez et al., 2016; Alvarez et al., 2019), and promoting anti-inflammatory effect in a DSS-induced colitis animal model (M.-J. Fábrega et al., 2017).

This work aims to identify the production of EVs by the potential probiotic strain *E. coli* CEC15 and characterize their functionality based on the EVs produced by the reference strain *E. coli* Nissle 1917.

2. MATERIAL AND METHODS

2.1. Strains growth

Two *Escherichia coli* strains were used in this work. We previously isolated the primo-colonizing *E. coli* CEC15 (CEC15) strain from freshly pooled fecal samples of 15-day-old suckling rodents (Tomas et al., 2015). The probiotic *E. coli* Nissle 1917 (EcN) strain was isolated from the commercial product Mutaflor. CEC15 and EcN were grown on Luria-Bertani (LB) medium (1% peptone, 0.5% yeast extract, and 0.5% NaCl) at 37°C under shaking conditions (150 rpm).

2.2. Extracellular vesicles preparation and standardization of purification protocol

A culture of each strain was inoculated in LB medium (10 mL, 8h, 37 °C, 150 rpm), reinoculated in 500 mL LB medium, and incubated overnight (16 h, 37 °C, 150 rpm). The cultures were centrifuged at 10,000 x g for 20min at 4°C and filtered (0.22 µM filter) to remove cells and large debris. The filtrate was concentrated using Amicon 100 kDa 15 mL concentration units (MERK, USA) until a final volume of 500 µL (1,000-fold concentration). The concentrate of EVs was purified using two methodologies.

2.2.1. Size Exclusion Chromatography purification

EVs in supernatant concentrate were purified using size exclusion chromatography (SEC) (iZon qEVoriginal / 70 nm columns) using PBS buffer as a carrier. The column was washed with 30 mL of PBS and it was drained until the PBS reached the top of the silica. The 500 µL obtained above was placed on the top of the column and, from this moment on, the bottom of the column was open and 14 aliquots of 500 µL were collected (after the 500 µL concentrate had entered the column, it was filled with PBS). Aliquots were individually analyzed.

2.2.2. OptiPrep™ Density Gradient purification

OptiPrep Density gradient (60% (w/v) solution of iodixanol in water (sterile)) was diluted to 50%, 45%, 40%, 35%, 30%, 25%, 20%, 15%, and 10% in DNase/RNase-free water. These dilutions (500 µL each) were placed in a ultracentrifuge tube, with care to not mix the fractions. At the top was placed the 500 µL concentrate obtained above. The gradient was then centrifuged at 140,000 x g for 16 h, 4 °C, and 10 fractions of 500 µL were carefully collected. Fractions were individually analyzed.

2.2.3. Biophysical characterization of EVs

EVs concentration and size were estimated from each aliquot / fraction obtained above, by nanoparticle tracking analysis (NTA) assay (NanoSight NS300, Malvern, UK). Transmission electron microscopy (TEM) was performed to evaluate the morphology, homogeneity and integrity of EVs, as described in (Luz et al., 2021), by negative staining with 2% uranyl acetate and observed at JEOL 1400 transmission electron microscope (JEOL Ltd., Japan), operating at 120 kV to examine EVs' shape and integrity. All aliquots from both purification strategies were analyzed by NTA analysis. The aliquots with better NTA results were evaluated by TEM. The aliquots with the larger quantity of EVs were pooled and further concentrated using Amicon 2mL 100 kDa concentration units to a final volume of 40 µL.

2.3. Whole-cell protein extraction

Strains were grown overnight in LB broth (10 mL, 16 h, 37 °C, 150 rpm) and centrifuged at 10,000 x g for 10min, the supernatant was removed and the pellet was washed twice with sterile PBS and centrifuged at 8,000 x g for 10 min. Bacterial pellets were re-suspended in 2 mL lysis solution (0.5 mL pH 7.5, 1 M Tris-HCl, 0.03 g SDS, 0.3 g DTT, 9.5 mL H₂O, and 1 mM phenylmethylsulphonyl fluoride (PMSF)), and incubated at -20 °C overnight. The frozen samples were thawed and dispersed by sonication (1 min, 1 HZ, 1W). The suspensions were transferred to 1.5 mL tubes with zirconia beads, and submitted to Precellys Evolution homogenizer (6 cycles of 30 s with 60 s intervals in between, 9,000 rpm). Samples were then centrifuged at 18,500 x g for 30min, the supernatants were carefully transferred to a new tube and stored at -20 °C until the time of use. Protein content was quantified by Qubit fluorometer (ThermoFisher, USA).

2.4. Proteomic analysis

Three independent replicates of EVs and whole-cell proteins (10 µg each), from each strain were separated on 12% SDS-PAGE and stained with Coomassie-blue (BIO-RAD, France). In-gel trypsin digestion was performed as described before (da Luz et al., 2022). Peptides were identified by mass spectrometry as described by (Tarnaud et al., 2020), followed by protein identification (maximum e-value of 0.05) from the MS/MS spectra with the X!TandemPipeline software (Langella et al., 2017). The peptides were searched against the genome sequences of the two strains described above with parameters as described in (de Rezende Rodvalho et al., 2020). A minimum of 3 peptides per protein was necessary for the validation of the sample and a protein was only considered present when it was identified in at least two of the three replicates. Protein cellular localization was performed using Cello2Go (Yu et al., 2014). Proteins were categorized into Clusters of Orthologous Groups (COG).

2.5. Modulation of Caco-2 cells

The human colon carcinoma cells (Caco2) were grown on DMEM high glucose (DMEM-HG) media supplemented with fetal bovine serum (FBS) at 10% and antibiotics Penicillin/Streptomycin (P/S) (Sigma: P0781 100x). The cells were seeded in a 75 cm² flask at a density of 1x10⁴ cells/cm² and incubated at 37 °C and 5% CO₂ until reached 80% confluence. The cells were washed twice with PBS and detached with trypsin 0.25% for 5 min at 37 °C, live cells were counted using the TC20 Automated Cell Counter (BIO-RAD) with trypan blue staining. A 6-well plate was prepared by seeding 1x10⁵ Caco2 cells/well, and it was kept at 37 °C and 5% CO₂ for 21 days until differentiation. The media was changed every 2 to 3 days for flasks and plates.

CEC15 and EcN strains were grown as described before. The cultures were diluted 10 and 100-fold and aliquoted in 1 mL microtubes. The strains were inactivated by heating at 60 °C for 1 hour. The microtubes were centrifuged 5000 x g for 10 min, the supernatant was removed and the pellet was re-suspended in 1 mL of DMEM-HG with antibiotics and FBS. On the day of the assay, the media was removed and cells were washed twice with sterile PBS. The PBS was then replaced for DMEM-HG (control), DMEM-HG + inactivated bacteria at MOI of 10 or 100, or DMEM-HG + EVs at the concentration of 1.10^9 or 1.10^{10} EVs/mL. The plate was incubated for 24 h at 37 °C and 5% CO₂. After incubation, the supernatant was removed and the cells were washed with PBS to remove the media and bacteria. Cells proceeded to RNA extraction and gene expression analysis. The assay was performed in three independent experiments.

2.6. RNA extraction and RT-qPCR assay

Total RNA was extracted using the RNeasy mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The cDNA was prepared from 1 µg of RNA using the qScript cDNA Synthesis Kit (Quantabio - Beverly, MA, EUA). The qPCR analysis was performed using the iQ™ SYBR® Green Supermix (BIO-RAD - Hercules, California, EUA) according to manufacture for a final volume of 20 µL and run in the CFX96 Real-Time system Thermal cycler (BIO-RAD - Hercules, California, EUA) with the following program: 95 °C for 3 min, 40 cycles of 95 °C for 10 s and 60 °C for 30 s, followed by a melting curve 55 – 95 °C increasing 0.5 °C per cycle. Data were analyzed by the method $2^{-\Delta\Delta CT}$ method using *GAPDH*, *B2M*, and *HPRT1* as reference genes. The list of primers used can be found in table 1.

Table 1. Sequence of primers used in this study.

Gene	Primer sequence	Reference
<i>GAPDH</i> *	(F) CTGGGCTACACTGAGCACC	(Jafarabadi et al., 2015)
	(R) AAGTGGTCGTTGAGGGCAATG	
<i>B2M</i> *	(F) GAGGCTATCCAGCGTACTCCA	(Saling et al., 2017)
	(R) CGGCAGGCATACTCATCTTTT	
<i>HPRT1</i> *	(F) CCTGGCGTCGTGATTAGTGAT	(Saling et al., 2017)
	(R) AGACGTTTCAGTCCTGTCCATAA	
<i>IL1B</i>	(F) ATGATGGCTTATTACAGTGGCAA	(Ding et al., 2020)
	(R) GTCGGAGATTCGTAGCTGGA	
<i>IL8</i>	(F) GTCGGAGATTCGTAGCTGGA	(Fang et al., 2018)
	(R) AACCCCTCTGCACCCAGTTTTTC	
<i>TLR7</i>	(F) TCCTTGGGGCTAGATGGTTTC	(Ye et al., 2021)
	(R) TCCACGATCACATGGTTCTTTG	
<i>TNF</i>	(F) CCTCTCTCTAATCAGCCCTCTG	(X. Li et al., 2016)
	(R) GAGGACCTGGGAGTAGATGAG	
<i>PTGS2</i>	(F) CTGGCGCTCAGCCATACAG	(Koike et al., 2022)
	(R) CGCACTTATACTGGTCAAATCCC	
<i>NOS2</i>	(F) TTCAGTATCACAACCTCAGCAAG	(Kong et al., 2017)
	(R) TGGACCTGCAAGTTAAAATCCC	

* Genes used as reference for RT-qPCR analysis.

3. RESULTS AND DISCUSSION

3.1. EVs production and standardization

Extraction and purification steps showed that both strains are able to produce EVs. After analysing all aliquots from OptiPrep and SEC purification, we could see that EVs purified with SEC present a higher concentration of EVs and with a more similar shape and size, as seen on Figure 1. Based on this results, from here on all EVs samples used were purified by SEC. From SEC purification, aliquots 7 - 9 were collected and pooled together, they were then further concentrated using Amicon 100 kDa 2 mL concentration units (MERK, USA) until a final volume of around 49 μ L. Hong and colleagues (Hong et al., 2019) performed a proteome comparison of both purification methods used here, density gradient centrifugation and SEC. In summary, distinct protein sets exhibited enrichment or reduction through both density gradient centrifugation and SEC purification. Specific proteins remained consistently under- or over-represented in EV samples purified via both density gradient centrifugation and SEC compared to their initial EV preparations (non purified). This consistency indicates that the additional purification steps indeed lead to the depletion or enrichment of protein cargo within the EVs and should be considered carefully to avoid study biases. A schematic representation of the full process can be seen on Figure 2.

3.2. EVs characterization and proteomic analysis

After SEC purification, CEC15 and EcN strains produced EVs with typical round cup-shape structure and monodisperse size, with a mean size of 128 ± 1.3 nm and modal size of 97.8 ± 1.8 nm for CEC15 (Figure 3A and 3B), and mean size of 165.9 ± 2.5 nm and modal size of 144.6 ± 3.7 nm for EcN strain (Figure 3C and 3D). EVs from EcN have been produced and purified by different methods in the literature, with average size varying from 90 - 210nm (Hong et al., 2019; Hu et al., 2020; J. Shi et al., 2023). EVs and whole-cell extracted proteins were submitted to SDS-PAGE to observe the band pattern. Figure 2E shows the differences in bands among the strains' whole-cell and EVs proteomes, it's possible to see a difference in composition on the two proteomes of the same strain and between strains. This differences were proven after proteome analysis, which showed that a total of 411 proteins were found expressed on CEC15 whole-cell proteome while EcN presented 403, the proteome of the EVs comprehend 120 and 177 proteins for CEC15 (Table 2) and EcN (Table 3) respectively. CEC15 whole-cell and EV proteome shared a total of 96 proteins, against 150 shared by EcN proteomes.

A total of 82 proteins were found exclusive in CEC15 proteome, 14 (12 chromosomal and 2 plasmidial) of which are not present on the genome of EcN. EcN, on the other hand, expressed 77 exclusive proteins, 19 of those are not present on the

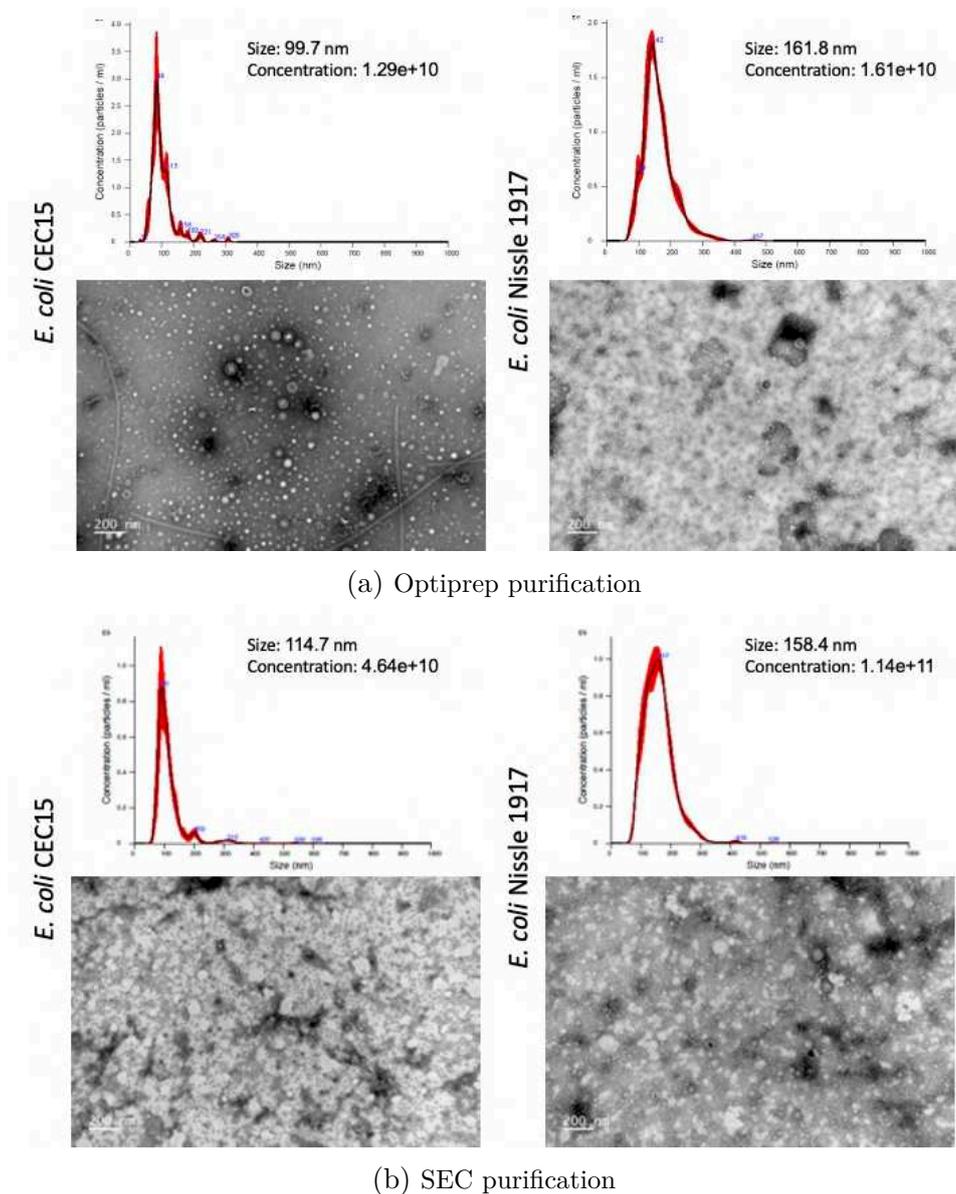


Figure 1: Physical characterization of *E. coli* EVs. (A) Nanoparticle tracking analysis (NTA) measurement of EVs size and concentration and transmission electron microscopy (TEM) pictures of EVs purified by OptiPrep density gradient centrifugation. (B) NTA measurements and TEM pictures of EVs purified by size exclusion chromatography (SEC).

CEC15 genome. From these exclusive proteins, 11 were only present on CEC15 EVs and 15 on EcN EVs. Figure 2F shows a Venn diagram showing the proteins that are shared among the four proteome.

The proteins found on EVs are wide varied with no group of proteins with specific functions taking the majority. It is important to note, however, the number of ribosomal proteins, secretion systems, permeases, and enzymes related to metabolic pathways. Another highlight is the presence of adhesins, specially Fimbriae proteins found on both EVs, which could promote adhesion of EVs to host cell as it has previously seen on the viable bacteria. Similar results were presented by Hu and colleagues (Hu et al., 2020), which revealed that numerous proteins present in vesicles, primarily originating from

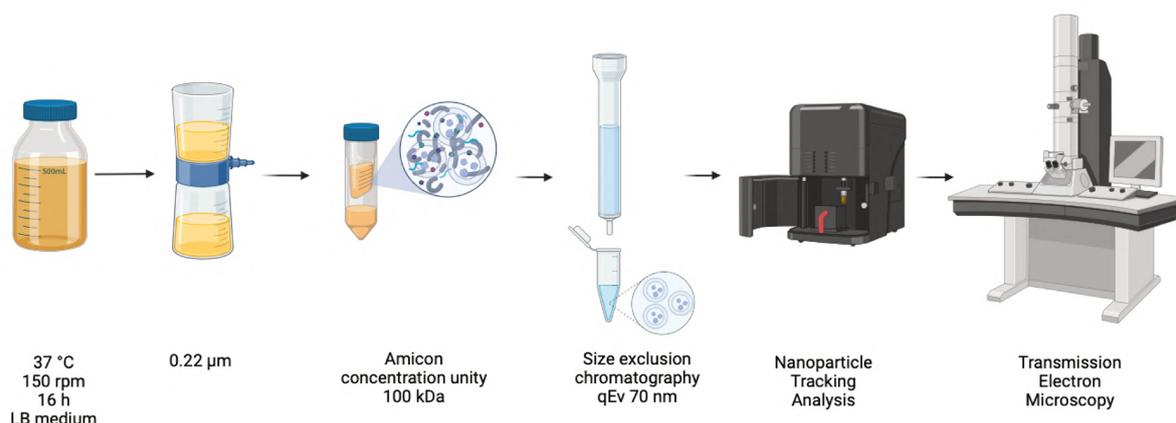


Figure 2: Schematic of the production, isolation, purification, and characterization steps of EVs in this study.

the outer membrane, could significantly contribute to the probiotic properties of EcN. These contributions encompass various aspects crucial for probiotic behavior, including intestinal adhesion and colonization (for example, flagellins like *flgA*, *flgE*, and *flgK*), bacterial survival within host niches (involving proteins associated with transport activities), antimicrobial capabilities (such as murein hydrolases like *mltA* and *mltC*), and immunomodulation of the host (involving various outer membrane proteins like *OmpA*, *OmpC*, and *OmpF*) (Hu et al., 2020). The presence of these diverse probiotic-related proteins in EcN EVs strongly implies that they might be instrumental in mediating the probiotic's effects on immune regulation and protection against diseases.

EcN EVs presents the bacteriocin Microcin H47, which could be useful for inhibition of other bacteria. Also a number of phage related proteins were found on EcN EVs, which could indicate the presence of active phages inside the EVs. The presence of phages, intact or defectives, inside EVs has been reported before in *Lactococcus lactis* and has been linked to stimulation of EVs formation (Liu et al., 2022). No production of any protein related to the synthesis of the genotoxic agent colibactin was detected in neither EVs or whole-cell proteome of EcN, what suggests that colibactin is not produced without environmental stimulation.

Table 2. Proteins identified in CEC15 EVs.

Locus-tag	Gene	Product
Cec15_00068	<i>slmA</i>	nucleoid occlusion factor SlmA
Cec15_00298		fimbrial protein
Cec15_00337	<i>mrcA</i>	peptidoglycan glycosyltransferase/peptidoglycan DD-transpeptidase MrcA
Cec15_00373	<i>yheU</i>	YheU family protein
Cec15_00379	<i>slyD</i>	peptidylprolyl isomerase
Cec15_00381	<i>fkpA</i>	FKBP-type peptidyl-prolyl cis-trans isomerase
Cec15_00386	<i>gspG</i>	type II secretion system major pseudopilin GspG

Cec15_00387	<i>gspF</i>	type II secretion system inner membrane protein GspF
Cec15_00389	<i>gspD</i>	type II secretion system secretin GspD
Cec15_00391*	<i>bfr</i>	bacterioferritin
Cec15_00392	<i>gspB</i>	putative general secretion pathway protein GspB
Cec15_00398	<i>rpsS</i>	30S ribosomal protein S19
Cec15_00399	<i>rplV</i>	50S ribosomal protein L22
Cec15_004010	<i>rplP</i>	50S ribosomal protein L16
Cec15_00403	<i>rpsQ</i>	30S ribosomal protein S17
Cec15_00405	<i>rplX</i>	50S ribosomal protein L24
Cec15_00408	<i>rpsH</i>	30S ribosomal protein S8
Cec15_00410	<i>rplR</i>	50S ribosomal protein L18
Cec15_00411	<i>rpsE</i>	30S ribosomal protein S5
Cec15_00412	<i>rpmD</i>	50S ribosomal protein L30
Cec15_00451		DeoR/GlpR family DNA-binding transcription regulator
Cec15_00469	<i>tldD</i>	metalloprotease TldD
Cec15_00474	<i>yhcO</i>	barstar family protein
Cec15_00475	<i>yhcN</i>	peroxide/acid stress response protein YhcN
Cec15_00552	<i>ubiT</i>	SCP2 domain-containing protein
Cec15_00584	<i>garD</i>	galactarate dehydratase
Cec15_00598*	<i>cyuA</i>	cysteine desulfidase
Cec15_00632	<i>higA</i>	type II toxin-antitoxin system antitoxin HigA
Cec15_00662	<i>yqiJ</i>	DUF1449 family protein
Cec15_00696*	<i>yqhD</i>	alcohol dehydrogenase
Cec15_00750	<i>pabC</i>	aminodeoxychorismate lyase
Cec15_00828		IS3 family transposase
Cec15_00907	<i>rpiA</i>	ribose-5-phosphate isomerase RpiA
Cec15_01033	<i>rlmM</i>	23S rRNA (cytidine(2498)-2'-O)-methyltransferase RlmM
Cec15_01034	<i>fucR</i>	L-fucose operon activator
Cec15_01072	<i>yqcE</i>	MFS transporter
Cec15_01122	<i>hycC</i>	formate hydrogenlyase subunit 3
Cec15_01238	<i>yfiE</i>	LysR family transcriptional regulator
Cec15_01277	<i>yphD</i>	ABC transporter permease
Cec15_01282	<i>trmJ</i>	tRNA (cytosine(32)/uridine(32)-2'-O)-methyltransferase TrmJ
Cec15_01362	<i>hisS</i>	histidine-tRNA ligase
Cec15_01417	<i>eutM</i>	ethanolamine utilization microcompartment protein EutM
Cec15_01484	<i>fryC</i>	PTS fructose transporter subunit IIC
Cec15_01532	<i>aroC</i>	chorismate synthase
Cec15_01586	<i>rbn</i>	ribonuclease BN
Cec15_01609	<i>rhmD</i>	L-rhamnonate dehydratase
Cec15_01782*	<i>pphC</i>	protein-serine/threonine phosphatase PphC
Cec15_01860*		ead/Ea22-like family protein
Cec15_01861*		hypothetical protein
Cec15_01962	<i>hprR</i>	response regulator transcription factor HprR
Cec15_01963	<i>hprS</i>	two-component system sensor histidine kinase HprS
Cec15_02029		Fur-regulated protein
Cec15_02055		DinI-like family protein
Cec15_02107	<i>yobH</i>	YobH family protein

Cec15_02162*	<i>topB</i>	DNA topoisomerase III
Cec15_02239	<i>ydiL</i>	YdiL family protein
Cec15_02267	<i>ydhQ</i>	AIDA repeat-containing protein
Cec15_02277	<i>mepH</i>	peptidoglycan DD-endopeptidase MepH
Cec15_02491*	<i>aldA</i>	aldehyde dehydrogenase
Cec15_02623	<i>ycjX</i>	YcjX family protein
Cec15_02666	<i>ymiA</i>	YmiA family putative membrane protein
Cec15_02758		prophage tail fiber N-terminal domain-containing protein
Cec15_02783	<i>rrrD</i>	lysozyme RrrD
Cec15_02784	<i>essD</i>	phage lysis protein EssD
Cec15_02897		class II D-tagatose-bisphosphate aldolase 2C non-catalytic subunit
Cec15_02925		FTR1 family protein
Cec15_02943	<i>cbpM</i>	chaperone modulator CbpM
Cec15_02951	<i>gnsA</i>	addiction module toxin 2C GnsA/GnsB family
Cec15_03015	<i>kdsB</i>	3-deoxy-manno-octulosonate cytidyltransferase
Cec15_03040	<i>lolA</i>	outer membrane lipoprotein chaperone LolA
Cec15_03082	<i>grxA</i>	glutaredoxin 1
Cec15_03112	<i>ybiR</i>	anion transporter
Cec15_03113	<i>mntR</i>	manganese-binding transcriptional regulator MntR
Cec15_03114	<i>mntS</i>	manganase accumulation protein MntS
Cec15_03122	<i>glnQ</i>	glutamine ABC transporter ATP-binding protein GlnQ
Cec15_03128	<i>ybiX</i>	PKHD-type hydroxylase YbiX
Cec15_03131	<i>ybiE</i>	protein YbiE
Cec15_03215	<i>sucA</i>	2-oxoglutarate dehydrogenase E1 component
Cec15_03216	<i>sdhB</i>	succinate dehydrogenase iron-sulfur subunit SdhB
Cec15_03341	<i>entS</i>	enterobactin transporter EntS
Cec15_03351	<i>hokE</i>	type I toxin-antitoxin system toxin HokE
Cec15_03360	<i>cusF</i>	Cu(+)/Ag(+) efflux RND transporter periplasmic metallochaperone CusF
Cec15_03377	<i>ppiB</i>	peptidylprolyl isomerase B
Cec15_03402	<i>tesA</i>	multifunctional acyl-CoA thioesterase I/protease I/lysophospholipase L1
Cec15_03405	<i>fetB</i>	iron export ABC transporter permease subunit FetB
Cec15_03523*		PIG-L deacetylase family protein
Cec15_03662	<i>rbsK</i>	ribokinase
Cec15_03670		cupin domain-containing protein
Cec15_03671		gluconate 5-dehydrogenase
Cec15_03677		putative zinc ribbon protein
Cec15_03688		ABC transporter substrate-binding protein
Cec15_03722	<i>gloB</i>	hydroxyacylglutathione hydrolase
Cec15_03723	<i>mltD</i>	murein transglycosylase D
Cec15_03724		methyltransferase domain-containing protein
Cec15_03779	<i>mrcB</i>	bifunctional glycosyl transferase/transpeptidase
Cec15_03961	<i>fimB</i>	type 1 fimbria switch DNA invertase FimB
Cec15_04004		DUF5983 family protein
Cec15_04007*	<i>uxuA</i>	mannonate dehydratase
Cec15_04031	<i>fecR</i>	ferric citrate uptake sigma factor regulator FecR
Cec15_04034	<i>fecC</i>	iron-dicitrate ABC transporter permease FecC
Cec15_04064	<i>arcA</i>	arginine deiminase

Cec15_04082*	<i>ytfF</i>	DMT family transporter
Cec15_04188	<i>yjdJ</i>	GNAT family N-acetyltransferase
Cec15_04191	<i>dcuS</i>	sensor histidine kinase
Cec15_04242	<i>nrfE</i>	heme lyase CcmF/NrfE family subunit
Cec15_04243	<i>nrfD</i>	cytochrome c nitrite reductase subunit NrfD
Cec15_04245	<i>nrfB</i>	cytochrome c nitrite reductase pentaheme subunit
Cec15_04246	<i>nrfA</i>	ammonia-forming nitrite reductase cytochrome c552 subunit
Cec15_04247	<i>acs</i>	acetate–CoA ligase
Cec15_04263	<i>aphA</i>	acid phosphatase AphA
Cec15_04299	<i>yjbE</i>	exopolysaccharide production protein YjbE
Cec15_04304		sugar-binding transcriptional regulator
Cec15_04362	<i>birA</i>	bifunctional biotin–[acetyl-CoA-carboxylase] ligase/biotin operon repressor BirA
Cec15_04452	<i>glnL</i>	nitrogen regulation protein NR(II)
Cec15_04497		DUF2877 domain-containing protein
Cec15_04499		DUF1116 domain-containing protein
Cec15_04507		LysR substrate-binding domain-containing protein
Cec15_04527		DUF3289 family protein
Cec15p_00067*		Serine protease pic autotransporter
Cec15p_00182*		Serine protease EspC

* exclusive proteins

Table 3. Proteins identified in EcN EVs.

Locus-tag	Gene	Product
EcN_00105		glycosyltransferase family A protein
EcN_00161	<i>yiaB</i>	inner membrane protein YiaB
EcN_00162	<i>yiaA</i>	inner membrane protein YiaA
EcN_00268	<i>tusA</i>	sulfurtransferase TusA
EcN_00315		fimbrial protein
EcN_00378	<i>nirB</i>	NADPH-nitrite reductase large subunit
EcN_00389	<i>yheU</i>	YheU family protein
EcN_00395	<i>slyD</i>	peptidylprolyl isomerase
EcN_00397	<i>fkpA</i>	FKBP-type peptidyl-prolyl cis-trans isomerase
EcN_00415	<i>gspH</i>	type II secretion system minor pseudopilin GspH
EcN_00416	<i>gspG</i>	type II secretion system major pseudopilin GspG
EcN_00417	<i>gspF</i>	type II secretion system inner membrane protein GspF
EcN_00419	<i>gspD</i>	type II secretion system secretin GspD
EcN_00422	<i>gspB</i>	putative general secretion pathway protein GspB
EcN_00428	<i>rpsS</i>	30S ribosomal protein S19
EcN_00430	<i>rpsC</i>	30S ribosomal protein S3
EcN_00431	<i>rplP</i>	50S ribosomal protein L16
EcN_00433	<i>rpsQ</i>	30S ribosomal protein S17
EcN_00435	<i>rplX</i>	50S ribosomal protein L24
EcN_00438	<i>rpsH</i>	30S ribosomal protein S8
EcN_00440	<i>rplR</i>	50S ribosomal protein L18
EcN_00441	<i>rpsE</i>	30S ribosomal protein S5

EcN_00442	<i>rpmD</i>	50S ribosomal protein L30
EcN_00489		DeoR/GlpR family DNA-binding transcription regulator
EcN_00491		substrate-binding domain-containing protein
EcN_00507	<i>tldD</i>	metalloprotease TldD
EcN_00512	<i>yhcO</i>	barstar family protein
EcN_00513	<i>yhcN</i>	peroxide/acid stress response protein YhcN
EcN_00546*	<i>kdsD</i>	arabinose-5-phosphate isomerase KdsD
EcN_00559	<i>yhbE</i>	DMT family transporter
EcN_00567	<i>glmM</i>	phosphoglucosamine mutase
EcN_00584*	<i>yhbW</i>	luciferase-like monooxygenase
EcN_00587	<i>ubiT</i>	SCP2 domain-containing protein
EcN_00656	<i>higA</i>	type II toxin-antitoxin system antitoxin HigA
EcN_00673	<i>rpsU</i>	30S ribosomal protein S21
EcN_00691	<i>yqiJ</i>	DUF1449 family protein
EcN_00793*	<i>gspH</i>	type II secretion system minor pseudopilin GspH
EcN_00812*		KpsF/GutQ family sugar-phosphate isomerase
EcN_00849*		N-acetylneuraminate epimerase
EcN_00850*		alpha/beta hydrolase
EcN_00889*	<i>papX</i>	HTH-type transcriptional regulator PapX
EcN_00901	<i>trmB</i>	tRNA (guanosine(46)-N7)-methyltransferase TrmB
EcN_00928*		prohibitin family protein
EcN_00950	<i>rpiA</i>	ribose-5-phosphate isomerase RpiA
EcN_01067	<i>rlmM</i>	23S rRNA (cytidine(2498)-2'-O)-methyltransferase RlmM
EcN_01068	<i>fucR</i>	L-fucose operon activator
EcN_01100	<i>yqcE</i>	MFS transporter
EcN_01147	<i>hycC</i>	formate hydrogenlyase subunit 3
EcN_01149	<i>hycE</i>	formate hydrogenlyase subunit HycE
EcN_01215*	<i>glaH</i>	glutarate dioxygenase GlaH
EcN_01218	<i>ratA</i>	type II toxin-antitoxin system toxin RatA
EcN_01241	<i>rluD</i>	23S rRNA pseudouridine(1911/1915/1917) synthase RluD
EcN_01259	<i>yfiE</i>	LysR family transcriptional regulator
EcN_01271	<i>era</i>	GTPase Era
EcN_01274	<i>acpS</i>	holo-ACP synthase
EcN_01278*	<i>yfhb</i>	phosphatidylglycerophosphatase C
EcN_01290	<i>yphF</i>	substrate-binding domain-containing protein
EcN_01292	<i>yphD</i>	ABC transporter permease
EcN_01300	<i>trmJ</i>	tRNA (cytosine(32)/uridine(32)-2'-O)-methyltransferase TrmJ
EcN_01310	<i>sseB</i>	enhanced serine sensitivity protein SseB
EcN_01319	<i>hisS</i>	histidine-tRNA ligase
EcN_01370	<i>eutM</i>	ethanolamine utilization microcompartment protein EutM
EcN_01430	<i>fryC</i>	PTS fructose transporter subunit IIC
EcN_01450	<i>emrY</i>	multidrug efflux MFS transporter permease subunit EmrY
EcN_01457		phase-variable autotransporter adhesin UpaE
EcN_01476	<i>aroC</i>	chorismate synthase
EcN_01536	<i>menD</i>	2-succinyl-5-enolpyruvyl-6-hydroxy-3-cyclohexene-1-carboxylic-acid synthase
EcN_01554	<i>rhmD</i>	L-rhamnonate dehydratase

EcN_01674	<i>cdd</i>	cytidine deaminase
EcN_01933*		type 4 pilus major pilin
EcN_01941	<i>hprR</i>	response regulator transcription factor HprR
EcN_01942	<i>hprS</i>	two-component system sensor histidine kinase HprS
EcN_02064		Fur-regulated protein
EcN_02067		phage terminase large subunit family protein
EcN_02102	<i>znuC</i>	zinc ABC transporter ATP-binding protein ZnuC
EcN_02105	<i>lpxM</i>	lauroyl-Kdo(2)-lipid IV(A) myristoyltransferase
EcN_02131*	<i>htpX</i>	protease HtpX
EcN_02134	<i>yobH</i>	YobH family protein
EcN_02176	<i>cdgl</i>	GGDEF domain-containing protein
EcN_02195	<i>sppA</i>	signal peptide peptidase SppA
EcN_02211	<i>ydjZ</i>	TVP38/TMEM64 family protein
EcN_02273	<i>ydiL</i>	YdiL family protein
EcN_02310	<i>mepH</i>	peptidoglycan DD-endopeptidase MepH
EcN_02392	<i>ydfG</i>	bifunctional NADP-dependent 3-hydroxy acid dehydrogenase/3-hydroxypropionate dehydrogenase YdfG
EcN_02528	<i>hslJ</i>	heat shock protein HslJ
EcN_02561	<i>ycjX</i>	YcjX family protein
EcN_02578	<i>pspA</i>	phage shock protein PspA
EcN_02582	<i>sapA</i>	ABC transporter substrate-binding protein SapA
EcN_02610	<i>ymiA</i>	YmiA family putative membrane protein
EcN_02622	<i>trpE</i>	anthranilate synthase component I
EcN_02691*	<i>ychF</i>	redox-regulated ATPase YchF
EcN_02704*	<i>ymgE</i>	GlsB/YeaQ/YmgE family stress response membrane protein
EcN_02724	<i>minD</i>	septum site-determining protein MinD
EcN_02729		hypothetical protein
EcN_02755*		phage minor tail protein L
EcN_02765		minor capsid protein E
EcN_02767		head decoration protein
EcN_02769*		phage head-stabilizing protein
EcN_02776	<i>rrrD</i>	lysozyme RrrD
EcN_02777	<i>essD</i>	phage lysis protein EssD
EcN_02780*	<i>ylcG</i>	YlcG family protein
EcN_02840	<i>pabC</i>	aminodeoxychorismate lyase
EcN_02865*	<i>flgN</i>	flagella biosynthesis chaperone FlgN
EcN_02871*	<i>grxB</i>	glutaredoxin 2
EcN_02907*		type IV toxin-antitoxin system YeeU family antitoxin
EcN_02962	<i>mchB</i>	microcin H47
EcN_03000		class II D-tagatose-bisphosphate aldolase 2C non-catalytic subunit
EcN_03020	<i>efeO</i>	iron uptake system protein EfeO
EcN_03021		FTR1 family protein
EcN_03039	<i>cbpM</i>	chaperone modulator CbpM
EcN_03047	<i>gnsA</i>	addiction module toxin 2C GnsA/GnsB family
EcN_03065	<i>yccX</i>	acylphosphatase
EcN_03127	<i>dmsC</i>	dimethyl sulfoxide reductase anchor subunit DmsC
EcN_03132	<i>lolA</i>	outer membrane lipoprotein chaperone LolA

