Surface layer protein SlpB mediates immunodulation and adhesion in the probiotic Propionibacterium freudenreichii CIRM-BIA 129.

Fillipe Luiz Rosa Do Carmo

UNIVERSITE ECOLOGIE
BRETAGNE GEOSCIENCES
LOIRE AGRONOMIE ALIMENTATION

AGRO CAMPUS OUEST

2018 - 14

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COMUE UNIVERSITE BRETAGNE LOIRE

ECOLE DOCTORALE N° 600
Ecole doctorale Ecologie, Géosciences, Agronomie et Alimentation
Spécialité : *Biochimie, biologie moléculaire et cellulaire*

Par
Fillipe Luiz ROSA DO CARMO

La protéine de couche de surface SlpB assure la médiation de l'immunomodulation et de l'adhésion chez le probiotique *Propionibacterium freudenreichii* CIRM-BIA 129

Thèse présentée et soutenue à AGROCAMPUS OUEST campus de Rennes, le 6 septembre 2018
Unité de recherche : UMR INRA AGROCAMPUS OUEST Science et Technologie du lait et de l'œuf (STLO)
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UNIVERSIDADE FEDERAL DE MINAS GERAIS
INSTITUTO DE CIÊNCIAS BIOLÓGICAS
DEPARTAMENTO DE BIOLOGIA GERAL
PROGRAMA DE PÓS-GRADUAÇÃO EM GENÉTICA

Tese de Doutorado

Papel da proteína de superfície SlpB na imunomodulação e adesão da linhagem probiótica *Propionibacterium freudenreichii* CIRM-BIA 129.

Orientado: Fillipe Luiz Rosa do Carmo

Orientadores: Prof./Dr. Vasco Ariston de Carvalho Azevedo

Dr. Gwénaël Jan

Belo Horizonte - Brasil
Rennes - France
2018

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Sous le label de l'Université Européenne de Bretagne

pour obtenir le diplôme de :

DOCTEUR DE L'INSTITUT SUPERIEUR DES SCIENCES AGRONOMIQUES, AGRO-ALIMENTAIRES, HORTICOLES ET DU PAYSAGE

Spécialité : Biochimie, Biologie moléculaire et cellulaire

Ecole Doctorale : VAS (Vie-Agro-Santé)

présentée par :

Fillipe Luiz Rosa do CARMO

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Thèse préparée à l'UMR Sciences et Technologies du Lait et l'OEuf,
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Fillipe Luiz Rosa do Carmo

Papel da proteína de superfície SlpB na imunomodulação e adesão da linhagem probiótica *Propionibacterium freudenreichii* CIRM-BIA 129.

Tese apresentada ao programa de Pós-Graduação em Genética do Departamento de Biologia Geral do Instituto de Ciências Biológicas da Universidade Federal de Minas Gerais como requisito parcial para obtenção do título de Doutor em Genética.

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Belo Horizonte – Brasil
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2018

Universidade Federal de Minas Gerais
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ATA DA DEFESA DE TESE

Fillipe Luiz Rosa do Carmo

110/2018
entrada
2°/2014
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Às dez horas do dia **06 de setembro de 2018**, reuniu-se, no INRA – Rennes - França, a Comissão Examinadora de Tese, indicada pelo Colegiado do Programa, para julgar, em exame final, o trabalho intitulado: **"Papel da proteína de superfície SlpB na adesão e imunomodulação da linhagem probiótica Propionibacterium freudenreichii CIRM-BIA 129"**, requisito para obtenção do grau de Doutor em **Genética**. Abrindo a sessão, o Presidente da Comissão, **Vasco Ariston de Carvalho Azevedo**, após dar a conhecer aos presentes o teor das Normas Regulamentares do Trabalho Final, passou a palavra ao candidato, para apresentação de seu trabalho. Seguiu-se a arguição pelos Examinadores, com a respectiva defesa do candidato. Logo após, a Comissão se reuniu, sem a presença do candidato e do público, para julgamento e expedição de resultado final. Foram atribuídas as seguintes indicações:

Prof./Pesq.	Instituição	CPF/Passaporte	Indicação
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Aristoteles Goes Neto	Brésil, Rapporteur - Relator	549.388.85-20	APROVADO
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Gwennola Ermel	Examinateur - Examinadora	05RR42214	Approvado
Nathalie Desmasures	Examinateur - Examinadora	13DC95528	Aprovado
Benoit Foligné	Examinateur - Examinador		Aprovado
Françoise Nau	Référent Agrocampus Ouest -Examinadora / Presidente da Banca		Aprovado
Yves Le Loir	DU de STLO, Membre invité - Membro convidado		APROVADO

Universidade Federal de Minas Gerais
Instituto de Ciências Biológicas
Programa de Pós-Graduação em Genética

Gwénaël Jan	INRA STLO, Directeur de thèse - Orientador		Aprovado

Pelas indicações, o candidato foi considerado: APROVADO
O resultado final foi comunicado publicamente ao candidato pelo Presidente da Comissão. Nada mais havendo a tratar, o Presidente encerrou a reunião e lavrou a presente ATA, que será assinada por todos os membros participantes da Comissão Examinadora.
Belo Horizonte, 06 de setembro de 2018.

Vasco Ariston de Carvalho Azevedo - Orientador _____
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Muriel Thomas _____
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"Papel da proteína de superfície SlpB na adesão e imunomodulação da linhagem probiótica Propionibacterium freudenreichii CIRM-BIA 129"

Fillipe Luiz Rosa do Carmo

Tese aprovada pela banca examinadora constituída pelos Professores:

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UFMG - Brésil, Directeur de these - Orientador

Aristóteles Góes Neto
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Gwénaël Jan
INRA STLO, Directeur de thèse – Orientador

Belo Horizonte, 06 de setembro de 2018.

“*Vi veri universum vivus vici*”

“*Pelo poder da verdade, eu, enquanto vivo, conquistei o universo*”

“Par le pouvoir de la vérité, j'ai, de mon vivant, conquis l'univers”

Faust - Johann Wolfgang von Goethe

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THESIS OUTPUTS

Published or submitted articles

DO CARMO, FILLIPE L. R.; CORDEIRO, BARBARA F., OLIVEIRA, EMILIANO R., RABAH, HOUEM., SILVA, SARA H., SAVASSI, BRUNA, FIGUEIROA, ALESSANDRA, FONSECA, CAIO., QUEIROZ, MARIA I., RODRIGUES, NUBIA M., SANDES, SÁVIO H.C., CANTINI, ÁLVARO N., LEMOS, LUISA., ALVES, JULIANA L.A., CAETANO., A. M. F., FERREIRA, ÊNIO., LE LOIR, YVES., JAN, GWÉNAËL., AZEVEDO, VASCO. **Surface layer protein SlpB is involved in *Propionibacterium freudenreichii* CIRM-BIA 129 ability to prevent 5-FU-induced mucositis.** (in submission)

DO CARMO, FILLIPE L. R.; SILVA, WANDERSON M., TAVRES, GUILHERME C., IBRAIM, IZABELA C., CORDEIRO, BARBARA F., OLIVEIRA, EMILIANO R., RABAH, HOUEM., CAUTY, CHANTAL., SILVA, SARA H., CANÁRIO, MARCUS V., CAETANO, ANA C. B., SANTOS, ROSELANE G., CARVALHO, RODRIGO D.D.O., JARDIN, JULIEN., PEREIRA, FELIPE L., FOLADOR, EDSON L., LE LOIR, YVES., FIGUEIREDO, HENRIQUE C.P., JAN, GWÉNAËL., AZEVEDO, VASCO. **Mutation of the surface layer protein SlpB has pleiotropic effects in the probiotic *Propionibacterium freudenreichii* 129.** v. 9, p. 1-22, 2018

CORDEIRO, BARBARA F., OLIVEIRA, EMILIANO R.,SILVA, SARA H., SAVASSI, BRUNA, ACURCIO, LEONARDO B., LEMOS, LUISA., ALVES, JULIANA L.A., ASSIS, HELDER C., VIERA, ANGÉLICA, T., CAETANO., A. M. F., FERREIRA, ÊNIO., LE LOIR, YVES., JAN, GWÉNAËL., GOULART, LUIZ R., AZEVEDO, VASCO, CARVALHO, RODRIGO D.O., **DO CARMO, FILLIPE L. R. Whey protein isolate-supplemented beverage fermented by *Lactobacillus casei* BL23 and *Propionibacterium freudenreichii* 138 in the prevention of mucositis in mice.** v. 9, p. 1-18, 2018

DO CARMO, FILLIPE L. R.; RABAH, H.; CARVALHO, R. D. O.; GAUCHER, F.; CORDEIRO, B. F.; SILVA, S. H.; LOIR, Y. L.; AZEVEDO, V.; JAN, G. **Extractable Bacterial Surface Proteins in Probiotic|Host Interaction.** Frontiers in Microbiology, v. 9, p. 1-12, 2018.

RABAH, HOUEM; MÉNARD, OLIVIA; GAUCHER, FLORIANE; **DO CARMO, FILLIPE L. R.**; DUPONT, DIDIER; JAN, GWÉNAËL. **Cheese matrix protects the immunomodulatory surface protein SlpB of Propionibacterium freudenreichii during *in vitro* digestion.** FOOD RESEARCH INTERNATIONAL, v. 1, p. 1, 2018.
Citações:11

DO CARMO, FILLIPE L. R.; DIAS CARVALHO, RODRIGO; JAN, GWÉNAËL., AZEVEDO, VASCO. **Therapeutic DNA Vaccines: The Final Step for Success.** JOURNAL OF BACTERIOLOGY & PARASITOLOGY, v. 08, p. 1000e125., 2017.

CARVALHO, RODRIGO D. DE OLIVEIRA; **DO CARMO, FILLIPE L. R.**; DE OLIVEIRA JUNIOR, ALBERTO; LANGELLA, PHILIPPE; CHATEL, JEAN-MARC; BERMÚDEZ-HUMARÁN, LUIS G.; AZEVEDO, VASCO; DE AZEVEDO, MARCELA S. **Use of Wild Type or Recombinant Lactic Acid Bacteria as an Alternative Treatment for Gastrointestinal Inflammatory Diseases: A Focus on Inflammatory Bowel Diseases and Mucositis**. Frontiers in Microbiology, v. 8, p. 1, 2017.

RABAH, HOUEM; **DO CARMO, FILLIPE L. R.**; JAN, GWÉNAËL. **Dairy Propionibacteria: Versatile Probiotics.** Microorganisms, v. 5, p. 24, 2017.

DO CARMO, FILLIPE L. R.; RABAH, HOUEM; HUANG, SONG; GAUCHER, FLORIANE; DEPLANCHE, MARTINE; DUTERTRE, STÉPHANIE; JARDIN, JULIEN; LE LOIR, YVES; AZEVEDO, VASCO; JAN, GWÉNAËL. ***Propionibacterium freudenreichii*** **Surface Protein SlpB Is Involved in Adhesion to Intestinal HT-29 Cells. Frontiers in Microbiology**, v. 8, p. 1, 2017.

MANCHA-AGRESTI, PAMELA; DRUMOND, MARIANA MARTINS; **DO CARMO, FILLIPE L. R.**; SANTOS, MONICA MORAIS; COELHO DOS SANTOS, JANETE SOARES; VENANZI, FRANCO; CHATEL, JEAN-MARC; LECLERCQ, SOPHIE YVETTE; AZEVEDO, VASCO. **A New Broad Range plasmid for DNA Delivery in Eukaryotic Cells Using Lactic Acid Bacteria: *in vitro* and in vivo assays.** Molecular Therapy - Methods & Clinical Development, v. 4, p. 83-91, 2016.

Agresti, P. C. M.; SOUSA, C. S.; **DO CARMO, F. L. R.**; OLIVEIRA JUNIOR, A. F.; AZEVEDO, V.; AZEVEDO, M. S. P. **Future Clinical Applications of the Potential use of Lactic Acid Bacteria as Vehicles to Deliver DNA Vaccines.** Austin Journal of Vaccines & Immunotherapeutics, v. 2, p. 1-10, 2015.

Book chapter

CARVALHO, R. D. O.; **DO CARMO, F. L. R.**; SILVA, S. H.; CORDEIRO, B. F.; VAZ, A.; GIMENEZ, E. G. T.; GOES-NETO, A.; GOULART, L.; JAN, G.; AZEVEDO, V. **Metagenomic approaches for investigating the role of the microbiome in Gut health and inflammatory diseases.** Metagenomics for Gut Microbes. 1ed.: INTECH, 2018, v. 1, p. 1-22.

CARVALHO, R. D. O.; **DO CARMO, F. L. R.**; OLIVEIRA JUNIOR, A. F.; LANGELLA, P.; CHATEL, J. M.; BERMUDEZ-HUMARAN, L.; AZEVEDO, V.; AZEVEDO, M. S. P. **Use of Wild Type or Recombinant Lactic Acid Bacteria as an Alternative Treatment for Gastrointestinal Inflammatory Diseases: A Focus on Inflammatory Bowel Diseases and Mucositis.** IMMUNOBIOTICS: INTERACTIONS OF BENEFICIAL MICROBES WITH THE IMMUNE SYSTEM.. 1ed.Tucumã: Frontiers, 2018, v. 1, p. 127-139.

DO CARMO, F. L. R.; RABAH, HOUEM; CORDEIRO, B. F.; SILVA, S. H.; JAN, GWÉNAËL; AZEVEDO, V.; CARVALHO, R. D. O. **Applications of Probiotic Bacteria and Dairy Foods in Health. Current Research in Microbiology.** 1ed.Wilmington: Open Access eBooks, 2017, v. 1, p. 1-33.

CARVALHO, R. D. O.; **DO CARMO, F. L. R.**; Agresti, P. C. M.; AZEVEDO, M. S. P.; SOUSA, C. S.; SILVA, S. H.; Saraiva, T. D. L.; DRUMOND, M. M.; SILVA, B. C.; AZEVEDO, V. **Novel Biotechnological and Therapeutic Applications for Wild type and Genetically Engineered Lactic Acid Bacteria**. Fermented foods of Latin America: from traditional knowledge to innovative Applications. 1ed.Boca Raton: CRC Press, 2016, v. 1, p. 295-323.

MANCHA AGRESTI, P.; **DO CARMO, F. L. R.**; CARVALHO, R. D. O.; AZEVEDO, M. S. P.; SILVA, A.; AZEVEDO, V. **DNA Technology, Genes and Genetic Engineering.** In: Zahoorullah S MD. (Org.). A Textbook of Biotechnology. 1ed., 2015, v., p. 1-12.

Patents

AZEVEDO, V.; MANCHA AGRESTI, P.; DRUMOND, M. M.; **DO CARMO, F. L. R.** Vetor plasmidial para entrega de DNA, composições vacinais e/ou terapêuticas e usos (**Plasmidial vector for DNA delivery, vaccine and / or therapeutic compositions and uses**). 2017, Brasil. Patente: Privilégio de Inovação. Número do registro: BR1020170277003, título: "Vetor plasmidial para entrega de DNA, composições vacinais e/ou terapêuticas e usos.", Instituição de registro: INPI - Instituto Nacional da Propriedade Industrial, Depositante (s): Universidade Federal de Minas Gerais; Fundação de Amparo à Pesquisa do Estado de Minas Gerais, Depósito: 21/12/2017.

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LIST OF ABBREVIATIONS

5-FU - 5-Fluorouracil

CB 129 - *Propionibacterium freudenreichii* CIRM-BIA 129

CB 138 - *Propionibacterium freudenreichii* CIRM-BIA 138

CB129Δ*slpB* - *Propionibacterium freudenreichii* CIRM-BIA 129 with chromosomal knockout of the *slpB* gene

CEUA - Brazilian Ethics Committee on Animal Use

CFU – Colony forming unit

IBD - Inflammatory Bowel disease

ICB - Institute of Biological Sciences

INRA - French National Institute for Agricultural Research

***L. casei* BL23** – *Lactobacillus casei* BL23

LAB – Lactic acid bacteria

MRS – de Man, Rogosa and Sharpe broth

PAB - Propionic acid bacteria

SCFAS - Short chain fatty acids

Slaps -S-Layer-Associated Proteins

Slps - S-layer proteins

SURFING - Starter SURFace against INflammation of the Gut

UFMG - Federal University of Minas Gerais

WPI - Whey protein isolate

YEL - Yeast Extract Sodium Lactate broth

RESUMO

Propionibacterium freudenreichii é uma bactéria Gram-positiva benéfica, tradicionalmente usada na maturação de queijos e que possui o status GRAS (*Generally recognized as safe*). Seus efeitos probióticos são associados a secreção de metabólitos benéficos (ácidos graxos de cadeia curta, vitaminas e fator bifidogênico). Recentemente, a linhagem *P. freudenreichii* CIRM-BIA 129 (CB 129) revelou possuir efeito imunomodulador que foi confirmado *in vivo* pela capacidade de proteger camundongos da colite induzida quimicamente. Esse efeito anti-inflamatório (específico de linhagem) é, pelo menos em parte, associado às proteínas de superfície (S-layer). Em bactérias gram-positivas, incluindo *P. freudenreichii*, algumas linhagem podem ser cobertas com uma camada externa de proteínas, conhecida como camada superficial paracristalina, que consiste em moléculas entrelaçadas de proteínas *S-layer* (Slps). As Slps, em diferentes espécies bacterianas, estão envolvidas em várias funções que favorecem o potencial probiótico, tais como adesão a células hospedeiras e ao muco, persistência no intestino ou na imunomodulação. No entanto, em *P. freudenreichii*, os mecanismos moleculares implícitos aos efeitos probióticos ainda são pouco descritos. O objetivo desse trabalho foi estudar o papel das proteínas de superfície da linhagem probiótica CB 129, que possui potencial imunomodulador associados a várias proteínas de superfície de Slps, incluindo a proteína SlpB. Neste estudo, a inativação do gene correspondente na linhagem mutante CB129Δ*slpB*, causou uma diminuição drástica da adesão a células epiteliais do intestino humano HT-29, confirmando o seu papel na adesão *in vitro*. A mutação do gene *slpB* ocasionou efeitos pleiotrópicos na linhagem CB129Δ*slpB*, que afetam as propriedades da superfície bacteriana, as cargas da superfície e a hidrofobicidade, bem como o proteoma total e de superfície em comparação com a linhagem selvagem CB 129. Foi avaliado a resposta imune ao estímulo pelas linhagens selvagem ou mutante em células intestinais de adenocarcinoma humano HT-29. A linhagem selvagem induziu a expressão da citocina IL-10, enquanto a linhagem CB129Δ*slpB* induziu uma maior expressão de IL-8 e uma menor expressão de IL-10. Para avaliar o papel central da proteína SlpB e seu potencial anti-inflamatório na linhagem *P. freudenreichii* CIRM-BIA 129, as duas linhagens foram avaliadas em um modelo murino de mucosite induzida por 5-Fluorouracil. A administração da linhagem selvagem preveniu a perda de peso, preservou a arquitetura das vilosidades e aumentou a densidade dos grânulos secretórios dentro das células de Paneth em camundongos com mucosite. Por outro lado, a CB129Δ*slpB* não apresentou esse efeito protetor, e a administração da CB129Δ*slpB* promoveu resposta pró-inflamatório, aumentando a danos ao epitélio intestinal, e os níveis de expressão de IL-17A. Além disso, a fim de aumentar o efeito probiótico frente ao quadro de mucosite, foi avaliado o efeito terapêutico de uma bebida fermentada por *P. freudenreichii*, no mesmo modelo murino de mucosite induzida por 5-FU. Essa bebida fermentada evitou a perda de peso, bem como danos teciduais no intestino, em camundongos inflamados por 5-FU. Desse modo, esse trabalho colocou em evidência o envolvimento da proteína SlpB da linhagem *P. freudenreichii* CIRM-BIA 129 e suas propriedades anti-inflamatórios e confirma o papel versátil da proteína SlpB nas interações entre probiótico/hospedeiro. Esse estudo abre novas perspectivas para a compreensão dos fatores determinantes dos efeitos probióticos das linhagens de bactérias *P. freudenreichii* e para a seleção dos melhores candidatos probióticos, bem como para o desenvolvimento de alimentos funcionais para auxiliar no tratamento de doenças como adjuvantes juntamente com os tratamentos apropriados.

Palavras-chave: Propionibacteria, probióticos, imunomodulação, proteína da camada S, mucosite.

ABSTRACT

Propionibacterium freudenreichii is a beneficial Gram-positive bacterium, traditionally used as a cheese ripening starter, with the GRAS status (Generally Recognized As Safe). It has probiotic abilities based on the release of beneficial metabolites (short-chain fatty acids, vitamins, bifidogenic factor). *P. freudenreichii* has revealed an immunomodulatory effect confirmed *in vivo* by the ability to protect mice from induced acute colitis. The anti-inflammatory effect is however highly strain-dependent and due, at least in part, to key surface compounds favouring probiotic effects. In gram-positive bacteria, including *P. freudenreichii,* some strains can be covered with an external proteinaceous layer called a surface-layer paracrystalin layer and formed by the self-assembly of surface-layer-proteins (Slps). Slps were shown, in different bacteria, to be involved in several probiotics traits, such as adhesion to host cells and mucus, persistence within the gut, or immunomodulation. However, in *P. freudenreichii*, the molecular mechanisms underpinning probiotic effects are still poorly characterized. The aim of this study is to investigate, in a *P. freudenreichii* probiotic strain, the surface protein that plays the main role in the probiotic interaction with the host. The *P. freudenreichii* CIRM-BIA 129 strain recently revealed promising immunomodulatory properties and possesses several Slps, including SlpB. In the presented work, inactivation of the corresponding gene, in the CB129Δ*slpB* mutant strain, caused a drastic decrease in adhesion to intestinal epithelial HT-29 cells, further evidencing the key role of Slps in cell adhesion. Mutation of *slpB* gene had a pleiotropic effect on CB129Δ*slpB*, affecting surface properties, surface extractable proteome, intracellular proteome, surface charges (ζ-potential) and hydrophobicity, compared to the parental wild type *P. freudenreichii* CIRM-BIA 129. Following observations about pleiotropic effects of the *slpB* gene mutation, we investigated immune response of HT-29 cells towards *P. freudenreichii* CIRM-BIA 129 and CB129Δ*slpB*. The wild type strain mainly induced expression of the immunomodulatory IL-10 by the cells. Interestingly, the mutant strain induced decreased expression of IL-10, yet increased expression of IL-8. To finally evaluate the role of the key surface protein SlpB in the ant-inflammatory property of *P. freudenreichii*, we examined both strains in a murine mucositis model induced by 5-Fluorouracil (5-FU). *P. freudenreichii* wild type strain was able to prevent weight loss, to preserve villous architecture and increased the secretory granules density inside Paneth cells in mucositis–affected mice. By contrast, the mutant CB129Δ*slpB* strain failed to exert such protective effect. Moreover, in healthy mice, administration of this strain promoted an inflammatory response by increasing intestinal epithelium damage and the expression of IL-17A. In addition, in order to enhance probiotic effect in the context of mucositis, we investigated the therapeutic effect of a new fermented beverage, fermented by *P. freudenreichii*, in the same mucositis 5-FU mice model.This fermented beverage prevented weight loss and intestinal damage in inflamed mice receiving 5-FU. This study evidenced the involvement of *P. freudenreichii* SlpB protein in its anti-inflammatory properties and confirmed the versatile role fulfilled by Slps in probiotic host interactions. It opens new avenues for the understanding of probiotic determinants in propionibacteria, for the selection of the best *P. freudenreichii* probiotic strains and for the development of functional fermented foods, targeting inflammatory diseases, and consumed in conjunction with the appropriate allopathic treatments.

Keywords: Propionibacteria, probiotic, immunomodulation, S-layer protein, mucositis.

RÉSUMÉ

Propionibacterium freudenreichii est une bactérie Gram-positive bénéfique, traditionnellement utilisée comme levain d'affinage fromager, qui bénéficie du statut GRAS (Generally Recognized As Safe). Elle a des aptitudes probiotiques qui reposent sur la sécrétion de métabolites bénéfiques (acides gras à chaine courte, vitamines, facteur bifidogène). *P. freudenreichii* a révélé un effet immunomodulateur qui a été confirmé *in vivo* par la capacité à protéger des souris d'une colite aigüe induite. L'effet anti-inflammatoire est cependant hautement souche-dépendant. Il est dû, au moins en partie, à des composés de surface clés qui favorisent ces effets probiotiques. Les bactéries Gram-positives, y compris *P. freudenreichii*, peuvent être recouvertes d'une couche extérieure protéique, appelée « surface-layer », paracristalline, et formée par l'autoassemblage de protéines dites de S-layer (Slps). Les Slps, dans différentes bactéries, sont impliquées dans plusieurs caractéristiques probiotiques, telles que l'adhésion aux cellules de l'hôte et au mucus, la persistance dans l'intestin, ou encore l'immunomodulation. Cependant, chez *P. freudenreichii*, les mécanismes moléculaires qui sous-tendent les effets probiotiques sont encore mal connus. Le but de cette étude est d'étudier, chez une souche probiotique de *P. freudenreichii*, la protéine de surface qui joue le principal rôle dans les interactions probiotiques avec l'hôte. La souche *P. freudenreichii* CIRM-BIA 129, récemment reconnue comme immunomodulatrice prometteuse, possède plusieurs protéines de surface Slps, y compris SlpB. Dans la présente étude, l'inactivation du gène correspondant, dans la souche mutante CB129Δ*slpB*, a provoqué une baisse drastique de l'adhésion aux cellules intestinales épithéliales HT-29, confirmant le rôle clé des Slps dans l'adhésion aux cellules. Cette mutation a eu des effets pléïotropes sur la souche CB129Δ*slpB*, affectant les propriétés de surface, les charges (potentiel ζ) et l'hydrophobie de surface, par rapport à la souche sauvage parentale *P. freudenreichii* CIRM-BIA 129. A la suite de l'observation de ces effets pléïotropes de la mutation du gène *SlpB*, nous avons étudié la réponse immune de cellules HT-29 stimulées par le sauvage ou par le mutant. Le sauvage induisait principalement l'expression de la cytokine immunomodulatrice IL-10. Le mutant induisait, de façon intéressante, moins d'IL-10, mais plus d'IL-8. Afin d'évaluer the rôle de cette protéines clé SlpB dans les effets antiinflammatoires de *P. freudenreichii*, nous avons testé ces deux souches dans un modèle murin de mucosite induite par le 5-Fluorouracile. La consommation de la souche sauvage prévenait la perte de poids, préservait l'architecture des villosités, ainsi que la densité des granules de sécrétion au sein des cellules de Paneth, chez les souris atteintes de mucosite. Par contre, le mutant n'avait pas cet effet protecteur. De plus, chez la souris saine, l'administration de cette souche mutante favorisait une réponse pro-inflammatoire en augmentant les dommages causés à l'épithélium intestinal, ainsi que l'expression de l'IL-17A. De plus, dans le but de favoriser l'effet probiotique dans le contexte de la mucosite, nous avons étudié l'effet thérapeutique d'une nouvelle boisson, fermentée par *P. freudenreichii*, dans le même modèle de mucosite induite par le 5-FU chez la souris. Cette boisson fermentée a prévenu la perte de poids, ainsi que les dommages intestinaux, chez les souris inflammées recevant le 5-FU. Cette étude a mis en évidence l'implication de la protéine SlpB de *P. freudenreichii* dans les propriétés anti-inflammatoires de cette bactérie et confirme le rôle versatile joué par les Slps dans les interactions probiotique/hôte. Elle ouvre des perspectives nouvelles pour la compréhension des déterminants probiotiques des bactéries propioniques laitières, pour la sélection des meilleures souches probiotiques de *P. freudenreichii*, et pour le développement d'aliments fermentés fonctionnels, ciblant les maladies inflammatoires, et consommés en accompagnement des traitements allopathiques appropriés.

Mots Clés : Propionibactéries, probiotique, immunomodulation, protéine de S-layer, mucosite.

CONTEXT OF RESEARCH

This work is supported by different funding agencies in Brazil (CNPq, CAPES) and in France (INRA, Région Bretagne). In this section, the context in which this project is inserted within the scope of the scientists involved, as well as the organizations that were essential for the development of this project, will be described.

The Council for Scientific and Technological Development (CNPq, Conselho Nacional de Desenvolvimento Científico e Tecnológico) is a public foundation in Brazil, founded in 1951 and linked to the Ministry of Science and Technology since 1985. The CNPq's mission is to promote and to stimulate the scientific and technological development of Brazil and to contribute to the formulation of national S&T policy through funding capacity building in S&T (at master, doctoral and specialization level) and financing research projects in key areas. The Federal Agency for Support and Evaluation of Higher Education (CAPES, Coordenação de Aperfeiçoamento de Pessoal de Nível Superior), supports the Ministry of Education (MEC) in formulating policies in the context of higher education and coordinates the Brazilian system of postgraduation. CAPES has an important role in funding international, research cooperation management and operating the agreements between Brazil and other countries.

Through various programs, the CNPq and CAPES support the development of research, strengthen research groups and institutions, and encourage international cooperation of Brazilian researchers. One such program is the Brazilian scientific mobility program Science Without Borders, which aims to promote the consolidation and expansion of science, technology, and innovation in Brazil through international exchange and mobility, by encouraging the establishment of international partnerships to make the interaction with foreign partners feasible.

The National Institute of Agricultural Research (INRA, Institut National de la Recherche Agronomique) was founded in 1946 and is a French Public Scientific and Technical Research Establishment, under the joint authority of the Ministries of Research and Agriculture. Aimed at leading projects of targeted research for sustainable agriculture, safeguarding the environment and increase healthy and high-quality food. INRA's mission is producing and disseminating knowledge, training future scientists, informing

public policy through collective scientific expert reports, foresight, and advanced studies, promoting innovation through technology transfer and partnerships, shaping national and European research strategies, and fostering debate regarding science's role in society.

INRA's Microbiology and the Food Chain Division (MICA, Microbiologie et Chaîne Alimentaire) department brings together INRA's expertise to take account of developments in the scientific, technological, environmental and societal contexts. MICA's objectives are targeted to promote the reduction of environmental impacts on food systems, health and safety of food chains, reduction of medicinal and antibiotic usage in livestock farming, development of agricultural raw material processing systems based on clean and efficient technologies, reduction of food losses and wastage, and research on alternatives to fossil fuels. The strategic plan of the MICA department is organized around three thematic fields (CT), each grouping several research priorities and being structured according to the fields of application: CT1 - Microbiology for a reasoned approach of the biotechnologies, CT2 - Microbiology for the quality, durability and food safety and CT3 - Microbiology for the maintenance of animal and human health.

Founded in 1927, the Federal University of Minas Gerais (UFMG, Universidade Federal de Minas Gerais) is recognized nationally and internationally for its excellence, both for its academic and scientific work and for the professional training it provides in a wide variety of subjects. It is recognized as one of the most important universities in Brazil, with outstanding work in teaching, research, and extension. A center of excellence in research, UFMG has leading projects in more than 800 groups formally registered in the CNPq and CAPES. Aimed at generating and disseminating scientific, technological and cultural knowledge, forming critical and ethical subjects with a solid scientific and humanistic base, as well as encouraging regional and national socio-economic development.

The Laboratory of Cellular and Molecular Genetics (LGCM, Laboratório de Genética Celular e Molecular) is part of the *Instituto de Ciências Biológicas* (ICB, Institute of Biological Sciences) at the UFMG. Founded in 1997, it has as mission to produce, share and disseminate knowledge, contributing to the formation of students and researchers.

Its focus is to understand the pathogenesis of diseases, find candidates for vaccines using different approaches and expertises (*in silico*, *in vitro* and *in vivo*), and to propose and develop treatments for diseases using the therapeutic potential of probiotic bacteria as well as recombinant proteins. The LGCM has a great importance in the global scenario, regarding research involving *Corynebacterium pseudotuberculosis*, genetically engineered microorganisms, and the therapeutic effects of probiotics.

The LGCM laboratory is coordinated by Prof. Dr. Vasco Ariston de Carvalho Azevedo, a graduate from veterinary school at the Federal University of Bahia in 1986. He obtained his Master's (1989) and Ph.D. (1993) degrees in microbial genetics, at Institut National Agronomique Paris-Grignon (INAPG) and Institut National de la Recherche Agronomique (INRA), France. He was a Postdoctoral researcher in the Microbiology Department of Medicine School in 1994 at the University of Pennsylvania, USA. Since 1995, he has been a Professor of the Federal University of Minas Gerais. He is a fellow of the Brazilian Academy of Sciences. His entire research history is composed by 370 publications, 3 books and 29 chapters. Professor Azevedo is a pioneer in the field of the genetics of Lactic Acid Bacteria and Corynebacterium pseudotuberculosis in Brazil. He has specialized and is currently researching bacterial genetics, genome, transcriptome, proteome and metabolome, development of new vaccines and diagnostics of infectious diseases.

The Science & Technologie du Lait & de l'Oeuf (STLO, Science and Technology of Milk and Eggs), or UMR1253 STLO, is a research unit, located in Brittany, Western France. It combines human resources from both the National Institute for Agricultural Research (INRA) and the Food and Agricultural Science University (AGROCAMPUS OUEST) to build a strong joint involvement in Education and Research benefitting society and industry with new scientific advances through diverse partnerships. STLO aims to develop public research expertise on the components of milk and eggs, as well as increase the quality and safety of fermented dairy products and ovoproducts.

Dr. Gwénaël Jan, as a Senior Scientist of INRA UMR STLO, has obtained a PhD in Life Sciences at the University of Rennes, in 1995, followed by a Post-doctoral Fellowship in molecular and cell biology at the Institute for Medical Sciences of the University of Aberdeen in the United Kingdom. Working at INRA since 1997, he has published more

than 80 articles, 3 international patents, and 6 book chapters. He dedicates his scientific carrier to the microbiology of fermented foods, with a particular interest in “2-in-1” bacteria, which exhibit both technological and probiotic abilities. In collaboration with French and international academics, stakeholders, and technical centers, he identified beneficial bacteria within the great biodiversity of traditional fermented food products. In 2011, he became a leader of a national research programme, the National Agency for Research (ANR): Starter SURFace against INflammation of the Gut (SURFING). The project’s purpose is to understand and exploit the effects and role of *Lactobacillus delbrueckii* and *Propionibacterium freudenreichii* surface proteins in the modulation of gut inflammation. This work revealed an unexpected probiotic potential in industrial dairy propionibacteria starters. It has furthermore identified key surface proteins that are involved in its immunomodulatory effects.

The collaboration between Brazil/UFMG and France/INRA comprises two French groups, INRA Agrocampus Ouest Rennes, under the coordination of research director Yves Le Loir, and INRA Jouy en Josas, coordinated by research director Jean-Marc Chatel, as well as three Brazilian groups from the UFMG, the Laboratory of Molecular and Cellular Genetics, coordinated by Vasco Azevedo, Laboratory of Immunopharmacology, coordinated by Mauro Teixeira, and Laboratory of Immunobiology, coordinated by Ana Maria Caetano. To structure collaboration between two research teams or laboratories (one in France and the other abroad) that already have joint publications, the creation of an International Associated Laboratory (LIA), a “laboratory without walls”, is required and was formalized through a contract signed by the heads of both organizations in 2017. The aims of the LIA “BactInflam”, involving UFMG and INRA, are to identify bacterial determinants involved in pro or anti-inflammatory processes in various contexts, including IBD and mastitis, to characterize inflammatory processes in diseases in the context of human and veterinary health and to develop new treatments.

In this context, I, Fillipe Luiz Rosa do Carmo, Chemistry Technician, Pharmacist, Master in Biosciences Applied to Pharmacy, Ph.D. candidate in Genetics at UFMG and Ph.D. candidate in Cell Biology, Molecular Biology and Biochemistry at AGROCAMPUS OUEST Rennes, was awarded a grant from the program “Science Without Borders” and properly fit in the cotutelle agreement between INRA and UFMG. I sought to study

Propionibacterium freudenreichii CIRM-BIA 129 (CB 129) alias ITG P20, a strain with a great probiotic potential, and focusing on CB 129 surface layer protein SlpB and its role in adhesion to the host and in immunomodulation abilities, leading to anti-inflammatory effects in the context of inflammatory bowel diseases.

GENERAL INTRODUCTION

Dairy products constitute an important source of beneficial nutrients and promote human well-being through a healthy diet. The global dairy market was evaluated around USD 413.8 billion in 2017 and is dominated by companies such as Nestle SA, Dairy Farmers of America, Fonterra Group Cooperative Limited, Danone, and Arla Foods Nestlé S.A. (ResearchAndMarkets, 2018). This market experienced a global growth of 1.8% during the past five years and is expected to grow at a CAGR (Compound Annual Growth Rate) of 7.35% during the period 2018-2023 (ResearchAndMarkets, 2018).

The dairy products market is linked to the growing demand for probiotics. In 2002, the Food and Agriculture Organization of the United Nations (FAO) and the World Health Organization (WHO) developed Guidelines for the Evaluation of Probiotics in Food and defined them, and in 2014. Hill *et al*. then corrected grammatically the definition of FAO/WHO as 'live microorganisms that, when administered in adequate amounts, confer a health benefit on the host (Hill et al., 2014). Bacteria used as probiotics usually have the GRAS status (Generally Recognized As Safe) conferred by the FAO (Food and Agriculture Organization of the United Nations) and are thus considered safe for human consumption (Nutrition).

Probiotics have been largely included in food delivery vehicles and in functional supplements, which are marketed and regulated as foods or functional foods. The combination of probiotics and other ingredients leads to "2-in-1" or multi-action products, which experience an increasing popularity. The global revenue generated by the Probiotics market was evaluated at USD 40.09 billion in 2017 and is expected to generate revenue of around USD 65.87 billion by the end of 2024, growing at a CAGR of around 7.35% between 2018 and 2024 (Zion Market Research™).

Researchers from both the academic and industrial fields were engaged in the last decades in investigations on probiotics, in order to unravel the mechanisms of probiotic action and to improve this action through “2-in-1” fermented products aiming at the prevention and/or treatment of human disorders. An archetypal example is the treatment of lactose intolerance by yogurt starters. Moreover, these news approaches to prevent Gastrointestinal (GI) disorders through probiotics will create significant demand for probiotics and will further boost the market of probiotic supplements and of fermented products including probiotics.

Mechanisms by which probiotics provide health benefits on the host can be mainly classified into three categories: probiotic metabolic effects, microbiota composition normalization and molecular interactions between probiotics and the host (Sánchez et al., 2017; Vieira et al., 2013). In addition, selection of probiotic strains should take into account criteria favoring *in situ* action. These include the robustness against different abiotic and biotic stresses, for example digestive stresses, adhesion to the intestinal epithelium, long persistence within the Gastrointestinal Tract (GIT) and ability to generate an immune response in the host (Carvalho et al., 2017; do Carmo et al., 2017a; Rabah et al., 2017).

The mostly claimed probiotic bacteria strains, which have long been studied with respect to beneficial effects, belong to the *Bifidobacterium* and *Lactobacillus* genera (O’Toole et al., 2017). However, there is a growing demand for new probiotics. In this context, dairy propionibacteria emerged as promising probiotic candidates, mainly because of their unexpected immunomodulatory effects recently revealed.

The main dairy propionibacteria species, *Propionibacterium freudenreichii*, was firstly described by E. von Freudenreich and S. Orla-Jensen. It is one of the major ingested bacteria in the French diet, due to its unavoidable presence in Emmental cheese as a cheese ripening agent (Cousin et al., 2010). *P. freudenreichii* is a dairy propionibacterium, is a Gram-positive Actinobacteria, characterized by a high production of propionic acid by a fermentative pathway named Wood-Werkman, which involves the transcarboxylase cycle (Thierry et al., 2011). Selected *P. freudenreichii* strains have demonstrated a remarkable robustness and tolerate harsh environments, such as the digestive tract constraints which include stomach acidity and bile salts (Rabah et al.,

2017). *P. freudenreichii* and *P. acidipropionici* received the GRAS status and were granted the “Qualified presumption of safety” (QPS) status (EFSA, 2009; Klinman et al., 2010). Moreover, dairy propionibacteria are the only GRAS bacteria producing food-grade vitamin B12 at the industrial scale (Thierry et al., 2011).

Several strains of *P. freudenreichii* have probiotic properties, which were demonstrated in mice models of inflammatory bowel diseases (IBDs). IBDs constitute a group of idiopathic and chronic inflammatory conditions of the GIT, including ulcerative colitis (CD) and Crohn’s disease (UC) (Carvalho et al., 2017; Rabah et al., 2017). In this context, a strain of *Propionibacterium freudenreichii* CIRM-BIA 129, alias ITG P20, recently attracted attention because of its anti-inflammatory properties (Le Maréchal et al., 2015; Plé et al., 2015, Plé et al., 2016). During 3 years, a national research project called SURFING (Starter SURFace against INFlammation of the Gut), funded by the French ANR agency (Agence Nationale de la Recherche), investigated immunomodulatory properties of 23 *P. freudenreichii* strains. It correlated such properties with proteins involved in this process, by using comparative genomics, transcriptomics and proteomics. It revealed key surface proteins, involved in its immunomodulatory effects. Surface layer protein SlpB, in particular, was proposed as a central actor in this process (Deutsch et al., 2017; Le Maréchal et al., 2015). Understanding how this main character acts will open perspectives to provides a new functional fermented product for preclinical and clinical studies aimed at prevention or treatment of inflammatory diseases by select one *P. freudenreichii* strain, in conjunction with the appropriate allopathic treatments.

INTRODUCTION GENERALE

Les produits laitiers constituent une source importante de nutriments bénéfiques et favorisent le bien-être via une alimentation saine. Le marché laitier mondial a été évalué à 413.8 milliards de USD en 2017. Il est dominé par des compagnies telles que Nestle SA, Dairy Farmers of America, Fonterra Group Cooperative Limited, Danone, et Arla Foods Nestlé S.A. (ResearchAndMarkets, 2018). Ce marché a vécu une croissance globale de 1.8% durant les cinq dernières années et l'on attend une croissance menant à une CAGR (Compound Annual Growth Rate) de 7.35% au cours de la période 2018-2023 (ResearchAndMarkets, 2018).

Le marché des produits laitiers est lié à la demande croissante de probiotiques. En 2002, la FAO et la WHO ont mis au point des instructions pour l'évaluation des probiotiques dans l'alimentation, avant de les définir en 2014. Hill *et al.* ont ensuite corrigé cette définition FAO/WHO en « microorganismes vivants qui, lorsqu'ils administrés en quantité adéquate, confèrent un bénéfice santé à l'hôte (Hill et al., 2014). Les bactéries utilisées comme probiotiques sont en générale dotée du statu GRAS (Generally Recognized As Safe) décerné par la FAO et sont donc considérés comme sûrs dans le cadre de l'alimentation humaine (Nutrition).

Les probiotiques ont été largement inclus dans des matrices alimentaires, ainsi que dans des suppléments fonctionnels, qui sont commercialisés et réglementés comme des aliments ou comme des aliments fonctionnels. La combinaison de probiotiques avec d'autres ingrédients conduit à des produits multifonctionnels qui connaissent une popularité croissante. Le bénéfice global généré par le marché des probiotiques a été évalué à 40.09 milliards de USD en 2017 et devrait générer des revenus de l'ordre de 65.87 milliards de USD à la fin de 2024, montant à une CAGR de l'ordre de 7.35% entre 2018 et 2024 (Zion Market Research™).

Les chercheurs des deux domaines, académique et industriel, ont participé durant les dernières décennies à de nombreuses études sur les probiotiques, dans le but de découvrir les mécanismes d'action probiotiques et d'améliorer cette action via des produits fermentés « 2-en-1 » destinés à la prévention et/ou au traitement de désordres

chez l'homme. Un exemple archétypique est le traitement de l'intolérance au lactose par des ferments lactiques sélectionnés comme levain du yaourt.

Les mécanismes permettant aux probiotiques d'exercer un effet bénéfique sur l'hôte sont principalement classés en trois catégories : les effets probiotiques métaboliques, la normalisation de la composition du microbiote, et les interactions moléculaires entre probiotique et hôte (Sánchez et al., 2017; Vieira et al., 2013). De plus, la sélection de souches probiotiques devrait tenir compte de critères favorisant l'action *in situ*. Ces critères comprennent la robustesse vis-à-vis de divers stress abiotiques et biotiques, comme par exemple les stress digestifs, l'adhésion à l'épithélium intestinal, la persistance dans le tractus digestif et la capacité à induire une réponse immune chez l'hôte (Carvalho et al., 2017; do Carmo et al., 2017a; Rabah et al., 2017).

Les souches bactériennes les plus décrites comme probiotiques, qui sont étudiées depuis longtemps pour leurs effets bénéfiques, appartiennent aux genres *Bifidobacterium* et *Lactobacillus* (O'Toole et al., 2017). Cependant, il y a une demande croissante pour de nouveaux probiotiques. Dans ce contexte, les bactéries propioniques laitières sont apparues comme des probiotiques prometteurs, principalement du fait de leurs effets immunomodulateurs récemment révélés.

La principale espèce de bactéries propioniques laitières, *Propionibacterium freudenreichii*, a été décrite pour la première fois par E. von Freudenreich et S. Orla-Jensen. C'est une des principales bactéries ingérées quotidiennement dans la diète française, en raison de sa présence obligatoire dans l'Emmental, en tant qu'agent d'affinage (Cousin et al., 2010). *P. freudenreichii* est une bactérie propionique laitière, une Actinobactérie Gram-Positive, caractérisée par une forte production d'acide propionique via une voie fermentaire appelée Wood-Werkman, qui implique le cycle transcarboxylase (Thierry et al., 2011). Des souches sélectionnées de cette bactérie ont démontré une robustesse remarquable et tolèrent des conditions rudes, comme les contraintes du tractus digestif, qui comprennent l'acidité stomacale et les sels biliaires (Rabah et al., 2017). *P. freudenreichii* et *P. acidipropionici* ont reçu le statu de bactérie GRAS, ainsi que celui de "Qualified presumption of safety" (QPS) (EFSA, 2009; Klinman et al., 2010). De plus, les bactéries propioniques laitières sont les seules bactéries

GRAS produisant de la vitamine B12 de qualité alimentaire, à l'échelle industrielle (Thierry et al., 2011).

Plusieurs souches de *P. freudenreichii* ont des propriétés probiotiques, qui ont été démontrées dans des modèles murins de maladies intestinales inflammatoires (IBD). Ces maladies constituent un groupe de conditions idiopathiques et inflammatoires chroniques de l'intestin, y compris la rectocolite hémorragique et la maladie de Crohn (Carvalho et al., 2017; Rabah et al., 2017). Dans ce contexte, une souche de *P. freudenreichii*, CIRM-BIA 129, alias ITG P20, a récemment attiré l'attention du fait de ses propriétés antiinflammatoires (Le Maréchal et al., 2015; Plé et al., 2015, 2016). Durant 3 ans, un projet de recherche national, nommé SURFING (Starter SURFace against INFlammation of the Gut), financé par l'ANR (Agence Nationale de la Recherche), a étudié les propriétés immunomodulatrices de 23 souches de *P. freudenreichii*. Il a corrélé de telles propriétés avec la présence de protéines impliquées dans ce processus, en utilisant les outils de génomique, transcriptomique et protéomique comparatives. Il a révélé des protéines de surface clés, impliquées dans les propriétés immunomodulatrices de cette bactérie. En particulier, la protéine de S-layer SlpB, a été citée comme un acteur central de ce processus (Deutsch et al., 2017; Le Maréchal et al., 2015). Le fait de comprendre comment cet acteur principal fonctionne va ouvrir des perspectives de mise au point d'un nouvel aliment fermenté fonctionnel pour des études précliniques et cliniques visant à prévenir ou à traiter des maladies inflammatoires grâce à des souches sélectionnées de *P. freudenreichii*, en complément des traitements allopathiques adaptés.

AIM OF THE PHD PROJECT

This research work investigated the functions fulfilled by surface-layer protein slpB of *Propionibacterium freudenreichii* CIRM-BIA 129. It focused on the involvement of this protein in adhesion to cultured human colon cells and in the immunomodulatory response, including probiotic/host interactions and anti-inflammatory effect, in the context of inflammatory conditions in mice models.

OBJECTIF DU PROJET DE THESE

Ce travail de recherche a étudié les fonctions remplies par la protéine de surface-layer SlpB chez *Propionibacterium freudenreichii* CIRM-BIA 129. Il a porté sur l'implication de cette protéine dans l'adhésion aux cellules épithéliales intestinales humaines en culture, ainsi que sur la réponse immunomodulatrice, y compris les interactions probiotique/hôte et les effets antiinflammatoires, dans le contexte de maladies inflammatoires dans des modèles murins.

RATIONALE OF THE PHD PROJECT

Modifications of the lifestyle in developed countries, including diet and lack of physical activity, in addition to increase of elderly people, lead to increased incidence of chronic diseases. Among these, many growing pathologies involve immune disorders, inflammation and dysbiosis. A typical example is the growing incidence of inflammatory bowel diseases, linked to genetic background, immune dysregulation and gut microbiota dysbiosis (Bouma and Strober, 2003). In addition, diseases such as mucositis, a severe inflammation of mucosal GI tract, affects 80% of patients undergoing oncological treatment based on chemotherapy and radiopharmaceuticals (Carvalho et al., 2017). Treatments do exist for IBD's and mucositis, but, in addition to a heavy burden to the health-care systems, they only provide remissions between relapses or cause side-effects which further increase treatment costs.

The pioneer work of gastroenterologists such as Prof P. Gionchetti evidenced the interest of using combinations of selected immunomodulatory strains of bacteria in conjunction with the treatment of IBD (Ghouri et al., 2014). Moreover, many research groups are initiating research using probiotic strains to relieve the symptoms of mucositis in mice model (Carvalho et al., 2017).

Microorganisms contained in fermented food products were traditionally selected on the basis of techno-functional rather than probiotic criteria. Probiotic bacteria can alleviate symptoms of inflammatory diseases (Bibiloni et al., 2005; Ghouri et al., 2014; Sood et al., 2009), but without techno-functional ability. However, anti-inflammatory properties were recently discovered in specific, selected strains of the main dairy starters, *Propionibacterium freudenreichii* (Foligné et al., 2010, 2013), *Lactobacillus delbrueckii* (Santos Rocha et al., 2012, 2014), *L. helveticus* (Rong et al., 2015; Yamashita et al., 2014) and *S. thermophilus* (Ménard et al., 2005; Rodríguez et al., 2010), but the mechanism of this probiotic effect remain in a black box.

The main hope in this context resides in the perspective to potentiate IBD and mucositis treatments, prolong remission, enhance life quality and limit the treatment costs. However, more clinical studies are needed to precise the role of food-grade bacteria in modulating inflammation, as well as the mechanisms involved in this probiotics effects.

PERTINENCE DU PROJET DE THESE

Dans les pays développés, les modifications de style de vie, y compris la diète et le manqué d'activité physique, ajoutée à l'augmentation du nombre de personnes âgées, a conduit à une augmentation de l'incidence de maladies chroniques. Parmi celles-ci, plusieurs pathologies croissantes impliquent des désordres immunitaires, une inflammation, et une dysbiose. Un exemple typique est l'incidence croissante des maladies inflammatoires chroniques de l'intestin (MICIs), liées au contexte génétique, à une dysrégulation immunitaire, et à une dysbiose du microbiote intestinal (Bouma and Strober, 2003). De plus, des maladies telles que la mucosite, un sévère inflammation de la muqueuse digestive, affecte 80% des patients subissant un traitement oncologique bas é sur la chimiothérapie ou la radiothérapie (Carvalho et al., 2017). Des traitements existent pour les MICI et pour la mucosite, mais, en plus d'une contrainte économique lourde pour les systèmes de santé publique, ils ne conduisent qu'à des rémissions entre des rechutes, ou provoquent à leur tous des effets secondaires qui augmentent encore le coût des traitements.

Les travaux pionniers de certains gastroentérologues tels que le Professeur P. Gionchetti ont mis en évidence l'intérêt d'utiliser des combinaisons de souches de bactéries immunomodulatrices sélectionnées, en accompagnement du traitement des MICI (Ghouri et al., 2014). De plus, plusieurs équipes de recherche ont entamé des recherches mettant en œuvre des souches probiotiques pour soulager les symptômes de la mucosite dans des modèles murins (Carvalho et al., 2017).

Les microorganismes contenus dans les produits alimentaires fermentés ont été traditionnellement sélectionnés sur la base de critères techno-fonctionnels, plutôt que probiotiques. Certaines bactéries probiotiques peuvent diminuer les symptômes de maladies inflammatoires (Bibiloni et al., 2005; Ghouri et al., 2014; Sood et al., 2009) , mais sans pour autant avoir d'aptitude techno-fonctionnelle. Cependant, des propriétés anti-inflammatoires ont récemment été découvertes dans des souches spécifiques, sélectionnées, de levains laitiers majeurs, *Propionibacterium freudenreichii* (Foligné et al., 2010, 2013), *Lactobacillus delbrueckii* (Santos Rocha et al., 2012, 2014), *L. helveticus* (Rong et al., 2015; Yamashita et al., 2014) et *S. thermophilus* (Ménard et al.,

2005; Rodríguez et al., 2010), mais les mécanismes de ces effets probiotiques restent dans une boite noire.

Le principal espoir, dans ce contexte, réside dans la perspective de potentialiser les traitements des MICIs et de la mucosite, de prolonger les rémissions, d'améliorer la qualité de vie et de limiter le coût des traitements. Cependant, il faudra d'autres études précliniques et cliniques pour préciser le rôle de bactéries alimentaires dans la modulation de l'inflammation, ainsi que les mécanismes impliqués dans ces effets probiotiques.

CHAPTER 1. LITERATURE REVIEW. STATE OF THE ART ON PROPIONIBACTERIA AS PROBIOTICS.

In this chapter, we provide a literature review about dairy propionibacteria, with a specific focus on *Propionibacterium freudenreichii*, a bacterium widely used in the food industry as a cheese-ripening starter, as a biopreservative and as a beneficial additive. *Propionibacterium* ssp. increasingly attract attention because of their promising probiotic properties. These last are linked with versatile attributes such as production of short chain fatty acids (SCFAs), of conjugated fatty acids, of bioactive surface proteins, of 1,4-dihydroxy-2-naphtoic acid (DHNA, the bifidogenic growth factor), of vitamin B12, as well as a great ability to endure digestive stressing conditions, adhesion to intestinal epithelial cells and immunomodulatory properties. We stress out the importance of improved growth conditions to enhance metabolites production. We indicate fermented food matrices as key means to improve probiotic properties of the bacteria comprised within the ingested product. In addition, we discuss the proposed molecular mechanisms that are responsible for the beneficial effects. *Propionibacterium* ssp. beneficial effects are described as promising tools in the aim to maintain homeostasis and immune regulation of the gut. We discuss possible implications for the host health, with new opportunities in the context of gastrointestinal disorders treatment including inflammatory disorders like the Inflammatory Bowel Diseases (IBD) as well as colorectal cancer (CRC).

The literature review has been published in Microorganisms as:
RABAH, HOUEM; ROSA DO CARMO, FILLIPE; JAN, GWÉNAËL. Dairy Propionibacteria: Versatile Probiotics. Microorganisms, v. 5, p. 24, 2017.

CHAPITRE 1. REVUE DE LITTERATURE. ETAT DE L'ART SUR LES PROPIONIBACTERIES COMME PROBIOTIQUES

Dans ce chapitre, nous apportons une revue de littérature sur les bactéries propiopniques laitières, avec une attention particulière à *Propionibacterium freudenreichii*, une bactérie largement utilisée dans l'industrie alimentaire comme levain d'affinage de fromages, comme bio-conservateur, et comme additif bénéfique. Les *Propionibacterium* ssp. Attirent de plus en plus l'attention, du fait de leur propriétés probiotiques prometteuses. Ces dernières sont liées à des aptitudes versatiles telles que la production d'acides gras à chaine courte (AGCCs), d'acides gras conjugués, de protéines de surface bioactives, d'acide 1,4-dihydroxy-2-naphtoïque (DHNA, le facteur de croissance bifidogène), de vitamine B12, ainsi qu'à une grande aptitude à endurer les conditions digestives stressantes, à adhérer aux cellules épithéliales intestinales et à des propriétés immunomodulatrices. Nous insistons sur l'importance des conditions de croissance pour améliorer la production de métabolites. Nous proposons les matrices alimentaires fermentées comme des moyens clés d'améliorer les propriétés probiotiques des propionibactéries comprise dans le produit ingéré. De plus, nous discutons les mécanismes moléculaires proposés comme responsables des effets bénéfiques. Les effets bénéfiques des propionibactéries sont décrits comme des outils prometteurs pour maintenir l'homéostasie et la régulation immune au niveau intestinal. Nous discutons les possibles implications pour la santé de l'hôte, avec de nouvelle opportunité dans le contexte du traitement de désordres intestinaux, y compris les troubles inflammatoires, tels que les maladies inflammatoires chroniques de 'intestin (MICI), de même que le cancer colorectal (CCR).

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Dairy Propionibacteria: Versatile Probiotics

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Abstract: Dairy propionibacteria are used as cheese ripening starters, as biopreservative and as beneficial additives, in the food industry. The main species, *Propionibacterium freudenreichii*, is known as GRAS (Generally Recognized As Safe, USA, FDA). In addition to another dairy species, *Propionibacterium acidipropionici*, they are included in QPS (Qualified Presumption of Safety) list. Additional to their well-known technological application, dairy propionibacteria increasingly attract attention for their promising probiotic properties. The purpose of this review is to summarize the probiotic characteristics of dairy propionibacteria reported by the updated literature. Indeed, they meet the selection criteria for probiotic bacteria, such as the ability to endure digestive stressing conditions and to adhere to intestinal epithelial cells. This is a prerequisite to bacterial persistence within the gut. The reported beneficial effects are ranked according to property's type: microbiota modulation, immunomodulation, and cancer modulation. The proposed molecular mechanisms are discussed. Dairy propionibacteria are described as producers of nutraceuticals and beneficial metabolites that are responsible for their versatile probiotic attributes include short chain fatty acids (SCFAs), conjugated fatty acids, surface proteins, and 1,4-dihydroxy-2-naphtoic acid (DHNA). These metabolites possess beneficial properties and their production depends on the strain and on the growth medium. The choice of the fermented food matrix may thus determine the probiotic properties of the ingested product. This review approaches dairy propionibacteria, with an interest in both technological abilities and probiotic attributes.

Keywords: dairy propionibacteria; probiotic; metabolites; inflammation; gut microbiota; delivery vehicle; functional food; fermented food

Introduction

Propionibacteria are high-GC content, non-motile, non-spore forming, anaerobic to aerotolerant, gram-positive bacteria, which belong to the actinomycetales order. They are therefore highly distinct from low GC content firmicutes, which include lactic acid bacteria. Actinomycetales comprise bacterial species with a mycelium-like aspect, found in various environments, including animal hosts and soil, and are known for a prolific production of small molecules, including antimicrobials. Propionibacteria morphology is described as pleomorphic rods, or small cocci, arranged in pairs, short

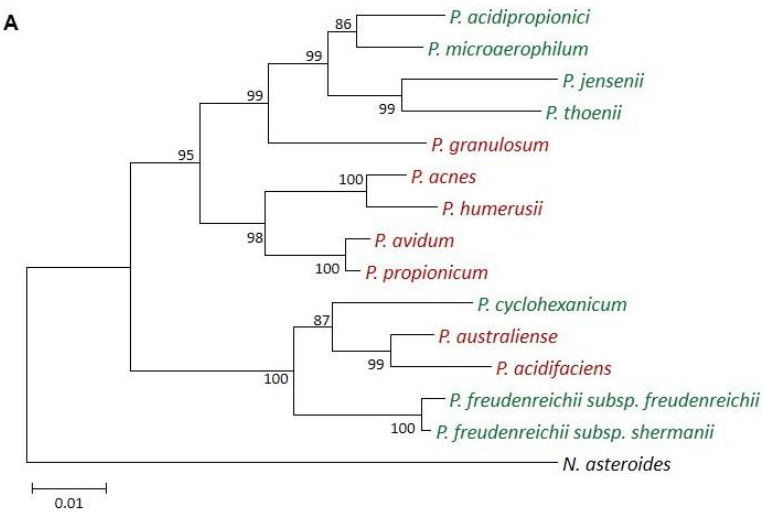
chains or clusters resembling “Chinese characters” [1].

The genus *Propionibacterium* comprises both cutaneous species (including the well-known *P. acnes*), which may act as opportunistic pathogens, and dairy species, which have no reported adverse effects. Figure 1 shows the different species as well as their phylogenetic repartition as described by McDowell et al. (2013) [2]. The dairy species *Propionibacterium freudenreichii* and *Propionibacterium* acidipropionici are clearly distinct from cutaneous species. *P. freudenreichii* has the GRAS (Generally Recognized As Safe) status in accordance with a long and documented history of safe use in food [3].

P. freudenreichii is widely cultivated and consumed by humans in fermented dairy products such as Swiss-type cheese and in food probiotic supplements. *P. freudenreichii* and *P. acidipropionici* have also been listed in the QPS (Qualified Presumption of Safety) list by the European food safety authority [3]. More generally, dairy propionibacteria have attracted attention as potent probiotics. A probiotic is defined as “a live microorganism which, when administrated in adequate amount, confers a health benefit on the host” [4]. Recent data also suggest the ability of some dairy propionibacteria metabolites to be used as prebiotics [5,6], such as 1,4-dihydroxy-2-naphtoic acid (DHNA): a selectively fermented ingredient that allows specific changes, both in the composition and/or activity in the gastrointestinal microflora that confer benefits [7–13].

Genome sequencing of P. freudenreichii and P. acidipropionici revealed the genetic basis of their great ability to adapt to various environments [14,15]. Moreover, they display a peculiar fermentative metabolism, which relies on propionic fermentation and may use various carbon and energy sources, releasing in the extracellular medium various beneficial metabolites. Recently, an accumulation of promising data, both *in vitro* and *in vivo*, evidenced a strong potential as probiotic bacteria in food, able to modulate beneficially the gut microbiota, metabolism, physiology and immunity through valuable metabolites [5]. This review will thus focus on their beneficial effects, their molecular mechanism of action and their applications.

A



B

Dairy (classical) propionibacteria No pathogenicity	**Cutaneous propionibacteria** Opportunistic pathogens
P. acidipropionici	*P. acidifaciens*
P. cyclohexanicum	*P. acnes*
P. thoenii	*P. australiense*
P. jensenii	*P. avidum*
P. microaerophilum	*P. granulosum*
P. freudenreichii subsp. *shemanii*	*P. propionicum*
subsp. *freudenreichii*	*P. humerusii*

Figure 1. (**A**) Propionibacteria minimum evolution phylogenetic tree based on 16S rDNA sequences. The 16S rDNA sequence of the Actinomycetale *Nocardia asteroides* was used as a distant outgroup to root the tree. Adapted from McDowell et al. [2]; (**B**) Repartition of *Propionibacterium* species in two distinct groups. The species formerly known as *P. inoccuum* and *P. lymphophilum* have been reclassified as *Propioniferax innocua* and *Propionimicrobium lymphophilum* respectively. *P. freudenreichii* received the GRAS (generally recognized as safe) status. Adapted from Cousin et al. [5]. Dairy species are presented in green and cutaneous ones in red.

Gut Persistence of Dairy Propionibacteria

Digestive Stress Tolerance

The Gastrointestinal tract is a complex ecosystem where physicochemical environment is unfavorable to exogenous microorganisms. A probiotic microorganism must be able to persist in the host gut, to deliver and produce beneficial metabolites. Therefore, tolerance to digestive stresses is one of the main factors limiting the use of microorganisms as live probiotic agent [16,17].
Gastric acid and bile salts are defense mechanisms encountered during intestinal transit whereas pancreatic secretions can also exert some antimicrobial activity via digestive enzymes. Dairy propionibacteria are particularly hardy and robust, compared to other probiotics, which is in accordance with their ecology. They show high tolerance *in vitro* to stimulated human upper gastrointestinal tract conditions, depending on species and strain type. The growth or delivery medium may also provide protection [18–24]. The tolerance response results in various modifications such as morphological changes or proteins expression. During exposure to acid and bile salts, *P. freudenreichii* expresses general stress proteins and induces regulatory genes involved in cellular response to membrane perturbation, oxidative stress and DNA damage [25–28]. *P. acidipropionici* showed the same high tolerance response to acid stress [29,30]. Microbiota competition for nutrients constitutes also a limiting factor for dairy propionibacteria persistence in the gut. However, dairy propionibacteria are able to metabolize various carbon and nitrogen sources, to produce reserve compounds such as polyphosphate, glycogen and trehalose, which also have an osmoprotectant role [22,31]. These results were reinforced by *in vivo* studies; *P. freudenreichii* was shown to maintain a metabolic activity, in addition to survival, within the human and animal digestive tracts [9,32–35]. Indeed, *P. freudenreichii* orients its genome expression towards the use of intestinally available substrates such as propanediol, gluconate and lactate, to sustain its metabolism, thus avoiding starvation during digestive transit [35]. Their concentration reached the adequate bacterial amount in the gut for probiotic applications.

Adhesion to the Gut Epithelium

Besides the ability to withstand digestive stresses, probiotic microorganisms should persist in the digestive tract to interact with host cells and exert their expected beneficial effects. Probiotics lifespan in the digestive tract will depend on their capacity to adhere to intestinal mucosa and on their growth rate. Propionibacteria species have a slow growth rate, so that adhesion and adaptation constitute the bottleneck of their beneficial effects within the host. Numerous studies showed the capacity of
P. acidipropionici and *P. freudenreichii* to adhere to human and animal intestinal cells [19,36,37], as well as to human and animal intestinal mucus [38–40]. Nonetheless, the adhesion rate evaluated *in vitro* varied between 0.03 to about 40%, depending on many factors as adhesion model used (cells or mucus), species types, strain type and growth or vehicle medium [19,23,24,38–40]. Figure 2 shows physical interaction of dairy propionibacteria with cultured human colon epithelial cells. *P.*

freudenreichii adhesion was evidenced even after heat inactivation [38]. Propionibacteria adhesion to intestinal cells leads to exclusion of invasive pathogenic bacteria by competitive adhesion or co-aggregation mechanisms. This concerns pathogens such as *Escherichia coli*, *Pseudomonas aeruginosa*, S*taphylococcus aureus* and *Salmonella enterica* [41–44]. Adhesion mechanisms remain poorly understood, but some preliminary experiments point out a role of surface proteins and teichoic acid in adhesion mechanisms [37]. Recently, a surface proteome study in *P. freudenreichii* evidenced the presence of two conserved proteins known to be involved in adhesion, in other bacterial species [45]. The first protein is Internalin A, which has Leucine Rich Repeat domains (LRR) known to be involved in protein/protein interaction. The second is BopA, belonging to the ABC superfamily, with an ATP binding cassette, showing homologies to a bifidobacteria adhesion protein. However, other components secreted may play a role in adhesion. Indeed *P. freudenreichii* was shown to secrete a lipopeptide having biosurfactant properties and an anti-adhesive effect on *P. aeruginosa* [43]. All these studies suggest the ability of dairy propionibacteria to adhere to the intestinal mucosa, allowing them to persist in the host. However, no *in vivo* analysis exist to assess specifically dairy propionibacteria adhesion, nonetheless some studies in animals and humans suggest there is only a transient colonization, since fecal propionibacteria population in human volunteers decreases after ceasing the ingestion of propionibacteria [34]. The beneficial effect of the promising metabolites produced by dairy propionibacteria would certainly be favored by their ability to tolerate digestive stresses and to adhere to epithelial cells, allowing close contact of the probiotic and the target cells.

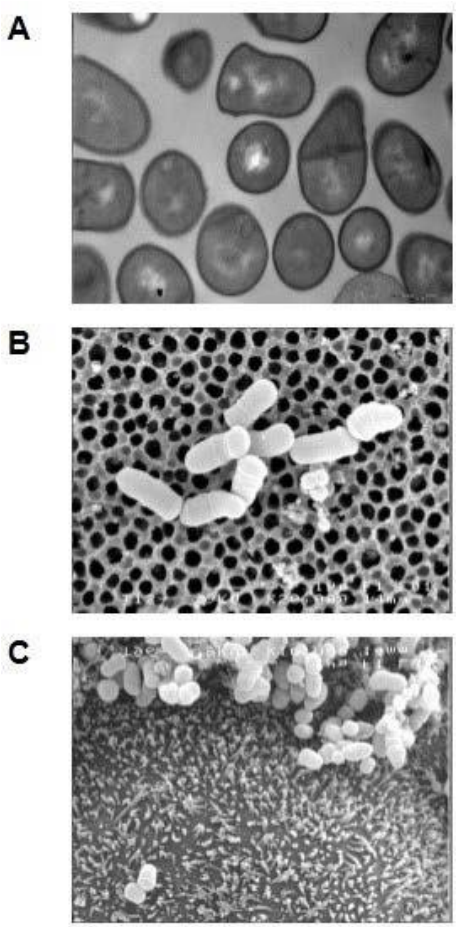


Figure 2. Electron microscopy analysis of *Propionibacterium freudenreichii*. The Propionibacteria were cultivated alone (**A**,**B**) or in contact with cultured human intestinal cell line Caco2 (**C**). Observation was made using either transmission (**A**) [46] or scanning (**B**) [26] and (**C**) (personal communication), electron microscopy.

Microbiota Modulation by Dairy Propionibacteria

The gut microbiota plays a role in several aspects of the host physiology, including metabolism, defense against pathogens, immune system maturation and brain development. An imbalance of the microbiota composition could be an initiator or a consequence of the development of much pathology, such as inflammatory diseases, colorectal cancer or *Clostridium difficile* infection. In disease contexts such as Inflammatory Bowel disease (IBD), patients present a lower microbiota diversity, which could initiate or exacerbate inflammatory disease [47]. Microbiota manipulation by fecal microbiota transplantation, prebiotic or probiotic consumption is a promising way to improve or to restore the microbiota diversity [47]. Modulation of the gut microbiota in animals and human being, as a result of *P. freudenreichii* and *P. acidipropionici* consumption, was reported in the context of colitis [7,13,48,49] and in healthy subjects [8,50–52]. These studies reported an increase in the genus of Bifidobacteria, which are well known for their positive health benefits to their host via their metabolic activities [53].

Dairy propionibacteria were also shown to decrease Bacteroides genus which possess an enterotoxin associated with the prevalence of IBD [54], and Clostridium genus, strains of which are associated with severe intestinal infections [47]. Modulation of the gut microbiota to favor symbiotic bacteria such as Bifidobacteria, and at the expense of opportunistic pathogens, is not fully understood. However, the bifidogenic effects described for dairy propionibacteria were attributed to the release of two small molecules, 1,4-dihydroxy-2-naphtoic acid (DHNA) and 2-amino-3-carboxy-1,4-naphthoquinone (ACNQ) [55]. DHNA is a vitamin K2 (or menaquinone) biosynthesis intermediate [56]. DHNA treatment was shown to restore *Lactobacillus* and *Enterobacteriacea* flora in dextran sulfate sodium (DSS)-induced-colitis in mice [13]. In addition, it induces expression of the anti-microbial C-type lectin Reg III protein family, which certainly affect microbial flora [48]. Elsewhere, ACNQ enhances the activity of NADH peroxidase and NADH oxidase in Bifidobacteria. It serves as an electron acceptor of NAD(P)H diaphorase and as an electron donor of NAD(P)H peroxidase [49,57,58]. Regeneration of these cofactors in Bifidobacteria is reported to enhance their growth. Indeed, consumption of dried cultures of the *P. freudenreichii* ET-3 strain, provided by the Japanese company Meiji, led to an enhanced population of Bifidobacteria within the human gut microbiota in healthy male and female human volunteers [50]. Similar modulation was obtained using a cell-free culture supernatant of *P. freudenreichii*, which was called bifidogenic growth stimulator (BGS), attesting the role of secreted components in the bifidogenic effects [49,51,57,59]. BGS was tested in humans at high doses, up to 45 tablets daily, without noticeable adverse effect, showing the potential and the safety of use of those components as prebiotics [60]. The use of dairy propionibacteria as an animal probiotic to modulate gut microbiota or pathogen infections is also a promising new application of dairy propionibacteria. Indeed, interesting results were obtained using *P. acidipropionici*, by slowing colonization by Bacteroides in the early stage of rearing chicks [52]. The presence of *P. acidipropionici* also limited the growth of *Bacteroidetes fragilis* and *Clostridium hystoliticum* groups in mice cecal slurries with and without fiber supplementation [61]. In addition, several strains of dairy propionibacteria were able to inhibit *in vitro Steptococcus bovis* in ruminal acidosis context [62].

Immunomodulation by Dairy Propionibacteria

Inflammatory diseases, such as inflammatory bowel disease (IBD), allergy, asthma or rheumatoid arthritis, are a public health problem and affect mainly the developed countries. These diseases are complex, and their precise etiology remains poorly understood. Risk factors related to the immune system, environment, genotype and especially the intestinal microbiota, seem to be involved. There is increasing indication of the potential of probiotics consumption, as a supplement to treatment, to limit the occurrence of some inflammatory diseases [63]. The present section focuses on immunomodulation by dairy propionibacteria in the context of IBD. However, clinical studies have demonstrated beneficial effects of dairy propionibacteria, in combination with other probiotic bacteria, to positively modulate the immune system.

IBD includes two main pathologies: ulcerative colitis (UC) and Crohn's disease (CD). They are thought to result from an abnormality of the immune response of the intestine with respect to certain components of the intestinal flora occurring in genetically predisposed individuals. There are arguments indicating that consumption of selected strains of probiotic microorganisms could play a favorable role in the treatment of UC [64,65]. *In vitro* and *in vivo* data suggest the ability of dairy propionibacteria, specifically *P. freudenreichii*, to modulate the gut immune system and alleviate the inflammation in the context of inflammatory bowel disease. In conventional mice, trinitrobenzene sulfonic acid (TNBS)-induced colitis was prevented by the consumption of *P. freudenreichii* in a strain-dependent manner. Strains inducing high levels of the regulatory cytokine interleukin 10 (IL10) in human peripheral blood mononuclear cells (PBMCs) were the most effective at alleviating TNBS-induced-colitis [66,67]. Immunomodulation exerted by selected strains of *P. freudenreichii* was further evidenced in pigs, with a decrease in plasma haptoglobulin and proinflammatory cytokines as IL-8 and tumor necrosis factor-α (TNFα) in gut mucosa, after lipopolysaccharides (LPS) stimulation ex vivo [8]. Recently, a probiotic mixture containing both *Lactobacillus rhamnosus* and *P. freudenreichii* was tested in humanized mice consuming a high-fat diet. It tended to down-regulate both intestinal and systemic pro-inflammatory changes induced by the diet [68]. When tested in irritable bowel syndrome patients (IBS), it alleviated the symptoms of IBS and stabilized the gut microbiota [69]. In a pilot study, patients with active ulcerative colitis receiving BGS experienced an improvement of the clinical activity index score [49,70]. Dairy propionibacteria exert anti-inflammatory effects through different components that seem to trigger different molecular mechanisms.

Surface Layer Proteins

Different propionibacteria compounds were reported for potential anti-inflammatory effects, including surface proteins called S-layer proteins (Slps). They form a surface-exposed proteinaceous network, which is present in many Gram-positive bacteria other than propionibacteria, as well as in archaebacteria [71]. Slps proteins are non-covalently anchored to the cell wall via S-layer homology domains (SLH). In *P. freudenreichii*, the annotation of the genome revealed the presence of seven genes encoding putative Slps proteins [14]. However, only three Slps proteins were identified by proteomic analysis (SlpA, SlpB and SlpE).The identified internalin A (InlA) also has SLH domains but is not considered as an Slp [45]. The family of genes encoding the Slps proteins exhibits a wide variety of sequences between species but also within the same species, in accordance with the great functional diversity of these proteins: adhesion, virulence factors, transport of molecules, masking of receptors to phages, and protection against environmental stresses [72,73]. In order to demonstrate the immunomodulatory properties of the *P. freudenreichii* Slps, selective extraction of these proteins by guanidine chloride was carried out. Treatment of PBMCs with this protein mixture induced the release of regulatory interleukin IL-10, in a dose-dependent manner, with

little or no secretion of pro-inflammatory factors (IL-12, TNF-α and IL6) [45]. Moreover, this extract, when applied in conjunction with a proinflammatory strain such as *Lactococcus lactis* MG1363, considerably reduces the induction of the proinflammatory cytokines IL-12, IFN-γ and TNF-α by this strain. This confirms that extractible surface proteins modulate the release of immunomodulatory cytokines. In order to identify the immunomodulatory properties of each surface protein, the *P. freudenreichii* CIRM-BIA 129 strain, which has a very marked anti-inflammatory profile, has been mutated for the *slp b and slp e* genes [74]. The mutations induced suppression of the anti-inflammatory effect of this strain on human PBMCs, this property seem to be a result not of the presence of one protein, but of a combination of several surface proteins [74]. Some of the strains of *P. freudenreichii* that fail to modulate the immune response are covered by an extracellular capsule of exopolysaccharides (EPS) [75,76]. Removal of this EPS (by mutational inactivation) unmasks surface proteins and confers immunomodulatory properties to the mutant [75,76]. This indicates a key role of surface proteins as Microbe-Associated Molecular Patterns (MAMPs) in this probiotic/host cross-talk, with promising anti-inflammatory applications. Indeed, as demonstrated for *Lactobacillus acidophilus* S-layer protein A, dairy propionibacteria S layer proteins are supposed to interact with immune cells as dendritic cells via specific receptors [77,78], inducing tolerance response leading to attenuated colonic inflammation.

Short Chain Fatty acids (SCFAs)

Different metabolites known for immune system modulation include the short chain fatty acids (SCFAs). SCFAs are produced mainly in the colon by colonic bacteria. Butyrate (C4), propionate (C3) and acetate (C2) are the major SCFAs produced by fiber or complex carbohydrate fermentation to be used as an energy source by mainly colonocytes and hepatocytes. Dairy propionibacteria produce mainly acetate and propionate in ratio 2:1 by anaerobic fermentation of carbohydrates or organic acids. There is a wealth of published scientific data on the central role of SCFAs in the regulation of the intestinal immune system [79–81]. Indeed, SCFAs impact on intestinal immunity will depend on the existing immune environment. Concerning dairy propionibacteria, a transcriptomic analysis of HT29 cells showed a modulation by *P. freudenreichii* or SCFAs treatments of NOD-like receptors and cytokine-cytokine receptor interaction gene expression, known to play a role in immune response [10]. In addition, an HDAC inhibitory activity was highlighted, which demonstrates the potential of dairy propionibacteria to modulate gut inflammation through SCFAs. HDACs inhibition activity degree varies with SCFAs nature (Butyrate > propionate > acetate) [80]. HDAC inhibition seems to be induced in part by SCFAs activated G protein–coupled receptors. Their activation by SCFAs modulates gut inflammation through regulation of activation, proliferation and differentiation of immune and epithelial cells [80–82].

Conjugated Fatty Acids

Another promising beneficial metabolic activity is the ability, shared by other probiotics, to convert free linoleic acid (LA, C18:2), α-linolenic (LNA, C18:3), γ-linolenic (GLA, C18:3) and stearidonic acids (SA, C18:4) into their respective conjugated fatty acid (CLA, CLNA, CGLA and CSA) [83–86]. Conjugated fatty acids (CFAs) are a mixture of a number of geometric and positional isomers of octadecadienoic acids. Until today, there have been a few studies reporting the ability of propionibacteria to produce some CFA isomers [83,84,86–88], however their biological effects have not been investigated. CFAs production by dairy propionibacteria is a way to cope with the inhibitory effect of fatty acids on bacterial growth [88]. Dairy propionibacteria, such as *P. freudenreichii*, produce the isomer cis-9, trans-11 octadecadienoic (Rumenic acid, RA) in culture

and in fermented dairy products from LA; and isomerize also the c12-double bond of LNA and γ-linolenic acid [84,85,87,89]. Nonetheless, animals and clinical studies report the anti-inflammatory effects of CLA and CLNA including those produced by dairy propionibacteria, at different level of modulation, according to isomer type, by acting as PPARγ agonists. PPARγ (peroxisome proliferator-activated receptor γ) is a nuclear receptor forming obligate heterodimer with retinoid X receptor (RXR). PPARγ activation by CFAs can regulate the expression of its target genes involved in adipogenesis, lipid metabolism, inflammation and maintenance of metabolic homeostasis [90,91]. It also interferes with other proteins and transcription factors such as NF-κB and AP-1 through repression mechanisms [91]. Indeed, CLA and CLNA decrease antigen-induced proinflammatory mediators [92–94], modulate immune cells proliferation and differentiation [90,95], and limit adverse effects of colonic inflammation [90,91,95,96]. For dairy propionibacteria, the administration of cheeses matrix containing *P. freudenreichii*, alone or in combination with lactic acid bacteria, was shown to increase *Pparg* mRNA levels in the colon of mice during TNBS-induced colitis [7,12]. This effect could be attributed to the presence of CFAs in fermented cheeses, but additional analysis is required to establish a link between the increase of *Pparg* gene expression and CFA production by dairy propionibacteria.

DHNA

DHNA, described above for its bifidogenic property, exerts an anti-inflammatory effect in different murine colitis models, as murine DSS-colitis and IL10-/- mice that develop spontaneous colitis. DHNA reduces the expression of cell adhesion molecules, as MAdCAM-1 or VCAM-1, depending on colitis model [11,13]. In IBD patients, those adhesion molecules are highly expressed, which aggravates the inflammation by increasing immune cells' infiltration of tissues. The lymphocyte infiltration observed in experimental mice colitis was clearly diminished by DHNA administration. In addition, DHNA reduces proinflammatory cytokine expression *in vivo* and *in vitro* within macrophage cells after endotoxin stimulation [48]. DHNA activate the aryl hydrocarbon receptor (AhR), an important transcriptional factor involved in inflammation. AhR activation seems to be involved in the inhibition of secretion of proinflammatory cytokines. Indeed, the inhibition of proinflammatory cytokine IL6 in LPS-stimulated macrophages was related to AhR activation by DHNA [48].

