La vectorisation de Propionibacterium freudenreichii CIRM-BIA 129 et de ses protéines immunomodulatrices par la matrice fromagère vers le tube digestif

Houem Rabah

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Par

« Houem RABAH»

« La vectorisation de *Propionibacterium freudenreichii* et de ses protéines immunomodulatrices par la matrice fromagère vers le tube digestif »

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Ecole Doctorale : « Ecologie, Géosciences, Agronomie et Alimentation (EGAAL)»

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قلۡ وَفَوۡقَ كُلِّ ذِي عِلۡمٍ عَلِيمٍ ﴿٧٦﴾

قلۡ Et au-dessus de tout homme détenant une science, il y a un savant plus docte que lui ﴿٧٦﴾

Sorah Yusuf ﴿Joseph﴾ – verset 76

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Symboles et abréviations

- AGCC/SCFA : acides gras à chaîne courte/short chain fatty acids
- CID : collision Induced Dissociation
- CIRM-BIA : centre international de ressources microbiennes et de bactéries d'intérêt alimentaire
- CNRZ : centre national de recherches zootechniques
- ConA : concanavalin A
- DHNA : 1.4-dihydroxy-2-naphthoic acid
- DSS : dextran sulfate sodium
- DTT : dithiothréitol
- ECD : electron capture dissociation
- EFSA: autorité européenne de sécurité des aliments
- ETD : electron-transfer dissociation
- FAO : organisation pour l'alimentation et l'agriculture/ food and agriculture organization
- FDA : food drug administration
- FOXP3 : forkhead box protein P3
- GALT :gut-associated lymphoid tissue
- GATA3 : GATA binding protein 3
- GRAS : generally recognized as safe
- HCD : high energy collision dissociation
- IFNγ : interferon gamma
- IgA : immunoglobuline A
- IL- : interleukine
- LGCM: laboratory of cellular and Molecular Genetic
- LPS : lipopolysaccharides
- MC/CD : maladie de Crohn/Crohn disease
- MICI/IBD : maladies inflammatoires intestinales/inflammatory bowel diseases
- MLNC : mensenteric lymph nodes cells

- MUF/UF : milk ultrafiltrate/ultrafiltrat de lait
- NuMecan: « nutrition, métabolismes et cancer »
- OMS/WHO : organisation mondiale de la santé/world health organization
- PBMC : peripheral blood mononuclear cells
- PBS: phosphate-buffered saline
- *Pf* : *Propionibacterium freudenreichii*
- PNGase F : peptide N-glycosidase F
- PRRs : pattern recognition receptors
- QPS : qualified presumption of safety
- RCH/UC : rectocolite hémorragique / ulcerative colitis
- RfpB : resuscitation-promoting factor
- RORγt : retinoid-related orphan receptor gamma t
- SDS-PAGE : sodium dodecyl sulfate-polyacrylamide gel electrophoresis
- SlpB : S-layer protein B
- Slps : S-layer proteins
- T-bet : T-box transcription factor
- TBS: Tris-buffered saline
- TFA : acide trifluoroacétique
- Th17 : T helper 17 cells
- Th2 : T helper 2 cells
- TNBS : acide 2, 4,6-trinitrobenzène sulfonique
- TNFα : tumor necrosis factor alpha
- Treg : regulatory T cells
- UFC/ CFU : unité formant une colonie/ colony-forming unit
- VBNC : viable but non-culturable
- YEL : yeast-extract-lactate
- YELA : yeast-extract-lactate agar

Introduction générale

Le lien entre l'alimentation et la santé a été d'ores et déjà établi depuis l'antiquité. De nombreuses études scientifiques ont mis la lumière l'impact de certains facteurs nutritionnels sur la survenue de nombreuses pathologies chroniques : maladies cardiovasculaires, cancers, ostéoporose, maladies métaboliques et maladies neurodégénératives. De plus, dans les pays industrialisés, le style de vie (hygiène, tabac, sédentarité, activité physique, …..) accélèrent l'émergence de ces maladies, ce qui affecte la durée et la qualité de vie, générant ainsi des coûts de santé publique croissants. En effet, lors de ces dernières décennies, le rôle central du microbiote intestinal dans notre santé a été mis en évidence, sa composition détermine la survenue de nombreuses pathologies, telles que l'obésité, le diabète de type 2 ou encore les maladies inflammatoires chroniques de l'intestin (MICI) (Prakash et al., 2011; Sommer and Bäckhed, 2013). L'impact du microbiote alimentaire, ingéré quotidiennement, fait partie des liens qui existent entre l'alimentation et la santé (Cotillard et al., 2013; David et al., 2013). La modulation du microbiote intestinal via des microorganismes probiotiques est aujourd'hui un champ d'investigation en plein essor. Un probiotique est un "microorganisme vivant qui, lorsqu'il est consommé en quantité adéquate, confère un bénéfice pour la santé de l'hôte" (FAO, WHO, 2006). Il s'agit en très grande majorité de bactéries ou de levures, dont l'effet sur la santé peut s'exprimer en termes d'actions préventives ou thérapeutiques vis-à-vis de certaines maladies. Par conséquent, le marché des probiotiques est en plein croissance (Probiotics Market by Source, Form, Application, End User, Region - 2022 | MarketsandMarkets). Des résultats prometteurs sont observés dans le cas des MICI (Derwa et al., 2017).

Dans ce contexte, les bactéries propioniques laitières, en particulier les souches de l'espèce *Propionibacterium freudenreichii*, pourraient avoir un rôle à jouer, suite à la découverte de leur propriétés anti-inflammatoires souche-dépendantes (Foligné et al., 2010). Les travaux au sein du laboratoire STLO (Science & technologie du lait & de l'œuf) ont mis en lumière les propriétés «2-en-1» de certaine souches de *P. freudenreichii*, c'est-à-dire des propriétés techno-fonctionnelles pour la fermentation d'aliments et des propriétés probiotiques. La sélection de souches de *P. freudenreichii* «2-en-1» permettrait ainsi de mettre au point des aliments fonctionnels fermentés adaptés à une fraction croissante de la population atteinte de MICI ou permettant de prévenir l'apparition de ces maladies chez des sujets à risque. Le fromage emmental pourrait être l'aliment de prédilection pour concevoir des

aliments fonctionnels laitiers fermentés par *P. freudenreichii*. En effet, la bactérie *P. freudenreichii* est consommée en grande quantité dans l'emmental (10^9 bactéries par gramme de produit). De plus, des fromages expérimentaux, fermentés par *P. freudenreichii* seul, ou en combinaison avec d'autres bactéries probiotiques, préviennent l'apparition de la colite chimiquement induite chez la souris (Plé et al., 2015, 2016). Cependant, nous ignorons comment la matrice fromagère impacte les propriétés probiotiques de *P. freudenreichii*, en particulier l'effet immunomodulateur orchestré, en partie, par des protéines de surface immunomodulatrices. En effet, les propriétés probiotiques peuvent être affectées du fait de l'interaction de la bactérie avec la matrice fromagère mais également avec l'écosystème microbien existant.

Ainsi, ce projet de thèse «**PropioCheeseGut**», intitulé «La vectorisation de *Propionibacterium freudenreichii* et de ses protéines immunomodulatrices par la matrice fromagère vers le tube digestif», a débuté dans le but d'apporter ces connaissances manquantes. La thèse bénéficiant d'une bourse CIFRE a été financée par l'association Bretagne Biotechnologies Alimentaires (BbA) et le Conseil Régional de Bretagne. Le travail a été encadré par Gwénaël Jan (INRA, UMR1253 STLO, Rennes, France) et s'inscrit dans les thématiques de recherche développés au sein du laboratoire STLO. Des collaborations avec l'institut NuMecan (Unité mixte INRA/INSERM, Saint Gilles) ainsi qu'avec le laboratoire LGCM (Université fédérale de Minas Gerais, Brésil) ont été établies pour répondre à la question de recherche : **Comment la matrice fromagère impacte-t-elle les propriétés immunomodulatrices de *P. freudenreichii* CIRM-BIA 129 à travers ses protéines de surface ?**

Le manuscrit présentant ce travail de thèse a été organisé sous forme de différents chapitres, qui sont les suivants :

- **Le chapitre 1** fait l'état de l'art des données de littérature sur l'utilisation des probiotiques comme adjuvant pour prévenir ou pour traiter les MICI, en particulier la rectocolite hémorragique. Il comprend une première revue de littérature publiée qui présente les propriétés probiotiques versatiles des bactéries propioniques laitières. Les mécanismes d'action anti-inflammatoire des microorganismes probiotiques sont présentés. Une deuxième revue de littérature publiée est intégrée à ce chapitre afin de présenter les fonctions multiples des protéines S-layer de bactéries probiotiques à Gram positif, y compris *P.*

freudenreichii, avec un focus particulier sur leur rôle dans l'interaction avec les cellules de l'hôte. Une attention particulière a été donnée à la notion d'aliments fermentés fonctionnels et l'effet matrice sur les propriétés probiotiques de *P. freudenreichii*.

- **Le chapitre 2** aborde la question de recherche, l'objectif et les stratégies optées pour répondre à la question de recherche.

Les chapitres suivants décrivent les résultats obtenus lors de la thèse, présentés sous forme d'articles :

- **Le chapitre 3** expose l'étude *in vitro* sur la vectorisation de *P. freudenreichii* CIRM-BIA 129 et de ses protéines de surface par la matrice fromagère durant le processus de digestion *in vitro*.

- **Le chapitre 4** traite des résultats obtenus en termes des effets de la vectorisation par la matrice fromagère sur les propriétés probiotiques de *P. freudenreichii* CIRM-BIA 129 *in vivo*, chez des porcelets sains.

- **Le chapitre 5** expose les résultats concernant l'effet matrice sur la viabilité *in vitro* de *P. freudenreichii* CIRM-BIA 129 dans l'environnement caecal, un milieu mimant les conditions environnmentales du côlon.

- **Le chapitre 6** présente les résultats précliniques sur l'effet anti-inflammatoire d'un emmental dit «probiotique» pour la prévention de la rectocolite hémorragique dans un modèle murin.

Enfin, le manuscrit se termine par une discussion générale, qui rappelle les principaux résultats présentés dans chaque chapitre de résultats, qui démontre aussi comment ces résultats répondent à la question de recherche, et qui discute les limites de ces résultats obtenus. Tout au long de la discussion, des perspectives sont également proposées pour la poursuite de ce projet.

Chapitre 1 : « Synthèse bibliographique »

1.1. Les maladies inflammatoires intestinales

1.1.1. Définition, prévalence et étiologie

Les maladies inflammatoires chroniques intestinales (MICI) comportent la maladie de Crohn (MC) et la rectocolite hémorragique (RCH). La MC se caractérise par une inflammation qui affecte n'importe quelle partie du tractus gastro-intestinal, de la bouche à l'anus. La RCH, quant à elle, correspond à l'atteinte inflammatoire de tout ou d'une partie du côlon. Elle est associée à un risque plus élevé de la survenue du cancer colorectal. Ces maladies évoluent par des poussées inflammatoires de durée et de fréquence variables, alternées par des phases de rémission (Observatoire National des MICI). Les pays développés d'Europe, d'Amérique du Nord, ainsi que d'Océanie présentent une prévalence élevée des MICI mais leur incidence s'est stabilisée au cours de ces dernières années (Figure 1) (Burisch et al., 2013; Kaplan, 2015). Cependant, l'incidence des MICI augmente dans les pays en cours d'industrialisation (Pays du Maghreb, Asie, Afrique du Sud...) (Burisch et al., 2013; Kaplan, 2015).

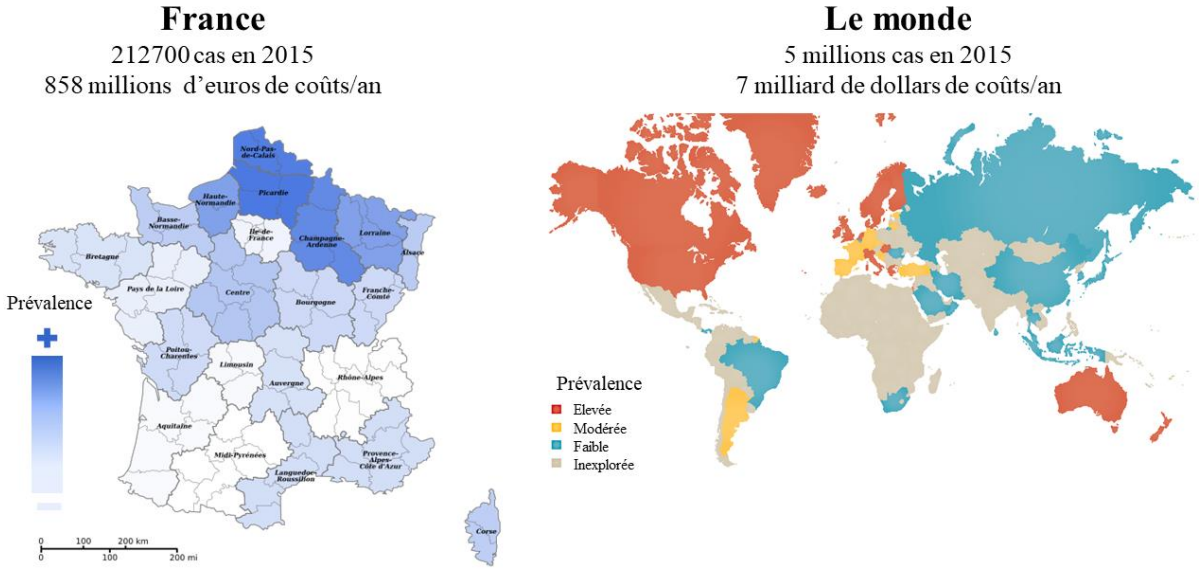

***Figure 1*. La prévalence ainsi que les coûts des maladies inflammatoires chroniques intestinales (MICI)**. Elles incluent la maladie de Crohn (MC) et la rectocolite hémorragique (RCH), les prévalences sont présentées pour l'année 2015 en France (Observatoire National des MICI), ainsi que dans le monde (Kaplan 2015).

En France, environ 25 nouveaux cas de MICI sont diagnostiqués chaque jour (Figure 1) (Observatoire National des MICI). Dans le monde occidental, les MICI affectent essentiellement la qualité de vie des malades et entraînent des coûts substantiels pour les systèmes de santé (Kaplan, 2015).

L'étiologie exacte de ces maladies n'est pas élucidée. Cependant, elles semblent impliquer plusieurs facteurs de risque liés au système immunitaire, à l'environnement, au génotype et tout particulièrement au microbiote intestinal. La mise en évidence d'une dysbiose chez les patients atteints de MICI montre qu'il peut s'agir d'une anomalie de la réponse du système immunitaire intestinal vis-à-vis de certains composants de la flore intestinale, survenant chez des individus génétiquement prédisposés (Vindigni et al., 2016; Zhang and Li, 2014). Contrairement aux individus sains porteurs d'une population bactérienne fécale stable dans le temps, les patients atteints de MICI possèdent un microbiote instable (Frank et al., 2007; Hartley et al., 1992). C'est ainsi qu'à côté des agents pathogènes classiques, le microbiote intestinal peut devenir pathogène par deux voies : l'expansion d'espèces « pro-inflammatoires » ou la diminution de la composante protectrice du microbiote (Duchmann et al., 1995; Packey and Sartor, 2009).

1.1.2. Symptômes et signes cliniques

Les symptômes généraux associés aux MICI sont des douleurs abdominales**,** des diarrhées fréquentes, parfois sanglantes (cas de la RCH), ou encore une atteinte de la région anale (fissure, abcès). Elles s'accompagnent souvent de fatigue, d'anémie, d'amaigrissement, de fièvre et voir de manifestations extra-intestinales (articulaires, cutanées, oculaires, hépatiques) (Bernstein, 2015; Matowicka-Karna, 2016). De nombreux marqueurs inflammatoires fécaux (lactoferrine, calprotectine fécale, …) ou sériques (Protéine C-réactive, sérum amyloïde A, cytokines pro-inflammatoires, ….) sont utilisés pour diagnostiquer les MICI et évaluer la sévérité de l'inflammation, en plus des examens endoscopiques (Langhorst et al., 2008; Matowicka-Karna, 2016).

1.1.3. Thérapies disponibles : la piste des probiotiques

Il n'existe pas de traitements curatifs permettant la guérison définitive des MICI. Les traitements conventionnels permettent seulement de diminuer l'intensité et la durée des poussées inflammatoires et d'allonger les durées de rémission. Il s'agit généralement d'anti-inflammatoires (Des dérivés salicylés ou des corticoïdes), des anti-TNFα, des immunosuppresseurs ou encore des antibiotiques. Des recommandations nutritionnelles sont parfois prescrites (Forbes et al., 2017). Dans certains cas sévères,

des interventions chirurgicales sont pratiquées. Les travaux pionniers du Pr. Gionchetti ont permis d'explorer la piste de moduler le microbiote intestinal via la consommation des microorganismes probiotiques immunomodulateurs afin d'atténuer l'inflammation intestinale (Bibiloni et al., 2005; Gionchetti et al., 2002). A ce jour, il y a des données suffisantes indiquant que la consommation de souches sélectionnées de probiotiques pourrait jouer un rôle favorable seulement pour lutter contre la RCH (Forbes et al., 2017). Ils permettraient de prévenir la RCH, ou d'induire la rémission et de la maintenir, en association ou non avec les traitements conventionnels de la RCH (Derwa et al., 2017; Forbes et al., 2017; Palumbo et al., 2016; Reiff and Kelly, 2010). La transplantation fécale est également une voie de thérapie en cours d'exploration, qui vise à implanter un microbiote sain pour atténuer l'inflammation intestinale (Wang et al., 2016).

1.2. Les probiotiques : des adjuvants pour prévenir ou traiter la rectocolite hémorragique

1.2.1. Méthodes d'évaluation des probiotiques anti-inflammatoires

Dans le contexte de RCH, l'effet anti-inflammatoire est recherché et résulte généralement d'interactions complexes entre le probiotique et la muqueuse intestinale. Pour cribler des souches anti-inflammatoires, des modèles *in vitro* cellulaires simplifiés sont mis en œuvre.

Des cultures monocouches de lignées cellulaires épithéliales intestinales du côlon, telles que les lignées HT-29 ou Caco-2, sont utilisées pour mimer les interactions du probiotique avec l'épithélium intestinal (Lea, 2015; Martínez-Maqueda et al., 2015). D'autres modèles, prenant en compte la complexité de la composition de l'épithélium intestinal, en termes d'architecture (Cultures en 2D ou 3D) et de composition cellulaire (cellules M, cellules de Goblet, cellules de Paneth ou encore des cellules entérodendocrines), sont développés (Araújo et al., 2016; Grabinger et al., 2014; Hill and Spence, 2017; Kernéis et al., 2000).

Les cellules immunitaires sont également utilisées pour comprendre les interactions entre le probiotique et l'hôte. Des lignées immortalisées de cellules immunes, telle que la lignée THP-1 dérivant de

monocytes humains; ainsi que des cultures primaires de cellules immunes du sang (Peripheral blood mononuclear cells, PBMC) sont également employées (Chanput et al., 2014; Groell et al., 2018).

Les modèles d'études précliniques pour évaluer le potentiel anti-inflammatoire sont essentiellement des modèles de colite murine. Les modèles de colite sont induits par de divers agents chimiques (Chassaing et al., 2014; Foligné et al., 2006; Randhawa et al., 2014). Ils incluent également des modèles d'animaux transgéniques, qui sont déficients pour un élément génétique impliqué dans la modulation de l'inflammation (Mizoguchi, 2012). Les modèles de colite induits chimiquement par l'acide 2, 4, 6-trinitrobenzène sulfonique (TNBS) ou le dextran sulfate sodium (DSS) sont les modèles de colite les plus communément utilisés pour évaluer le potentiel d'utilisation d'une souche probiotique dans un contexte de RCH (Martín et al., 2017).

1.2.2. Répertoire des probiotiques anti-inflammatoires

Les nombreuses études précliniques ont permis d'identifier une diveristé de microorganismes probiotiques présentant des potentialités anti-inflammatoires dans le contexte de la RCH (Martín et al., 2017). Les propriétés anti-inflammatoires mises en évidence sont généralement souches-dépendantes. Ces microorganismes probiotiques englobent majoritairement des bactéries à Gram positif, telles que les bactéries lactiques et les bifidobactéries; ainsi que des levures, telles que *Saccharomyces boulardii* (Martín et al., 2017). Parmi ces microorganismes, on peut distinguer des espèces originellement identifiées comme espèces commensales, telle que *Faecalibacterium prausnitzii;* et d'autres espèces qui un long historique d'utilisation dans notre alimentation.

Les effets protecteurs de ces probiotiques en tant qu'adjuvants aux traitements conventionnels ont été validés pour certains d'entre eux au niveau clinique (Tableau 1) (Probiotic Chart). Les formulations probiotiques existantes peuvent inclure plusieurs souches probiotiques qui apportent des fonctionnalités complémentaires. La Société Européenne de Nutrition Clinique et Métabolisme (ESPEN) recommande en effet la formulation probiotique VSL#3 ainsi que *Escherichia coli* Nissle 1917 pour prolonger les durées de rémission des patients atteints de la RCH.

Produit probiotique	Souches	Indication	UFC-posologie/jour	Etape d'essai clinique	Références
FlorastorMax®	***Saccharomyces boulardii lyo CNCM I-745***	**MICI-Rectocolite hémorragique-Adjuvants aux traitements conventionnels**	1.10^{10} UFC/sachet-1 sachet 5.10^{9} UFC/sachet-1 à 2 sachets	***III***	(Guslandi et al., 2000)
Mutaflor®	***Echerichia coli Nissle* 1917**		$2.5\text{-}25.10^{9}$ UFC /capsule 1-2 capsule	***I***	(Henker et al., 2008; Kruis et al., 1997, 2004)
Visbiome Extra Strength™	***L. acidophilus* DSM24735** ***L. paracasei* DSM24733** ***L. delbrueckii subsp. bulgaricus* DSM24734** ***L. plantarum* DSM24730** ***B. longum* DSM24736** ***B. breve* DSM24732** ***S. thermophilus* DSM2473** ***B. infantis* DSM24737**		$4.50.10^{11}$ UFC/sachet-1 à 2 sachets $1.12.10^{11}$ UFC/capsule-1 à 4 capsules	***I***	(Sood et al., 2009; Tursi et al., 2004, 2010)
VSL#3®	***L. acidophilus* SD5212** ***L. casei* SD5218** ***L. bulgaricus* SD5210** ***L. plantarum* SD5209** ***B longum* SD5219** ***B. infantis* SD5220** ***B. breve* SD5206** ***S. thermophilus* SD5207**		9.10^{11} UFC/sachet-1 sachet $4{,}50.10^{11}$ UFC/sachet-1 à 2 sachets $1{,}12.10^{11}$ UFC/capsule-1 à 4 capsules	***I***	(Sood et al., 2009; Tursi et al., 2004, 2010)

***Tableau 1*. Liste des formules probiotiques ayant été évaluées au niveau clinique pour prolonger les durées de rémission de patients atteints de rectocolite hémorragique**. Les différentes formules sont commercialisées et les souches utilisées ont le statut GRAS délivré par la FDA et/ou le numéro délivré par Health Canada (http://usprobioticguide.com/).

I : Preuves obtenues à partir d'au moins un essai randomisé correctement conçu.

II : Preuves obtenues d'essais contrôlés bien conçus sans randomisation. Des résultats significatifs dans des essais non contrôlés pourraient également être considérés.

III : Avis d'autorités respectées, fondées sur des études clinique, des études descriptives ou des rapports de comités d'experts.

1.2.3. Revue de littérature : les propriétés probiotiques versatiles des bactéries propioniques laitières

Récemment, les souches de *Propionibacterium freudenreichii* sont rajoutées à cette liste de probiotiques ayant présenté des effets pour réduire la sévérité de la colite murine (Foligné et al., 2010; Plé et al., 2015, 2016). Connu pour leur long historique d'usage en tant que ferment fromager, *P. freudenreichii* a récemment été décrit comme une bactérie commensale immunomodulatrice (Colliou et al., 2017).

Propionibacterium freudenreichii est une actinobactérie bénéfique, qui possède un statut GRAS (Generally Recognized as Safe) délivré par la FDA aux Etats Unis, ainsi qu'un statut QPS (Qualified Presumption of Safety) en Europe. Elle est exploitée par l'industrie agroalimentaire pour différentes utilisations, principalement pour la fabrication de fromages à pâte pressée cuite (Thierry et al., 2011a). Dans la littérature, les souches de *P. freudenreichii* sont décrites comme des bactéries ayant de multiples propriétés probiotiques plus ou moins confirmées au niveau clinique, tels que des effets anti-cancéreux, la modulation du microbiote intestinal et des effets immunomodulateurs.

La revue résume les caractéristiques probiotiques souches-dépendantes des propionibactéries laitières rapportées par la littérature. En effet, les propionibactéries répondent aux critères de sélection des bactéries probiotiques, tels que la capacité de tolérance aux stress digestifs et d'adhésion aux cellules épithéliales intestinales. Ceci est un prérequis pour leur persistance dans le tractus digestif. Dans cette revue, les effets bénéfiques rapportés sur *P. freudenreichii* et sur *P. acidipropionici* sont classés en fonction de l'effet probiotique induit : modulation du microbiote intestinal, immunomodulation et des effets anti-cancéreux. Les mécanismes moléculaires proposés pour expliquer ces effets probiotiques sont discutés.

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Review

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

1. Introduction

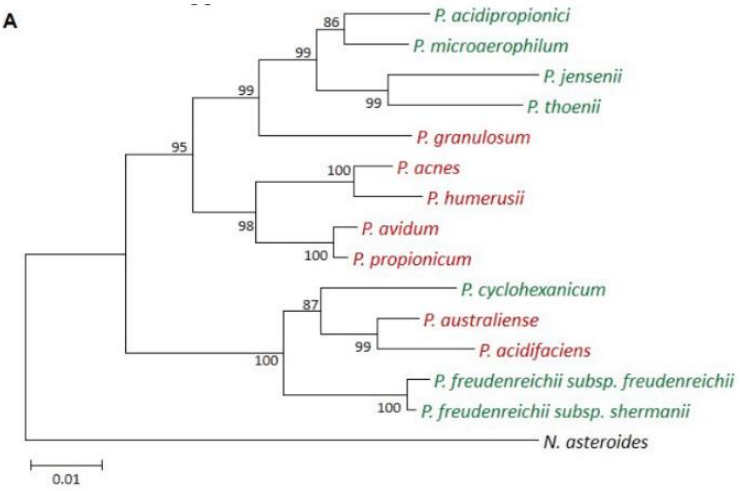
Propionibacteria are high-GC content, non-motile, non-spore forming, anaerobic to aerotolerant, gram-positive bacteria, which belong to the actinomycetales order. They are therefore highly distinct from low GC content firmicutes, which include lactic acid bacteria. Actinomycetales comprise bacterial 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.

2. Gut Persistence of Dairy Propionibacteria

2.1. 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.

2.2. 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*, *Staphylococcus 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.

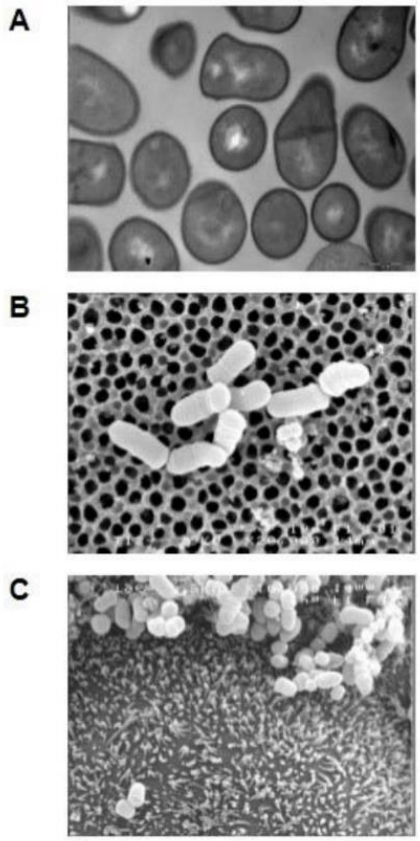


Figure 2. Electron microscopy analysis of *Propionibacterium freudenreichii*. The Propionibacteria were cultivated alone (**A**,**B**) or in contact with cultured human intestinal cell line Caco2 (**C**). Observation was made using either transmission (**A**) [46] or scanning (**B**) [26] and (**C**)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

3. 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].

4. 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.

4.1. 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.

4.2. 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].

4.3. 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.

4.4. 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].

5. 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].

5.1. 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].

5.2. 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.

5.3. 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.

6. 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].

7. Technological Applications of Dairy Propionibacteria

7.1. 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].

7.2. 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].

7.3. 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.

8. 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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***Nota Bene* :** Reclassification d'espèces sélectionnées du genre *Propionibacterium* dans les nouveaux genres proposés : *Acidipropionibacterium* gen. nov., *Cutibacterium* gen. nov. et *Pseudopropionibacterium* gen. nov.

Dans la revue précédente, nous avons distingué les espèces de propionibactéries dites « laitières » et « cutanées » qui appartenaient au même genre *Propionibacterium*. Une nouvelle reclassification des espèces de *Propionibacterium* est proposée sur la base de l'homologie de la séquence de l'ARNr 16S (Figure 2) (Scholz and Kilian, 2016). La reclassification proposée est en cohérence avec les autres caractéristiques des espèces, telles que l'habitat des espèces, la topologie génomique, le contenu en GC de l'ADN et les schémas de pertes et d'acquisitions de gènes dans les propionibactéries cutanées, au cours de leur adaptation à l'hôte humain (Scholz and Kilian, 2016).

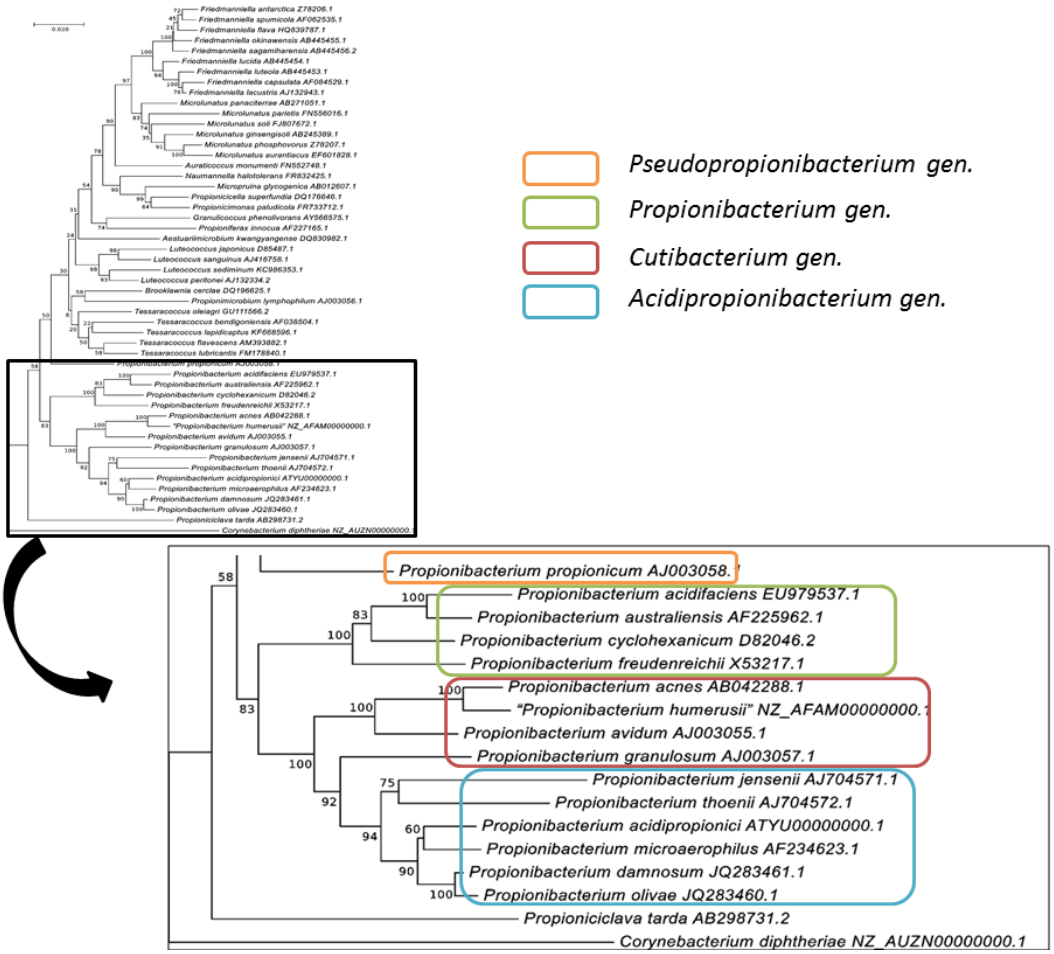

Figure 2. **Arbre phylogénétique à évolution minimale des espèces de la famille des Propionibacteriaceae basé sur les séquences d'ARNr 16S.** La séquence d'ARNr 16S de l'Actinomycetale *Corynebacterium diphteriae* a été utilisée pour construire l'arbre phylogénétique. La répartition des différentes espèces de *Propionibacterium* en 4 genres distincts est précisée pour chaque espèce. Le schéma a été adapté de Scholz et Kilian,. (2016).

1.3. Les mécanismes moléculaires d'action des probiotiques

1.3.1. Interactions complexes entre les probiotiques et l'hôte

Une meilleure compréhension des mécanismes moléculaires déclenchés par les probiotiques, impliqués dans l'atténuation de l'inflammation, aidera à mieux interpréter les données cliniques et facilitera au mieux la sélection de meilleures souches probiotiques pour lutter contre la RCH.

La muqueuse intestinale est constituée d'épithélium intestinal et elle est également associée aux tissus lymphoïdes du tube digestif (Gut-Associated Lymphoid tissue, GALT) (Bron et al., 2012). Les vaisseaux lymphatiques drainent le tissu conjonctif sous-jacent à l'épithélium intestinal (Lamina propia), où de diverses cellules immunitaires y résident, telles que les cellules présentatrices d'antigène (cellules dendritiques, DC) et d'autres cellules immunitaires (lymphocytes T et lymphocytes B). Ils connectent la Lamina Propia et les plaques de Peyer, qui sont des follicules lymphoïdes contenant diverses cellules immunes, aux ganglions mésentériques intestinaux, le cœur de l'immunité adaptative (Figure 3). Cette structure complexe est le siège de la première réponse de défense ou de tolérance contre les microorganismes présents dans la lumière intestinale (Bron et al., 2012).

Le « dialogue » permanent entre la muqueuse intestinale et le microbiote, y compris les probiotiques ingérés, résulte d'une part des interactions avec les cellules épithéliales intestinales qui sont en interaction avec les cellules immunes présentes dans les GALT. D'autre part, il résulte également des interactions directes entre les cellules immunes (cellules dendritiques) et le microbiote (Figure 3).

Pour les sections qui suivent, pour des soucis de simplification, les données présentées concernent seulement les probiotiques bactériens, et non les probiotiques levures, qui présentent en partie des mécanismes moléculaires d'action similaires aux probiotiques bactériens (Pothoulakis, 2009).

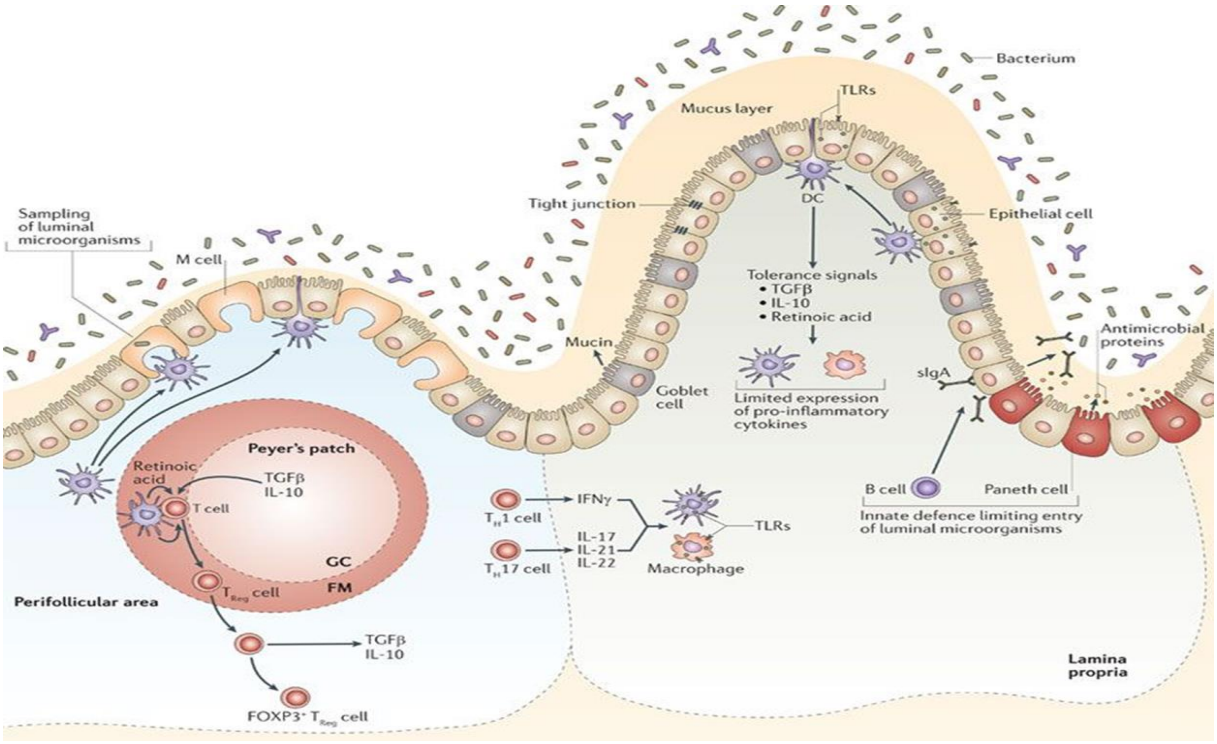

Figure 3. **Représentation schématique de l'architecture complexe de la muqueuse intestinale qui est constituée de l'épithélium intestinal et de tissus lymphoïdes (Gut-Associated Lymphoid tissue, GALT).** L'épithélium intestinal est constitué d'entérocytes, de cellules de Paneth en rouge (Production de peptides antimicrobiens), de cellules caliciformes (Production de mucus), de cellules M en jaune (présentation d'antigènes) et de cellules entéroendocrines. Les cellules immunitaires présentes dans les GALT sont en étroite interaction avec l'épithélium intestinal et le microbiote intestinal. Le schéma a été repris de la revue de littérature publiée par Bron et al., (2012).

1.3.2. Métabolites immunomodulateurs sécrétés

De nombreux métabolites issus du métabolisme du microbiote ou du probiotique ingéré sont responsables des propriétés immunomodulatrices, permettant ainsi l'atténuation de l'inflammation intestinale et le maintien l'homéostasie intestinale (Engevik and Versalovic, 2017; Suchodolski and Jergens, 2016). Les propriétés immunomodulatrices exercées par ces métabolites peuvent agir de manière directe sur les cellules épithéliales intestinales, ou sur les cellules immunes ou encore sur ces deux types de cellules. En plus, les métabolites peuvent agir sur l'inflammation via la modulation du microbiote intestinal (Engevik and Versalovic, 2017).

Certains métabolites sont communément produits par différentes espèces probiotiques et possèdent des mécanismes d'action moléculaires homologues. Parmi ces composés, on retrouve les acides gras à chaîne courte (Machiels et al., 2014; Sun et al., 2017) ou les acides gras conjugués (Bassaganya-Riera et al., 2012a, 2012b; Evans et al., 2010); ou encore des vitamines, telles que les vitamines B (Levit et al., 2017, 2018; Thomas et al., 2016) ou des précurseurs de la vitamine K (Okada, 2006). D'autres composants sont plus spécifiques d'une souche donnée et contribuent également à l'effet immunomodulateur d'un probiotique, parmi ces composés, on retrouve : des bactériocines (Yin et al., 2018), des protéines sécrétées (Bermúdez-Humarán et al., 2015) et des microvésicules extracellulaires (Alvarez et al., 2016; Fábrega et al., 2017; Rubio et al., 2017).

Le nombre et le type de métabolites produits sont espèce et souche–dépendants. Ainsi, l'effet anti-inflammatoire d'une souche probiotique donnée résultera d'un composé donné ou d'un ensemble de métabolites sécrétés. Dans le cas des souches de *P. freudenreichii*, nombre de ces métabolites cités sont en effet produits de manière souche-dépendante (voir section 1.2.3).

1.3.3. Composants immunomodulateurs de la paroi

La surface de la paroi bactérienne semble être un site clé dans ces mécanismes d'interaction entre les probiotiques et les cellules de l'hôte. Elle est constituée de diverses molécules, de natures biochimiques très diverses (protéines, lipides, glycanes, …), qui présentent des propriétés immunogènes (Figure 4). Ces composants sont appelés MAMPs, pour Microbe-Associated Molecular Pattern. Les MAMPs, incluent diverses molécules telles que le peptidoglycane, des exopolysaccharides, des acides téichoïques et lipotéichoïques, des lipopolysaccharides, …. (Figure 4).

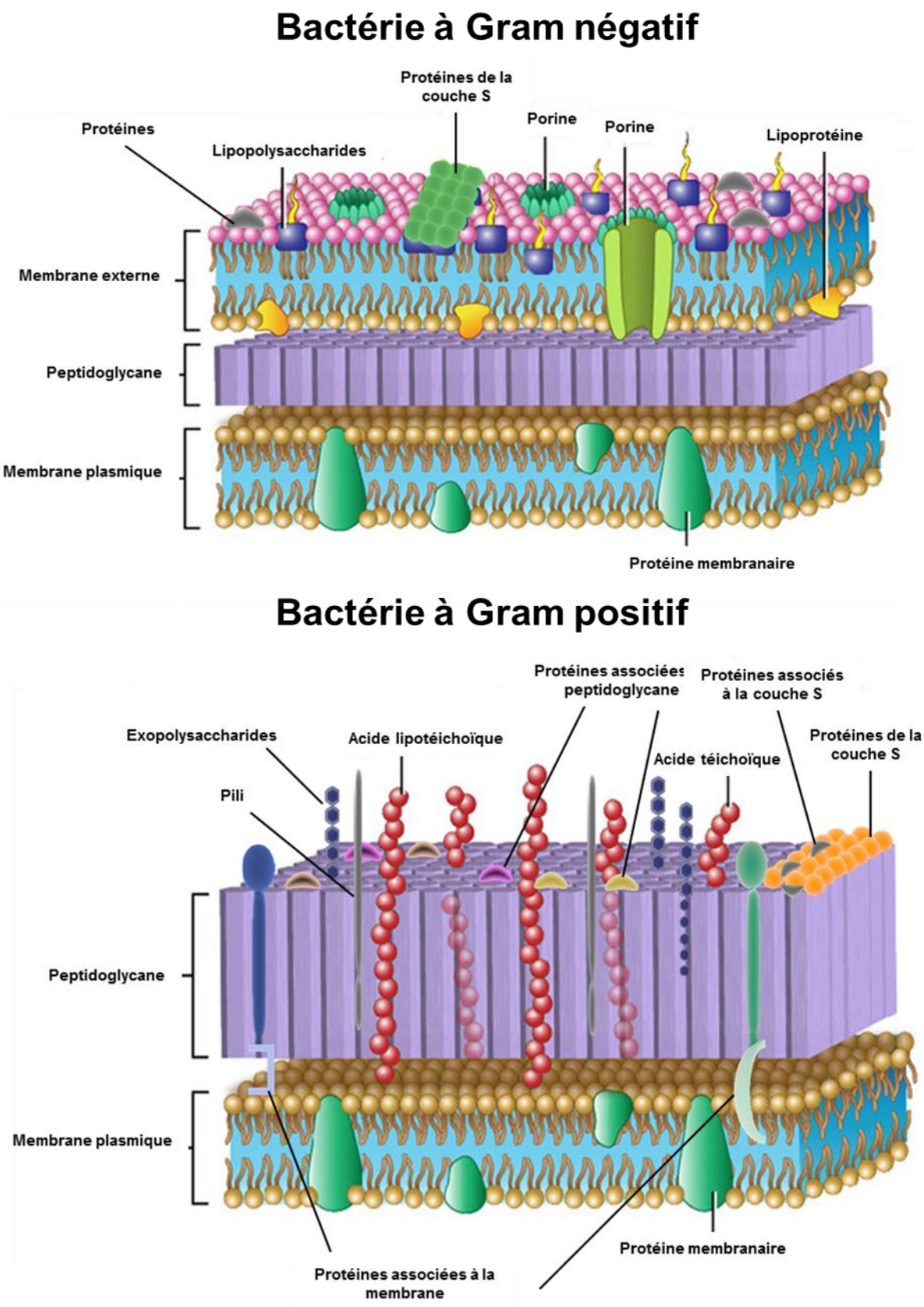

Figure 4. **Représentation schématique de la composition pariétale des bactéries à Gram négatif et à Gram positif.** Les images (Behance) ont été modifiées selon des revues de littérature publiées (Sengupta et al. 2013; Delcour et al. 1999; Sleytr et al. 2014). Les différents MAMPs cités dans la section 1.3.3 sont indiqués.

Les MAMPs, selon leur nature, sont reconnus de manière spécifique par des récepteurs de l'immunité innée appelés PRRs, pour Pattern recognition receptors. Les PRRs sont présents sur les cellules immunitaires ainsi que sur les cellules épithéliales intestinales. Parmi ces récepteurs de l'immunité qui peuvent interagir avec des composants bactériens, on distingue les récepteurs Toll-Like Receptors (TLR) (Gao and Li 2017), les récepteurs Nucleotide-binding and oligomerization domain (NOD)-like (NLRs) (Kim et al., 2016; Kobayashi et al., 2005) et les récepteurs C-type Lectin (CLRs) (Geijtenbeek and Gringhuis, 2016; Hou et al., 2017).

L'interaction entre un PRR donné avec un MAMP donne lieu à une cascade de signalisation cellulaire aboutissant à la production de cytokines. Le cocktail de cytokines produit déterminera ainsi la différenciation des lymphocytes T (immunité adaptative) et régira ainsi la réponse immunitaire globale intestinale (Figure 5). La réponse immunitaire résultera en un mécanisme de défense ou de tolérance en fonction du MAMP détecté. D'autres fonctionnalités de l'homéostasie intestinale peuvent être également altérées: production d'IgA, production de mucus, production de peptides antimicrobiens…..

En effet, chez les espèces de *Bifidobacterium* et de *Lactobacillus*, les études montrent le rôle des exopolysaccharides dans la modulation de la réponse immunitaire (Balzaretti et al., 2017; Laiño et al., 2016; Murofushi et al., 2015; Shao et al., 2014; Verma et al., 2018; Wu et al., 2010). Les acides téichoïques et lipotéichoïques sont également des médiateurs des effets immunomodulateurs de certaines espèces probiotiques (Kim et al., 2008; Lebeer et al., 2012; Mohamadzadeh et al., 2011; Smelt et al., 2013) .

Les protéines de surface, liées de manières covalentes ou non, contribuent également à l'effet immunomodulateur de certains probiotiques. Parmi ces protéines, on distingue les pili (Ganguli et al., 2015), des protéines associées au peptidoglycane, des protéines constitutives de la couche S (Protéines S-layer, Slps) ou encore des protéines associés à la couche S (Lebeer et al., 2018; Sengupta et al., 2013). Dernièrement, un intérêt croissant a été porté sur les protéines de la couche S, principalement chez les espèces de *Lactobacillus*, pour leur rôle dans l'interaction avec l'hôte (Gerbino et al., 2015a). En effet, Chez *L. acidophilus*, l'interaction entre la protéine SlpA et un récepteur de type CLRs est déterminante pour son effet protecteur contre la colite murine (Lightfoot et al., 2015).

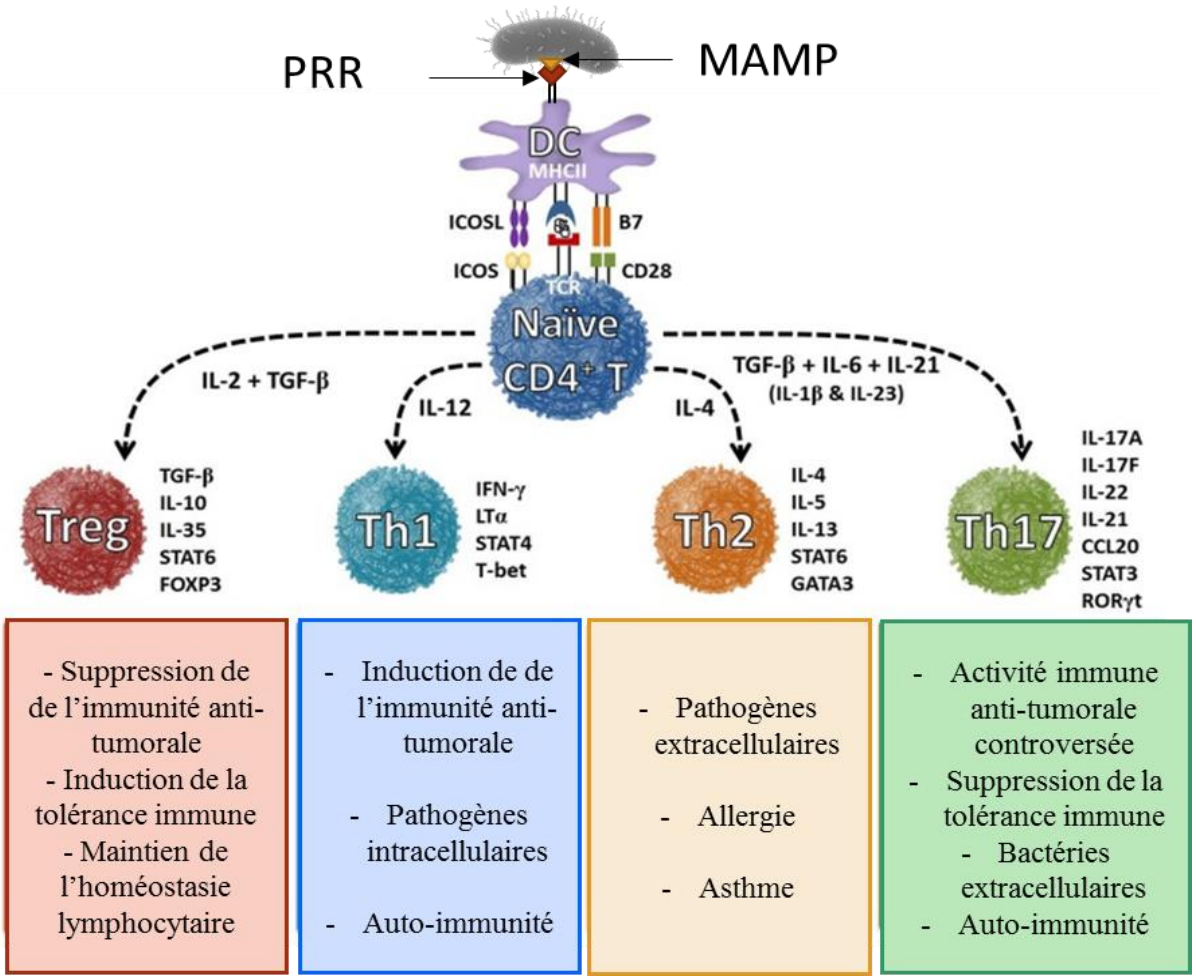

Figure 5. **Représentation schématique de la différenciation des lymphocytes T naïfs pilotée par les cellules dendritiques (DC) suite à une interaction avec un motif de la paroi bactérienne.** Selon les cytokines produites par les DC, les lymphocytes T (LT) peuvent être différenciés principalement en lymphocytes régulateurs (Treg), en lymphocytes T auxiliaires 1 (Th1), en lymphocytes T auxiliaires 2 (Th2) ou en lymphocytes T auxiliaires 17. Les lymphocytes T activés sécrètent des cocktails de cytokines spécifiques ainsi que des facteurs de transcription spécifiques de chaque population (Foxp3, T-bet, GATA3 et RORγt). La réponse immunitaire sera déterminée en fonction des orientations Treg/Th17 et Th1/Th2. Le schéma a été adapté à partir d'une revue de littérature publiée (Bailey et al. 2014).

1.3.4. Revue de littérature: l'état de l'art sur les fonctions des protéines de surface extractibles

Certaines bactéries à Gram positif, y compris certaines bactéries probiotiques, sont recouvertes d'une couche protéique appelée la couche S (S-layer). Décrite comme une couche paracristalline et formée par l'auto-assemblage de protéines S-layer (Slps), cette structure optionnelle est particulière. D'autres protéines, y compris des protéines associées à la couche S (SLAP), et d'autres protéines liées de manière non covalente à la surface, peuvent également être extraites avec cette structure de surface.

Chez, *P. freudenreichii*, le projet de recherche ANR SURFING (Starter SURFace against INflammation of the Gut) a mis en évidence la présence de cinq protéines de surface de type S-layer chez la souche anti-inflammatoire CIRM-BIA 129: SlpA, SlpB, SlpE, Internaline like (Inl like) et Large Surface Protein A (LspA) (Le Maréchal et al., 2015). Elles sont en partie responsables de l'effet immunomodulateur, via l'induction de cytokines par les cellules immunitaires du sang (Le Maréchal et al., 2015). Récemment, le rôle d'une protéine de surface S-layer a été démontré dans l'effet protecteur de *P. freudenreichii* UF1 contre l'entérocolite nécrosante chez les nouveaux nés.

Dans cette revue, nous explorons ainsi les multiples fonctions des protéines S-layer bactériennes, ainsi que leur rôle dans l'interaction entre les bactéries probiotiques à Gram positif, y compris les propionibactéries, et les cellules de l'hôte.

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REVIEW
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Extractable Bacterial Surface 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.

OCCURRENCE, 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

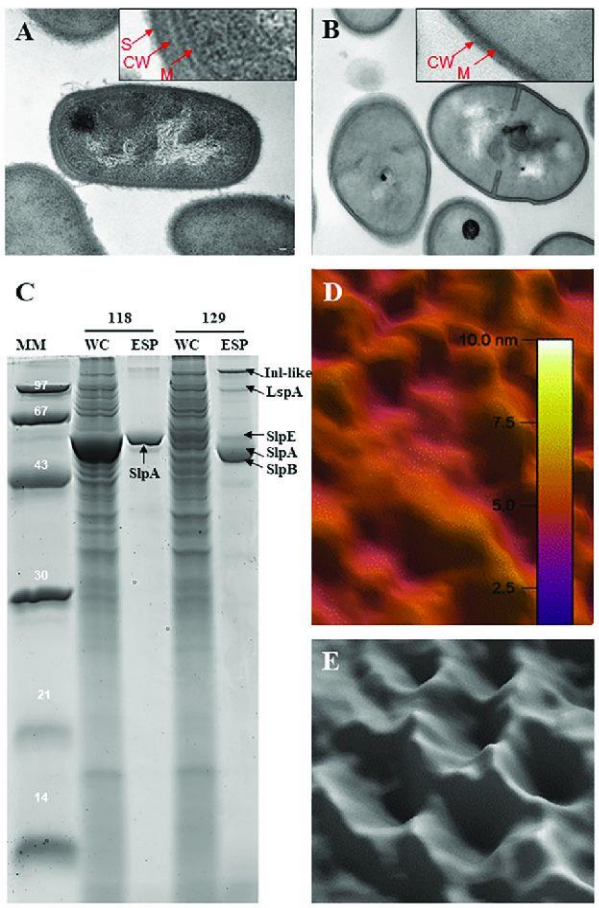

FIGURE 1 | Occurrence of an S-layer is strain-dependent in *Propionibacterium freudenreichii*. *P. freudenreichii* CIRM-BIA 118 is covered by an outermost surface layer **(A)** that is removed by extraction using the chaotropic agent guanidine **(B)**. Red arrows indicate the membrane (M), cell wall (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 Extractable Surface-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; Waśko 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,

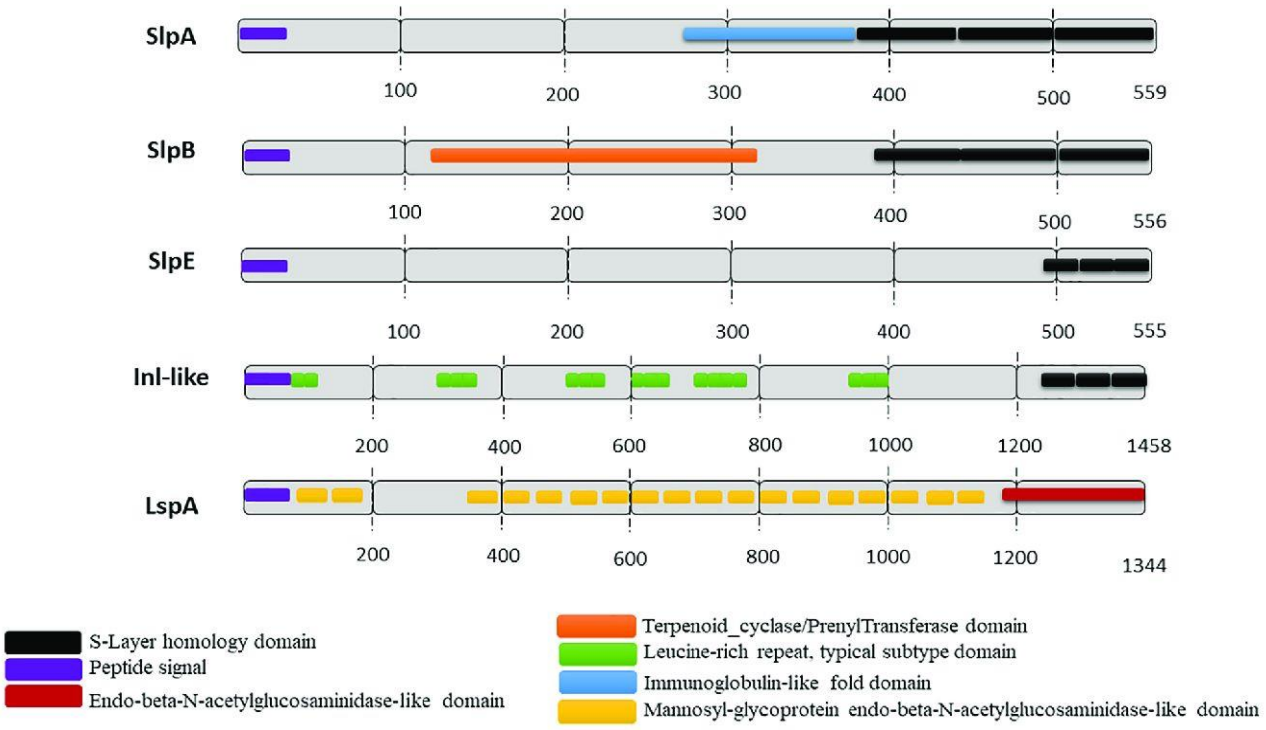

FIGURE 2 | Predicted functional domains in *P. freudenreichii* extractable surface proteins. The functional domains were predicted using InterPro (EMBI/NCBI) for the five extractable surface proteins identified in different *P. freudenreichii* strains (Le Maréchal et al., 2015). S-layer protein A (SlpA), 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; Waśko 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,

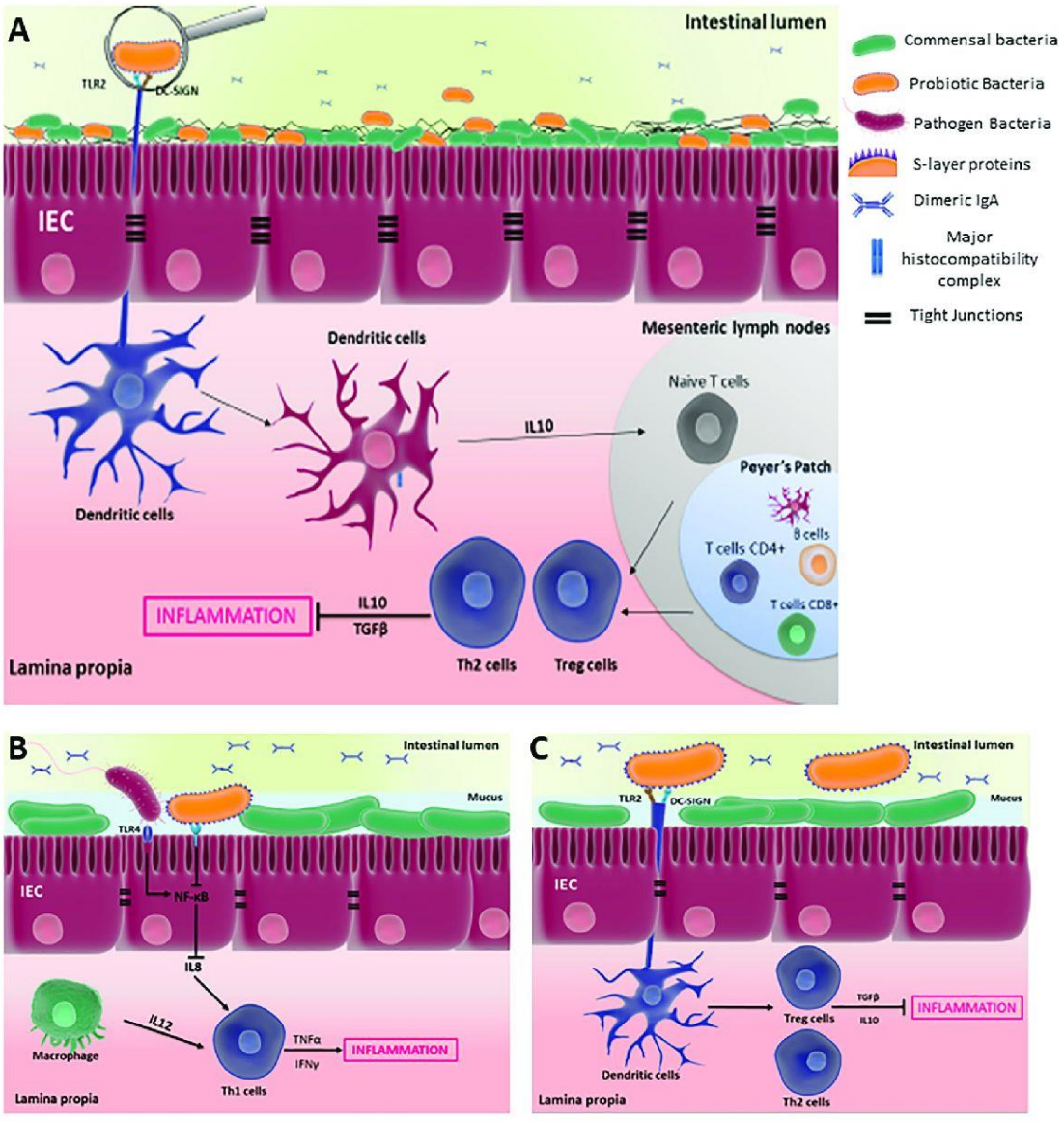

FIGURE 3 | Cross-talk between probiotic bacteria and the host, mediated by IECs and immunes cells, within the gut associated lymphoid tissues (GALT). **(A)** An overview of the interaction of antigen-presenting-cells such as DCs with probiotic bacterian, which initiates a tolerance response by inducing 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 *L. 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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1.4. Les aliments fonctionnels fermentés : des adjuvants pour prévenir ou traiter la rectocolite hémorragique

1.4.1. Avantage des probiotiques « 2-en-1 »

Certains probiotiques, dont les effets préventifs contre la RCH ont été démontrés, possèdent des propriétés dites «2-en-1 ». Il s'agit souvent de microorganismes utilisés dans la fermentation de diverses matrices alimentaires, et qui exercent également un fort potentiel probiotique. La mise au point d'aliments laitiers fermentés fonctionnels via ces bactéries « 2-en-1 » est un levier prometteur pour moduler le microbiote intestinal dans le but de prévenir ou d'aider à la rémission de la RCH. Les aliments fonctionnels sont définis comme «ingrédients similaires en apparence, ou pouvant être un aliment conventionnel, qui est consommé dans le cadre d'une diète normale, et qui a été démontré comme ayant des bénéfices physiologiques et/ou pouvant réduire le risque de maladies chroniques, au-delà des fonctions nutritionnelles de base» (Diplock et al., 1999; Saris et al., 1998).