EcN_03175	<i>grxA</i>	glutaredoxin 1
EcN_03205	<i>ybiR</i>	anion transporter
EcN_03206	<i>mntR</i>	manganese-binding transcriptional regulator MntR
EcN_03207	<i>mntS</i>	manganase accumulation protein MntS
EcN_03218	<i>mcbA</i>	DUF1471 family periplasmic protein McbA
EcN_03219	<i>fiu</i>	catecholate siderophore receptor Fiu
EcN_03221	<i>ybil</i>	C4-type zinc finger protein Ybil
EcN_03299	<i>sucA</i>	2-oxoglutarate dehydrogenase E1 component
EcN_03365*	<i>gltI</i>	glutamate/aspartate ABC transporter substrate-binding protein GltI
EcN_03420	<i>entS</i>	enterobactin transporter EntS
EcN_03453	<i>ybcJ</i>	ribosome-associated protein YbcJ
EcN_03454	<i>ybcI</i>	metal-dependent hydrolase
EcN_03456	<i>ppiB</i>	peptidylprolyl isomerase B
EcN_03458*		hypothetical protein
EcN_03481	<i>tesA</i>	multifunctional acyl-CoA thioesterase I/protease I/lysophospholipase L1
EcN_03721		PapB/FocB family fimbrial expression transcriptional regulator
EcN_03733		DNA-binding protein
EcN_03740	<i>fucP</i>	L-fucose:H ⁺ symporter permease
EcN_03741	<i>rbsK</i>	ribokinase
EcN_03749		cupin domain-containing protein
EcN_03750		gluconate 5-dehydrogenase
EcN_03753		AlpA family transcriptional regulator
EcN_03757		putative zinc ribbon protein
EcN_03764		hypothetical protein
EcN_03768		ABC transporter substrate-binding protein
EcN_03801	<i>rnhA</i>	ribonuclease HI
EcN_03804	<i>mltD</i>	murein transglycosylase D
EcN_03805		methyltransferase domain-containing protein
EcN_03825	<i>arfB</i>	alternative ribosome rescue aminoacyl-tRNA hydrolase ArfB
EcN_03828	<i>rof</i>	Rho-binding antiterminator
EcN_03868	<i>mrcB</i>	bifunctional glycosyl transferase/transpeptidase
EcN_03891	<i>yadG</i>	ABC transporter ATP-binding protein
EcN_03923	<i>yacG</i>	DNA gyrase inhibitor YacG
EcN_03950*	<i>leuL</i>	leu operon leader peptide
EcN_03963*		hypothetical protein
EcN_04080*	<i>btsT</i>	pyruvate/proton symporter BtsT
EcN_04113*	<i>fimH</i>	type 1 fimbria D-mannose specific adhesin FimH
EcN_04131		hypothetical protein
EcN_04164		DUF5983 family protein
EcN_04190	<i>fecR</i>	ferric citrate uptake sigma factor regulator FecR
EcN_04224	<i>arcA</i>	arginine deiminase
EcN_04238	<i>nrdD</i>	anaerobic ribonucleoside-triphosphate reductase
EcN_04355	<i>yjdJ</i>	GNAT family N-acetyltransferase
EcN_04357	<i>dcuS</i>	sensor histidine kinase
EcN_04413	<i>nrfE</i>	heme lyase CcmF/NrfE family subunit
EcN_04414	<i>nrfD</i>	cytochrome c nitrite reductase subunit NrfD
EcN_04416	<i>nrfB</i>	cytochrome c nitrite reductase pentaheme subunit

EcN_04417	<i>nrfA</i>	ammonia-forming nitrite reductase cytochrome c552 subunit
EcN_04418	<i>acs</i>	acetate-CoA ligase
EcN_04434	<i>aphA</i>	acid phosphatase AphA
EcN_04477	<i>yjbE</i>	exopolysaccharide production protein YjbE
EcN_04482		sugar-binding transcriptional regulator
EcN_04492		hypothetical protein
EcN_04538	<i>birA</i>	bifunctional biotin-[acetyl-CoA-carboxylase] ligase/biotin operon repressor <i>BirA</i>
EcN_04546	<i>trmA</i>	tRNA (uridine(54)-C5)-methyltransferase TrmA
EcN_04649	<i>glnL</i>	nitrogen regulation protein NR(II)
EcN_04677	<i>tatD</i>	3'-5' ssDNA/RNA exonuclease TatD
EcN_04679*	<i>tatB</i>	Sec-independent protein translocase protein TatB
EcN_04694		DUF2877 domain-containing protein
EcN_04696		DUF1116 domain-containing protein
EcN_04698		glutamyl-tRNA amidotransferase
EcN_04700		LysR substrate-binding domain-containing protein
EcN_04719		DUF3289 family protein

* exclusive proteins

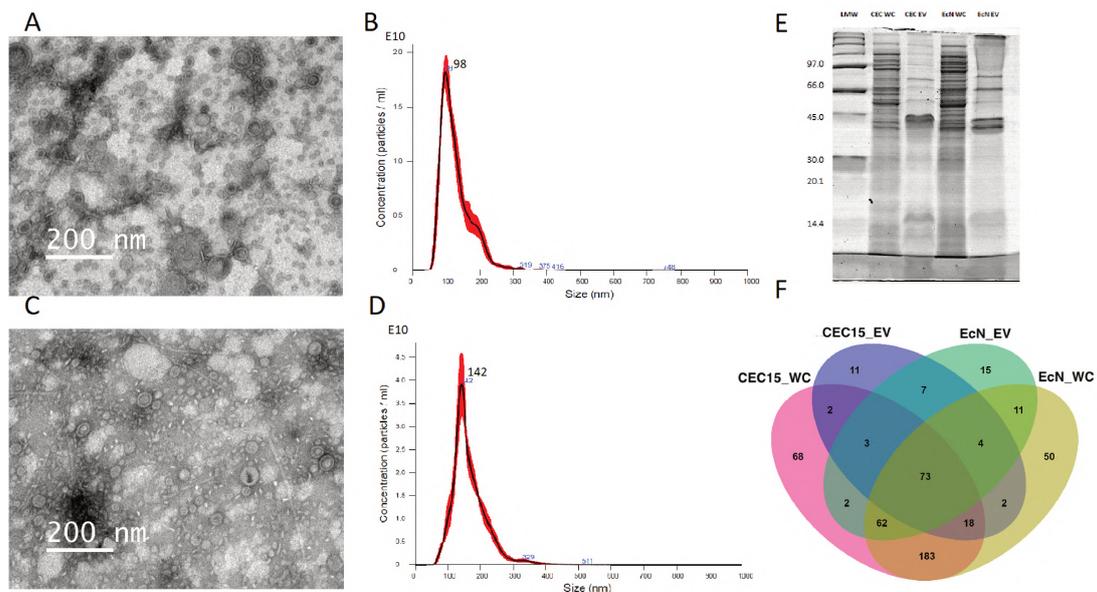


Figure 3: Characterization of extracellular vesicles produced by *E. coli* strains. (A,C) TEM and (B,D) NTA measurements of of EVs produced by CEC15 and EcN, respectively. (E) Comparative SDS-PAGE of the cellular and EV proteome of the two *E. coli* strains. (F) Venn diagram of the exclusive and shared proteins between bacteria whole-cell (WC) and vesicles (EV) of CEC15 and EcN.

These proteins can be categorized into COGs (Clusters of Orthologous Groups) based on their function. When compared the relative quantity of proteins in each COG category between WC and EVs it's possible to see some clear differences especially on category M (Cell wall/membrane/envelope biogenesis), which corresponds to the proteins that compose the membranes of bacteria, being in a larger proportion on EVs than in the whole-cell. Another clear difference can be seen in category J (Translation),

higher proportionally in the whole cell. In general, both strains and their respective EVs present a similar pattern of COG categories distribution (Figure 4A) and are in accordance with what was present above, and as have been described before by Hu et al. (Hu et al., 2020). As for the localization of the proteins, EVs have a shift towards the relative presence of more membranes and periplasm proteins than the whole-cell, with this being more accentuated on the CEC15 strain EVs, which has the largest percentage of periplasmic, outer-membrane, and extracellular proteins (Figure 4B).

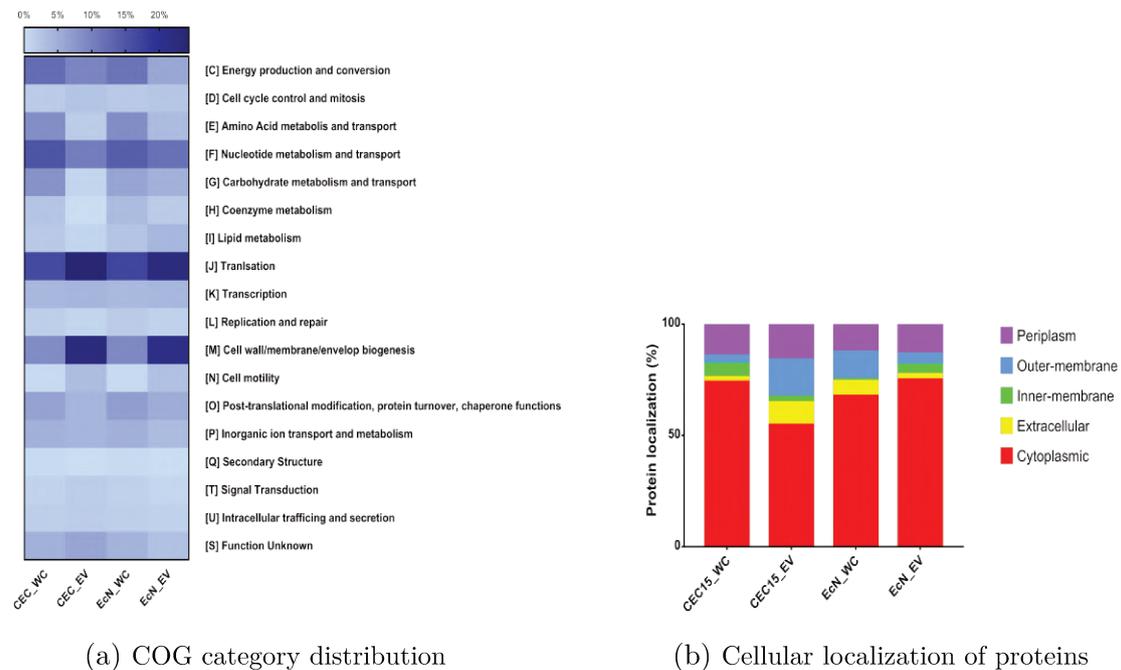


Figure 4: Profile characterization of CEC15 and EcN whole-cell and EVs' proteomes.

3.3. EVs immunomodulation of Caco2 cells

The EVs and the bacterial cell inactivated by heat of both strains were tested on Caco-2 cells for their ability to modulate the gene expression of key factors for immunoregulation and epithelial integrity. The 4 conditions tested, EVs at the concentration of $1 \cdot 10^{10}$ EVs/mL, EVs at the concentration of $1 \cdot 10^9$ EVs/mL, inactivated bacteria at MOI100, and inactivated bacteria at MOI10, were able to promote some degree of modulation in the majority of the genes tested (Figure 5), notably the increased expression of Toll-like receptor 7 (*TLR7*) by inactivated bacteria of CEC15 (Figure 5E) and, in contrast, by EVs from EcN (Figure 5F), and the increased expression of Interleukin 8 (*IL8*) by EVs and inactivated bacteria at high concentrations from CEC15 (5 and 7-fold) (Figure 5C) and EVs from EcN (12-fold) (Figure 5D). IL-8 plays a crucial role in various neutrophil functions, including recruitment, granule release activation, superoxide generation induction, and increased expression of adhesion molecules (Peveri et al., 1988; Schröder, 1989). Previous research has established that EcN can elevate

IL8 expression in different human intestinal epithelial cell lines, such as Caco-2 cells. This elevation is primarily associated with EcN's flagella (Sabharwal et al., 2016), its capsule (K5) (Hafez et al., 2009), and other unidentified factors (Wan et al., 2018).

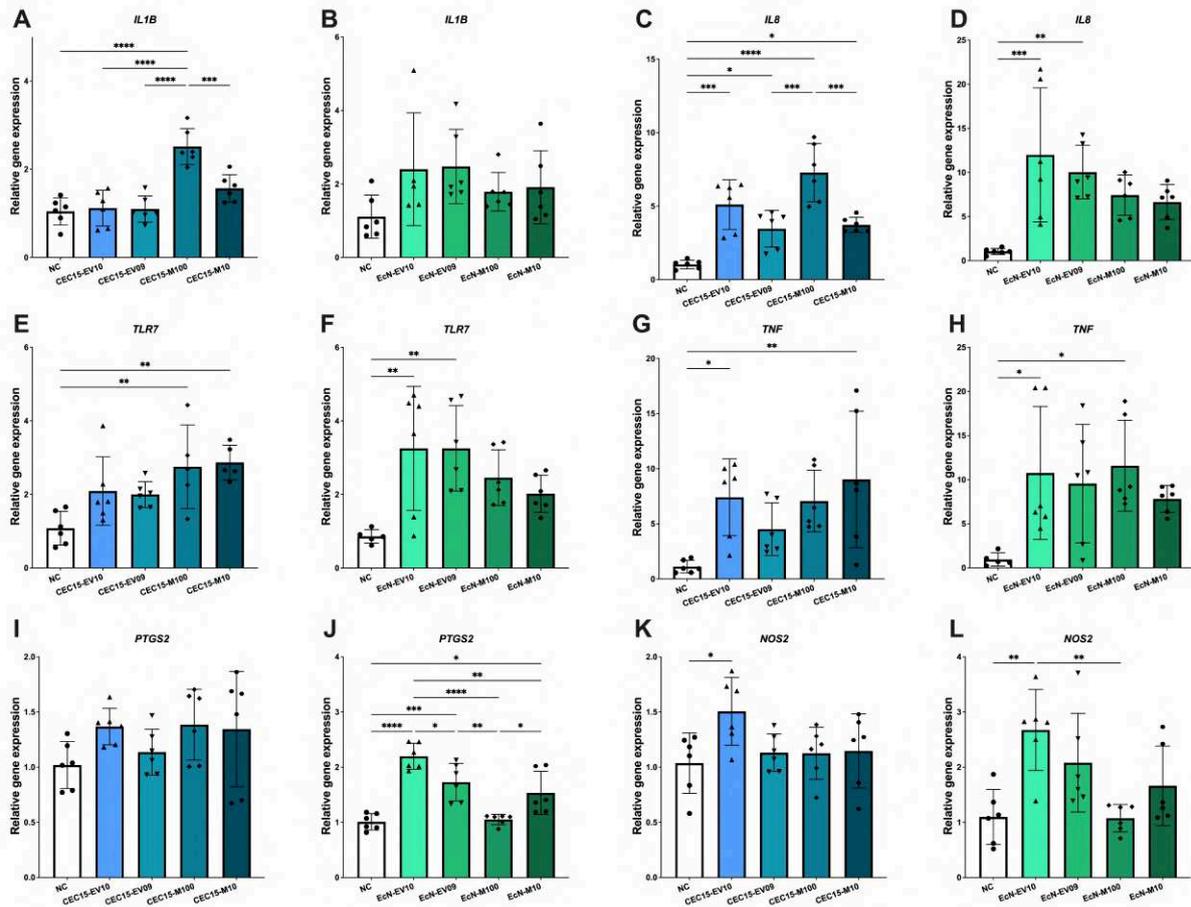


Figure 5: Modulation of immunomodulatory genes expression in Caco2 cells. The relative gene expression of genes related to immunomodulation and intestinal barrier (*IL1B* [A and B], *IL8* [C and D], *TLR7* [E and F], *TNF* [G and H], *PTGS2* [I and J], and *NOS2* [K and L] on CEC15- and EcN-treated cells, respectively, were evaluated with the *GAPDH*, *B2M*, and *HPRT1* genes as reference ($2^{-\Delta\Delta ct}$). Statistical analysis were performed by One-way ANOVA with Tukey's post-test on GraphPad Prism 7.0. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$. NC - negative control; CEC15-EV10 - CEC15 EVs 10^{10} particles; CEC15-EV09 - CEC15 EVs 10^9 particles; CEC15-M100 - CEC15 treatment at MOI 100; CEC15-M10 - CEC15 treatment at MOI 10; EcN-EV10 - EcN EVs 10^{10} particles; EcN-EV09 - EcN EVs 10^9 particles; EcN-M100 - EcN treatment at MOI 100; EcN-M10 - EcN15 treatment at MOI 10

The Interleukin 1 β (*IL1B*) gene expression was increased under CEC15 inactivated bacteria MOI100 stimulation (Figure 5A) while no modulation was observed for the EcN strain (Figure 5B). In a healthy state, IL1 β production plays a critical role in neutrophil recruitment and stimulation of degranulation, as well as in promoting the production of monocytes/macrophages. This mediates innate immunity training, promotes mucus secretion, induces proliferation, and enhances the surface coagulability of barrier cells (Pyrrillou et al., 2020).

EcN and CEC15 EVs at high concentration alone were able to stimulate the production of Nitric Oxide Synthase 2 (*NOS2*), 3-fold for EcN (Figure 5L) and 1.5-fold for

CEC15 (Figure 5K). The Prostaglandin-endoperoxide synthase 2 (*PTGS2*) was highly stimulated by EcN's EVs (Figure 5J) and lower MOI while no increase was observed for CEC15 (Figure 5I). There is increasing evidence to suggest that *PTGS2*, may play a critical role as an early event in the development of colorectal cancer. *PTGS2* is found to be over-expressed in 80-90% of colorectal adenocarcinoma and in 40-50% of pre-malignant adenomas (Prescott and Fitzpatrick, 2000; Williams and DuBois, 1996). Studies involving mice have also shown that the inactivation of the *PTGS2* gene leads to a reduction in intestinal tumorigenesis. These findings suggest that *PTGS2* may be a potential target for the prevention and treatment of colorectal cancer (Popolo et al., 2000).

Expression of Tumor necrosis factor (*TNF*) was elevated by EVs at high concentration from both strains by 10-fold (Figure 5G and 5H). In numerous inflammatory conditions like Crohn's disease (CD), *TNF* is recognized as a pivotal factor in provoking inflammation within the intestines. A suggested proinflammatory mechanism implicated in intestinal inflammation is the *TNF*-triggered enhancement of permeability in tight junctions (TJ) of the intestinal epithelium. This heightened permeability can further intensify the inflammatory reaction within the gastrointestinal tract (Ma et al., 2005).

4. FINAL REMARKS

These preliminary results demonstrate that, as expected, CEC15 produces extracellular vesicles with similar characteristics as the ones produced by EcN. CEC15 EVs contain a large quantity of genes in common with EcN EVs, in special the ones that could be considered to have an impact on probiotic activity. As the *in vitro* assay have shown, the EVs are able to promote immunomodulation. This is an indicative that the EVs from these two strains can carry the beneficial effect of the strain and be used as form of treatment instead of the live bacteria. Besides promising, more studies, in special studies in animal models of intestinal inflammatory diseases, are necessary to have stronger evidence on the effect of these EVs.

CHAPTER 6 - ADDITIONAL WORK.

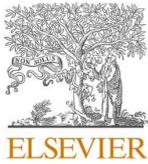
ROLE OF FAT AND PROTEINS ON THE PROBIOTIC EFFECT OF *P. freudenreichii*

In this chapter, we provide a complementary original research paper that emphasises the importance of the fermentation matrix in the modulatory effect of a probiotic. In this research, we tested again the effects of the probiotic strain *Propionibacterium freudenreichii* CIRM-BIA 129 on a murine model of DSS-induced colitis. *P. freudenreichii* have already shown its beneficial effects on this model before, yet, the results presented small differences when the treatment was administered with a different matrix. Here we decided to test the effects of milk proteins and fat on the modulatory effect of the strain. The strain was grown on milk permeate ultra-filtrated (very low protein and very low fat), skim milk (low fat), and whole milk (normal contents of protein and fat).

The study shows that milk proteins has little effect on this probiotic modulation. However the fat, present in the whole milk, was able to improve the modulation of *P. freudenreichii* CIRM-BIA 129 in comparison with the other formulations.

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Fat matters: Fermented whole milk potentiates the anti-colitis effect of *Propionibacterium freudenreichii*

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ABSTRACT

Inflammatory bowel diseases (IBD) constitute a growing concern in western countries. They coincide with gut microbiota dysbiosis, including a loss of immunomodulatory bacteria. Accordingly, probiotic products containing selected immunomodulatory bacterial strains mitigate IBD. Selected strains of *Propionibacterium freudenreichii* display promising modulatory properties and prevent colitis in animal models. Dairy matrices protect propionibacteria immunomodulatory surface antigens during digestive transit. However, the functional role of the dairy matrix components in such fermented dairy products remains unknown. In the present work this study, *P. freudenreichii* CIRM-BIA129, a probiotic strain known for its anti-inflammatory properties, was used to ferment whole milk, skim milk or skim milk ultrafiltrate. The preventive potential of fermented products was tested in DSS-induced mice colitis, in comparison with their unfermented counterparts. *P. freudenreichii*-fermented milk prevented colitis. Dairy fat in the fermented product potentiated the anti-colitis effects of the probiotic. This work opens new perspectives for developing immunomodulatory functional fermented foods.

1. Introduction

1.1. Inflammatory diseases constitute a growing concern.

Many developed countries presently experience an epidemic of chronic inflammatory disease, which can be, at least in part, attributed to a deleterious shift in lifestyle and in diet. Indeed, consumption of Western diet combined with overnutrition and sedentary lifestyle evoke a state of chronic metabolic inflammation, referred to as metaflammation (Christ et al., 2019). This in turn contributes to an increased incidence of non-communicable diseases, which include inflammatory bowel diseases (IBD) such as Crohn's disease (CD) and ulcerative colitis (UC). This constitutes a rising public health problem with global epidemic dimensions responsible for a growing burden of care. The

causes of such an epidemic are complex. Dietary factors such as overconsumption of fat and protein, yet low intake of fruits and vegetables, are probably involved (Hou et al., 2011). Indeed, high-fiber and fruit intakes are associated with decreased risk of IBD, including CD and UC (Hou et al., 2011). By contrast, a review of clinical evidence associated fermented dairy products with an anti-inflammatory activity. This association was particularly significant in subjects with metabolic disorders (Bordoni et al., 2017). In addition to the diet, dysbiosis of the gut microbiota may also play a key role in such inflammatory ailments. Indeed, exacerbated immune response in IBD involves an interplay between the gut microbiota, host genetic factors, and environmental factors.

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1.2. Dysbiosis coincides with inflammatory ailments.

In many inflammatory diseases, including IBD, the gut microbiota is modified, in comparison to that of healthy volunteers. This modification constitutes dysbiosis, which can be defined as “a disturbance to gut microbiota homeostasis due to an imbalance in the flora itself, changes in their functional composition and metabolic activities, or changes in their local distribution” (DeGruttola et al., 2016). Indeed, dysbiosis of the gut microbiota was described in IBD patients, including in CD (Sokol et al., 2008) (Matsuoka and Kanai, 2015) and in UC (Zhang et al., 2007) (Kostic et al., 2014). Dysbiosis is more pronounced in CD patients than in UC ones, with a more reduced diversity and a less stable microbial community (Pascal et al., 2017). Indeed, increasing human diseases are often associated with a modern western lifestyle and with a reduction in the intestinal microbiota diversity (Mosca et al., 2016). This includes metabolic diseases and cancer, but also immune disorders such as CD, UC, allergy and celiac disease. Loss of microbial diversity in this context is characterized by a reduction of the population of commensal bacteria with a recognized immunomodulatory role. This includes reduced population of *Faecalibacterium prausnitzii*, a butyrate-producing bacterium with immunomodulatory anti-inflammatory properties (Quévrain et al., 2016); (Touch et al., 2022). This also includes species of Bifidobacteria, also recognized as key immunomodulators (Tojo et al., 2014). In line with this, a meta-analysis of available clinical data indicated that the consumption of a mix of 8 strains of probiotic bacteria, VSL#3, which includes bifidobacteria and lactic acid bacteria, mitigates the symptoms of IBD in humans (Sniffen et al., 2018). This evidences the key importance of a research work aimed at the identification of food-grade bacteria able to down-regulate inflammation in the context of colitis.

1.3. Propionibacteria exhibit promising immunomodulatory properties.

Dairy propionibacteria, including the main species *Propionibacterium freudenreichii*, are generally recognized as safe (GRAS) bacteria, with the qualified presumption of safety (QPS) status, which are consumed both in Swiss-type cheeses and in probiotic food supplements. They constitute promising immunomodulatory probiotic candidates that may be useful to mitigate symptoms of colitis. In a pioneer study, selected strains of *P. freudenreichii* were shown to induce the modulatory IL-10 in human immune cells *ex vivo* (PBMC). These strains, when administered in a preventive way, were further shown to prevent acute colitis induced by TNBS in BALB/c mice (Foligné et al., 2010). A wider screening of *P. freudenreichii* strains then revealed that this ability is highly dependent on the strain, with a great variability of the immunomodulatory properties (Foligné et al., 2013). These last depend on the presence of specific key immunomodulatory surface proteins, and extraction of such proteins suppresses propionibacteria ability to induce IL-10 (Le Marechal et al., 2015). Moreover, mutational inactivation of key genes (*slpB*, *slpE*) encoding such surface layer proteins also suppresses this immunomodulatory ability (Deutsch et al., 2017). The *P. freudenreichii* CIRM-BIA129 was thus selected as the most modulatory one, exposing SlpB and SlpE proteins at its surface. It prevents acute colitis induced by TNBS (Plé et al., 2015), as well as colitis induced by DSS (Rabah, 2020) and mucositis induced by the cancer chemotherapy drug 5-fluorouracyl (2018). Inactivation of the *slpB* gene in this strain suppresses adhesion to human intestinal epithelial cells, as well as protective effect towards colitis and mucositis (do Carmo et al., 2017); 2019). In line with this, cloning and expression of this gene in *Lactococcus lactis* confers to this lactic acid bacterium enhanced anti-inflammatory properties (Belo et al., 2021). In humans, a clinical study revealed that propionibacteria were members of the gut microbiota of breast-fed preterm infants, while they were absent of that from formula-fed ones, in which necrotizing enterocolitis (NEC), a leading and intractable cause of mortality in preterm infants, is more frequent (Colliou et al., 2017). Accordingly, these authors isolated a *P. freudenreichii* strain from a healthy infant and described its ability to confer protection against pathogen infection and

to increase intestinal Th17 cells in mice. In line with this, a pilot clinical study indicated that the consumption of whey cultures of *P. freudenreichii* improved the clinical activity index score in patients with active UC (Mitsuyama et al., 2007). These authors suggested that this healing effect was mediated by a secreted compound, found in culture supernatant and referred to as bifidogenic growth stimulator (BGF), produced by *P. freudenreichii* (Suzuki et al., 2006). These data strongly suggest that, in addition to being a GRAS and QPS dairy starter with a long history of safe use, *P. freudenreichii* also constitutes a human symbiont and a promising immunomodulatory probiotic.

1.4. The dairy matrix may play a role in the modulatory effect.

We previously addressed the role of the dairy matrix in the modulation of *P. freudenreichii* probiotic activity. Using an *in vitro* gastrointestinal digestion system, the cheese matrix was shown to protect the immunomodulatory SlpB protein from digestive proteolysis (Rabah, Ménard, et al., 2018). Both the viability of the propionibacterium and the integrity of the SlpB were preserved during digestion of the cheese, by contrast with a liquid culture. In young weaned piglets, the cheese matrix modulated *P. freudenreichii* immunomodulatory properties (Rabah, Ferret-Bernard, et al., 2018). Compared to liquid cultures, cheese containing *P. freudenreichii* enhanced Treg and Th2 cells in piglets PBMC and MLNC. Consumption of such a cheese reduced severity of subsequent DSS-induced colitis in mice, including weight loss, disease activity index, score, as well as induction of Tumor Necrosis Factor α (TNF α), Interferon γ (IFN γ) and Interleukin-17 (IL-17) (Rabah, 2020). Such data indicate the interest of using a fermented dairy product such as cheese for the vectorization of propionibacteria to the gut. It is easy to hypothesize that the dairy constituents, including proteins and lipids, protect these bacteria from the insults of low gastric pH, of digestive enzymes and of bile salts. However, the precise role of these constituents in the overall probiotic effect of the fermented food remains elusive. Do dairy lipids and proteins play a role, *per se*, as immunomodulators? Indeed, bioactive milk proteins reportedly exert anti-inflammatory effects (Chatterton et al., 2013) and so do casein hydrolysates (Chen et al., 2022). Dietary fats, depending on their structure, may exert opposite effects on intestinal inflammation (Basson et al., 2020).

We therefore assessed the question of the respective role of propionibacteria, of milk protein and of milk fat in the anti-colitis effect of *P. freudenreichii*-fermented milk. To do so, whole milk, skim milk and milk ultrafiltration permeate were fermented by *P. freudenreichii* CIRM-BIA129. These fermented products, as well as their unfermented control, were administered to mice prior to colitis induction. The research work indicate that the most protective product is the whole milk fermented by *P. freudenreichii* CIRM-BIA129, suggesting a protective role of milk fat.

2. Materials & methods

2.1. Growth of dairy propionibacteria

The strain *Propionibacterium freudenreichii* CIRM-BIA129, equivalent to ITG P20, was initially isolated from a Swiss-type cheese. It was kindly provided by CNIEL (Centre National Interprofessionnel de l'Économie Laitière) and maintained by the CIRM-BIA microbiological resource center (Centre International de Ressources Microbiennes, Bactéries d'Intérêt Alimentaire, Rennes, France). Starting from a frozen stock, precultures were grown in liquid Yeast Extract Lactate (YEL) broth containing 0.1 M lactate and 10 g.L⁻¹ yeast extract as described (do Carmo et al., 2017); 2019)(Malik et al., 1968). CIRM-BIA129 was incubated at 30 °C during 3 days, without agitation, until stationary phase was reached.

2.2. Preparation of fermented dairy products

Dairy media for propionibacteria growth were prepared as previously described (Cousin et al., 2012). Milk ultrafiltration (UF) permeate was prepared as follows. Raw cow milk was skimmed using a cream separator (Westfalia, Chateau-Thierry, France). It was then separated using an UF pilot equipment (T.I.A., Bollene, France) equipped with an organic spiral membrane with a molecular weight cut-off of 5 kDa (Koch International, Lyon, France). The temperature during the ultrafiltration process was maintained at 55 °C. The collected UF permeate was supplemented with 0.1 M food-grade sodium lactate (Sigma-Aldrich, St. Louis, MO, USA) and 10 g.L⁻¹ food-grade grade casein hydrolysate (Casein Peptone Plus, Organotechnie, La Courneuve, France) prior to sterilization using a 0.2 µm filtration unit (Nalgene, Roskilde, Denmark) and stored at 4 °C. UHT skim milk and whole milk (Agrilait, France) were purchased from a local supermarket and supplemented with 0.1 M food-grade sodium lactate (Sigma-Aldrich) and 10 g.L⁻¹ food-grade grade casein hydrolysate, as was UF permeate. Starting from YEL pre-cultures, UF pre-cultures were grown prior to inoculation of dairy media (UF, skim milk, whole milk). Growth was at 30 °C during 3 days, without agitation, until stationary phase was reached, corresponding to bacterial densities of 10⁹ CFU g.L⁻¹, as described previously (Cousin et al., 2012).

2.3. Animal experiments

C57Bl/6 mice (n = 80), male, 6 weeks old, were obtained from the “Biotério central” of the Federal University of Minas Gerais (Belo Horizonte, Minas Gerais, Brazil). They were randomly divided into 8 groups of 10 animals. They were kept in microisolators at a controlled temperature (25 °C ± 1), with a 12 h/12 h light/dark cycle and *ad libitum* access to food (standard chow pellets – Nuvilab Brazil) and autoclaved drinking water. During 14 days, mice received treatments indicated in Table 1. They were daily gavaged with 200 µL of sterile phosphate buffered saline (PBS), filter-sterilised milk UF, UHT sterile skim milk, UHT sterile whole milk, or with 200 µL of *P. freudenreichii* culture in milk UF, in skim milk, or in whole milk. During the last 7 days, colitis was induced, in all groups except the negative control (NC) group, by replacing drinking water by a 2.5% dextran sulphate sodium (DSS) solution in autoclaved water (DSS 40 kDa, TdB Consultancy, Uppsala, Sweden). On the 15th day, animals were euthanized by anesthetic overdose (Ketamine 270 mg/Kg and xylazine 30 mg/Kg). The Ethics Committee on Animal Experimentation of the Universidade Federal de Minas Gerais (CEUA-UFGM, Brazil) approved all experimental procedures realized in this work by the protocol no. 148/2020. Total blood was collected for permeability assay. The colon was collected and measured, washed with sterile PBS, and divided into 2 sections for histology and for gene expression analysis.

2.4. Intestinal permeability

Intestinal permeability was assessed as described previously (do Carmo et al., 2019). On the last day, mice were gavaged with 100 µL of a solution of radiolabelled Diethylenetriamine Pentaacetic Acid (DTPA) containing 18.5 MBq of ^{99m}technetium (^{99m}Tc-DTPA). After 4 h, the

Table 1

Groups codes and treatment description.

Group code	Treatment administered	Colitis induction
NC	PBS	No
DSS	PBS	DSS 2.5%
DSS-PUF	Milk Ultra Filtrated	DSS 2.5%
DSS-SM	Skim Milk	DSS 2.5%
DSS-WM	Whole Milk	DSS 2.5%
DSS-UF129	Fermented milk Ultra Filtrated	DSS 2.5%
DSS-SM129	Fermented Skim Milk	DSS 2.5%
DSS-WM129	Fermented Whole Milk	DSS 2.5%

animals were anesthetized as described above and blood was collected. The radioactivity was counted in a gamma radiation counter (Wizard, PerkinElmer) according to the instructions of the manufacturer. Results were expressed as a percentage of dose per g of blood, using the following equation: % dose = (cpm in blood/cpm in administrated dose) × 100 (cpm: counts per minute) (Generoso et al., 2011).

2.5. Assessment of colitis severity

Food and liquid consumption were daily measured for each isolator and animals were weighed individually. During the last three days, feces were analyzed for consistency and for the presence of blood using the Feca-Cult test, (Inlab, Brazil). The disease activity index (DAI) was determined as indicated in Table 2, according to the 3 main clinical symptoms of colitis: diarrhoea, rectal bleeding, and weight loss, following a scoring previously described (Murthy et al., 1993). The size of PBS-washed colons was measured.