Anti-Cancerous Effect

According to WHO, cancers represent a leading cause of morbidity and mortality worldwide, with approximately 8.2 million deaths caused by cancers in 2012. Colorectal cancer, the fourth most common cause of cancer death, is considered to be a Westernized disease with high incidence rates (in North America, Australia, New Zealand and Europe (>40 cases per 100,000). On one hand, significant associations between unhealthy dietary factors and colorectal cancer risk have been demonstrated by several studies [97,98]. On the other hand, gut microbiota appears to govern gut inflammation and colorectal cancer development, specifically through its metabolites. Managing gut microbiota modulation by probiotic and prebiotic consumption may modify gut microbiota metabolism to achieve the objective of preventing colorectal cancer in western societies [97,98].

Short Fatty Acids

Dairy propionibacteria, including strains of *P. acidipropionici*, *P. freudenreichii* subsp *freudenreichii* and *P. freudenreichii* subsp *shermanii*, have been shown to possess the ability to induce apoptosis in colorectal and gastric cancer cells *in vitro* (HT29, Caco2, and HGT-1 cancerous cell lines) [10,99–101] and *in vivo* within human microbiota associated rats [102]. Propionate and

acetate, produced by dairy propionibacteria, were identified as the main actors of this effect [10,99–101]. The pro-apoptotic action, exerted on cultured colon cancer cells and confirmed in an animal model of carcinogenesis, was studied in details at the cellular and molecular level. As demonstrated for butyrate, the anti-cancerous effect of propionibacterial SCFA consists in inducing apoptosis. The apoptotic intrinsic pathway is activated: SCFAs act on the mitochondria adenine nucleotide transclactor (ANT) pore, causing mitochondria depolarization and permeabilisation, leakage of cytochrome C and caspase activation [99–101]. Furthermore, Cousin et al. (2016) showed that these SCFAs, in combination with TNF-Related Apoptosis-Inducing Ligand (TRAIL) treatment, increased its cytotoxicity, by enhancing TRAIL-R2/DR5 expression in HT29 cells. TRAIL is a death receptor, a tumor necrosis factor receptor superfamily member, which mediates apoptosis by activating the extrinsic apoptotic death pathway [7]. In parallel, such combinations lead to a modulation of genes expression involved in apoptosis, decreasing $FLIP_L$ and XIAP expression, which are two apoptosis inhibitors regulating extrinsic and intrinsic cell death pathways, respectively [10]. In addition, inhibition of histone deacetylase (HDAC) in HT29 cells by SCFAs leads to growth inhibition and cell cycle arrest by expression of p21, which was observed at transcriptomic and proteomic levels [10].

Conjugated Fatty Acids

In vitro and *in vivo* studies showed the anti-proliferative effect of conjugated fatty acids, including those produced by dairy propionibacteria [84,86–89], on various cancerous cells such as melanoma, colorectal, breast, prostate and hepatoma cell lines [92]. The anti-proliferative kinetic is time and dose dependent. Furthermore, the mechanism and effectiveness vary with the CFAs type—conjugated linolenic acid is more efficient than conjugated linoleic acid—and with isomer type of a particular CFA [93,95]. For some CFAs isomers, the anti-proliferative mechanisms on cancerous cells were characterized at a molecular level: cycle cell arrest, lipid peroxidation and activation of apoptotic pathways are induced by CFA treatment *in vitro* and *in vivo*.

Concerning human studies, lower serum levels of CLA in post-menopausal Finnish women was associated with high cancer occurrence, and milk consumption was inversely related to breast cancer risk. In addition, diverse clinical trials suggested the beneficial impact of conjugated fatty acids for prevention or treatment of cancers. As a consequence, biohydrogenation of polyunsaturated fatty acids by probiotic bacteria such as dairy propionibacteria opens the possibility of developing dairy or vegetable fermented food from ingredients rich in polyunsaturated fatty acid, designed for high-risk cancer populations.

Bioremediation against Carcinogenic Components

Dairy propionibacteria, among other probiotic bacteria, have the ability to bind *in vitro* and *in vivo* to heavy metals or toxins associated with high risk of cancers, which may contribute to reduced risk factors for cancer development. *P. acidipropionici* showed capacity to remove plant lectins such as concanavalin A and jacalin from colonic cells, which reduced lectins' toxicity for intestinal cells [103]. Furthermore, selected strains of dairy propionibacteria and of lactic acid bacteria block the intestinal absorption of aflatoxin B1 and remove heavy metals such as cadmium and lead, in a strain-dependent manner [104–108]. The detoxification effect on aflatoxin B1 of dairy propionibacteria was attested by a clinical trial on the population of Southern china continuously exposed to aflatoxin contamination [109].

No clinical evidence on propionibacteria consumption within colorectal cancer (CRC) patients exists, however *P. freudenreichii* uptake by humans increases SCFAs in feces, suggesting the possibility of modulating gut SCFAs concentration with the aim of preventing CRC occurrence.

When tested in healthy men, a probiotic mixture containing both lactic acid bacteria and propionibacteria [110,111], led to a reduction in fecal α-glucosidase, which is associated with carcinogenesis.

Impact of Vectorization on Probiotic Properties

Probiotics are commonly consumed under the form of dried microorganisms, in capsules or tablets. The development of functional foods fermented by dairy propionibacteria is a promising research area. The health benefits of dairy propionibacteria are strain-dependent, but the delivery vehicle also plays a crucial role, which remains barely investigated. Indeed, the matrix affects the metabolites amount or/and the bacterial capacity to persist in the gut. As demonstrated by Cousin et al (2012), the anti-inflammatory effect of *P. freudenreichii* was enhanced upon growth in milk ultrafiltrate medium [9], which could be explained by an enhanced Slp expression compared to a classical growth medium. The biohydrogenation of polyunsaturated acids by dairy propionibacteria may enhance CFAs content in fermented dairy products, but could be limited by the low polyunsaturated acid availability in the matrix. Natural sources of conjugated fatty acids are very limited and are relatively low; the addition of CFA chemically synthesized cannot remedy to some isomers deficiency in foods. Vegetal matrices such as soya, rich in CL and CLN, could be employed to develop new foods enriched in conjugated fatty acids, by dairy propionibacteria fermentation. Similarly, SCFAs production is proportionally related to the amount of fermentable substrates in the medium. Dairy products are naturally rich in lactose, both lactate and lactose can be used by dairy propionibacteria. The delivery vehicle also affects the tolerance response of dairy propionibacteria to digestive stresses and adhesion to cells, depending on its biochemical composition, its physical microstructure and the existing microbial ecosystem [23,24], which directly affect the viable bacterial amount reaching the gut. Growth of dairy propionibacteria on stressful mediums such as fermented dairy products confers a high tolerance to acid and bile salt stresses *in vitro* and *in vivo* [20]. In addition, dairy products with a high osmotic pressure enhance storage of trehalose, glycogen and polyphosphate, which could improve nutrients deficiency tolerance in the gut [22]. Some clinical trials confirmed the matrix effect; a probiotic mixture including *P. freudenreichii* was also tested in humans either in conventional capsules, in yogurt or in cheese. The highest fecal quantity of *P. freudenreichii* was yielded by yogurt [112]. Accordingly, yogurt was shown in a French human study to favor not only survival, but also metabolic intestinal activity of
P. freudenreichii [34].

Technological Applications of Dairy Propionibacteria

Swiss-Cheese Manufacturing

Dairy propionibacteria, especially *P. freudenreichii*, are mainly employed as a ripening starter for Swiss-type cheese manufacturing, such as Emmental cheese. They contribute to their characteristic flavor and openings [1,113,114]. The openings are due to the production of carbon dioxide (CO_2), produced during fermentation of lactate and aspartate. These latter are generated in cheese during degradation of lactose and proteins, respectively, by lactic acid bacteria. The ability and the intensity of aspartate metabolism in *P. freudenreichii* are strain-dependent; a high aspartate metabolism leads to a higher proportion of CO_2. This high aspartate catabolism can be associated with an opening imperfection during Emmental cheese ripening, resulting in the formation of slits and cracks [1,113]. The typical Swiss-type cheese flavor is due mainly to the presence of dairy propionibacteria that produces flavor compounds by three metabolism pathways: lactate and aspartate fermentation,

fat hydrolysis, and amino acid catabolism. The lactate and aspartate fermentations lead to the production of organic acids, mainly propionate, succinate, and acetate acid, which are considered to be principal flavor compounds. Free fatty acids are also important for cheese flavor, the lipolytic activity during cheese ripening is mainly due to dairy propionibacteria in strain-dependent manner [113,115]. The third compound is the branched-chain volatile molecules, which are formed from branched-chain amino acid catabolism. The two branched-chain compounds produced by *P. freudenreichii* are 2-methylbutanoic acid and isovaleric acid. In Emmental cheeses, *P. freudenreichii* reaches a high population, with counts over 10^9 cfu/g of cheese, depending in ripening period. The high tolerance of *P. freudenreichii* to different stresses allows this population to be reached. Indeed, during the cheese manufacturing process, dairy propionibacteria face different stresses, such as high and low temperature, acidification, osmotic stress induced by NaCl; their robustness, compared to the other dairy species, would be responsible for the prevalence of this species in Swiss-type cheeses [113]. Dairy propionibacteria can also be implemented in low amounts in various cheeses without openings to enhance flavor formation [1].

Production of Nutritional Molecules

Dairy propionibacteria produce several nutritional molecules essential to human health such as B vitamins (including cobalamin and folic acid). Indeed, *P. freudenreichii* is the only B12 producer known to be a GRAS bacteria [114]. Vitamin B12 (or cobalamin) is synthesized as a cofactor for propionic acid fermentation. Vitamin B12 is an essential vitamin, required for maintaining healthy nerve cells, for the production of cell's genetic material and energy, and for other important functions. Vitamin B12 has been industrially produced for a long time by chemical synthesis, which requires more than 70 steps by a chemical method [116]. This production method is too difficult and expensive, compared to the biosynthesis dairy propionibacteria [116,117]. The pathway of vitamin B12 synthesis in *P. freudenreichii* has been completely characterized, and important efforts have been made to improve vitamin B12 biosynthesis by implementing random mutagenesis, genetic engineering and by optimizing fermentation conditions [116,117]. The DHNA, described above as a Vitamin K precursor, has also a potential application as prebiotic to enhance intestinal Bifidobacteria population. There is no industrial production of DHNA, nonetheless some studies investigated how to improve DHNA production by manipulating fermentation conditions [56,114].

Production of Antimicrobial Molecules

Propionibacterium spp strains are widely used as food biopreservatives for their antimicrobial activity. They were shown to suppress the growth of mold and undesirable microorganisms in many foods, which prolong their shelf-life [1,6,16,116,117]. Propionic acid is the main anti-microbial molecule produced by dairy propionibacteria. It is commercially available as a Microgard™ product, composed of skim milk fermented by *P. freudenreichii* subsp *shermanii* [116]. The microbial production of propionic acid is limited by parameters including low productivity and low conversion efficiency. However, *P. acidipropionici* species were shown to produce a high amount of propionic acid, by glycerol fermentation, without acetic acid production [117]. Other organic acids are also considered as anti-microbial molecules, including acetic, succinic and lactic acids [116]. In *P. jensenii*, 2-pyrrolidone-5-carboxylic acid, 3-phenyllactic acid, hydroxyphenyl lactic acid and 3-phenyllactic acid were shown to have antimicrobial activity [117]. In addition, different bacteriocins produced by both dairy and cutaneous propionibacteria have been reported and characterized. Bacteriocins are antimicrobial peptides or proteins and are active against other propionibacteria, lactic acid bacteria, other Gram positive bacteria, Gram negative bacteria, yeast and molds. To date, there is no bacteriocin from dairy propionibacteria recognized as GRAS by FDA;

more investigation is needed to evaluate their potential application as food biopreservatives or bacteriocin-producer probiotics to inhibit intestinal pathogens.

Conclusion

The studies reviewed here allowed the development of different tools to screen and elucidate the beneficial properties of dairy propionibacteria strains. Not only the phenotypic traits, but also the molecular bases of probiotic effects are being made available. Dairy propionibacteria are used for various purposes and eaten in various food products. Strains used in the food industry are screened based on technological properties but not on health properties. On the other hand, the technological abilities of probiotic bacteria to produce a fermented food product are rarely studied. Screening of wide collections of propionibacteria for technological and probiotic properties should lead to development of new functional foods. Indeed, specific populations with health problems linked to developed countries life style (intolerance, allergy, inflammation, cancer) will need specific diets. In this context, propionibacteria can play a key role via the modulation of key parameters such as inflammation.

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References

Thierry, A.; Falentin, H.; Deutsch, S.M.; Jan, G. *Propionibacterium* spp. In *Encyclopedia of Dairy Sciences*; Fox, P.F., McSweeney, P.L., Fuquay, J.W., Eds.; Academic Press: San Diego, CA, USA, 2011; Volume 1, pp. 403–411.

McDowell, A.; Nagy, I.; Magyari, M.; Barnard, E.; Patrick, S. The Opportunistic Pathogen Propionibacterium acnes: Insights into Typing, Human Disease, Clonal Diversification and CAMP Factor Evolution. *PLoS ONE* **2013**, *8*, e70897. [CrossRef] [PubMed]

EFSA Panel on Biological Hazards (BIOHAZ) Scientific Opinion on the maintenance of the list of QPS biological agents intentionally added to food and feed (2013 update): QPS 2013 update. *EFSA J.* **2013**, *11*, 3449.

Food and Agriculture Organization. *WHO Probiotics in Food: Health and Nutritional Properties and Guidelines for Evaluation*; FAO: Rome, Italy, 2006.

Cousin, F.J.; Mater, D.D.G.; Foligne, B.; Jan, G. Dairy propionibacteria as human probiotics: A review of recent evidence. *Dairy Sci. Technol.* **2010**, *91*, 1–26. [CrossRef]

Altieri, C. Dairy propionibacteria as probiotics: Recent evidences. *World J. Microbiol. Biotechnol.* **2016**, *32*, 172. [CrossRef] [PubMed]

Plé, C.; Richoux, R.; Jardin, J.; Nurdin, M.; Briard-Bion, V.; Parayre, S.; Ferreira, S.; Pot, B.; Bouguen, G.; Deutsch, S.-M.; et al. Single-strain starter experimental cheese reveals anti-inflammatory effect of *Propionibacterium freudenreichii* CIRM BIA 129 in TNBS-colitis model. *J. Funct. Foods* **2015**, *18*, 575–585. [CrossRef]

Cousin, F.J.; Foligné, B.; Deutsch, S.-M.; Massart, S.; Parayre, S.; Le Loir, Y.; Boudry, G.; Jan, G. Assessment of the Probiotic Potential of a Dairy Product Fermented by *Propionibacterium freudenreichii* in Piglets. *J. Agric. Food Chem.* **2012**, *60*, 7917–7927. [CrossRef] [PubMed]

Cousin, F.J.; Louesdon, S.; Maillard, M.-B.; Parayre, S.; Falentin, H.; Deutsch, S.-M.; Boudry, G.; Jan, G. The first dairy product exclusively fermented by *Propionibacterium freudenreichii*: A new vector to study probiotic potentialities in vivo. *Food Microbiol.* **2012**, *32*, 135–146. [CrossRef] [PubMed]

Cousin, F.J.; Jouan-Lanhouet, S.; Théret, N.; Brenner, C.; Jouan, E.; Moigne-Muller, G.L.; Dimanche-Boitrel, M.-T.; Jan, G.; Cousin, F.J.; Jouan-Lanhouet, S.; et al. The probiotic *Propionibacterium freudenreichii* as a new adjuvant for TRAIL-based therapy in colorectal cancer. *Oncotarget* **2016**, *7*, 7161–7178. [PubMed]

Okada, Y.; Tsuzuki, Y.; Narimatsu, K.; Sato, H.; Ueda, T.; Hozumi, H.; Sato, S.; Hokari, R.; Kurihara, C.; Komoto, S.; et al. 1,4-Dihydroxy-2-naphthoic acid from *Propionibacterium freudenreichii* reduces inflammation in interleukin-10-deficient mice with colitis by suppressing macrophage-derived proinflammatory cytokines.
J. Leukoc. Biol. **2013**, *94*, 473–480. [CrossRef] [PubMed]

Plé, C.; Breton, J.; Richoux, R.; Nurdin, M.; Deutsch, S.-M.; Falentin, H.; Hervé, C.; Chuat, V.; Lemée, R.; Maguin, E.; et al. Combining selected immunomodulatory *Propionibacterium freudenreichii* and *Lactobacillus delbrueckii* strains: Reverse engineering development of an anti-inflammatory cheese. *Mol. Nutr. Food Res.* **2016**, *60*, 935–948. [CrossRef] [PubMed]
Okada, Y. *Propionibacterium freudenreichii* component 1.4-dihydroxy-2-naphthoic acid (DHNA) attenuates dextran sodium sulphate induced colitis by modulation of bacterial flora and lymphocyte homing. *Gut* **2006**, *55*, 681–688. [CrossRef] [PubMed]
Falentin, H.; Deutsch, S.-M.; Jan, G.; Loux, V.; Thierry, A.; Parayre, S.; Maillard, M.-B.; Dherbécourt, J.; Cousin, F.J.; Jardin, J.; et al. The Complete Genome of *Propionibacterium freudenreichii* CIRM-BIA1T, a Hardy Actinobacterium with Food and Probiotic Applications. *PLoS ONE* **2010**, *5*, e11748. [CrossRef] [PubMed]
Parizzi, L.P.; Grassi, M.C.B.; Llerena, L.A.; Carazzolle, M.F.; Queiroz, V.L.; Lunardi, I.; Zeidler, A.F.; Teixeira, P.J.; Mieczkowski, P.; Rincones, J.; et al. The genome sequence of Propionibacterium acidipropionici provides insights into its biotechnological and industrial potential. *BMC Genom.* **2012**, *13*, 562. [CrossRef] [PubMed]
Amund, O.D. Exploring the relationship between exposure to technological and gastrointestinal stress and probiotic functional properties of lactobacilli and bifidobacteria. *Can. J. Microbiol.* **2016**, *62*, 715–725. [CrossRef] [PubMed]
Corcoran, B.; Stanton, C.; Fitzgerald, G.; Ross, R. Life under Stress: The Probiotic Stress Response and How it may be Manipulated. *Curr. Pharm. Des.* **2008**, *14*, 1382–1399. [CrossRef] [PubMed]
Huang, Y.; Adams, M.C. In vitro assessment of the upper gastrointestinal tolerance of potential probiotic dairy propionibacteria. *Int. J. Food Microbiol.* **2004**, *91*, 253–260. [CrossRef] [PubMed]
Campaniello, D.; Bevilacqua, A.; Sinigaglia, M.; Altieri, C. Screening of *Propionibacterium* spp. for potential probiotic properties. *Anaerobe* **2015**, *34*, 169–173. [CrossRef] [PubMed]
Gagnaire, V.; Jardin, J.; Rabah, H.; Briard-Bion, V.; Jan, G. Emmental Cheese Environment Enhances *Propionibacterium freudenreichii* Stress Tolerance. *PLoS ONE* **2015**, *10*, e0135780. [CrossRef] [PubMed]
Martinovic, A.; Brede, M.E.; Vegarud, G.E.; Østlie, H.M.; Narvhus, J.; Skeie, S.B. Survival of lactic acid and propionibacteria in low- and full-fat Dutch-type cheese during human digestion ex vivo. *Lett. Appl. Microbiol.* **2016**, *62*, 404–410. [CrossRef] [PubMed]
Huang, S.; Rabah, H.; Jardin, J.; Briard-Bion, V.; Parayre, S.; Maillard, M.-B.; Le Loir, Y.; Chen, X.D.; Schuck, P.; Jeantet, R.; et al. Hyperconcentrated Sweet Whey, a New Culture Medium That Enhances *Propionibacterium freudenreichii* Stress Tolerance. *Appl. Environ. Microbiol.* **2016**, *82*, 4641–4651. [CrossRef] [PubMed]
Ranadheera, C.S.; Evans, C.A.; Adams, M.C.; Baines, S.K. Effect of dairy probiotic combinations on in vitro gastrointestinal tolerance, intestinal epithelial cell adhesion and cytokine secretion. *J. Funct. Foods* **2014**, *8*, 18–25. [CrossRef]
Ranadheera, C.S.; Evans, C.A.; Adams, M.C.; Baines, S.K. In vitro analysis of gastrointestinal tolerance and intestinal cell adhesion of probiotics in goat's milk ice cream and yogurt, In vitro analysis of gastrointestinal tolerance and intestinal cell adhesion of probiotics in goat's milk ice cream and yogurt. *Food Res. Int.* **2012**, *49*, 619–625. [CrossRef]
Anastasiou, R.; Leverrier, P.; Krestas, I.; Rouault, A.; Kalantzopoulos, G.; Boyaval, P.; Tsakalidou, E.; Jan, G. Changes in protein synthesis during thermal adaptation of *Propionibacterium freudenreichii* subsp. shermanii. *Int. J. Food Microbiol.* **2006**, *108*, 301–314. [CrossRef] [PubMed]
Jan, G.; Leverrier, P.; Pichereau, V.; Boyaval, P. Changes in Protein Synthesis and Morphology during Acid Adaptation of *Propionibacterium freudenreichii*. *Appl. Environ. Microbiol.* **2001**, *67*, 2029–2036. [CrossRef] [PubMed]
Leverrier, P.; Dimova, D.; Pichereau, V.; Auffray, Y.; Boyaval, P.; Jan, G. Susceptibility and Adaptive Response to Bile Salts in *Propionibacterium freudenreichii*: Physiological and Proteomic Analysis. *Appl. Environ. Microbiol.* **2003**, *69*, 3809–3818. [CrossRef] [PubMed]
Leverrier, P.; Vissers, J.P.C.; Rouault, A.; Boyaval, P.; Jan, G. Mass spectrometry proteomic analysis of stress adaptation reveals both common and distinct response pathways in *Propionibacterium freudenreichii*. *Arch. Microbiol.* **2004**, *181*, 215–230. [CrossRef] [PubMed]
Guan, N.; Shin, H.; Chen, R.R.; Li, J.; Liu, L.; Du, G.; Chen, J. Understanding of how Propionibacterium acidipropionici respond to propionic acid stress at the level of proteomics. *Sci. Rep.* **2014**, *4*, 6951. [CrossRef] [PubMed]
Guan, N.; Liu, L.; Shin, H.; Chen, R.R.; Zhang, J.; Li, J.; Du, G.; Shi, Z.; Chen, J. Systems-level understanding of how Propionibacterium acidipropionici respond to propionic acid stress at the microenvironment levels: Mechanism and application. *J. Biotechnol.* **2013**, *167*, 56–63. [CrossRef] [PubMed]
Cardoso, F.S.; Gaspar, P.; Hugenholtz, J.; Ramos, A.; Santos, H. Enhancement of trehalose production in dairy propionibacteria through manipulation of environmental conditions. *Int. J. Food Microbiol.* **2004**, *91*, 195–204. [CrossRef]
Lan, A.; Bruneau, A.; Philippe, C.; Rochet, V.; Rouault, A.; Hervé, C.; Roland, N.; Rabot, S.; Jan, G. Survival and metabolic activity of selected strains of *Propionibacterium freudenreichii* in the gastrointestinal tract of human microbiota-associated rats. *Br. J. Nutr.* **2007**, *97*, 714. [CrossRef] [PubMed]
Argañaraz-Martínez, E.; Babot, J.D.; Apella, M.C.; Perez Chaia, A. Physiological and functional characteristics of Propionibacterium strains of the poultry microbiota and relevance for the development of probiotic products. *Anaerobe* **2013**, *23*, 27–37. [CrossRef] [PubMed]
Hervé, C.; Fondrevez, M.; Chéron, A.; Barloy-Hubler, F.; Jan, G. Transcarboxylase mRNA: A marker which evidences P.

freudenreichii survival and metabolic activity during its transit in the human gut. *Int. J. Food Microbiol.* **2007**, *113*, 303–314. [CrossRef] [PubMed]
Saraoui, T.; Parayre, S.; Guernec, G.; Loux, V.; Montfort, J.; Le Cam, A.; Boudry, G.; Jan, G.; Falentin, H. A unique in vivo experimental approach reveals metabolic adaptation of the probiotic *Propionibacterium freudenreichii* to the colon environment. *BMC Genom.* **2013**, *14*, 911. [CrossRef] [PubMed]
Zárate, G.; Palacios, J.M.; Villena, J.; Zúñiga-Hansen, M.E. Inhibition of enteropathogens adhesion to human enterocyte-like HT-29 cells by a dairy strain of *Propionibacterium acidipropionici*. *Benef. Microbes* **2016**, *7*, 431–441. [CrossRef] [PubMed]
Zárate, G.; Morata De Ambrosini, V.; Perez Chaia, A.; González, S. Some factors affecting the adherence of probiotic Propionibacterium acidipropionici CRL 1198 to intestinal epithelial cells. *Can. J. Microbiol.* **2002**, *48*, 449–457. [CrossRef] [PubMed]
Ouwehand, A.C.; Tölkkö, S.; Kulmala, J.; Salminen, S.; Salminen, E. Adhesion of inactivated probiotic strains to intestinal mucus. *Lett. Appl. Microbiol.* **2000**, *31*, 82–86. [CrossRef] [PubMed]
Tuomola, E.M.; Ouwehand, A.C.; Salminen, S.J. Human ileostomy glycoproteins as a model for small intestinal mucus to investigate adhesion of probiotics. *Lett. Appl. Microbiol.* **1999**, *28*, 159–163. [CrossRef] [PubMed]
Ganan, M.; Martinez-Rodriguez, A.J.; Carrascosa, A.V.; Vesterlund, S.; Salminen, S.; Satokari, R. Interaction of *Campylobacter* spp. and Human Probiotics in Chicken Intestinal Mucus: Adhesion of *Campylobacter* and Interaction with Probiotics. *Zoonoses Public Health* **2013**, *60*, 141–148. [CrossRef] [PubMed]
Darilmaz, D.O.; Beyatli, Y.; Yuksekdag, Z.N. Aggregation and Hydrophobicity Properties of 6 Dairy Propionibacteria Strains Isolated from Homemade Turkish Cheeses. *J. Food Sci.* **2012**, *77*, M20–M24. [CrossRef] [PubMed]
Vesterlund, S. Staphylococcus aureus adheres to human intestinal mucus but can be displaced by certain lactic acid bacteria. *Microbiology* **2006**, *152*, 1819–1826. [CrossRef] [PubMed]
Hajfarajollah, H.; Mokhtarani, B.; Noghabi, K.A. Newly Antibacterial and Antiadhesive Lipopeptide Biosurfactant Secreted by a Probiotic Strain, *Propionibacterium freudenreichii*. *Appl. Biochem. Biotechnol.* **2014**, *174*, 2725–2740. [CrossRef] [PubMed]
Nair, D.V.T.; Kollanoor-Johny, A. Effect of *Propionibacterium freudenreichii* on *Salmonella* multiplication, motility, and association with avian epithelial cells. *Poult. Sci.* **2016**. [CrossRef] [PubMed]
Le Maréchal, C.; Peton, V.; Plé, C.; Vroland, C.; Jardin, J.; Briard-Bion, V.; Durant, G.; Chuat, V.; Loux, V.; Foligné, B.; et al. Surface proteins of *Propionibacterium freudenreichii* are involved in its anti-inflammatory properties. *J. Proteom.* **2015**, *113*, 447–461. [CrossRef] [PubMed]
Falentin, H.; Deutsch, S.-M.; Loux, V.; Hammani, A.; Buratti, J.; Parayre, S.; Chuat, V.; Barbe, V.; Aury, J.-M.; Jan, G.; et al. Permanent draft genome sequence of the probiotic strain *Propionibacterium freudenreichii* CIRM-BIA 129 (ITG P20). *Stand. Genom. Sci.* **2016**, *11*, 6. [CrossRef] [PubMed]
Hudson, L.E.; Anderson, S.E.; Corbett, A.H.; Lamb, T.J. Gleaning Insights from Fecal Microbiota Transplantation and Probiotic Studies for the Rational Design of Combination Microbial Therapies. *Clin. Microbiol. Rev.* **2017**, *30*, 191–231. [CrossRef] [PubMed]
Fukumoto, S.; Toshimitsu, T.; Matsuoka, S.; Maruyama, A.; Oh-oka, K.; Takamura, T.; Nakamura, Y.; Ishimaru, K.; Fujii-Kuriyama, Y.; Ikegami, S.; et al. Identification of a probiotic bacteria-derived activator of the aryl hydrocarbon receptor that inhibits colitis. *Immunol. Cell Biol.* **2014**, *92*, 460–465. [CrossRef] [PubMed]
Mitsuyama, K.; Masuda, J.; Yamasaki, H.; Kuwaki, K.; Kitazaki, S.; Koga, H.; Uchida, M.; Sata, M. Treatment of Ulcerative Colitis with Milk Whey Culture with *Propionibacterium freudenreichii*. *J. Intest. Microbiol.* **2007**, *21*, 143–147.
Hojo, K.; Yoda, N.; Tsuchita, H.; Ohtsu, T.; Seki, K.; Taketomo, N.; Murayama, T.; Iino, H. Effect of Ingested Culture of *Propionibacterium freudenreichii* ET-3 on Fecal Microflora and Stool Frequency in Healthy Females. *Biosci. Microflora* **2002**, *21*, 115–120. [CrossRef]
Seki, K.; Nakao, H.; Umino, H.; Isshiki, H.; Yoda, N.; Tachihara, R.; Ohuchi, T.; Saruta, H.; Suzuki, K.; Mitsuoka, T. Effects of Fermented Milk Whey Containing Novel Bifidogenic Growth Stimulator Produced by *Propionibacterium* on Fecal Bacteria, Putrefactive Metabolite, Defecation Frequency and Fecal Properties in Senile Volunteers Needed Serious Nursing-Care Taking Enteral Nutrition by Tube Feeding. *J. Intest. Microbiol.* **2004**, *18*, 107–115.
Martínez, E.A.; Babot, J.D.; Lorenzo-Pisarello, M.J.; Apella, M.C.; Chaia, A.P. Feed supplementation with avian *Propionibacterium acidipropionici* contributes to mucosa development in early stages of rearing broiler chickens. *Benef. Microbes* **2016**, *7*, 687–698. [CrossRef] [PubMed]
O'Callaghan, A.; van Sinderen, D. Bifidobacteria and Their Role as Members of the Human Gut Microbiota. *Front. Microbiol.* **2016**, *7*. [CrossRef] [PubMed]
Wexler, H.M. Bacteroides: The Good, the Bad, and the Nitty-Gritty. *Clin. Microbiol. Rev.* **2007**, *20*, 593–621. [CrossRef] [PubMed]
Isawa, K.; Hojo, K.; Yoda, N.; Kamiyama, T.; Makino, S.; Saito, M.; Sugano, H.; Mizoguchi, C.; Kurama, S.; Shibasaki, M.; et al. Isolation and Identification of a New Bifidogenic Growth Stimulator Produced by *Propionibacterium freudenreichii* ET-3. *Biosci. Biotechnol. Biochem.* **2002**, *66*, 679–681. [CrossRef] [PubMed]

Furuichi, K.; Hojo, K.; Katakura, Y.; Ninomiya, K.; Shioya, S. Aerobic culture of *Propionibacterium freudenreichii* ET-3 can increase production ratio of 1,4-dihydroxy-2-naphthoic acid to menaquinone. *J. Biosci. Bioeng.* **2006**, *101*, 464–470. [CrossRef] [PubMed]
Kaneko, T. A Novel Bifidogenic Growth Stimulator Produced by *Propionibacterium freudenreichii*. *Biosci. Microflora* **1999**, *18*, 73–80. [CrossRef]
Yamazaki, S.; Kano, K.; Ikeda, T.; Isawa, K.; Kaneko, T. Role of 2-amino-3-carboxy-1,4-naphthoquinone, a strong growth stimulator for bifidobacteria, as an electron transfer mediator for NAD(P)+ regeneration in Bifidobacterium longum. *Biochim. Biophys. Acta BBA Gen. Subj.* **1999**, *1428*, 241–250. [CrossRef]
Kaneko, T.; Mori, H.; Iwata, M.; Meguro, S. Growth Stimulator for Bifidobacteria Produced by *Propionibacterium freudenreichii* and Several Intestinal Bacteria. *J. Dairy Sci.* **1994**, *77*, 393–404. [CrossRef]
Uchida, M.; Tsuboi, H.; Takahashi, M.; Nemoto, A.; Seki, K.; Tsunoo, H.; Martyres, S.; Roberts, A. Safety of high doses of *Propionibacterium freudenreichii* ET-3 culture in healthy adult subjects. *Regul. Toxicol. Pharmacol.* **2011**, *60*, 262–267. [CrossRef] [PubMed]
Lorenzo-Pisarello, M.J.; Gultemirian, M.L.; Nieto-Peñalver, C.; Perez Chaia, A. Propionibacterium acidipropionici CRL1198 influences the production of acids and the growth of bacterial genera stimulated by inulin in a murine model of cecal slurries. *Anaerobe* **2010**, *16*, 345–354. [CrossRef] [PubMed]
Luo, J.; Ranadheera, C.S.; King, S.; Evans, C.A.; Baines, S.K. Potential influence of dairy propionibacteria on the growth and acid metabolism of Streptococcus bovis and Megasphaera elsdenii. *Benef. Microbes* **2016**, *8*, 111–119. [CrossRef] [PubMed]
Sánchez, B.; Delgado, S.; Blanco-Míguez, A.; Lourenço, A.; Gueimonde, M.; Margolles, A. Probiotics, gut microbiota, and their influence on host health and disease. *Mol. Nutr. Food Res.* **2017**, *61*, 1600240. [CrossRef] [PubMed]
Wasilewski, A.; Zielińska, M.; Storr, M.; Fichna, J. Beneficial Effects of Probiotics, Prebiotics, Synbiotics, and Psychobiotics in Inflammatory Bowel Disease. *Inflamm. Bowel Dis.* **2015**, *21*, 1674–1682. [CrossRef] [PubMed]
DuPont, A.; Richards, D.M.; Jelinek, K.A.; Krill, J.; Rahimi, E.; Ghouri, Y. Systematic review of randomized controlled trials of probiotics, prebiotics, and synbiotics in inflammatory bowel disease. *Clin. Exp. Gastroenterol.* **2014**, *7*, 473. [CrossRef] [PubMed]
Foligné, B.; Breton, J.; Mater, D.; Jan, G. Tracking the microbiome functionality: Focus on *Propionibacterium* species. *Gut* **2013**, *62*, 1227–1228. [CrossRef] [PubMed]
Foligne, B.; Deutsch, S.-M.; Breton, J.; Cousin, F.J.; Dewulf, J.; Samson, M.; Pot, B.; Jan, G. Promising Immunomodulatory Effects of Selected Strains of Dairy Propionibacteria as Evidenced In Vitro and In Vivo. *Appl. Environ. Microbiol.* **2010**, *76*, 8259–8264. [CrossRef] [PubMed]
Oksaharju, A.; Kooistra, T.; Kleemann, R.; van Duyvenvoorde, W.; Miettinen, M.; Lappalainen, J.; Lindstedt, K.A.; Kovanen, P.T.; Korpela, R.; Kekkonen, R.A. Effects of probiotic Lactobacillus rhamnosus GG and *Propionibacterium freudenreichii* ssp. shermanii JS supplementation on intestinal and systemic markers of inflammation in ApoE*3Leiden mice consuming a high-fat diet. *Br. J. Nutr.* **2013**, *110*, 77–85. [CrossRef] [PubMed]
Kajander, K.; Myllyluoma, E.; Rajilić-Stojanović, M.; Kyrönpalo, S.; Rasmussen, M.; Järvenpää, S.; Zoetendal, E.G.; De Vos, W.M.; Vapaatalo, H.; Korpela, R. Clinical trial: Multispecies probiotic supplementation alleviates the symptoms of irritable bowel syndrome and stabilizes intestinal microbiota. *Aliment. Pharmacol. Ther.* **2008**, *27*, 48–57. [CrossRef] [PubMed]
Suzuki, A.; Mitsuyama, K.; Koga, H.; Tomiyasu, N.; Masuda, J.; Takaki, K.; Tsuruta, O.; Toyonaga, A.; Sata, M. Bifidogenic growth stimulator for the treatment of active ulcerative colitis: A pilot study. *Nutrition* **2006**, *22*, 76–81. [CrossRef] [PubMed]
Lortal, S.; Rouault, A.; Cesselin, B.; Sleytr, U.B. Paracrystalline surface layers of dairy propionibacteria. *Appl. Environ. Microbiol.* **1993**, *59*, 2369–2374. [PubMed]
Fagan, R.P.; Fairweather, N.F. Biogenesis and functions of bacterial S-layers. *Nat. Rev. Microbiol.* **2014**, *12*, 211–222. [CrossRef] [PubMed]
Ilk, N.; Egelseer, E.M.; Sleytr, U.B. S-layer fusion proteins—Construction principles and applications. *Curr. Opin. Biotechnol.* **2011**, *22*, 824–831. [CrossRef] [PubMed]
Deutsch, S.-M.; Mariadassou, M.; Nicolas, P.; Parayre, S.; Le Guellec, R.; Chuat, V.; Peton, V.; Le Maréchal, C.; Burati, J.; Loux, V.; et al. Identification of proteins involved in the anti-inflammatory properties of *Propionibacterium freudenreichii* by means of a multi-strain study. *Sci. Rep.* **2017**, *7*, 46409. [CrossRef] [PubMed]
Deutsch, S.-M.; Parayre, S.; Bouchoux, A.; Guyomarc'h, F.; Dewulf, J.; Dols-Lafargue, M.; Bagliniere, F.; Cousin, F.J.; Falentin, H.; Jan, G.; et al. Contribution of Surface—Glucan Polysaccharide to Physicochemical and Immunomodulatory Properties of *Propionibacterium freudenreichii*. *Appl. Environ. Microbiol.* **2012**, *78*, 1765–1775. [CrossRef] [PubMed]
Deutsch, S.M.; Bivic, P.L.; Herve, C.; Madec, M.N.; LaPointe, G.; Jan, G.; Loir, Y.L.; Falentin, H. Correlation of the Capsular Phenotype in *Propionibacterium freudenreichii* with the Level of Expression of gtf, a Unique Polysaccharide Synthase-Encoding Gene. *Appl. Environ. Microbiol.* **2010**, *76*, 2740–2746. [CrossRef] [PubMed]
Lightfoot, Y.L.; Selle, K.; Yang, T.; Goh, Y.J.; Sahay, B.; Zadeh, M.; Owen, J.L.; Colliou, N.; Li, E.; Johannssen, T.; et al. SIGNR3-dependent immune regulation by Lactobacillus acidophilus surface layer protein A in colitis. *EMBO J.* **2015**, *34*, 881–895. [CrossRef] [PubMed]

Konstantinov, S.R.; Smidt, H.; de Vos, W.M.; Bruijns, S.C.M.; Singh, S.K.; Valence, F.; Molle, D.; Lortal, S.; Altermann, E.; Klaenhammer, T.R.; et al. S layer protein A of Lactobacillus acidophilus NCFM regulates immature dendritic cell and T cell functions. *Proc. Natl. Acad. Sci. USA* **2008**, *105*, 19474–19479. [CrossRef] [PubMed]
Puertollano, E.; Kolida, S.; Yaqoob, P. Biological significance of short-chain fatty acid metabolism by the intestinal microbiome. *Curr. Opin. Clin. Nutr. Metab. Care* **2014**, *17*, 139–144. [CrossRef] [PubMed]
Tan, J.; McKenzie, C.; Potamitis, M.; Thorburn, A.N.; Mackay, C.R.; Macia, L. Chapter Three—The Role of Short-Chain Fatty Acids in Health and Disease. In *Advances in Immunology*; Alt, F.W., Ed.; Academic Press: Cambridge, MA, USA, 2014; Volume 121, pp. 91–119.
Kim, C.H.; Park, J.; Kim, M. Gut microbiota-derived short-chain Fatty acids, T cells, and inflammation. *Immune Netw.* **2014**, *14*, 277–288. [CrossRef] [PubMed]
Zimmerman, M.A.; Singh, N.; Martin, P.M.; Thangaraju, M.; Ganapathy, V.; Waller, J.L.; Shi, H.; Robertson, K.D.; Munn, D.H.; Liu, K. Butyrate suppresses colonic inflammation through HDAC1-dependent Fas upregulation and Fas-mediated apoptosis of T cells. *AJP Gastrointest. Liver Physiol.* **2012**, *302*, G1405–G1415. [CrossRef] [PubMed]
McIntosh, F.M.; Shingfield, K.J.; Devillard, E.; Russell, W.R.; Wallace, R.J. Mechanism of conjugated linoleic acid and vaccenic acid formation in human faecal suspensions and pure cultures of intestinal bacteria. *Microbiology* **2009**, *155*, 285–294. [CrossRef] [PubMed]
Jiang, J.; Björck, L.; Fonden, R. Production of conjugated linoleic acid by dairy starter cultures. *J. Appl. Microbiol.* **1998**, *85*, 95–102. [CrossRef] [PubMed]
Xu, S.; Boylston, T.D.; Glatz, B.A. Conjugated Linoleic Acid Content and Organoleptic Attributes of Fermented Milk Products Produced with Probiotic Bacteria. *J. Agric. Food Chem.* **2005**, *53*, 9064–9072. [CrossRef] [PubMed]
Hennessy, A.A.; Barrett, E.; Paul Ross, R.; Fitzgerald, G.F.; Devery, R.; Stanton, C. The Production of Conjugated α-Linolenic, γ-Linolenic and Stearidonic Acids by Strains of Bifidobacteria and Propionibacteria. *Lipids* **2012**, *47*, 313–327. [CrossRef] [PubMed]
Gorissen, L.; Leroy, F.; De Vuyst, L.; De Smet, S.; Raes, K. Bacterial Production of Conjugated Linoleic and Linolenic Acid in Foods: A Technological Challenge. *Crit. Rev. Food Sci. Nutr.* **2015**, *55*, 1561–1574. [CrossRef] [PubMed]
Rainio, A.; Vahvaselkä, M.; Suomalainen, T.; Laakso, S. Reduction of linoleic acid inhibition in production of conjugated linoleic acid by *Propionibacterium freudenreichii* ssp. shermanii. *Can. J. Microbiol.* **2001**, *47*, 735–740. [CrossRef] [PubMed]
Fontes, A.L.; Pimentel, L.L.; Simões, C.D.; Gomes, A.M.P.; Rodríguez-Alcalá, L.M. Evidences and perspectives in the utilization of CLNA isomers as bioactive compound in foods. *Crit. Rev. Food Sci. Nutr.* **2017**, *57*, 2611–2622. [CrossRef] [PubMed]
Yuan, G.-F.; Chen, X.-E.; Li, D. Conjugated linolenic acids and their bioactivities: A review. *Food Funct.* **2014**, *5*, 1360. [CrossRef] [PubMed]
Yuan, G.; Chen, X.; Li, D. Modulation of Peroxisome Proliferator-Activated Receptor gamma (PPAR γ) by Conjugated Fatty Acid in Obesity and Inflammatory Bowel Disease. *J. Agric. Food Chem.* **2015**, *63*, 1883–1895. [CrossRef] [PubMed]
Kim, J.H.; Kim, Y.; Kim, Y.J.; Park, Y. Conjugated Linoleic Acid: Potential Health Benefits as a Functional Food Ingredient. *Annu. Rev. Food Sci. Technol.* **2016**, *7*, 221–244. [CrossRef] [PubMed]
Churruca, I.; Fernández-Quintela, A.; Portillo, M.P. Conjugated linoleic acid isomers: Differences in metabolism and biological effects. *BioFactors* **2009**, *35*, 105–111. [CrossRef] [PubMed]
Draper, E.; DeCourcey, J.; Higgins, S.C.; Canavan, M.; McEvoy, F.; Lynch, M.; Keogh, B.; Reynolds, C.; Roche, H.M.; Mills, K.H.G.; et al. Conjugated linoleic acid suppresses dendritic cell activation and subsequent Th17 responses. *J. Nutr. Biochem.* **2014**, *25*, 741–749. [CrossRef] [PubMed]
Hennessy, A.A.; Ross, P.R.; Fitzgerald, G.F.; Stanton, C. Sources and Bioactive Properties of Conjugated Dietary Fatty Acids. *Lipids* **2016**, *51*, 377–397. [CrossRef] [PubMed]
Viladomiu, M.; Hontecillas, R.; Bassaganya-Riera, J. Modulation of inflammation and immunity by dietary conjugated linoleic acid. *Eur. J. Pharmacol.* **2016**, *785*, 87–95. [CrossRef] [PubMed]
Bultman, S.J. Interplay between diet, gut microbiota, epigenetic events, and colorectal cancer. *Mol. Nutr. Food Res.* **2017**, *61*, 1500902. [CrossRef] [PubMed]
O'Keefe, S.J.D. Diet, microorganisms and their metabolites, and colon cancer. *Nat. Rev. Gastroenterol. Hepatol.* **2016**, *13*, 691–706. [CrossRef] [PubMed]
Jan, G.; Belzacq, A.-S.; Haouzi, D.; Rouault, A.; Métivier, D.; Kroemer, G.; Brenner, C. Propionibacteria induce apoptosis of colorectal carcinoma cells via short-chain fatty acids acting on mitochondria. *Cell Death Differ.* **2002**, *9*, 179–188. [CrossRef] [PubMed]
Lan, A.; Lagadic-Gossmann, D.; Lemaire, C.; Brenner, C.; Jan, G. Acidic extracellular pH shifts colorectal cancer cell death from apoptosis to necrosis upon exposure to propionate and acetate, major end-products of the human probiotic propionibacteria. *Apoptosis* **2007**, *12*, 573–591. [CrossRef] [PubMed]
Cousin, F.J.; Jouan-Lanhouet, S.; Dimanche-Boitrel, M.-T.; Corcos, L.; Jan, G. Milk Fermented by *Propionibacterium freudenreichii* Induces Apoptosis of HGT-1 Human Gastric Cancer Cells. *PLoS ONE* **2012**, *7*, e31892. [CrossRef] [PubMed]
Lan, A.; Bruneau, A.; Bensaada, M.; Philippe, C.; Bellaud, P.; Rabot, S.; Jan, G. Increased induction of apoptosis by

Propionibacterium freudenreichii TL133 in colonic mucosal crypts of human microbiota-associated rats treated with 1,2-dimethylhydrazine. *Br. J. Nutr.* **2008**, *100*, 1251. [CrossRef] [PubMed]
Zarate, G.; Perez Chaia, A. Dairy bacteria remove in vitro dietary lectins with toxic effects on colonic cells. *J. Appl. Microbiol.* **2009**, *106*, 1050–1057. [CrossRef] [PubMed]
Halttunen, T.; Collado, M.C.; El-Nezami, H.; Meriluoto, J.; Salminen, S. Combining strains of lactic acid bacteria may reduce their toxin and heavy metal removal efficiency from aqueous solution: Toxin removal with LAB combination. *Lett. Appl. Microbiol.* **2007**, *46*, 160–165. [CrossRef] [PubMed]
Lee, Y.K.; El-Nezami, H.; Haskard, C.A.; Gratz, S.; Puong, K.Y.; Salminen, S.; Mykkänen, H. Kinetics of adsorption and desorption of aflatoxin B1 by viable and nonviable bacteria. *J. Food Prot.* **2003**, *66*, 426–430. [CrossRef] [PubMed]
Gratz, S.; Mykkänen, H.; El-Nezami, H. Aflatoxin B1 binding by a mixture of Lactobacillus and Propionibacterium: In vitro versus ex vivo. *J. Food Prot.* **2005**, *68*, 2470–2474. [CrossRef] [PubMed]
Gratz, S.; Mykkanen, H.; Ouwehand, A.C.; Juvonen, R.; Salminen, S.; El-Nezami, H. Intestinal Mucus Alters the Ability of Probiotic Bacteria To Bind Aflatoxin B1 In Vitro. *Appl. Environ. Microbiol.* **2004**, *70*, 6306–6308. [CrossRef] [PubMed]
El-Nezami, H.; Mykkänen, H.; Kankaanpää, P.; Salminen, S.; Ahokas, J. Ability of Lactobacillus and Propionibacterium strains to remove aflatoxin B, from the chicken duodenum. *J. Food Prot.* **2000**, *63*, 549–552. [CrossRef] [PubMed]
El-Nezami, H.S.; Polychronaki, N.N.; Ma, J.; Zhu, H.; Ling, W.; Salminen, E.K.; Juvonen, R.O.; Salminen, S.J.; Poussa, T.; Mykkänen, H.M. Probiotic supplementation reduces a biomarker for increased risk of liver cancer in young men from Southern China. *Am. J. Clin. Nutr.* **2006**, *83*, 1199–1203. [PubMed]
Kekkonen, R.A.; Holma, R.; Hatakka, K.; Suomalainen, T.; Poussa, T.; Adlercreutz, H.; Korpela, R. A Probiotic Mixture Including Galactooligosaccharides Decreases Fecal-Glucosidase Activity but Does Not Affect Serum Enterolactone Concentration in Men during a Two-Week Intervention. *J. Nutr.* **2011**, *141*, 870–876. [CrossRef] [PubMed]
Hatakka, K.; Holma, R.; El-Nezami, H.; Suomalainen, T.; Kuisma, M.; Saxelin, M.; Poussa, T.; Mykkänen, H.; Korpela, R. The influence of Lactobacillus rhamnosus LC705 together with *Propionibacterium freudenreichii* ssp. shermanii JS on potentially carcinogenic bacterial activity in human colon. *Int. J. Food Microbiol.* **2008**, *128*, 406–410. [CrossRef] [PubMed]
Saxelin, M.; Lassig, A.; Karjalainen, H.; Tynkkynen, S.; Surakka, A.; Vapaatalo, H.; Järvenpää, S.; Korpela, R.; Mutanen, M.; Hatakka, K. Persistence of probiotic strains in the gastrointestinal tract when administered as capsules, yoghurt, or cheese. *Int. J. Food Microbiol.* **2010**, *144*, 293–300. [CrossRef] [PubMed]
Thierry, A.; Deutsch, S.-M.; Falentin, H.; Dalmasso, M.; Cousin, F.J.; Jan, G. New insights into physiology and metabolism of *Propionibacterium freudenreichii*. *Int. J. Food Microbiol.* **2011**, *149*, 19–27. [CrossRef] [PubMed]
Zárate, G. Dairy Propionibacteria: Less Conventional Probiotics to Improve the Human and Animal Health. In *Probiotic in Animals*; InTechOpen: Rijeka, Croatia, 2012; ISBN 978-953-51-0777-4.
Yee, A.L.; Maillard, M.-B.; Roland, N.; Chuat, V.; Leclerc, A.; Pogačić, T.; Valence, F.; Thierry, A. Great interspecies and intraspecies diversity of dairy propionibacteria in the production of cheese aroma compounds. *Int. J. Food Microbiol.* **2014**, *191*, 60–68. [CrossRef] [PubMed]
Piao, Y.; Yamashita, M.; Kawaraichi, N.; Asegawa, R.; Ono, H.; Murooka, Y. Production of vitamin B12 in genetically engineered *Propionibacterium freudenreichii*. *J. Biosci. Bioeng.* **2004**, *98*, 167–173. [CrossRef]
Wang, P.; Zhang, Z.; Jiao, Y.; Liu, S.; Wang, Y. Improved propionic acid and 5,6-dimethylbenzimidazole control strategy for vitamin B12 fermentation by *Propionibacterium freudenreichii*. *J. Biotechnol.* **2015**, *193*, 123–129. [CrossRef] [PubMed]

CHAPTER 2. LITERATURE REVIEW. SATE OF THE ART ON SURFACE PROTEINS AS MEDIATORS OF PROBIOTIC'S ACTIVITY.

We have seen that *Propionibacterium freudenreichii* can be used as a probiotic bacterium, since this peculiar bacterium has versatile technological applications as well as cheese ripening starter, adding flavor, producing nutraceutical compounds that may enhance or maintain human health through dietary modulation (RABAH; ROSA DO CARMO; JAN, 2017). Moreover, in the previous section, we reported probiotic properties of this genus, including stress tolerance, adhesion to intestinal epithelial cells, which contributes to persistence in the host, and immunomodulatory properties.

Interestingly, the beneficial properties of *Propionibacterium freudenreichii* may depend on the presence of key surface layer proteins. Indeed, such proteins were associated to probiotic effects in the *Lactobacillus* genus and some of them mediate key interactions with the host (HYNÖNEN; PALVA, 2013). Gram-positive bacteria, depending on the species, and sometime on the strain, can be covered by an outermost para-crystalline proteinaceous layer, non-covalently attached to the cell surface, called surface-layer, or S-layer (HOUWINK, 1953; SLEYTR et al., 2014). This macromolecular monolayer called S-layer is found in many bacterial species. However, the S-layer protein sequence is highly variable among bacteria species (HOUWINK, 1953; SLEYTR et al., 2014). In addition, these S-layer proteins have been associated with stress tolerance in harsh environmental conditions such as within the GI tract (DO CARMO et al., 2017a). Moreover, certain S-layer proteins mediate the cross-talk between these bacteria and the host through adhesion process, consequently favoring delivery of beneficial metabolites and of nutraceutical compounds, as well as through the modulation of GIT functions (HYNÖNEN; PALVA, 2013).

Recent studies have shown that the most anti-inflammatory *P. freudenreichii* strain, namely CIRM-BIA 129, exhibits five distinct surface proteins extractable by guanidine: SlpA, SlpB, SlpE, Internaline like A (Inl A) and Large surface protein A (Lsp A). This set of proteins is linked to *P. freudenreichii* immunomodulatory properties (DEUTSCH et al., 2017; LE MARÉCHAL et al., 2015). As a matter of fact, such versatile S-layer proteins were shown to play a role in the regulation of inflammatory processes in GIT disorders. Thus, selecting probiotic bacteria that possess desired S-layer proteins may allow

determining functionalities and thus beneficial effects of food products or supplements, in future biotechnological applications.

In this following section, we explore probiotic bacteria's S-layer proteins properties and their involvement in the interface between bacterium and host/host cells.

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CHAPITRE 2. REVUE DE LITTERATURE. ETAT DE L'ART SUR LES PROTEINES DE SURFACE COMME MEDIATEURS DE L'ACTIVITE PROBIOTIQUE.

Nous avons vu que *Propionibacterium freudenreichii* peut être utilisé comme bactéries probiotique, dans la mesure où cette bactérie particulière trouve des applications technologiques diverses, telles que l'affinage de fromages, la production de composés d'arôme, de composés nutraceutiques qui peuvent améliorer ou préserver la santé humaine à travers la modulation de la diète. (RABAH; ROSA DO CARMO; JAN, 2017). De plus, dans la section précédente, nous rapportons les propriétés probiotiques des propionibactéries, y compris la tolérance aux stress, l'adhésion aux cellules épithéliales intestinales humaines, ce qui contribue à la persistance chez l'hôte, ainsi qu'au propriétés immunomodulatrices.

De façon intéressante, les propriétés bénéfiques de *Propionibacterium freudenreichii* peuvent dépendre de la présence de certaines protéines de surface clés. En effet, de telles protéines ont été associées aux effets probiotiques chez des lactobacilles et certaines jouent le rôle de médiateur dans les interactions clés entre probiotique et hôte (HYNÖNEN; PALVA, 2013). Les bactéries Gram-positives, selon l'espèce, et même parfois la souche, peuvent être recouvertes par une couche externe paracristalline, attaché de façon non-covalente à la surface de la cellule, appelées surface-layer, ou S-layer (HOUWINK, 1953; SLEYTR et al., 2014). Cette monocouche macromoléculaire se retrouve dans de nombreuses espèces bactériennes. Cependant, la séquence des protéines S-layer est hautement variable parmi les espèces bactériennes. (HOUWINK, 1953; SLEYTR et al., 2014). De plus, certaines protéines S-layer ont été associées à la tolérance au stress en conditions environnementales rudes, telles que dans le tube digestif (DO CARMO et al., 2017a). Enfin, certaines protéines S-layer permettent le dialogue entre bactérie probiotique et hôte, via le procédé d'adhésion, qui favorise à son tour la libération de métabolites bénéfiques et de composés nutraceutiques, de même que via la modulation de fonctions clés du tractus gastro-intestinal (HYNÖNEN; PALVA, 2013).

Les études récentes ont montré que la souche la plus anti-inflammatoire de *P. freudenreichii*, nommée CIRM-BIA 129, expose cinq protéines de surface distinctes, extraites par la guanidine : SlpA, SlpB, SlpE, Internaline like A (Inl A) et Large surface protein A (Lsp A). Cet ensemble de protéines est relié aux propriétés

immunomodulatrices de *P. freudenreichii* (DEUTSCH et al., 2017; LE MARÉCHAL et al., 2015). En effet, ce type de protéines de surface versatiles jouent un rôle dans la modulation des processus inflammatoires, dans le contexte de désordres intestinaux. Dès lors, la sélection de souches bactériennes qui possèdent les protéines de S-layer souhaitées pourrait permettre d'orienter les fonctionnalités, et donc les effets bénéfiques, de produits alimentaires ou de suppléments fonctionnels, dans des applications biotechnologiques futures.

Dans la section qui suite, nous explorons les propriétés des protéines de S-layer bactériennes, ainsi que leur implication dans l'interface entre la bactérie probiotique et l'hôte / les cellules de l'hôte.

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REVIEW
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Extractable BacterialSurface Proteins in Probiotic–Host Interaction

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Some Gram-positive bacteria, including probiotic ones, are covered with an external proteinaceous layer called a surface-layer. Described as a paracrystalline layer and formed by the self-assembly of a surface-layer-protein (Slp), this optional structure is peculiar. The surface layer *per se* is conserved and encountered in many prokaryotes. However, the sequence of the corresponding Slp protein is highly variable among bacterial species, or even among strains of the same species. Other proteins, including surface layer associated proteins (SLAPs), and other non-covalently surface-bound proteins may also be extracted with this surface structure. They can be involved a various functions. In probiotic Gram-positives, they were shown by different authors and experimental approaches to play a role in key interactions with the host. Depending on the species, and sometime on the strain, they can be involved in stress tolerance, in survival within the host digestive tract, in adhesion to host cells or mucus, or in the modulation of intestinal inflammation. Future trends include the valorization of their properties in the formation of nanoparticles, coating and encapsulation, and in the development of new vaccines.

Keywords: surface layer protein, probiotic, immunomodulation, host, adhesion

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Introduction

Probiotics are live microorganisms, traditionally regarded as safe for human consumption that, when ingested in sufficient numbers, confer a health benefit to the host (FAO/WHO, 2006). Probiotic microorganisms comprise mainly Gram-positive bacteria including LAB, bifidobacteria, enterococci, and propionibacteria. Some yeasts and Gram-negative bacteria may also be considered for probiotic use. Potential applications of probiotics involve the prevention and treatment of diarrhea caused by rotavirus, allergy and eczema, IBD; and the improvement of intestinal comfort, lactose intolerance, infection by *Helicobacter pylori*, and metabolic diseases (Syngai et al., 2016; Evivie et al., 2017). LAB constitute a large family of Gram-positive bacteria which are extensively implemented in the fermentation of a wide variety of food products. They include a variety

Abbreviations: APF, aggregation-promoting-like factor; CLR, C-type lectin receptor; CWBD, cell wall binding domain; DC, dendritic cell; DC-SIGN, dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin; FAO, Food and Agriculture Organization; GALT, gut-associated lymphoid tissue; IBD, inflammatory bowel disease; IEC, intestinal epithelial cell; LAB, lactic acid bacteria; MAMP, microbe-associated molecular pattern; PBMC, peripheral blood mononuclear cell; PRR, pattern recognition receptor; SCWP, secondary cell-wall polymer; SLAP, S-layer associated protein; SLH, S-layer homology domain; Slp, surface-layer protein; TLR, toll-like receptor; WHO, World Health Organization.

of probiotic species: *Lactobacillus brevis*, *L. bulgaricus*, *L. plantarum*, *L. rhamnosus*, *L. casei*, *L. helveticus, L. salivarius*, *L. reuteri*, *L. johnsonii, L. fermentum,* and *L. acidophilus* (Avall-Jääskeläinen and Palva, 2005). Propionibacteria, in particular *Propionibacterium freudenreichii* strains, are emergent probiotics, also used as ripening starter in Emmental cheese manufacturing, and as vitamins producers. These propionibacteria recently revealed potent beneficial effects, including the modulation of colon cancer cells proliferation and of colon inflammation (Rabah et al., 2017). Several molecular mechanisms behind these probiotics' beneficial effects are being elucidated. They involve modulation of the gut microbiota composition, stimulation of the epithelial barrier function, and induction of immune responses (Lebeer et al., 2008; Rabah et al., 2017). In addition, the role of bacterial surface compounds of Gram-positive bacteria includes the modulation of the gut immune system firstly, and then the systemic immune system, by mediating a cross-talk between the host and bacteria, whether they are commensals or probiotics. Such bacterial surface compounds constitute MAMPs; such as proteins, glycoproteins, lipoproteins, lipoteichoic acids, lipopolysaccharides and flagellins, which interact with the host PRRs, resulting in immune system modulation. Recently, several studies revealed the key role of surface-bound proteins, which are non-covalently attached to the cell wall, and are optionally present in certain probiotic bacteria. The surface-bound proteins may belong to a Slp lattice, an outermost macromolecular monolayer. First described in 1953 by Houwink, it consists of a paracrystalline bidimensional array made up of a Slp, which was first found on *Spirillum* sp. cell surface (Houwink, 1953; Sleytr et al., 2014). Slps are extracted using chaotropic agents such as guanidine chloride and lithium chloride (Koval and Murray, 1984). These agents may also extract other proteins, either associated to the S-layer lattice, or anchored to the cell wall through non-covalent interaction domains. These proteins include CWBDs, lysin motif domain (LysM), GW modules or SLH domains (Desvaux et al., 2006). Several studies revealed the involvement of surface-bound proteins in the bacteria/host interaction, leading to beneficial effects such as immune modulation, but the molecular mechanisms are still not fully understood. Indeed, they fulfill various crucial functions in bacteria, such as contribution to determination or maintenance of cell shape, molecular sieve, enzyme activities, contribution to adhesion, coaggregation, modulation of gut immune cells, protection against environmental stresses and antimicrobial peptides (Hynönen and Palva, 2013). The purpose of this review is to discuss involvement of non-covalently surface-bound proteins in Gram-positive probiotics' functionalities and thus in their beneficial effects, and their future biotechnological applications.

Occurence, location, and structure of S-layer proteins

S-Layer Proteins

S-layers are present in Archaea, Gram-positive and Gram-negative bacteria (Sára and Sleytr, 1996, 2000), they exhibit a thickness of 5–25 nm (Sára and Sleytr, 1996, 2000) and are highly porous (Sára and Sleytr, 1996; Sleytr and Beveridge, 1999). The S-layer paracrystalline lattice can be organized in different symmetry: oblique (p1, p2), tetragonal (p4), or hexagonal (p3, p6) symmetry (Lortal et al., 1993; Sleytr, 1997; Sleytr and Beveridge, 1999; Mobili et al., 2010). In Gram-positive bacteria, the S-layer lattice is generally composed of a single protein (Fagan and Fairweather, 2014; Pum and Sleytr, 2014; Sleytr et al., 2014), and is attached to peptidoglycan-bound SCWPs by non-covalent interactions (Fagan and Fairweather, 2014; Sleytr et al., 2014). The non-covalent anchorage of Slps may be mediated by different modules (Fagan and Fairweather, 2014). Three SLH domains can fold into a pseudo-trimer and cooperate in the binding to SCWPs. This is the most widely distributed anchorage of Slps, found in many *Bacillus* species and in the probiotic *Propionibacterium freudenreichii* (Le Maréchal et al., 2015). Another conserved anchorage mechanism is mediated via three modules of cell- wall binding domain 2 (CWB2), found in many *Clostridium* species, and binding to cell wall compounds that are still not fully elucidated (Fagan and Fairweather, 2014). By contrast, Slps from members of the *Lactobacillus* species are devoid of such motif and are anchored by a conserved CWBD, which can be C-terminal (*L. acidophilus*, *L. crispatus*) or N-terminal (*L. brevis*), while the opposite part of the protein, more variable, is involved in the self-assembly (Hynönen and Palva, 2013). The *L. acidophilus* SlpA C-terminal binding domain, which represents one-third of the protein, interacts with negatively charged SCWPs and with neutral polysaccharides (Sleytr et al., 2014).

Slps possess a molecular weight ranging from 25 to 200 kDa (Avall-Jääskeläinen and Palva, 2005) and are typically rich in acidic and hydrophobic amino acids (Sára and Sleytr, 1996, 2000; Sleytr and Beveridge, 1999; Pum and Sleytr, 2014), exhibiting a generally low isoelectric point (pI), with the exception of *Lactobacillus* Slps which have a high pI. In *P. freudenreichii*, five extractable surface proteins were identified using guanidine: SlpA, SlpB, SlpE, Internaline A (Inl A) and Large surface protein A (lsp A) (Le Maréchal et al., 2015; Deutsch et al., 2017). All these surface proteins are expressed quantitatively and qualitatively differently between different strains (Deutsch et al., 2017). However, only SlpA (illustrated in **Figure 1**) is considered as a true S-layer protein since its high expression level leads to the self-assembly of a SlpA-composed surface layer in *P. freudenreichii* strains CIRM-BIA 118 and CIRM-BIA 508 (alias CNRZ 722) (Lortal et al., 1993; de sa Peixoto et al., 2015). The SlpB protein, also presenting three SLH domains in its C-terminal domain (**Figure 2**), constitutes the major extractable surface protein in other *P. freudenreichii* strains, including CIRM BIA 129 (Le Maréchal et al., 2015). This suggests SlpB is a true S-layer protein, although the occurrence of a surface paracrystalline layer was not evidenced in these strains.

Glycosylation is the major covalent modification observed in Slps from Gram-positive bacteria. It was previously reported in *L. kefiri* and *L. buchneri* (Mobili et al., 2010). The glycosylation rate in Gram-positive bacteria Slps, leading to modification of 2–4 amino acid residues, is much lower than in Archaea. SlpB of *L. buchneri* shows four glycosylation sites consisting

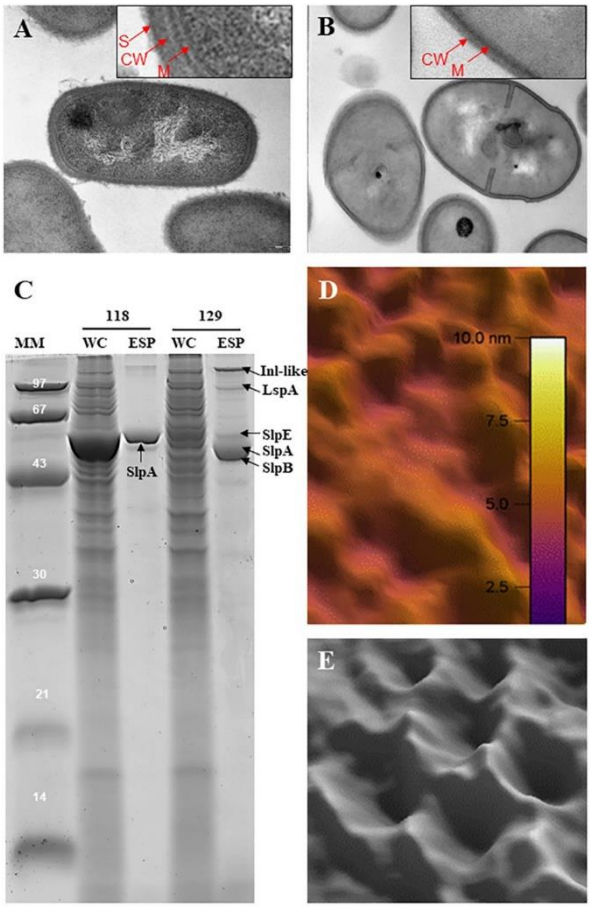

FIGURE 1 | Occurrence of an S-layer is strain-dependent in *Propionibacterium freudenreichii*. *P. freudenreichii* CIRM-BIA 118 is covered by an outermost surface layer (A) that is removed by extraction using the chaotropic agent guanidine (B). Red arrows indicate the membrane (M), cell wall (CW), and S-layer (S). The CIRM-BIA 118 guanidine extract was analyzed by SDS-PAGE (C), showing a major band close to 58 kDa corresponding to the S-layer protein A, as identified by MS/MS (Le Maréchal et al., 2015). Extracted SlpA was dialyzed against HEPES/NaCl buffer and deposited on mica and recrystallized prior to atomic force microscopy imaging (de sa Peixoto et al., 2015). (D) Typical amplitude image obtained with purified Slp. (E) Close-up view of a Gaussian-filtered 40 nm × 40 nm phase image of recrystallized Slp showing a hexagonal arrangement. By contrast, *P. freudenreichii* CIRM-BIA 129 does not exhibit this S-layer (data not shown). However, extractable surface proteins (C), in this strain, include Inl-like protein, 145 kDa, LspA (96 kDa), SlpE (59 kDa), SlpA (58 kDa), and SlpB (56 kDa). MM, molecular mass markers; WC, whole-cell SDS protein extract; ESP, extractable surface proteins guanidine extract.

in *O*-glycosylation of seven glucose residues (Anzengruber et al., 2014). Slps glycosylations may be *N*- or *O*-anchored to the peptide skeleton and consist in about 50 identical units containing neutral hexoses, pentoses, heptoses or deoxyhexose and amino sugars. The Slp of *L. kefiri* is O- and N-glycosylated, with 5-8 glucose units carrying galacturonic acid (Cavallero et al., 2017). Little is known about structure–function relationships of S-layer glycan moieties (Messner et al., 2008; Schuster and Sleytr, 2015). These covalent modifications, however, may be critical for the cross-talk between bacteria carrying Slps and the host through PRRs, as demonstrated for *L. kefiri* (Prado Acosta et al., 2016).

Other ExtractableSurface-Bound Proteins

Being non-covalently anchored to the cell wall, surface-bound proteins are extracted from intact bacteria by the action of chaotropic agents such as lithium chloride and guanidine chloride. Thus, bacterial strains that do possess a true S-layer are characterized by the fact that extraction leads to the isolation of one single molecular protein species, able to re-assemble into a characteristic lattice. However, a thorough proteomic study of this extracted fraction evidenced other proteins, in addition to Slps, in *L. acidophilus*, for example, showing that Slps constitute an anchor for several other extractable surface-bound proteins called SLAPs (Johnson et al., 2013). Such proteins, identified in *Lactobacillus* species, have different functions, including interaction with the host (Johnson et al., 2013, 2016, 2017; Wa´sko et al., 2014; Zhang et al., 2016). In the absence of an S-layer, other non-covalently surface-bound proteins may exist and be extracted using chaotropic agents. They present different anchorage domains including SLH domains (Desvaux et al., 2006).

Surface proteome analysis of many *P. freudenreichii* strains revealed the presence of two other proteins, SlpE and Inl-like, a protein showing homology with InlA (internalin A), which exhibit C-terminal SLH domains, with a lower level of expression (**Figures 1**, **2**). They are detected in strains with or without a true SlpA surface-layer, suggesting that they are not true Slps. Regarding the protein lspA (large surface protein A), it is predicted to have a mannosyl-glycoprotein endo-beta-*N*- acetylglucosamidase-like domain and no SLH domain. Similarly, several reports further evidenced extractable surface-bound proteins in probiotic lactobacilli, and designated them as Slps, based on the presence of SLH domains, although these proteins were not shown to constitute a true paracrystalline thick surface layer. These last were, however, taken into consideration on the present review, provided that they play a role in probiotic/host interaction.

Probiotic-Host interaction via extractable surface proteins

The interaction between probiotic surface components and host cells may lead to modulation of gut functions (Velasquez- Manoff, 2015). Commensal bacteria colonizing the gut have co-evolved with their host and developed molecular interaction mechanisms involved in adherence, epithelial barrier function and in immune system development (Zaneveld et al., 2008; Vindigni et al., 2016). Therefore, immune cells and IECs are able to recognize several surface components (MAMPs) of autochthonous microbiota members, including lactobacilli and bifidobacteria, but also of allochthonous (food-borne) bacteria including lactobacilli, lactococci, and propionibacteria (Carvalho et al., 2017; Rook et al., 2017). The beneficial effect of probiotic bacteria, including activation of receptor-dependent pathways,

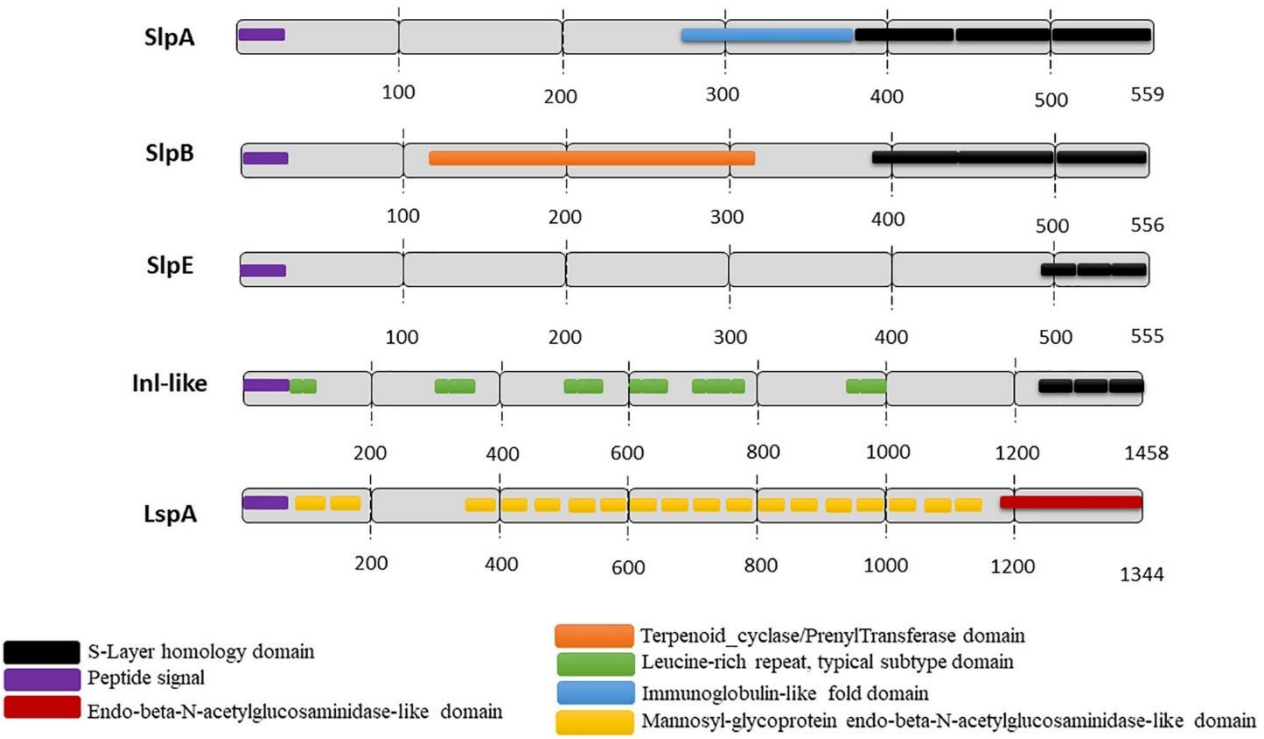

FIGURE 2 | Predicted functional domains in *P. freudenreichii* extractable surface proteins. The functional domains were predicted using InterPro (EMBI/NCBI) for the five extractable surface proteins identified in different *P. freudenreichii* strains (Le Maréchal et al., 2015). S-layer protein A (SlpA), S-layer protein B (SlpB), S-layer protein E (SlpE) and Internaline-like (Inl-like) all exhibit three C-terminal conserved SLH domains allowing interaction with the peptidoglycan cell wall. By contrast, LspA exhibits no SLH domain, but *N*-acetylglucosaminidase-like domains suggesting a role in peptidoglycan metabolism.

is most probably favored by its ability to adhere to target cells. In this context, PRRs, including TLRs, expressed by enterocytes, are able to recognize MAMP, including extractable surface proteins.

Extractable Surface Proteins Are Involved in Adhesion to Epithelial Cells and Extracellular Matrix Proteins

In order to exert a beneficial effect on the host, probiotic bacteria must have the ability to tolerate digestive stresses and interact with host cells [Food and Agriculture Organization of the United Nations and World Health Organization (FAO/WHO), 2002]. The adhesion of probiotic bacteria allows extending their persistence in the digestive tract, thus favoring a probiotic action. To understand how cell surface compounds from both partners can contribute to bacteria/cell adhesion, *in vitro* assays have been extensively used. This led to the development of recognized *in vitro* techniques (Blum et al., 1999; Vesterlund et al., 2005). This includes the use of IECs lines such as Caco-2 (Hirakata et al., 1998) and HT-29 (Maoret et al., 1989; Gagnon et al., 2013; Martínez-Maqueda et al., 2015). Extracellular components are also used for adhesion, including laminin, fibronectin, collagen, and proteoglycan. Intestinal mucus, an extracellular matrix composed of large glycoproteins (mucins), water, electrolyte, produced by goblet cells, may also be used. Extracellular components are reported to play a major role in modulating adhesion of microorganisms to epithelial surface (Otte and Podolsky, 2004; Johansson et al., 2011). Adhesion of probiotics to the gut mucosa may result in reduced colonization by pathogens, via competitive exclusion.

Several *in vitro* studies evidenced the involvement of extractable surface proteins, including Slps, in probiotic lactobacilli adhesion to mucus, and also to IECs. Gene inactivation of Slp genes was used in this purpose. Indeed, in *Lactobacillus acidophilus* NCFM, a knock-out mutant of the main S-layer protein, SlpA, evidenced its central role in adhesion to DCs and to their DC-SIGN receptors (Konstantinov et al., 2008). Inactivation of this gene also leads to reduced adhesion to cultured IEC (Buck et al., 2005). In the same strain, a key role of several surface layer associated proteins in adhesion was confirmed when the mutation of AcmB (β-*N*-acetylglucosaminidase) (Johnson et al., 2016), and of APF (Goh and Klaenhammer, 2010), led to a reduced binding to mucin, laminin and collagen and to IECs. By contrast, the deletion of the SLAP Serine Protease Homolog PrtX in *L. acidophilus* NCFM increases adhesion to mucin and fibronectin, which may result from the liberation of binding sites from the S-layer proteinaceous matrix (Johnson et al., 2017). In *L. salivarius* REN, inactivation of cbpA, encoding a SLAP choline-binding protein A, showed reduced adhesion to cultured

IEC (Wang et al., 2017). In other strains of *L. acidophilus*, the high expression of SlpA was correlated with a high capacity to adhere to Caco-2 cells (Ashida et al., 2011). However, it was reported that mutation of *slp* genes have pleiotropic effects, including the loss of exposure of a variety of SLAPs, making it difficult to conclude the specific role of each protein species.

A role of Slps in adhesion was also suggested by investigations using extracted surface proteins. Indeed, surface extractable proteins from *L. acidophilus*, *L. brevis*, *L. helveticus,* and *L. kefiri* block DC-SIGN receptors *in vitro* and prevent adhesion of pathogenic bacteria to DC-SIGN expressing cells (Prado Acosta et al., 2016). Furthermore, lithium extraction of *L. acidophilus* fb214 surface proteins reduces adhesion to cultured IECs (Meng et al., 2014). In *L. acidophilus* NCFM, FbpB is a SLAP, showing a fibronectin-binding domain, which mediates adhesion to mucin and fibronectin *in vitro* (Hymes et al., 2016). Accordingly, *L. brevis* ATCC 8287 surface layer shows a high affinity to laminin and to fibronectin, and its removal affects *L. brevis* adhesion to intestinal cells and to extracellular matrix proteins (Hynönen et al., 2002; de Leeuw et al., 2006; Uroić et al., 2016). Extracted surface bound proteins from probiotic lactobacilli were shown by different authors to bind *in vitro* to host cells proteins and extracellular matrix (Chen et al., 2007; Johnson-Henry et al., 2007; Carasi et al., 2014; Wa´sko et al., 2014; Prado Acosta et al., 2016; Zhang et al., 2016). However, such results should be considered with care, as Slps are poorly soluble, forming aggregates in aqueous environments, which renders interpretation of results difficult.

Adhesion to IECS and to mucus was also reported for dairy propionibacteria (Cousin et al., 2012). Nonetheless, the adhesion rates were highly variable, depending on the adhesion model used, the strain and the growth conditions (Cousin et al., 2010; Rabah et al., 2017). A comparative study of *P. freudenreichii* strains, in terms of 1) adhesion rate and 2) surface proteome, led to the identification of propionibacterial SlpB as a potent adhesin, which was further confirmed by *slpb* gene inactivation (de Carmo et al., 2017). One protein, Inl-like (**Figure 2**) contains several leucine-rich repeats (LRRs), predicted to be involved in protein binding. It shows homologies with InlA, which functions as an adhesin in *Listeria monocytogenes*. However, no functional characterization was undertaken to study the role of this protein in *P. freudenreichii* adhesion. Furthermore; the S-layer protein A of *P. freudenreichii* is predicted to have an Immunoglobulin-like fold domain, found in some surface proteins in pathogen bacteria (Buts et al., 2003; Lin et al., 2010), where it is reported to play a role in adhesion to host cells.

The *in vitro* investigations reported here indicate a role of Slps and other associated proteins in adhesion to mucus components and to IECs, which is a prominent feature for probiotic bacteria to trigger beneficial effects within the gut mucosa. Nonetheless, *in vivo* studies are needed to confirm the role of Slps in adhesion, and thus in the persistence of probiotic bacteria within the gut. In addition, Slps-mediated adhesion to mucus and IECs is poorly understood, but is thought to lead to inhibition of pathogenic microorganisms adhesion (Hynönen and Palva, 2013; Sleytr et al., 2014).