Il existe quelques aliments fonctionnels commercialisés, et qui ont été ainsi évalués au niveau clinique pour de nombreuses indications (Tableau 2) (Probiotic Chart). Il existe des preuves solides de l'efficacité de ces aliments fonctionnels, notamment pour la prévention de diarrhées d'origine infectieuse. Cependant, jusqu'à aujourd'hui, il n'existe pas d'aliments fonctionnels pour le co-traitement ou la prévention de la RCH, en Europe ou ailleurs dans le monde (Tableau 2). Il est à noter que des bactéries probiotiques non « 2-en-1 » peuvent être incluses également dans les aliments fonctionnels (Tableau 2).

Les souches de *P. freudenreichii*, possédant également ces propriétés « 2-en-1 », sont des bactéries de prédilection pour développer des aliments fonctionnels laitiers fermentés, tels que les fromages à pâte pressée cuite. En effet, les produits laitiers fermentés font partie du régime quotidien dans les pays industrialisés, et présentent un moyen pertinent pour la vectorisation de bactéries probiotiques. En France, 72 % des Français consomment tous les jours des produits laitiers et font partie des plus grands consommateurs de fromage avec une consommation de 27,2 Kg de fromage/ an en 2016 (L'économie laitière en chiffres – édition 2018).

Aliment	Souche	Indication	Sujets	Etape d'essai clinique	Références
DanActive Actimel®	***L. casei sp. Paracasei CNCM I-1518***	Prévention de la diarrhée associée à la prise d'antibiotiques	Adultes	*I*	(Hickson et al., 2007)
		Diarrhée de cause infectieuse	Enfants	*II*	(Merenstein et al., 2010; Pedone et al., 1999, 2000)
		Effets immunomodulateurs divers	Adultes	*I*	(Boge et al., 2009; Guillemard et al., 2010b, 2010a; Marcos et al., 2004; Meyer et al., 2006; Ortiz-Andrellucchi et al., 2008; Tiollier et al., 2007; Turchet et al., 2003)
		Adjuvant associé au traitement conventionnel pour l'éradication d'*Helicobacter pylori*	Enfants	*I*	(Sýkora et al., 2005)
		Effets immunomodulateurs divers	Enfants	*I*	(Giovannini et al., 2007; Lin et al., 2009; Merenstein et al., 2010)
Yakult®	***L.casei Shirota***	Constipation	Adultes	*I*	(Koebnick et al., 2003; Tilley et al., 2014)
		Humeur et anxiété/stress (Il ne s'agit pas d'un substituant au traitement conventionnel)	Adultes	*II*	(Kato-Kataoka et al., 2016; Rao et al., 2009)
		Effets immunomodulateurs divers	Adultes	*I*	(Dong et al., 2013; Gleeson et al., 2011; Ivory et al., 2008; Nagao et al., 2000)
Activia®	***B. (animalis) lactis CNCM I-2494***	Constipation	Adultes	*I*	(Yang et al., 2008)
		Syndrome de l'intestin irritable (IBS)	Adultes	*I*	(Agrawal et al., 2009; Guyonnet et al., 2007)
GoodBelly® ProbioticJuice Drinks	***L. plantarum 299v***	Syndrome de l'intestin irritable (IBS)	Adultes	*I*	(Ducrotté et al., 2012; Niedzielin et al., 2001; Nobaek et al., 2000)

GoodBelly® Oat Drink Straight Shot		Prévention de la diarrhée associée à *Clostridium difficile*	Adultes	*III*	(Klarin et al. 2008)
GoodBelly® Oat Drink Super Shot		Prévention de la diarrhée associée à la prise d'antibiotiques	Adultes	*III*	(Lönnermark et al., 2010)

Tableau 2. **Liste des aliments fonctionnels disponibles dans le commerce dont les effets probiotiques ont été étudiés chez l'homme.** Les souches utilisées possèdent le statut GRAS délivré par la FDA et/ou le numéro délivré par Health Canada (http://usprobioticguide.com/).

I : Preuves obtenues à partir d'au moins un essai randomisé correctement conçu.

II : Preuves obtenues d'essais contrôlés bien conçus sans randomisation. Des résultats significatifs dans des essais non contrôlés pourraient également être considérés.

III : Avis d'autorités respectées, fondées sur des études cliniques, des études descriptives ou des rapports de comités d'experts.

1.4.2. Effet de la matrice fromagère sur les propriétés probiotiques de *P. freudenreichii*

Lors de la vectorisation d'un probiotique via des aliments fermentés, les interactions complexes existantes entre la matrice de vectorisation et la flore microbienne peuvent affecter ses propriétés immunomodulatrices. Ainsi, il est essentiel de caractériser ces interactions afin de comprendre les mécanismes d'action du probiotique *in situ*.

Quelques données sont disponibles sur l'effet matrice sur les propriétés probiotiques de *P. freudenreichii* (voir section 1.2.3). Les études menées sur *P. freudenreichii* suggèrent que la microstructure physique et la composition chimique du milieu de culture et/ou de vectorisation peuvent affecter la physiologie bactérienne, y compris sa tolérance aux stress digestifs et son activité métabolique dans le côlon (Cousin et al., 2012; Gagnaire et al., 2015; Leverrier et al., 2005).

En ce qui concerne l'impact de la matrice fromagère sur les propriétés anti-inflammatoires de *P. freudenreichii*, très peu de données sont disponibles. Les études *in vivo* montrent que des fromages fonctionnels fermentés par *P. freudenreichii* CIRM-BIA 129, seule ou en combinaison avec *Lactobacillus delbrueckii* subsp. *lactis* CNRZ327, peuvent prévenir l'apparition de la colite chimiquement induite chez les souris (Plé et al., 2015, 2016). Cependant, nous ignorons si les effets anti-inflammatoires des fromages fonctionnels ainsi développés sont optimisés ou atténués, en comparaison à une vectorisation par un milieu liquide, de qualité alimentaire tel qu'un lait ou un ultrafiltrat de lait. Aucune étude n'a été menée sur la tolérance aux stress digestifs ou sur l'adaptation au côlon chez *P. freudenreichii*, inclus dans une matrice fromagère. De plus, il a été démontré que les protéines immunomodulatrices de *P. freudenreichii* sont bien exprimées dans la matrice fromagère (Plé et al., 2015), mais leur devenir dans le tube digestif est inconnu, telle que leur protéolyse ou leur expression dans le côlon.

De plus, outre les impacts pouvant résulter de l'interaction entre *P. freudenreichii* et la matrice fromagère, des interactions pourraient exister entre *P. freudenreichii* et l'écosystème microbien existant dans le fromage, et affecter ainsi ses propriétés probiotiques.

Chapitre 2 : « Objectif et stratégie »

2.1. Question de recherche

Tel que indiqué dans les deux revues de littérature, les propriétés probiotiques de *Propionibacterium freudenreichii* (***Pf***), dont l'effet immunomodulateur exercé par les protéines S-layer (Slps), sont souche-dépendantes. L'étude de l'effet matrice sur le potentiel probiotique de *P. freudenreichii* reste peu exploré. Dans la perspective de développer des fromages dit «fonctionnels» fermentés par *P. freudenreichii*, il est fondamental de comprendre comment la vectorisation par la matrice fromagère influence les propriétés probiotiques de *P. freudenreichii*. Les études menées jusqu'à aujourd'hui nous ont permis d'avancer l'hypothèse que le potentiel immunomodulateur de *P. freudenreichii* sera modulé par la matrice fromagère, par comparaison à une culture liquide ; par le fait d'une une variation de la quantité vivante atteignant le côlon d'une part, et d'autre part par le fait d'une variation en termes de protéome de surface, en particulier les protéines Slps. En fonction de la matrice de vectorisation, l'activité métabolique *in situ* et donc la production de métabolites anti-inflammatoires sera modulée. La réponse immunitaire de l'hôte se fera également en fonction de la composition du protéome de surface.

La question de recherche principale, objet de la thèse, est donc la suivante :

Comment la matrice fromagère impacte-t-elle les propriétés immunomodulatrices de *P. freudenreichii* à travers ses protéines Slps ?

2.2. Objectif et stratégie

Le projet de thèse vise à déterminer l'impact de la matrice fromagère sur les propriétés probiotiques, en particulier les propriétés immunomodulatrices de *P. freudenreichii* et de ses protéines S-layer, pour une vectorisation vers le tube digestif. Nous avons fait le choix de travailler principalement sur une seule souche, ***Pf*** CIRM-BIA 129 ; il s'agit de la souche ayant le meilleur potentiel anti-inflammatoire *in vitro* et ayant été évaluée *in vivo* dans un modèle de colite murine (Foligné et al., 2010, 2013; Le Maréchal et al., 2015; Plé et al., 2015). De plus, dans la première partie du projet de thèse, nous avons fait le choix d'étudier l'effet de la matrice fromagère en utilisant un fromage monosouche développé précédemment (Plé et al., 2015), afin de s'affranchir des interactions qui peuvent exister entre *P. freudenreichii* et la

flore microbienne d'un fromage emmental. La question de recherche a été déclinée en plusieurs sous-questions présentées sous forme de chapitres. Pour y répondre, différentes expérimentations ont été menées, s'inscrivant dans un schéma général de stratégie (Figure 6), détaillé ci-dessous :

1) Dans le **chapitre 3**, la question principale étudiée était : **quel est l'effet de la matrice fromagère sur les propriétés immunomodulatrices de *P. freudenreichii* CIRM-BIA 129 lors de la digestion *in vitro* ?** Pour cela, la tolérance aux stress digestifs de *P. freudenreichii* ainsi que la protéolyse des protéines de surface ont été comparées, entre une vectorisation par la matrice fromagère et une vectorisation par une culture sur l'ultrafiltrat de lait. Le modèle de digestion statique *in vitro*, ainsi que le modèle dynamique de digestion DIDGI® ont été utilisés. Dans une démarche complémentaire, l'effet immunomodulateur des peptides issus de la protéolyse des protéines Slps sur des cellules épithéliales intestinales HT-29 a été déterminé, et il a été comparé à l'effet immunomodulateur exercé par *P. freudenreichii* CIRM-BIA 129 et des protéines Slps intactes.
2) Dans le **chapitre 4,** au vue des résultats *in vitro*, nous avons souhaité répondre à la question suivante : **est-ce que la matrice fromagère module les propriétés immunomodulatrices *in vivo* de *P. freudenreichii* CIRM-BIA 129 ?** Pour y répondre**,** l'effet de la matrice fromagère sur la protéolyse *in vivo* des Slps en comparaison à une culture sur l'ultrafiltrat de lait a été recherché dans un modèle de porcelets. Ensuite, l'effet de la matrice fromagère sur l'activité métabolique *in vivo* ainsi que les propriétés immunomodulatrices de *P. freudenreichii* a été examiné chez les porcelets, par comparaison à une culture sur l'ultrafiltrat de lait. La réponse immunitaire des porcelets a été évaluée au niveau intestinal et au niveau systémique en termes de réponse cytokinique et de modulation des phénotypes des lymphocytes T. La réponse *ex vivo* des cellules immunitaires intestinales et systémiques a été également analysée en réponse à des stimuli pro-inflammatoires.
3) Dans le **chapitre 5**, nous avons souhaité explorer, **comment le milieu de croissance influence-t-il la survie de *P. freudenreichii* CIRM-BIA 129 dans le milieu caecal ?** Un milieu caecal a été préparé à partir des contenus caecaux récupérés lors de l'abattage des porcelets (Chapitre 4)

afin de mimer l'environnement colique. Nous avons ainsi étudié la survie de *P. freudenreichii,* provenant de deux milieux de culture (YEL et ultrafiltrat de lait), en plus du fromage monosouche. Nous avons également étudié la survie de ***Pf*** en fonction de la protéolyse des protéines de surface afin de comprendre l'influence des protéines Slps sur l'adaptation de ***Pf*** au milieu caecal. De plus, nous avons étudié la survie de ***Pf*** et de ***Pf*** KO SlpB dans le milieu caecal. L'expression *de novo* des Slps a été également examinée.

4) **Dans le chapitre 6**, la question principale posée était : **quel est l'effet d'un emmental « probiotique » dans un modèle de colite chimiquement induite chez la souris ?** Dans cette perspective, nous avons souhaité évaluer des souches lactiques et propioniques anti-inflammatoires pour élaborer un emmental aux propriétés immunomodulatrices souhaitées. Nous avons sélectionné les souches : ***Pf*** CIRM-BIA 129, *Streptococcus thermophilus* LMD-9 et de *Lactobacillus delbrueckii* subsp. *lactis* CNRZ327. L'effet probiotique d'un fromage emmental a été évalué dans un modèle de colite murine, en le comparant à celui du fromage expérimental monosouche, précédemment testé (Plé et al., 2015).

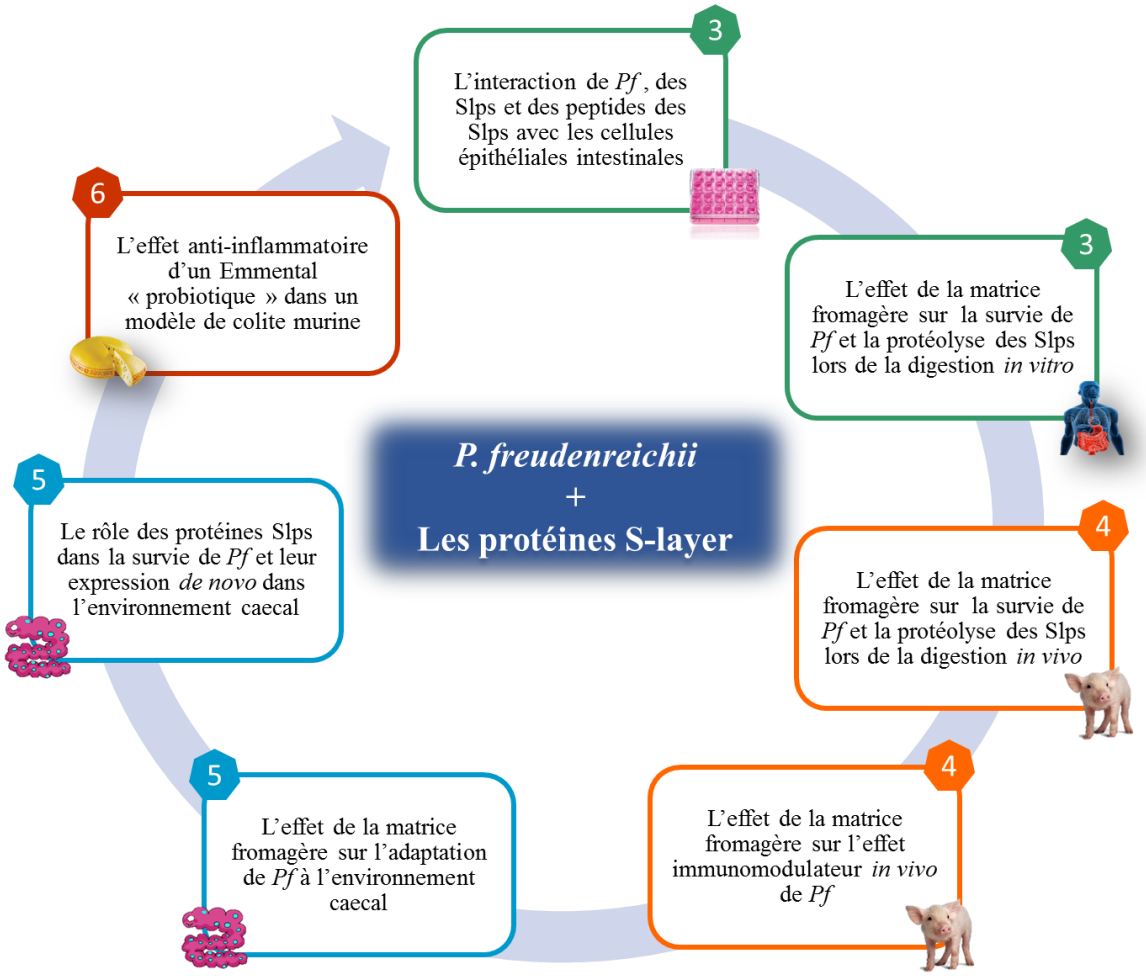

Figure 6. **Schéma de la stratégie générale mise en œuvre lors du projet de thèse pour répondre à la question de recherche : « Comment la matrice fromagère impacte-t-elle les propriétés immunomodulatrices de *P. freudenreichii* à travers ses protéines Slps ? ».** La question de recherche a été déclinée en sept questions intermédiaires de recherche s'intéressant aux effets probiotiques de *P. freudenreichii* CIRM-BIA 129 et de ses protéines Slps, suite à une vectorisation par la matrice fromagère. Le numéro de chapitre incluant les sous questions traitées est annoté sur la figure sous forme d'hexagones.

Chapitre 3 : « L'étude *in vitro* de la vectorisation de *P. freudenreichii* et de ses protéines S-layer par la matrice fromagère »

3.1. Introduction

Les études précédentes ont montré que les protéines de surface S-layer (Slps), qui jouent un rôle clé dans les interactions de *P. freudenreichii* (***Pf***) avec les cellules de l'hôte, sont sensibles aux protéases digestives (Deutsch et al., 2017; Do Carmo et al., 2017; Le Maréchal et al., 2015). De plus, lors du projet ANR SURFING, il a été montré que les protéines de surface S-layer de *P. freudenreichii* CIRM-BIA 129 sont bien exprimées dans le fromage de type monosouche (Plé et al., 2015). Cependant, nous ignorons la cinétique de protéolyse des protéines Slps lors de la digestion, ainsi que s'il y a un effet de matrice sur cette cinétique. De plus, il est connu que la tolérance de *P. freudenreichii* aux stress digestifs est matrice-dépendante (Cousin et al., 2012; Leverrier et al., 2005), mais elle n'a jamais été investiguée lors d'une vectorisation par une matrice fromagère (Gagnaire et al., 2015). Ces effets hypothétiquement matrice-dépendants peuvent affecter les propriétés immunomodulatrices de ***Pf*** *in situ*.

Cette partie du travail de thèse a donc pour objectif d'évaluer l'impact de la matrice fromagère sur la tolérance de *P. freudenreichii* aux stress digestifs d'une part, et d'autre part d'évaluer l'effet protecteur de la matrice fromagère vis-à-vis de la protéolyse digestive des protéines Slps, par comparaison aux matrices de vectorisation liquides. Cependant, dans une démarche préliminaire, nous avons souhaité explorer si les peptides libérés lors de la protéolyse des Slps sont bioactifs et s'ils peuvent induire, tout comme les protéines entières, un effet anti-inflammatoire sur les cellules de l'hôte (Figure 7).

Pour répondre à cet ensemble de questions, nous avons comparé l'effet immunomodulateur de *P. freudenreichii* CIRM-BIA 129, de Slps, et des peptides issus de la protéolyse digestive des Slps, sur des cellules épithéliales intestinales HT-29, en présence ou en absence de stimulus pro-inflammatoire (Lipopolysaccharide, LPS). Ensuite, nous avons utilisé en premier temps un modèle de digestion *in vitro* statique. Nous avons testé trois matrices de vectorisation ayant des concentrations en protéines laitières croissantes : une culture sur l'ultrafiltrat de lait (Cousin, et al. 2012), un lait fermenté, et un fromage monosouche (Plé et al., 2015). Dans ce modèle de digestion statique, nous avons testé deux souches différentes : la souche d'intérêt *P. freudenreichii* CIMR-BIA 129, qui exprime majoritairement la protéine SlpB, ainsi que la souche *P. freudenreichii* CIRM-BIA 118, qui exprime majoritairement la

protéine SlpA. Ensuite, en raison de nombreuses limites du modèle statique de digestion, nous avons utilisé le modèle dynamique de digestion DIDGI® (Ménard et al., 2014). Ce dernier permet d'obtenir un suivi de viabilité et de protéolyse en cinétique et d'avoir un réglage précis du pH gastrique, ce qui était difficile à mettre en place lors du modèle statique de digestion.

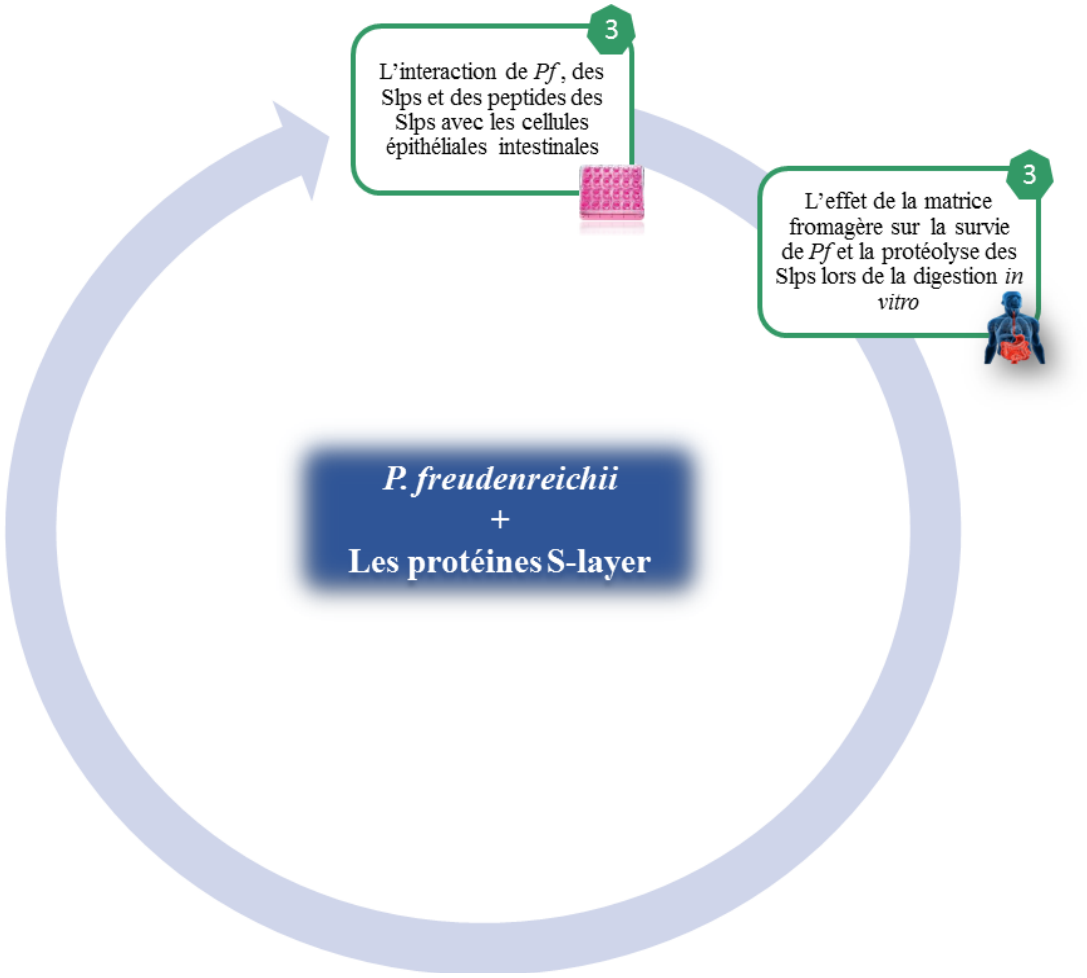

Figure 7. **Quel est l'effet de la matrice fromagère sur les propriétés immunomodulatrices de *P. freudenreichii* CIRM-BIA 129 lors de la digestion *in vitro* ?** Le chapitre 3 (en vert) présente les résultats des expérimentations *in vitro* visant, à comprendre la pertinence de protéger les protéines Slps contre la protéolyse digestive et d'évaluer l'effet protecteur de la matrice fromagère sur *P. freudenreichii* (***Pf***) et sur ses protéines Slps. Ainsi, les questions abordées dans ce chapitre 3 sont:

1. Est-ce que les peptides issus de la protéolyse des protéines Slps sont bioactifs ?
2. Est-ce que la matrice fromagère améliore la tolérance de ***Pf*** aux stress digestifs ?
3. Est-ce que la matrice fromagère protège les protéines Slps de la protéolyse digestive ?

Les résultats de cette étude ont fait l'objet de plusieurs communications scientifiques :

- 2 posters ont été présentés :

i) Au 4éme symposium international sur Propionibactéries et Bifidobactéries, Cork-Irlande, Septembre 2016;

ii) A la conférence internationale de probiotiques et de protéines antimicrobiennes, Barcelone-Espagne, Novembre 2017;

-Une communication orale à la 12éme conférence internationale sur les probiotiques, prébiotiques, microbiote intestinal et santé (IPC2018), Budapest-Hongrie, Juin 2018;

- Ce travail a été publié dans le journal « Food Research International », comme suit:

Rabah H, Ménard O, Gaucher F, Do Carmo FLR, Dupont D, Jan G**. Cheese matrix protects the immunomodulatory surface protein SlpB of *Propionibacterium freudenreichii* during *in vitro* digestion.** Food Res Int. 2018 Apr 1; 106:712–21**.**

3.2. Article 1 : la matrice fromagère protège la protéine de surface immunomodulatrice SlpB de *P. freudenreichii* durant la digestion *in vitro*

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Cheese matrix protects the immunomodulatory surface protein SlpB of *Propionibacterium freudenreichii* during *in vitro* digestion

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ABSTRACT

Propionibacterium freudenreichii is a traditional Swiss-type cheeses starter and constitutes an emergent probiotic, exerting several beneficial effects, including anti-inflammatory modulation of gut inflammation. This feature relies on several metabolites and on surface proteins, with a prominent role of the surface protein SlpB. In this study, we firstly investigated the relevance to avoid SlpB digestive proteolysis, by comparing the effect of i) *P. freudenreichii* CIRM-BIA 129, ii) its native Slps, or iii) peptides resulting from Slps digestive proteolysis, with respect to modulation of HT-29 cells response to a lipopolysaccharide (LPS) challenge. The anti-inflammatory effect exerted by *P. freudenreichii* CIRM-BIA 129 and by its native surface proteins (Slps) on HT-29 cells was abolished by digestive proteolysis. This result confirmed the importance to protect immunomodulatory surface proteins from digestive proteolysis in order to allow gut immune system modulation. Thus, we examined the effect of dairy matrices on *P. freudenreichii* viability and on SlpB integrity during digestion. In comparison with liquid matrices, the cheese matrix provided an enhanced tolerance towards digestive stresses and protection of SlpB towards proteolysis, during two *in vitro* digestion models: static and dynamic. Taken together, these results show that cheese is an adequate delivery vehicle for *P. freudenreichii* immunomodulatory proteins. This opens perspectives for the development of fermented dairy functional foods aimed at target populations at high risk for diet-related diseases with an inflammatory component.

1. Introduction

Propionibacterium freudenreichii is an emergent probiotic bacterium, belonging to dairy propionibacteria, within the group of Actinomycetales. It possesses the GRAS (Generally Recognized As Safe, USA, FDA) and QPS (Qualified Presumption of Safety, EFSA, European Union) status (EFSA Panel on Biological Hazards (BIOHAZ), 2013). Dairy propionibacteria, including *P. freudenreichii*, are typically exploited in the food industry for the production of vitamins and for the manufacture of Swiss-type cheeses, including Emmental (Rabah, Rosa do Carmo, and Jan, 2017; Thierry et al., 2011). Recently, several beneficial effects exerted by *P. freudenreichii* were reported, including anti-inflammatory modulation in the context of gut inflammation (Cousin, Mater, Foligné, and Jan, 2010; Rabah et al., 2017). Dairy products fermented by *P. freudenreichii*, alone or in combination with the probiotic *Lactobacillus delbrueckii*, were efficient to attenuate a chemically-induced colitis in mice (Benoît Foligné et al., 2016; Plé et al., 2015; Plé et al., 2015). Accordingly, a pilot clinical study indicates healing of ulcerative colitis in patients, as a result of consumption of dried *P. freudenreichii* whey culture (Mitsuyama et al., 2007; Suzuki et al., 2006). These data highlight the probiotic potential of *P. freudenreichii* to promote remission from IBD. The anti-inflammatory properties of *P. freudenreichii* are strain-dependent (Foligné, Breton, Mater, and Jan, 2013) and mediated by several metabolites, such as 1, 4-dihydroxy-2-naphtoic acid (DHNA), short-chain fatty acids (SCFAs) (Rabah et al., 2017) and by immunomodulatory surface proteins which present structural characteristics of S-layer proteins (Slps) (Deutsch et al., 2017; Le Maréchal et al., 2015). S-layer proteins belong to the outermost S-layer lattice, non-covalently anchored to the cell wall, and alternately present in Gram-positive and Gram-negative bacteria (Gerbino, Carasi, Mobili, Serradell, and Gómez-Zavaglia, 2015). They display a variety of functions such as adhesion, sieving of molecules, and protection against environmental stresses (Gerbino et al., 2015; Lortal, Rouault, Cesselin, and Sleytr, 1993). In the

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reference *P. freudenreichii* CIRM-BIA 1 strain, nine genes encoding putative S-layer proteins were identified within the genome (Falentin et al., 2010). In contrast with SlpA from the strain *P. freudenreichii* CIRM-BIA 118, which is confirmed as a true S-layer protein (de sa Peixoto et al., 2015; Lortal et al., 1993), the protein designated previously as S-layer protein B (SlpB) from *P. freudenreichii* CIRM-BIA 129 was not shown to form a true surface layer. However, it was detected by surface proteomics (Le Maréchal et al., 2015) and our studies pointed out the key role of SlpB in *P. freudenreichii's* anti-inflammatory properties and in interaction with immune cells (Deutsch et al., 2017; Le Maréchal et al., 2015). SlpB is also involved in the adhesion to human intestinal epithelial cells (Carmo et al., 2017). These results indicate a prominent role of the surface protein SlpB as a Microbe-Associated Molecular Pattern (MAMP) in this probiotic/host cross-talk, with promising anti-inflammatory applications. This is consistent with previous studies indicating that Lactobacilli S-layer proteins interact with immune cells, such as dendritic cells, *via* Pathogen-Recognition Receptors (PRRs), within the gut, inducing tolerance response leading to attenuated colonic inflammation (Konstantinov et al., 2008; Lightfoot et al., 2015). During *P. freudenreichii* consumption, susceptibility of its bioactive surface proteins to proteolysis within the digestive tract thus becomes a crucial question, not yet addressed. The delivery vehicle may determine the transit of intact surface proteins through the host intestinal tract. We investigated the efficacy of the "Swiss-type" cheese matrix as a protective delivery vehicle of anti-inflammatory potential of *P. freudenreichii* to target intact surface proteins to the colon. Indeed, cheese environment enhances propionibacteria tolerance towards acid and bile salts stresses (Gagnaire, Jardin, Rabah, Briard-Bion, and Jan, 2015). Furthermore, the presence of a proteinaceous matrix may exert a protective effect towards enzymatic proteolysis, allowing undamaged bacteria and surface proteins to reach the digestive tract. In the present study, we first assessed the impact of SlpB proteolytic degradation on its immunomodulatory properties, using on colon epithelial HT-29 cells. We then investigated the potential of cheese matrix, as a vehicle, to deliver intact anti-inflammatory *P. freudenreichii* MAMP to the colon. In that aim, we compared efficacy of dairy liquid matrices and of a solid Swiss-type cheese matrix, using two *in vitro* digestion models.

2. Material and method

2.1. Bacterial strains and dairy matrices preparation

The *Propionibacterium freudenreichii* strain CIRM-BIA 129 was provided as certified pure cultures by the CIRM-BIA International Biological Resource Center (Centre International de Ressources Microbiennes-Bactéries d'Intérêt Alimentaire, INRA, Rennes, France) and routinely cultivated in YEL (Yeast Extract Lactate medium). Milk ultrafiltrate and milk media were prepared by adding 5 g/L of casein peptone (Organotechnie, France) and 100 mM of sodium DL-lactate (Sigma), as described previously (Cousin et al., 2012). *P. freudenreichii* was grown at 30 °C, without agitation, in microaerophilic conditions until stationary phase (72 h). Single-strain cheese was prepared with *P. freudenreichii* CIRM-BIA 129 using a process close to 'Swiss-type' cheeses as described previously (Plé, Richoux, et al., 2015). Briefly, *P. freudenreichii* was cultivated in sterile whole milk, supplemented with cream, casein powder, casein peptone and sodium lactate, until stationary phase. After coagulation, (chy-max®Extra, Chr. Hansen),the curd was cut, heated (40 °C, 10 min), molded, pressed, dried under laminar flow, wrapped and storage at 4 °C. The biochemical composition of the cheese was: dry matters 58 g/100 g, lipids 28 g/100 g, proteins 29 g/100 g, carbohydrates 0 g/100 g, and calcium 840 mg/100 g (Plé, Richoux, et al., 2015). The propionibacteria amounts reached 3.10^9 CFU/mL in milk ultrafiltrate, 7.10^9 CFU/mL in milk and 1.10^{10} CFU/g in single-strain cheese.

2.2. Surface proteins extraction and peptides preparation

Surface proteins (Slps) extraction was performed as described previously (Le Maréchal et al., 2015). Briefly, *P. freudenreichii* CIRM-BIA 129 cultures in milk ultrafiltrate (40 mL) were centrifuged (7000 ×*g*, 10 min) to remove the supernatants and the pellets were washed twice with cold PBS buffer. The pellets were then suspended in guanidine chloride-5 M (4 mL, Sigma), and incubated at 50 °C during 15 min. These suspensions were centrifuged (20,000 ×*g*, 20 min) to recover the supernatants. The proteins extracts were dialyzed against water during 24 h and then against PBS. Slps-derived peptides were prepared by treating 50 µg of Slps with 2.5 µg of trypsin corresponding to an activity of 17.8 U/µg (Promega). The samples were incubated for 8 h at 37 °C. The complete digestion of Slps was checked by SDS-PAGE (data not shown). Resulting peptides were recovered by filtration through, successively, 20 kDa and 10 kDa-CutOff filters (Vivaspin, Sartorius stedim biotech) without dilution, nor concentration.

2.3. HT-29 cells challenging

HT-29 cells were routinely cultivated 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 and the growth medium was changed every 2 days. HT-29 cells were cultured until complete confluence, 1.10^6 cell/mL-well. Prior to challenging cells, complete medium was replaced with antibiotic-free medium and HT-29 cells were then subjected to the different treatments according to (Duary, Batish, and Grover, 2014). Briefly, cells were stimulated 3 h with 100 ng/mL lipopolysaccharide (LPS from *E. coli*, Sigma), or 7 h with probiotic preparations. These last were: *P. freudenreichii* CIRM-BIA 129 (1.10^7 CFU/mL-well, corresponding to an MOI of 10, as determined previously (Benoît Foligné et al., 2010), or Surface proteins (Slps) (50 µg/mL-well), or peptides (50 µg/mL-well). After different stimulation conditions, the HT-29 cells was monitored and the viability percentage are listed in Table S1.

2.4. 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). Table 1 lists interleukine and chemokine genes analyzed (Duary et al., 2014). 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 (280 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 min at 95 °C and 40 cycles of 2 steps consisting of 5 s at 95 °C and 30 s at 60 °C. The relative quantification of the mRNA levels of the target genes was determined using CFX Manager. The transcript level was normalized to the transcript level of GAPDH and actin genes (housekeeping gene, see Table S1). 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).

2.5. Static digestion model

Digestion of three fermented dairy matrices was carried out simultaneously using the consensus static *in vitro* digestion protocol as described previously (Minekus et al., 2014), see Table 2. In detail, simulated salivary (SSF) simulated gastric (SGF) and simulated duodenal

Table 1
Primer sequences of target genes of HT-29 cells analyzed by Reverse transcription PCR.

Gene	Forward primer (5′-3′)	Reverse primer (5′-3′)	Product size (bp)	Tm (°C)
β-actin	tgg ctg ggt gtt gaa ggt ct	agc acg gca tcg tca cca act	238	83
GAPDH	caa cga cca ctt tgt caa gc	ttc ctc ttg tgc tct tgc tg	140	85.9
Il-8	tgg ctc tct tgg cag cct tc	tgc acc cag ttt tcc ttg gg	238	83
Il12p35	ttc acc act ccc aaa acc tgc	gag gcc agg caa ctc cca tta	225	84.5
Tnf-alpha	agc cca tgt tgt agc aaa cc	tga ggt aca ggc cct ctg at	134	89.5
Il-10	aaa gaa ggc atg cac agc tc	aag cat gtt agg cag gtt gc	132	90.0
Tgf-β2	atg ccc gta ttt atg gag tt	att gtc att ttg gtc ttg cc	234	83

Table 2
In vitro static digestion parameters. Three fermented matrices; the milk ultrafiltrate and single-strain cheese fermented by *P. freudenreichii* CIRM-BIA 129 were subjected to *in vitro* static digestion. The parameters were set as described by Minekus et al. (2014).

	Oral phase	Gastric phase	Duodenal phase
Time (minutes)	2	120	120
Temperature (°C)	37		
pH	Sample pH	3	7
Enzymes	–	Porcine pepsin (2000 U/mL)	Porcine pancreatin Trypsin (100 U/mL)
Bile salt	–	–	Bovine Bile 10 mM

(SDF) fluids were prepared as preconized. For single-strain cheese, milk and ultrafiltrate milk, the bacterial population was adjusted to 5.10^{10} CFU. The samples (5 g) were mixed with SSF in the stomacher (AWEL®). $CaCl_2$ (25 µL) was added to a concentration of 0.3 M and the mixtures were incubated 2 min at 37 °C under agitation (100 rpm). The derived bolus was mixed with 8 mL of SGF, the pH was quickly adjusted to 3 (3 M HCl), and $CaCl_2$ (5 µL) was added. Porcine pepsin (EC 3.4.23.1, Sigma P6887, 2823 U/mg) was added at a final concentration of 2000 U/mL when the temperature was over 36 °C. The gastric phase digestion was performed at 37 °C for 2 h under agitation (100 rpm) and was stopped using pepstatin A (5 µg/mL, Sigma). Afterwards, the pH was quickly adjusted to 7 and SDF was added to the digestate, as $CaCl_2$ (40 µL) and 10 mM bovine bile (3.1 µmol/mg, Sigma). Porcine pancreatin (Sigma P7545, trypsin activity: 6.57 U/mg) was added for a final concentration in trypsin of 100 U/mL. The intestinal phase digestion was performed at 37 °C for 2 h at pH 7, and it was stopped by adding the protease inhibitor Pefabloc (0.1 M) at 50 µL/mL of sample (Sigma). Samples were treated for bacterial proteins analysis and CFU counting to determine survival of dairy propionibacteria.

2.6. Dynamic digestion model

The previously described *in vitro* dynamic digestion system DIDGI® (Ménard et al., 2014) was used. *In vitro* dynamic digestion of dairy samples was conducted using the same parameters for single-strain cheese and fermented milk ultrafiltrate, *i.e.* parameters of cheese digestion in the aim to determine the matrix effect (Table 3). The digestion was monitored by the software 'StoRM'. To control the transport time of the digestate in each compartment, a power exponential mathematical equation is used as for chyme delivery as described previously (Ménard et al., 2014). The equation parameters including the half time of delivery ($t_{1/2}$) and time of the coefficient describing the shape of the curve (β) was obtained from an exhaustive review on cheese meal digestion conditions and set at $t_{1/2}$ = 85 min or 200 min and β = 1.8 or 2.5 for gastric or intestinal phase, respectively (Lvova et al., 2012). The pH curve in the stomach was taken from literature describing cheese digestion (Lvova et al., 2012; Pitino et al., 2012). Gastric pH monitored a linear regression $pH = 1.68 * 4.32e^{(-t/45)}$, where t is time. Intestinal pH was kept constant at 6.5. Digestive enzymes and bile were diluted in 150 mM NaCl, pH 6.5. After rehydration, all the digestive enzymes were kept on ice throughout the experiment in

Table 3
In vitro dynamic digestion parameters. Two matrices, milk ultrafiltrate and single-strain cheese fermented by *P. freudenreichii* CIRM-BIA 129 were subjected to *in vitro* dynamic digestion, by using the same parameters.

	Stomach	Duodenum
Volume ingested (mL)	60 Diluted or mixed with 60 mL NaCl 150 mM	–
Fasted volume (mL)	24 Pepsin 625 U/mL diluted in NaCl 150 mM pH = 3	–
pH curve	$pH = 1.68 * 4.32e^{(-t/45)}$	6.8
Secretions	Pepsin 2000 U/mL Flow 0.25 mL/min	Bile 4% for 30 min 2% for 30–240 min Flow 0.5 mL/min Pancreatin 7% Flow 0.25 mL/min
Transit time	$t_{1/2}$ (min) 70 β 1.8	$t_{1/2}$ (min) 200 β 2.5

order to avoid autolysis. In the stomach, porcine pepsin diluted at 1250 U of pepsin/mL was added at a constant flow rate of 0.25 mL/min. In the small intestine compartment, a 4% (for 30 min) and 2% (30 min to 240 min) solutions of bovine bile and a 7% solution of porcine pancreatin were added at a constant flow rate of 0.5 mL/min and 0.25 mL/min, respectively. Digestion experiments were performed in duplicates. For gastric digestion, samples were collected in the gastric compartment at 30, 60, 80, 120, and 180 min. For the intestinal digestion, samples were collected at the output of intestinal compartment, at 120, 150, 195, and 240 min. Samples remaining in the small intestine duodenum were also collected to be analyzed (samples D). For bacterial survival monitoring, the bacterial viability was calculated per mL of the digested matrix by considering the emptying effect. For proteins proteolysis monitoring, pefabloc or pepstatin A were added at digested samples in order to inhibit proteolysis. The proteins quantity analyzed for each collected sample were normalized for considering emptying effect during the digestion.

2.7. Survival monitoring

Different aliquots of digestate were submitted to serial 1/10 dilutions in isotonic peptone water (0.9% NaCl, 0.1% peptic digest of meat, Biokar Diagnostics, Beauvais, France, adjusted to pH 7.0) and dilutions were plated on both YEL-agar plates (YELA) and in lithium-glycerol-agar plates (LGA) for CFU counting. Percent survival was then determined by comparing digested and undigested samples. Similar results were obtained using YELA (maximal recovery) and LGA (selective medium).

2.8. SDS–PAGE and western-blotting

Digestate from milk and milk ultrafiltrate were centrifuged, the bacterial pellet was washed twice with citrate water ($C_6H_5Na_3O_7$-$2H_2O$, 20 g/L, pH = 7.5). For cheese digestate, samples were grinded with 9 volumes of cold citrate water in Stomacher (AWEL®). Samples were

centrifuged and the pellet washed several times successively with citrate water. Lysis solution (Tris-HCl 50 mM pH 7.5, SDS 0.3%, DTT 200 mM) was added to bacterial pellet and samples were frozen prior to sonication and cell lysis using Precellys® Evolution homogenizer. Resulting SDS extracts were recovered by centrifugation (21,000 ×*g*, 4 °C, 20 min) and analyzed by one-dimensional electrophoresis SDS-PAGE. The proteins extracts from triplicate experiments were pooled and the proteins amount was determined using 2-D Quant Kit (GE Healthcare). Alternatively, surface protein extracts were separated by 12% SDS–PAGE and transferred to PVDF membranes (GE Healthcare). After blocking with 3% nonfat 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 anti-SlpB primary antibodies purified from rabbit sera (AGRO-BIO, France) 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).

2.9. Statistical analysis

All the experiments were performed with three technical replicates and three biological replicates. The results were expressed as means ± standard deviations (SD). Statistical analyses were performed in GraphPad Prism Software (Prism 7 for Windows) using one-way or two-way ANOVA with Dunnet or Tukey *post hoc* analyses for multiple comparisons.

3. Results

3.1. Proteolytic degradation of Slps abolishes their anti-inflammatory effects on HT-29 cells

3.1.1. Pro-inflammatory cytokines expression

We analyzed the expression of three pro-inflammatory interleukins, IL-8, IL-12 and Tumor Necrosis Factor (TNF)-α, in HT-29 cultured human colon cells. These cells were stimulated by 1) *P. freudenreichii*, 2) extracted Slps, 3) peptides resulting from Slps proteins trypsin-proteolysis, 4) LPS from *E. coli*, 5) *P. freudenreichii* + LPS, 6) Slps + LPS and 7) peptides + LPS (Fig. 1). In the absence of pro-inflammatory stimulation, IL-8 expression was not affected by *P. freudenreichii* (0.872 ± 0.366), nor by Slps (1.768 ± 0.746) nor by peptides (2.689 ± 1.795), compared to control non-treated cells (Fig. 1A). The LPS stimulation induced a significant increase of IL-8 expression (5.859 ± 0.856). This increase was attenuated by *P. freudenreichii* (2.695 ± 0.943) and by Slps (3.096 ± 0.166), but not by Slps-peptides (5.587 ± 1.945) (Fig. 1A). In the same way, neither *P. freudenreichii* (0.911 ± 0.707), nor Slps (0.812 ± 0.251), nor peptides (1.277 ± 0.317) induced TNF-α expression, in comparison to the control (Fig. 1B). In the presence of proinflammatory LPS, increased expression of TNF-α (5.728 ± 2.955) was attenuated by *P. freudenreichii* (2.42 ± 0.280) and by Slps (1.894 ± 1.121). However peptides were unable to attenuate this LPS-induced TNF-α expression (4.013 ± 0.337) (Fig. 1B). Similarly, IL-12 expression was not significantly changed by *P. freudenreichii* (1.372 ± 0.503), nor by Slps (1.131 ± 0.353), nor by peptides (1.397 ± 0.814). The LPS-induced expression of IL-12 (2.809 ± 0.765) was attenuated by *P. freudenreichii* (1.09 ± 0.579) and native Slps (1 ± 0.397). It tended to decrease in the presence of peptides, (1.788 ± 0.859), but this decrease was non-significant (Fig. 1C).

3.1.2. Regulatory cytokines expression

We also analyzed the expression of two regulatory interleukin IL-10 and transforming growth factor (TGF)-β by RT-PCR (Fig. 2). Neither Slps (1.036 ± 0.2194), nor peptides (1.57 ± 0.5259), nor LPS (0.8164 ± 0.2569) induced a significant change of the IL-10 expression, in comparison to the control ($p > 0.05$). Only *P. freudenreichii* cells induced a significant increase of IL-10 (2.343 ± 0.3992) (Fig. 2A). Induction of IL-10 by *P. freudenreichii* was also observed in the presence of LPS (2.368 ± 0.8191) (Fig. 2A). However, the presence of Slps (1.823 ± 1.003) and peptides (1.306 ± 0.1546) did not change significantly IL-10 expression on HT-29 cells stimulated by LPS (Fig. 2A). For TGF-β expression, only Slps induced a significant increase of expression (3.513 ± 1.07), in comparison to the control (Fig. 2B). However, peptides from digested Slps failed to do so (1.437 ± 0.76), (Fig. 2B). In the presence of LPS, here as well, only native Slps induced an increase of TGF-β expression (3.196 ± 0.71), while peptides did not (1.353 ± 0.68) (Fig. 2B).

3.2. Cheese matrix enhances bacterial survival during in vitro digestion

3.2.1. In vitro static digestion

The survival of *P. freudenreichii* CIRM-BIA 129 was evaluated during digestion of three matrices: fermented milk ultrafiltrate, fermented milk and single-strain cheese; in an *in vitro* static digestion model. The aim of the study was to evaluate the matrix effect on dairy propionibacteria tolerance towards digestive stresses. During digestion, we assessed the survival rate at three stages: 1) after the oral phase, 2) after successive oral and gastric phases and 3) after successive oral, gastric and intestinal phases. The survival rates after the oral remained unaffected, whatever the food matrix, and were identical to the untreated samples. They are thus not represented in Fig. 3. After the succession of oral and gastric phases, the survival rate of propionibacteria provided in cheese was significantly higher (110.5% ± 27.8%) than when provided in fermented milk (65.9% ± 28.8, $p < 0.05$) or in fermented ultrafiltrate (47.2% ± 29.6%, $p < 0.01$) (Fig. 3A). The propionibacteria survival rates were not significantly different between fermented ultrafiltrate and fermented milk ($p > 0.05$) (Fig. 3A). Similarly, after the complete digestion, survival in cheese (62.0% ± 27.7%) was significantly higher than in ultrafiltrate (27.1% ± 10.9%, $p < 0.05$) (Fig. 3B). However, the survival rate of propionibacteria in milk (32.0% ± 14.0%) and in cheese were not significantly different ($p > 0.05$), and neither were survival rates between milk ultrafiltrate and milk ($p > 0.05$) (Fig. 3B).

3.2.2. In vitro dynamic digestion

In the *in vitro* dynamic DIDGI® digestion system, two different matrices were tested, milk ultrafiltrate and single-strain cheese, both fermented by the strain CIRM-BIA 129. We used the same parameters during *in vitro* dynamic digestion, those determined for cheese matrix digestion. The survival rates were highly different, so that we represented the survival in logarithmic scale (Fig. 4). During the gastric phase (0 to 180 min), the propionibacteria in cheese showed a significantly higher viability than in milk ultrafiltrate (Fig. 4A). At the end of the gastric phase, we did not observe a significant loss of propionibacteria viability in cheese (0.22 log CFU/mL) (Fig. 4A). For milk ultrafiltrate matrix, a significant loss of viability (3.3 log CFU/mL) was observed at the end of gastric phase. At the small intestine output (120 min to 240 min), we observed the same difference in propionibacteria viability (Fig. 4B). The viability of propionibacteria in cheese decreased by 0.57 log CFU/mL, while in fermented milk ultrafiltrate the viability decreased by 2.9 log (Fig. 4B). The viability of propionibacteria provided within cheese was drastically higher at the end of the intestinal phase, with a difference of 3 log CFU/mL (Fig. 4C).

3.3. Cheese matrix protects surface protein SlpB from proteolysis

3.3.1. In vitro static digestion

P. freudenreichii CIRM-BIA 129 was selected as the most anti-inflammatory strain of *P. freudenreichii* in our previous screening of strains on human PBMCs. It produces high amounts of the

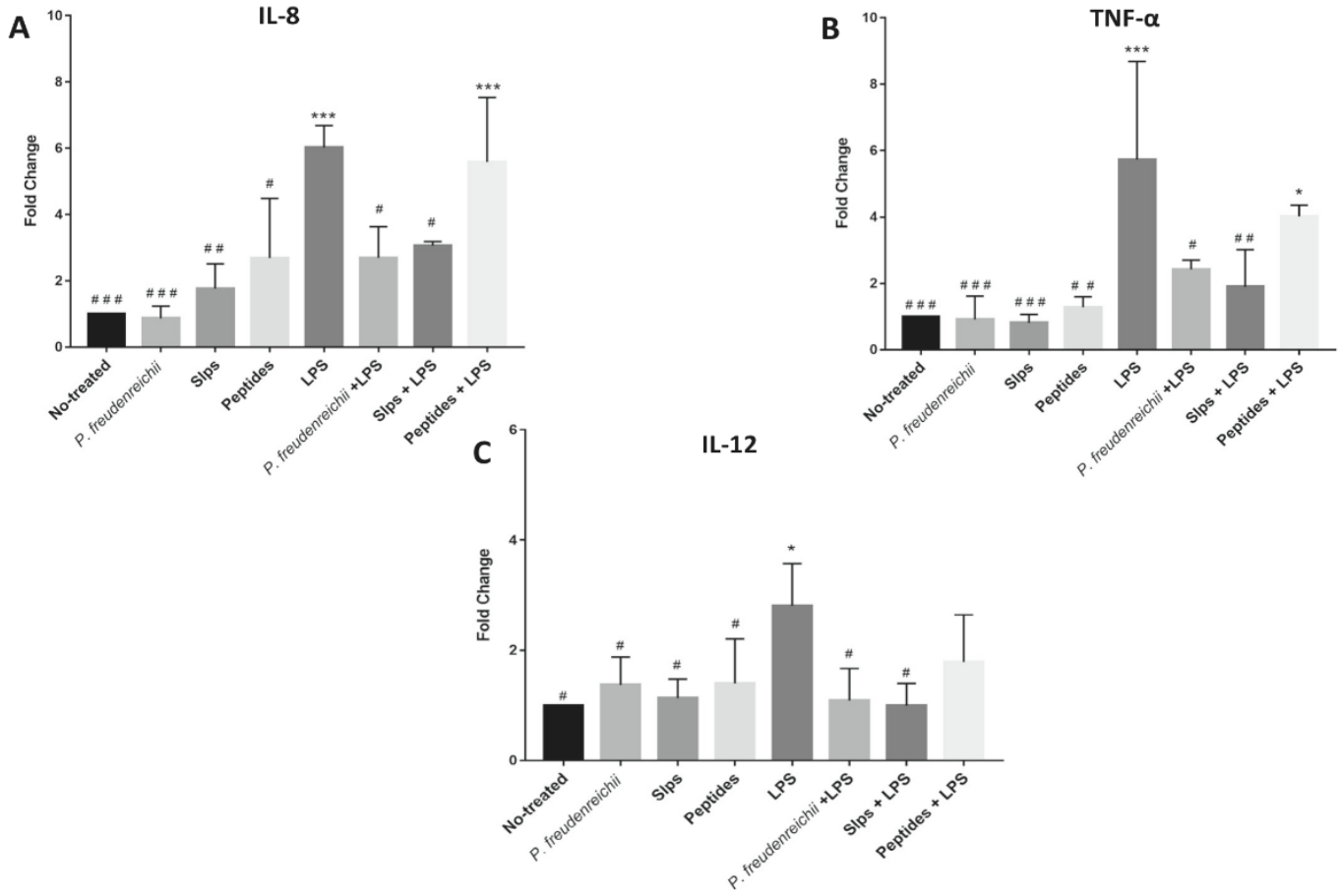

Fig. 1. Pro-inflammatory cytokines expression on HT-29 cells. HT-29 cells were stimulated by lipopolysaccharides (LPS), *P. freudenreichii* CIRM-BIA 129, S-layer proteins (Slps) and peptides resulting from Slps proteolysis. **A**) IL-8, **B**) TNF-α and **C**) IL-12 expression was monitored by RT-PCR. Data are represented as mean ± standard deviation for triplicate experiments. * $p < 0.05$ compared with Non-treated condition. # $p < 0.05$ compared with LPS condition.

immunomodulatory surface protein SlpB, the size of which is 54 kDa. Proteolysis of SlpB during *in vitro* static digestion was monitored by SDS-PAGE & Western blotting (Fig. 5). This was done at four times, 1) before digestion (T0), 2) after oral phase (T1), 3) after oral and gastric phases (T2); and 4) after successive oral, gastric and intestinal phases (T3). In the case of liquid matrices, fermented milk ultrafiltrate and fermented milk, SlpB disappeared after the gastric phase, as evidenced by SDS PAGE (Fig. 5A & 5C) and western blotting (Fig. 5B & 5D). During cheese digestion, however, we observed the presence of SlpB after the gastric phase and after complete digestion (Fig. 5E & 5F). At

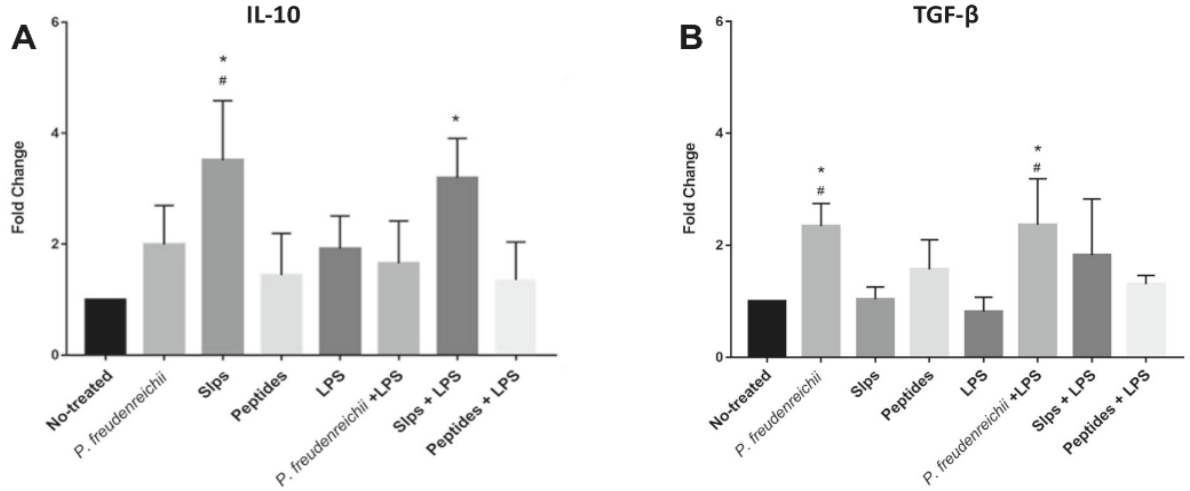

Fig. 2. Regulatory cytokines expression on HT-29 cells. HT-29 cells were stimulated by lipopolysaccharides (LPS), *P. freudenreichii* CIRM-BIA 129, S-layer proteins (Slps) and peptides resulting from Slps proteolysis. **A**) IL-10, and **B**) TGF-β expression was monitored by RT-PCR. Data are represented as mean ± standard deviation for triplicate experiments. * $p < 0.05$ compared with Non-treated condition. # $p < 0.05$ compared with LPS condition.