2.6. Histology

Colon sections were gently washed in PBS, rolled in a distal-proximal configuration, fixed in neutral buffered 10%(v/v) formalin solution (Sigma-Aldrich) and processed for histological analysis. Hematoxylin phloxine saffron (HPS) and Alcian Blue stained sections of the paraffin-embedded proximal colonic tissue were used for histological analysis. Tissue damage was determined by two blinded investigators quantifying the destruction of mucosal architecture, the presence and degree of cellular infiltration, the extent of muscle thickening and the goblet cell depletion. The destruction of mucosal architecture was scored as 0–3 (0: none, 1: 1/3 basal, 2: 2/3 basal and 3: loss of crypt and epithelium). Then, presence and degree of cellular infiltration was rated as 0–3 (0: none, 1: infiltrate around crypt basis, 2: extensive infiltration reaching the muscularis mucosae and 3: infiltration of the submucosa). Next, the extent of muscle thickening was scored as 0–3 (0: none, 1: mild, 2: moderate and 3: extensive thickening). Finally, presence or absence of goblet cell depletion was determined as 0: goblet cells presence and 1: loss of goblet cells. An extension factor of 1–4 was implemented when the criteria measured reached 25, 50, 75 and 100% of the tissue evaluated.

Furthermore, the crypt depths and the number of goblet cells per crypt were determined based on histology images. Five pictures from random sections of the colon were collected per animal. For the crypt measurements, 20 crypts were measured from each image and the mean was determined for each animal. The number of goblet cells crypt was counted on 10 crypts per image, and the average was used for the analysis.

2.7. Gene expression analysis

After collection, colon samples were stored in RNAlater RNA-later (Invitrogen, USA) at –20C until further RNA extraction. The RNA extraction was performed using the RNeasy Mini Kit (QIAGEN, USA) according to the manufacturer’s instructions. Purified RNA (1 µg) was then used to produce the cDNA with the QSCRIPT cDNA synthesis kit (Quantabio, USA) for a final volume of 20 µL. The cDNA incubation program consisted of 5 min at 22C, 30 min at 42C, and 85C for 5 min.

Table 2

Disease Activity Index.

Score	Weight loss (%)	Stool consistence*	Occult/Gross Bleeding
0	None	Normal	Absent
1	0–5		
2	5–10	Loose	Occult blood
3	10–15		
4	>15	Diarrhea	Gross bleeding

Both the RNA and the produced cDNA were quantified in a nanodrop spectrophotometer (Thermo Scientific, USA). qPCR was carried out in a 16 µL volume containing 15 ng cDNA, specific primers (300 nM), and 8 µL IQTM SYBR Green Supermix (Bio-Rad). Reactions were run on a CFX96 real-time system (Bio-Rad, France) using the following cycling parameters: DNA polymerase activation and DNA denaturation 95 °C for 5 min, 40 cycles of denaturation at 94 °C for 15 s, and extension at 60 °C for 30 s. Melting curve analysis was included to check the amplification of single PCR products. The primers used are listed in Table 3. All genes were analysed in duplicate and the amount of mRNA was normalized by using the geometrical mean value of four reference genes: *actB* (beta-actin), *gapdh* (glyceraldehyde 3-phosphate dehydrogenase), *ppiA* (peptidylprolyl isomerase A) and *tuba* (beta-tubulin). The online tool Ref-FINDER (<https://www.heartcure.com.au/reffinder/>) was used to identify the most stably expressed genes in the experimental conditions tested from a panel of six gene candidates. The values corresponding to the amount of amplified gene compared to the NC control group (2- $\Delta\Delta$ CT) were expressed as mean \pm SEM. The differences among the groups were assessed by the Kruskal-Wallis test followed by Dunn's multiple comparisons test performed using GraphPad Prism version 9.5.1 for macOS, GraphPad Software, San Diego, California USA, <https://www.graphpad.com>.

3. Results

3.1. Consumption of *P. freudenreichii*-fermented milk attenuated the clinical signs of DSS-induced colitis.

The animal study is depicted in Fig. 1A. We first explored

Table 3
Primers used in this study.

Gene	Primer Sequence	Reference
<i>ACTB</i> *	Forward	CCTTCTGGGTATGGAATCCTGT
	Reverse	CACTGTGTGGCATAGAGGCTCTTAC
<i>GAPDH</i> *	Forward	TGACCTCAACTACATGGTCTACA
	Reverse	CTTCCCATTCTCGGCCCTTG
<i>TUBA</i> *	Forward	TGTCCTGGACAGGATTCCGC
	Reverse	CTCCATCAGCAGGGAGGTG
<i>PP1A</i> *	Forward	GGCAAAATGCTGGACCAAAAC
	Reverse	CATTCTGGACCCAAAACG
<i>TNF</i>	Forward	CTGTAGCCCACTCGTAGC
	Reverse	TTGAGATCCATGCGCTTG
<i>IL6</i>	Forward	GAGGATACCACTCCCAACAGACC
	Reverse	AAGTGCATCATCGTTTCATACA
<i>IL1B</i>	Forward	GCAACTGTTCTGAACTCAACT
	Reverse	ATCTTTTGGGGTCCGTCAACT
<i>NOS2</i>	Forward	CAGCTGGGCTGTACAAACCTT
	Reverse	CATTGGAAGTGAAGCGTTTCG
<i>PPARG</i>	Forward	CAGGCTCCACTATGGAGTTC
	Reverse	GGCAGTAAAGATCACACCTATCA
<i>OCN</i>	Forward	CAGCCTTCTGCTTCATCG
	Reverse	GTCGGGTTCACTCCCATTA
<i>CLDN1</i>	Forward	ATCCATAGGAAAGGCCCTTCAGCA
	Reverse	TACATGTAGGGCAACCAAGTGCCT
<i>CLDN5</i>	Forward	ACGGGAGGAGCGCTTTAC
	Reverse	GTTGGCGAACAGCAGAG

* Reference genes.

macroscopic markers of DSS-induced colitis, in the presence or in the absence of the administration of the probiotic *P. freudenreichii* CIRM-BIA129 (Fig. 1A). This last was consumed either under the form of a fermented milk ultrafiltrate (DSS-UF129), of a fermented skim milk (DSS-SM129), or of a fermented whole milk (DSS-WM129). In addition, to evaluate the impact of the fermentation by CIRM-BIA129 versus that of the vehicle food matrix, mice were also treated with the unfermented dairy products (DSS-UF, DSS-SM and DSS-WM). As expected, addition of 2.5 % DSS to the drinking water led to clinical signs such as an increased disease activity index (DAI) score, taking into account weight loss, stool consistence and gross bleeding (Fig. 1B), as well as colon shortening (Fig. 1C and D). Consumption of the unfermented sterile dairy products tested here had no significant effect on the clinical signs of DSS-induced colitis. Indeed, DAI remains increased, in the context of colitis, whether mice consumed sterile UF, skim milk, or whole milk. Nevertheless, consumption of the *P. freudenreichii*-fermented dairy product alleviated the clinical signs of DSS-induced colitis, as indicated by a DAI score intermediate between the NC and the DSS, and no significant difference between the DSS-UF129, the DSS-WM129 groups, and the NC groups (Fig. 1B). In the same manner, colon shortening was not modified by the unfermented sterile dairy products tested, but significantly decreased in DSS-UF129 and DSS-SM129 groups when compare to the DSS group (Fig. 1C and D). These data show that consumption of *P. freudenreichii*-fermented dairy products reduced colitis and lowered colon damage.

3.2. Consumption of *P. freudenreichii*-fermented milk attenuated the histological symptoms of DSS-induced colitis.

At a microscopic scale, the histopathological analysis revealed a length-wide destruction of the colonic epithelium, with considerable modification of the mucosal architecture, loss of crypts, cellular infiltration and thickening of the muscular layer, as a result of colitis induction (DSS), when compared to healthy control intestinal tissue (NC) (Fig. 2). As a result of this destruction, the histopathological score (Fig. 3A), based on the mucosal architecture, the presence and degree of cellular infiltration, the extent of muscle and the presence or absence of goblet cells, was increased in the DSS group from 2.04 + -0.85 in the control NC group to 6.68 + -3.37 in the DSS group. Accordingly, the depth of the crypts (Fig. 3B) and the number of goblet cells per crypt (Fig. 3C) were significantly reduced as a result of DSS treatment. None of the unfermented dairy products was able to reduce this score significantly, although results indicate a trend towards a reduced score, when compared to the DSS group. However, crypts depth and density of goblet cells remained low. Considering the *P. freudenreichii*-fermented dairy products, only the consumption of the fermented whole milk (DSS-WM129 group) significantly restored a low score, down to 2.2 + -1.41 (Fig. 2A and B). Furthermore, the crypt depth, which was dramatically reduced by DSS, was partially restored by the consumption of both fermented skim milk (DSS-SM129) and fermented whole milk (DSS-WM129) (Fig. 2C). The number of goblet cells, also drastically reduced in DSS-induced colitis group (9.05 + -1.08), was partially prevented by the two fermented products SM129 and WM129 (11.04 + -1.98 and 13.67 + -2.3, respectively), although only DSS-WM129 did not differ from the healthy control (14.22 + -3.29) (Fig. 2D).

3.3. Consumption of *P. freudenreichii*-fermented milk attenuated the pathological increase in intestinal permeability.

We further investigated the permeability of the gut by monitoring the passage of radiolabelled Diethylenetriamine Pentaacetic Acid (DTPA) from the gut content into the blood stream. Gut permeability was drastically increased as a result of DSS treatment (Fig. 2E). Indeed, the DTPA fraction monitored in the blood was 0.33%+0.1 in control NC mice, while it was 0.74%+0.18 in the DSS one. Consumption of unfermented sterile dairy products resulted in a trend towards reduced gut permeability, especially with whole milk, but this trend failed to reach

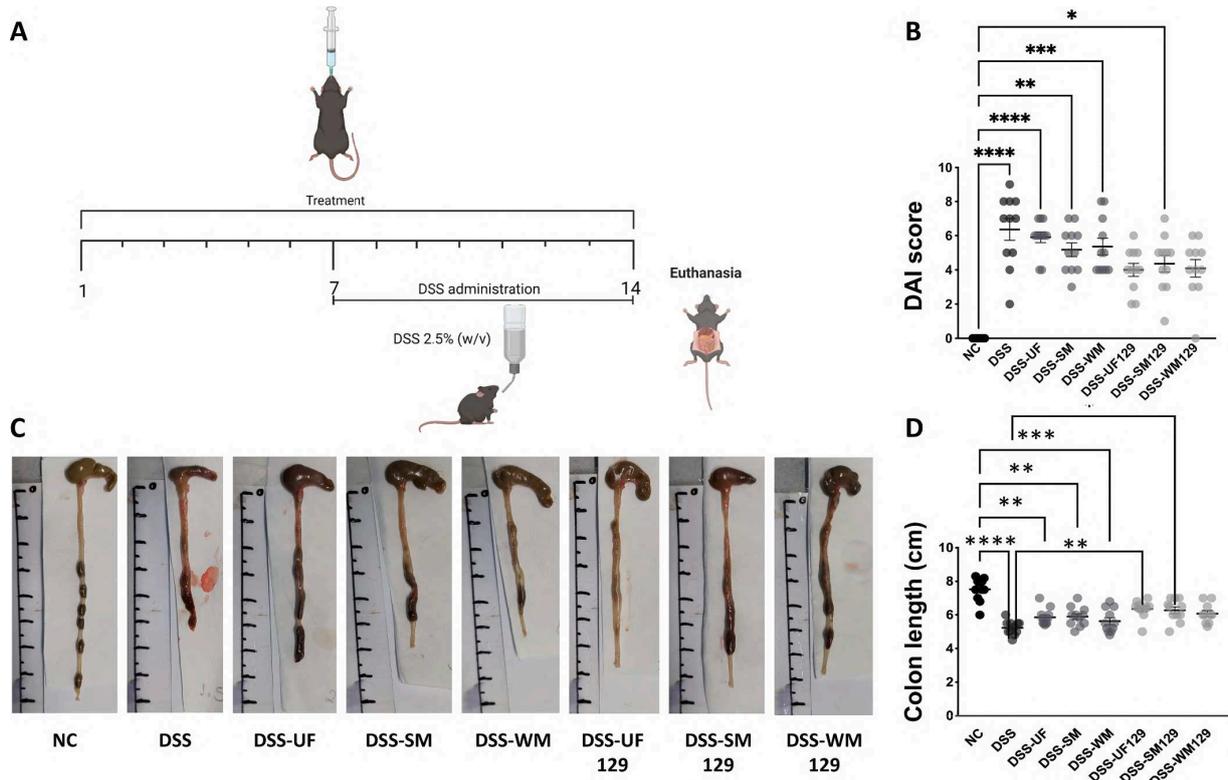


Fig. 1. *P. freudenreichii*-fermented milk alleviated DSS-induced experimental colitis in mice. (A) Design of the experimental protocol to investigate the effect of dairy matrices, fermented or not by *P. freudenreichii* CIRM-BIA129, in an experimental model of colitis in mice. Mice were gavaged during the 14 days of the experiment with 200 μ L of sterile phosphate buffered saline (PBS), sterile milk ultrafiltrate (UF), sterile skim milk (SM), sterile whole milk (WM), or with 200 μ L of *P. freudenreichii* CIRM-BIA129 culture in milk ultrafiltrate (UF129), in skim milk (SM129), or in whole milk (WM129). Mice had access to sterilised drinking water for negative control mice (NC) or to 2.5 % DSS in drinking water. (B) Disease activity index (DAI) was determined according to the 3 main clinical symptoms of colitis: diarrhoea, rectal bleeding, and weight loss. (C) Representative examples of colon morphology. (D) Colon size. Data represent mean \pm SEM of 10 mice per group. Kruskal-Wallis test followed by Dunn's multiple comparisons test was performed. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$.

statistical significance. Consumption of *P. freudenreichii*-fermented dairy products however significantly prevented this increase in intestinal permeability (Fig. 2E) mitigating the permeability back to basal levels. In particular, the fermented whole milk reduced this DTPA fraction to 0.25% \pm 0.14.

3.4. Consumption of *P. freudenreichii*-fermented milk attenuate DSS-induced gene expression changes in the colon.

The impact of DSS treatment, of unfermented dairy products, and of *P. freudenreichii*-fermented dairy products was also assessed with respect to gene expression in colonic tissue. As expected, DSS-induced colitis drastically modulated the expression of genes coding for immunomodulatory and barrier related proteins. Indeed, the barrier related genes *ocln*, *cldn1* and *cldn5* were up to 11-fold less expressed in the DSS group than in the NC group (Fig. 4). Likewise, the expression of the inflammatory genes *tnf* and *il1b* increased 37 and 52 times in the DSS group, respectively (Fig. 5). The expression of *il6* was also highly induced by DSS, but changes were not statistically significant against the NC group. We also found that the expression of *ppary*, coding for the peroxisome proliferator activated receptor gamma, was down-regulated by DSS, while the abundance of *nos2* coding for the nitric oxide synthase 2 was 80-fold higher in the DSS group than in the NC group. Interestingly, the consumption of fermented whole milk (DSS-WM-129) was able to prevent alterations of the expression of all these genes, except *tnf*. The other fermented preparations also appeared to exhibit similar effects, but in a

lesser extent than DSS-WM-129. Moreover, while DSS-WM-129 prevented the effects of DSS on the expressions of *ocln*, *nos2* and *il1b*, their expressions in the DSS-WM group were similar to those in the DSS group, showing the impact of the fermentation. Altogether, these results showed that DSS treatment resulted in dramatic changes in the expressions of many colonic epithelium genes, changes that were prevented by *P. freudenreichii* fermented preparations, notably fermented whole milk.

4. Discussion

The food matrix containing the probiotic *P. freudenreichii* has been shown to play a determinant role in its probiotic effects. When consumed in a fermented dairy product such as a fermented milk or a cheese, *P. freudenreichii* reaches higher live populations in the gut, when compared to isolated bacteria (Hervé et al., 2007; Rabah, Ferret-Bernard, et al., 2018), and affords protection towards induced colitis (Plé et al., 2015). The structure of the food matrix, resulting from the network involving proteins, lipids, and minerals, clearly plays a protective role towards the probiotic and its key surface molecules involved in the modulatory effects. In the current research work, we sought the respective role of dairy proteins and of dairy fat in the anti-colitis effect of *P. freudenreichii* CIRM-BIA129, a strain known for its immunomodulatory potential. This work indicates that dairy fat plays a beneficial role in the *in vivo* monitored anti-inflammatory effect of *P. freudenreichii*-fermented milk, giving new evidence for the need to consider the impact of the food delivery vehicle on the efficacy of probiotics further arguing

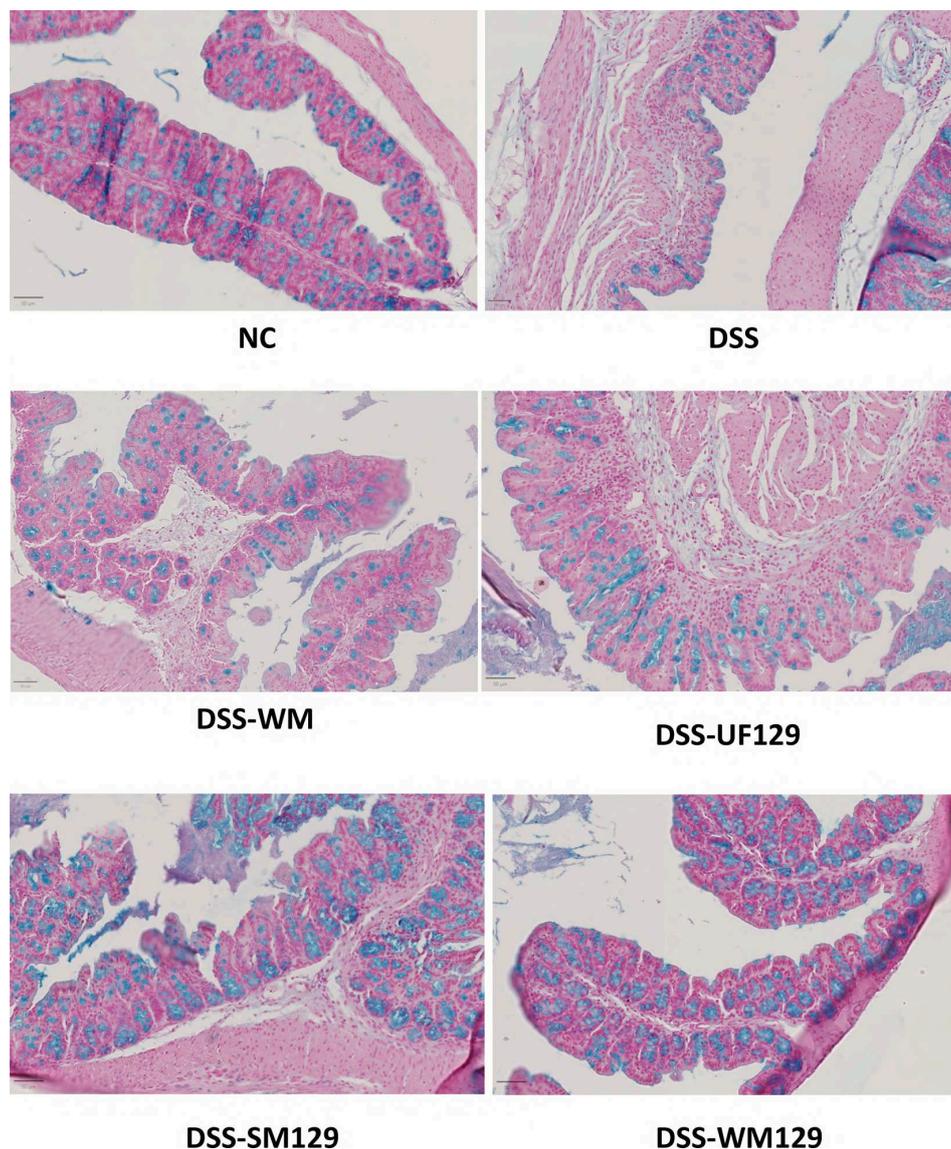


Fig. 2. *P. freudenreichii*-fermented milk alleviated DSS-induced colon histological damages. Representative images of Hematein Phloxin Safran (HPS) and Alcian Blue stained colon tissues (magnification 200 x) illustrate damages caused by DSS, when compared to control animals (NC). Damages were lower in mice consuming *P. freudenreichii*-fermented whole milk (DSS-WM129). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

for a focus on how to improve the effectiveness (Lé et al., 2022).

Several strains of *P. freudenreichii* were already described as modulators of inflammation and of chemically induced colitis in mice models. A first study reported a promising protective effect of propionibacteria cultures in mice with TNBS-induced colitis (Foligné et al., 2010). In the same model, cheese containing *P. freudenreichii*, either as a sole bacterium (Plé et al., 2015), or in conjunction with selected immunomodulatory strains of the dairy starters *S. thermophilus* and *L. delbrueckii*, also afforded protection towards TNBS-induced colitis (Plé et al., 2016). Such a cheese furthermore protected mice from DSS-induced colitis (Rabah, 2020). In these studies, consumption of *P. freudenreichii* limited colon shortening and thickening, reduced disease activity index, histopathological score, colonic myeloperoxidase (MPO) activity, as well as pathological increase of gut permeability. In the research work

presented here, consumption of milk fermented by *P. freudenreichii* CIRM-BIA129 was further shown to exert a preventive effect towards colitis induced by DSS. As a macroscopic indication, the shortening of the colon length was prevented by this consumption, whatever the food matrix. However, the disease activity index, which is increased during colitis, was reduced by the consumption of *P. freudenreichii*-fermented milk, yet not of fermented ultrafiltrate, which consists in the aqueous phase of milk, devoid of fat and of proteins. Furthermore, as a microscopic indication, the histopathological score was reduced as a result of fermented whole milk consumption, while the other fermented products failed to do so. Hence, the food matrix components, including dairy fat, play a role in the protective effect of *P. freudenreichii*-fermented products.

In our study, consumption of *P. freudenreichii*-fermented milk

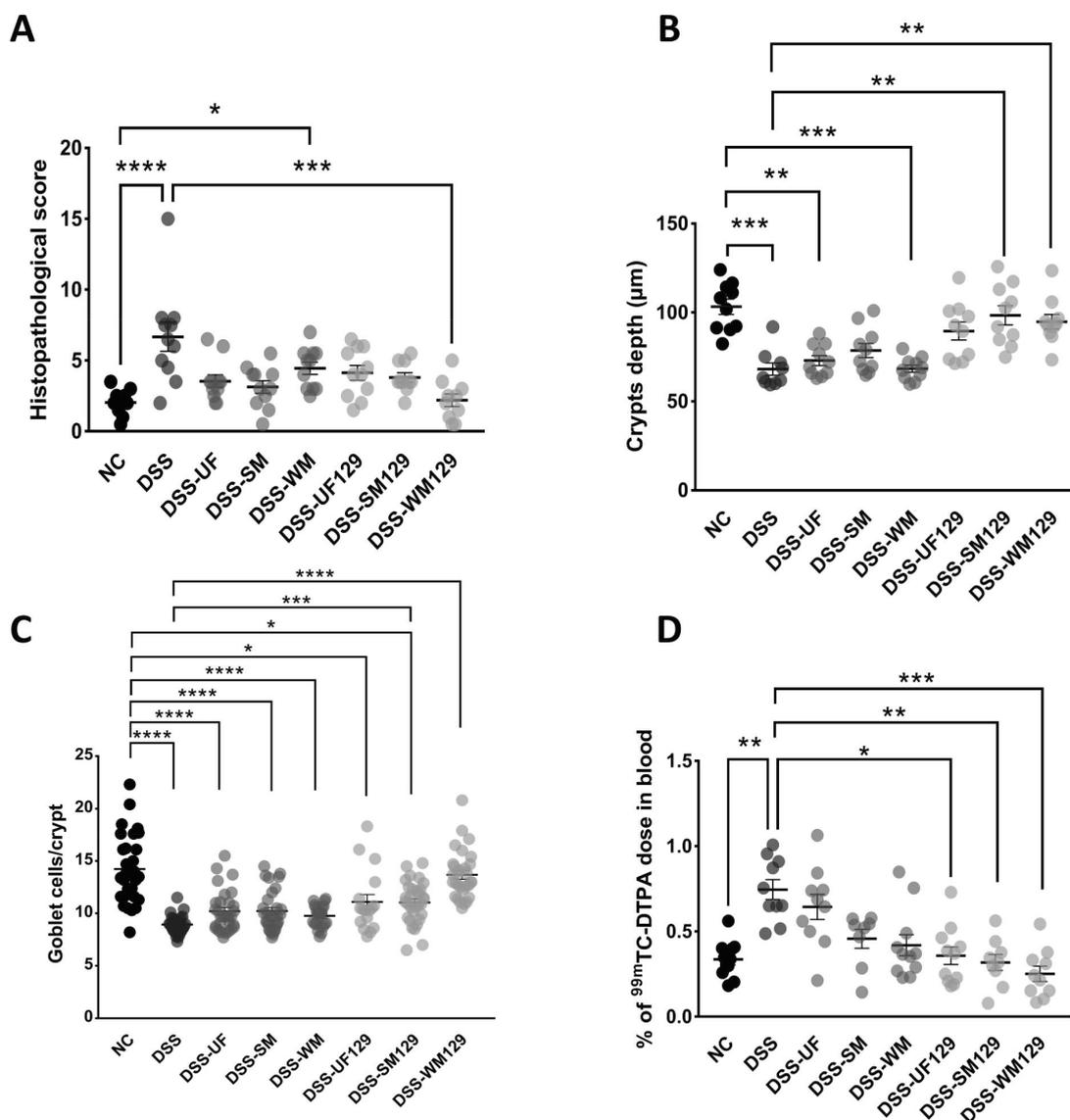


Fig. 3. *P. freudenreichii*-fermented milk alleviated DSS-induced colon histopathological injury and increased permeability. (A) Histological score were evaluated by quantifying the destruction of mucosal architecture, cellular infiltration, muscle thickening and loss of goblet cells. (B) The depth of colon crypts was measured for 50 crypts per animal. The mean value was used for comparison between groups. (C) Goblet cells were measured as the ratio goblet cells/crypt, 50 crypts per animal. The average was used for comparison between groups. (D) On the last day, mice were gavaged with a solution of radiolabelled ^{99m}Tc-DTPA. After 4 h, the animals were anesthetized and radioactivity determined in the blood. Data represent mean \pm SEM of 10 mice per group. Kruskal-Wallis test followed by Dunn's multiple comparisons test was performed. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$.

prevented the pathological increase of gut permeability upon colitis induction by DSS. Indeed, passage of the radiolabelled DTPA to the blood stream, which was drastically increased by DSS-induced colitis, was limited by the consumption of all the *P. freudenreichii*-fermented products. As key actors of gut integrity, intestinal epithelial cells, under the mucus layer, are tightly bound by tight junctions (TJ). TJ proteins, including ZO-1, occludin and claudin, are main actors of the integrity of the gut epithelial barrier and play a crucial role in intestinal homeostasis and tightness. Indeed, reduced expression of TJ proteins coincides with permeability increase in a leaky gut and has been described in animal models of colitis (Camilleri, 2019). We thus investigated the expression of genes encoding TJ proteins. Indeed, expression of the genes encoding occluding, claudin-5 and claudin-1 were repressed in DSS-treated mice

in the present work, which is in line with the observed increased gut permeability. By contrast, consumption of *P. freudenreichii*-fermented whole milk restored expression of genes encoding TJ proteins. This is consistent with the concomitant restoration of intestinal integrity as shown by DTPA monitoring. Damages of the intestinal epithelial TJ result in abnormal increase of gut permeability. Pro-inflammatory metabolites of the gut microbiota, including lipopolysaccharide, in turn translocate through the intestinal barrier and elicit an inflammatory response. This last in turn exacerbates gut barrier defects in a vicious circle. Identifying food components able to restore gut barrier integrity thus constitutes a major quest. Protection of the gut barrier integrity, in the context of DSS-induced colitis, was already reported for probiotic bacteria such as *Limosilactobacillus reuteri* (H. L. (Lee et al., 2022);

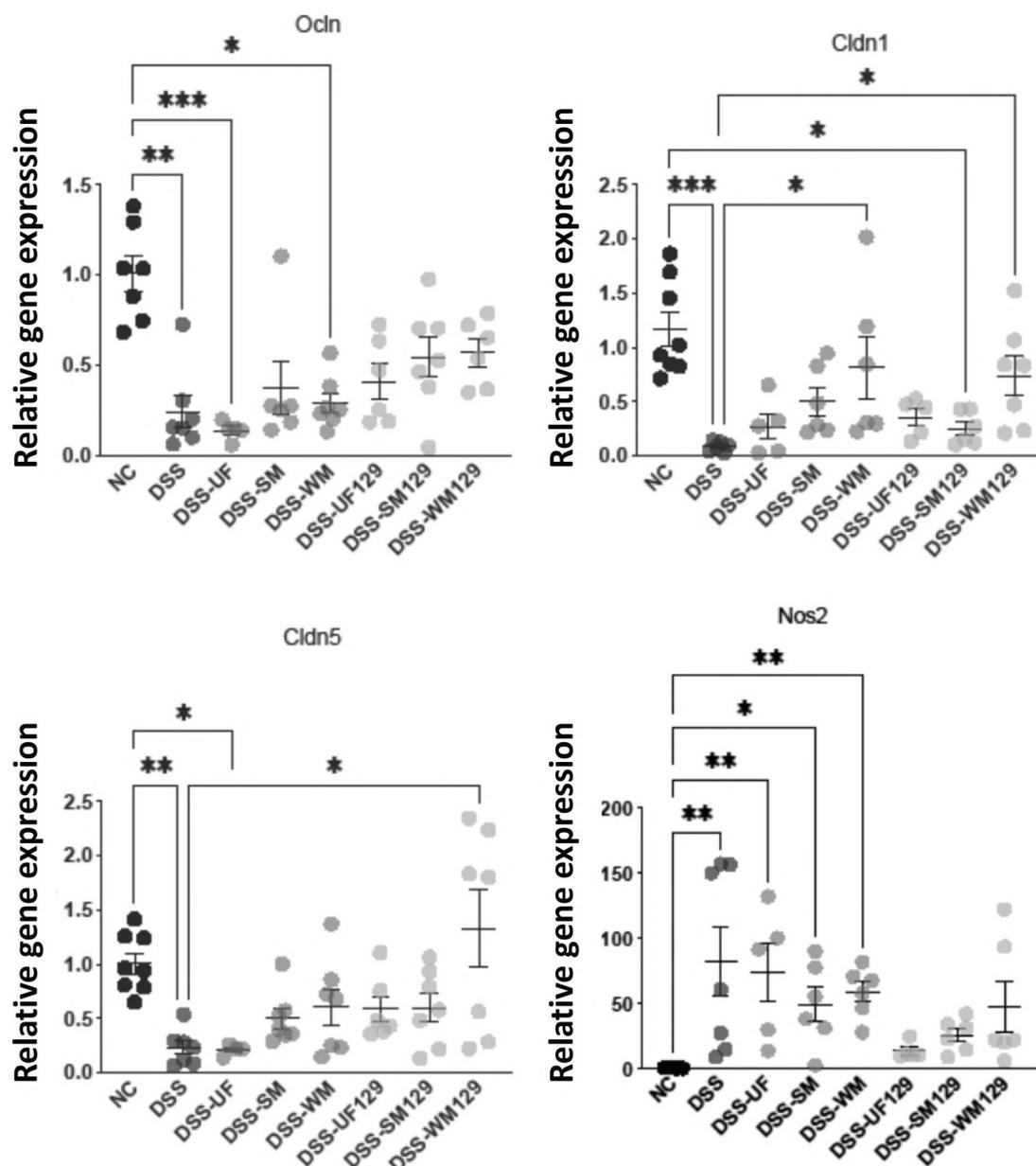


Fig. 4. *P. freudenreichii*-fermented milk modulated expression of key genes in the colon. The expression of key genes was monitored in colon extracts. Data are presented for genes Ocln, Cldn1, Cld5, Nos2, Il1b, Il6, Tnf and Pparg. Relative expression was determined using Actb, Gapdh and Tuba as reference normalisation genes. Kruskal-Wallis test followed by Dunn's multiple comparisons test was performed. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$.

Lactobacillus casei (Zakostelska et al., 2011); *Limosilactobacillus fermentum* (Kaur et al., 2022); *Bifidobacterium breve* (Niu et al., 2022); *B. bifidum* (Shang et al., 2022) and *B. longum* (Y. (Chen et al., 2021)). In these reports, the probiotic bacteria were also shown to restore the expression of tight junction protein genes. Such an effect was evidenced here as a result of *P. freudenreichii*-fermented products. Moreover, we show here that milk components, and in particular dairy fat, potentiate this protective effect. Indeed, the amount of propionibacteria was the same, i.e. 10^9 CFU $g.L^{-1}$, so that the differential effect of fermented products cannot be attributed to differential population of propionibacteria.

In the different animal studies dealing with probiotics and cited

above, restoration of the gut barrier integrity was intimately linked to the ability of the probiotic bacterium to modulate inflammation. The inflammatory response includes the induction of the production of pro-inflammatory cytokines, which in turn contribute to the progression of colitis. Several probiotic bacteria, including dairy starters, were reported to mitigate the inflammatory response by preventing such inductions (Illikoud et al., 2022) and by repressing the TLR4-NF- κ B pathway (Y. (Chen et al., 2021)). In our study, DSS upregulated the pro-inflammatory cytokine IL-1 β , indicating the induction of gut inflammation. IL-1 β is a key factor in the pathogenesis of colitis and its upregulation is associated with ulcerative colitis (Bergemalm et al., 2021). Such induction exacerbates gut inflammation through the dysregulation

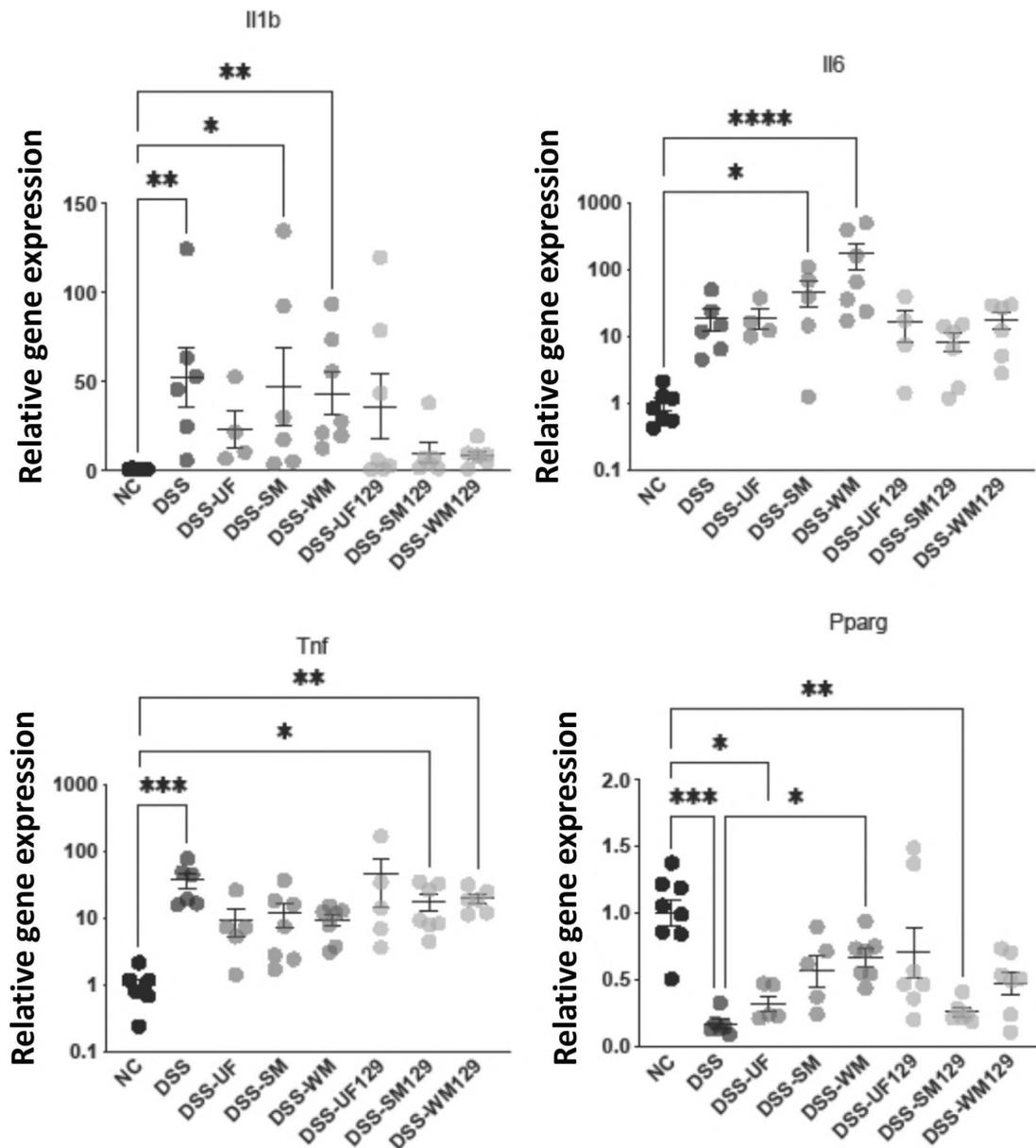


Fig. 5. *P. freudenreichii*-fermented milk modulated expression of key genes related to inflammation in the colon. The expression of key genes was monitored in colon extracts. Data are presented for genes Ocln, Cldn1, Cld5, Nos2, Il1b, Il6, Tnf and Pparg. Relative expression was determined using Actb, Gapdh and Tuba as reference normalisation genes. Kruskal-Wallis test followed by Dunn's multiple comparisons test was performed. * p < 0.05, ** p < 0.01, *** p < 0.001 and **** p < 0.0001.

of macrophage polarization. However, consumption of *P. freudenreichii*-fermented milk prevented this IL-1 β induction, in line with previous probiotic studies. Immunomodulation was accordingly reported in *P. freudenreichii*, including an ability to induce IL-10 production in human immune cells (PBMC), and to prevent induction of pro-inflammatory cytokines *in vivo* in the context of colitis (Foligné et al., 2010) (Plé et al., 2015). Our study further demonstrates that an appropriate food delivery vehicle is necessary for this potential to give rise to protection against colitis. Indeed, protection was significant here only when *P. freudenreichii* was provided within a fermented milk, yet not in a UF culture. Accordingly, fermented whole milk afforded protection, while

unfermented whole milk failed to do so.