Inhibition of Pathogens by Extractable Surface Proteins

Inhibition of bacterial or viral infections is another beneficial application reported for probiotic bacteria, in which extractable surface proteins may play an important role. Indeed, adhesion of probiotic bacteria to the intestinal mucosa, via surface extractable adhesins interacting with host PRRs, may result in the inhibition of pathogens invasion by competitive exclusion. As an example, *L. helveticus* R0052 inhibits adhesion of entero-hemorrhagic *Escherichia coli* to caco-2 cells and so does its lithium surface proteins extract (Johnson-Henry et al., 2007), which coaggregates with several pathogen species (Beganović et al., 2011; Waśko et al., 2014). In addition, Slps of *L. crispatus* ZJ001 (Chen et al., 2007), *L. kefir* (Golowczyc et al., 2007), *L. salivarius,* and *L. reuteri* (Zhang et al., 2013) were proposed to be responsible for competitive exclusion of bacterial pathogens such as *E. coli* and *Salmonella* species. A similar effect was shown for *L. acidophilus* Slps which inhibited adherence and invasion of Caco-2 cells by *Salmonella enterica* serovar Typhimurium (Li et al., 2010, 2011) and protected cells by restoring the transepithelial resistance. This resistance is a recognized marker of barrier integrity of tight junctions, in epithelial cell lines (Klingberg et al., 2005). These Slps inhibited the mitogen-activated protein kinase (MAPK) signaling pathways induced by *S. typhimurium* (Li et al., 2011). In the same way, *L. plantarum* CGMCC 1258 MIMP protein, an extractable surface protein, limits pathogen invasion by inducing the expression of Claudin-1, Occludin, JAM-1, and ZO-1, by restoring tight junction damage and thus epithelium integrity (Qin et al., 2009; Liu et al., 2011a,b). Indeed, the intestinal mucosa tightness, relying on tight junctions (or "zonula occludens"), is impaired in several inflammatory and infections gut diseases (Vélez et al., 2007; Sengupta et al., 2013).

A potential role of extractable surface proteins covering *L. acidophilus* ATCC4356, *L. brevis* ATCC14869, *L. helveticus* ATCC12046 or *L. kefiri* JCM5818 against bacterial infection of cells was observed (Prado Acosta et al., 2016). Cells expressing the DC-specific DC-SIGN receptor exhibited a reduced susceptibility to bacterial infections, as a result of pre-treatment with Lactobacilli Slps. Moreover, pre-treatment of the pathogens (both Gram-negative and mycobacterial models) with Slps from *L. acidophilus* ATCC 4356 and *L. brevis* ATCC 14869 reduces pathogens viability but also prevents infection (Prado Acosta et al., 2016). DC-SIGN is a CLR present in DCs and macrophages, it is involved in the recognition of several viruses and other microbes, a key step in the entry of pathogens into the cell. Indeed, *L. acidophilus* ATCC4356 Slps stimulate the activation of mouse DCs by activating IFN-I signaling pathway, which inhibit invasion of DCs by influenza virus H9N2 (Gao et al., 2016). In addition, *L. acidophilus* ATCC4356 Slps inhibit the Junin virus (JUNV) infection by interacting with DC-SIGN (Martínez et al., 2012).

These data suggest a role of probiotics extractable surface proteins, including Slps, in the prevention of host colonization

by pathogens responsible for infectious diarrhea. Once again, data obtained using extracted surface bound proteins should be considered with care and these hypotheses should be confirmed *in vivo*.

Immunomodulatory Properties of Extractable Surface Proteins

Selected strains of LAB, including *L. acidophilus*, and of PROPIONIBACTERIA, including *P. freudenreichii*, exert anti-inflammatory properties in the context of colitis, by modulating gut immunity. Intestinal homeostasis is tightly governed by regulatory immune mechanisms, which are established by interactions involving commensal/probiotic bacteria and host PRRs, including CLRs and TLRs. The disruption of such regulatory mechanisms may result in IBD. **Figure 3A** illustrates the cross-talk between probiotic bacteria and the host, mediated by IECs and immunes cells within the GALT, which initiates an immune responses according to the MAMPs recognized by various PRRs. This hypothetical schema is mainly based on *in vitro* investigations.

Detailed studies revealed the crucial role of Slps in host–probiotic interactions mediated by intestinal cells,

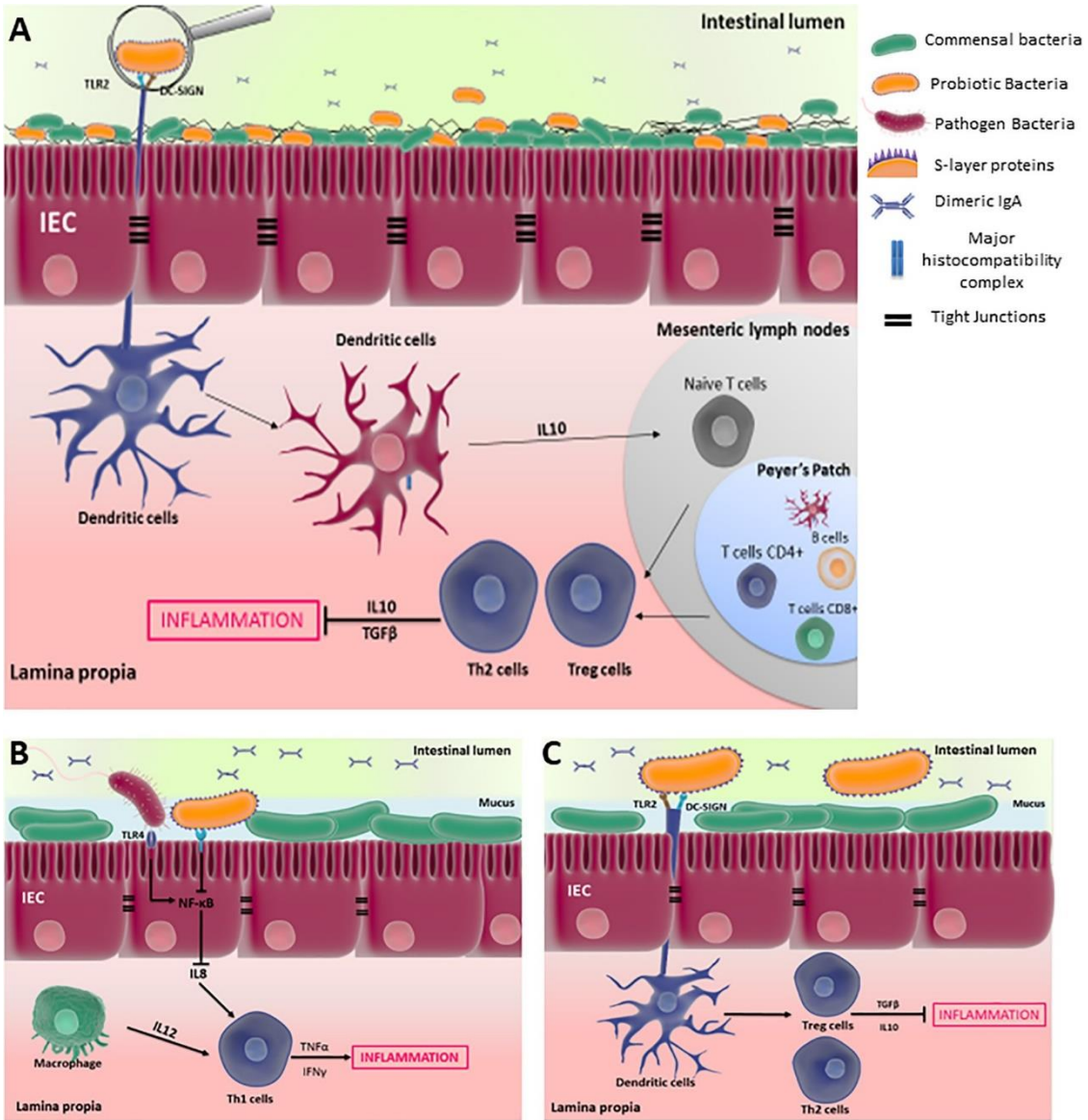

FIGURE 3 | Cross-talk between probiotic bacteria and the host, mediated by IECs and immunes cells, within the gut associated lymphoid tissues (GALT). An overview of the interaction of antigen-presenting-cells such as DCs with probiotic bacterian, which initiates a tolerance response by inducing Treg/Th2 anti-inflammatory response; while DCs-pathogenic bacteria interaction induces a Th1/Th17 proinflammatory response. (B) S-layer proteins inhibit the proinflammatory response of epithelial cells by reducing NF-κB activity, which is induced by pathogenic bacteria; (C) S-layer proteins are recognized by DCs via DC-SIGN and TLR2 receptors, inducing tolerance response in the GALT. These hypothetical schemata are mainly based on *in vitro* investigations.

which are an important protagonist at the forefront to maintain gut immunity homeostasis. *L. helveticus* MIMLh5 anti-inflammatory effects on Caco-2 cells is mediated by its SlpA and reduces activation of NF-κB (Taverniti et al., 2013).

L. acidophilus contains three different Slps, SlpA, SlpB, and SlpX, which interact with PRRs and modulate the immune response.

L. acidophilus Slps decrease interleukin (IL) 8 secretion in Caco-2 cells stimulated by *S. typhimurium* (Li et al., 2011). IL-8 cytokine is an important proinflammatory mediator secreted by intestinal cells, as well as by activated macrophages, leading, in synergy with IL-12, to the development of T helper (Th1) cells in the intestinal mucosa (Sanchez-Muñoz et al., 2008). **Figure 3B** illustrates how probiotics, via the recognition of Slps, may reduce activation of NF-κB and therefore expression of IL8 in IECs, limiting the proinflammatory response induced by pathogen.

Besides the interaction with IECs, Slps interact with antigen-presenting cells such as DCs, which reside in the Peyer's patch, lamina propria and mesenteric lymph nodes. As schematized in **Figure 3A**, DCs are the main stimulators of naive T cells, which distinguishes them from all other antigen presenting cells. Depending on the microbial stimulus encountered, DCs promote the differentiation of naïve T cells toward Th1, Th2, unpolarized T cells, Th17 or T regulatory cell responses. Investigation of the role of *L. acidophilus* Slps provided insights into immune cells-Slps interactions and the resulting immune response within the gut. The high expression of SlpA in *L. acidophilus* L92 was correlated with high induction of IL-12p70 secretion during splenocytes stimulation (Ashida et al., 2011). However, SlpA of *L. acidophilus* was reported to confer anti-inflammatory traits to the strain. Indeed, mutation of *L. acidophilus* NCFM SlpA results in a chromosomal inversion leading to dominant expression of SlpB. This mutant induces higher levels of proinflammatory cytokines such as IL-12p70, tumor necrosis factor-α (TNFα), and IL-1, compared to the wild-type strain, in DCs. However, both SlpA and SlpB activated TLR-2 at similar levels, an interaction that appeared to be crucial for the activating of IL-4-producing T cells (Konstantinov et al., 2008). The protective role of SlpA in the context of colitis was further elucidated. *L. acidophilus* NCK2187, which solely expresses SlpA, and its purified SlpA, both bind to the C type lectin SIGNR3 to induce regulatory signals that result in alleviation of colitis (Lightfoot et al., 2015). However, such protection was not observed in Signr3-/- mice, suggesting that the SlpA/SIGNR3 interaction plays a key regulatory role in the healing of colitis (Lightfoot et al., 2015). Other extractable surface proteins are involved in *L. acidophilus* NCFM immunomodulatory properties (Johnson et al., 2013, 2017). Indeed, mutation of the S-layer-associated serine protease homolog (Prtx) in *L. acidophilus* NCFM enhanced stimulation of IL-6, IL-12, and IL-10, compared to wild-type, when exposed to mouse DCs (Johnson et al., 2017). The authors suggest that PrtX may degrade certain cytokines, so that amounts of cytokines are higher when using the mutant. Mutation of Lba-1029, a putative SLAP in *L. acidophilus* NCFM, revealed its role in a pro-inflammatory response in murine DCs, including TNFα secretion (Johnson et al., 2013). SLAPs may thus take part in probiotics immunological properties. Slps of *L. helveticus* NS8, isolated from fermented koumiss, had no effect on the basal production of IL-10 in mouse macrophage cell line RAW264.7, but decreased IL12 expression triggered by LPS stimulation, suggesting an anti-inflammatory potential (Rong et al., 2015). Lactobacilli Slps contribute to the anti-inflammatory effect of probiotic bacteria within GALT, by interacting with DCs via different PRRs, which initiate the differentiation of Treg cells as illustrated by **Figure 3C**. Contrastingly, *L. helveticus* MIMLh5 and its SlpA act as stimulators of the innate immune system by triggering the expression of proinflammatory mediators such as TNFα and cyclooxygenase 2 (COX-2) in the human macrophage cell line U937 via TLR2 recognition (Taverniti et al., 2013). In the same experiments, this purified SlpA did not affect the expression of the anti-inflammatory cytokine interleukin-10 (Taverniti et al., 2013). Similarly, *L. brevis* Slps induce TNFα production in monocyte-derived dendritic cells (moDCs) (Uroić et al., 2016).

Immunomodulation was also reported for PROPIONIBACTERIA. As an example, strain-dependent immunomodulatory properties were evidenced *in vitro* using human PBMCs (Foligné et al., 2013; Deutsch et al., 2017) and then confirmed *in vivo* in a mouse colitis model (Foligné et al., 2010). Extractable surface proteins were involved in this modulatory effect (Le Maréchal et al., 2015). Indeed, the extracted proteins induce regulatory IL-10, in a dose-dependent manner, in PBMCs, with little or no secretion of pro-inflammatory factors (IL-12, TNFα, and IL6) (Le Maréchal et al., 2015). In addition, they reduce the proinflammatory response triggered by the proinflammatory strain *Lactococcus lactis* MG1363 in PBMCs (Le Maréchal et al., 2015). Inactivation of the gene encoding SlpB suppress IL-10 induction by *P. freudenreichii*, and so does inactivation of SlpE (Deutsch et al., 2017). In the same time, expression of other surface proteins, including SlpF and moonlighting proteins, is also correlated with this anti-inflammatory trait in *P. freudenreichii*. By contrast, *P. freudenreichii* strains expressing high level of SlpA, a true S-layer protein, exert no immunomodulatory effect. The authors suggest that the immunomodulatory properties of *P. freudenreichii* strains result from a combination of several surface proteins (Deutsch et al., 2017). By contrast with lactobacilli, mechanisms involved in the interaction between PRRs and PROPIONIBACTERIA surface proteins remain unknown.

In conclusion, probiotic bacteria, via Slps, may have an immunomodulatory effect mediated by C-type lectin and TLR receptors within GALT. However, the effective role of these proteins should be confirmed *in vivo* in order to give tools to fight gut inflammation.

Protective role of probiotics extractable surface proteins

Bacterial surface layers are generally recognized as the outermost structure of the bacterial cell (Gerbino et al., 2015a). They are thus widely considered to play a key role as an interface between intra- and extra-cellular compartments, and thus between the

bacterium and its environment or its host. This interface was most likely developed as a consequence of the selective pressure generated by these interactions. They may act as a physical barrier against external factors (Sleytr and Messner, 1988), and also prevent the release of cellular molecules (Sleytr and Messner, 1988). Indeed, they are described as a molecular sieve, forming a highly porous structure with pores exhibiting identical morphology and size, within a bacterial strain, with some variations among strains. The porosity of this layer occupies a surface area that can go up to 70% (Sleytr et al., 2001; Avall- Jääskeläinen and Palva, 2005). Studies on permeability have shown that some S-layers prevent the entry of molecules with molecular weights exceeding 10,000–15,000 kDa, providing the strain with a selective advantage (Lortal et al., 1993).

The presence of surface layers was reportedly linked with enhanced tolerance toward stresses. Presence of an S-layer was reported to decrease *L. helveticus* susceptibility to mutanolysin (Lortal et al., 1992). S-layers were furthermore shown to resist to extreme environmental conditions, even in extremophile bacteria, and to digestive assaults including variations in pH, bile salts, proteases and simulated gastrointestinal conditions (Smit et al., 2001; Chen et al., 2007; Eslami et al., 2013). In accordance, coating of liposomes with S-layer proteins from *brevis* and from *L. kefiri* increased their stability upon exposure to bile salts, pancreatic extract and pH, as compared to uncoated liposomes (Hollmann et al., 2007). On the other hand, removal of Slps caused enhanced *L. hilgardii* susceptibility toward bacteriolytic enzymes and physicochemical stress (Dohm et al., 2011). Furthermore, removal of the surface layer using the chaotropic agent lithium chloride drastically affects survival of *L. acidophilus* and of *L. helveticus* in simulated gastric and intestinal conditions (Frece et al., 2005; Meng et al., 2014). In accordance with a physiological role of S-layer proteins in defense mechanisms, their expression is induced by stimuli participating in digestive stress. In *L. acidophilus*, exposition to bile increases expression of SlpA in the ATCC 4356 strain (Khaleghi et al., 2010). The same authors further evidenced similar induction of this protein by acidic pH and heat stress (Khaleghi and Kasra, 2012). Among bile constituents, bile salts were further evidenced as the stimuli responsible for S-layer induction in *L. acidophilus* IBB 8001 (Grosu-Tudor et al., 2016). The induction of Slps expression may thus take part in a general strategy to adapt and survive harsh environmental conditions encountered in the environment, in the digestive tract, or in the succession thereof (Butler et al., 2013; Gerbino et al., 2015a). However, contrasting informations result from experimental gene inactivation of S-layer proteins in probiotic bacteria. Such mutations were indeed reported to drastically affect interactions with the host, including adhesion (do Carmo et al., 2017; Wang et al., 2017) and immune modulation (Konstantinov et al., 2008; Deutsch et al., 2017). However, their impact on probiotics stress tolerance is still elusive. Deletion of the *slpa* gene caused alterations in cell envelope structure and defect in resistance to solvent and shear stresses in the environmental extremophilic bacterium *Deinococcus radiodurans* (Rothfuss et al., 2006). In the probiotic *L. acidophilus*, the auxiliary S-layer protein SlpX, identified as a protein associated with the S-layer complex, plays a role in its permeability. Indeed, inactivation of SlpX affects the growth rate and the tolerance to bile salts in the NCK1962 mutant, as well as its relative overexpression in NCK1377, a SlpB- dominant strain that lacks SlpA protein. The NCK1962 slpX- negative mutant is more susceptible to SDS, yet more resistant to bile, than the wild type (Goh et al., 2009). Aggregation- promoting factors (Apf) are proteins considered as "S-layer-like" and identified at the surface of several lactobacilli. They share several characteristics with *Lactobacillus* S-layer proteins, such as their relative abundance on the cell surface, extractability by lithium chloride (LiCl), amino acid composition, predicted physical properties like high pI and indispensability for growth (Ventura et al., 2002; Jankovic et al., 2003). Mutation of the corresponding *apf* gene in *L. acidophilus* resulted in enhanced susceptibility to SDS, to bile and to intestinal and gastric juices (Goh and Klaenhammer, 2010). This suggests reduced survival during transit through the digestive tract. Finally, S-layers may also play a role in detoxification. The biosorption of toxic compounds, including uranium (Hennig et al., 2001) and heavy metals (Velásquez and Dussan, 2009) was reported for telluric bacteria belonging to the *Bacillus* species. It was attributed to their S-layers (Merroun et al., 2005). Such heavy metal biosorption was later reported for *L. kefir* (Gerbino et al., 2015b) with a key role of S-layer proteins. Biosorption of heavy metals and of mycotoxins was reported in *L. rhamnosus* and in *P. freudenreichii* (Ibrahim et al., 2006; Halttunen et al., 2008). These last were shown in a clinical study to reduce the biologically effective dose of aflatoxin exposure and may thereby offer an effective dietary approach to decrease the risk of liver cancer (El-Nezami et al., 2006).

Biotechnological applications

The peculiar property of Slps to auto-assemble and to form reproducible supramolecular aggregates that are reputed irreversible and resistant to physicochemical assaults naturally led to the idea to use them in the field of (nano)biotechnology (Hynönen et al., 2002; Sleytr et al., 2011). Such monomolecular arrays provide well-defined structures, depending on the physicochemical properties of the glycoprotein, which constitutes the closed, isoporous lattice, and for which a wide biodiversity exists, among bacteria. This led to investigate the application of re-crystalized Slps to develop ultrafiltration membranes with very accurate molecular cutoffs, good stability afforded by intramolecular cross-linking, low membrane fouling and tunable surface properties in terms of net charges and hydrophilicity. Furthermore, chemical modifications and genetic engineering allow the immobilization of functional molecules, including enzymes, ligands, antigens and antibodies, while retaining the self-assembly properties of Slps. Some Slps being known to spontaneously produce preformed nanoparticles, on native surface-layers, functionalized Slps nanoparticles were made, including metallic and semiconductor nanoparticles. Other applications include Slps as supporting structures for functional lipid membranes, or for vaccine development. Indeed, conjugate vaccines with Slps and antigens, haptens or recombinant

allergens, gave promising results in vaccination trials, due to intrinsic adjuvant properties of some SlpS. For a review on Slps biotechnological applications, se the review by Sleytr et al. (2014).

Conclusion

Extractable surface proteins, with various properties, have been described in several species and strains of probiotic bacteria. The peculiar properties of extractable surface proteins, including abundant expression, self-assembly, surface location, resistance to physicochemical assaults, immunomodulation, adhesion and toxic remediation, offer the possibility to orientate the biological properties of fermented food products and of probiotic food supplements. S-layer proteins have a great potential in the field of nanobiotechnology, because of their ability to form repetitive protein arrays by spontaneous association (Hynönen et al., 2002; Sleytr et al., 2011). This applies to vaccine candidates, to surface display of epitope, of proteins with therapeutic or biotechnologic interest (Michon et al., 2016). This opens promising perspectives in the field of gut disorders, including IBS and IBD, infectious diseases, as well as oral vaccination. Future trends include engineering of Slps for specific, efficient and cost-effective targeting of desired antigens and other medically important molecules.

AUTHOR CONTRIBUTIONS

GJ, YLL, and VA supervised the work and corrected the manuscript. FLRdC and HR did the main part of the bibliographical survey. All the authors took part in the writing of the manuscript.

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References

Anzengruber, J., Pabst, M., Neumann, L., Sekot, G., Heinl, S., Grabherr, R., et al. (2014). Protein O-glucosylation in *Lactobacillus buchneri*. *Glycoconj. J.* 31, 117–131. doi: 10.1007/s10719-013-9505-7

Ashida, N., Yanagihara, S., Shinoda, T., and Yamamoto, N. (2011). Characterization of adhesive molecule with affinity to Caco-2 cells in *Lactobacillus acidophilus* by proteome analysis. *J. Biosci. Bioeng.* 112, 333–337. doi: 10.1016/j.jbiosc.2011. 06.001

Avall-Jääskeläinen, S., and Palva, A. (2005). *Lactobacillus* surface layers and their applications. *FEMS Microbiol. Rev.* 29, 511–529. doi: 10.1016/j.femsre.2005. 04.003

Beganović, J., Frece, J., Kos, B., Leboš Pavunc, A., Habjanič, K., and Sušković, J. (2011). Functionality of the S-layer protein from the probiotic strain *Lactobacillus helveticus* M92. *Antonie Van Leeuwenhoek* 100, 43–53. doi: 10.1007/s10482-011-9563-4

Blum, S., Reniero, R., Schiffrin, E. J., Crittenden, R., Mattila-Sandholm, T., Ouwehand, A. C., et al. (1999). Adhesion studies for probiotics: need for validation and refinement. *Trends Food Sci. Technol.* 10, 405–410. doi: 10.1016/ S0924-2244(00)00028-5

Buck, B. L., Altermann, E., Svingerud, T., and Klaenhammer, T. R. (2005). Functional analysis of putative adhesion factors in *Lactobacillus acidophilus* NCFM. *Appl. Environ. Microbiol.* 71, 8344–8351. doi: 10.1128/AEM.71.12. 8344-8351.2005

Butler, È., Alsterfjord, M., Olofsson, T. C., Karlsson, C., Malmström, J., and Vásquez, A. (2013). Proteins of novel lactic acid bacteria from *Apis mellifera mellifera*: an insight into the production of known extra-cellular proteins during microbial stress. *BMC Microbiol.* 13:235. doi: 10.1186/1471-2180-13-235

Buts, L., Bouckaert, J., De Genst, E., Loris, R., Oscarson, S., Lahmann, M., et al. (2003). The fimbrial adhesin F17-G of enterotoxigenic *Escherichia coli* has an immunoglobulin-like lectin domain that binds N-acetylglucosamine. *Mol. Microbiol.* 49, 705–715.

Carasi, P., Ambrosis, N. M., De Antoni, G. L., Bressollier, P., Urdaci, M. C., and Serradell, M. L. Á. (2014). Adhesion properties of potentially probiotic *Lactobacillus kefiri* to gastrointestinal mucus. *J. Dairy Res.* 81, 16–23. doi: 10.1017/S0022029913000526

Carvalho, R. D., Breyner, N., Menezes-Garcia, Z., Rodrigues, N. M., Lemos, L., Maioli, T. U., et al. (2017). Secretion of biologically active pancreatitis-associated protein I (PAP) by genetically modified dairy *Lactococcus lactis* NZ9000 in the prevention of intestinal mucositis. *Microb. Cell Fact.* 16:27. doi: 10.1186/s12934-017-0624-x

Cavallero, G. J., Malamud, M., Casabuono, A. C., Serradell, M. L. Á., and Couto, A. S. (2017). A glycoproteomic approach reveals that the S-layer glycoprotein of *Lactobacillus kefiri* CIDCA 83111 is O- and N-glycosylated. *J. Proteomics* 162, 20–29. doi: 10.1016/j.jprot.2017.04.007

Chen, X., Xu, J., Shuai, J., Chen, J., Zhang, Z., and Fang, W. (2007). The S-layer proteins of *Lactobacillus crispatus* strain ZJ001 is responsible for competitive exclusion against *Escherichia coli* O157:H7 and *Salmonella* typhimurium. *Int. J. Food Microbiol.* 115, 307–312. doi: 10.1016/j.ijfoodmicro.2006.11.007

Cousin, F. J., Deutsch, S.-M., Perez Chaia, A., Foligné, B., and Jan, G. (2012). Interactions between probiotic dairy propionibacteria and the intestinal epithelium. *Curr. Immunol. Rev.* 8, 216–226. doi: 10.2174/15733951280067 1976

Cousin, F. J., Mater, D. D. G., Foligné, B., and Jan, G. (2010). Dairy propionibacteria as human probiotics: a review of recent evidence. *Dairy Sci. Technol.* 91, 1–26. doi: 10.1051/dst/2010032

de Leeuw, E., Li, X., and Lu, W. (2006). Binding characteristics of the *Lactobacillus brevis* ATCC 8287 surface layer to extracellular matrix proteins. *FEMS Microbiol. Lett.* 260, 210–215. doi: 10.1111/j.1574-6968.2006.00313.x

de sa Peixoto, P., Roiland, C., Thomas, D., Briard-Bion, V., Le Guellec, R., Parayre, S., et al. (2015). Recrystallized S-layer protein of a probiotic propionibacterium: structural and nanomechanical changes upon temperature or pH shifts probed by solid-state NMR and AFM. *Langmuir* 31, 199–208. doi: 10.1021/la503735z

Desvaux, M., Dumas, E., Chafsey, I., and Hébraud, M. (2006). Protein cell surface display in Gram-positive bacteria: from single protein to macromolecular protein structure. *FEMS Microbiol. Lett.* 256, 1–15. doi: 10.1111/j.1574-6968. 2006.00122.x

Deutsch, S.-M., Mariadassou, M., Nicolas, P., Parayre, S., Le Guellec, R., Chuat, V., et al. (2017). Identification of proteins involved in the anti-inflammatory properties of *Propionibacterium freudenreichii* by means of a multi-strain study. *Sci. Rep.* 7:46409. doi: 10.1038/srep46409

de Carmo, F. L. R., Rabah, H., Huang, S., Gaucher, F., Deplanche, M., Dutertre, S., et al. (2017). *Propionibacterium freudenreichii* surface protein SlpB is involved in adhesion to intestinal HT-29 cells. *Front. Microbiol.* 8:1033. doi: 10.3389/fmicb.2017.01033

do Carmo, F. L. R., Rabah, H., Huang, S., Gaucher, F., Deplanche, M., Dutertre, S., et al. (2017). *Propionibacterium freudenreichii* surface protein SlpB is involved in adhesion to intestinal HT-29 cells. *Front. Microbiol.* 8:1033. doi: 10.3389/fmicb.2017.01033

Dohm, N., Petri, A., Schlander, M., Schlott, B., König, H., and Claus, H. (2011). Molecular and biochemical properties of the S-layer protein from the wine bacterium *Lactobacillus hilgardii* B706. *Arch. Microbiol.* 193, 251–261. doi: 10.1007/s00203-010-0670-9

El-Nezami, H. S., Polychronaki, N. N., Ma, J., Zhu, H., Ling, W., Salminen, E. K., et al. (2006). Probiotic supplementation reduces a biomarker for increased risk of liver cancer in young men from Southern China. *Am. J. Clin. Nutr.* 83, 1199–1203.

Eslami, N., Kermanshahi, R. K., and Erfan, M. (2013). Studying the stability of S-layer protein of *Lactobacillus acidophilus* ATCC 4356 in simulated gastrointestinal fluids using SDS-PAGE and circular dichroism. *Iran. J. Pharm. Res.* 12, 47–56.

Evivie, S. E., Huo, G.-C., Igene, J. O., and Bian, X. (2017). Some current applications, limitations and future perspectives of lactic acid bacteria as probiotics. *Food Nutr. Res.* 61:1318034. doi: 10.1080/16546628.2017.1318034

Fagan, R. P., and Fairweather, N. F. (2014). Biogenesis and functions of bacterial S-layers. *Nat. Rev. Microbiol.* 12, 211–222. doi: 10.1038/nrmicro3213 FAO/WHO (2006). *Probiotics in Food: Health and Nutritional Properties and Guidelines for Evaluation*. Report of a Joint FAO/WHO Working Group on Drafting Guidelines for the Evaluation of Probiotics in Food. Rome: FAO.

Foligné, B., Breton, J., Mater, D., and Jan, G. (2013). Tracking the microbiome functionality: focus on *Propionibacterium* species. *Gut* 62, 1227–1228. doi: 10.1136/gutjnl-2012-304393

Foligné, B., Deutsch, S.-M., Breton, J., Cousin, F. J., Dewulf, J., Samson, M., et al. (2010). Promising immunomodulatory effects of selected strains of dairy propionibacteria as evidenced *in vitro* and *in vivo*. *Appl. Environ. Microbiol.* 76, 8259–8264. doi: 10.1128/AEM.01976-10

Food and Agriculture Organization of the United Nations, and World Health Organization (FAO/WHO) (ed.) (2002). *Probiotics in Food: Health and Nutritional Properties and Guidelines for Evaluation*. Rome: Food and Agriculture Organization of the United Nations.

Frece, J., Kos, B., Svetec, I. K., Zgaga, Z., Mrsa, V., and Suskovic, J. (2005). Importance of S-layer proteins in probiotic activity of *Lactobacillus acidophilus* M92. *J. Appl. Microbiol.* 98,285–292.

Gagnon, M., Zihler Berner, A., Chervet, N., Chassard, C., and Lacroix, C. (2013). Comparison of the Caco-2, HT-29 and the mucus-secreting HT29-MTX intestinal cell models to investigate *Salmonella* adhesion and invasion. *J. Microbiol. Methods* 94, 274–279. doi: 10.1016/j.mimet.2013.06.027

Gao, X., Huang, L., Zhu, L., Mou, C., Hou, Q., and Yu, Q. (2016). Inhibition of H9N2 virus invasion into dendritic cells by the S-Layer protein from *L. acidophilus* ATCC 4356. *Front. Cell. Infect. Microbiol.* 6:137. doi: 10.3389/fcimb.2016.00137

Gerbino, E., Carasi, P., Mobili, P., Serradell, M. A., and Gómez-Zavaglia, A. (2015a). Role of S-layer proteins in bacteria. *World J. Microbiol. Biotechnol.* 31, 1877–1887. doi: 10.1007/s11274-015-1952-9

Gerbino, E., Carasi, P., Araujo-Andrade, C., Tymczyszyn, E. E., and Gómez-Zavaglia, A. (2015b). Role of S-layer proteins in the biosorption capacity of lead by *Lactobacillus kefir*. *World J. Microbiol. Biotechnol.* 31, 583–592. doi: 10.1007/s11274-015-1812-7

Goh, Y. J., Azcarate-Peril, M. A., O'Flaherty, S., Durmaz, E., Valence, F., Jardin, J., et al. (2009). Development and application of an upp-based counterselective gene replacement system for the study of the S-layer protein SlpX of *Lactobacillus acidophilus* NCFM. *Appl. Environ. Microbiol.* 75, 3093–3105. doi: 10.1128/AEM.02502-08

Goh, Y. J., and Klaenhammer, T. R. (2010). Functional roles of aggregation-promoting-like factor in stress tolerance and adherence of *Lactobacillus acidophilus* NCFM. *Appl. Environ. Microbiol.* 76, 5005–5012. doi: 10.1128/ AEM.00030-10

Golowczyc, M. A., Mobili, P., Garrote, G. L., Abraham, A. G., and De Antoni, G. L. (2007). Protective action of *Lactobacillus kefir* carrying S-layer protein against *Salmonella enterica* serovar Enteritidis. *Int. J. Food Microbiol.* 118, 264–273. doi: 10.1016/j.ijfoodmicro.2007.07.042

Grosu-Tudor, S.-S., Brown, L., Hebert, E. M., Brezeanu, A., Brinzan, A., Fadda, S., et al. (2016). S-layer production by *Lactobacillus acidophilus* IBB 801 under environmental stress conditions. *Appl. Microbiol. Biotechnol.* 100, 4573–4583. doi: 10.1007/s00253-016-7355-5

Halttunen, T., Collado, M. C., El-Nezami, H., Meriluoto, J., and Salminen, S. (2008). Combining strains of lactic acid bacteria may reduce their toxin and heavy metal removal efficiency from aqueous solution. *Lett. Appl. Microbiol.* 46, 160–165. doi: 10.1111/j.1472-765X.2007.02276.x

Hennig, C., Panak, P. J., Reich, T., Rossberg, A., Raff, J., Selenska-Pobell, S., et al. (2001). EXAFS investigation of uranium (VI) complexes formed at *Bacillus cereus* and *Bacillus sphaericus* surfaces. *Radiochim. Acta* 89, 625–631.

Hirakata, Y., Izumikawa, K., Yamaguchi, T., Igimi, S., Furuya, N., Maesaki, S., et al. (1998). Adherence to and penetration of human intestinal Caco-2 epithelial cell monolayers by *Pseudomonas aeruginosa*. *Infect. Immun.* 66, 1748–1751.

Hollmann, A., Delfederico, L., Glikmann, G., De Antoni, G., Semorile, L., and Disalvo, E. A. (2007). Characterization of liposomes coated with S-layer proteins from lactobacilli. *Biochim. Biophys. Acta* 1768, 393–400. doi: 10.1016/j.bbamem.2006.09.009

Houwink, A. L. (1953). A macromolecular mono-layer in the cell wall of *Spirillum* spec. *Biochim. Biophys. Acta* 10, 360–366.

Hymes, J. P., Johnson, B. R., Barrangou, R., and Klaenhammer, T. R. (2016). Functional analysis of an S-layer-associated fibronectin-binding protein in *Lactobacillus acidophilus* NCFM. *Appl. Environ. Microbiol.* 82, 2676–2685. doi: 10.1128/AEM.00024-16

Hynönen, U., and Palva, A. (2013). *Lactobacillus* surface layer proteins: structure, function and applications. *Appl. Microbiol. Biotechnol.* 97, 5225–5243. doi: 10.1007/s00253-013-4962-2

Hynönen, U., Westerlund-Wikström, B., Palva, A., and Korhonen, T. K. (2002). Identification by flagellum display of an epithelial cell- and fibronectin-binding function in the SlpA surface protein of *Lactobacillus brevis*. *J. Bacteriol.* 184, 3360–3367.

Ibrahim, F., Halttunen, T., Tahvonen, R., and Salminen, S. (2006). Probiotic bacteria as potential detoxification tools: assessing their heavy metal binding isotherms. *Can. J. Microbiol.* 52, 877–885.

Jankovic, I., Ventura, M., Meylan, V., Rouvet, M., Elli, M., and Zink, R. (2003). Contribution of aggregation-promoting factor to maintenance of cell shape in *Lactobacillus gasseri* 4B2. *J. Bacteriol.* 185, 3288–3296.

Johansson, M. E. V., Ambort, D., Pelaseyed, T., Schütte, A., Gustafsson, J. K., Ermund, A., et al. (2011). Composition and functional role of the mucus layers in the intestine. *Cell. Mol. Life Sci.* 68, 3635–3641. doi: 10.1007/s00018-011- 0822-3

Johnson, B., Selle, K., O'Flaherty, S., Goh, Y. J., and Klaenhammer, T. (2013). Identification of extracellular surface-layer associated proteins in *Lactobacillus acidophilus* NCFM. *Microbiology* 159,2269–2282. doi: 10.1099/mic.0.070755-0

Johnson, B. R., Hymes, J., Sanozky-Dawes, R., Henriksen, E. D., Barrangou, R., and Klaenhammer, T. R. (2016). Conserved S-layer-associated proteins revealed by exoproteomic survey of S-layer-forming lactobacilli. *Appl. Environ. Microbiol.* 82, 134–145. doi: 10.1128/AEM.01968-15

Johnson, B. R., O'Flaherty, S., Goh, Y. J., Carroll, I., Barrangou, R., and Klaenhammer, T. R. (2017). The S-layer associated serine protease homolog PrtX impacts cell surface-mediated microbe-host interactions of *Lactobacillus acidophilus* NCFM. *Front. Microbiol.* 8:1185. doi: 10.3389/fmicb.2017.01185

Johnson-Henry, K. C., Hagen, K. E., Gordonpour, M., Tompkins, T. A., and Sherman, P. M. (2007). Surface-layer protein extracts from *Lactobacillus helveticus* inhibit enterohaemorrhagic *Escherichia coli* O157:H7 adhesion to epithelial cells. *Cell. Microbiol.* 9, 356–367. doi: 10.1111/j.1462-5822.2006. 00791.x

Khaleghi, M., and Kasra, R. (2012). "Effect of environmental stresses on S-Layer production in *Lactobacillus acidophilus* ATCC 4356," in *Advances in Applied Biotechnology*, ed. M. Petre (Rijeka: InTech).

Khaleghi, M., Kermanshahi, R. K., Yaghoobi, M. M., Zarkesh-Esfahani, S. H., and Baghizadeh, A. (2010). Assessment of bile salt effects on S-layer production, *slp* gene expression and some physicochemical properties of *Lactobacillus acidophilus* ATCC 4356. *J. Microbiol. Biotechnol.* 20, 749–756.

Klingberg, T. D., Pedersen, M. H., Cencic, A., and Budde, B. B. (2005). Application of Measurements of transepithelial electrical resistance of intestinal epithelial

cell monolayers to evaluate probiotic activity. *Appl. Environ. Microbiol.* 71, 7528–7530. doi: 10.1128/AEM.71.11.7528-7530.2005

Konstantinov, S. R., Smidt, H., de Vos, W. M., Bruijns, S. C. M., Singh, S. K., Valence, F., et al. (2008). S-layer protein A of *Lactobacillus acidophilus* NCFM regulates immature dendritic cell and T cell functions. *Proc. Natl. Acad. Sci. U.S.A.* 105, 19474–19479. doi:10.1073/pnas.0810305105

Koval, S. F., and Murray, R. G. (1984). The isolation of surface array proteins from bacteria. *Can. J. Biochem. Cell Biol. Rev. Can. Biochim. Biol. Cell.* 62, 1181–1189.

Le Maréchal, C., Peton, V., Plé, C., Vroland, C., Jardin, J., Briard-Bion, V., et al.

(2015). Surface proteins of *Propionibacterium freudenreichii* are involved in its anti-inflammatory properties. *J. Proteomics* 113, 447–461. doi: 10.1016/j.jprot.2014.07.018

Lebeer, S., Vanderleyden, J., and De Keersmaecker, S. C. J. (2008). Genes and molecules of lactobacilli supporting probiotic action. *Microbiol. Mol. Biol. Rev.* 72, 728–764. doi: 10.1128/MMBR.00017-08

Li, P., Ye, X., Wang, Z., Yu, Q., and Yang, Q. (2010). Effects of S-layer proteins from *Lactobacillus* against *Salmonella* typhimurium adhesion and invasion on Caco-2 cells. *Wei Sheng Wu Xue Bao* 50, 1226–1231.

Li, P., Yu, Q., Ye, X., Wang, Z., and Yang, Q. (2011). *Lactobacillus* S-layer protein inhibition of *Salmonella*-induced reorganization of the cytoskeleton and activation of MAPK signalling pathways in Caco-2 cells. *Microbiol. Read. Engl.* 157, 2639–2646. doi: 10.1099/mic.0.049148-0

Lightfoot, Y. L., Selle, K., Yang, T., Goh, Y. J., Sahay, B., Zadeh, M., et al.

(2015). SIGNR3-dependent immune regulation by *Lactobacillus acidophilus* surface layer protein A in colitis. *EMBO J.* 34, 881–895. doi:

10.15252/embj.201490296 Lin, Y.-P., McDonough, S. P., Sharma, Y., and Chang, Y.-F. (2010). The terminal immunoglobulin-like repeats of LigA and LigB of *Leptospira* enhance their binding to gelatin binding domain of fibronectin and host cells. *PLoS One*

5:e11301. doi: 10.1371/journal.pone.0011301

Liu, Z., Shen, T., Chen, H., Zhou, Y., Zhang, P., Ma, Y., et al. (2011a). Functional characterization of MIMP for its adhesion to the intestinal epithelium. *Front. Biosci.* 16:2106–2127.

Liu, Z., Shen, T., Zhang, P., Ma, Y., and Qin, H. (2011b). *Lactobacillus plantarum* surface layer adhesive protein protects intestinal epithelial cells against tight junction injury induced by enteropathogenic *Escherichia coli*. *Mol. Biol. Rep.* 38, 3471–3480. doi: 10.1007/s11033-010-0457-8

Lortal, S., Rouault, A., Cesselin, B., and Sleytr, U. B. (1993). Paracrystalline surface layers of dairy propionibacteria. *Appl. Environ. Microbiol.* 59, 2369–2374.

Lortal, S., Van Heijenoort, J., Gruber, K., and Sleytr, U. B. (1992). S-layer of *Lactobacillus helveticus* ATCC 12046: isolation chemical characterization and re-formation after extraction with lithium chloride. *J. Gen. Microbiol.* 138, 611–618.

Maoret, J. J., Font, J., Augeron, C., Codogno, P., Bauvy, C., Aubery, M., et al. (1989). A mucus-secreting human colonic cancer cell line. Purification and partial characterization of the secreted mucins. *Biochem. J.* 258, 793–799. doi: 10.1042/bj2580793

Martínez, M. G., Prado Acosta, M., Candurra, N. A., and Ruzal, S. M. (2012). S-layer proteins of *Lactobacillus acidophilus* inhibits JUNV infection. *Biochem. Biophys. Res. Commun.* 422, 590–595. doi: 10.1016/j.bbrc.2012.05.031

Martínez-Maqueda, D., Miralles, B., and Recio, I. (2015). "HT29 cell line," in *The Impact of Food Bioactives on Health*, eds K. Verhoeckx, P. Cotter, I. López-Expósito, C. Kleiveland, T. Lea, and A. Mackie (Cham: Springer International Publishing), 113–124. doi: 10.1007/978-3-319-16104-4_11

Meng, J., Zhu, X., Gao, S.-M., Zhang, Q.-X., Sun, Z., and Lu, R.-R. (2014).

Characterization of surface layer proteins and its role in probiotic properties of three *Lactobacillus* strains. *Int. J. Biol. Macromol.* 65, 110–114. doi: 10.1016/j.ijbiomac.2014.01.024

Merroun, M. L., Raff, J., Rossberg, A., Hennig, C., Reich, T., and Selenska-Pobell, S. (2005). Complexation of uranium by cells and S-layer sheets of *Bacillus sphaericus* JG-A12. *Appl. Environ. Microbiol.* 71, 5532–5543. doi: 10.1128/AEM.71.9.5532-5543.2005

Messner, P., Steiner, K., Zarschler, K., and Schäffer, C. (2008). S-layer nanoglycobiology of bacteria. *Carbohydr. Res.* 343, 1934–1951. doi: 10.1016/j.carres.2007.12.025

Michon, C., Langella, P., Eijsink, V. G. H., Mathiesen, G., and Chatel, J. M. (2016). Display of recombinant proteins at the surface of lactic acid bacteria: strategies and applications. *Microb. Cell Fact.* 15:70. doi: 10.1186/s12934-016-0468-9

Mobili, P., Gerbino, E., Tymczyszyn, E., and Gómez-Zavaglia, A. (2010). S-layers in lactobacilli: structural characteristics and putative role in surface and probiotic properties of whole bacteria. *Curr. Res. Technol. Educ. Top. Appl. Microbiol. Microb. Biotechnol.* 22, 1224–1234.

Otte, J.-M., and Podolsky, D. K. (2004). Functional modulation of enterocytes by gram-positive and gram-negative microorganisms. *Am. J. Physiol. Gastrointest. Liver Physiol.* 286, G613–G626. doi: 10.1152/ajpgi.00341.2003

Prado Acosta, M., Ruzal, S. M., and Cordo, S. M. (2016). S-layer proteins from *Lactobacillus* sp. inhibit bacterial infection by blockage of DC-SIGN cell receptor. *Int. J. Biol. Macromol.* 92, 998–1005. doi: 10.1016/j.ijbiomac.2016.07.096

Pum, D., and Sleytr, U. B. (2014). Reassembly of S-layer proteins.

Nanotechnology

25:312001. doi: 10.1088/0957-4484/25/31/312001

Qin, H., Zhang, Z., Hang, X., and Jiang, Y. (2009). *L. plantarum* prevents enteroinvasive *Escherichia coli*-induced tight junction proteins changes in intestinal epithelial cells. *BMC Microbiol.* 9:63. doi: 10.1186/1471-2180- 9-63

Rabah, H., Rosa do Carmo, F. L., and Jan, G. (2017). Dairy propionibacteria: versatile probiotics. *Microorganisms* 5:E24. doi: 10.3390/microorganisms5020024

Rong, J., Zheng, H., Liu, M., Hu, X., Wang, T., Zhang, X., et al. (2015). Probiotic and anti-inflammatory attributes of an isolate *Lactobacillus helveticus* NS8 from Mongolian fermented koumiss. *BMC Microbiol.* 15:196. doi: 10.1186/s12866- 015-0525-2

Rook, G., Bäckhed, F., Levin, B. R., McFall-Ngai, M. J., and McLean, A. R. (2017). Evolution, human-microbe interactions, and life history plasticity. *Lancet* 390, 521–530. doi: 10.1016/S0140-6736(17)30566-4

Rothfuss, H., Lara, J. C., Schmid, A. K., and Lidstrom, M. E. (2006). Involvement of the S-layer proteins Hpi and SlpA in the maintenance of cell envelope integrity in *Deinococcus radiodurans* R1. *Microbiol. Read. Engl.* 152, 2779–2787. doi: 10.1099/mic.0.28971-0

Sanchez-Muñoz, F., Dominguez-Lopez, A., and Yamamoto-Furusho, J. K. (2008). Role of cytokines in inflammatory bowel disease. *World J. Gastroenterol.* 14, 4280–4288. doi: 10.3748/wjg.14.4280

Sára, M., and Sleytr, U. B. (1996). Crystalline bacterial cell surface layers (S-layers): from cell structure to biomimetics. *Prog. Biophys. Mol. Biol.* 65, 83–111. doi: 10.1016/S0079-6107(96)00007-7

Sára, M., and Sleytr, U. B. (2000). S-Layer proteins. *J. Bacteriol.* 182, 859–868.

Schuster, B., and Sleytr, U. B. (2015). Relevance of glycosylation of S-layer proteins for cell surface properties. *Acta Biomater.* 19, 149–157. doi: 10.1016/j.actbio. 2015.03.020

Sengupta, R., Altermann, E., Anderson, R. C., McNabb, W. C., Moughan, P. J., and Roy, N. C. (2013). The role of cell surface architecture of lactobacilli in host-microbe interactions in the gastrointestinal tract. *Mediators Inflamm.* 2013:237921. doi: 10.1155/2013/237921

Sleytr, U. B. (1997). I. Basic and applied S-layer research: an overview. *FEMS Microbiol. Rev.* 20, 5–12. doi: 10.1111/j.1574-6976.1997.tb00301.x

Sleytr, U. B., and Beveridge, T. J. (1999). Bacterial S-layers. *Trends Microbiol.* 7, 253–260.

Sleytr, U. B., and Messner, P. (1988). Crystalline surface layers in procaryotes. *J. Bacteriol.* 170, 2891–2897.

Sleytr, U. B., Sára, M., Pum, D., and Schuster, B. (2001). Characterization and use of crystalline bacterial cell surface layers. *Prog. Surf. Sci.* 68, 231–278. doi: 10.1016/S0079-6816(01)00008-9

Sleytr, U. B., Schuster, B., Egelseer, E.-M., and Pum, D. (2014). S-layers: principles and applications. *FEMS Microbiol. Rev.* 38, 823–864. doi: 10.1111/1574-6976. 12063

Sleytr, U. B., Schuster, B., Egelseer, E. M., Pum, D., Horejs, C. M., Tscheliessnig, R., et al. (2011). Nanobiotechnology with S-layer proteins as building blocks. *Prog. Mol. Biol. Transl. Sci.* 103, 277–352. doi: 10.1016/B978-0-12-415906-8.00 003-0

Smit, E., Oling, F., Demel, R., Martinez, B., and Pouwels, P. H. (2001). The S-layer protein of *Lactobacillus acidophilus* ATCC 4356: identification and characterization of domains responsible for S-protein assembly and cell wall binding. *J. Mol. Biol.* 305, 245–257. doi: 10.1006/jmbi.2000.4258

Syngai, G. G., Gopi, R., Bharali, R., Dey, S., Lakshmanan, G. M. A., and Ahmed, G. (2016). Probiotics - the versatile functional food ingredients. *J. Food Sci. Technol.* 53, 921–933. doi: 10.1007/s13197-015-2011-0

Taverniti, V., Stuknyte, M., Minuzzo, M., Arioli, S., De Noni, I., Scabiosi, C., et al. (2013). S-layer protein mediates the stimulatory effect of *Lactobacillus helveticus* MIMLh5 on innate immunity. *Appl. Environ. Microbiol.* 79, 1221–1231. doi: 10.1128/AEM.03056-12

Uroić, K., Novak, J., Hynönen, U., Pietilä, T. E., Leboš Pavunc, A., Kant, R., et al.

(2016). The role of S-layer in adhesive and immunomodulating properties of probiotic starter culture *Lactobacillus brevis* D6 isolated from artisanal smoked fresh cheese. *Food Sci. Technol.* 69, 623–632. doi: 10.1016/j.lwt.2016.02.013

Velásquez, L., and Dussan, J. (2009). Biosorption and bioaccumulation of heavy metals on dead and living biomass of *Bacillus sphaericus*. *J. Hazard. Mater.* 167, 713–716. doi: 10.1016/j.jhazmat.2009.01.044

Velasquez-Manoff, M. (2015). Gut microbiome: the peacekeepers. *Nature* 518, S3–S11. doi: 10.1038/518S3a

Vélez, M. P., De Keersmaecker, S. C. J., and Vanderleyden, J. (2007). Adherence factors of *Lactobacillus* in the human gastrointestinal tract. *FEMS Microbiol. Lett.* 276, 140–148. doi: 10.1111/j.1574-6968.2007.00908.x

Ventura, M., Jankovic, I., Walker, D. C., Pridmore, R. D., and Zink, R. (2002). Identification and characterization of novel surface proteins in *Lactobacillus johnsonii* and *Lactobacillus gasseri*. *Appl. Environ. Microbiol.* 68, 6172–6181.

Vesterlund, S., Paltta, J., Karp, M., and Ouwehand, A. C. (2005). Measurement of bacterial adhesion-in vitro evaluation of different methods. *J. Microbiol. Methods* 60, 225–233. doi: 10.1016/j.mimet.2004.09.013

Vindigni, S. M., Zisman, T. L., Suskind, D. L., and Damman, C. J. (2016). The intestinal microbiome, barrier function, and immune system in inflammatory bowel disease: a tripartite pathophysiological circuit with implications for new therapeutic directions. *Ther. Adv. Gastroenterol.* 9, 606–625. doi: 10.1177/1756283X16644242

Wang, R., Jiang, L., Zhang, M., Zhao, L., Hao, Y., Guo, H., et al. (2017). The Adhesion of *Lactobacillus salivarius* REN to a Human intestinal epithelial cell line requires S-layer proteins. *Sci. Rep.* 7:44029. doi: 10.1038/srep44029

Waśko, A., Polak-Berecka, M., Paduch, R., and Jóźwiak, K. (2014). The effect of moonlighting proteins on the adhesion and aggregation ability of *Lactobacillus helveticus*. *Anaerobe* 30C, 161–168. doi: 10.1016/j.anaerobe.2014.10.002

Zaneveld, J., Turnbaugh, P. J., Lozupone, C., Ley, R. E., Hamady, M., Gordon, J. I., et al. (2008). Host-bacterial coevolution and the search for new drug targets. *Curr. Opin. Chem. Biol.* 12, 109–114. doi: 10.1016/j.cbpa.2008.01.015

Zhang, W., Wang, H., Liu, J., Zhao, Y., Gao, K., and Zhang, J. (2013). Adhesive
ability means inhibition activities for *Lactobacillus* against pathogens and S-layer protein plays an important role in adhesion. *Anaerobe* 22, 97–103. doi: 10.1016/j.anaerobe.2013.06.005

Zhang, Y., Xiang, X., Lu, Q., Zhang, L., Ma, F., and Wang, L. (2016). Adhesions of extracellular surface-layer associated proteins in *Lactobacillus* M5-L and Q8-L. *J. Dairy Sci.* 99, 1011–1018. doi: 10.3168/jds.2015-10020

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CHAPTER 3 – ORIGINAL ARTICLE - *Propionibacterium freudenreichii* SURFACE PROTEIN SLPB IS INVOLVED IN ADHESION TO INTESTINAL HT-29 CELLS.

Propionibacterium freudenreichii has shown strain-dependent beneficial effects, including modulation of colon physiology by strain metabolites releasing, which are described as nutraceuticals compounds (RABAH, ROSA DO CARMO, JAN, 2017). For optimal effect on target cells, the local action of immunomodulatory metabolites and molecular motifs is favored by the ability of probiotics to adhere to host cells (DO CARMO et al., 2018). Dairy propionibacteria were shown to adhere to mice intestinal epithelial cells both *ex vivo* and *in vivo* (ZARATE, 2012), as well as to human intestinal cell lines *in vitro* (HUANG; ADAMS, 2003; MOUSSAVI; ADAMS, 2010). Moreover, adhesion is reportedly a key criterion in strain selection, since it is described as the initial step in the colonization of the host (HAVENAAR; BRINK; VELD, 1992, p. havenar; PREISING et al., 2010; RIEDEL et al., 2006). Adhesion depends on crucial surface compounds, including surface proteins (LEBEER; VANDERLEYDEN; DE KEERSMAECKER, 2010).

S-layer proteins were firstly described in 1953 by Houwink and were first found on *Spirillum* sp. cell surface, and subsequently found in many probiotic bacteria such as L*actobacillus* species (HOUWINK, 1953; SLEYTR et al., 2014). Over the decades, through a range of studies, S-layer proteins have been shown to exert versatile roles in crucial bacteria functions, such as determination or maintenance of cell shape, molecular sieve, enzymatic activities, adhesion, coaggregation, modulation of gut immune cells, protection against environmental stresses and antimicrobial peptides (HYNÖNEN; PALVA, 2013).

During the SURFing project, five extractable surface proteins, SlpA, SlpB, SlpE, Internaline-like A (Inl A) and Large surface protein A (lsp A), were identified in *P. freudenreichii*: CIRM-BIA 129. Then, through an integrative approach, SlpB protein, a major extractable surface protein in *P. freudenreichii* CIRM-BIA 129, was pointed out as a key with a pivotal role (DEUTSCH et al., 2017; LE MARÉCHAL et al., 2015). However, molecular investigations on dairy propionibacteria are lacking. Thus, the precise role of each of these 5 surface proteins is still unclear.

Elucidating the molecules involved in adhesion mechanisms constitutes a fundamental step in the understanding of interactions between the bacterium and the host. Considering the central role that S-layer proteins can play in other bacteria, we thus investigated this process poorly characterized in propionibacteria. In this section, we thus aimed at deciphering the mechanisms involved in *P. freudenreichii* adhesion to intestinal epithelial cells. This work opens new perspectives for the selection of adhesive strains. This may in turn enhance the initial steps of host colonization, favour probiotic/host crosstalk, allow local delivery of nutraceutical compounds, and modulate host inflammatory response through immunomodulatory effects.

In this work, we then aimed to investigate the involvement of *P. freudenreichii* surface layers proteins in adhesion to intestinal epithelial cells.

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CHAPITRE 3 – ARTICLE ORIGINAL ARTICLE - *Propionibacterium freudenreichii* SURFACE PROTEIN SlpB IS INVOLVED IN ADHESION TO INTESTINAL HT-29 CELLS.

Propionibacterium freudenreichii a montré des effets bénéfiques dépendant de la souche, y compris la modulation de la physiologie du côlon via la libération de métabolites, dont certains sont décrits comme des nutraceutiques (RABAH; ROSA DO CARMO; JAN, 2017). Pour un effet optimal sur les cellules-cibles, l'action locale des métabolites et des motifs moléculaire immunomodulateurs est favorisée par la capacité de probiotiques à adhérer aux cellules de l'hôte (DO CARMO et al., 2018). Les bactéries propioniques laitières adhèrent aux cellules épithéliales intestinales murines, *ex vivo* et *in vivo* (ZARATE, 2012), ainsi qu'à des lignées de cellules épithéliales intestinales humaines en culture (HUANG; ADAMS, 2003; MOUSSAVI; ADAMS, 2010). De plus, l'adhésion est, d'après la littérature, un critère clé de sélection de souches, étant donné que cela constitue la première étape de la colonisation de l'hôte (HAVENAAR; BRINK; VELD, 1992, p. havenar; PREISING et al., 2010; RIEDEL et al., 2006). L'adhésion dépend de composés de surface clés, y compris les protéines de surface (LEBEER; VANDERLEYDEN; DE KEERSMAECKER, 2010).

Les protéines de S-layer ont été décrites pour la première fois en 1953 par Houwink. Elles ont été trouvées à la surface cellulaire de *Spirillum* sp., puis par la suite retrouvées chez différentes bactéries probiotiques telles que des lactobacilles (HOUWINK, 1953; SLEYTR et al., 2014). Au cours des dernières décennies, à travers une série d'études, les protéines S-layer se sont vues attribuer des rôles divers dans des fonctions bactériennes cruciales, telles que la détermination et le maintien de la morphologie cellulaire, le tamisage moléculaire, certaines activités enzymatiques, l'adhésion, la co-agrégation, la modulation des cellules immunes de l'hôte, la protection vis-à-vis des stress environnementaux et des peptides antimicrobiens (HYNÖNEN; PALVA, 2013).

Au cours du projet ANR SURFING, cinq protéines de surface extractibles, SlpA, SlpB, SlpE, Internaline-like A (Inl A) et Large surface protein A (Lsp A), ont été identifiées chez *P. freudenreichii* CIRM-BIA 129. Puis, par une approche intégrative, SlpB, protéine de surface extractible majeure chez *P. freudenreichii* CIRM-BIA 129, a été identifiée comme protéine clé jouant un rôle pivot (DEUTSCH et al., 2017; LE MARÉCHAL et al.,

2015). Cependant, les études moléculaires portant sur les propionibactéries manquent encore. Ainsi, le rôle précis de chacune de ces cinq protéines est encore incertain.

L'élucidation des molécules impliquées dans les mécanismes d'adhésion constitue une étape fondamentale dans la compréhension des interactions entre la bactérie et l'hôte. Etant donné le rôle central joué par certaines protéines de S-layer chez d'autre bactéries, nous avons donc tenté d'élucider les mécanismes impliqués dans l'adhésion de *P. freudenreichii* aux cellules épithéliales intestinales. Ce travail ouvre de nouvelles perspectives pour la sélection de souches adhésives. Cela pourrait ensuite améliorer les étapes initiales de colonisation de l'hôte, favoriser les dialogue probiotique/hôte, permettre la libération locale de composés nutraceutiques, et moduler l'inflammation de l'hôte via des effets immunomodulateurs.

Dans ce travail, nous avons étudiés l'implication des protéines de surface de *P. freudenreichii* dans le rôle de l'adhésion aux cellules épithéliales intestinales.

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Propionibacterium freudenreichii Surface Protein SlpB Is Involved in Adhesion to Intestinal HT-29 Cells

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Propionibacterium freudenreichii is a beneficial bacterium traditionally used as a cheese ripening starter and more recently for its probiotic abilities based on the release of beneficial metabolites. In addition to these metabolites (short-chain fatty acids, vitamins, and bifidogenic factor), *P. freudenreichii* revealed an immunomodulatory effect confirmed *in vivo* by the ability to protect mice from induced acute colitis. This effect is, however, highly strain-dependent. Local action of metabolites and of immunomodulatory molecules is favored by the ability of probiotics to adhere to the host cells. This property depends on key surface compounds, still poorly characterized in propionibacteria. In the present study, we showed different adhesion rates to cultured human intestinal cells, among strains of *P. freudenreichii*. The most adhesive one was *P. freudenreichii* CIRM-BIA 129, which is known to expose surface-layer proteins. We evidenced here the involvement of these proteins in adhesion to cultured human colon cells. We then aimed at deciphering the mechanisms involved in adhesion. Adhesion was inhibited by antibodies raised against SlpB, one of the surface-layer proteins in *P. freudenreichii* CIRM-BIA 129. Inactivation of the corresponding gene suppressed adhesion, further evidencing the key role of *slpB* product in cell adhesion. This work confirms the various functions fulfilled by surface-layer proteins, including probiotic/host interactions. It opens new perspectives for the understanding of probiotic determinants in propionibacteria, and for the selection of the most efficient strains within the *P. freudenreichii* species.

Keywords: adhesion, immunomodulation, surface proteins, probiotic, SlpB

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Introduction

Propionibacterium freudenreichii is a GRAS (Generally Recognized As Safe) actinobacterium consumed in high amounts in fermented dairy products. It is a beneficial bacterium used in the food industry for the production of vitamins, for cheese ripening, and for its probiotic properties (Cousin et al., 2010). Probiotics are defined as "living microorganisms which when administered in adequate amounts confer a health benefit on the host" (Food and Agriculture Organization of the United Nations and World Health Organization, 2002). *P. freudenreichii* indeed revealed probiotic

traits including modulation of intestinal inflammation (Mitsuyama et al., 2007; Foligné et al., 2010, 2013), as well as properties linked to the production of beneficial metabolites such as short-chain fatty acids (Jan et al., 2002; Lan et al., 2007; Cousin et al., 2012b), vitamins and the bifidogenic compound 1,4-dihydroxy-2-naphthoic acid (DHNA) (Bouglé et al., 1999; Kaneko, 1999; Hojo et al., 2002; Ouwehand et al., 2002; Seki et al., 2004; Mitsuyama et al., 2007).

Microorganisms that live in or transit through the digestive tract of humans may establish a symbiotic relationship with the host, thus promoting intestinal homeostasis (de Souza and Fiocchi, 2016). Consumption of *P. freudenreichii* selected strains can enhance human complex intestinal microbiota through the increase of other beneficial bacteria populations, such as bifidobacteria (Bouglé et al., 1999; Kaneko, 1999; Hojo et al., 2002; Ouwehand et al., 2002; Seki et al., 2004; Mitsuyama et al., 2007). In contrast, out of normal physiological conditions, the digestive microbiota may be involved in a variety of immune and inflammatory disorders (Vitetta et al., 2014). One example is inflammatory bowel diseases (IBD), chronic inflammatory disorders that severely affect the digestive tract and may lead, in the long term, to the irreversible deterioration of their structure and function (Belkaid and Hand, 2014; Vitetta et al., 2015). Cheese containing *P. freudenreichii,* in conjunction with *Lactobacillus delbrueckii* (Plé et al., 2016) or as a single strain (Plé et al., 2015), was recently shown to exert immunomodulatory effects, to protect mice against TNBS- induced colitis, to alleviate the severity of symptoms and to modulate local and systemic inflammation markers. Such cheese is currently tested in a pilot clinical trial (ClinicalTrials.gov, 2017). Interestingly, removal of propionibacteria surface- layer (S-layer) proteins, which are non-covalently anchored to the cell surface via an S-layer homology (SLH) domain, suppressed the induction of anti-inflammatory cytokines (Foligné et al., 2010). By contrast, some *P. freudenreichii* strains that possess an extracellular polysaccharide capsule fail to immunomodulate, while mutagenetic suppression of this capsule confers immunomodulatory activity (Deutsch et al., 2012).

Surface proteins of *P. freudenreichii* ITG P20 [*Centre International de Ressources Microbiennes-Bactéries d'Intérêt Alimentaire* (CIRM-BIA) 129], which is used as a cheese ripening starter (Richoux et al., 1998; Thierry et al., 2004), were investigated by a combination of proteomic methods previously developed for bacteria and eukaryotic cells (Lortal et al., 1993; Mayrhofer et al., 2006; Rodríguez-Ortega et al., 2006; Berlec et al., 2011; Bøhle et al., 2011; Bensi et al., 2012; Ythier et al., 2012; Michaux et al., 2013). This investigation demonstrated the involvement of certain S-layer proteins in immunomodulation (Bryson et al., 2006; Le Maréchal et al., 2015). Surface proteins, susceptible to enzymatic shaving and to guanidine extraction, were shown to be involved in the ability of *P. freudenreichii* to modulate the release of cytokines by human immune cells (Le Maréchal et al., 2015). However, the respective role of the different bacterial S-layer proteins was not fully elucidate. Immunomodulation is favored by the ability of specific strains to adhere to the host cells and mucus (Tuomola et al., 1999; Ouwehand et al., 2000; Huang and Adams, 2003; Thiel et al., 2004; Le Maréchal et al., 2015). Indeed, the local action of metabolites and of immunomodulatory molecules is favored by the ability of probiotics to adhere to the host cells. Dairy propionibacteria were shown to adhere to mice intestinal epithelial cells both *ex vivo* and *in vivo* (Zarate, 2012) as well as to cultured human intestinal cell lines *in vitro* (Huang and Adams, 2003; Moussavi and Adams, 2010). However, the precise mechanisms are poorly characterized in *P. freudenreichii*. Adhesion moreover constitutes a key criterion in strain selection and is described as the initial step for colonization of the host (Havenaar et al., 1992, havenar; Riedel et al., 2006; Preising et al., 2010), depending on crucial surface compounds, including surface proteins (Lebeer et al., 2010).

The identification of adhesion mechanisms and molecules is a fundamental step in the elucidation of the bacterium/host cross-talk (van de Guchte et al., 2012). This was lacking in probiotic dairy propionibacteria. The aim of our study was thus to identify *P. freudenreichii* protein(s) involved in adhesion to human intestinal epithelial cells.

Materials and methods

Bacterial Strains and Culture Conditions

The *P. freudenreichii* wild-type (WT) strains, genetically modified strain and plasmids used in this study are listed in **Table 1**. All strains in this study were obtained from the collection of the CIRM-BIA (STLO, INRA Rennes, France). All *P. freuderenichii* WT strains were grown at 30°C in YEL broth (Malik et al., 1968) without agitation or in cow's milk ultrafiltrate supplemented with 50 mM of sodium L-lactate (galaflowSL60, Société Arnaud, Paris, France) and 5 g/L of casein hydrolysate (Organotechnie, La Courneuve, France), sterilized by 0.2 μm filtration (Nalgene, Roskilde, Denmark) as described previously (Cousin et al., 2012a). For genetically modified strains, YEL and Milk Ultrafiltrate culture media were supplemented with chloramphenicol (10 μg ml^{-1}). The growth of *P. freudenreichii* strains was monitored spectrophotometrically by measuring the optical density at 650 nm (OD650), as well as by counting colony-forming units (CFUs) in YEL medium (Malik et al., 1968) containing 1.5% agar. *P. freudenreichii* strains was harvested in a stationary phase (76 h, 10^9 CFU/mL, determined by plate counts) by centrifugation (6,000 *g*, 10 min, 4°C). *Escherichia coli* strain DH5α was grown in Luria–Bertani medium at 37°C, and cells carrying DNA plasmid were selected by addition of ampicillin (100 μg ml^{-1}).

Enzymatic Shaving of Surface Proteins

One hundred microliter of propionibacteria stationary phase culture (see above) were harvested by centrifugation (6,000*g*, 10 min, 4°C) and washed in an equal volume of PBS [pH 8.5] containing 5 mM DTT before resuspension in 1/10 volume of the same buffer. Sequencing grade modified trypsin (V5111, Promega, Madison, WI, United States) was dissolved in the same buffer (qsp 0.2 g/L) and added to the bacterial suspension. "Shaving" was performed for 1 h at 37°C in a 0.5 mL reaction volume containing 5×10^9 bacteria and 4 μg of trypsin,

TABLE 1 | *Propionibacterium freudenreichii* wild-type strains, their genetically modified derivatives and plasmids used in the study.

Strains and plasmids	Relevant genotype and phenotype	Source or reference
Strains *P. freudenreichii*[a]		
CB 118	Wild-type; SlpA, SlpB, SlpE, and InlA proteins detected in guanidine extract[b]	CIRM-BIA
CB 121	Wild-type; InlA and LspA proteins detected in guanidine extract	CIRM-BIA
CB 129	Wild-type; SlpA, SlpB, SlpE, InlA, and LspA proteins detected in guanidine extract	CIRM-BIA
CB 134	Wild-type; SlpA, SlpE, InlA, and LspA proteins detected in guanidine extract	CIRM-BIA
CB 136	Wild-type; SlpA, SlpB, InlA, and LspA proteins detected in guanidine extract	CIRM-BIA
CB 508	Wild-type; SlpA, SlpB, SlpE, InlA, and LspA proteins detected in guanidine extract	CIRM-BIA
CB 527	Wild-type; Absence of surface layer proteins in guanidine extract	CIRM-BIA
CB 129*OslpB*	Cm^r; CIRM-BIA 129 with chromosomal insertion of pUC:*OslpB*:CmR in the *slpB* sequence; SlpB protein absent in guanidine extract	This Study
Plasmids		
pUC:slpB	pUC18; Amp; harboring *slpB* partial gene sequence for inactivation	This Study
pUC:*O*slpB:CmR	pUC18 carrying a chloramphenicol resistance gene and harboring *slpB* partial gene sequence	This Study

[a] *CB, CIRM-BIA, Centre International de Ressources Microbiennes–Bactéries d'Intérêt Alimentaire, INRA, UMR 1253, Science et Technologie du Lait et de l'Oeuf, Rennes, France.* [b] *Guanidine Hydrochloride treatment used to extract surface layer associated proteins non-covalently bound to the surface is described in "Materials and Methods."*

with gentle agitation (180 rpm). Bacteria were removed by centrifugation (8,000 *g*, 10 min, 20°C) and subjected to three washes in PBS prior to adhesion assay.

Cell Line and Culture Conditions

The human colon adenocarcinoma cell line HT-29 was obtained from ATCC (American Type Culture Collection, Rockville, MD, United States). These cells was cultured under conditions of 37°C, 5% CO_2, and 90% relative humidity in DMEM High Glucose with L-Glutamine with Sodium Pyruvate (PAN, Dominique Dutscher, Brumath, France) supplemented with 10% heat-inactivated fetal calf serum (FCS) (PAN, Dominique Dutscher, Brumath, France) and antibiotics or not (for adhesion assays).

Electroporation and Inactivation of the slpB Gene in P. freudenrenichii CIRM-BIA 129 by Suicide Vector

Inactivation of *P. freudenreichii* gene was adapted from Deutsch et al. (2012) with some modifications. For insertional inactivation of a *slpB* gene, a 520-bp DNA fragment homologous to pUC: nucleotides 30–550 of the 5′ region of the slpB coding region By in P. freudenreichii CIRM-BIA 129 genome was synthesized Genscript Inc.[1] with restriction sites *Xba*I-*slpB*-5′ and *BamH*I-*slpB*-3' resulting in pUC: Δ*slpB* plasmid. The pUC: Δ*slpB* plasmidic DNA was digested with *XbaI* and *BamH*I, purified, and cloned in plasmid pUC:CmR digested by the same enzymes,

which resulted in the suicide vector pUC: Δ*slpB*:CmR, which was confirmed by sequencing. See **Supplementary Figure S2**. Electrocompetent *P. freudenreichii* CIRM-BIA 129 cells was prepared as previously described (Deutsch et al., 2012) with slight modifications. They were cultured in YEL medium supplemented with 0.5 M sucrose and 2% glycine until the early exponential growth phase (OD 0.1), harvested (6,000 *g*, 10 min, 4°C). The pellet was washed extensively in ice-cold 0.5 M sucrose and resuspended in electroporation buffer containing 0.5 M sucrose with 10% glycerol and 1 mM potassium acetate (pH 5.5). For electroporation, a 100-µl aliquot of the electrocompetent cells was mixed with 3 µg of pUC: Δ*slpB*:*Cm*R plasmid DNA in a cooled electroporation cuvette. The electroporation of *P. freudenreichii* CIRM-BIA 129 was performed with a Gene Pulser Xcell™ (Bio-Rad Laboratories, Richmond, CA, United States) at 20 kV/cm, 200-*K* resistance, and 25-µF capacitance. Immediately after the pulse, 900 µL of YEL containing 0.5 M sucrose, 20 mM $MgCl_2$, and 2 mM $CaCl_2$ were added before incubation, 24 H at 30°C under microaerophilic conditions. Cells were plated, and incubated 7 days at 30°C under anaerobic conditions, on YEL medium containing 1.5% agar (YELA) supplemented with 10 µg ml^{-1} of chloramphenicol in order to select *P. freudenreichii* mutants harboring inserted Δ*slpB*:*Cm*R. The *P. freudenreichii* CIRM-BIA 129 Δ*slpB* (CB129Δ*slpB*) mutant strain was further checked by proteomics for the absence of intact SlpB surface proteins as indicated in the "Results" section. Moreover, the stability of the insertion was checked after three independent cultures in YEL and Milk Ultrafiltrate media without chloramphenicol.

In Vitro Adhesion Assays

Adhesion of *P. freudenreichii* (WT and mutant) to the human colon adenocarcinoma cell line HT-29 was examined by adding 10^8 live propionibacteria (washed twice in PBS, numerated by CFU conting, ratio 100 bacteria:1 HT-29 cell, MOI 100) to 10^6 cells in DMEM culture medium without antibiotics. Adhesion assay was conducted by incubation of bacteria/cell at 37°C for 60 min under conditions, 5% CO_2 and 90% relative humidity. Cells were washed twice with prewarmed PBS pH 7.4, and the subsequently supernatant was removed, and 400 µL of trypsin-EDTA (Invitrogen) was added to each well, before incubation for 5 min at 37°C and to trypsin inactivation by adding 800 µL of DMEM culture medium without antibiotics. Cells were harvested (3,000 *g*, 3 min) and lysed with 0.1% Triton X-100 before serial dilutions and plating on YELA. Finally, plates were incubated at 30°C for 5 days under anaerobic conditions. A rate of adhesion was calculated as follows: (bacterial count after adhesion experiment/bacterial population added on to HT29 cells). The CIRM-BIA 129 WT strain was then used as a reference in this work, with a % adhesion of 100, and used to normalize all other

to normalize all other adhesion rates as a percentage of CIRM- BIA 129 WT adhesion. Each adhesion assay was conducted in technical and biological triplicates. To test involvement of surface proteins in adhesion, propionibacteria were subjected (or not) to enzymatic shaving (see section "Enzymatic Shaving of Surface Proteins") before adhesion assay. To confirm this hypothesis, propionibacteria were incubated 60 min at 37°C with 50 µg of *P. freudenreichii* CIRM-BIA 129 guanidine extracted S-layer associated proteins, in solution in PBS, under agitation, before adhesion. This amount (50 µg) was determined after preliminary experiments to determine amounts efficient in restoring adhesion. For specific inhibition of adhesion by antibodies directed against SlpB, propionibacteria were incubated in PBS pH 7.4 with immunoglobulins purified from rabbit anti-SlpB serum (AGRO-BIO, France) in 1:10.000 dilution, under agitation, 60 min at 37°C. Propionibacteria were washed twice with PBS pH 7.4 before adhesion assay.

The adhesion ratio of CB 129 strain alone was used as a reference to calculate the adhesion rates of different strains and treatments.

Internalization of bacteria was determined as previously described (Bouchard et al., 2013) 2-h post contact following an additional 2-h incubation step with DMEM supplemented with gentamicin (100 µg/ml) to kill extracellular bacteria. Subsequently, HT-29 cells monolayers were washed three times with PBS, treated with trypsin, centrifuged for 5 min at 800 *g*, and lysed in 0.01% Triton to allow the numeration of internalized propionibacteria population only.

Bacterial Cell Adhesion Observation by Microscopy

Observation of *P. freudenreichii* adhesion to cultured human colon epithelial cells was as described previously for lactobacilli (Tiptiri-Kourpeti et al., 2016), with modifications for propionibacteria. Briefly, propionibacteria, cultured as described above, were washed and resuspended in PBS, prior to the addition of 20 µM CFSE (carboxyfluorescein succinimidyl ester, cell trace proliferation kit for flow cytometry ref C34554 Thermo Fisher Scientific, Waltman, MA, United States), freshly prepared as a 1,000 × solution in DMSO and kept in the dark. Incorporation of CFSE was allowed for 30 min at 30°C in the dark, prior to washing and resuspension of propionibacteria in YEL medium, 30°C. Hydrolysis of CFSE by intracellular esterase activity was allowed 30 min at 30°C in the dark to generate intracellular fluorescence. Fluorescence was checked on an epifluorescence microscope (BX-51, Olympus, equipped with a U-MWB2 fluorescence filter cube). HT-29 cells were cultured in Lab-Tek chamber slide (Thermo Fisher Scientific) and labeled bacteria were added to a 1/100 ratio (1×10^6 cells, 1×10^8 bacteria, in 1 mL of DMEM) before incubation, 1 h, 37°C. After two washes with PBS, the plasma membrane of cells was then labeled with the exogenous head-labeled phospholipid fluorescent probe *N*-(Lissamine rhodamine B sulfonyl) dioleoyl phosphatidylethanolamine (Rh-DOPE, Avanti Polar Lipids Inc., Birmingham, England) used at a concentration of 1 µg/mL in DMEM, for 10 min. Cell layer was then washed twice with PBS and slides mounted in DAPI (4,6-diamidino-2-phenylindole)-containing mounting medium (Vectashield mounting medium for fluorescence Vector ref *H-1200*). Cells were observed using a confocal Leica SP8 and a 63/1.4 oilHC PL APO CS objective. Images were acquired using LAS-AF (Leica, Wetzlar, Germany) software. For scanning electron microscopy, HT-29 cells were cultured in Corning® Transwell® polycarbonate membrane cell culture inserts on polycarbonate 0.4 µm pore size filtration membrane. Adhesion was conducted as described above. Membranes were then removed, washed in PBS, fixed 48 h by 2% (wt/vol) glutaraldehyde in 0.1 M sodium cacodylate buffer [pH 6.8] and rinsed in the same buffer. Samples were dehydrated with ethanol (10, 25, 50, 75, 95, and finally 100%), critical-point dried by the CO_2 method and coated with gold. Cells were examined and photographed with a Philips XL 20 scanning electron microscope operating at 10 kV.

Bacterial Cell Adhesion Determination by Cytometric Analysis

Determination of *P. freudenreichii* adhesion to cultured human colon epithelial cells was performed as described previously for lactobacilli (Tiptiri-Kourpeti et al., 2016). Cells were cultured in DMEM as described above to confluence. CFSE-labeled bacteria were added as described above before a 1-h incubation at 37°C. Cells were trypsinized and analyzed by fluorescence cytometry using an excitation wavelength of 488 and emission at 585 nm (Accuri C6 Becton Dickinson, Le Pont-de-Claix, France). Data were collected from 50,000 cells and analysis was performed with CFlow software.

Guanidine Extraction of Surface Layer Associated Proteins Non-covalently Bound to the Cell Wall

Propionibacteria cultures in stationary phase (76-h) were collected by centrifugation (8,000 *g*, 10 min, 4°C) for extraction of S-layer proteins by Guanidine Hydrochloride (GuaHCl) (Le Maréchal et al., 2015). The bacterial pellet was washed two times with an equal volume of PBS buffer pH 7.4. This pellet was resuspended in 5 M GuaHCl to a final OD_{650} of 20 then incubated 15 min at 50°C, and subsequently, the suspension was centrifuged (21.000 *g*, 20 min, 30°C). The cells were eliminated, and the supernatant was dialyzed extensively against PBS buffer pH 7.4 (for adhesion assays) or 0.1% SDS (for SDS– PAGE analysis) for 24 h at 4°C using Slide-A-Lyer® Dialysis Cassette (ThermoScientific, Rockford, IL, United States). This procedure was applied in three independent cultures.