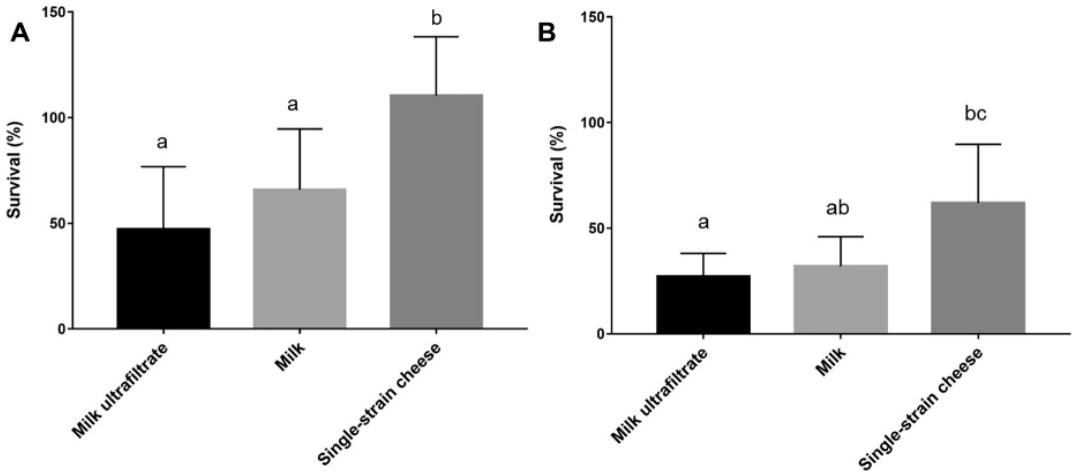

Fig. 3. *P. freudenreichii* CIRM-BIA 129 viability after *in vitro* static digestion. Three fermented matrices were subjected to *in vitro* static digestion, viable propionibacteria were enumerated by plate counting at different digestion stage. **A)** percentage of survival after succession of oral and gastric phases; **B)** percentage of survival after complete digestion (oral, gastric and intestinal phases). The results are expressed as percentage of survival, and as mean ± standard deviation for triplicate experiments. Different letters above the columns indicate significant differences ($p < 0.05$).

the end of digestion, only cheese allowed detection of intact SlpB (Fig. 5E & 5F).

3.3.2. *In vitro dynamic digestion*

The dynamic digestion model further gave a kinetic analysis of surface protein SlpB proteolysis during digestion of fermented milk ultratfiltrate and of cheese (Fig. 6). During digestion of the fermented milk ultrafiltrate (Fig. 6A), SlpB was detected until 60 min of digestion in the stomach but disappeared after 80 min of digestion (Fig. 6A & 6B). However, during cheese digestion, SlpB was detected until 120 min of gastric digestion (Fig. 6C & 6D). During the intestinal phase, the surface protein SlpB in milk ultrafiltrate was not detected at the output of the duodenum. By contrast, during cheese digestion, SlpB was detected at the output of duodenum until 150 min of digestion. SlpB was not detected in the duodenal compartment at the end of digestion phases, for both matrices, cheese and milk ultrafiltrate.

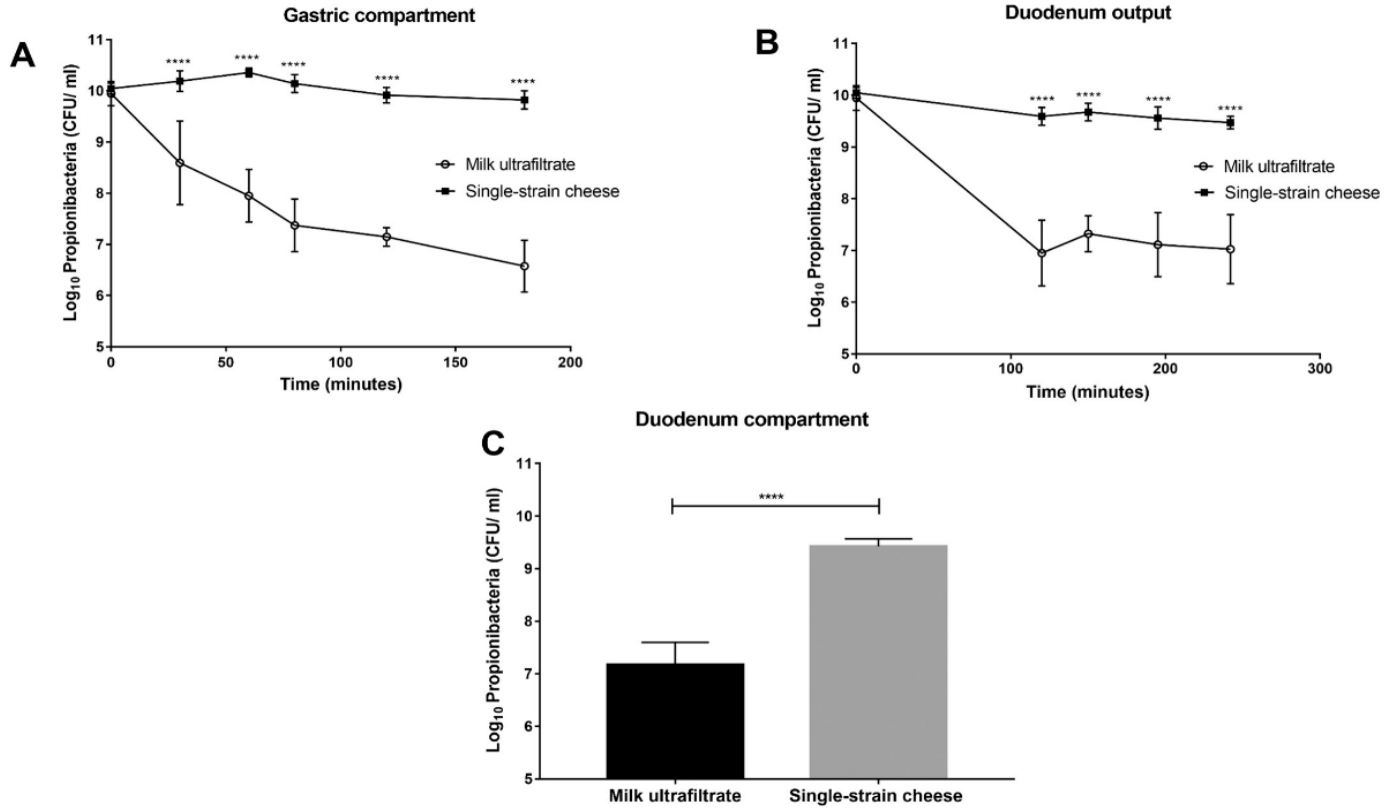

Fig. 4. *P. freudenreichii* CIRM-BIA 129 viability during *in vitro* dynamic digestion. Two matrices fermented by *P. freudenreichii* CIRM-BIA 129 were subjected to *in vitro* dynamic digestion. Viable propionibacteria were monitored by plate counting at different times during digestion. **A)** the evolution of propionibacteria viability during the gastric phase is shown; **B)** the evolution of propionibacteria viability at the output of the duodenal compartment; **C)** the propionibacteria viability in the duodenal compartment at the end of the digestion. The results are expressed in logarithmic scale as mean ± standard deviation for duplicate experiments. *$p < 0,05$.

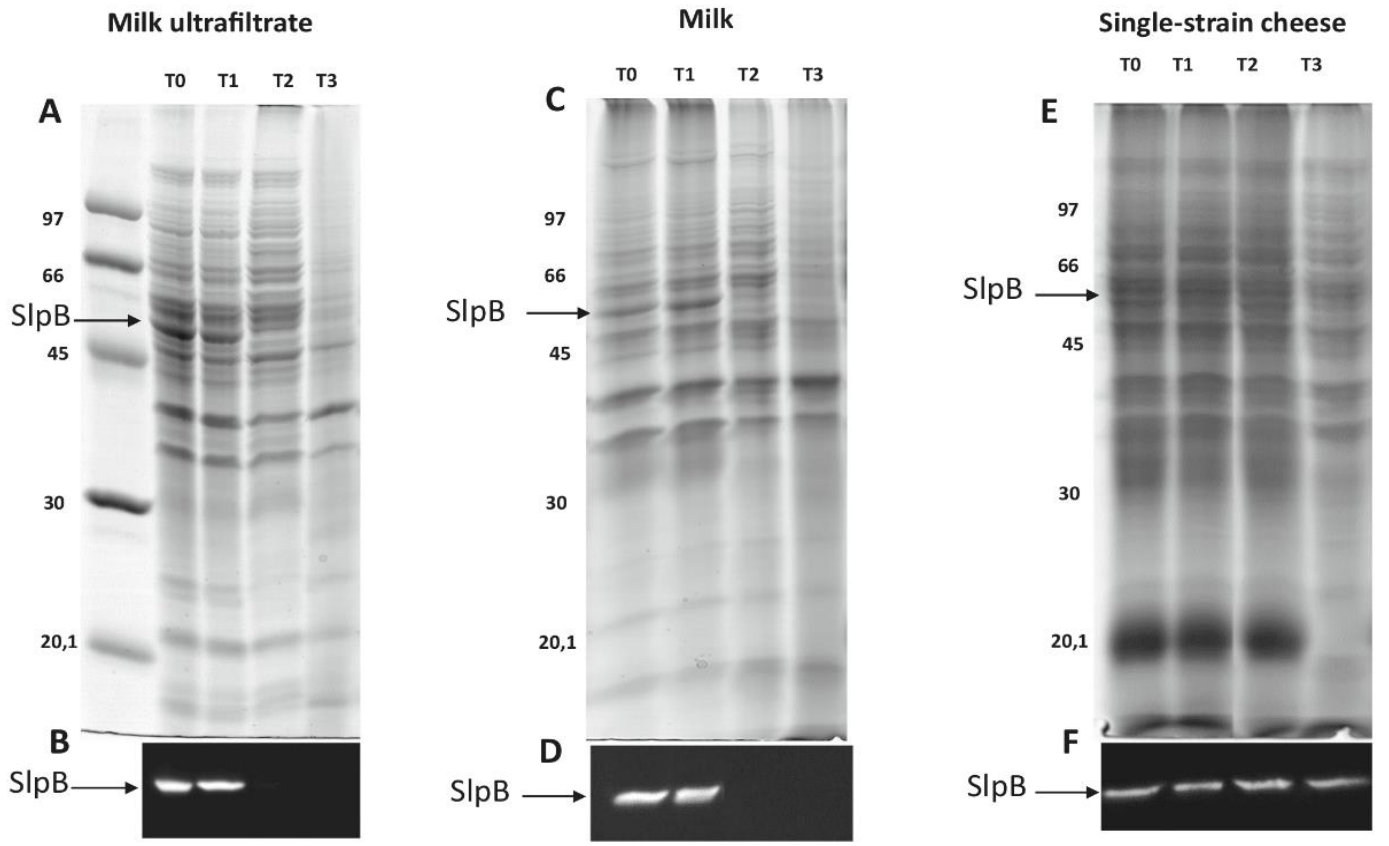

Fig. 5. *P. freudenreichii* CIRM-BIA 129 SlpB proteolysis during *in vitro* static digestion. Three different matrices, fermented by *P. freudenreichii* CIRM-BIA 129, were tested, **A-B)** Milk ultrafiltrate, **C-D)** milk and ***E*-F)** Single-strain cheese. Samples were collected at different times: **(T0)** before digestion, **(T1)** at the end of oral phase, **(T2)** after the succession of oral and gastric phases, **(T3)** and after the complete digestion (oral, gastric and intestinal phases). Total proteins extracts from bacterial pellets were prepared; then analyzed by 12% SDS PAGE electrophoresis (A, C and E) and by SlpB western blotting (B, D and F).

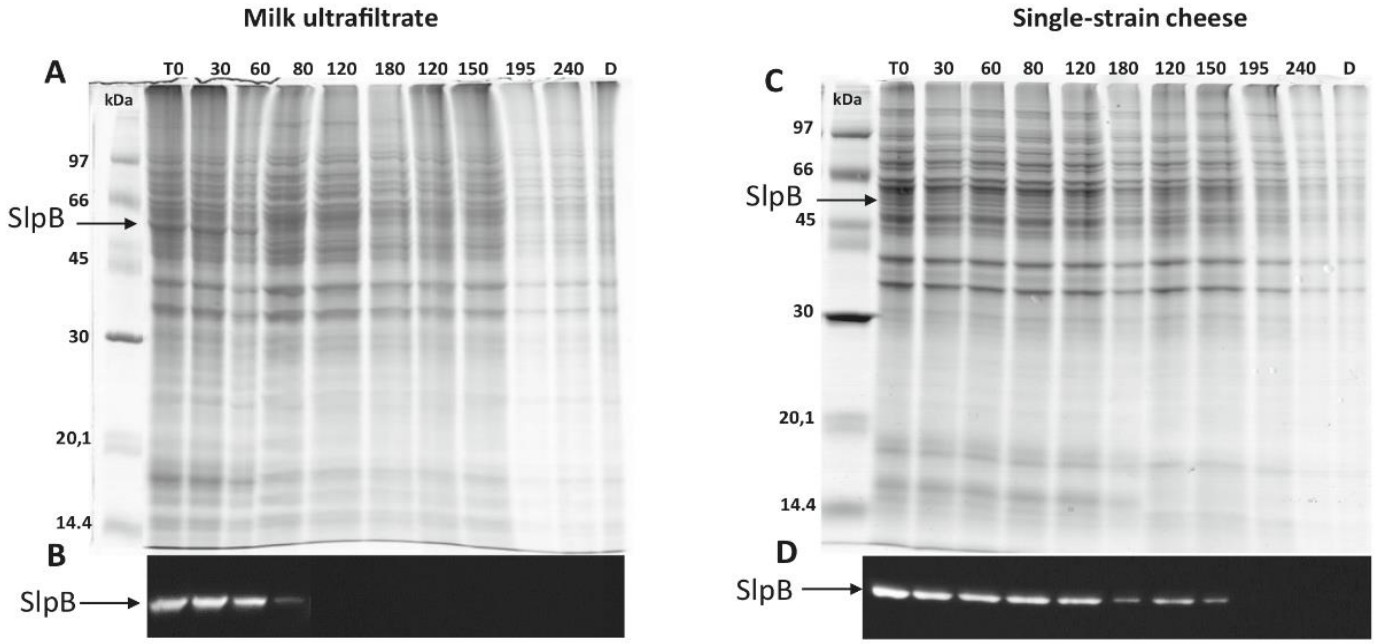

Fig. 6. *Propionibacterium freudenreichii* CIRM-BIA 129 SlpB proteolysis during *in vitro* dynamic digestion. Two different matrices fermented by *P. freudenreichii* CIRM-BIA 129 were tested, milk ultrafiltrate **(A,B)** and single-strain cheese **(C, D)**. Samples collected from 30 to 180 min, samples correspond to the proteolysis assessed in the gastric compartment. Samples collected from 120 to 240 min correspond to the proteolysis assessed at the output of duodenum compartment. Samples D correspond to samples remaining in the duodenum compartment at the end of the digestion. Total proteins extracts from bacterial pellets were prepared; then analyzed by 12% SDS PAGE electrophoresis **(A, B)** and by SlpB western blotting **(C, D)**.

4. Discussion

The anti-inflammatory effect of *P. freudenreichii* and the key role of its surface proteins SLPs were clearly evidenced using human peripheral blood mononuclear cells (Deutsch et al., 2017; Foligné et al., 2013; Le Maréchal et al., 2015). By contrast, little is known about the mechanism allowing *P. freudenreichii* to attenuate inflammation within the gut during *in vivo* induced colitis. Indeed, a single-strain cheese fermented by *P. freudenreichii* CIRM-BIA 129 was able to reduce inflammation during trinitrobenzene sulfonic acid (TNBS)-induced colitis model in mice (Plé, Breton, et al., 2015; Plé, Richoux, et al., 2015). *P. freudenreichii* produces several beneficial metabolites which have anti-inflammatory effects, or metabolites inducing microbiota changes which may modulate inflammation (Rabah et al., 2017). Until the presented study, there was no evidence that *P. freudenreichii* interacts with innate immunity cells, as demonstrated for L. *acidophilus* NCFM (Konstantinov et al., 2008; Lightfoot et al., 2015). Besides nutrients absorption, intestinal epithelial cells (IECs) play a major role in gut homeostasis by communicating the microbial environment to professional immune cells resident within gut-associated lymphoid tissues (Peterson and Artis, 2014). Previously, *P. freudenreichii* was shown to adhere to IECs *via* Slps (Carmo et al., 2017). The present report constitutes the first evidence that the direct interaction of *P. freudenreichii* and its Slps with IECs results in an anti-inflammatory response, mainly by inhibiting LPS-induced pro-inflammatory response. Similar responses were reported for probiotic *Lactobacillus* species (Cammarota et al., 2009; Duary et al., 2014; Li, Yu, Ye, Wang, and Yang, 2011; Rolny, Tiscornia, Racedo, Pérez, and Bollati-Fogolín, 2016; Vizoso Pinto et al., 2009), but few researches evidenced that Slps are responsible in part for this effect (Li et al., 2011; Vizoso Pinto et al., 2009). *P. freudenreichii*, as well as its extractable Slps, decreased LPS-induced expression of IL-8 *in vitro*. This is consistent with previous *in vivo* results, *P. freudenreichii* consumption decreased IL-8 expression in pigs intestinal explants stimulated by LPS (Cousin et al., 2012). IL-8 is a pro-inflammatory chemokine which, additionally to others signals, triggers neutrophil recruitment to the lamina propria, and interacts with intraepithelial T cells through activation of CD1 and CD8 + T cells (Kucharzik et al., 2005; Singer and Sansonetti, 2004). In accordance with previous data (Cousin, Foligné, et al., 2012; Le Maréchal et al., 2015), *P. freudenreichii* and its Slps decreased TNF-α expression in LPS-stimulated IECs. TNF-α is a pro-inflammatory cytokine which controls the production of inflammatory mediators and different modalities of cell death in intestinal inflammation (Bai, Ouyang, Zhang, Wang, and Li, 2004; Schmitz et al., 1999). Only Slps induced a significant increased expression of TFG-β, which is a key cytokine during periods of active inflammation, modulating IECs restitution and preventing mucosal-injury (Babyatsky, Rossiter, and Podolsky, 1996; Jarry et al., 2008; Peterson and Artis, 2014). Moreover, only *P. freudenreichii* induced a significant induction of IL-10, which plays a crucial role in preventing epithelial damage during inflammation (Denning et al., 2000; Jarry et al., 2008). The anti-inflammatory effect, in particularly the inhibition of the pro-inflammatory response to LPS, was not observed following treatment with peptides resulting from Slps proteolysis. However, more investigations are needed to characterize the surface protein moiety involved. Nonetheless, our results indicate the necessity to protect surface proteins from digestive proteolysis to enhance *P. freudenreichii* immunomodulatory functionalities.

P. freudenreichii is a well-known cheese starter used in Swiss-type cheese. Choosing a cheese matrix as a delivery vehicle to design functional food fermented by probiotic strains, thus opens new perspectives to reduce high risk factor for life-style related diseases, such as IBD. One main prerequisite for the efficient incorporation of probiotic microorganism in the existing microbiota is its survival to stressing GIT conditions. We assessed the propionibacteria viability during *in vitro* static digestion, by comparing three matrices: fermented milk ultrafiltrate, fermented milk or single-strain cheese. Stress tolerance, in *P. freudenreichii*, is strain-dependent (Fig. S1), in accordance with previous studies (Cousin, Foligné, et al., 2012; Cousin, Louesdon, et al., 2012; Huang et al., 2016; Lan et al., 2007; Leverrier, Fremont, Rouault, Boyaval, and Jan, 2005). The cheese matrix enhanced the survival rate during the gastric and intestinal phases of static digestion, in comparison to milk and to ultrafilttrate. Contrasting results exist about the protective effect of dairy matrices on probiotic bacteria. Some studies indicate cheese matrix as a good vehicle for various probiotic species (Adouard et al., 2015; Martinovic et al., 2016; Ortakci, Broadbent, McManus, and McMahon, 2012; Pitino et al., 2012; Ricciardi, Blaiotta, Di Cerbo, Succi, and Aponte, 2014), while other studies suggest the opposite (Adouard et al., 2016; Hernández-Galán et al., 2017). The cheese ripening time, and thus the bacterial adaptation mechanisms, influence the tolerance towards digestive stresses (Martinovic et al., 2016; Ricciardi et al., 2014). Indeed, some bacteria fail to resist to digestive stresses, when included in cheese matrix, which led to the hypothesis that the stressful environment of the cheese may weaken bacteria (Adouard et al., 2016; Hernández-Galán et al., 2017; Martinovic et al., 2016). However, in propionibacteria, it is proven that the cheese environment enhances tolerance response towards different digestive stresses (Gagnaire et al., 2015). Furthermore, the buffering effect of the dairy proteins and lipids limits the damaging effects of gastric secretions and bile salts (Fig. S2A) (Martinovic et al., 2016; Pitino et al., 2012; Ricciardi et al., 2014). Indeed, one of the limits of the *in vitro* static model is the absence of a continuous control of pH during gastric phase. We thus assessed the bacterial viability using *in vitro* dynamic digestion, by applying the same parameters for the two matrices, cheese and fermented milk ultratfiltrate. The dynamic model of digestion provides a continuous control of the stomach pH (Fig. S2B). Despite the continuous pH control during gastric phase, propionibacteria from cheese still present a superior tolerance to acid challenge than those from milk utltrafiltrate. Nonetheless, additional to acid stress, the long emptying time for milk ultratfiltrate may explain in part also the great survival loss in this medium. During the intestinal phase, propionibacteria survival was enhanced by cheese matrix at the output and in the of the duodenum compartment, in comparison to milk ultrafiltrate culture. Taken together, the *in vitro* static and dynamic models results indicate a protective effect of the matrix on *P. freudenreichii* survival towards digestive stresses.

This study shows clearly the expression of immunomodulatory SlpB in cheese, but it is not possible to compare Slps expression between the liquid culture and cheese. Indeed, the bacterial pellet extraction from cheese doesn't totally recover the bacterial population. The experiment shows the protective effect of *P. freudenreichii* CIRM-BIA 129 SlpB by the cheese matrix from digestive proteolysis, similar results were obtained for another *P. freudenreichii* strain during *in vitro* static digestion (Fig. S3). An *in vivo* study demonstrated the molecular activity and physiological adaptation *of P. freudenreichii* within the colon, which constitutes a prerequisite to *P. freudenreichii* probiotic potential expression (Cousin, Louesdon, et al., 2012; Saraoui et al., 2013). There is no study indicating that *P. freudenreichii* expresses Slps within the gut, however some studies suggest that surface proteins expression, including S-layer proteins, may be induced to enhance long-term survival and tolerance to environmental stresses (Aburjaile et al., 2016). Nonetheless, the present work evidences a protective effect of the cheese matrix towards digestive proteolysis of Slps in the stomach and duodenum compartments. It should thus deliver *P. freudenreichii* with intact Slps to the colon, able to adhere to IEC, *via* the adhesion role of Slps (Carmo et al., 2017).

5. Conclusion

These *in vitro* results provide new insights into the matrix effect on *P. freudenreichii* probiotic functionalities. The cheese matrix offers significant protection against the digestion stresses by enhancing survival, and by protecting surface layer proteins from proteolysis. However, *in*

vivo investigations are necessary to study the matrix effect during digestion, to follow Slps expression and to evaluate the matrix effect on immunomodulation by *P. freudenreichii* within the gut. Such data accordingly will allow the development of new functional foods for the delivery of *P. freudenreichii* to the gastrointestinal tract of humans in the aim to help prevention or treatment of life-style related diseases with an inflammatory component.

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Supplemental data

Condition	Cells viability (%)
Untreated	97,8 ± 1,0
LPS	95,2 ± 1,0
P. freudenreichii	98,3 ± 1,0
***P. freudenreichii* + LPS**	97,6 ± 1,1
Slps	97,6 ± 0,8
Slps+LPS	98,4 ± 0,5
Peptides	94,2 ± 5,4
Peptides+LPS	97,5 ± 0,6

Table S1. Percentage of HT-29 cells viability during different stimulation conditions. Trypan blue staining was used for distinguish dead cells from alive cells. The results are expresses as percentage of viability from triplicate experiments.

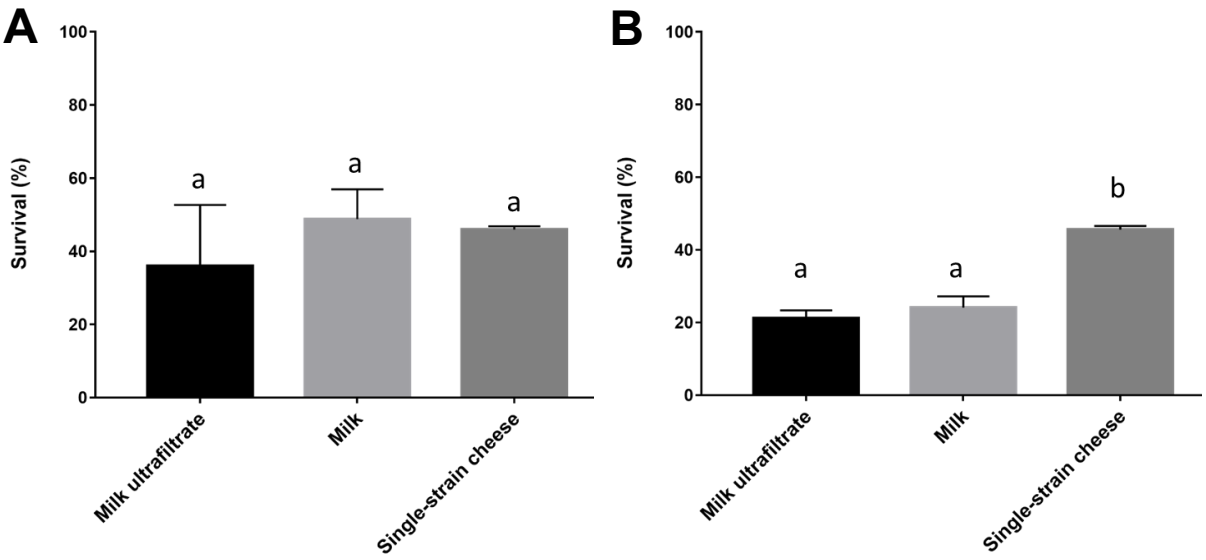

Figure S1. ***P. freudenreichii*** **CIRM-BIA 118 viability during** ***in vitro*** **static digestion**. Three fermented media were subjected to *in vitro* static digestion, viable propionibacteria were enumerated by plate counting at different digestion stage. **A)** Percentage of survival after the succession of oral and gastric phases; **B)** percentage of survival after complete digestion (oral, gastric and intestinal phases). The results are expressed as percentage of survival, and as mean ± standard deviation for triplicate experiments. Different letters above the columns indicate significant differences ($p < 0.05$).

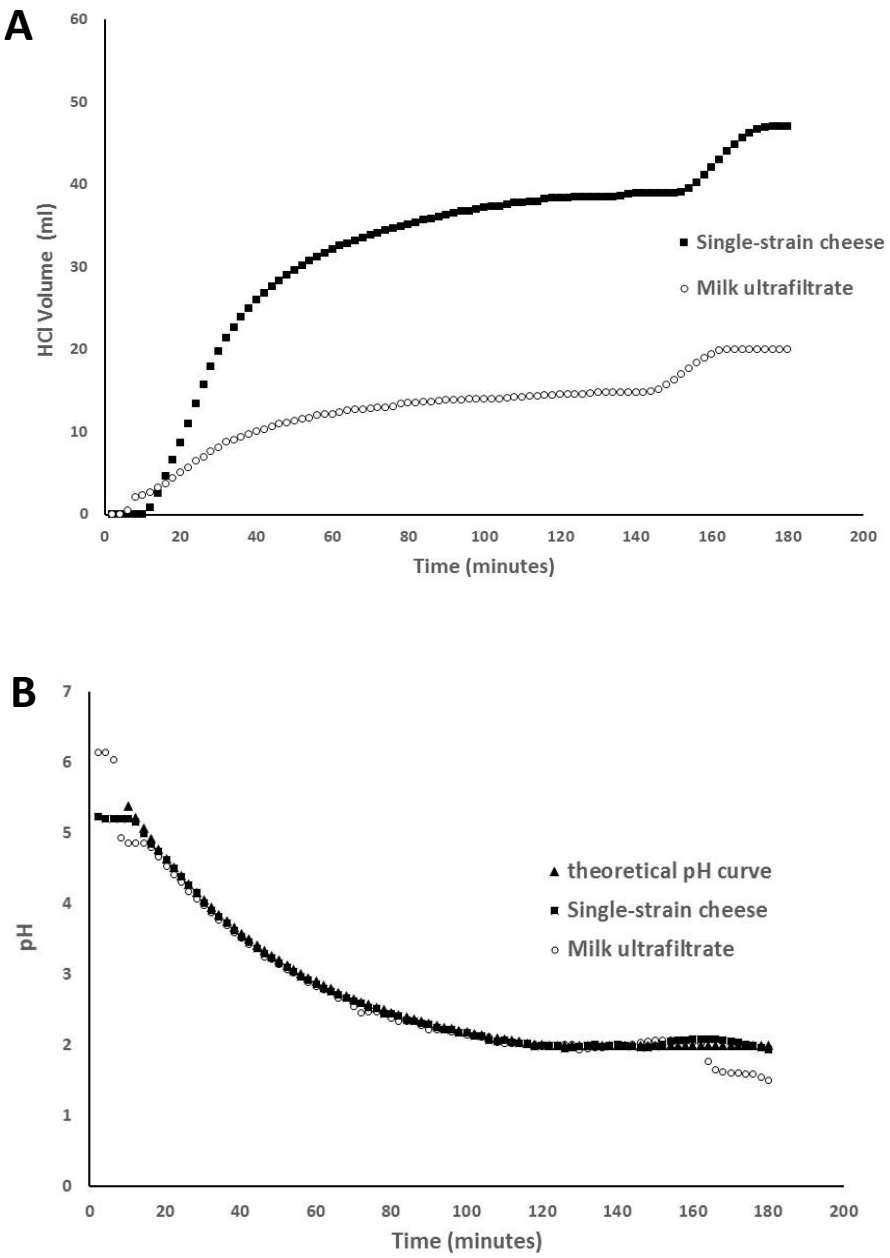

Figure S2. pH monitoring during gastric phase in DIDGI® experiments. **A)** The HCl volume accumulated in the gastric compartment in the aim to reach pH=2. **B)** Acidification curve in gastric compartment for the two matrices tested, milk ultrafiltrate and single-strain cheese, as well the theoretical pH curve programmed.

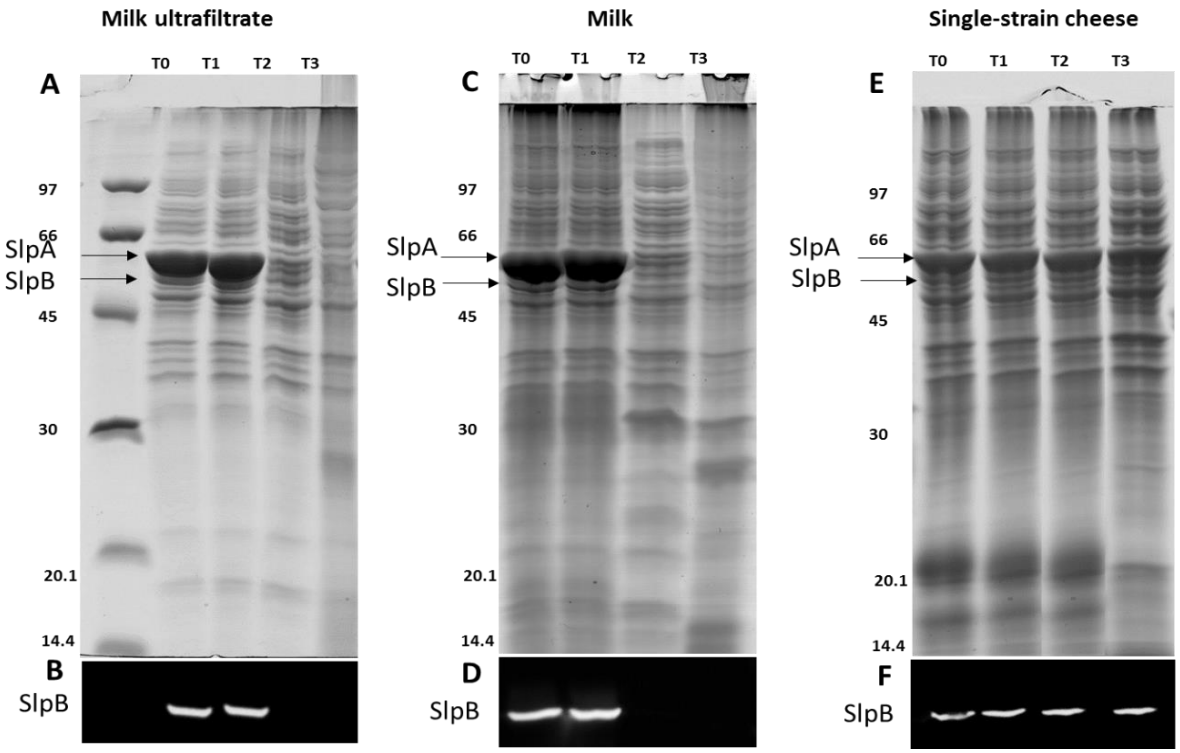

Figure S3. *P. freudenreichii* CIRM-BIA 118 SlpB proteolysis during *in vitro* static digestion. Three different matrices fermented by *P. freudenreichii* CIRM-BIA 118 were tested, **A-B)** Milk ultrafiltrate, **C-D)** milk; and **E-F)** single-strain cheese. Samples were collected at different times: **(T0)** before digestion, **(T1)** at the end of oral phase, **(T2)** after the succession of oral and gastric phases, **(T3)** and after the complete digestion (oral, gastric and intestinal phases). Total proteins extracts from bacterial pellets were prepared; then analyzed by 12% SDS PAGE electrophoresis (A, C and E) and by SlpB western blotting (B, D and F).

3.3. Conclusion

Dans ce chapitre 3, nous avons mis en évidence que l'effet anti-inflammatoire de *P. freudenreichii* CIRM-BIA 129, ainsi que des protéines Slps, sur les cellules épithéliales intestinales HT-29. En effet, *P. freudenreichii*, ainsi que les protéines Slps diminuent l'expression de cytokines pro-inflammatoires (IL-8 et TNFα) induites par la présence du LPS. De plus, *P. freudenreichii* CIRM-BIA 129 induit une sécrétion d'IL-10 sur les cellules HT-29 qu'elles aient été stimulées ou pas par le LPS, et seules les protéines de surface induisent l'expression du facteur régulateur TGFβ. Cependant, les peptides issus de la protéolyse des Slps n'ont pas eu un effet anti-inflammatoire similaire aux protéines Slps intactes. En effet, les peptides des Slps ne diminuent pas l'expression des cytokines pro-inflammatoires (IL-8 et TNFα) induites par la présence de LPS. En tenant en compte des résultats obtenus à l'issue de cette expérimentation, il est difficile de conclure sur la bioactivité des peptides issus de la protéolyse des Slps. En effet, il est probable que certains peptides pourraient être bioactifs, mais ils sont minoritaires dans la fraction testée sur les cellules épithéliales intestinales. Ces résultats montrent l'importance de protéger les protéines Slps de la protéolyse digestive, afin que *P. freudenreichii* puisse induire une réponse anti-inflammatoire via les protéines Slps au niveau des cellules épithéliales intestinales.

Les modèles de digestion *in vitro* statique et dynamique ont montré l'effet protecteur de la matrice fromagère vis-à-vis de la protéolyse digestive de la protéine SlpB de *P. freudenreichii* CIRM-BIA 129, contrairement à des matrices de vectorisation laitières, comme le lait ou l'ultrafiltrat de lait. De plus, les résultats confirment que la tolérance de *P. freudenreichii* aux stress digestifs est souche- et matrice-dépendante. En effet, la matrice fromagère améliore la survie de *P. freudenreichii* CIRM-BIA 129 aux stress digestifs. L'amélioration de la survie de *P. freudenreichii* par la matrice fromagère est liée probablement aux différents facteurs tels que :

- La protection physique des bactéries et de leurs protéines de surface dans les particules digérées du fromage
- La présence des protéines qui jouent un rôle tampon contre le stress acide
- La présence de lipides qui peuvent limiter les dommages induits par les sels biliaires

- L'accumulation d'osmoprotecteurs (tréhalose, glycogène, glutamate ou glycine bétaïne)

Les résultats *in vitro* suggèrent également que la vectorisation de *P. freudenreichii* CIRM-BIA 129 par la matrice fromagère pourrait moduler différemment les propriétés immunomodulatrices de ***Pf*** CIRM-BIA 129. En effet, la protéolyse complète des protéines Slps se déroulait dans le compartiment gastrique lors de la vectorisation par l'ultrafiltrat de lait. Suite à une vectorisation par la matrice fromagère, la protéolyse des Slps aurait lieu à la fin de la phase intestinale. En effet, cela suggère que le système immunitaire ne percevra pas la surface de *P. freudenreichii* de manière similaire et cela pourrait induire une réponse immunitaire différente selon la présence ou l'absence des protéines Slps. De plus, l'effet probiotique de *P. freudenreichii in situ* dépendra également de la production de nombreux métabolites bénéfiques. Dans ce cas, il pourrait y avoir une plus grande production de métabolites bénéfiques par *P. freudenreichii* suite à une vectorisation par la matrice fromagère, par comparaison à une vectorisation par une matrice laitière liquide.

Chapitre 3 : à retenir

- ***P. freudenreichii* CIRM-BIA 129, ainsi que ses protéines Slps, induisent un effet anti-inflammatoire *in vitro* sur les cellules épithéliales intestinales HT-29**
- **La protéolyse des protéines Slps abolit leur effet anti-inflammatoire *in vitro* sur les cellules épithéliales intestinales HT-29**
- **La matrice fromagère améliore la tolérance de *Pf* CIRM-BIA 129 aux stress digestifs lors de la digestion *in vitro***
- **la matrice fromagère limite la protéolyse digestive des protéines Slps lors de la digestion *in vitro***

Chapitre 4 : « L'étude *in vivo* de la vectorisation de *P. freudenreichii* et de ses protéines de surface par la matrice fromagère »

4.1. Introduction

Nous avons démontré précédemment que la protéolyse *in vitro* des protéines Slps abolissait leur propriétés anti-inflammatoires *in vitro* sur des cellules épithéliales du côlon (HT-29), d'où la nécessité de les protéger de la protéolyse digestive. L'étude *in vitro* a révélé l'effet protecteur de la matrice fromagère pour améliorer la tolérance de *P. freudenreichii* CIRM-BIA 129 aux stress digestifs, par comparaison à une culture sur du lait ou sur l'ultrafiltrat de lait. De plus, seule la matrice fromagère a limité la protéolyse des protéines de surface lors de la digestion *in vitro*. Suite à ces résultats *in vitro*, nous avons émis l'hypothèse que l'effet immunomodulateur de *P. freudenreichii* CIRM-BIA 129 serait matrice-dépendant.

Nous avons souhaité, par conséquent, évaluer l'impact de la matrice fromagère sur les propriétés immunomodulatrices de *P. freudenreichii* CIRM-BIA 129 chez des porcelets sains (Figure 8). Trois groupes de porcelets (âgés de 6 semaines) ont été gavés pendant 2 semaines avec *P. freudenreichii* CIRM-BIA 129, sous forme de culture sur l'ultrafiltrat de lait (PF-culture) ou sous forme de fromage (PF-fromage) fermentés par la souche CIRM-BIA 129, et comparés à un groupe contrôle gavé avec une matrice fromagère stérile. La dose journalière prise en bactéries par les porcelets a été fixée à 10^{11} UFC /jour.

L'activité métabolique de *P. freudenreichii* CIRM-BIA 129 *in vivo* a été évaluée en mesurant la concentration fécale en acides gras à chaîne courte (AGCC) et en déterminant sa capacité bifidogène. L'effet bifidogène de ***Pf*** est en effet bien connu et dépend de la synthèse du DHNA par ***Pf*** (Isawa et al., 2002; Seki et al., 2004). Pour déterminer l'impact de la matrice de vectorisation sur l'immunomodulation par *P. freudenreichii* CIRM-BIA 129, nous avons analysé la sécrétion basale de cytokines et les phénotypes des lymphocytes T dans les cellules immunitaires des ganglions lymphatiques mésentériques intestinaux (MLNC) et du sang périphérique (PBMC) des porcelets. Ensuite, pour rechercher un rôle protecteur de la consommation de *P. freudenreichii* contre l'inflammation, nous avons évalué les réponses des MLNC et des PBMC, *ex vivo*, à la stimulation exogène par un lipopolysaccharide (LPS) ou par la concanavaline A (une lectine, ConA).

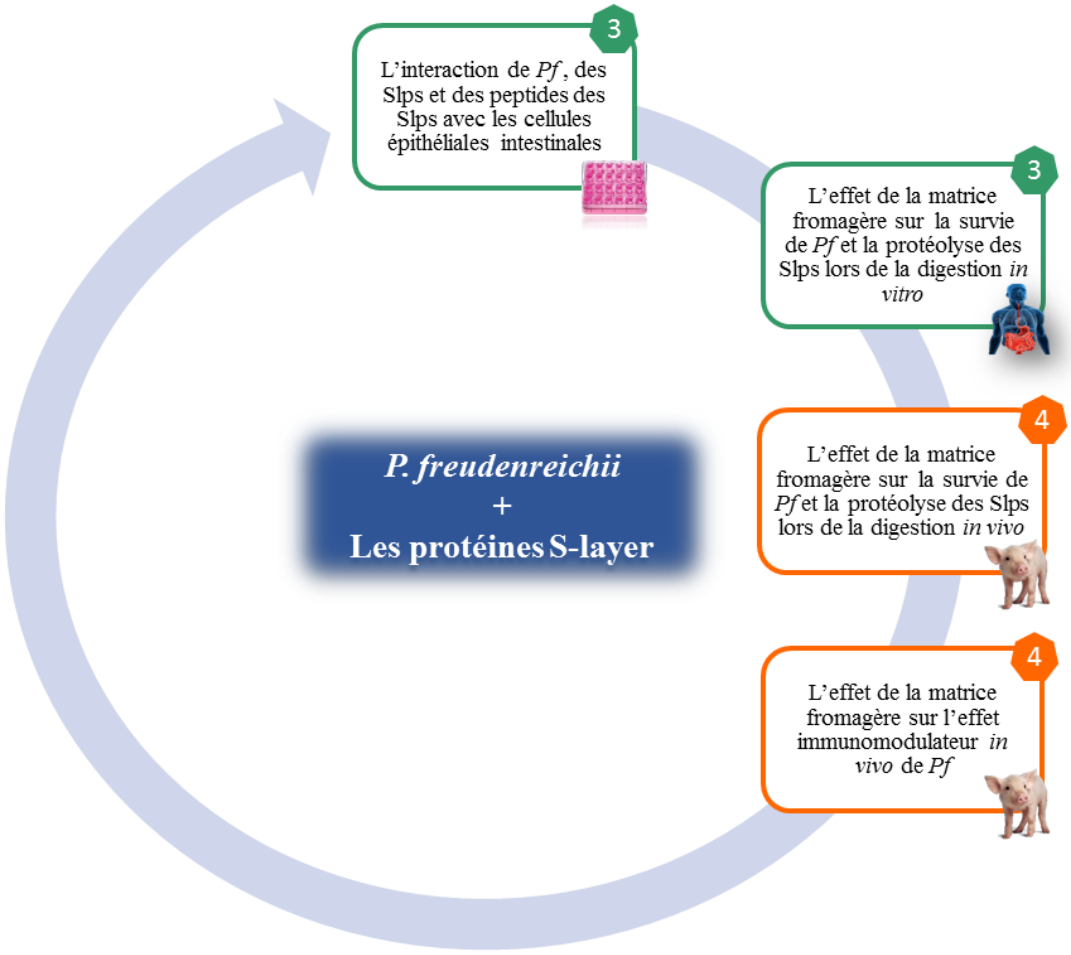

Figure 8. **Est-ce que la matrice fromagère module les propriétés immunomodulatrices *in vivo* de *P. freudenreichii* CIRM-BIA 129?** Le chapitre 4 (en orange) présente les expérimentations effectuées pour comprendre l'impact de la matrice fromagère sur l'effet immunomodulateur *in vivo* de *P. freudenreichii* (***Pf***). Ainsi, les questions abordées dans ce chapitre 4 sont :

1. La matrice fromagère améliore-elle la survie de ***Pf*** *in vivo* ?
2. La matrice fromagère protège-elle les protéines Slps de la protéolyse digestive *in vivo* ?
3. L'effet immunomodulateur de ***Pf*** est-il matrice-dépendant ?

Les résultats de cette étude ont fait l'objet de plusieurs communications scientifiques :

- **3 posters ont été présentés :**

i) Au 10^{éme} symposium « Cheese », Rennes-France, Avril 2018;

ii) Aux 4èmes rencontres du GIS Nutrition Alimentation Métabolisme et Santé (NAMS), Rennes-France, Octobre 2018.

iii) Au congrès mondial de la fédération internationale du lait (IDF world dairy summit), les 16-21 octobre 2018 à Daejeon, Corée du Sud;

- **Ce travail a été publié dans le journal Frontiers in Microbiology, comme suit:**

  Rabah H, Ferret-Bernard S, Huang S, Le Normand L, Cousin FJ, Gaucher F, Jeantet R, Boudry G and Jan G. (2018). **The Cheese Matrix Modulates the Immunomodulatory Properties of *Propionibacterium freudenreichii* CIRM-BIA 129 in Healthy Piglets**. Front. Microbiol. 9:2584. doi: 10.3389/fmicb.2018.02584.

4.2. Article 2 : la matrice fromagère module les propriétés immunomodulatrices de *P. freudenreichii* CIRM-BIA 129 chez des porcelets sains

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The Cheese Matrix Modulates the Immunomodulatory Properties of *Propionibacterium freudenreichii* CIRM-BIA 129 in Healthy Piglets

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Propionibacterium freudenreichii is a beneficial bacterium, used as a cheese starter, which presents versatile probiotic properties. These properties are strain-dependent. We hypothesized they may also be delivery vehicle-dependent. In this study, we thus explored in healthy piglets how the cheese matrix affects the immunomodulatory properties of *P. freudenreichii*. During 2 weeks, three groups of weaned piglets consumed, respectively, *P. freudenreichii* as a liquid culture (PF-culture), *P. freudenreichii* under the form of a cheese (PF-cheese), or a control sterile cheese matrix (Cheese-matrix). The *in vivo* metabolic activity of *P. freudenreichii* was assessed by determining short chain fatty acids (SCFA) concentration and bifidobacteria population in feces. Whatever the delivery vehicle, *P. freudenreichii* was metabolically active in piglets' colon and enhanced both bifidobacteria and SCFA in feces. *P. freudenreichii* consumption decreased the secretion of TNFα and of IL-10 by peripheral blood mononuclear cells (PBMC). It did not alter IL-10, IFNγ, IL-17, and TNFα secretion in mesenteric lymph node immune cells (MLNC). PF-cheese enhanced significantly Treg phenotype, while PF-culture decreased significantly Th17 phenotype in PBMC and MLNC. Remarkably, only PF-cheese induced an increase of Th2 phenotype in PBMC and MLNC. *Ex vivo* stimulation of PBMC and MLNC by Lipopolysaccharides and Concanavalin A emphasized the difference in the immunomodulatory responses between PF-culture and PF-cheese group, as well as between PBMC and MLNC. This study shows the importance to consider the delivery vehicle for probiotic administration. It confirms the anti-inflammatory potential of *P. freudenreichii*. It opens new perspectives for the use propionibacteria-fermented products as preventive agents for inflammatory bowel diseases and intestinal infectious diseases.

Keywords: probiotics, *P. freudenreichii*, delivery vehicle, cheese matrix, immunomodulation, T lymphocytes phenotype, PBMC, MLNC

INTRODUCTION

Propionibacterium freudenreichii is a beneficial bacterium, belonging to the Actinomycetales order. It has been recognized as safe (GRAS status) in the United States of America, and qualified presumption of safety (QPS status) in Europe. *P. freudenreichii* is a cheese starter used in Swiss-type cheeses manufacture such as Emmental. It moreover revealed versatile, strain-dependent, probiotic functionalities (Thierry et al., 2011; Rabah et al., 2017). These properties are strain-dependent and result from the production of several beneficial metabolites by propionibacteria (Cousin et al., 2011; Rabah et al., 2017). Short chain fatty acids (SCFA), especially propionic and acetic acids derived from lactate fermentation by propionibacteria, have anti-inflammatory and anti-cancerous effects on colonic intestinal cells (Jan et al., 2002; Lan et al., 2008; Cousin et al., 2012b, 2016). *P. freudenreichii* produces also bifidogenic factors, including 1,4-dihydroxy-2-naphtoic acid (DHNA),which enhances bifidobacteria growth and reduces inflammation in intestinal epithelial cells (Isawa et al., 2002; Okada, 2006; Suzuki et al., 2006; Rabah et al., 2017). Finally, the surface proteome of *P. freudenreichii* is involved in host-bacteria interaction, with a prominent role of non-covalently surface-bound proteins such as S-layer proteins (Slps) (Le Maréchal et al., 2015; Colliou et al., 2017; Deutsch et al., 2017; Do Carmo et al., 2018). The development of dairy fermented products as functional foods by screening specific starter bacteria which possess both probiotic and food fermentation abilities is a promising perspective. It will provide an alternative tool to prevent several inflammatory diseases, as inflammatory bowel diseases (IBD). A functional food is defined as an "ingredient that affects beneficially one or more target functions in the body, beyond adequate nutritional effects, in a way that is relevant to either an improved state of health and well-being and/or reduction of risk disease" (Saris et al., 1998; Diplock et al., 1999). Understanding how *P. freudenreichii* impacts health, specifically intestinal health, is crucial to develop functional dairy foods. Indeed, effects of dairy products, fermented solely by propionibacteria, or in combination with lactic acid bacteria, already revealed beneficial effects in a mice model of colitis (Plé et al., 2015, 2016; Foligné et al., 2016). The ingestion of these fermented dairy foods reduced the severity of chemically induced colitis. Recently, *P. freudenreichii* was shown to be part of the human milk microbiota and to participate in the prevention of necrotizing enterocolitis (NEC) in preterm infants (Colliou et al., 2017). These detailed studies highlighted the potential of *P. freudenreichii* consumption to prevent intestinal inflammatory diseases. However, investigations on the impact of the delivery vehicle on the probiotic functionalities of *P. freudenreichii* in healthy subjects are lacking. Here, we investigated the influence of the cheese matrix on *in vivo* probiotic functionalities of *P. freudenreichii*. Indeed, dairy matrices were shown to enhance propionibacteria tolerance toward digestive stresses, via overexpression of a panel of proteins involved in acid and bile salts stress responses (Leverrier et al., 2005; Saxelin et al., 2010; Gagnaire et al., 2015). In addition, the high concentration of dairy proteins in cheese plays a role as buffering agent toward gastric acids, in addition to the presence of lipids, which limits the toxic effect of bile salts on bacterial membranes (Rabah et al., 2017, 2018). Such tolerance may favor propionibacteria survival, enhance their metabolic activity, and consequently their immunomodulatory effects, within the gut. Furthermore, adhesion and immunomodulation, both mediated by S-layer proteins, may be promoted, since the cheese matrix protects these proteins from digestive proteolysis *in vitro* (Do Carmo et al., 2017; Rabah et al., 2018). In this study, we compared the effect of two delivery vehicles, a single-strain cheese (PF-cheese) and fresh culture in milk ultrafiltrate (PF-culture), in healthy piglets. Both delivery vehicles were fermented by the strain *P. freudenreichii* CIRM-BIA 129, which has been selected previously as the most anti-inflammatory one (Foligné et al., 2010, Foligné et al., 2013). The impact of the delivery vehicle on *in vivo* metabolic activity and on immunomodulation by *P. freudenreichii* was assessed in healthy piglets. Then, to seek a functional role of *P. freudenreichii* consumption by healthy animals against inflammation, we assessed piglets' immune cell responses to exogenous proinflammatory stimulations.

MATERIALS AND METHODS

Bacterial Strain and Dairy Matrices Preparation

The strain *P. freudenreichii* CIRM-BIA 129 (equivalent to ITGP20 strain) was provided by the French Dairy Interbranch Organization (Centre National Interprofessionnel de l'Economie Laitière, CNIEL) and maintained by the International Centre for Microbial Resources (Centre International de Ressources Microbiennes-Bactéries d'Intérêt Alimentaire, CIRM-BIA). Dairy propionibacteria were routinely cultivated at 30°C in yeast-extract-lactate medium (YEL). For the PF- culture, *P. freudenreichii* CIRM-BIA 129 was grown in milk ultrafiltrate supplemented with 100 mM sodium DL-lactate (50% in H_2O, Sigma) and 5 g/L casein hydrolysate (Organotechnie, La Courneuve, France) (Cousin et al., 2012c) at 30°C, without agitation, in microaerophilic conditions until stationary phase (60 h of incubation). PF-cheese is a single-strain cheese fermented by *P. freudenreichii* CIRM-BIA 129 as described previously (Plé et al., 2015). The biochemical composition of the cheese was: dry matter 58 g/100 g, lipids 28 g/100 g, proteins 29 g/100 g, carbohydrates 0 g/100 g, and calcium 840 mg/100 g (Plé et al., 2015). The cheese matrix is a sterile dairy matrix prepared in the same way as the single strain cheese. Glucono delta-lactone was used to acidify the sterile supplemented milk before cheese matrix manufacturing procedure, as described previously (Plé et al., 2015). The propionibacteria amounts reached 5.10^9 CFU/ml in PF-culture, and 1.10^{10} CFU/g in PF-cheese.

Ethics Statement

The experimental protocol was performed in accordance with recommendations of the French law (2001-464 29/05/01)

and EEC (86/609/CEE) for the care and use of laboratory animals. The protocol was approved by the ethical committee on animal experimentation of Rennes (France), under the certificate of authorization to experiment 2017010922379066-V2. Pigs were sacrificed by electronarcosis followed by exsanguination, and every effort was made to minimize animal suffering.

Animal Procedures and Immune Cell Isolation

Twenty one [(Pietrain × Landrace) × (Large White)] 8-week old piglets (13.3 ± 0.4 kg) from the experimental herd of INRA St-Gilles (UEPR, France) were used. Three groups of seven piglets were constituted: (1) Cheese matrix (10 g), (2) PF-culture (1.10^{11} CFU of *P. freudenreichii*) and (3) PF-cheese (1.10^{11} CFU of *P. freudenreichii*). PF-cheese and cheese matrix were mixed by a turrax (Ultra-turrax T8 IKA, Fischer Scientific, 20,000 tr/min-2 min) in four volumes of sterile physiological water. Piglets were gavaged using syringes every morning (between 9.00 and 10.00 am) during 14 days. They were fed with a standard pig diet that was given at 10.00 am. Food was removed from the cage at 4:00 pm to monitor daily food intake. Animals were fasted from 4:00 pm to 9:00 am but had free access to water. Piglets were weighed five times (d0, d1, d4, d7, d10, and d14) and fecal samples were collected at day 0, day 7, and day 14. At the end of the 14-day treatment period, pigs were sacrificed 30 min after their last gavage by electronarcosis then exsanguination. Blood was collected in sterile BD vacutainer®CPT™ tubes (containing sodium heparin as well as Ficoll™Hypaque™ density fluid and a polyester gel barrier) at room temperature. Following centrifugation at 1500 *g* for 20 min without brake, we isolated the peripheral blood mononuclear cells (PBMC) by carefully pipetting the interface above the gel barrier. Additional washing in Hank's balanced saline solution (HBSS), supplemented with 200 UI/ml penicillin and 200 µg/ml streptomycin, and centrifugation steps resulted in a suspension of concentrated mononuclear cells. After laparotomy, 4 g of intestinal mesenteric lymph nodes (MLN) were placed in ice-cold HBSS for mononuclear cell isolation, as already described (Ferret-Bernard et al., 2010).

Quantification of Fecal Propionibacteria, Lactobacilli and Bifidobacteria Populations

Propionibacteria, Lactobacilli, and Bifidobacteria were quantified in feces collected at day 0 (before treatment) and day 14 (end of the treatment). Samples were analyzed in duplicate. For propionibacteria quantification, QIAamp DNA Stool Kit was used to extract DNA, as described previously (Hervé et al., 2007; Cousin et al., 2012a). For lactobacilli and bifidobacteria quantification, DNeasy Blood & Tissue Kits (Qiagen) was used to extract DNA from pure cultures of *Bifidobacterium longum* CIRM-BIA 1336 or *Lactobacillus pentosus* CIRM-BIA 660. Propionibacteria concentrations were measured by qPCR of 5S subunit gene of transcarboxylase as described previously (**Supplementary Table S1**) (Hervé et al., 2007; Cousin et al., 2012a). Briefly, ten-fold dilutions of the *P. freudenreichii* CIRM-BIA 129 were prepared in saline solution and enumerated using YELA medium. One hundred microliters of each dilution was then added to 200 mg of feces from naive pig (exempt of propionibacteria) and thoroughly mixed. DNA was then extracted in the same way as unknown samples. A standard curve was generated and results are expressed as log [bacteria] per gram of sample. For lactobacilli and bifidobacteria quantification, 10-fold serial dilutions of target genomic DNA extracted from pure cultures of *B. longum* CIRM-BIA 1336 or *L. pentosus* CIRM-BIA 660 were performed (**Supplementary Table S1**). The linear equation for the standard curve was then used to interpolate the numbers of copies present in the unknown samples.