Altogether, our results indicate that milk constituents, including dairy proteins and fat, potentiate the anti-colitis effects of the probiotic *P. freudenreichii*. Interestingly, consumption of unfermented whole milk also restored a gut permeability similar to that of healthy control mice, while being less anti-inflammatory than the fermented whole milk. In line with this, a previous report evidenced that the probiotic *L. casei* BL23, when provided within a fermented milk, alleviated symptoms of colitis in mice, while it failed to do so when provided in a nutrient-free buffer (B- (Lee et al., 2015)). This raises the question of the potentiating effect of milk constituents in fermented milk anti-colitis effects.

Interestingly, milk phospholipids were shown to alleviate DSS-induced colitis and to prevent goblet cell depletion (Wang et al., 2019). The protective effect of milk polar lipids was then shown to be more pronounced if mice received a western high-fat diet (Garcia et al., 2022). Milk fat globule membranes (MFGM) were shown to maintain the gut mucosal barrier, to inhibit oxidative stress and to attenuate colitis and hepatic injury in DSS-treated mice (Wu et al., 2022). Milk extracellular vesicles (mEVs) are bioactive constituents of milk and play a role in maintaining intestinal health. Their consumption alleviates the symptoms of DSS-induced acute colitis and limits the induction of pro-inflammatory cytokines (Du, Wang, et al., 2022). Furthermore, two subsets of mEVs contribute differently to colitis healing, one by modulating innate immunity and the other by decreasing inflammation (Benmoussa et al., 2019). This protective effect of mEVs was further shown to involve modulation of intestinal gene expression and of the gut microbiota in favour of bifidobacteria (Du, Wang, et al., 2022). Exosomes deriving from both bovine and human milk were further shown to mitigate DSS-induced colitis, to reduce expression of pro-inflammatory cytokines and to provide miRNAs involved in gene expression regulation (Reif et al., 2020). Milk fat globule-EGF factor 8 (MFG-E8) is known to play a role in maintaining the integrity of the gut mucosa. Accordingly, administration of this protein alleviates colitis and promotes mucosal repair (Chogle et al., 2011). Feeding mice with casein was further shown to facilitate recovery from DSS-induced colitis (Yu et al., 2021). Dietary intervention revealed that consumption of milk proteins limited the colon shortening and the IL-1 β levels, while increasing the gut concentration of bifidobacteria, in DSS-induced colitis (Ma et al., 2021). Indeed, several probiotics revealed an anti-colitis effect when provided within a fermented milk, an effect which may be stronger than when the probiotic is consumed without a food matrix (Lee et al., 2015). As a possible explanation, immunomodulation often relies on key surface proteins or glycoproteins acting as MAMPs (microbial associated molecular pattern) and such MAMPs are susceptible to proteolysis during digestion. The dairy matrix protects such molecules from proteolysis, which may enhance their probability to interact with the host immune response. Concerning *P. freudenreichii*, its anti-colitis effect was accordingly particularly strong, in a previous study, when using Emmental cheese as food matrix delivery vehicle, with a significant effect on macroscopic, histological and immunological markers (Rabah, 2020). This cheese, as a Swiss-type (pressed cooked) cheese, is particularly rich in dairy proteins and lipids. Thus, different dairy matrices, with different structures, may potentiate the effects of probiotic bacteria. A special attention should thus be paid to protein and lipid constituent of the food matrix, when developing innovative fermented functional foods.

5. Conclusion

As a conclusion, this work demonstrates the key role of milk, including milk fat, in potentiating the anti-colitis potential of *P. freudenreichii*. This opens new perspectives for the development of fermented functional foods, which should consider the presence of the different components of milk, as well as the food structure, in addition to bacterial fermentation.

Ethics statement

The Ethics Committee on Animal Experimentation of the Universidade Federal de Minas Gerais (CEUA-UFMG, Brazil) approved all experimental procedures realized in this work by the protocol no. 148/2020.

CRedit authorship contribution statement

Marine Mantel: Investigation. **Tales da Silva:** Investigation. **Rafael Gloria:** Investigation. **Danièle Vassaux:** Investigation. **Kátia Duarte Vital:** . **Valbert Nascimento Cardoso:** . **Simone Odília Antunes Fernandes:** Investigation. **Éric Guédon:** Data curation. **Yves Le Loir:**

Supervision. **Ana Maria Caetano Faria:** . **Malvyne Rolli-Derkinderen:** Supervision. **Vasco Azevedo:** Supervision. **Gwénaél Jan:** Conceptualization, Formal analysis, Investigation, Supervision, Validation, Writing – original draft, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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CHAPTER 7 - GENERAL DISCUSSION AND FINAL CONSIDERATIONS

The use of probiotics for prevention and treatment of diseases has become a common practice nowadays. However, the indiscriminate use and the lack of rigid legislation around the world has become an increasing health problem. In this manner, the correct identification, characterization, and functional evaluation of each strain aiming to be commercialized as a probiotic, for human or animal use, is vital. With this in mind, the aim of this thesis was to use genomic tools, *in vitro* analysis, *in vivo* assays, using animal models, and comparative analysis to characterize the probiotic properties of the strain *E. coli* CEC15 and, also, evaluate how the food matrix in which the probiotic is produced can modulate its beneficial effects. Beneficial effects of the CEC15 strain has been demonstrated before (Escribano-Vazquez et al., 2019; Tomas et al., 2015) but the strain was yet to be characterized and the mechanisms understood.

1. What have we discovered so far?

1.1. Genomic, *in vitro*, and animal studies demonstrated the safety and probiotic effect of CEC15

In the first part of this study we have performed a throughout characterization of the probiotic strain the *E. coli* CEC15. From the genome to *in vivo* studies, we have shown that CEC15 is safer at a genomic level than the reference strain *E. coli* Nissle 1917, presents a wide variety of genes related to resistance to acid stress, which may have a direct correlation to the high survival rate in *in vitro* digestion assay. We have also found numerous genes associated to the adhesive properties of the strain, which were observed through proteomic analysis and are associated to the high level of adhesion to Caco2 cells. CEC15 also showed a reduced number of antibiotic resistance genes, with a phenotype of sensitivity to the majority of antibiotic tested, we have also noted that no gene related to antibiotic resistance was close enough to a transposable element to have the risk of transfer to other bacteria. We observed that CEC15 does not present hemolytic activity. CEC15 has also demonstrate safety in mice, where it was administrated in high dosages (10^{10} CFU/day/mouse) and no harmful effect was observed after 12 days of administration, with weight gain and modulation of intestinal epithelial architecture reducing intestinal permeability. CEC15 was also contested with a inflammatory model of 5-fluorouracyl(5FU)-induced intestinal mucositis in mice, demonstrating promising results. CEC15 was able to reduce weight loss and protect the intestinal epithelial architecture in the form of reduced histopathological score and

reduced intestinal permeability. CEC15 was also able to reduced infiltrate of neutrophils and, thus, reduce the inflammatory process. In parallel we tested the effects of the reference strain EcN in the same animal model and we did not see a protection with EcN administration. With these results we have demonstrated the safety of the strain and established the beneficial effect of CEC15 in a intestinal inflammatory model.

1.2. CEC15 postbiotic preparations presents promising results on animal studies of intestinal inflammation

In the second part of this thesis, we evaluated the beneficial activity of the postbiotic forms of CEC15 and EcN, for comparison purposes, in the form of heat-inactivated bacteria and cell-free supernatant. the viable strains, heat-inactivated strains, and cell-free supernatants were tested against the same model of 5-FU-induced intestinal mucositis performed in the first section. In this study we have observed the same effects for the viable strains that were demonstrated above, confirming the protective effect of CEC15 strain and the inability of protection of EcN. As for the postbiotic preparations, we have observed a beneficial effect, in special of heat-inactivated CEC15. Is important to note here that EcN cell-free supernatant has been tested before in similar models (in rats) and have demonstrated promising results (Prisciandaro et al., 2011; Wang et al., 2017), although here it does not compare to the effect of CEC15. Inactivated CEC15 presented the best results, simmilar in most cases to the viable strain. Inactivated CEC15 promoted reduced weight loss, preservation of intestinal length and villus height, reduced histopathological score, preservation of goblet cells, reduced permeability, reduction of *IL1B* gene expression and increase of the *TJP1* and *CLDN1* tight junction proteins gene expression. These results helped to consolidate CEC15 as a beneficial strain for the protection / treatment of intestinal inflammatory diseases. It also shows that inactive bacteria, and even the metabolites produced, has potential to exert similar beneficial effects to the strain of origin.

1.3. E. coli probiotic strains presents a high genomic diversity

In the third section of this study we performed a comparative genomic analysis of CEC15 against seven other probiotic *E. coli* strains, EcN, and 6 strains from the commercial probiotic Symbioflor2 (*E. coli* G1/2, G3/10, G4/9, G5, G6/7, and G8). Phylogenetically we have demonstrated that the strains belongs to three different phylogroups of *E. coli*, being CEC15 classified as B2, EcN as B1, and the Symbioflor strains in the A phylogroup. Pan genome analysis have demonstrate the high genetic variability of the strains, with about a third (2533) of the total number of genes (8316) being classified as unique genes, and, thus, not shared among the strains. The core genome, shared by all eight strains, contained 3336 genes. Functional analysis of the strains, by COG and KEEG classifications, also demonstrated a large number of unique genes in

the categories most present. The presence of genomic islands, bacteriocins, adhesins, and antibiotic resistance genes were also evaluated for the strains. CEC15 presented one of the lowest number of genomic islands, after the G4/9 strain. As for the majority of the PAI found on all strains, no highly pathogenicity-related gene was found, with the exception of PAI 9 on EcN which contains the genetic cluster for colibactin production. The majority of genes found in PAIs were either with function unknown (hypothetical genes) or transposable elements with no gene in between, making it difficult to evaluate the potential threat of them. All strains seem to be able to produce, at least, one type of bacteriocins, all of them sharing the genetic components to produce botromycin. Analysis of antibiotic resistance genes presence revealed a wide range in the number of genes for each strain, ranging from 15 to 143 genes, with the majority being classified as efflux pumps, followed by antibiotic target alteration. This results have highlighted the genetic variability of these strains, enhancing our comprehension of the genetic structure of *E. coli*.

1.4. Extracellular vesicles from CEC15 can play an important role on immunomodulation

The fourth part of this thesis comprehends a characterization study of extracellular vesicles (EVs) produced by CEC15. In the light of the effects promoted by postbiotic formulations of CEC15, we decided to evaluate the production of EVs by CEC15, their proteome content, and their potential immunomodulatory effects. EVs were successfully produced and presented normal size and dispersion. EVs were shown to contain / be composed of a large number of proteins, furthermore, we have detected proteins in EVs that were not detected in the bacteria proteome. A great number of ribosomal proteins, secretion systems, permeases, and carbohydrates metabolizing enzymes were found in the proteome of EV. Another class of proteins present on EVs was the adhesins, in special the presence of fimbriae, which would allow EVs to adhere on their target. Post characterization, we evaluated the effect of EVs on the gene expression modulation of genes of Caco2 cells, and we observed a overexpression of *IL8*, *TNF*, and *NOS2* after co-incubation with CEC15 EVs. This demonstrate a potential immunomodulatory of EVs and opens a new path of study on the role of EVs on the probiotic effect of CEC15.

1.5. Food matrix has an impact on the effect of probiotics (in special Propionibacterium freudenreichii)

Finally, in the last section of this work, we performed a comparative analysis to evaluate the effect of the food matrix in the probiotic effect of well characterized probiotic strain, the *Propionibacterium freudenreichii* CIRM-BIA 129 in an intestinal inflammatory model of colitis induced by DSS administration. This model and strain were

chosen due to consolidated results in previous works Rabah et al., 2020, which would allow us to focus on the modulatory effect of the food matrix. We tested the effects of *P. freudenreichii* grown in whole milk, skim milk, and milk permeate ultrafiltrate to evaluate the effects of milk proteins and fat on probiotic activity. The fermented preparations were given to mice daily and, in the end, we could observe a improved result on the protection against inflammation caused by DSS administration in animals which received *P. freudenreichii*-fermented whole milk. The results shows a greater protection of intestinal epithelium architecture with preservation of goblet cells and reduced permeability, followed by an increase in the expression of tight junction genes and reduction of pro-inflammatory genes. This work has demonstrated how the probiotic growth matrix is able to modulate its probiotic activity being able to greatly enhancing it.

2. What is yet to be known?

In the pursuit of advancing our understanding of probiotics and harnessing their potential for improving human health, this study has laid a solid foundation by comprehensively exploring the safety, probiotic effects, genomic diversity, and immunomodulatory properties of the *E. coli* probiotic strain CEC15. Additionally, it has shed light on how the food matrix can modulate the probiotic activity, exemplified by *Propionibacterium freudenreichii* in various milk formulations. However, the journey of unraveling the intricacies of probiotics and their application is far from complete. This study sets the stage for future research, presenting critical directions for exploration and advancement. In this section, we delineate potential avenues for future works, encompassing investigations that can deepen our insights, refine probiotic applications, and potentially revolutionize how probiotics are employed to optimize human health. Through these proposed future works, we aim to contribute to a more profound understanding of probiotics, further propelling the field towards novel and targeted probiotic-based therapies.

2.1. Long term safety and the mechanisms involved in CEC15 probiotic effect

We have demonstrated here the safety of CEC15 based on genomic features and after short term administration to healthy mice animal model. Future research should focus on conducting long-term safety assessments of CEC15, including chronic exposure studies, to ascertain its safety over extended periods. Derived from conventional toxicology protocols used to evaluate the safety of minor compounds, prolonged chronic investigations involving animal models are conventionally characterized by a time frame of six months to one year. These studies incorporate prior experiential understanding or tendencies related to impacted organs, the potential for toxicity reversal,

levels of observed effects (or lack thereof), and the quantification of clinical risk at anticipated doses for extended-term therapeutic use (Colerangle, 2017; Food and drug administration, 2007).

In taking CEC15 into further clinical trials, it is imperative to broaden the scope of safety assessments to encompass a diverse array of populations and age groups. This expansive approach aims to ensure a comprehensive understanding of CEC15's safety profile across various demographics. Long-term observational studies, conducted longitudinally, can offer valuable insights into persistent safety and potential long-term effects associated with CEC15 administration. Addressing the safety challenge involves defining relevant outcomes and pathways that traverse a spectrum of targeted organisms and their metabolic potentials within the complex microbial community of the gut (Merenstein et al., 2023; Veiga et al., 2020). This task necessitates identifying critical features such as thresholds that influence diversity indices and microbial community alterations. These features could serve as indicators of concern, signaling deviations from the norm or perturbations in community structures, such as those linked to pro-inflammatory states, pro-obesity tendencies, disrupted immune homeostasis, metabolic syndrome, diabetes predispositions, or other additional conditions. Validated metagenomic markers may be essential to unravel these intricate relationships, underscoring the need for foundational research in this domain (Bilal et al., 2022; Veiga et al., 2020).

Applying this preclinical framework to the realm of next-generation live biotherapeutics, which lack an established history of safe usage, implies that conducting 12-month studies in human subjects aligns with the anticipated standards for novel molecular agents or targets (Merenstein et al., 2023). Especially in cases where limited or no post-market experience exists, integrating these long-term safety studies into Phase III clinical trials is prudent and aligns with regulatory expectations. This approach facilitates a robust evaluation of safety and enables the early identification of any potential adverse effects associated with the use of these innovative live biotherapeutics (Merenstein et al., 2023).

Additionally, a deeper exploration of CEC15's genomic characteristics and its interplay with the human gut microbiome could shed light on how these factors influence its safety and probiotic efficacy. A intricate molecular interplay occurs among microbiota, dietary constituents, and host cells, orchestrating immune and metabolic functions in the host (Furusawa et al., 2015). Some probiotic established outcomes include inhibition of pathogens and amelioration of diarrhea, which have been linked to the production of organic acids (Fukuda et al., 2011), antibacterial peptides (Moroni et al., 2006), quorum-sensing inhibitors (Cotar et al., 2010), pathogen displacement (Ruas-Madiedo et al., 2006), and attenuation of virulence (Tanner et al., 2016). Furthermore, they can regulate host functions and ferment complex polysaccharides from

the diet (Bermudez-Brito et al., 2013; Furusawa et al., 2015; Heuvelin et al., 2010; Heuvelin et al., 2009; Ménard et al., 2005). Unraveling the specific molecules that mediate cross-talk between CEC15 and the host can elucidate their beneficial effects and pave the way for microbiome-targeted therapies for human diseases.

2.2. Key bioactive compounds associated with beneficial effects of CEC15's postbiotic preparations

Postbiotics are described as "a preparation of inanimate microorganisms and/or their components that confers a health benefit on the host" (Salminen et al., 2021). They are derived from deactivated commensal bacteria and encompass deactivated microbial cells, supernatants devoid of cells, and essential constituents, typically rendered inactive through heat. Despite being lifeless, they impart comparable, and in certain cases, even greater health advantages when compared to probiotics, a phenomenon often termed the "probiotic paradox" (Adams, 2010). We have demonstrated here in this work the potential beneficial effects of CEC15 postbiotic preparations. Although promising, future research might investigate the mechanisms of action behind the observed beneficial effects of heat-inactivated CEC15. Also, the identification and isolation of bioactive compounds within the postbiotic preparation could lead to the development of therapeutic agents with targeted anti-inflammatory properties.

While postbiotics do not harbor live microorganisms, they exhibit advantageous effects on the microbiome's health. Additionally, it is hypothesized that postbiotics can modulate the intestinal microbiome in a manner akin to probiotics, while mitigating potential associated risks (Klemashevich et al., 2014). Postbiotics seem to pose minimal adverse effects, while maintaining comparable efficacy to probiotics, albeit without direct comparative studies between substances belonging to these respective groups (Żólkiewicz et al., 2020).

The effectiveness of postbiotics is believed to stem from the interplay between the host and the substances generated by the microbiome as a result of microbial growth triggered by the presence of postbiotics. These substances encompass a wide array of microbial byproducts, including metabolites, proteins, lipids, carbohydrates, vitamins, organic acids, cell wall components, and other intricate molecules (Aguilar-Toalá et al., 2018; Konstantinov et al., 2013). Additionally, it is plausible that active compounds within the postbiotic formulation may permeate the mucus layers and directly stimulate epithelial cells (Piqué et al., 2019). Moreover, the loss of viability and cell breakdown could potentially lead to more intricate beneficial effects, such as immunomodulation (Piqué et al., 2019).

The effectiveness of supernatants and cell fractions from various microbial cultures varies significantly. In a study conducted by Lee and colleagues (M. J. Lee et al., 2002), varying levels of cytokine production by macrophages were observed when

treated with different fractions of *B. bifidum* BGN4, including intact cells, cell-free extracts, purified cell wall, and culture supernatant. The intact cells exhibited the highest expression of tumor necrosis factor alpha (TNF- α), while the cell-free extracts triggered the highest production of interleukin-6 (IL-6). Similarly, in a related study by Ji and Kim (Ji and Kim, 2006), it was found that intact cells and different fractions of BGN4 significantly stimulated the production of IL-10 and IL-6. Furthermore, BGN4 cell-free extracts induced notable morphological modifications in macrophages, enhancing their phagocytosis properties compared to macrophages treated with intact cells and the cell wall of BGN4.

Recent research indicates that supernatants obtained from bacterial cultures of *Lactobacilaceae* family and *Bifidobacterium* genera demonstrate antibacterial capabilities, specifically by impeding the invasion of enteroinvasive *E. coli* strains into enterocytes *in vitro* (Khodaii et al., 2017). While these antibacterial effects may be attributed to hindering the adhesion of pathogenic bacterial strains, possibly due to competition for receptor sites, the supernatants could also exert a localized impact on the intestinal environment, cell barrier, and the expression of protective genes (Khodaii et al., 2017). Consequently, cell-free bacterial supernatants hold promise as effective anti-infectious agents, particularly in the context of treating diarrhea.

Supernatants of *L. acidophilus* and *L. casei* exhibit anti-inflammatory and antioxidant properties when applied to intestinal epithelial cells, macrophages, and neutrophils. These effects manifest through the reduction of pro-inflammatory cytokine tumor necrosis factor α (TNF α) secretion and an increase in the secretion of the anti-inflammatory cytokine interleukin 10 (IL10) (Marco et al., 2018). Moreover, supernatants from *L. casei* and *L. rhamnosus* GG cultures have demonstrated the ability to hinder the invasion of colon cancer cells (Escamilla et al., 2012).

Specifically, research has demonstrated that orally administered lyophilized BLs (bacterial lysates) successfully reach Peyer's patches in the small intestine. Here, they stimulate dendritic cells (DCs), which then activate T and B lymphocytes (Kearney et al., 2015). These mature lymphocytes migrate to the mucous membrane of the respiratory tract, initiating the stimulation of the innate immune system and promoting the secretion of IgA (Kearney et al., 2015). Notably, the safety of BLs' utilization has been validated through numerous clinical studies encompassing various diseases, such as recurrent upper respiratory tract infections in children (Schaad et al., 2002).

The previously mentioned researches illustrated distinct immune responses exhibited by intact cells and various fractions of the identical microorganism. These findings highlight the need of throughout testing of new postbiotics, such as the CEC15 here studied, to better evaluate its mechanisms of action and efficacy in different conditions.

2.3. Role of *E. coli* probiotic strains' unique genes on promoting beneficial effects

The comparative genomic analysis of *E. coli* probiotic strains revealed a large number of unique genes in each strain, which could be related to their differential beneficial effects as we have also seen here when we compared CEC15 and EcN in *in vitro* and *in vitro* studies. Expanding the functional analysis of unique genes in different *E. coli* probiotic strains can offer insights into their potential contributions to health benefits.

Historically, genome analyses primarily emphasized predicted metabolic traits, heavily leaning on functional annotation, genome composition, and synteny (Klaenhammer et al., 2008). Nevertheless, these conventional approaches might fall short in addressing some of the most intriguing inquiries. In the study of probiotic bacteria genome sequences, our understanding of probiotic mechanisms and their interactions with the host GIT is advancing through the integration of functional genomic techniques (Johnson and Klaenhammer, 2014).

In *L. acidophilus* NCFM, a gene (*lba1524*) responsible for encoding a functional histidine protein kinase (HPK) was intentionally disrupted, resulting in a mutant strain that displayed heightened sensitivity to acid stress compared to the original parental strain. Additionally, a transcriptomic analysis utilizing DNA microarray technology was conducted, comparing the *lba1524* mutant with the wild-type strain. This analysis revealed a significant impact on 80 genes (Azcarate-Peril et al., 2005). Notably, among the genes that showed increased expression in the HPK mutant, the *LuxS* homolog, associated with the autoinducer-2 quorum sensing compound, was particularly upregulated. This upregulation of the *LuxS* homolog is of significance as it plays a crucial role in survival within gastric juices and adhesion to intestinal epithelial cell lines (B. Buck et al., 2009; Lebeer et al., 2008).

Functional genomic investigations on multi-drug resistant (MDR) transporters within probiotic strains of *L. reuterii* and *L. acidophilus* have revealed their involvement in bile tolerance, as demonstrated in studies by Whitehead et al. (Whitehead et al., 2008) and Pfeiler and Klaenhammer (Pfeiler and Klaenhammer, 2009). Additionally, recent research utilizing *in silico* genome analysis has identified and functionally characterized a MDR transporter gene, *betA* (bile efflux transporter), in *B. longum*, as documented by Gueimonde et al. (Gueimonde et al., 2009). When *betA* was expressed in *E. coli*, it enhanced bile tolerance by actively expelling bile salts.

During *in silico* genome screening of *L. acidophilus* NCFM, five potential adhesion cell surface proteins were identified. These included a fibronectin binding protein (*FbpA*), an S-layer protein (*SlpA*), a mucin-binding protein (*Mub*), and two R28 homologues associated with streptococcal adhesion, as documented by Buck et al. in (B. L. Buck et al., 2005). Subsequent mutational analysis revealed that *FbpA*, *Mub*, and *SlpA*

all played significant roles in facilitating adhesion to Caco-2 epithelial cell lines.

An analysis of the genomes of two *L. rhamnosus* strains revealed a significant finding in *L. rhamnosus* GG: the presence of a genomic island housing three pilin-encoding genes (*spaCBA*) that are secreted and sortase-dependent, as elucidated by Kankainen et al. in (Kankainen et al., 2009). Subsequent immunoblotting and immunogold electron microscopy provided visual confirmation of the pili being bound to the cell wall. Moreover, through mutational analysis of the *spaC* gene, it was observed that the adherence capability of *L. rhamnosus* GG to human intestinal mucus was lost, underscoring the critical role of these distinctive pili structures in adherence and retention within the GIT (Kankainen et al., 2009).

Additionally, targeted studies could investigate the role of specific genes in promoting probiotic effects, allowing for the engineering of strains with enhanced functionalities.

2.4. EVs' cargo composition and roles on immunomodulation

We have shown in this study that CEC15 is able to produce extracellular vesicles. Besides, characterization of the EVs' proteome has revealed a few important proteins related to adhesion, translation and metabolism. We believe that future research should focus on understanding the cargo content of CEC15 EVs and how it influences immune responses. Additionally, isolating and characterizing specific proteins, nucleic acids, or metabolites within EVs can unveil the key immunomodulatory components.

Commensal and pathogenic gram-negative bacteria utilize diverse mechanisms to establish communication with host cells. One such mechanism involves the production of membrane vesicles, enabling the transfer of cargo to distant targets within the host (Ashrafian, Behrouzi, et al., 2019; Ashrafian, Shahriary, et al., 2019; Behrouzi et al., 2018; Kulp and Kuehn, 2010).

The secretion of EVs by *Bacteroides fragilis* and *Akkermansia muciniphila* has been shown to enhance immunomodulatory effects and inhibit gut inflammation in mouse models of experimental colitis (Kang et al., 2013; Shen et al., 2012). EVs produced by commensal strains are emerging as pivotal elements in signaling processes within the intestinal mucosa. Recent studies have demonstrated that EVs from EcN and vesicles produced by other commensal *E. coli* strains are internalized by epithelial cells using clathrin-mediated endocytosis, influencing signaling to the immune system through the intestinal epithelial barrier (Cañas et al., 2016). Research has confirmed that EVs from the probiotic EcN and other commensal *E. coli* strains transfer mediators that activate host immunity. EVs from *E. coli* strains exhibit immunomodulatory effects in various in vitro models of the intestinal barrier and human colonic explants, regulating the expression of antimicrobial peptides and inflammatory biomarkers towards a more calming profile (M. J. Fábrega et al., 2016).

It is crucial to emphasize the notable correlation observed between cellular localization and the quantity of proteins isolated from EVs. Specifically, when protein localization was categorized as 'membrane,' the proteome appeared to be more limited, ranging from 11 to 192 proteins. Conversely, when the most abundant protein representation originated from the cytoplasm, the proteome was broader, spanning from 11 to 1286 proteins. Notably, in instances where the proteome exceeded 300 proteins, 'cytoplasm' emerged as the predominant classification. This pattern could be attributed to two primary factors—the sensitivity of the research techniques employed and the extent of contamination of the EVs' proteome with proteins sourced from bacterial cells that produce these structures (J. Lee et al., 2016; Nagakubo et al., 2020; Shao et al., 2018).

A deficiency in sensitivity could result in the exclusion of proteins with low representation in the EVs' proteome. Nevertheless, these proteins may possess crucial functions for the microorganisms secreting them (Ahmed and Rice, 2005; Pedersen et al., 2003). Conversely, an excessively high representation of proteins within the EVs' proteome may indicate potential contamination, underscoring the necessity to enhance the purification step of the obtained EVs (Klimentová and Stulík, 2015; Nagakubo et al., 2020). Balancing sensitivity is thus essential to ensure accurate representation and meaningful insights into the roles and functions of proteins within extracellular vesicles.

As outlined in the review by Nagakubo et al. (Nagakubo et al., 2020), certain researchers view the abundant presence of ribosomal proteins (30S and 50S) in EVs as a potential sign of proteome contamination originating from the cytoplasm. However, it's important to note that other studies have presented evidence supporting the extracellular secretion of ribosomal proteins. These proteins, despite their typical cytoplasmic origin, may serve crucial functions beyond ribosomal activity in the physiology of microorganisms. These functions include roles in biofilm formation (Graf et al., 2019) and conferring resistance against antibiotics that target translation processes (Kesavan et al., 2020; Sung et al., 2021). Therefore, the presence of ribosomal proteins in the EVs' proteome might not solely indicate contamination but could signify significant extracellular functions for these proteins.

In the EVs secreted by EcN, a significant portion of the proteins were represented adhesive proteins, including fimbrial subunits (FocA, Fim1C, FocF, FocG, and FocH), flagellar subunits (FliC, FliD, FlgA, FlgE, FlgK, and FlgL), and outer membrane proteins (OmpA, OmpC, OmpF, and NmpC). Many researchers emphasize that the presence of adhesins anchored on the surface of Gram-negative bacterial EVs is a crucial factor aiding intestinal colonization (S. I. Kim et al., 2018; Rolhion et al., 2010; Taheri et al., 2018). Another important group of proteins identified in these EVs was associated with peptidoglycan and cell membrane rearrangement, including murein hydrolases (MltA, MltB, and MltC), murein-interacting protein MipA, and peptidoglycan-

associated lipoproteins (Pal, TolB, Sat, LpoA, YbaY, and SlyB). The rearrangement of murein and cell membranes is highlighted as essential steps in EV biogenesis, justifying the presence of proteins related to these processes within EVs (Avila-Calderón et al., 2021). Additionally, researchers point out the potential role of murein hydrolases enclosed within EVs in competitive interactions against other bacteria (Clarke, 2018). To summarize, the primary constituents of the EVs' proteome from *E. coli* Nissle 1917 encompass adhesins and proteins associated with peptidoglycan rearrangement, vital for efficient host colonization and protection against pathogenic microorganisms.

Fabrega et al. (M. J. Fábrega et al., 2016) discovered that the presence of lipopolysaccharides (LPS) in *E. coli*-derived EVs may account for the activation of IL-6, IL-8, and TNF. Conversely, the upregulation of IL-10 appears to be linked to other factors present in the vesicles. In parallel, Morishita et al. (Morishita et al., 2021; Morishita et al., 2022) demonstrated that EVs-mediated cytokine production is closely tied to their internalization. They observed a reduction in the release of TNF and IL-6 from cells treated with EVs when endocytosis and TLR2 inhibitors were present, except for RAW264.7 cells, where TNF reduction was not observed even after blocking clathrin-mediated endocytosis and micropinocytosis. This suggests the involvement of multiple pathways in the interaction between EVs and cells. Understanding these primary effectors is crucial for comprehending the mechanisms of immunomodulation in the host.

It is evident that a substantial portion of the research on EVs produced by probiotics primarily examines the biological impact of these structures within the gastrointestinal system. This focus is logical and justifiable, given the ingestible administration of probiotic EVs, emphasizing their intuitive action within the digestive system (Chang et al., 2020; Cieślik et al., 2022). However, it remains essential to acknowledge that EVs, owing to their nanoscale dimensions, have the potential to reach diverse tissues within the host. Consequently, exploring their effects on various types of human cells beyond the gastrointestinal tract is equally crucial (Hendrix and Wever, 2022; Viswanathan and Muthusamy, 2022).

Studies, like the above mentioned ones, are essential to a precise characterization of the mechanistic activity of probiotic strains EVs. Additionally, in-depth studies exploring the receptor-ligand interactions between EVs and immune cells can elucidate the precise pathways through which EVs exert their effects, aiding in the development of targeted immunomodulatory therapies.

2.5. The intricate interplay between probiotics and different food matrices, and the consequences for the final consumer

The beneficial effect of some probiotic strains are well characterized in many different diseases models and clinical trials. An important point to be noted, however, is that the form this probiotic is presented and the matrix that compose the probiotic prod-

uct can influence greatly the effects of this probiotic strain. Here we have demonstrated the effect of milk fat and proteins in the improvement of *P. freudenreichii* CIRM-BIA 129 anti-colitis effect.

While the advantages of the probiotic matrix were once at the forefront, there has been a notable shift in focus towards specific strains and their targeted effects on diseases. Consequently, the influence of the matrix has been somewhat overshadowed (Sanders et al., 2010). However, it's essential to recognize that sensory attributes and ease of use are pivotal aspects of probiotic matrices, significantly impacting consumer acceptance. Moreover, matrices can have implications for probiotic shelf-life, survival in the GIT, and overall clinical efficacy (Sanders et al., 2010).

In fact, the matrix itself can exert a beneficial effect on the host (Sanders et al., 2010). Achieving a careful balance among sensory properties, ease of use, GIT survival, clinical efficacy, and host benefits is critical in the development of probiotic products. The role of carrier matrices in achieving this balance is paramount (Sanders et al., 2010). Unfortunately, the probiotic-matrix relationship has not received adequate attention, serving as a barrier to innovation and highlighting the need for new guidelines in this domain (van den Nieuwboer et al., 2014).

In any probiotic product, two critical factors come into play: the attributes of the probiotic strain itself and the characteristics of the matrix, which can either positively or negatively impact both the strain and the host. Probiotic bacteria can be incorporated into a food product in two main ways. The first approach involves growing the probiotics within the final product, typically through fermentation processes (e.g., transforming milk into yogurt). The second approach entails adding dried or encapsulated probiotic microorganisms to the product (e.g., in infant formulae and juices (Makinen et al., 2012)). During the processing stage, factors such as fermentation temperature and oxygen exposure significantly influence probiotic growth.