One-Dimensional SDS–Polyacrylamide Gel Electrophoresis (1-DE) and Western Blotting

Extracts of S-layer proteins in 0.1% SDS were diluted in SDS sample buffer and then heat-denatured 10 min at 95°C. One-dimensional polyacrylamide gel electrophoresis (10.0%) was conducted according to Laemmli (Laemmli, 1970) on a Mini-PROTEAN® Tetra Cell (Bio-Rad, Hercules, CA, United

States) and the gels were stained using Coomassie Blue Bio- Safe reagent (Bio-Rad). Alternatively, S-layer protein associated extracts were separated by 10% SDS–PAGE and transferred to PVDF membranes (GE Healthcare). After blocking with 3% non-fat dry milk diluted in TBS (Tris 10 mM, NaCl 0.15 M, 0.3% tween 20), the membranes were incubated overnight at 4°C with primary antibodies purified from rabbit sera (AGRO-BIO, France). These were obtained by injecting the following slpB peptide to rabbits: IDATVDKQNSKGGFGWGG and used at the dilution 1:10,000. After washing, membranes were incubated with secondary antibodies: anti-rabbit IgG conjugated with horseradish peroxidase (1:15,000, AGRO-BIO, France) for 2 h at room temperature. Bound antibodies were visualized with ECL Plus system (GE Healthcare, Vélizy, France) and blots were scanned using the Syngene GBox (Ozyme, Saint-Quentin-en- Yvelines, France). The specificity of anti-SlpB western blotting was checked (**Supplementary Figure S1**). A single band was observed only in strains expressing SlpB and the labeling pattern was distinct from that of anti-SlpA and anti-SlpE western blotting.

Data Analysis

All the experiments were performed with three technical replicates and three biological replicates, and the results were expressed as means standard deviations (SD). Statistical analyses were performed in R Statistical Software (Foundation for Statistical Computing, Vienna, Austria) using ANOVA with Tukey *post hoc* analyses for multiple comparisons.

Results

Surface Layer Associated Proteins and Adhesion to Cultured Human Colon Cells Are Variable among Strains of *P. freudenreichii*

Seven strains of *P. freudenreichii* from the CIRM-BIA collection (**Table 1**), CB 118, CB 121, CB 129, CB 134, CB 136, CB 508, and CB 527, have been selected based on preliminary proteomic screening as they all displayed different surface proteomes as shown by their S-layer associated protein pattern after guanidine treatment (**Figure 1A**). The five proteins, previously identified in CB 129 (SlpA, SlpB, SlpE, InlA, and LspA, see Le Maréchal et al., 2015), and thought to play a role in interactions with the host, are indicated in the figure. Preliminary results pointed out SlpB as a potential key surface protein in *P. freudenreichii.* We thus developped antibodies in order to confirm this. Western blot analysis using these antibodies further confirmed variability of surface proteins (**Figure 1B**). SlpB was detected in four strains out of seven, with different intensities. The variability of S-layer associated proteins suggested possible variations regarding interactions with host cells. The seven strains were further compared with respect to adhesion to HT-29 cultured colon cells (**Figure 1C**). The CB129 strain, exhibited the highest adhesion rate (6.44 CFU/1 HT-29 cell) and was used as the reference (100% adhesion) for comparison with the other strains

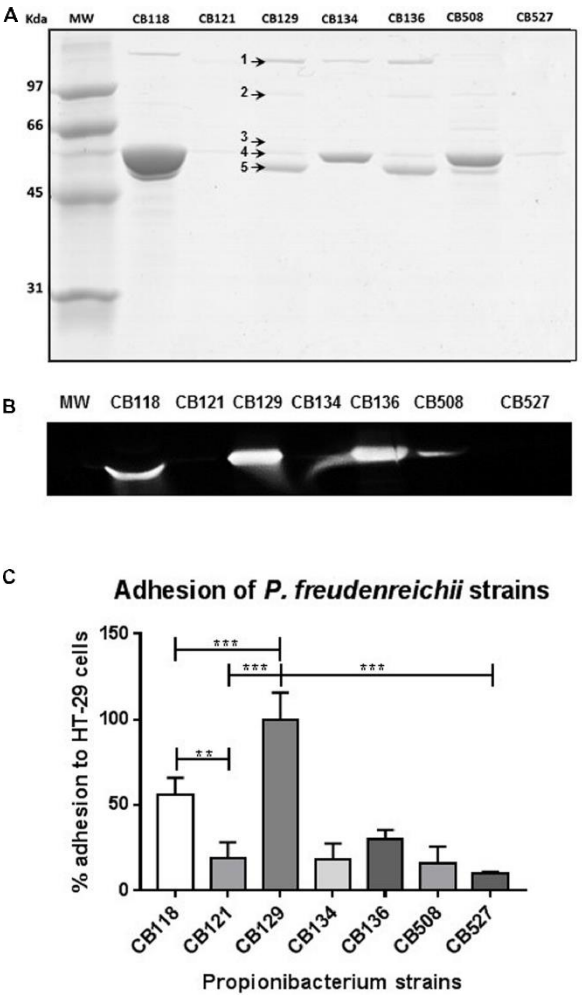

FIGURE 1 | Variability of surface proteome and of adhesion among strains of *Propionibacterium freudenreichii*. (A) Guanidine-extracted surface layer associated proteins are variable. Seven strains of *P. freudenreichii* were cultured in milk ultrafiltrate and subjected to guanidine-extraction followed by SDS–PAGE (10%) gel electrophoretic analysis of the extracts. Gels were either Coomassie-Blue-stained (A) or transferred to a PVDF membrane. Surface proteins previously identified by mass spectrometry as InlA, LspA, SlpE, SlpA and SlpB in strain CB129 are indicated by 1, 2, 3, 4, and 5, respectively. (B) Western Blotting detection of surface layer protein SlpB. PVDF membranes were treated using rabbit antibodies raised against *P. freudenreichii* surface layer protein SlpB. (C) Adhesion to cultured human colon epithelial cells is variable. HT-29 cells were cultured to confluence in DMEM prior to co-incubation. Each well (1×10^6 HT-29 cells) was added with 1×10^8 colony-forming unit (CFU) of *P. freudenreichii*. Co-incubation was 60 min at 37°C in DMEM. After thorough washing with PBS, adhered bacteria were enumerated by CFU plate counting in trypsinized cells. Numbers of the strains used are indicated. Asterisks represent statistically significant differences between strains and were indicated as follows: $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$. Adhesion is presented as a percent of the reference CB129 *P. freudenreichii* strain. Original gels and western blots, uncropped, are provided in Supplementary Figure S1.

(100.0% ± 1. 17). Indeed, CB129 showed a significant difference ($p < 0.001$) with the other *P. freudenreichii* strains tested under the same experimental conditions. The CB118 strain exhibited a

lower but significant adherence percentage of 56.0%±10.0 and also displayed SlpB. All the other strains exhibited low adhesion rates without significant differences among them, although CB136 (30.0% ± 5.0), which also displays SlpB, tended to be more adhesive than the rest of this subset. Finally, the lowest adhesion rate was recorded for CB527, 10.0%±1.0, for which no surface protein was detected, in accordance with (Deutsch et al., 2017). Different propionibacteria: HT-29-cells ratios were tested for adhesion (100:1, 500:1, and 1,000:1, in technical and biological triplicates) with similar results in adhesion rates ranking. At the MOI of 100:1 used in this study, no internalization of *P. freudenreichii* was observed (data not shown) using the gentamicin method used by our team to monitor staphylococci internalization (Bouchard et al., 2013).

P. freudenreichii CB129 Interacts with Cultured Human Colon Cells

Adhesion of *P. freudenreichii* to HT-29 cells being demonstrated, we further looked at such an interaction, using three-dimensional confocal microscopy. As seen in **Figure 2A**, the sections close to the bottom of the slide culture chamber mainly exhibited the blue fluorescence of the HT-29 nuclei, stained with DAPI, a poorly fluorescent cytoplasm, surrounded by a red-stained plasma membrane (lowest images in **Figure 2A**). Ascending within this "z-stack," higher sections showed dots with intense red fluorescence, corresponding to cell membranes, indicative of colonocytes microvilli constituting the brush border. Higher sections showed co-localization of these red dots with green- fluorescent propionibacteria, caused by CFSE metabolization within propionibacteria. More precisely, propionibacteria appeared as aggregates, in the intercellular space of the epithelial HT-29 monolayer. This localization of propionibacteria in contact with cells is further illustrated in the reconstituted 3-D view (**Figure 2B**). Interaction of propionibacteria with cultured human colonocytes was further illustrated by scanning electron microscopy of co-cultures on cell culture inserts (**Figure 2C**). This revealed localization of propionibacteria at the surface of cells, in contact with the brush border.

P. freudenreichii CB129 Adhesion to Cultured Human Colon Cells Involves Surface Proteins

To determine whether the presence of surface proteins is involved in the adhesion of *P. freudenreichii* to HT-29 cells, the method

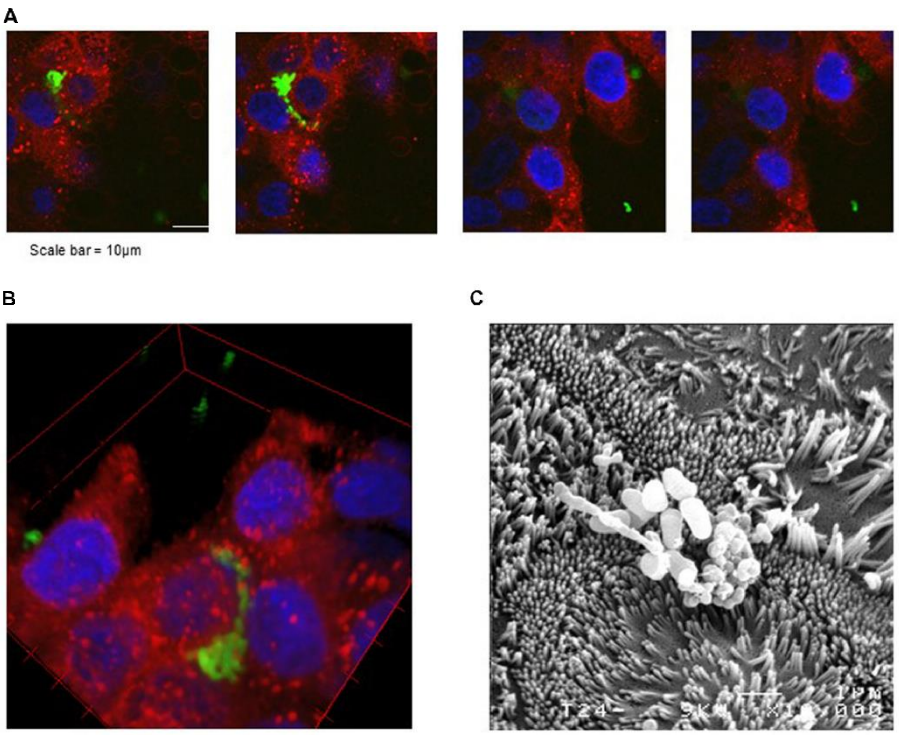

FIGURE 2 | Microscopy imaging of *P. freudenreichii Centre International de Ressources Microbiennes–Bactéries d'Intérêt Alimentaire* (CIRM-BIA) 129 adhesion to cultured human colon epithelial cells. HT-29 cells were cultured to confluence in DMEM on a slide chamber prior to interaction. *P. freudenreichii* was cultured in fermented milk ultrafiltrate prior to intracellular labeling of live bacteria using CFSE. Labeled bacteria were then co-incubated with colon cells in a slide chamber prior to washing with PBS and to staining of plasma membrane using Rh-DOPE and mounting in DAPI-containing mounting medium. (A,B) Blue fluorescence evidences colon cells nuclei, red fluorescence their plasma membrane and green fluorescence CFSE-labeled propionibacteria. (A) Z-stack, i.e., confocal images acquired at different "z" altitudes in the labeled preparation. (B) Reconstituted 3-D image showing a cluster of propionibacteria at surface of cells. (C) Scanning electron microscopy observation of propionibacteria adhesion to cultured colon epithelial cells. The same co-incubation was performed in a polycarbonate membrane cell culture insert prior to fixation and scanning electron microscopy observation.

of enzymatic shaving using trypsin was applied, before adhesion assay. A significant reduction ($p < 0.001$) was observed in the adhesion rate: 21.77± 8.10% for shaved bacteria, compared to the positive control consisting of propionibacteria (**Figure 3A**). Western blot analysis also indicated absence of SlpB at the surface of *P. freudenreichii* as a result of shaving (**Figure 3B**). To further confirm the role of surface proteins in adhesion,

P. freudenreichii CB129 cells, shaved or not, were incubated with 50 µg of extracted surface proteins. This guanidine extract from the CB129 strain was previously dialyzed against PBS and quantified by Bradford assay. It contained the five proteins (SlpA, SlpB, SlpE, InlA, and LspA, see **Figure 1A**) in PBS buffer pH 7.4. Adhesion assay was then conducted. This incubation increased the rate of adhesion of *P. freudenreichii* CB129 to HT-29 cells, from 100.00% 8.93 to 317.07% 46.68. Furthermore, adhesion rate, which was strongly diminished by enzymatic shaving (33.99%± 14.30), was restored by this incubation (157.44% ±18.31, **Figure 3C**). This further experiment confirmed the key role of at least one of these surface proteins in adhesion.

Surface Protein SlpB Plays a Key Role in Adhesion to Cultured Human Colon Cells

In a second approach to inhibit adhesion and to precise the role of specific surface proteins, *P. freudenreichii* was incubated with antibodies raised against SlpB, at a dilution of 1:10,000, before adhesion assay. This resulted in a significant reduction following incubation with the anti-SlpB antibodies 39.95%±6.92 ($p < 0.001$), (**Figure 4A**). We then further focused on SlpB and inactivated its gene in *P. freudenreichii* CB129. The mutant

P. freudenreichii CB129Δ*slpB* was obtained by insertion of the pUC:Δ*slpB*:*Cm*R suicide plasmid as described in the "Materials and Methods" section (**Supplementary Figure S2**). The stability of the mutant was validated after growth in the presence or absence of chloramphenicol by checking for the absence of SlpB production. As shown in **Figure 4B**, one protein band (about 55 kDa in size) was lacking in the mutant S-layer associated proteins guanidine extract (line 2), when compared to the WT parental strain (line 1). Western Blot analysis using antibodies raised against the SlpB protein (**Figure 4C**) confirmed that this protein was effectively mutated in the mutant (line 2) when compared to the parental strain (line 1). Efficient and specific inactivation of the *slpB* gene was further established by mass spectrometry analysis of guanidine-extracted S-layer proteins. Indeed, the SlpA, SlpB, and SlpE proteins were clearly identified in the WT CB129 strains, while only SlpA and SlpE were detected in the mutant *P. freudenreichii* CB129Δ*slpB* strain (**Table 2**).

Adhesion to HT-29 cells was then assessed by CFU counting and the mutant CB129Δ*slpB* strain was impaired in adhesion (20.66% ± 8.32) when compared to the WT control (100.00%± 7.37) (**Figure 4A**, $p < 0.001$). To confirm this result, adhesion of *P. freudenreichii* to HT-29 cells, using CFSE- stained propionibacteria, was quantified by flow cytometry. Cells were treated with CFSE-labeled propionibacteria, WT or CB129Δ*slpB* mutant, for 1 h, before cytometric monitoring of cell fluorescence (**Figures 4D–F**). A shift in fluorescence intensity

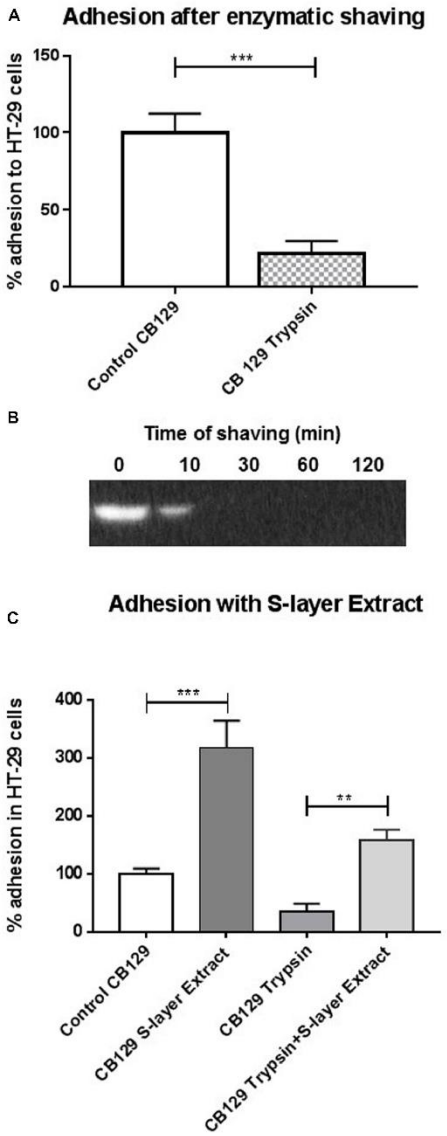

FIGURE 3 | Involvement of *P. freudenreichii* surface proteins in adhesion. (A) Trypsin shaving reduces *P. freudenreichii* CIRM-BIA 129 adhesion. Human colon cells were cultured in DMEM prior to co-incubation with propionibacteria. Used propionibacteria were either untreated (control) or submitted to trypsin shaving of surface proteins for 60 min (trypsin). Adhered bacteria were enumerated by CFU plate counting in trypsinized cells. (B) Trypsin shaving reduces presence of SlpB protein in *P. freudenreichii* CIRM-BIA 129 in different times of incubation. *P. freudenreichii* CIRM-BIA 129 show after different incubations time with trypsin (T_{zero} min, T_{30} min, T_{60} min, and T_{120} min) a decreased amount of SlpB in Western Blot analysis with anti-SlpB antibodies. (C) Addition of extracted surface layer proteins enhances *P. freudenreichii* CIRM-BIA 129 adhesion. Human colon cells were cultured prior to co-incubation with propionibacteria. Used propionibacteria were either shaved for 60 min (trypsin) or untreated (control). They were then added with surface layer guanidine extract (50 µg of proteins) or not. Adhesion was quantified by plate CFU counting of propionibacteria after trypsinization of colon cells. Adhesion is presented as a percent of the reference CIRM-BIA 129 *P. freudenreichii* strain. Asterisks represent statistically significant differences between strains and were indicated as follows: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. Adhesion is presented as a percent of the reference CB129 *P. freudenreichii* strain.

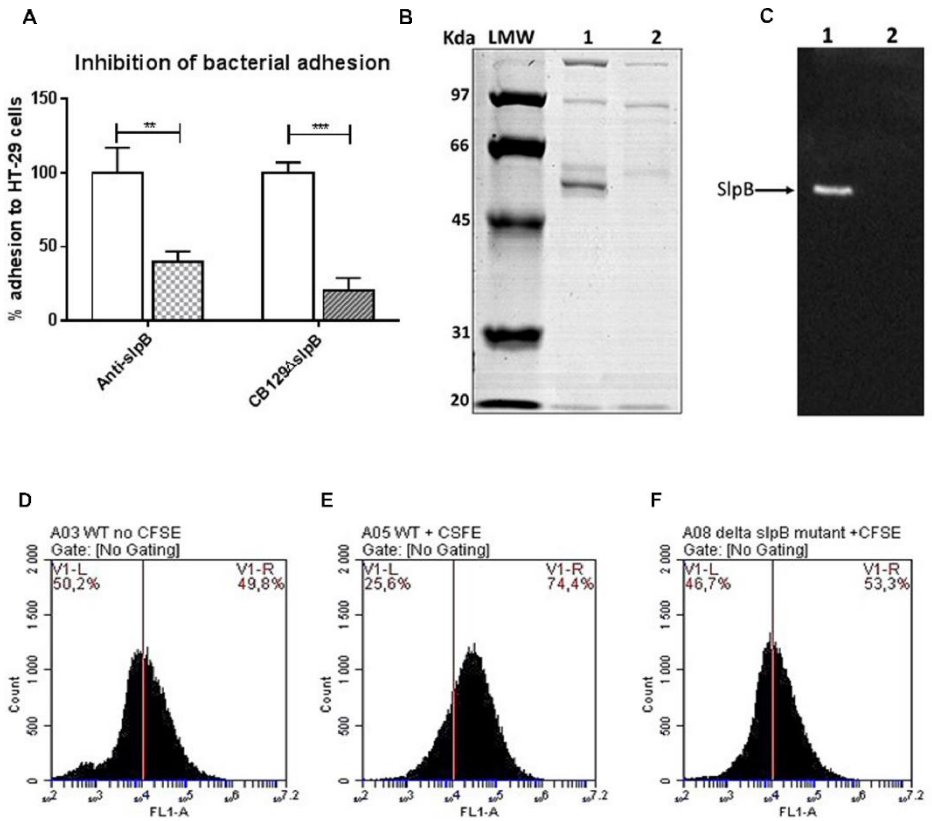

FIGURE 4 | Key role of surface layer protein SlpB in adhesion. (A) Antibodies reduce *P. freudenreichii* adhesion and so does *slpB* gene inactivation. Human colon cells were cultured in DMEM prior to co-incubation with propionibacteria. *P. freudenreichii* CIRM-BIA 129 was treated with antibodies raised against SlpB prior to adhesion assay. As an alternative, the *slpB* gene was inactivated in *P. freudenreichii* CIRM-BIA 129 prior to adhesion assay. Adhesion is presented as a percent of the reference CB129 *P. freudenreichii* strain. (B) Guanidine-extracted surface layer associated proteins were compared by SDS–PAGE in *P. freudenreichii* CIRM-BIA 129 wild-type (line 1) and in the corresponding mutant CB129*OslpB* (line 2). (C) The corresponding PVDF membrane was subjected to Western Blotting using rabbit antibodies raised against *P. freudenreichii* surface layer protein SlpB (B). (D,E) Fluorescently labeled live *P. freudenreichii* CIRM-BIA 129 adheres to cultured human colon epithelial HT-29 cells. The adhesion of CFSE-labeled propionibacteria was detected by the shift in FL1 intensity (E), compared to HT-29 cells with unlabelled propionibacteria (D). Cells (10^6) were co-incubated with 10^8 CFU of CFSE-stained propionibacteria for 1 h. At least 50.000 cells per sample were analyzed. As an alternative, labeled *P. freudenreichii* mutant CB129*OslpB* was co-incubated with HT-29 cells (F). Original gels and western blots, uncropped, are provided in Supplementary Figure S1. Asterisks represent statistically significant differences between strains and were indicated as follows: $*p < 0.05$; $**p < 0.01$; $***p < 0.001$. Adhesion is presented as a percent of the reference CB129 *P. freudenreichii* strain.

TABLE 2 | Surface-layer proteins identified after Guanidine Hydrochloride extraction.

		Wild-type strain			Delta SlpB strain		
Gene name	Locus tag	Log (*e*-value)[a]	Cover (%)[b]	Peptides[c]	Log (*e*-value)[a]	Cover (%)[b]	Peptides[c]
SlpB	PFCIRM129_00700	−263.2	74	34	0	0	0
SlpE	PFCIRM129_05460	−125.6	50	19	−138.8	55	18
SlpA	PFCIRM129_09350	−174.3	75	24	−143.5	68	22

[a] *The e-value is the probability that a given peptide score will be achieved by incorrect matches from a database search. Protein e-value is the product of individual peptide e-value. Protein identifications were automatically validated when they showed at least two unique peptides with an e-value below 0.05 corresponding to log (e-value) < 1.3.* [b] *The percentage of the protein amino acid sequence covered by tandem mass spectrometry identification of peptides.* [c] *Number of unique peptide sequence identified with an individual e-value < 0.01 for this protein.*

(FL1) was observed as a result of fluorescent *P. freudenreichii* CB129 adhesion to cells (**Figure 4E**) when compared with control cells without bacteria (**Figure 4D**). This indicates an increase of fluorescence emission at 488 nm, corresponding to 6-carboxyfluorescein succinimidyl harbored by adhering bacteria, as described previously for lactobacilli (Tiptiri-Kourpeti et al., 2016). By contrast, the mutant CB129*OslpB* strain failed to reproduce this fluorescence shift in HT-29 cells, and the pattern (**Figure 4F**) was similar to that of HT-29 without bacteria (**Figure 4A**). Altogether, these results confirm the key role of the

SlpB surface protein in adhesion of *P. freudenreichii* to HT-29 cells.

Discussion

Adhesion is a key determinant of host/bacterium interactions, whether pathogenic or probiotic. Adhesion of probiotic bacteria to host intestinal cells may favor important effects including modulation of mucus secretion (Mack et al., 2003), of defensin production (Schlee et al., 2007, 2008), or the local action of beneficial metabolites. It can improve competitive exclusion of pathogens by adhesion competition (Servin, 2004; Lebeer et al., 2008) and constitutes a key factor for several clinical applications of probiotics in the prevention and treatment of gastrointestinal disorders and of IBD. It may involve, on the bacterial side, various microorganism-associated molecular patterns (MAMPs) including flagellin, fimbriae (also called pili) or other surface proteins including moonlighting proteins and S-layer proteins (Lebeer et al., 2010).

Surface-layer proteins constitute a field of research that deserves further investigation. Although anchored to the cell wall via conserved SLH domains, their extracellular protruding part is highly variable, poorly conserved amongst bacterial species and strains. A previous paradigm described S-layers as a macromolecular paracrystalline network formed by the self-assembly of numerous copies of one monomeric protein or glycoprotein and constituting an extracellular S-layer in many bacteria (Sleytr, 1997; Sára and Sleytr, 2000). This was later challenged by studies on *Lactobacillus acidophilus* showing that a S-layer can contain various S-layer proteins or SLPs (Hymes et al., 2016). These proteins are in fact versatile molecules that may play an important role in growth and survival, maintenance of cell integrity, enzyme display, molecular sieving, co-aggregation, immunomodulation, as well as adhesion and persistence within the animal host (Lebeer et al., 2010; Fagan and Fairweather, 2014). In *P. freudenreichii*, such proteins were shown to be involved in immunomodulatory interactions with the host (Le Maréchal et al., 2015), a property highly strain-dependent (Mitsuyama et al., 2007; Foligné et al., 2010, 2013). Indeed, a functional role in immunomodulation by *P. freudenreichii* was recently attributed to a set of proteins: SlpB, SlpE, two putative S-layer proteins with SLH domains, and HsdM3, predicted as cytoplasmic (Deutsch et al., 2017).

Variability of *P. freudenreichii* surface proteins may thus be related to variability in functional properties. In this context, we have selected in the present work seven *P. freudenreichii* strains with different patterns evidenced in a preliminary study.

We confirm here that *P. freudenreichii* S-layer proteins are variable, and so is its ability to adhere to cultured human epithelial cells, as determined by quantitative culturing (Mack et al., 1999), which suggests a functional link between variations in the surface protein pattern. *P. freudenreichii* CIRM-BIA 129, shown to alleviate symptoms of acute colitis in mice, displays S-layer associated proteins and the highest adhesion ability, whatever the bacteria/cell ratio (100:1; 500:1; and 1,000:1). Moreover, at a ratio of 100/1, no internalization was observed. This suggests that propionibacteria either do not internalize into cultured HT-29 cells, or do not suvive within the cells. Cultured colon epithelial HT-29 cells do not produce mucus in our conditions. This suggests that *P. freudenreichii* interacts with epithelial cell surface compounds rather than mucins, a property previously reported for the probiotic *L. acidophilus* (Johnson et al., 2013). Interestingly, CB129 was shown to restore expression of ZO-1, a key protein of tight junctions which expression was impaired in colitis (Plé et al., 2015), as part of its anti-inflammatory effect. Adhesion close to these junctions may favor the local action of propionibacteria via local release of propionate, the major metabolite of propionibacteria, which was shown to improve intestinal barrier function and to restore expression of ZO-1 in DSS-treated mice (Tong et al., 2016). Accordingly, protection toward inflammation-induced barrier defects was reported for the probiotic product VSL#3 (Krishnan et al., 2016).

Enzymatic shaving of surface proteins reduced adhesion and was previously shown to hydrolyze at least 16 distinct proteins (Le Maréchal et al., 2015). Dramatic inhibition of adhesion was observed following blockage with antibodies raised against SlpB. Interruption of the *slpB* gene in CB129 strain also resulted in a drastic reduction ($P < 0.01$) in adhesion. Moreover, addition of purified S-layer proteins restored the adhesion that was suppressed in *P. freudenreichii* by enzymatic shaving. Altogether, these results indicate a role of *P. freudenreichii* S-layer protein, including SlpB, in adhesion, as was reported for the SlpA protein in *L. acidophilus* NCFM (Buck et al., 2005).

This study evidenced a key role of one of the *P. freudenreichii* S-layer proteins in adhesion to human intestinal cells. Understanding determinants of probiotic action is a key challenge. It opens new avenues for the screening of most promising propionibacteria strains, by monitoring their expression, and for the development of new functional products containing them. It is particularly relevant in the context of pathogens competitive exclusion and inflammation remediation.

AUTHOR CONTRIBUTIONS

GJ and FdC designed the research. GJ, YL, and VA supervised the work. FdC, HR, SH, FG, MD, SD, and JJ took part to the experiments. FdC and GJ wrote the manuscript. YL and VA corrected the manuscript.

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Supplementary material

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2017.01033/full#supplementary-material

FIGURE S1 | Specificity of the anti-SlpB antibodies. The whole gels (I, II) and whole blots (II, IV) corresponding to Figures 1, 4 are shown. A single band reacting with anti-SlpB antibodies is evidenced. Moreover, specific inactivation of slpB gene leads to disappearance of this reactive band (IV). Finally, western blot using anti-SlpB antibodies reveals the SlpB protein only in strains which harbor the corresponding slpB gene, as indicated by the Table (V). In supplemental western blots of the same extracts (VI), sera directed against SlpA and SlpE evidence a distinct pattern. In particular, the two close Coomassie-stained bands, 58 and 56 kDa, were identified by western blot (this work) and by mass spectrometry (Le Maréchal et al., 2015) as SlpA and slpB, respectively.

FIGURE S2 | Interruption of *slpB* gene using suicide vector pUC:*ΔslpB*:*CmR*. (A–C) Schematic view of homologous recombination producing a mutant CB 128*ΔslpB*. Disruption of *slpB* gene in CB 129 WT by suicide vector pUC:*CmR* harboring 520-bp of *slpB*. Mutant strain show a chloramphenicol resistance by insertion of cassette containing *CmR*. (D) Targeting sequence used to inactivate. Partial sequence of *slpB* gene in CB 129 WT and sequence used to homologous recombination (red). The primers annealing site are indicated as underlined bases and oligonucleotides sequence are shown in figure.

References

Belkaid, Y., and Hand, T. (2014). Role of the microbiota in immunity and inflammation. *Cell* 157, 121–141. doi: 10.1016/j.cell.2014.03.011

Bensi, G., Mora, M., Tuscano, G., Biagini, M., Chiarot, E., Bombaci, M., et al. (2012). Multi high-throughput approach for highly selective identification of vaccine candidates: the group a *Streptococcus* case. *Mol. Cell. Proteomics* 11, M111.015693. doi: 10.1074/mcp.M111.015693

Berlec, A., Zadravec, P., Jevnikar, Z., and Štrukelj, B. (2011). Identification of candidate carrier proteins for surface display on *Lactococcus lactis* by theoretical and experimental analyses of the surface proteome. *Appl. Environ. Microbiol.* 77, 1292–1300. doi: 10.1128/AEM.02102-10

Bøhle, L. A., Riaz, T., Egge-Jacobsen, W., Skaugen, M., Busk, ØL., Eijsink, V. G., et al. (2011). Identification of surface proteins in *Enterococcus faecalis* V583. *BMC Genomics* 12:135. doi:10.1186/1471-2164-12-135

Bouchard, D. S., Rault, L., Berkova, N., Le Loir, Y., and Even, S. (2013). Inhibition of *Staphylococcus aureus* invasion into bovine mammary epithelial cells by contact with live *Lactobacillus casei*. *Appl. Environ. Microbiol.* 79, 877–885. doi: 10.1128/AEM.03323-12

Bouglé, D., Roland, N., Lebeurrier, F., and Arhan, P. (1999). Effect of propionibacteria supplementation on fecal bifidobacteria and segmental colonic transit time in healthy human subjects. *Scand. J. Gastroenterol.* 34, 144–148.

Bryson, K., Loux, V., Bossy, R., Nicolas, P., Chaillou, S., van de Guchte, M., et al. (2006). AGMIAL: implementing an annotation strategy for prokaryote genomes as a distributed system. *Nucleic Acids Res.* 34, 3533–3545. doi: 10.1093/ nar/gkl471

Buck, B. L., Altermann, E., Svingerud, T., and Klaenhammer, T. R. (2005). Functional analysis of putative adhesion factors in *Lactobacillus acidophilus* NCFM. *Appl. Environ. Microbiol.* 71, 8344–8351. doi: 10.1128/AEM.71.12. 8344-8351.2005

ClinicalTrials.gov (2017). *Interest of Propionibacterium Freudenreichii for the Treatment of Mild to Moderate Ulcerative Colitis*. Available at: https://clinicaltrials.gov/ct2/show/NCT02488954 [accessed October 11 2016].

Cousin, F. J., Foligné, B., Deutsch, S.-M., Massart, S., Parayre, S., Le Loir, Y., et al. (2012a). Assessment of the probiotic potential of a dairy product fermented by *Propionibacterium freudenreichii* in piglets. *J. Agric. Food Chem.* 60, 7917–7927. doi: 10.1021/jf302245m

Cousin, F. J., Jouan-Lanhouet, S., Dimanche-Boitrel, M.-T., Corcos, L., and Jan, G. (2012b). Milk fermented by *Propionibacterium freudenreichii* induces apoptosis of HGT-1 human gastric cancer cells. *PLoS ONE* 7:e31892. doi: 10.1371/journal.pone.0031892

Cousin, F. J., Mater, D. D. G., Foligne, B., and Jan, G. (2010). Dairy propionibacteria as human probiotics: a review of recent evidence. *Dairy Sci. Technol.* 91, 1–26. doi: 10.1051/dst/2010032

de Souza, H. S. P., and Fiocchi, C. (2016). Immunopathogenesis of IBD: current state of the art. *Nat. Rev. Gastroenterol. Hepatol.* 13, 13–27. doi: 10.1038/nrgastro.2015.186

Deutsch, S. M., Mariadassou, M., Nicolas, P., Parayre, S., Le Guellec, R., Chuat, V., et al. (2017). Identification of proteins involved in the anti-inflammatory properties of *Propionibacterium freudenreichii* by means of a multi-strain study. *Sci. Rep* 7:46409. doi: 10.1038/srep46409

Deutsch, S.-M., Parayre, S., Bouchoux, A., Guyomarc'h, F., Dewulf, J., Dols-Lafargue, M., et al. (2012). Contribution of surface **β**-glucan polysaccharide to physicochemical and immunomodulatory properties of *Propionibacterium freudenreichii*. *Appl. Environ. Microbiol.* 78, 1765–1775. doi: 10.1128/AEM. 07027-11

Fagan, R. P., and Fairweather, N. F. (2014). Biogenesis and functions of bacterial S-layers. *Nat. Rev. Microbiol.* 12, 211–222. doi: 10.1038/nrmicro3213

Foligné, B., Breton, J., Mater, D., and Jan, G. (2013). Tracking the microbiome functionality: focus on Propionibacterium species. *Gut* 62, 1227–1228. doi: 10.1136/gutjnl-2012-304393

Foligné, B., Deutsch, S.-M., Breton, J., Cousin, F. J., Dewulf, J., Samson, M., et al. (2010). Promising immunomodulatory effects of selected strains of dairy propionibacteria as evidenced in vitro and in vivo. *Appl. Environ. Microbiol.* 76, 8259–8264. doi: 10.1128/AEM.01976-10

Food and Agriculture Organization of the United Nations and World Health Organization (eds) (2002). *Probiotics in Food: Health and Nutritional Properties and Guidelines for Evaluation*. Rome: Food and Agriculture Organization.

Havenaar, R., Brink, B. T., and Huisin't Veld, J. H. J. (1992). "Selection of strains for probiotic use," in *Probiotics*, eds R Fuller (Dordrecht: Springer), 209–224.

Hojo, K., Yoda, N., Tsuchita, H., Ohtsu, T., Seki, K., Taketomo, N., et al. (2002). Effect of ingested culture of *Propionibacterium freudenreichii* ET-3 on fecal microflora and stool frequency in healthy females. *Biosci. Microflora* 21, 115–120. doi: 10.12938/bifidus1996.21.115

Huang, Y., and Adams, M. C. (2003). An in vitro model for investigating intestinal adhesion of potential dairy propionibacteria probiotic strains using cell line C2BBe1. *Lett. Appl. Microbiol.* 36, 213–216.

Hymes, J. P., Johnson, B. R., Barrangou, R., and Klaenhammer, T. R. (2016). Functional analysis of an S-Layer-Associated fibronectin-binding protein in *Lactobacillus acidophilus* NCFM. *Appl. Environ. Microbiol.* 82, 2676–2685. doi: 10.1128/AEM.00024-16

Jan, G., Belzacq, A.-S., Haouzi, D., Rouault, A., Métivier, D., Kroemer, G., et al. (2002). Propionibacteria induce apoptosis of colorectal carcinoma cells via short-chain fatty acids acting on mitochondria. *Cell Death. Differ.* 9, 179–188. doi: 10.1038/sj.cdd.4400935

Johnson, B., Selle, K., O'Flaherty, S., Goh, Y. J., and Klaenhammer, T. (2013). Identification of extracellular surface-layer associated proteins in *Lactobacillus acidophilus* NCFM. *Microbiol. Read. Engl.* 159, 2269–2282. doi: 10.1099/mic.0.070755-0

Kaneko, T. (1999). A novel bifidogenic growth stimulator produced by *Propionibacterium freudenreichii*. *Biosci. Microflora* 18, 73–80. doi: 10.12938/bifidus1996.18.73

Krishnan, M., Penrose, H. M., Shah, N. N., Marchelletta, R. R., and McCole, D. F. (2016). VSL#3 probiotic stimulates T-cell Protein tyrosine phosphatase-mediated recovery of IFN-**γ**-induced intestinal epithelial barrier defects. *Inflamm. Bowel Dis.* 22, 2811–2823. doi: 10.1097/MIB.0000000000000954

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227, 680–685. doi: 10.1038/227680a0

Lan, A., Lagadic-Gossmann, D., Lemaire, C., Brenner, C., and Jan, G. (2007). Acidic extracellular pH shifts colorectal cancer cell death from apoptosis to necrosis upon exposure to propionate and acetate, major end-products of the human probiotic propionibacteria. *Apoptosis Int. J. Program. Cell Death* 12, 573–591. doi: 10.1007/s10495-006-0010-3

Le Maréchal, C., Peton, V., Plé, C., Vroland, C., Jardin, J., Briard-Bion, V., et al. (2015). Surface proteins of *Propionibacterium freudenreichii* are involved in its anti-inflammatory properties. *J. Proteomics* 113, 447–461. doi: 10.1016/j.jprot.2014.07.018

Lebeer, S., Vanderleyden, J., and De Keersmaecker, S. C. J. (2008). Genes and molecules of lactobacilli supporting probiotic action. *Microbiol. Mol. Biol. Rev.* 72, 728–764. doi: 10.1128/MMBR.00017-08

Lebeer, S., Vanderleyden, J., and De Keersmaecker, S. C. J. (2010). Host interactions of probiotic bacterial surface molecules: comparison with commensals and pathogens. *Nat. Rev. Microbiol.* 8, 171–184. doi: 10.1038/nrmicro2297

Lortal, S., Rouault, A., Cesselin, B., and Sleytr, U. B. (1993). Paracrystalline surface layers of dairy propionibacteria. *Appl. Environ. Microbiol.* 59, 2369–2374.

Mack, D. R., Ahrne, S., Hyde, L., Wei, S., and Hollingsworth, M. A. (2003). Extracellular MUC3 mucin secretion follows adherence of *Lactobacillus* strains to intestinal epithelial cells in vitro. *Gut* 52, 827–833.

Mack, D. R., Michail, S., Wei, S., McDougall, L., and Hollingsworth, M. A. (1999). Probiotics inhibit enteropathogenic E. coli adherence in vitro by inducing intestinal mucin gene expression. *Am. J. Physiol.* 276, G941–G950.

Malik, A. C., Reinbold, G. W., and Vedamuthu, E. R. (1968). An evaluation of the
taxonomy of *Propionibacterium*. *Can. J. Microbiol.* 14, 1185–1191.

Mayrhofer, C., Krieger, S., Allmaier, G., and Kerjaschki, D. (2006). DIGE compatible labelling of surface proteins on vital cells in vitro and in vivo. *Proteomics* 6, 579–585. doi: 10.1002/pmic.200500104

Michaux, C., Saavedra, L. F. R., Reffuveille, F., Bernay, B., Goux, D., Hartke, A., et al. (2013). Cold-shock RNA-binding protein CspR is also exposed to the surface of *Enterococcus faecalis*. *Microbiol. Read. Engl.* 159, 2153–2161. doi: 10.1099/mic.0.071076-0

Mitsuyama, K., Masuda, J., Yamasaki, H., Kuwaki, K., Kitazaki, S., Koga, H., et al. (2007). Treatment of ulcerative colitis with milk whey culture with *Propionibacterium freudenreichii*. *J. Intest. Microbiol.* 21, 143–147. doi: 10.11209/jim.21.143

Moussavi, M., and Adams, M. C. (2010). An in vitro study on bacterial growth interactions and intestinal epithelial cell adhesion characteristics of probiotic combinations. *Curr. Microbiol.* 60, 327–335. doi: 10.1007/s00284-009-9545-1

Ouwehand, A. C., Lagström, H., Suomalainen, T., and Salminen, S. (2002). Effect of probiotics on constipation, fecal azoreductase activity and fecal mucin content in the elderly. *Ann. Nutr. Metab.* 46, 159–162.

Ouwehand, A. C., Tölkkö, S., Kulmala, J., Salminen, S., and Salminen, E. (2000). Adhesion of inactivated probiotic strains to intestinal mucus. *Lett. Appl. Microbiol.* 31, 82–86.

Plé, C., Breton, J., Richoux, R., Nurdin, M., Deutsch, S.-M., Falentin, H., et al. (2016). Combining selected immunomodulatory *Propionibacterium freudenreichii* and *Lactobacillus delbrueckii* strains: reverse engineering development of an anti-inflammatory cheese. *Mol. Nutr. Food Res.* 60, 935–948. doi: 10.1002/mnfr.201500580

Plé, C., Richoux, R., Jardin, J., Nurdin, M., Briard-Bion, V., Parayre, S., et al. (2015). Single-strain starter experimental cheese reveals anti-inflammatory effect of *Propionibacterium freudenreichii* CIRM BIA 129 in TNBS-colitis model. *J. Funct. Foods* 18(Part A), 575–585. doi: 10.1016/j.jff.2015.08.015

Preising, J., Philippe, D., Gleinser, M., Wei, H., Blum, S., Eikmanns, B. J., et al. (2010). Selection of bifidobacteria based on adhesion and anti-inflammatory capacity in vitro for amelioration of murine colitis. *Appl. Environ. Microbiol.* 76, 3048–3051. doi: 10.1128/AEM.03127-09

Richoux, R., Faivre, É., and Kerjean, J.-R. (1998). Effet de la teneur en NaCl sur la fermentation du lactate par *Propionibacterium freudenreichii* dans des minifromages à pâte cuite. *Le Lait* 78, 319–331. doi: 10.1051/lait:1998332

Riedel, C. U., Foata, F., Goldstein, D. R., Blum, S., and Eikmanns, B. J. (2006). Interaction of bifidobacteria with Caco-2 cells-adhesion and impact on expression profiles. *Int. J. Food Microbiol.* 110, 62–68. doi: 10.1016/j.ijfoodmicro.2006.01.040

Rodríguez-Ortega, M. J., Norais, N., Bensi, G., Liberatori, S., Capo, S., Mora, M., et al. (2006). Characterization and identification of vaccine candidate proteins through analysis of the group A *Streptococcus* surface proteome. *Nat. Biotechnol.* 24, 191–197. doi: 10.1038/nbt1179

Sára, M., and Sleytr, U. B. (2000). S-Layer proteins. *J. Bacteriol.* 182, 859–868.

Schlee, M., Harder, J., Köten, B., Stange, E. F., Wehkamp, J., and Fellermann, K. (2008). Probiotic lactobacilli and VSL#3 induce enterocyte beta-defensin 2. *Clin. Exp. Immunol.* 151, 528–535. doi: 10.1111/j.1365-2249.2007.03587.x

Schlee, M., Wehkamp, J., Altenhoefer, A., Oelschlaeger, T. A., Stange, E. F., and Fellermann, K. (2007). Induction of human beta-defensin 2 by the probiotic *Escherichia coli* Nissle 1917 is mediated through flagellin. *Infect. Immun.* 75, 2399–2407. doi: 10.1128/IAI.01563-06

Seki, K., Nakao, H., Umino, H., Isshiki, H., Yoda, N., Tachihara, R., et al. (2004). Effects of fermented milk whey containing novel bifidogenic growth stimulator produced by propionibacterium on fecal bacteria, putrefactive metabolite, defecation frequency and fecal properties in senile volunteers needed serious nursing-care taking enteral nutrition by tube Feeding. *J. Intest. Microbiol.* 18, 107–115. doi: 10.11209/jim.18.107

Servin, A. L. (2004). Antagonistic activities of lactobacilli and bifidobacteria against microbial pathogens. *FEMS Microbiol. Rev.* 28, 405–440. doi: 10.1016/j.femsre. 2004.01.003

Sleytr, U. B. (1997). I. Basic and applied S-layer research: an overview. *FEMS Microbiol. Rev.* 20, 5–12. doi: 10.1111/j.1574-6976.1997.tb00301.x

Thiel, A., Eikmanns, B., Salminen, S., and Ouwehand, A. C. (2004). In vitro adhesion of propionibacteria to human intestinal mucus [dairy products]. *Ital. J. Food Sci*. Available at: http://agris.fao.org/agris-search/search.do?recordID=IT2005600093 [Accessed October 11, 2016].

Thierry, A., Richoux, R., and Kerjean, J.-R. (2004). Isovaleric acid is mainly produced by *Propionibacterium freudenreichii* in Swiss cheese. *Int. Dairy J.* 14, 801–807. doi: 10.1016/j.idairyj.2004.02.002

Tiptiri-Kourpeti, A., Spyridopoulou, K., Santarmaki, V., Aindelis, G., Tompoulidou, E., Lamprianidou, E. E., et al. (2016). *Lactobacillus casei* exerts anti-proliferative effects accompanied by apoptotic cell death and up-regulation of TRAIL in colon carcinoma cells. *PLoS ONE* 11:e0147960. doi: 10.1371/journal.pone.0147960

Tong, L.-C., Wang, Y., Wang, Z.-B., Liu, W.-Y., Sun, S., Li, L., et al. (2016). Propionate ameliorates dextran sodium sulfate-induced colitis by improving intestinal barrier function and reducing inflammation and oxidative stress. *Front. Pharmacol.* 7:253. doi: 10.3389/fphar.2016.00253

Tuomola, E. M., Ouwehand, A. C., and Salminen, S. J. (1999). Human ileostomy glycoproteins as a model for small intestinal mucus to investigate adhesion of probiotics. *Lett. Appl. Microbiol.* 28, 159–163.

van de Guchte, M., Chaze, T., Jan, G., and Mistou, M.-Y. (2012). Properties of probiotic bacteria explored by proteomic approaches. *Curr. Opin. Microbiol.* 15, 381–389. doi: 10.1016/j.mib.2012.04.003

Vitetta, L., Briskey, D., Alford, H., Hall, S., and Coulson, S. (2014). Probiotics, prebiotics and the gastrointestinal tract in health and disease. *Inflammopharmacology* 22, 135–154. doi: 10.1007/s10787-014-0201-4

Vitetta, L., Hall, S., and Coulson, S. (2015). Metabolic interactions in the gastrointestinal tract (GIT): host, commensal, probiotics, and bacteriophage influences. *Microorganisms* 3, 913–932. doi: 10.3390/microorganisms3040913

Ythier, M., Resch, G., Waridel, P., Panchaud, A., Gfeller, A., Majcherczyk, P., et al.
(2012). Proteomic and transcriptomic profiling of *Staphylococcus aureus* surface LPXTG-proteins: correlation with agr genotypes and adherence phenotypes. *Mol. Cell. Proteomics MCP* 11, 1123–1139. doi: 10.1074/mcp.M111.014191

Zarate, G. (2012). "Dairy propionibacteria: less conventional probiotics to improve the human and animal Health," in *Probiotic in Animals*, ed. E. Rigobelo (Rejika: InTech).

Conflict of Interest Statement: 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.

Supplemental Figure 1.

I

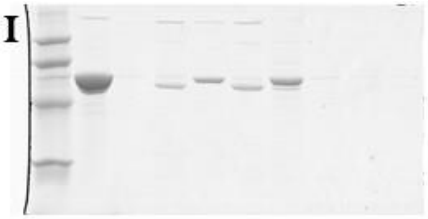


Fig.1.A,
Whole gel

II

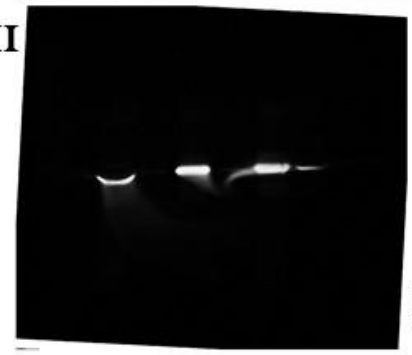


Fig.1.B,
Whole blot

III

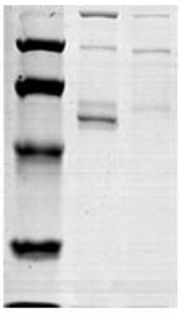


Fig.4.B,
Whole gel

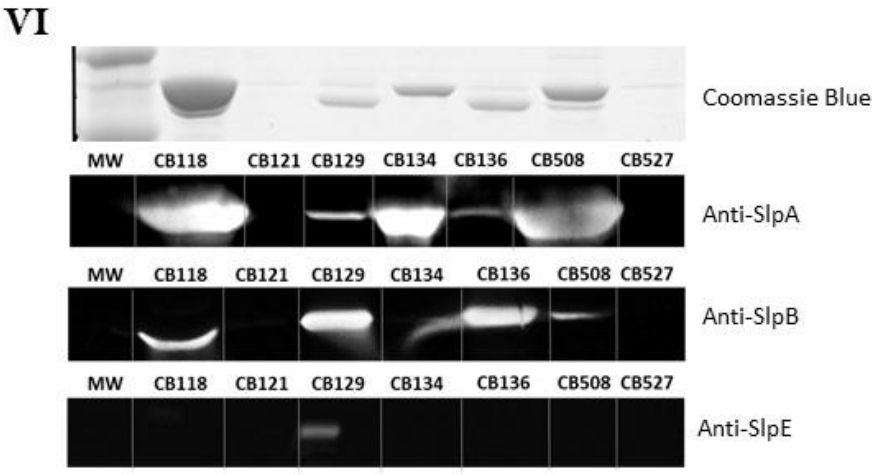
IV

Fig.4.C,
Whole blot

V

	Propionibacterium freudenreichii CIRM BIA strain number						
	CB118	CB121	CB129	CB134	CB136	CB508	CB527
slpB gene[1]	PFCIRM118_02660	NP[2]	PFCIRM129_00700	NP[2]	PFCIRM136_01810	PFCIRM508_02155	NP[2]
1 : when the gene is present, its locus tag is shown							
2 : gene not present							

VI

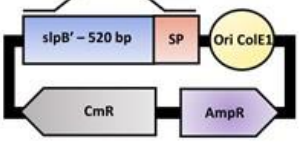


Supplemental Figure1. Specificity of the anti-SlpB antibodies.

Supplemental Figure 2.

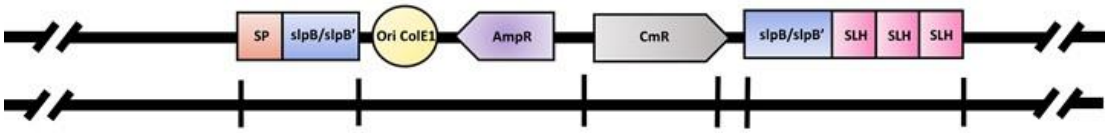
A. *slpB* gene targeting in CB 129 strain

B. Vector targeting



pUC:Δ*slpB*:CmR

C. Targeted interruption of *slpB* gene in CB 129 strain



D. CB 129 *slpB* gene sequence targeting for inactivation

```
1    atgtccgtca  ggaagagcct  gaccgggatg  gcgctggggc  ttgcctcac
                                         Primer XbaI slpB Forward
51   catcaccccg  ctcgccggcg  cggttccggc  gtcagccgac  accgcaccgg
101  ccccaaggga  tgccatcacc  aaggcagccg  attggctggt  gaatgattac
151  aacaccaatt  gtcttggcga  caagcagaca  agttatagct  gctcgaacgg
201  cggcctggcc  gatgtcatcc  tggccctgtc  atccaccggt  gacgcgaaat
                 Sequence for slpB gene inactivation (520 bp)
251  atgccgacga  gatctccacc  atgatgacga  atttggcacc  gcaggtggcc
301  agctacacga  aggacaatgc  gggcgctacc  gccaagatca  tcatcactgt
351  cattgccgcc  catcagaaac  cgagtgcctt  tgggggggaat  gacctggtgg
401  gccagttgca  ggcactgaac  gcggagaacc  ccgccggtgg  cggggcatgg
451  ggaccgcagt  tgtcgatggt  ggctctcacc  cgcgccgggg  agaccgtgcc
501  cgaggcactg  atcgatgcga  cagtggacaa  gcaaaacagc  aagggcggct
                                         Primer BamHI slpB Reverse
551  tcggctgggg  cggcgacacg  ggcgatggcg  acaacaccgc  atggcca...
```

Primer XbaI slpB Forward = 5' CCCCTCTAGAGCGCTGGGGCTTGCC 3'
Primer BamHI slpB Reverse = 5' CCCGGATCCAGCCGCCCTTGCTG 3'

Supplemental Figure2. Interruption of *slpB* gene using suicide vector pUC:Δ*slpB*:*CmR*.

CHAPTER 4 – ORIGINAL ARTICLE - MUTATION OF THE SURFACE LAYER PROTEIN SlpB HAS PLEIOTROPIC EFFECTS IN THE PROBIOTIC *Propionibacterium freudenreichii*

Extracellular components, including S-layer, are reported to be determinants of probiotic action and to play a major role in modulating adhesion of microorganisms to epithelial surfaces in the host (JOHANSSON et al., 2011; OTTE; PODOLSKY, 2004). The initial step of colonization is mediated by adhesion of probiotic bacteria to epithelial cells. Adhesion further extends their persistence within the digestive tract, favouring probiotic action. The surface layer associated proteins shape the bacterial cell surface and determine key surface parameters such as hydrophobic and hydrophilic profiles, both correlated to the adhesion process to intestinal epithelial cells (GUO et al., 2010). The identification and the understanding of the precise contribution of cell surface compounds to bacteria/cell adhesion is a fundamental step in the elucidation of the bacterium/host cross-talk (VAN DE GUCHTE et al., 2012; WILSON et al., 2001).

In addition to adhesion to host cells, S-layer proteins may also act as a physical barrier against external factors (SLEYTR; MESSNER, 1988). Expression of certain S-layers is reportedly linked to *in vitro* monitored tolerance towards extreme environmental conditions, such as variations in pH, bile salts, proteases and simulated gastrointestinal conditions (CHEN et al., 2007; ESLAMI; KERMANSHAHI; ERFAN, 2013; SMIT et al., 2001). It was noticed that removal of S-layer proteins from the surface of *Lactobacillus hilgardii* increases its susceptibility towards bacteriolytic enzymes and physicochemical stress (DOHM et al., 2011).

The *P. freudenreichii* CIRM-BIA 129 possess several S-layer proteins mediating immunomodulation and adhesion (DO CARMO et al., 2017b; LE MARÉCHAL et al., 2015). A mutant *P. freudenreichii* CIRM-BIA 129Δ*slpB* (CB129Δ*slpB*) strain exhibits decreased adhesion to intestinal epithelial HT-29 cells. Moreover, trypsin-shaved cells of *P. freudenreichii* lose both their S-layer proteins and their ability to adhere to cells. Incubation of these shaved cells with guanidine-extracted S-layer proteins restored this adhesion. This results makes a statement of *P. freudenreichii* CIRM-BIA 129 S-layer protein, mainly SlpB, involvement in adhesion, as was reported for the SlpA protein in *Lactobacillus acidophilus* NCFM (BUCK et al., 2005).

In the present study, we thus aimed at deciphering the impact of the single mutation of *P. freudenreichii* CIRM-BIA 129 *slpB* gene on physicochemical properties, tolerance to harsh environment and bacterial physiology.

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CHAPITRE 4 – ARTICLE DE RECHERCHE ORIGINAL - MUTATION OF THE SURFACE LAYER PROTEIN SlpB HAS PLEIOTROPIC EFFECTS IN THE PROBIOTIC *Propionibacterium freudenreichii.*

Les composants extracellulaires, y compris les protéines S-layer, sont considérés comme des déterminants majeurs de l'effet probiotique, jouant un rôle majeur dans l'adhésion des microorganismes aux surface épithéliales de l'hôte (JOHANSSON et al., 2011; OTTE; PODOLSKY, 2004). L'étape initiale de la colonisation est assurée par l'adhésion des bactéries probiotiques aux cellules intestinale. L'adhésion, de plus, prolonge leur persistance dans le tractus digestif, favorisant ainsi l'action probiotique. Les protéines associées à la surface-layer donnent la frome à la surface bactérienne et déterminent des paramètres clés de surface tels que profils hydrophiles et hydrophobes, corrélés aux processus d'adhésion aux cellules intestinales épithéliales (GUO et al., 2010). L'identification et la compréhension de la contribution précise de composés cellulaires de surface à l'adhésion bactérie/cellule constitue une étape fondamentale de l'élucidation du dialogue bactérie/hôte (VAN DE GUCHTE et al., 2012; WILSON et al., 2001).

En plus de l'adhésion aux cellules de l'hôte, les protéines de S-layer peuvent également agir comme une barrière physique contre des facteurs externes (SLEYTR; MESSNER, 1988). L'expression de certaines S-layer est reconnue comme liée à la tolérance vis-à-vis de conditions extrêmes telles que la variation du pH, les sels biliaires, les protéases, ainsi que les conditions gastro-intestinales simulées *in vitro* (CHEN et al., 2007; ESLAMI; KERMANSHAHI; ERFAN, 2013; SMIT et al., 2001). Il a été rapporté que l'élimination des protéines S-layer de la surface de *Lactobacillus hilgardii* augmente sa sensibilité vis-à-vis d'enzymes bactériolytiques et de stress physicochimiques (DOHM et al., 2011).

La souche *P. freudenreichii* CIRM-BIA 129 possède plusieurs protéines S-layer qui déterminent l'immunomodulation et l'adhésion (DO CARMO et al., 2017b; LE MARÉCHAL et al., 2015). Un mutant *P. freudenreichii* CIRM-BIA 129Δ*slpB* (CB129Δ*slpB*) montre une adhésion réduite aux cellules épithéliales intestinales HT-29 ; De plus, des cellules de *P. freudenreichii* CIRM-BIA 129, rasées à l'aide de trypsine, perdent à la fois leur couche de surface et leur capacité à adhérer aux mêmes cellules. L'incubation de ces bactéries rasées avec des protéines S-layer extraites à l'aide de trypsine restaure cette adhésion. Ce résultat indique que les protéines de type S-layer,

principalement SlpB, sont impliquées dans l'adhésion de *P. freudenreichii* CIRM-BIA 129, comme cela a été rapporté pour la protéine SlpA de *Lactobacillus acidophilus* NCFM (BUCK et al., 2005).

Dans l'étude présentée ci-après, nous avons donc cherché à élucider l'impact de la mutation simple du gène *slpB* de *P. freudenreichii* CIRM-BIA 129 sur certaines propriétés physiologiques, sur la tolérance aux conditions environnementales stressantes.

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Mutation of the surface layer protein slpb has pleiotropic effects in the probiotic *propionibacterium freudenreichii* cirm-bia 129

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do Carmo FLR, Silva WM, Tavares GC, Ibraim IC, Cordeiro BF, Oliveira ER, Rabah H, Cauty C, da Silva SH, Canário Viana MV, Caetano ACB, dos Santos RG, de Oliveira Carvalho RD, Jardin J, Pereira FL, Folador EL, Le Loir Y, Figueiredo HCP, Jan G and Azevedo V (2018) Mutation of the Surface Layer Protein SlpB Has Pleiotropic Effects in the Probiotic Propionibacterium freudenreichii CIRM-BIA 129. Front. Microbiol. 9:1807. doi: 10.3389/fmicb.2018.01807

Propionibacterium freudenreichii is a beneficial Gram-positive bacterium, traditionally used as a cheese-ripening starter, and currently considered as an emerging probiotic. As an example, the *P. freudenreichii* CIRM-BIA 129 strain recently revealed promising immunomodulatory properties. Its consumption accordingly exerts healing effects in different animal models of colitis, suggesting a potent role in the context of inflammatory bowel diseases. This anti-inflammatory effect depends on surface layer proteins (SLPs). SLPs may be involved in key functions in probiotics, such as persistence within the gut, adhesion to host cells and mucus, or immunomodulation. Several SLPs coexist in *P. freudenreichii* CIRM-BIA 129 and mediate immunomodulation and adhesion. A mutant *P. freudenreichii* CIRM-BIA 129Δ*slpB* (CB129Δ*slpB*) strain was shown to exhibit decreased adhesion to intestinal epithelial cells. In the present study, we thoroughly analyzed the impact of this mutation on cellular properties. Firstly, we investigated alterations of surface properties in CB129Δ*slpB*. Surface extractable proteins, surface charges (ζ-potential) and surface hydrophobicity were affected by the mutation. Whole-cell proteomics, using high definition mass spectrometry, identified 1,288 quantifiable proteins in the wild-type strain, i.e., 53% of the theoretical proteome predicted according to *P. freudenreichii* CIRM-BIA 129 genome sequence. In the mutant strain, we detected 1,252 proteins, including 1,227 proteins in common with the wild-type strain. Comparative quantitative analysis revealed 97 proteins with significant differences between wild-type and mutant strains. These proteins are involved in various cellular process like signaling, metabolism, and DNA repair and replication. Finally, *in silico* analysis predicted that *slpB* gene is not part of an operon, thus not affecting the downstream genes after gene knockout. This study, in accordance with the various roles attributed in the literature to SLPs, revealed a pleiotropic effect of a single slpB mutation, in the probiotic P. freudenreichii. This suggests that SlpB may be at a central node of cellular processes and confirms that both nature and amount of SLPs, which are highly variable within the P. freudenreichii species, determine the probiotic abilities of strains.

Keywords: bacteria genomic, bacteria proteomic, surface layer protein, HDMSE, shotgun proteomic

INTRODUCTION

Probiotic bacteria are defined as "living microorganisms which when administered in adequate amounts confer a health benefit on the host" (Food and Agriculture Organization of the United Nations and World Health Organization, 2002). This term was further used by International Scientific Association for Probiotics and Prebiotics (ISAP) (Hill et al., 2014). Clinical proofs of efficiency were indeed obtained, in the context of antibiotic- and *Clostridium difficile*-associated diarrhea (Rondanelli et al., 2017), lactose intolerance (Oak and Jha, 2018), irritable bowel syndrome (IBS) (Ford et al., 2014), and ulcerative colitis, one of the disorders that constitute Inflammatory bowel disease (IBD) (Plaza-Díaz et al., 2017). The mechanisms underpinning these effects mainly belong to three categories: (i) metabolic effects,
(ii) modulation of the gut microbiota, and (iii) probiotic/host molecular interactions. Although lactobacilli and bifidobacteria were mainly considered for probiotic usage, promising effects were also reported for dairy propionibacteria (Rabah et al., 2017). The probiotic properties of dairy propionibacteria are strain-dependent and include microbiota modulation, apoptosis modulation in colonic cells and immunomodulation. Some of these probiotic abilities were validated at the clinical level. Microbiota modulation by dairy propionibacteria result in a bifidogenic effect (Roland et al., 1998; Seki et al., 2004; Suzuki et al., 2006). The corresponding molecular mechanisms were elucidated, and two molecules are shown to be involved in bifidogenic effect: 1,4-dihydroxy-2-naphtoic acid (DHNA) and 2-amino-3-carboxy-1,4-naphthoquinone (ACNQ) (Isawa et al., 2002; Furuichi et al., 2006). The pro-apoptotic effect of dairy propionibacteria was evidenced using *in vitro* cellular models (Jan et al., 2002) and animals models (Lan et al., 2008). This effect is mainly due to the production of the short chain fatty acids (SCFA) acetate and propionate by dairy propionibacteria (Lan et al., 2007; Cousin et al., 2016). The anti-inflammatory effect was suggested in IBD patients (Mitsuyama et al., 2007) and confirmed in animal colitis models (Foligné et al., 2010; Plé et al., 2015, 2016). Immunomodulatory properties are due to several metabolites as SCFAs and to cells wall component (Rabah et al., 2017). Indeed, surface proteins considered as microorganism-associated molecular patterns (MAMP) play a pivotal role in interaction with host's immune system (Deutsch et al., 2012; Le Maréchal et al., 2015). This includes SlpB and SlpE, surface proteins anchored to the cell wall via surface- layer homology (SLH) domains (Deutsch et al., 2017; do Carmo et al., 2018). Indeed, mutation of *slpB* and *slpE* genes clearly affected the immunomodulatory properties of *P. freudenreichii* (Deutsch et al., 2017). We have recently shown that SlpB is involved both in immunomodulation and in adhesion to cultured human intestinal epithelial cells (do Carmo et al., 2017). In probiotic bacteria, extractable surface proteins play several role in bacterium/host interaction, protection against environmental stresses, inhibition of pathogens, survival within the host digestive tract, and determination or maintenance of cell shape (Hynönen and Palva, 2013; do Carmo et al., 2018). In this study, we investigated the impact of *slpB* gene mutation on the physiology of *P. freudenreichii* CIRM-BIA 129 using a proteomic approach. In this purpose, we investigated alterations in extractable surface proteins and in the whole-cell proteome. We compared wild-type CIRM-BIA 129 with mutant CB129Δ*slpB*. We report pleiotropic effects of this single mutation on physicochemical properties of this propionibacteria.

MATERIALS AND METHODS

Bacterial Strains and Culture Conditions

The wild-type *P. freudenreichii* CIRM-BIA 129 (WT) strain and genetically modified *P. freudenreichii* CIRM-BIA 129Δ*slpB* strain (CB129Δ*slpB*) (do Carmo et al., 2017) were grown at 30°C in Yeast Extract Lactate (YEL) broth (Malik et al., 1968). For the CB129ΔslpB, YEL culture media were supplemented with chloramphenicol (10 μg.mL^{-1}). The growth of *P. freudenreichii* strains was monitored spectrophotometrically by measuring the optical density at 650 nm ($OD_{650\ nm}$), as well as by counting colony-forming units (CFUs) in YEL medium containing 1.5% agar. *P. freudenreichii* strains were harvested in a stationary phase (76 h, 2 × 10^9 CFU.mL^{-1}, determined by plate counts) by centrifugation (8,000 × g, 10 min, 4°C).

Inventory of Extractable Surface Proteins Using Guanidine Hydrochloride and MS/MS

Proteins were guanidine-extracted, trypsinolysed and subjected to mass spectrometry (Le Maréchal et al., 2015). Peptides were separated by Nano-LC-MS/MS using a Dionex U3000- RSLC nano-LC system fitted to a Qexactive mass spetrometer (Thermo Scientific, San Jose, CA, USA) equipped with a nano- electrospray ion source (ESI) (Proxeon Biosystems A/S, Odense, Denmark). Peptides were identified from MS/MS spectra using the X!Tandem pipeline 3.4.3 software (Langella et al., 2017) for search into two concatenated databases: (i) a homemade database containing all the predicted proteins of the *P. freudenreichii* CIRM-BIA 129 used in this study and (ii) a portion of the UniProtKB database corresponding to taxonomy 754252:
P. freudenreichii subsp. *shermanii* (strain ATCC 9614/CIP 103027/CIRM-BIA1).

Zeta Potential Analysis

Electrophoretic mobility (zeta potential) was determined according to the well-described protocol of Schär-Zammaretti and Ubbink (2003). Bacteria were harvested from a 5 mL stationary phase culture by centrifugation (8.000 × g, 10 min, room temperature) and washed twice with a PBS buffer pH 7.0. Cell count of the final suspensions was approximately 10^8 CFU/ml. The pellet was resuspended in a 10 mM KH_2PO_4 solution (pH 7.0). The electrophoretic mobility was measured by using a ZetaSizer nanoZS (Malvern Instruments, Malvern, United Kingdom) and a glass capillary Zetasizer Nanoseries DTS 1061 (Malvern Instruments, Malvern, United Kingdom) as the electrophoretic cell. Electrophoretic mobilities were converted to the **ζ**-potential using the Helmholtz-Smoluchowski equation (Schär-Zammaretti and Ubbink, 2003). All experiments were done in biological and technical triplicates.

Cell Surface Hydrophobicity Analysis

The Microbial Adhesion To Hydrocarbons (MATH) assay was performed as described by Kos et al. (2003). The optical density of the stationary phase bacteria was adjusted to an $OD_{650\ nm} = 1$. The samples were centrifuged for 5 min, 10,000 × g at room temperature and the pellets washed twice with the same volume of PBS pH 7.0 prior to resuspension in 15 mL of 0.1M KNO_3, pH 6.2. An aliquot of each bacterial suspension (4 ml) was mixed with 1 mL of the solvent (Xylene, chloroform and ethyl acetate), incubated for 5 min at room temperature and mixed by vortex during 120 s. Subsequently, samples were incubated during 60 min to allow phases separation, the aqueous phase was carefully removed and absorbance ($OD_{600\ nm}$) was determined as above. Cell surface hydrophobicity in terms of per cent (H %) was calculated using the following formula: H % = (1–A1/A0) × 100. All experiments were done in biological and technical triplicates.

Transmission Electron Microscopy Assay

Cultures were grown on YEL medium to an $OD_{650\ nm}$ of 1. Transmission electron microscopy was executed after bacteria were washed with PBS and fixed overnight at 4°C in 0.1 M sodium cacodylate buffer (pH 7.2) containing 2% glutaraldehyde. Fixed bacteria were rinsed and stored at 4°C in cacodylate buffer containing 0.2 M sucrose. They were then postfixed 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. Thin sections (70 nm) were collected on 200-mesh cooper grids and counterstained with lead citrate before examination. The thickness of the cell wall was determined using the imageJ software in both strains analyzed by Transmission Electron Microscopy (TEM) as described (Foligné et al., 2010; Deutsch et al., 2012).

Stress Conditions Challenge

P. freudenreichii strains in stationary phase were subjected to lethal doses of different stresses. The acid challenge was carried out at pH 2.0 for 1 h as described (Jan et al., 2000). The bile salts stress was induced by adding 1.0 g/l of bile salts for 60 s as described (Leverrier et al., 2003). For the thermic stress, bacteria were heated for 30 min at 63°C. Viable cells were determined by serial dilutions of samples made up in peptone water (0.1% bacteriological peptone, Kasvi, Brazil), adjusted to pH 7.0 and containing 0.9% NaCl, into YEL medium containing 1.5% agar. CFU were counted after 6 days of anoxic incubation at 30°C (Anaerocult® A - Merck Millipore). All experiments were done in biological and technical triplicates.

Whole-Cell Protein Extraction and Preparation of Total Bacterial Lysates

The optical density of the stationary phase bacteria was adjusted to an $OD_{650\ nm} = 1$. The cultures were centrifuged for 5 min, 10,000 × g at room temperature and the bacterial pellets from biological triplicates were resuspended in 1 mL of lysis buffer containing 42% urea, 15% thiourea, 4% SDC (sodium deoxycholate), 12.5 mM Tris-HCl pH 7.5 and 1.5% dithiothreitol (DTT) with 10 **µ**L of protease inhibitor (GE HealthCare, Pittsburgh, USA). Next, whole-cell proteins were extracted as described (Silva et al., 2014) and quantified by Qubit 2.0 fluorometer (Invitrogen, Carlsbad, USA). 100 **µ**g of each protein extract were denatured with 0.2% of RapiGest SF solution (Waters, Milford, USA) at 80°C for 15 min, reduced with 100 mM DTT at 60°C for 30 min, and alkylated with 300 mM iodoacetamide at room temperature in a dark room for 30 min (Leibowitz et al., 2017). Subsequently, proteins were enzymatically digested with 10 **µ**l of trypsin at 0.5 **µ**g.**µ**L^{-1} (Promega, Madison, USA), and the digestion stopped with the addition of 10 **µ**L of 5% trifluoroacetic acid (TFA) (Sigma Aldrich, Saint Louis, USA) (Silva et al., 2017). Tryptic peptides were subjected to SDC removal (Lin et al., 2010), desalted using C18 MacroSpin Columns (Harvard Apparatus, Holliston, USA), according to the manufacturer's instructions, and dried under vacuum in the Eppendorf™ Vacufuge™ Concentrator (Eppendorf, Hamburg, Germany) (Wong et al., 2013). Prior to injection, the peptides were resuspended in 20 mM ammonium formate (Sigma Aldrich) and transferred to Waters Total Recovery vials (Waters).

LC-HDMS^E Analysis and Data Processing

Quantitative proteomics analyses were conducted with Bidimensional Nano Ultra-Performance Liquid Chromatography (nanoUPLC) tandem Nano Electrospray High Definition Mass Spectrometry (nanoESI-HDMS^E) both using a 1-h reverse-phase (RP) gradient from 7 to 40% (v/v) acetonitrile (0.1% v/v formic acid) and a 500 nL.min^{-1} nanoACQUITY UPLC 2D Technology system (Waters) (Gilar et al., 2005). A nanoACQUITY UPLC High Strength Silica (HSS) T3 1.8 **µ**m, 75 **µ**m × 150 mm column (pH 3) was used in conjunction with a RP Acquity UPLC Nano Ease XBridge BEH130 C18 5 **µ**m, 300 **µ**m × 50 mm nanoflow column (pH 10) (Silva et al., 2017). Typical on-column sample loads were 500 ng of total protein digests for each sample of the 5 fractions (500 ng per fraction/load).

The measurements for all samples by mass spectrometer was operated in resolution mode with a typical m/z resolving power of at least 25,000 Full Width at Half Maximum (FWHM) and an ion mobility cell that was filled with helium gas and a cross-section resolving power at least 40 K/O K. The effective

resolution with the conjoined ion mobility was 25,000 FWHM. Analyses were performed using nano-electrospray ionization in positive ion mode nanoESI (+) and a NanoLock-Spray (Waters) ionization source. The lock mass channel was sampled every 30 s. The mass spectrometer was calibrated with an MS/MS spectrum of [Glu1]-Fibrinopeptide B human (Glu-Fib) solution (100 fmol.μL^{-1}) that was delivered through the reference sprayer of the NanoLock-Spray source. The double-charged ion ($[M + 2H]^{2+}$ = 785.8426) was used for initial single-point calibration, and MS/MS fragment ions of Glu-Fib were used to obtain the final instrument calibration.

The multiplexed data-independent acquisition (DIA) scanning with added specificity and selectivity of a non-linear "T-wave" ion mobility ($HDMS^E$) device was performed with a Synapt G2-Si HDMS mass spectrometer (Waters) (Giles et al., 2011). Synapt G2-Si HDMS was automatically planned to switch between standard MS (3 eV) and elevated collision energies $HDMS^E$ (19–45 eV) applied to the transfer "T-wave" collision-induced dissociation cell with nitrogen gas. The trap collision cell was adjusted to 1 eV, using a millisecond scan time that was previously adjusted based on the linear velocity of the chromatographic peak that was delivered through nanoACQUITY UPLC (Waters). A minimum of 20 scan points was generated for each single peak, both in low-energy and high- energy transmission at an orthogonal acceleration time-of-flight (oa-TOF) and a mass range from m/z 50 to 2,000.

Mass spectrometric analysis of tryptic peptides was performed using a mass spectrometer equipped with a T-Wave-IMS device (Waters) in MS^E mode following the method previously described (Distler et al., 2014). Stoichiometric measurements based on scouting runs of the integrated total ion account prior to analysis were performed to ensure standardized molar values across all samples. Therefore, the tryptic peptides of each strain were injected with the same amount on the column. The radio frequency (RF) offset (MS profile) was adjusted such that the nanoESI-$HDMS^E$ data were effectively acquired from m/z 400 to 2000, which ensured that any masses less than m/z 400 that were observed in the high energy spectra with arose from dissociations in the collision cell (Silva et al., 2017).

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE (Vizcaíno et al., 2016) partner repository with the dataset identifier PXD009804.

Proteins Identification and Quantification

$HDMS^E$ raw data were processed using Progenesis QI for Proteomics (QIP) v.2.0 (Nonlinear Dynamics, Newcastle, UK) as described by Kuharev et al. (2015). For proteins identification, the peptides were searching against a *P. freudenreichii* strain CIRM-BIA 129 database as described above. The reversed sequences were joined together to the original sequences using ProteinLynx Global Server (PLGS) v 3.0.2 (Waters) database management tool. The reversed sequences were used to calculate the false positive rate during identification process. Next, the following parameters were used for peptide identification: digest reagent = trypsin; maximum missed cleavage = one; maximum protein mass = 600 kDa; modifications: carbamidomethyl of cysteine (fixed), acetyl N-terminal (variable), phosphoryl (variable), oxidation of methionine (variable); search tolerance parameters: peptide tolerance = 10 ppm, fragment tolerance = 20 ppm, maximum false discovery rate (FDR) = 1%.

The protein-level quantitation was performed with Relative Quantitation using Hi-N algorithm. Proteins identified with at least two peptides and presents in at least two of the three biological replicates were considered (Silva et al., 2014). The proteins list was exported by the function "export protein measurements" and was used to subsequent bioinformatics analysis. Proteins were considered to be differentially expressed between mutant and wild type if there were a significant ($p < 0,05$, ANOVA) change in expression ≥ 2-fold (log2 ratio ≥ 1.0). A volcano plot was generated to visualize the differentially expressed proteins across these strains.

Extraction of Genomic DNA of the CB129Δ*slpB* Strain

Genomic DNA was extracted from CB129ΔslpB culture grown in YEL medium supplemented with chloramphenicol (10 $\mu g\ ml^{-1}$), during the phase (76 h at 30°C). Samples was centrifuged at 4°C and 8,000 × g for 10 min. Bacterial pellets were resuspended in 1 ml Tris/EDTA/RNase [10 mM Tris/HCl (pH 7.0), 10 mM EDTA (pH 8.0), 300 mM NaCl, 50 $\mu g\ ml^{-1}$] with 50 mg of Glass beads VK01 and cell lysis occurred in Precellys 24 by 2 cylces of 15 s at 6,500 rpm. DNA was purified using phenol/chloroform/isoamyl alcohol and precipitated with ethanol according with Sambrook and Russell (2001). DNA concentrations were determined spectrophotometrically in Thermo Scientific NanoDrop 1000.

Genome Sequencing, Assembly and Annotation of the CB129Δ*slpB* Strain

CB129ΔslpB strain sequencing libraries were constructed using 100 ng of genomic DNA. The gDNA was sheared with the Ion Shear™ Plus Reagents Kit and barcoded using the Ion Xpress Fragment Library kit and Ion Xpress™ Barcode Adapters (Life Technologies, USA), according to the manufacturer's protocol. Size selection of ~400 bp was performed with 2% E-Gel® SizeSelect™ Agarose Gels (Invitrogen, USA) and quantified with the Ion Library Quantitation Kit. The libraries were amplified with the OneTouch Template 400 kit on the Ion One Touch™ 2 (Life Technologies) and enriched on the Ion OneTouch™ ES (Life Technologies). Genomic libraries were enriched using Ion PI™ Hi-Q™ Sequencing Polymerase in the Ion 318™ v2 Chip, according to the manufacturer's protocols, and they were sequenced using Ion Torrent Personal Genome Machine (PGM). The amplification processes were performed using Ion PGM™ Hi-Q™ Sequencing 400 Polymerase with required 1,100 flows. Finally, signal processing was performed using Torrent Suite 4.2.1 to conclude the sequencing process.

De novo assembly was conducted using the software Newbler v 2.9 (Roche 454, USA). The assembled contigs were oriented to generate a scaffold using CONTIGuator v 2.7 (Galardini et al., 2011) and the strains *P. freudenreichii* CIRM-BIA 1 (FN806773.1) and *P. freudenreichii* JS17 (LT618789) as reference.

The *P. freudenreichii* CIRM-BIA 1 strain (without the *slpB* gene) was used for comparative analysis as it is a reference from INRA strain collection strain and *P. freudenreichii* JS17 strain was used due to the presence of the s-layer gene *slpB*. CLC Genomics Workbench 7.0 (Qiagen, USA) was used to map the raw reads against the reference genome and to generate the consensus sequence used to the gap filling. The plasmid that integrated within and disrupted the *slpB* gene was not found in the scaffold, but its sequence was found within the contigs that were excluded during the scaffold generation. It was manually inserted to the scaffold by mapping its ends on the *slpB* gene and using the overlap sequences as coordinates for the insertion. The insertion was validated by mapping the reads on the assembly and checking for mismatches on the regions flanking the plasmid. The genome of CB129Δ*slpB* strain was annotated automatically using RAST pipeline (Aziz et al., 2008; Brettin et al., 2015).

Bioinformatics Analyses

The predicted proteins of CB129Δ*slpB* and WT strain were analyzed using the SurfG+ v1.0 tool (Barinov et al., 2009) to predict sub-cellular localization. It enabled the classification of proteins within the following categories: cytoplasmic (CYT), membrane (MEM), potentially surface-exposed (PSE) and secreted (SEC). The prediction of orthologous groups by functional category the sequences was performed using Cluster of Orthologous Genes (COG) database version 2014db (Galperin et al., 2015). The COG database search was performed using an *in-house* script (available at https://github.com/aquacen/blast_ cog). The number of predicted proteins in relation to subcellular localization and functional category were visualized in plots generated using TIBCO SpotFire software 7.0 (TIBCO, Boston, USA) from the protein list exported of QIP. The Interactivenn web-based tool (Heberle et al., 2015) was used to evaluate the shared proteins among strains through Venn diagram.