Short Chain Fatty Acids Analysis

Short chain fatty acid concentration was determined in fecal samples at days 7 and 14. Immediately after collection, fecal samples were diluted in ortho-phosphoric acid (50% V/V) to stop fermentation and samples were stored at −20°C until analysis. SCFA were separated on a BP20 (SGE) column and quantified by a flame ionization detector as previously described (Jouany et al., 1981). Isocaproic acid was used as an internal standard. Samples were analyzed in duplicate, and the results are expressed as micromolar per g of feces.

PBMC and MLNC Stimulation

Immune cells were suspended in complete RPMI 1640 medium (Sigma) supplemented with 10% fetal calf serum (FCS), 100 IU/ml penicillin and 100 mg/ml streptomycin to achieve cell concentration of 5×10^6 cells/ml for PBMC and 10×10^6 cells/ml for MLNC in 96-well flat-bottomed plates. Cells were stimulated for 72 h at 37°C, under an atmosphere containing 5% CO_2, in unstimulated condition (complete RPMI alone) or in presence of 200 µg/ml of *P. freudenreichii*' S-layer proteins (Slps). Slps were extracted (Le Maréchal et al., 2015), partially purified by size exclusion chromatography as previously described (De sa Peixoto et al., 2015) and proteins were concentrated by filtration using VivaSpin-10 kDa. PBMC and MLNC were also cultivated in presence of 10 µg/ml of Lipopolysaccharides (LPS) from *Escherichia coli* 0111:B4, or in presence of 0.5 µg/ml of Concanavalin A (ConA, sub-optimal concentration), or a combination of Slps + LPS and Slps + ConA. Culture supernatants of PBMC and MLNC were harvested and stored at −20°C until assayed for cytokine detection. Remaining cells were re-suspended in FCS 10% DMSO (Hybri-max, Sigma) and stored at −150°C until mRNA extraction.

Cytokine Patterns of PBMC and MLNC

Concentrations of IL-10, IFNγ, and TNFα were measured in culture supernatants of PBMC and MLNC, using capture sandwich ELISA porcine ELISA kit (R&D Systems, Lille, France) according to the manufacturer's instructions. IL-17 concentration was measured also using capture sandwich swine ELISA kit (VetSet™, Kingfisher Biotech, United States). Cytokine concentrations after stimulation were given in pictograms per ml of supernatant.

RT-qPCR

Quantitative PCR was performed to determine *Tbet*, *GATA3*, *FOXP3*, and *RORγt* mRNA levels in PBMC and MLNC. Primers used for mRNA quantification are listed in **Supplementary Table S1**. Total RNA from cells was isolated by Trizol reagent (Invitrogen Ambion), and cDNA was synthesized using a qScript cDNA synthesis kit (Quanta Biosciences). Amplification was performed as previously described (Rabah et al., 2018). The transcripts level of the target genes was normalized to the transcript level of *hprt* gene (Ledger et al., 2004) (housekeeping gene, see **Supplementary Table S1**). These primers were described previously (Muráni et al., 2007; Delroisse et al., 2008; Hernández et al., 2009; Young et al., 2012; Hermann-Bank et al., 2013; Zhu et al., 2014). *Hprt* expression was not affected by the tested matrices. The results are expressed as expression level ($2^{-\Delta CT}$), in duplicate analysis for each piglets (n = 7) for the three groups.

Statistical Analysis

We analyzed all data with non-parametric tests after checking the non-Gaussian distribution of data. The effect the consumption of *P. freudenreichii* on the fecal propionibacteria amounts was analyzed with the Mann–Whitney test by comparing only PF-culture and PF-cheese groups. The difference in lactobacilli and bifidobacteria was analyzed separately by comparing the concentration at day 0 to the concentrations determined at day 14 using non-parametric ANOVA with Dunn's multiple comparison test as a *post hoc* test. The same test was also performed to compare the three groups in term of SCFA concentration, basal cytokine concentrations and gene expression. To analyze cytokine secretion after ConA and LPS stimulation, 2-way non-parametric ANOVA was performed with the sidak' s multiple comparison test as a *post hoc* test. Statistical significance was set at $p < 0.05$. Calculations were performed using GraphPad Prism Software (Prism 7 for Windows). All data were expressed as mean values and standard error of the mean (SEM) ($n = 7$).

RESULTS

Piglet Growth and Food Intake

Food intake was similar among the three experimental groups (**Supplementary Figure S1A**). Piglets had a similar growth without significant difference between experimental groups (**Supplementary Figure S1B**). Feed efficiency was also similar among experimental groups (**Supplementary Figure S1C**). No health problems was encountered during the 14-day experimental period.

Quantification of Fecal Population of Propionibacteria, Bifidobacteria, and Lactobacilli

Fecal propionibacteria population, as determined by qPCR, was undetectable before treatment (day 0, data not shown). Propionibacteria remained also undetectable in the cheese matrix piglets group's feces (**Figure 1A**). After 2 weeks of gavage, propionibacteria reached 7.0 ± 0.1 log/g of feces in the PF-culture piglets and 6.9 ± 0.1 log/g of feces in the PF-cheese piglets, with no significant difference between the two groups ($p > 0.05$). The impact of *P. freudenreichii* consumption on piglet's microbiota was investigated, focusing on two genera, using qPCR. Lactobacilli and bifidobacteria populations between the different treated groups at day 14 were compared to the population level at day 0. *P. freudenreichii* ingestion significantly enhanced bifidobacteria in feces (**Figure 1B**). By contrast, none of the treatments induced significant change in the population of lactobacilli, regardless of the delivery vehicle (**Figure 1B**).

SCFA Quantification in Piglet Feces

To assess the *in vivo* metabolic activity of *P. freudenreichii*, SCFA concentration were determined in feces at days 7 and 14. At day 7, SCFA concentrations in feces were equivalent among the different groups (**Supplementary Figure S2**). However, at day 14, *P. freudenreichii* consumption enhanced significantly total SCFA concentration in feces, regardless

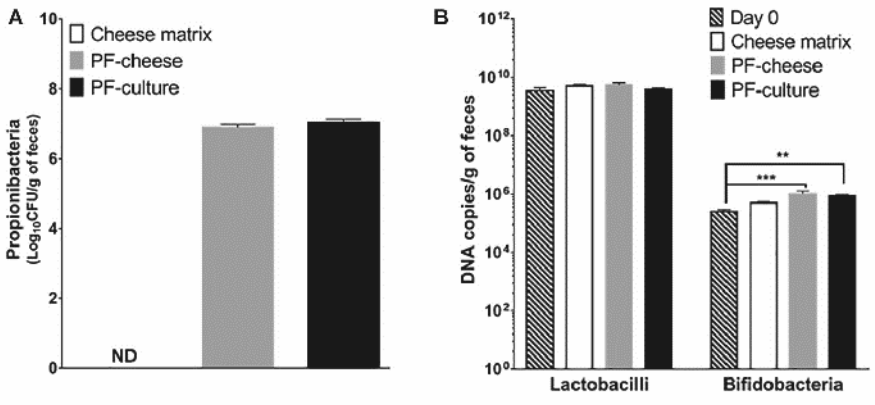


FIGURE 1 | Propionibacteria total population **(A)**, lactobacilli and bifidobacteria **(B)** total population in feces were assessed at day 14 in the different piglets groups: cheese matrix, PF-culture or PF-cheese fermented both by *P. freudenreichii* CIRM-BIA 129 (PF). Propionibacteria, lactobacilli, and bifidobacteria concentrations were determined by qPCR in feces. Results are represented as means ± SEM (n = 7). $^{**}P < 0.005$ and $^{***}P < 0.0005$.

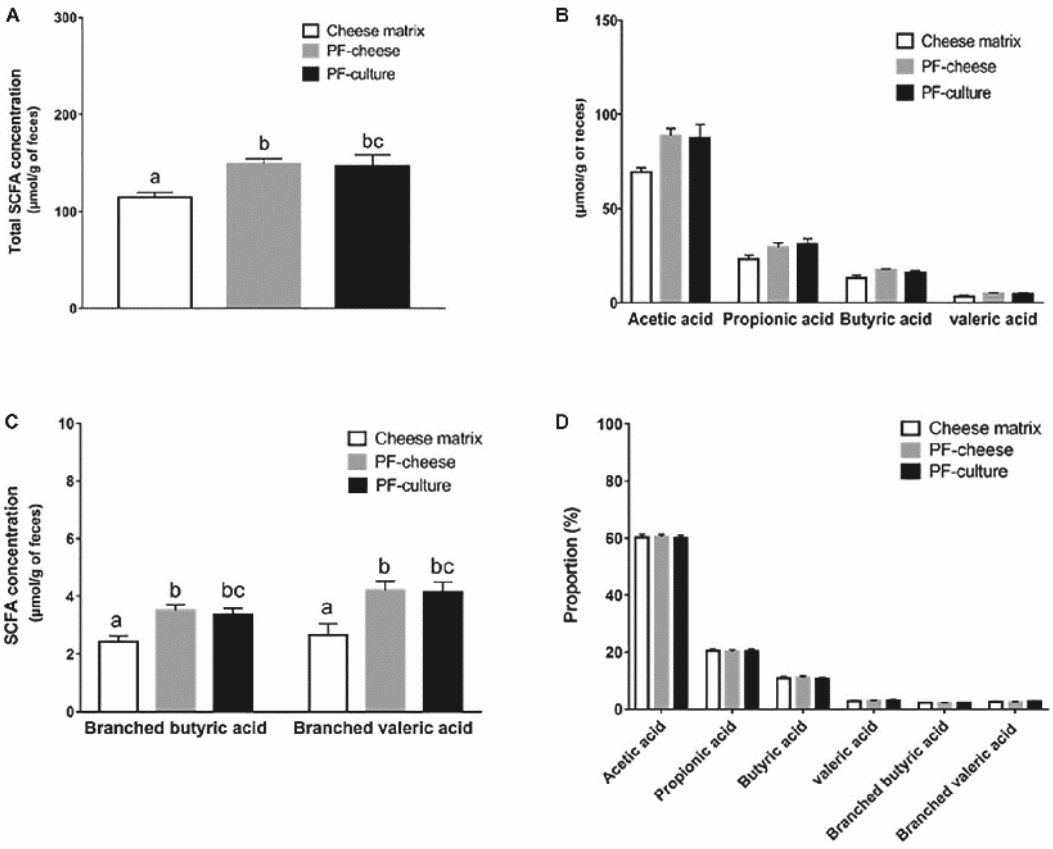


FIGURE 2 | Analysis of short chain fatty acids (SCFA) concentration in feces contents at day 14 of the three piglets groups fed with: cheese matrix, PF-culture or PF-cheese fermented by *P. freudenreichii* CIRM-BIA 129 (PF). The total SCFA concentration **(A)**, the concentration of each SCFA type (C2, C3, C4, and C5) **(B)**, and the concentration of branched short chain fatty acids **(C)** were measured by gas-phase chromatography, thus the proportion of each type of SCFA **(D)** was determined. Results are represented as means ± SEM (n = 7). Letters denotes significant difference, $P < 0.05$.

of the delivery matrix (PF-culture or PF-cheese, p = 0.98) (**Figure 2A**). *P. freudenreichii* consumption tended to increase acetic acid concentration, compared to the cheese matrix group (p = 0.062 for PF-culture group and p = 0.069 for PF-cheese group) (**Figure 2B**). By contrast, propionic, butyric or valeric acid concentrations were not significantly enhanced (**Figure 2B**). Regarding the concentrations of branched chain fatty acids (BCFA), PF-culture piglets and PF-cheese piglets displayed increased fecal branched valeric and butyric acids concentration, compared to cheese matrix group (**Figure 2C**). In addition, compared to cheese matrix group, no significant difference in BCFA concentrations between the PF-culture and the PF-cheese groups were observed (**Figure 2C**). The proportions of the different SCFA were not modified by the experimental treatments (**Figure 2D**).

Anti-inflammatory Properties of *P. freudenreichii* Slps Proteins on Naive Swine PBMC and MLNC

To verify the effect of *P. freudenreichii* Slps on naive swine immune cells as already demonstrated in other species (Le Maréchal et al., 2015), PBMC and MLNC from cheese matrix piglets were stimulated by Slps in the presence of LPS or ConA. Slps significantly increased IL-10 secretion by both PBMC and MLNC (**Figures 3A,B**). In the presence of ConA, Slps did not influence secretion of IL-10 by both cells types (**Figures 3A,B**). In the presence of LPS, Slps tended to increase IL-10 secretion by PBMC (p = 0.0763) (**Figure 3A**). Slps significantly reduced LPS or ConA-induced TNFα secretion by PBMC and MLNC (**Figures 3C,D**). Only ConA triggered a high secretion of IFNγ by PBMC, which was significantly reduced by the co-stimulation with Slps (**Figures 3E,F**).

Basal Cytokines Secretion by PBMC and MLNC

Propionibacterium freudenreichii consumption decreased basal secretion of IL-10 by PBMC, compared to cheese matrix group, whatever the delivery vehicle (**Figure 4A**). Basal TNFα secretion by PBMCs was significantly lower in PF-culture and PF-cheese groups, compared to cheese matrix group (**Figure 4C**). Finally, basal secretion of IFNγ and IL-17 by PBMC was similar in all piglet groups (**Figures 4B,D**). By contrast, in MLNC, IL-10, TNFα, IL-17, and IFNγ secretion were similar among the different experimental groups (**Supplementary Figures S3A–D**).

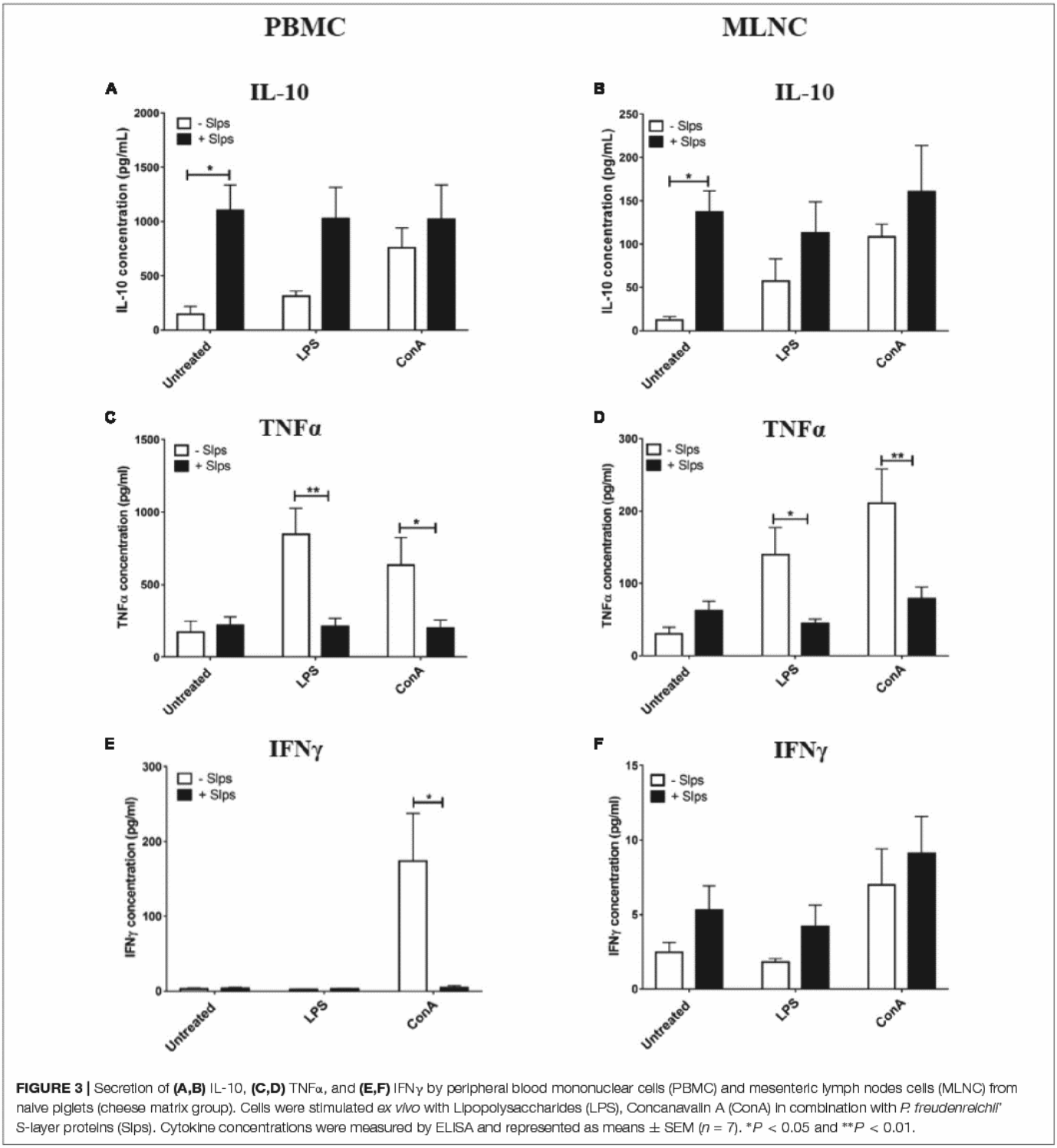


FIGURE 3 | Secretion of **(A,B)** IL-10, **(C,D)** TNFα, and **(E,F)** IFNγ by peripheral blood mononuclear cells (PBMC) and mesenteric lymph nodes cells (MLNC) from naive piglets (cheese matrix group). Cells were stimulated *ex vivo* with Lipopolysaccharides (LPS), Concanavalin A (ConA) in combination with *P. freudenreichii*' S-layer proteins (SIps). Cytokine concentrations were measured by ELISA and represented as means ± SEM ($n = 7$). $^{*}P < 0.05$ and $^{**}P < 0.01$.

T Lymphocytes Phenotype in PBMC and MLNC

Phenotype of T lymphocytes population in PBMC and MLNC was assessed by analyzing the expression of transcriptions factors: Tbet, GATA3, Foxp3, and RORγt, respectively, specific of Th1, Th2, Treg, and Th17 lymphocytes populations. Tbet expression in PBMC was similar among the different piglets groups (**Figure 5A**). PF-cheese, but not PF-culture consumption, increased significantly GATA3 expression in PBMC compared to the cheese matrix group (**Figure 5B**). The Th1/Th2 ratio was significantly lower in PF-cheese piglets, compared to cheese matrix ones, without significant difference with PF-culture piglets

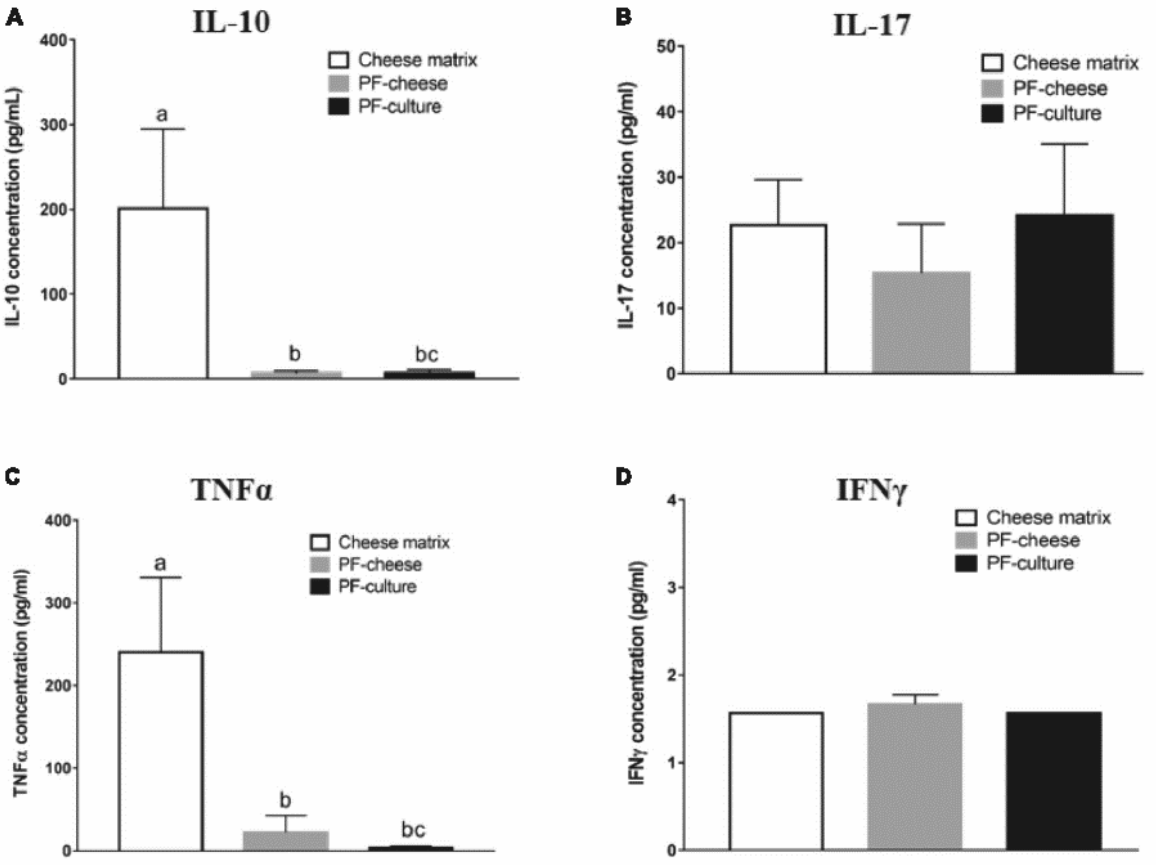


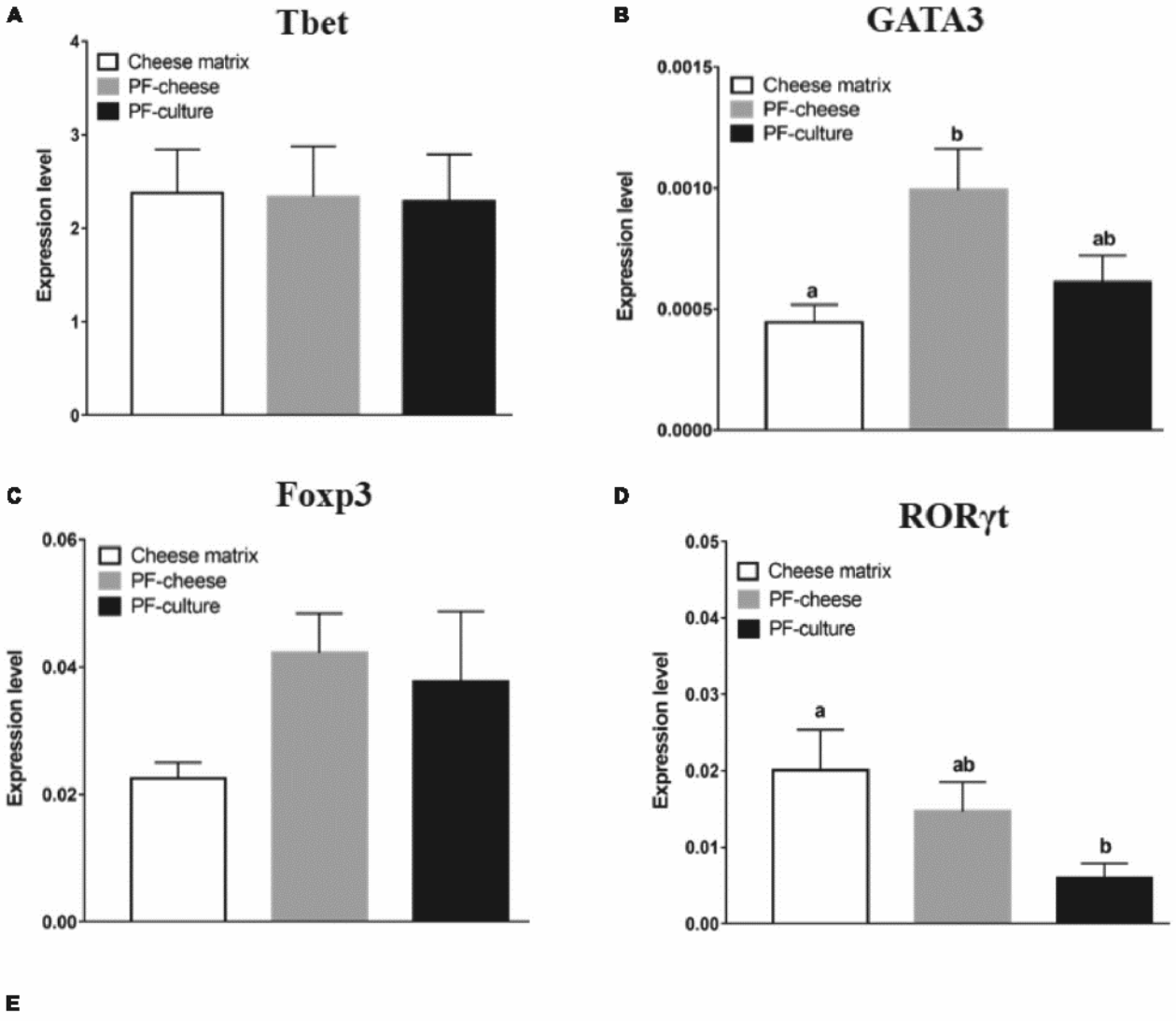
FIGURE 4 | Basal secretion of **(A)** IL-10, **(B)** IL-17, **(C)** TNFα, and **(D)** IFNγ by PBMC of different piglets groups fed with: cheese matrix, PF-culture or PF-cheese both fermented by *P. freudenreichii* CIRM-BIA 129 (PF). Cytokine concentrations were determined by ELISA and represented as means ± SEM ($n = 7$). Letters denotes significant difference, $P < 0.05$.

(**Figure 5E**). PBMC from PF-cheese piglets tended to express more Foxp3, compared to cheese matrix ones ($p = 0.0636$) (**Figure 5C**). PBMC from PF-culture piglets, but not from PF-cheese ones, had a significantly lower expression of RORγt compared to cheese-matrix piglets (**Figure 5D**). By determining Treg/Th17 ratio, we observed that only PF-culture piglets displayed a significant increase of Treg/Th17 ratio, compared to the cheese matrix piglets (**Figure 5E**). The Treg/Th17 ratio tended also to be higher in PF-cheese piglets, compared to cheese matrix ones ($p = 0.0666$) (**Figure 5E**).

Tbet expression was similar in MLNC from the different piglet groups (**Figure 6A**). Consumption of PF-cheese significantly increased GATA3 expression in MLNC, compared to the cheese matrix group, but without significant difference compared with PF-culture group (**Figure 6B**). MLNC from PF-cheese piglets displayed greater Foxp3 expression, compared to MLNC from both cheese matrix and PF-culture groups (**Figure 6C**). Consumption of PF-cheese significantly decreased Th1/Th2 ratio, compared to PF-culture group, but without significant difference with cheese matrix group. RORγt expression was lower in MLNC from PF-culture piglets compared to PF-cheese and cheese matrix groups (**Figure 6D**). Consumption of *P. freudenreichii*, regardless of the delivery vehicle, significantly increased the Treg/Th17 ratio (**Figure 6E**).

Cytokine Secretion by PBMC and MLNC in Response to *ex vivo* Stimuli

The LPS stimulation of PBMC induced a significant increase of IL-10 secretion, compared to untreated cells, only by PBMC from PF-culture and PF-cheese groups (**Figure 7A**). LPS stimulation induced also a significant increase of TNFα secretion by PBMC, compared to untreated cells, in all groups (**Figure 7B**). However, IFNγ secretion by PBMC was not triggered by LPS in any group (**Figure 7C**). ConA stimulation of PBMC induced a significant increase of IL-10 secretion compared to untreated cells, with no difference between groups (**Figure 7D**). ConA stimulation triggered a significant increase of TNFα secretion compared to untreated cells, only in PBMC from PF-cheese group. PBMC from PF-culture group tended also ($p = 0.0676$) to secrete more TNFα in response to ConA stimulation (**Figure 7E**). Stimulation by ConA induced a significant increase of IFNγ secretion, compared to untreated cells, only in PBMC from cheese matrix group (**Figure 7F**).



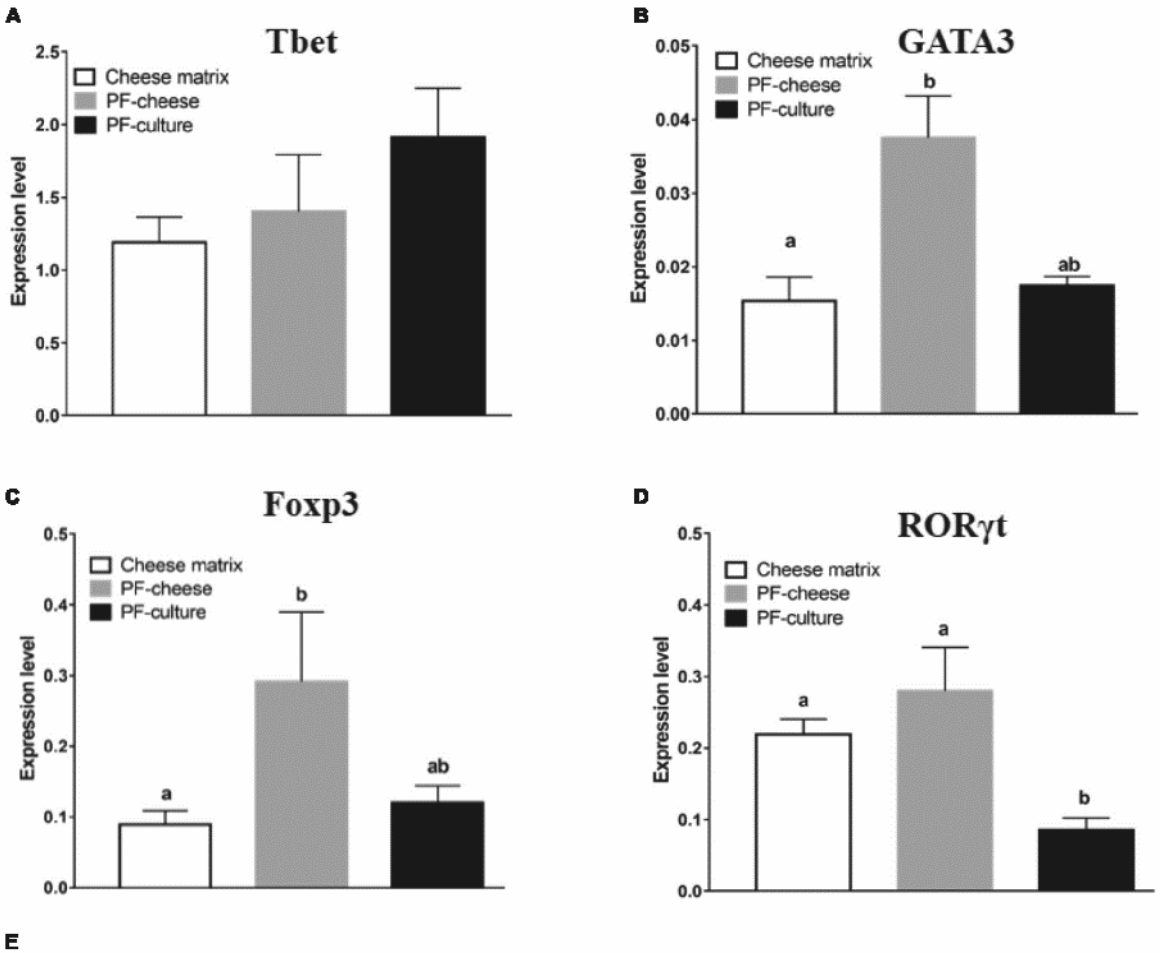
E

Groups	Th1/Th2 ratio	Treg/Th17 ratio
Cheese-matrix	7180 ± 1475 ^{a}	1.20 ± 0.32 ^{a}
PF-cheese	2600 ± 801.3 ^{b}	5,45 ± 2,08 ^{ab}
PF-culture	4008 ± 721.5 ^{ab}	9.65 ± 3.45 ^{b}

FIGURE 5 | Basal expression of **(A)** Tbet, **(B)** GATA3, **(C)** FOXP3, and **(D)** in PBMC from different piglets groups fed with cheese matrix, PF-culture or PF-cheese both fermented by *P. freudenreichii* CIRM-BIA 129 (PF). The level of expression was determined by RT-PCR. Ratios of Th1/Th2 and Treg/Th17 **(E)** were determined by calculating, respectively, Tbet/GATA3 and Foxp3/RORγt ratios. Results are represented as means ± SEM ($n = 7$). Letters denotes significant difference, $P < 0.05$.

The LPS Stimulation of MLNC increased significantly IL-10 secretion compared to untreated cells, only in MLNC from PF-culture group (**Figure 8A**). It induced also a significant increase of TNFα secretion by MLNC from cheese matrix and PF-culture piglets but not PF-cheese ones (**Figure 8B**). In addition, LPS stimulation induced a slight but significant secretion of IFNγ compared to untreated cells, only in MLNC from PF-cheese group (**Figure 8C**).

ConA triggered a significant rise of IL-10 secretion only in MLNC from PF-cheese group (**Figure 8D**). ConA stimulation of MLNC from all groups induced a significant secretion of TNFα compared to untreated cells (**Figure 8E**). Similarly, ConA



E

Groups	Th1/Th2 ratio	Treg/Th17 ratio
Cheese matrix	71.19 ± 12,99 ^{ab}	0.27 ± 0.11 ^{a}
PF-cheese	41.23 ± 14,2 ^{a}	1.35 ± 0.33 ^{b}
PF-culture	159.2 ± 51,27 ^{b}	1.22 ± 0.32 ^{b}

FIGURE 6 | Basal expression of **(A)** Tbet, **(B)** GATA3, **(C)** FOXP3, and **(D)** RORγt were analyzed in MLNC from different piglets groups fed with: cheese matrix, PF-culture or PF-cheese both fermented by *P. freudenreichii* CIRM-BIA 129 (PF). The level expression was determined by RT-PCR. Ratios of Th1/Th2 and Treg/Th17 **(E)** were determined by calculating, respectively, Tbet/GATA3 and Foxp3/RORγt ratios. Results are represented as means ± SEM (n = 7). Letters denotes significant difference, $P < 0.05$.

stimulation significantly increased IFNγ secretion compared to untreated cells, only in MLNC from PF-cheese piglets (**Figure 8F**).

DISCUSSION

The aim of this study was to investigate the impact of the delivery vehicle on the probiotic functionalities of *P. freudenreichii* in healthy piglets. For this purpose, three groups of piglets were fed during 2 weeks, with sterile cheese matrix, or a fresh culture of *P. freudenreichii* (PF-culture, 10^{11} CFU/day) or a single-strain cheese (PF-cheese, 10^{11} CFU/day) fermented solely by *P. freudenreichii* CIRM-BIA 129.

We investigated firstly if *P. freudenreichii* was metabolically active in piglet colon. *P. freudenreichii* metabolically active enhanced SCFA concentration in rats (Lan et al., 2007) and increased bifidobacteria in humans' fecal contents

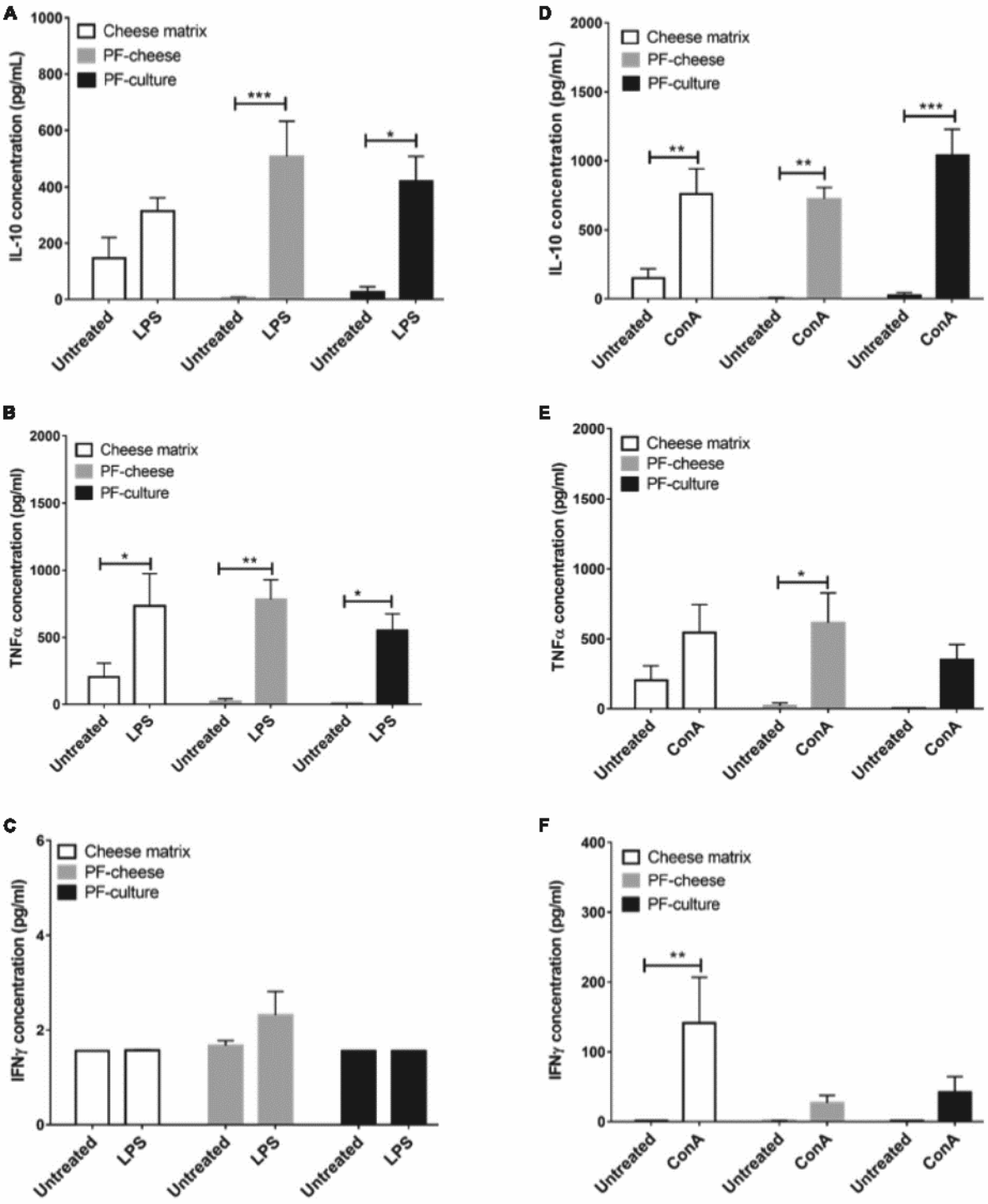


FIGURE 7 | Secretion of IL-10, TNFα, and IFNγ of stimulated PBMC from different piglets groups fed with: cheese matrix, PF-culture or PF-cheese fermented by *P. freudenreichii* CIRM-BIA 129 (PF). Cells were stimulated ex vivo with **(A–C)** LPS or **(D–F)** Concanavalin A (ConA). Cytokine concentrations were measured by ELISA and represented as means ± SEM ($n = 7$). $^{*}P < 0.05$, $^{**}P < 0.005$, and $^{***}P < 0.001$.

(Bouglé et al., 1999). The ability of a probiotic to be metabolically active in the colon depends on its adaptability toward colonic environment. *In vitro* studies suggested that propionibacteria digestive stresses tolerance is strain-dependent (Leverrier et al., 2005 ; Cousin et al., 2012c), but also matrix-dependent (Leverrier et al., 2005; Cousin et al., 2012c; Huang et al., 2016; Rabah et al., 2018). By contrast, our study demonstrated that both vehicles delivered metabolically

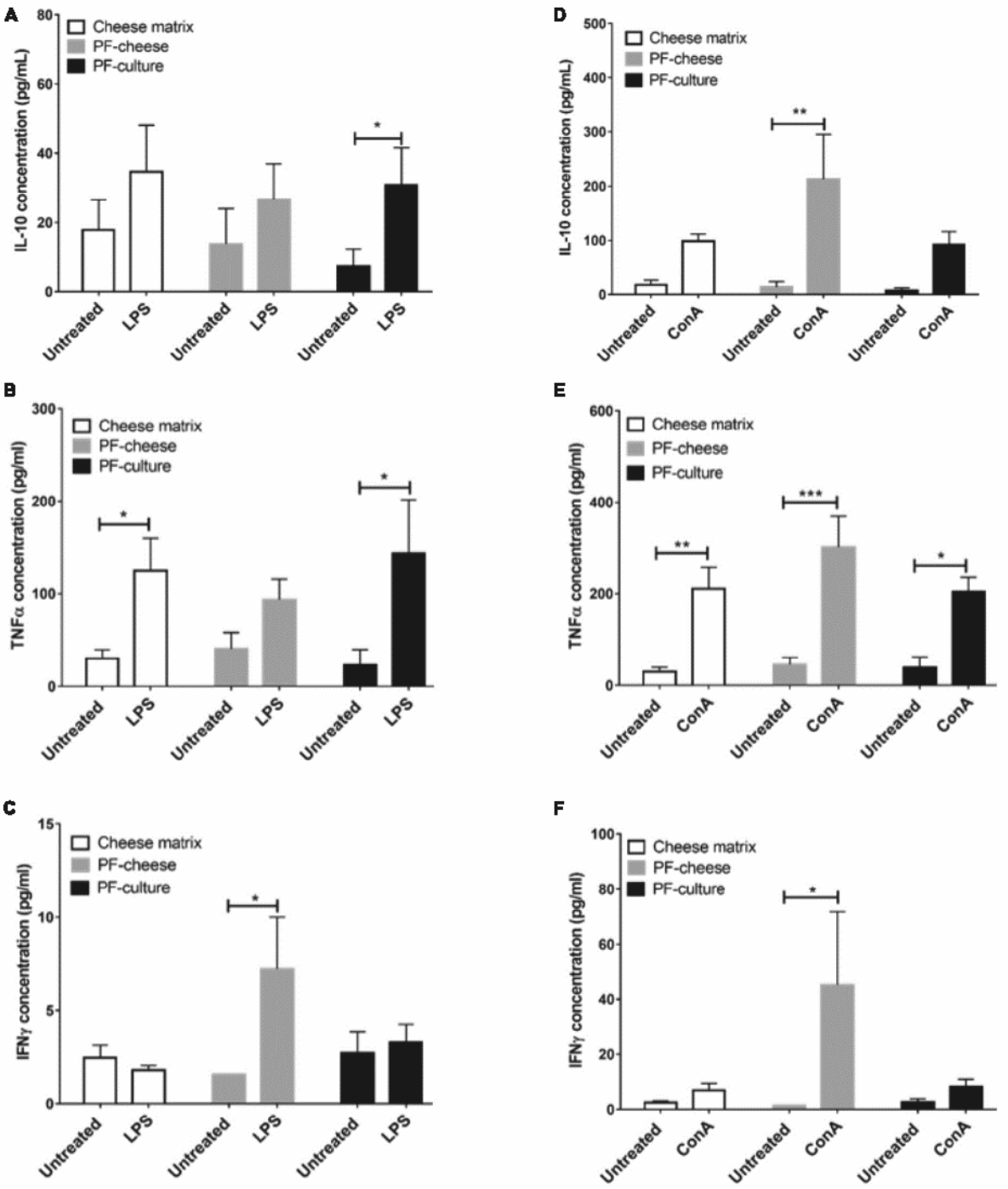


FIGURE 8 | Secretion of IL-10, TNFα, and IFNγ of stimulated MLNC from different piglets groups fed with: cheese matrix, PF-culture or PF-cheese fermented by *P. freudenreichii* CIRM-BIA 129 (PF). Cells were stimulated ex vivo with **(A–C)** Lipopolysaccharides (LPS) or **(D–F)** Concanavalin A (ConA). Cytokine concentrations were measured by ELISA and represented as means ± SEM ($n = 7$). $^{*}P < 0.05$, $^{**}P < 0.005$, $^{***}P < 0.001$.

active *P. freudenreichii* to the piglets' colon. To attest of the *in vivo* metabolic activity of *P. freudenreichii*, we investigated its bifidogenic effect. Indeed, numerous studies demonstrated that *P. freudenreichii* has a bifidogenic effect (Bouglé et al., 1999; Hojo et al., 2002; Colliou et al., 2017) due to the *in situ* production of several metabolites (Yamazaki et al., 1999; Isawa et al., 2002; Kouya et al., 2007). *P. freudenreichii* consumption enhanced bifidobacteria population, without affecting lactobacilli population, in fecal contents, compared to cheese matrix group. *P. freudenreichii* metabolic activity was also assessed by analyzing

fecal SCFA concentration. In our study, *P. freudenreichii* CIRM-BIA 129 significantly increased total SCFA concentration, in particularly BCFA concentrations. The ability of *P. freudenreichii* to produce BCFA has already been demonstrated (Dherbécourt et al., 2008). These results are consistent with a previous transcriptomic study demonstrating that *P. freudenreichii*, within the colon, represses the wood-werkman cycle pathway (suggesting decreased propionic and acetic acid production), while inducing branched-chain amino acid degradation (suggesting an increase of BCFA production) (Saraoui et al., 2013). The modulation of SCFA content could also result indirectly from the increase of bifidobacteria population (Ríos-Covián et al., 2016; Hemalatha et al., 2017; LeBlanc et al., 2017). In a previous study, *P. freudenreichii* CIRM-BIA 129, with a dose of 1.10^{10} CFU/day, did not change colonic SCFA concentration in healthy piglets (Cousin et al., 2012a). However, in our study, piglets receiving 10^{11} CFU of *P. freudenreichii* per day showed an increase of fecal SCFA concentration. These results suggest the major importance of the dose of live propionibacteria when seeking *in vivo* beneficial metabolic effects.

We examined *ex vivo* the immunomodulatory effect of extracted *P. freudenreichii* Slps on PBMC and MLNC from naive piglets (cheese matrix group). As already demonstrated with human PBMC (Le Maréchal et al., 2015; Deutsch et al., 2017), Slps induced high IL-10 secretion in swine PBMC. We further extended this observation to swine MLNC. In addition, Slps inhibited the secretion of TNFα induced by LPS and ConA stimulation, in both cell types. Slps also inhibited ConA-induced IFNγ secretion in PBMC. Indeed, these results are consistent with a previous study, showing that *P. freudenreichii* CIRM-BIA 129 Slps decreased IFNγ induced by the pro-inflammatory *Lactococcus lactis* MG1363 strain in human PBMC (Le Maréchal et al., 2015). The molecular mechanisms by which propionibacteria Slps suppress induction of pro-inflammatory cytokines triggered by ConA and LPS remain unexplained. Few studies showed the ability of Slps from *P. freudenreichii* (Colliou et al., 2017) or from other probiotics (Konstantinov et al., 2008; Martínez et al., 2012; Prado Acosta et al., 2016) to bind to C-type lectins receptors of monocytes. More investigations are, however, needed to elucidate the detailed molecular mechanism.

To explore the immunomodulatory effects of *P. freudenreichii* consumption at the systemic level, immune cells from blood (PBMC) were isolated. At the basal state, cytokines secretion and T lymphocytes phenotypes were analyzed. *P. freudenreichii* consumption, whatever the delivery vehicle, modulated Treg/Th17 ratio, compared to cheese matrix group. Nevertheless, PF-culture decreased significantly Th17 phenotype, compared to cheese matrix group. Contrastingly, PF-cheese tended to enhance Treg phenotype, compared to the cheese matrix group. In addition, only PF-cheese significantly modulated PBMC Th1/Th2 ratio toward a Th2 phenotype. However, basal cytokine secretions were not consistent with T lymphocytes PBMC phenotypes investigated by qPCR. *P. freudenreichii* consumption, in both delivery vehicles, decreased basal PBMC secretion of IL-10 (a cytokine produced by Treg and Th2 cells) and of TNFα (a cytokine produced by Th1 cells), compared to cheese matrix group. Basal cytokine secretion is a global response of T, B cells and innate immune cells (macrophages and dendritic cells), which may explain these discrepancies. Moreover, we did not evaluate IL-4 basal secretion, a marker of Th2 T cells. Finally *in vitro* studies showed *P. freudenreichii* CIRM-BIA 129 as an inducer of IL-10 and TNFα in human PBMC (Kekkonen et al., 2008; Le Maréchal et al., 2015; Deutsch et al., 2017), which is in contradiction to the *in vivo* results obtained in this study. This result suggests that chronic ingestion of *P. freudenreichii* affects differently immune cells than *ex vivo* acute stimulation. Noteworthy, *P. freudenreichii* consumption inhibited IFNγ secretion by PBMC in response to ConA stimulation, regardless of the delivery vehicle. Further research is needed to understand this delivery vehicle-induced switch in TNFα/IFNγ secretion.

The intestinal immune response to *P. freudenreichii* consumption was also investigated. *P. freudenreichii*, regardless of the delivery vehicle, did not affect basal secretion of IL-10, IL-17, TNFα, or IFNγ. Once again, cytokine secretion patterns were not consistent with the phenotype of T lymphocytes in the different piglet groups. The effects of *P. freudenreichii* on T lymphocytes phenotypes of MLNC were similar to that of PBMC. PF-cheese enhanced Treg phenotype and PF-culture decreased Th17 phenotype, compared to cheese matrix group. Moreover, PF-cheese enhanced Th2 phenotype, compared to cheese matrix group, but without significant difference with the PF-culture group. MLNC responses to LPS and ConA stimulation showed different responses between PF-cheese and PF-culture groups from that of PBMC. Indeed, in response to LPS stimulation, PF-culture consumption enhanced IL-10 and TNFα secretion by MLNC, compared to untreated cells, while PF-cheese consumption induced a slight, yet significant increase in IFNγ secretion. In addition, only MLNC from PF-cheese group showed a high secretion of IL-10 and IFNγ, compared to untreated cells. ConA in naive MLNC did not induce secretion of IFNγ. Moreover, there was no significant difference between the three groups in Th1 phenotypes, an IFNγ-secreting phenotype (Takaoka and Yanai, 2006; Kak et al., 2018). IFNγ may be also secreted by others innate immune cells (Takaoka and Yanai, 2006; Kak et al., 2018). This result suggests that PF-cheese modulates innate immune cells differently from PF-culture. IFNγ plays a primordial role in immune cells to fight intestinal infections: it activates macrophages and Th1 expansion, which induces an effective immune response against pathogens (Kak et al., 2018). In addition, only PF-cheese enhanced Th2 phenotype. Thus, the secretion of IFNγ may be a way to control Th2 cells expansion. These results suggest the potential of using *P. freudenreichii* CIRM-BIA 129 in functional foods to prevent intestinal infections, as already shown with *Listeria monocytogenes* infection in mice model (Kato-Mori et al., 2010; Colliou et al., 2018). This should be confirmed in the future by *in vivo* pathogens challenge experiments in piglets.

Altogether, *P. freudenreichii* showed an anti-inflammatory effect on the systemic and intestinal immune system by enhancing Treg and Th2 phenotypes or decreasing Th17 phenotype, depending on the delivery vehicle. Th2 and Treg responses triggered by *B. breve* was shown to protect mice from chemically induced colitis (Zheng et al., 2014). This may partially explain the protective role of *P. freudenreichii* in cheese against

colitis (Plé et al., 2015, 2016; Foligné et al., 2016). Previously, *P. freudenreichii* P.UF1 was shown to increase Th17 population, to sustain Treg population and to decrease Th1 population, via a S-layer protein, in mice (Colliou et al., 2017, 2018).

In this study, since a similar metabolic activity of *P. freudenreichii* between the two delivery vehicles was observed, we assumed also that the matrix-dependent immunomodulatory effect may be related to surface proteins that would be protected by the cheese matrix (Rabah et al., 2018). More investigations are needed to confirm this hypothesis and to elucidate the molecular mechanism triggered by *P. freudenreichii* CIRM-BIA 129 to interact with host immune system.

CONCLUSION

The present study demonstrated that *P. freudenreichii* exerts an anti-inflammatory effect, regardless of the delivery vehicle. The difference between vehicles in term of immunomodulatory modulation was obvious after *ex vivo* stimulation of immunes cells by LPS and ConA, in particularly at the intestinal level. Our study shows that the delivery vehicle should be carefully considered. It opens the perspective to use *P. freudenreichii* in cheeses to prevent IBD or intestinal infectious diseases.

AUTHOR CONTRIBUTIONS

GB, GJ, and SF-B designed the study. HR and SH prepared all matrices. HR, SH, FG, RJ, SF-B, LLN, GB, and GJ participated in animal experiment and laboratory analyses. HR and GB analyzed data and prepared figures. HR wrote the manuscript with the help of GB, FC, SF-B, and GJ. RJ, GB, and GJ supervised the project. All authors read and approved the final 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.2018.02584/full#supplementary-material

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Conflict of Interest Statement: FG was employed by company Bioprox.

The remaining 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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Supplemental data

		Sequence (5′→3′)	Size (bp)	Accession no.	Reference
Propionibacteria 5S ssu transcarboxylase	F	ATTCCATCGCCCTGAAGGA	103	AY861355.1	Hervé et al., (2007)
	R	TTGATCTGCGTCTTCTGGCC			
Family Bifidobacteriaceae 16S	F	CGCGTCYGGTGTGAAAG	244	-	Delroisse et al.,(2006)
	R	CCCCACATCCAGCATCCA			
Genus Lactobacillus 23S	F	GCGGTGAAATTCCAAACG	216	-	Hermann-Bank et al., (2013)
	R	GGGACCTTAACTGGTGAT			
Tbet	F	TGGACCCAACTGTCAATTGCT	76	NM_001315722.1	Hernández et al., (2008)
	R	ACGGCTGGGAACGGGATA			
GATA3	F	TGCGGGCTCTACCACAAAAT	240	DQ450901.1	Muráni et al,. (2007)
	R	TAACCCGAGTAAAATGTGC			
RORc	F	GAAGTGGTGCTGGTCAGGAT	140	AK238874	Young et al,. (2012)
	R	CGGGAGAAGTCAAAGATGGA			
Foxp3	F	GGTGCAGTCTCTGGAACAAC	148	NM_001128438	Zhu et al,. (2014)
	R	GGTGCCAGTGGCTACAATAC			
HPRT	F	GGTGATAGATCCATTCCTATGACTGTAGA	104	U69731	Ledger et al., 2004)
	R	TGAGAGATCATCTCCACCAATTACTT			

Table S1. Specific primer sequences in order to target bacterial groups and species of high interest in the swine intestine as well as different transcriptional factors playing a key role for T cells differenciation.

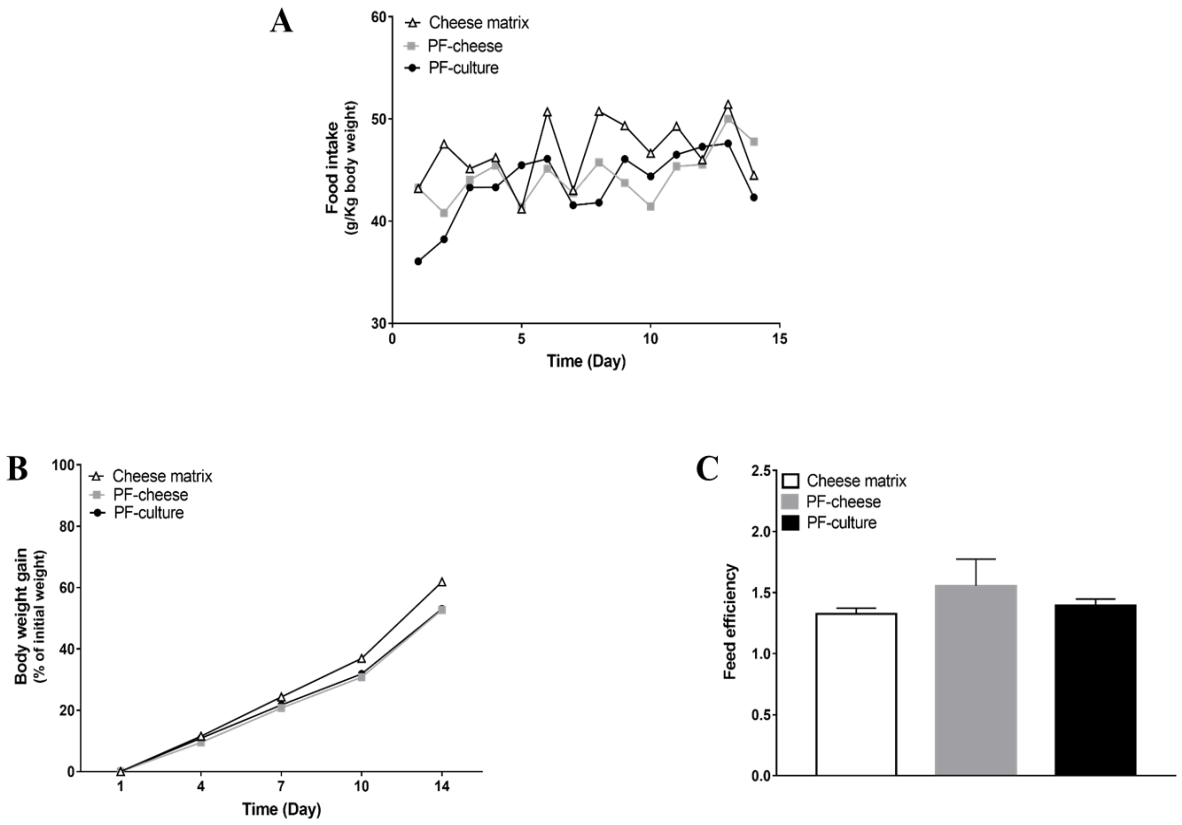

Fig.S1. Food intake, weight gain and feed efficiency of piglets fed with cheese matrix or fermented matrices by *P. freudenreichii* CIRM-BIA 129 (PF): PF-cheese or PF-culture. Food intake **(A)** was measured daily for 2 weeks and weight gain **(B)** was measured at four times (d1, d4, d7, d10 and d14) during the same period. The feed efficiency **(C)** corresponds to the ratio between the total food intake and the weight gain at day 14. The results are represented as means ± SEM (n=7). $P<0.05$.