Milk, as a matrix for probiotic incorporation, contains various components including whey proteins (β -lactoglobulin, α -lactalbumin), caseins (α s1, α s2, β , and κ), immunoglobulins, bovine serum albumin, lactoferrin, lactoperoxidase, alkaline phosphatase, catalase, and plasmin (Burgain et al., 2014; Livney, 2010). Additionally, milk contains fat globules consisting of triacylglycerol and other components like carotenoids, vitamins A, D, E, and K, as well as active molecules such as phospholipids, sphingolipids, cholesterol, and milk fat globule membrane proteins. Milk is also rich in minerals such as calcium, inorganic phosphate, citrate, and others (e.g., magnesium, sodium, potassium, and chloride). Water-soluble vitamins like vitamin C, B1, and B2 are present in dairy products (Burgain et al., 2014). Lactose (α -lactose or β -lactose), the major disaccharide in dairy products, serves as a substrate for probiotics, potentially enhancing their efficacy (Varcoe et al., 2002). Bacteria seem to have an affinity for milk fat globules, possibly providing protection during gastrointestinal

transit (Burgain et al., 2014). For example, higher fat and whey protein content in simulated gastric conditions leads to significantly higher probiotic cell counts (Ziaro and Zaręba, 2015).

Saxelin et al. (Saxelin et al., 2010) demonstrated that the matrix used for administering probiotics significantly affects gastrointestinal survival in a strain-dependent manner. In a study comparing yogurt, cheese, and capsule matrices, higher fecal recovery rates were observed for *B. animalis* BB-12 and *Propionibacterium freudenreichii* shermanii JS in yogurt, but not for *L. rhamnosus* GG and *L. rhamnosus* LC705. However, caution is needed in interpreting these results due to substantial dosing differences between the matrices. Participants in the yogurt group had significantly higher daily CFU consumption of *B. animalis* BB-12 and *Propionibacterium freudenreichii* shermanii JS compared to the cheese and capsule groups. A similar trend was observed for *L. rhamnosus* GG and *L. rhamnosus* LC705. Rochet et al. (Rochet et al., 2008) investigated *B. animalis* DN-173 010 in lyophilized and fermented formats, finding slightly higher survival in lyophilized form but with higher doses.

The alleviation of colitis in a mouse model through the administration of a probiotic strain appears to be influenced by the addition of milk. When administering *L. casei* BL23 in milk versus a phosphate-buffered saline solution (PBS), mice with induced colitis exhibited a significantly lower change in body weight, highlighting the potential positive impact of the milk matrix (B. Lee et al., 2015). Collins et al. (Collins et al., 2002) observed higher fecal excretion levels of *L. salivarius* UCC118 in fresh milk compared to fermented milk, while Varcoe et al. (Varcoe et al., 2002) found no significant differences in fecal lactobacilli counts after administering *L. acidophilus* NCFM in different matrices. Finally, Klingberg and Budde (Klingberg and Budde, 2006) reported more individuals with isolated *L. plantarum* MF 1298 after consuming probiotic sausage compared to lyophilized probiotic powder.

There is a lack of extensive data evaluating the probiotic matrix effect on clinical efficacy. Most clinical studies primarily compare two identical matrices with differing probiotic content rather than examining the impact of the matrix itself. Certain clinical trials, however, have substantiated the matrix effect. For instance, a probiotic blend that incorporated *P. freudenreichii* was administered to humans through various forms, including conventional capsules, yogurt, or cheese. Notably, the highest fecal levels of *P. freudenreichii* were observed in individuals who consumed the probiotic in yogurt form (Saxelin et al., 2010). This finding is in line with a French study demonstrating that yogurt not only enhances the survival of *P. freudenreichii* but also promotes its metabolic activity within the intestinal tract (Hervé et al., 2007).

In a study assessing if probiotic administration could expedite recovery from acute diarrhea, children were given *L. rhamnosus* GG in either fermented milk or freeze-dried powder for 5 days. Both treatments significantly reduced the duration of

diarrhea by approximately 1 day compared to the control; however, no matrix-specific effects were observed (Isolauri et al., 1991). Chiang et al. (Chiang et al., 2000) and Sheih et al. (Sheih et al., 2001) reported an increase in immune cell activity following the intake of *B. lactis* HN019 in either low-fat milk or lactose-hydrolyzed low-fat milk, with the latter favoring higher natural killer (NK)-cell activity. In a study by Hütt et al. (Hütt et al., 2015), systolic and diastolic blood pressure (BP) values were lowered after a three-week administration of *L. plantarum* TENSIA in both a probiotic cheese matrix (1×10^{10} CFU/day) and a yogurt matrix (6×10^9 CFU). The diastolic BP reductions from baseline were significant for both matrices. The systolic BP reduction from baseline was only significant for the cheese matrix, with a strong trend observed for the yogurt matrix (Hütt et al., 2015). It's important to note that participants in the cheese group consumed a larger daily dose of *L. plantarum* (1×10^{10} CFU) compared to the yogurt group (6×10^9 CFU), potentially accounting for the observed difference between matrices.

Current probiotics research often prioritizes studying strain-specific effects over considering the impact of carrier matrices. However, both probiotic strains and their carrier matrices significantly influence the quality and efficacy of a probiotic product. Matrix components like fats and proteins can affect probiotic viability and efficacy, and in vivo studies highlight strain-specific effects on gastrointestinal survival. Striking a balance among these factors, along with considering sensory properties, is essential for product functionality and consumer acceptance. To drive innovation, studies comparing multiple matrices with the same probiotic strain are needed. It's important to shift from solely valuing strain-specific effects to assessing strain functionality within a specific carrier matrix, guiding health claims based on the product as a whole.

Future studies could also assess a broader range of food matrices to comprehensively understand how various components within these matrices influence the viability, stability, and bioactivity of probiotics, exploring the mechanisms that govern the interaction between probiotics and different food components will allow for the design of optimal food matrices that maximize probiotic potency and viability. Furthermore, investigating consumer acceptability and preferences for probiotic-fortified food products will guide the development of palatable and effective delivery systems.

These future perspectives will guide forthcoming research endeavors, aiding in the refinement of probiotic applications and the development of innovative strategies to maximize their potential for improving human health.

3. Conclusions

In conclusion, this comprehensive study illuminates the multifaceted nature of probiotic strain CEC15, an *E. coli* variant. The research robustly demonstrates the safety and probiotic potential of CEC15, surpassing even the established reference

strain *E. coli* Nissle 1917 in terms of genomic stability and beneficial traits. Both live CEC15 and its postbiotic forms, particularly heat-inactivated CEC15, exhibit promising protective effects against intestinal mucositis, emphasizing the therapeutic potential of inactive bacteria and their metabolites. Additionally, the study delves into the genetic diversity of *E. coli* probiotic strains, underscoring the complexity of their genomic makeup. Furthermore, the investigation into extracellular vesicles (EVs) from CEC15 unveils their potential immunomodulatory role, introducing a new dimension to understanding the probiotic effect. Lastly, the study underscores the profound impact of the food matrix, as evidenced by the enhanced probiotic activity of *Propionibacterium freudenreichii* in specific milk formulations. These insights collectively deepen our understanding of probiotics, paving the way for optimized formulations and tailored applications in promoting intestinal health and ameliorating inflammatory conditions.

1. Introduction

Ces dernières années, on observe un intérêt croissant pour l'utilisation des probiotiques en tant que moyen de restaurer et de maintenir l'état de santé du sujet, notamment en ce qui concerne le maintien d'une microbiote intestinale saine. Les probiotiques sont définis comme des "micro-organismes vivants qui, lorsqu'ils sont administrés en quantités adéquates, confèrent un bénéfice pour la santé à l'hôte" (Hill et al., 2014). Ils peuvent faire partie de la composition de divers produits alimentaires tels que les produits laitiers fermentés (par exemple, le yaourt) et les légumes fermentés (par exemple, la choucroute), ainsi que des compléments alimentaires. Le concept de probiotiques n'est pas nouveau et ses origines remontent au début du XXe siècle lorsque le lauréat du prix Nobel, Elie Metchnikoff, a émis l'hypothèse que la consommation de produits laitiers fermentés contribuait à la longévité des paysans bulgares (Mercenier et al., 2003). Depuis lors, des recherches approfondies ont été menées pour explorer les bénéfices potentiels pour la santé des probiotiques et leurs mécanismes d'action. Les avantages potentiels des probiotiques dépassent le seul aspect de la santé intestinale. Des études ont montré que les probiotiques pouvaient avoir des effets positifs sur le système immunitaire, la santé mentale et la santé de la peau, entre autres domaines. En conséquence, le marché des probiotiques a connu une croissance exponentielle, les consommateurs recherchant des produits probiotiques pour améliorer leur bien-être global (Grumet et al., 2020).

Les souches de bactéries probiotiques le plus souvent revendiquées, qui font l'objet d'études approfondies depuis longtemps en ce qui concerne leurs effets bénéfiques, appartiennent aux genres *Bifidobacterium* et à la famille des *Lactobacillaceae* (O'Toole et al., 2017). Cependant, il existe une demande croissante pour de nouvelles souches de probiotiques. Dans ce contexte, l'identification de souches parmi celles déjà reconnues comme probiotiques et celles commensales de l'intestin, comme c'est le cas des probiotiques de nouvelle génération, devient une stratégie prometteuse pour répondre à cette demande.

La bactérie *Escherichia coli* est bien connue pour sa pathogénicité et pour être également un membre commun de la microbiote intestinale humaine et animale, en tant que bactérie commensale. Cependant, un membre de cette espèce, *E. coli* Nissle 1917, est utilisé comme probiotique depuis plus d'un siècle, avec des effets significatifs dans le traitement de la diarrhée intestinale infectieuse (T. M. Wassenaar, 2016), et de nombreuses études ont montré ses effets bénéfiques, tant chez l'homme que chez les modèles animaux, sur des maladies telles que les maladies inflammatoires de l'intestin

(rectocolite hémorragique et maladie de Crohn) (Schultz, 2008) et les allergies (Weise et al., 2011). D'autres souches d'*E. coli* ont également montré des effets bénéfiques chez l'homme, en particulier les souches composant le produit probiotique Synbioflor2 (T. Wassenaar et al., 2014) et le colinfant newborn (Kocourková et al., 2007). Une souche nouvellement isolée d'*E. coli*, CEC15, a démontré des effets bénéfiques sur le remodelage de la structure du tissu intestinal chez les rats (Tomas et al., 2015) et a protégé les souris contre la rectocolite hémorragique induite par l'administration d'acide dinitrobenzène sulfonique (DNBS) (Escribano-Vazquez et al., 2019). Cependant, des études supplémentaires sont nécessaires pour confirmer et comprendre les effets bénéfiques de cette souche chez l'hôte.

La caractérisation phénotypique et génotypique des souches constitue une étape cruciale dans la compréhension et l'identification des probiotiques (Shokryazdan et al., 2017). Un aspect essentiel de cette caractérisation implique de classer taxonomiquement les souches probiotiques afin de déterminer leur relation génétique avec d'autres micro-organismes. Les méthodes traditionnelles, telles que les traits phénotypiques, sont utiles pour une identification préliminaire (FAO/WHO, 2002). Cependant, l'avènement des techniques moléculaires, comme le séquençage du génome entier (WGS), a révolutionné la caractérisation des probiotiques. Cette méthode permet aux chercheurs de nommer les souches probiotiques en fonction de la séquence des gènes présents dans leur ADN. En comparant la séquence des gènes à une base de données de référence, les chercheurs peuvent classer précisément la souche probiotique (Ranjan et al., 2016).

La probiogénomique est un domaine émergent qui se concentre sur la compréhension de la composition génomique des micro-organismes probiotiques. Les génomes des probiotiques renferment des informations précieuses sur leurs capacités métaboliques et leurs propriétés fonctionnelles (R. D. O. Carvalho et al., 2022). En analysant le contenu génétique des souches probiotiques, les chercheurs peuvent identifier les gènes spécifiques responsables des fonctions probiotiques, fournissant des éclairages sur leurs bénéfices potentiels pour la santé (Castro-López et al., 2021). Le séquençage du génome et les outils bioinformatiques jouent un rôle crucial en probiogénomique. La génomique comparative permet aux chercheurs de comparer les génomes de différentes souches probiotiques, mettant en évidence les similitudes et les différences dans leur contenu génétique. Cette approche comparative aide à identifier les gènes conservés associés aux activités probiotiques et, inversement, à comprendre les caractéristiques fonctionnelles propres à chaque souche (Kazou et al., 2018). La probiogénomique contribue également à l'optimisation des formulations probiotiques. En identifiant les gènes essentiels impliqués dans la fonctionnalité probiotique, les chercheurs peuvent modifier les souches probiotiques pour améliorer leur efficacité (Ventura et al., 2012). De plus, la probiogénomique contribue à répondre aux

préoccupations liées à la stabilité génétique des probiotiques pendant la production et le stockage, garantissant que le produit final conserve ses bénéfices pour la santé initialement prévus (Ventura et al., 2009).

Un autre aspect essentiel de la caractérisation des probiotiques réside dans la garantie de leur sécurité pour la consommation humaine. Les probiotiques doivent être non pathogènes et dépourvus de traits nocifs. Des évaluations approfondies de la sécurité, comprenant des études toxicologiques et une analyse du génome, sont réalisées pour vérifier l'absence de gènes nuisibles ou de facteurs de virulence. Les considérations de sécurité sont cruciales, en particulier pour les populations vulnérables telles que les nourrissons, les femmes enceintes et les personnes immunodéprimées (Sanders et al., 2010). En outre, les propriétés fonctionnelles exhibées par les probiotiques sont primordiales pour leur caractérisation. Divers tests *in vitro* sont réalisés pour évaluer leur potentiel probiotique. Ces tests comprennent leur capacité à survivre aux conditions difficiles du tractus gastro-intestinal, telles que l'acidité et la bile, ainsi que leur capacité à adhérer aux cellules épithéliales intestinales (Coelho-Rocha et al., 2023). De plus, les études *in vitro* peuvent identifier la présence de gènes fonctionnels responsables des activités probiotiques, comme la production de composés antimicrobiens ou la modulation des réponses immunitaires. Ces tests sont réalisés dans des conditions de laboratoire contrôlées, permettant aux chercheurs de simuler certains aspects de l'environnement gastro-intestinal (de Jesus et al., 2021). Un aspect primordial des tests *in vitro* réside dans l'évaluation de la survie probiotique dans des conditions gastriques. L'environnement acide et difficile de l'estomac peut être préjudiciable à la viabilité probiotique. Ainsi, les chercheurs soumettent les souches probiotiques à des conditions gastriques simulées, surveillant leurs taux de survie pour déterminer leur résilience (Vera-Pingitore et al., 2016). Un autre paramètre essentiel étudié *in vitro* est l'adhésion probiotique aux cellules épithéliales intestinales. L'adhésion est une condition préalable à l'exercice de leurs effets bénéfiques dans l'intestin. Divers modèles de culture cellulaire sont utilisés pour évaluer l'adhésion probiotique, fournissant des données précieuses sur leur potentiel à coloniser et interagir avec la muqueuse intestinale (Saadat et al., 2019). De plus, les tests *in vitro* incluent l'évaluation de l'activité antimicrobienne des probiotiques contre les micro-organismes pathogènes. Les probiotiques peuvent produire des composés antimicrobiens qui inhibent la croissance des bactéries nocives. Ces activités antagonistes sont cruciales pour maintenir la santé intestinale et prévenir les infections (Lindgren and Dobrogosz, 1990).

Pour évaluer davantage les fonctions probiotiques, des modèles *in vivo* peuvent aider à simuler de véritables maladies et l'efficacité des probiotiques chez les organismes vivants, tels que les modèles animaux et les sujets humains. Ces études fournissent des preuves réelles des effets probiotiques sur la santé intestinale et le bien-être global (Rousseau et al., 2020). Les modèles animaux, comprenant les rongeurs

et d'autres espèces mammifères, sont couramment utilisés pour les tests probiotiques *in vivo*. Ces modèles permettent aux chercheurs d'évaluer les impacts probiotiques sur la composition de la microbiote intestinale, les réponses immunitaires et d'autres paramètres physiologiques (Rousseau et al., 2020). Les résultats des études sur les animaux fournissent des connaissances précieuses et orientent la conception des essais cliniques chez l'homme. Les essais cliniques chez l'homme représentent la norme de référence pour évaluer l'efficacité des probiotiques. Ces essais consistent à administrer des probiotiques à des sujets humains et à évaluer leurs effets sur des conditions de santé spécifiques (Zawistowska-Rojek and Tyski, 2018). Les essais cliniques randomisés, en double aveugle et contrôlés par placebo, sont préférés pour minimiser les biais et obtenir des résultats fiables. Les essais cliniques ont démontré l'efficacité des probiotiques dans diverses conditions telles que la diarrhée, le syndrome du côlon irritable et les allergies (Pogačar et al., 2022).

Après avoir confirmé l'effet bénéfique d'une souche probiotique, il est nécessaire de déterminer comment cette souche sera administrée à l'hôte et comment la matrice dans laquelle le probiotique sera contenu peut affecter son activité. Les produits probiotiques peuvent se présenter sous forme de suspensions, sous forme desséchée en capsules et sous forme de préparations alimentaires fermentées telles que le lait fermenté, les viandes fermentées, les légumes fermentés et les fromages (Ozen and Dinleyici, 2015). Il a déjà été démontré que la présentation du probiotique peut affecter la manière dont il module l'hôte, il est donc important de réévaluer l'activité probiotique lorsqu'il est administré sous une forme différente de celle initialement testée (Mantel et al., 2023).

Alors que la recherche sur les probiotiques continue d'évoluer, il est essentiel de rester vigilant dans l'évaluation critique des preuves scientifiques étayant les revendications probiotiques. Bien que les probiotiques présentent une immense promesse, tous les produits probiotiques sur le marché n'ont pas été rigoureusement testés ou prouvés pour fournir les avantages pour la santé revendiqués (de Simone, 2019). Par conséquent, la poursuite de la recherche, la publication transparente des résultats des études et la collaboration entre les chercheurs, l'industrie et les organismes de réglementation sont cruciales pour libérer tout le potentiel des probiotiques. Enfin, le champ émergent des probiotiques offre des opportunités passionnantes pour exploiter le pouvoir du microbiote intestinal pour améliorer la santé. En comprenant davantage les complexités du microbiote intestinale et les mécanismes d'action des probiotiques, nous pouvons débloquent de nouvelles approches thérapeutiques pour relever divers défis de santé (Shokryazdan et al., 2017). Avec les progrès dans les études sur les probiotiques, l'avenir promet beaucoup pour que ces micro-organismes bénéfiques contribuent de manière significative à la santé et au bien-être humains.

2. Pertinence de la thèse

Les changements dans le mode de vie des habitants des nations développées, incluant des facteurs tels que l'alimentation, l'inactivité physique et le vieillissement de la population, contribuent à une augmentation des maladies chroniques. Une part importante de ces problèmes de santé croissants concerne des problèmes liés au système immunitaire, à l'inflammation et aux déséquilibres de la microbiote intestinale. Un exemple flagrant de cette tendance est l'augmentation de la prévalence des maladies inflammatoires de l'intestin, qui sont liées à la prédisposition génétique, aux irrégularités du système immunitaire et aux perturbations de l'équilibre de la microbiote intestinale (Bouma and Strober, 2003). De plus, des affections telles que la mucosite, une inflammation sévère de la muqueuse du tractus gastro-intestinal, touchent environ 80% des patients subissant des traitements contre le cancer impliquant la chimiothérapie et les radiopharmaceutiques (R. O. Carvalho et al., 2017). Bien que des traitements soient disponibles pour des affections comme les maladies inflammatoires de l'intestin et la mucosite, ces interventions sollicitent considérablement les systèmes de santé et ne procurent qu'un soulagement temporaire entre les récurrences, provoquant parfois des effets indésirables qui augmentent encore les dépenses de traitement.

Les bactéries probiotiques ont montré le potentiel d'atténuer les symptômes des maladies inflammatoires (Bibiloni et al., 2005; Ghouri et al., 2014; Sood et al., 2009). Cependant, il est bien connu que cet effet dépend de la souche et ne peut pas être extrapolé à d'autres souches de la même espèce sans un examen approfondi de leurs caractéristiques et de leurs effets bénéfiques. Cela souligne l'importance d'une identification et caractérisation correctes d'une souche probiotique potentielle non seulement sur le plan phénotypique, mais aussi au niveau génomique.

3. Objectives

L'objectif principal de cette thèse est de caractériser pleinement la souche nouvellement isolée *Escherichia coli* CEC15 en tant que souche probiotique potentielle, en évaluant des aspects liés à la toxicité, à la survie lors du passage dans le tractus gastro-intestinal, à l'adhérence à la paroi intestinale, aux effets sur un hôte sain et sa microbiote, ainsi qu'à l'effet de la souche dans un modèle murin de mucosite induite par le 5-FU, en utilisant des cellules viables, des bactéries inactivées par la chaleur et le surnageant sans cellules fermentées. Pour atteindre cet objectif principal, celui-ci a été segmenté en objectifs spécifiques ultérieurs :

1. Identifier la souche en utilisant son génome et effectuer la classification dans les phylogroupes d'*E. coli* ;

2. Appliquer des approches *in silico* pour identifier les gènes liés à la production de facteurs de résistance aux antibiotiques, de toxines, de facteurs de résistance à la digestion gastro-intestinale, de protéines d'adhésion et de la présence d'éléments mobiles ;
3. Évaluer la survie de la souche dans un protocole de digestion simulée ;
4. Déterminer le niveau d'adhérence aux cellules épithéliales du côlon humain (Caco2) *in vitro* ;
5. Évaluer la sécurité de la souche à forte dose pendant une longue période chez les souris saines;
6. Évaluer l'immunomodulation de CEC15 inactivé par la chaleur et du surnageant sans cellules sur Caco2 *in vitro* ;
7. Étudier l'activité immunomodulatrice de CEC15 viable dans un modèle murin de mucosite intestinale induite par le 5-FU ;
8. Étudier la modulation de CEC15 viable dans le microbiote des souris saines et des souris atteintes de mucosite intestinale ;
9. Évaluer l'immunomodulation de CEC15 inactivé par la chaleur et du surnageant sans cellules dans un modèle murin de mucosite intestinale induite par le 5-FU ;
10. Comparer la souche CEC15 avec la souche de référence *E. coli* Nissle 1917 sur ses caractérisations *in silico* et *in vitro* et leurs effets sur les modèles *in vivo* susmentionnés ;
11. Réaliser une étude génomique comparative entre CEC15 et toutes les souches d'*E. coli* ayant présenté des effets bénéfiques ;
12. Évaluer la production de vésicules extracellulaires par *E. coli* CEC15 et *E. coli* Nissle 1917, et les caractériser par un dosage protéomique et l'immunomodulation des cellules Caco2 ;
13. Évaluer l'impact du milieu de croissance sur l'immunomodulation de l'effet d'une souche probiotique en utilisant la souche *Propionibacterium freudenreichii* CIRM-BIA 129 comme test dans un modèle murin de colite ulcéreuse induite par DSS.

Les objectifs 1-8 et 10 sont abordés dans le chapitre 3, qui présente un article de recherche original utilisant la probiogénomique et des tests *in vitro* pour caractériser la souche CEC15, ainsi que des modèles *in vivo* pour évaluer sa sécurité et son effet protecteur.

Les objectifs 9 et 10 sont abordés dans le chapitre 4, qui présente un article de recherche original où nous comparons les effets des bactéries inactivées par la chaleur et du surnageant sans cellules par rapport aux bactéries viables (pour CEC15 et Nissle 1917) dans un modèle murin de mucosite intestinale induite par le 5-FU.

Les objectifs 11 et 12 sont abordés dans le chapitre 5, qui présente des résultats supplémentaires de la thèse et comprend une analyse génomique comparative entre les souches bénéfiques d'*E. coli* et sur la production et la caractérisation des vésicules extracellulaires par *E. coli* CEC15 et *E. coli* Nissle 1917.

L'objectif 13 est abordé dans le chapitre 6, qui présente un article de recherche original où nous comparons les effets de *P. freudenreichii* CIRM-BIA 129 sur un modèle murin de colite ulcéreuse induite par DSS en fermentant trois milieux différents : perméat de lait ultrafiltré, lait écrémé et lait entier.

Une discussion générale des résultats, les conclusions et les perspectives sont présentées dans le chapitre 7.

4. Résultats

4.1. Article de recherche 1 - Comprehensive probiogenomics analysis of the commensal *Escherichia coli* CEC15 as a potential probiotic strain

Le tractus gastro-intestinal humain abrite une communauté complexe de micro-organismes jouant un rôle crucial dans la santé et la maladie. Les bactéries probiotiques sont depuis longtemps reconnues pour leurs bienfaits sur la santé, mais leurs effets sont aujourd'hui reconnus comme étant hautement spécifiques à l'espèce voire à la souche. En conséquence, la recherche s'est tournée vers l'identification et la sélection de candidats probiotiques par le biais d'un domaine appelé probiogénomique. La probiogénomique combine des technologies "omiques" avancées telles que la génomique, la transcriptomique et la métabolomique avec des tests fonctionnels, permettant ainsi une compréhension exhaustive des probiotiques potentiels. Cela permet une plongée profonde dans les mécanismes des activités probiotiques et améliore le processus de sélection. En réponse à ce besoin, nous avons effectué une dissection fonctionnelle et intégrée multi-"omiques" de la souche commensale *E. coli* CEC15, en la comparant à la souche probiotique établie *E. coli* Nissle 1917 (EcN).

Leur analyse phylogénomique a établi que CEC15 appartient à une lignée associée aux commensaux plutôt qu'aux agents pathogènes. Alors qu'EcN a un historique d'utilisation probiotique, des préoccupations concernant sa sécurité ont été soulevées en raison de la présence d'un groupe de gènes de génotoxine. L'analyse génomique de CEC15 a dissipé de telles inquiétudes, car elle ne possédait pas ce groupe de gènes et d'autres gènes associés à la virulence et à la résistance aux antibiotiques

présents chez EcN. Outre les avantages génomiques, CEC15 a présenté des traits probiotiques clés, essentiels pour l'activité probiotique et la colonisation, tels que la tolérance à l'acidité, l'adhérence épithéliale, l'immunomodulation des cellules intestinales, et la stabilisation de la perméabilité intestinale et du microbiote chez la souris.

Comparé à EcN, CEC15 a modulé l'expression de davantage de gènes anti-inflammatoires dans les cellules intestinales, mettant en évidence une fonction de barrière muqueuse améliorée *in vivo*. Cela était évident dans un modèle murin de mucosite intestinale chimiothérapique, où CEC15 atténuait notablement la perte de poids, les fuites, la dysbiose du microbiote, l'inflammation et les lésions tissulaires. En revanche, EcN n'a pas fourni ce niveau de protection, soulignant l'avantage comparatif de CEC15.

En résumé, ces résultats positionnent CEC15 en tant que principal candidat probiotique avec un génome unique adapté à l'adaptation intestinale et un profil fonctionnel distinct visant à renforcer l'homéostasie épithéliale. Nous proposons que son arrière-plan génomique phylogénétiquement distinct contribue à une résilience accrue au stress et à une tonalité immunomodulatrice. En élucidant les mécanismes sous-jacents aux avantages de CEC15 par rapport à EcN grâce à une approche probiogénomique, cette étude ouvre la voie à des thérapies ciblées utilisant des probiotiques de nouvelle génération.

4.2. Article de recherche 2 - Postbiotic effect of *Escherichia coli* CEC15 and *Escherichia coli* Nissle 1917 on a murine model of 5-FU-induced intestinal mucositis

La mucosite intestinale est un effet secondaire courant et sévère de la chimiothérapie anticancéreuse, provoquant une inflammation et des lésions du tractus gastro-intestinal. Trouver des moyens de prévenir ou de traiter la mucosite pourrait améliorer considérablement la qualité de vie et les résultats chez les patients atteints de cancer. Les probiotiques ont montré des promesses, mais des questions subsistent quant à leur efficacité et leur sécurité. Cette étude a évalué les souches probiotiques d'*E. coli* CEC15 et Nissle 1917 (EcN), ainsi que leurs formes tuées par la chaleur et leurs surnageants sans cellules ("postbiotiques"), dans un modèle murin de mucosite induite par le 5-FU.

Les résultats ont clairement mis en évidence les avantages de CEC15 par rapport à EcN pour atténuer la perte de poids, le raccourcissement intestinal, les lésions tissulaires et l'inflammation provoqués par le 5-FU. CEC15, sous forme viable ou inactivée, s'est révélé efficace en empêchant l'infiltration des neutrophiles, l'augmentation de la perméabilité et la perte de cellules caliciformes. De plus, CEC15 a montré une immunomodulation supérieure, normalisant l'expression de cytokines telles que l'IL-1 β , l'IL-17 et le NF- κ B. En revanche, EcN a montré une protection limitée dans la plupart

des tests. Ses formes postbiotiques atténuent certaines modifications histologiques mais ne réduisent pas l'infiltration des neutrophiles ni la perméabilité.

Cette étude comparative met en lumière l'importance des différences spécifiques aux souches dans les effets probiotiques. Elle suggère qu'*E. coli* CEC15 pourrait constituer une thérapie probiotique prometteuse contre la mucosite induite par la chimiothérapie et d'autres affections gastro-intestinales, les formes vivantes et tuées par la chaleur offrant une protection. Les conclusions soulignent le potentiel des postbiotiques en tant qu'alternative sûre aux probiotiques vivants, en particulier pour les patients immunodéprimés. Une analyse approfondie des mécanismes d'action de CEC15 et des essais cliniques chez l'homme constituera des étapes importantes pour le développement de thérapies visant à prévenir la mucosite débilite pendant le traitement du cancer.

4.3. Résultats supplémentaires

4.3.1. Génomique comparée

Les caractéristiques des micro-organismes probiotiques varient, notamment leur origine, leur taxonomie, leur dosage optimal et leurs avantages spécifiques pour la santé, en fonction de la souche particulière utilisée. Parmi les micro-organismes Gram-négatifs ayant des propriétés probiotiques, la souche d'*E. coli* Nissle 1917 (EcN) fait l'objet de recherches approfondies et est un produit médicamenteux agréé en Allemagne depuis plus d'un siècle. Cependant, malgré son potentiel, l'abondance réelle et le rôle d'*E. coli* dans le microbiote intestinal demeurent des sujets d'intérêt. *E. coli* est présent dans l'iléon et le côlon, mais n'est pas l'espèce la plus prévalente. Les souches d'*E. coli* servent de base à plusieurs produits probiotiques disponibles sur le marché, dont Mutaflor (EcN), Symbioflor 2 (contenant 6 souches) et Colinfant. En plus de ces souches, *E. coli* CEC15 a montré des résultats prometteurs dans la protection contre les maladies intestinales telles que la colite et la mucosite intestinale chez les modèles animaux.

Les progrès dans la technologie de séquençage à haut débit et la génomique comparative ont permis des investigations approfondies sur les génomes des bactéries probiotiques, offrant des informations précieuses sur leurs caractéristiques génomiques, leurs relations phylogénétiques et leurs gènes fonctionnels liés à l'adaptation au nichoir et à l'activité probiotique à travers diverses souches. Cependant, en dehors d'EcN, les génomes des souches probiotiques d'*E. coli* ont été peu caractérisés et leurs propriétés fonctionnelles distinctes restent à explorer. Dans cette étude, nous menons une analyse génomique comparative de huit génomes de souches d'*E. coli* disponibles (EcN, CEC15 et les six souches de Symbioflor 2) pour identifier les caractéristiques génomiques qui pourraient contribuer à leurs effets bénéfiques.

Cette analyse approfondie explore la génomique de huit souches distinctes d'*E. coli*, mettant en lumière leurs relations évolutives et leurs variations génomiques. L'analyse phylogénomique illustre le regroupement des souches en différentes clades en fonction des gènes uniques partagés. Il est à noter que les souches probiotiques forment des groupes phylogénomiquement éloignés par rapport aux souches pathogènes et commensales, soulignant une hétérogénéité substantielle parmi les souches d'*E. coli*.

Une exploration approfondie du pangenome de ces souches révèle des schémas génomiques intéressants. Le pangenome comprend un génome de base, un nuage (gènes uniques) et une coquille (partagée entre des sous-ensembles de souches). Par exemple, les souches probiotiques d'*E. coli* possèdent un nombre significatif de gènes uniques, indiquant des fonctionnalités distinctes potentielles. La distribution et la composition de ces gènes mettent en lumière la diversité génomique et les adaptations évolutives spécifiques à chaque souche. De plus, la présence de divers gènes exclusifs, tels que ceux liés à la résistance aux antibiotiques et aux voies métaboliques, souligne le répertoire génétique varié au sein de ces souches.

Un aspect critique de l'analyse génomique concerne l'identification et la caractérisation des îlots génomiques (GEIs). Ces îlots représentent des segments du génome pouvant jouer un rôle crucial dans le métabolisme, la pathogénicité ou l'adaptation environnementale. L'identification des îlots métaboliques liés à l'utilisation des substrats offre des perspectives sur les capacités métaboliques potentielles des souches. De plus, la présence d'îlots de pathogénicité avec des gènes associés aux facteurs de virulence et aux systèmes de sécrétion souligne la diversité génétique et les caractéristiques pathogènes potentielles au sein de ces souches d'*E. coli*.

L'exploration des gènes de résistance aux antibiotiques dans ces génomes fournit des aperçus précieux sur leurs mécanismes potentiels de résistance. La présence de multiples gènes de résistance associés à divers antibiotiques suggère une gamme diversifiée de mécanismes de résistance aux antibiotiques utilisés par ces souches. Notamment, la comparaison des profils de gènes de résistance entre différentes souches révèle des variations dans les types et les nombres de gènes de résistance, soulignant l'importance de comprendre les profils spécifiques de résistance aux antibiotiques propres à chaque souche. Cette analyse génomique approfondie ouvre des perspectives pour de futures recherches visant à comprendre les implications fonctionnelles de ces caractéristiques génomiques et leur pertinence potentielle dans divers contextes biologiques.

4.3.2. Production de vésicules extracellulaires

Les probiotiques, micro-organismes vivants offrant des avantages pour la santé lorsqu'ils sont administrés en quantités suffisantes, ont fait l'objet d'études

approfondies visant à comprendre leurs mécanismes d'effets bénéfiques. Ces mécanismes incluent la production de substances antimicrobiennes telles que les bactériocines, la modulation du microbiote intestinale, le maintien et l'augmentation de la barrière épithéliale intestinale, la production de mucine, l'inhibition des voies NF-kB, la production d'acides gras à chaîne courte essentiels pour la santé intestinale et l'immunomodulation. Diverses bactéries telles que les Lactobacilles, Lactocoques, Bifidobactéries, Propionibactéries, *E. coli* Nissle 1917 et *Saccharomyces* spp. ont montré des effets immunorégulateurs dans le tractus gastro-intestinal. Récemment, les vésicules extracellulaires (VEs) dérivées de certaines souches probiotiques ont attiré l'attention pour leur rôle dans l'immunomodulation et l'activité antimicrobienne. Les VEs, bicouches lipidiques sécrétées par les organismes vivants, transportent des molécules biologiquement actives entre les cellules et jouent des rôles dans la pathogénèse, l'homéostasie du microbiote, le transfert de gènes et la modulation des cellules hôtes.