Protein-protein interaction (PPI) network was constructed using interolog mapping methodology and metrics according to Folador et al. (2014). To generate a preview of the interaction network was generated using Cytoscape version 2.8.3 (Shannon et al., 2003) with a spring-embedded layout. To indicate the reliability of our predicted PPIs in the database STRING, the network was selected using the score 500 (0.5). In the PPI network, the interactions with score close to 500 are with red or yellow lines and, above 700 in dark green lines. The score indicating how much the pair of proteins in the interaction is similar (homologous) to the interaction according to the database. In the PPI, they interact with at least 65% identity with at least 65% coverage.

A circular map comparing the chromosome of CB129Δ*slpB* with *P. freudenreichii* CIRM-BIA 1 and JS17 strains was generated using BLAST Ring Image Generator(BRIG) software v0.95 (Alikhan et al., 2011). Operon prediction in CB129Δ*slpB* strain was performed using FGENESB (http://www.softberry.com).

Statistical Analyses

Growth curve, MATH assay, Zeta potential measure, and stress challenges were performed with three technical replicates and three biological replicates. The results were expressed as means ± standard deviations. Statistical analyses were performed in GraphPad Prism Software version 7 (GraphPad Software) using Student's *t*-test, one-way or two-way ANOVA with SIDAK's or Tukey *post-hoc* analyses for multiple comparisons. Asterisks represent statistically significant differences and were indicated as follows: $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

RESULTS

Impact of slpB Mutation on *P. freudenreichii* Extractable Surface Proteins

SLPs play a key role in probiotic/host interactions and we have shown that such interactions are impaired in an *slpB* mutant. Electrophoretic analysis of guanidine extracts confirmed the disappearance of the corresponding SlpB protein (do Carmo et al., 2017). In the present study, we further investigated these extractable fractions in order to decipher the impact of such a single mutation on the inventory of SLPs, and more widely, of extractable surface proteins, including surface layer associated proteins (SLAPs). Using nanoLC-MS/MS, we identified 40 surface extractable proteins in CB129Δ*slpB* strain, yet 33 in the parental wild-type CIRM BIA 129 one (**Table 1**). The core of extractable proteins, non-covalently bound to the cell wall, common to mutant and parental strains, was composed of 23 proteins, including solute-binding protein of the ABC transport system (BopA), internalin A (InlA), surface protein with SLH Domain E (SlpE), and surface- Layer Protein A (SlpA). Moreover, it comprised a series of cytoplasmic proteins involved in different biological processes like Heat shock 70 kDa protein 1 (HSP70 1), Clp chaperone, GroL1 and GroL2, Elongation factor Tu, and subunits of Methylmalonyl-CoA mutase and subunits of Methylmalonyl- CoA carboxytransferase. Among extractable proteins specific of the CB129Δ*slpB*, we identified proteins involved in metabolic processes like Coenzyme A transferase involved in acetyl-CoA metabolic process and Pyruvate phosphate dikinase Pyruvate synthase involved in pyruvate metabolic process. Furthermore, this specific subset also comprised another protein involved in stress response (HSP70 2). As expected, the SlpB protein was found only in the parental wild type CIRM BIA 129, yet not in the CB129Δ*slpB* mutant.

Impact of slpB Mutation on *P. freudenreichii* ζ-Potential and Cell Surface Hydrophobicity

Propionibacterial SLPs, with a low isoelectric point, confer negative charges to the cell surface. In order to identify if the net surface charge was altered in the mutant strain, we conducted **ζ**-potential and cell surface hydrophobicity assays in both strains.

As shown in the **Figure 1A**, the WT strain exhibited a zeta potential of -21.73 ± 1.63 mV, reflecting a negative net charge, in accordance with the low isoelectric point of *P. freudenreichii* SlpB protein. By contrast, mutation of *slpB* gene significantly affected the zeta potential of the CB129Δ*slpB* strain, which

TABLE 1 | Proteins identified in the extraction of surface proteins non-covalently bound to the cell wall using guanidine hydrochloride of CB 129 wild-type and CB129Δ*slpB* strains[1].

Strain							Wild-type						CB129Δ*slpB*				
Group	Sub-group	locus_tag	Protein description[c]	SurfG+[d]	COG	MW[f]	log(*e*-	Coverage[h]	Uniques[i]	Specific	emPAI[k]	log(*e*-	Coverage[h]	Uniques[i]	Specific	emPAI[k]	
ID[a]	ID[b]			letter[e]		value)[g]			uniques[j]			value)[g]		uniques[j]			
a1	a1.a1	PFCIRM129_05460	Surface protein with SLH domain (S-layer protein E)	SEC	O	59.2	−125.6	50	19	18	47.3	−138.8	55	18	17	41.8	
a1	a1.a2	PFCIRM129_00700	Surface layer protein B (S-layer protein B)	SEC	O	56.8	−263.2	74	34	33	37274.9	–	–	–	–	–	
a1	a1.a3	PFCIRM129_09350	Surface layer protein A (S-layer protein A)	SEC	O	58.3	−174.3	75	24	23	16.0	−143.5	68	22	21	9.0	
a2	a2.a1	PFCIRM129_12235	Internaline A	SEC	S	145.5	−464.8	67	53	–	89.1	−426.3	65	49	–	42.3	
a3	a3.a1	PFCIRM129_03680 & PFCIRM129_03685	MERGED=TRUE	–	–	95.9	−186.7	43	18	–	283.8	−196.6	47	20	–	431.9	
a4	a4.a1	PFCIRM129_10100	60 kDa chaperonin 2 (Protein Cpn60 2) (groEL protein 2) (Heat shock protein 60 2)	CYT	O	56.4	−82.5	38	13	–	5.3	−112.6	49	18	–	14.8	
a5	a5.a1	PFCIRM129_07835	60 kDa chaperonin 1 (Protein Cpn60 1) (groEL protein 1) (Heat shock protein 60 1)	CYT	O	56	−89.2	38	12	–	2.2	−134.7	60	21	–	7.5	
a6	a6.a1	PFCIRM129_06355	Chaperone clpB 2 (ATP-dependent Clp protease B2) (Clp chaperone)	CYT	O	94.2	−39.6	19	10	9	1.2	−103.0	29	18	–	3.0	
a7	a7.a1	PFCIRM129_06315	Chaperone protein dnaK 1 (Heat shock protein 70 1) (Heat shock 70 kDa protein 1) (HSP70 1)	CYT	O	65.3	−24.0	13	5		0.6	−61.2	34	15	12	3.8	
a7	a7.a2	PFCIRM129_08775	Chaperone protein dnaK 2 (Heat shock protein 70 2) (Heat shock 70 kDa protein 2) (HSP70 2)	CYT	O	67.1	–	–	–	–	–	−43.7	23	10	7	2.0	
a9	a9.a1	PFCIRM129_08275	Elongation factor Tu	CYT	J	43.6	−43.7	33	7	–	2.0	−32.4	28	7	–	3.4	
b11	b11.a1	PFCIRM129_11405	30S ribosomal protein S1	CYT	J	53.5	−5.4	7	2	–	0.3	−58.9	27	8	–	1.6	
b12	b12.a1	PFREUD_01840	Pyruvate synthase/Pyruvate-flavodoxin oxidoreductase	CYT	C	136.4	–	–	–	–	–	−67.5	16	14	–	1.2	
b13	b13.a1	PFCIRM129_10305	Methylmalonyl-CoA carboxytransferase 5S subunit. (transcarboxylase 5S) 505 bp	CYT	C	55.5	−23.3	16	5	–	0.7	−37.1	23	9	–	1.8	
b14	b14.a1	PFCIRM129_06950	Trigger factor (TF)	CYT	O	57.3	−8.3	6	2	–	0.3	−36.7	20	6	–	2.0	

(Continued)

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TABLE 1 | Continued

Strain							Wild-type					CB129Δ*slpB*				
Group	Sub-group	locus_tag	Protein description[c]	SurfG+[d]	COG	MW[f]	log(*e*-	Coverage[h]	Uniques[i]	Specific	emPAI[k]	log(*e*-	Coverage[h]	Uniques[i]	Specific	emPAI[k]
ID[a]	ID[b]			letter[e]		value)[g]			uniques[j]		value)[g]			uniques[j]		
b15	b15.a1	PFCIRM129_07240	Methylmalonyl-CoA mutase large subunit (Methylmalonyl-CoA mutase alpha subunit) (MCM-alpha) (MUTB-(R)-2-Methyl-3-oxopropanoyl-CoA CoA-carbonylmutase)	CYT	I	80.1	−15.6	7	4	–	0.4	−38.3	15	8	–	1.0
b16	b16.a1	PFCIRM129_06070	Enolase 1	CYT	G	45.9	−26.5	20	5	–	1.1	−41.5	25	7	–	1.7
b17	b17.a1	PFCIRM129_07235	Methylmalonyl-CoA mutase small subunit (Methylmalonyl-CoA mutase beta subunit) (MCB-beta)	CYT	I	69.5	−16.2	9	4	–	0.4	−59.4	26	9	–	1.2
b19	b19.a1	PFCIRM129_10180	Iron-sulfur protein	CYT	C	57.2	−26.1	18	6	–	1.1	−16.7	8	3	–	0.4
b20	b20.a1	PFCIRM129_08670	Cell-wall peptidases, NlpC/P60 family SEC protein	SEC	M	58.7	−51.6	22	8	–	1.7	−9.5	6	2	–	0.4
b21	b21.a1	PFCIRM129_09300	FAD-dependent pyridine nucleotide-disulphide oxidoreductase:4Fe-4S ferredoxin, iron-sulfur binding:Aromatic-ring hydroxylase	CYT	C	59.7	–	–	–	–	–	−42.1	20	8	–	1.2
b22	b22.a1	PFCIRM129_00205	Succinate dehydrogenase flavoprotein subunit	CYT	C	74.7	−17.1	5	3	–	0.3	−20.8	6	4	–	0.5
b23	b23.a1	PFCIRM129_08495	NADH-quinone oxidoreductase chain G (NADH dehydrogenase I, chain G)	CYT	C	84.8	−22.3	6	3	–	0.2	−28.3	9	5	–	0.4
b24	b24.a1	PFCIRM129_09980	Peptidyl-prolyl cis-trans isomerase	SEC	O	35.9	−23.0	22	4	–	5.8	−11.5	7	2	–	1.2
b25	b25.a1	PFCIRM129_10295	Methylmalonyl-CoA carboxytransferase 12S subunit (EC2.1.3.1) (Transcarboxylase 12S subunit). 610 bp	CYT	I	56.3	−31.2	11	5	–	0.7	−15.2	7	3	–	0.4
b26	b26.a1	PFCIRM129_11300	Glyceraldehyde-3- phosphate dehydrogenase / erythrose 4 phosphate dehydrogenase	CYT	G	37.7	−48.7	39	9	–	2.9	–	–	–	–	–

(Continued)

TABLE 1 | Continued

Strain							Wild-type					CB129Δ*slpB*				
Group	Sub-group	locus_tag	Protein description[c]	SurfG+[d] letter[e]	COG	MW[f] value)[g]	log(*e*-	Coverage[h]	Uniques[i] uniques[j]	Specific	emPAI[k]	log(*e*- value)[g]	Coverage[h]	Uniques[i] uniques[j]	Specific	emPAI[k]
ID[a]	ID[b]															
b27	b27.a1	PFCIRM129_05155	ATP synthase subunit alpha (ATPase subunit alpha) (ATP synthase F1 sector subunit alpha)	CYT	C	58.8	−7.9	5	2	–	0.2	−17.9	11	5	–	0.6
b30	b30.a1	PFREUD_10490	ATP synthase subunit beta (ATPase subunit beta) (ATP synthase F1 sector subunit beta)	CYT	C	52.4	−12.0	9	3	–	0.4	−15.0	12	4	–	0.7
b31	b31.a1	PFCIRM129_11080 & PFCIRM129_11085	MERGED=TRUE	–	–	35.4	–	–	–	–	–	−17.1	20	4	–	0.9
b32	b32.a1	PFCIRM129_10995	Glycerol kinase (ATP:glycerol 3-phosphotransferase) (Glycerokinase) (GK)	CYT	C	55.6	–	–	–	–	–	−17.7	11	5	–	1.3
b33	b33.a1	PFCIRM129_01440	Coenzyme A transferase (Putative succinyl-CoA or butyryl-CoA:coenzyme A transferase)	CYT	C	55.6	–	–	–	–	–	−14.5	7	3	–	0.5
b34	b34.a1	PFCIRM129_11710 & PFCIRM129_11715	MERGED=TRUE	–	–	58.8	−12.1	4	2	–	0.4	−30.6	13	5	–	1.2
b35	b35.a1	PFCIRM129_05730	D-lactate dehydrogenase	CYT	C	63.6	−9.5	9	3	–	0.3	−14.4	11	4	–	0.4
b36	b36.a1	PFCIRM129_00390	Cysteine synthase 2	CYT	E	33.5	−40.2	38	6	–	1.7	–	–	–	–	–
b37	b37.a1	PFCIRM129_08120	Solute binding protein of the ABC transport system	SEC	E	61.4	−7.3	7	3	–	0.4	−11.9	4	2	–	0.3
b38	b38.a1	PFCIRM129_05105	Hypothetical protein	CYT	–	64	–	–	–	–	–	−17.0	10	5	–	0.6
b40	b40.a1	PFCIRM129_01500	Pyruvate phosphate dikinase	CYT	G	95.7	–	–	—-	–	–	−11.2	3	2	–	0.1
b41	b41.a1	PFCIRM129_03550	Alanine dehydrogenase	CYT	E	39.3	–	–	–	–	–	−5.8	6	2	–	0.4
b43	b43.a1	PFCIRM129_10420	iolA (Myo-inositol catabolism IolA protein) (Methylmalonic acid semialdehyde dehydrogenase)	CYT	C	52.7	–	–	–	–	–	−9.1	6	2	–	0.3
b44	b44.a1	PFCIRM129_08025	Resuscitation-promoting factor	SEC	L	37.7	−15.9	11	2	–	0.9	–	–	–	–	–
b45	b45.a1	PFREUD_14570	Polyribonucleotide nucleotidyltransferase (Polynucleotide phosphorylase) (PNPase) (Guanosine pentaphosphate synthetase)	CYT	J	79.3	–	–	–	–	–	−9.5	3	2	–	0.2
b46	b46.a1	PFCIRM129_08280	Elongation factor G (EF-G)	CYT	J	76.5	–	–	–	–	–	−5.4	2	2	–	0.2

(Continued)

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TABLE 1 | Continued

Strain							Wild-type						CB129Δ*slpB*				
Group	Sub-group	locus_tag	Protein description[c]	SurfG+[d]	COG	MW[f]	log(*e*-	Coverage[h]	Uniques[i]	Specific	emPAI[k]	log(*e*-	Coverage[h]	Uniques[i]	Specific	emPAI[k]	
ID[a]	ID[b]			letter[e]		value)[g]			uniques[j]		value)[g]			uniques[j]			
b48	b48.a1	PFCIRM129_08935	FAD linked oxidase domain protein	CYT	C	100.4	–	–	–	–	–	−18.2	5	3	–	0.2	
b49	b49.a1	PFCIRM129_08300	DNA-directed RNA polymerase beta chain (RNAP beta subunit) (Transcriptase beta chain) (RNA polymerase subunit beta)	CYT	K	128.5	–	–	–	–	–	−8.8	3	2	–	0.1	
b50	b50.a1	PFCIRM129_00200	Succinate dehydrogenase	CYT	C	27	−8.4	10	2	–	0.6	–	–	–	–	–	
b51	b51.a1	PFCIRM129_10175	Hypothetical protein	CYT	S	23.1	−11.5	16	2	–	0.7	–	–	–	–	–	

[a] *The Group to which the protein belongs. All the proteins in a group have at least one peptide in common.*
[b] *The Sub-Group to which the protein belongs. All the proteins in a sub-group are identified with the same valid peptides.*
[c] *Protein description as it appears in the header of the fasta file.*
[d] *SurfG+ localization prediction.*
[e] *Cluster of Orthologous Group category – A, RNA processing and modification; B, Chromatin Structure and dynamics; C, Energy production and conversion; D, Cell cycle control and mitosis; E, Amino Acid metabolis and transport; F, Nucleotide metabolism and transport; G, Carbohydrate metabolism and transport; H, Coenzyme metabolis; I, Lipid metabolism; J, Translation; K, Transcription; L, Replication and repair; M, Cell wall/membrane/envelope biogenesis; N, Cell motility; O, Post-translational modification; P, Inorganic ion transport and metabolism; Q, Secondary Structure; T, Signal Transduction; U, Intracellular trafficking and secretion; Y, Nuclear structure; Z, Cytoskeleton; R, General Functional Prediction only; S, Function Unknown.*
[f] *Molecular weight of the protein expressed in KDa.*
[g] *Protein e-value expressed in log. Statistical value representing the number of times this protein would be identified randomly. Calculated as the product of unique peptide e-values in the sample.*
[h] *Percentage of protein sequence covered by identified peptides.*
[i] *The number of unique peptide sequence assigned to this protein.*
[j] *The number of unique peptide sequence specific to this subgroup of proteins. It is only available if there are more than one subgroup within a group.*
[k] *The Exponentially Modified Protein Abundance Index (emPAI) computation (Ishihama et al., 2005).*
[1] *Part of these results were previously published in do Carmo et al. (2017).*

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was −6.75 ± 0.55 mV, showing a reduced electronegativity, in accordance with a disorganization of the S-layer at the bacterial cell surface. As shown in **Figure 1B**, the wild type strain also showed a high affinity to the hydrocarbons tested, whereas the CB129Δ*slpB* mutant showed a decreased adhesion, whatever the hydrocarbon used in the assay. Adhesion, respectively to mutant and WT strains, were as follow: to Xylol, 0.33 ± 0.52% and 43.67 ± 6.31%, to Chloroform 16.5 ± 10.7% and 75 ± 5.88, and to Ethyl Acetate 5.33 ± 7.17% and 43.83 ± 5.74%. Cell surface properties being drastically affected, we then sought morphological changes caused by the mutation (**Figure 2**). Both strains exhibited a similar cell wall thickness, 24.33 ± 0.4154 nm and 24.90 ± 0.4154 nm, respectively. No significant difference in term of bacteria morphology, cell wall thickness and shape was observed between the two strains using transmission electron microscopy.

Impact of slpB Mutation on *P. freudenreichii* Growth and Stress Tolerance

A single mutation, inactivating a key gene, may affect bacterial fitness and thus probiotic efficacy. We therefore monitored *P. freudenreichii* growth and tolerance toward acid, bile salts and heat challenges, in the wild type and in the mutant. The growth curves showed a similar pattern for both strains (**Figure 3A**). The bacterial count at the stationary phase end was also equivalent for both strains, with a viable population count of 1.63×10^9 CFU.mL^{-1} and 1.75×10^9 CFU.mL^{-1} for the wild type and the mutant strains, respectively. Tolerance toward stress challenges is reported in **Figure 3B**. In the case of acid stress, we observed a significant decrease in viability for the CB129Δ*slpB* strain 0.71 ± 0.13% (7.3×10^6 CFU.mL^{-1}) compared to the WT strain 5.76 ± 1.48% (5.76×10^7 CFU.mL^{-1}). During the bile salts stress, we observed the same trend in the tolerance. Indeed, the survival rate for the CB129Δ*slpB* strain was significantly decreased 0.37 ± 0.24% (3.71×10^6 CFU.mL^{-1}), compared to the WT strain 2.19 ± 1.01% (2.19×10^7 CFU.mL^{-1}). The same stands for heat challenge, with a reduced survival in CB129Δ*slpB* 0.71 ± 0.16% (9.01×10^6 CFU.mL^{-1}) compared to WT strain 5.76 ± 1.35% (5.86×10^7 CFU.mL^{-1}).

Impact of slpB Mutation on *P. freudenreichii* Qualitative and Quantitative Proteome

Considering the major alterations in surface extractable proteins, bacteria cell surface physicochemical properties, and stress tolerance, a qualitative and quantitative analysis of the total proteome was performed to elucidate the impact of the *slpB* gene knockout in the mutant strain. A total of 1,288 quantifiable proteins (53.26% of predicted proteome) wherein 1,253 proteins (reported in **Figure 4A**) were identified (**Table S1**). In the WT strain 1,227 proteins were found, whereas in the CB129Δ*slpB* strain, we detected 1,252 proteins. Comparative analysis revealed a core-proteome, composed by 1,226 proteins, shared by both strains (**Figure 4A**). Differences in protein abundance were observed by proteomic quantitative analysis (**Figure 4B**). A

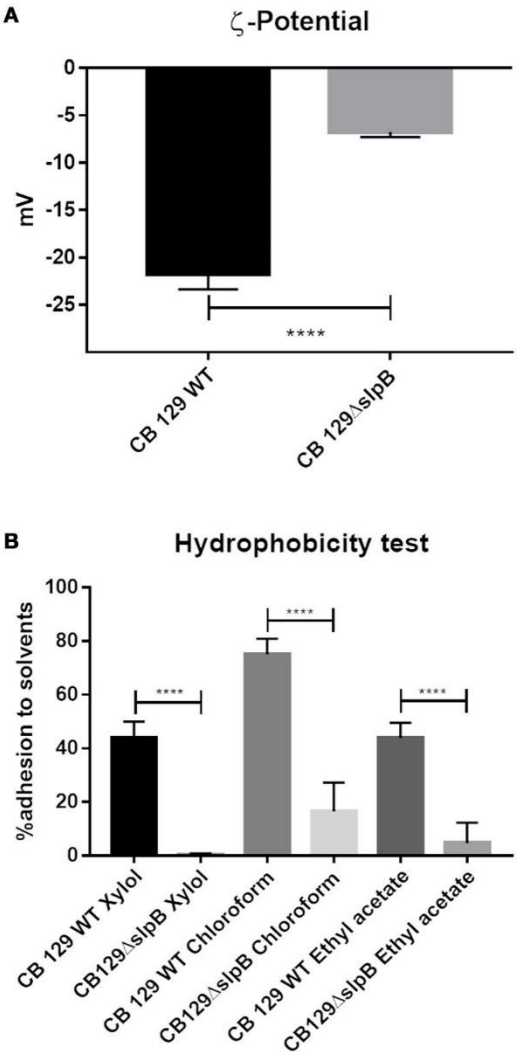

FIGURE 1 | Mutation of *slp*B gene drastically affects surface proprieties in *P. freudenreichii* CIRM-BIA 129. (A) The surface net charge was determined by measuring the ζ-potential. (B) The surface hydrophobicity was determined by quantifying adhesion to solvents (Xylol, Chloroform, and Ethyl Acetate). Wild-type (WT) and mutant CB129Δ*slpB* strains were compared. Bar represents the mean SD of three biological replicates and three technical replicates. The asterisks (****) denotes the statistical significance of the represented value between CB 129 WT and CB129Δ*slpB* ($p > 0.0001$).

total of 97 proteins (4.2% of the predicted proteome) of these common proteins showed differences in the level of expression among strains, including 36 up-regulated and 61 down-regulated proteins in CB129Δ*slpB* in comparison with the WT strain (**Table 2**).

According to the predicted subcellular localization of the 1,253 proteins identified, 1,081 proteins are CYT (61% of predicted proteome), 71 are MEM (18% of predicted proteome), 77 are PSE (41% of predicted proteome) and 24 are SEC (38% of predicted proteome). In the analysis of non-differentially expressed proteins, we classified 1,001 as CYT proteins, 67 as MEM proteins, 70 as PSE proteins and 22 as SEC proteins

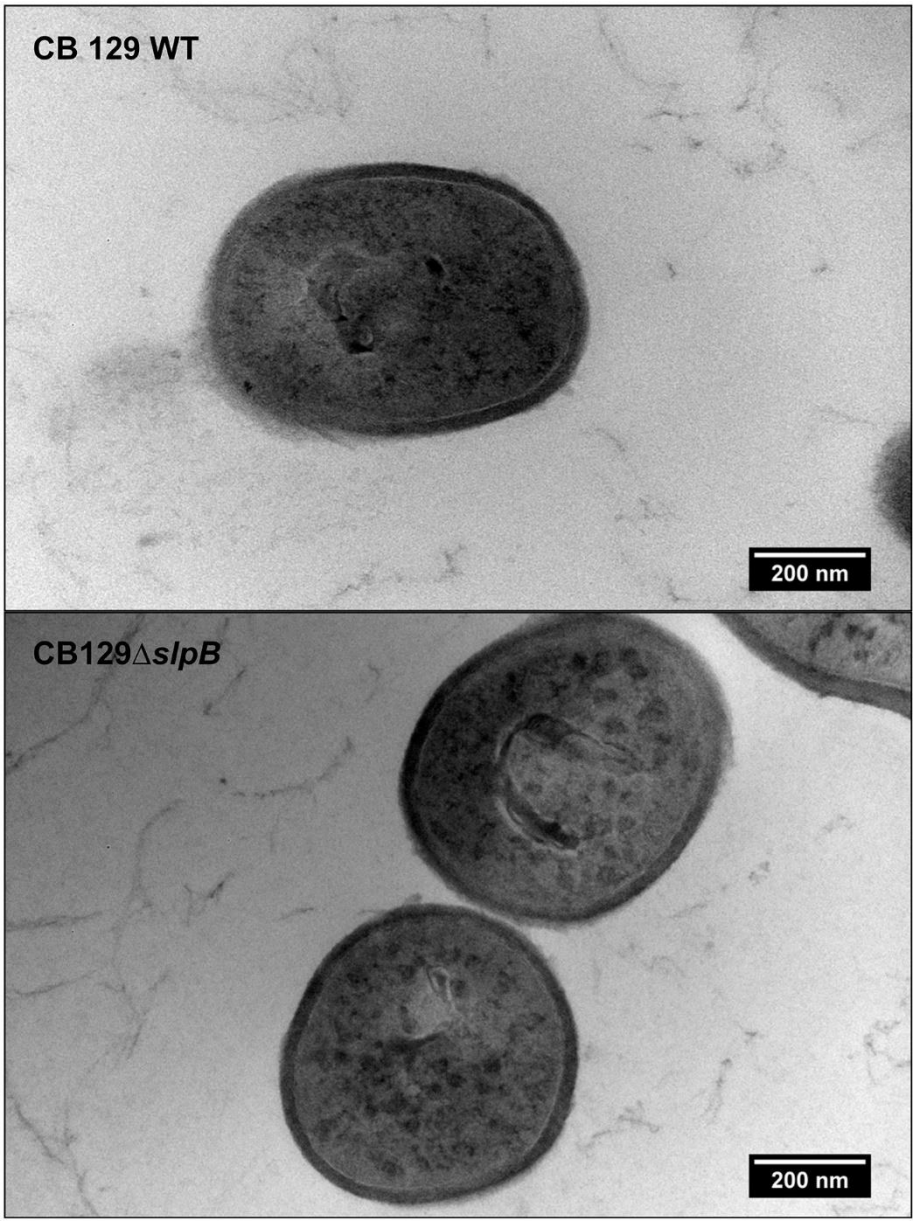

FIGURE 2 | Mutation of *slp*B gene does not affect envelope thickness in *P. freudenreichii* CIRM-BIA 129. Wild-type (WT) and mutant CB129Δ*slpB* strains were analyzed by transmission electron microscopy (TEM). No difference in morphology and cell wall tickeness was found.

(**Figure 4C**). Meanwhile, between the *P. freudenreichii* WT and the CB129Δ*slpB* strains, from 97 proteins differentially expressed, the subcellular localization were predicted as follow: 81 CYT, 2 MEM, 7 PSE, and 7 SEC proteins (**Figure 4C**).

According to COG functional classifications, the differentially expressed proteins were classified into 20 biological processes (**Figure 5A**). A general category of differentially regulated proteins in CB129Δ*slpB* strain core proteome showed 27 proteins involved in information storage and processing, 25 associated to metabolism and, 18 proteins related to cellular processes and signaling (**Figure 5A**). Proteins that mediate different biological process were dysregulated in the mutant strain. As seen in **Figure 5B**, 11 proteins were classified as having general functions, 10 proteins related to process of replication, recombination and repair, other 10 proteins linked to posttranslational modification, chaperones, protein turnover, and 9 proteins involved in the transcription process. The differentially expressed proteins between wild-type and mutant strains detected in each functional category are shown in **Table 2**. In addition, we detected proteins exclusive to the proteome of each strain. WT strain exhibits a unique exclusive protein, the Putative carboxylic ester hydrolase, which is involved in metabolism,

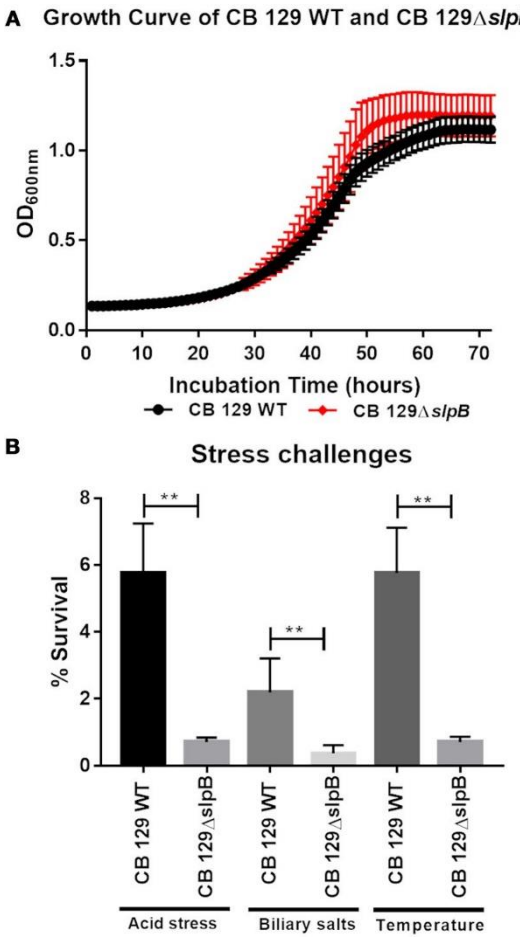

FIGURE 3 | Mutation of *Slp*B drastically affects stress tolerance in *P. freudenreichii* CIRM-BIA 129. (A) The growth curve of Wild-type (WT) and mutant CB129Δ*slpB* strains was determined at 30°C in YEL broth until stationary phase (72 h). Growth was monitored by $OD_{650\,nm}$ as a function of time. No statistically significant difference was found in growth curve between strains. (B) Wild-type (WT) and mutant CB129Δ*slpB* strains were subjected to acid, bile salts and thermal challenges. Viable propionibacteria were enumerated by plate counting before and after each challenge. Asterix represent statistically significant differences between strains and were indicated as follows: $^{**}p < 0.01$.

metabolism, especially in hydrolase activity. Interestingly, 27 proteins were found exclusively in the mutant strain, they are involved in several processes like metabolism and replication, recombination and repair (**Table S1**).

slpB Gene Mutagenesis and Whole-Genome Co-localization

Complete genome of CB129Δ*slpB* (BioProject - PRJNA476583, Accession - CP030279) strain was sequenced and assembled in a circular chromosome, which exhibits a length of 2.6815.18 bp, with a G+C content of 67.28%, and a total of 2,479 CDSs, 6 rRNA genes (5S, 16S, and 23S), and 45 tRNA genes. The circular map showed a high similarity when comparing CB129Δ*slpB* with the CIRM-BIA 1 and the JS17 reference strains (**Figure 6A**). **Figure 6B** shows the localization of the plasmid inserted within the *slpB* gene during its knockout and **Figures S1**, **S2** shows the read mapping before and after the insertion. Genomic analyses of genetic context, i.e., the sequences upstream and downstream the *slpB* gene, confirmed that this locus is not part of an operon and thus should not affect the expression of downstream genes or upstream genes. Complete genome sequence of CB129Δ*slpB* strain further ruled out any homologous recombination (HR) in other genome sites.

Protein-Protein Interaction (PPI)

We performed a PPI network to evaluate the interactions among the proteins differentially regulated in WT and CB129Δ*slpB* strains (**Figure 7**). The interactome analysis revealed 118 interactions between identified proteins. In PPI network, we observed that upregulated proteins, such as DNA-directed RNA polymerase alpha chain (PFCIRM129_08045), and 50S ribosomal protein L2 (PFCIRM129_08225), which exhibit high interaction, are involved in Transcription and Translation, respectively. Moreover, downregulated proteins such as GTP binding signal recognition particle protein (PFCIRM129_00245), DNA polymerase III alpha subunit (PFCIRM129_04260) and Enolase 2 (PFCIRM129_06035) showing high interaction, are involved in metabolism, DNA repair and main glycolytic pathway, respectively.

DISCUSSION

Propionibacterium freudenreichii CIRM-BIA 129 has emerged as a probiotic strain with a great immunomodulatory potential in the context of inflammatory bowel disease, according to promising results obtained in animal models (Plé et al., 2015, 2016). Recently, our group has studied the role of the surface SlpB protein of *P. freudenreichii* CIRM-BIA 129 in adhesion to the intestinal epithelial cells, a probiotic property linked to beneficial effects. Knocking-out of the *slpB* gene evidenced a direct involvement of this protein in the adhesion to HT-29 cells. Electrophoretic analysis of guanidine extracts confirmed the disappearance of the corresponding SlpB protein (do Carmo et al., 2017). Surface layer proteins are associated to several functions (do Carmo et al., 2018). Therefore, in order to better understand the impact of this mutation, we performed a more thorough proteomic analysis by applying nanoLC-MS/MS to these extracts. Differences were found between the parental wild type CIRM BIA 129 and the isogenic CB129Δ*slpB* mutant strains of *P. freudenreichii*, in terms of surface extractable proteins. As shown in **Table 1**, proteins previously identified in CB 129 WT strain guanidine-extracted proteins (Le Maréchal et al., 2015) were detected in both strains, including in particular, surface proteins anchored in the peptidoglycan cell wall via surface layer homology (SLH) domains, such as SlpA, SlpB, SlpE, and InlA like as previously reported by Carmo and collaborators (do Carmo et al., 2017). However, this set of SLH domain-containing proteins was reduced in the mutant strain guanidine-extracted proteins, with the expected absence of SlpB protein, thus validating the directed mutagenesis. Analysis

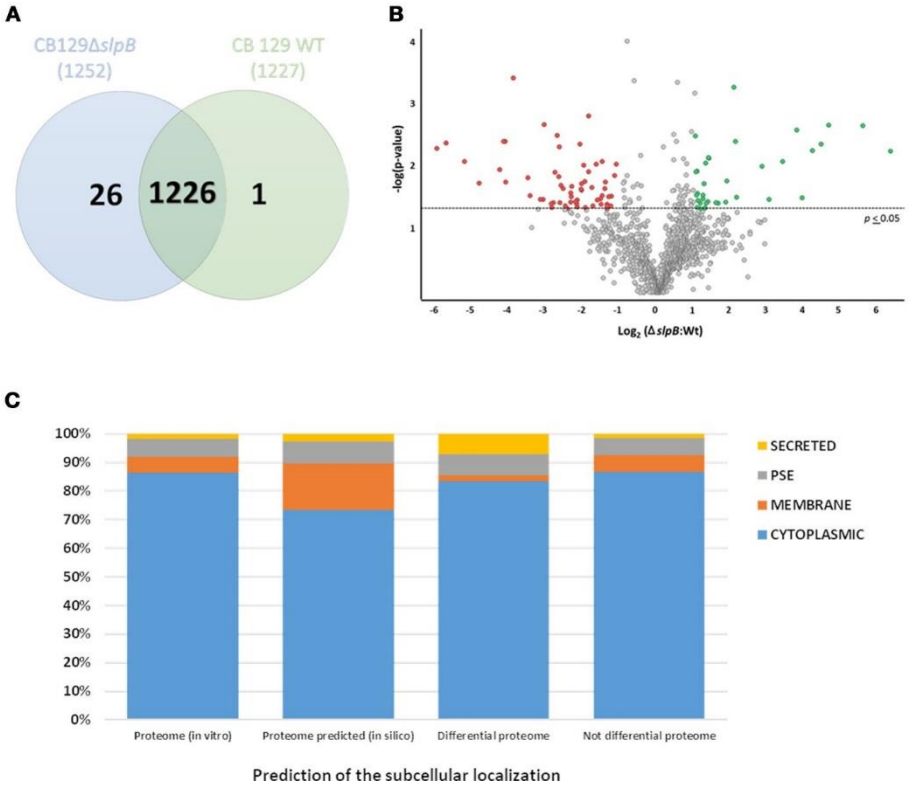

FIGURE 4 | Label-free quantification of proteins from *P. freudenreichii* CIRM-BIA 129 and CB 129Δ*slpB* strain. (A) Distribution of the proteins identified in the proteome of WT and CB129Δ*slpB* strains, represented by a Venn diagram. (B) Volcano Plot showing Log(2) Fold Change of the differentially expressed proteins in CB129Δ*slpB* strain in relation to WT strain. Green (up-regulated proteins) and red circles (down-regulated proteins) represent proteins statistically different ($p \leq 0.05$, ANOVA) in abundance between strains by 2-fold or more. (C) Prediction of the subcellular localization of the proteins identified by LC/MS and organized as cytoplasmic (CYT), membrane (MEM), potentially surface-exposed (PSE) or secreted proteins (SEC).

of CB129Δ*slpB* strain guanidine-extracted proteins, identified several proteins, including chaperones, such as ClpB, DnaK, and GroEL, and Enolase (carbohydrate metabolism) involved in stress tolerance, as previously reported for *Propionibacterium* ssp. strains by enzymatic shaving of the surface proteins using trypsin (Jan et al., 2000; Gagnaire et al., 2015; Huang et al., 2016). Another noticeable difference was the higher number of guanidine-extracted proteins, in the mutant strain, compared to the wild type strain. This included proteins usually described as cytoplasmic: enzymes of the central carbon metabolic pathways, such as pyruvate synthase, or the two subunits of the methylmalonyl-CoA mutase, a recognized cytoplasmic marker, previously described as an extracellular marker of autolysis (Valence et al., 2000). Interestingly, the HSP 70 cytoplasmic stress-related protein present at the surface of the mutant strain could be responsible for preventing protein denaturation. It is as such considered a factor of virulence and pathogenesis in some specific pathogens (Ghazaei, 2017), in *Neisseri meingitidis* (Knaust et al., 2007) and in *Mycobacterium* spp. (Das Gupta et al., 2008). This appeals further investigation, as it suggests a profound modification of the envelope structure and cell surface properties of the mutant strain. SLAPs are known to determine key parameters of the surface layer of bacteria, in terms of charge and hydrophobicity (Wilson et al., 2001). Not only amino acid residues, but also covalent modification may endow the S-layer lattice with a strong negative charge. Thus, we determined the surface charge in both *P. freudenreichii* WT and CB129Δ*slpB* strains by measuring the zeta potential, which reflects the mobility rate of cells within an electric field. A lower negative value is reportedly linked with higher hydrophobicity, consequently improving adhesion (de Wouters et al., 2015). Likewise, considering the presence of surface proteins and their role in zeta potential, van der Mei *et al*. have shown that some wild type strains, like the *L. acidophilus* ATCC4356, with SLPs, are more negatively charged at pH 7 than strains without SLPs, such as *L. johnsonii* LMG9436 and *L. gasseri* LMG9203 (van der Mei et al., 2003). We thus further investigated the hydrophobicity of the cell surface, a parameter thought to be correlated with *in vitro* adhesion of bacteria to mucin, collagen, fibronectin, and to human epithelial cells (Duary et al., 2011). The cell surface hydrophobic and hydrophilic properties have been studied in lactic acid bacteria (Sandes et al., 2017) and can be correlated to the adhesion process to intestinal epithelial cells of apolar surface proteins (Guo et al., 2010). Using the MATH assay, we showed that the CB129Δ*slpB* strain has a strongly decreased ability to adhere to xylol, as well as to chloroform and to ethyl acetate solvents, indicating a change in the global properties of the cell surface, affecting adhesion to surfaces.

TABLE 2 | Differentially regulated proteins at CB129Δ*slpB* in relation to CB 129 wild-type.

Accession	Score	Description	LOG(2) ratio fold-change	Anova (p)	COG biological process
UP-REGULATED PROTEINS					
PFCIRM129_09610	41.9018	Protein of unknown function	6.16	0.006	Coenzyme transport and metabolism and Signal transduction mechanisms
PFCIRM129_09540	37.0751	Protein of unknown function	5.43	0.003	Transcription
PFCIRM129_09590	102.7882	Protein of unknown function	4.53	0.002	Cell wall/membrane/envelope biogenesis
PFCIRM129_09465	51.2086	Protein of unknown function	4.33	0.005	–
PFCIRM129_09585	90.2656	Protein of unknown function	4.10	0.006	General function prediction only
PFCIRM129_04060	38.8837	Guanylate kinase, Guanosine monophosphate kinase (GMP kinase)	3.83	0.033	Nucleotide transport and metabolism
PFCIRM129_09570	44.4682	Protein of unknown function	3.69	0.003	Cell motility
PFCIRM129_07005	243.5985	DNA ligase (NAD+)	3.32	0.009	Replication, recombination and repair
PFCIRM129_01620	59.7705	Stomatin/prohibitin	2.96	0.036	Posttranslational modification, protein turnover, chaperones
PFCIRM129_10485	35.443	Spermidine synthase	2.76	0.011	Amino acid transport and metabolism
PFCIRM129_10870	30.3436	Protein of unknown function	2.09	0.033	General function prediction only
PFCIRM129_09930	56.3355	Hypothetical protein	2.06	0.004	Posttranslational modification, protein turnover, chaperones
PFCIRM129_09935	87.1427	Aldo/keto reductase	2.02	0.001	Secondary metabolites biosynthesis, transport and catabolism
PFCIRM129_08225	203.0233	50S ribosomal protein L2	1.84	0.018	Translation, ribosomal structure and biogenesis
PFCIRM129_05110	60.8023	Nuclease of the RecB family	1.80	0.039	Replication, recombination and repair
PFCIRM129_02560	52.268	Transcriptional regulator	1.61	0.041	Coenzyme transport and metabolism
PFCIRM129_08430	75.8136	Pyruvate flavodoxin/ferredoxin oxidoreductase	1.55	0.040	Energy production and conversion
PFCIRM129_09920	380.2718	Hypothetical secreted protein	1.37	0.008	Translation, ribosomal structure and biogenesis
PFCIRM129_04715	57.0906	Hypothetical protein	1.36	0.008	Signal transduction mechanisms
PFCIRM129_09175	100.5874	NAD-dependent epimerase/dehydratase	1.33	0.038	General function prediction only
PFCIRM129_12405	136.3375	UDP-glucose 4-epimerase	1.29	0.041	Cell wall/membrane/envelope biogenesis
PFCIRM129_01790	34.8378	3-dehydroquinate dehydratase	1.27	0.010	Amino acid transport and metabolism
PFCIRM129_07890	128.6333	Putative O-sialoglycoprotein endopeptidase	1.26	0.048	Translation, ribosomal structure and biogenesis
PFCIRM129_00585	212.5378	Polyphosphate glucokinase	1.24	0.048	Transcription and Carbohydrate transport and metabolism
PFCIRM129_07790	140.7785	Cysteine synthase 1	1.23	0.020	Amino acid transport and metabolism
PFCIRM129_09600	51.9916	Protein of unknown function	1.21	0.034	Replication, recombination and repair
PFCIRM129_11300	522.5826	Glyceraldehyde-3-phosphate dehydrogenase/erythrose 4 phosphate dehydrogenase	1.21	0.030	Carbohydrate transport and metabolism
PFCIRM129_00690	23.8969	Protein of unknown function	1.14	0.049	Function unknown
PFCIRM129_01510	22.4775	Carbohydrate or pyrimidine kinases PfkB family	1.14	0.040	Carbohydrate transport and metabolism
PFCIRM129_03870	27.4108	Glutamine-dependent NAD(+) synthetase	1.08	0.036	General function prediction only
PFCIRM129_00225	85.9906	16S rRNA processing protein	1.06	0.028	Translation, ribosomal structure and biogenesis
PFCIRM129_11255	221.963	Pyridoxal biosynthesis lyase pdxS	1.06	0.047	Coenzyme transport and metabolism

(Continued)

TABLE 2 | Continued

Accession	Score	Description	LOG(2) ratio fold-change	Anova (p)	COG biological process
PFCIRM129_03920	293.1815	Pyridine nucleotide-disulphide oxidoreductase	1.05	0.013	Energy production and conversion
PFCIRM129_07930	409.5736	Glucosamine–fructose-6- phosphate aminotransferase (Hexosephosphate aminotransferase, D-fructose-6-phosphate amidotransferase)	1.04	0.031	Cell wall/membrane/envelope biogenesis
PFCIRM129_11805	158.7382	Magnesium (Mg2+) transporter	1.03	0.013	Inorganic ion transport and metabolism
PFCIRM129_08045	417.9752	DNA-directed RNA polymerase alpha chain (RNAP alpha subunit) (Transcriptase alpha chain) (RNA polymerase subunit alpha)	1.01	0.004	Transcription
DOWN-REGULATED PROTEINS					
PFCIRM129_06035	66.5616	Enolase 2	−1.09	0.010	Carbohydrate transport and metabolism
PFCIRM129_06325	41.3227	Trypsin-like serine protease	−1.13	0.015	Posttranslational modification, protein turnover, chaperones
PFCIRM129_00315	221.1159	Beta-lactamase-like:RNA- metabolizing metallo-beta-lactamase	−1.21	0.031	Translation, ribosomal structure and biogenesis
PFCIRM129_04530	19.2011	Hypothetical protein	−1.23	0.045	Function unknown
PFCIRM129_06605	17.9867	Metal-dependent hydrolase	−1.29	0.032	General function prediction only
PFCIRM129_10030	162.9893	DNA repair protein	−1.32	0.042	Replication, recombination and repair
PFCIRM129_06500	87.9342	Hypothetical protein	−1.33	0.048	Nucleotide transport and metabolism
PFCIRM129_10650	33.3856	Hypothetical protein	−1.33	0.045	Cell wall/membrane/envelope biogenesis
PFCIRM129_03835	79.2532	Pyrazinamidase/nicotinamidase	−1.37	0.019	Coenzyme transport and metabolism and Signal transduction mechanisms
PFCIRM129_10070	83.9023	Hypothetical protein	−1.39	0.024	General function prediction only
PFCIRM129_00245	381.851	GTP binding signal recognition particle protein	−1.45	0.031	Intracellular trafficking, secretion, and vesicular transport
PFCIRM129_05955	85.1759	Peptide-methionine (S)-S-oxide reductase	−1.46	0.009	Posttranslational modification, protein turnover, chaperones
PFCIRM129_09830	327.7897	Aspartyl/glutamyl-tRNA(Asn/Gln) amidotransferase subunit B (Asp/Glu-ADT subunit B)	−1.49	0.042	Translation, ribosomal structure and biogenesis
PFCIRM129_09395	77.9918	Protein of unknown function	−1.50	0.035	Replication, recombination and repair
PFCIRM129_07355	38.1315	Hypothetical protein	−1.59	0.036	Amino acid transport and metabolism
PFCIRM129_02750	19.5802	Anti-sigma factor	−1.61	0.010	Transcription
PFCIRM129_09840	37.8042	Glutamyl-tRNA(Gln) amidotransferase subunit C (Aspartyl/glutamyl-tRNA(Asn/Gln) amidotransferase subunit C)	−1.71	0.045	Translation, ribosomal structure and biogenesis
PFCIRM129_12290	113.782	Hypothetical protein	−1.73	0.023	Translation, ribosomal structure and biogenesis
PFCIRM129_02880	157.3643	Zn dependant peptidase	−1.82	0.002	General function prediction only
PFCIRM129_01675	171.214	Flavin-containing amine oxidase	−1.82	0.013	Amino acid transport and metabolism
PFCIRM129_00465	78.5201	Thiamine biosynthesis protein	−1.90	0.047	Coenzyme transport and metabolism
PFCIRM129_09980	56.8929	Peptidyl-prolyl cis-trans isomerase	−1.91	0.019	Posttranslational modification, protein turnover, chaperones
PFCIRM129_02370	174.428	L-aspartate oxidase (LASPO) (Quinolinate synthetase B)	−1.94	0.010	Coenzyme transport and metabolism

(Continued)

TABLE 2 | Continued

Accession	Score	Description	LOG(2) ratio fold-change	Anova (p)	COG biological process
PFCIRM129_05120	33.1399	Putative carboxylic ester hydrolase	−1.99	0.020	Lipid transport and metabolism
PFCIRM129_04475	54.968	Transporter	−2.01	0.026	Function unknown
PFCIRM129_12425	80.5954	Protein of unknown function FUZZYLOCATION=TRUE	−2.02	0.025	Transcription
PFCIRM129_04980	227.0852	D-alanine–D-alanine ligase (D-alanylalanine synthetase)	−2.05	0.005	Cell wall/membrane/envelope biogenesis and General function prediction only
PFCIRM129_11215	88.6965	Dioxygenase	−2.12	0.047	Inorganic ion transport and metabolism and Secondary metabolites biosynthesis, transport and catabolism
PFCIRM129_10195	96.8755	Transcriptional regulator	−2.12	0.036	Transcription
PFCIRM129_08985	30.4822	Hypothetical protein	−2.13	0.042	General function prediction only
PFCIRM129_04260	287.3443	DNA polymerase III alpha subunit	−2.15	0.038	Replication, recombination and repair
PFCIRM129_02065	15.789	Ferrous iron uptake protein A 9.a.8.1.x	−2.25	0.022	Inorganic ion transport and metabolism
PFCIRM129_04725	106.5589	Hypothetical protein	−2.27	0.032	Cell wall/membrane/envelope biogenesis
PFCIRM129_05460	489.2107	Surface protein with SLH domain	−2.29	0.039	Posttranslational modification, protein turnover, chaperones
PFCIRM129_04925	12.884	Hypothetical protein	−2.29	0.028	Carbohydrate transport and metabolism
PFCIRM129_10690	9.166	Protein of unknown function	−2.37	0.049	Function unknown
PFCIRM129_05620	65.4691	MscS transporter, small conductance mechanosensitive ion channel	−2.43	0.044	Cell wall/membrane/envelope biogenesis
PFCIRM129_06895	73.9719	Thiredoxine like membrane protein	−2.49	0.024	Posttranslational modification, protein turnover, chaperones
PFCIRM129_10610	181.5134	Phosphocarrier, HPr family	−2.54	0.021	Signal transduction mechanisms and Carbohydrate transport and metabolism
PFCIRM129_02565	36.2455	Hypothetical protein	−2.57	0.039	Defense mechanisms
PFCIRM129_00850	58.5524	Cation-transporting ATPase	−2.59	0.005	Inorganic ion transport and metabolism
PFCIRM129_02970	142.4983	Hypothetical protein	−2.60	0.016	Energy production and conversion
PFCIRM129_00010	145.5914	Argininosuccinate lyase (Arginosuccinase)	−2.65	0.004	Amino acid transport and metabolism
PFCIRM129_02590	36.8971	Hypothetical transmembrane protein	−2.71	0.013	Inorganic ion transport and metabolism
PFCIRM129_02910	44.2268	Hypothetical protein	−2.74	0.039	Replication, recombination and repair
PFCIRM129_10040	39.9232	Hypothetical protein	−2.78	0.048	Carbohydrate transport and metabolism
PFCIRM129_12235	1098.1026	Internaline A	−2.80	0.041	Posttranslational modification, protein turnover, chaperones
PFCIRM129_00040	20.5108	N-acetyl-gamma-glutamyl- phosphate reductase (AGPR) (N- acetyl-glutamate semialdehyde dehydrogenase) (NAGSA dehydrogenase)	−2.99	0.002	Amino acid transport and metabolism
PFCIRM129_03005	41.8204	Hypothetical protein	−3.01	0.035	Secondary metabolites biosynthesis, transport and catabolism
PFCIRM129_05445	69.2875	Transcriptional Regulator, TetR family	−3.09	0.036	Transcription
PFCIRM129_02960	83.486	Cold shock-like protein CspA	−3.36	0.031	Transcription

(Continued)

TABLE 2 | Continued

Accession	Score	Description	LOG(2) ratio fold-change	Anova (p)	COG biological process
PFCIRM129_00705	46.3689	Surface protein of unknown function	−3.42	0.016	–
PFCIRM129_08670	192.0452	Cell-wall peptidases, NlpC/P60 family secreted protein	−3.80	0.000	General function prediction only
PFCIRM129_03390	45.4963	Superfamily II RNA helicase	−4.01	0.019	Replication, recombination and repair
PFCIRM129_06155	35.4	Hypothetical protein	−4.03	0.004	Carbohydrate transport and metabolism
PFCIRM129_06085	371.7356	Transcription-repair coupling factor	−4.07	0.004	Replication, recombination and repair and Transcription
PFCIRM129_01360	47.9803	NUDIX hydrolase	−4.17	0.012	Nucleotide transport and metabolism
PFCIRM129_11775	48.0011	Surface protein D with SLH domain	−4.70	0.020	Posttranslational modification, protein turnover, chaperones
PFCIRM129_00700	461.2371	Surface layer protein B (S-layer protein B)	−5.10	0.009	Posttranslational modification, protein turnover, chaperones
PFCIRM129_11140	154.2908	Type I restriction-modification system DNA methylase	−5.58	0.005	Defense mechanisms
PFCIRM129_04135	15.2209	Uncharacterized ATPase related to the helicase subunit of the holliday junction resolvase	−5.82	0.006	Replication, recombination and repair

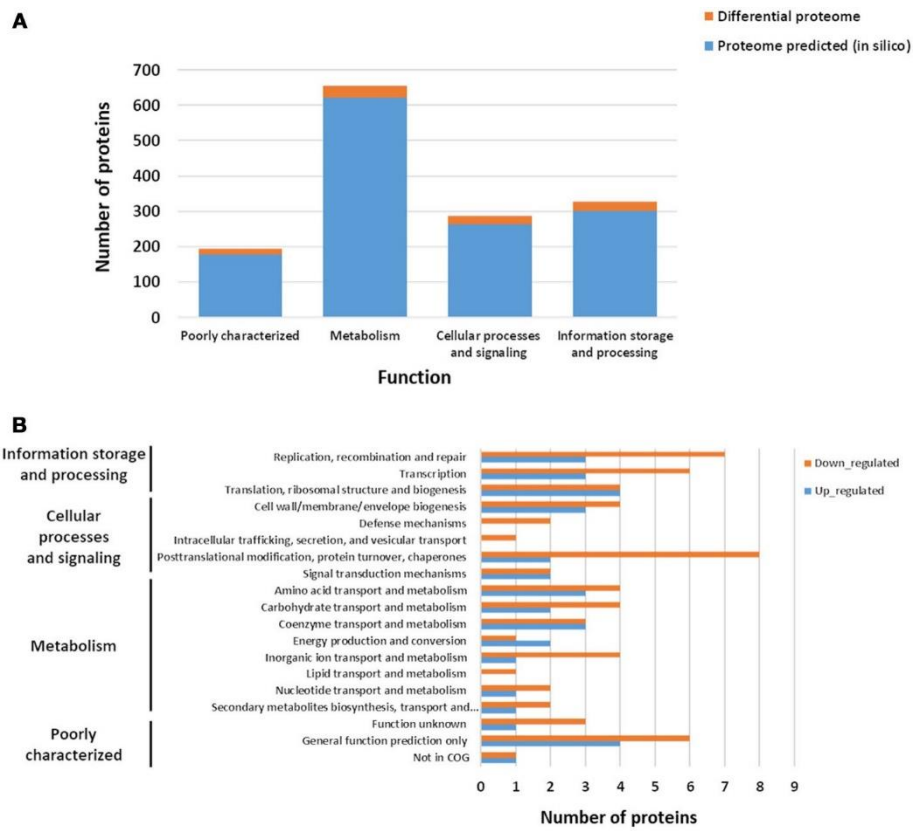

FIGURE 5 | Repartition of differential proteins in biological processes. (A) Functional distribution of the predicted theoretical proteome and of the experimental differential proteins when comparing Wild-type (WT) and mutant CB129Δ*slpB* strains. (B) Repartition of differential proteins in Biological processes. Functional distribution and Biological process were predicted based on the functional classifications of COG database.

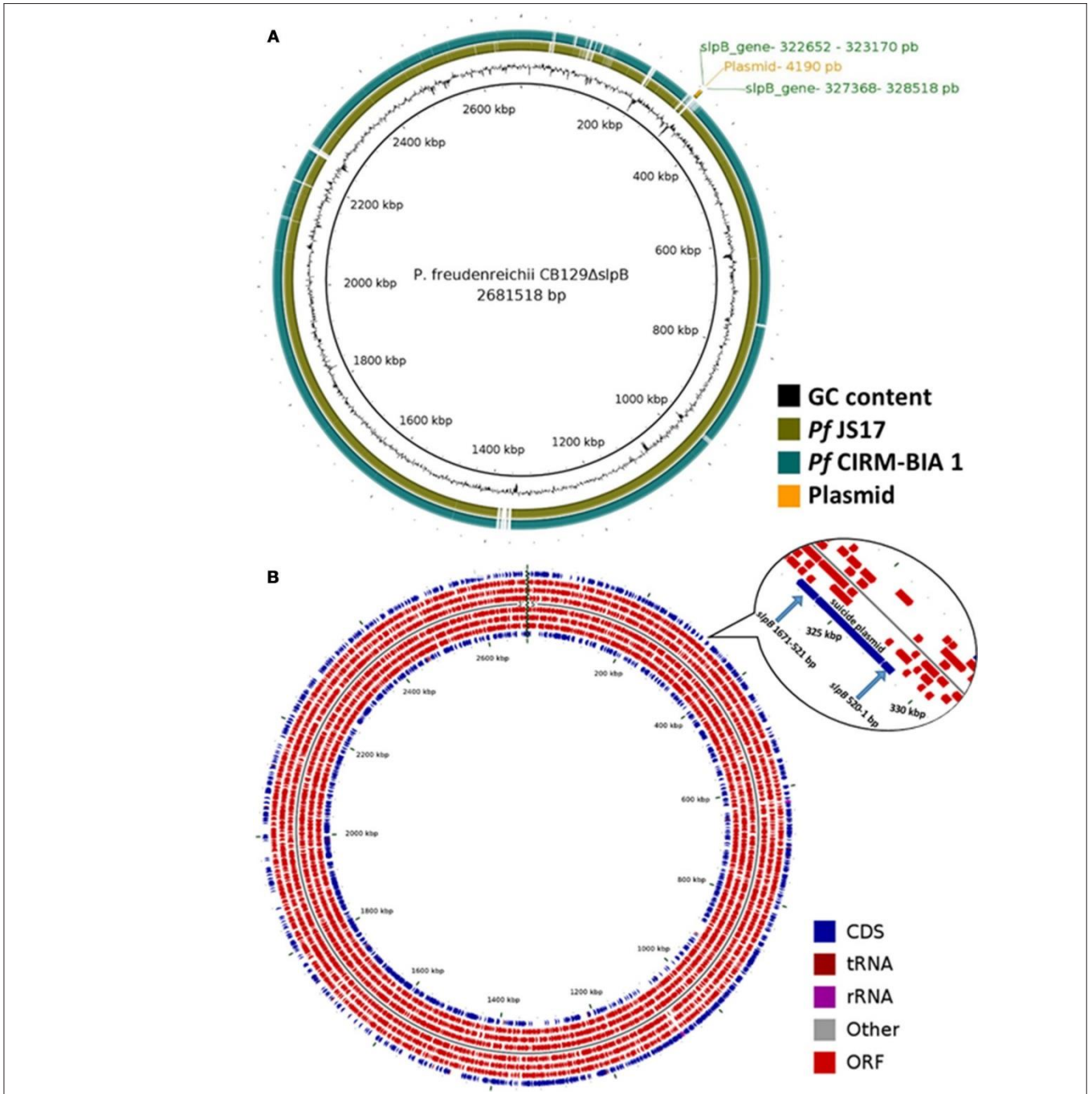

FIGURE 6 | Comparative genomic map generated with BRIG and Map of Circular genome generated with CGview. (A) *P. freudenreichii* CIRM-BIA 1 and *P.freudenreichii* JS17 were aligned using CB129Δ*slpB* strain as a reference. (B) In the outermost ring the genes localization in genome, followed by CDS, tRNAs, rRNAs, other RNAs, and CDSs. The insertion site of the plasmid for the *slpB* gene mutation is visualized in the zoom image.

These results corroborate with the previous study showing a decreased adhesion to HT-29 human intestinal epithelial cells (do Carmo et al., 2017). Hydrophobicity and ζ-potential are factors correlated with bacterial adhesion to the epithelial cells, which are guided by charge and hydrophobicity of the bacterial surface.

The presence of surface layers being reportedly linked to tolerance toward stresses (do Carmo et al., 2018), we decided to investigate the impact of such a mutation on the CB129Δ*slpB* strain tolerance toward stress challenges that are relevant for the selection of new probiotics. The ability to survive acid stress in the stomach and bile salts stress in the duodenum during the passage

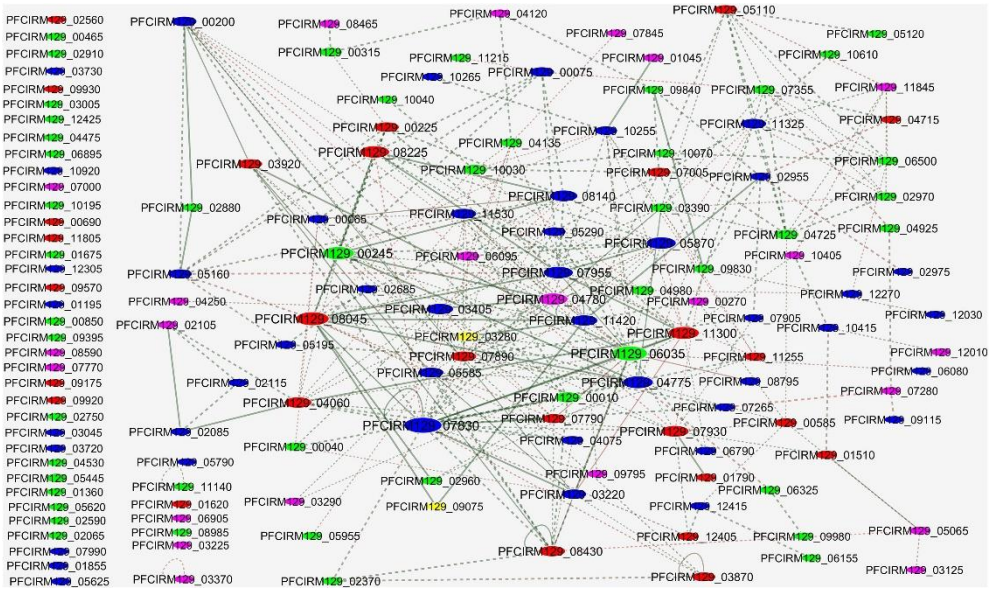

FIGURE 7 | Protein-protein interactions of the proteins identified as differentially expressed in CB129Δ*slpB*. The sizes of the nodes represent the degree of interaction for each gene/protein; the major nodes demonstrate greater interactions. Red, up-regulated; Blue, unchanged; Green, down-regulated; Yellow, Exclusive identified at WT strain; Purple, Exclusive identified at CB129Δ*slpB* strain.

through the digestive tract, is important for probiotic interaction with the host (Rabah et al., 2017). Accordingly, *in vitro* assays can be used to simulate digestive stresses, mimicking the exposure to acidic conditions (pH 2.0) or to biliary salts (1 $g.L^{-1}$) (Jan et al., 2000). For *P. freudenreichii*, commonly used as a cheese starter, the heat stress tolerance constitutes a relevant technological ability of this strain (Rosa do Carmo et al., 2017). Overall, we observed a large decrease in tolerance to the environmental stresses, confirming a role of SlpB in toughness. In the guanidine- extracted proteins of the mutant strain, the chaperones and heat shock proteins, DnaK1, DnaK2, ClpB 2, GroE1, and GroE2 were found. Inside the cell, they are responsible for protein folding and are correlated to acid and bile adaptation (Leverrier et al., 2005; Gagnaire et al., 2015). Here, they were found at the surface of the CB129Δ*slpB* mutant, which was more susceptible to extreme acid stress and temperature, compared to wild type strain. Previous work showed that *L. acidophilus* ATCC 4356 adapts to harsh environments by increasing the expression of the s-layer SlpA protein upon bile, acidic pH and heat stress exposition (Khaleghi et al., 2010; Khaleghi and Kasra, 2012). Moreover, changes in the cell surface properties could alter the transmembrane protein complex responsible for the extrusion of protons from the cytoplasm, which are responsible for surviving environmental stresses (Ruiz et al., 2013; Rosa do Carmo et al., 2017).

Profound modifications of *P. freudenreichii* physiology and surface properties suggested that modifications, wider than the disappearance of a single protein, occurred as a result of *slpB* gene inactivation. To understand this impact of the mutation, a comparative proteomic analysis was performed to identify significant alterations in the whole proteome profile of mutant of the mutant strain, using label-free quantitative proteomic analysis. Prediction of sub cellular localization using the SurfG+ tool (Barinov et al., 2009) evidenced changes in all the categories (CYT, MEM, PSE and SEC) in the differential proteome of CB129Δ*slpB*. In addition, differential proteome was functionally classified using COG, showing a functional implication of differential proteins in cellular processes such as signaling, information storage, processing, and metabolism. Specifically, this study showed that the moonlighting enolase and NlpC/P60 are both exported (Frohnmeyer et al., 2018), as it was recently observed in the cutaneous *Propionibacterium acnes* strain (Jeon et al., 2017). These moonlighting proteins were downregulated in CB129Δ*slpB*. Interestingly, in the *Bifidobacterium* and *Lactobacillus* genera, moonlighting proteins, such as enolase, also play a role in immunomodulation and adhesion (Sánchez et al., 2010; Kainulainen and Korhonen, 2014; Vastano et al., 2016). Furthermore, in the PPI network we observed high interactions between the downregulated Enolase (PFCIRM129_06035), reportedly involved in human gut colonization and stress adaptation (Ruiz et al., 2009), with other proteins involved in several other processes, including metabolism and DNA repair. Moreover, all surface layer-associated proteins SlpA, SlpD, SlpE, and InlA were downregulated in CB129Δ*slpB*. These proteins form a protective layer on the surface of the bacteria, and have been associated with environmental stress tolerance (Fagan and Fairweather, 2014). As seen previously, a decreased amount of these proteins could be directly associated with stress susceptibility and with altered hydrophobicity. SLAPs can directly influence these properties (Pum et al., 2013), and consequently alter adhesion to epithelial cells (do Carmo et al., 2017).

We performed the complete genome DNA sequencing of the CB129Δ*slpB*, which, in turn, allowed us to evaluate whether the *slpB* gene disruption had major consequences on the mutant strain genome. The *slpB* gene is not part of an operon, which suggests that homologous recombination using the suicide plasmid pUC: Δ*slpB*:*Cm*R (do Carmo et al., 2017) did not affect the expression of upstream and downstream genes. Analysis of the genetic context, upstream and downstream, revealed that the homologous recombination process was site- specific, and not affecting other genes in the genome of the mutant strain CB129Δ*slpB*. However, we were unable to evaluate possible rearrangements in the genome of CB129ΔslpB, which could have affected the transcription of other genes. Therefore, more studies are necessary to explore whether any probiotic potential was lost after the single mutation of the *slpB* gene in *Propionibacterium freudenreichii* CIRM-BIA 129 strain.

CONCLUSION

This study evidenced the pleiotropic impact of the surface layer protein *slpB* mutation in the probiotic strain *Propionibacterium freudenreichii* CIRM-BIA 129 in relation to its physicochemical proprieties, stress challenges, surfaceome and whole cell quantitative proteome. It confirmed the key role of SLPs and strongly suggests that expression of specific ones, such as *P. freudenreichii* SlpB, should be used as criteria for selecting strains with probiotic potential.

AUTHOR CONTRIBUTIONS

FC performed *in vitro* assays, microscopy, proteomic assays and data interpretation. WS, FP, GT, and ROC performed proteomic assays, data interpretation and bioinformatics analyses. BC, EO, and SS performed *in vitro* assays. II and HR data interpretation. EF performed PPI network. CC performed microscopy. MC, AC, and RS performed genomics and data interpretation. VA, GJ, HF, and YL contributed to the supervision, analysis, and interpretation of data and were major contributors to revising the manuscript. All authors contributed in writing the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2018.01807/full#supplementary-material

Figure S1 | Verification of assembly error by read mapping. The plasmid pUC:Δ*slpB*:*Cm*R was not inserted in the *slpB* gene during de novo genome assembly. The read mapping on the *slpB* gene shows misalignments upstream and downstream insertion site, confirming the assembly error. The read mapping was performed using in CLC Genomics Workbench 7.0.

Figure S2 | Assembly curation and validation by read mapping. The manual insertion of plasmid pUC: Δ*slpB*:*Cm*R in the *slpB* gene was validated by read mapping. The correct read alignments upstream (A) and downstream (B) the plasmid validate the manual insertion. The read mapping was performed using in CLC Genomics Workbench 7.0.

Table S1 | Total list of proteins identified in the core-proteome of CB 129 wild-type and CB129ΔslpB.

REFERENCES

Alikhan, N.-F., Petty, N. K., Ben Zakour, N. L., and Beatson, S. A. (2011). BLAST ring image generator (BRIG): simple prokaryote genome comparisons. *BMC Genomics* 12:402. doi: 10.1186/1471-2164-12-402

Aziz, R. K., Bartels, D., Best, A. A., DeJongh, M., Disz, T., Edwards, R. A., et al. (2008). The RAST server: rapid annotations using subsystems technology. *BMC Genomics* 9:75. doi: 10.1186/1471-2164-9-75

Barinov, A., Loux, V., Hammani, A., Nicolas, P., Langella, P., Ehrlich, D., et al. (2009). Prediction of surface exposed proteins in *Streptococcus pyogenes*, with a potential application to other Gram-positive bacteria. *Proteomics* 9, 61–73. doi: 10.1002/pmic.200800195

Brettin, T., Davis, J. J., Disz, T., Edwards, R. A., Gerdes, S., Olsen, G. J., et al. (2015). RASTtk: a modular and extensible implementation of the RAST algorithm for building custom annotation pipelines and annotating batches of genomes. *Sci. Rep.* 5:8365. doi: 10.1038/srep08365

Cousin, F. J., Jouan-Lanhouet, S., Théret, N., Brenner, C., Jouan, E., Le Moigne-Muller, G., et al. (2016). The probiotic *Propionibacterium freudenreichii* as a new adjuvant for TRAIL-based therapy in colorectal cancer. *Oncotarget* 7, 7161–7178. doi: 10.18632/oncotarget.6881

Das Gupta, T., Bandyopadhyay, B., and Das Gupta, S. K. (2008). Modulation of DNA-binding activity of *Mycobacterium tuberculosis* HspR by chaperones. *Microbiology* 154, 484–490. doi: 10.1099/mic.0.2007/012294-0

de Wouters, T., Jans, C., Niederberger, T., Fischer, P., and Rühs, P. A. (2015). Adhesion potential of intestinal microbes predicted by physico-chemical characterization methods. *PLoS ONE* 10:e0136437. doi: 10.1371/journal.pone.0136437

Deutsch, S.-M., Mariadassou, M., Nicolas, P., Parayre, S., Le Guellec, R., Chuat, V., et al. (2017). Identification of proteins involved in the anti-inflammatory properties of *Propionibacterium freudenreichii* by means of a multi-strain study. *Sci. Rep.* 7:46409. doi: 10.1038/srep46409

Deutsch, S. M., Parayre, S., Bouchoux, A., Guyomarc'h, F., Dewulf, J., Dols-Lafargue, M., et al. (2012). Contribution of surface β-glucan polysaccharide to physicochemical and immunomodulatory properties of *Propionibacterium freudenreichii*. *Appl. Environ. Microbiol.* 78, 1765–1775. doi: 10.1128/AEM.07027-11

Distler, U., Kuharev, J., Navarro, P., Levin, Y., Schild, H., and Tenzer, S. (2014). Drift time-specific collision energies enable deep-coverage data-independent acquisition proteomics. *Nat. Methods* 11, 167–170. doi: 10.1038/nmeth.2767

do Carmo, F. L. R., Rabah, H., De Oliveira Carvalho, R. D., Gaucher, F., Cordeiro, B. F., da Silva, S. H., et al. (2018). Extractable bacterial surface proteins in probiotic–host interaction. *Front. Microbiol.* 9:645. doi: 10.3389/fmicb.2018.00645

do Carmo, F. L. R., Rabah, H., Huang, S., Gaucher, F., Deplanche, M., Dutertre, S., et al. (2017). *Propionibacterium freudenreichii* surface protein SlpB is involved in adhesion to intestinal HT-29 cells. *Front. Microbiol.* 8:1033. doi: 10.3389/fmicb.2017.01033

Duary, R. K., Rajput, Y. S., Batish, V. K., and Grover, S. (2011). Assessing the adhesion of putative indigenous probiotic *lactobacilli* to human colonic epithelial cells. *Ind. J. Med. Res.* 134, 664–671. doi: 10.4103/0971-5916.90992

Fagan, R. P., and Fairweather, N. F. (2014). Biogenesis and functions of bacterial S-layers. *Nat. Rev. Microbiol.* 12, 211–222. doi: 10.1038/nrmicro3213

Folador, E. L., Hassan, S. S., Lemke, N., Barh, D., Silva, A., Ferreira, R. S., et al. (2014). An improved interolog mapping-based computational prediction of protein–protein interactions with increased network coverage. *Integr. Biol.* 6, 1080–1087. doi: 10.1039/C4IB00136B

Foligné, B., Deutsch, S. M., Breton, J., Cousin, F. J., Dewulf, J., Samson, M., et al. (2010). Promising immunomodulatory effects of selected strains of dairy propionibacteria as evidenced *in vitro* and *in vivo*. *Appl. Environ. Microbiol.* 76, 8259–8264. doi: 10.1128/AEM.01976-10

Food and Agriculture Organization of the United Nations and World Health Organization (eds.). (2002). *Probiotics in Food: Health and Nutritional Properties and Guidelines for Evaluation*. Rome: Food and Agriculture Organization of the United Nations : World Health Organization.

Ford, A. C., Quigley, E. M. M., Lacy, B. E., Lembo, A. J., Saito, Y. A., Schiller, L. R., et al. (2014). Efficacy of prebiotics, probiotics, and synbiotics in irritable bowel syndrome and chronic idiopathic constipation: systematic review and meta-analysis. *Am. J. Gastroenterol.* 109, 1547–1561; quiz 1546, 1562. doi: 10.1038/ajg.2014.202

Frohnmeyer, E., Deptula, P., Nyman, T. A., Laine, P. K. S., Vihinen, H., Paulin, L., et al. (2018). Secretome profiling of *Propionibacterium freudenreichii* reveals highly variable responses even among the closely related strains. *Microb. Biotechnol.* 11, 510–526. doi:10.1111/1751-7915.13254

Furuichi, K., Hojo, K., Katakura, Y., Ninomiya, K., and Shioya, S. (2006). Aerobic culture of *Propionibacterium freudenreichii* ET-3 can increase production ratio of 1,4-dihydroxy-2-naphthoic acid to menaquinone. *J. Biosci. Bioeng.* 101, 464–470. doi: 10.1263/jbb.101.464

Gagnaire, V., Jardin, J., Rabah, H., Briard-Bion, V., and Jan, G. (2015). Emmental cheese environment enhances *Propionibacterium freudenreichii* stress tolerance. *PLoS ONE* 10:e0135780. doi: 10.1371/journal.pone.0135780

Galardini, M., Biondi, E. G., Bazzicalupo, M., and Mengoni, A. (2011). CONTIGuator: a bacterial genomes finishing tool for structural insights on draft genomes. *Source Code Biol. Med.* 6:11. doi: 10.1186/1751-0473-6-11

Galperin, M. Y., Makarova, K. S., Wolf, Y. I., and Koonin, E. V. (2015). Expanded microbial genome coverage and improved protein family annotation in the COG database. *Nucleic Acids Res.* 43, D261–D269. doi: 10.1093/nar/gku1223

Ghazaei, C. (2017). Role and mechanism of the Hsp70 molecular chaperone machines in bacterial pathogens. *J. Med. Microbiol.* 66, 259–265. doi: 10.1099/jmm.0.000429

Gilar, M., Olivova, P., Daly, A. E., and Gebler, J. C. (2005). Two-dimensional separation of peptides using RP-RP-HPLC system with different pH in first and second separation dimensions. *J. Sep. Sci.* 28, 1694–1703. doi: 10.1002/jssc.200500116

Giles, K., Williams, J. P., and Campuzano, I. (2011). Enhancements in travelling wave ion mobility resolution. *Rapid Commun. Mass Spectrom. RCM* 25, 1559–1566. doi: 10.1002/rcm.5013

Guo, X.-H., Kim, J.-M., Nam, H.-M., Park, S.-Y., and Kim, J.-M. (2010). Screening lactic acid bacteria from swine origins for multistrain probiotics based on *in vitro* functional properties. *Anaerobe* 16, 321–326. doi: 10.1016/j.anaerobe.2010.03.006

Heberle, H., Meirelles, G. V., da Silva, F. R., Telles, G. P., and Minghim, R. (2015). InteractiVenn: a web-based tool for the analysis of sets through Venn diagrams. *BMC Bioinformatics* 16:169. doi: 10.1186/s12859-015-0611-3

Hill, C., Guarner, F., Reid, G., Gibson, G. R., Merenstein, D. J., Pot, B., et al. (2014). Expert consensus document: the international scientific association for probiotics and prebiotics consensus statement on the scope and appropriate use of the term probiotic. *Nat. Rev. Gastroenterol. Hepatol.* 11, 506–514. doi: 10.1038/nrgastro.2014.66

use of the term probiotic. *Nat. Rev. Gastroenterol. Hepatol.* 11, 506–514. doi: 10.1038/nrgastro.2014.66

Huang, S., Rabah, H., Jardin, J., Briard-Bion, V., Parayre, S., Maillard, M.-B., et al. (2016). Hyperconcentrated sweet whey, a new culture medium that enhances *Propionibacterium freudenreichii* stress tolerance. *Appl. Environ. Microbiol.* 82, 4641–4651. doi: 10.1128/AEM.00748-16

Hynönen, U., and Palva, A. (2013). *Lactobacillus* surface layer proteins: structure, function and applications. *Appl. Microbiol. Biotechnol.* 97, 5225–5243. doi: 10.1007/s00253-013-4962-2

Isawa, K., Hojo, K., Yoda, N., Kamiyama, T., Makino, S., Saito, M., et al. (2002). Isolation and identification of a new bifidogenic growth stimulator produced by *Propionibacterium freudenreichii* ET-3. *Biosci. Biotechnol. Biochem.* 66, 679–681. doi: 10.1271/bbb.66.679

Ishihama, Y., Oda, Y., Tabata, T., Sato, T., Nagasu, T., Rappsilber, J., et al. (2005). Exponentially modified protein abundance index (emPAI) for estimation of absolute protein amount in proteomics by the number of sequenced peptides per protein. *Mol. Cell. Proteomics MCP* 4, 1265–1272. doi: 10.1074/mcp.M500061-MCP200

Jan, G., Belzacq, A.-S., Haouzi, D., Rouault, A., Métivier, D., Kroemer, G., et al. (2002). Propionibacteria induce apoptosis of colorectal carcinoma cells via short-chain fatty acids acting on mitochondria. *Cell Death Differ.* 9, 179–188. doi: 10.1038/sj.cdd.4400935

Jan, G., Rouault, A., and Maubois, J.-L. (2000). Acid stress susceptibility and acid adaptation of *Propionibacterium freudenreichii* subsp. *shermanii*. *Le Lait* 80, 325–336. doi: 10.1051/lait:2000128

Jeon, J., Mok, H. J., Choi, Y., Park, S. C., Jo, H., Her, J., et al. (2017). Proteomic analysis of extracellular vesicles derived from *Propionibacterium acnes*. *PROTEOMICS - Clin. Appl.* 11:1600040. doi: 10.1002/prca.201600040

Kainulainen, V., and Korhonen, T. K. (2014). Dancing to another tune- adhesive moonlighting proteins in bacteria. *Biology* 3, 178–204. doi: 10.3390/biology3010178

Khaleghi, M., and Kasra, R. (2012). "Effect of environmental stresses on S-layer production in *Lactobacillus acidophilus* ATCC 4356," in *Advances in Applied Biotechnology*, ed. M. Petre (London: InTech), 209–224.