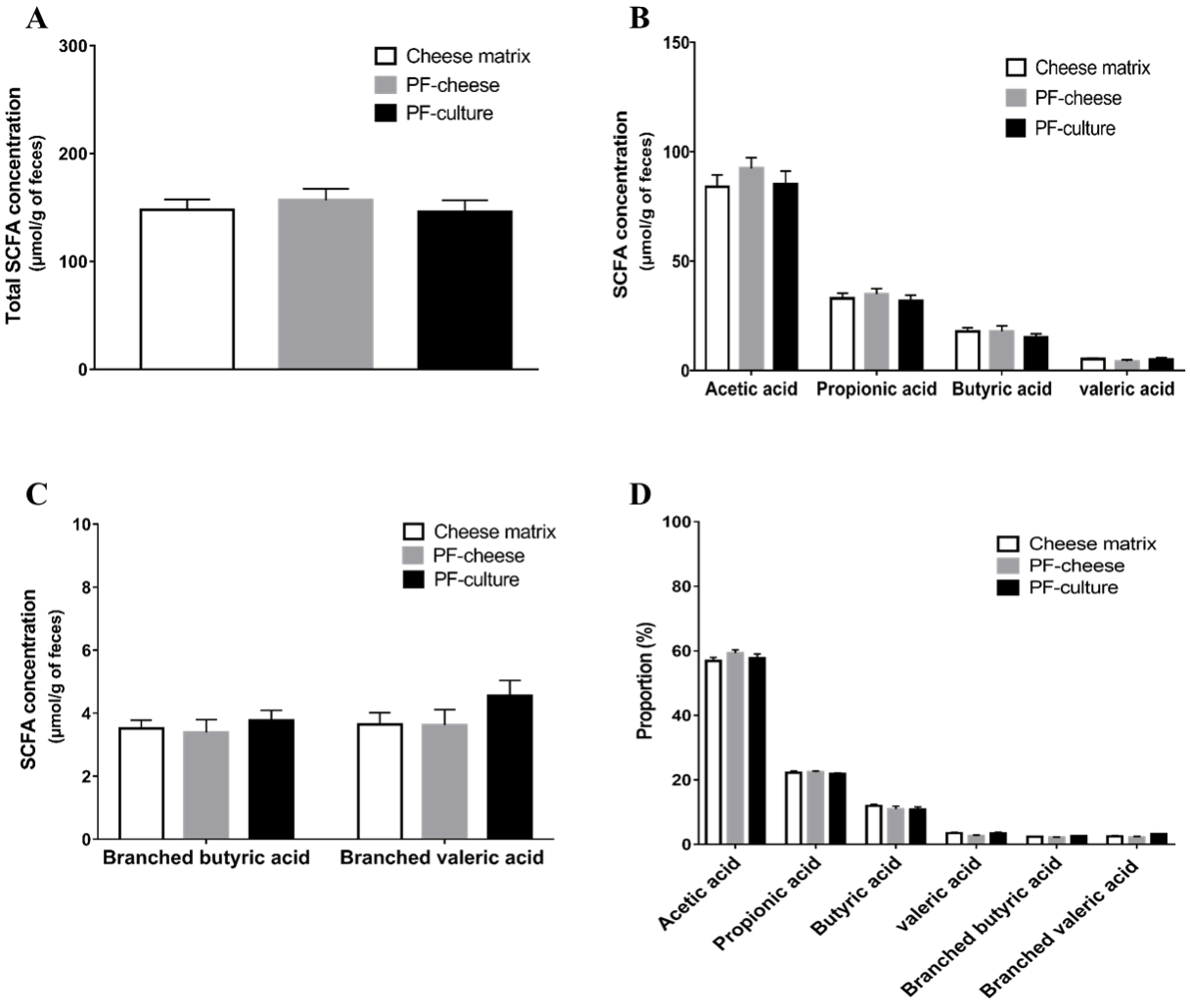

Fig.S2. Short chain fatty acids (SCFA) concentration in faeces contents at day 7 of the three piglets groups fed with: PF-culture or PF-cheese fermented by *P. freudenreichii* CIRM-BIA 129 (PF). The total SCFA concentration **(A)**, the concentration of each SCFA type (C2, C3, C4, C5) **(B),** and the concentration of branched short chain fatty acids **(C)** were measured by gas-phase chromatography, and the proportion of each type of SCFAs **(D)** was determined. Results are represented as means ± SEM (n=7). P <0.05.

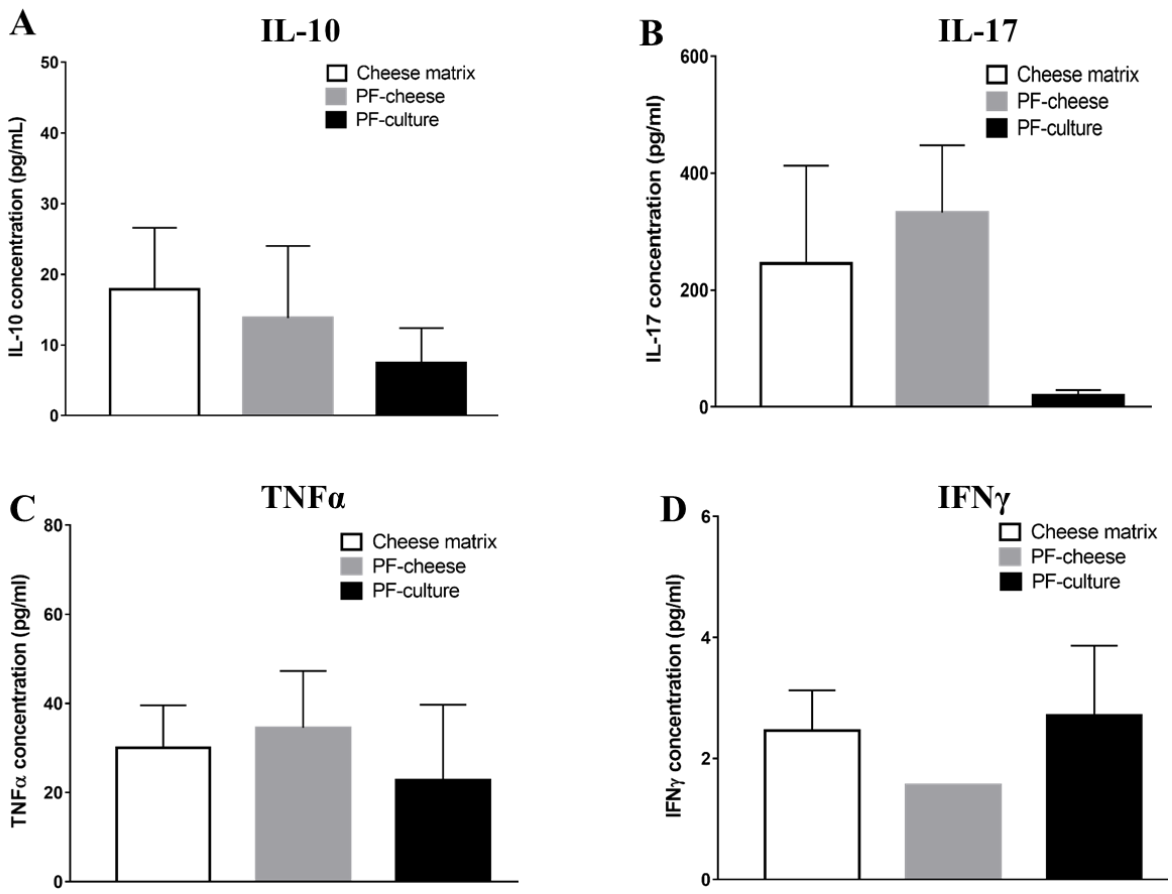

Fig.S3. Basal secretion of IL-10 **(A)**, IL-17 **(B)**, TNFα **(C)** and IFNγ **(D)** were measured in mesenteric lymph nodes cells (MLNC) cells of different piglets groups fed with: cheese matrix, or PF-culture or PF-cheese both fermented by *P. freudenreichii* CIRM-BIA 129 (PF). Cytokine concentrations were determined by ELISA and represented as means ± SEM of two analyses per piglet (n=7). $P < 0.05$.

4.3. Conclusion

Dans ce chapitre 4, nous avons émis l'hypothèse que l'effet probiotique, en particulier l'effet immunomodulateur, serait matrice-dépendant. En effet, dans un premier temps, l'étude *in vitro* suggérait, par le fait de l'amélioration de la tolérance aux stress digestifs, que la matrice fromagère apporterait une quantité plus significative de bactéries vivantes au côlon que l'ultrafiltrat de lait. Cependant, l'étude *in vivo* montre que, sans différence significative entre les deux matrices vectrices, l'ingestion de *P. freudenreichii* augmentait la concentration en acides gras totaux à chaîne courte et présentait un effet bifidogène. Cela suggère que les deux matrices apportent la même quantité de bactéries métaboliquement actives au tractus digestif des porcelets.

Dans un deuxième temps, comme la protéolyse des protéines Slps est matrice-dépendante, nous avons émis l'hypothèse que l'effet immunomodulateur serait également matrice-dépendant. L'étude *in vivo* a montré que, quelle que soit la matrice vectrice, *P. freudenreichii* présente des effets anti-inflammatoires. L'ingestion de *P. freudenreichii* modulait seulement le profil cytokinique des cellules immunes du sang (PBMC), sans différence significative entre les deux matrices vectrices. On observait une diminution de la sécrétion d'IL-10 et de TNFα par les PBMC. Cependant, *P. freudenreichii* modulait les phénotypes des lymphocytes T de manière matrice-dépendante. En effet, l'ingestion de *P. freudenreichii* sous forme d'ultrafiltrat de lait diminuait le phénotype Th17 ; alors que l'ingestion de *P. freudenreichii* sous forme de fromage augmentait les phénotypes Treg et Th2, à la fois au niveau intestinal et systémique. Le profil cytokinique des cellules immunes n'était pas en cohérence avec l'orientation induite des lymphocytes T. En effet, les cellules isolées sont un mélange de lymphocytes B et T, ainsi que des cellules de l'immunité innée (macrophages, cellules dendritiques). Nous émettons l'hypothèse que l'ingestion de *P. freudenreichii* a très probablement modulé également la réponse des autres types cellules de l'immunité.

Les deux matrices vectrices apportaient la même quantité de bactéries métaboliquement actives, ce qui suggère que la différence d'immunomodulation entre les deux matrices est très probablement liée à la composition du protéome de surface, qui est en interaction directe avec le système immunitaire.

Cependant, cette hypothèse devrait être confirmée par d'autres expérimentations, puisque nous n'avons pas réussi à détecter les protéines Slps dans les contenus digestifs des différents groupes.

La différence en termes d'immunomodulation a été observée également lors de la stimulation *ex vivo* des cellules immunes par le LPS ou la Concanavaline A. La réponse des cellules MLNC et PBMC au même stimulus était différente et le stimulus induisait une réponse différente, selon la matrice ingérée par les porcelets.

De plus, nous avons confirmé que l'effet anti-inflammatoire des protéines Slps sur les cellules PBMC en présence de stimuli proinflammatoires, mais de manière originale, sur les cellules immune intestinales (MLNC).

Chapitre 4 : à retenir

- **L'ultrafiltrat de lait ainsi que le fromage apportent des bactéries propioniques métaboliquement actives au tractus digestif des porcelets :**
  - ***P. freudenreichii* augmente la concentration fécale en acides gras à chaîne courte, en particulier les acides gras branchés à chaîne courte**
  - ***P. freudenreichii* augmente la population fécale en bifidobactéries**
- ***P. freudenreichii* diminue la sécrétion d'IL-10 et de TNFα par les PBMC, sans différence entre les deux matrices vectrices**
- ***P. freudenreichii* présente des effets anti-inflammatoires matrice-dépendants*:***
  - **Sous forme d'ultrafiltrat de lait, *Pf* diminue le phénotype Th17**
  - **Sous forme de fromage, *Pf* augmente les phénotypes Treg et Th2**
- **Les stimulations *ex vivo* des MLNC et des PBMC confirment l'effet immunomodulateur matrice-dépendant de *P. freudenreichii***

Chapitre 5 : « L'étude *in vitro* de l'effet du milieu de croissance sur l'adaptation de *P. freudenreichii* à l'environnement caecal »

5.1. Introduction

Dans le chapitre précédent, les vecteurs alimentaires, l'ultrafiltrat de lait et le fromage, semblent apporter la même quantité de bactéries métaboliquement actives au tractus digestif des porcelets. Cependant, l'analyse *in vitro* montrait que la matrice fromagère apporterait une quantité plus importante de bactéries vivantes au côlon par l'amélioration de la tolérance aux stress digestifs. La quantité de bactéries vivantes atteignant le côlon va dépendre évidement de la tolérance des bactéries aux stress gastriques et duodénaux, mais également de leur capacité à survivre dans le côlon. Ce dernier aspect n'a pas été pris en compte par les modèles de digestion *in vitro*. Par conséquent, nous supposons que les bactéries vectorisées par l'ultrafiltrat de lait ou par le fromage ne possèdent pas les mêmes capacités d'adaptation aux conditions environnementales existantes dans le côlon.

De plus, l'immunomodulation par *P. freudenreichii* était matrice-dépendante *in vivo*. Nous avons émis l'hypothèse qu'il existe une différence, en termes de protéome de surface, entre les bactéries vectorisées par l'ultrafiltrat de lait et celles vectorisées par le fromage. Nous ignorons si *P. freudenreichii* exprime *de novo* ses protéines de surface dans le côlon après la protéolyse de celles-ci plus tôt dans les parties supérieures du tractus digestif (Estomac et intestin grêle). En effet, chez différentes espèces de *Lactobacillus*, les protéines de type S-layer jouent un rôle dans la résistance à de multiples stress, dont les stress digestifs, et participent à la persistance des bactéries dans le tractus digestif de l'hôte (Gerbino et al., 2015b). Chez *P. freudenreichii*, nous supposons que les protéines Slps pourraient également avoir un rôle dans sa capacité d'adaptation à l'environnement colique.

Pour répondre à ces différentes questions, notre stratégie était de reconstituer un milieu caecal à partir de contenus caecaux de porcelets pour mimer les conditions environnementales qui existent dans le gros intestin (Figure 9). Différents culots de *P. freudenreichii* CIRM-BIA 129, provenant de cultures en phase stationnaire sur YEL ou sur l'ultrafiltrat de lait, ainsi que d'un fromage monosouche, ont subi un rasage enzymatique des protéines de surface (Shaving) ou un traitement témoin, sans trypsine. Les différents culots ont été incorporés dans l'eau caecale et incubés 50 heures en anaérobiose et à 37°C. La survie par dénombrement sur YELA, la densité optique, ainsi que la viabilité par la coloration de fluorescence

LIVE/DEAD ont été suivies en cinétique. L'expression *de novo* de la protéine SlpB a été analysée par Western Blot.

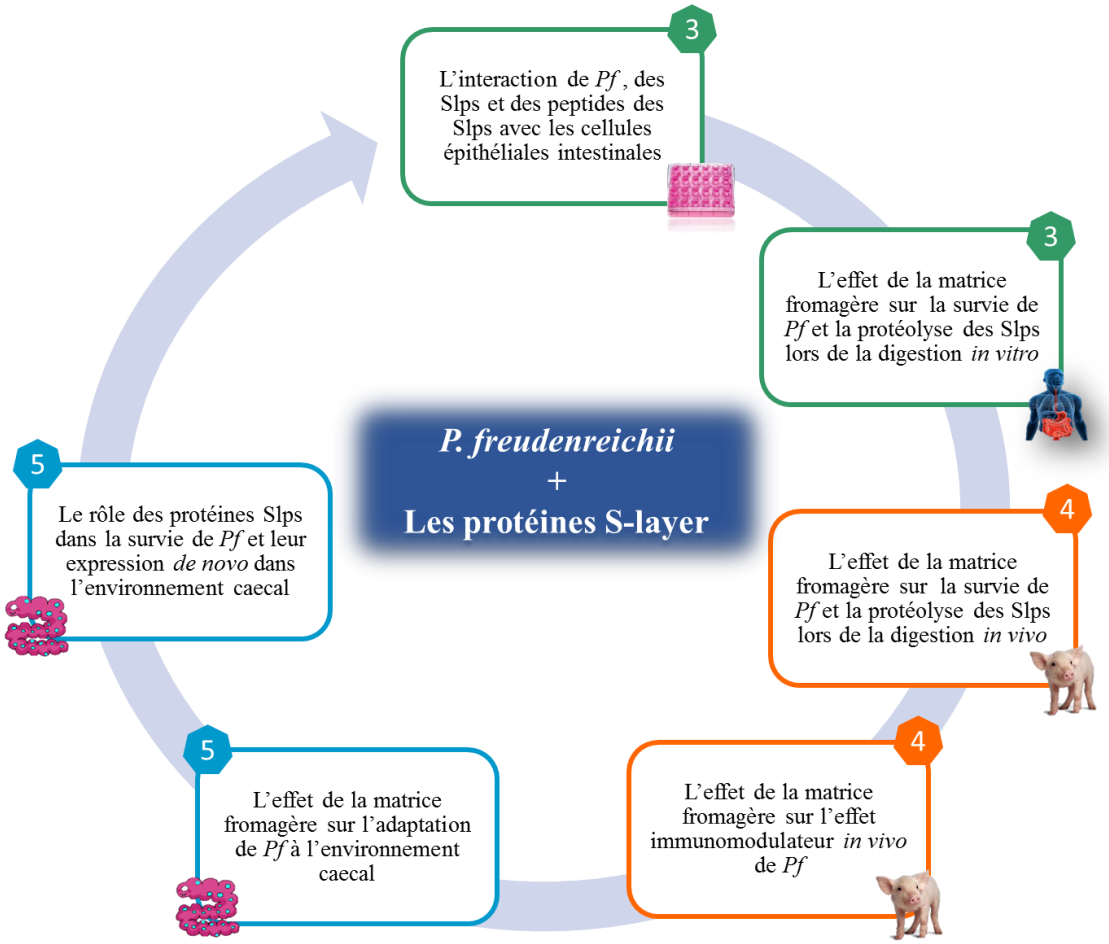

Figure 9. **Comment le milieu de croissance influence-t-il la survie de *P. freudenreichii* CIRM-BIA 129 dans le milieu caecal ?** Le chapitre 5 (en bleu) présente les travaux menés sur la compréhension de l'adaptation de *P. freudenreichii* (***Pf***) à l'environnement caecal et l'influence du milieu de croissance sur ces mécanismes. Ainsi, les questions abordées dans ce chapitre 5 sont :

1. Est-ce que le milieu de croissance a un impact sur la survie de ***Pf*** dans le milieu caecal ?
2. Est-ce que la protéolyse digestive des protéines de surface a un impact sur la survie de ***Pf*** dans le milieu caecal ?
3. Quelle est l'activité métabolique de ***Pf*** dans le milieu caecal ?

Les résultats de cette étude ont fait l'objet d'une communication par poster:

Au congrès national de la société française de microbiologie (SFM), Paris-France, Octobre 2018.

L'article présentant les résultats de ce chapitre est en cours préparation et à soumettre au journal « Applied Microbiology and Biotechnology »

5.2. Article 3 : l'influence du milieu de croissance sur la capacité *in vitro* d'adaptation de *Propionibacterium freudenreichii* CIRM-BIA 129 à l'environnement caecal

Impact of the growth medium on *in vitro P. freudenreichii* CIRM-BIA 129 adaptation ability to the caecal environment

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Introduction

Propionibacterium freudenreichii is a beneficial microorganism, which possesses the GRAS and the QPS status (Cousin et al., 2011; Thierry et al., 2011a). Indeed, it is used traditionally as a cheese starter and it also displays versatile probiotic properties (Cousin et al., 2011; Rabah et al., 2017). These probiotic properties include inter alia, anti-inflammatory effects demonstrated by *in vitro* and *in vivo* studies, leading respectively to the induction of IL-10 in immune cells (Deutsch et al., 2017; Le Maréchal et al., 2015) and to the prevention of murine induced colitis (Foligné et al., 2013; Le Maréchal et al., 2015; Plé et al., 2015, 2016). Several beneficial metabolites as short chain fatty acids and 1.4-dihydroxy-2-naphthoic acid (DHNA) are responsible for the anti-inflammatory properties of *P. freudenreichii* (Rabah et al., 2017). The surface proteome, particularly S-layer proteins, are also crucial to its immunomodulatory properties (Colliou et al., 2017; Deutsch et al., 2017; Le Maréchal et al., 2015). To exert the corresponding probiotic effects, bacteria must be able to deliver or even to produce their beneficial metabolites by persisting in the colon environment. The survival of a probiotic bacterium within the digestive tract depends on its tolerance to digestive stresses, but also on its adaptation ability towards colonic environment. In the colon segment, different stresses are encountered as pH variations, antimicrobial peptides, bile salts presence, but mainly nutrients shortage. *P. freudenreichii* CIRM-BIA1 was shown *in vivo* to have the ability to adapt to the colonic environment (Saraoui et al., 2013). However, the delivery vehicle or the growth medium may influence the ability of *P. freudenreichii* to adapt to nutrients shortage in the colon. Indeed, the growth of *P. freudenreichii* in a stressful and enriched medium led to the accumulation of intracellular energy storage molecules and compatible solutes, which may enhance the tolerance response to digestive stresses and nutrient shortage in the colon (Dalmasso et al., 2012; Huang et al., 2016; Rabah et al., 2018b). In addition, in the case of *P. freudenreichii* CIRM-BIA 129, the tolerance response towards digestive stresses was shown to be affected by the mutation of the *Slpb* gene (Do Carmo et al., 2018b). This study suggests that Slps may play a key role in the enhanced tolerance response towards digestive stresses. Indeed, these proteins are described in the literature to have versatile functions: such as contribution to determination or maintenance of cell shape, molecular sieve, enzymatic activities, contribution to adhesion, coaggregation, modulation of gut immune cells

and protection against environmental stresses (Do Carmo et al., 2018a; Le Maréchal et al., 2015). In *Lactobacillus* species, Slps expression may be modulated by the environmental stresses as osmotic or digestive stresses (Grosu-Tudor et al., 2016; Khaleghi et al., 2010; Khaleghi and Kasra, 2012; Palomino et al., 2016). However, we still ignore the role of S-layer proteins on *P. freudenreichii* adaptation ability to the colonic environment. We have previously shown that the food delivery vehicle may protect Slps from *in vitro* simulated digestive proteolysis (Rabah et al., 2018b). In this study, we thus examined how the growth medium affects the *in vitro* adaptation ability of *P. freudenreichii* CIRM-BIA 129 in the colon environment. Secondly, we aimed to investigate how the proteolysis of surface proteins impacts *P. freudenreichii* CIRM-BIA 129 survival in the colon, with a particular interest to the Slps proteins. In this purpose, we prepared a caecal medium from porcine cecum contents to mimic the environment conditions in the large bowel.

Materials and methods

Preparation and composition analysis of the caecal medium

Cecum contents from 8 weeks piglets were recovered and stored at -20°C until caecal medium preparation (Rabah et al., 2018a). The content were then centrifuged for 15 min at 10 000 x g and the supernatant was autoclaved (115°C, 15 min). The sterilized supernatant was centrifuged for 30 min at 20 000 x g and sterilized by filtration using 0.2 µm Qiagen filter. The sterile caecal medium was stored at -20°C until utilization. For biochemical analysis of this medium, it was then diluted with 0.04 NH_2SO_4 and centrifuged at 8 000 ×g for 30-min at 4°C to remove the proteins pellet, the supernatant was filtrated and sugar and organic acids separated by high performance liquid chromatography (HPLC). An Aminex A-6 ion exchange column (Dionex, Sunnyvale, CA) operating at 55°C with 0.01 NH_2SO_4 as the eluent at a flow rate of 1 $ml.min^{-1}$. For sugar and organic acids quantification, both UV (210 nm) and refractometric detectors were used, as described previously (Aburjaile et al., 2016b).

Bacterial strains and culture conditions

P. freudenreichii CIRM-BIA 129 (equivalent to ITGP20 strain) and *P. freudenreichii* CIRM-BIA 129 KO SlpB were routinely cultivated at 30°C, respectively in Yeast Extract-Lactate medium (YEL) or

YEL supplemented with chloramphenicol (10 µg.mL-1) until stationary phase (Do Carmo et al., 2018b). *P. freudenreichii* CIRM-BIA 129 was grown in milk ultrafiltrate (MUF) supplemented with 100 mM of sodium DL-lactate (60% in H_2O, Sigma) and 5 g/L of casein hydrolysate (Organotechnie, La Courneuve, France) at 30°C until stationary phase. Finally, *P. freudenreichii* CIRM-BIA 129 was used to ferment a single-strain cheese as described previously (Plé et al., 2015).

Enzymatic Shaving of Surface Proteins

Bacterial pellets of *P. freudenreichii* CIRM-BIA 129 were extracted from YEL, MUF were recovered by centrifugation (8 000 × g, 10 min, 4°C) and washed with phosphate buffer saline (PBS). Similar procedure was performed for *P. freudenreichii* CIRM-BIA 129 KO SlpB grown in YEL medium. Bacterial pellets from cheese were recovered by serial extractions and washing steps, as previously described (Plé et al., 2015). Bacterial pellets were submitted to enzymatic shaving of surface proteins as described previously (Le Maréchal et al., 2015). Briefly, bacterial pellets corresponding to 20 OD units were suspended in 1 mL of PBS (pH 8.5) containing 5 mM of DTT. Sequencing grade modified trypsin (4µg) (Promega, Madison, WI, United States) was added to the bacterial suspension. Shaving was performed for 1 h at 37°C, with gentle agitation (180 rpm). As a negative shaving control, bacterial pellets were submitted to an incubation of 1h at 37°C, with gentle agitation and without adding trypsin. Bacteria were recovered by centrifugation (8 000 × g, 10 min, 37°C) and washed twice by PBS buffer.

Survival monitoring in the caecal medium

Bacterial pellets from different media were suspended in the caecal medium, in a volume to obtain an optical density at 650 nm (OD_{650nm}) of 4. These different bacterial suspension were incubated at 37°C in anaerobic condition for 50 h. Survival kinetics were followed simultaneously by OD_{650nm} measurements and by CFU counting every 5 hours. The survival rates during incubation time were determined by calculating the ratio between CFU number at each point and the initial CFU number. In all cases, two technical replicates and three biological replicates were performed.

LIVE/DEAD fluorescence staining

P. freudenreichii viability in the caecal medium was evaluated after 5, 25 or 50 hours of incubation, using LIVE/DEAD staining (Invitrogen, Carlsbad, USA). Briefly, bacteria were centrifuged and washed with PBS. The bacterial pellets were suspended in PBS in order to obtain an OD_{650nm} of 4 and doubled-stained with propidium iodide and SYTO 9 according to the instructions of LIVE/DEAD Light kit manufacturer's instructions. The bacteria were then analyzed using an Olympus BX51 microscope, equipped with a U-M51005 epifluorescence cube and an Olympus 100x/1.25 oil objective (Nikon D300), and CAMERA CONTROL PRO 2 software (Nikon Corporation, Tokyo, Japan). For each sample, 30 photos were analyzed for each time point.

Whole-cell protein extraction

Whole-cell protein extraction were performed from different pellets before and after incubation in the caecal medium for each condition, as described previously (Rabah et al., 2018b). Pellets were subjected to centrifugation at 8 000 x g for 10 min at 37°C. Bacterial pellets were washed three times in 10 mL of PBS and centrifuged at 8 000 x g for 10 minutes at 37°C. Pellets were suspended in 1 mL of lysis buffer containing 0.1 M Tris-HCl, 0.003 g SDS, 0.03 g DTT, add 1 mM phenylmethylsulfonyl fluoride (PMSF). The samples were then sonicated and cells were lysed using zirconium beads in the Precellys® Evolution homogenizer. Subsequently, cell lysis extracts were subjected to centrifugation at 20 000 x g for 20 min at 4°C. At the end of this process, proteins extracts were collected. The protein concentration was quantified by using 2-D Quant Kit (GE Healthcare Bio-Sciences AB) according to the manufacturer's recommendations.

Western Blotting

Whole-cells protein extracts were analyzed by Western Blot to detect the SlpB protein. SDS-PAGE was conducted according to the Laemmli procedure (Laemmli, 1970) on a Mini-PROTEAN® Tetra Cell (Bio-Rad, Hercules, CA, United States). Gels were transferred to PVDF membranes (Polyvinylidene difluoride) (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 anti-SlpB purified from rabbit sera (AGRO-BIO, France) (Do Carmo et al., 2017; Rabah et al., 2018b).

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).

Statistical analyses

To analyze the survival rate of *P. freudenreichii* in the caecal medium from different growth media and after different treatment, we used 2 way ANOVA with the Sidak' s multiple comparison test as a post hoc test. We compared for each treatment in each growth medium, the survival rate after 50 hours incubations in the caecal medium to the initial survival rate (normalized to 100%). The difference in the survival rate between different pellets after 50 hours of incubation was analysed using one-way ANOVA with a Tukey test or student test. Statistical significance was set at $p<0.05$. Calculations were performed using GraphPad Prism Software (Prism 7 for Windows). All data were expressed as mean values ± standard errors of the mean (SD) (n=7).

Results

P. freudenreichii CIRM-BIA 129 survival in the caecal medium according to the growth medium

The optical density for the different cultures oscillated during incubation time (Fig. 1A). No significant difference was observed between the initial and the final OD, regardless of pellets origin (Fig. 1B). However, *P. freudenreichii* survival rate as determined by CFU counting was significantly diminished in the caecal medium (Fig. 1C). The survival rate was 33 ± 10.2 % for the YEL pellet ($p<0.0001$), 44.1 ± 8.4 for the MUF pellet ($p<0.0001$), and 23.5 ± 4.9 % for the cheese pellet ($p<0.0001$), by comparison to the initial survival rate (Fig. 1D). There was also a significant difference in the survival rate between pellets from MUF and cheese after 50 hours incubation ($p<0.01$) (Fig. 1D). In addition, the coloration by LIVE/DEAD fluorescence were performed, few or no dead bacteria were observed, regardless of the pellet origin, after different times of incubation in the caecal medium (Fig. 1E).

Effect of surface proteins proteolysis on P. freudenreichii CIRM-BIA 129 survival in the caecal medium

The proteolysis of surface proteins may occur during digestion in a matrix-dependent manner, as previously mentioned. We thus aimed to investigate how the surface protein proteolysis impacts *P. freudenreichii* adaptation ability in the caecal medium. We examined the survival of *P. freudenreichii* from YEL, MUF or cheese media without or with shaving treatment. For all media, the OD of *P. freudenreichii* cultures oscillated during the incubation period in the caecal medium (Fig. 2 A, C, E), as previously observed for non-treated pellets. As previously observed, the initial and the final OD were similar, regardless of the pellet origin or the treatment (Fig. 2 B, D, and F). However, the shaving treatment enhanced significantly the survival rate of *P. freudenreichii*, whatever the growth medium (Fig. 3 A, C, E). Indeed, for bacteria grown in YEL, the survival rate was 36.1 ± 7.6 % for shaving negative control and 86.8 ± 9.6 % for the shaved YEL pellet (Fig. 3 B). For bacteria from MUF, the survival rate was 37.3 ± 6.6 % for the negative shaving control and 75.4 ± 13.1 % for shaved MUF pellet after the incubation period (Fig. 3 D). For bacteria from cheese, the survival rate was 25.9 ± 5.7 % for the negative shaving control and 62.2 ± 7.5 % for shaved cheese pellet after incubation period (Fig. 3 E). As was previously observed, after LIVE/DEAD fluorescence staining, no dead bacteria were observed after 5, 25 or 50 hours of incubation, regardless of the pellet origin and the treatment (Fig. 4).

De novo expression of S-layer protein SlpB by P. freudenreichii CIRM-BIA 129 in the caecal medium

To examine if the enhanced survival rate of *P. freudenreichii* is specifically related to the absence of surface proteins, we assessed if S-layer proteins, in particularly the major protein SlpB *de novo* expression after incubation in the caecal medium. We examined SlpB expression in YEL, MUF and cheese pellets by Western Blot (Fig. 5A, C, E). For the non-treated pellets and the negative shaving control ones, the expression of SlpB protein was not affected by the incubation in the caecal medium (Fig. 5B, D, F). However, *P. freudenreichii* did not express *de novo* the protein SlpB after surface proteins proteolysis in the caecal medium, as no immunoreactive band was detected compared to the positive control (Fig. 5B, D, F).

P. freudenreichii CIRM-BIA 129 KO SlpB survival in the caecal medium

As observed for the wild-type strain, the OD of the mutant cultures oscillated during incubation time, regardless of the treatment (Fig. 6A). The final OD were not significantly different from the initial OD (Fig. 6B). However, the shaving treatment, as well the negative control shaving treatment enhanced the mutant survival, compared to non-treated pellets (Fig 6C). The final survival rates were 56.30 ± 7.40 % for the non-treated pellets, 95.48 ± 9.52 % for the control negative shaving pellets and 92.27 ± 8.56 % for the shaved pellets (Fig. 6D). As was previously observed for the wild-type strain, no dead bacteria were observed the by LIVE/DEAD fluorescence staining after 5, 25 or 50 hours of incubation in the caecal medium (Fig. 6E).

Discussion

In this study, we aimed to deepen the understanding of *P. freudenreichii* CIRM-BIA 129 adaptation mechanisms to the gut environment. Indeed, the adaptation ability of *P. freudenreichii* to the colon environment was already demonstrated (Saraoui et al., 2013). However, we ignore if this ability depends on the growth medium. We hypothesized that the growth medium may modulate the adaptation ability of *P. freudenreichii* to the colon environment, which in turn can influence the *in situ* probiotic functionalities, such as the production of beneficial metabolites (Rabah et al., 2017). For this purpose, we recreated a medium mimicking the large bowel environment from porcine cecum contents previously recovered (Rabah et al., 2018a). The survival of *P. freudenreichii* from different growth media was monitored by optical density, CFU enumeration and by observation of bacteria viability using LIVE/DEAD fluorescence staining.

P. freudenreichii survival decreased drastically in the caecal medium, whatever the growth medium tested: the milk ultrafiltrate, cheese or YEL. However, the OD was stable and the fluorescence images did not show dead bacteria at different times of incubation in the caecal medium. The gluconate, which can be used as a carbon source, was totally consumed after 50 hours of incubation in the caecal medium (Fig. S1), in accordance with previous reports indicating *P. freudenreichii* ability to use gluconate as a carbon and energy source (Loux et al., 2015), including in the intestinal conditions (Saraoui et al., 2013).

Indeed, during a previous *in vivo* study, *P. freudenreichii* CIRM-BIA 1 down-regulated genes involved in proteins synthesis and induced genes involved in cell division after one day of incubation in pigs 'colon, with a survival rate of 66 % (Saraoui et al., 2013). In addition, *P. freudenreichii* CIRM-BIA 129 was shown also previously to adapt to a long nutrient shortage in YEL medium by using a viable but non-cultivable state (VBNC) (Aburjaile et al., 2016a). We speculate thus that *P. freudenreichii* uses a viable but non-cultivable state to adapt to the caecal environment, regardless of the growth medium. The state of dormancy is described as "a reversible state of low metabolic activity in which cells can persist for extended periods without division" (Smeulders et al., 1999). The cells are considered as 'viable but non-culturable' cells (VBNC) and the resuscitation of VBNC cells is allowed in favorable conditions (Oliver, 2010; Ramamurthy et al., 2014; Smeulders et al., 1999). Consequently, we made two hypothesis to explain ours results. The first hypothesis is that 100 % of bacteria are in a dormancy state in the caecal medium and that the resuscitation mechanisms are completely independent from the adaptation mechanisms in the caecal medium. The second hypothesis is that the percentage of survival rate determined corresponds to viable bacteria and that the remainder of bacteria are in VBNC state.

These survival mechanisms seem to depend on the growth medium. The growth in milk ultrafiltrate or in hyperconcentrated sweet whey may confer to *P. freudenreichii* an advantage linked to the accumulation of carbon and energy storage components, such as polyphosphates, compared to the growth in cheese (Dalmasso et al., 2012; Huang et al., 2016). Surprisingly, the proteolysis of surface proteins seems to influence also the survival of *P. freudenreichii* in the caecal medium. We assumed that the absence of Slps proteins, in particularly the protein SlpB, is involved in this mechanism. Indeed, *P. freudenreichii* CIRM-BIA 129 did not express the protein SlpB after surface proteins proteolysis, regardless of the growth medium. In addition, the KO SlpB mutant strain showed a better survival ability compared to the wild-type strain. Moreover, the shaving treatment induced also an enhanced survival ability, as observed for the wild-type strain. The SlpB mutation was shown to have a pleiotropic effect on the whole-cell proteome of *P. freudenreichii*, while the *slpb* gene is not located in an operon structure (Do Carmo et al., 2018b). Taken together, these results suggest that other surface proteins may be involved in the enhanced survival, and that SlpB protein may have a regulatory role in adaptation

mechanisms of *P. freudenreichii* to different stresses. Furthermore, the fact that *P. freudenreichii* does not expresses *de novo* the protein SlpB does not reject the possibility that *P. freudenreichii* express *de novo* other surface proteins which may be involved in host-bacteria interaction, and in the modulation of *P. freudenreichii* immunomodulatory effect (Rabah et al., 2018a, 2018b) .

A particular surface protein identified previously attracted our attention, the Resuscitation-promoting factor B (RpfB). RpfB is already identified in the proteins samples resulting from *P. freudenreichii* shaving treatment and was predicted as a secreted protein (Le Maréchal et al., 2015). RpfB is a conserved protein among the actinobacteria phylum (Schroeckh and Martin, 2006) and shown to have a peptidoglycan hydrolase activity (Kana and Mizrahi, 2010; Mukamolova et al., 2006; Telkov et al., 2006). Indeed, data suggest that the activation of dormant cells requires peptidoglycan hydrolysis to facilitate cell division or releases lysis products that function as anti-dormancy signals (Kana and Mizrahi, 2010; Keep et al., 2006).We assumed that the shaving treatment enhanced the expression level of this protein or activated RpfB hydrolase activity. This explanation is in favor of our first hypothesis.

To understand the survival mechanism of *P. freudenreichii* in the caecal medium, the metabolic activity of different treated pellets from different media will be assessed. A comparative quantitative proteomic analysis will address the adaption molecular mechanisms of *P. freudenreichii* in the caecal medium, according to the growth medium and to the shaving treatment. Indeed, viable cells and VBNC may present different proteomic profiles, as demonstrated previously for other bacterial species (Heim et al., 2002; Zhao et al., 2016). The comparative proteomic analysis will also allow a qualitative analysis of the surface proteome of *P. freudenreichii* after shaving treatment in the caecal medium. All results will allow us to understand the growth medium influence on the probiotic properties of *P. freudenreichii* exerted in the digestive tract.

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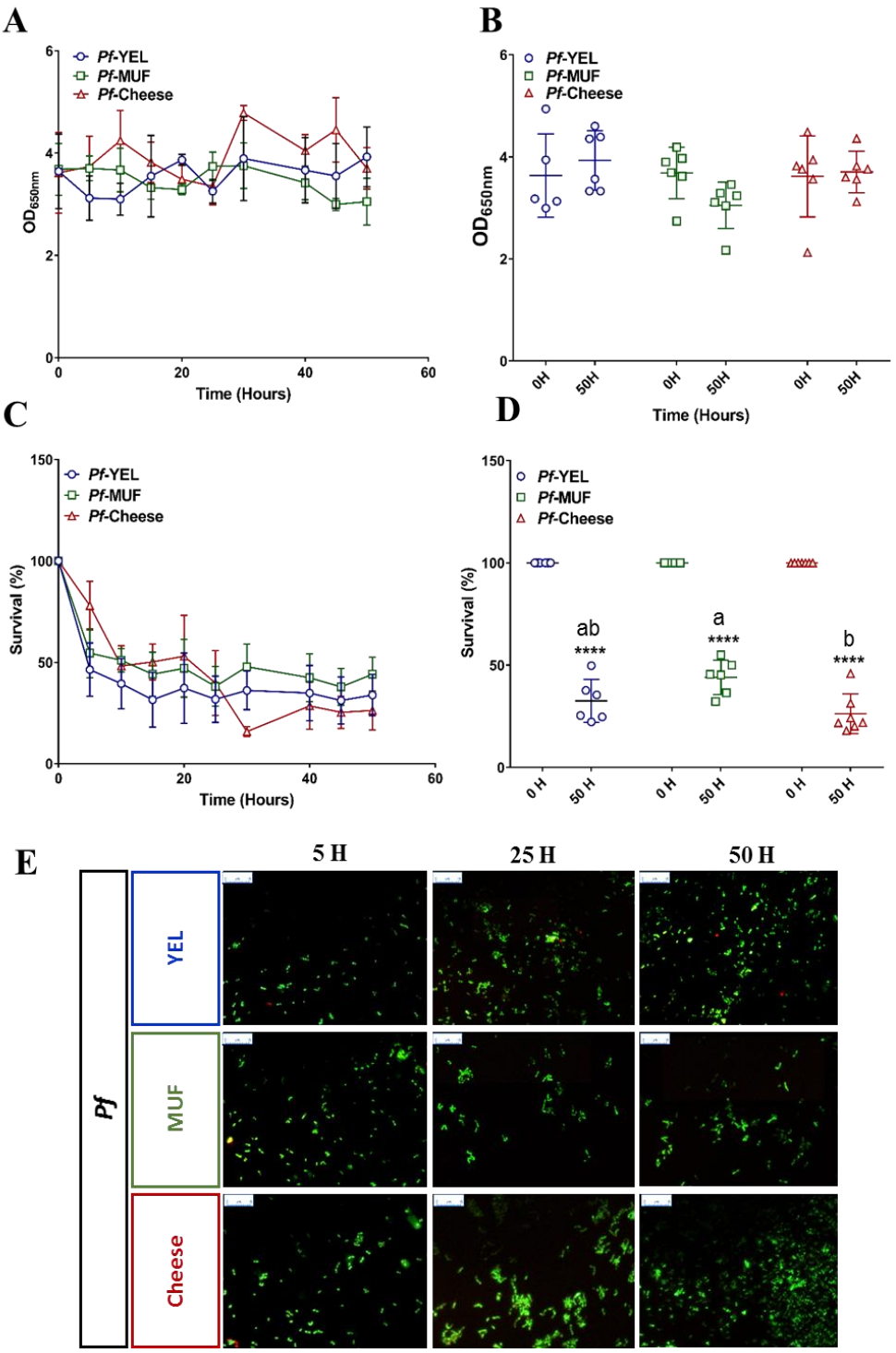

Fig. 1. Survival of *P. freudenreichii* CIMR-BIA 129 in the caecal medium. Pellets from YEL, MUF or cheese media were incubated in the caecal medium during 50 hours in anaerobic conditions at 37°C. Survival was monitored by OD_{650nm} **(A, B)** and by CFU enumeration by plate counting **(C, D)**. Representative Epifluorescence microscopy photos of *P. freudenreichii* CIMR-BIA from different growth media after LIVE/DEAD staining after 5, 25 or 50 hours of incubation in the caecal medium are shown **(E)**. Results are the mean values of triplicate experiments. Error bars represent the standard deviation. **** P< 0.0001. Letters indicate significant difference between the three different pellets after incubation in the caecal medium (P<0.05).

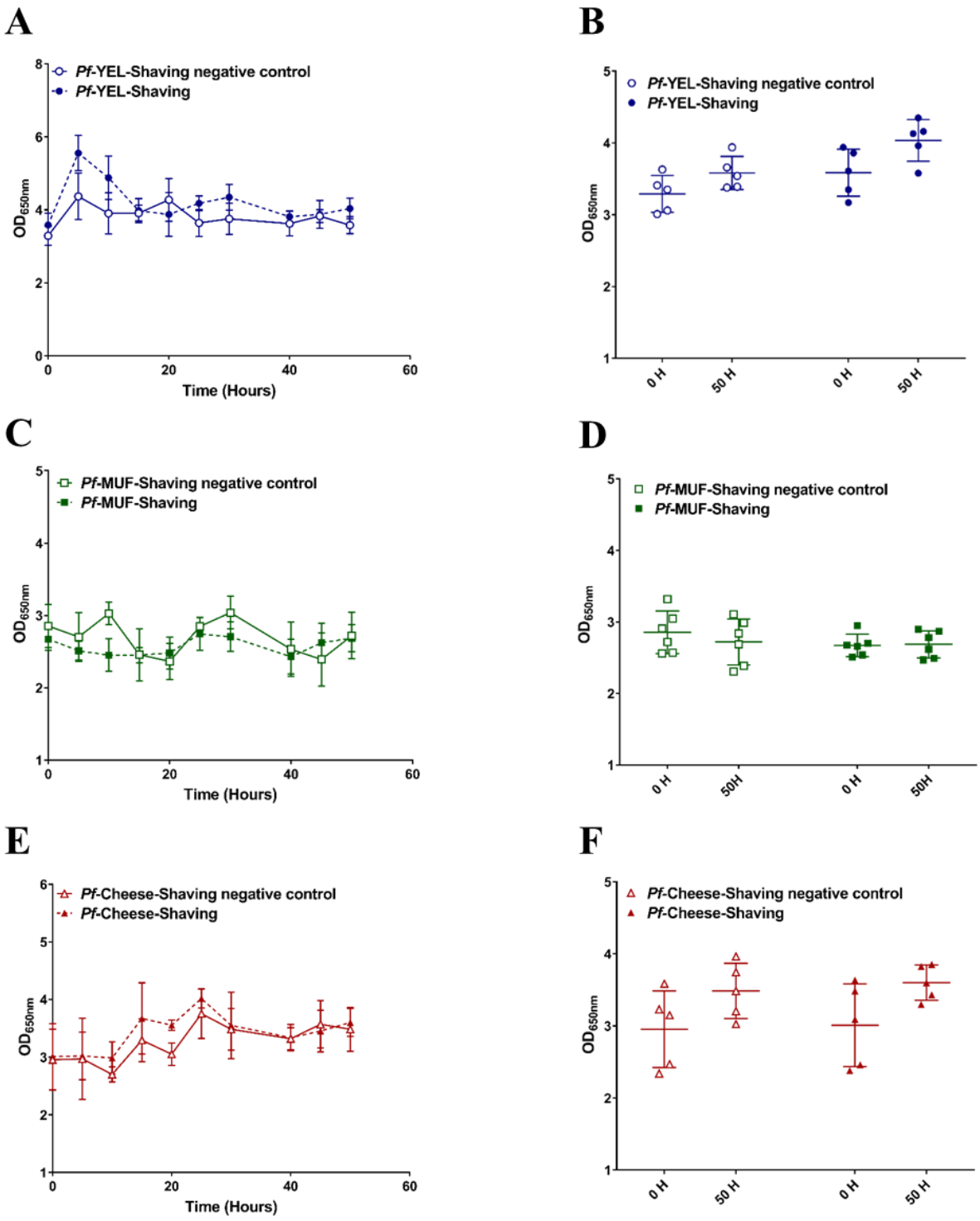

Fig. 2. Survival of *P. freudenreichii* CIMR-BIA 129 in the caecal medium monitored by optical density (OD_{650nm}) measurement. Pellets from YEL (**A**, **B**), MUF (**C**, **D**) or cheese (**E**, **F**) were submitted to a negative shaving control or shaving treatments to proteolysis surface proteins. Cultures were incubated in the caecal medium for 50 hours in anaerobic conditions at 37°C. Results are the mean values of triplicate experiments. Error bars represent the standard deviation.

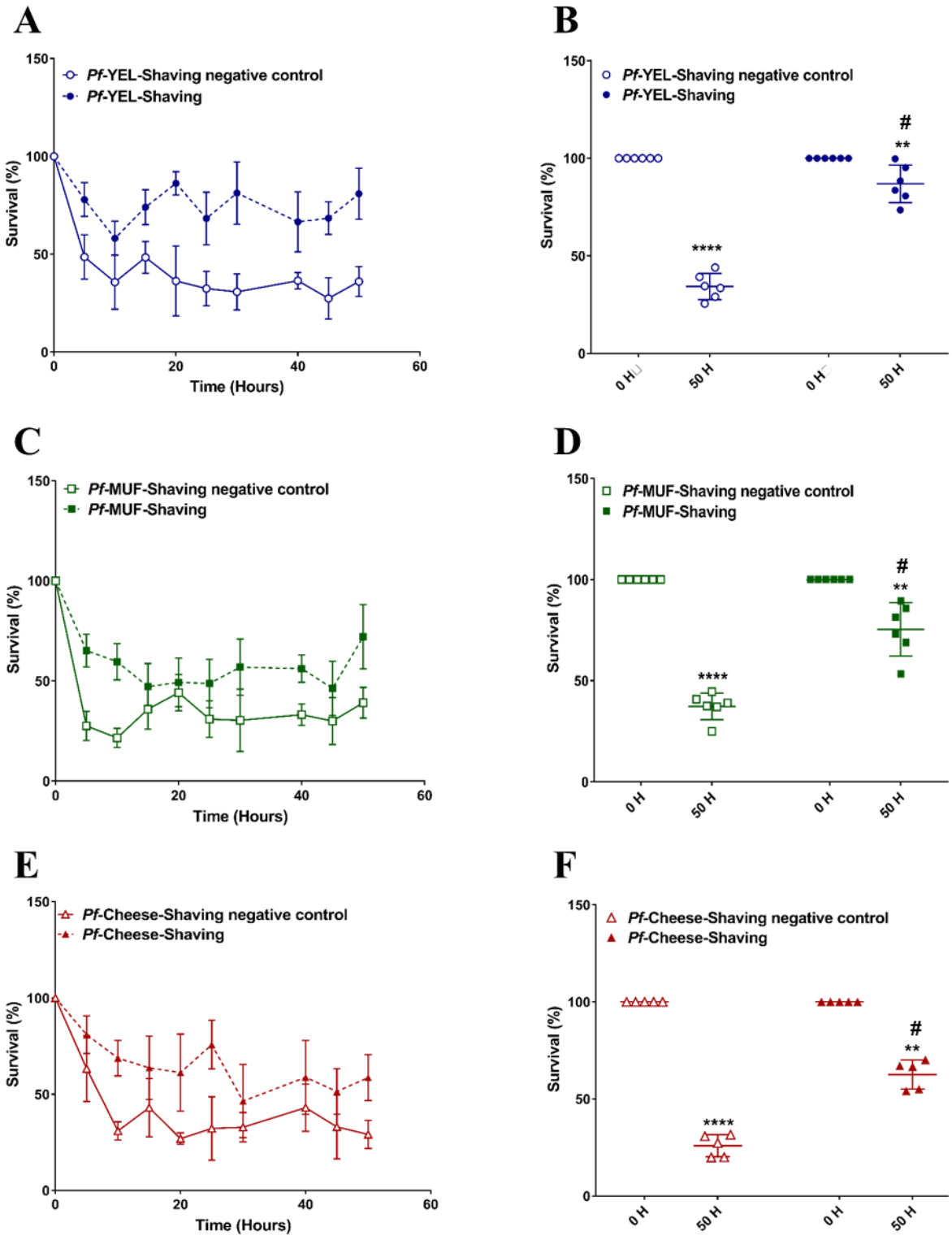

Fig. 3. Survival of *P. freudenreichii* CIMR-BIA 129 in the caecal medium monitored by CFU counting. Pellets from YEL (**A**, **B**), MUF (**C**, **D**) or cheese (**E**, **F**) were submitted to a negative shaving control or shaving treatments to proteolysis surface proteins. Cultures were incubated in the caecal medium for 50 hours in anaerobic conditions at 37°C. Results are the mean values of triplicate experiments. Error bars represent the standard deviation. ** P<0.01, **** P< 0.0001. # indicate significant difference between the three different pellets after incubation in caecal medium (P<0.05).

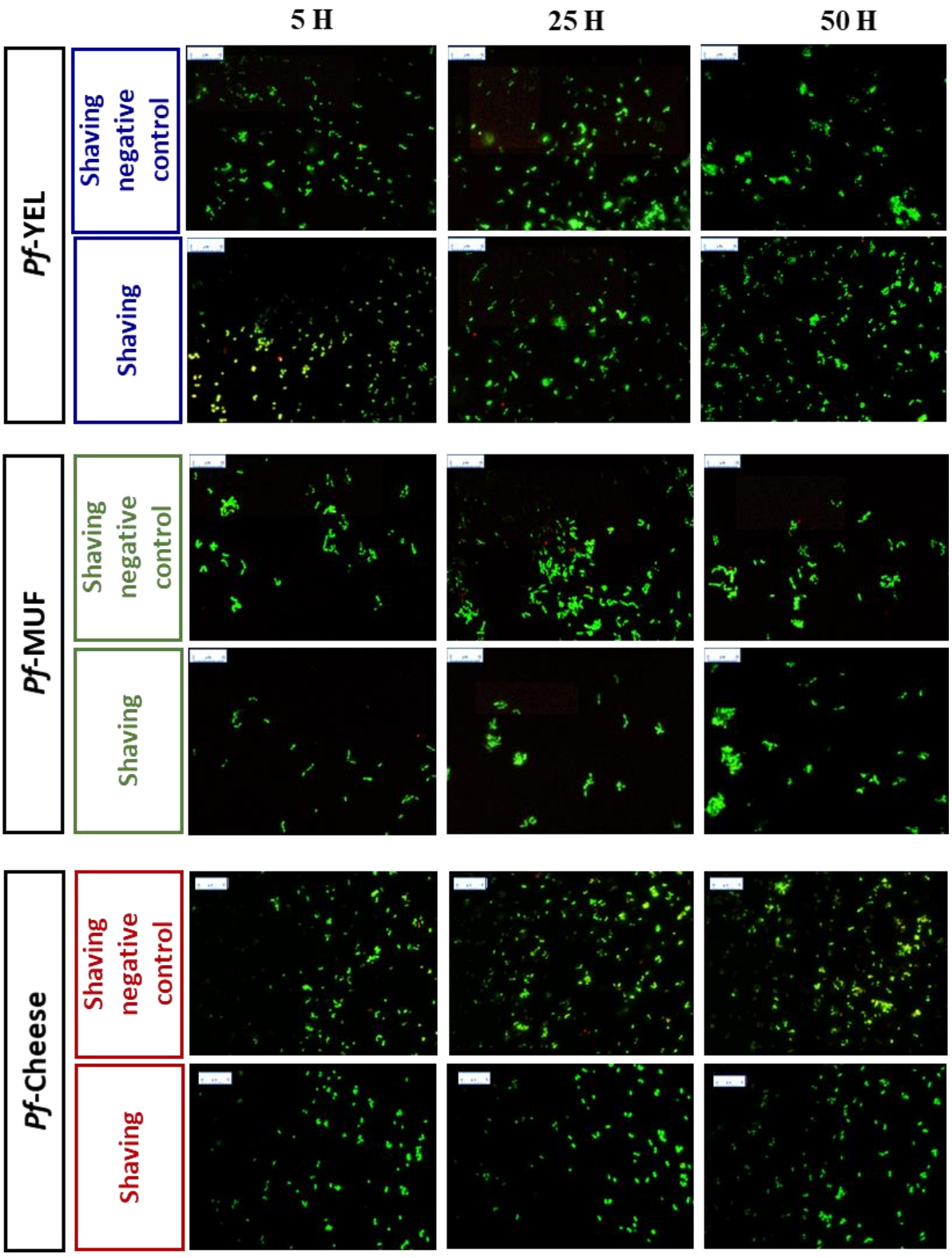

Fig. 4. Representative Epifluorescence microscopy photos of *P. freudenreichii* CIMR-BIA 129 from YEL, MUF or cheese, stained with LIVE/DEAD staining after 5, 25 or 50 hours of incubation in the caecal medium with or without shaving treatment.

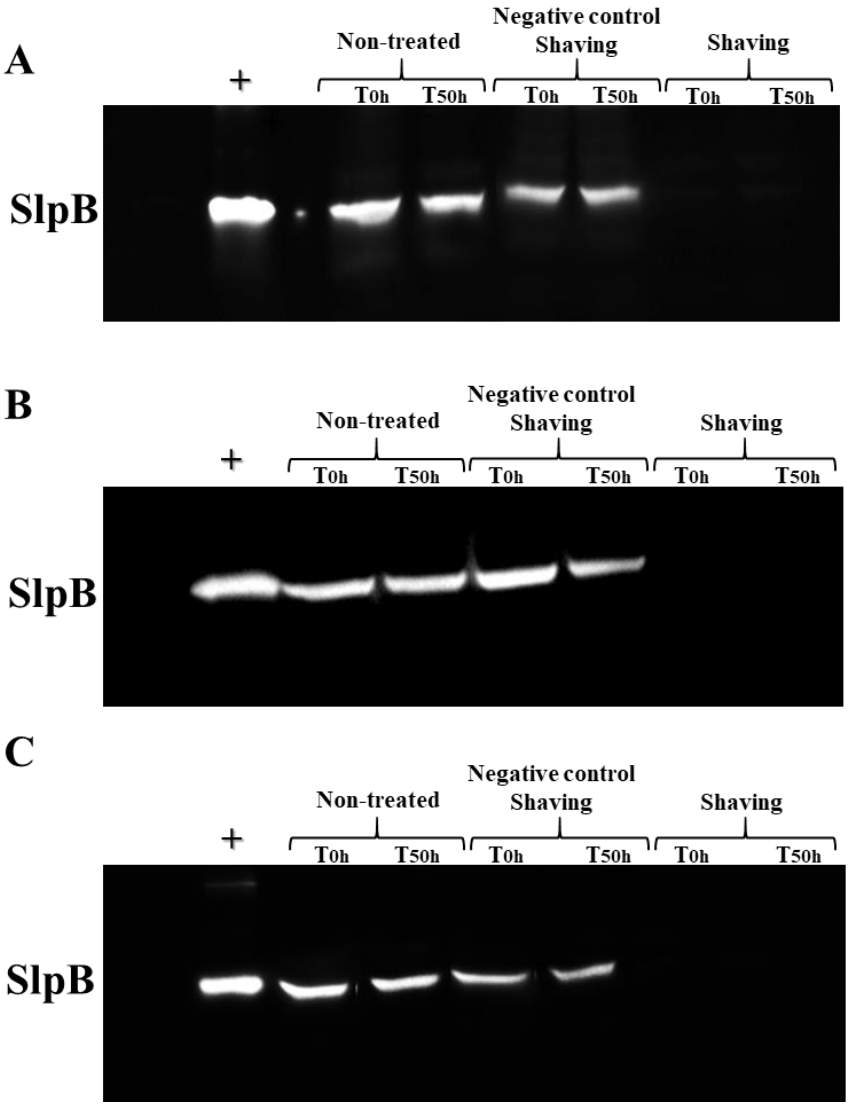

Fig. 5. Analysis of S-layer protein B (SlpB) expression by *P. freudenreichii* CIMR-BIA 129 from YEL, MUF and cheese after incubation in the caecal medium. Pellets from YEL (**A**), MUF (**B**) or cheese (**C**) were incubated 50 hours in the caecal medium, after no treatment, negative shaving control or shaving treatments. Whole-cell protein extracts from different cultures were analyzed by Western Blot detection, using an anti-body directed against SlpB. A surface layer proteins extract was loaded as a positive control (+).