Les bactéries à Gram négatif et à Gram positif produisent différents types de VEs avec un potentiel significatif pour des applications cliniques, bien que les études sur les VEs des bactéries probiotiques soient limitées. Les VEs de souches spécifiques telles que les Lactobacilles, les Bifidobactéries et *Propionibacterium freudenreichii* CIRM-BIA 129 ont montré des effets immunorégulateurs similaires à ceux de leur bactérie parentale vivante, ce qui indique leur potentiel pour des applications thérapeutiques. De plus, on a observé que les VEs provenant de la souche *E. coli* Nissle 1917 influencent les réponses immunitaires dans les cellules épithéliales intestinales, modulent le microbiote et le métabolome intestinaux, renforcent l'immunomodulation et l'activité antimicrobienne dans les macrophages, régulent le dysfonctionnement de la barrière intestinale et présentent des effets anti-inflammatoires dans un modèle animal de colite induite par le SSS. La recherche actuelle vise à identifier et caractériser les VEs produits par la souche probiotique potentielle *E. coli* CEC15, en les comparant aux VEs de la souche de référence *E. coli* Nissle 1917 afin de comprendre leur fonctionnalité.

L'étude a porté sur l'analyse des vésicules extracellulaires (EVs) produites par les souches bactériennes CEC15 et EcN après purification. Les EVs ont montré des structures typiques en forme de coupe ronde avec des distributions de taille spécifiques pour chaque souche. Les protéines présentes dans les EVs étaient diverses, comprenant des protéines ribosomales, des systèmes de sécrétion, des perméases, des enzymes liées aux voies métaboliques et des adhésines telles que les protéines de la Fimbriae. Les EVs d'EcN contenaient la bactériocine Microcin H47 et des protéines liées aux phages, inhibant potentiellement d'autres bactéries et suggérant la présence de phages actifs à l'intérieur des EVs. L'étude n'a trouvé aucune preuve de la synthèse de colibactine dans les EVs d'EcN ou dans le protéome cellulaire entier sans stimula-

tion environnementale. Les protéines ont été catégorisées en fonction de leur fonction dans des Clusters of Orthologous Groups (COGs), montrant des différences dans la distribution entre les EVs et les cellules entières, en particulier dans les catégories liées à la biogenèse de la paroi cellulaire/membrane et à la traduction. Les tests sur les cellules Caco-2 ont révélé que tant les EVs que les cellules bactériennes inactivées par la chaleur modulaient l'expression des gènes liés à l'immunorégulation et à l'intégrité épithéliale. Notamment, les EVs d'EcN et les cellules bactériennes de CEC15 à fortes concentrations stimulaient l'expression de gènes clés associés à l'inflammation et au cancer colorectal. L'expression du TNF était notablement augmentée par les EVs des deux souches, ce qui pourrait contribuer à des affections inflammatoires telles que la maladie de Crohn.

4.4. Article de recherche 3 - Fat matters: Fermented whole milk potentiates the anti-colitis effect of *Propionibacterium freudenreichii*

Les maladies inflammatoires de l'intestin (MII) se sont révélées être une préoccupation de santé pressante dans les pays occidentaux, coïncidant avec des changements significatifs dans la composition du microbiote intestinal et un déclin des bactéries immunomodulatrices. Ce scénario évolutif nécessite une compréhension approfondie et des stratégies de gestion efficaces pour soulager le fardeau des MII. Les chercheurs se sont tournés vers l'exploration du potentiel de souches spécifiques de *Propionibacterium freudenreichii*, reconnaissant leurs propriétés modulatrices prometteuses qui peuvent prévenir efficacement la colite dans les modèles animaux. Ces découvertes offrent un aperçu d'espoir pour des interventions potentielles ciblant les MII. De manière cruciale, les matrices laitières, y compris le lait entier, ont été identifiées comme des transporteurs protecteurs des antigènes de surface immunomodulateurs des propionibactéries lors de la digestion. Cela souligne l'importance de la composition du lait dans la préservation de la viabilité et de l'efficacité de ces bactéries bénéfiques.

Dans une quête visant à dévoiler les possibilités d'amélioration des effets anti-colite de *Propionibacterium freudenreichii*, nous nous sommes penchés sur *P. freudenreichii* CIRM-BIA129, une souche probiotique acclamée pour ses propriétés anti-inflammatoires. Cette souche probiotique a été exploitée pour fermenter différents milieux laitiers - lait entier, lait écrémé et ultrafiltrat de lait - afin d'évaluer leur potentiel dans la prévention de la colite.

Les résultats de cette expérience de fermentation étaient prometteurs. Le lait fermenté enrichi en *P. freudenreichii* a démontré une capacité notable à prévenir la colite. De manière intrigante, la présence de matières grasses laitières dans le produit fermenté a été identifiée comme un important activateur de ses effets anti-colite. Cela souligne l'importance de prendre en considération la matrice laitière et ses composants pour optimiser les avantages potentiels pour la santé issus des aliments fonctionnels

fermentés.

De plus, cette recherche s'est plongée dans les subtilités de la perméabilité intestinale, un aspect crucial dans les MII. L'étude a révélé que les produits laitiers fermentés par *P. freudenreichii* prévenaient efficacement l'augmentation alarmante de la perméabilité intestinale généralement induite par la colite. Cela renforce davantage le potentiel de tels produits fermentés dans l'atténuation des MII.

5. Discussion

L'utilisation de probiotiques pour la prévention et le traitement des maladies est devenue courante de nos jours. Cependant, l'utilisation indiscriminée et le manque de réglementation rigide dans le monde entier posent un problème croissant pour la santé. Ainsi, l'identification correcte, la caractérisation et l'évaluation fonctionnelle de chaque souche visant à être commercialisée en tant que probiotique, pour une utilisation humaine ou animale, sont vitales. Dans cette optique, l'objectif de cette thèse était d'utiliser des outils génomiques, des analyses *in vitro*, des tests *in vivo* utilisant des modèles animaux et des analyses comparatives pour caractériser les propriétés probiotiques de la souche *E. coli* CEC15 et évaluer comment la matrice alimentaire dans laquelle le probiotique est produit peut moduler ses effets bénéfiques. Les effets bénéfiques de la souche CEC15 ont déjà été démontrés (Escribano-Vazquez et al., 2019; Tomas et al., 2015), mais la souche n'avait pas encore été caractérisée et les mécanismes n'étaient pas compris.

1. Qu'avons-nous découvert jusqu'à présent ?

1.1. Les études génomiques, *in vitro* et sur animaux ont démontré la sécurité et l'effet probiotique de CEC15

Dans la première partie de cette étude, nous avons effectué une caractérisation approfondie de la souche probiotique *E. coli* CEC15. Du génome aux études *in vivo*, nous avons montré que CEC15 est plus sûre au niveau génomique que la souche de référence *E. coli* Nissle 1917, présentant une grande variété de gènes liés à la résistance au stress acide, ce qui peut être directement lié au taux de survie élevé dans le test de digestion *in vitro*. Nous avons également trouvé de nombreux gènes associés aux propriétés adhésives de la souche, observés par analyse protéomique et associés au fort niveau d'adhérence aux cellules Caco2. CEC15 a également montré un nombre réduit de gènes de résistance aux antibiotiques, avec un phénotype de sensibilité à la majorité des antibiotiques testés. Nous avons également noté qu'aucun gène lié à la résistance aux antibiotiques n'était assez proche d'un élément transposable pour présenter un risque de transfert à d'autres bactéries. Nous avons observé que CEC15 ne présentait pas d'activité hémolytique. CEC15 a également démontré sa sécurité chez

les souris, où elle a été administrée à des doses élevées (10^{10} UFC/jour/souris) sans effet nocif observé après 12 jours d'administration, avec gain de poids et modulation de l'architecture épithéliale intestinale réduisant la perméabilité intestinale. CEC15 a également été testée dans un modèle inflammatoire de mucosité intestinale induite par le 5-fluorouracile (5FU) chez la souris, démontrant des résultats prometteurs. CEC15 a pu réduire la perte de poids et protéger l'architecture épithéliale intestinale sous forme de score histopathologique réduit et de perméabilité intestinale réduite. CEC15 a également réduit l'infiltrat de neutrophiles et, ainsi, le processus inflammatoire. En parallèle, nous avons testé les effets de la souche de référence EcN dans le même modèle animal et nous n'avons pas observé de protection avec l'administration d'EcN. Avec ces résultats, nous avons démontré la sécurité de la souche et établi l'effet bénéfique de CEC15 dans un modèle inflammatoire intestinal.

1.2. Les préparations postbiotiques de CEC15 présentent des résultats prometteurs dans les études animales sur l'inflammation intestinale

Dans la deuxième partie de cette thèse, nous avons évalué l'activité bénéfique des formes postbiotiques de CEC15 et d'EcN, à des fins de comparaison, sous forme de bactéries inactivées par la chaleur et de surnageant sans cellules. Les souches viables, les souches inactivées par la chaleur et les surnageants sans cellules ont été testés contre le même modèle de mucosité intestinale induite par le 5-FU réalisé dans la première section. Dans cette étude, nous avons observé les mêmes effets pour les souches viables que ceux démontrés ci-dessus, confirmant l'effet protecteur de la souche CEC15 et l'incapacité de protection d'EcN. En ce qui concerne les préparations postbiotiques, nous avons observé un effet bénéfique, en particulier avec la souche CEC15 inactivée par la chaleur. Il est important de noter ici que le surnageant sans cellules d'EcN a été testé auparavant dans des modèles similaires (chez les rats) et a montré des résultats prometteurs (Prisciandaro et al., 2011; Wang et al., 2017), bien que dans cette étude, il ne soit pas comparable à l'effet de CEC15. La souche CEC15 inactivée a présenté les meilleurs résultats, similaires dans la plupart des cas à la souche viable. La souche CEC15 inactivée a favorisé une perte de poids réduite, la préservation de

la longueur intestinale et de la hauteur des villosités, un score histopathologique réduit, la préservation des cellules caliciformes, une perméabilité réduite, une réduction de l'expression du gène *IL1B* et une augmentation de l'expression des gènes des protéines de jonction serrée *TJP1* et *CLDN1*. Ces résultats ont contribué à consolider CEC15 en tant que souche bénéfique pour la protection/traitement des maladies inflammatoires intestinales. Cela montre également que les bactéries inactivées, et même les métabolites produits, ont le potentiel d'exercer des effets bénéfiques similaires à ceux de la souche d'origine.

1.3. Les souches probiotiques d'*E. coli* présentent une grande diversité génomique

Dans la troisième section de cette étude, nous avons réalisé une analyse génomique comparative de CEC15 par rapport à sept autres souches probiotiques d'*E. coli*, EcN, et six souches du probiotique commercial Symbioflor2 (*E. coli* G1/2, G3/10, G4/9, G5, G6/7, et G8). Phylogénétiquement, nous avons démontré que les souches appartiennent à trois phylogroupes différents d'*E. coli*, CEC15 étant classée comme B2, EcN comme B1, et les souches Symbioflor dans le phylogroupe A. L'analyse du pan-génome a démontré la grande variabilité génétique des souches, environ un tiers (2533) du nombre total de gènes (8316) étant classés comme gènes uniques et, par conséquent, non partagés entre les souches. Le génome de base, partagé par toutes les huit souches, contenait 3336 gènes. L'analyse fonctionnelle des souches, selon les classifications COG et KEEG, a également montré un grand nombre de gènes uniques dans les catégories les plus présentes. La présence d'îlots génomiques, de bactériocines, d'adhésines et de gènes de résistance aux antibiotiques a également été évaluée pour les souches. CEC15 a présenté l'un des plus faibles nombres d'îlots génomiques, après la souche G4/9. Pour la majorité des PAI trouvés dans toutes les souches, aucun gène lié à une grande pathogénicité n'a été trouvé, à l'exception du PAI 9 sur EcN, qui contient le cluster génétique pour la production de colibactine. La majorité des gènes trouvés dans les PAI étaient soit de fonction inconnue (gènes hypothétiques) soit des éléments transposables sans gène entre eux, ce qui rend difficile l'évaluation de leur menace potentielle. Toutes les souches semblent être capables de produire, au moins, un type de bactériocines, toutes partageant les composants génétiques pour produire la bottromycine. L'analyse de la présence des gènes de résistance aux antibiotiques a révélé une large gamme dans le nombre de gènes pour chaque souche, allant de 15 à 143 gènes, la majorité étant classifiée comme des pompes à efflux, suivies de l'altération de la cible antibiotique. Ces résultats ont mis en évidence la variabilité génétique de ces souches, améliorant notre compréhension de la structure génétique d'*E. coli*.

1.4. Les vésicules extracellulaires de CEC15 peuvent jouer un rôle important dans l'immunomodulation

La quatrième partie de cette thèse comprend une étude de caractérisation des vésicules extracellulaires (VE) produites par CEC15. À la lumière des effets induits par les formulations postbiotiques de CEC15, nous avons décidé d'évaluer la production de VE par CEC15, leur contenu protéique et leurs effets immunomodulateurs potentiels. Les VE ont été produites avec succès et présentaient une taille et une dispersion normales. Il a été démontré que les VE contenaient/un étaient composées d'un grand nombre de protéines, de plus, nous avons détecté des protéines dans les

VE qui n'avaient pas été détectées dans le protéome des bactéries. Un grand nombre de protéines ribosomales, de systèmes de sécrétion, de perméases et d'enzymes métabolisant les glucides ont été trouvés dans le protéome des VE. Une autre classe de protéines présente dans les VE était celle des adhésines, en particulier la présence de fimbriae, ce qui permettrait aux VE d'adhérer à leur cible. Après la caractérisation, nous avons évalué l'effet des VE sur la modulation de l'expression génique des gènes des cellules Caco2, et nous avons observé une surexpression de *IL8*, *TNF* et *NOS2* après co-incubation avec les VE de CEC15. Cela démontre un potentiel immunomodulateur des VE et ouvre une nouvelle voie d'étude sur le rôle des VE dans l'effet probiotique de CEC15.

*1.5. La matrice alimentaire a un impact sur l'effet des probiotiques (en particulier *Propionibacterium freudenreichii*)*

Enfin, dans la dernière section de ce travail, nous avons réalisé une analyse comparative pour évaluer l'effet de la matrice alimentaire sur l'effet probiotique d'une souche probiotique bien caractérisée, *Propionibacterium freudenreichii* CIRM-BIA 129, dans un modèle inflammatoire intestinal de colite induite par l'administration de DSS. Ce modèle et cette souche ont été choisis en raison des résultats consolidés dans des travaux précédents (Rabah et al., 2020), ce qui nous a permis de nous concentrer sur l'effet modulateur de la matrice alimentaire. Nous avons testé les effets de *P. freudenreichii* cultivé dans du lait entier, du lait écrémé et de l'ultrafiltrat de perméat de lait pour évaluer les effets des protéines du lait et des matières grasses sur l'activité probiotique. Les préparations fermentées ont été administrées aux souris quotidiennement et, à la fin, nous avons pu observer un résultat amélioré concernant la protection contre l'inflammation causée par l'administration de DSS chez les animaux qui ont reçu du lait entier fermenté par *P. freudenreichii*. Les résultats montrent une meilleure protection de l'architecture de l'épithélium intestinal avec préservation des cellules caliciformes et une perméabilité réduite, suivie d'une augmentation de l'expression des gènes des jonctions serrées et d'une réduction des gènes pro-inflammatoires. Ce travail a démontré comment la matrice de croissance probiotique est capable de moduler son activité probiotique, pouvant ainsi l'améliorer considérablement.

2. Qu'est-ce qui reste à connaître ?

Dans la quête d'avancer notre compréhension des probiotiques et d'exploiter leur potentiel pour améliorer la santé humaine, cette étude a posé des bases solides en explorant de manière exhaustive la sécurité, les effets probiotiques, la diversité génomique et les propriétés immunomodulatrices de la souche probiotique *E. coli* CEC15. De plus, elle a mis en lumière comment la matrice alimentaire peut moduler l'activité probiotique, comme illustré par *Propionibacterium freudenreichii* dans di-

verses formulations de lait. Cependant, le parcours visant à élucider les subtilités des probiotiques et leur application est loin d'être achevé. Cette étude prépare le terrain pour les recherches futures, en présentant des orientations cruciales pour l'exploration et l'avancement. Dans cette section, nous détaillons les avenues potentielles pour les travaux futurs, englobant des investigations qui peuvent approfondir nos connaissances, affiner les applications probiotiques et potentiellement révolutionner la manière dont les probiotiques sont utilisés pour optimiser la santé humaine. À travers ces futures propositions de travaux, nous visons à contribuer à une compréhension plus profonde des probiotiques, propulsant davantage le domaine vers des thérapies probiotiques novatrices et ciblées.

2.1. Sécurité à long terme et mécanismes impliqués dans l'effet probiotique de CEC15

Nous avons démontré ici la sécurité de CEC15 sur la base de ses caractéristiques génomiques et après une administration à court terme à un modèle animal de souris en bonne santé. Les recherches futures devraient se concentrer sur la réalisation d'évaluations de sécurité à long terme de CEC15, y compris des études d'exposition chronique, afin de garantir sa sécurité sur des périodes prolongées. Découlant des protocoles classiques de toxicologie utilisés pour évaluer la sécurité des composés mineurs, les études chroniques prolongées impliquant des modèles animaux sont caractérisées de manière conventionnelle par une période de six mois à un an. Ces études intègrent une compréhension expérimentale préalable ou des tendances liées aux organes impactés, au potentiel de réversibilité de la toxicité, aux niveaux d'effets observés (ou leur absence) et à la quantification du risque clinique aux doses anticipées pour une utilisation thérapeutique à long terme (Colerangle, 2017; Food and drug administration, 2007).

Lors de l'introduction de CEC15 dans de futurs essais cliniques, il est impératif d'élargir le champ des évaluations de sécurité pour englober une gamme diversifiée de populations et de groupes d'âge. Cette approche vise à garantir une compréhension complète du profil de sécurité de CEC15 dans diverses démographies. Des études observationnelles à long terme, menées de manière longitudinale, peuvent offrir des aperçus précieux sur la sécurité persistante et les effets potentiels à long terme associés à l'administration de CEC15. Aborder le défi de la sécurité implique de définir des résultats et des voies pertinents qui traversent un spectre d'organismes ciblés et de leurs potentiels métaboliques au sein de la communauté microbienne complexe de l'intestin (Merenstein et al., 2023; Veiga et al., 2020). Cette tâche nécessite d'identifier des caractéristiques critiques telles que les seuils influençant les indices de diversité et les altérations de la communauté microbienne. Ces caractéristiques pourraient servir d'indicateurs de préoccupation, signalant des déviations par rapport à la norme ou des perturbations dans les structures de la communauté, telles que celles

liées aux états pro-inflammatoires, aux tendances pro-obésité, à la perturbation de l'homéostasie immunitaire, au syndrome métabolique, aux prédispositions au diabète, ou à d'autres conditions supplémentaires. Des marqueurs métagénomiques validés peuvent s'avérer essentiels pour élucider ces relations complexes, soulignant la nécessité de recherches fondamentales dans ce domaine (Bilal et al., 2022; Veiga et al., 2020).

Appliquer ce cadre préclinique au domaine des biothérapies vivantes de nouvelle génération, qui ne bénéficient pas d'une histoire établie d'utilisation sûre, implique que la réalisation d'études de 12 mois sur des sujets humains est conforme aux normes anticipées pour les nouveaux agents ou cibles moléculaires novateurs (Merenstein et al., 2023). Surtout dans les cas où une expérience post-commercialisation limitée ou inexistante existe, intégrer ces études de sécurité à long terme dans les essais cliniques de phase III est judicieux et conforme aux attentes réglementaires. Cette approche facilite une évaluation solide de la sécurité et permet d'identifier précocement d'éventuels effets indésirables associés à l'utilisation de ces biothérapies vivantes novatrices (Merenstein et al., 2023).

De plus, une exploration approfondie des caractéristiques génomiques de CEC15 et de son interaction avec le microbiote intestinal humain pourrait éclairer la manière dont ces facteurs influent sur sa sécurité et son efficacité probiotique. Une interaction moléculaire complexe se produit entre le microbiote, les constituants alimentaires et les cellules hôtes, orchestrant les fonctions immunitaires et métaboliques chez l'hôte (Furusawa et al., 2015). Certains résultats établis des probiotiques comprennent l'inhibition des pathogènes et l'amélioration de la diarrhée, qui ont été liées à la production d'acides organiques (Fukuda et al., 2011), de peptides antibactériens (Moroni et al., 2006), d'inhibiteurs de la quorum-sensing (Cotar et al., 2010), de déplacement des pathogènes (Ruas-Madiedo et al., 2006), et d'atténuation de la virulence (Tanner et al., 2016). De plus, ils peuvent réguler les fonctions de l'hôte et fermenter les polysaccharides complexes de l'alimentation (Bermudez-Brito et al., 2013; Furusawa et al., 2015; Heuvelin et al., 2010; Heuvelin et al., 2009; Ménard et al., 2005). Élucider les molécules spécifiques qui médiatisent le dialogue entre CEC15 et l'hôte peut élucider leurs effets bénéfiques et ouvrir la voie à des thérapies ciblant le microbiote pour les maladies humaines.

2.2. Principaux composés bioactifs associés aux effets bénéfiques des préparations postbiotiques de CEC15

Les postbiotiques sont définis comme «une préparation de microorganismes inanimés et/ou de leurs composants conférant un bénéfice pour la santé de l'hôte» (Salminen et al., 2021). Ils proviennent de bactéries commensales désactivées et englobent des cellules microbiennes désactivées, des surnageants dépourvus de cel-

lules et des constituants essentiels, habituellement rendus inactifs par la chaleur. Bien qu'étant dépourvus de vie, ils confèrent des avantages pour la santé comparables, et dans certains cas même supérieurs, aux probiotiques, phénomène souvent appelé le « paradoxe probiotique » (Adams, 2010). Nous avons démontré dans ce travail le potentiel bénéfique des préparations postbiotiques de CEC15. Bien que prometteurs, les recherches futures pourraient examiner les mécanismes d'action derrière les effets bénéfiques observés de CEC15 inactivé par la chaleur. De plus, l'identification et l'isolement des composés bioactifs au sein de la préparation postbiotique pourraient conduire au développement d'agents thérapeutiques ciblant les propriétés anti-inflammatoires.

Bien que les postbiotiques n'hébergent pas de microorganismes vivants, ils manifestent des effets bénéfiques sur la santé du microbiome. De plus, il est hypothétique que les postbiotiques peuvent moduler le microbiome intestinal de manière similaire aux probiotiques, tout en atténuant les risques potentiels associés (Klemashevich et al., 2014). Les postbiotiques semblent présenter peu d'effets indésirables, tout en maintenant une efficacité comparable aux probiotiques, bien qu'aucune étude comparative directe entre les substances appartenant à ces groupes respectifs n'ait été menée (Żółkiewicz et al., 2020).

L'efficacité des postbiotiques est censée découler de l'interaction entre l'hôte et les substances générées par le microbiome à la suite de la croissance microbienne déclenchée par la présence de postbiotiques. Ces substances englobent une vaste gamme de sous-produits microbiens, notamment des métabolites, des protéines, des lipides, des glucides, des vitamines, des acides organiques, des composants de paroi cellulaire et d'autres molécules complexes (Aguilar-Toalá et al., 2018; Konstantinov et al., 2013). De plus, il est plausible que les composés actifs au sein de la formulation postbiotique puissent pénétrer les couches de mucus et stimuler directement les cellules épithéliales (Piqué et al., 2019). De plus, la perte de viabilité et la dégradation cellulaire pourraient potentiellement conduire à des effets bénéfiques plus complexes, tels que l'immunomodulation (Piqué et al., 2019).

L'efficacité des surnageants et des fractions cellulaires de diverses cultures microbiennes varie considérablement. Dans une étude menée par Lee et ses collègues (M. J. Lee et al., 2002), des niveaux variables de production de cytokines par les macrophages ont été observés lorsqu'ils étaient traités avec différentes fractions de *B. bifidum* BGN4, comprenant des cellules intactes, des extraits sans cellules, des parois cellulaires purifiées et des surnageants de culture. Les cellules intactes ont présenté la plus forte expression du facteur de nécrose tumorale alpha (TNF- α), tandis que les extraits sans cellules ont induit la plus forte production d'interleukine-6 (IL-6). De même, dans une étude connexe menée par Ji et Kim (Ji and Kim, 2006), il a été constaté que les cellules intactes et différentes fractions de BGN4 stimulaient de manière signi-

ficative la production d'IL-10 et d'IL-6. De plus, les extraits sans cellules de BGN4 ont induit des modifications morphologiques notables dans les macrophages, améliorant leurs propriétés de phagocytose par rapport aux macrophages traités avec les cellules intactes et la paroi cellulaire de BGN4.

Des recherches récentes indiquent que les surnageants obtenus à partir de cultures bactériennes de la famille *Lactobacilaceae* et du genre *Bifidobacterium* démontrent des capacités antibactériennes, en particulier en entravant l'invasion de souches d'*E. coli* entéroinvasives dans les entérocytes *in vitro* (Khodaii et al., 2017). Bien que ces effets antibactériens puissent être attribués à l'entrave de l'adhérence de souches bactériennes pathogènes, probablement en raison de la compétition pour les sites récepteurs, les surnageants pourraient également avoir un impact localisé sur l'environnement intestinal, la barrière cellulaire et l'expression de gènes protecteurs (Khodaii et al., 2017). Par conséquent, les surnageants bactériens sans cellules se présentent comme des agents anti-infectieux prometteurs, notamment dans le contexte du traitement de la diarrhée.

Les surnageants de *L. acidophilus* et de *L. casei* présentent des propriétés anti-inflammatoires et antioxydantes lorsqu'ils sont appliqués aux cellules épithéliales intestinales, aux macrophages et aux neutrophiles. Ces effets se manifestent par la réduction de la sécrétion de la cytokine pro-inflammatoire facteur de nécrose tumorale α (TNF α) et par une augmentation de la sécrétion de la cytokine anti-inflammatoire interleukine 10 (IL10) (Marco et al., 2018). De plus, les surnageants des cultures de *L. casei* et de *L. rhamnosus* GG ont démontré la capacité à entraver l'invasion des cellules cancéreuses du côlon (Escamilla et al., 2012).

Plus précisément, des recherches ont démontré que les lysats bactériens (BLs) lyophilisés administrés par voie orale atteignent avec succès les plaques de Peyer dans l'intestin grêle. Ici, ils stimulent les cellules dendritiques (DC), qui activent ensuite les lymphocytes T et B (Kearney et al., 2015). Ces lymphocytes matures migrent vers la muqueuse des voies respiratoires, amorçant la stimulation du système immunitaire inné et favorisant la sécrétion d'IgA (Kearney et al., 2015). Il est à noter que la sécurité de l'utilisation des BLs a été validée par de nombreuses études cliniques portant sur diverses maladies, telles que les infections récurrentes des voies respiratoires supérieures chez les enfants (Schaad et al., 2002).

Les recherches précédemment mentionnées ont illustré des réponses immunitaires distinctes manifestées par les cellules intactes et diverses fractions du même microorganisme. Ces résultats soulignent la nécessité de tester de manière approfondie de nouveaux postbiotiques, tels que le CEC15 étudié ici, afin d'évaluer au mieux ses mécanismes d'action et son efficacité dans différentes conditions.

2.3. Rôle des gènes uniques des souches probiotiques d'*E. coli* dans la promotion

d'effets bénéfiques

L'analyse génomique comparative des souches probiotiques d'*E. coli* a révélé un grand nombre de gènes uniques dans chaque souche, ce qui pourrait être lié à leurs effets bénéfiques différentiels, comme nous l'avons également observé ici lors de la comparaison entre CEC15 et EcN dans des études *in vitro* et *in vitro*. Élargir l'analyse fonctionnelle des gènes uniques dans différentes souches probiotiques d'*E. coli* peut fournir des informations sur leurs contributions potentielles aux avantages pour la santé.

Historiquement, les analyses génomiques se sont principalement concentrées sur les traits métaboliques prédits, s'appuyant fortement sur l'annotation fonctionnelle, la composition génomique et la synténie (Klaenhammer et al., 2008). Cependant, ces approches conventionnelles peuvent ne pas suffire à répondre à certaines des questions les plus intrigantes. Dans l'étude des séquences génomiques de bactéries probiotiques, notre compréhension des mécanismes probiotiques et de leurs interactions avec le tube digestif de l'hôte progresse grâce à l'intégration de techniques génomiques fonctionnelles (Johnson and Klaenhammer, 2014).

Dans *L. acidophilus* NCFM, un gène (*Iba1524*) responsable de l'encodage d'une histidine protéine kinase fonctionnelle (HPK) a été intentionnellement perturbé, ce qui a abouti à une souche mutante présentant une sensibilité accrue au stress acide par rapport à la souche parentale d'origine. De plus, une analyse transcriptomique utilisant la technologie des puces à ADN a été réalisée, comparant le mutant *Iba1524* avec la souche sauvage. Cette analyse a révélé un impact significatif sur 80 gènes (Azcarate-Peril et al., 2005). Notamment, parmi les gènes ayant montré une surexpression dans le mutant HPK, l'homologue de LuxS, associé au composé de signalisation de quorum autoinducteur-2, a été particulièrement régulé à la hausse. Cette régulation à la hausse de l'homologue de LuxS est significative car elle joue un rôle crucial dans la survie dans les sucs gastriques et l'adhérence aux lignées cellulaires épithéliales intestinales (B. Buck et al., 2009; Lebeer et al., 2008).

Les investigations génomiques fonctionnelles sur les transporteurs multi-résistants (MDR) au sein des souches probiotiques de *L. reuterii* et de *L. acidophilus* ont révélé leur implication dans la tolérance à la bile, comme le démontrent les études de Whitehead et al. (Whitehead et al., 2008) et Pfeiler et Klaenhammer (Pfeiler and Klaenhammer, 2009). De plus, des recherches récentes utilisant une analyse génomique *in silico* ont identifié et caractérisé fonctionnellement un gène de transporteur MDR, *betA* (transporteur d'efflux de la bile), dans *B. longum*, comme documenté par Gueimonde et al. (Gueimonde et al., 2009). Lorsque *betA* était exprimé dans *E. coli*, il améliorait la tolérance à la bile en expulsant activement les sels biliaires.

Lors du criblage génomique *in silico* de *L. acidophilus* NCFM, cinq protéines de

surface cellulaire d'adhérence potentielles ont été identifiées. Il s'agissait d'une protéine de liaison à la fibronectine (*FbpA*), d'une protéine de couche S (*SlpA*), d'une protéine de liaison aux mucines (*Mub*), et de deux homologues de R28 associés à l'adhésion streptococcique, comme documenté par Buck et al. dans (B. L. Buck et al., 2005). Une analyse mutagénique ultérieure a révélé que *FbpA*, *Mub* et *SlpA* jouaient tous des rôles importants dans la facilitation de l'adhésion aux lignées cellulaires épithéliales Caco-2.

Une analyse des génomes de deux souches de *L. rhamnosus* a révélé une découverte importante dans *L. rhamnosus* GG : la présence d'une île génomique abritant trois gènes codant pour des pilines (*spaCBA*) qui sont sécrétés et dépendent de la sortase, comme élucidé par Kankainen et al. dans (Kankainen et al., 2009). Des analyses immunoblotting et d'immunomicroscopie électronique ont confirmé visuellement que les pili étaient liés à la paroi cellulaire. De plus, grâce à une analyse mutagénique du gène *spaC*, il a été observé que la capacité d'adhérence de *L. rhamnosus* GG au mucus intestinal humain était perdue, soulignant le rôle crucial de ces structures de pili distinctives dans l'adhérence et la rétention dans le tube digestif (Kankainen et al., 2009).

De plus, des études ciblées pourraient enquêter sur le rôle de gènes spécifiques dans la promotion d'effets probiotiques, permettant ainsi l'ingénierie de souches aux fonctionnalités améliorées.

2.4. Composition du contenu des EVs et leur rôle dans l'immunomodulation

Nous avons démontré dans cette étude que CEC15 est capable de produire des vésicules extracellulaires (EVs). De plus, la caractérisation du protéome des EVs a révélé quelques protéines importantes liées à l'adhésion, à la traduction et au métabolisme. Nous pensons que les recherches futures devraient se concentrer sur la compréhension du contenu cargo des EVs de CEC15 et sur la manière dont il influence les réponses immunitaires. De plus, l'isolement et la caractérisation de protéines spécifiques, d'acides nucléiques ou de métabolites au sein des EVs peuvent révéler les composants clés de l'immunomodulation.

Les bactéries gram-négatives commensales et pathogènes utilisent divers mécanismes pour établir une communication avec les cellules hôtes. L'un de ces mécanismes implique la production de vésicules membranaires, permettant le transfert de contenu vers des cibles distantes au sein de l'hôte (Ashrafian, Behrouzi, et al., 2019; Ashrafian, Shahriary, et al., 2019; Behrouzi et al., 2018; Kulp and Kuehn, 2010).

La sécrétion d'EVs par *Bacteroides fragilis* et *Akkermansia muciniphila* a été montrée pour renforcer les effets immunomodulateurs et inhiber l'inflammation intestinale dans des modèles murins de colite expérimentale (Kang et al., 2013; Shen et al., 2012). Les EVs produits par des souches commensales émergent comme des élé-

ments pivots dans les processus de signalisation au sein de la muqueuse intestinale. Des études récentes ont démontré que les EVs d'EcN et les vésicules produites par d'autres souches commensales d'*E. coli* sont internalisées par les cellules épithéliales par endocytose médiée par la clathrine, influençant la signalisation au système immunitaire à travers la barrière épithéliale intestinale (Cañas et al., 2016). Des recherches ont confirmé que les EVs de la souche probiotique EcN et d'autres souches commensales d'*E. coli* transfèrent des médiateurs qui activent l'immunité de l'hôte. Les EVs de souches d'*E. coli* présentent des effets immunomodulateurs dans divers modèles *in vitro* de la barrière intestinale et d'explants coliques humains, régulant l'expression de peptides antimicrobiens et de biomarqueurs inflammatoires vers un profil apaisant (M. J. Fábrega et al., 2016).

Il est crucial de souligner la corrélation notable observée entre la localisation cellulaire et la quantité de protéines isolées des EVs. Plus précisément, lorsque la localisation des protéines était catégorisée comme 'membranaire', le protéome semblait être plus limité, allant de 11 à 192 protéines. En revanche, lorsque la représentation en protéines la plus abondante provenait du cytoplasme, le protéome était plus vaste, s'étendant de 11 à 1286 protéines. À noter que dans les cas où le protéome dépassait 300 protéines, le 'cytoplasme' émergeait comme la classification prédominante. Cette tendance pourrait être attribuée à deux facteurs principaux : la sensibilité des techniques de recherche employées et le degré de contamination du protéome des EVs par des protéines provenant des cellules bactériennes produisant ces structures (J. Lee et al., 2016; Nagakubo et al., 2020; Shao et al., 2018).

Un déficit de sensibilité pourrait entraîner l'exclusion de protéines peu représentées dans le protéome des EVs. Cependant, ces protéines peuvent avoir des fonctions cruciales pour les micro-organismes qui les sécrètent (Ahmed and Rice, 2005; Pedersen et al., 2003). À l'inverse, une représentation excessive de protéines dans le protéome des EVs peut indiquer une contamination potentielle, soulignant la nécessité d'améliorer l'étape de purification des EVs obtenus (Klimentová and Stulík, 2015; Nagakubo et al., 2020). Équilibrer la sensibilité est donc essentiel pour garantir une représentation précise et des insights significatifs sur les rôles et les fonctions des protéines au sein des vésicules extracellulaires.

Comme indiqué dans la revue par Nagakubo et al. (Nagakubo et al., 2020), certains chercheurs considèrent la présence abondante de protéines ribosomales (30S et 50S) dans les EVs comme un signe potentiel de contamination du protéome d'origine cytoplasmique. Cependant, il est important de noter que d'autres études ont présenté des preuves soutenant la sécrétion extracellulaire de protéines ribosomales. Ces protéines, malgré leur origine cytoplasmique typique, peuvent avoir des fonctions cruciales au-delà de l'activité ribosomale dans la physiologie des micro-organismes. Ces fonctions incluent des rôles dans la formation de biofilms (Graf et al., 2019) et la confédération

de résistance contre les antibiotiques ciblant les processus de traduction (Kesavan et al., 2020; Sung et al., 2021). Par conséquent, la présence de protéines ribosomales dans le protéome des EVs pourrait ne pas indiquer uniquement une contamination, mais pourrait signifier des fonctions extracellulaires significatives pour ces protéines.