Khaleghi, M., Kermanshahi, R. K., Yaghoobi, M. M., Zarkesh-Esfahani, S. H., and Baghizadeh, A. (2010). Assessment of bile salt effects on s-layer production, slp gene expression and some physicochemical properties of *Lactobacillus acidophilus* ATCC 4356. *J. Microbiol. Biotechnol.* 20, 749–756. doi: 10.4014/jmb.0906.06050

Knaust, A., Weber, M. V. R., Hammerschmidt, S., Bergmann, S., Frosch, M., and Kurzai, O. (2007). Cytosolic proteins contribute to surface plasminogen recruitment of *Neisseria meningitidis*. *J. Bacteriol.* 189, 3246–3255. doi: 10.1128/JB.01966-06

Kos, B., Susković, J., Vuković, S., Simpraga, M., Frece, J., and Matosić, S. (2003). Adhesion and aggregation ability of probiotic strain *Lactobacillus acidophilus* M92. *J. Appl. Microbiol.* 94, 981–987. doi: 10.1046/j.1365-2672.2003.01915.x

Kuharev, J., Navarro, P., Distler, U., Jahn, O., and Tenzer, S. (2015). In-depth evaluation of software tools for data-independent acquisition based label-free quantification. *Proteomics* 15, 3140–3151. doi: 10.1002/pmic.201400396

Lan, A., Bruneau, A., Bensaada, M., Philippe, C., Bellaud, P., Rabot, S., et al. (2008). Increased induction of apoptosis by *Propionibacterium freudenreichii* TL133 in colonic mucosal crypts of human microbiota- associated rats treated with 1,2-dimethylhydrazine. *Br. J. Nutr.* 100, 1251–1259. doi: 10.1017/S0007114508978284

Lan, A., Lagadic-Gossmann, D., Lemaire, C., Brenner, C., and Jan, G. (2007). Acidic extracellular pH shifts colorectal cancer cell death from apoptosis to necrosis upon exposure to propionate and acetate, major end-products of the human probiotic propionibacteria. *Apoptosis Int. J. Program. Cell Death* 12, 573–591. doi: 10.1007/s10495-006-0010-3

Langella, O., Valot, B., Balliau, T., Blein-Nicolas, M., Bonhomme, L., and Zivy, M. (2017). X!TandemPipeline: a tool to manage sequence redundancy for protein inference and phosphosite identification. *J. Proteome Res.* 16, 494–503. doi: 10.1021/acs.jproteome.6b00632

Le Maréchal, C., Peton, V., Plé, C., Vroland, C., Jardin, J., Briard-Bion, V., et al. (2015). Surface proteins of *Propionibacterium freudenreichii* are involved in its anti-inflammatory properties. *J. Proteomics* 113, 447–461. doi: 10.1016/j.jprot.2014.07.018 Leibowitz, M. P., Tavares, G. C., Pereira, F. L., Perdigão, C., Azevedo, V., and Figueiredo, H. C. P. (2017). Shotgun label-free proteomic analyses of the oyster parasite Perkinsus marinus. J. Proteomics Genomics Res. 2:13. doi: 10.14302/issn.2326-0793.JPGR-17-1571

Leverrier, P., Dimova, D., Pichereau, V., Auffray, Y., Boyaval, P., and Jan, G. (2003). Susceptibility and adaptive response to bile salts in Propionibacterium

freudenreichii: physiological and proteomic analysis. Appl. Environ. Microbiol. 69, 3809–3818.
Leverrier, P., Fremont, Y., Rouault, A., Boyaval, P., and Jan, G. (2005). In vitro tolerance to digestive stresses of propionibacteria: influence of food matrices. Food Microbiol. 22, 11–18. doi: 10.1016/j.fm.2004.05.003
Lin, Y., Liu, Y., Li, J., Zhao, Y., He, Q., Han, W., et al. (2010). Evaluation and optimization of removal of an acid-insoluble surfactant for shotgun analysis of membrane proteome. Electrophoresis 31, 2705–2713. doi: 10.1002/elps.201000161
Malik, A. C., Reinbold, G. W., and Vedamuthu, E. R. (1968). An evaluation of the taxonomy of Propionibacterium. Can. J. Microbiol. 14, 1185–1191.
Mitsuyama, K., Masuda, J., Yamasaki, H., Kuwaki, K., Kitazaki, S., Koga, H., et al. (2007). Treatment of ulcerative colitis with milk whey culture with Propionibacterium freudenreichii. J. Intest. Microbiol. 21, 143–147. doi: 10.11209/jim.21.143
Oak, S. J., and Jha, R. (2018). The effects of probiotics in lactose intolerance: a systematic review. Crit. Rev. Food Sci. Nutr. 9, 1–9. doi: 10.1080/10408398.2018.1425977
Plaza-Díaz, J., Ruiz-Ojeda, F. J., Vilchez-Padial, L. M., and Gil, A. (2017). Evidence of the anti-inflammatory effects of probiotics and synbiotics in intestinal chronic diseases. Nutrients 9:E555. doi: 10.3390/nu9060555
Plé, C., Breton, J., Richoux, R., Nurdin, M., Deutsch, S.-M., Falentin, H., et al. (2016). Combining selected immunomodulatory Propionibacterium freudenreichii and Lactobacillus delbrueckii strains: reverse engineering development of an anti-inflammatory cheese. Mol. Nutr. Food Res. 60, 935–948. doi: 10.1002/mnfr.201500580
Plé, C., Richoux, R., Jardin, J., Nurdin, M., Briard-Bion, V., Parayre, S., et al. (2015). Single-strain starter experimental cheese reveals anti-inflammatory effect of Propionibacterium freudenreichii CIRM BIA 129 in TNBS-colitis model. J. Funct. Foods 18, 575–585. doi: 10.1016/j.jff.2015.08.015
Pum, D., Toca-Herrera, J. L., and Sleytr, U. B. (2013). S-layer protein self-assembly.
Int. J. Mol. Sci. 14, 2484–2501. doi: 10.3390/ijms14022484
Rabah, H., Rosa do Carmo, F. L., and Jan, G. (2017). Dairy Propionibacteria: versatile probiotics. Microorganisms 5:E24. doi: 10.3390/microorganisms5020024
Roland, N., Bougle, D., Lebeurrier, F., Arhan, P., and Maubois, J. L. (1998). Propionibacterium freudenreichii stimulates the growth of Bifidobacterium bifidum in vitro and increases fecal bifidobacteria in healthy human volunteers. Int. Dairy J. 8, 587–588.
Rondanelli, M., Faliva, M. A., Perna, S., Giacosa, A., Peroni, G., and Castellazzi, A. M. (2017). Using probiotics in clinical practice: where are we now? A review of existing meta-analyses. Gut Microbes 8, 521–543. doi: 10.1080/19490976.2017.1345414
Rosa do Carmo, F. L., Rabah, H., Fernandes Cordeiro, B., Da Silva, S. H., Jan, G., Azevedo, V. A., et al. (2017). "Applications of Probiotic Bacteria and Dairy Foods in Health," in Current Research in Microbiology (Wilmington, DE). Available online at: https://hal.archives-ouvertes.fr/hal-01583804
Ruiz, L., Couté, Y., Sánchez, B., de los Reyes-Gavilán, C. G., Sanchez, J.-C., and Margolles, A. (2009). The cell-envelope proteome of Bifidobacterium longum in an in vitro bile environment. Microbiology 155, 957–967. doi: 10.1099/mic.0.024273-0
Ruiz, L., Margolles, A., and Sánchez, B. (2013). Bile resistance mechanisms in Lactobacillus and Bifidobacterium. Front. Microbiol. 4:396. doi: 10.3389/fmicb.2013.00396
Sambrook, J., and Russell, D. W. (2001). Molecular Cloning: a Laboratory Manual. 3rd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.
Sánchez, B., Urdaci, M. C., and Margolles, A. (2010). Extracellular proteins secreted by probiotic bacteria as mediators of effects that promote mucosa-bacteria interactions. Microbiol. Read. Engl. 156, 3232–3242. doi: 10.1099/mic.0.044057-0
Sandes, S., Alvim, L., Silva, B., Acurcio, L., Santos, C., Campos, M., et al. (2017). Selection of new lactic acid bacteria strains bearing probiotic features
from mucosal microbiota of healthy calves: looking for immunobiotics through in vitro and in vivo approaches for immunoprophylaxis applications. Microbiol. Res. 200, 1–13. doi: 10.1016/j.micres.2017. 03.008
Schär-Zammaretti, P., and Ubbink, J. (2003). The cell wall of lactic acid bacteria: surface constituents and macromolecular conformations. Biophys. J. 85, 4076–4092. doi: 10.1016/S0006-3495(03)74 820-6
Seki, K., Nakao, H., Umino, H., Isshiki, H., Yoda, N., Tachihara, R., et al. (2004). Effects of fermented milk whey containing novel bifidogenic growth stimulator produced by Propionibacterium on fecal bacteria, putrefactive metabolite, defecation frequency and fecal properties in senile volunteers needed serious nursing-care taking enteral nutrition by tube feeding. J. Intest. Microbiol. 18, 107–115. doi: 10.11209/jim.18.107
Shannon, P., Markiel, A., Ozier, O., Baliga, N. S., Wang, J. T., Ramage, D., et al. (2003). Cytoscape: a software environment for integrated models of biomolecular interaction networks. Genome Res. 13, 2498–2504. doi: 10.1101/gr.1239303
Silva, W. M., Carvalho, R. D., Soares, S. C., Bastos, I. F., Folador, E. L., Souza, G. H., et al. (2014). Label-free proteomic analysis to confirm the predicted proteome of Corynebacterium pseudotuberculosis under nitrosative stress mediated by nitric oxide. BMC Genomics 15:1065. doi: 10.1186/1471-2164-15-1065
Silva, W. M., Folador, E. L., Soares, S. C., Souza, G. H. M. F., Santos, A. V., Sousa, C. S., et al. (2017). Label-free quantitative proteomics of Corynebacterium pseudotuberculosis isolates reveals differences between Biovars ovis and equi strains. BMC Genomics 18:451. doi: 10.1186/s12864-017-3835-y
Suzuki, A., Mitsuyama, K., Koga, H., Tomiyasu, N., Masuda, J., Takaki, K., et al. (2006). Bifidogenic growth stimulator for the treatment of active ulcerative colitis: a pilot study. Nutrition 22, 76–81.doi: 10.1016/j.nut.2005.04.013
Valence, F., Deutsch, S. M., Richoux, R., Gagnaire, V., and Lortal, S. (2000). Autolysis and related proteolysis in Swiss cheese for two Lactobacillus helveticus strains. J. Dairy Res. 67, 261–271. doi: 10.1017/S0022029900004118
van der Mei, H., van de Belt-Gritter, B., Pouwels, P., Martinez, B., and Busscher,
H. (2003). Cell surface hydrophobicity is conveyed by S-layer proteins—a study in recombinant lactobacilli. Colloids Surf. B Biointerfaces 28, 127–134. doi: 10.1016/S0927-7765(02)00144-3
Vastano, V., Pagano, A., Fusco, A., Merola, G., Sacco, M., and Donnarumma,
G. (2016). The Lactobacillus plantarum Eno A1 enolase is involved in immunostimulation of Caco-2 cells and in biofilm development. Adv. Exp. Med. Biol. 897, 33–44. doi: 10.1007/5584_2015_5009
Vizcaíno, J. A., Csordas, A., del-Toro, N., Dianes, J. A., Griss, J., Lavidas, I., et al. (2016). 2016 update of the PRIDE database and its related tools. Nucleic Acids Res. 44, D447–D456. doi: 10.1093/nar/gkv1145
Wilson, W. W., Wade, M. M., Holman, S. C., and Champlin, F. R. (2001). Status of methods for assessing bacterial cell surface charge properties based on zeta potential measurements. J. Microbiol. Methods 43, 153–164. doi: 10.1016/S0167-7012(00)00224-4
Wong, C. C. L., Cociorva, D., Miller, C. A., Schmidt, A., Monell, C., Aebersold, R., et al. (2013). Proteomics of Pyrococcus furiosus (Pfu): identification of extracted proteins by three independent methods. J. Proteome Res. 12, 763–770. doi: 10.1021/pr300840j

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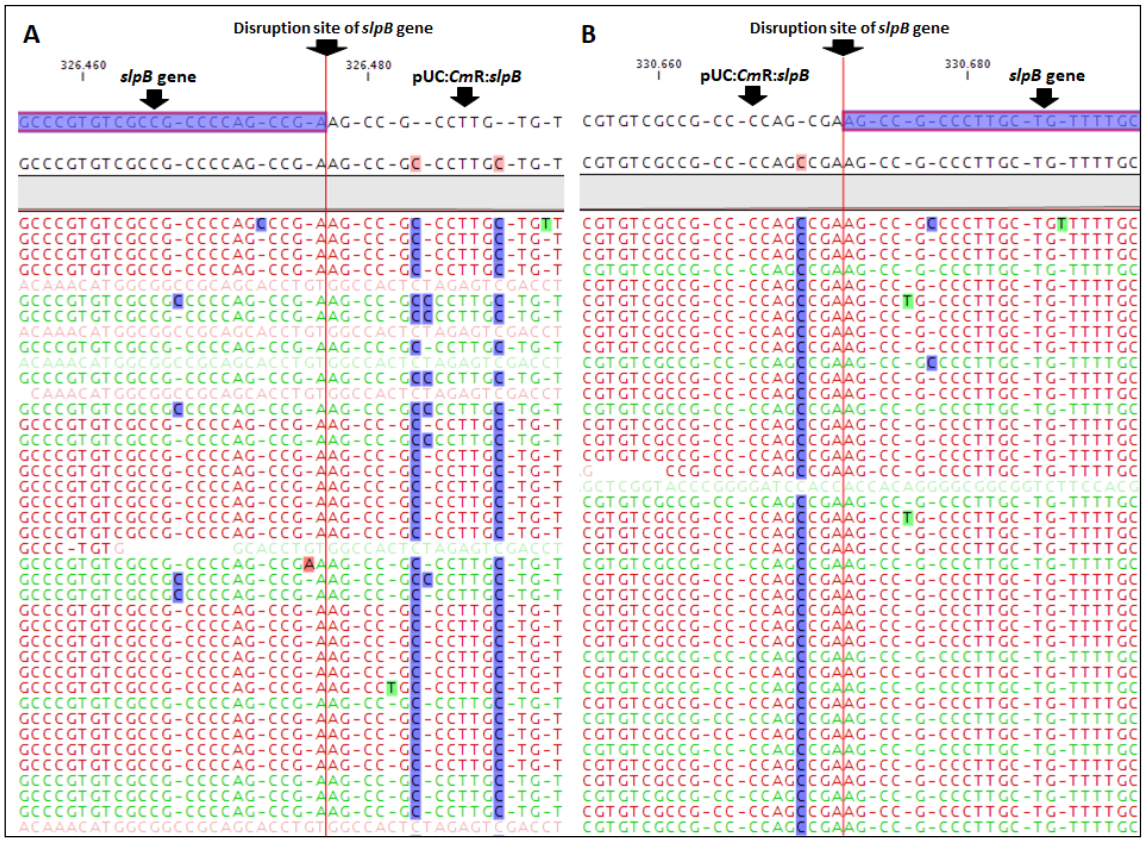
Supplemental figure 1

Supplemental figure 2



CHAPTER 5 – ARTICLE DE RECHERCHE ORIGINAL – SURFACE LAYER PROTEIN SlpB IS INVOLVED IN *Propionibacterium freudenreichii* CIRM-BIA 129 ABILITY TO PREVENT 5-FU-INDUCED MUCOSITIS

In our work we found that there was a great and pleiotropic impact of the *slpB* gene mutation in *P. freudenreichii* CB129Δ*slpB* strain, affecting adhesion to intestinal epithelial cells, physicochemical properties, tolerance to harsh environments, and whole cell quantitative proteome, when compared to the parental wild type probiotic strain *P. freudenreichii* CIRM-BIA 129. Following the discoveries and insights into the ability of probiotic strains to modulate gut microbiota and immune system (COUSIN et al., 2012), previous work unravelled the immunomodulatory role of a specific protein in an inflammatory context, both *in vitro* and *in vivo*.

In several Lactobacilli harbouring an S-layer, these proteins are responsible for anti-inflammatory effects, stimulating pathways that reduce inflammation. For example, the SlpA of *L. helveticus* MIMLh5 reduces activation of NF-κB in Caco-2 cells (TAVERNITI et al., 2013). Further, Slps of the probiotic *L. acidophilus* decrease interleukin IL- 8 secretion in Caco-2 cells stimulated by the proinflammatory *S. typhimurium* (LI et al., 2011). Finally, SlpA of *L. acidophilus* NCK2187 mediates regulatory signals, which in turn alleviate colitis severity in a mice model (LIGHTFOOT et al., 2015). In accordance, removing S-layer proteins from the surface of *P. freudenreichii* CIRM-BIA 129 suppressed the induction of anti-inflammatory cytokines in human PBMC's (FOLIGNÉ et al., 2010).

A set of S-layer proteins of *P. freudenreichii* CIRM-BIA 129 triggers immunomodulatory effect, and plays a central role in the adhesion process. It is thus of a paramount importance to investigate whether the immunomodulatory effect can be associated with a specific protein (DO CARMO et al., 2017b; LE MARÉCHAL et al., 2015). All the results obtained during previous investigations in the STLO laboratory focused on the central role of protein SlpB in the immunomodulatory strain *P. freudenreichii* CIRM-BIA 129 and culminate to realize an *actum finalem* and the claim that this protein has a multifaceted effect in probiotic interactions with the host. Recently, dairy propionibacteria have been shown to be effective in preventing inflammatory bowel diseases in mice, so we

decided to investigate their probiotic ability in another inflammatory model, which responds to a wide appeal, worldwide, in the field of human health preservation.

Mucositis affects a large proportion of patients who are undergoing cancer treatment with antineoplastic such as 5-Flourouracil (5-FU). Mucositis is characterized as a severe inflammation that affects the Alimentary Tract (AT) (SONIS, 2004a, 2004b). Moreover, mucositis induced by 5-FU treatment causes severe weight loss, shortening of intestinal villi and an inflammation of the mucosa, together with increased predisposition to local and systemic secondary infections (CARVALHO et al., 2017). These side effects increase the patient's hospital stay and increase the cancer treatment costs. Meanwhile, treatments available for mucositis are poorly effective and provide serious side effects, and some studies, have proposed the use of probiotic bacterial strains, as promising candidates for mucositis treatment (CARVALHO et al., 2017).

In the present work, we decided to evaluate the probiotic capacity of the *P. freudenreichii* CIRM-BIA 129 in a mice model of mucositis and investigate the importance of SlpB in the prevention against inflammatory mucositis damage, using knockout strain CB129Δ*slpB*.

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CHAPITRE 5 – ARTICLE DE RECHERCHE ORIGINAL – SURFACE LAYER PROTEIN SlpB IS INVOLVED IN *Propionibacterium freudenreichii* CIRM-BIA 129 ABILITY TO PREVENT 5-FU-INDUCED MUCOSITIS.

Dans ce travail, nous avons constaté que la simple mutation du gène *slpB* avait un grand et pléïotrope effet sur la souche mutante *P. freudenreichii* CB129Δ*slpB*, affectant l'adhésion aux cellules épithéliales intestinales, les propriétés physicochimiques, la tolérance au stress, ainsi que le protéome cellualire total, en comparaison avec la souche parentale sauvage *P. freudenreichii* CIRM-BIA 129. Après la découverte et l'étude de la capacité de souches probiotiques à moduler le microbiote et l'immunité, au niveau intestinal (COUSIN et al., 2012), des études précédentes ont confirmé et élucidé le rôle spécifique d'une protéine de type S-layer dans une action antiinflammatoire, que ce soit *in vitro* ou *in vivo*.

Chez plusieurs souches de lactobacilles arborant une S-layer, ces protéines sont responsables des effets immunomodulateurs, stimulant des voies de signalisation qui réduisent l'inflammation. Par exemple, la protéine SlpA de *L. helveticus* MIMLh5 réduit l'activation de NF-κB dans les cellules Caco-2 (TAVERNITI et al., 2013). De plus, les Slps du probiotique *L. acidophilus* réduisent la sécrétion d'IL-8 dans les cellules Caco-2 stimulées par la bactéries proinflammatoire *S. typhimurium* (LI et al., 2011). Enfin, la SlpA de *L. acidophilus* NCK2187 induit des signaux de régulation, qui à leur tour limitent la sévérité de la colite dans un modèle murin (LIGHTFOOT et al., 2015). De même, le fait de retirer les protéines de S-layer de la surface de *P. freudenreichii* CIRM-BIA 129 supprimait l'induction de cytokines antiinflammatoires par des PBMCs humains (FOLIGNÉ et al., 2010).

Un ensemble de protéines de S-layer de *P. freudenreichii* CIRM-BIA 129 induit un effet immunomodulateur, et joue un rôle central dans le processus d'adhésion. Il est donc d'une importance majeure de déterminer si cet effet peut être attribuer à une protéine spécifique (DO CARMO et al., 2017b; LE MARÉCHAL et al., 2015). Tous les résultats obtenus précédemment au laboratoire STLO se concentrent sur le rôle central de la protéine slpB dans la souche immunomodulatrice *P. freudenreichii* CIRM-BIA 129 et concluent au fait que cette protéine a un effet « multi-facette » dans les interactions probiotiques avec l'hôte. Récemment, les bactéries propioniques laitières se sont révélées protectrices

dans le contexte de maladies inflammatoires intestinales chez la souris. Nous avons donc décidé d'étudier leur efficacité probiotique dans un autre modèle inflammatoire, qui correspond à une large demande, à l'échelle mondiale, dans le domaine de la préservation de la santé humaine.

La mucosite affecte une large part des patients qui subissent un traitement du cancer avec un antinéoplasique tel que le 5-Fluorouracil (5-FU). La mucosite consiste en une inflammation sévère qui affecte la totalité du tractus digestif (SONIS, 2004a, 2004b). De plus, la mucosite induite par la chimiothérapie au 5-FU provoque des pertes de poids sévères, un raccourcissement des villosités intestinales et une inflammation de la muqueuse, ainsi qu'une prédisposition accrue aux infections locales et systémiques (CARVALHO et al., 2017). Ces effets secondaires augmentent le temps d'hospitalisation des patients ainsi que le coût du traitement du cancer. En même temps, les traitements de la mucosite sont peu efficaces et provoquent à leur tour de sérieux effets secondaires. Certaines études ont proposé l'utilisation de bactéries probiotiques comme candidats prometteurs dans le cadre du traitement de la mucosite (CARVALHO et al., 2017).

Dans le travail présenté ici, nous avons décidé d'évaluer l'efficacité probiotique de *P. freudenreichii* CIRM-BIA 129 dans un modèle murin de mucosite et d'étudier l'importance de SlpB dans la prévention des dommages inflammatoires, en utilisant la souche mutante inactivée CB129Δ*slpB*.

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SURFACE LAYER PROTEIN B IS ESSENTIAL FOR *Propionibacterium freudenreichii* CIRM-BIA 129 TO PREVENT MUCOSITIS IN 5-FU MICE MODEL.

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Running Title: propionibacterial against 5-FU induced mucositis

Abstract

Propionibacterium freudenreichii CIRM-BIA 129 (*P. freudenreichii* WT) is a probiotic bacterium, which exerts immunomodulatory effects. Its consumption alleviates symptoms of induced colitis in mice. Surface layer proteins (Slp) play a key role in this anti-inflammatory effect and are responsible for induction of the immunomodulatory IL-10 in immune cells. Mutation of *slpB* gene leads to a decreased adhesion to epithelial cells. Considering the key role of surface proteins in probiotic/host interactions, we decided to investigate the impact of *slpB* gene mutation on epithelial immunomodulation mediated by *P. freudenreichii*. In an *in vitro* assay, *P. freudenreichii* WT reduced LPS-induction of IL-8 and TNF-α cytokines in LPS-stimulated HT-29 cells. The mutant strain *P. freudenreichii* Δ*slpB*, lacking the SlpB protein, failed to do so. Therefore, presence of the SlpB protein is essential for the regulation of a pro-inflammatory response *in vitro*. We thus conducted *in vivo* investigations to compare probiotic efficacy of both strains in a 5-FU-induced mucositis mice model. Mucositis affects a large proportion of patients who are undergoing cancer treatment with antineoplastic drugs such as 5-Fluorouracil. This drug's side effect causes severe weight loss, villi shortness and inflammation of the mucosa. Consumption of *P. freudenreichii* WT prevented weight loss and shortening of intestinal villi, reduced inflammation and consequently histopathological scores. By contrast, the *P. freudenreichii* Δ*slpB* mutant failed to modulate these parameters. At the molecular level, the probiotic effect of *P. freudenreichii* WT was concomitant with the regulation of key markers, including Claudin-1 *(Cld1)* and IL-17a genes, and IL-12 and IL-1β cytokines. *P. freudenreichii* Δ*slpB* mutant displayed opposite regulatory effect on *Cld1* expression and on IL-12 levels. This work emphasizes the importance of *P. freudenreichii* S-layer protein SlpB in reducing mucositis. New perspectives are open for the development of probiotic products aimed at healing the side effects of chemotherapy using GRAS bacteria possessing S-layer proteins with immunomodulatory properties.

keywords: mucositis, probiotic, S-layer, immunomodulation, cancer.

Introduction

Propionibacterium freudenreichii represents the main species of dairy propionibacteria. It is a gram-positive, non-motile, non-spore forming and anaerobic to aerotolerant beneficial bacteria, which plays an important role in food transformation, particularly for cheese ripening [1]. It has been listed in the Qualified Presumption of Safety list by the European food safety authority, and they also have the GRAS (Generally Recognized As Safe) status [2]. Dairy propionibacteria are peculiar bacteria with a great probiotic potential. They produce the short chain fatty acids (SCFAs) acetate and propionate, and other beneficial metabolites such as 1,4-dihydroxy-2-naphthoic acid (DHNA) and 2-amino-3-carboxy-1,4-naphthoquinone (ACNQ), which were described as bifidogenic growth stimulators, and vitamin B9 and B12 [1].

Probiotic effects of *P. freudenreichii* also include the modulation of the gut microbiota and of the gut immune system [2]. In 2012, Cousin and collaborators demonstrated that dairy propionibacteria induce the production of the regulatory cytokine IL-10 in *ex vivo* model, and decrease the proinflammatory cytokines, such as IL-8 and tumor necrosis factor-α (TNFα), in the gut mucosa of piglets after lipopolysaccharides (LPS) stimulation [3].

P. freudenreichii strains, isolated or associated with other probiotic bacteria, have also been shown to attenuate colitis induced by trinitrobenzene sulfonic acid (TNBS), in conventional mice [4]. *P. freudenreichii* was also reported to reduce intestinal and systemic proinflammatory alterations, caused by a high-fat diet, in a mice model [5]. Moreover, dairy propionibacteria strains may alleviate symptoms and stabilize the intestinal microbiota in patients with irritable bowel syndrome [6]. Altogether, these studies attracted attention on *P. freudenreichii* as a promising probiotic to potentiate the treatment of inflammatory diseases.

P. freudenreichii strain ITGP20, equivalent to CIRM-BIA 129 (*P. freudenreichii* WT) strain was used for the development of two experimental cheeses, one single-stran, and one in association with *Lactobacillus delbrueckii* subsp *lactis* CNRZ327. Both cheeses gave promising results and alleviated TNBS-induced colitis in mice model [7,8]. Regarding the anti-inflammatory effects of this peculiar bacterium, a study showed that specific surface layer associated proteins were linked with the probiotic effects of selected *P. freudenreichii* WT strain [9]. Focusing on *P. freudenreichii* WT, a surface proteomic analysis of Slps proteins identified

three surface-exposed ones, designated SlpA, SlpB and SlpE [9]. Interestingly, after extraction of S-layer proteins from *P. freudenreichii* WT strain, the bacteria lost their ability to induce anti-inflammatory cytokines in human PBMCs [9]. Moreover, in *P. freudenreichii* WT, Carmo and collaborators confirmed that the surface protein SlpB is involved in adhesion to cultured human intestinal epithelial cells HT-29 [10], and mutation of the *slpB* gene caused drastic changes in surface properties [11].

In this context, the great probiotic potential of *P. freudenreichii* in the context of inflammatory bowel diseases [7,8], and the presence of a characterized surface layer protein SlpB with immunomodulatory activity [12] and directly linked to adhesion to cells human epithelial cells [10]. Led us to challenge this bacterium in another animal model involving inflammation: chemotherapy-induced mucositis [13].

Mucositis is a severe inflammation that affects the Alimentary Tract (AT) of individuals undergoing cancer treatment based on radiotherapy or chemotherapy, such as 5-Flourouracil (5-FU) [14]. Disease is characterized by pathological changes in the small bowel. This includes the presence of degenerate enterocytes, leukocyte infiltrate in the lamina propria, increased mucus production and degeneration of goblet cells, atrophy of villi, hypoplasia and apoptosis of intestinal crypts [15–17]. The currently available treatments of mucositis are not effective and have serious side effects. In this context, some studies have proposed the use of probiotic bacterial strains, as promising candidates in the treatment or prevention of inflammatory conditions such as mucositis [13,18].

The aim of this study is to evaluate the probiotic effects of *P. freudenreichii* CIRM-BIA 129 to protect mice against inflammatory mucositis damages induced by 5-FU, and to further investigate the associated role of protein SlpB in immunomodulatory effect *in vitro* and *in vivo*.

Materials and Methods

Bacterial strains and culture conditions

The wild-type strain *P. freudenreichii* strain ITGP20, equivalent to CIRM-BIA 129 (*P. freudenreichii* WT), was provided by the CNIEL (Centre National Interprofessionnel de l'Economie Laitière) and maintained by the CIRM-BIA

(International Centre for Microbial Resources – Food Associated Bacteria). This strain, as well as the genetically modified *P. freudenreichii* Δ*slpB* strain (*P. freudenreichii* Δ*slpB*) [10] , were grown at 30°C in Yeast Extract Lactate (YEL) broth[19]. For the *P. freudenreichii* Δ*slpB* mutant, YEL culture media were supplemented with chloramphenicol (10 µg.mL^{-1}). The growth of *P. freudenreichii* strains was monitored spectrophotometrically by measuring the optical density at 650 nm (OD_{650nm}), as well as by counting colony-forming units (CFUs) in YEL medium, according to Malik and collaborators [19], containing 1.5% agar. *P. freudenreichii* strains were harvested in a stationary phase (76 h, 2 x 10^9 CFU.mL^{-1}, determined by plate counts) by centrifugation (8,000 × g, 10 min, 4°C).

HT-29 cells challenging

HT-29 cells were routinely grown in T-25 flasks in complete medium DMEMc (10% (v/v) fetal calf serum, 100 U/mL penicillin and 100 µg/mL streptomycin sulphate) at 37°C with 5% CO_2. Trypsin/EDTA (Gibco, Saint Aubin, France) was used to release adherent cells for subculturing. For the experiment, cells were seeded in 12-well plates (1 ml of medium/well) and the growth medium was changed every 2 days. HT-29 cells were grown until complete confluence, 1.10^6 cell.mL^{-1}-well. Prior to challenging cells, complete medium was replaced with antibiotic-free medium. HT-29 cells were subjected to the different treatments: 7h with 100 ng/mL of Lipopolysaccharide (LPS from E. coli 0111: B4, Sigma), or 7h with *P. freudenreichii* WT, or 7h with *P. freudenreichii* Δ*slpB*. In parallel, cells were also subjected to co-treatments for 7 h: LPS in combination with *P. freudenreichii* WT or *P. freudenreichii* Δ*slpB*, ratio 10 bacteria:1 HT-29 cell, MOI 10 (1 x 10^7 CFU.mL^{-1}-weel). The percentage of HT-29 cells viability after the different stimulation conditions was checked by trypan blue staining and the cells viability was not affected.

Total RNA isolation and gene expression analysis by qRT-PCR

Cellular RNA was isolated with Trizol reagent (Invitrogen Ambion), and cDNA was synthesized using a qScript cDNA synthesis kit (Quanta Biosciences). Amplification was performed on a CFX96 real-time system (Bio-Rad, Marne la Coquette, France). Real-time PCR reactions were set up in CFX96 real-time system (Bio-Rad, Marne la Coquette, France). Each PCR reaction was performed

in a 16 µL reaction mixture containing 5 µL SYBR Green PCR Master Mix (Biorad), 5 µL of properly diluted cDNA (350 ng of cDNA for all genes), 3 µL mixture of each primer at 300 nM. The negative controls (with no DNA template, only primer pair, water and SYBR Green PCR Master Mix) for each primer set were included in each run. Amplification was carried out on a CFX96 Real-Time System (Bio-Rad) for 3 minutes at 95°C and 40 cycles of 2 steps consisting of 5 seconds at 95°C and 30 seconds at 60°C. The relative quantification of the mRNA levels of the target genes was determined using CFX Manager Software. The transcript level was normalized to the transcript level of GAPDH and β-actin genes (housekeeping gene). Finally, the results are presented as fold change using $2^{-\Delta\Delta CT}$ method for an unknown sample versus the control (untreated HT-29 cells). Primers sequence used in this study are listed in Table 1.

Evaluation probiotic proprieties of *P. freudenreichii* wild-type and mutant strain to prevent of mucositis induced by 5-FU in a mice model.

Animals

Conventional female BALB/c mice, between 6 and 8 weeks of age, were obtained at Federal University of Minas Gerais (UFMG–Belo Horizonte, Brazil). These Mice were kept in a temperature-controlled room with *ad libitum* access to water and standard chow diet. The study was approved by the Ethics Committee on Animal Experimentation of the Federal University of Minas Gerais (CEUA-UFMG, Brazil, protocol 366).

Prevention treatment, mucositis induction, and experimental groups

For treatment, grouped mice received, during 10 days by continuous feeding, a culture medium YEL (control), or cultured strains in YEL, *P. freudenreichii* WT or *P. freudenreichii* Δ*slpB*, both containing 10^9 CFU mL^{-1} of bacteria. Grouped mice that received only water without treatment was used as a group's control. To induce mucositis, mice received a single intraperitoneal injection of 5-FU (Fauldfluor – Libbs) (300 mg/kg) on day 11 and were euthanized on day 14, according to [20]. An injection of saline (NaCl 0.9%) was used as a control group. Mice were randomly divided into the following eight groups (6 mice per groups) in three experimental replicates: Animals from group 1–4 were injected with 0.9% saline on day 10 (noninflamed groups); (1) Naive: received saline; (2) YEL: received culture media; (3) *P. freudenreichii* WT: received *P. freudenreichii* WT

culture; (4) *P. freudenreichii* ∆*slpB:* received *P. freudenreichii* ∆*slpB* culture. Mice from group 5-8 were injected with 5-FU (inflamed groups): (5) receive saline; (6) received YEL; (7) received *P. freudenreichii* WT culture, and (8) received *P. freudenreichii* ∆*slpB* culture.

Histological analysis

For histomorphological analysis, the distal portion of the mice ileum was collected after the euthanasia and washed with PBS. Afterwards, rolls were prepared and immersed in formaldehyde solution (4%) for tissue fixation. This material was embedded in paraffin, and a 4 µm section of samples were placed on a glass slide and stained with hematoxylin and eosin (HE). Histological inflammation score was determined as described by Soares and collaborators [21], measuring three major histological changes in mucositis disease: (i) intensity of the infiltrate of mononuclear and polymorphonuclear cells in the *lamina propria*, (ii) presence of ulceration and erosion and (iii) alterations in mucosal architecture. The score was given according to the severity of the lesion in the tissues: absent (0), mild (1), moderate (2) and severe (3). For morphometric analysis, ten images of the ileum of each animal were randomly captured and analyzed using ImageJ software (version 1.8.0). Granular density of Paneth cells was determined by measuring the intracellular area occupied by secretory granules [20]. Villi height and crypt depth were measured vertically from the tip of villi to the base of the adjacent crypt. Villus height/crypt depth ratio from the intestinal epithelium was also measured [20].

Measurement of Secretory IgA

For measurement of secretory IgA (sIgA), the small bowel of all euthanized mice were washed using PBS. These materials were vortexed, and centrifuged for 30 min at 850 g at 4° C. Afterwards, the supernatant was transferred to a test tube and used for tested by enzyme-linked immunosorbent assay (ELISA) for IgA concentration as previously described by [20]. The results were measured in concentration of sIgA (µg) per ml of intestinal fluid, according to the standard curve.

Tissue preparation and cytokine quantification by ELISA

For the quantification of cytokines, the ileum were weighed and homogenized in PBS containing Tween-20 0.05% (Sigma-Aldrich, St. Louis, MO, USA), phenylmethylsulfonyl fluoride 0.1 mM (Sigma- Aldrich, St. Louis, MO, USA), benzethonium chloride 0.1 mM (Sigma-Aldrich, St. Louis, MO, USA), EDTA 10 mM (Synth, São Paulo, São Paulo, Brazil), and aprotinin A 20 KIU (Sigma-Aldrich, St. Louis, MO, USA). Afterwards, this material was homogenized, centrifuged at 3,000 g for 10 min and the supernatants collected for cytokine assay. Plates were coated with purified monoclonal antibodies reactive with cytokines IL- 10, IL-12 p70 and IL-1β/IL-1F2 (R&D Systems, Inc, USA), overnight at 4°C. Then, plate wells were washed, supernatants were added, and plates were again incubated overnight at 4°C. On the third day, biotinylated monoclonal antibodies against cytokines (R&D Systems, Inc, USA) were added on the plates and incubated for 2 h, at room temperature. Colour was developed at room temperature with 100 µl/well of orthophenylenediamine (1 mg/ml) and 0.04% (v/v) H_2O_2 substrate in sodium citrate buffer. The reaction was interrupted by the addition of 20 µl/well of 2N H_2SO_4. The absorbance was measured at 492 nm using a Microplate Reader Model 680 (BIO-RAD).

Relative expression of cytokines in ileum

Quantitative expression of genes in ileum tissue was made according Oliveira and collaborators [22]. First, small fragments (1 cm approximately) of ileum were collected and stored in RNAlater (Ambion, Austin, USA) at −80 °C until RNA extraction. Total RNA was isolated using an RNeasy mini kit (Qiagen, Hilden, Germany) according to the manufacturer's recommended protocol. Residual genomic DNA was digested and removed using DNase I (Invitrogen, Waltham, MA, USA) treatment. Samples were then treated with Turbo DNA-free Kit® (Ambion), according to manufacturer's instruction, for DNA removal. cDNA of each sample was produced with High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, USA), according to its manual instructions. Quantitative PCR (qPCR) was performed using iTaq universal SYBR green supermix (Biorad, Hercules, CA, USA) and gene specific-primers for Muc2, Claudin-1 (Cld1), Tjp1, Ocludin, iNOS and IL-17a [23–26] as well as housekeeping genes for β-actin and GAPDH [23]. Amplification reactions were performed in a final

volume of 10 µl, using 5 µl of SYBR green supermix and 10 ng of cDNA. The amplification program consisted of the following steps: 95°C for 30 sec, and 40 cycles of 95°C for 15 sec and 60°C for 30 sec on an ABI PRISM 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA). Expression levels in control group (with no treatment) were used as calibration data. Results are shown graphically as fold changes in gene expression, using the means and standard deviations of target cytokine expression amount ($2^{-\Delta\Delta Ct}$) according to Hellemans, Mortier, De Paepe, Speleman, and Vandesompele (2007) [27].

Statistical analyses

The results were reported as the mean ± standard deviation. Parametric data's were analyzed using One-Way ANOVA followed by the Tukey or Sidak post-test. Non-parametric data's were analyzed using Kruskal-Wallis data followed by the Dunns post-test. Graphs and statistical analyzes were performed in GraphPad Prism version 7.00 for Windows (GraphPad Software, San Diego, California, U.S.A.). Asterisks represent statistically significant differences between strains and were indicated as follows: *p < 0.05; **p < 0.01; ***p < 0.001 and ****p < 0.0001.

Results

Propionibacterium freudenreichii WT, yet not the *P. freudenreichii* Δ*slpB* mutant, prevents LPS-induced inflammation in HT-29 cells

We investigated the anti-inflammatory potential of *P. freudenreichii* WT, and the impact of the mutation of the *slpB* gene on this potential. We thus exposed HT-29 cells to both strains. Cells were in control conditions, or stressed by proinflammatory Lipopolysaccharide (LPS) from *E. coli*. We thus monitored changes in the relative expression of genes involved in the inflammatory process (Fig.1).

CB 129 WT induced expression of IL-10 cytokines (Fig. 1A), with significant differences, 3.18±0.85 (*p < 0.0001*), compared to all conditions analyzed and to control non-treated cells. The mutant *P. freudenreichii* Δ*slpB* failed to induce IL-10 expression (0.96±0.27), by contrast with CB 129 WT strain. LPS did not change IL-10 expression, with or without co-stimulation with *P. freudenreichii* WT or *P. freudenreichii* Δ*slpB*. In HT-29 cells stressed by LPS, IL-8 was strongly

induced (Fig. 1B). This induction was inhibited by the presence of *P. freudenreichii* WT (3.91±0.53), with a significant difference with LPS alone 11.36±2.56 ($p < 0.0001$). However, the mutant *P. freudenreichii* Δ*slpB* strain lost this ability to down-regulate IL-8 after LPS stimulation. As a control, CB 129 WT did not induce IL-8 expression, while the mutant *P. freudenreichii* Δ*slpB* did (3.72±1.24), when compared to *P. freudenreichii* WT (1.26±0.51) or to untreated control.

The same trend was observed for IFN-α and TNF-α. Analysis of IFN-α expression (Fig. 1C) revealed that *P. freudenreichii* Δ*slpB* induced this cytokine (4.09±1.35) at a level similar to LPS (3.65±1.01), while *P. freudenreichii* WT did not (1.79±0.64). Furthermore, *P. freudenreichii* WT was capable to down-regulate IFN-α expression (2.08±0.42) significantly ($p<0.01$) in LPS stimulated-HT-29 cells, while *P. freudenreichii* Δ*slpB* was not (4.3±1.35). Expression of the pro-inflammatory TNF-α (Fig. 1D) was increased by LPS 14.37±3.17, however not by *P. freudenreichii* WT (1.44±0.75). *P. freudenreichii* WT down-regulated, in LPS stimulated-HT-29 cells, expression of TNF-α (7.14±0.97), compared to LPS 14.37±3.17 ($p<0.0001$) and to *P. freudenreichii* Δ*slpB* 14.95±4.33 ($p<0.0001$). In contrast, *P. freudenreichii* Δ*slpB* lost this ability to down-regulate TNF-α expression.

We then monitored expression of TLR2 and TLR9 receptors. LPS had no effect on TLR2 expression (Fig. 2A). However, *P. freudenreichii* WT induced high levels of TLR2, 4.01±2.36, in comparison with untreated cells ($p < 0.01$) or LPS stimulated-HT-29 cells ($p < 0.05$). Addition of LPS to HT-29 cells followed by treatment with *P. freudenreichii* WT completely suppressed TLR2 induction ($p < 0.01$). The mutant *P. freudenreichii* Δ*slpB* had no significant effect on TLR2 expression.

Neither LPS, nor *P. freudenreichii* WT modified TLR9 expression significantly, compared to the control (Fig. 2B). However, a combination thereof, *P. freudenreichii* WT+LPS 0.76±0.36, induced a significant decrease ($p<0.05$), compared to *P. freudenreichii* WT 1.57±0.35. The *P. freudenreichii* Δ*slpB* did affect expression of TLR9. Meanwhile, co-stimulation with LPS and *P. freudenreichii* Δ*slpB* induced a high level of TLR9 expression (2.11±0.17).

Regarding tight junction gene ZO-1 (Fig. 2C) we did not find any significant differences between controls untreated cells and stimulated cells. Interestingly,

the mutant strain *P. freudenreichii* Δ*slpB* + LPS (Fig. 2D) was able to increase the levels ($p<0.001$) of Muc2 1.52±0.20 gene expression when compared to CB129 WT + LPS 0.75±0.14.

***Propionibacterium freudenreichii* WT, yet not the *P. freudenreichii* Δ*slpB* mutant, improves mucosal preservation in the ileum of mice treated with 5-FU.**

To evaluate if probiotic administration decreases the symptoms of mucositis, weight was monitored before and after 5-FU administration for the control (saline) and mucositis (5-FU) groups (Fig. 3). No weight alteration was observed between groups before 5-FU injection. However, a reduction in weight was clearly observed after 5-FU injection, when compared to untreated groups (Fig. 3A). In this last group, *P. freudenreichii* WT strain consumption significantly limited weight loss: 13%±1.15 ($p<0.001$) compared to group receiving water 20.94%±3.21 (Fig 3B). By contrast, the mutant *P. freudenreichii* Δ*slpB* failed to limit weight loss (19.34%±2.58 compared to *P. freudenreichii* WT group, $p<0.05$).

Regarding histopathological analysis, control groups injected with saline showed no significant difference in ileum mucosal pattern, whether they consumed water, YEL culture medium, or a YEL culture of *P. freudenreichii* WT (Fig. 4A). However, consumption of the mutant strain *P. freudenreichii* Δ*slpB* increased histopathological score, leading to epithelium flattening, areas of erosion and ulceration in the ileum mucosa (Fig. 4B). Moreover, submucosa and muscular layer were thicker than the other control groups (water, YEL and *P. freudenreichii* WT). In submucosa layer, vessels were dilated, and edema intense. Some areas presenting focal hemorrhage in the muscular layer. Furthermore, the epithelium showed an inflammatory cell infiltration, predominantly polymorphonuclear and mononuclear cells migration, characteristic of defense cell infiltration.

In inflamed (5-FU) groups consuming controls (water and YEL), histopathological parameters were clearly changed regarding the morphological structure of the ileum. This corresponded to increased submucosa and muscular layer, villi shortening, increased number of inflammatory cells, with diffuse mononuclear polymorphonuclear inflammatory infiltrate in the *lamina propria* (Fig. 4B). Consumption of *P. freudenreichii* WT significantly reduced histopathological scores, compared to control inflamed groups (water and YEL) $p<0.001$ (Fig. 4A).

This corresponded to reduced infiltration, ulceration and alterations of intestinal mucosa (Fig. 4B). The mutant *P. freudenreichii* Δ*slpB*, by contrast, failed to alleviate the tissue damages caused by 5-FU (Fig. 4B).

In addition, we evaluated the height of the villi and the depth of the crypts (Fig. 5). There were no significant differences between the control groups injected with saline. The inflamed groups, injected with 5-FU, showed a reduction in villus height. Consumption of *P. freudenreichii* WT partially restored this height (114µm±17.92, $p>0.0001$), compared to groups receiving either water or *P. freudenreichii* Δ*slpB* 80.13µm±16.96 (Fig. 5A). No significant difference was observed between the inflamed or non-inflamed groups in terms of crypt depth (Fig. 5B).

In summary, consumption of *P. freudenreichii* WT, but not of mutant *P. freudenreichii* Δ*slpB,* improved villous architecture preservation by increasing villus height (Fig. 5A), villus / crypt ratio (Fig. 5C), and granular density within Paneth (Fig. 5D) in inflamed mice. Moreover, consumption of mutant *P. freudenreichii* Δ*slpB* per se resulted in some inflammatory changes in the ileum.

Propionibacterium freudenreichii reduces Secretory IgA production

Concentration of secretory IgA (SIgA) in the small intestine of mice, inflamed and non-inflamed, was measured (Fig. 6). Injection of 5-FU increased SIgA, in comparison with untreated mice. This induction was totally suppressed by consumption of both strains. Indeed, both *P. freudenreichii* WT and *P. freudenreichii* Δ*slpB* decreased the amount of SIgA in the inflamed and non-inflamed groups.

Genes expression in the mice ileum

In healthy mice and in mice receiving 5-FU, no significant difference was found regarding expression of Muc2 gene (Fig. 7A). Consumption of *P. freudenreichii* WT significantly increased Cld1 gene expression levels in mice injected with 5-FU, compared to water ($p<0.001$), YEL ($p<0.05$) or the mutant *P. freudenreichii* Δ*slpB* ($p<0.001$) (Fig. 7B). *P. freudenreichii* Δ*slpB* failed to do so. Expression levels of ZO-1 only show significant differences ($p<0.001$) between YEL and *P. freudenreichii* WT in heathy mice (saline) (Fig. 7C). Expression of Occludin was monitored and no significant difference was founded (Fig. 7D). Interestingly,

consumption of *P. freudenreichii* Δ*slpB* significantly increased level of iNOS, compared to groups receiving water ($p<0.05$) and *P. freudenreichii* WT ($p<0.05$), in healthy mice (Saline) (Fig. 7E). In addition, consumption of the mutant strain induced high expression levels of IL-17 in healthy mice ($p<0.0001$) (Fig. 7F). In inflamed mice receiving water, 5-FU triggered a significant induction of IL-17 2.81±1.00, compared to the mice receiving YEL 1.17±.058 ($p<0.01$) and CB 129 WT 0.97±0.34 ($p<0.01$), but difference was found between *P. freudenreichii* Δ*slpB* 1.81±0.51 (5-FU) and *P. freudenreichii* Δ*slpB* 3.08±2.06 (Saline).

Cytokine ELISA measurements in the ileum

Cytokines were quantified by ELISA in the intestinal mucosa of the groups inflamed (5-FU) and non-inflamed (Saline) (Fig. 8). In healthy conditions (saline), consumption of *P. freudenreichii* WT enhanced production of the immunomodulatory cytokine IL-10, while the *P. freudenreichii* Δ*slpB* mutant failed to do so (Fig. 8A). In mucositis conditions (5-FU), the disease drastically induced all the measured cytokines. Consumption of P. freudenreichii WT prevented the induction of IL-12 and of IL-1β. Regarding IL-10, in the inflamed mice (5-FU), a significant difference was found between the Naive group and the *P. freudenreichii* Δ*slpB* group, which presented lower concentration of IL-10 in the intestinal tissue (Fig. 8A). Interestingly, IL-12 concentration is elevated in the inflamed mice (5-FU), except in the group which received the *P. freudenreichii* WT strain, where a significant reduction was recorded ($p<0.0001$) (Fig. 8B). Both *P. freudenreichii* WT and *P. freudenreichii* Δ*slpB* strains are shown to be able to reduce IL-1β cytokine levels in the inflamed mice (5-FU) with significant difference to group receiving water (*P. freudenreichii* WT $p<0.01$; *P. freudenreichii* Δ*slpB* $p<0.01$) (Fig. 8C).

In addition, by analyzing the ratio of IL-10 to IL-12 (Fig. 9), we observed that in healthy mice, consumption of *P. freudenreichii* WT strain increased the IL-10/IL-12 ratio with significant differences ($p<0.05$), while the mutant failed to do so. In 5-FU induced mucositis mice, the *P. freudenreichii* WT strain showed the same trend, with significant difference among others groups (water $p<0.01$, YEL $p<0.05$ and *P. freudenreichii* Δ*slpB* $p<0.01$) (Fig. 8D).

Discussion

Propionibacterium freudenreichii strain ITGP20, equivalent to CIRM-BIA 129 (*P. freudenreichii* WT) has recently been recognized as a promising probiotic, with healing effects, studied *in vitro* and *in vivo* in a colitis mice model induced by TNBS [4,7–9,12]. This probiotic capacity is favored with the beneficial role played by the metabolites it produces (propionate, ACNQ, DHNA, vitamin K2 and B12) [1]. Recently, the probiotic effects of *P. freudenreichii* WT were associated with S-layer surface proteins responsible for its *in vitro* anti-inflammatory potential [9,12].

S-layer proteins (Slps) are non-covalently anchored to the bacterial cell wall via S-layer homology domains (SLH), forming a surface-exposed proteinaceous network [28,29]. Various S-layer proteins play various roles in bacteria and may be involved in virulence or immunomodulatory effects, in transport of molecules, and in protection against environmental stresses [30]. In probiotic bacteria, S-layer proteins can mediate a cross-talk between the host and bacteria, and play a key role in beneficial effects [30]. The surface proteins SlpB and SlpE were further shown to be responsible for immunomodulatory interactions with the host, mainly through the induction of IL-10 production [12]. This immunomodulatory ability is related to different molecular mechanisms exerted by the bacteria, including adhesion to human intestinal epithelial cells [31]. Since, bacterial adhesion to host cells is an important step of host colonization and can be mediated by surface proteins including S-layer proteins (Slps) [30,32]. This was reported in *L. acidophilus* NCFM, where S-layer protein SlpA contributes to interactions with human cells [31].

In the *P. freudenreichii* WT strain, the SlpB protein is associated in adhesion to intestinal human cells, as confirmed by knocking out of this gene in the strain *P. freudenreichii* Δ*slpB* [10]*.* Moreover, mutation of the *slpB* gene in *P. freudenreichii* Δ*slpB* caused pleotropic effects, and consequently, this strain lost characteristics relevant to probiotic potential, such as surface properties and environmental stress tolerance [11]. Therefore, to further investigate the role of SlpB in the host interaction, we decided to investigate the anti-inflammatory potential of both strains, *P. freudenreichii* WT and *P. freudenreichii* Δ*slpB*, in an assay using LPS-stimulated HT-29 cells.

As reported by Rabah and collaborators (2017), *P. freudenreichii* WT can reduce inflammatory response in HT-29 stimulated by *E. coli* LPS. Indeed, the *P.*

freudenreichii WT has the ability to induce the release of IL-10, and to repress that of IL-8 [33]. IL-10 induction is a determining factor for the probiotics and play a crucial role in the prevention of damage during inflammation process [34,35]. Repression of IL-8 expression is another one, because this cytokine triggers the recruitment of neutrophils in addition to further pro-inflammatory signals in the *lamina propria* [36,37]. Interestingly, the *P. freudenreichii* Δ*slpB* strain was shown here to lose this ability to induce IL-10 expression, probably due to absence of the SlpB protein and to reduced adhesion [10]. SlpB absence may also be responsible for the inability to down-regulate IL-8, another important actor with a pro-inflammatory action [38]. This is also observed concerning TNF-α, a pro-inflammatory cytokine, which controls the production of another inflammatory mediator. *P. freudenreichii* WT repressed TNF-α expression in an inflammatory context (LPS-stimulated HT-29), as was related about other probiotics strain [39–43]. However, the mutant strain *P. freudenreichii* Δ*slpB* lost this anti-inflammatory potential.

The bacteria/host interactions may modulate Toll-like receptors (TLRs), and such modulation may participate in the anti-inflammatory activity of probiotics [44,45]. *P. freudenreichii* WT was shown here to enhance TLR2 expression in HT-29 cells without LPS stimulation, while the mutant failed to do so. By contrast, *Lactobacillus rhamnosus* LGG decreased the expression of TLR2 and TLR-9 in HT-29 cells exposed to *Salmonella* or LPS [43,46]. Each probiotic strain has its specific properties and some of them reportedly induce expression of pro and anti-inflammatory cytokines in HIECs. Following our results *in vitro*, a relevant *in vivo* inflammatory model was needed, in order to validate whether a mutation of the *slpB* gene would affect its probiotic abilities.

Mucositis is an inflammatory disease that significantly affects cancer patients undergoing chemotherapy with antineoplastic such as 5-fluorouracil (5-FU). Available treatments for mucositis have limitations and probiotics are considered in this context, because of their reported effects to alleviates inflammation process by different mechanism [18]. During mucositis, mucosal integrity is impaired, due to alterations in tissue architecture, shortening of villi, increased crypt depth and presence of ulcerations [14]. The *P. freudenreichii* WT probiotic strain was able to alleviate the tissue damages caused by 5-FU, to preserve villus height and consequently to decrease histopathological scores. In addition, limited

recruitment of inflammatory cells infiltrating was observed in the *lamina propria*. Moreover, *P. freudenreichii* WT increased secretory granules density inside Paneth cells, which are involved in antimicrobial gastrointestinal function [20,47,48]. Weight loss was also attenuated by *P. freudenreichii* WT consumption, in agreement with other studies showing the efficacy of other probiotics to regulate this parameter [48–50]. Levels of Secretory IgA in the small bowel, a defense mechanism of the host towards the inflammatory process, increase during mucositis. Decreased levels of SIgA can be correlated with the integrity of the epithelial barrier and consequently protection against pathogens [20,51,52]. We show here decreased sIgA levels in mice receiving propionibacteria, in contrast with control mice receiving water. However, mutation of *slpB* gene did not affect sIgA, showing that the absence of SlpB protein does not affect release of IgA in the intestinal content.

The structure of tight junctions is an essential factor of the integrity of the epithelial barrier [53,54]. The expression of genes encoding ZO-1 and Muc2 proteins remained unchanged. However, treatment with probiotic *P. freudenreichii* WT increased Cld1 gene expression in the 5-FU inflamed group. Claudin-1 is involved in tight junctions formation and in epithelial cells intercellular adhesion [55]. CB 129 WT consumption reduced ileal levels of IL-12 and IL-1β cytokines, which were elevated in the mucositis model [56]. Immunomodulatory IL-10 was also enhanced in healthy mice consuming *P. freudenreichii* WT, a marker of anti-inflammatory effect also reported for *Lactobacillus acidophilus* [57]. The increase in the IL-10/IL-12 ratio, proposed as an anti-inflammatory probiotic effect marker [58], was observed here as a result of *P. freudenreichii* WT consumption in healthy mice, moreover this increase was significant in mice with mucositis. It is plausible that IL-10 played a key role in *P. freudenreichii* WT preventing the inflammatory events driven by 5-FU, given the importance of this cytokine in gut homeostasis. The effect of *P. freudenreichii* WT in IL-10 upregulation was no longer observed at the end of 5-FU treatment since the *P. freudenreichii* WT probiotic bacteria already prevented the mucositis inflammation. The same observation could be related to SIgA. Levels of SIgA increase, when inflammatory stimuli threaten the integrity of the mucosa, as a protective mechanism to avoid further damage [51]. When measured at the end of 5-FU treatment, SIgA is also decreased in the

group treated with *P. freudenreichii* WT, probably because inflammation was already under control.

Mice receiving *P. freudenreichii* Δ*slpB* exhibited a histopathological score different from those receiving the probiotic strain *P. freudenreichii* WT, but closer to that of mucositis control groups. The mutant lost the ability to maintain architectural integrity of the ileum mucosa. In addition to losing its anti-inflammatory capacity, the mutant strain induced inflammation in the ileum of healthy mice, in accordance with its inefficacy to protect form mucositis. It failed to reduce the abrupt weight loss caused by 5-FU, in contrast with the probiotic strain. Expression of Cld1, decreased in mucositis, was restored by consumption of the probiotic *P. freudenreichii* WT, but not by the mutant bacteria. Mutation of *slpB* gene in the mutant strain avoid the restoration of expression of Cld1 and, consequently can correlated to tight junction damage caused in 5-FU mice that receiving mutant strain. Accordingly, S-layer proteins are associated with the induction of the expression of tight junctions gene as Claudin-1, Occludin, JAM-1, and ZO-1 [30]. Moreover, consumption of the mutant increased expression of iNOS, which is linked to inflammatory process [59]. As the ileum contains a great number of IL-17 producing cells [60], and considering the observed modulation of iNOS, IL-17 increase in response to mutant *P. freudenreichii* Δ*slpB* may contribute to the onset of the inflammatory condition in healthy mice and to its inefficacy to alleviate mucositis induced by 5-FU.

Conclusion

This work demonstrates, by *in vitro* and *in vivo* approaches, that the mutation of the surface layer protein *slpB* gene affected directly the probiotic potential and therapeutics effects of *Propionibacterium freudenreichii* strain ITGP20, equivalent to CIRM-BIA 129. This is mainly evidenced by the fact that *P. freudenreichii* Δ*slpB* loses its ability to regulate pro-inflammatory cytokines in LPS stimulated HT-29 cells, and to alleviate 5-FU induced mucositis in mice model. Understanding the mechanism of protective effects opens new perspectives for the utilization of this strain to alleviate the inflammatory process caused by mucositis. Moreover, this study will open new opportunities to investigate the efficacy of food products containing dairy propionibacteria in different inflammatory conditions

Author Contributions

FLRdC performed animal experimentation regarding mucositis pre-treatment, interpreted the data regarding and was a major contributor in writing the manuscript. BFC, SHS, BMS, AF, LL, JLA, CCF, MIAQ and SHCS were major contributors in animal experimentation, cytokines measurement, quantitative PCR and performed, analyzed and interpreted the secretory IgA quantification essay. EF and NMR performed, analyzed and interpreted the morphometric analysis and histological analysis from ileum slides. HR performed *in vitro* analysis and data interpretation. AMCF, AC, YLL, GJ and VA contributed to data interpretation and were a major contributor in writing the manuscript. GJ and YLL were responsible for ceding the strains, contributed to data interpretation and were major contributors in writing the manuscript. GJ and VA, have equally contributed in the supervision, experiments performance, analysis and interpretation of immunological data and as major contributors in writing the manuscript.

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Data availability statement

All datasets generated or analyzed during the current experimental study are available from the corresponding author on reasonable request.

Ethical approval and informed consent

This project was approved by the Ethics Committee on Animal Use at Federal University of Minas Gerais (CEUA/UFMG) with protocol no. 366/2012, related to the present study is in agreement with the Ethical Principles in Animal Experimentation, and was approved in 11/04/2013.

Conflict of Interest Statement

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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References

1. Rabah, H., do Carmo, F. L. R. & Jan, G. Dairy Propionibacteria: Versatile Probiotics. *Microorganisms* **5,** (2017).

2. Cousin, F. J., Mater, D. D. G., Foligne, B. & Jan, G. Dairy propionibacteria as human probiotics: A review of recent evidence. *Dairy Sci. Technol.* (2010).

3. Cousin, F. J. *et al.* Assessment of the Probiotic Potential of a Dairy Product Fermented by *Propionibacterium freudenreichii* in Piglets. *J. Agric. Food Chem.* **60,** 7917–7927 (2012).

4. Foligné, B. *et al.* Promising immunomodulatory effects of selected strains of dairy propionibacteria as evidenced in vitro and in vivo. *Appl. Environ. Microbiol.* **76,** 8259–8264 (2010).

5. Oksaharju, A. *et al.* Effects of probiotic *Lactobacillus rhamnosus* GG and *Propionibacterium freudenreichii* ssp. *shermanii* JS supplementation on intestinal and systemic markers of inflammation in ApoE*3Leiden mice consuming a high-fat diet. *Br. J. Nutr.* **110,** 77–85 (2013).

6. Kajander, K. *et al.* Clinical trial: multispecies probiotic supplementation alleviates the symptoms of irritable bowel syndrome and stabilizes intestinal microbiota. *Aliment. Pharmacol. Ther.* **27,** 48–57 (2008).

7. Plé, C. *et al.* Single-strain starter experimental cheese reveals anti-inflammatory effect of *Propionibacterium freudenreichii* CIRM BIA 129 in TNBS-colitis model. *J. Funct. Foods* **18, Part A,** 575–585 (2015).

8. Plé, C. *et al.* Combining selected immunomodulatory *Propionibacterium freudenreichii* and *Lactobacillus delbrueckii* strains: Reverse engineering

development of an anti-inflammatory cheese. *Mol. Nutr. Food Res.* **60,** 935–948 (2016).

9. Le Maréchal, C. *et al.* Surface proteins of *Propionibacterium freudenreichii* are involved in its anti-inflammatory properties. *J. Proteomics* **113,** 447–461 (2015).

10. do Carmo, F. L. R. *et al. Propionibacterium freudenreichii* Surface Protein SlpB Is Involved in Adhesion to Intestinal HT-29 Cells. *Front. Microbiol.* **8,** (2017).

11. do Carmo, F. L. R. *et al.* Mutation of the surface layer protein SlpB has pleiotropic effects in the probiotic *Propionibacterium freudenreichii* 129. *Front. Microbiol.* **9,** (2018).

12. Deutsch, S.-M. *et al.* Identification of proteins involved in the anti-inflammatory properties of *Propionibacterium freudenreichii* by means of a multi-strain study. *Sci. Rep.* **7,** 46409 (2017).

13. do Carmo, F. L. R. *et al.* Applications of Probiotic Bacteria and Dairy Foods in Health. in *Current Research in Microbiology* 1–33 (Open Access eBooks 919 North Market Street Suite 425 Wilmington, DE 19801, 2017).

14. Sonis, S. T. The pathobiology of mucositis. *Nat. Rev. Cancer* **4,** 277–284 (2004).

15. Antunes, M. M. *et al.* Pretreatment With L-Citrulline Positively Affects the Mucosal Architecture and Permeability of the Small Intestine in a Murine Mucositis Model. *JPEN J. Parenter. Enteral Nutr.* **40,** 279–286 (2016).

16. Chang, C.-T. *et al.* 5-Fluorouracil induced intestinal mucositis via nuclear factor-κB activation by transcriptomic analysis and in vivo bioluminescence imaging. *PloS One* **7,** e31808 (2012).

17. Stringer, A. M. Interaction between host cells and microbes in chemotherapy-induced mucositis. *Nutrients* **5,** 1488–1499 (2013).

18. Carvalho, R. D. D. O. *et al.* Use of wild type or recombinant Lactic Acid Bacteria as an alternative treatment for gastrointestinal inflammatory diseases: A focus on Inflammatory Bowel Diseases and Mucositis. *Front. Microbiol.* **8,** (2017).

19. Malik, A. C., Reinbold, G. W. & Vedamuthu, E. R. An evaluation of the taxonomy of *Propionibacterium*. *Can. J. Microbiol.* **14,** 1185–1191 (1968).

20. Carvalho, R. D. *et al.* Secretion of biologically active pancreatitis-associated protein I (PAP) by genetically modified dairy *Lactococcus lactis* NZ9000 in the prevention of intestinal mucositis. *Microb. Cell Factories* **16,** 27 (2017).

21. Soares, P. M. G. *et al.* Gastrointestinal dysmotility in 5-fluorouracil-induced intestinal mucositis outlasts inflammatory process resolution. *Cancer Chemother. Pharmacol.* **63,** 91–98 (2008).

22. Oliveira, J. S. *et al.* In vitro and in vivo evaluation of two potential probiotic lactobacilli isolated from cocoa fermentation (Theobroma cacao L.). *J. Funct. Foods* **47,** 184–191 (2018).

23. Giulietti, A. *et al.* An Overview of Real-Time Quantitative PCR: Applications to Quantify Cytokine Gene Expression. *Methods* **25,** 386–401 (2001).

24. Hu, G.-Q. *et al.* AIM2 contributes to the maintenance of intestinal integrity via Akt and protects against *Salmonella* mucosal infection. *Mucosal Immunol.* **9,** 1330–1339 (2016).

25. Tokumasu, R. *et al.* Dose-dependent role of claudin-1 in vivo in orchestrating features of atopic dermatitis. *Proc. Natl. Acad. Sci.* **113,** E4061–E4068 (2016).

26. Wlodarska, M. *et al.* Antibiotic Treatment Alters the Colonic Mucus Layer and Predisposes the Host to Exacerbated *Citrobacter rodentium* -Induced Colitis. *Infect. Immun.* **79,** 1536–1545 (2011).

27. Hellemans, J., Mortier, G., De Paepe, A., Speleman, F. & Vandesompele, J. qBase relative quantification framework and software for management and automated analysis of real-time quantitative PCR data. *Genome Biol.* **8,** R19 (2007).

28. Gerbino, E., Carasi, P., Mobili, P., Serradell, M. A. & Gómez-Zavaglia, A. Role of S-layer proteins in bacteria. *World J. Microbiol. Biotechnol.* **31,** 1877–1887 (2015).

29. Sleytr, U. B., Schuster, B., Egelseer, E.-M. & Pum, D. S-layers: principles and applications. *FEMS Microbiol. Rev.* **38,** 823–864 (2014).

30. do Carmo, F. L. R. *et al.* Extractable Bacterial Surface Proteins in Probiotic–Host Interaction. *Front. Microbiol.* **9,** (2018).

31. Buck, B. L., Altermann, E., Svingerud, T. & Klaenhammer, T. R. Functional Analysis of Putative Adhesion Factors in *Lactobacillus acidophilus* NCFM. *Appl. Environ. Microbiol.* **71,** 8344–8351 (2005).

32. Preising, J. *et al.* Selection of bifidobacteria based on adhesion and anti-inflammatory capacity in vitro for amelioration of murine colitis. *Appl. Environ. Microbiol.* **76,** 3048–3051 (2010).

33. Rabah, H. *et al.* Cheese matrix protects the immunomodulatory surface protein SlpB of *Propionibacterium freudenreichii* during in vitro digestion. *Food Res. Int. Ott. Ont* **106,** 712–721 (2018).

34. Denning, T. L. *et al.* Expression of IL-10 receptors on epithelial cells from the murine small and large intestine. *Int. Immunol.* **12,** 133–139 (2000).

35. Jarry, A. *et al.* Mucosal IL-10 and TGF-β play crucial roles in preventing LPS-driven, IFN-γ–mediated epithelial damage in human colon explants. *J. Clin. Invest.* (2008). doi:10.1172/JCI32140

36. Kucharzik, T. Acute induction of human IL-8 production by intestinal epithelium triggers neutrophil infiltration without mucosal injury. *Gut* **54,** 1565–1572 (2005).

37. Singer, M. & Sansonetti, P. J. IL-8 is a key chemokine regulating neutrophil recruitment in a new mouse model of Shigella-induced colitis. *J. Immunol. Baltim. Md 1950* **173,** 4197–4206 (2004).

38. Bai, A.-P., Ouyang, Q., Zhang, W., Wang, C.-H. & Li, S.-F. Probiotics inhibit TNF-alpha-induced interleukin-8 secretion of HT29 cells. *World J. Gastroenterol. WJG* **10,** 455–457 (2004).

39. Cammarota, M. *et al.* In vitro evaluation of Lactobacillus plantarum DSMZ 12028 as a probiotic: Emphasis on innate immunity. *Int. J. Food Microbiol.* **135,** 90–98 (2009).

40. Duary, R. K., Batish, V. K. & Grover, S. Immunomodulatory activity of two potential probiotic strains in LPS-stimulated HT-29 cells. *Genes Nutr.* **9,** 398 (2014).

41. Li, P., Yu, Q., Ye, X., Wang, Z. & Yang, Q. Lactobacillus S-layer protein inhibition of Salmonella-induced reorganization of the cytoskeleton and activation of MAPK signalling pathways in Caco-2 cells. *Microbiology* **157,** 2639–2646 (2011).

42. Rolny, I. S., Tiscornia, I., Racedo, S. M., Pérez, P. F. & Bollati-Fogolín, M. *Lactobacillus delbrueckii* subsp *lactis* CIDCA 133 modulates response of human epithelial and dendritic cells infected with *Bacillus cereus* . *Benef. Microbes* **7,** 749–760 (2016).

43. Vizoso Pinto, M. G. *et al.* Lactobacilli stimulate the innate immune response and modulate the TLR expression of HT29 intestinal epithelial cells in vitro. *Int. J. Food Microbiol.* **133,** 86–93 (2009).

44. Mogensen, T. H. Pathogen Recognition and Inflammatory Signaling in Innate Immune Defenses. *Clin. Microbiol. Rev.* **22,** 240–273 (2009).

45. Paolillo, R., Romano Carratelli, C., Sorrentino, S., Mazzola, N. & Rizzo, A. Immunomodulatory effects of *Lactobacillus plantarum* on human colon cancer cells. *Int. Immunopharmacol.* **9,** 1265–1271 (2009).

46. Gao, K. *et al.* Immunomodulation and signaling mechanism of *Lactobacillus rhamnosus* GG and its components on porcine intestinal epithelial cells stimulated by lipopolysaccharide. *J. Microbiol. Immunol. Infect.* **50,** 700–713 (2017).

47. Kumar, M., Kissoon-Singh, V., Coria, A. L., Moreau, F. & Chadee, K. Probiotic mixture VSL#3 reduces colonic inflammation and improves intestinal barrier function in Muc2 mucin-deficient mice. *Am. J. Physiol.-Gastrointest. Liver Physiol.* **312,** G34–G45 (2017).

48. Trindade, L. M. *et al.* Oral administration of Simbioflora® (synbiotic) attenuates intestinal damage in a mouse model of 5-fluorouracil-induced mucositis. *Benef. Microbes* **9,** 477–486 (2018).

49. Bowen, J. M. *et al.* VSL#3 probiotic treatment reduces chemotherapy-induced diarrhea and weight loss. *Cancer Biol. Ther.* **6,** 1449–1454 (2007).

50. Kato, S. *et al.* Probiotic *Bifidobacterium bifidum* G9-1 attenuates 5-fluorouracil-induced intestinal mucositis in mice via suppression of dysbiosis-related secondary inflammatory responses. *Clin. Exp. Pharmacol. Physiol.* **44,** 1017–1025 (2017).

51. Lycke, N. Y. & Bemark, M. The regulation of gut mucosal IgA B-cell responses: recent developments. *Mucosal Immunol.* **10,** 1361–1374 (2017).

52. Schmucker, D. L., Owen, R. L., Outenreath, R. & Thoreux, K. Basis for the Age-related Decline in Intestinal Mucosal Immunity. *Clin. Dev. Immunol.* **10,** 167–172 (2003).

53. Corridoni, D. *et al.* Probiotic Bacteria Regulate Intestinal Epithelial Permeability in Experimental Ileitis by a TNF-Dependent Mechanism. *PLoS ONE* **7,** e42067 (2012).

54. Mennigen, R. *et al.* Probiotic mixture VSL#3 protects the epithelial barrier by maintaining tight junction protein expression and preventing apoptosis in a murine model of colitis. *Am. J. Physiol.-Gastrointest. Liver Physiol.* **296,** G1140–G1149 (2009).

55. Henderson, P., van Limbergen, J. E., Schwarze, J. & Wilson, D. C. Function of the intestinal epithelium and its dysregulation in inflammatory bowel disease: *Inflamm. Bowel Dis.* **17,** 382–395 (2011).

56. Mi, H. *et al.* Bifidobacterium Infantis Ameliorates Chemotherapy-Induced Intestinal Mucositis Via Regulating T Cell Immunity in Colorectal Cancer Rats. *Cell. Physiol. Biochem.* **42,** 2330–2341 (2017).

57. Justino, P. F. C. *et al.* Regulatory role of *Lactobacillus acidophilus* on inflammation and gastric dysmotility in intestinal mucositis induced by 5-fluorouracil in mice. *Cancer Chemother. Pharmacol.* **75,** 559–567 (2015).

58. Foligne, B. *et al.* Correlation between in vitro and in vivo immunomodulatory properties of lactic acid bacteria. *World J. Gastroenterol.* **13,** 236–243 (2007).

59. Suschek, C. V., Schnorr, O. & Kolb-Bachofen, V. The role of iNOS in chronic inflammatory processes in vivo: is it damage-promoting, protective, or active at all? *Curr. Mol. Med.* **4,** 763–775 (2004).

60. Niess, J. H., Leithäuser, F., Adler, G. & Reimann, J. Commensal gut flora drives the expansion of proinflammatory CD4 T cells in the colonic lamina propria under normal and inflammatory conditions. *J. Immunol. Baltim. Md 1950* **180,** 559–568 (2008).

Figures legends

Figure 1. *Propionibacterium freudenreichii* Δ*slpB* mutant strain induces expression of pro-inflammatory cytokines in HT-29 cells. mRNA relative expression of cytokines **(A)** IL-10, **(B)** IL-8, **(C)** TNF-α, **(D)** IFN-α, in HT-29 cells, stimulated by lipopolysaccharides (LPS), CB 129 WT, CB 129Δ*slpB*, or combinations thereof, was monitored by RT-PCR. Asterisks represent statistically significant differences between strains and were indicated as follows: ∗p < 0.05; ∗∗p < 0.01; ∗∗∗p < 0.001, and ∗∗∗∗p < 0.0001 (n = 6).

Figure 2. *Propionibacterium freudenreichii* WT strain modulates *in vitro* expression of in Toll-like receptors (TLRs) in HT-29 cells. mRNA relative expression of TLR2 (**A**), TLR9 (**B**), ZO-1 (**C**) and of MUC2 (**D**) genes in HT-29 cells stimulated by lipopolysaccharides (LPS), CB 129 WT, CB 129Δ*slpB*, or combinations thereof, was monitored by RT-PCR. Asterisks represent statistically significant differences between strains and were indicated as follows: ∗p < 0.05; ∗∗p < 0.01; ∗∗∗p < 0.001, and ∗∗∗∗p < 0.0001 (n = 6).

Figure 3. *Propionibacterium freudenreichii* WT strain prevents weight loss in 5-FU-treated mice. (A)Time-course of body weight for mice treated with culture media YEL (control), probiotic strain *P. freudenreichii* 129 WT and mutant strain *P. freudenreichii* Δ*slpB* or without treatment (water) (n = 18). **(B)** Weigh**t** loss observed after 5-FU injection and differences across groups. Asterisks represent statistically significant differences as follows: * p <0.05; ** p <0.01; and *** p <0.001 (n = 18).

Figure 4. *Propionibacterium freudenreichii* WT strain alleviates mucosal damage in the ileum of in 5-FU-treated mice while mutant strain *P. freudenreichii* Δ*slpB* causes inflammation in healthy mice. (A) Histopathological score obtained in healthy and inflamed mice treated (n = 12), and **(B)** Representative images of H&E-stained of mice mucosal ileum histopathology (n = 12). The image acquisition was done with objective

magnification at 20x. Scale bar=100µm. Asterisks represent statistically significant differences as follows: * p <0.05; ** p <0.01; *** p <0.001; **** p <0.0001.

Figure 5. *Propionibacterium freudenreichii* WT strain protects villus architecture and Paneth cells secretory granules density during 5-FU-induced mucositis. Morphometric analysis of villus height **(A)** and crypt depth **(B)** of mice treated with culture media YEL (control), probiotic strain *P. freudenreichii* WT and mutant strain *P. freudenreichii* Δ*slpB* or without treatment (water) following 5-FU or saline administration and ratio villus height /crypt depth **(C)**. Microscopic morphometric analysis of Paneth cell secretory granules **(D)** of mice treated with culture media YEL (control), probiotic strain *P. freudenreichii* WT and mutant strain *P. freudenreichii* Δ*slpB* or without treatment (water) following 5-FU or saline administration.Values were obtained using objective magnification at 40x by measuring ten random images of the ileum of mice. Asterisks represent statistically significant differences as follows: * p <0.05; ** p <0.01; *** p <0.001; **** p <0.0001 (n = 6-9).

Figure 6. Secretory immunoglobulin A (IgA) in intestinal small bowel content. Quantification of immunoglobulin A secretion (sIgA) in the small intestine of healthy or inflamed mice. Asterisks represent statistically significant differences as follows: * p <0.05; ** p <0.01; *** p <0.001; **** p <0.0001 (n = 6).

Figure 7. *Propionibacterium freudenreichii* Δ*slpB* induces expression of IL-17 and of inducible NOS (*iNOS*) in healthy mice. mRNA relative expression of genes **(A)** Muc2, **(B)** Cld1, **(C)** ZO-1, **(D)** Occludin, **(E)** iNOS, and **(F)** IL-17 in mice treated with culture media YEL (control), probiotic strain *P. freudenreichii* WT and mutant strain *P. freudenreichii* Δ*slpB* or without treatment (water) following 5-FU or saline administration. Expression levels was monitored by RT-PCR. Asterisks represent statistically significant differences between strains and were indicated as follows: $*p < 0.05$; $**p < 0.01$; $***p < 0.001$, and $****p < 0.0001$ (n = 6).

Figure 8. *Propionibacterium freudenreichii* WT strain reduces the pro-inflammatory cytokine IL-12 production during 5-FU-induced mucositis. The secreted levels of **(A)** IL-10, **(B)** IL-12, **(C)** IL-1β, and **(D)** IL-10/IL-12 ratio were determined in the supernatant of homogenized ileum mice tissue using an ELISA. Mice was treated with culture media YEL (control), probiotic strain *P. freudenreichii* WT and mutant strain *P. freudenreichii* Δ*slpB* or without treatment (water) following 5-FU or saline administration. Asterisks represent statistically significant differences between strains and were indicated as follows: $*p < 0.05$; $**p < 0.01$; $***p < 0.001$, and $****p < 0.0001$ (n = 4).

Table

Table 1. List of primers used in the *in vitro* study.

GENE	PRIMER	SEQUENCE (5′→3′)	PRODUCT SIZE (bp)	Reference
β-actin	β-actinF	TGG CTG GGT GTT GAA GGT CT	238	(Duary et al., 2014)
	β-actinR	AGC ACG GCA TCG TCA CCA ACT		
Gapdh	GapdhF	CAA CGA CCA CTT TGT CAA GC	140	
	GapdhR	TTC CTC TTG TGC TCT TGC TG		
IL-8	IL-8F	TGG CTC TCT TGG CAG CCT TC	238	
	IL-8R	TGC ACC CAG TTT TCC TTG GG		
TNFα	TNFαF	AGC CCA TGT TGT AGC AAA CC	134	
	TNFαR	TGA GGT ACA GGC CCT CTG AT		
IL-10	IL-10F	AAA GAA GGC ATG CAC AGC TC	132	
	IL-10R	AAG CAT GTT AGG CAG GTT GC		
IFNα	IFNαF	CTG AAA CCA TCC CTG TCC TC	147	
	IFNαR	CAC AGG CTT CCA GGT CAT TC		
MUC2	MUC2F	CAG CAC CGA TTG CTG AGT TG	140	
	MUC2R	GCT GGT CAT CTC AAT GGC AG		
ZO1	ZO1F	GAA TGA TGG TTG GTA TGG TGC G	191	(Carrasco-Pozo et al., 2013)
	ZO1R	TCA GAA GTG TGT CTA CTG TCC G		
TLR9	TLR9F	GAG CGC AGT GGC AGA CTG GGT G	132	(Vizoso Pinto et al., 2009)
	TLR9R	CAC AGG TTC TCA AAG AGG GT		
TLR2	TLR2F	GCA GAA GCG CTG GGG AAT GG	300	
	TLR2R	GGA TGC CTA CTG GGT GGA GAA		

Figures

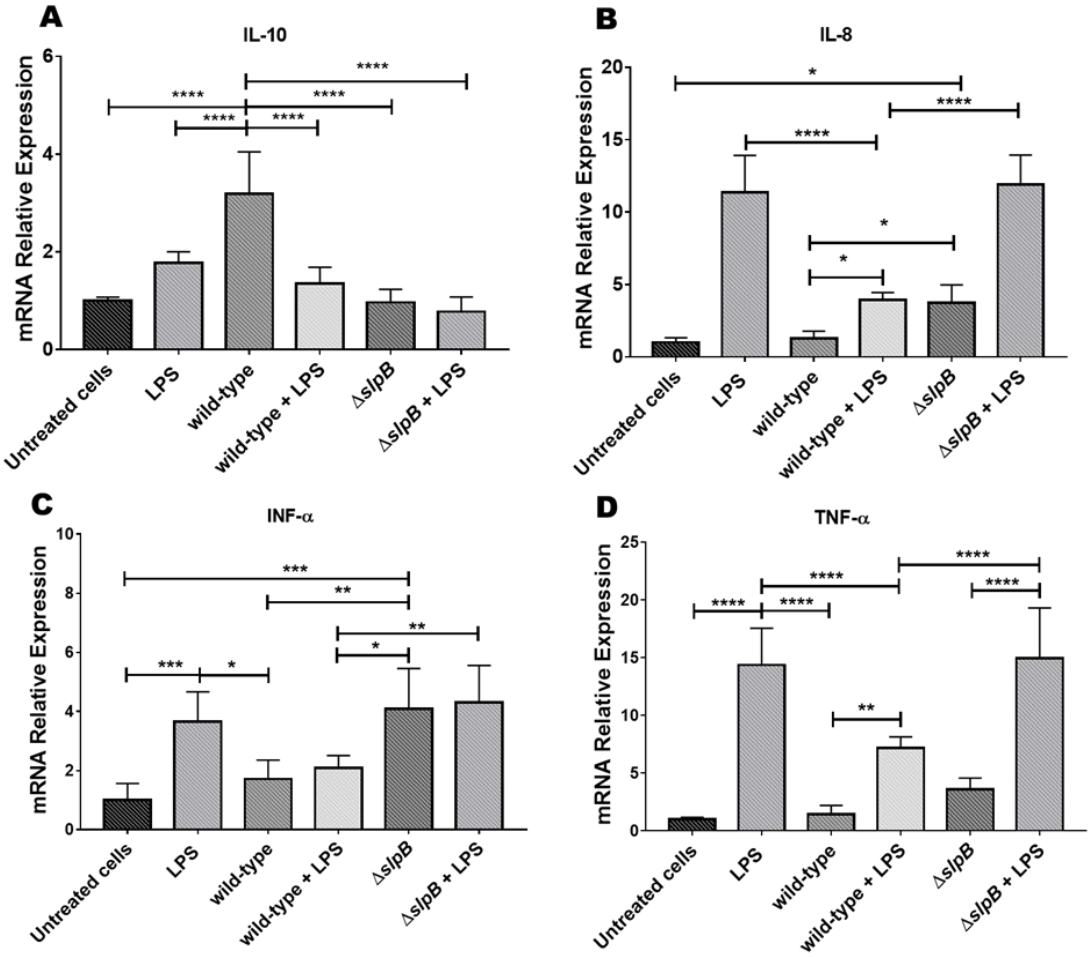


Figure 1.