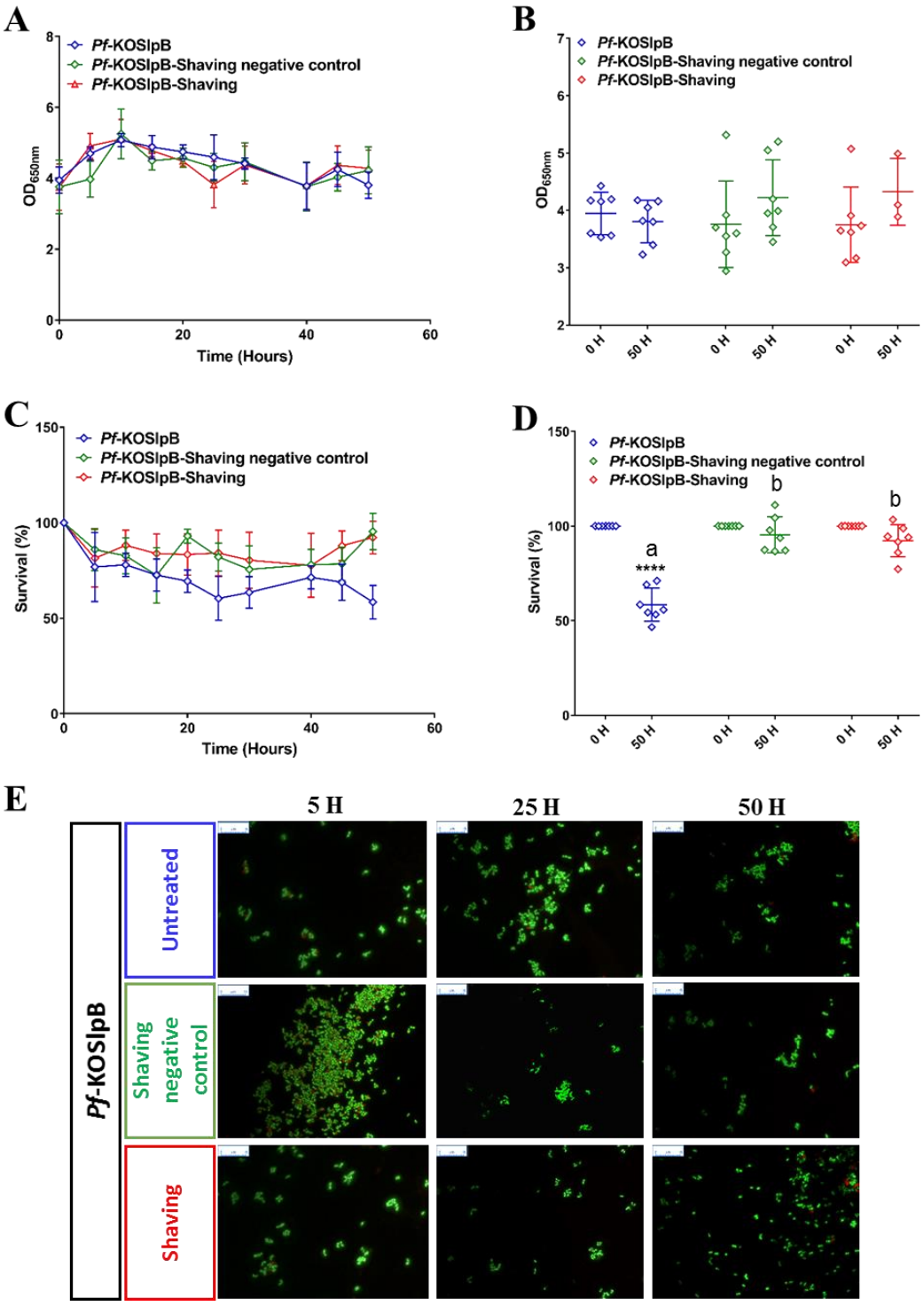

Fig. 6. Survival monitoring of *P. freudenreichii* CIMR-BIA 129 KO SlpB in the caecal medium. Pellets from YEL medium were submitted to shaving or to a negative shaving control treatment. Culture were incubated in the caecal medium during 50 hours in anaerobic conditions at 37°C. Growth was monitored by OD_{650nm} (**A**, **B**) and by CFU enumeration (**C**, **D**). Representative Epifluorescence microscopy photos of *P. freudenreichii* CIMR-BIA 129 KO SlpB after LIVE/DEAD staining of different pellets (**E**). Results are the mean values of triplicate experiments. Error bars represent the standard deviation. **** P< 0.0001. Letters indicate significant difference between the three different pellets after incubation in the caecal medium (P<0.05).

Supplemental data

Measured Parameter	
Gluconate (g/L)	**72.45** ± 13.1
Galactose (g/L)	**0.41** ± 0.075
Lactic acid (g/L)	**0.03** ± 0,008
Valeric acid (g/L)	**0.27** ± 0.07
Butyric acid (g/L)	**1.20** ± 0.2892
Propionic acid (g/L)	**2.82** ± 0.55
Acetic acid (g/L)	**3.71** ± 0.67
Pyruvate (g/L)	**0.003** ± 0,0005
Citrate (g/L)	**0.02** ± 0.004
Osmotic pressure (osmol)	**0.0147** ± 0.001
pH	**6** ± 0.11

Table S1. The initial caecal medium composition in organic acids and sugars was assessed by HPLC analyses. The osmotic pressure and pH were also measured.

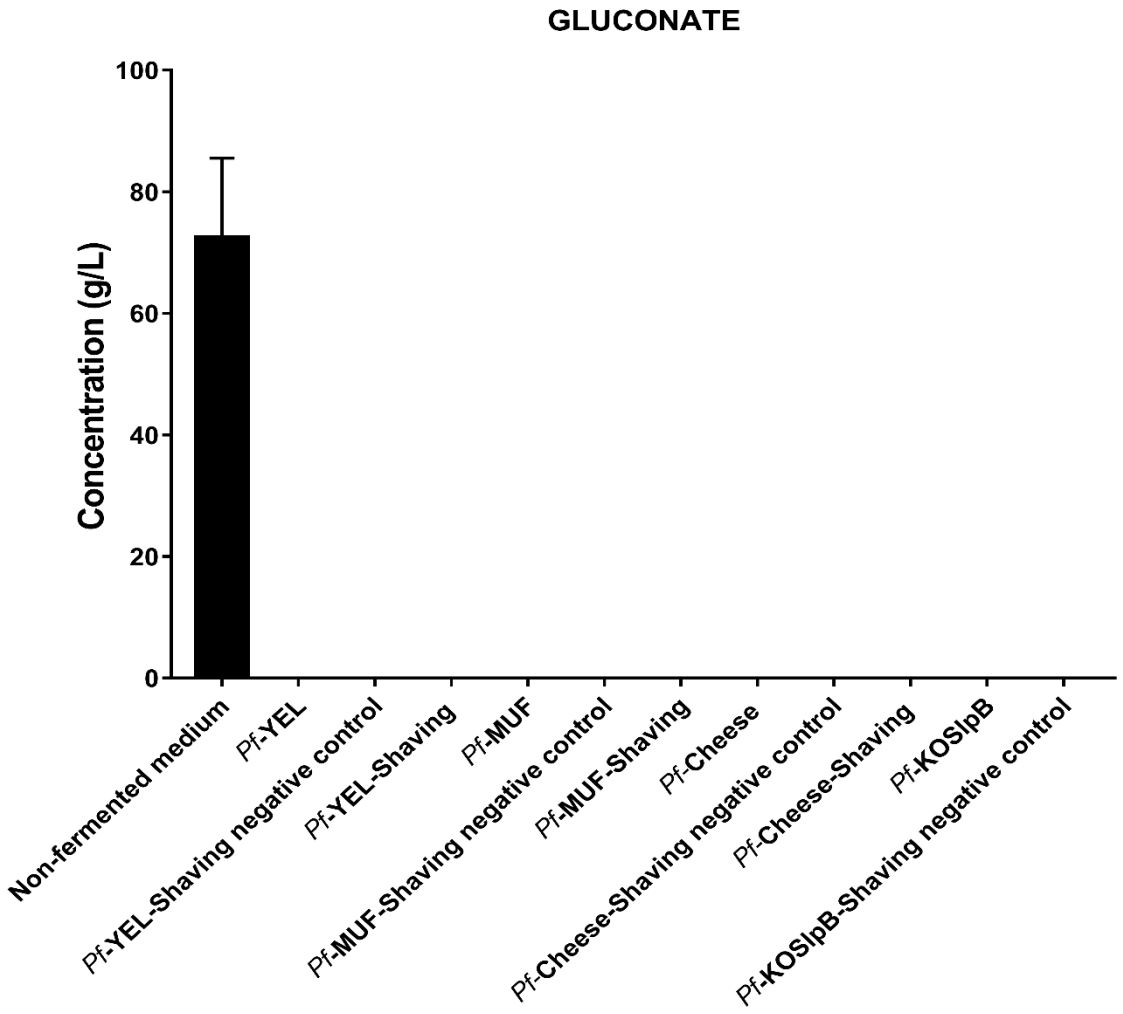

Fig.S1. The consumption of gluconate, as a carbon source, by *P. freudenreichii* CIRM-BIA 129 and *P. freudenreichii* CIRM-BIA 129 KO SlpB in the caecal medium. The *P. freudenreichii* CIRM-BIA 129 were extracted from different media: milk ultrafiltrate culture, YEL culture and fermented cheese. The *P. freudenreichii* CIRM-BIA 129 KO SlpB pellet were from YEL culture. The different pellets were submitted to different treatments: non-treated, shaving negative control or shaving treatments to proteolysis the surface proteins. Gluconate concentration was determined before and after incubation in the caecal medium by HPLC analysis.

5.3. Conclusion

Dans ce chapitre, nous avons souhaité comprendre d'une part si les mécanismes d'adaptation de *P. freudenreichii* (***Pf***) à l'environnement colique sont matrice-dépendants. D'autre part, nous avons souhaité confirmer l'hypothèse que l'effet immunomodulateur matrice-dépendent observé *in vivo* est lié essentiellement à une différence de protéines de surface, en particulier les protéines Slps. En effet, les résultats de digestion *in vitro* ont montré que la vectorisation par la matrice fromagère apporte une proportion de bactéries dont le protéome de surface est intacte, la protéine SlpB y étant présente. Cependant, lors de l'expérimentation sur les porcelets, la protéine SlpB n'a pas pu être détectée dans les contenus digestifs par Western Blot pour des problèmes méthodologiques.

Nous avons ainsi étudié l'adaptation de ***Pf*** CIRM-BIA 129 et de la souche ***Pf*** CIRM-BIA 129 KO SlpB à l'environnement caecal en fonction du milieu de croissance et de la protéolyse des protéines de surface. La source de carbone majoritaire, le gluconate, a été totalement consommée, par la souche sauvage ou mutante, confirmant ainsi la capacité de ***Pf*** à dégrader ce composé abondant dans le tractus digestif des animaux. Cela permet de valider l'utilisation de ce milieu comme modèle pour mimer l'environnement colique. Cependant, ce modèle présente des limites, du fait de la disponibilité du gluconate. Dans le côlon, il serait constamment renouvelé et pourrait être consommé par d'autres flores du microbiote intestinal (Chang et al., 2004).

Les résultats montrent que ***Pf*** possède une capacité d'adaptation à l'environnement caecal matrice-dépendante (ultrafiltrat de lait > fromage) et semble adopter un état viable non cultivable (VBNC), quel que soit le milieu de croissance. En effet, nous ignorons si la proportion de bactéries viables dénombrées correspond à une population viable ou à une population VBNC qui a retrouvé un état viable sur le milieu gélosé riche YELA. De plus, la protéolyse des protéines de surface améliore la capacité de survie de *P. freudenreichii* dans le milieu caecal, quel que soit le milieu de croissance. Des observations similaires ont été faites sur la souche mutante pour le gène *slpb*, qui a présenté une meilleure capacité de survie que la souche sauvage. Ces résultats montrent que les phénomènes d'adaptation de ***Pf*** sont également liés à la présence de certaines protéines de surface, telle que la protéine SlpB ou RfpB. De plus, nous

avons constaté qu'une fois que la protéine SlpB est protéolysée, ***Pf*** ne synthétise pas *de novo* cette protéine dans l'environnement caecal. Ce résultat pourrait expliquer partiellement la différence en termes d'immunomodulation observée *in vivo* entre une vectorisation par l'ultrafiltrat de lait ou une vectorisation par le fromage. Néanmoins, cela n'indique pas forcément que les autres protéines ne sont pas synthétisées *de novo* après leur protéolyse. Pour cela, une analyse de protéomique comparative est en cours entre les différentes bactéries provenant de différents milieux de culture, et ayant subi ou non un traitement de rasage des protéines de surface. L'analyse protéomique devrait répondre aux questions suivantes :

- Quels sont les mécanismes d'adaptation de ***Pf*** au milieu caecal en fonction du milieu croissance ?
- ***Pf*** synthétise-t-il *de novo* les autres protéines de surface après la protéolyse de ces dernières ?
- ***Pf*** adopte-il un état viable non cultivable pour s'adapter au milieu caecal ?

Une analyse métabolique est également en cours pour évaluer l'activité métabolique de ***Pf*** dans le milieu caecal, notamment la production des acides gras à chaîne courte et le facteur DHNA. Ces résultats viendront compléter ce projet de publication.

Chapitre 5 : à retenir

- ***Pf*** **est métaboliquement actif et s'adapte en consommant du gluconate**
- ***Pf*** **semble adopter un état viable non cultivable pour s'adapter à l'environnement caecal**
- **Les mécanismes d'adaptation de *Pf* semblent dépendre du milieu de croissance, ainsi que de la protéolyse des protéines de surface**
- ***Pf*** **ne synthétise pas *de novo* la protéine SlpB dans l'environnement caecal après sa protéolyse**

Chapitre 6 : « L'étude *in vivo* de l'effet anti-inflammatoire d'un emmental «probiotique» pour la prévention de la colite murine »

6.1. Introduction

Dans la première partie de ce projet de thèse, nous avons montré que la matrice fromagère, tout comme une culture sur l'ultrafiltrat de lait, sont de bons vecteurs pour les propriétés immunomodulatrices de *P. freudenreichii*, mais avec des effets immunomodulateurs différents (Chapitre 4). Cependant, dans la perspective de développer des fromages fonctionnels fermentés par les bactéries propioniques probiotiques, l'emmental semble être un candidat potentiel pour vectoriser d'importantes quantités de bactéries propioniques jusqu'au côlon.

Au sein de l'emmental, différentes espèces bactériennes coexistent et sont sélectionnées pour obtenir les caractéristiques finales souhaitées en termes technologiques et organoleptiques, afin de respecter un cahier des charges bien précis (Legifrance). En effet, les bactéries lactiques sont utilisées, en plus des bactéries propioniques, pour l'élaboration de l'emmental. En fin d'affinage, les bactéries propioniques sont à environ 10^9 CFU/g alors que les bactéries lactiques sont à environ 10^6 CFU/g. Nous avons émis initialement l'hypothèse que la présélection de souches de *Lactobacillus sp*, de *S. thermophilus* ainsi que de *P. freudenreichii*, selon leurs propriétés probiotiques et immunomodulatrices, aura un effet synergique sur l'efficacité anti-inflammatoire d'un fromage emmental (Figure 10).

Nous avons fait le choix de sélectionner une combinaison de trois souches, en s'appuyant sur les données bibliographiques disponibles, sans faire au préalable un criblage de différentes combinaisons possibles sur un modèle *in vitro* (Stimulation des cellules PBMC). Les souches sélectionnées ont été : *S. thermophilus* LMD-9, *L. delbrueckii* subsp. *lactis* CNRZ327 et ***Pf*** CIRM-BIA 129 (Foligné et al., 2013; Plé et al., 2015). Des propriétés immunomodulatrices ont en effet été rapportées pour les souches LMD9 (Junjua et al., 2016), CNRZ327 (Santos Rocha et al., 2012, 2014) et CIRM-BIA 129 (Plé et al., 2015). En effet, au-delà des propriétés probiotiques propres à chaque souche, on suppose que des interactions peuvent exister entre les différentes bactéries, pouvant ainsi affecter leurs propriétés probiotiques mutuelles. Un fromage emmental a été donc fabriqué à l'échelle industrielle en utilisant ces souches et son effet anti-inflammatoire a été évalué sur un modèle murin de colite. Un protocole de prévention contre la colite induite par le dextran sodium sulfate (DSS) a été mis place. Ainsi, des souris C57BL/6

femelles âgées de 6 à 8 semaines ont été utilisées et ont été gavées par différents régimes : 1) PBS, 2) une matrice fromagère stérile, 3) un fromage monosouche fermenté par ***Pf*** CIRM-BIA 129, ou 4) un fromage emmental « probiotique ». Les souris ont reçu par gavage les différentes matrices pendant 7 jours, avant l'induction de la colite par l'administration du DSS. Les marqueurs macroscopiques ont été évalués pendant 7 jours, le temps de l'ingestion du DSS par les souris. Après sacrifice des souris au 15^{éme} jour, les différents marqueurs macroscopiques et microscopiques ont été évalués afin d'examiner la sévérité de la colite.

Figure 10. **Quel est l'effet d'un emmental « probiotique » dans un modèle de colite chimiquement induite chez la souris ?** Le chapitre 6 (en rouge) expose les résultats de l'effet protecteur d'un fromage emmental dit « probiotique » pour prévenir la colite dans un modèle murin.

L'article présentant les résultats de ce chapitre est en préparation et sera soumis au Journal of Functionnal Food.

6.2. Article 4 : l'effet protecteur d'un emmental probiotique dans la prévention de la colite murine induite par le DSS

Effect of probiotic Emmental cheese on DSS-induced colitis in mice

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Introduction

Functional foods are defined as “ingredients that affect beneficially one or more target functions in the body, beyond adequate nutritional effects, in a way that is relevant to either an improved state of health and well-being and/or reduction of the risk a disease” (Diplock et al., 1999; Saris et al., 1998). The dairy fermented foods, including cheeses, constitute a large part of our daily diet. Recently, several investigations showed that some starter bacteria, typically employed in dairy fermented foods, can exert probiotic properties such as microbiota modulation, anti-cancerous and anti-inflammatory effects, in strain-dependent manner. A probiotic is defined as “a live microorganism which, when administrated in adequate amount, confers a health benefit on the host” (FAO, WHO, 2006). The perspective to develop functional dairy fermented foods by using probiotic starter bacteria, presents a promising manner to reduce the risk of diseases, which are related to lifestyle and diet. Some lactic acid bacteria and dairy propionibacteria strains were characterized for their immunomodulatory properties, specifically in inflammatory bowel disease (IBD) (Rabah et al., 2017; Santaolalla and Abreu, 2012). IBD includes two main pathologies: ulcerative colitis (UC) and Crohn’s disease (CD). They are supposed 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 (Ko and Auyeung, 2014; Vindigni et al., 2016; Zhang and Li, 2014). There are arguments indicating that ingested probiotic microorganisms could play a favorable role in the treatment of UC (Derwa et al., 2017; DuPont et al., 2014). Consumption of food-grade microorganisms selected for their probiotic application, alone or in combination with conventional drugs, has been shown to induce and/or to enhance remission in UC patients. New evidences revealed the anti-inflammatory potential of *P. freudenreichii*, which is a dairy *Propionibacterium*, and routinely added, in association with lactic acid bacteria, as a starter in the production of Swiss-type cheeses such as Emmental cheese (Thierry et al., 2011b). They contribute to the Emmental characteristic flavor and opening. Propionibacteria and lactic acid bacteria produce valuable metabolites with anti-inflammatory properties such as short fatty acids, DHNA, conjugated fatty acids and surface proteins, which are produced in food grade matrices such as cheese (Rabah et al., 2017). Indeed, the consumption of experimental cheese fermented by *P. freudenreichii*, alone or with *Lactobacillus delbrueckii* subsp. *lactis*, protected mice from acute colitis induced by TNBS (Plé et al.,

2015, 2016). Analogous functionalities were reported for lactic acid bacteria, strains also used for Emmental cheese manufacturing (George et al., 2018). Regarding all those data, we hypothesized that selection of specific lactic acid bacteria (LAB) and dairy propionibacteria strains with immunomodulatory properties could be used to prepare a potentially probiotic Emmental cheese with anti-inflammatory activities (Plé et al., 2015, 2016; Santos Rocha et al., 2012, 2014). Therefore, the aim of this study was to evaluate the beneficial properties of a Emmental through the fermentation of three selected anti-inflammatory strains: *P. freudenreichii* CIRM-BIA 129 (Plé et al., 2015), *Lactobacillus delbrueckii* subsp. *lactis* CNRZ327 (Santos Rocha et al., 2014), *Streptococcus thermophilus* LMD-9 (Junjua et al., 2016), by using *in vivo* murine DSS-induced colitis. This Emmental cheese was produced on a local industrial production chain, in order to be as close possible to industrial cheese.

Material and methods

Bacterial strains

Lactobacillus delbrueckii subsp. *lactis* CNRZ327, *Propionibacterium freudenreichii* CIRM-BIA 129 (equivalent to ITGP20 strain) and *Streptococcus thermophilus* LMD-9, were provided by the microbiological resource center CIRM BIA (Centre International de Ressources Microbiennes Bacteries d'Intérêt Alimentaire). They were routinely cultured respectively in MRS, YEL or M17 culture media, according to CIRM-BIA standard procedure.

Cheeses manufacturing

The experimental single-strain cheese solely fermented by *P. freudenreichii* CIRM-BIA 129 was prepared as previously described (Plé et al., 2015, 2016). The biochemical composition of the cheese was: dry matter 58 g/100g, lipids 28 g/100g, proteins 29 g/100g, carbohydrates 0 g/100g, and calcium 840 mg/100g (Plé et al., 2015). The cheese matrix is a sterile dairy matrix prepared in the same way as the single-strain cheese. Gluconodeltalactone was used to acidify the sterile supplemented milk before cheese matrix manufacturing procedure, as described previously (Plé et al., 2015). Propionibacteria were enumerated on Yeast-Extract-Lactate agar (YELA). Coliforms, mesophilic flora, thermophilic flora,

yeast, and molds, all below 10 CFU/G, were also enumerated (Table S2). The probiotic Emmental cheese was manufactured by Entremont Alliance© Company (Malestroit, France) using their production standard process. *Lactobacillus delbrueckii* subsp. *lactis* CNRZ327, *Streptococcus thermophilus* LMD-9 and *P. freudenreichii* CIRM-BIA 129 were used as starters. The propionibacteria, lactic acid bacteria and specific contaminant flora were enumerated according to Entremont Alliance© standards (Table S2). The identity of strains used as starter were checked by PFGE analyses according to CIRM-BIA procedures (Table S3). *P. freudenreichii* CIRM-BIA 129 was the only propionibacteria strain present in the Emmental cheese (Table S3). Other strains of *L. delbrueckii* were present but the strain CNRZ327 was predominant. For *S. thermophilus*, four different strains were found and the strain LMD-9 was not the predominant one (Table S3).

Animals, Feeding procedure and Dextran sulfate sodium induced colitis

Female C57BL6 mice (6-8 weeks old,) were obtained from Federal University of Minas Gerais (UFMG–Belo Horizonte, Brazil) and the study was approved by the Brazilian Ethics Committee on Animal Use (CEUA). They were randomly divided into groups of six and housed in a controlled environment (with a temperature of 25°C, a 12 h/12 h light/dark cycle and ad libitum access to food and water). For each preventive treatment (PBS, cheese matrix, single-strain cheese or Emmental cheese), two groups were constituted of 6 mice, one of which was treated to induce colitis. Mice were fed by gavage for seven consecutive days: 500 µl of PBS buffer, 100 mg of the sterile dairy matrix, and 100 mg of single-strain cheese and 400 mg of Emmental cheese. *P. freudenreichii* daily intake was set to 10^9 CFU, for the single-strain cheese and the Emmental cheese. Cheeses were grinded by ultrathurax in phosphate buffer saline (PBS). Then, DSS-colitis was induced by adding 3% dextran sulfate sodium (DSS) (36,000-50,000 M.Wt, MP Biomedicals) to the drinking water for 7 days.

Assessment of DSS-induced colitis

The severity of colitis was assessed during 7 days before animals sacrifice. Weight loss, stool consistency and blood content were assessed. The disease activity index (DAI) score was calculated based on these markers, as previously described (Cooper et al., 1993). For each parameter, a score was

given: absent (0), mild (1), moderate (2) and severe (3). The DAI correspond to the sum of different scores.

Quantification of secretory IgA

After mice sacrifice, the small bowel was collected and the content was washed by PBS. The intestinal contents were vortexed and centrifuged (850 g, 30 min, 4°c). The supernatants were recovered and IgA concentration was assessed by ELISA, as previously described (Carvalho et al., 2017).

Real-time quantitative PCR and gene expression analysis

Colon fragments were collected and stored in RNAlater at -80°C until RNA extraction. Total RNA was isolated using RNeasy mini kit according to manufacturer's instructions. Residual DNA was digested by adding DNase I. Samples were then treated with Turbo DNA free Kit®. cDNA for each sample was produced with high capacity cDNA Reverse Transcription kit. Quantitative PCR was performed using iTaq universal SYBR green supermix and by using gene specific primers for colonic cells (Table 1). Actin and GAPDH genes were used as housekeeping genes. Amplification reactions were performed on an ABI PRISM 7900HT Sequence detection system. Expression levels are represented as fold changes ($2^{-\Delta\Delta Ct}$), using the means and standard deviation of target genes.

Tissues preparation and cytokines quantification by ELISA

Colon fragments were homogenized in PBS solution containing 0.05 % tween-20, 0.1 mM phenylmethylsulfonyl fluorid, 0.1 mM benzethonium chloride, 10 mM EDTA and 20 KIU aprotinin. Tissues mixtures were centrifuged (3 000 g, 10 min) and supernatants were collected for ELISA assays. Plates were coated with purified monoclonal antibodies anti IL-10, IL-1β, IL-12p70, IL-17, IFNγ, TGFβ, TNFα and IL-6, overnight at 4°C. Plates were washed by TBS (Tris-buffered saline) and supernatant from homogenized colon tissues were added. Plates then were incubated overnight at 4°C. After plates washing, biotinylated monoclonal antibodies against different cytokines were added to coated plates and incubated for 2 hours at room temperature. The revelation were performed by adding 100 µl/well of a citrate buffer containing orthophenyldiamine (1 mg/ml) and 0.04 % (v/v) H_2O_2. The

revelation reaction were stopped by the addition of 2N H_2SO_4 solution. The absorbance was measured at 492 nm.

Statistical analysis

The protective effect of the different cheeses in the DSS-induced colitis preventions was analyzed using one-way ANOVA with Tukey multiple comparisons test. Statistical significance was set at $p<0.05$. Calculations were performed using GraphPad Prism Software (Prism 7 for Windows). All data were expressed as mean values and standard deviation (SD) (n=7).

Results

Mortality, body weight loss, colon length

DSS-induced colitis caused significant body weight loss in all mice groups, compared to the healthy group (PBS) (Fig1. A). However, the ingestion of the single-strain cheese and of the Emmental cheese attenuated the body weight loss, compared to the PBS-DSS and the cheese matrix-DSS groups (Fig1. A). The DSS-induced colitis increased the disease activity index (DAI), which considers the body weight loss, the severity of diarrhea and the presence of blood in feces (Fig1. B). The cheese matrix intake did not reduce the DAI, whereas the ingestion of the single-strain cheese or of the Emmental cheese significantly attenuated the DAI, compared to the PBS-DSS group (Fig1. B). The DSS-induced colitis caused colon shortening in all mice groups (Fig1. C). The intake of the cheese matrix, the single-strain cheese or the Emmental cheese did not prevent the colon shortening (Fig1. C). Altogether, these results show that the single-strain cheese or the Emmental cheese intake reduced the severity of DSS-induced colitis, without significant difference between the two cheeses.

IgA secretory production

Concentration of secretory IgA in the small intestine of all mice groups was assessed by ELISA quantification. The DSS-induced colitis increased significantly the IgA secretion (Fig2). The cheese matrix intake did not attenuate IgA secretion, whereas the consumption of single-strain cheese and the Emmental cheese reduced significantly IgA secretion, compared to the PBS-DSS mice group (Fig2).

There was no significant difference in IgA secretion between the single-strain cheese and the Emmental cheese (Fig2).

Colonic oxidative stress and epithelial barrier

The gene expression of different marker of the colonic oxidative stress and epithelial barrier integrity were assessed by quantitative RT-PCR (Fig3.). No significant changes were observed in expression of genes involved in epithelial barrier integrity, as *claudind1*, *mucin 2*, *zonula occluden1* and *zonula occluden2* genes (Fig3. A, B, C, D). However, the DSS-induced colitis induced a significant decrease of *occludin* gene expression, which is prevented significantly only by the Emmental cheese intake and not by the cheese matrix intake (Fig3. E). The DSS-induced colitis induced an oxidative stress in colonic cells as showed by the induction of nitrite oxide synthase (iNOS) gene expression (Fig3. F). The cheese matrix attenuated significantly iNOS expression in the colonic cells, compared to the PBS-DSS group (Fig3. F). The single-strain cheese intake tended to decrease iNOS expression, compared to the PBS-DSS group (Fig3. F). There was no significant differences between the Emmental cheese and the healthy or the PBS-DSS groups (Fig3. F).

Pro-inflammatory and anti-inflammatory gene expression in colon

The expression of pro-inflammatory and anti-inflammatory cytokines genes expression in the colonic cells was assessed in all mice groups (Fig4.). The DSS-induced colitis did not modify IL-10 and TGFβ expression, compared to the healthy group (Fig4. A, B). Similarly, the ingestion of the cheese matrix and of the single-strain cheese did not change IL-10 and TGFβ expression (Fig4. A, B). However, the ingestion of the Emmental cheese enhanced IL-10 expression during DSS colitis induction, compared to all mice groups (Fig4. A). The DSS-induced colitis did not induce significant change in IL-1β and TNFα expression in colonic cells, compared to the healthy group (Fig4. C, D). However, the single-strain cheese intake enhanced IL-1β expression during DSS colitis, compared to the healthy group (Fig4. C). The cheese matrix, the single-strain cheese and the Emmental cheese attenuated significantly TNFα expression, compared to the healthy group (Fig4. D). In addition, the DSS-induced colitis increased IFNγ expression in the colonic cells, compared to the healthy group (Fig4. E). The cheese matrix, the

single-strain cheese and the Emmental cheese attenuated significantly IFNγ expression, compared to the healthy group (Fig4. E). There was no significant change of IL-21 expression in all mice groups (Fig4. F).

Pro-inflammatory and anti-inflammatory cytokines concentration in colon

The concentration of pro-inflammatory and anti-inflammatory cytokines in the colonic tissues was assessed in all mice groups by ELISA quantification (Fig5.). The DSS-induced colitis increased IL-10 secretion, compared to the healthy group (Fig5. A). The cheese matrix intake further enhanced secretion of IL-10 during DSS-induced colitis, compared to the healthy group (Fig5. A). However, fermented cheeses ingestion reduced IL-10 induction (Fig5. A). The DSS-induced colitis increased TGFβ secretion in the PBS-DSS and the cheese matrix-DSS groups (Fig5. B). The Emmental cheese and the single-strain cheese ingestion didn't influence significantly TGFβ secretion, compared to the DSS-PBS and the healthy groups (Fig5. B). In case of IL-6, the DSS-induced colitis increased significantly its secretion, compared to the healthy group (Fig5. C). The Emmental cheese intake attenuated significantly IL-6 secretion, compared to the PBS-DSS group (Fig5. C). Similar observations were found for IFNγ secretion, only the Emmental cheese attenuated IFNγ secretion induced by the DSS-induced colitis (Fig5. D). The DSS-induced colitis increased IL-17 secretion, compared to the healthy group, which is exacerbated by the cheese matrix intake (Fig5. E). Only the Emmental cheese tended ($p=0.07$) to decrease IL-17 secretion, compared to the PBS-DSS group (Fig5. E). The DSS-induced colitis induced a significant increase of TNFα only in the cheese matrix group Fig5. F). The DSS-induced colitis did not induce significant change in IL-12 secretion, compared to the healthy group (Fig5. G). Cheeses intake did not either alter significantly IL-12 secretion, compared to healthy group (Fig5. G). Finally, the DSS-induced colitis increased IL-1β secretion, compared to the healthy group, which is not attenuated by the Emmental cheese intake (Fig5. H).

Discussion

In this study, we aimed to investigate the protective effect of a "probiotic" Emmental cheese towards DSS-induced colitis. The probiotic Emmental cheese was manufactured at an industrial scale and starter

bacteria were selected for their immunomodulatory properties previously described, which are: *P. freudenreichii* CIRM-BIA 129 (Plé et al., 2015), *L. delbrueckii* subsp. *lactis* CNRZ327 (Santos Rocha et al., 2014)and *S. thermophilus* LMD9 (Junjua et al., 2016). Indeed, we compared its effects to those of an experimental single-strain cheese, fermented only by *P. freudenreichii* CIRM-BIA 129 and to a germ-free cheese matrix (Plé et al., 2016). We induced colitis in C57BL6 mice by adding 3% (w/v) DSS in drinking water for 7 consecutive days after daily gavage by different cheeses for 7 days. DSS triggered a strong colonic inflammation accompanied by weight loss, diarrhea and rectal bleeding. No mortality was observed. The Emmental cheese and the single-strain cheese attenuated the DSS-colitis severity by reducing the colitis inflammation markers. These results are consistent with the previous studies. *P. freudenreichii* CIRM-BIA 129 alone, or in combination with lactic acid bacteria, attenuates TNBS and DSS-induced colitis (Foligné et al., 2010, 2013; Plé et al., 2015, 2016). However, neither of the two fermented cheeses prevented the colon shortening triggered by the DSS. Similarly, in a previous study, the administration of *P. freudenreichii* CIRM-BIA 129 in combination with lactic acid bacteria did not prevent colon shortening during DSS-induced colitis (Foligné et al., 2016).

The DSS-induced colitis increased the IgA secretion in the small bowel, which is attenuated by the single-strain cheese and the Emmental cheese. The IgA secretion increase is a marker of ileum microbiota modulation and gut barrier disturbance by the DSS-induced colitis. Here, we supposed that IgA secretion decrease is due to an attenuate inflammation triggered by the fermented cheeses. This suggests also that DSS ingestion would affect not only the large intestine but also the small intestine (Ohtsuka and Sanderson, 2003; Perše and Cerar, 2012). Indeed, disruption of gut barrier integrity is a key step in the inflammatory gut disease. The DSS-induced colitis induced a significant increase of nitric oxide synthase (iNOS) expression in the colon tissues and was attenuated by the cheese matrix and the fermented cheeses. However only the Emmental cheese restored the occludin expression in the colon tissues, while its repression participates in the colitis severity (Ahl et al., 2016).

Cytokines are major mediators of colitis pathogenesis. We thus assessed the cytokines expression and concentration in the colonic tissues. Results of cytokine expression were partially consistent with cytokine secretion results. The DSS-induced colitis increased the secretion (not the expression) of IL-

10 and TGFβ, which is consistent with cytokine pathogenesis in UC patients (Roda et al., 2011). This increase was attenuated by the cheese matrix administration. Moreover, the Emmental cheese and the single-strain cheese further reduced the secretion of IL-10 and TGFβ, compared to the PBS-DSS control. However, only the Emmental cheese increased IL-10 expression in the colonic tissue, compared to all other groups. IL-10 is an anti-inflammatory cytokine which inhibits the production of IL-1β, IL-6, and TNF-α. The increase of IL-10 was shown to be effective only when it is induced before DSS-colitis induction (Cardoso et al., 2018). *L. delbrueckii* subsp. *lactis* CNRZ327, as well *P. freudenreichii* CIRM-BIA 129 enhanced Treg FOXP3+ cells subset in animal models, which produce high amount of IL-10 (Rabah et al., 2018a; Santos Rocha et al., 2014). Similarly, TGFβ is an anti-inflammatory mediator which is highly synthesized by mononuclear cells of UC patients (Roda et al., 2011). Similarly, we supposed that the fermented cheeses attenuated inflammation, so that TGFβ secretion is diminished after colitis induction.

The DSS-induced colitis increased the secretion of IFNγ and IL-6 and tended to increase the secretion of TNFα. At the expression level, only IFNγ expression was induced by the DSS, compared to the healthy group. The germ-free cheese matrix administration did not affect the expression of TNFα but decreased the expression of IFNγ, compared to the PBS-DSS group. However, the cheese matrix did not decrease the secretion of IFNγ, TNFα and IL-6 triggered by the DSS. The single-strain cheese and the Emmental cheese decreased the expression of IFNγ gene but only the Emmental cheese decreased the IFNγ secretion, compared to the PBS-DSS group. Similarly, the Emmental cheese decreased the secretion of IL-6, IFNγ, IL-6 and TNFα are pro-inflammatory cytokines highly secreted in the gut mucosa of UC patients (Roda et al., 2011). TNF-α is produced by APCs and macrophages and induced the secretion of IL-6 and IFNγ, the typical cytokine of Th1 cells subset. IL-6 and TGFβ secretion can induce Th17 cells, which are involved in the IBD pathogenesis (Letterio and Roberts, 1998; Wen et al., 2015). Indeed, IL-17, the Th17 cells cytokine, was increased by the DSS-induced colitis and tended to be attenuated ($p=0.06$) by the Emmental cheese administration only. The DSS-induced colitis increases only IL-1β secretion (not expression) compared to the healthy group. Neither cheese attenuated IL-1β

secretion. However, the single-strain cheese increased its expression and the Emmental cheese increased its secretion, compared to the healthy group.

Taken together, these results show that the preventive effects triggered by the Emmental cheese or the single-strain cheese are induced by different mechanisms. Although, the propionic acid bacteria in Emmental cheese were predominant compared to the lactic acid bacteria which underwent lysis phenomena during cheese ripening (Lazzi et al., 2016; Pang et al., 2017; Valence et al., 2000). Indeed, killed lactic acid bacteria were shown to have similar or attenuated ability to reduce chemically-induced colitis (Foligné et al., 2006; Sang et al., 2015; Thakur et al., 2016; Ueno et al., 2011). The anti-inflammatory properties of lactic acid bacteria are probably induced by cell wall componentS of lysed or dead cells. The interactions between propionic acid and lactic acid bacteria are poorly characterized and may modulate their probiotic properties. As an example, in one hand, the proteolytic activity exerted by the lactic acid bacteria can affect propionic acid bacteria immunomodulatory surface proteome and metabolism. This interaction may affect the production of beneficial metabolites by propionic bacteria and their immunomodulatory effects (Baer, 1995; Thierry.A, et al., 2011). In other hand, the proteolytic characteristic of lactic acid bacteria may lead to the liberation of bioactive peptides from caseins proteolysis, which can participate to the anti-inflammatory property of an Emmental cheese (Brown et al., 2017; Politis and Chronopoulou, 2008). Further studies are needed to well understand interactions, which exist between the propionic and lactic acid bacteria, in particularly how it affects their probiotic attributes. These studies will provide screening criteria to choose the most effective lactic and propionic acid bacteria to develop anti-inflammatory functional foods.

As a conclusion, the Emmental cheese fermented by preselected immunomodulatory starter bacteria was able to reduce the DSS-induced colitis severity in a mice model. This protective effect seems to result from a synergy between lactic acid and propionic acid bacteria.

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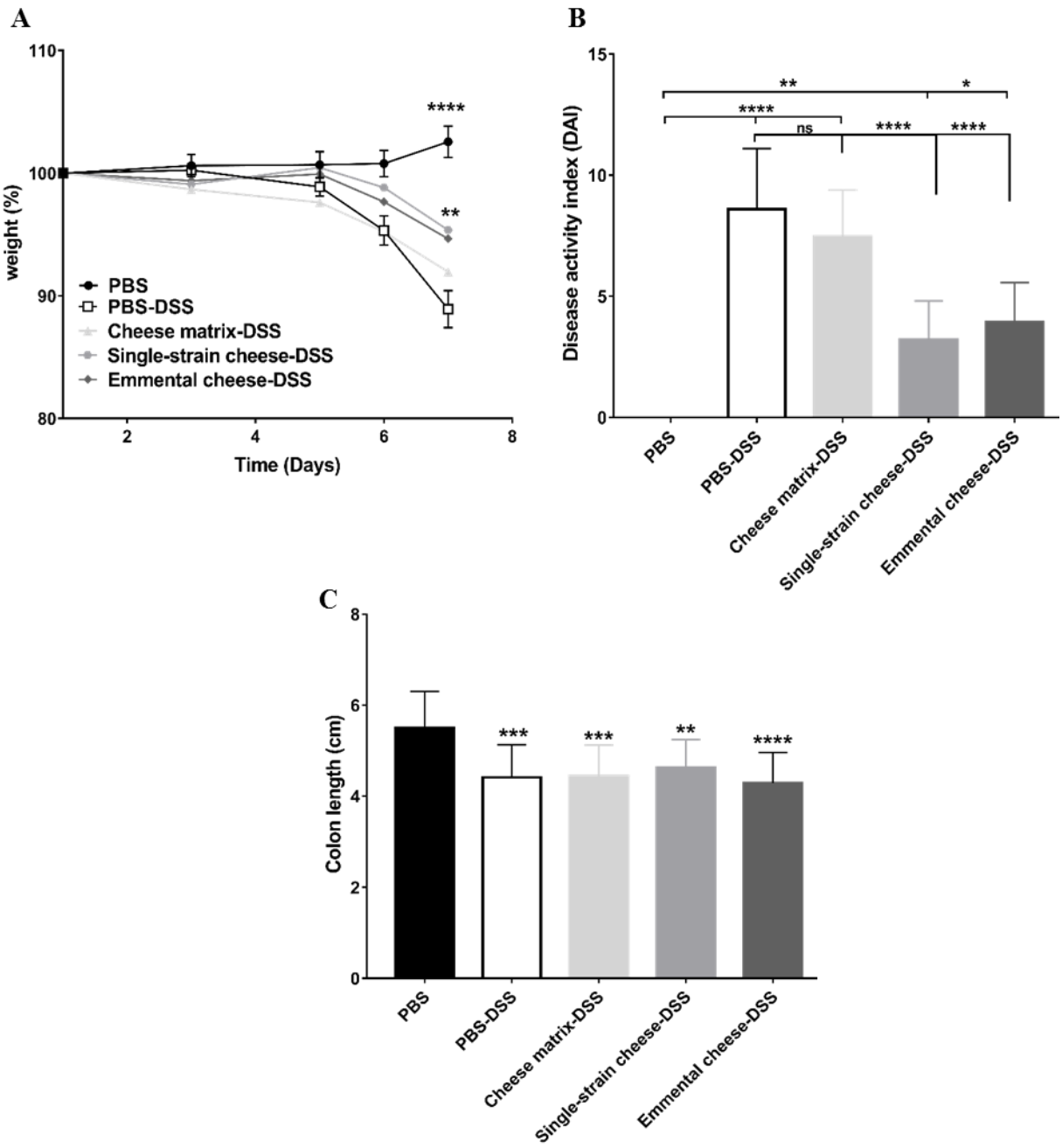

Fig1. Impact of cheese matrix, single-strain cheese and Emmental cheese on the severity of DSS-induced colitis. **(A)** Body weight (as a percentage of the initial weight) in the healthy group (PBS); and after DSS colitis induction in PBS, cheese matrix, single-strain cheese and Emmental cheese groups. **(B)** Disease activity index. **(C)** Colon length. The data represent the mean ± SD of 18 mice per group. Multiple comparisons were performed, *p<0.05, *p<0.05 ** p<0.01, ***p<0.001 **** p<0.0001.

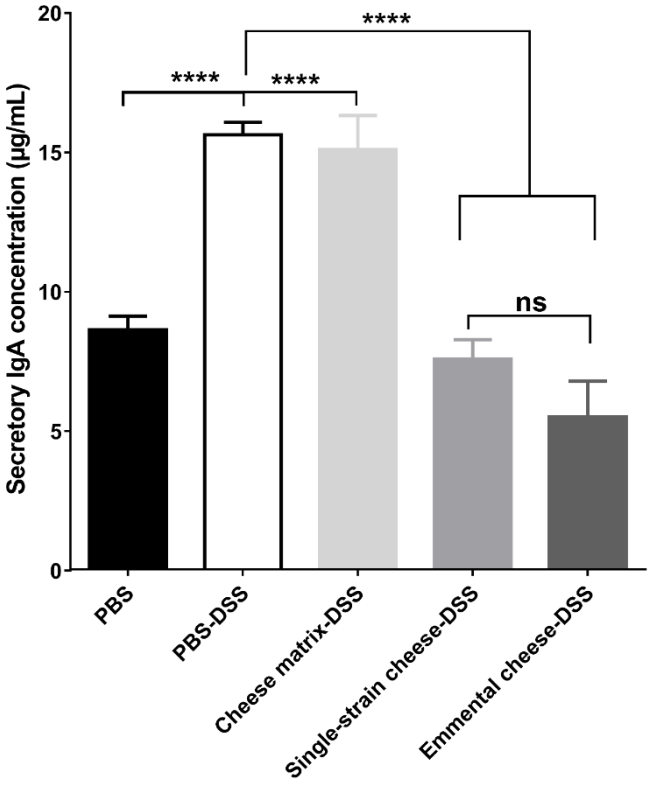

Fig2. Impact of cheese matrix, single-strain cheese and Emmental cheese intake on small bowel IgA secretion. Secretory IgA concentration in the small bowel was determined by ELISA quantification. The data represent the mean ± SD of 18 mice per group. Multiple comparisons were performed, *p<0.05, *p<0.05 ** p<0.01, ***p<0.001 **** p<0.0001.

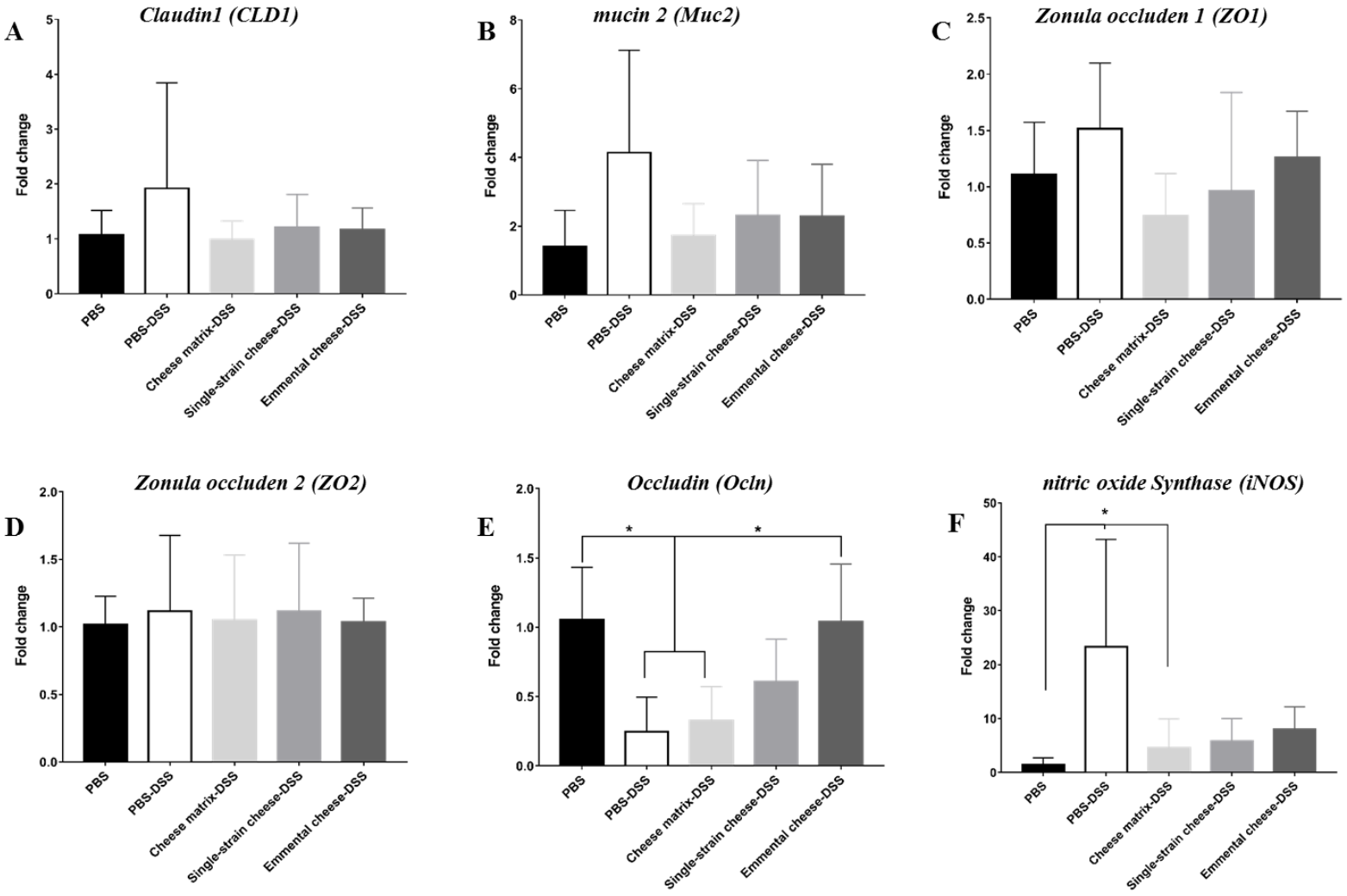

Fig3. Impact of cheese matrix, single-strain cheese and Emmental cheese on the epithelial colonic cells barrier and on the colonic oxidative stress during DSS-induced colitis. Colonic mRNA expression levels of **(A)** CLD1, **(B)** Muc2, **(C)** ZO1, **(D)** ZO2 **(E)** Ocln and **(F)** iNOS genes are analysed. The data represent the mean ± SD of 18 mice per group. Multiple comparisons were performed, *p<0.05, *p<0.05 ** p<0.01, ***p<0.001 **** p<0.0001.

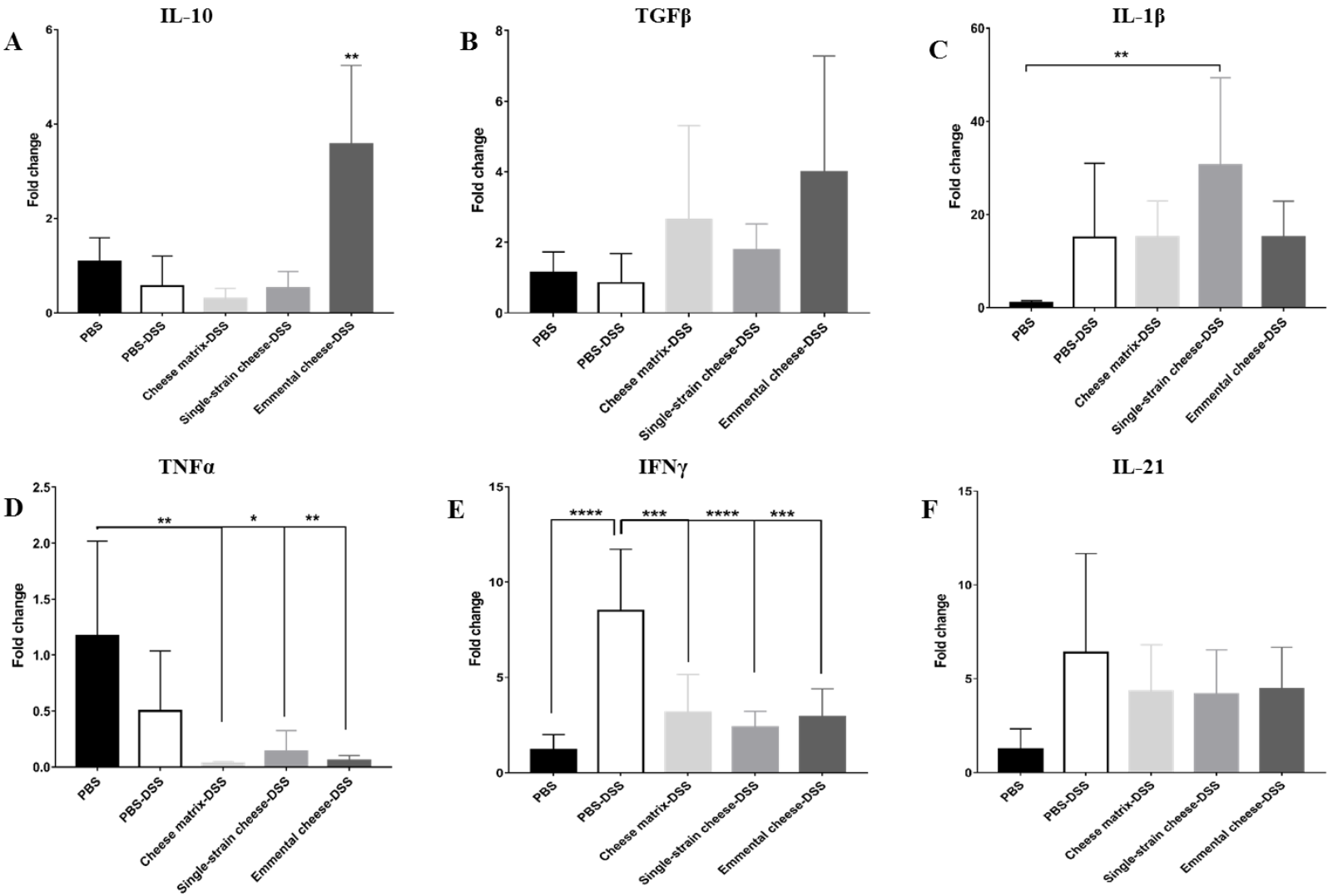

Fig4. Impact of cheese matrix, single-strain cheese and Emmental cheese on cytokines expression in the colonic tissues during DSS-induced colitis. Colonic mRNA expression levels of **(A)** IL-10, **(B)** TGFβ**, (C)** IL-1β, **(D)** TNFα**, (E)** IFNγ**,** and **(F)** IL-21 were determined. Data represent the mean ± SD of 18 mice per group. Multiple comparisons were performed, *p<0.05, *p<0.05 ** p<0.01, ***p<0.001 **** p<0.0001.

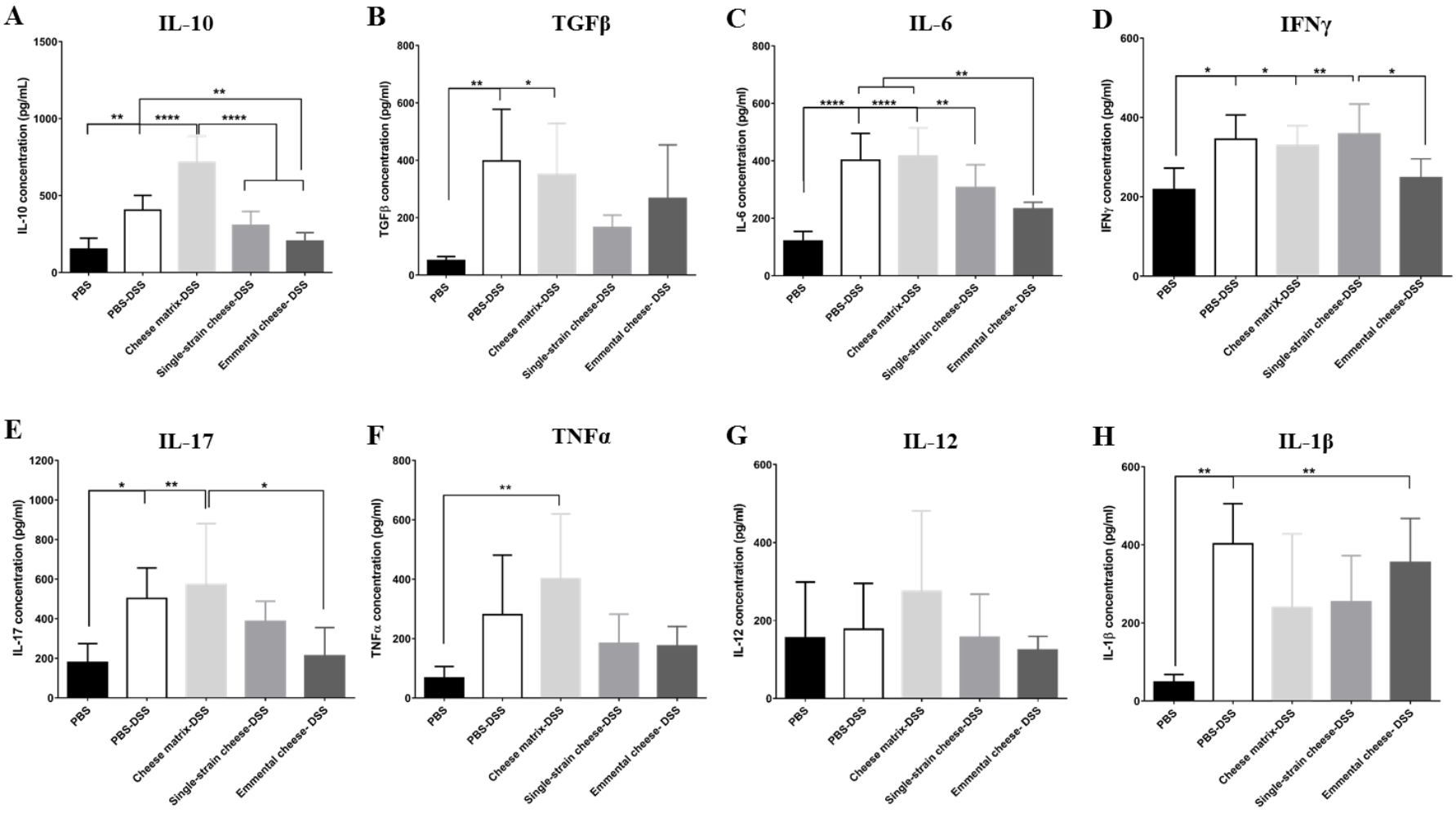

Fig5. Impact of cheese matrix, single-strain cheese and Emmental cheese on secreted cytokines in the colonic tissues during DSS-induced colitis. Cytokines concentration of **(A)** IL-10, **(B)** TGFβ, **(C)** IL-6, **(D)** IFNγ, **(E)** IL-17, **(F)** TNFα**, (G)** IL-12 and **(H)** IL-1β were quantified by ELISA. Data represent the mean ± SD of 18 mice per group. Multiple comparisons were performed, *p<0.05, *p<0.05 ** p<0.01, ***p<0.001 **** p<0.0001.

Supplemental data

Primer		Sequence 5'-3'	Reference
Ocln	Forward	GGACCCTGACCACTATGAAACAGACTA	(Yamazaki et al., 2011)
	Reverse	TAGGTGGATATTCCCTGACCCAGTC	
CLD1	Forward	CTGGAAGATGATGAGGTGCAGAA	
	Reverse	CTAATGTCGCCAGACCTGAA	
Muc2	Forward	GCTGACGAGTGGTTGGTGAATG	(Wlodarska et al., 2011)
	Reverse	GATGAGGTGGCAGACAGGAGAC	
ZO1	Forward	AGCTCATAGTTCAACACAGCCTCCAG	(Yamazaki et al., 2011)
	Reverse	CTTCCACAGCTGAAGGACTCACAG	
ZO2	Forward	GGAGACCAGATTCTGAAGGTGAACACA	
	Reverse	CCTTTGGGGATTTCTAGCAGGTAGAGGAC	
iNOS	Forward	CAGCTGGGCTGTACAAACCTT	(Giulietti et al., 2001)
	Reverse	CATTGGAAGTGAAGCGTTTCG	
Actb	Forward	AGAGGGAAATCGTGCGTGAC	
	Reverse	CAATAGTGATGACCTGGCCGT	
Gapdh	Forward	TCACCACCATGGAGAAGGC	
	Reverse	GCTAAGCAGTTGGTGGTGCA	
IL1beta	Forward	CAACCAACAAGTGATATTCTCCATG	
	Reverse	GATCCACACTCTCCAGCTGCA	
IL6	Forward	GAGGATACCACTCCCAACAGACC	
	Reverse	AAGTGCATCATCGTTGTTCATACA	
IL10	Forward	GGTTGCCAAGCCTTATCGGA	
	Reverse	ACCTGCTCCACTGCCTTGCT	
IL12b	Forward	GGAAGCACGGCAGCAGAATA	
	Reverse	AACTTGAGGGAGAAGTAGGAATGG	
IL17a	Forward	GCTCCAGAAGGCCCTCAGA	
	Reverse	AGCTTTCCCTCCGCATTGA	
Infg	Forward	TCAAGTGGCATAGATGTGGAAGAA	
	Reverse	TGGCTCTGCAGGATTTTCATG	
Tgfb1	Forward	TGACGTCACTGGAGTTGTACGG	
	Reverse	GGTTCATGTCATGGATGGTGC	
Tnfa	Forward	CATCTTCTCAAAATTCGAGTGACAA	
	Reverse	TGGGAGTAGACAAGGTACAACCC	

Table S1. Specific primer sequences in order to target murine genes analysed in the study.