Dans les EVs sécrétées par EcN, une partie importante des protéines était représentée par des protéines adhésives, y compris les sous-unités fimbriales (FocA, Fim1C, FocF, FocG et FocH), les sous-unités flagellaires (FliC, FliD, FlgA, FlgE, FlgK et FlgL) et les protéines de la membrane externe (OmpA, OmpC, OmpF et NmpC). De nombreux chercheurs soulignent que la présence d'adhésines ancrées à la surface des EVs bactériens à Gram négatif est un facteur crucial facilitant la colonisation intestinale (S. I. Kim et al., 2018; Rolhion et al., 2010; Taheri et al., 2018). Un autre groupe important de protéines identifiées dans ces EVs était associé au réarrangement du peptidoglycane et de la membrane cellulaire, y compris les murein-hydrolases (MltA, MltB et MltC), la protéine MipA interagissant avec le murein, et les lipoprotéines associées au peptidoglycane (Pal, TolB, Sat, LpoA, YbaY et SlyB). Le réarrangement du murein et des membranes cellulaires est mis en avant comme des étapes essentielles dans la biogenèse des EVs, justifiant la présence de protéines liées à ces processus au sein des EVs (Avila-Calderón et al., 2021). De plus, les chercheurs soulignent le rôle potentiel des murein-hydrolases enfermées dans les EVs dans les interactions compétitives avec d'autres bactéries (Clarke, 2018). En résumé, les principaux constituants du protéome des EVs d'*E. coli* Nissle 1917 englobent les adhésines et les protéines associées au réarrangement du peptidoglycane, essentielles pour une colonisation efficace de l'hôte et une protection contre les micro-organismes pathogènes.

Fabrega et al. (M. J. Fábrega et al., 2016) ont découvert que la présence de lipopolysaccharides (LPS) dans les EVs dérivées d'*E. coli* pouvait expliquer l'activation de l'IL-6, de l'IL-8 et du TNF. À l'inverse, l'uprégulation de l'IL-10 semble être liée à d'autres facteurs présents dans les vésicules. Parallèlement, Morishita et al. (Morishita et al., 2021; Morishita et al., 2022) ont démontré que la production de cytokines médiée par les EVs est étroitement liée à leur internalisation. Ils ont observé une réduction de la libération de TNF et d'IL-6 des cellules traitées par les EVs en présence d'inhibiteurs de l'endocytose et de TLR2, à l'exception des cellules RAW264.7, où la réduction de TNF n'a pas été observée même après blocage de l'endocytose médiée par la clathrine et de la micropinocytose. Cela suggère l'implication de multiples voies dans l'interaction entre les EVs et les cellules. Comprendre ces effecteurs principaux est crucial pour comprendre les mécanismes d'immunomodulation chez l'hôte.

Il est évident qu'une partie substantielle de la recherche sur les EVs produits par les probiotiques examine principalement l'impact biologique de ces structures au sein du système gastro-intestinal. Cette focalisation est logique et justifiable, étant donné l'administration ingérable des EVs probiotiques, mettant en avant leur action intuitive

dans le système digestif (Chang et al., 2020; Ciešlik et al., 2022). Cependant, il reste essentiel de reconnaître que les EVs, en raison de leurs dimensions nanométriques, ont le potentiel d'atteindre divers tissus de l'hôte. Par conséquent, explorer leurs effets sur différents types de cellules humaines au-delà du tractus gastro-intestinal est tout aussi crucial (Hendrix and Wever, 2022; Viswanathan and Muthusamy, 2022).

Des études, comme celles mentionnées ci-dessus, sont essentielles pour une caractérisation précise de l'activité mécanistique des EVs des souches probiotiques. De plus, des études approfondies explorant les interactions récepteur-ligand entre les EVs et les cellules immunitaires peuvent élucider les voies précises par lesquelles les EVs exercent leurs effets, contribuant au développement de thérapies immunomodulatrices ciblées.

2.5. L'interaction complexe entre les probiotiques et différentes matrices alimentaires et ses conséquences pour le consommateur final

Les effets bénéfiques de certaines souches de probiotiques sont bien caractérisés dans de nombreux modèles de maladies différents et lors d'essais cliniques. Il est cependant important de noter que la forme sous laquelle ce probiotique est présenté et la matrice qui compose le produit probiotique peuvent grandement influencer les effets de cette souche probiotique. Nous avons ici démontré l'effet des matières grasses et des protéines du lait dans l'amélioration de l'effet anti-colite de *P. freudenreichii* CIRM-BIA 129.

Alors que les avantages de la matrice probiotique étaient autrefois au premier plan, l'attention s'est notablement déplacée vers des souches spécifiques et leurs effets ciblés sur les maladies. Par conséquent, l'influence de la matrice a été quelque peu éclipsée (Sanders et al., 2010). Cependant, il est essentiel de reconnaître que les attributs sensoriels et la facilité d'utilisation sont des aspects cruciaux des matrices probiotiques, influant de manière significative sur l'acceptation du consommateur. De plus, les matrices peuvent avoir des implications sur la durée de conservation des probiotiques, leur survie dans le tractus gastro-intestinal (TGI) et leur efficacité clinique globale (Sanders et al., 2010).

En fait, la matrice elle-même peut exercer un effet bénéfique sur l'hôte (Sanders et al., 2010). Parvenir à un équilibre judicieux entre les propriétés sensorielles, la facilité d'utilisation, la survie dans le TGI, l'efficacité clinique et les avantages pour l'hôte est essentiel dans le développement des produits probiotiques. Le rôle des matrices porteuses dans la réalisation de cet équilibre est primordial (Sanders et al., 2010). Malheureusement, la relation probiotique-matrice n'a pas reçu l'attention nécessaire, ce qui constitue un frein à l'innovation et souligne la nécessité de nouvelles directives dans ce domaine (van den Nieuwboer et al., 2014).

Dans tout produit probiotique, deux facteurs cruciaux entrent en jeu : les attributs

de la souche probiotique elle-même et les caractéristiques de la matrice, qui peuvent influencer positivement ou négativement à la fois la souche et l'hôte. Les bactéries probiotiques peuvent être incorporées dans un produit alimentaire de deux manières principales. La première approche implique la croissance des probiotiques au sein du produit final, généralement par le biais de processus de fermentation (par exemple, la transformation du lait en yaourt). La deuxième approche consiste à ajouter des micro-organismes probiotiques séchés ou encapsulés au produit (par exemple, dans les formules pour nourrissons et les jus (Makinen et al., 2012)). Au cours de l'étape de traitement, des facteurs tels que la température de fermentation et l'exposition à l'oxygène influent considérablement sur la croissance des probiotiques.

Le lait, en tant que matrice pour l'incorporation de probiotiques, contient divers composants, notamment les protéines du lactosérum (β -lactoglobuline, α -lactalbumine), les caséines (α s1, α s2, β et κ), les immunoglobulines, l'albumine sérique bovine, la lactoferrine, la lactopéroxydase, la phosphatase alcaline, la catalase et la plasmine (Burgain et al., 2014; Livney, 2010). De plus, le lait contient des globules gras composés de triacylglycérol et d'autres composants tels que les caroténoïdes, les vitamines A, D, E et K, ainsi que des molécules actives telles que les phospholipides, les sphingolipides, le cholestérol et les protéines de la membrane des globules gras du lait. Le lait est également riche en minéraux tels que le calcium, le phosphate inorganique, le citrate et autres (par exemple, le magnésium, le sodium, le potassium et le chlorure). Les vitamines hydrosolubles comme la vitamine C, B1 et B2 sont présentes dans les produits laitiers (Burgain et al., 2014). Le lactose (α -lactose ou β -lactose), le principal disaccharide des produits laitiers, sert de substrat aux probiotiques, améliorant potentiellement leur efficacité (Varcoe et al., 2002). Les bactéries semblent avoir une affinité pour les globules gras du lait, fournissant peut-être une protection lors du transit gastro-intestinal (Burgain et al., 2014). Par exemple, une teneur plus élevée en matières grasses et en protéines du lactosérum dans des conditions gastriques simulées conduit à des nombres de cellules probiotiques significativement plus élevés (Ziarno and Zaręba, 2015).

Saxelin et al. (Saxelin et al., 2010) ont démontré que la matrice utilisée pour l'administration des probiotiques affecte significativement la survie gastro-intestinale de manière dépendante de la souche. Lors d'une étude comparant les matrices de yaourt, de fromage et de capsule, des taux de récupération fécale plus élevés ont été observés pour *B. animalis* BB-12 et *Propionibacterium freudenreichii* shermanii JS dans le yaourt, mais pas pour *L. rhamnosus* GG et *L. rhamnosus* LC705. Cependant, il convient d'interpréter ces résultats avec prudence en raison de différences substantielles de dosage entre les matrices. Les participants du groupe du yaourt avaient une consommation quotidienne significativement plus élevée en UFC de *B. animalis* BB-12 et de *Propionibacterium freudenreichii* shermanii JS par rapport aux groupes du

fromage et de la capsule. Une tendance similaire a été observée pour *L. rhamnosus* GG et *L. rhamnosus* LC705. Rochet et al. (Rochet et al., 2008) ont étudié *B. animalis* DN-173 010 sous forme lyophilisée et fermentée, constatant une survie légèrement plus élevée sous forme lyophilisée mais avec des doses plus élevées.

L'atténuation de la colite dans un modèle murin par l'administration d'une souche probiotique semble être influencée par l'ajout de lait. Lors de l'administration de *L. casei* BL23 dans du lait par rapport à une solution saline tamponnée au phosphate (PBS), les souris atteintes de colite induite ont présenté un changement de poids corporel significativement plus faible, soulignant l'impact potentiellement positif de la matrice de lait (B. Lee et al., 2015). Collins et al. (Collins et al., 2002) ont observé des niveaux d'excrétion fécale plus élevés de *L. salivarius* UCC118 dans le lait frais par rapport au lait fermenté, tandis que Varcoe et al. (Varcoe et al., 2002) n'ont trouvé aucune différence significative dans les comptes de lactobacilles fécaux après l'administration de *L. acidophilus* NCFM dans différentes matrices. Enfin, Klingberg et Budde (Klingberg and Budde, 2006) ont rapporté que davantage de personnes avaient isolé *L. plantarum* MF 1298 après avoir consommé des saucisses probiotiques par rapport à de la poudre probiotique lyophilisée.

Il existe un manque de données approfondies évaluant l'effet de la matrice probiotique sur l'efficacité clinique. La plupart des études cliniques comparent principalement deux matrices identiques avec une teneur probiotique différente au lieu d'examiner l'impact de la matrice elle-même. Cependant, certaines études cliniques ont confirmé l'effet de la matrice. Par exemple, un mélange probiotique incorporant *P. freudenreichii* a été administré à des humains sous différentes formes, notamment des capsules classiques, du yaourt ou du fromage. Notamment, les niveaux fécaux les plus élevés de *P. freudenreichii* ont été observés chez les individus ayant consommé le probiotique sous forme de yaourt (Saxelin et al., 2010). Cette constatation est en phase avec une étude française démontrant que le yaourt améliore non seulement la survie de *P. freudenreichii*, mais favorise également son activité métabolique dans le tractus intestinal (Hervé et al., 2007).

Dans une étude visant à déterminer si l'administration de probiotiques pouvait accélérer la récupération de la diarrhée aiguë, des enfants ont reçu *L. rhamnosus* GG soit sous forme de lait fermenté, soit sous forme de poudre lyophilisée pendant 5 jours. Les deux traitements ont réduit significativement la durée de la diarrhée d'environ 1 jour par rapport au témoin ; cependant, aucun effet spécifique de la matrice n'a été observé (Isolauri et al., 1991). Chiang et al. (Chiang et al., 2000) et Sheih et al. (Sheih et al., 2001) ont rapporté une augmentation de l'activité des cellules immunitaires après la prise de *B. lactis* HN019 soit dans du lait faible en matières grasses, soit dans du lait faible en matières grasses lactosé.

Ce dernier favorisait une activité plus élevée des cellules tueuses naturelles

(NK). Dans une étude menée par Hütt et al. (Hütt et al., 2015), les valeurs de pression artérielle systolique et diastolique ont été réduites après une administration de trois semaines de *L. plantarum* TENSIA dans une matrice de fromage probiotique (1×10^{10} UFC/jour) et dans une matrice de yaourt (6×10^9 UFC). Les réductions de la pression artérielle diastolique par rapport à la ligne de base étaient significatives pour les deux matrices. La réduction de la pression artérielle systolique par rapport à la ligne de base était uniquement significative pour la matrice de fromage, une tendance forte étant observée pour la matrice de yaourt (Hütt et al., 2015). Il est important de noter que les participants du groupe du fromage consommaient une plus grande dose quotidienne de *L. plantarum* (1×10^{10} UFC) par rapport au groupe du yaourt (6×10^9 UFC), ce qui pourrait potentiellement expliquer la différence observée entre les matrices.

Les recherches actuelles sur les probiotiques privilégient souvent l'étude des effets spécifiques des souches au détriment de la considération de l'impact des matrices porteuses. Cependant, les souches probiotiques et leurs matrices porteuses influencent significativement la qualité et l'efficacité d'un produit probiotique. Les composants de la matrice tels que les graisses et les protéines peuvent affecter la viabilité et l'efficacité des probiotiques, et des études in vivo mettent en évidence les effets spécifiques des souches sur la survie gastro-intestinale. Trouver un équilibre entre ces facteurs, tout en tenant compte des propriétés sensorielles, est essentiel pour la fonctionnalité du produit et son acceptation par le consommateur. Pour stimuler l'innovation, des études comparant de multiples matrices avec la même souche probiotique sont nécessaires. Il est important de passer d'une valorisation uniquement des effets spécifiques des souches à une évaluation de la fonctionnalité des souches dans une matrice porteuse spécifique, guidant les allégations de santé basées sur le produit dans son ensemble.

Les études futures pourraient également évaluer un éventail plus large de matrices alimentaires pour comprendre de manière exhaustive comment divers composants au sein de ces matrices influent sur la viabilité, la stabilité et la bioactivité des probiotiques. Explorer les mécanismes qui régissent l'interaction entre les probiotiques et les différents composants alimentaires permettra de concevoir des matrices alimentaires optimales maximisant la puissance et la viabilité des probiotiques. De plus, l'étude de l'acceptabilité et des préférences des consommateurs pour les produits alimentaires enrichis en probiotiques guidera le développement de systèmes de distribution efficaces et plaisants.

Ces perspectives futures orienteront les recherches à venir, contribuant à peaufiner les applications probiotiques et à développer des stratégies innovantes pour maximiser leur potentiel pour améliorer la santé humaine.

6. Conclusions

En conclusion, cette étude approfondie met en lumière la nature complexe de la souche probiotique CEC15, une variante d'*E. coli*. La recherche démontre de manière robuste la sûreté et le potentiel probiotique de CEC15, surpassant même la souche de référence établie *E. coli* Nissle 1917 en termes de stabilité génomique et de caractéristiques bénéfiques. À la fois CEC15 vivant et ses formes postbiotiques, en particulier CEC15 inactivé par la chaleur, présentent des effets protecteurs prometteurs contre la mucosité intestinale, mettant en avant le potentiel thérapeutique des bactéries inactives et de leurs métabolites. De plus, l'étude plonge dans la diversité génétique des souches probiotiques d'*E. coli*, soulignant la complexité de leur constitution génomique. Par ailleurs, l'exploration des vésicules extracellulaires (EVs) provenant de CEC15 dévoile leur rôle potentiel d'immunomodulation, introduisant ainsi une nouvelle dimension à la compréhension de l'effet probiotique. Enfin, l'étude souligne l'impact profond de la matrice alimentaire, comme en témoigne l'activité probiotique renforcée de *Propionibacterium freudenreichii* dans des formulations spécifiques de lait. Ces connaissances approfondies enrichissent notre compréhension des probiotiques, ouvrant la voie à des formulations optimisées et à des applications adaptées pour promouvoir la santé intestinale et améliorer les affections inflammatoires.

The work developed during this Ph.D. thesis resulted in the scientific products enumerated below:

1. Research articles as first author

da Silva, Tales Fernando; Glória, Rafael De Assis; De Sousa, Thiago Jesus; Americo, Monique Ferrary; Freitas, Andria Dos Santos; Viana, Marcus Vinicius Canário; De Jesus, Luís Cláudio Lima; Da Silva Prado, Ligia Carolina; Daniel, Nathalie; Ménard, Olivia; Cochet, Marie-Françoise; Dupont, Didier; Jardin, Julien; Borges, Amanda Dias; Fernandes, Simone Odília Antunes; Cardoso, Valbert Nascimento; Brenig, Bertram; Ferreira, Enio; Profeta, Rodrigo; Aburjaile, Flavia Figueira; Carvalho, Rodrigo Dias de Oliveira; Langella, Philippe; Le Loir, Yves; Cherbuy, Claire; Jan, Gwénaël; Azevedo, Vasco; Guédon, Éric. **Comprehensive probiogenomics analysis of the commensal *Escherichia coli* CEC15 as a potential probiotic strain.** BMC Microbiol v. 23, 2023.

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2. Research articles as co-author

Andrade, Maria Emília Rabelo; Trindade, Luisa Martins; Leocádio, Paola Caroline Lacerda; Leite, Jacqueline Isaura Alvarez; Dos Reis, Diego Carlos; Cassali, Geovanni Dantas; **Da Silva, Tales Fernando**; De Oliveira Carvalho, Rodrigo Dias; De Carvalho Azevedo, Vasco Ariston; Cavalcante, Gregório Grama; De Oliveira, Jamil Silvano; Fernandes, Simone Odília Antunes; Generoso, Simone Vasconcelos; Cardoso, Valbert Nascimento. **Association of Fructo-oligosaccharides and Arginine Improves Severity of Mucositis and Modulate the Intestinal Microbiota.** Probiotics and Antimicrobial Proteins, v. 1, p. 1-17, 2023. (*The contributions to this article included processing of metagenomic samples and writing of the manuscript*)

Da Cruz Ferraz Dutra, Joyce; Passos, Marcele Fonseca; García, Glen Jasper Yupanqui; Gomes, Rosimeire Floripes; Magalhães, Tomas Andrade; Dos Santos Freitas, Andria; Laguna, Juliana Guimaraes; Da Costa, Francielly Morais Rodrigues; **Da Silva,**

Tales Fernando; Rodrigues, Luciano Santos; Américo, Monique Ferrary; Campos, Gabriela Munis; Pereira, Gilberto; Vandenbeghe, Luciana; Soccol, Carlos Ricardo; Góes-Neto, Aristóteles; De Carvalho Azevedo, Vasco Ariston. **Anaerobic digestion using cocoa residues as substrate: Systematic review and meta-analysis.** *Energy for Sustainable Development*, v. 72, p. 265-277, 2023. *(The contributions to this article included writing and revision of the manuscript)*

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3. Books and book chapters

Amancio, Mariana Bonifácio; **da Silva, Tales Fernando**; García, Glen Jasper Yupanqui; Fernandes, Geraldo Wilson; Oki, Yumi; Goes-Neto, Aristóteles; Azevedo, Vasco Ariston de Carvalho. **Análise da prospecção tecnológica por meio de revisão sistemática seguida de meta análise das patentes do gênero *Baccharis***. Atena Editora, 2023. *(The contributions to this article included writing and revision of the manuscript)*

da Silva, Tales Fernando; Batista, Viviane Lima; Coelho-Rocha, Nina Dias; Drumond, Mariana Martins; Mancha-Agresti, Pamela; Azevedo, Vasco. **Eixo intestino-osso: disbiose intestinal e alterações ósseas**. In: Dan Linetzky Waitzberg; Rafaela Malagoli Rocha; Alan Hiltner Alemida. (Org.). *MICROBIOTA GASTROINTESTINAL - Da disbiose ao tratamento*. 1ed. Rio de Janeiro: Atheneu, 2021, v. 1, p. 1-.

Barroso, F. A. L.; De Jesus, L. C. L.; **Da Silva, T.F.** ; Freitas, A. S.; Américo, M. F.; Fernandes, L. J. S.; Gloria, R. A.; Campos, G. M.; Carvalho, R. D. O.; Santos, T. M.; Laguna, J. G.; Azevedo, V. A. C.. **LAB as cell factories: A synthetic biology approach for plasmid DNA and recombinant protein production**. In: Didier Montet; Ramesh C. Ray; Vasco Ariston de Carvalho Azevedo; Spiro Paramithiotis. (Org.). *Lactic Acid Bacteria as Cell Factories - Synthetic Biology and Metabolic Engineering*.

1ed.Kidlington: Elsevier, 2023, v. 1, p. 1-16. *(The contributions to this article included writing and revision of the manuscript)*

Aburjaile, F. F.; De Jesus, Luis Cláudio Lima; **Da Silva, Tales Fernando** ; Drumond, Mariana Martins; Carvalho, R. D. O.; Azevedo, V.; Mancha-Agresti, P. D. C.. **Lactic acid bacteria in gut microbiota, probiotics and disease prevention.** In: Ramesh C. Ray; Spiro Paramithiotis; Vasco Ariston de Carvalho Azevedo; Didier Montet. (Org.). Lactic acid bacteria in food biotechnology: Innovation and functional aspects. 1ed.Cambridge: Elsevier, 2022, v. 1, p. 1-. *(The contributions to this article included writing and revision of the manuscript)*

De Jesus, Luis Cláudio Lima; **Da Silva, Tales Fernando** ; Gloria, R. A.; Freitas, A. S.; Américo, M. F.; Fernandes, L. J. S.; Campos, G. M.; Gomes, G. C.; Santos, R. C. V.; Carvalho, R. D. O.; Barh, D.; Azevedo, V.. **Lactic acid bacteria based beverages in the promotion of gastrointestinal tract health.** In: Debasis Bagchi; Bernard William Downs. (Org.). Microbiome, Immunity, Digestive Health and Nutrition: Epidemiology, Pathophysiology, Prevention and Treatment. 1ed.: Academic Press, 2022, v. 1, p. 373-386. *(The contributions to this article included writing and revision of the manuscript)*

Batista, Viviane Lima; **Da Silva, Tales Fernando** ; de Jesus, Luis Cláudio Lima; Tapiacosta, Ana Paula; Drumond, Mariana Martins; Azevedo, Vasco; Mancha-Agresti, Pamela. **Lactic Acid Bacteria as Delivery Vehicle for Therapeutics Applications.** In: Pfeifer B.A.; Hill A.. (Org.). Methods in Molecular Biology. 1ed.New York: Springer US, 2021, v. 2183, p. 447-459. *(The contributions to this article included writing and revision of the manuscript)*

Freitas, A. S.; Fernandes, L. J. S.; Coelho-Rocha, N. D.; De Jesus, L. C. L.; De Rezende Rodvalho, V; **Da Silva, Tales Fernando**; Carvalho, R. D. O.; Azevedo, V.. **Immunomodulatory and antiinflammatory mechanisms of probiotics.** In: Adriano Brandelli. (Org.). Probiotics. 1ed.London: Elsevier, 2021, v. 1, p. 1-532. *(The contributions to this article included writing and revision of the manuscript)*

Cláudio Lima de Jesus, Luís; Alvarenga Lima, Fernanda; Dias Coelho-Rocha, Nina; **Fernando da Silva, Tales** ; Paz, Júlia; Azevedo, Vasco; Mancha-Agresti, Pamela; Martins Drumond, Mariana. **Recombinant Probiotics and Microbiota Modulation as a Good Therapy for Diseases Related to the GIT.** In: Liana Claudia Salanta. (Org.). The Health Benefits of Foods - Current Knowledge and Further Development [Working Title]. 1ed.: IntechOpen, 2019, v. , p. 1-. *(The contributions to this article included writing and revision of the manuscript)*

4. Conference oral presentations

Da Silva, T.; De Rezende Rodovalho, V; Silva Rosa Da Luz, B; Rosa Do Carmo, F.; Nicolas, A.; Jardin, J.; Briard-Bion, V.; Jan, G.; Le Loir, Y.; Azevedo, Vasco; Guedon, E.. **Immunomodulatory role of *Propionibacterium freudenreichii* extracellular vesicles.** 2022. (FoodMicro congress, Athens - Greece).

da Silva, Tales; Assis, Rafael; Aburjaile, Flavia; Sousa, Thiago; Viana, Marcus; Carvalho, Rodrigo; de Jesus, Luis; Freitas, Andria; Americo, Monique; Campus, Gabriela; Menard, Olivia; Cochet, Marie-Françoise; Dupont, Didier; Jan, Gwénaël; Cherbuy, Claire; Guédon, Éric; Azevedo, Vasco. **Genomic and probiotic traits of the commensal *Escherichia coli* CEC15 strain.** 2022. (BactInflam webinar, Rennes - France)

5. Conference poster presentations

Da Silva, T. F.; Gloria, R. A. ; Sousa, T. J. ; Américo, M. F. ; Freitas, A. S. ; Aburjaile, F. F. ; Jan, G. ; Guedon, E. ; Azevedo, V. . Evaluation of Commensal *Escherichia coli* CEC15 Strain as a Potential Probiotic: *In Silico*, *In Vitro*, and *In Vivo* Analysis. 2023. (68th Brazilian Congress of Genetics, Ouro Preto, Brazil)

6. Research articles accepted

Silva, Brendhal Almeida; Amarante, Victor Santos; Xavier, Rafael Gariglio Clark; Colombo, Salene Angelini; **da Silva, Tales Fernando;** Brenig, Bertram; Aburjaile, Flavia Figueira; Azevedo, Vasco Ariston de Carvalho; Silveira, Rodrigo Silveira. **Characterization of ESBL/AmpC-producing extraintestinal *Escherichia coli* (ExPEC) in dogs treated at a Veterinary Hospital in Brazil.** *Research in Veterinary Science*. Acceptance date: 03 Dec. 2023.

7. Research articles submitted

da Silva, Tales Fernando; Glória, Rafael de Assis; Américo, Monique Ferrary; Freitas, Andria dos Santos; de Jesus, Luis Claudio Lima; Barroso, Fernanda Alvarenga Lima; Laguna, Juliana Guimarães, Coelho-Rocha, Nina Dias; Tavares, Laisa Macedo; Le Loir, Yves; Jan, Gwénaël; Guédon, Éric; Azevedo, Vasco Ariston de Carvalho. **From genome to bedside: The road of probiotics from characterization to commercialization.** *Probiotics and antimicrobial proteins*. Submission date: 22 Sep. 2023

Glória, Rafael de Assis; **da Silva, Tales Fernando;** Gomes, Tomás Andrade Magalhães; Vital, Kátia Duarte; Fernandes, Simone Odília Antunes; Cardoso, Valbert Nascimento; Ferreira, Ênio; Cherbuy, Claire; Le Loir, Yves; Jan, Gwénaël; Guédon, Éric; Azevedo, Vasco Ariston de Carvalho. **Posbiotic effect of *Escherichia coli* CEC15**

and *Escherichia coli* Nissle 1917 on a murine model of 5-FU-induced intestinal mucositis. *Probiotics and antimicrobial proteins*. Submission date: 10 Nov. 2023

Terra, Daniel Antonio Albuquerque; Carvalho, Rodrigo Dias de Oliveira; **da Silva, Tales Fernando**; Freitas, Andria; Amarante, Victor Santos; Góes-Neto, Aristóteles; Azevedo, Vasco; Vilela, Eduardo Garcia; Coelho, Luiz Gonzaga Vaz; Silva, Rodrigo Otavio. **Bacterial microbioma change after fecal transplantation for recurrent *Clostridioides difficile* infection in the Brazilian center.** *Brazilian Journal of Microbiology*. Submission date: 18 Aug. 2023.

Laguna, Juliana Guimarães; Freitas, Andria; Barroso, Fernanda Alvarenga Lima; De Jesus, Luís Cláudio Lima; de Vasconcelos, Octávio Augusto Greco Gomes; Quaresma, Ludmila Silva; Américo, Monique Ferrary; Campos, Gabriela; Glória, Rafael De Assis; Dutra, Joyce da Cruz Ferraz; **Da Silva, Tales Fernando**; Vital, Kátia Duarte; Fernandes, Simone O.; Souza, Ramon O.; Martins, Flaviano; Ferreira, Enio; Santos, Túlio Marcos; Birbrair, Alexander; de Oliveira, Marcos Felipe Andrade; Faria, Ana Maria Caetano; Carvalho, Rodrigo Dias De Oliveira; Venanzi, Franco Maria; Le Loir, Yves; Jan, Gwénaél; Guédon, Eric; and Azevedo, Vasco Ariston De Carvalho. **p62 Protein-Based Gene Therapy Modulates the Immune Responses in DSS-induced Colitis in Mice.** *Frontiers in Microbiology, section Food Microbiology*. Submission date: 07 Oct. 2023

Gonçalves, Nélio; Laguna, Juliana; Moreira, Guilherme; Moreira, Tiago; Meneses, Rodrigo; Küster, Paulo; **da Silva, Tales**; Saliba, Eloisa; Barbosa, Geraldo; Queiroz, Clarindo ; Peruzzo, Gabriela ; Azevedo, Vasco A; Macedo, Andrea. **Dietary oil supplementation alters the expression of target genes of the PPAR- α nuclear receptor potentially preventing hepatic fat accumulation in dairy cattle.** *Journal of Dairy Science*. Submission date: 08 Nov. 2023

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ANNEXES

This section contains supplementary information for this thesis. For research articles, supplementary images and supplementary tables are available online, with their electronic addresses provided.

Supplementary files for Research article 1 (Chapter 3) can be found online at:
<http://doi.org/10.6084/m9.figshare.23657679>.

Supplementary files for Additional results (Chapter 5) can be found online at:
<http://doi.org/10.6084/m9.figshare.24188553>.

Moreover, this annexes section includes the published research articles and book chapters as co-author, the papers currently submitted, and the certificates for oral and banner presentations.



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Anaerobic digestion using cocoa residues as substrate: Systematic review and meta-analysis

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ABSTRACT

This systematic review analyses the potential of cocoa residues for energy conversion using anaerobic digestion and evaluates strategies for optimizing methane production yields, retrieving information from papers published on anaerobic digestion of cocoa residues in journals indexed in Scopus, Web of Science, and PubMed databases. The articles were selected according to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) method. The effects of operational and compositional variables were evaluated by multivariate (PCA) and bivariate (correlation) statistics. A total of twenty-two studies were selected for this systematic review, and five of them were considered for meta-analysis. This search evidenced a lack of studies on the anaerobic digestion of cocoa residues, leading to, for example, high heterogeneity in methane yields between studies (163 to 382 mL CH₄ gVS⁻¹). Operational and compositional conditions in the anaerobic digestion were comparable (statistically significant correlations), and anaerobic digestion was considered efficient in the treatment of cocoa residues. In the meta-analysis, optimizing strategies did not significantly affect methane yields. Nonetheless, pretreatment using organic solvents can lead to inhibitory effects on the process. Anaerobic co-digestion can be considered a satisfactory strategy depending on the origin of the co-substrate. In conclusion, this review has worldwide relevance for the waste treatment sector and regarding the search for sustainable energy alternatives. To the best of our knowledge, this study was the first one that used quantitative methods such as multivariate bivariate statistical approaches and meta-analysis to answer questions related to the energy conversion of cocoa residues using anaerobic digestion.

Introduction

Cocoa is the fruit from cocoa trees (*Theobroma cacao*) and has significant economic importance in the food, pharmaceutical, and cosmetic industries (Darand, Oghaz, Amani, & Hadi, 2021; Karim et al., 2016; Puchol-miquel, Palomares, Barat, & Perez-esteve, 2021; Zou et al.,

2012). In the last 40 years, a continuous increase in cocoa production, mainly in developing countries, has been observed (Voora, Bermúdez, & Larrea, 2019) and, due to this increased production, higher volumes of waste were generated (Mansur, Tago, Masuda, & Abimanyu, 2014; Vásquez et al., 2019a). Ivory Coast is the world's largest cocoa producer, accounting for 39 % (Simone & Pereira, 2021) while Brazil ranks

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Association of Fructo-oligosaccharides and Arginine Improves Severity of Mucositis and Modulate the Intestinal Microbiota

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Abstract

Mucositis is defined as inflammatory and ulcerative lesions along of the gastrointestinal tract that leads to the imbalance of the intestinal microbiota. The use of compounds with action on the integrity of the intestinal epithelium and their microbiota may be a beneficial alternative for the prevention and/or treatment of mucositis. So, the aim of this study was to evaluate the effectiveness of the association of fructo-oligosaccharides (FOS) and arginine on intestinal damage in experimental mucositis. BALB/c mice were randomized into five groups: CTL (without mucositis + saline), MUC (mucositis + saline), MUC + FOS (mucositis + supplementation with FOS—1st until 10th day), MUC + ARG (mucositis + supplementation with arginine—1st until 10th day), and MUC + FOS + ARG (mucositis + supplementation with FOS and arginine—1st until 10th day). On the 7th day, mucositis was induced with an intraperitoneal injection of 300 mg/kg 5-fluorouracil (5-FU), and after 72 h, the animals were euthanized. The results showed that association of FOS and arginine reduced weight loss and oxidative stress ($P < 0.05$) and maintained intestinal permeability and histological score at physiological levels. The supplementation with FOS and arginine also increased the number of goblet cells, collagen area, and GPR41 and GPR43 gene expression ($P < 0.05$). Besides these, the association of FOS and arginine modulated intestinal microbiota, leading to an increase in the abundance of the genera *Bacteroides*, *Anaerostipes*, and *Lactobacillus* ($P < 0.05$) in relation to increased concentration of propionate and acetate. In conclusion, the present results show that the association of FOS and arginine could be important adjuvants in the prevention of intestinal mucositis probably due to modulated intestinal microbiota.

Keywords Mucositis · 5-Fluorouracil · Fructo-oligosaccharides · Arginine

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Evaluation of Probiotic Properties of Novel Brazilian *Lactiplantibacillus plantarum* Strains

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Abstract

Beneficial effects of *Lactiplantibacillus plantarum* strains have been widely reported. Knowing that the effects of probiotic bacteria are strain-dependent, this study aimed to characterize the probiotic properties and investigate the gastrointestinal protective effects of nine novel *L. plantarum* strains isolated from Bahia, Brazil. The probiotic functionality was first evaluated in vitro by characterizing bile salt and acidic tolerance, antibacterial activity, and adhesion to Caco-2 cells. Antibiotic resistance profile, mucin degradation, and hemolytic activity assays were also performed to evaluate safety features. In vivo analyses were conducted to investigate the anti-inflammatory effects of the strains on a mouse model of 5-Fluorouracil-induced mucositis. Our results suggest that the used *L. plantarum* strains have good tolerance to bile salts and low pH and can inhibit commonly gastrointestinal pathogens. Lp2 and Lp11 strains also exhibited high adhesion rates to Caco-2 cells (13.64 and 9.05%, respectively). Phenotypical resistance to aminoglycosides, vancomycin, and tetracycline was observed for most strains. No strain showed hemolytic or mucolytic activity. Seven strains had a protective effect against histopathological and inflammatory damage induced by 5-FU. Gene expression analysis of inflammatory markers showed that five strains upregulated interleukin 10 (*IL10*), while four downregulated both interleukin 6 (*IL6*) and interleukin 1b (*IL1b*). Additionally, all strains reduced eosinophilic and neutrophilic infiltration; however, they could not prevent weight loss or reduced liquid/food intake. Altogether, our study suggests these Brazilian *L. plantarum* strains present good probiotic characteristics and safety levels for future applications and can be therapeutically adjuvant alternatives to prevent/treat intestinal mucositis.