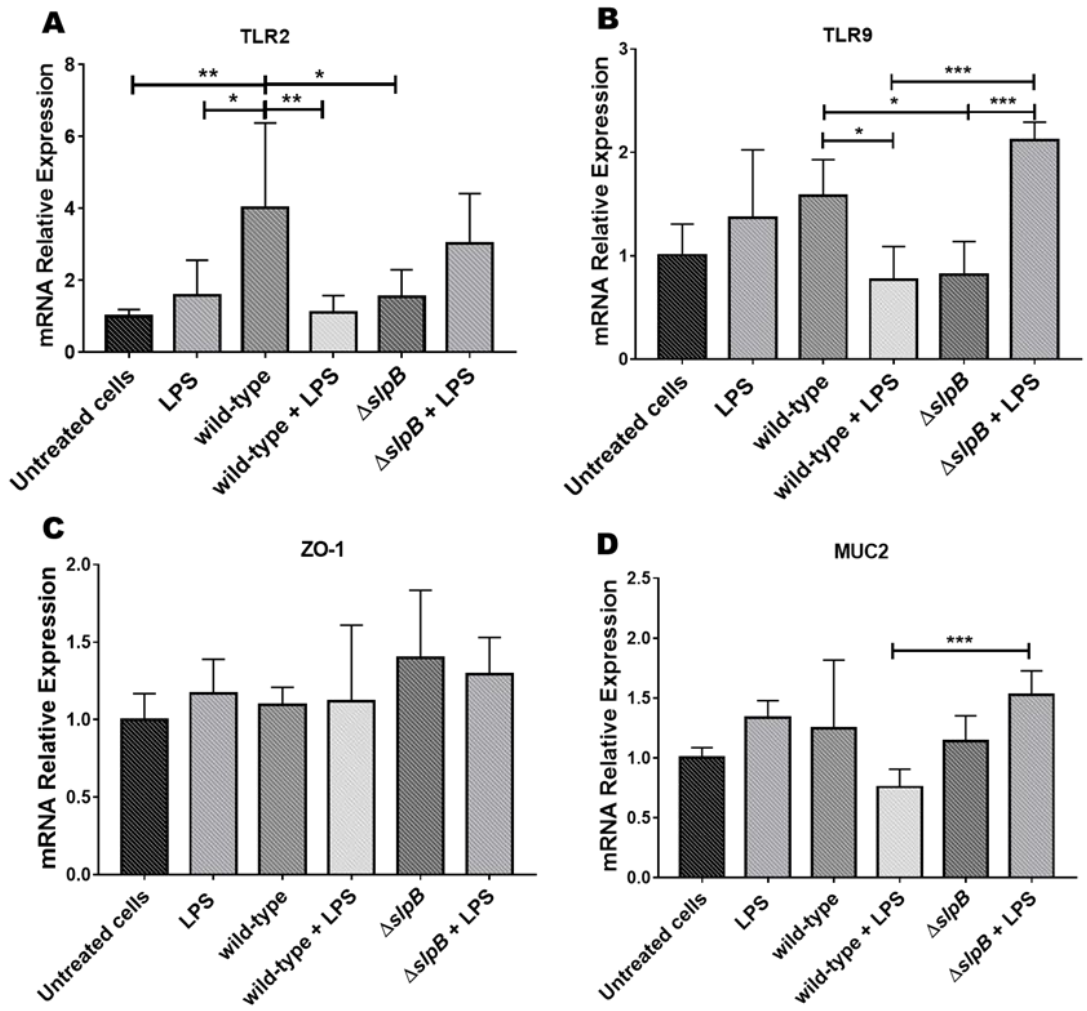

A
TLR2
mRNA Relative Expression
0
2
4
6
8
**
*
*
**
Untreated cells
LPS
wild-type
wild-type + LPS
ΔslpB
ΔslpB + LPS
B
TLR9
mRNA Relative Expression
0
1
2
3
***
*
*
***
Untreated cells
LPS
wild-type
wild-type + LPS
ΔslpB
ΔslpB + LPS
C
ZO-1
mRNA Relative Expression
0.0
0.5
1.0
1.5
2.0
Untreated cells
LPS
wild-type
wild-type + LPS
ΔslpB
ΔslpB + LPS
D
MUC2
mRNA Relative Expression
0.0
0.5
1.0
1.5
2.0
2.5
***
Untreated cells
LPS
wild-type
wild-type + LPS
ΔslpB
ΔslpB + LPS

Figure 2.

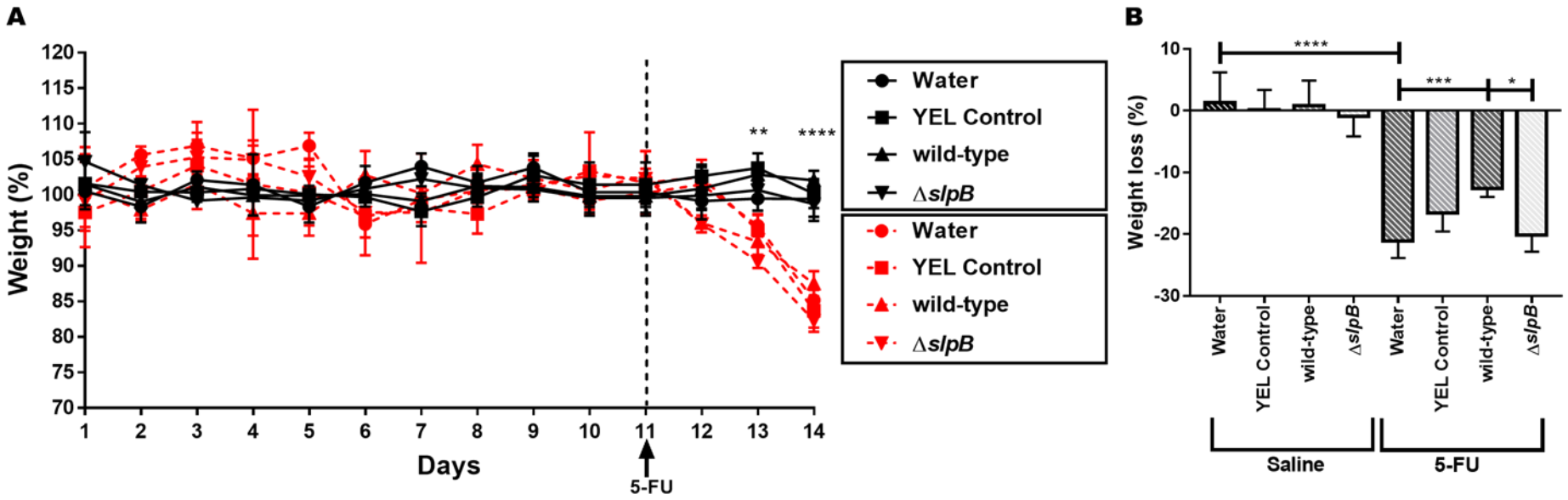

A
Weight (%)
Days
5-FU
**
****
Water
YEL Control
wild-type
ΔslpB
B
Weight loss (%)
****
***
*
Saline
5-FU

Figure 3.

131

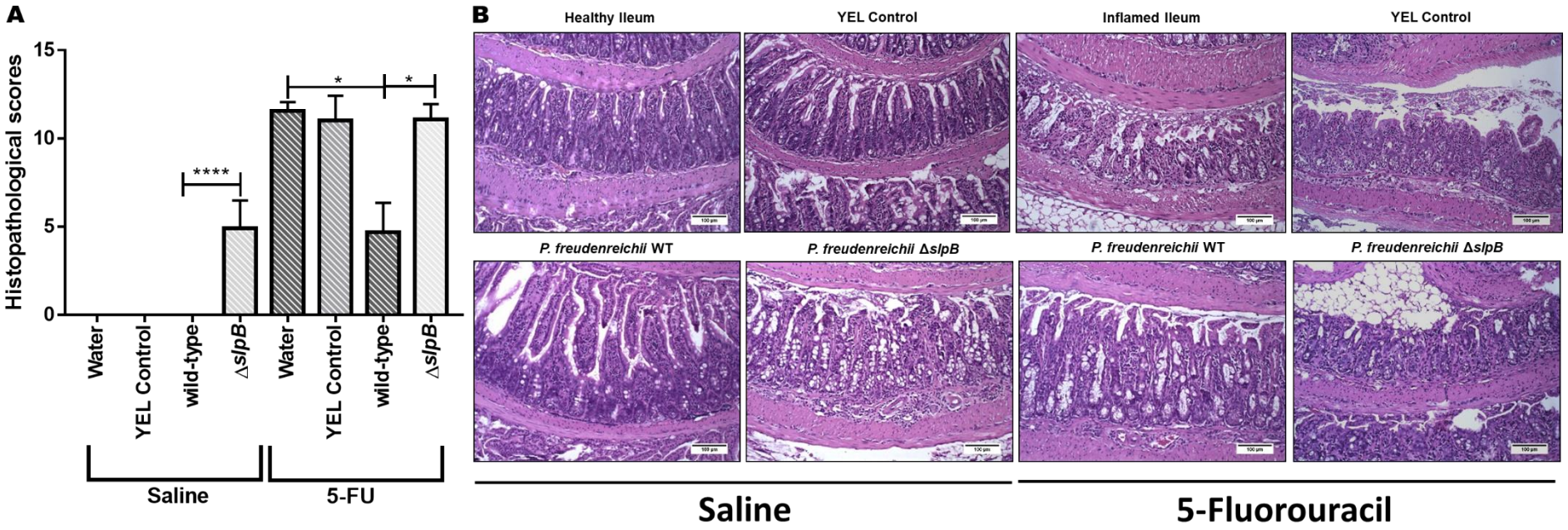
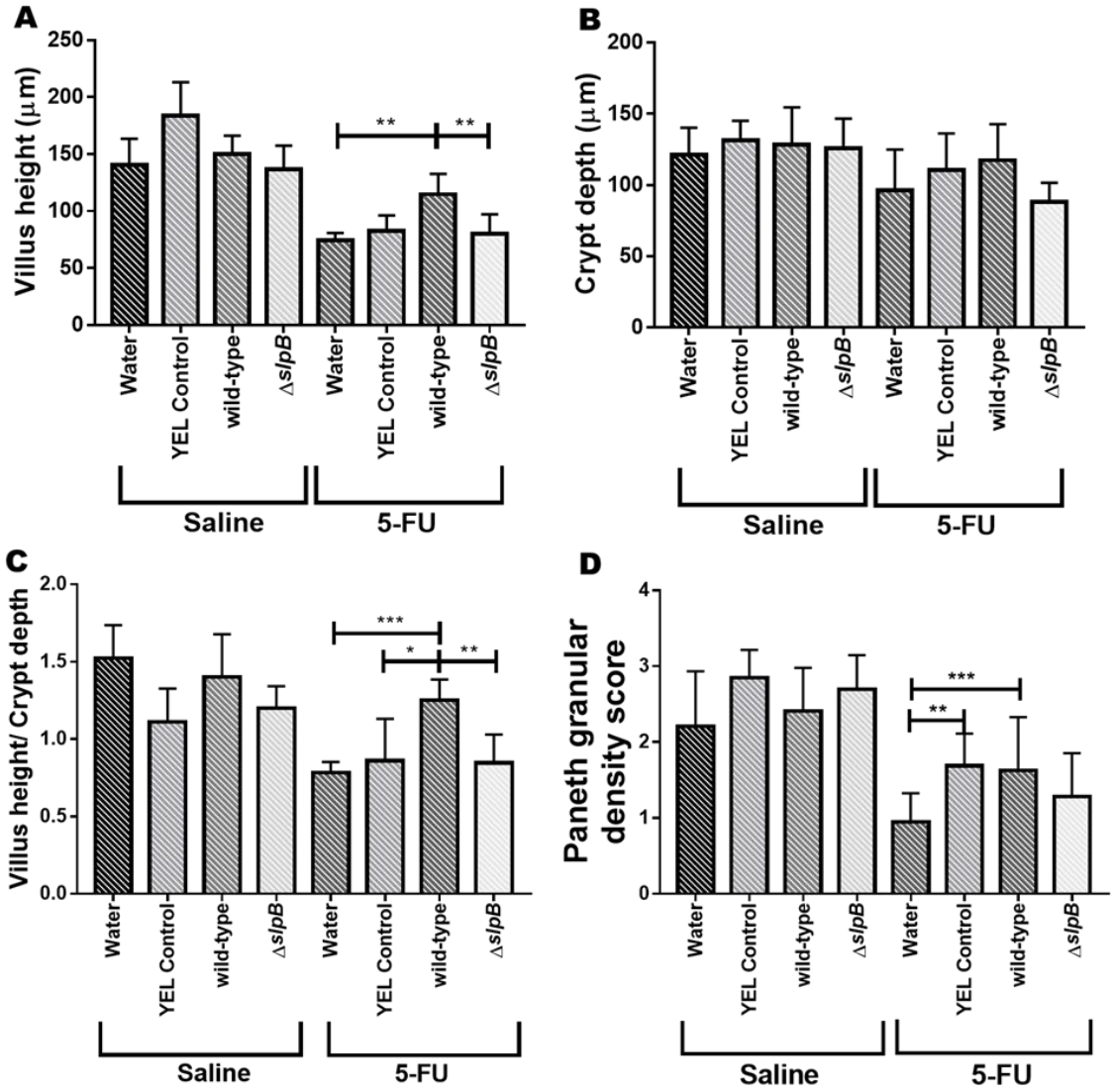

A
Histopathological scores
15
10
5
0
****
*
*
Water
YEL Control
wild-type
ΔslpB
Water
YEL Control
wild-type
ΔslpB
Saline
5-FU
B
Healthy Ileum
YEL Control
Inflamed Ileum
YEL Control
P. freudenreichii WT
P. freudenreichii ΔslpB
P. freudenreichii WT
P. freudenreichii ΔslpB
Saline
5-Fluorouracil

Figure 4.

132



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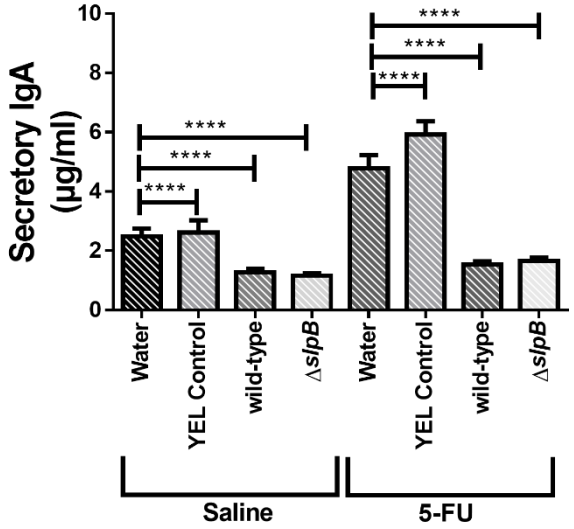

Figure 6.

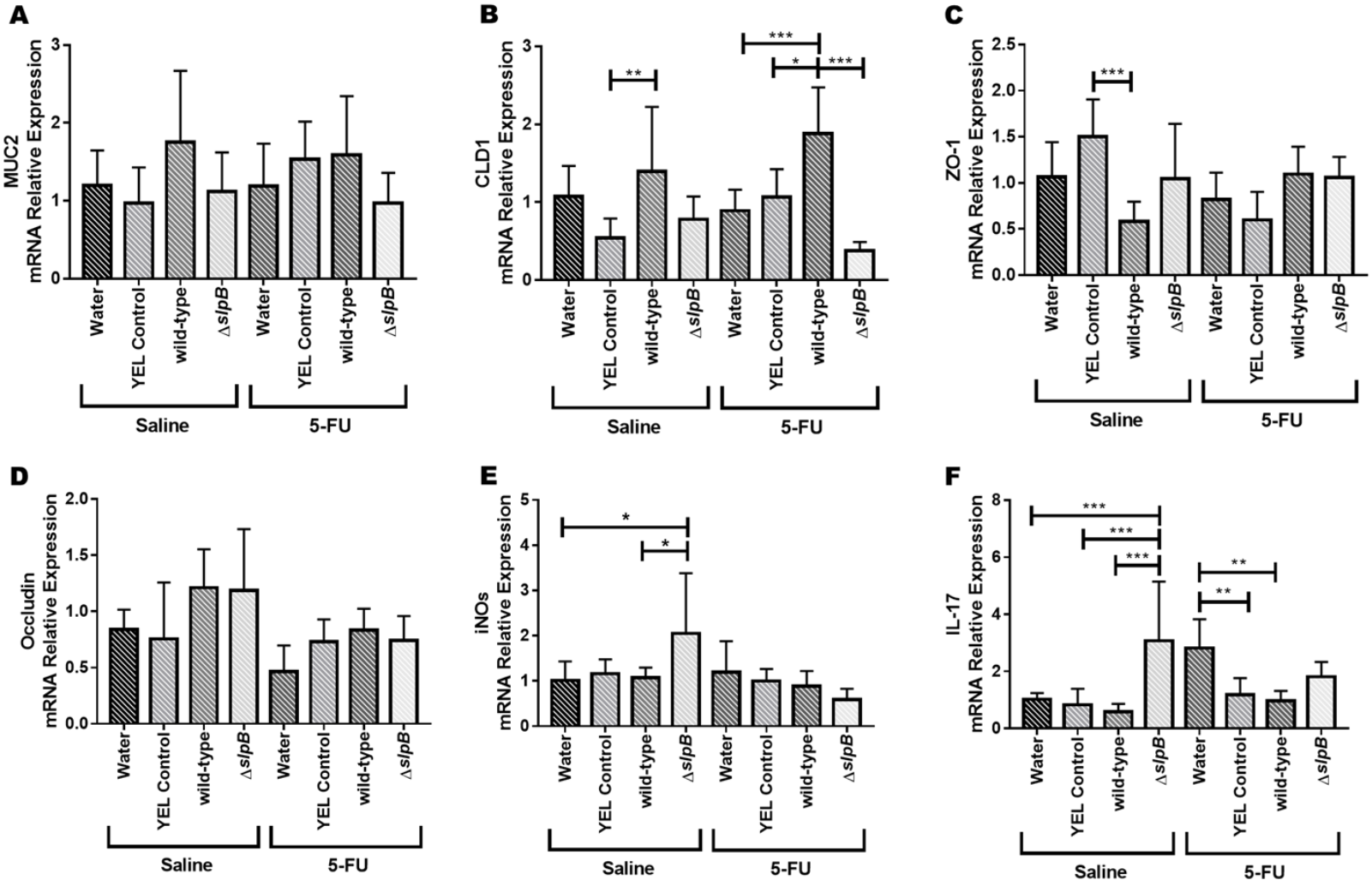
A
MUC2
mRNA Relative Expression
B
CLD1
mRNA Relative Expression
C
ZO-1
mRNA Relative Expression
D
Occludin
mRNA Relative Expression
E
iNOs
mRNA Relative Expression
F
IL-17
mRNA Relative Expression
Water
YEL Control
wild-type
ΔslpB
Saline
5-FU

Figure 7.

134

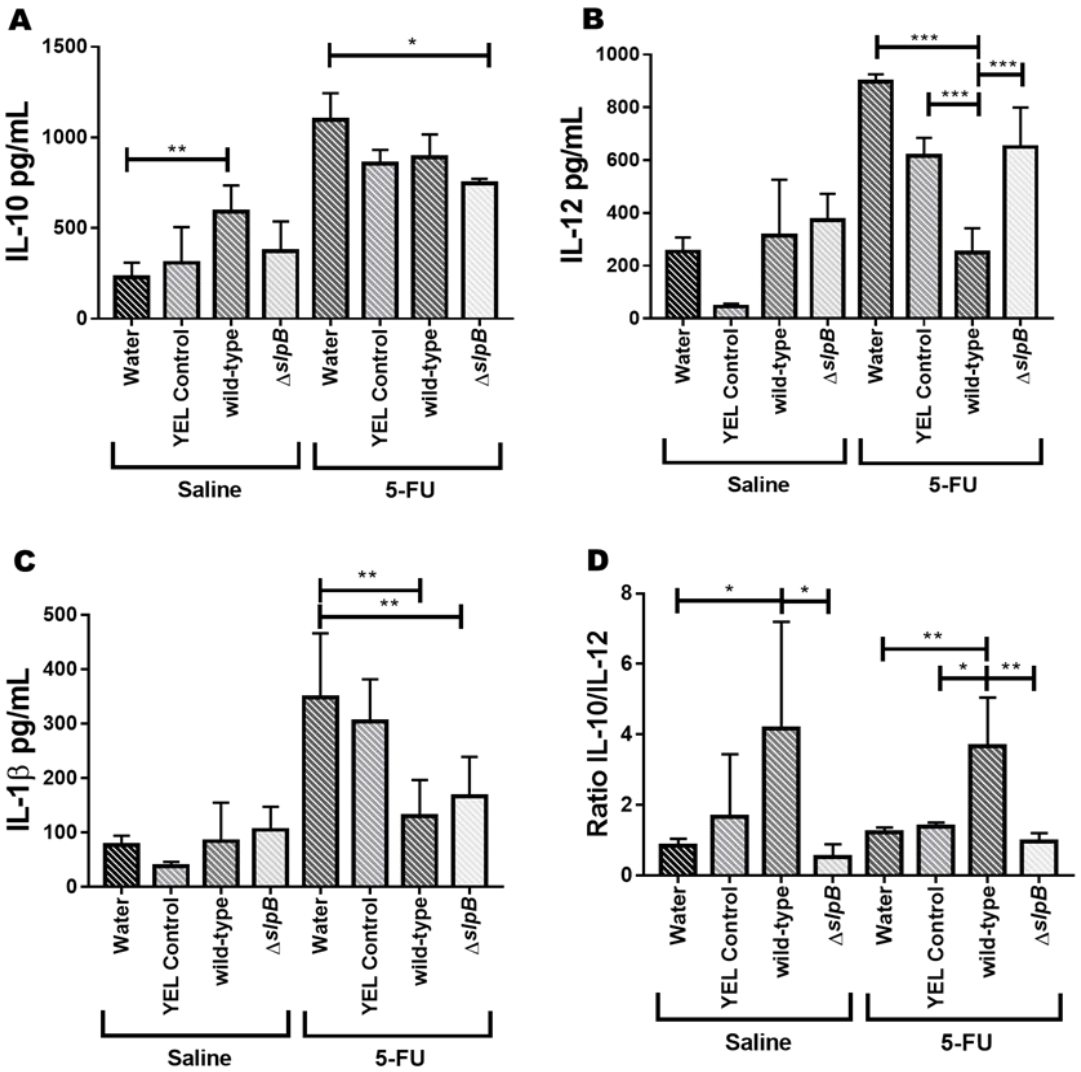
A
IL-10 pg/mL
1500
1000
500
0
**
*
Water
YEL Control
wild-type
ΔslpB
Saline
5-FU
B
IL-12 pg/mL
1000
800
600
400
200
0
***
***
***
C
IL-1β pg/mL
500
400
300
200
100
0
**
**
D
Ratio IL-10/IL-12
8
6
4
2
0
*
*
**
*
**

Figure 8.

CHAPTER 6 – ORIGINAL ARTICLE - WHEY PROTEIN ISOLATE-SUPPLEMENTED BEVERAGE FERMENTED BY *Lactobacillus casei* BL23 AND *Propionibacterium freudenreichii* 138 IN THE PREVENTION OF MUCOSITIS IN MICE

As demonstrated in the previous section, the probiotic strain *P. freudenreichii* CIRM-BIA 129 was able to decrease mucosal inflammation in a mucositis mice model induced by 5-FU. This opens a new perspective to explore the dairy propionibacteria potential in a pathological situation other than colitis.

Regarding previous studies, probiotic bacteria such as members of the *Lactobacillus* genus have shown the potential to prevent histological damage in mice with mucositis induced by 5-FU. This was evidenced using selected strains like *Lactobacillus acidophilus* and *Lactobacillus casei* variety *rhamnosus*, taken separately (JUSTINO et al., 2015; YEUNG et al., 2015), or in association with the other bacteria *Bifidobacterium bifidum* and *Lactobacillus acidophilus* (YEUNG et al., 2015). Moreover, Trindade and collaborators recently showed that a commercial formulation known as Simbioflora®, which contains *Lactobacillus paracasei, Lactobacillus rhamnosus, Lactobacillus acidophilus* and *Bifidobacterium lactis* in combination with the prebiotic fructooligosaccharide (FOS), was able to reduce mucosal damage in the same mucositis model (5-FU) (TRINDADE et al., 2018). In a certain way, probiotic potential of the selected strains can be potentiated by using in prebiotic together in a formulation.

Developing functional foods is a promising research and development area, mainly by incorporation of select probiotic strains with proven therapeutic potential in preclinical studies (BELL; FERRÃO; FERNANDES, 2017). In addition, fermented beverages, fermented by probiotic bacteria, have functional food status. Experimental evidences reveal that fermented milk, taking advantage of increased production of beneficial metabolites, enhance the therapeutic effects in the context of intestinal inflammatory disorders (DO CARMO et al., 2017a; SHIBY; MISHRA, 2013). Furthermore, protective matrices, such as whey proteins, were able to increase this effect by increasing viability of the probiotic bacteria used in the fermentation (BEAULIEU; DUPONT; LEMIEUX, 2007; MARTEAU, 2006).

In a timely manner, dairy propionibacteria produces metabolites which can be considered prebiotics, such as the bifidogenic DHNA and ACNQ, and vitamin B9 and B12. These metabolites have been shown to be effective in murine models of

inflammatory diseases and therefore constitute good candidates for the treatment of mucositis as well (COUSIN et al., 2012, 2016; PLÉ et al., 2015). Selected *P. freudenreichii* strains could also be promising candidates for the treatment of this emergent disease. Mucositis occurrence is indeed rising today, due to the high cancer rates in the world population and consequently to the treatments based on chemotherapies.

In this section, we investigate the therapeutic effect of administration of whey protein isolate-supplemented beverage fermented by *Lactobacillus casei* BL23 or by *P. freudenreichii* CIRM-BIA 138 strains. This innovative beverage was able to exert beneficial effects in mice submitted to experimental mucositis.

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CHAPTER 6 – ARTICLE DE RECHERCHÉ ORIGINAL- WHEY PROTEIN ISOLATE-SUPPLEMENTED BEVERAGE FERMENTED BY *Lactobacillus casei* BL23 AND *Propionibacterium freudenreichii* 138 IN THE PREVENTION OF MUCOSITIS IN MICE

Comme l'ont montré les sections précédentes, la souche probiotique *P. freudenreichii* CIRM-BIA 129 est capable de réduire l'inflammation de la muqueuse induite dans un modèle murin de mucosite induite par le 5-FU. Ceci ouvre de nouvelles perspectives pour l'exploration du potentiel de bactéries propioniques laitières dans une autre situation pathologique.

Quant à d'autres études précédentes, des bactéries probiotiques telles que des lactobacilles, en particulier, ont également montré le potentiel de prévenir les dommages histologiques dans le même modèle murin de mucosite induite par le 5-FU. C'est le cas d'études mettant en œuvre des souches telles que *Lactobacillus acidophilus* et *Lactobacillus casei* variété *rhamnosus* , séparément (JUSTINO et al., 2015; YEUNG et al., 2015), ou associés à d'autres bactéries *Bifidobacterium bifidum* et *Lactobacillus acidophilus* (YEUNG et al., 2015).

De plus, Trindade et al. ont montré récemment qu'une formulation commerciale connue sous le nom de Simbioflora®, qui contient *Lactobacillus paracasei, Lactobacillus rhamnosus, Lactobacillus acidophilus* et *Bifidobacterium lactis*, en combinaison avec le prébiotique fructooligosaccharide (FOS), était capable de réduire les dommages provoqués sur la muqueuse dans le même modèle de mucosite (TRINDADE et al., 2018). D'une certaine façon, le potentiel probiotique des souches bactériennes sélectionnées peut être potentialisé par l'utilisation d'un prébiotique, en conjonction avec les probiotiques, dans une formulation.

Le développement de nouveaux aliment fonctionnels constitue un domaine de recherche et développement prometteur, principalement par l'exploitation de de souches probiotiques sélectionnées et caractérisées, avec un potentiel thérapeutique démontré dans des études précliniques (BELL; FERRÃO; FERNANDES, 2017). De plus, les boissons fermentées par des bactéries probiotiques constituent des aliments fonctionnels. Il existe des évidences expérimentales que le lait fermenté améliore la production de métabolites bénéfiques, et donc de l'effet probiotique, dans le contexte de désordres intestinaux inflammatoires. (DO CARMO et al., 2017a; SHIBY; MISHRA,

2013). De plus, des matrices protectrices, telles que les protéines laitières sériques, sont capables d'augmenter cet effet en améliorant la viabilité du probiotique utilisé dans la fermentation (BEAULIEU; DUPONT; LEMIEUX, 2007; MARTEAU, 2006).

Les bactéries propioniques laitières produisent des métabolites qui peuvent être considérés probiotiques, le DHNA et l'ACNQ, qui favorisent la croissance des bifidobactéries, et les vitamines B9 et B12. Ces métabolites se sont révélés efficaces dans un modèle murin de maladie inflammatoire et constituent donc de bons candidats pour le traitement de la mucosite (COUSIN et al., 2012, 2016; PLÉ et al., 2015). Des souches sélectionnées de *P. freudenreichii* constituent également de bons candidats pour le traitement de cette maladie en augmentation, étant donnée son occurrence liée au taux élevés de cancers dans la population mondiale, et donc aux chimiothérapies.

Dans cette section, nous avons étudié les effets thérapeutiques d'une boisson fermentée supplémentée en protéines sériques, fermentée par *Lactobacillus casei* BL23 ou *P. freudenreichii* CIRM-BIA 138. Ces boissons ont permis d'exercer un effet bénéfique chez des souris soumises à une mucosite expérimentale causée par le 5-FU.

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ORIGINAL RESEARCH
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Whey Protein Isolate-Supplemented Beverage, Fermented By *Lactobacillus Casei* BI23 And *Propionibacterium Freudenreichii* 138, In The Prevention Of Mucositis In Mice

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Mucositis is a clinically important gastrointestinal inflammatory infirmity, generated by antineoplastic drugs cytotoxic effects. The inflammatory process caused by this disease frequently leads to derangements in the alimentary tract and great malaise for the patient. Novel strategies are necessary for its prevention or treatment, as currently available treatments of mucositis have several limitations in relieving its symptoms. In this context, several research groups have investigated the use of probiotic bacteria, and in particular dairy bacterial strains. Compelling evidences reveal that milk fermented by certain probiotic bacteria has the capacity to ameliorate intestinal inflammatory disorders. In addition, innovative probiotic delivery strategies, based on probiotics incorporation into protective matrices, such as whey proteins, were able to increase the therapeutic effect of probiotic strains by providing extra protection for bacteria against environmental stresses. Therefore, in this study, we evaluated the role of the whey protein isolate (WPI), when added to skim milk fermented by *Lactobacillus casei* BL23 (*L. casei* BL23) or by *Propionibacterium freudenreichii* CIRM-BIA138 (*P. freudenreichii* 138), as a protective matrix against *in vitro* stress challenges. In addition, we investigated the therapeutic effect of these fermented beverages in a murine model of mucositis induced by 5-Fluorouracil (5-FU). Our results demonstrated that milk supplementation with 30% (w/v) of WPI increases the survival rate of both strains when challenged with acid, bile salts, high temperature and cold storage stresses, compared to fermented skim milk without the addition of WPI. Moreover, treatment with the probiotic beverages prevented weight loss and intestinal damages in mice receiving 5-FU. We conclude that the presence of WPI maximizes the anti-inflammatory effects of *L. casei* BL23, but not for *P. freudenreichii* 138, suggesting that whey protein enhancement of probiotic activity might be strain-dependent.

Keywords: *Lactobacillus*, mucositis, probiotics, *Propionibacterium*, stress tolerance, whey protein isolate

INTRODUCTION

Mucositis is a severe inflammation that affects the entire extension of the Alimentary Tract (AT) of individuals undergoing malignancy treatment based on chemotherapy or radiotherapy (Sonis, 2004). One of the main drugs associated with this condition is 5-Fluorouracil (5-FU). This is a antimetabolic drug commonly prescribed for the treatment of head, neck and gastrointestinal cancer (Longley et al., 2003). 5-FU unfortunately presents non-specific cytotoxicity toward cells, inhibiting the proliferation of both cancer cells and normal cells with high replication rates, such as the enterocytes of the gastrointestinal tract (GIT) (Carvalho et al., 2017a). A series of clinical symptoms, such as nausea, weight loss, vomiting, severe abdominal pain and diarrhea are commonly reported in patients receiving 5- FU during cancer treatment (Bastos et al., 2016). Moreover, mucositis frequently increases predisposition to local and systemic secondary infections, thus generating additional costs and extending the patient's hospitalization time (Carvalho et al., 2017a). Mucositis is characterized by pathological changes in the small bowel. These changes include the presence of degenerate enterocytes (Ciorba et al., 2016), submucosal vessel damage, leukocyte infiltrate in the *lamina propria*, with accumulation of neutrophils and eosinophils (Antunes et al., 2016), increased mucin production and degeneration of goblet cells (Stringer, 2013), atrophy of villi, hypoplasia and apoptosis of intestinal crypts (Chang et al., 2012). Currently, there is no treatment that is completely successful in the prevention and treatment of mucositis. However, there has been a growing interest in the use of probiotics as promising candidates for the treatment of this disease (Carvalho et al., 2017a).

Probiotics are included in a variety of products, including fermented foods, dietary supplements, formulas for newborns and infants, as well as various pharmaceutical formulations (Cousin et al., 2011). Currently, fermented beverages by one or more bacteria, have gained the functional food status, which makes them an important part of our diet as well as our main daily source of beneficial microbes (Leroy and De Vuyst, 2014; Carmo et al., 2017).

Selected strains of lactic acid bacteria (LAB) were reported as probiotic with beneficial effects provided by different mechanisms of action and can be used in functional foods withal (Carvalho et al., 2017b; Eales et al., 2017; Tang et al., 2017). Some studies have shown that the administration of lactobacilli strains can reduced some parameters of mucositis in mice model induced by 5-FU, such as prevent weight loss, attenuate the diarrhea and intestinal damage (Justino et al., 2015). *L. casei* BL23 has also been considered as a good probiotic strain, according to results obtained in others inflammatory models. Some studies demonstrated that *L. casei* BL23 was able to alleviates colitis symptoms in a dextran sulfate sodium (DSS) model (Foligne et al., 2007; Rochat et al., 2007) and the ability of these strain to attenuate intestinal inflammation can be enhanced using a protection matrix (Lee et al., 2015b). Other important group of bacteria widely used in the food industry, particularly in Swiss-type cheese manufacture, is propionic acid bacteria (PAB). *Propionibacterium freudenreichii* represents the main species thereof and is listed in the Qualified Presumption of Safety list (QPS) by the European food safety authority and it has recently been considered a promising probiotic (Rabah et al., 2017). *P. freudenreichii* produces metabolites are considered as prebiotics, such as 1,4- dihydroxy-2-naphtoic acid (DHNA) and 2-amino-3-carboxy- 1,4-naphthoquinone (ACNQ), both associated to bifidogenic effects. Furthermore, *P. freudenreichii* is the only GRAS bacterial species producing food-grade vitamin B12 at the industrial scale (Rabah et al., 2017). Selected *P. freudenreichii* strains have been associated with therapeutical effects based on *in vitro* and *in vivo* properties to attenuate colitis model induced by trinitrobenzene sulfonic acid (TNBS) (Cousin et al., 2012a, 2016; Plé et al., 2015). A dairy propionibacteria, *P. freudenreichii* 138, demonstrated a pro-apoptosis capacity, in HTG-1 human gastric cancer cells without toxicity effects in healthy human cells (Cousin et al., 2012a, 2016). Moreover, Cousin et al. (2012b) shown the persistence of *P. freudenreichii* 138 in piglets colon, withal metabolic activity and producing propionate, which is a SCFA with probiotic properties (Cousin et al., 2012b).

An extremely important factor for the therapeutic effects of probiotics is the ability of the bacteria to survive during transit through the GIT or during industrial processes (Cousin et al., 2012b; Rabah et al., 2017). Digestion indeed imposes harsh conditions including gastric acid and presence of bile salts, which may severely affect bacterial viability (Leroy and De Vuyst, 2014; Huang et al., 2016a). A probiotic microorganism must, however, tolerate these stresses for a long persistence in the host and for an enhanced beneficial effect (Carmo et al., 2017). In the aim to maximize the tolerance of bacteria to stressful environments and thus to increase their probiotic ability, the food matrix used in the manufacture of fermented products plays a key role as a protective medium (Gagnaire et al., 2015; Lee et al., 2015a). As an example, milk proteins, as well as whey protein isolates, constitute very promising protective matrices for probiotics, besides being an efficient delivery vehicles to target protective molecules and microorganisms to digestive epithelium (Livney, 2010; Cousin et al., 2012a; Vargas et al., 2015; Huang et al., 2016a). Whey proteins have been recognized for their various functional and nutritional properties. The functional properties are mainly due to their physical, chemical and structural characteristics and the nutritional value is directly linked to the concentration of essential amino acids (Yadav et al., 2015). Some studies have also demonstrated the potential of whey proteins to enhance the survival and viability of probiotic bacteria during production and storage (Marshall, 2004; Madureira et al., 2007; Almeida et al., 2009; Huang et al., 2016b; Baruzzi et al., 2017; Dąbrowska et al., 2017).

The aims of this work were (i) to evaluate whether whey protein isolate is a good protective matrix for *L. casei* BL23 and *P. freudenreichii* CIRM-BIA 138, against adverse environmental conditions and (ii) to investigate the therapeutic effect of administration of whey protein isolate-supplemented beverage, fermented by both strains, in the prevention of mucositis induced by 5-FU.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions

Lactobacillus casei BL23 strain was kindly supplied by the UMR1219 Micalis Institute (INRA-AgroParisTech, Jouy-En-Josas, France) and the *P. freudenreichii* CIRM-BIA138 (alias ITG P9) strain by the Biological Resource Center (International Center of Microbial Resources, INRA, Rennes, France). *L. casei* BL23 was grown in MRS broth at 37°C for 24 h, without shaking. *P. freudenreichii* 138 was grown in YEL culture medium at 30°C for 72 h, without agitation (Malik et al., 1968).

Dairy Beverage Formulation and Supplementation With Whey Protein Isolate

The fermented beverage was prepared using skimmed milk (SM) powder 12% w/v (Itambé, Brazil). For cultivation of *L. casei* BL23 (SMLC), the SM was supplemented with yeast extract (Kasvi Curitiba, Brazil) and glucose (Merck, Germany) (Tharmaraj and Shah, 2003). For cultivation of *P. freudenreichii* 138 (SMPF), the milk was supplemented with casein peptone (5g/L) (KASVI, Curitiba, Brazil) and sodium lactate (50 m/M) (Sigma, St. Louis, MO, United States) (Cousin et al., 2012b). Both milk were autoclaved at 110°C for 15 min. The SMs was supplemented with whey protein isolate (WPI), natural flavor, 90% protein (Vulgo Supplements, Brazil) at concentrations of 5, 15, and 30% w/v, and strains cultivated in skimmed milk without WPI was used as control. The same growth conditions used to cultivate the strains in MRS or YEL were applied for growth in fermented beverages.

Stress Challenges

Samples of 10 ml from the stationary-phase of *L. casei* BL23 and *P. freudenreichii* 138 in culture media or in skim milk, supplemented or not with WPI were subjected to acid, bile salts, and heat challenges (Huang et al., 2016b). For acid stress, the samples were incubated in MRS broth or YEL broth, previously adjusted to pH 2.0 using HCl, at 37°C for 60 min. Briefly, for bile salts stress, the samples were incubated in MRS broth or YEL broth containing 1.0 g/L of bile salts (an equimolar mixture of cholate and deoxycholate, Sigma Chemical, St. Louis, MO, United States) and then, incubated at 37°C for 60 min. Finally, to simulate the pasteurization temperature established by the International Dairy Foods Association (IDFA), the samples were incubated in their specific culture media pre-heated to 63°C for 30 min. After stresses challenges, aliquots of each sample were subjected to 1:10 serial dilutions using peptone water (9 g/L peptone, 5 g/L NaCl) and plated on MRS agar or YEL agar medium. Plates of *L. casei* BL23 were incubated for 48 h at 37°C. Plates of *P. freudenreichii* 138 were incubated for 144 h (6 days) at 30°C in jars containing anaerobiosis generator (Anaerocult A®, Merck Millipore). The number of viable bacteria was determined by counting of colony forming unit (CFU) after incubation. The bacteria survival rate (%) through each stress condition was calculated through the following equation (Ferreira et al., 2017):

$$\text{Survival Rate}\,(\%): \frac{Log\ N}{(\log N_0 \times 100)} \quad (1)$$

Where N refers to the number of bacteria population (CFU mL^{-1}) in culture medium after stress challenges, and N_0 refers to the number of initial population (CFU mL^{-1}) before the stress challenges.

Bacterial Survival During Storage at 4◦C

The long-term survival of *L. casei* BL23 and *P. freudenreichii* 138 in dairy beverage supplemented with 30% of WPI was assessed during the storage process at 4°C for 90 days kept away from light (Huang et al., 2016b). For the evaluation of bacterial survival during cold storage, plate seeding was performed on days 0 (pre-storage time), 7, 14, 21, 30, 60, and 90 after storage. The *L. casei* BL23 and *P. freudenreichii* 138 plates were incubated according to their specific conditions on agar media (see above). The number of viable bacteria during storage was determined by counting CFU in the culture after incubation. The evolution of acidification of stored samples was also screened in the same days. To evaluate if both fermented beverages could survive GIT stress conditions after being stored at 4°C, we performed acid stress and biliary stress *in vitro* challenges for the dairy beverage supplemented with 30% of WPI. Acid and bile salts stresses were performed on days 30, 60, and 90 after the storage start.

Evaluation of Therapeutic Effects of Beverages Fermented by *L. casei* BL23 or *P. freudenreichii* 138 in a Mice Model of Mucositis

Animals

Conventional female BALB/c mice between 6 and 8 weeks of age were obtained at Federal University of Minas Gerais (UFMG– Belo Horizonte, Brazil). Mice were kept in a temperature- controlled room with *ad libitum* access to water and standard chow diet. The study was approved by the Ethics Committee on Animal Experimentation of the Federal University of Minas Gerais (CEUA-UFMG, Brazil, protocol 366).

Probiotic Treatment, Mucositis Induction, and Experimental Groups

For probiotic treatment, mice received 0.5 ml of fermented beverages supplemented or not with 30% of WPI via gastric gavage, during 13 days. In order to induce mucositis, mice received a single intraperitoneal injection of 5-FU (Fauldfluor – Libbs) (300 mg/kg) on day 11, and were euthanized 72 h after induction of mucositis, in 14th of experimental day (Carvalho et al., 2017a). An injection of saline (NaCl 0.9%) was used in control groups. After euthanasia, a longitudinal abdominal incision was performed to remove the intestine for further analyses. Body weight of mice was determined throughout the experiment. For *in vivo* experimentation, BALB/c mice were divided into sixteen groups according to **Table 1**. All experiments

TABLE 1 | Experimental groups and the respective treatments.

Non-inflamed groups Injection of 300 mg/kg of saline (0.9% NaCl)		Inflamed groups Injection of 300 mg/kg of 5-FU	
Group	Treatment	Group	Treatment
Water	H_2O	Water	H_2O
SMLC + WPI BL23 + WPI	Skim milk specific for *L. casei*	SMLC + WPI BL23 + WPI	Skim milk specific for *L. casei*
SMPF + WPI *P. freudenreichii* 138 + WPI	Skim milk specific for	SMPF + WPI *P. freudenreichii* 138 + WPI	Skim milk specific for
SMLC + BL23 BL23 fermented by *L. casei* BL23	Skim milk specific for *L. casei*	SMLC + BL23 BL23 fermented by *L. casei* BL23	Skim milk specific for *L. casei*
SMLC + WPI + BL23 BL23 fermented by *L. casei* BL23 + WPI	Skim milk specific for *L. casei*	SMLC + WPI + BL23 BL23 fermented by *L. casei* BL23 + WPI	Skim milk specific for *L. casei*
SMPF + 138 *P. freudenreichii* 138 fermented by *P. freudenreichii* 138	Skim milk specific for	SMPF + 138 *P. freudenreichii* 138 fermented by *P. freudenreichii* 138	Skim milk specific for
SMPF + WPI + 138 *P. freudenreichii* 138 fermented by *P. freudenreichii* 138 + WPI	Skim milk specific for	SMPF + WPI + 138 *P. freudenreichii* 138 fermented by *P. freudenreichii* 138 + WPI	Skim milk specific for
Association (Assoc) SMLC + WPI + BL23 and SMPF + WPI + 138	Equal mixture of	Association (Assoc) SMLC + WPI + BL23 and SMPF + WPI + 138	Equal mixture of

All groups were gavaged daily, with 0.5 ml of the appropriate treatments, for 13 days.

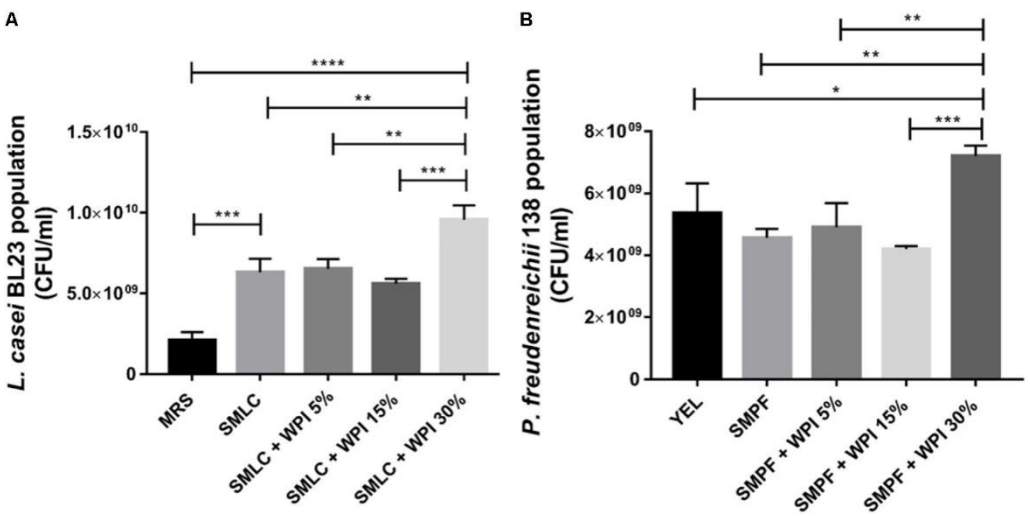

FIGURE 1 | Different concentrations of WPI in skim milk impacted on *Lactobacillus casei* BL23 and *Propionibacterium freudenreichii* 138 growth. (A) Colony-forming unit (CFU) counting of *L. casei* BL23, after 24 h of incubation at 37°C and (B) Population of *P. freudenreichii* 138, after 78 h of anaerobic incubation at 30°C were assessed by CFU counts. These assays were performed in triplicate technique and biological triplicate. Asterisks represent statistically significant differences and were indicated as follows: $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$.

were performed simultaneously therefore, the same control groups were used for all experimental probiotic assays. Each group containing 6–9 animals. All beverages contained 10^9 CFU mL^{-1} bacteria.

Histological Analysis

The distal portion of the small bowel (ileum) from the mice was collected and washed with PBS. Afterwards, rolls were prepared for histomorphological analysis. Histological materials were immersed in 4% buffered formaldehyde solution for tissue fixation. Then, the material was embedded in paraffin, and a 4 µm section of each sample was placed on a glass slide and stained with Hematoxylin-Eosin (HE). The histological score was determined using a score that measures the intensity of the infiltrate of mononuclear and polymorphonuclear cells in the *lamina propria* of the ileum, the presence of ulceration and erosion and changes in mucosal architecture (Soares et al., 2008). For each parameter a classification was given according to the

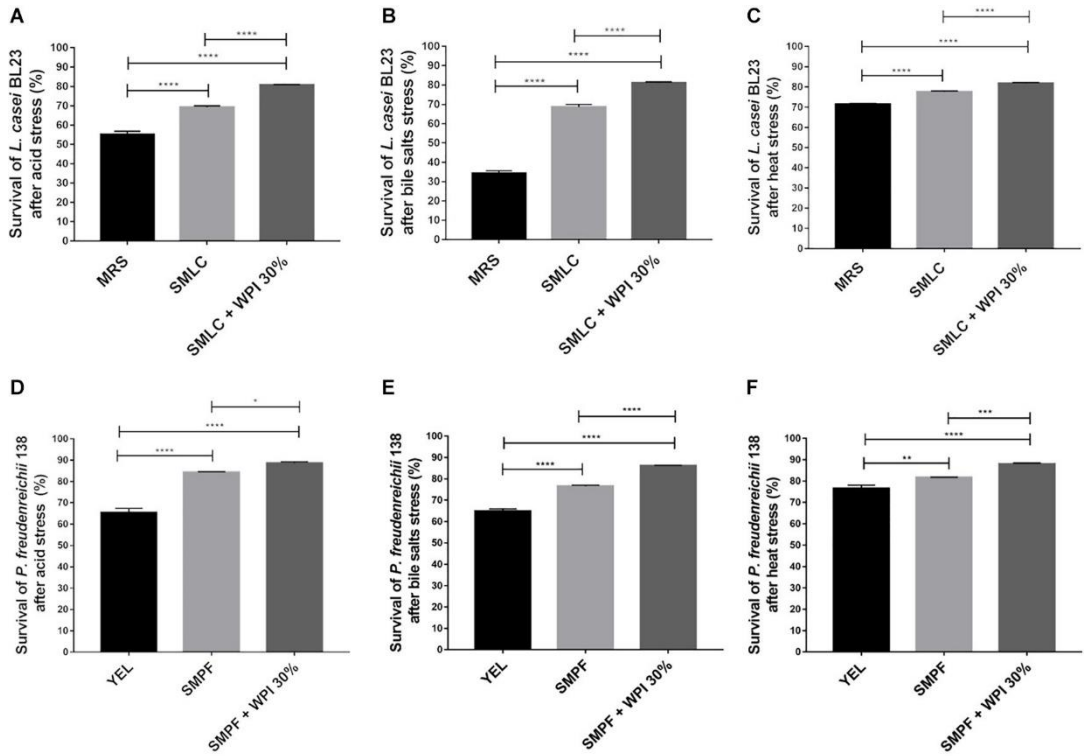

FIGURE 2 | Whey protein isolate (WPI) confers stress tolerance on *L. casei BL23* and *P. freudenreichii* 138. *L. casei* BL23 was cultured for 24 h in the indicated growth media until stationary phase and then subjected to (A) acid stress (pH 2 for 60 min at 37°C); (B) bile salts stress (1 g/liter for 60 min at 37°C) or (C) heat stress (63°C for 30 min). *P. freudenreichii* 138 was cultured for 72 h in each culture media until stationary phase, and then subjected to (D) acid stress, (E) bile salts stress, or (F) heat stress. Viable bacteria were enumerated by counting colonies in the challenged and control cultures and then, expressed as percent survival (means ± standard deviations). These assays were performed in triplicate technique and biological triplicate. Asterisks represent statistically significant differences and were indicated as follows: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

severity of the lesion in the tissues: absent (0), mild (1), moderate (2) and severe (3). For morphometric analysis, 10 images of the ileum of each animal were randomly captured and analyzed using ImageJ software (version 1.8.0). Villi height and the crypt depth were measured vertically from the tip of villi to the base of the adjacent crypt. Additional cuts in the paraffinized samples from the ileum were stained by the Periodic Acid-Schiff (PAS), technique to determine the number of goblet cells in the tissues (Prisciandaro et al., 2011). Ten random field images of each sample were made using the 40 objective and the intact goblet cells were counted using ImageJ software (version 1.8.0) and expressed as the number of cells per high-power field (hpf) (40 × , 108.2 μm^2).

Measurement of Secretory IgA

Levels of secretory IgA (sIgA) were determined by enzyme-linked immunosorbent assay (ELISA) in small bowel intestinal fluids (Carvalho et al., 2017a). Microtiter plates (Nunc-Immuno Plates, MaxiSorp) were coated with anti-IgA antibodies (Southern Biotechnology, Birmingham, AL, United States) for 18 h at 4°C. The plates were washed with saline (NaCl 0.9%) added with Tween 20 (0.05%) and blocked with 200 μl PBS-casein (0.05%) for 1 h at room temperature. Intestinal fluid samples were diluted in PBS-casein (0.25%) and then added to the plate. After incubation for 1 h at room temperature, the wells were washed and biotin-conjugated anti-mouse IgA antibody (Southern Biotechnology) diluted in PBS-casein (0.25%) (1: 10,000). The plates were incubated for 1 h at 37°C and anti- IgA conjugated to streptavidin peroxidases (1:10,000) were added (Southern Biotechnology). After 1 h of incubation, 100 μl of orthophenylenediamine (OPD) (Sigma, St. Louis, MO, United States) and H_2O_2 (0.04%) were added to each well. Plates were kept away from light until the coloration developed. The reaction was stopped by addition of 2 N H_2SO_4. Reading was performed on a plate reader (Bio-Rad Model 450 Microplate Reader) at 492 nm absorbance. The results were measured in concentration of sIgA (μg) per ml of intestinal fluid, according to the standard curve.

Statistical Analyses

The results were reported as the mean ± standard deviation and analyzed using Student's *t*-test, Holm–Sidak *t*-test, One- Way ANOVA or Two-Way ANOVA followed by the Tukey or Sidak post-test. Non-parametric data's were analyzed using Kruskal-Wallis data followed by the Dunns post-test. Graphs and statistical analyzes were performed in GraphPad Prism

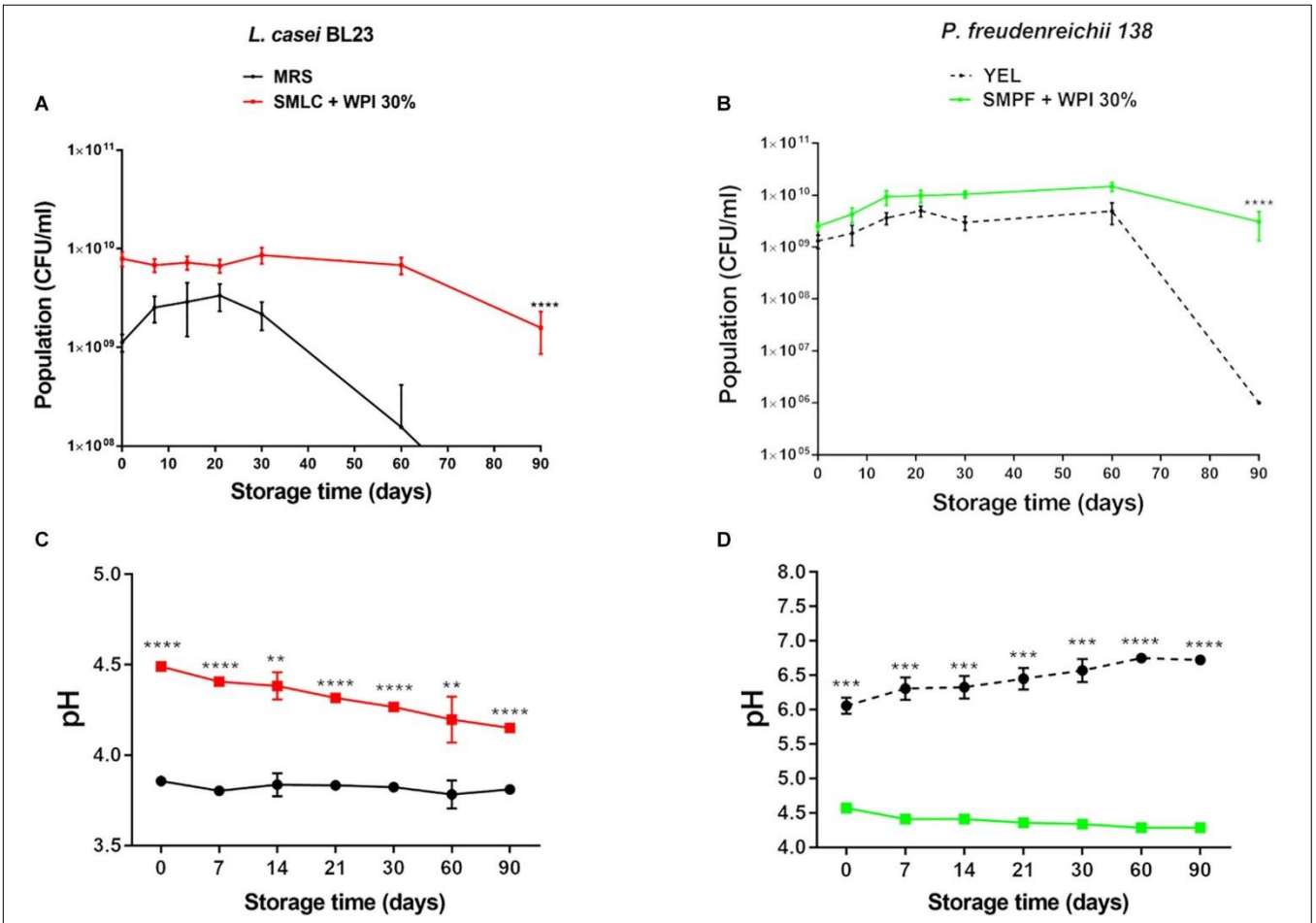

FIGURE 3 | Viability of *L. casei BL23* and *P. freudenreichii* 138 are maintained during 90 days of cold storage when are cultured in skim milk supplemented with WPI. Final population (CFU ml^{-1}) of (A) *L. casei* BL23, and (B) *P. freudenreichii* 138 in the indicated culture media. Bacteria were culture until the stationary phase and then storage at 4°C for 90 days. Viable bacteria were enumerated by counting the colonies on days 0, 7, 14, 21, 30, 60, and 90 after storage. (C,D) Variation of pH values throughout storage days. Asterisks represent statistically significant differences as follows: $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$.

version 7.00 for Windows (GraphPad Software, San Diego, CA, United States). *P*-values under 0.05 were considered significant.

RESULTS

Different Concentrations of Whey Protein Isolate Alter the Growth of Bacteria

The final population of *L. casei* BL23 and of *P. freudenreichii* 138 was monitored after growth in skim milk supplemented with different concentrations of WPI (**Figure 1**). We observed a significant increase in the CFU counting of *L. casei* BL23 when cultivated in SMLC after 24 h (6.3 10^9 CFU mL^{-1}), in comparison with MRS medium (2.1 10^9 CFU mL^{-1}). Moreover, the largest final population of *L. casei* BL23 was found in skim milk supplemented with 30% WPI (SMLC WPI 30%) (9.5 10^9 CFU mL^{-1}). The final population of *P. freudenreichii* 138 also showed a similar result (**Figure 1B**). In this case, the highest population (7.2 × 10^9 CFU mL^{-1}) was obtained in skim milk supplemented with 30% of WPI (SMPF WPI 30%). This condition allowed the highest growth of the propionibacteria, when compared with all other culture media used.

Whey Protein Isolate Improves the Tolerance of Bacteria Toward Environmental Stresses

The survival rate of *L. casei* BL23 and *P. freudenreichii* 138 was evaluated after acid stress, bile salts stress and high-temperature stress (**Figure 2**). *L. casei* BL23 showed enhanced survival rate after acid stress, when cultured in SMLC medium (69.2%), compared to the MRS control (55.1%) (**Figure 2A**). The survival was further increased when SMLC was supplemented with 30% WPI, leading to the highest survival rate of *L. casei* BL23 (80.6%). This value was significantly higher than skim milk supplemented with 5% and 15% of WPI (data not shown). The population after acid stress in SMLC + WPI 30% was 8.5 × 10^7 CFU mL^{-1}. A similar result was observed for *P. freudenreichii* 138 (**Figure 2D**). After acid stress, the highest

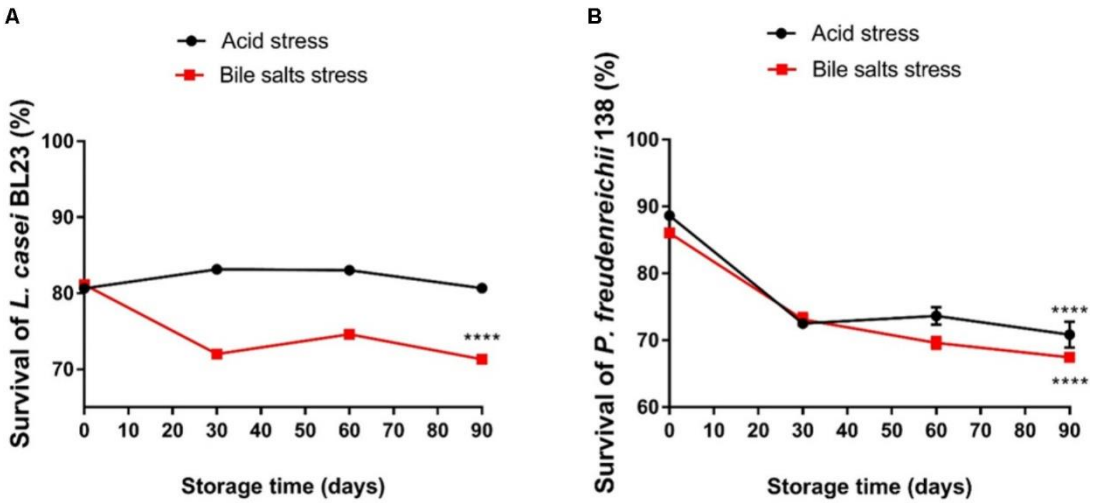

FIGURE 4 | *Lactobacillus casei* BL23 (A) and *P. freudenreichii* 138 (B) maintain the ability to tolerate acid stress and bile salts stress even during storage when cultured in skim milk supplemented with WPI. Viable bacteria were enumerated by counting colonies on cultures after acid and bile salt stress on days 0, 30, 60, and 90 post-storage and then expressed as percent survival (means ± standard deviation). The assays were performed in triplicate technique and biological triplicate. Asterisks represent statistically significant differences as follows: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

tolerance was observed when *P. freudenreichii* 138 was grown in skim milk supplemented with 30% WPI (SMPF + WPI 30%) (88.6%), compared with skim milk without supplementation (SMPF) (84.3%) or YEL medium (65.3%). In addition, the survival rate of *P. freudenreichii* 138 grown in milk added with 5% of WPI or 15% are lower than supplementation with 30% (data not shown). The population of *P. freudenreichii* 138 after acid stress in SMPF WPI 30% was 7 ± 6 10^8 CFU mL^{-1}. Likewise, our results show enhanced tolerance toward bile salts stress when *L. casei* BL23 and *P. freudenreichii* 138 were cultured in beverage containing 30% of WPI (**Figures 2B,E**). After temperature stress, we observed that both strains presented a high tolerance to 63°C, independent of culture medium. However, the highest survival rate was obtained when bacteria were cultured in milk added with 30% of WPI (**Figures 2C,F**).

Bacteria Remained Viable in the Fermented Milk Supplemented With WPI During Storage at 4◦C

Changes in bacterial population in fermented skim milk supplemented with whey protein were monitored during storage at 4°C for 90 days (**Figures 3A,B**). The viability of both strains, in skim milk contained 30% of WPI, remained practically unchanged, with a small CFU reduction after 90 days. After cold storage, the *L. casei* BL23 population in the skim milk culture media was maintained at 1.5 10^9 CFU mL^{-1}, while the *P. freudenreichii* 138 population at 7.4 10^9 CFU mL^{-1}. In contrast, viable *L. casei* BL23 and *P. freudenreichii* 138 was significantly decreased when grown in culture media (MRS and YEL, respectively) over the entire storage time, presenting a final population below 2×10^2 CFU mL^{-1}, for *L. casei* BL23 and 1.0×10^6 CFU mL^{-1} for *P. freudenreichii* 138.

Figures 3C,D represents the evolution of the pH values in the culture of both strains during the storage. We observed a decrease in pH values in beverages containing WPI. In the order hand, in controls the pH values did not decline over the storage time, remaining constant for *L. casei* BL23 while a slight increase in the pH value was detected for *P. freudenreichii* 138. **Figures 4A,B** represent the survival rates of *L. casei* BL23 and *P. freudenreichii* 138, upon acid and bile salt stresses, following storage at 4°C. The survival rate of *L. casei* remained almost constant between days 0 and 90 of storage (**Figure 4A**). For *P. freudenreichii* 138, the acid and bile salts tolerance slightly decreased, between days 0 and 30 (**Figure 4B**). However, propionibacteria survival remained around 70% after 90 days of storage.

Probiotic Beverage Fermented by L. casei BL23 or P. freudenreichii 138 Reduces the Weight Loss in Mice With Mucositis

Time-course of the weight of mice during the 14 experimental days is shown in **Figure 5**. None of the treatments with probiotic beverages was able to significantly alter the weight of mice during the first 11 days of gavage preceding mucositis induction. As expected, mice receiving 5-FU began to lose weight soon after the drug injection on day 11 (**Figures 5A,C,E**). Interestingly, we observed that the treatment with probiotic beverage fermented by *L. casei* BL23 (**Figure 5B**) or fermented by *P. freudenreichii* 138 (**Figure 5D**) was able to reduce the weight loss of inflamed mice, in the presence or absence of WPI in the medium. However, the association of both bacterial strains were not efficient to further limit the weight loss, compared to mice that received no probiotic treatment (**Figure 5F**).

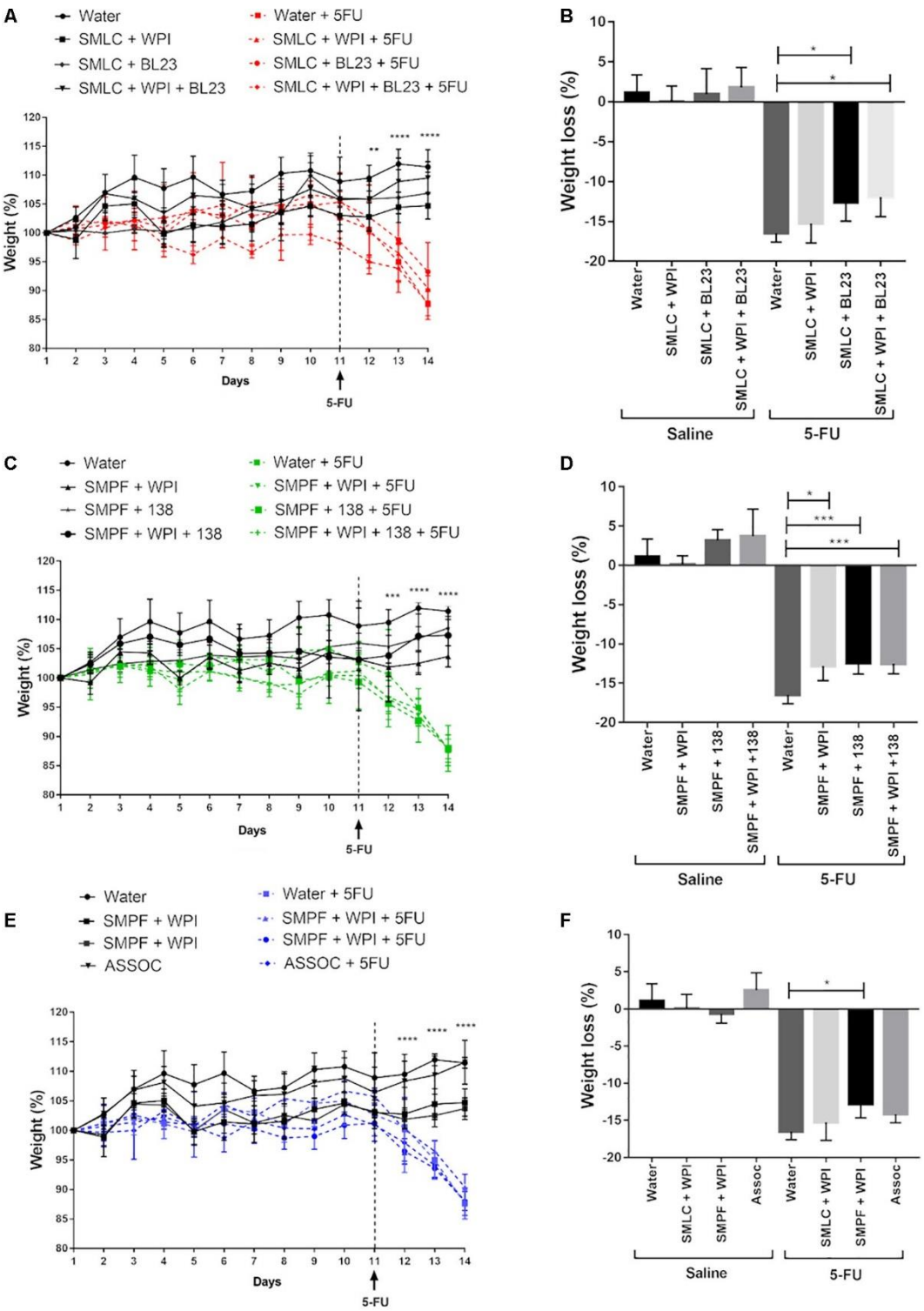

FIGURE 5 | Time-course of body weight for mice treated with (A) probiotic beverage fermented by *L. casei* BL23; (C) probiotic beverage fermented by *P. freudenreichii* 138 and (E) probiotic beverage fermented by association with *L. casei* BL23 and *P. freudenreichii* 138. (B,D,F) Weight loss observed after 5-FU injection and differences across groups. $N = 6–9$. Mice were weighted daily during 14 days. Asterisks represent statistically significant differences as follows: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

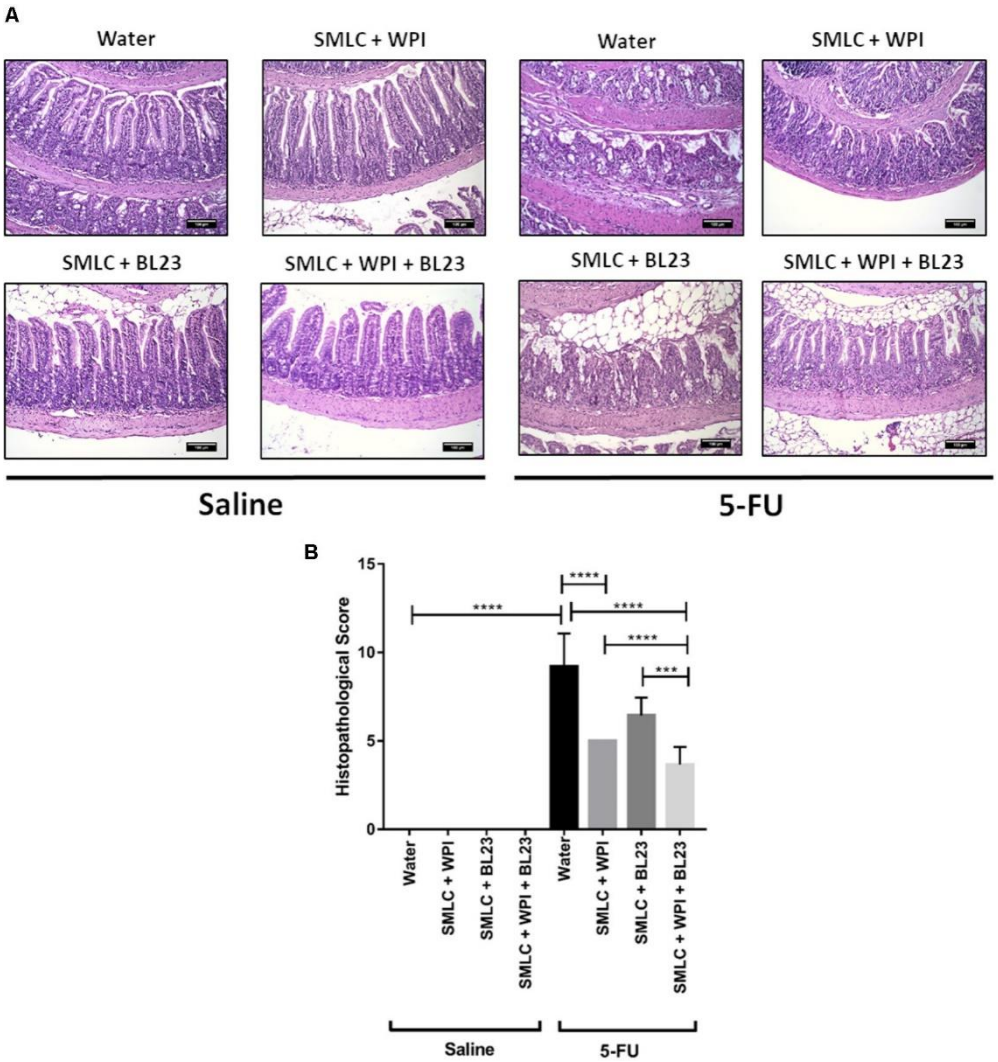

FIGURE 6 | Administration of skim milk supplemented with WPI fermented by *L. casei* BL23 prevents mucosal damage in mice. (A) Representative H&E-stained images from mucosal histopathology and (B) histopathological score obtained in mice treated. The image acquisition was done with a 20× magnification objective. Scale bar = 100 μm. Same control groups were used for all experimental probiotic assays. Asterisks represent statistically significant differences as follows: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

Beverage Supplemented With Whey Protein Isolate Improves Mucosal Preservation in the Inflamed Mice

Histological analysis revealed a mucosal pattern within normal limits in all groups injected with saline (0.9% NaCl), showing that the probiotics beverages did not alter gut mucosal morphology (**Figures 6–8**). On the other hand, mice submitted to mucositis demonstrated alterations in the morphological structure of the ileum, which was evidenced by an increase in the histopathological parameters. This reflected mainly inflammatory cell infiltration in the *lamina propria*, submucosa and muscular layer, and a prominent alteration in villus structure. However, mice treated with probiotic beverages showed decreased mucosal damage, compared to inflamed mice that did not receive any probiotic treatment. Moreover, supplementation with WPI was able to improve the anti-inflammatory effects of *L. casei* BL23 beverage (**Figure 6**) but not for *P. freudenreichii* (**Figure 7**). Mice treated with the association of *L. casei* BL23 and *P. freudenreichii* 138 in skim milk, presented a reduced histopathological score compared to mice receiving only water, however, the histopathological scores obtained are no better than the treatment using the individually fermented milks by *L. casei* BL23 or *P. freudenreichii* 138 (**Figure 8**).

Treatment With Probiotic Beverages Prevented Villus Shortening and Degeneration of Goblet Cells

Morphometric analysis was carried out to evaluate epithelial integrity. A decrease in villus height and in crypt depth was

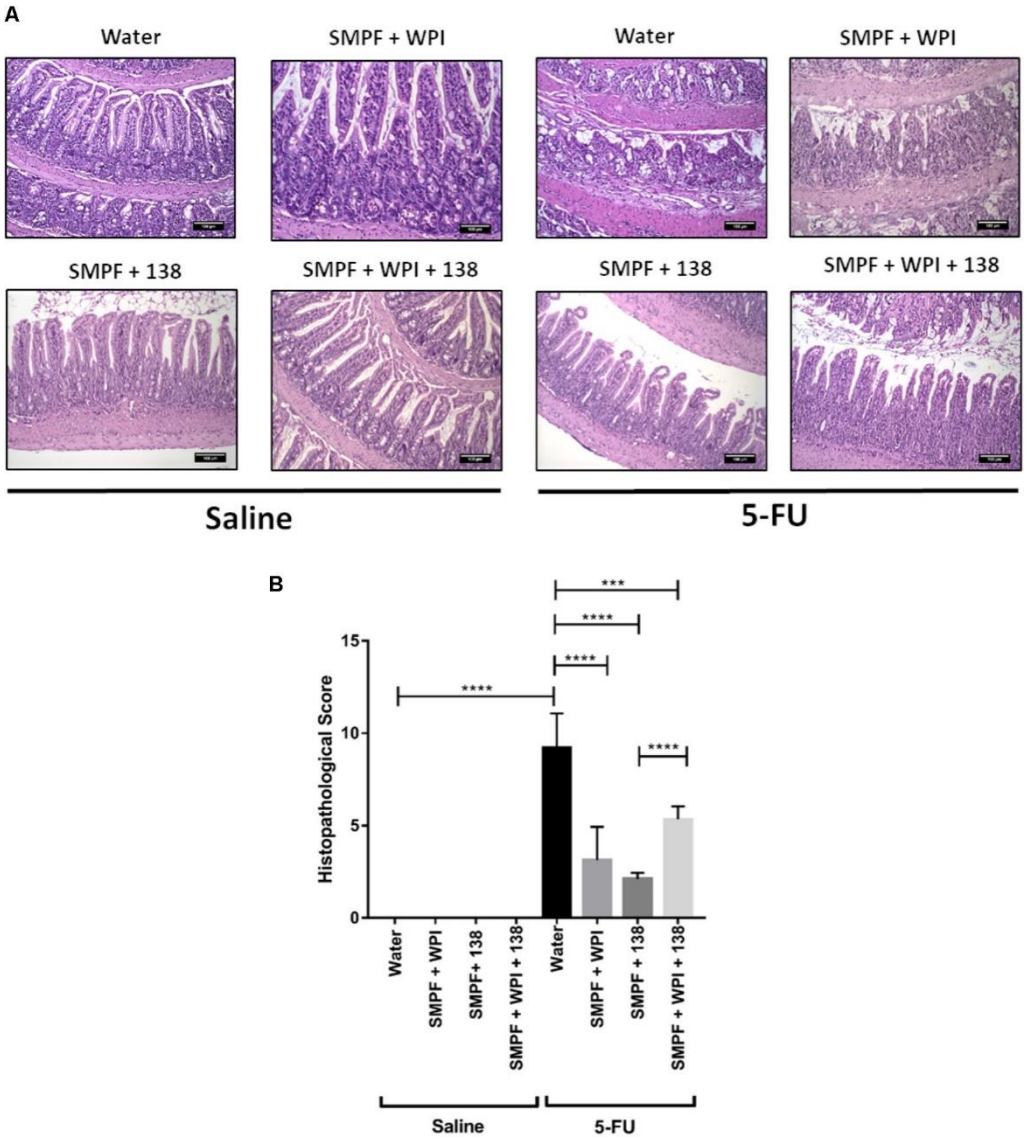

FIGURE 7 | Administration of skim milk without supplemented with whey protein isolate and fermented by *P. freudenreichii* 138 prevents mucosal damage in mice. (A) Representative H&E-stained images from mucosal histopathology and (B) histopathological score obtained in mice. The image acquisition phase was done with a 20× magnification objective. Scale bar = 100 μm. Same control groups were used for all experimental probiotic assays. Asterisks represent statistically differences as follows: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

observed in mice after 5-FU injection (66 μm) (**Figure 9**). Treatment with probiotic beverages showed increased villus height, especially in groups treated with SMLC WPI BL23 (129.2 μm) (**Figure 9A**) and SMPF 138 (176.04 μm) (**Figure 9C**). No difference was found in crypt depths either in treated or untreated mice. As expected, the mucositis induction resulted in substantial decrease in goblet cells number (9.08 goblet cell/hpf) (**Figure 10**) when compared to the groups injected with 0.9% saline (51.4 goblet cell/hpf). In the other hand, administration of probiotics beverages prevented the degeneration of goblet cells in the mice ileum. The highest goblet cell count was found in mice treated with SMLC WPI BL23 (34.7 goblet cell/hpf) (**Figure 10A**) and SMPF 138 (27.9 goblet cell/hpf) (**Figure 10B**).