	Emmental cheese		Single-Strain cheese		Cheese matrix	
P. freudenreichii	CFU / g	4.10^9	CFU / g	1.10^{10}	-	-
Thermophilic *Lactobacillus*	CFU / g	1.10^6	-		-	-
Streptococcus thermophilus	CFU / g	4.10^6	-		-	-
E. Coli	CFU / g	<10	-		-	-
Staphylococcus	CFU / g	<10	-		-	-
Listeria	CFU /25g	abs	-		-	-
Salmonella	CFU /25g	abs	-		-	-
Total coliform Bacteria	-	-	CFU / g	<10	CFU / g	<10
Total thermophilic Bacteria	-	-	CFU / g	<10	CFU / g	<10
Total mesophilic Bacteria	-	-	CFU / g	<10	CFU / g	<100
Yeasts and molds	-	-	CFU / g	<10	CFU / g	<10

Table S2. Microbiological analysis of the Emmental cheese, the single-strain cheese and the cheese matrix. *P. freudenreichii* enumeration was performed in the Emmental cheese and the Single-strain cheese. *Lactobacillus sp* and *Streptococcus thermophilus* numeration was done only in the Emmental cheese. Different microbiological analysis were performed to numerate contaminating flora in the Emmental cheese in one hand, and in the single-strain cheese and the cheese matrix in the other hand.

	Number of colonies collected	Different strains identified	Colony corresponding to the original strain
P. freudenreichii	10	1	10/10
L. delbrueckii	10	4	6/10
S. thermophilus	10	4	1/10

Table S3. PFGE analysis of different colonies isolated from petri dishes done for *P. freudenreichii*, *Lactobacillus* and *S. thermophilus* enumeration in the Emmental cheese. The identity of strains were checked by analyzing 10 different colonies isolated.

6.3. Conclusion

Dans ce chapitre, nous avons souhaité évaluer l'effet anti-inflammatoire d'un fromage emmental, dit « probiotique ». Les souches ferments de cet emmental ont été présélectionnées pour leurs propriétés immunomodulatrices : *S. thermophilus* LMD-9, *L. delbrueckii* subsp. *lactis* CNRZ327 et *P. freudenreichii* CIRM-BIA 129. L'effet anti-inflammatoire de ce fromage a été examiné *in vivo* dans un modèle de colite chimiquement induite par le DSS chez la souris. Cet effet anti-inflammatoire du fromage emmental « probiotique » a été comparé à celui d'un fromage monosouche fermenté par *P. freudenreichii* CIRM-BIA 129 et à celui d'une matrice fromagère de caséines.

Le fromage monosouche, ainsi que le fromage emmental, ont réduit la sévérité de la colite chimiquement induite, comme indiquent les diminutions de la perte de poids, des saignements rectaux et de la survenue des diarrhées. Cependant, le rétrécissement du côlon n'a pas été atténué par l'ingestion du fromage monosouche ou du fromage emmental. En évaluant l'expression, ainsi que la sécrétion de plusieurs cytokines, le fromage monosouche ainsi que le fromage emmental ne modulaient pas de la même manière leur expression et leur sécrétion. Notamment, l'ingestion du fromage monosouche n'a pas permis d'atténuer les sécrétions des cytokines IL-6 et IFNγ induites par le DSS. L'analyse du microbiote intestinal est en cours de réalisation et permettra de savoir si les deux fromages fermentés ont des effets différents sur sa composition.

L'effet probiotique de l'emmental semble résulter des effets anti-inflammatoires cumulés des bactéries propioniques et lactiques. Les interactions qui peuvent exister entre ces bactéries pourraient influencer également leurs propriétés anti-inflammatoires, et ainsi celles de l'emmental. De plus, l'emmental ayant subi le processus d'affinage (Protéolyse), ce qui n'est pas le cas du fromage monosouche, on suppose que l'interaction entre les bactéries lactiques et la matrice fromagère peut moduler cet effet anti-inflammatoire. Différents peptides bioactifs peuvent être libérés par la protéolyse des caséines par les bactéries lactiques.

Chapitre 6 : à retenir

- **L'emmental « probiotique », tout comme le fromage monosouche, ont réduit la sévérité d'une colite chimiquement induite dans un modèle murin**
- **Au niveau des marqueurs microscopiques, l'emmental « probiotique » semble avoir un meilleur effet préventif contre la colite chimiquement induite**
- **L'emmental « probiotique » semble ne pas réduire la sévérité de la colite de manière similaire à celle induite par le fromage monosouche**

Discussion générale & perspectives

1. Le fromage comme vecteur pour les propriétés immunomodulatrices de *P. freudenreichii* CIRM-BIA 129

Le projet de thèse avait pour objectif de comprendre l'impact de la matrice fromagère sur les propriétés probiotiques de *P. freudenreichii* CIRM-BIA 129 (***Pf***), et en particulier les propriétés immunomodulatrices à travers ses protéines de surface Slps. Nous avons utilisé des approches *in vivo* et *in vitro* complémentaires pour comparer et comprendre les différences en termes de propriétés immunomodulatrices de *P. freudenreichii* CIRM-BIA 129 lors d'une vectorisation par une culture sur ultrafiltrat de lait ou par une matrice fromagère. Nous avons avancé l'hypothèse que les propriétés immunomodulatrices de ***Pf*** seraient matrice-dépendantes, du fait de la variation de la quantité de bactéries vivantes vectorisées vers le côlon et d'une différence en termes de composants de surface due à la protéolyse éventuelle des protéines Slps.

1.1. Effet de la matrice fromagère sur la viabilité de *P. freudenreichii* dans le tube digestif

Nous avons montré que la matrice fromagère améliore la tolérance de ***Pf*** aux stress gastriques et duodénaux lors de la digestion *in vitro*, par comparaison à l'ultrafiltrat de lait. Cependant, l'étude *in vivo* montre que les deux matrices vectrices apportent des quantités similaires de bactéries métaboliquement actives au tractus digestif des porcelets. Cela suggère que ***Pf*** produit des quantités similaires de métabolites bénéfiques, quelle que soit la matrice de vectorisation.

Il est vrai que les modèles de digestion *in vitro* utilisés présentaient des limites. En effet, le modèle de digestion *in vitro* statique ne comporte pas de régulation du pH lors de la phase gastrique, ce qui peut affecter la viabilité bactérienne (Minekus et al., 2014). Lors de la digestion de la matrice fromagère, le pH initialement ajusté à 3 n'était pas stable lors de la digestion, à l'inverse de l'ultrafiltrat de lait. De plus, dans le modèle de digestion *in vitro* dynamique, nous avons fait le choix d'utiliser des paramètres de digestion mis au point pour des matrices fromagères solides : le temps de vidange utilisé est nettement supérieur à des temps physiologiques observés pour des matrices liquides, d'où une perte de viabilité

considérable pour l'ultrafiltrat de lait. L'ensemble de ces limites pourrait expliquer l'incohérence trouvée entre les résultats *in vitro* et *in vivo.*

Cependant, la quantité de bactéries vivantes atteignant le côlon dépend également de la capacité d'adaptation de *P. freudenreichii* à l'environnement colique, et non seulement de sa tolérance aux stress gastriques et duodénaux. Pour explorer cet aspect, nous avons évalué l'impact du milieu de croissance sur la capacité d'adaptation de ***Pf*** au milieu caecal, qui mime l'environnement colique. Nous avons observé que la viabilité de ***Pf*** diminue dans le milieu caecal, quel que soit le milieu de croissance. Cependant, les mécanismes d'adaptation sont peu clairs pour le moment. Les analyses de protéomique comparative et de métabolique permettront de valider une des deux hypothèses émises dans le chapitre 5. Néanmoins, nous avons constaté que le milieu de croissance, ainsi que la protéolyse des protéines de surface, influencent la viabilité de *P. freudenreichii* dans le milieu caecal. Les résultats suggèrent que les bactéries cultivées sur l'ultrafiltrat de lait auraient une moindre tolérance aux stress gastriques et duodénaux, mais une meilleure capacité d'adaptation au côlon. Pour les bactéries provenant du fromage, elles auraient une meilleure capacité de tolérance aux stress digestifs mais auraient une moindre capacité d'adaptation au côlon.

De manière générale, ces résultats mettent en lumière l'importance d'utiliser des modèles de digestion pertinents pour simuler correctement la dynamique de digestion gastrique et intestinale en fonction de la rhéologie et la composition du vecteur, et d'inclure en plus un compartiment mimant les conditions coliques, afin d'évaluer l'activité *in situ* d'un microorganisme probiotique. Ces modèles permettront d'évaluer à la fois la tolérance aux stress gastriques et duodénaux et la capacité d'adaptation du probiotique à l'environnement colique, tel que le modèle SHIME (Simulator of the Human Intestinal Microbial Ecosystem) (Molly et al., 1994).

1.2. Effet de la matrice fromagère sur l'effet immunomodulateur de *P. freudenreichii*

Dans un deuxième temps, nous avons émis l'hypothèse que les variations de propriétés immunomodulatrices de ***Pf*** seraient liées à une différence de composition en composants de surface, due à la protéolyse éventuelle des protéines Slps. En effet, les résultats *in vivo* montrent que les propriétés

immunomodulatrices de ***Pf*** sont matrice-dépendantes. Les différences, en termes d'immunomodulation par ***Pf***, entre les deux matrices vectrices, semblent être liées à une différence en composants de surface, puisque les deux matrices vectrices apportent des quantités similaires de bactéries métaboliquement actives vers le côlon des porcelets.

En effet, les résultats de stimulation des cellules épithéliales intestinales suggèrent que la protéolyse des Slps abolisse leur propriété anti-inflammatoire. Parallèlement, nous avons montré que la matrice fromagère limite la protéolyse des protéines Slps lors de la digestion *in vitro*. L'analyse des contenus digestifs ne nous a pas permis de discriminer les deux matrices par la présence de SlpB. De plus, comme il a été démontré que ***Pf*** peut s'adapter à l'environnement du côlon, nous nous sommes interrogés sur la possibilité que ***Pf*** puisse exprimer *de novo* ses protéines Slps après la protéolyse de celles-ci. Pour corroborer cette hypothèse selon laquelle la différence en termes d'immunomodulation observée sur l'étude *in vivo* est liée essentiellement à une différence en termes de composants de surface, nous avons étudié l'expression *de novo* de la protéine SlpB dans le milieu caecal après sa protéolyse. En effet, en cas de protéolyse de la protéine SlpB lors de la digestion, ***Pf*** ne l'exprimera pas *de novo* dans le milieu caecal. Ce résultat n'exclue pas que ***Pf*** puisse exprimer d'autres protéines de surface qui peuvent interagir également avec le système immunitaire de l'hôte. En plus, comme démontré pour des espèces de *Bifidobacterium* et de *Lactobacillus*, des composants de surface, tels que les acides lipotéichoïques ou le peptidoglycane, peuvent également exercer un effet immunomodulateur (Sarkar and Mandal, 2016; Shida et al., 2009; Smelt et al., 2013). En effet, la stimulation *in vitro* de cellules PBMC montre une capacité d'immunomodulation différente de ***Pf*** ayant perdu les protéines de surface par protéolyse digestive, par comparaison à ***Pf*** intact. La différence d'immunomodulation consistait essentiellement en la perte de la capacité d'induction de l'interleukine IL-10 (Le Maréchal et al., 2015).

Pour récapituler, les deux matrices, l'ultrafiltrat de lait et le fromage, apportent des quantités similaires de bactéries métaboliquement actives au côlon des porcelets. Cependant, les deux matrices présentent *in vivo* des effets anti-inflammatoires matrice-dépendants. En effet, nous pensons que cela est dû à une variation en composants de surface. La vectorisation de ***Pf*** par la matrice fromagère amène une grande proportion de ***Pf*** intact vers le côlon, alors que la vectorisation de ***Pf*** par l'ultratfiltrat de lait engendre une

protéolyse de la protéine SlpB qui ne sera pas exprimée *de novo* dans le côlon. Néanmoins, l'analyse de protéomique nous permettra de voir si certaines protéines de surface sont exprimées ou non *de novo* après leur protéolyse digestive, ce qui peut affecter également l'immunomodulation exercée par ***Pf***.

2. Compréhension des mécanismes immunomodulateurs de *P. freudenreichii* CIRM-BIA 129

2.1. L'effet immunomodulateur de *P. freudenreichii*

Nous avons montré que ***Pf*** CIRM-BIA 129 ainsi que les protéines Slps, induisent une réponse anti-inflammatoire sur les cellules épithéliales intestinales HT-29, en présence de stimulus pro-inflammatoire. Ces résultats sont cohérents avec les effets observés sur les cellules immunes du sang (PBMC). De plus, nous avons montré que les protéines Slps ont également un effet anti-inflammatoire sur les cellules immunes des ganglions mésentériques intestinaux des porcelets, suite à une stimulation *ex vivo*. La stimulation des cellules HT-29 par la souche ***Pf*** CIRM-BIA 129 KO SlpB montre que ce mutant présente un profil pro-inflammatoire lors de la stimulation des cellules HT-29, par comparaison à la souche sauvage (Annexe 1). Cependant, comme la mutation de la protéine SlpB a eu un effet pléiotrope sur la composition du protéome de surface (Do Carmo et al., 2018b), il est donc difficile d'attribuer exclusivement l'effet pro-inflammatoire de ***Pf*** CIRM-BIA 129 KO SlpB à l'absence de la protéine SlpB. L'étude de protéomique portant sur l'adaptation de ***Pf*** à l'environnement caecal va nous fournir des données cruciales sur la composition du protéome de surface pour comprendre cet effet immunomodulateur à travers ces protéines clés. En effet, si l'analyse montre, ou non, que d'autres protéines de surface sont exprimées *de novo* dans l'environnement caecal, cela suggère qu'en effet l'immunomodulation exercée par *P. freudenreichii* résulte d'une combinaison de protéines de surface, tel que il a été suggéré précédemment (Deutsch et al., 2017) ; mais elle peut être due également à des composants de la paroi. En effet, d'autres souches de ***Pf*** ont montré un effet anti-inflammatoire *in vitro* mais possèdent un protéome de surface (notamment en termes de composition en protéines Slps) différent de la souche ***Pf*** CIRM-BIA 129 (Deutsch et al., 2017; Foligné et al., 2013). Cependant, il n'est pas exclu d'aller plus loin dans l'élucidation des mécanismes moléculaires d'action des protéines Slps, ainsi que leur interaction avec l'immunité de l'hôte. Il faudra que les approches expérimentales menées pour

comprendre cet effet immunomodulateur intègrent de façon réelle ce qui se passe lors de l'ingestion de la bactérie.

1) ***Perspective : élargir l'étude des mécanismes immunomodulateurs de Pf à d'autres protéines de surfaces ou à des composants de la paroi***

De plus, nous avons montré que l'ingestion de ***Pf*** induit une différenciation des lymphocytes T différente selon la matrice de vectorisation. Lors de l'étude *in vivo* et en fonction des stimulations *ex vivo* menées, nous avons également émis l'hypothèse que l'ingestion de ***Pf*** modulerait également l'immunité innée. Dans une démarche additionnelle, on pourrait étudier la différenciation des lymphocytes T en mettant en contact les bactéries, ayant subi un shaving ou non, ainsi que des protéines Slps, en contact des cellules dendritiques et/ou de macrophages qui seront ultérieurement mis en contact avec des lymphocytes T naïfs. Des études *in vivo* sont également nécessaires pour aller plus loin dans les mécanismes d'action de ***Pf*** dans l'induction de la différenciation des lymphocytes T. Ainsi, on pourrait confirmer si la différence, en termes de différenciations des lymphocytes T induites par ***Pf*** *in vivo*, est liée au protéome de surface. Cependant, une observation majeure a été faite lors de l'expérimentation réalisée sur les porcelets, la consommation chronique de *P. freudenreichii* n'induit pas la même pas réponse immunitaire sur les cellules PBMC qu'une stimulation transitoire *in vitro*. ***Pf*** induit une sécrétion d'IL-10 lors de la stimulation *in vitro* de PBMC ou de MLNC, alors que l'ingestion chronique de ***Pf*** diminue la sécrétion d'IL-10 par les cellules PBMC. Il s'agit d'un résultat important à souligner lorsque des études *in vivo* sont menées et qui montre qu'il est nécessaire de trouver des modèles *in vitro* plus représentatifs de l'ensemble des évènements se déroulant dans le tube digestif.

2) ***Perspective : étudier les interactions entre Pf avec ses protéines de surface et les cellules de l'immunité innée et leurs conséquences sur la différenciation des lymphocytes T in vitro ainsi qu'in vivo***

De plus, les résultats obtenus à l'issue de l'étude *in vivo* sur les porcelets, ouvrent la perspective intéressante d'aller étudier *in vivo* l'effet matrice-dépendant de *P. freudenreichii* pour prévenir une maladie inflammatoire donnée. En effet, les résultats des phénotypes de Lymphocytes T *in vivo* montrent

que, selon la matrice de vectorisation, ***Pf*** pourrait plus ou moins efficacement prévenir une inflammation d'origine différente (Allergie, diarrhée infectieuse,……).

3) ***Perspective : étudier l'effet matrice sur la prévention de différents modèles de maladies inflammatoires qui peuvent être prévenues par l'ingestion de Pf***

2.2. La caractérisation structurale de la protéine SlpB pour son rôle immunomodulateur

Lors de ce projet de thèse, nous avons réussi à préciser de manière partielle les modifications covalentes de la protéine SlpB, révélant en particulier sa nature glycoprotéique (Annexe 2). Cette étude préliminaire permettra d'aller plus loin dans l'identification des sites modifiés, ainsi que l'architecture des sucres présents sur la protéine. Une stratégie pour atteindre cet objectif est proposée (Annexe 2). L'ensemble de ces expérimentations ouvriront de nombreuses perspectives afin de comprendre les mécanismes d'interaction de cette protéine avec les récepteurs de l'immunité innée. Il est également possible d'envisager de déterminer la structure tridimensionnelle de SlpB par cristallographie ou par des outils bio-informatiques, afin de pouvoir faire des études de type « Docking » pour déterminer les récepteurs pouvant potentiellement interagir avec cette protéine. Cette perspective permettra également d'identifier des peptides bioactifs qui peuvent être utilisés comme agents thérapeutiques anti-inflammatoires dans un contexte de maladies inflammatoires. Il faudra également envisager d'élargir ces études aux autres protéines Slps des bactéries propioniques.

4) ***Perspective : identifier les récepteurs de l'hôte interagissant avec la protéine SlpB et étudier les cascades de réaction induites dans les cellules présentatrices d'antigène, telles que les cellules M ou les cellules dendritiques***

5) ***Perspective : étudier de manière plus précise les modifications covalentes de la protéine SlpB et étudier leur rôle dans l'interaction avec les récepteurs de l'immunité innée***

3. Compréhension de la physiologie et des mécanismes d'adaptation de *P. freudenreichii* CIRM-BIA 129

3.1. Les mécanismes d'adaptation de *P. freudenreichii* aux stress digestifs

L'analyse par protéomique, en cours, apportera un éclairage sur les mécanismes d'adaptation à l'environnement caecal. Les résultats seront comparés aux résultats de l'analyse transcriptomique effectuée *in vivo* sur la souche ***Pf*** CIRM-BIA 1 (Saraoui et al., 2013). Cette précédente étude montrait que des mécanismes sont régulés de manière similaire que dans le cas de l'adaptation de la souche de ***Pf*** CIRM-BIA 138 à une carence longue en nutriments dans le milieu YEL (Aburjaile et al., 2016b).

Les résultats préliminaires de l'étude sur l'adaptation de ***Pf*** à l'environnement caecal suggèrent que ces mécanismes d'adaptation sont matrice-dépendants. L'étude *in vivo* menée sur l'ingestion de ***Pf*** CIRM-BIA 138 a montré que, selon que la bactérie est apportée sous forme lyophilisée ou sous forme de lait fermenté, cette souche n'avait pas la même activité métabolique *in situ* (Cousin et al., 2012). Cela montre que, outre le choix de la souche, le milieu de croissance et le procédé technologique sont des éléments à prendre en compte pour optimiser l'effet probiotique de ***Pf*** *in situ*. La souche ***Pf*** CIRM-BIA 129 est une souche lactate (+) et lactose (+), l'abondance de la source de carbone dans les milieux laitiers conduit à l'accumulation de molécules telles que le tréhalose, le glycogène ou les polyphosphates, qui peuvent être utilisées lors de la survie ***Pf*** dans l'environnement caecal. L'accumulation de ces molécules est très probablement matrice-dépendante, ce qui conduit à des capacités d'adaptation différentes aux conditions coliques. Il existe quelques données sur l'accumulation de ces molécules et leur rôle dans la tolérance de ***Pf*** aux différents stress, tels que les stress osmotique, digestif et thermique (Dalmasso et al., 2012; Huang et al., 2016). Cependant, les études sur le rôle de ces molécules dans la tolérance de ***Pf*** à la carence en nutriments n'existent pas et peuvent être conduites dans le futur pour comprendre au mieux l'influence du milieu de croissance sur les mécanismes d'adaptation de ***Pf*** au tractus digestif.

6) Perspective : étudier les phénomènes moléculaires responsables de l'influence du milieu de croissance sur l'adaptation de Pf à l'environnement colique

L'étude de l'adaptation de ***Pf*** au milieu caecal suggère que ***Pf*** adopte un état viable non cultivable (VBNC). L'état de dormance de ***Pf*** CIRM-BIA 129 semble être un mécanisme d'adaptation à une carence en nutriments, tel que suggéré précédemment (Aburjaile et al., 2016a). L'analyse protéomique permettra de confirmer ou d'infirmer cette hypothèse. Des études supplémentaires sont nécessaires afin d'investiguer si pendant d'autres types de stress, *P. freudenreichii* adopte également cet état VBNC. En effet, le passage d'un état viable non cultivable dans des situations de stress a été bien décrit chez des espèces pathogènes non sporulées du phylum des actinobactéries (Koltunov et al., 2010; Nikitushkin et al., 2013), mais peu ou quasiment jamais chez des bactéries probiotiques non sporulées.

7) Perspective : étudier les phénomènes moléculaires de l'induction de la dormance chez Pf

La survie, ainsi que l'état de dormance, semblent également dépendre des protéines de surface. En effet, nous supposons que la protéine « Resuscitation promoting factor » (RpfB) est impliquée dans ces phénomènes observés. RpfB est impliquée dans le passage d'un état de dormance vers un état viable cultivable par son activité d'hydrolyse du peptidoglycane. L'étude protéomique et l'étude structurale *in silico* de la protéine RpfB nous permettront d'élucider son mécanisme d'action. Une autre protéine de surface décrite comme une peptidase de la paroi peut avoir également un rôle dans ces phénomènes de dormance et de survie, NlpC/P60 (Le Maréchal et al., 2015). Tout comme la protéine RpfB, il s'agit d'une hydrolase du peptidoglycane (Anantharaman and Aravind, 2003; Keep et al., 2006) .

8) Perspective : étudier les phénomènes moléculaires de ressuscitation de Pf après passage par un état viable non cultivable, notamment préciser le rôle des protéines RpfB et NlpC/P60

3.2. Les mécanismes de glycosylation et de phosphorylation chez *P. freudenreichii*

Jusqu'aujourd'hui, seules les protéines Slps sont décrites comme des protéines glycosylées, et seule la protéine SlpB a été décrite comme une protéine phosphorylée et glycosylée (Annexe 2). En effet, peu d'études ont été menées dans ce domaine. Précédemment, au sein de l'équipe Microbio du STLO, l'activité glycotransférase d'une enzyme codée par le gène *gtf* a été caractérisée pour son implication dans la production des exopolysaccharides. Nous ignorons si ce type d'enzymes peut être également impliqué

dans les mécanismes de glycosylation de diverses protéines chez *P. freudenreichii*. Il est vrai que la présence de ces motifs glycanes sur les protéines Slps peut intervenir dans la modulation de la réponse immunitaire innée envers *P. freudenreichii*. Cependant, pour la bactérie, il est probable que ces mécanismes de glycosylation de ces protéines interviennent dans d'autres processus indispensables pour sa survie. De la même manière, il a été décrit précédemment que ces bactéries possèdent des phosphokinases. Cependant, nous ignorons tout sur le phosphoprotéome de *P. freudenreichii*. En effet, les phénomènes de phosphorylation et de déphosphorylation des protéines sont très liés à des mécanismes d'adaptation aux stress environnementaux, ainsi qu'aux phénomènes de dormance. Par ailleurs, la protéine SlpB porte un groupement phosphate, il se peut qu'elle ait également un rôle dans la transmission de signaux cellulaires en cas de stress environnementaux. Diverses perspectives sont possibles pour l'étude sur le glycoprotéome et le phosphoprotéome des souches de *P. freudenreichii*.

9) Perspective : identifier les enzymes impliquées dans la glycosylation des protéines chez Pf

10) Perspective : étudier le phosphoprotéome de Pf lors de stress modèles (thermique, osmotique, oxydatif ou carence nutritif)

4. Conception d'un emmental fonctionnel pour la prévention ou le traitement de la rectocolite hémorragique

4.1. Les limites scientifiques

a) Manque de données sur les interactions entre les bactéries lactiques et propioniques

Dans la deuxième partie de ce projet de thèse, nous avons émis initialement l'hypothèse que la présélection de souches de *Lactobacillus*, de *S. thermophilus* ainsi que de *P. freudenreichii*, selon leurs propriétés probiotiques, aurait un effet synergique sur l'efficacité anti-inflammatoire d'un fromage emmental, produit à l'échelle industrielle. En effet, nous avons observé qu'un emmental « probiotique » était aussi efficace qu'un fromage monosouche expérimental pour prévenir la colite induite chimiquement chez la souris. Cependant, les mécanismes de prévention induits étaient différents selon les deux fromages. L'effet probiotique de l'emmental peut être attribué à différents effets résultant individuellement de chaque souche probiotique utilisée, aux interactions qui peuvent exister entre les

différentes espèces bactériennes présentes et aux interactions qui existent entre la matrice fromagère et les espèces bactériennes présentes. Les interactions des bactéries lactiques d'une part, et les bactéries propioniques d'autre part, sont partiellement étudiées. Les résultats obtenus montrent la nécessité d'aller plus loin dans la compréhension des interactions entre les bactéries lactiques et propioniques, et leurs conséquences à la fois sur leurs propriétés technologiques et leurs proproétés probiotiques mutuelles. Ces études permettront de fournir des critères de choix pour la sélection de combinaisons de bactéries lactiques et propioniques pour la conception de fromages fonctionnels.

11) Perspective : étudier les interactions entre les bactéries lactiques et propioniques et leurs conséquences à la fois sur leurs propriétés technologiques et probiotiques

b) Manque de preuves cliniques

En effet, le fromage emmental fonctionnel dont nous avons sélectionné les ferments pour leurs propriétés présentait une un effet protecteur contre la colite. Cependant, des études cliniques sont indispensables pour confirmer ces résultats précliniques. En effet, une étude pilote a été menée en amont de ce projet de thèse, en évaluant l'effet d'une préparation fromagère à base de poudre issue du fromage emmental « probiotique » et qui a été ensuite fermentée par la souche ***Pf*** CIRM-BIA 129. L'étude a été menée avec la collaboration du CHU de Pontchaillou avec la supervision du Pr. Guillaume Bouguen. L'ingestion de ce fromage a été évaluée pour aider à la rémission de patients atteints de rectocolite hémorragique légère à modérée. Les patients ont gardé leur traitement habituel et ont consommé de manière quotidienne cette préparation fromagère pendant 8 semaines. Le défaut de recrutement n'a pas permis de tirer des conclusions claires sur cette étude. Cependant, un tiers des patients ont présenté une réponse à la prise du formage probiotique et 16 % des patients étaient en rémission. Aucun effet délétère du fromage n'a été observé pendant cette étude. Il est donc nécessaire de mener d'autres essais cliniques de plus grande ampleur, pour évaluer l'efficacité du fromage emmental « probiotique » pour prolonger la durée de rémission des patients atteints de RCH.

12) Perspective : mener des études cliniques de plus grandes ampleurs sur l'effet de fromages fonctionnels fermentés par Pf sur la rémission de RCH

c) La pertinence des protocoles précliniques

Cependant, cette étude clinique pilote a mis en lumière une problématique plus généralement observée dans les études cliniques évaluant des effets probiotiques dans le cadre des maladies inflammatoires intestinales. Les résultats obtenus en préclinique sont souvent incohérents avec les résultats cliniques. En effet, les protocoles d'administration utilisés en préclinique sont souvent des protocoles de prévention et non de co-traitement. Dans le cas des études cliniques, les protocoles de prévention sont en général indisponibles pour évaluer un effet probiotique d'un microorganisme ou d'un aliment fonctionnel. En effet, la rectocolite hémorragique résulte d'une inflammation multifactorielle, un probiotique administré à titre préventif ou à titre curatif n'aura pas la même efficacité vis-à-vis de l'atténuation de l'inflammation, comme suggéré par quelques études scientifiques (Hayes et al., 2014; Zheng et al., 2016). Les protocoles préventifs pour évaluer l'efficacité d'un probiotique dans les modèles animaux sont plus faciles à mettre en œuvre que les protocoles curatifs. Il faudra toute une réflexion sur l'utilisation de ces modèles précliniques afin de les rapprocher le plus possible de la réalité clinique. De plus, cet aspect pose la problématique du modèle murin en tant que tel, du fait des différences en termes du microbiote intestinal et du système immunitaire qui peuvent exister avec ceux de l'homme. Il est possible de penser à utiliser des modèles précliniques dont le système immunitaire et/le microbiote intestinal est proche de l'homme, tel que le modèle porc ou des rongeurs gnotobiotiques dont le microbiote intestinal est d'origine humaine.

13) Perspective : améliorer ou concevoir des modèles précliniques pour évaluer les effets anti-inflammatoires d'un probiotique ou d'un aliment fonctionnel

d) Choix de la population cible

Cette dualité du traitement probiotique en tant que traitement curatif ou préventif soulève une autre question cruciale lors du développement d'aliments fonctionnels, qui est « à qui destine-t-on ces fromages fonctionnels anti-inflammatoires ? »

Dans un premier cas, supposant que ces fromages sont destinés à une population cible déjà atteinte de rectocolite hémorragique, l'efficacité de l'ingestion de ce fromage n'est pas garantie car cette maladie est multifactorielle et il est difficile qu'une sélection donnée de souches sera efficace pour tous les patients.

En effet, aujourd'hui, au vue des données disponibles, un traitement probiotique sur-mesure doit être établi selon l'état du patient, la composition de son microbiote intestinal et les facteurs de risque génétique. Dans l'autre cas, l'approche est d'inciter les industriels à utiliser des souches sélectionnées pour à la fois leurs propriétés technologiques et probiotiques, afin de de valoriser des produits laitiers fermentés déjà existant, dans l'objectif de prévenir de diverses maladies liées à l'alimentation et à la sédentarité. Les deux approches mèneront à se questionner sur des contraintes technologiques et réglementaires différentes pour l'utilisation de ces fromages fonctionnels.

4.2. Les limites technologiques

Lors de ce projet de thèse, nous avons fait le choix des souches levains selon leurs propriétés anti-inflammatoires décrites. Auparavant, nous avons insisté sur l'importance de comprendre les interactions de ***Pf***, de *Streptococcus thermophilus* et de *Lactobacillus* sp, dans l'objectif d'effectuer un criblage pour sélectionner différentes combinaisons de ces bactéries. Dans un premier temps, les différentes combinaisons atypiques de bactéries lactiques et propioniques peuvent conduire à des réajustements des procédés de fabrication. Dans les fabrications artisanales, il est plus facile d'adapter le procédé de fabrication aux souches levains, ce qui n'est pas le cas d'une manière générale dans les industries. Dans un deuxième temps, le nombre de combinaisons à produire est en fonction du type de population cible qu'on souhaite atteindre. S'il s'agit de fabrication ponctuelle pour une population restreinte de patients, on peut supposer qu'un nombre restreint de combinaisons bactériennes est suffisant. Cependant, s'il s'agit de produire des fromages fonctionnels à titre préventif à destination de toute la population, on suppose que le nombre de combinaisons nécessaires est plus important, afin de remédier aux problématiques des phages, tout particulièrement pour les bactéries lactiques. De plus, dans ce dernier cas, les fromages de type emmental sont soumis à un cahier de charge précis à respecter, en termes d'ouverture des yeux, de goût et de texture (Legifrance). Malgré de telles perspectives, la problématique de la réglementation européenne est le frein majeur pour le développement de tels fromages, quelle que soit la population cible.

14) Perspective : mener des études d'interaction entre les différentes bactéries « 2-en-1 » dans le fromage emmental afin de comprendre les variations pouvant survenir sur le procédé de fabrication

4.3. Les limites réglementaires en Europe

La recherche clinique sur le rôle du microbiote intestinal ainsi que sa modulation par l'administration de probiotiques ou d'aliments fonctionnels dans une grande variété de maladies, est en plein essor. Dans d'autres pays développés, tels que la Suisse, le Japon ou le Canada, les effets des probiotiques sur la santé, pour lesquels des preuves scientifiques ont été fournies, peuvent être communiqués aux consommateurs potentiels. En Europe, la réglementation est plus stricte. Jusqu'en 2013, les résultats d'évaluations sur des allégations de santé concernant les probiotiques, conduite par l'autorité européenne de sécurité des aliments (EFSA), ont abouti à des refus. Toutes les demandes récentes ont été également rejetées (Dronkers et al., 2018; Guarner et al., 2011; van Loveren et al., 2012).

Le type de population à laquelle sont destinés ces fromages fonctionnels déterminera le type d'allégation à demander auprès des autorités réglementaires. Les revendications vont être effectuées sur deux types de populations, une population cible restreinte ou une population en bonne santé. En effet, il existe trois types d'allégations qui peuvent être accordées à un produit alimentaire par l'EFSA (van Loveren et al., 2012). (1) Les allégations nutritionnelles qui utiliseraient les termes « riche », « pauvre » ou « enrichi » en un nutriment en particulier. (2) Les allégations de santé qui affirment, suggèrent ou impliquent l'existence d'une relation entre, d'une part, une denrée alimentaire ou l'un de ses composants et, d'autre part, la santé. Cela pourrait être le cas de fromages fonctionnels destinés à des patients en rémission de la colite. (3) Les allégations relatives à la réduction d'un risque de maladie constituent des allégations de santé qui suggèrent qu'une denrée ou l'un de ses composants réduit sensiblement un facteur de risque de développement d'une maladie humaine. Pour des effets préventifs de fromages fonctionnels, une allégation de santé relative à une réduction d'un risque de développement de colite est envisageable. Dans ce cas, il est nécessaire de trouver des biomarqueurs généralement acceptés en fonction du risque de maladie.

Pour cela, il est essentiel de comprendre les mécanismes d'actions des probiotiques ou des aliments fonctionnels fermentés afin de mieux interpréter les données précliniques et cliniques, qui vont fournir des preuves solides pour l'obtention des allégations pour leur utilisation.

En conclusion, ce travail de thèse montre que les propriétés immunomodulatrices de *P. freudenreichii* CIRM-BIA 129 sont matrice-dépendantes, en plus d'être souche-dépendantes. Malgré de nombreuses problématiques à résoudre, ce travail de thèse montre le potentiel des fromages fonctionnels fermentés par *P. freudenreichii*, tel que l'emmental, pour prévenir la rectocolite hémorragique ou atténuer les symptômes.

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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 A (InlA) all exhibit three C-terminal conserved surface-layer-homology (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.

Figure 3. **Adhesion of probiotic bacteria to the gut mucosa mediated by extractable surface proteins.** Probiotic bacteria may adhere to intestinal mucus, to intestinal epithelial cells, or co-aggregate with other bacteria. Such interactions may lead to inhibition of pathogenic bacteria by probiotic ones, via competitive exclusion or co-aggregation, preventing the pro-inflammatory response induced by pathogen at the gut level. Extractable surface proteins, being the outermost components, are involved in such interactions in some probiotic bacteria.

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Article 1:

Figure. 1. Pro-inflammatory cytokines expression on HT-29 cells. HT-29 cells were stimulated by lipopolysaccharides (LPS), *P. freudenreichii* CIRM-BIA 129, S-layer proteins (Slps) and peptides resulting from Slps proteolysis. A) IL-8, B) TNF-α and C) IL-12 expression was monitored by RT-PCR. Data are represented as mean ± standard deviation for triplicate experiments. * $p < 0.05$ compared with Non-treated condition. # $p < 0.05$ compared with LPS condition.

Figure. 2. Regulatory cytokines expression on HT-29 cells. HT-29 cells were stimulated by lipopolysaccharides (LPS), *P. freudenreichii* CIRM-BIA 129, S-layer proteins (Slps) and peptides resulting from Slps proteolysis. A) IL-10, and B) TGF-β expression was monitored by RT-PCR. Data are represented as mean ± standard deviation for triplicate experiments. * $p < 0.05$ compared with Non-treated condition. # $p < 0.05$ compared with LPS condition.

Figure. 3. *P. freudenreichii* CIRM-BIA 129 viability after *in vitro* static digestion. Three fermented matrices were subjected to *in vitro* static digestion, viable propionibacteria were enumerated by plate counting at different digestion stage. A) Percentage of survival after succession of oral and gastric phases; B) percentage of survival after complete digestion (oral, gastric and intestinal phases). The results are expressed as percentage of survival, and as mean ± standard deviation for triplicate experiments. Different letters above the columns indicate significant differences ($p < 0.05$).

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during the same period. The feed efficiency (**C**) corresponds to the ratio between the total food intake and the weight gain at day 14. The results are represented as means ± SEM (n=7). * P<0.05.

Figure.S2. Short chain fatty acids (SCFA) concentration in faeces contents at day 7 of the three piglets groups fed with: PF-culture or PF-cheese fermented by *P. freudenreichii* CIRM-BIA 129 (PF). The total SCFA concentration **(A)**, the concentration of each SCFA type (C2, C3, C4, C5) **(B),** and the concentration of branched short chain fatty acids **(C)** were measured by gas-phase chromatography, and the proportion of each type of SCFAs **(D)** was determined. Results are represented as means ± SEM (n=7). P <0.05.

Figure.S3. Basal secretion of IL-10 **(A)**, IL-17 **(B)**, TNFα **(C)** and IFNγ **(D)** were measured in mesenteric lymph nodes cells (MLNC) cells of different piglets groups fed with: cheese matrix, or PF-culture or PF-cheese both fermented by *P. freudenreichii* CIRM-BIA 129 (PF). Cytokine concentrations were determined by ELISA and represented as means ± SEM of two analyses per piglet (n=7). P < 0.05.

Article 3:

Figure. 1. Survival of *P. freudenreichii* CIMR-BIA 129 in the caecal medium. Pellets from YEL, MUF or cheese media were incubated in the caecal medium during 50 hours in anaerobic conditions at 37°C. Survival was monitored by OD_{650nm} **(A, B)** and by CFU enumeration by plate counting **(C, D)**. Representative Epifluorescence microscopy photos of *P. freudenreichii* CIMR-BIA from different growth media after LIVE/DEAD staining after 5, 25 or 50 hours of incubation in the caecal medium are shown **(E)**. Results are the mean values of triplicate experiments. Error bars represent the standard deviation. **** P< 0.0001. Letters indicate significant difference between the three different pellets after incubation in the caecal medium (P<0.05).

Figure. 2. Survival of *P. freudenreichii* CIMR-BIA 129 in the caecal medium monitored by optical density (OD_{650nm}) measurement. Pellets from YEL (**A**, **B**), MUF (**C**, **D**) or cheese (**E**, **F**) were submitted to a negative shaving control or shaving treatments to proteolysis surface proteins. Cultures were incubated in the caecal medium for 50 hours in anaerobic conditions at 37°C. Results are the mean values of triplicate experiments. Error bars represent the standard deviation.

Figure. 3. Survival of *P. freudenreichii* CIMR-BIA 129 in the caecal medium monitored by CFU counting. Pellets from YEL (**A**, **B**), MUF (**C**, **D**) or cheese (**E**, **F**) were submitted to a negative shaving control or shaving treatments to proteolysis surface proteins. Cultures were incubated in the caecal medium for 50 hours in anaerobic conditions at 37°C. Results are the mean values of triplicate experiments. Error bars represent the standard deviation. ** P<0.01, **** P< 0.0001. # indicate significant difference between the three different pellets after incubation in caecal medium (P<0.05).

Figure. 4. Representative Epifluorescence microscopy photos of *P. freudenreichii* CIMR-BIA 129 from YEL, MUF or cheese, stained with LIVE/DEAD staining after 5, 25 or 50 hours of incubation in the caecal medium with or without shaving treatment.

Figure. 5. Analysis of S-layer protein B (SlpB) expression by *P. freudenreichii* CIMR-BIA 129 from YEL, MUF and cheese after incubation in the caecal medium. Pellets from YEL (**A**), MUF (**D**) or cheese (**C**) were incubated 50 hours in the caecal medium, after no treatment, negative shaving control or shaving treatments. Whole-cell protein extracts from different cultures were analyzed by Western Blot detection, using an anti-body directed against SlpB. A surface layer proteins extract was loaded as a positive control (+).

Figure. 6. Survival monitoring of *P. freudenreichii* CIMR-BIA 129 KO SlpB in the caecal medium. Pellets from YEL medium were submitted to shaving or to a negative shaving control treatment. Culture were incubated in the caecal medium during 50 hours in anaerobic conditions at 37°C. Growth was monitored by OD_{650nm} (**A**, **B**) and by CFU enumeration (**C**, **D**). Representative Epifluorescence microscopy photos of *P. freudenreichii* CIMR-BIA 129 KO SlpB after LIVE/DEAD staining of different pellets (**E**). Results are the mean values of triplicate experiments. Error bars represent the standard deviation. **** P< 0.0001. Letters indicate significant difference between the three different pellets after incubation in the caecal medium (P<0.05).

Figure.S1. The consumption of gluconate, as a carbon source, by *P. freudenreichii* CIRM-BIA 129 and *P. freudenreichii* CIRM-BIA 129 KO SlpB in the caecal medium. The *P. freudenreichii* CIRM-BIA 129 were extracted from different media: milk ultrafiltrate culture, YEL culture and fermented cheese. The *P. freudenreichii* CIRM-BIA 129 KO SlpB pellet were from YEL culture. The different pellets were submitted to different treatments: non-treated, shaving negative control or shaving treatments to proteolysis the surface proteins. Gluconate concentration was determined before and after incubation in the caecal medium by HPLC analysis.

Article 4:

Figure 1. Impact of cheese matrix, single-strain cheese and Emmental cheese on the severity of DSS-induced colitis. **(A)** Body weight (as a percentage of the initial weight) in the healthy group (PBS); and after DSS colitis induction in PBS, cheese matrix, single-strain cheese and Emmental cheese groups. **(B)** Disease activity index. **(C)** Colon length. The data represent the mean ± SD of 18 mice per group. Multiple comparisons were performed, *p<0.05, *p<0.05 ** p<0.01, ***p<0.001 **** p<0.0001.

Figure 2. Impact of cheese matrix, single-strain cheese and Emmental cheese intake on small bowel IgA secretion. Secretory IgA concentration in the small bowel was determined by ELISA quantification. The data represent the mean ± SD of 18 mice per group. Multiple comparisons were performed, *p<0.05, *p<0.05 ** p<0.01, ***p<0.001 **** p<0.0001.

Figure 3. Impact of cheese matrix, single-strain cheese and Emmental cheese on the epithelial colonic cells barrier and on the colonic oxidative stress during DSS-induced colitis. Colonic mRNA expression levels of **(A)** CLD1, **(B)** Muc2, **(C)** ZO1, **(D)** ZO2 **(E)** Ocln and **(F)** iNOS genes are analysed. The data

represent the mean ± SD of 18 mice per group. Multiple comparisons were performed, *p<0.05, *p<0.05 ** p<0.01, ***p<0.001 **** p<0.0001.

Figure 4. Impact of cheese matrix, single-strain cheese and Emmental cheese on cytokines expression in the colonic tissues during DSS-induced colitis. Colonic mRNA expression levels of **(A)** IL-10, **(B)** TGFβ**, (C)** IL-1β, **(D)** TNFα**, (E)** IFNγ**,** and **(F)** IL-21 were determined. Data represent the mean ± SD of 18 mice per group. Multiple comparisons were performed, *p<0.05, *p<0.05 ** p<0.01, ***p<0.001 **** p<0.0001.

Figure 5. Impact of cheese matrix, single-strain cheese and Emmental cheese on secreted cytokines in the colonic tissues during DSS-induced colitis. Cytokines concentration of **(A)** IL-10, **(B)** TGFβ, **(C)** IL-6, **(D)** IFNγ, **(E)** IL-17, **(F)** TNFα**, (G)** IL-12 and **(H)** IL-1β were quantified by ELISA. Data represent the mean ± SD of 18 mice per group. Multiple comparisons were performed, *p<0.05, *p<0.05 ** p<0.01, ***p<0.001 **** p<0.0001.

ANNEXES

Annexe 1 : l'étude de la réponse inflammatoire *in vitro* de la souche mutante *P. freudenreichii* CIRM-BIA 129 KO SlpB versus la souche sauvage *P. freudenreichii* CIRM-BIA 129 sur les cellules épithéliales intestinales HT-29

L'effet immunomodulateur de la souche mutante de *P. freudenreichii* ***(Pf)*** CIRM-BIA 129 pour la protéine SlpB (KO) a été étudié *in vitro* sur des cultures de cellules épithéliales intestinales HT-29 ; en suivant le protocole présenté dans l'article 1 (voir section 3.2). L'effet immunomodulateur de cette souche mutante a été comparé à l'effet immunomodulateur de la souche sauvage. Les stimulations des cellules par les deux souches ont été réalisées en présence ou en absence de lipopolysaccharide d'*Escherichia coli*. Le niveau d'expression des gènes codant pour des cytokines ou des marqueurs de l'inflammation a été déterminé par RT-PCR quantitative.

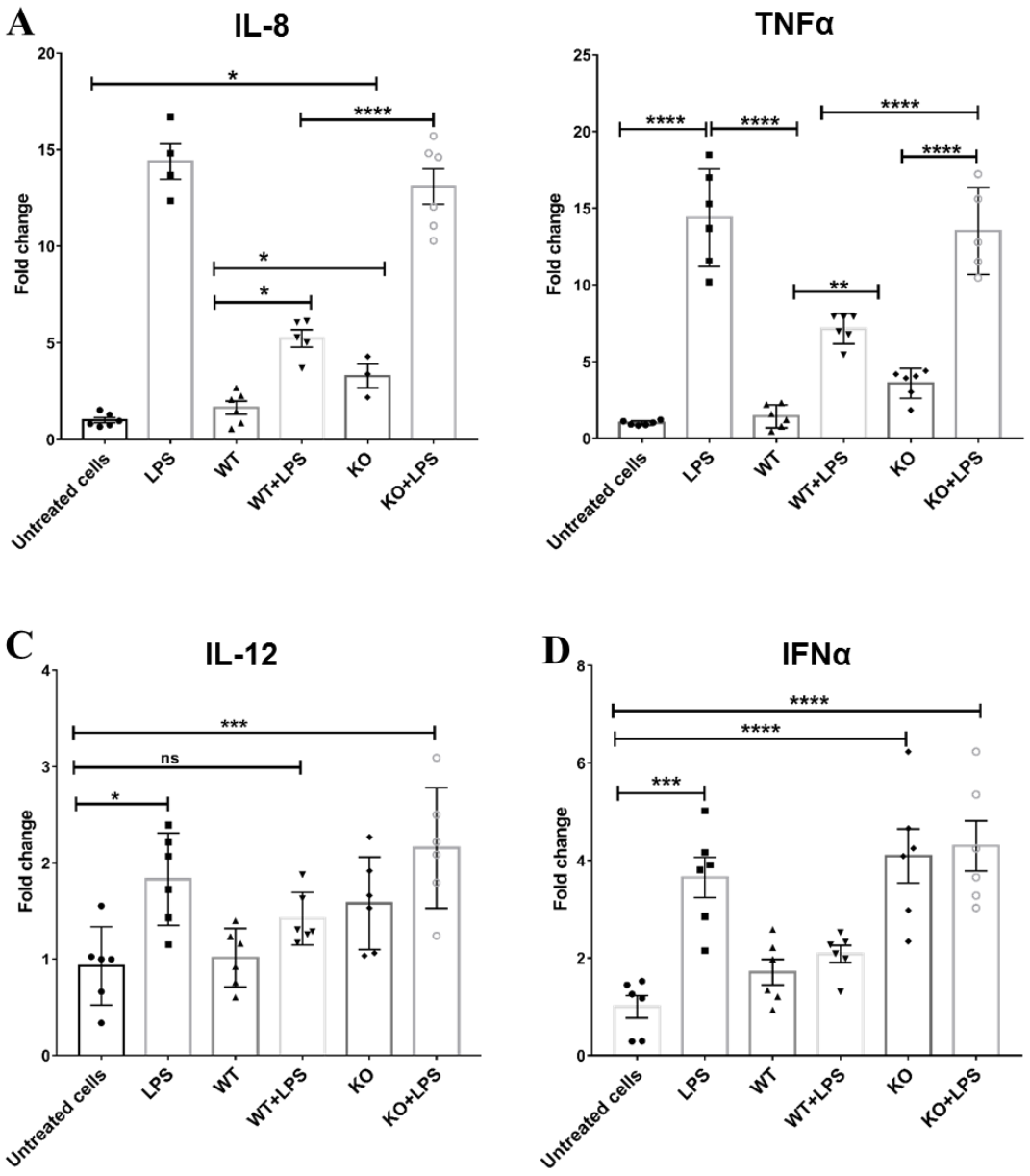


Figure 1. Le niveau d'expression des gènes codant pour des cytokines pro-inflammatoires : **(A)** IL-8, **(B)** TNFα, **(C)** IL-12 et **(D)** IFNα. Les cellules HT-29 ont été stimulées par la souche sauvage ***Pf*** CIRM-BIA 129 (WT) ou la souche mutante ***Pf*** CIRM-BIA 129 KO SlpB (KO), en présence ou en absence de lipopolysaccharide (LPS).

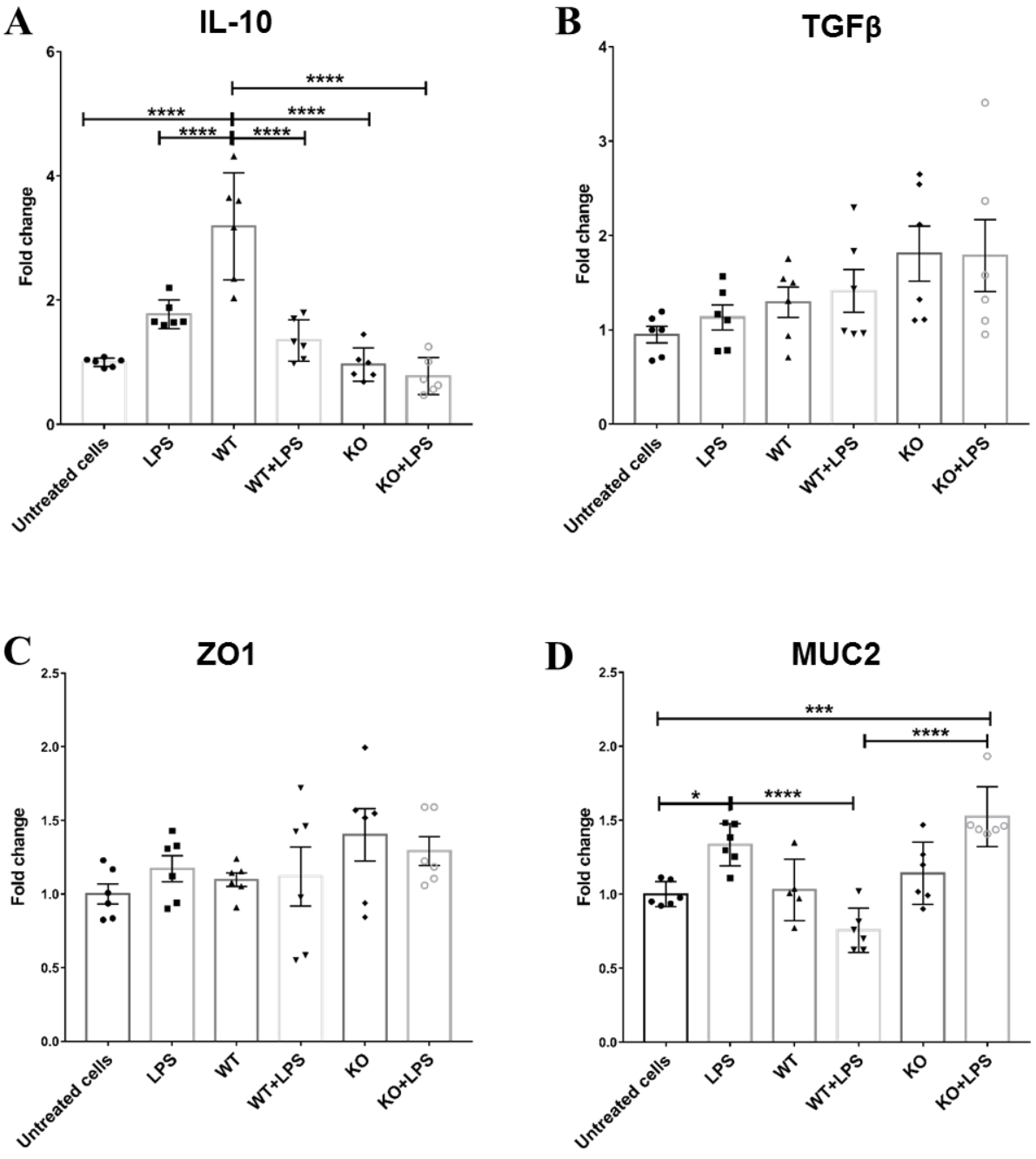


Figure 2. Le niveau d'expression de différents gènes codant pour des cytokines anti-inflammatoires, ainsi que des gènes marqueurs de l'intégrité cellulaire : **(A)** IL-10, **(B)** TGFβ, **(C)** ZO1 et **(D)** MUC2. Les cellules HT-29 ont été stimulées par la souche sauvage ***Pf*** CIRM-BIA 129 (WT) ou la souche mutante ***Pf*** CIRM-BIA 129 KO SlpB (KO), en présence ou en absence de lipopolysaccharide (LPS).

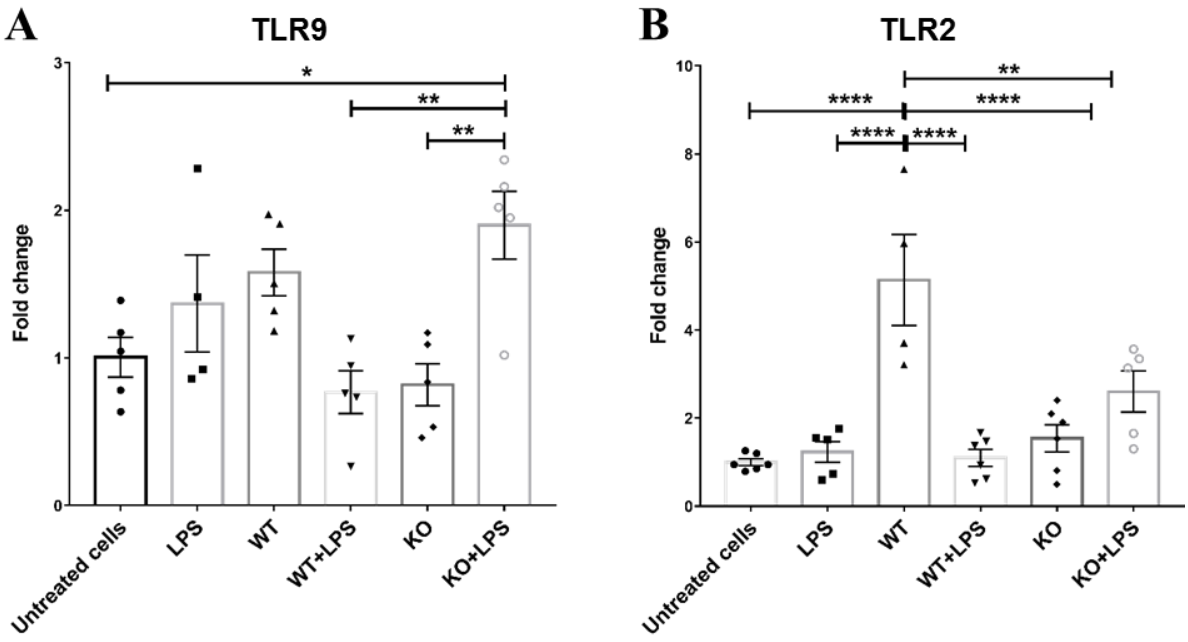


Figure 3. Le niveau d'expression des gènes codant pour les récepteurs **(A)** TLR9 et **(B)** TLR2. Les cellules HT-29 ont été stimulées par la souche sauvage ***Pf*** CIRM-BIA 129 (WT) ou la souche mutante ***Pf*** CIRM-BIA 129 KO SlpB (KO), en présence ou en absence de lipopolysaccharides (LPS).

Annexe 2 : l'étude des modifications covalentes de la protéine immunomodulatrice SlpB de *P. freudenreichii* CIRM-BIA 129

Introduction

1. Les différents types de modifications covalentes des protéines bactériennes

Les modifications post-traductionnelles ou les modifications covalentes impliquées dans le polymorphisme protéique sont nombreuses, et elles sont décrites dans les trois domaines du monde vivant : les eucaryotes, les bactéries et les archées (Cain et al., 2014; Schäffer and Messner, 2017). Elles affectent la structure primaire de la chaîne peptidique et/ou la protéine mature de manière réversible ou irréversible. Les principales modifications covalentes décrites chez les bactéries sont :

1) La glycosylation, le processus de modification post-traductionnel le plus fréquent dans le monde vivant, il s'agit d'une modification irréversible impliquée dans la localisation, la stabilité des protéines, ainsi que la reconnaissance protéines-protéine ou protéine-ligand. À ce jour, on distingue principalement deux types de glycosylation. La N-glycosylation correspondant à un greffage par une glycotransférase d'un glycane sur un résidu aspargine (le motif Asparagine-X-Serine/Thréonine, où X peut être un acide aminé quelconque sauf une proline). L'O-glycosylation s'effectue sur des résidus tyrosine, sérine ou thréonine. En addition de ces deux catégories, plusieurs autres types de glycosylation ont été décrits, mais de manière moins fréquente (la phosphoglycosylation, la C-mannosylation, ou la S-glycosylation) (Abu-Qarn et al., 2008; Cain et al., 2014).
2) La phosphorylation des protéines s'agit d'une modification réversible impliquée dans de nombreuses fonctions incluant la transmission de signaux cellulaires, d'activation/inactivation d'enzymes, de modulation des interactions protéine-protéine. La phosphorylation des protéines s'agit d'un ajout par des Kinases d'un groupement PO_4^{3-} sur un résidu tyrosine, sérine, thréonine, histidine, aspartate, arginine ou encore cystéine (Cain et al., 2014).
3) L'acétylation d'une protéine consiste en une addition d'un groupe acétyle au groupement amine des chaînes latérales d'une lysine ou d'un acide aminé de l'extrémité N-terminale de la protéine.

En plus des effets stériques, l'acétylation modifie également la charge de la protéine (Cain et al., 2014).

4) L'acylation est le processus par lequel une chaîne hydrocarbonée est ajoutée à la protéine (Cain et al., 2014).