Keywords Lactic acid bacteria · Probiotic potential · Stress tolerance · Antimicrobial activity · Anti-inflammatory effect · Intestinal mucositis

Introduction

Probiotics are “live microorganisms which, when administered in adequate amounts, confer a health benefit on the host” [1]. Currently, the use of these microorganisms

for human consumption has received increasing attention due to the discovery of new scientific evidence regarding their functional properties and beneficial effects on the host [2]. Most probiotic strains belong to the Lactic Acid Bacteria (LAB) group [3]. Among them, *Lactiplantibacillus*

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Growth differentiation factor 11 delivered by dairy *Lactococcus lactis* strains modulates inflammation and prevents mucosal damage in a mice model of intestinal mucositis

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Mucositis is an inflammation of the gastrointestinal mucosa that debilitate the quality of life of patients undergoing chemotherapy treatments. In this context, antineoplastic drugs, such as 5-fluorouracil, provokes ulcerations in the intestinal mucosa that lead to the secretion of pro-inflammatory cytokines by activating the NF- κ B pathway. Alternative approaches to treat the disease using probiotic strains show promising results, and thereafter, treatments that target the site of inflammation could be further explored. Recently, studies reported that the protein GDF11 has an anti-inflammatory role in several diseases, including *in vitro* and *in vivo* results in different experimental models. Hence, this study evaluated the anti-inflammatory effect of GDF11 delivered by *Lactococcus lactis* strains NCDO2118 and MG1363 in a murine model of intestinal mucositis induced by 5-FU. Our results showed that mice treated with the recombinant lactococci strains presented improved histopathological scores of intestinal damage and a reduction of goblet cell degeneration in the mucosa. It was also observed a significant reduction of neutrophil infiltration in the tissue in comparison to positive control group. Moreover, we observed immunomodulation of inflammatory markers *Nfkb1*, *Nlrp3*, *Tnf*, and upregulation of *Il10* in mRNA expression levels in groups treated with recombinant strains that help to partially explain the ameliorative effect in the mucosa. Therefore, the results found in this study suggest that the use of recombinant *L. lactis* (pExu:gdf11) could offer a potential gene therapy for intestinal mucositis induced by 5-FU.

KEYWORDS

intestinal inflammation, 5-fluorouracil, lactic acid bacteria, DNA delivery, gene therapy



Synergistic synbiotic containing fructooligosaccharides and *Lactobacillus delbrueckii* CIDCA 133 alleviates chemotherapy-induced intestinal mucositis in mice

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Abstract

Intestinal mucositis is a commonly reported side effect in oncology patients undergoing chemotherapy and radiotherapy. Probiotics, prebiotics, and synbiotics have been investigated as alternative therapeutic approaches against intestinal mucositis due to their well-known anti-inflammatory properties and health benefits to the host. Previous studies showed that the potential probiotic *Lactobacillus delbrueckii* CIDCA 133 and the prebiotic Fructooligosaccharides (FOS) alleviated the 5-Fluorouracil (5-FU) chemotherapy-induced intestinal mucosa damage. Based on these previous beneficial effects, this work evaluated the anti-inflammatory property of the synbiotic formulation containing *L. delbrueckii* CIDCA 133 and FOS in mice intestinal mucosa inflammation induced by 5-FU. This work showed that the synbiotic formulation was able to modulate inflammatory parameters, including reduction of cellular inflammatory infiltration, gene expression downregulation of *Tlr2*, *Nfkb1*, and *Tnf*, and upregulation of the immunoregulatory *Il10* cytokine, thus protecting the intestinal mucosa from epithelial damage caused by the 5-FU. The synbiotic also improved the epithelial barrier function by upregulating mRNA transcript levels of the short chain fatty acid (SCFA)-associated GPR43 receptor and the occludin tight junction protein, with the subsequent reduction of paracellular intestinal permeability. The data obtained showed that this synbiotic formulation could be a promising adjuvant treatment to be explored against inflammatory damage caused by 5-FU chemotherapy.

Keywords Chemotherapy · Intestinal damage · Synbiotic · Immunomodulation · Intestinal barrier

Laísa Macedo Tavares and Luís Cláudio Lima de Jesus have contributed equally to this work.

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Isolation and Identification of Potential Probiotic Bacteria from Human Milk

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Abstract

Breast milk was long considered a sterile environment, but now it is known to harbor many bacteria that will shape the newborn microbiota. The benefits of breastfeeding to newborn health are, on some level, related to the presence of beneficial bacteria in human milk. Therefore, this study aims to investigate and isolate potential probiotics present in human milk that might be associated with improved health in infants, being potential candidates to be used in simulated human milk formula. Milk samples of 24 healthy mothers were collected at three time points: 30 min (colostrum), 5–9 days (transitional milk), and 25–30 days (mature milk) postpartum. Samples were evaluated by culturing, and the isolated bacteria were identified by MALDI-TOF MS and 16S DNA sequencing. In vitro screening for probiotics properties was performed, and the potential probiotics were mono-associated with germ-free mice to evaluate their ability to colonize the gastrointestinal tract. The microorganisms were submitted to the spray-drying process to check their viability for a potential simulated milk formula production. Seventy-seven bacteria were isolated from breast milk pertaining to four bacterial genera (*Staphylococcus*, *Streptococcus*, *Leuconostoc*, and *Lacticaseibacillus*). Four potential probiotics were selected: *Lacticaseibacillus rhamnosus* ($n=2$) and *Leuconostoc mesenteroides* ($n=2$). Isolates were able to colonize the gastrointestinal tract of germ-free mice and remained viable after the spray-drying process. In conclusion, breast milk harbors a unique microbiota with beneficial microorganisms that will impact the newborn gut colonization, being an essential source of probiotic candidates to be used in a formula of simulated maternal milk.

Keywords Breast milk · Microbiota · *Lacticaseibacillus* · *Leuconostoc* · Probiotics

Introduction

Breastfeeding is a highly recommended type of nutrition for infants due to the composition of breast milk, which is rich in nutrients and antibodies, contributing to child health [1].

Breast milk has three stages of lactation: colostrum, transitional milk, and mature milk. Colostrum is produced up to 5 days postpartum in low quantities, rich in immunoglobulin A, lactoferrin, leucocytes, and developmental factors such as epidermal growth factor [2]. Its principal function is immunological and trophic rather than nutritional [2]. The transitional milk occurs between 5 and 14 days after delivery, and after 4 to 6 weeks, the milk is considered fully mature.

Quésia S. Damaceno and Bruno Gallotti These authors contributed equally to this work

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The human milk was long considered a sterile environment where the health benefits of its consumption were attributed to the presence of bioactive molecules in its composition, but now it is known that the human milk harbors a significant number of bacteria that will contribute to shaping the newborn microbiota [3, 4]. The origin of this microbiota is not well known. However, some hypotheses have been raised as coming from the maternal skin, from the newborn oral microbiota, due to the high degree of return of milk to the breast ducts during breastfeeding, or from bacteria belonging to the maternal intestinal microbiota that could reach the mammary

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Lactobacillus delbrueckii CIDCA 133 Ameliorates Chemotherapy-Induced Mucositis by Modulating Epithelial Barrier and TLR2/4/Myd88/NF- κ B Signaling Pathway

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Intestinal mucositis promoted by the use of anticancer drugs is characterized by ulcerative inflammation of the intestinal mucosa, a debilitating side effect in cancer patients undergoing treatment. Probiotics are a potential therapeutic option to alleviate intestinal mucositis due to their effects on epithelial barrier integrity and anti-inflammatory modulation. This study investigated the health-promoting impact of *Lactobacillus delbrueckii* CIDCA 133 in modulating inflammatory and epithelial barrier markers to protect the intestinal mucosa from 5-fluorouracil-induced epithelial damage. *L. delbrueckii* CIDCA 133 consumption ameliorated small intestine shortening, inflammatory cell infiltration, intestinal permeability, villus atrophy, and goblet cell count, improving the intestinal mucosa architecture and its function in treated mice. Upregulation of *Muc2*, *Cldn1*, *Hp*, *F11r*, and *Il10*, and downregulation of markers involved in NF- κ B signaling pathway activation (*Tlr2*, *Tlr4*, *Nfkb1*, *Il6*, and *Il1b*) were observed at the mRNA level. This work suggests a beneficial role of *L. delbrueckii* strain CIDCA 133 on intestinal damage induced by 5-FU chemotherapy through modulation of inflammatory pathways and improvement of epithelial barrier function.

Keywords: 5-FU-induced mucositis, probiotics, *Lactobacillus delbrueckii*, anti-inflammatory cytokines, intestinal permeability, tight junction proteins

The contributions to this article included animal handling, samples processing, and writing of the manuscript



Lyophilized Symbiotic Mitigates Mucositis Induced by 5-Fluorouracil

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Mucositis is an adverse effect of cancer chemotherapies using 5-Fluorouracil (5-FU). It is characterized by mucosal inflammation, pain, diarrhea, and weight loss. Some studies reported promising healing effects of probiotic strains, when associated with prebiotics, as adjuvant treatment of mucositis. We developed a lyophilized symbiotic product, containing skimmed milk, supplemented with whey protein isolate (WPI) and with fructooligosaccharides (FOS), and fermented by *Lactobacillus casei* BL23, *Lactiplantibacillus plantarum* B7, and *Lactocaseibacillus rhamnosus* B1. In a mice 5-FU mucositis model, this symbiotic lyophilized formulation was able to reduce weight loss and intestinal permeability. This last was determined *in vivo* by quantifying blood radioactivity after oral administration of ^{99m}Tc-DTPA. Finally, histological damages caused by 5-FU-induced mucositis were monitored. Consumption of the symbiotic formulation caused a reduced score of inflammation in the duodenum, ileum, and colon. In addition, it decreased levels of pro-inflammatory cytokines IL-1 β , IL-6, IL-17, and TNF- α in the mice ileum. The symbiotic product developed in this work thus represents a promising adjuvant treatment of mucositis.

Keywords: probiotic, chemotherapy, prebiotic, immunomodulant effects, symbiotic

INTRODUCTION

Mucositis consists of an inflammation, mainly of the small bowel, that affects individuals submitted to cancer chemotherapy treatments, such as 5-Fluorouracil (5-FU) (Sonis, 2004). It includes mucosal injury, inflammation, diarrhea, and weight loss. It may lead to mucosal lesions and/or ulcerations throughout the gastrointestinal tract (Rodríguez-Caballero et al., 2012). Mucositis markers include the presence of leukocyte infiltrate in the lamina propria, degenerate enterocytes (Ciorba et al., 2016), accumulation of neutrophils and eosinophils (Antunes et al., 2016), increased degeneration of goblet cells (Stringer, 2013), as well as atrophy of villi (Chang et al., 2012).

There is presently no effective treatment for the prevention or alleviation of symptoms of mucositis. Furthermore, the use of chemotherapeutics causes severe dysbiosis (imbalance in the intestinal microbiota) which in turn worsens intestinal inflammation (van der Velden et al., 2014). In this context, development of alternative or adjuvant treatments is needed. Indeed, the use of probiotics as

The contributions to this article included processing of samples, qPCR analysis, and writing of the manuscript



Methane production from lignocellulosic biomass using hydrothermal pretreatment

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Abstract

Lignocellulosic biomass is a promising raw material for energy production due to its low cost and sustainability. In this work, *Egeria densa*, *Typha domingensis*, *Eichhornia crassipes*, and *Cyperus papyrus* ‘Nanus’ aquatic macrophytes were evaluated as a potential to produce methane, and their biochemical transformations were investigated. All substrate produced biogas by anaerobic fermentation. The values obtained to methane yield were 170.47, 132.42, 121.90, and 180.57 mLCH₄ gSV⁻¹, respectively. The hydrolysis rate was higher using *E. densa* biomass. Total volatile acid concentrations were 32.56 mg L⁻¹ (*E. densa*), 77.35 mg L⁻¹ (*T. domingensis*), 22.8 (*E. crassipes*), and 12.4 mg L⁻¹ (*Cyperus papyrus* ‘Nanus’). The more significant microbiological activity was observed in the reactors using *E. densa* (RE) and *T. domingensis* (RT). The total and volatile solids removal order was RE > RT > Rec (*E. crassipes*) > RP (*Cyperus papyrus* ‘Nanus’). So, for recovery of the bioenergy step, the *E. densa* biomass obtained the most promising results, and it was selected for hydrothermal pretreatment (HPT). After HPT, the biomass was more suitable for bioprocessing, increasing the carbohydrates available. There was a reduction in the crystallinity index due to the low lignin content and increased type II cellulose amorphous fraction. Also, the samples increased thermal stability (374 °C and 388 °C) and showed morphology with fibers partially disorganized. The methane yield was 4.2% higher as the time increased of the HPT. And the material became more vulnerable to attack by methanogenic microorganisms, resulting in greater energy production, with a potential of *E. densa* to generate methane.

Keywords Aquatic macrophytes · Hydrothermal pretreatment · Lignin · Methane · Hydrolysis

1 Introduction

Aquatic macrophytes constitute a significant problem in aquatic environments. Although those plants show essential functions in the aquatic biosystem, like high uptake capacity to store pollutants (heavy metals and nutrients) [1, 2], they have a high growth rate and versatility to develop in nutritious environments [3–5]. These characteristics favor the overpopulation of these plants, which threatens biodiversity and harbors disease-causing agents generate, causing a problem for hydroelectric power generation, waterborne transportation, fishing activities, and eutrophication [6–9]. Eutrophication is characterized by reducing dissolved oxygen and the disposal of organic matter and nutrients stored by macrophytes in environments [8], thus causing the death of aquatic organisms [10] and making these means anthropologically unusable. In this manner, the control of excessive biomass should be constant and effective to avoid

Statement of novelty In this study, the products generated and the capacity to generate biogas, through anaerobic digestion, of four types of aquatic plants (*E. densa*, *E. crassipes*, *T. domingensis*, and *C. papyrus* ‘Nanus’) were evaluated. This strategy is being tested for the first time on the species *Cyperus Papyrus* ‘Nanus,’ an aquatic plant widely used in wetlands. However, the most promising aquatic plant (*E. densa*) was selected for a less aggressive pretreatment (hydrothermal). Studying the morphological, structural, and thermal effects on *E. densa* biomass is unprecedented. This biomass has the potential to produce clean energy.

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Novel Strategies for Efficient Production and Delivery of Live Biotherapeutics and Biotechnological Uses of *Lactococcus lactis*: The Lactic Acid Bacterium Model

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Lactic acid bacteria (LAB) are traditionally used in fermentation and food preservation processes and are recognized as safe for consumption. Recently, they have attracted attention due to their health-promoting properties; many species are already widely used as probiotics for treatment or prevention of various medical conditions, including inflammatory bowel diseases, infections, and autoimmune disorders. Some LAB, especially *Lactococcus lactis*, have been engineered as live vehicles for delivery of DNA vaccines and for production of therapeutic biomolecules. Here, we summarize work on engineering of LAB, with emphasis on the model LAB, *L. lactis*. We review the various expression systems for the production of heterologous proteins in *Lactococcus* spp. and its use as a live delivery system of DNA vaccines and for expression of biotherapeutics using the eukaryotic cell machinery. We have included examples of molecules produced by these expression platforms and their application in clinical disorders. We also present the CRISPR-Cas approach as a novel methodology for the development and optimization of food-grade expression of useful substances, and detail methods to improve DNA delivery by LAB to the gastrointestinal tract. Finally, we discuss perspectives for the development of medical applications of recombinant LABs involving animal model studies and human clinical trials, and we touch on the main safety issues that need to be taken into account so that bioengineered versions of these generally recognized as safe organisms will be considered acceptable for medical use.

Keywords: *Lactococcus lactis*, genetic engineering, biotherapeutic molecules, mucosal immunity, safe for consumption

Probiotic potential of novel Brazilian *Lactobacillus crispatus* strains

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ABSTRACT. Lactobacilli are the predominant bacterial species colonizing the vaginal surfaces of healthy women, where they play a protective role against opportunistic and polymicrobial infections, such as bacterial vaginosis. Several *Lactobacillus* species, especially *L. crispatus*, have been prospected for probiotic applications due to their potential antimicrobial and anti-inflammatory capacities. During the last decade, several genomic studies have been investigating the genetics of *L. crispatus* strains in an effort to identify novel probiotic strains and evaluate their potential for improving human and animal health. This mini review highlights the main genes associated with *L. crispatus* protective mechanisms in four novel strains of this species that we recently isolated from healthy Brazilian women of reproductive age. Among the probiotic features of these strains, the roles of a pyruvate oxidase-encoding gene, lactate synthesis related enzymes, bacteriocin genes, and genomic islands, are reviewed, and the next steps for confirming their activity are indicated.

INTRODUCTION

First identified in 1894 by a German physician named Doderlein, lactobacilli have been reported as the dominant bacterial species that colonize the vaginal epithelium of women of reproductive age (Tachedjian et al., 2018). *Lactobacillus crispatus* is the most frequently isolated microorganism from this environment; it plays an important role in



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Pyrolysis and anaerobic digestion of the *Egeria Densa* biomass in different pretreatment conditions for potential use as a substrate in the generation of bioproducts

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ABSTRACT

In recent years, the interest in clean and sustainable energy technologies has grown significantly. As an alternative to fossil fuels, organic raw materials can reduce the emission of pollutants and greenhouse gases, positively impacting the environment. Submerged aquatic plants, such as *Egeria densa*, have become an interesting alternative. Due to the high capacity for proliferation and its physicochemical characteristics, *E. densa* has potential in the generation of bio-oil and biogas for thermochemical and biological processes, respectively. Here, the authors study the pyrolysis of lignocellulosic biomass and the feasibility of methane gas production through anaerobic digestion, after different pretreatments. The design of experiments (DOE) was used to investigate mass loss, biogas yield, and extractives content. Changes in surface morphology (coloration, roughness, and porosity), during pretreatment steps, were evaluated by colorimetric assays and scanning electronic microscopy. The kinetics of biogas production was modeled through a first-order equation. According to DOE results, the degradation of lignocellulose and the efficiency of methane production were favored by the alkaline pretreatment. The maximum yield of methane at 35 °C was 231.82 mL of CH₄ g⁻¹ (VS). Changes in the chemical constitution of biomass pretreated with acid, via thermochemical strategy, were observed in colorimetric and microstructural tests, justified by the increase in the concentration of extracts. The maximum mass loss was 80 % (pyrolysis) after biomass was submitted to an acid catalyst. The results showed that the physically-chemically pre-treated aquatic macrophyte *E. densa* is a renewable source in bio-oil and methane production.

1. Introduction

The growing demand for clean energy sources, the fossil fuel crisis, and the consequent increase in fuel prices have risen the need for numerous researches aiming for alternative energy matrices [1–5]. Biomass and lignocellulosic waste are promising and sustainable sources, found in different sectors of society – e.g., agriculture, industry –, having high availability and low cost. They are defined as a contemporary vegetable input, formed by photosynthetic capture of solar energy and stored in the form of chemical energy [6], becoming susceptible to decomposition by the action of different microorganisms.

Lately, submerged plants, such as the *Egeria densa*, have raised interest as a renewable energy source [2,7–9]. They are usually invasive species, with a high growth rate and disordered proliferation [10,11]. The excessive growth of invasive plants generates negative environmental impacts on lakes and reservoirs, due to eutrophication, requiring low-cost and environmental-friendly treatments [8,12]. Examples of such treatments include the use of microbial electrochemical technologies, pyrolysis, and anaerobic digestion.

Microbial electrochemical technologies (MET) is a promising science that aims to obtain energy from organic waste and directly produce electricity and valuable chemicals from microorganisms capable of

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Article

Effective Biocorrosive Control in Oil Industry Facilities: 16S rRNA Gene Metabarcoding for Monitoring Microbial Communities in Produced Water

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Abstract: Microbiologically influenced corrosion (MIC) or biocorrosion is a complex biological and physicochemical process. Strategies for monitoring MIC are frequently based on microbial cultivation methods, while microbiological molecular methods (MMM) are not well-established in the oil industry in Brazil. Thus, there is a high demand for the development of effective protocols for monitoring biocorrosion with MMM. The main aim of our study was to analyze the physico-chemical features of microbial communities occurring in produced water (PW) and in enrichment cultures in oil pipelines of the petroleum industry. In order to obtain strictly comparable results, the same samples were used for both culturing and metabarcoding. PW samples displayed higher phylogenetic diversity of bacteria and archaea whereas PW enrichments cultures showed higher dominance of bacterial MIC-associated genera. All samples had a core community composed of 19 distinct genera, with MIC-associated *Desulfovibrio* as the dominant genus. We observed significant associations between the PW and cultured PW samples, with a greater number of associations found between the cultured sulfate-reducing bacteria (SRB) samples and the uncultured PW samples. When evaluating the correlation between the physicochemical characteristics of the environment and the microbiota of the uncultivated samples, we suggest that the occurrence of anaerobic digestion metabolism can be characterized by well-defined phases. Therefore, the detection of microorganisms in uncultured PW by metabarcoding, along with physico-chemical characterization, can be a more efficient method compared to the culturing method, as it is a less laborious and cost-effective method for monitoring MIC microbial agents in oil industry facilities.

Keywords: petroleum; produced water; microbiologically influenced corrosion; oil industry; metabarcoding

Chapter

Recombinant Probiotics and Microbiota Modulation as a Good Therapy for Diseases Related to the GIT

Luís Cláudio Lima de Jesus, Fernanda Alvarenga Lima, Nina Dias Coelho-Rocha, Tales Fernando da Silva, Júlia Paz, Vasco Azevedo, Pamela Mancha-Agresti and Mariana Martins Drumond

Abstract

Many diseases that affect the gastrointestinal tract (GIT) have great influence on the quality of life of the majority of patients. Many probiotic strains are being highly studied as a promising candidate due to their beneficial effect reported in the GIT. With the purpose of increasing the beneficial characteristics of some probiotics strains and, consequently, to improve further the reported results, many probiotic strains expressing or encoding different proteins, with anti-inflammatory activities, have been developed. These recombinant strains have been reported as good candidates for the treatment of different pathological conditions, especially colitis and mucositis disease since they have been shown to have positive results and good perspectives for GIT inflammation. Thus, this chapter will first address the aspects of the gastrointestinal tract in humans as well as its microbiota. In a second moment, it will discuss about chronic diseases, mainly the intestinal ones. Finally, it will discuss about probiotics, especially concerning on lactic acid bacteria (LAB), and its action in the prevention and treatment of these diseases. At the final part, we will point out aspects on the development of recombinant strains and the results found in the literature on disease models.

Keywords: *L. lactis*, *Lactobacillus*, DNA vaccine, heterologous protein

1. The human gastrointestinal tract

The human gastrointestinal tract is formed by a complex ecosystem which includes the gastrointestinal epithelium, immune cells, and resident microbiota [1] and comprehends one of the biggest existent interfaces between the host, environmental factors, and antigens in the human body.

The intestine encompasses a broad variety of microorganisms (bacteria, archaea, eukarya, and viruses) [2] from more than 3500 different species [3, 4] that coevolved with the host in a mutually beneficial relationship [5, 6]. The composition and density of bacterial populations in adult individuals differ considerably over the GIT. The area

Chapter 16

Immunomodulatory and antiinflammatory mechanisms of probiotics

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16.1 Introduction

The gastrointestinal immune system known as gut-associated lymphoid tissue (GALT) is the largest specialized immunological anatomic structure in the body, and it is constantly exposed to external compounds and microbes, playing a crucial role in the host defense and homeostasis (Ballan, Battistini, Xavier-Santos, & Saad, 2020). It must be able to carry out the dual role of both tolerating the presence of a huge number of commensal microbiota and protecting the intestinal mucosa against pathogenic microorganism and dietary antigens (Allaire et al., 2018; Wershil & Furuta, 2008). In order to maintain the balance in the colonizing bacterial communities, the innate immunity and adaptive immunity work together to recognize a variety of antigens and coordinate protection mechanisms in the intestines (Kato, Shimpei, Mikako, & Sidonia, 2014).

16.1.1 Mucosal immunity and intestinal epithelium

Apart from immune responses, the very first line of defense against pathogens for host protection is the mucosal barrier, which comprises both cellular and enzymatic components (Farhadi, Banan, Fields, & Keshvarzian, 2003). Starting in the mouth and running through the entire upper gastrointestinal (GI) tract, low pH, bile salts, and proteolytic enzymes such as pepsin, trypsin and pancreatic proteases fulfill the role of facilitating the digestion process by breaking down big molecules into small ones. Besides helping digestion, this process allows conversion of potentially immunogenic proteins into small length peptides, therefore less immunogenic (Justin & Dhamoon, 2020).

The intestinal epithelial cells (IECs) are the basis of the mucosal barrier and are generally found in the whole intestines in different specialized forms. The majority of IECs bordering the intestinal lumen are absorptive enterocytes, which are responsible for nutrient and water absorption and provide a physical barrier against pathogens, being joined together by tight junctions, maintaining low intestinal permeability (Mowat & Agace, 2014). Among the main cellular components of the mucus barrier are the goblet cells, specialized IECs, which produce mucin glycoproteins that cover the adjacent epithelium (Kim et al., 2016). This barrier prevents pathogen and antigen invasion, and works as a binding site for secretory IgA (sIgA), which is thereafter directly transported through transcytosis by IECs across the epithelial barrier into the intestinal lumen (Peterson & Artis, 2014). Despite being a component of adaptive immune responses, sIgA is an important immunoglobulin that functions as an inhibitor of microbial attachment to the underlying epithelium, avoiding their translocation to the lumen, thus supporting barrier function (Lamont, 1992).

Barrier function against pathogens is also enhanced by the presence of Paneth cells, specialized IEC type that are found at the base of intestinal crypts and release many antimicrobial peptides, such as α -defensins, lysozyme, and secretory phospholipase (Gallo & Hooper, 2012). These antimicrobial factors, in special α -defensins, have been demonstrating to exert inhibitory activity against Gram-positive and Gram-negative bacteria, fungi, viruses, and protozoa (Vaishnava, Behrendt, Ismail, Eckmann, & Hooper, 2008; Wehkamp & Stange, 2006).

Besides promoting barrier function, specialized IEC lineages also help regulate local and systemic immunity through a cross talk between innate and adaptive cells (Peterson & Artis, 2014), carried out by activating the cell signaling cascade,

LAB as cell factories: A synthetic biology approach for plasmid DNA and recombinant protein production



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1.1 Introduction

Lactic Acid Bacteria (LAB) are a heterogeneous group of microorganisms that occupy a variety of ecological niches, such as fruits, decomposing vegetable materials, silages, sewage, dairy products, meat, fermented foods, cereals, beet, pickled vegetables, potatoes, juices, and animal or human body cavities (mouth, genitals, or intestinal tracts) (Liu et al., 2014; König and Fröhlich, 2017). LAB are characterized as Gram-positive, facultatively anaerobic, acid-tolerant, static, nonspore-forming, and rod or cocci-shaped. Moreover, they present low guanine and cytosine (G+C) content in the genome (Bintsis, 2018).

Historically, LAB have been defined by their metabolic characteristics, according to their ability to convert carbohydrates to lactic acid as the primary metabolic product of the glycolytic cycle ($\geq 50\%$) (George et al., 2018). Due to this property, LAB are usually used as starter cultures for fermentation processes of various foods to preserve them longer (Joardder and Masud, 2019). Additionally, since LAB are commonly consumed by animals and humans, two status have been established to classify the safety of these microorganisms: Generally Recognized As Safe (GRAS) according to the Food and Drug Administration (FDA) of the United States, and the Qualified Presumption of Safety (QPS) by the European Food Safety Authority (EFSA) (Bintsis, 2018).

Advances in biotechnology and molecular biology have opened up new possibilities for LAB, including their use as vectors to express/deliver heterologous proteins, antigens, vaccines, and biotechnology drugs (Peirotén and Landete, 2020). Several expression systems have been developed, constituting the basis of all expression



Chapter 24

Lactic Acid Bacteria as Delivery Vehicle for Therapeutics Applications

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Abstract

Lactic acid bacteria comprise a large group of Gram-positive organisms capable of converting sugar into lactic acid. They have been studied due to their therapeutic potential on the mucosal surface. Among the species, *Lactococcus lactis* is considered the model bacterium and it has been explored as an important vehicle for providing therapeutic molecules and antigens in the mucosa. They can be genetically engineered to produce a variety of molecules as well as deliver heterologous DNA and protein. DNA vaccines consist of the administration of a bacterial plasmid under the control of a eukaryotic promoter encoding the antigen of interest. The resulting proteins are capable of stimulating the immune system, becoming a promising technique for immunization against a variety of tumors and infection diseases and having several advantages compared to conventional nucleic acid delivery methods (such as bioballistic delivery, electroporation, and intramuscular administration).

Key words Lactic acid bacteria, DNA vaccine, DNA delivery, Mucosal administration, *Lactococcus lactis*

1 Introduction

Lactic acid bacteria (LAB) comprises a large group of gram-positive and nonsporulating microorganisms with fermentative properties, having the capacity to convert sugar into lactic acid. The majority of LAB have a “Generally Recognized as Safe” (GRAS) status according to the United States Food and Drug Administration (US FDA), meeting the criteria to be considered safe for human consumption [1].

LAB has been intensively studied due to their potential therapeutic effects on mucosal surfaces and can also be genetically engineered to efficiently produce a large variety of molecules either for the delivery of DNA or heterologous proteins [2–5]. In this context, among the various species of LAB, *Lactococcus lactis* is

Lactic acid bacteria in gut microbiota, probiotics and disease prevention

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1 Introduction: Microbiota: Health and disease

1.1 Intestinal microbiota

Microbiota is the collection of microorganisms in a defined region and/or period. The human microbiota corresponds to approximately 1–2 kg, being weight-like compared to the brain (roughly 1.5 kg). Depending on where in the body they are found, the species of microorganisms vary widely in their functions (Cummings and Macfarlane, 1997). It is estimated that the microbiota population can exceed 10^{14} , surpassing the number of cells from the host itself (Gill et al., 2006). Intestinal microbiota comprehends microorganisms habiting from the duodenum to the rectum producing vitamins, short-chain fatty acids (SCFA), nutrients, and closely interacting with the host, influencing the metabolic and health status of the host (David et al., 2014).

Environmental factors can have a significant impact on the microbiota composition. Since birth or, as some studies have found, since the creation of the womb, the way the host interacts with the environment can determine which groups of organisms will prevail and how they will affect the health of the host (Thursby and Juge, 2017). Babies born by c-section have different microbiota of the ones from a vaginal birth; the same individual has different microbiota compositions in various stages of life, based on the types of food they eat, the beings

Chapter 26

Lactic acid bacteria–based beverages in the promotion of gastrointestinal tract health

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26.1 Introduction

Humanity has known fermented beverages since the beginning of civilizations, and they are recognized as beneficial for therapeutic use. It was an innovation that was introduced in prehistoric agricultural societies, with evidence of fermented beverage production found in China 7000 B.C. and in Mesopotamia 5000 B.C. Fermented milk has been used in the Middle East, Egypt, Greece, and Italy since 7000–5000 B.C., while in Asia, at approximately the same time, the primary consumption was products that were fermented from rice (Gasbarrini et al., 2016). Since then, modern technology has been employed to investigate the health effects that are provided by fermented products, with increasing research in discovering microorganism strains able to confer additional health improvement in daily consumption (Gasbarrini et al., 2016).

Fermented beverages must contain a sugar source and a natural microbiota or surrounding microorganisms that are responsible for the natural fermentation process (Otes and Ozyurt, 2019). *Lactobacillus* and *Bifidobacterium* comprise most species present in dairy fermented beverages and food (Tamang et al., 2016a,b). However, other bacterial genera (*Enterococcus*, *Lactococcus*, *Pediococcus*, *Bacillus*, *Brevibacterium*, *Leuconostoc*, and *Propionibacterium*) and yeasts, such as *Saccharomyces*, were also isolated and are used in fermented beverages worldwide (Hittinger et al., 2018; Tamang et al., 2016a, 2016b).

The health benefits for the gastrointestinal tract (GIT) of fermented beverages were recognized even before the existence of microorganisms was discovered. Fermented beverages provide vital nutrients to a well-balanced diet: calcium, proteins, potassium, phosphorus, vitamins, and lipids. Also, the acid that is produced slowly helps digestion. Besides nutrients, fermented beverages contain microorganisms that transform the raw material properties that enhance sensorial quality, degrade toxic components and nonnutritive factors, fortify and deliver bioactive compounds, and produce antioxidant and antimicrobial components (Hittinger et al., 2018). The consumption of dairy products was associated with a reduction in the risk of type 2 diabetes, better digestion, attenuation of irritable bowel syndrome, fatty acid liver disease through immunomodulation, and even a decrease in cholesterol levels (Chandan et al., 2017). Many of these effects are derived from Lactic Acid Bacteria (LAB), the mainly microorganism group used in the production of fermented products.

26.2 Lactic Acid Bacteria

Lactic Acid Bacteria are a heterogeneous group of gram-positive, facultative anaerobic microorganisms (Pachla et al., 2018), generally nonmotile, nonsporulating, catalase-negative, and able to produce lactic acid as the primary metabolic end product of carbohydrate fermentation (Bintsis, 2018). They can adapt to different environments, which could

Titre : Caractérisation probiotique de la souche bénéfique d'*Escherichia coli* CEC15 et rôle de la matrice alimentaire dans la modulation de l'activité probiotique

Mot clés : Probiotiques; Probiogénomique; *Escherichia coli*; Postbiotiques

Résumé : Les probiotiques sont des micro-organismes bénéfiques, administrés en quantités suffisantes, connus pour avoir un impact positif sur l'hôte. Il existe une gamme diversifiée de micro-organismes probiotiques, en particulier les bactéries, disponibles sur le marché. Cependant, l'effet probiotique varie en fonction de la souche spécifique, et tous les membres d'une souche probiotique reconnue n'exhibent pas nécessairement des effets bénéfiques. Une nouvelle souche d'*Escherichia coli*, CEC15, isolée des selles de rat nouveau-né, a montré des effets bénéfiques sur la santé intestinale dans des modèles animaux. L'analyse approfondie du génome et du phénotype de CEC15 a révélé des caractéristiques prometteuses. La souche a présenté une résistance au pas-

sage gastro-intestinal, une forte adhérence aux cellules intestinales et aucun effet nocif lorsqu'elle a été administrée à des animaux sains. Dans un modèle murin de mucosite intestinale induite par la 5-fluorouracile, CEC15 a protégé les structures intestinales et réduit la perte de poids. De plus, les formes postbiotiques de CEC15, en particulier la forme CEC15 inactivée par la chaleur, ont montré des effets protecteurs similaires à la souche viable. Comparé à la souche probiotique bien connue EcN, CEC15 a affiché des résultats supérieurs dans divers essais, ce qui indique son potentiel en tant que souche probiotique pour une utilisation chez l'homme. Cependant, des recherches supplémentaires et des essais cliniques sont essentiels pour valider l'efficacité de CEC15 chez les sujets humains.

Title: Probiotic characterization of the beneficial *Escherichia coli* CEC15 strain and role the of food matrix in probiotic activity modulation

Keywords: Probiotics; Probiogenomics; *Escherichia coli*; Postbiotics

Abstract: Probiotics are beneficial microorganisms, administered in sufficient quantities, known to have a positive impact on the host. There is a diverse range of probiotic microorganisms, particularly bacteria, available in the market. However, the probiotic effect varies depending on the specific strain, and not all members of a recognized probiotic strain necessarily exhibit beneficial effects. A novel *Escherichia coli* strain, CEC15, isolated from newborn rat feces, has displayed beneficial effects on intestinal health in animal models. In-depth analysis of CEC15's genome and phenotype revealed promising traits. The strain exhibited resistance to gastrointestinal pas-

sage, high adhesion to intestinal cells, and no harmful effects when administered to healthy animals. In a murine model of 5-Fluorouracyl-induced intestinal mucositis, CEC15 protected intestinal structures and reduced weight loss. Moreover, postbiotic forms of CEC15, particularly heat-inactivated CEC15, demonstrated similar protective effects as the viable strain. In comparison to the well-known probiotic strain EcN, CEC15 showed superior outcomes in various assays, indicating its potential as a probiotic strain for human use. However, further research and clinical trials are essential to validate the effectiveness of CEC15 in human subjects.