Administration of Probiotic Beverages Did Not Change the Secretory IgA Production

Figure 11 indicates the concentration of IgA secreted in the small intestine of healthy animals or after induction of 5-FU mucositis, treated or not with *L. casei* BL23, with *P. freudenreichii* 138, or with the association of the two strains. Our results showed that

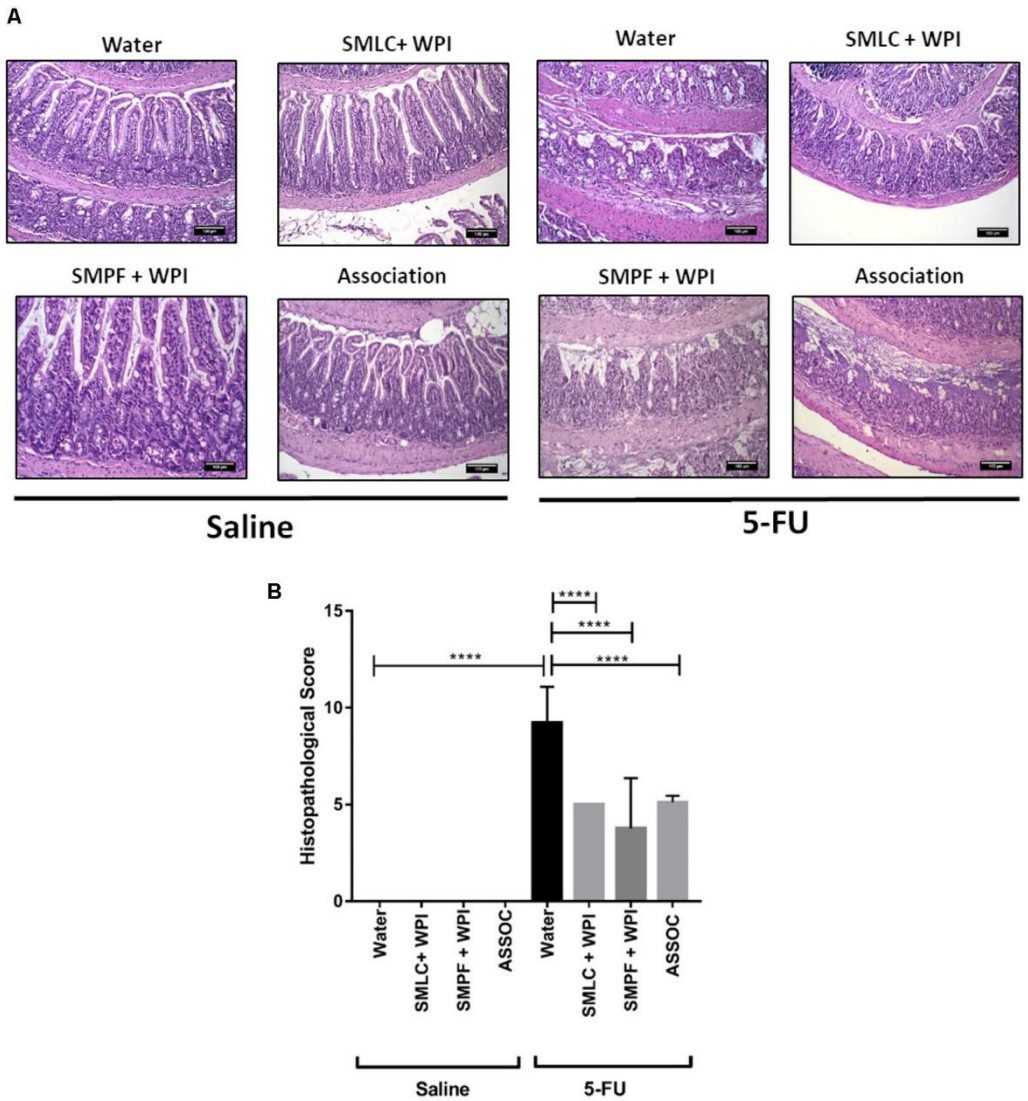

FIGURE 8 | Association of skim milk fermented by *L. casei* BL23 and skim milk fermented by *P. freudenreichii* 138 does not provide additional effect in prevents mucosal damage. (A) Representative H&E-stained images from mucosal histopathology and (B) histopathological score obtained in the animals treated with different beverages. The image acquisition phase was done with a 20× magnification objective. Scale bar = 100 μm. Same control groups were used for all experimental probiotic assays. Asterisks represent statistically significant differences as follows: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

there was no significant difference across the groups evaluated in this study.

DISCUSSION

Mucositis is a gastrointestinal inflammation that affects the quality of life of patients undergoing malignancy treatments (Sonis, 2004). Currently, classical therapies available for the prevention and treatment of the disease are not very effective and therefore new options have been suggested, such as the use of probiotic bacteria (Carvalho et al., 2017a). Several of these probiotics required the addition of protective matrices, in order to confer a protection via an efficient delivery of probiotic bacteria to the GIT, and enhance the therapeutics effects in the disease context (Carmo et al., 2017). The present study investigated the effects of whey protein isolate as a protective matrix for two bacterial strains and study the probiotic potential of these fermented beverages in a mucositis mice model induced by 5-FU. Our data show that the addition of whey protein isolate booste d the growth of *L. casei* BL23 and of *P. freudenreichii* 138. This result may be due to a larger amount of nutrients provided by milk constituents and by WPI in the beverages, including carbohydrate and nitrogen sources, as they are essential for the energy metabolism of both *L. casei* BL23 and *P. freudenreichii* 138 (Cousin et al., 2011; Pessione, 2012). This result corroborates previous studies showing that other bacteria experienced enhanced growth as a result of whey proteins addition

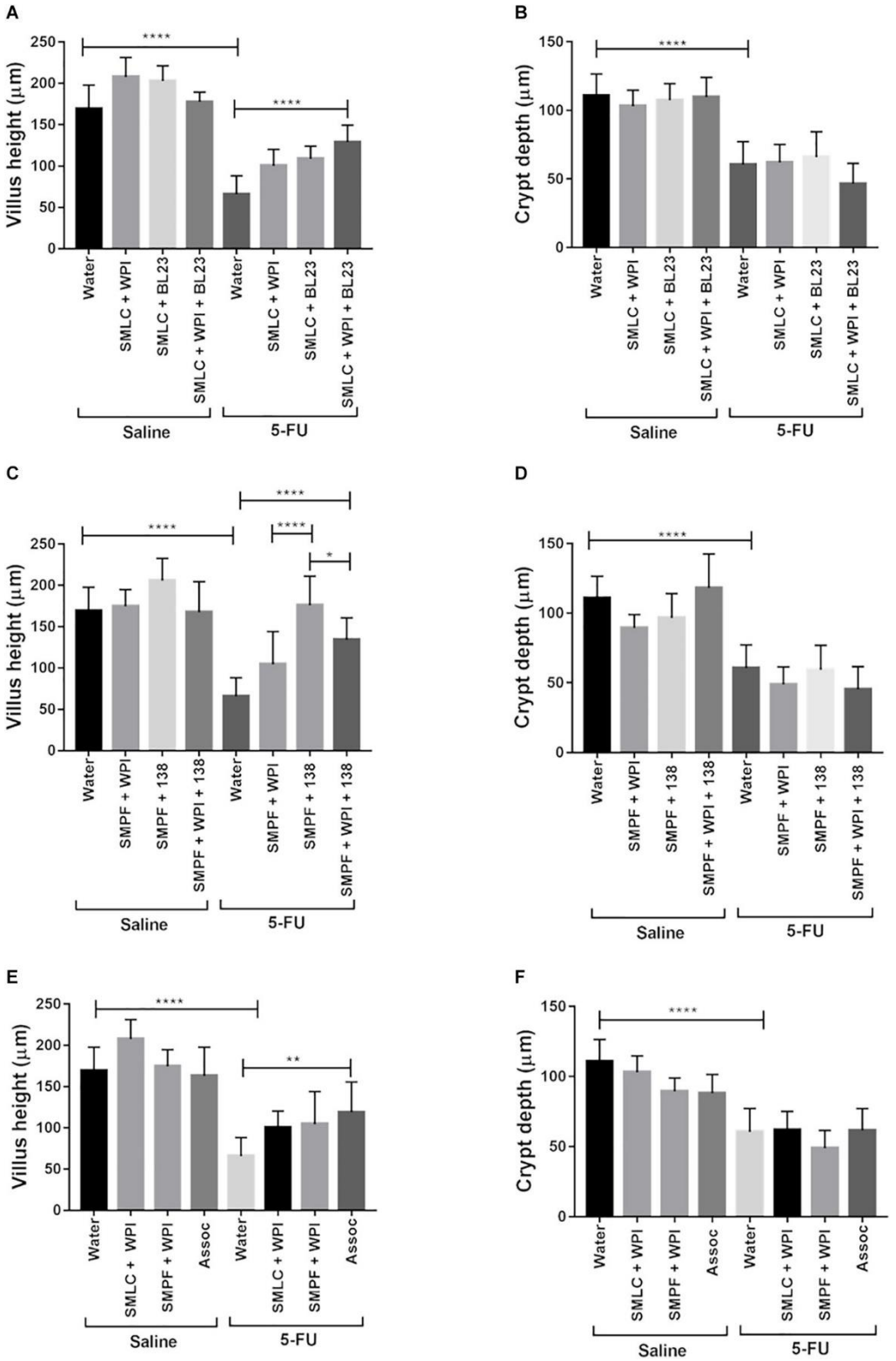

FIGURE 9 | Administration of probiotic beverages improves villus architecture. Morphometric analysis of villus height and crypt depth of animals treated with (A,B) beverages fermented by *L. casei* BL23; (C,D) beverages fermented by *P. freudenreichii* 138 or (E,F) beverages fermented by the association of both bacteria following 5-FU or saline administration. Values were obtained by measuring ten random images of the ileum of mice. $N = 6, 9$. Asterisks represent statistically significant differences as follows: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

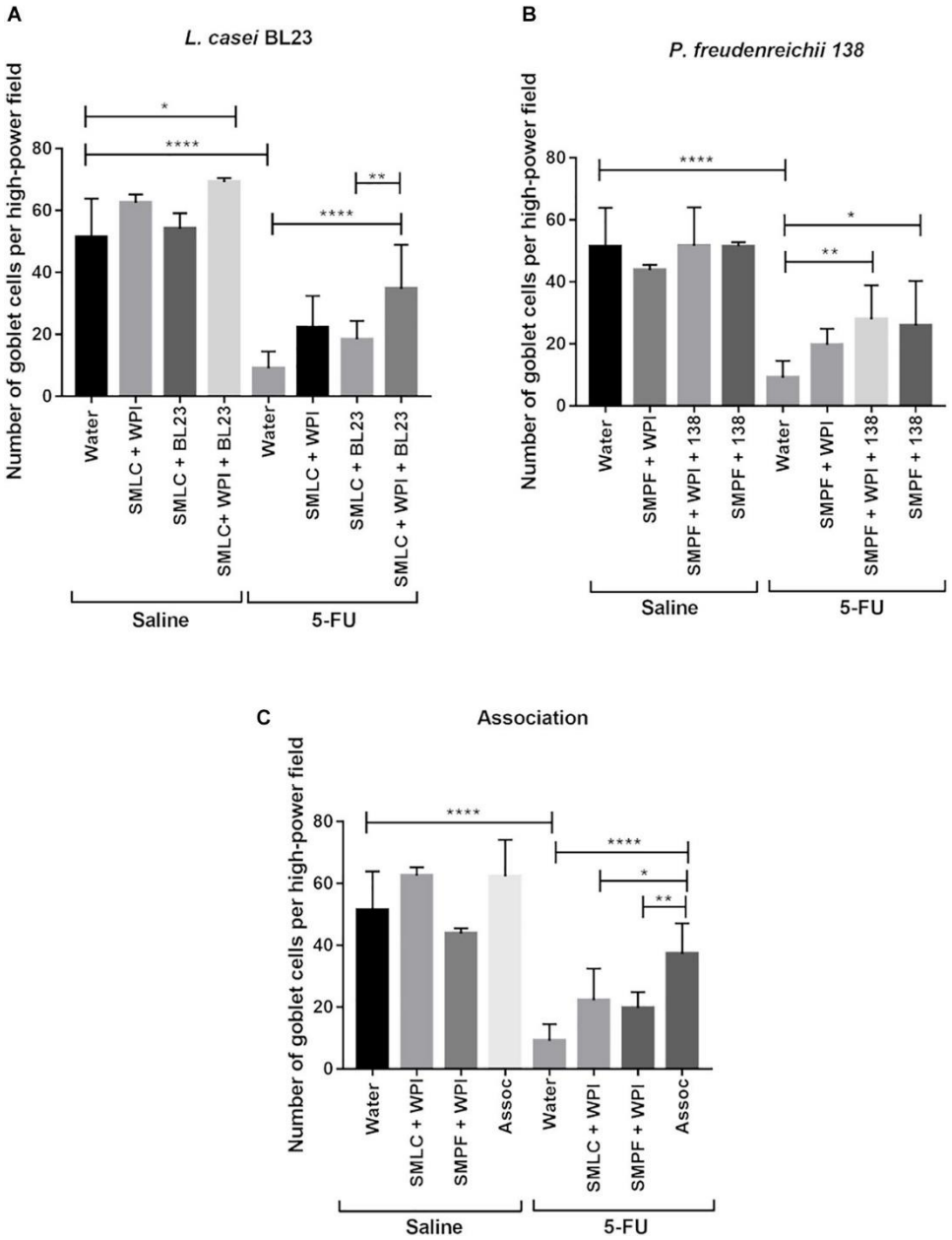

FIGURE 10 | Administration of probiotic beverages prevented the marked degeneration of goblet cells in the mice ileum. Quantification of intact goblet cells in the animals ileum treated with (A) beverages fermented by *L. casei* BL23; (B) beverages fermented by *P.freudenreichii* 138 or (C) beverages fermented by the association of both bacteria following 5-FU or saline administration. Values were obtained by counting intact cells in ten random field images of mice. Results were expressed as means ± standard deviation. $N = 6–9$. Asterisks represent statistically significant differences as follows: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

addition (Almeida et al., 2009; Skryplonek and Jasińska, 2015; Huang et al., 2016b; Baruzzi et al., 2017).

Due the fact that the products to be considered as a probiotic must be in sufficient quantities of viable bacteria in your local of action (Cousin et al., 2012a; Rabah et al., 2017), we decided to investigate the efficiency of WPI as a protective matrix for bacteria. For this, we evaluated the survival rate of *L. casei* BL23 and of *P. freudenreichii* 138 strains in environments that simulated stomach pH (pH 2), in the presence of bile salts in GIT and at high temperature. Our results demonstrated that the tolerance of *L. casei* BL23 and of *P. freudenreichii* 138 to these stressing conditions was significantly higher in the presence of WPI. Therefore, beverages containing WPI also showed a high viable cell counts after these stresses. Thus, skim milk supplemented with WPI is an effective matrix for the two strains used in this work, considering acid, bile salts

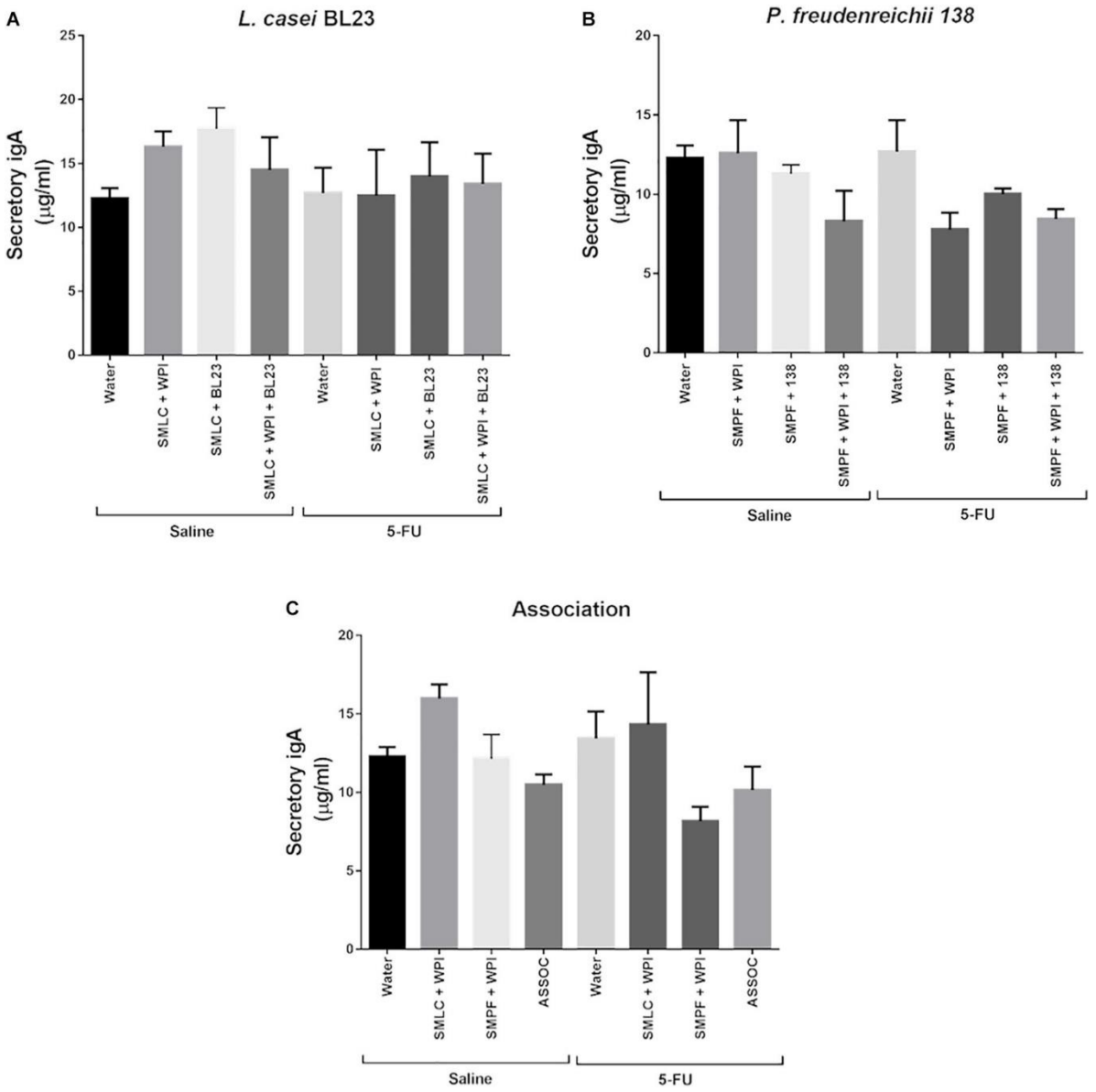

FIGURE 11 | Administration of probiotic beverages did not alters the secretory IgA levels. Quantification of immunoglobulin A secretion (sIgA) in the small intestine of healthy or inflammation mice, treated for 14 days with (A) *L. casei* BL23 (B) *P. freudenreichii* 138, or (C) association of *L. casei* BL23 and *P. freudenreichii* 138. Results were expressed as means ± standard deviation. *N* = 6–9.

and high-temperature stresses. Similar results were described by Vargas et al. (2015), who demonstrated that the probiotic strains *Streptococcus thermophilus* and *L. bulgaricus* presented a higher tolerance to the same stress conditions in the presence of WPI in the culture medium (Vargas et al., 2015). Cousin et al. (2012b) have also shown that *Propionibacterium* strains survived better in acid and biliary stresses when included in a dairy matrix (Cousin et al., 2012b). In a study by Huang et al. (2016b) was also demonstrated that the *P. freudenreichii* CIRM-BIA 129 tolerates simulated GIT stresses when included in hyperconcentrated sweet whey (Huang et al., 2016b). Studies suggest that dairy proteins protect probiotic bacteria via a process called coacervation. In this process, the proteins form microspheres that pack the microorganism inside, thus forming a kind of barrier that protects them from adverse environmental conditions (Silva et al., 2015; Coghetto et al., 2016).

The current definition of probiotic stipulates that microorganisms should be consumed alive (WHO, 2002; Hill et al., 2014). Thus, the survival of bacteria at low temperatures is an important parameter for the development of an effective probiotic product and fermented dairy products are generally stored at 4°C (Cousin et al., 2012b). Both bacteria tested in this work remained viable during at least 90 days at 4°C when cultured in skim milk supplemented with WPI, reaching up

to 10^9 CFU mL^{-1}, after 90 days. Furthermore, it is possible to suggest that these bacteria were also metabolically active as a pH decrease was observed, due to production of lactic acid and propionic acid (main fermentation products of *L. casei* and *P. freudenreichii,* respectively) during cold storage. In addition, stress tolerance of *L. casei* BL23 and of *P. freudenreichii* 138 was maintained upon cold storage in skim milk plus WPI. Therefore, the amount of nutrients provided by milk plus WPI was sufficient to sustain survival of the bacteria over 90 days of cold storage, in accordance with previous reports (Cousin et al., 2012b; Vargas et al., 2015; Moslemi et al., 2016; Shori, 2016; Baruzzi et al., 2017). In our probiotic beverages, WPI supplementation increases the bacteria's survival rate to environmental stresses, which is an essential parameter for therapeutic effects (Rabah et al., 2017). Since, *L. casei* BL23 is able to reduce inflammation parameters in colitis model (Rochat et al., 2007; Watterlot et al., 2010), and *P. freudenreichii* 138 has been shown some probiotic effects *in vitro* and *ex vivo* model (Cousin et al., 2012a,b), which is interesting to check their potentials in other disease models. In this context, we tested whether the probiotic beverages were able to exert beneficial effects in mice submitted to experimental mucositis and whether the addition of WPI would enhance these probiotic effects. 5-FU treatment caused weight loss, shortening of intestinal villi and an inflammation of mucosa in mice, in accordance with the literature (Carvalho et al., 2017a). Moreover, probiotic beverages fermented by *L. casei* BL23 and *P. freudenreichii* 138 were able to decrease 5-FU-induced intestinal inflammation in BALB/c mice, with preservation of the mucosal integrity and reduced weight loss. Same results were observed by oral administration of Simbioflora®, that containing *L. paracasei*, *L. rhamnosus*, *L. acidophilus* and *Bifidobacterium lactis* plus fructooligosaccharide in a 5-FU-mucositis mice model and in a treatment using a probiotic mixture, named VSL#3, in mucositis model induced by Irinotecan in rats (Bowen et al., 2007; Trindade et al., 2018). Our study also shows that addition of WPI improved *L. casei* BL23 beneficial effects in the ileum, but not for *P. freudenreichii* 138. Another important feature evaluated in this study was the number of goblet cells throughout the tissue. Goblet cells are responsible for producing a layer of mucus that covers the entire surface of the intestinal epithelium and is mainly composed of high molecular weight glycoproteins known as mucins (Johansson et al., 2013). This mucus prevents the direct adhesion of microorganisms to the epithelium and their translocation to the internal layers of the intestine, besides being important for the lubrication of the intestinal walls and for the protection of the epithelium against digestive acidic fluids and toxins (Kim and Khan, 2013). Previous studies have described that the intestine cells need a series of amino acids, mainly threonine, cysteine, and serine, for the synthesis of this mucus in healthy conditions (Faure et al., 2006). However, during inflammatory processes such as mucositis, a superactivation of the goblet cells occurs, aiming to increase the protection of the epithelium damaged by the inflammatory process (Stringer et al., 2007, 2009a). Consequently, the requirement for amino acids by the cells is increased. However, these amino acids are usually insufficient during the inflammation, compromising adequate mucus barrier functioning (Stringer et al., 2009b). The demand for threonine, cysteine, and serine can be adequately supplied by the diet in order to increase the availability of these amino acids (Faure et al., 2006). WPI used in this study is rich in these three amino acids. Our probiotic beverages prevented the degeneration of goblet cells, suggesting that the presence of milk and WPI may have increased the availability of these amino acids, increasing the production of mucus and consequently improving the framework of protection and tissue repair observed in the histological analyses. Similar results were shown in a probiotic treatment with *Saccharomyces cerevisiae* UFMG A-905 in a murine model of irinotecan-induced mucositis (Bastos et al., 2016), as well as in a mice treated with a mixture of *L. acidophilus* and *Bifidobacterium bifidum* in a 5-FU-induced intestinal mucositis model (Yeung et al., 2015). Prisciandaro et al. (2011) also shown that a *Escherichia coli* Nissle 1917 (EcN) probiotic derived supernatants was able to partially maintained acidic-mucin producing goblet cells in the jejunum and neutral mucin producing goblet cells in the ileum, in 5-FU mucositis model in mice (Prisciandaro et al., 2011). Furthermore, due to the capacity to preserve globet cells and consequently to maintenance of mucin production, this is possible that adhesion of *L. casei* BL23 and *P. freudenreichii* 138 strains to the intestinal epithelial cells can be enhanced in mouse GIT, leading to an increase the probiotic therapeutic effect (Ouwehand and Salminen, 2003).

In summary, our results indicate that the developed probiotic beverages have anti-inflammatory effects in mucositis. Thus, we sought to investigate the role of IgA in the regulation of inflammatory conditions in these mice. IgA is the main antibody type found in mucosal secretions (Pabst et al., 2016) and has many important functions such as modulation of intestinal microbiota and mucosal protection against invading pathogens (Lycke and Bemark, 2017). These functions are naturally important in intestinal mucositis because this disease is associated with dysfunctions related to imbalances in the intestinal microbial community (Clemente et al., 2012). Destruction of the physical barrier that covers the GIT facilitates the invasion of pathogens from the lumen (Bischoff et al., 2014). However, none of the probiotic beverages used in this study was able to alter IgA production, ruling out a probiotic effect via stimulation of sIgA. Accordingly, administration of another probiotic species, *Lactococcus lactis* NCDO, in a DSS-induced colitis model, does not enhance the levels of sIgA (Luerce et al., 2014).

CONCLUSION

We have demonstrated that the supplementation of skim milk with 30% of whey protein isolate is a good matrix to provide protection for the *L. casei* BL23 and for *P. freudenreichii* 138 against environmental stresses. Furthermore, both probiotic beverages developed here were efficient in preventing mucositis induced by 5-Fluorouracil in BALB/c mice. *L. casei* BL23 protective effect was further enhanced by the addition of WPI. The benefits of adding WPI for the prevention of mucositis thus depends on the bacterial strain used.

AUTHOR CONTRIBUTIONS

BC performed the *in vitro* analysis, animal experimentation regarding mucositis pre-treatment with the probiotic strains, interpreted the data regarding the immunological parameters that were assessed, and was a major contributor in the writing of the manuscript. EO and BS were major contributors in the animal experimentation. EF and SS performed, analyzed, and interpreted the histological analysis from ileum slides. JA analyzed and interpreted the morphometric analysis. LA performed the *in vitro* analysis and data interpretation. LL and HA performed, analyzed, and interpreted the secretory IgA quantification assay. AF, AV, LG, and VA contributed to the data interpretation and were major contributors in the writing of the manuscript. GJ and YLL were responsible for ceding the strains, contributed to the data interpretation, and were major contributors in the writing of the manuscript. RC and FC have contributed equally in the supervision, performing experiments, analysis and interpretation of the immunological data, and were major contributors equally in the writing of the manuscript.

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REFERENCES

Almeida, K. E., Tamime, A. Y., and Oliveira, M. N. (2009). Influence of total solids contents of milk whey on the acidifying profile and viability of various lactic acid bacteria. *LWT Food Sci. Technol.* 42, 672–678. doi: 10.1016/j.lwt.2008. 03.013

Antunes, M. M., Leocádio, P. C. L., Teixeira, L. G., Leonel, A. J., Cara, D. C., Menezes, G. B., et al. (2016). Pretreatment with l-citrulline positively affects the mucosal architecture and permeability of the small intestine in a murine mucositis model. *J. Parenter. Enter. Nutr.* 40, 279–286. doi: 10.1177/ 0148607114567508

Baruzzi, F., de Candia, S., Quintieri, L., Caputo, L., and De Leo, F. (2017). Development of a synbiotic beverage enriched with bifidobacteria strains and fortified with whey proteins. *Front. Microbiol.* 8:640. doi: 10.3389/fmicb.2017. 00640

Bastos, R. W., Pedroso, S. H. S. P., Vieira, A. T., Moreira, L. M. C., França, C. S., Cartelle, C. T., et al. (2016). *Saccharomyces cerevisiae* UFMG A-905 treatment reduces intestinal damage in a murine model of irinotecan-induced mucositis. *Benef. Microbes* 7, 549–558. doi: 10.3920/BM2015.0190

Bischoff, S. C., Barbara, G., Buurman, W., Ockhuizen, T., Schulzke, J., Serino, M., et al. (2014). Intestinal permeability – a new target for disease prevention and therapy. *BMC Gastroenterol.* 14:189. doi: 10.1186/s12876-014-0189-7

Bowen, J. M., Stringer, A. M., Gibson, R. J., Yeoh, A. S., Hannam, S., and Keefe, D. M. (2007). VSL#3 probiotic treatment reduces chemotherapy-induced diarrhoea and weight loss. *Cancer Biol. Ther.* 6, 1445–1450. doi: 10.4161/cbt.6. 9.4622

Carmo, F. L. R., Rabah, H., Cordeiro, B., Silva, S., Jan, G., Azevedo, V. A., et al. (2017). "Applications of probiotic bacteria and dairy foods in health," in *Current Research in Microbiology Applications*, eds B. Sivasankari, G. Tomazetto, and M. Verma (Wilmington, DE: Open Access eBooks), 1–33.

Carvalho, R. D., Breyner, N., Garcia, Z. M., Rodrigues, N. M., Lemos, L., Maioli, T. U., et al. (2017a). Secretion of biologically active pancreatitis associated protein I (PAP) by genetically modified dairy *Lactococcus lactis* NZ9000 in the prevention of intestinal mucositis. *Microb. Cell Fact.* 16, 1–11. doi: 10.1186/ s12934-017-0624-x.

Carvalho, R. D., do Carmo, F. L. R., de Oliveira Junior, A., Langella, P., Chatel, J.-M., Bermúdez-Humarán, L. G., et al. (2017b). Use of wild type or recombinant lactic acid bacteria as an alternative treatment for gastrointestinal inflammatory diseases: a focus on inflammatory bowel diseases and mucositis. *Front. Microbiol.* 8:800. doi: 10.3389/fmicb.2017.00800

Chang, C. T., Ho, T. Y., Lin, H., Liang, J. A., Huang, H. C., Li, C. C., et al. (2012). 5-fluorouracil induced intestinal mucositis via nuclear factor-kB activation by transcriptomic analysis and in vivo bioluminescence imaging. *PLoS One* 7:e31808. doi: 10.1371/journal.pone.0031808

Ciorba, M., Hellemeier, C., Stenson, W., and Parikh, P. (2016). Probiotics to prevents gastrointestinal toxicity from cancer therapy: an interpretative review and call to action. *Curr. Opin. Support. Palliat. Care* 1848, 3047–3054. doi: 10.1016/j.bbamem.2015.02.010.Cationic

Clemente, J. C., Ursell, L. K., Parfrey, L., and Knight, R. (2012). The impact of the gut microbiota on human health: an integrative view. *Cell* 148, 1258–1270. doi: 10.1016/j.cell.2012.01.035

Coghetto, C. C., Brinques, G. B., and Ayub, M. A. Z. (2016). Probiotics production and alternative encapsulation methodologies to improve their viabilities under adverse environmental conditions. *Int. J. Food Sci. Nutr.* 67, 929–943. doi: 10.1080/09637486.2016.1211995

Cousin, F. J., Jouan-Lanhouet, S., Dimanche-Boitrel, M. T., Corcos, L., and Jan, G. (2012a). Milk fermented by *Propionibacterium freudenreichii* induces apoptosis of HGT-1 human gastric cancer cells. *PLoS One* 7:e31892. doi: 10.1371/journal. pone.0031892

Cousin, F. J., Louesdon, S., Maillard, M. B., Parayre, S., Falentin, H., Deutsch, S. M., et al. (2012b). The first dairy product exclusively fermented by *Propionibacterium freudenreichii*: a new vector to study probiotic potentialities in vivo. *Food Microbiol.* 32, 135–146. doi: 10.1016/j.fm.2012.05.003

Cousin, F. J., Jouan-Lanhouet, S., Theret, N., Brenner, C., Jouan, E., Le Moigne-Muller, G., et al. (2016). The probiotic *Propionibacterium freudenreichii* as a new adjuvant for TRAIL-based therapy in colorectal cancer. *Oncotarget* 7, 7161–7178. doi: 10.18632/oncotarget.6881

Cousin, F. J., Mater, D. D. G., Foligne, B., and Jan, G. (2011). Dairy propionibacteria as human probiotics: a review of recent evidence. *Dairy Sci. Technol.* 91, 1–26. doi: 10.1051/dst/2010032

Dąbrowska, A., Babij, K., Marek, S., and Chrzanowska, J. (2017). Viability and growth promotion of starter and probiotic bacteria in yogurt supplemented with whey protein hydrolysate during refrigerated storage. *Postep. Hig. Med. Dosw.* 71, 952–959. doi: 10.5604/01.3001.0010.5866

Eales, J., Gibson, P., Whorwell, P., Kellow, J., Yellowlees, A., Perry, R. H. J., et al. (2017). Systematic review and meta-analysis: the effects of fermented milk with *Bifidobacterium lactis* CNCM I-2494 and lactic acid bacteria on gastrointestinal discomfort in the general adult population. *Ther. Adv. Gastroenterol.* 10, 74–88. doi: 10.1177/1756283X16670075

Faure, M., Mettraux, C., Moennoz, D., Godin, J.-P., Vuichoud, J., Rochat, F., et al. (2006). Specific amino acids increase mucin synthesis and microbiota in dextran sulfate sodium-treated rats. *J. Nutr.* 136, 1558–1564. doi: 10.1093/jn/136.6.1558

Ferreira, A. A., Huang, S., Perrone, ÍT., Schuck, P., Jan, G., and Carvalho, A. F. (2017). Tracking Amazonian cheese microbial diversity: development of a noriginal, sustainable, and robust starter by freeze drying/spray drying. *J. Dairy Sci.* 100, 6997–7006. doi: 10.3168/jds.2016-12418

Foligne, B., Nutten, S., Grangette, C., Dennin, V., Goudercourt, D., Poiret, S., et al. (2007). Correlation between in vitro and in vivo immunomodulatory properties

of lactic acid bacteria. *World J. Gastroenterol*. 13, 236–243. doi: 10.3748/wjg.v13.i2.236

Gagnaire, V., Jardin, J., Rabah, H., Briard-Bion, V., and Jan, G. (2015). Emmental cheese environment enhances *Propionibacterium freudenreichii* stress tolerance. *PLoS One* 10:e0135780. doi:10.1371/journal.pone.0135780

Hill, C., Guarner, F., Reid, G., Gibson, G. R., Merenstein, D. J., Pot, B., et al. (2014). Expert consensus document: the international scientific association for probiotics and prebiotics consensus statement on the scope and appropriate use of the term probiotic. *Nat. Rev. Gastroenterol. Hepatol.* 11, 506–514. doi: 10.1038/nrgastro.2014.66

Huang, S., Cauty, C., Dolivet, A., Le Loir, Y., Chen, X. D., Schuck, P., et al. (2016a). Double use of highly concentrated sweet whey to improve the biomass production and viability of spray-dried probiotic bacteria. *J. Funct. Foods* 23, 453–463. doi: 10.1016/j.jff.2016.02.050

Huang, S., Rabah, H., Jardin, J., Briard-Bion, V., Parayre, S., Maillard, M. B., et al. (2016b). Hyperconcentrated sweet whey, a new culture medium that enhances *Propionibacterium freudenreichii* stress tolerance. *Appl. Environ. Microbiol.* 82, 4641–4651. doi: 10.1128/AEM.00748-16

Johansson, M. E. V., Sjövall, H., and Hansson, G. C. (2013). The gastrointestinal mucus system in health and disease. *Nat. Rev. Gastroenterol. Hepatol.* 10, 352–361. doi: 10.1038/nrgastro.2013.35

Justino, P. F. C., Melo, L. F. M., Nogueira, A. F., Morais, C. M., Mendes, W. O., Franco, A. X., et al. (2015). Regulatory role of *Lactobacillus acidophilus* on inflammation and gastric dysmotility in intestinal mucositis induced by 5-fluorouracil in mice. *Cancer Chemother. Pharmacol.* 75, 559–567. doi: 10.1007/s00280-014-2663-x

Kim, J., and Khan, W. (2013). Goblet cells and mucins: role in innate defense in enteric infections. *Pathogens* 2, 55–70. doi: 10.3390/pathogens2010055

Lee, B., Tachon, S., Eigenheer, R. A., Phinney, B. S., and Marco, M. L. (2015a). *Lactobacillus casei* low-temperature, dairy-associated proteome promotes persistence in the mammalian digestive tract. *J. Proteome Res.* 14, 3136–3147. doi: 10.1021/acs.jproteome.5b00387

Lee, B., Yin, X., Griffey, S. M., and Marco, M. L. (2015b). Attenuation of colitis by *Lactobacillus casei* BL23 is dependent on the dairy delivery matrix. *Appl. Environ. Microbiol.* 81, 6425–6435. doi: 10.1128/AEM.01360-15

Leroy, F., and De Vuyst, L. (2014). Fermented food in the context of a healthy diet: how to produce novel functional foods? *Curr. Opin. Clin. Nutr. Metab. Care* 17, 574–581. doi: 10.1097/MCO.0000000000000108

Livney, Y. D. (2010). Milk proteins as vehicles for bioactives. *Curr. Opin. Colloid Interface Sci.* 15, 73–83. doi: 10.1016/j.cocis.2009.11.002

Longley, D. B., Harkin, D. P., and Johnston, P. G. (2003). 5-Fluorouracil: mechanisms of action and clinical strategies. *Nat. Rev. Cancer* 3, 330–338. doi: 10.1038/nrc1074

Luerce, T. D., Gomes-Santos, A. C., Rocha, C. S., Moreira, T. G., Cruz, D. N., Lemos, L., et al. (2014). Anti-inflammatory effects of *Lactococcus lactis* NCDO 2118 during the remission period of chemically induced colitis. *Gut Pathog.* 6, 1–11. doi: 10.1186/1757-4749-6-33

Lycke, N. Y., and Bemark, M. (2017). The regulation of gut mucosal IgA B-cell responses: recent developments. *Mucosal Immunol.* 10, 1361–1374. doi: 10.1038/mi.2017.62

Madureira, A. R., Pereira, C. I., Gomes, A. M. P., Pintado, M. E., and Xavier Malcata, F. (2007). Bovine whey proteins - overview on their main biological properties. *Food Res. Int.* 40, 1197–1211. doi: 10.1016/j.foodres.2007.07.005

Malik, A. C., Reinbold, G. W., and Vedamuthu, E. R. (1968). An evaluation of the taxonomy of *Propionibacterium*. *Can. J. Microbiol.* 14, 1185–1191. doi: 10.1139/m68-199

Marshall, K. (2004). Therapeutic applications of whey protein. *Altern. Med. Rev.* 9, 136–156.

Moslemi, M., Mazaheri Nezhad Fard, R., Hosseini, S. M., Homayouni-Rad, A., and Mortazavian, A. M. (2016). Incorporation of Propionibacteria in fermented milks as a probiotic. *Crit. Rev. Food Sci. Nutr.* 56, 1290–1312. doi: 10.1080/10408398.2013.766584

Ouwehand, A. C., and Salminen, S. (2003). In vitro adhesion assays for probiotics and their in vivo relevance: a review. *Microb. Ecol. Health Dis.* 15, 175–184. doi: 10.1080/08910600310019886

Pabst, O., Cerovic, V., and Hornef, M. (2016). Secretory IgA in the coordination of establishment and maintenance of the microbiota. *Trends Immunol.* 37, 287–296. doi: 10.1016/j.it.2016.03.002

Pessione, E. (2012). Lactic acid bacteria contribution to gut microbiota complexity: lights and shadows. *Front. Cell. Infect. Microbiol.* 2:86. doi: 10.3389/fcimb.2012.00086

Plé, C., Richoux, R., Jardin, J., Nurdin, M., Briard-Bion, V., Parayre, S., et al. (2015). Single-strain starter experimental cheese reveals anti- inflammatory effect of *Propionibacterium freudenreichii* CIRM BIA 129 in TNBS-colitis model. *J. Funct. Foods* 18, 575–585. doi: 10.1016/j.jff.2015. 08.015

Prisciandaro, L. D., Geier, M. S., Butler, R. N., Cummins, A. G., and Howarth, G. S. (2011). Probiotic factors partially improve parameters of 5- fluorouracil-induced intestinal mucositis in rats. *Cancer Biol. Ther.* 11, 671–677. doi: 10.4161/cbt.11.7.14896

Rabah, H., Rosa do Carmo, F., and Jan, G. (2017). Dairy Propionibacteria: versatile probiotics. *Microorganisms* 5:E24. doi: 10.3390/microorganisms502 0024

Rochat, T., Bermúdez-Humarán, L., Gratadoux, J. J., Fourage, C., Hoebler, C., Corthier, G., et al. (2007). Anti-inflammatory effects of *Lactobacillus casei* BL23 producing or not a manganese-dependant catalase on DSS-induced colitis in mice. *Microb. Cell Fact.* 6, 1–10. doi: 10.1186/1475-2859-6-22

Shori, A. B. (2016). Influence of food matrix on the viability of probiotic bacteria: a review based on dairy and non-dairy beverages. *Food Biosci.* 13, 1–8. doi: 10.1016/j.fbio.2015.11.001

Silva, T. M., Rodrigues, L. Z., Codevilla, C. F., Silva, C., and Menezes, C. R. (2015). Coacervação complexa?: uma técnica para a encapsulação de probióticos. *Ciência Nat.* 37, 49–55. doi:10.5902/2179-460X19714

Skryplonek, K., and Jasińska, M. (2015). Fermented probiotic beverages based on acid whey. *Acta Sci. Pol. Technol. Aliment.* 14, 397–405. doi: 10.17306/J.AFS.2015.4.39

Soares, P. M. G., Mota, J. M. S. C., Gomes, A. S., Oliveira, R. B., Assreuy, A. M. S., Brito, G. A. C., et al. (2008). Gastrointestinal dysmotility in 5-fluorouracil- induced intestinal mucositis outlasts inflammatory process resolution. *Cancer Chemother. Pharmacol.* 63, 91–98. doi: 10.1007/s00280-008-0715-9

Sonis, S. T. (2004). A biological approach to mucositis. *J. Support. Oncol.* 2, 21–36.

Stringer, A. M. (2013). Interaction between host cells and microbes in chemotherapy-induced mucositis. *Nutrients* 5, 1488–1499. doi: 10.3390/nu5051488

Stringer, A. M., Gibson, R. J., Bowen, J. M., Logan, R. M., Ashton, K., Yeoh, A. S. J., et al. (2009a). Irinotecan-induced mucositis manifesting as diarrhoea corresponds with an amended intestinal flora and mucin profile. *Int. J. Exp. Pathol.* 90, 489–499. doi: 10.1111/j.1365-2613.2009.00671.x

Stringer, A. M., Gibson, R. J., Logan, R. M., Bowen, J. M., Yeoh, A. S. J., Hamilton, J., et al. (2009b). Gastrointestinal microflora and mucins may play a critical role in the development of 5-fluorouracil-induced gastrointestinal mucositis. *Exp. Biol. Med.* 234, 430–441. doi: 10.3181/0810-RM-301

Stringer, A. M., Gibson, R. J., Bowen, J. M., Logan, R. M., Yeoh, A. S.-J., and Keefe, D. M. K. (2007). Chemotherapy-induced mucositis: the role of gastrointestinal microflora and mucins in the luminal environment. *J. Support. Oncol.* 5, 259–267.

Tang, Y., Wu, Y., Huang, Z., Dong, W., Deng, Y., Wang, F., et al. (2017). Administration of probiotic mixture DM#1 ameliorated 5-fluorouracil induced intestinal mucositis and dysbiosis in rats. *Nutrition* 33, 96–104. doi: 10.1016/j.nut.2016.05.003

Tharmaraj, N., and Shah, N. P. (2003). Selective enumeration of Lactobacillus delbrueckii ssp. *bulgaricus, Streptococcus thermophilus, Lactobacillus acidophilus,* bifidobacteria, *Lactobacillus casei, Lactobacillus rhamnosus,* and propionibacteria. *J. Dairy Sci.* 86, 2288–2296. doi: 10.3168/jds.S0022- 0302(03)73821-1

Trindade, L. M., Martins, V. D., Rodrigues, N. M., Souza, E. L. S., Martins, F. S., Costa, G. M. F., et al. (2018). Oral administration of Simbioflora® (synbiotic) attenuates intestinal damage in a mouse model of 5-fluorouracil- induced mucositis. *Benef. Microbes* 9, 477–486. doi: 10.3920/BM2017. 0082

Vargas, L. A., Olson, D. W., and Aryana, K. J. (2015). Whey protein isolate improves acid and bile tolerances of *Streptococcus thermophilus* ST-M5 and Lactobacillus delbrueckii ssp. *bulgaricus* LB-12. *J. Dairy Sci.* 98, 2215–2221. doi: 10.3168/jds. 2014-8869

Watterlot, L., Rochat, T., Sokol, H., Cherbuy, C., Bouloufa, I., Lefèvre, F., et al. (2010). Intragastric administration of a superoxide dismutase-producing recombinant *Lactobacillus casei* BL23 strain attenuates DSS colitis in mice. *Int. J. Food Microbiol.* 144, 35–41. doi: 10.1016/j.ijfoodmicro.2010.03.037

WHO (2002). *Guidelines for the Evaluation of Probiotics in Food*. London: World Health Organization, 1–11. doi: 10.1111/j.1469-0691.2012.03873

Yadav, J. S. S., Yan, S., Pilli, S., Kumar, L., Tyagi, R. D., and Surampalli, R. Y. (2015). Cheese whey: a potential resource to transform into bioprotein, functional/nutritional proteins and bioactive peptides. *Biotechnol. Adv.* 33, 756–774. doi: 10.1016/j.biotechadv.2015.07.002

Yeung, C. Y., Chan, W. T., Jiang, C. B., Cheng, M. L., Liu, C. Y., Chang, S. W., et al. (2015). Amelioration of chemotherapy-induced intestinal mucositis by orally administered probiotics in a mouse model. *PLoS One* 10:e0141402. doi: 10.1371/journal.pone.0138746

Conflict of Interest Statement: 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.

GENERAL CONCLUSION AND PERSPECTIVES

The purpose of this thesis work was to highlight the adhesion and immunomodulatory properties, and the corresponding mechanisms, in probiotic dairy propionibacteria, which so far were still scarcely explored. These bacteria are widely used for two main applications: as ripening agents in the production of Emmental type cheeses, and now as probiotics with remarkable immunomodulatory properties which were thought to be associated with surface proteins (RABAH; ROSA DO CARMO; JAN, 2017).

One step at a time.

In the first step of this work we investigated adhesion properties in dairy propionibacteria. After an initial screening of 7 strains of *Propionibacterium freudenreichii* that possess different sets and expression levels of extractable surface proteins, it was observed that the *Propionibacterium freudenreichii* CIRM-BIA 129 (CB 129) strain possessed the highest rate of adherence *in vitro* to cultured human intestinal epithelial cells (HIECs), compared to other strains. Previous studies have shown that this strain, CB 129, has a great *in vitro* and *in vivo* anti-inflammatory potential (LE MARÉCHAL et al., 2015; PLÉ et al., 2015, 2016). This strain, which is more adhesive to HIECs, has five extractable surface proteins, as shown using the chaotropic agent guanidine, including the SlpB protein. Previously works associated SlpB protein with the anti-inflammatory potential of the CB 129 strain, and thus we focused on the slpB protein. After interruption of the *slpB* gene in the CB 129 strain, we observed a drastic decreased adhesion to HIECs. Moreover, the mutant strain, also known as CB129Δ*slpB*, had its adhesion potential restored by the addition of S-layer proteins, purified from the parental wild-type strain. In accordance, trypsin-shave *P. freudenreichii* CIRM-BIA 129 cells lost adhesion ability, which was restored by purified S-layer proteins, in the same way (DO CARMO et al., 2017b).

Posteriorly, in the second step was investigated if the SlpB protein is associated with other processes. Since surface layer proteins possess a range of roles in bacterial cells, we properly investigated other probiotic properties linked to surface properties and to tolerance towards harsh environmental conditions. In the mutant CB129Δ*slpB* strain, a change was observed in surface properties. Hydrophobicity and ζ-potential were decreased, in comparison to the wild-type strain CB 129. Furthermore, tolerance

to acid stress, temperature and bile salts were reduced in the CB129Δ*slpB* mutant strain. Using proteomic approaches, modifications were found in the surface-exposed proteome and in the whole-cell proteome, mainly affecting proteins involved in signalling, information storage, processing, and metabolism process. Genomic DNA of the CB129Δ*slpB* was sequenced, it was observed that the mutation of the *slpB* gene was not inserted within an operon and not directly affect the transcription of other proteins. However, the mutation caused a pleotrophic effect, probably through drastic impact on the surface layer architecture.

In a third step, to explore the effect of the mutation of SlpB on the immunomodulatory effects, we conducted *in vitro* experiments in HIECS submitted to a bacterial stimulation. In this assay, CB 129 wild type strain induced expression of the immunomodulatory IL-10. However, CB129Δ*slpB* mutant strai, increased transcriptional levels pro-inflammatory cytokine IL-8. This mutant was no more able to induce expression of anti-inflammatory cytokine IL-10 in HIEC. The loss of this *in vitro* immunomodulatory potential in the mutant CB129Δ*slpB* strain, and the increased expression of proinflammatory cytokine, enabled us to question whether we would obtain the same trend *in vivo* in a severe model of inflammation. In a mucositis mice model induced by 5-FU, the CB 129 wild type strain alleviates mucosal inflammation, prevents weight loss, and preserves villous architecture. By contrast, the mutant CB129Δ*slpB* strain is not able to protect mice from inflammation induced by 5-FU. Moreover, the administration of the mutant strain promoted an inflammatory mucosal response in the ileum of healthy mice. These data support the assertion that SlpB is a key protein for anti-inflammatory property of *P. freudenreichii* CIRM-BIA 129.

Then we took one more step. We focused on the development of a probiotic fermented product using a probiotic dairy propionibacteria to test in mucositis mice model. The *Propionibacterium freudenreichii* 138 (CB 138) probiotic strain was chosen because of its potential to induce apoptosis in HGT-1 human gastric cancer cells and its ability to produce propionic acid inside the gut (COUSIN et al., 2012). Treatment with the probiotic beverage fermented by CB 138 was able to prevent weight loss and to reduce intestinal damages in mice submitted to 5-FU. Thus, this probiotic fermented beverage, fermented by dairy propionibacteria, can be an interesting tool to prevent mucositis in patients who will receive chemotherapy.

And the next steps?

The results obtained during this thesis work, and the tools developed, allow monitoring of surface proteins, used as a selection criterion in the search for new probiotic strains. Since the surface proteins of the CB 129 strain have the ability to increase the adherence to epithelial cells, they may be used as vaccine adjuvants.

In order to elucidate the mechanisms involved in SlpB protein interaction with target cells, *in vitro* assays will be performed using dendritic cells and macrophages (TH1, TH2, or regulatory effector T cells). This will also allow understanding how CB129Δ*slpB* has become a proinflammatory strain (KONSTANTINOV et al., 2008). In addition, to check another probiotic potential, dairy propionibacteria strains, as well as the CB129 mutant strain, should be challenged in competitive inhibition by exclusion of bacterial pathogens such as *E. coli* enteropathogenic, *Listeria monocytogenes*, and *Salmonella* ssp (GUEIMONDE et al., 2006).

To explore results obtained in *in vivo* inflammatory models, the mechanisms by which CB 129 and CB 138 strains were able to protect mice submitted to 5-FU-mucositis will be investigated and correlated with the mechanisms allowing strain CB 129 to reduce inflammation in murine models of colitis (PLÉ et al., 2015, 2016). Moreover, metagenomic studies of feces from healthy and sick mice treated with probiotic strains will be performed to check for changes in the faecal microbiome. Following the observation that a new fermented milk by CB 138 alleviates mucositis 5-Fu in a mice model, the protection of results through a patent for using probiotic *P. freudenreichii* fermented milk for cancer patients to prevent side-effects is in progress. Clinical studies will be proposed for using the probiotic beverage to prevent mucositis in patients who will receive chemotherapy.

In conclusion, I, Fillipe Luiz Rosa do Carmo, through the collaboration of INRA and UFMG teams and laboratories that are part of the International Associated Laboratory, bring new solid data, using several approaches, to fill gaps of information concerning the pivotal role of *Propionibacterium freudenreichii* CIRM-BIA 129 S-layer protein SlpB. This allows to infer that the SlpB mutation caused profound modifications in *P. freudenreichii* CIRM-BIA 129 physiology and surface properties, adhesion, immunomodulation *in-vitro* and *in-vivo*, and consequently pleiotropic effects affecting

probiotic properties. In addition, a new perspective was added to the probiotic potential of dairy propionibacteria for clinical trials in patients with mucositis.

CONCLUSION GENERALE ET PERSPECTIVES

Le but de ce travail de thèse était de mettre en évidence les propriétés d'adhésion et immunomodulatrices, ainsi que les mécanismes correspondants, des bactéries propioniques laitières probiotiques. Ces propriétés sont encore peu explorées, pourtant ces bactéries sont largement utilisées pour deux applications principales : en tant qu'agents de maturation dans la production de fromages de type Emmental et, depuis quelques années en tant que probiotiques aux propriétés immunomodulatrices remarquables. Des travaux récents de l'équipe ont permis d'associer ces dernères propriétés aux protéines de surface (RABAH; ROSA DO CARMO; JAN, 2017).

Des avancées pas à pas.

Dans la première étape de ce travail, nous avons étudié les propriétés d'adhésion des bactéries propioniques laitières. Après un premier criblage de 7 souches de *Propionibacterium freudenreichii* possédant différents types de protéines de surface extractibles et différents niveaux d'expression de ces protéines, il a été observé que la souche de *P. freudenreichii* CIRM-BIA 129 (CB129) présentait le taux d'adhésion *in vitro* le plus élevé sur cellules épithéliales intestinales humaines (CEIH), comparativement aux autres souches. Des études antérieures ont montré que cette souche, CB129, possède un grand potentiel anti-inflammatoire *in vitro* et *in vivo* (LE MARÉCHAL et al., 2015; PLÉ et al., 2015, 2016). Cette souche CBB129, plus adhésive aux CEIH, possède cinq protéines de surface extractibles, comme le montre la méthode d'extraction à la guanidine, un agent chaotropique. Ces protéines comprennent notamment la protéine SlpB qui a précédemment été associée au potentiel anti-inflammatoire de la souche CB 129. Nous nous sommes donc concentrés sur cette protéine SlpB. L'interruption du gène *slpB* dans la souche CB 129 a permis d'observer une diminution drastique de l'adhésion aux CEIH. De plus, la souche mutante, CB129Δ*slpB*, avait son potentiel d'adhésion restauré par l'addition de la protéine SlpB purifiée à partir de la souche parentale non mutée. Ces résultats démontrent l'implication de la protéine de surface SlpB dans la capacité d'adhésion de la souche *P. freudenreichii* CB 129 aux CEIH (DO CARMO et al., 2017).

Ensuite, dans une deuxième étape, l'implication éventuelle de la protéine SlpB dans d'autres processus a été investiguée. Étant donné que les protéines de surface couvrent une large gamme de rôles chez les bactéries, nous avons étudié d'autres propriétés probiotiques et de tolérance à certaines conditions environnementales stressantes. Dans la souche CB129Δ*slpB* mutante, nous avons observé un changement dans les propriétés de surface : l'hydrophobicité et le potentiel zeta étaient diminués par rapport à la souche CB129 sauvage. De plus, la tolérance au stress acide, à la température et aux sels biliaires était réduite dans la souche mutante CB129Δ*slpB*. Une analyse protéomique a révélé des modifications dans le protéome exposé à la surface et dans le protéome des cellules entières, affectant principalement les protéines impliquées dans la signalisation, le stockage de l'information, le traitement et certains processus métaboliques. Le génome du mutant CB129Δ*slpB* a été séquencé. Ceci a montré que la mutation du gène *slpB* n'affectait pas un opéron et n'affectait pas directement la transcription d'autres protéines. Cependant, la mutation a provoqué un effet pléotrophique, probablement à travers un impact drastique sur l'architecture de la surface bactérienne.

Dans une troisième étape, pour explorer l'effet de la mutation *slpB* sur les propriétés immunomodulatrices, nous avons mené des expériences *in vitro* sur CEIH soumises à une stimulation bactérienne. Dans ce test, la souche CB 129 sauvage a induit l'expression d'IL-10, une cytokine anti-inflammatoire. A l'inverse, le mutant CB129Δ*slpB* a provoqué une augmentation du niveau d'expression de la cytokine pro-inflammatoire IL-8 et n'était plus capable d'induire l'expression d'IL-10 dans les CEIH. La perte de ce potentiel immunomodulateur *in vitro* chez la souche mutante CB129Δ*slpB* et l'augmentation de l'expression de cytokine pro-inflammatoire nous a amené à nous demander si nous obtiendrions la même tendance *in vivo* dans un modèle sévère d'inflammation. Dans un modèle de souris mucosite induite par le 5-FU, la souche CB129 sauvage soulage l'inflammation des muqueuses, empêche la perte de poids et préserve l'architecture des villosités. Au contraire, la souche CB129Δ*slpB* n'est pas capable de protéger les souris de l'inflammation induite par le 5-FU. De plus, l'administration de la souche mutante a induit une réponse inflammatoire de la muqueuse dans l'iléon des souris contrôles saines. Ces données soutiennent l'affirmation que SlpB est une protéine clé pour la propriété anti-inflammatoire de *P. freudenreichii* CIRM-BIA 129.

Ensuite, pour aller plus loin vers l'application, nous nous sommes concentrés sur le développement d'un produit fermenté probiotique en utilisant une bacterie propionique laitière probiotique pour le tester ensuite sur le modèle de mucosite induite chez la souris. La souche probiotique *P. freudenreichii* 138 (CB138) a été choisie en raison de son potentiel, précédemment démontré par l'équipe, à induire l'apoptose dans des cellules cancéreuses humaines HGT-1 et sa capacité à produire de l'acide propionique dans l'intestin (COUSIN et al., 2012). Le traitement avec la boisson probiotique fermentée par CB138 a permis de prévenir la perte de poids et de réduire les dommages intestinaux chez les souris soumises au 5-FU. Ainsi, cette boisson probiotique fermentée par des bactéries propioniques laitières peut être un outil intéressant pour prévenir la mucosite chez les patients qui vont recevoir une chimiothérapie.

Et les prochaines étapes ?

Les résultats obtenus au cours de cette thèse et les outils développés permettent d'utiliser certaines protéines de surface comme critère de sélection dans la recherche de nouvelles souches probiotiques. Puisque certaines protéines de surface de la souche CB129 ont la capacité d'augmenter l'adhésion aux CEIH, elles pourraient être utilisées comme adjuvants de vaccin.

Afin d'élucider les mécanismes impliqués dans l'interaction de la protéine SlpB avec des cellules cibles autres que les CEIH, des dosages *in vitro* seront effectués en utilisant des cellules dendritiques et des macrophages (TH1, TH2, ou des cellules T effectrices régulatrices). Cela permettra également de comprendre pourquoi CB129Δ*slpB* est pro-inflammatoire (KONSTANTINOV et al., 2008). En outre, pour vérifier un autre type de potentiel probiotique, les souches de bactéries propioniques laitières, ainsi que la souche mutante CB129Δ*slpB*, pourraient être tester lors d'expérience d'inhibition de pathogènes bactériens tels que *Escherichia coli* entéropathogène, *Listeria monocytogenes* et *Salmonella* ssp par exclusion et compétition pour les sites d'adhésion (GUEIMONDE et al., 2006).

Pour explorer les résultats obtenus dans des modèles inflammatoires *in vivo*, les mécanismes par lesquels les souches CB 129 et CB 138 peuvent protéger les souris soumises à la mucosite induite par le 5-FU seront étudiés et corrélés aux mécanismes

permettant à la souche CB129 de réduire l'inflammation dans les modèles murins de la colite (PLE et al., 2015, 2016). De plus, des études métagénomiques des fèces de souris saines et malades traitées avec des souches probiotiques seront effectuées pour vérifier les changements dans le microbiome fécal. Suite à l'observation qu'un nouveau lait fermenté par CB138 soulage la mucosite induite au 5-Fu dans un modèle murin, la protection des résultats est en cours par un brevet pour l'utilisation de lait fermenté par des souches de *P. freudenreichii* probiotiques chez des patients cancéreux pour prévenir les effets secondaires de chimiothérapie. Des études cliniques seront proposées pour utiliser la boisson probiotique pour prévenir la mucosite chez les patients qui recevront une chimiothérapie.

En conclusion, grâce à la collaboration des équipes INRA et UFMG et dans le cadre du Laboratoire International Associé « BactInflam », mon travail de thèse apporte de nouvelles données solides, en utilisant plusieurs approches, pour combler les lacunes dans nos connaissances sur le rôle pivot de la protéine SlpB de *P. freudenreichii* CIRM-BIA 129. Ceci a permis de démontrer que la mutation *slpB* provoque des modifications profondes de la physiologie et des propriétés de surface de *P. freudenreichii* CIRM-BIA 129, ainsi que de l'adhésion, de l'immunomodulation *in vitro* et *in vivo*. Des effets pléiotropes affectant les propriétés probiotiques. De plus, une nouvelle perspective a été ajoutée au potentiel probiotique des propionibactéries laitières pour les essais cliniques chez les patients atteints de mucite.

REFERENCE

BEAULIEU, J.; DUPONT, C.; LEMIEUX, P. Anti-inflammatory potential of a malleable matrix composed of fermented whey proteins and lactic acid bacteria in an atopic dermatitis model. **Journal of Inflammation (London, England)**, v. 4, p. 6, 21 mar. 2007.

BELL, V.; FERRÃO, J.; FERNANDES, T. Nutritional Guidelines and Fermented Food Frameworks. **Foods (Basel, Switzerland)**, v. 6, n. 8, 7 ago. 2017.

BIBILONI, R. et al. VSL#3 probiotic-mixture induces remission in patients with active ulcerative colitis. **The American Journal of Gastroenterology**, v. 100, n. 7, p. 1539–1546, jul. 2005.

BOUMA, G.; STROBER, W. The immunological and genetic basis of inflammatory bowel disease. **Nature Reviews. Immunology**, v. 3, n. 7, p. 521–533, jul. 2003.

BUCK, B. L. et al. Functional Analysis of Putative Adhesion Factors in Lactobacillus acidophilus NCFM. **Applied and Environmental Microbiology**, v. 71, n. 12, p. 8344–8351, dez. 2005.

CARVALHO, R. D. D. O. et al. Use of wild type or recombinant Lactic Acid Bacteria as an alternative treatment for gastrointestinal inflammatory diseases: A focus on Inflammatory Bowel Diseases and Mucositis. **Frontiers in Microbiology**, v. 8, 2017.

CHEN, X. et al. The S-layer proteins of Lactobacillus crispatus strain ZJ001 is responsible for competitive exclusion against Escherichia coli O157:H7 and Salmonella typhimurium. **International Journal of Food Microbiology**, v. 115, n. 3, p. 307–312, 20 abr. 2007.

COUSIN, F. J. et al. Dairy propionibacteria as human probiotics: A review of recent evidence. **Dairy Science & Technology**, 2 ago. 2010.

COUSIN, F. J. et al. Milk fermented by Propionibacterium freudenreichii induces apoptosis of HGT-1 human gastric cancer cells. **PloS One**, v. 7, n. 3, p. e31892, 2012.

COUSIN, F. J. et al. The probiotic Propionibacterium freudenreichii as a new adjuvant for TRAIL-based therapy in colorectal cancer. **Oncotarget**, v. 7, n. 6, p. 7161–7178, 2016.

DEUTSCH, S.-M. et al. Identification of proteins involved in the anti-inflammatory properties of Propionibacterium freudenreichii by means of a multi-strain study. **Scientific Reports**, v. 7, p. 46409, 13 abr. 2017.

DO CARMO, F. L. R. et al. Applications of Probiotic Bacteria and Dairy Foods in Health. In: **Current Research in Microbiology**. [s.l.] Open Access eBooks 919 North Market Street Suite 425 Wilmington, DE 19801, 2017a. p. np.

DO CARMO, F. L. R. et al. Propionibacterium freudenreichii Surface Protein SlpB Is Involved in Adhesion to Intestinal HT-29 Cells. **Frontiers in Microbiology**, v. 8, 8 jun. 2017b.

DO CARMO, F. L. R. et al. Extractable Bacterial Surface Proteins in Probiotic–Host Interaction. **Frontiers in Microbiology**, v. 9, 4 abr. 2018.

DOHM, N. et al. Molecular and biochemical properties of the S-layer protein from the wine bacterium Lactobacillus hilgardii B706. **Archives of Microbiology**, v. 193, n. 4, p. 251–261, abr. 2011.

EFSA. Panel on Biological Hazards (BIOHAZ) - Scientific Opinion on the maintenance of the list of QPS microorganisms intentionally added to food or feed (2009 update): QPS 2009 update. **EFSA Journal**, v. 7, n. 12, p. 1431, dez. 2009.

ESLAMI, N.; KERMANSHAHI, R. K.; ERFAN, M. Studying the Stability of S-Layer Protein of Lactobacillus Acidophilus ATCC 4356 in Simulated Gastrointestinal Fluids Using SDS-PAGE and Circular Dichroism. **Iranian Journal of Pharmaceutical Research : IJPR**, v. 12, n. Suppl, p. 47–56, 2013.

FOLIGNÉ, B. et al. Promising immunomodulatory effects of selected strains of dairy propionibacteria as evidenced in vitro and in vivo. **Applied and Environmental Microbiology**, v. 76, n. 24, p. 8259–8264, dez. 2010.

FOLIGNÉ, B. et al. Tracking the microbiome functionality: focus on Propionibacterium species. **Gut**, v. 62, n. 8, p. 1227–1228, ago. 2013.

GHOURI, Y. A. et al. Systematic review of randomized controlled trials of probiotics, prebiotics, and synbiotics in inflammatory bowel disease. **Clinical and Experimental Gastroenterology**, v. 7, p. 473–487, 2014.

GUEIMONDE, M. et al. Adhesion and competitive inhibition and displacement of human enteropathogens by selected lactobacilli. **Food Research International**, v. 39, n. 4, p. 467–471, maio 2006.

GUO, X.-H. et al. Screening lactic acid bacteria from swine origins for multistrain probiotics based on in vitro functional properties. **Anaerobe**, v. 16, n. 4, p. 321–326, ago. 2010.

HAVENAAR, R.; BRINK, B. T.; VELD, J. H. J. H. I. 'T. Selection of strains for probiotic use. In: **Probiotics**. [s.l.] Springer Netherlands, 1992. p. 209–224.

HILL, C. et al. Expert consensus document. The International Scientific Association for Probiotics and Prebiotics consensus statement on the scope and appropriate use of the term probiotic. **Nature Reviews. Gastroenterology & Hepatology**, v. 11, n. 8, p. 506–514, ago. 2014.

HOUWINK, A. L. A macromolecular mono-layer in the cell wall of Spirillum spec. **Biochimica Et Biophysica Acta**, v. 10, n. 3, p. 360–366, mar. 1953.

HUANG, Y.; ADAMS, M. C. An in vitro model for investigating intestinal adhesion of potential dairy propionibacteria probiotic strains using cell line C2BBe1. **Letters in Applied Microbiology**, v. 36, n. 4, p. 213–216, 2003.

HYNÖNEN, U.; PALVA, A. Lactobacillus surface layer proteins: structure, function and applications. **Applied Microbiology and Biotechnology**, v. 97, n. 12, p. 5225–5243, jun. 2013.

JOHANSSON, M. E. V. et al. Composition and functional role of the mucus layers in the intestine. **Cellular and molecular life sciences: CMLS**, v. 68, n. 22, p. 3635–3641, nov. 2011.

JUSTINO, P. F. C. et al. Regulatory role of Lactobacillus acidophilus on inflammation and gastric dysmotility in intestinal mucositis induced by 5-fluorouracil in mice. **Cancer Chemotherapy and Pharmacology**, v. 75, n. 3, p. 559–567, mar. 2015.

KLINMAN, D. M. et al. FDA guidance on prophylactic DNA vaccines: analysis and recommendations. **Vaccine**, v. 28, n. 16, p. 2801–2805, 1 abr. 2010.

KONSTANTINOV, S. R. et al. S layer protein A of Lactobacillus acidophilus NCFM regulates immature dendritic cell and T cell functions. **Proceedings of the National Academy of Sciences**, v. 105, n. 49, p. 19474–19479, 9 dez. 2008.

LE MARÉCHAL, C. et al. Surface proteins of Propionibacterium freudenreichii are involved in its anti-inflammatory properties. **Journal of Proteomics**, v. 113, p. 447–461, 15 jan. 2015.

LEBEER, S.; VANDERLEYDEN, J.; DE KEERSMAECKER, S. C. J. Host interactions of probiotic bacterial surface molecules: comparison with commensals and pathogens. **Nature Reviews. Microbiology**, v. 8, n. 3, p. 171–184, mar. 2010.

LI, P. et al. Lactobacillus S-layer protein inhibition of Salmonella-induced reorganization of the cytoskeleton and activation of MAPK signalling pathways in Caco-2 cells. **Microbiology (Reading, England)**, v. 157, n. Pt 9, p. 2639–2646, set. 2011.

LIGHTFOOT, Y. L. et al. SIGNR3-dependent immune regulation by Lactobacillus acidophilus surface layer protein A in colitis. **The EMBO Journal**, v. 34, n. 7, p. 881–895, 1 abr. 2015.

MARTEAU, P. Probiotics, prebiotics, synbiotics: ecological treatment for inflammatory bowel disease. **Gut**, v. 55, n. 12, p. 1692–1693, dez. 2006.

MÉNARD, S. et al. Bifidobacterium breve and Streptococcus thermophilus secretion products enhance T helper 1 immune response and intestinal barrier in mice. **Experimental Biology and Medicine (Maywood, N.J.)**, v. 230, n. 10, p. 749–756, nov. 2005.

MOUSSAVI, M.; ADAMS, M. C. An in vitro study on bacterial growth interactions and intestinal epithelial cell adhesion characteristics of probiotic combinations. **Current Microbiology**, v. 60, n. 5, p. 327–335, maio 2010.

NUTRITION, C. FOR F. S. AND A. **Generally Recognized as Safe (GRAS)**. WebContent. Disponível em: <https://www.fda.gov/Food/IngredientsPackagingLabeling/GRAS/>. Acesso em: 28 jun. 2018.

O'TOOLE, P. W.; MARCHESI, J. R.; HILL, C. Next-generation probiotics: the spectrum from probiotics to live biotherapeutics. **Nature Microbiology**, v. 2, n. 5, maio 2017.

OTTE, J.-M.; PODOLSKY, D. K. Functional modulation of enterocytes by gram-positive and gram-negative microorganisms. **American Journal of Physiology. Gastrointestinal and Liver Physiology**, v. 286, n. 4, p. G613-626, abr. 2004.

PLÉ, C. et al. Single-strain starter experimental cheese reveals anti-inflammatory effect of Propionibacterium freudenreichii CIRM BIA 129 in TNBS-colitis model. **Journal of Functional Foods**, v. 18, p. 575–585, 2015.

PLÉ, C. et al. Combining selected immunomodulatory *Propionibacterium freudenreichii* and *Lactobacillus delbrueckii* strains: Reverse engineering development of an anti-inflammatory cheese. **Molecular Nutrition & Food Research**, v. 60, n. 4, p. 935–948, abr. 2016.

PREISING, J. et al. Selection of bifidobacteria based on adhesion and anti-inflammatory capacity in vitro for amelioration of murine colitis. **Applied and Environmental Microbiology**, v. 76, n. 9, p. 3048–3051, maio 2010.

RABAH, H.; ROSA DO CARMO, F. L.; JAN, G. Dairy Propionibacteria: Versatile Probiotics. **Microorganisms**, v. 5, n. 2, 13 maio 2017.

RESEARCHANDMARKETS. **Global Dairy Market Analysis, Growth, Trends & Forecast 2018-2023**. Disponível em: <https://www.businesswire.com/news/home/20180515005739/en/Global-Dairy-Market-Analysis-Growth-Trends-Forecast>. Acesso em: 28 jun. 2018.

RIEDEL, C. U. et al. Interaction of bifidobacteria with Caco-2 cells-adhesion and impact on expression profiles. **International Journal of Food Microbiology**, v. 110, n. 1, p. 62–68, 1 jul. 2006.

RODRÍGUEZ, C. et al. Therapeutic effect of Streptococcus thermophilus CRL 1190-fermented milk on chronic gastritis. **World Journal of Gastroenterology**, v. 16, n. 13, p. 1622–1630, 7 abr. 2010.

RONG, J. et al. Probiotic and anti-inflammatory attributes of an isolate Lactobacillus helveticus NS8 from Mongolian fermented koumiss. **BMC microbiology**, v. 15, p. 196, 2 out. 2015.

SÁNCHEZ, B. et al. Probiotics, gut microbiota, and their influence on host health and disease. **Molecular Nutrition & Food Research**, v. 61, n. 1, p. 1600240, jan. 2017.

SANTOS ROCHA, C. et al. Anti-inflammatory properties of dairy lactobacilli. **Inflammatory Bowel Diseases**, v. 18, n. 4, p. 657–666, abr. 2012.

SANTOS ROCHA, C. et al. Local and systemic immune mechanisms underlying the anti-colitis effects of the dairy bacterium Lactobacillus delbrueckii. **PloS One**, v. 9, n. 1, p. e85923, 2014.

SHIBY, V. K.; MISHRA, H. N. Fermented milks and milk products as functional foods--a review. **Critical Reviews in Food Science and Nutrition**, v. 53, n. 5, p. 482–496, 2013.

SLEYTR, U. B. et al. S-layers: principles and applications. **FEMS Microbiology Reviews**, v. 38, n. 5, p. 823–864, set. 2014.

SLEYTR, U. B.; MESSNER, P. Crystalline surface layers in procaryotes. **Journal of Bacteriology**, v. 170, n. 7, p. 2891–2897, jul. 1988.

SMIT, E. et al. The S-layer Protein of Lactobacillus acidophilus ATCC 4356: Identification and Characterisation of Domains Responsible for S-protein Assembly and Cell Wall Binding. **Journal of Molecular Biology**, v. 305, n. 2, p. 245–257, 12 jan. 2001.

SONIS, S. T. A biological approach to mucositis. **The Journal of Supportive Oncology**, v. 2, n. 1, p. 21–32; discussion 35-36, fev. 2004a.

SONIS, S. T. The pathobiology of mucositis. **Nature Reviews. Cancer**, v. 4, n. 4, p. 277–284, abr. 2004b.

SOOD, A. et al. The probiotic preparation, VSL#3 induces remission in patients with mild-to-moderately active ulcerative colitis. **Clinical Gastroenterology and Hepatology: The Official Clinical Practice Journal of the American Gastroenterological Association**, v. 7, n. 11, p. 1202–1209, 1209.e1, nov. 2009.

TAVERNITI, V. et al. S-layer protein mediates the stimulatory effect of Lactobacillus helveticus MIMLh5 on innate immunity. **Applied and Environmental Microbiology**, v. 79, n. 4, p. 1221–1231, fev. 2013.

THIERRY, A. et al. New insights into physiology and metabolism of Propionibacterium freudenreichii. **International Journal of Food Microbiology**, v. 149, n. 1, p. 19–27, 1 set. 2011.

TRINDADE, L. M. et al. Oral administration of Simbioflora® (synbiotic) attenuates intestinal damage in a mouse model of 5-fluorouracil-induced mucositis. **Beneficial Microbes**, v. 9, n. 3, p. 477–486, 25 abr. 2018.

VAN DE GUCHTE, M. et al. Properties of probiotic bacteria explored by proteomic approaches. **Current Opinion in Microbiology**, v. 15, n. 3, p. 381–389, jun. 2012.

VIEIRA, A. T.; TEIXEIRA, M. M.; MARTINS, F. S. The Role of Probiotics and Prebiotics in Inducing Gut Immunity. **Frontiers in Immunology**, v. 4, 2013.

WILSON, W. W. et al. Status of methods for assessing bacterial cell surface charge properties based on zeta potential measurements. **Journal of Microbiological Methods**, v. 43, n. 3, p. 153–164, jan. 2001.

YAMASHITA, M. et al. Lactobacillus helveticus SBT2171, a cheese starter, regulates proliferation and cytokine production of immune cells. **Journal of Dairy Science**, v. 97, n. 8, p. 4772–4779, 2014.

YEUNG, C. Y. et al. Amelioration of chemotherapy-induced intestinal mucositis by orally administered probiotics in a mouse model. **PLoS ONE**, v. 10, n. 9, p. 1–16, 2015.

ZARATE, G. Dairy Propionibacteria: Less Conventional Probiotics to Improve the Human and Animal Health. In: RIGOBELO, E. (Ed.). . **Probiotic in Animals**. [s.l.] InTech, 2012.

ZION MARKET RESEARCH™. **Probiotics Market Size, Share, Growth | Forecast (2018-0223)**. Disponível em: <https://www.zionmarketresearch.com/>. Acesso em: 28 jun. 2018.

UNIVERSITE BRETAGNE LOIRE / ECOLOGIE GEOSCIENCES AGRONOMIE ALIMENTATION

AGRO CAMPUS OUEST

Titre : La protéine de couche de surface SlpB assure la médiation de l'immunomodulation et de l'adhésion chez le probiotique *Propionibacterium freudenreichii* CIRM-BIA 129.

Mots clés : propionibactéries, probiotique, immunomodulation, protéine de S-layer, mucosite.

Résumé : *Propionibacterium freudenreichii* est une bactérie Gram-positive bénéfique, traditionnellement utilisée comme levain d'affinage fromager, qui bénéficie du statut GRAS (Generally Recognized As Safe). *P. freudenreichii* a révélé un effet immunomodulateur qui a été confirmé *in vivo* par la capacité à protéger des souris d'une colite aigüe induite. L'effet anti-inflammatoire est cependant hautement souche-dépendant. Il est dû, au moins en partie, à des composés de surface clés qui favorisent ces effets probiotiques. Les bactéries Gram-positives, y compris *P. freudenreichii*, peuvent être recouvertes d'une couche extérieure protéique, appelée « surface-layer », paracristalline, et formée par l'autoassemblage de protéines dites de S-layer (Slps). Les Slps, dans différentes bactéries, sont impliquées dans plusieurs caractéristiques probiotiques, telles que l'adhésion aux cellules de l'hôte et au mucus, la persistance dans l'intestin, ou encore l'immunomodulation. Le but de cette étude est d'étudier, chez une souche probiotique de *P. freudenreichii*, la protéine de surface qui joue le principal rôle dans les interactions probiotiques avec l'hôte. La souche *P. freudenreichii* CIRM-BIA 129, récemment reconnue comme immunomodulatrice prometteuse, possède plusieurs protéines de surface Slps, y compris SlpB. Dans la présente étude, l'inactivation du gène correspondant, dans la souche mutante CB129Δ*slpB*, a provoqué une baisse drastique de l'adhésion aux cellules intestinales épithéliales HT-29, confirmant le rôle clé des Slps dans l'adhésion aux cellules. P. freudenreichii CIRM-BIA 129. Nous avons étudié la réponse immune de cellules HT-29 stimulées par le sauvage ou par le mutant. Le sauvage induisait principalement l'expression de la cytokine immunomodulatrice IL-10. Le mutant induisait, de façon intéressante, moins d'IL-10, mais plus d'IL-8. Afin d'évaluer the rôle de cette protéines clé SlpB dans les effets antiinflammatoires de P. freudenreichii, nous avons testé ces deux souches dans un modèle murin de mucosite induite par le 5-Fluorouracile. La consommation de la souche sauvage prévenait la perte de poids, préservait l'architecture des villosités, ainsi que la densité des granules de sécrétion au sein des cellules de Paneth, chez les souris atteintes de mucosite. Cependant, le mutant n'avait pas cet effet protecteur. Cette étude a mis en évidence l'implication de la protéine SlpB de *P. freudenreichii* dans les propriétés anti-inflammatoires de cette bactérie et confirme le rôle versatile joué par les Slps dans les interactions probiotique/hôte.

Title : Surface layer protein SlpB mediates immunodulation and adhesion in the probiotic *Propionibacterium freudenreichii* CIRM-BIA 129.

Keywords : Propionibacteria, probiotic, immunomodulation, S-layer protein, mucositis.

Abstract: *Propionibacterium freudenreichii* is a beneficial Gram-positive bacterium, traditionally used as a cheese ripening starter, with the GRAS status (Generally Recognized As Safe). *P. freudenreichii* has revealed an immunomodulatory effect confirmed in vivo by the ability to protect mice from induced acute colitis. The anti-inflammatory effect is however highly strain-dependent and due, at least in part, to key surface compounds favouring probiotic effects. Gram-positive bacteria, including P. freudenreichii, can be covered with an external proteinaceous layer called a surface-layer paracrystalin layer and formed by the self-assembly of surface-layer-proteins (Slps). Slps were shown, in different bacteria, to be involved in several probiotics traits, such as adhesion to host cells and mucus, persistence within the gut, or immunomodulation. The aim of this study is to investigate, in a *P. freudenreichii* probiotic strain, the surface protein that plays the main role in the probiotic interaction with the host. The P. freudenreichii CIRM-BIA 129 strain recently revealed promising immunomodulatory properties and possesses several Slps, including SlpB. In the presented work, inactivation of the corresponding gene, CB129Δ*slpB* a mutant strain, caused a drastic decrease in adhesion to intestinal epithelial HT-29 cells, further evidencing the key role of Slps in cell adhesion. we investigated immune response of HT-29 cells towards *P. freudenreichii* CIRM-BIA 129 and CB129Δ*slpB*. The wild type strain mainly induced expression of the immunomodulatory IL-10 by the cells. Interestingly, the mutant strain induced decreased expression of IL-10, yet increased expression of IL-8. To finally evaluate the role of the key surface protein SlpB in the ant-inflammatory property of *P. freudenreichii*, we examined both strains in a murine mucositis model induced by 5-Fluorouracil (5-FU). *P. freudenreichii* wild type strain was able to prevent weight loss, to preserve villous architecture and to preserve secretory granules density inside Paneth cells in mucositis–affected mice. By contrast, the mutant CB129Δ*slpB* strain failed to exert such protective effect. This study evidenced the involvement of *P. freudenreichii* SlpB protein in its anti-inflammatory properties and confirmed the versatile role fulfilled by Slps in probiotic host interactions.