2. Les modifications covalentes des protéines S-layer

Les protéines Slps (« S-Layer Proteins») constituent la couche S, une surface paracristalline structurée qui enveloppe la surface de certaines bactéries à Gram négatif et à Gram positif. Elle était initialement identifiée et bien caractérisée chez les archées (Beveridge et al., 1997; Gerbino et al., 2015b; Rodrigues-Oliveira et al., 2017). Cette structure joue un rôle important dans la croissance et la survie de ces microorganismes. Les nombreuses fonctions des Slps comprennent le maintien de l'intégrité de la cellule, la lutte contre des agents pathogènes, l'interaction avec l'hôte et son système immunitaire (Beveridge et al., 1997; Do Carmo et al., 2018a; Engelhardt, 2007). La caractérisation biochimique des protéines S-layer, à la fois chez les bactéries ainsi que chez les archées, a montré que la glycosylation est la modification covalente majeure présente sur ce type de protéines (Schäffer and Messner, 2004, 2017). La présence de telles modifications covalentes sur les protéines S-layer impacte la formation de la couche S et ses propriétés de surface (Schäffer and Messner, 2004). Cependant, il existe une différence en termes du taux de glycosylation observé entre les archées et les bactéries. En effet, les structures des N-glycosylations des Slps des archées présentent une grande diversité en termes de taille des glycanes; de degré de ramification; de présence de sucres uniques et de la nature de l'acide aminé modifié (Anzengruber et al., 2014; Cavallero et al., 2017; Schäffer and Messner, 2004). Le nombre de résidus modifiés ainsi que la longueur des chaînes glycanes (50 unités identiques) sont moindres chez les protéines S-layer des bactéries. Les glycosylations des protéines S-layer, décrites essentiellement chez les espèces lactiques, peuvent être ancrées en N ou O sur le squelette peptidique et peuvent se composer d'hexoses, de pentoses, d'heptoses ou de désoxyhexose neutres et d'osamines, tels que le N-acétylgalactosamine ou le N-acétylglucosamine (Anzengruber et al., 2014; Åvall-Jääskeläinen, 2005; Cavallero et al., 2017; Mobili et al., 2010).

3. Les modifications covalentes des protéines S-layer de *P. freudenreichii*

Chez *P. freudenreichii*, l'analyse des extraits de protéines Slps provenant de différentes souches a révélé la présence d'isoformes de point isoélectrique pour les protéines SlpA, SlpB, SlpE et l'internaline like (Inl). L'analyse par spectrométrie de masse a permis de démontrer la présence de modifications covalentes (Figure 1), notamment des sites probables de glycosylation, à la fois des N-glycosylations et d'O-glycosylations (Rabah, H., 2015). En plus, en ce qui concerne la protéine SlpB, un site potentiel de phosphorylation a été également mis en évidence. Dans la littérature, aucune protéine portant des modifications covalentes telle qu'une glycosylation ou une phosphorylation n'a été identifiée ou caractérisée chez les espèces des propionibactéries laitières.

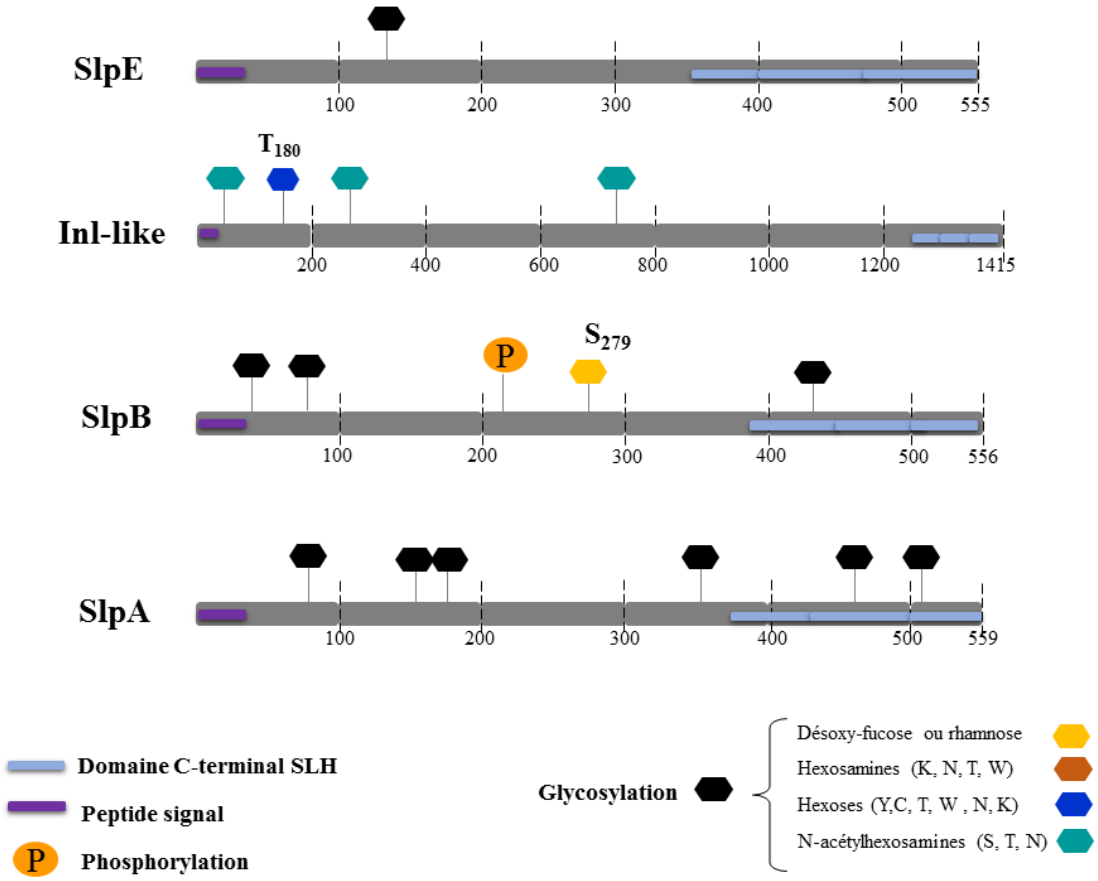


Figure 1. Les modifications covalentes ainsi que les sites modifiés identifiés par spectrométrie de masse. Les différentes isoformes des protéines SlpA, SlpB, SlpE et Internaline-like ont été prélevées sur les gels bidimensionnels. Les peptides issus de la protéolyse par trypsine des différentes isoformes ont été analysés par LC-MS/MS. Des sites potentiels de glycosylation ainsi que de phosphorylation ont été identifiés.

4. Le rôle des modifications covalentes des protéines S-layer dans les interactions bactérie/hôte

Il existe de nombreuses études relevant l'importance des modifications covalentes des protéines de surface chez les bactéries pathogènes lors des interactions avec les cellules de l'hôte. Cependant, les études émergentes soulignent également l'importance de la glycosylation des protéines dans l'interaction du microbiote ou du probiotiques avec l'hôte (Do Carmo et al., 2018a; Latousakis and Juge, 2018). En effet, les modifications covalentes peuvent induire des changements de conformation des sites actifs ou de liaison des ligands, ce qui affecte la relation structure/fonction des protéines. Les protéines S-layer de *P. freudenreichii* et d'autres espèces bactériennes probiotiques jouent un rôle essentiel dans l'interaction avec le système immunitaire de l'hôte. En effet, elles constituent des MAMP (microbe-associated molecular pattern), par lesquelles le probiotique module le système immunitaire de l'hôte (Colliou et al., 2017; Do Carmo et al., 2018a; Lightfoot et al., 2015). Les modifications covalentes de type glycosylation peuvent donc être déterminantes pour l'interaction entre les protéines Slps et l'hôte via les récepteurs des cellules de l'hôte, appelés Pattern Recognition Receptors (PRRs), comme cela a été démontré pour *Lactobacillus kefiri* (Malamud et al., 2018). En effet, les récepteurs de type C-lectin sont des PRRs qui peuvent potentiellement interagir avec les motifs glycanes des Slps. Chez *P. freudenreichii* UF1, une protéine de surface possédant un domaine fonctionnel semblable à la protéine SlpB interagit avec le récepteurs DC-SIGN (dendritic cell- specific intercellular adhesion molecule-3 grabbing non- integrin) (Colliou et al., 2017). Cependant, les auteurs n'ont pas examiné la présence probable de modifications covalentes de type glycosylation sur cette protéine S-layer de la souche *P. freudenreichii* UF1.

Objectif et stratégie

L'analyse préliminaire n'a pas permis d'identifier de manière affirmative les sites ainsi que les structures exactes des modifications covalentes des protéines Slps. En effet, chez une seule forme de protéine, un peptide donné est détecté sous sa forme non-glycosylée et glycosylée dans le même échantillon, et les formes glycosylées sont moins abondantes, ce qui a rendu leur identification plus compliquée. De plus, certaines protéines telles que SlpE, elle est présente en faible concentration dans l'extrait analysé, ce qui

a rendu l'identification des peptides glycosylés par spectrométrie de masse encore plus difficile. Par conséquent, l'analyse était insuffisante pour caractériser de manière précise les modifications covalentes des protéines S-layer de *P. freudenreichii*, en particulier la protéine SlpB.

L'objectif de cette étude était de déterminer les sites de modifications covalentes présents sur la protéine SlpB; ainsi que leur nature et leur structure. Dans un premier temps, nous avons examiné la nature des glycanes présents sur la protéine SlpB par le lectinblot et confirmé la présence de phosphorylation par une méthode de coloration spécifique des phosphoprotéines. Ensuite, nous avons mis en place une stratégie d'analyse par spectrométrie de masse en préparant des échantillons peptidiques +/-glycosylés ou phosphorylés des différentes isoformes de la protéine SlpB prélevé sur des gels bidimensionnels (Figure 2). L'analyse de ces échantillons a été effectuée par la spectrométrie de masse LC-MS/MS, au sein de la plateforme de protéomique de l'institut Jacques Monod (Paris). Pour cela; différentes échantillons ont été préparés en suivant une stratégie (Figure 2), tirée de l'article de Li-Juan Huang et al,.(2014) (Huang et al., 2014).

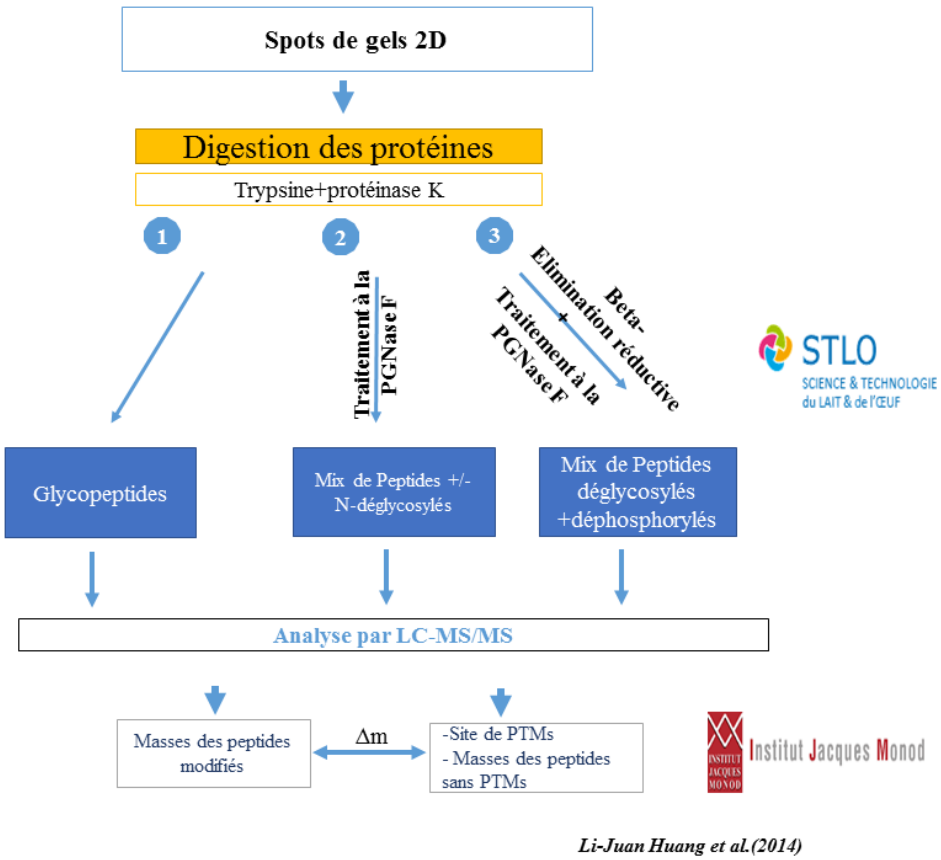


Figure 2. La stratégie expérimentale mise en œuvre pour identifier les sites des modifications covalentes ainsi que les types de glycanes présents sur la protéine SlpB.

Matériels et méthodes

Extraction des protéines de surface par le chlorure de guanidine

Une extraction des protéines Slps a été réalisée en utilisant l'agent chaotrope, le chlorure de guanidine, tel que décrit précédemment (Le Maréchal et al., 2015). Plusieurs cultures successives sur l'ultrafiltrat de lait ont été réalisées afin de constituer un stock de protéines Slps. Après centrifugation, les culots bactériens sont lavés deux fois par le tampon PBS (Phosphate Buffer saline). Les culots correspondants à une densité optique de 40 sont ensuite repris dans 2 mL de solution de chlorure de guanidine à 5 M. Les mélanges sont incubés pendant 15 minutes à 50°C. Les surnageants contenant les protéines Slps sont récupérés par centrifugation. Les extraits protéiques successifs obtenus sont dialysés contre de l'eau déminéralisée, puis contre du PBS grâce à des cassettes de dialyse Slide-A-Lyzer® 10KDa (Thermo). La concentration protéique des différents extraits protéiques obtenus a été déterminée en utilisant le kit 2-D Quant Kit et en suivant les instructions du fabricant (GE Healthcare, Vélizy, France).

Lectinblot

Le lectinblot est une technique d'identification et de caractérisation de motifs glucidiques présents sur des protéines, et qui se repose sur les interactions lectine-glycane. Selon les caractéristiques des lectines, différents types de sucres peuvent être reconnus (Cao et al., 2013). Les extraits protéiques de Slps ont été séparés par SDS-PAGE sur un système d'électrophorèse Mini-PROTEAN 3 (Biorad), en suivant le protocole de Laemmeli (Laemmli, 1970). La migration a été effectuée à un voltage constant jusqu'à la sortie du front de migration indiqué par le bleu de bromophénol. Les gels ont été ensuite transférés sur des membranes PVDF (Polyvinylidene difluoride) (GE Healthcare) en utilisant le système Trans-Blot® Electrophoretic Transfer Cell (250 mA, 30 V, 1h30). Les membranes ont été incubées à température ambiante dans une solution de blocage (0,1 M de Tris-HCl, 0,25 % gélatine, 10 % d'éthanolamine, pH=9). Après rinçage avec une solution de TBS (Tris-buffered saline, 10 mM de Tris-HCl, 0,15 M de NaCl, pH=7,5), les membranes ont été incubées toute la nuit à une température ambiante avec les différentes lectines biotinylées (BIOTINYLATED LECTIN KIT I, Vector Laboratories, Inc). Les différentes lectines ont été diluées dans une solution de dilution (50 mM de Tris-HCl, 0.25% de gélatine, 0,05% de tween 20, 0,15 M de NaCl, 5 mM EDTA, pH=7,5) à une concentration de 10 µg/ml. Les

membranes ont été ensuite rincées, puis incubées avec une solution de R.T.U (Horseradish Peroxidase Streptavidin, Vector Laboratories, Inc). Les membranes ont été révélées en utilisant le système ECL plus® (GE Healthcare, Vélizy, France) et ensuite scannées en utilisant la GBox Syngene (Ozyme, Saint-Quentin-en-Yvelines, France).

Coloration au Pro-Q® Diamond

Les extraits protéiques de Slps, environ 25 µg ont été analysés par SDS-PAGE, tel que décrit précédemment. Un standard de poids moléculaire contenant deux phosphoprotéines (PeppermintStick™, Molecular Probes, Inc., Invitrogen) a été utilisé en tant qu'un témoin positif. Les gels obtenus ont été fixés toute la nuit dans une solution contenant 50% méthanol et 10% d'acide acétique, afin de s'assurer que tout le SDS est éliminé du gel. Les gels sont ensuite rincés (3 rinçage de 10 minutes) à l'eau ultra-pure afin d'éliminer toute trace d'acide acétique ou de méthanol. Les gels sont ensuite incubés pendant 2 h dans une solution Pro-Q® Diamond (Molecular Probes, Inc., Invitrogen), puis ils ont été décolorés par une solution de décoloration (20% acétonitrile, 50 mM acétate de sodium, pH=4) pour une durée d'une heure. Au final, les gels décolorés sont rincés deux fois à l'eau ultra-pure. Les gels ont été visualisés en utilisant la GBox Syngene (Ozyme, Saint-Quentin-en-Yvelines, France), avec une longueur d'excitation de 532 nm, selon les instructions du fabricant.

Electrophorèse bidimensionnelle

La quantité protéique en Slps nécessaire à la réalisation d'un gel bidimensionnels a été précipitée avec le kit 2D-Clean Up (GE Healthcare, Vélizy, France) Les protéines sont ensuite réhydratées dans 100 µL de tampon Destreak (GE Healthcare, Vélizy, France), contenant 2% d'IPG Buffer pH 4-7 (GE Healthcare, Vélizy, France). La focalisation isoélectrique a été effectuée sur des bandelettes linéaires Immobiline DryStrip Gels de 13 cm, avec un gradient de pH allant de 4 à 7 (GE Healthcare) sur un système Multiphore II (GE Healthcare, Vélizy, France). Ces bandelettes sont préalablement réhydratées une nuit dans 450 µL de tampon Destreak additionné d'IPG Buffer (pH 4-7, 2%). Les échantillons sont déposés à l'extrémité acide des bandelettes à l'aide de cupules. La migration s'est effectuée en 24 h selon un programme permettant une augmentation progressive de la tension et une accumulation de 60 KVh. À la fin de la focalisation, les bandelettes sont incubées sous agitation successivement dans les

solutions d'équilibration I et II (50 mM de Tris-HCl-pH=6,8, 6 M d'urée, 30% de glycérol, 1% de SDS, avec 0,3% de DTT pour la solution I et 4,5% d'iodoacétamide pour la solution II) pendant 10 min. Cette étape permet de dénaturer davantage les protéines en réduisant les ponts disulfures avec le DTT et en alkylant les fonctions SH libérées avec l'iodoacétamide. Cela permet également de complexer les protéines avec le SDS et d'éliminer certains constituants de la première dimension.

Les bandelettes sont adaptées en haut d'un gel SDS-PAGE et immobilisées avec de l'agarose de pontage (125 mM de Tris-HCl-pH=6,8, 0,1% de SDS et 0,5% d'agarose) qui servira également de gel de concentration. La seconde migration est réalisée dans un système PROTEAN II xi (Biorad). Après coloration des gels au bleu de Coomassie, les différentes isoformes de la protéine SlpB ont été prélevées (Tableau 1).

Préparation des fractions des peptides

a) Digestion peptidique

Les bouts de gels sont lavés avec une solution d'acétonitrile à 50 % afin d'éliminer le bleu de Coomassie. Les morceaux de gel décolorés à ce stade, ont été séchés au SpeedVac. Un mix d'une solution de trypsine et d'une solution de protéinase K a été préparé contenant 4 µL de trypsine (soit 0,8 µg) + 1 µL de protéinase K (soit 0,2 µg) et déposé sur les bouts de gels déshydratés. Les bouts de gels sont incubés 15 minutes à température ambiante pour qu'ils soient réhydratés. Ils ont été ensuite recouverts avec 25 µL de tampon de carbonate d'ammonium (50 mM). Les gels sont ensuite incubés toute une nuit à 37°C. La réaction de protéolyse est arrêtée en ajoutant 3 µL d'acide trifluoroacétique (TFA) à 5 %.

b) Extraction des peptides

Les surnageants présents autour des gels (pour chaque spot) ont été récupérés (SN1). Ensuite, 25 µL d'acétonitrile (ACN) à 50 % sont ajoutés, après agitation pendant 15 min (table vibrante ~250 tr/min), les surnageants (SN2) ont été récupérés. De nouveau, 25 µL d'ACN à 50 % aux bouts de gels afin de récupérer une troisième fraction (SN3). L'ensemble des surnageants prélevés (SN1+SN2+SN3) a été concentré sous vide sans aller à sec.

c) N-déglycosylation des peptides

La solution de PNGase F a été ajoutée (5 unités/ spot de gel) aux peptides extraits précédemment (Etape b), soit les échantillons N°4 à 9 (Tableau 1). Les échantillons sont incubés 2 h à une température ambiante. La réaction est arrêtée par l'ajout de TFA à 5%.

d) O-déglycosylation et déphosphorylation par le kit β-elimination

Les volumes des échantillons N°7 à 9 ont été précisément mesurés. Le réactif de β-élimination (Sigma) a été ensuite ajouté, à environ 20% du volume de l'échantillon ; soit 20 µL de réactif pour 100 µL d'échantillon. Les échantillons 7 à 9 sont ainsi incubés 18 heures à 25°C (Tableau 1). La réaction est arrêtée par l'ajout de 10 µL de TFA à 5%.

N° d'échantillon	N° du gel	Protéine S-layer	N° du spot	Traitement avec PNGase F	Traitement avec β-élimination reagent Mixture
1	1	SlpB	B2		
2			B3		
3			B4		
4	2		B2	x	
5			B3	x	
6			B4	x	
7	3		B2	x	x
8			B3	x	x
9			B4	x	x

Tableau 1. Le récapitulatif des échantillons préparés. Les différents traitements subis par les peptides sont indiqués, après une «digestion in gel» des spots de SlpB par un mélange de trypsine et de protéinase K.

Analyse par spectrométrie de Masse

En addition des différents traitements enzymatiques et chimiques effectués des échantillons, l'analyse par spectrométrie de masse a consisté à utiliser différents modes de fragmentation (Moradian et al., 2014; Yu et al., 2018) :

1) Le premier type de fragmentation des peptides est la fragmentation par collision-induced dissociation (CID), il s'agit d'une fragmentation des ions moléculaires en phase gazeuse. Dans le cas des phosphorylations, la fragmentation CID des peptides contenant des phosphosérines et phosphothréonines conduitt souvent à une perte d'H_3PO_4 via une β-élimination en phase gazeuse.

2) Le deuxième type de fragmentation des peptides est la dissociation par collision de haute énergie (HCD). Le schéma de fragmentation du HCD est caractérisé par une énergie d'activation plus élevée et un temps d'activation plus court, par comparaison à une fragmentation CID. Les O-glycanes sont habituellement complétement éliminés via un réarrangement en phase gazeuse.
3) La fragmentation par ETD (Electron-transfer dissociation) permettrait théoriquement d'identifier les modifications post-traductionnelles labiles au CID. Idéalement, elle peut fournir à la fois des informations sur la séquence et sur la localisation des sites de modification.
4) La fragmentation par ECD (Electron-capture dissociation) est plus préférable lorsqu'il s'agit de peptides ayant des phosphorylations multiples, car elle peut provoquer une fragmentation efficace du squelette peptidique tout en laissant la modification covalente intacte.

Ainsi, l'identification précise du type de modification et du site de modification peut être très difficile en envisageant un seul type de fragmentation des peptides modifiés (Moradian et al., 2014; Yu et al., 2018). L'analyse de spectrométrie de masse à l'institut Jacques Monod a consisté à combiner les 4 types de fragmentation. Des informations différentes et complémentaires peuvent être ainsi obtenues sur la structure primaire ou les modifications chimiques présentes sur les peptides analysés.

Résultats & Discussion

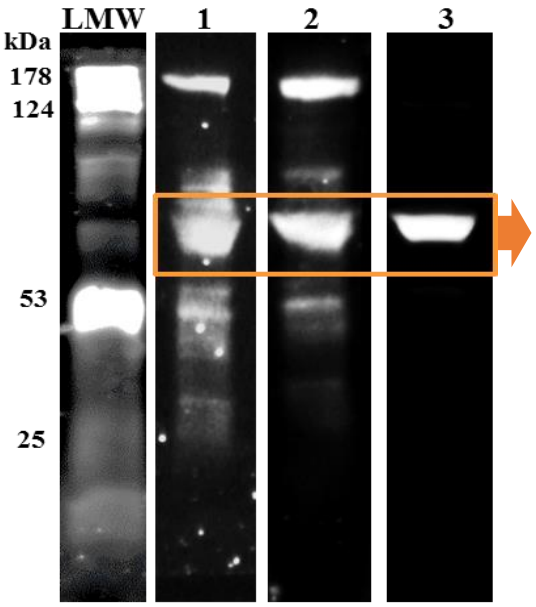
Ce volet de travail a consisté à caractériser les modifications covalentes de la protéine SlpB par des outils de protéomique. L'analyse par le lectinblot des extraits de protéines de surface de *P. freudenreichii* CIRM-BIA 129 confirme en effet la présence de glycosylations sur la protéine SlpB (Figure 3). Un jeu de sept lectines différentes a été testé, seulement trois d'entre elles ont réagi positivement à la protéine SlpB : la lectin *Dolichos biflorus* agglutinin (DBA) qui reconnait le motif αGalNAc, la lectine *Ulex europaeus* agglutinin 1(UEA 1) qui reconnait le motif α-fucose, ainsi que la lectine *Arachis hypogaea* (peanut) agglutinin (PNA) qui reconnait le motif Galβ3GalNAc (Figure 3). Il est noté, n'ayant pas testé l'ensemble des lectines, la protéine SlpB peut évidemment porter d'autres types de glycanes.

Cependant, l'analyse par spectrométrie de masse effectuée n'a pas abouti à un résultat concluant. En effet, la digestion non spécifique par la protéinase K a conduit à la génération de peptides trop courts, ce qui a compliqué l'alignement des séquences peptidiques sur la séquence protéique. Au lieu, nous

avons procédé à l'analyse *de novo* des données générées lors de la première expérience (Rabah, H., 2015). L'analyse des données a permis de mettre en évidence 4 sites de glycosylation, dont 3 qui sont localisés sur la partie C-terminale de la protéine (Domaine SLH) (Figure 5). Il s'agit d'O-glycosylations sur la sérine 283, la sérine 429, la thréonine 415 ; ainsi qu'une N-glycosylation sur l'asparagine 462 (Figure 5). Les types de glycanes mis en évidence sont en cohérence avec les observations faites sur les lectinblot. Cependant, la structure exacte des glycanes reste inconnue. Chez *Cutinibacterium acnes* (*Propionibacterium acnes*), DsA1, une protéine de liaison au fibrinogène a été décrite comme glycosylée portant des résidus GalNAc (Grange et al., 2017). Très peu de connaissances existent sur les mécanismes de glycosylation chez les propionibactéries laitières. En effet, une glycosyltransférase a été bien caractérisée chez les souches de *P. freudenreichii* pour son rôle dans la production des exopolysaccharides (Deutsch et al., 2008, 2010). L'expression élevée du gène *gtf* a été corrélée à la présence d'une capsule d' exopolysaccharides parmi les souches présentant le phénotype agglutination (+) (Deutsch et al., 2010). Néanmoins, un niveau d'expression plus bas du gène *gtf* a été mis en évidence chez les souches présentant le phénotype agglutination (-), ce qui suggère que ces enzymes pourraient être indispensables pour d'autres fonctions bactériennes, probablement la synthèse des glycoprotéines. En effet, les glycosyltransférases des actinomycètes sont décrites comme étant versatiles, en ce qui concerne le donneur de sucre, mais également dans une moindre mesure, en ce qui concerne l'accepteur des sucres (Salas and Méndez, 2007).

La coloration au Pro-Q® Diamond des extraits Slps avant ou après le traitement à la phosphatase alkaline montre bien la présence de phosphorylation seulement sur la protéine SlpB (Figure 4). L'analyse des données de spectrométrie de masse a également confirmé la présence de phosphorylation sur la thréonine 174 (Figure 5). En ce qui concerne la phosphorylation, seulement des Serine kinases ont été déjà décrites chez les espèces des propionibactéries laitières (Cagen and Friedmann, 1972), mais il n'existe pas d'étude du phosphoprotéome chez *P. freudenreichii*. Le rôle de la présence d'une telle modification covalente sur la protéine SlpB est inconnu. Néanmoins, l'outil InterPro a prédis la présence d'un domaine dit « Terpenoid cyclases/protein prenyltransferase alpha-alpha toroid » sur la protéine SlpB. Les protéines présentant ce domaine sont identifiées comme des enzymes impliquées notamment

dans le transfert d'un groupement farnésyle sur des résidus cystèines de protéines, nécessaire pour les ancrages des protéines de surface à la membrane cellulaire. Une seule protéine S-layer du pathogène *Aeromonas hydrophila*, appartenant au phylum de Proteobactérie, a été décrite comme une protéine phosphorylée (une tyrosine phosphorylation) (Thomas and Trust, 1995).



SlpB	Lectines		Sucres reconnus
	Piste 1	Dolichos biflorus agglutinin	αGalNAc
	Piste 2	Ulex europaeus agglutinin 1	αFucose
	Piste 3	Peanut agglutinin	Galβ3GalNAc

Figure 3. LectinBlot de l'extrait Slps de *P.freudenreichii* CIRM-BIA 129. La réactivité des différentes lectines biotinylées, (1) *Dolichos biflorus* agglutinin (DBA), (2) *Ulex europaeus* agglutinin, (3) *Arachis hypogaea* (Peanut); vis-à-vis de la protéine SlpB a été examinée.

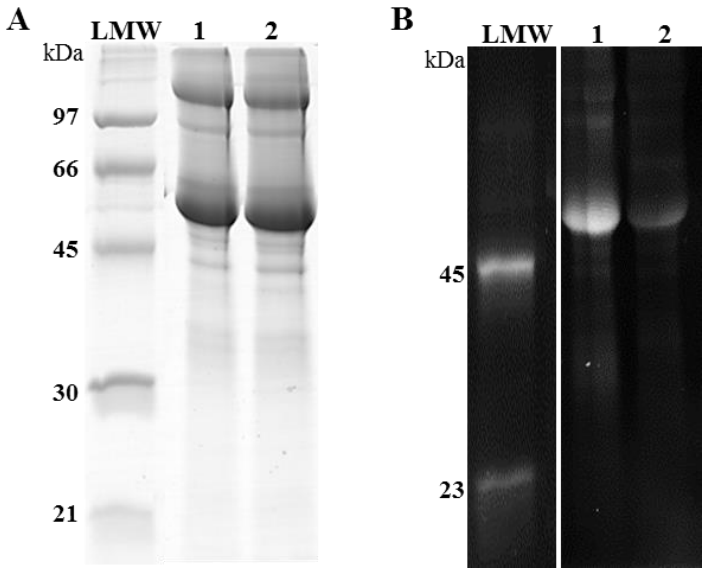


Figure 4. Analyse de l'extrait Slps de *P.freudenreichii* CIRM-BIA 129 +/- traité à la phosphatase alkaline. **(1)** le marqueur moléculaire, **(2)** l'extrait de protéines Slps et **(3)** le même extrait traité à la phosphatase alkaline. Les gels sont colorés au Biosafe **(A)** ou au Pro-Q® Diamond **(B),** qui révèlent respectivement les protéines totales et les phosphoprotéines dans les extraits Slps.

Les auteurs attribuent à cette modification un rôle structural probable ou de transduction de signaux environnementaux. En effet, la phosphorylation est décrite comme un mécanisme impliqué dans la dormance, la division cellulaire, l'adaptation aux stress environnementaux, mais également impliquée dans l'interaction avec les cellules hôtes (virulence), chez de nombreuses espèces d'actinobactéries (Mijakovic and Macek, 2012; Parandhaman et al., 2014; Rioseras et al., 2018; Schmidt et al., 2014). Pour la protéine SlpB, il est probable que la phosphorylation a un rôle dans l'activation ou la désactivation de son activité enzymatique prédite.

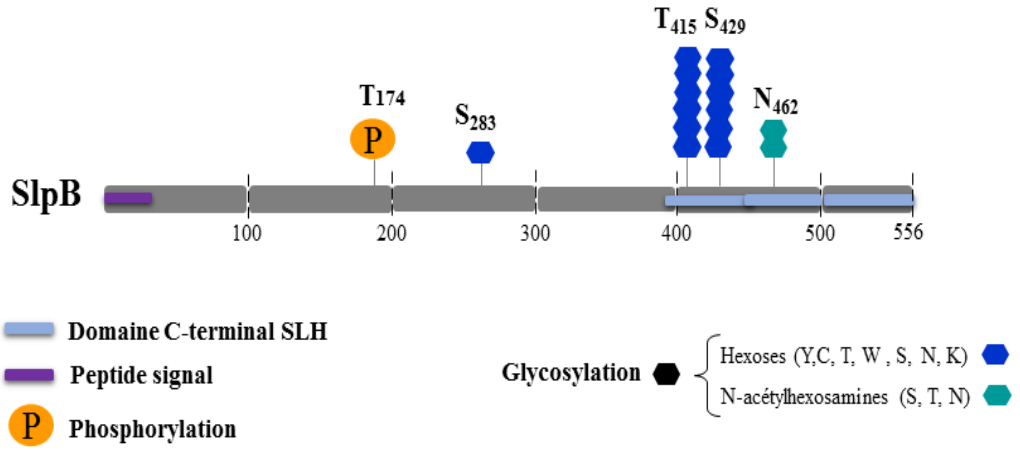


Figure 5. Les sites de modifications covalentes identifiés sur la protéine SlpB, ainsi que la structure partielle des glycosylations.

Néanmoins, quand bien même l'ensemble des résultats obtenus semblent cohérents; il faut prendre en compte ces résultats avec quelques précautions. En effet, il est crucial d'envisager comme perspective une étude supplémentaire pour caractériser de manière fine les modifications covalentes présentes sur la protéine SlpB. Une stratégie d'expérimentation est proposée (Figure 6). Les méthodes d'enrichissement des glycopeptides par chromatographie d'interaction hydrophobe (HILIC) ou des phosphopeptides par chromatographie d'affinité (IMAC) permettra d'augmenter le rapport signal sur bruit (Zhao and Jensen, 2009). Pour l'analyse des sites de N-glycosylation, l'utilisation de l'endoglycosidase H peut être envisagée, car elle laisse une signature HexNAc sur les peptides plutôt qu'un écart de 1 Da pour la PNGase F (Colangelo and Orlando, 2001). La détermination exacte de la

structure et la nomenclature des sucres présents sur SlpB permettra d'utiliser les enzymes de déglycosylation adéquates pour étudier, par exemple, le rôle des glycosylations dans l'interaction de SlpB avec les récepteurs des cellules de l'hôte. En plus de la détermination des sites modifiés, elle permettra quant à elle de modéliser la structure tridimensionnelle de SlpB, et faire du docking, dans la perspective de déterminer les ligands potentiels de la protéine SlpB.

Conclusion

Les différentes analyses menées ont permis d'identifier la présence de modifications covalentes de type glycosylation et phosphorylation sur la protéine SlpB. Elles ont permis également d'obtenir des informations partielles sur la nature des glycanes ainsi que les sites potentiels d'acides aminés modifiés. Cependant, des analyses supplémentaires sont indispensables pour identifier la structure des glycanes et confirmer les sites modifiés. Cette perspective permettra de comprendre le mécanisme d'action de la protéine SlpB dans son rôle d'immunomodulation. En plus, l'identification de phosphoprylation sur une telle protéine, ouvre la perspective de l'étude du phosphoprotéome de *P. freudenreichii* en lien avec ses propriétés probiotiques.

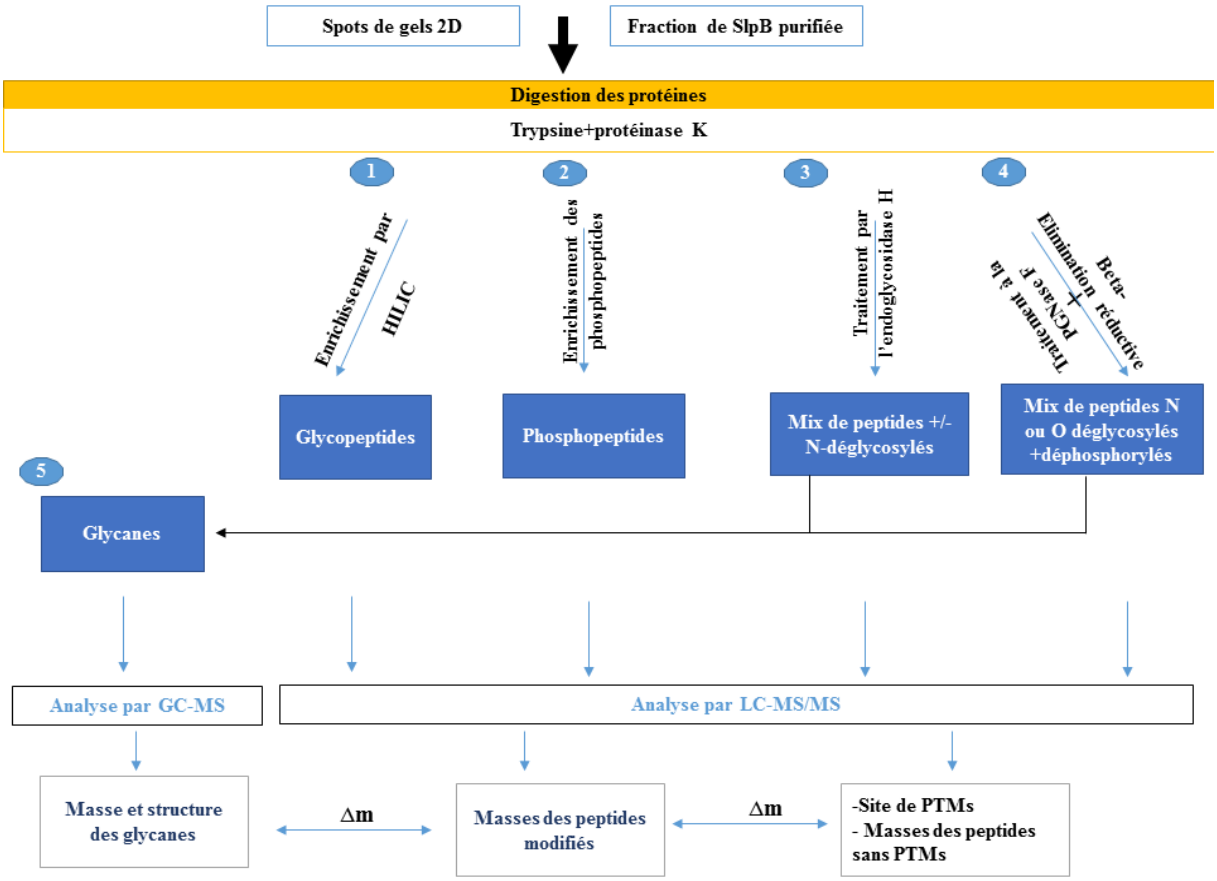


Figure 6. La stratégie à considérer pour déterminer la structure exacte des groupements glycanes, ainsi que les sites d'acides aminés modifiés sur la protéine SlpB.

Annexe 3 : publications scientifiques

❖ Publications publiées dans des journaux à comité de lecture :

- **Rabah H**, Do Carmo FLR, Jan G. Dairy Propionibacteria: Versatile Probiotics. Microorganisms. 2017 May 13; 5(2):24.
- **Rabah H**, Ménard O, Gaucher F, do Carmo FLR, Dupont D, Jan G. Cheese matrix protects the immunomodulatory surface protein SlpB of *Propionibacterium freudenreichii* during *in vitro* digestion. Food Res Int. 2018 Apr 1; 106:712–21.
- **Do Carmo FLR *, Rabah H ***, De Oliveira Carvalho RD, Gaucher F, Cordeiro BF, Da Silva SH, et al. Extractable Bacterial Surface Proteins in Probiotic-Host Interaction. Front Microbiol. 2018. 9:645. doi: 10.3389/fmicb.2018.00645.
- -**Rabah H**, Ferret-Bernard S, Huang S, Le Normand L, Cousin FJ, Gaucher F, Jeantet R, Boudry G and Jan G. (2018). The Cheese Matrix Modulates the Immunomodulatory Properties of *Propionibacterium freudenreichii* CIRM-BIA 129 in Healthy Piglets. Front. Microbiol. 9:2584. doi: 10.3389/fmicb.2018.02584.
- **Huang, S ***, **Rabah H ***, Ferret-Bernard S, Le Normand L, Gaucher F, Guerin S, Nogret I, Le Loir Y, Chen XD, Jan G, Boudry G, Jeantet R. Propionic fermentation by the probiotic *Propionibacterium freudenreichii* to functionalize whey. Journal of Functional Foods. https://doi.org/10.1016/j.jff.2018.11.043

❖ Publications en préparation :

- **Rabah H,** Jardin J, Briard-Bion V, Maillard MB, Gaucher F, Do Carmo FLR, Jan G. **(In preparation).** The growth medium influence on *in vitro Propionibacterium freudenreichii* adaptation ability to caecal environment. Target Journal: Applied Microbiology and Biotechnology.
- **Rabah H *, Do Carmo FLR *et al,. (In preparation).** Effect of probiotic Emmental cheese on DSS-induced colitis on mice. Target Journal: Applied Microbiology and Biotechnology.

*contribution égale au travail

Annexe 4 : communications scientifiques

❖ Communication orale

Rabah, H., Ménard, O., Gaucher, F., Do Carmo, FLR., Dupont, D., Jan, G. Cheese matrix protects the immunomodulatory surface protein SlpB of *Propionibacterium freudenreichii* during *in vitro* digestion. 12th International Scientific Conference on Probiotics, Prebiotics, Gut Microbiota and Health, Budapest-Hungary, June 2018.

Le 1er prix du "young scientist award" à IPC2018 pour la présentation orale, ex aequo avec Stefano Colombo (Italie)

❖ Communications par poster

1) **Rabah, H**., Do Carmo, FLR., Jan, G. Is cheese a good delivery vehicle for *Propionibacterium freudenreichii* anti-inflammatory property? Symposium on Propionibacteria and Bifidobacteria, Cork-Ireland, Septembre 2016.
2) **Rabah, H**., Ménard, O., Gaucher, F., Do Carmo, FLR., Dupont, D., Jan, G. Cheese matrix as a delivery vehicle for immunomodulatory proteins of *Propionibacterium freudenreichii*. International Probiotics and Antimicrobial Proteins Conference, Barcelone-Spain, November 2017.
3) **Rabah, H**., Ferret-Bernard, S., Huang, S., Le Normand, L., Cousin, FJ., Gaucher, F., Jeantet, R., Boudry, G., Jan, G. The delivery vehicle modulates the immunomodulatory effect of *Propionibacterium freudenreichii* in healthy piglets. The 10th cheese symposium, Rennes-France, April 2018.

Le 2éme prix du meilleur poster présenté par les doctorants

4) **Rabah, H**., Gaucher, F., Do Carmo, FLR., Maillard, MB., Jan, G. *In vitro* assessment of *Propionibacterium freudenreichii* adaptation to caecal environment. Congrès national de la société française de microbiologie, Paris-France, Octobre 2018.

5) **Rabah, H**., Ferret-Bernard, S., Huang, S., Le Normand, L., Cousin, FJ., Gaucher, F., Jeantet, R., Boudry, G., Jan, G. The cheese matrix modulates the immunomodulatory effect of *Propionibacterium freudenreichii* in healthy piglets. les 4èmes rencontres du GIS Nutrition Alimentation Métabolisme et Santé, Rennes-France, Octobre 2018. Au congrès mondial de la fédération internationale du lait (IDF world dairy summit), les 16-21 octobre 2018 à Daejeon, Corée de Sud;

Le prix du meilleur poster présenté par les participants du congrès GIS.

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Is cheese a good delivery vehicle for *Propionibacterium freudenreichii* anti-inflammatory property?

Rabah H [1, 2], Carmo FLR[1,2,3], Jan G [1, 2]

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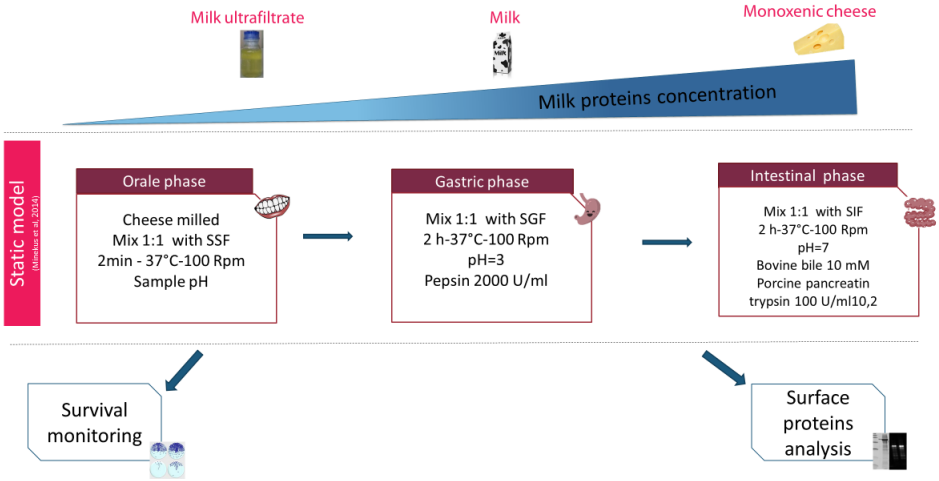
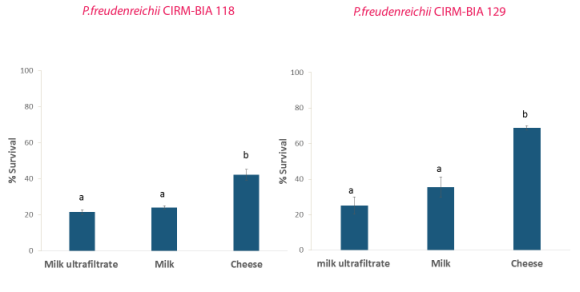
Propionibacterium freudenreichii is a beneficial Actinobacterium (GRAS & QPS status). It exhibits promising strain-dependent immunomodulatory properties (Interleukin 10 induction in immune cells), involving key surface proteins, particularly the surface-layer protein B (SlpB) (Le Maréchal et al., 2014). Furthermore, it reduces chemically-induced colitis severity in mice (Plé et al., 2016). This highlights the probiotic potential of *P.freudenreichii* to help Inflammatory Bowel Diseases (IBD) treatment. In this study, we investigate the potential of a monoxenic cheese model as a delivery vehicle for anti-inflammatory potential of *P.freudenreichii* to digestive tract, by comparing it to milk ultrafiltrate and milk cultures. We show here that the cheese matrix protects both propionibacteria viability and surface proteins from proteolysis during *in vitro* mimicked digestion.

Experimental Strategy: *In vitro* static digestion challenge

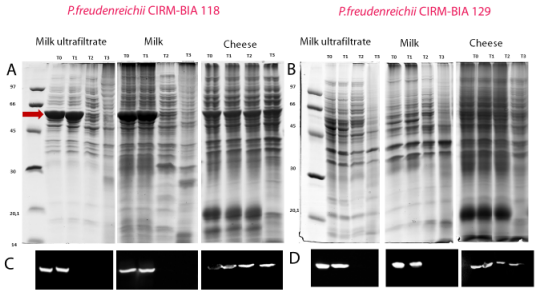


Bacterial viability: enhanced by cheese



***P.freudenreichii* viability after *in vitro* static digestion.** Three dairy products, fermented by *Propionibacteria freudenreichii* CIRM-BIA 118 or CIRM-BIA 129, were subjected to *in vitro* static digestion, viable propionibacteria were enumerated by plate counting before and after *in vitro* digestion. The results are expressed as percent survival. The error bars represent the standard deviations for triplicate experiments. Different letters above the columns indicate significant differences (P0.01) from Tukey test .

Surface layer Proteins: preserved by cheese



S-layer proteins proteolysis during static *in vitro* digestion. Three dairy products, fermented by *P.freudenreichii* CIRM-BIA 118 or CIRM-BIA 129 underwent *in vitro* digestion. Samples were collected at different times: before digestion (T0), at the end of oral phase (T1), at the end of gastric phase (T2) and at the end of intestinal phase (T3). Total proteins extracts from bacterial pellets were prepared; then analyzed by 12% SDS PAGE electrophoresis (A,B) and by western blotting using anti-SlpB antibodies (C, D). The arrow indicate *P.freudenreichii* main S-layer protein migration distance.

Conclusion & Perspectives

This study evidences that a cheese matrix is the best vehicle to deliver for anti-inflammatory surface proteins of Propionibacterium freudenreichii to the colon. It will be confirmed by in vivo experiments monitoring both protection of the probiotic and immunomodulatory effects in recognized models of inflammatory gut diseases. This will lead to the development of functional dairy food products, fermented by selected strains of dairy propionibacteria, for target populations.

The 4th Symposium on Propionibacteria and Bifidobacteria

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CHEESE MATRIX AS A DELIVERY VEHICLE FOR IMMUNOMODULATORY PROTEINS OF *PROPIONIBACTERIUM FREUDENREICHII*

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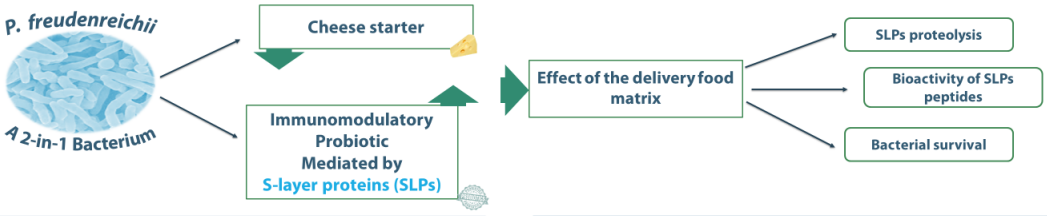
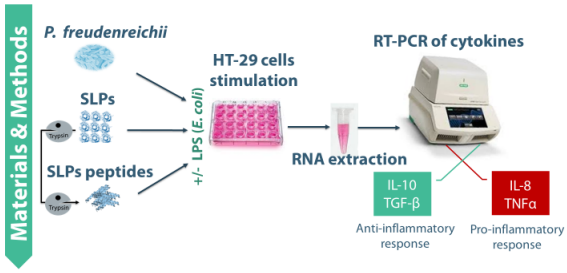
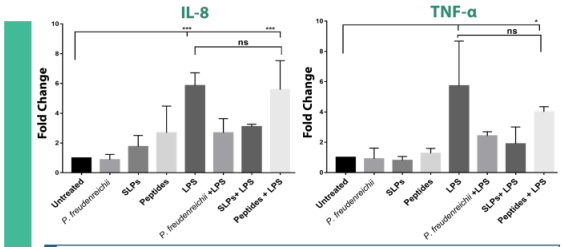
Context

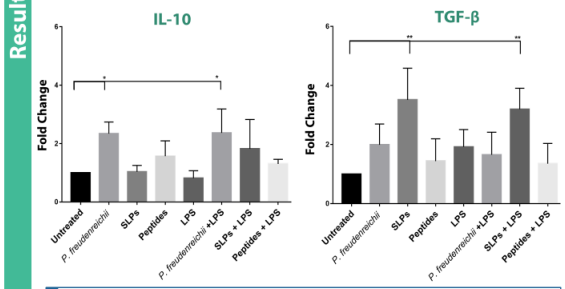


SLPs proteolysis abolishes its anti-inflammatory properties



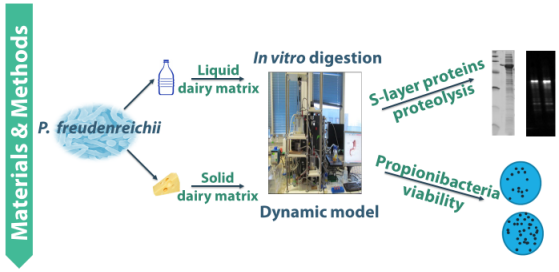


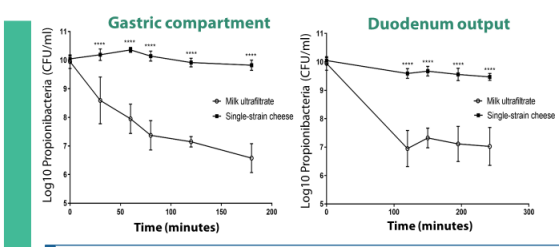
P. freudenreichii and its SLPs, but not SLPs peptides, inhibit pro-inflammatory cytokines expression in the presence of LPS



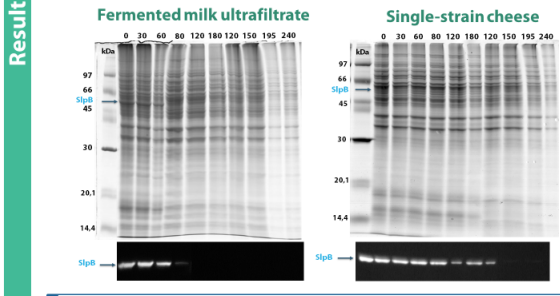
P. freudenreichii and its SLPs, but not SLPs peptides, induce anti-inflammatory cytokines expression in the presence of LPS

Cheese matrix is a suitable delivery vehicle for *P. freudenreichii*





Cheese matrix protects bacterial viability during *in vitro* dynamic digestion



Cheese matrix protects SLPs from digestive proteolysis during *in vitro* dynamic digestion

Concluding remarks

- It is crucial to prevent SLPs proteolysis in order to preserve their anti-inflammatory properties
- Cheese matrix is a suitable delivery vehicle for anti-inflammatory properties of *P. freudenreichii*
- These results shall be confirmed by *in vivo* experiments (Piglet model)
- These results provide new insights into potential functional foods fermented by dairy propionibacteria

THE DELIVERY VEHICLE MODULATES THE IMMUNOMODULATORY EFFECT OF *PROPIONIBACTERIUM FREUDENREICHII* IN HEALTHY PIGLETS

Houem Rabah [1 2], Stéphanie Ferret-Bernard[3], Song Huang[1], Laurence Le Normand[3], Fabien Cousin [4], Floriane Gaucher[1] , Romain Jeantet[1], Gaelle Boudry[3], Gwénaël Jan [1]

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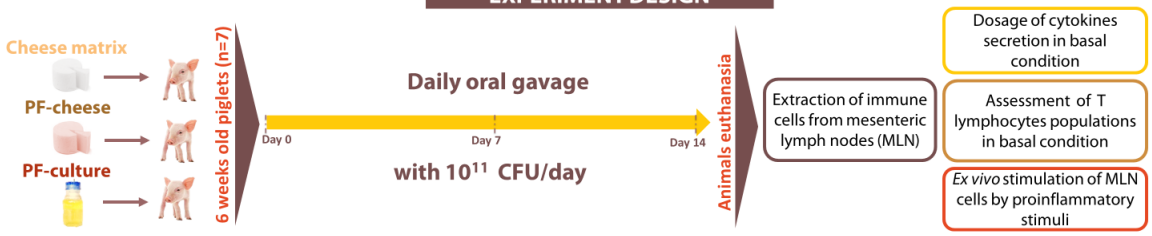
CONTEXT

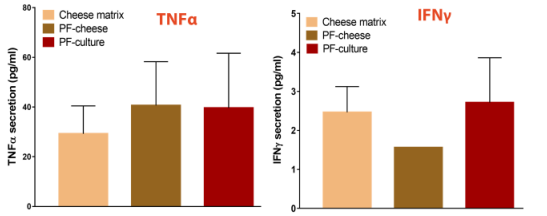
Propionibacterium freudenreichii (**PF**) is a «2-in-1 bacterium»: it is used as a cheese ripening starter and as a probiotic ingredient. Its promising strain-dependent immunomodulatory properties are due to several metabolites including short chain fatty acids, bifidogenic molecules and surface proteins (**Slps**). In this study, we investigated the impact of the delivery vehicle on the immunomodulatory effect of *P. freudenreichii* in healthy piglets. We compared two delivery vehicles, milk utlratfiltrate and a cheese matrix, both fermented by a selected anti-inflammatory strain, *P. freudenreichii* CIRM-BIA129 (ITG P20).

EXPERIMENT DESIGN



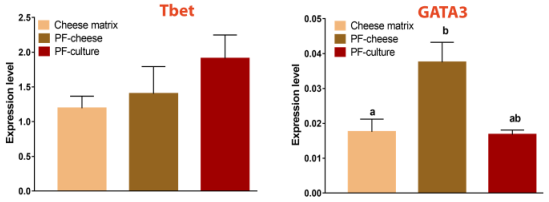
RESULTS

1 PF doesn't modulate the basal secretion of TNFα and IFNγ



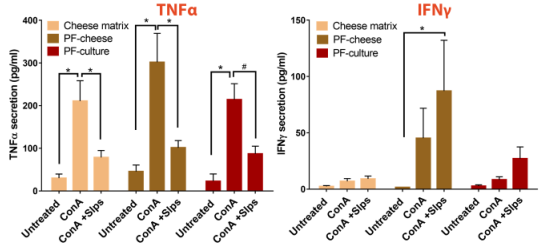
Immunes cells from MLN extracted from the three piglets groups were cultivated during 72h and the basal secretion of TNFα and IFNγ were assessed by ELISA.

2 PF-cheese enhances Th2 response while PF-culture enhances Th1 response



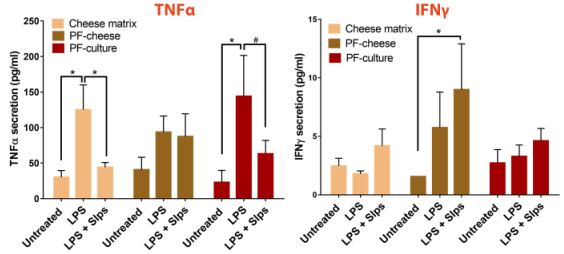
Total mRNA of immunes cells from MLN were extracted. The expression level of Tbet and Gata3 were assessed. Tbet and GATA3 are markers of Th1 and Th2 lymphocytes, respectively. Th1/Th2 orientation was determined by calculating Tbet/GATA3 ratio.

3 PF-cheese enhances IFNγ secretion after ConA and Slps co-stimulation



Immunes cells from MLN of the three piglets groups were cultivated and stimulated during 72h, with concanavalin A (ConA) alone or in the presence of Slps proteins. The secretion of TNFα and IFNγ were determined by ELISA.

4 PF-cheese decreases TNFα secretion and enhances IFNγ secretion after LPS ans Slps co-stimulation



Immunes cells from MLN of the three piglets groups were cultivated and stimulated during 72h, with lipopolysaccharides (LPS) alone or in the presence of Slps proteins. The secretion of TNFα and IFNγ were determined by ELISA.

CONCLUSION

1. The delivery vehicle change Th1/Th2 orientation exerted by PF
2. PF-cheese enhances Th2 response of the gut immunity
3. PF-cheese enhances IFNγ response to proinflammatory stimuli: anti-infection effect in the gut
4. Slps proteins of PF play a role in its anti-infection effect

IN VITRO ASSESSMENT OF *PROPIONIBACTERIUM FREUDENREICHII* ADAPTATION TO CAECAL ENVIRONMENT

HOUEM RABAH [1 2], FLORIANE GAUCHER [1 3], FILLIPE LUIZ ROSA DO CARMO [1 4], MARIE-BERNADETTE MAILLARD [1], GWÉNAËL JAN [1]

CONTEXT

P. freudenreichii

PF

A 2-in-1 Bacterium

Flavor compounds — Cheese starter

Probiotic — Immunomodulatory proteins (Slps); Short chain fatty acids; Bifidogenic factors

Delivery vehicle

- **Tolerance towards digestive stresses**
  - The tolerance response to digestive stresses depends on the delivery vehicle
- **Slps proteolysis**
  - Slps digestive proteolysis depends on the delivery vehicle
- **Adaptation to colon environment**

Does the delivery vehicle affect *Pf* adaptation ability to caecal environment ?

STRATEGY

Yeast extract-lactate (YEL) — *Pf* 129, *Pf* 129 KO SlpB

Milk ultrafiltrate (UF) — *Pf* 129

Single-strain cheese (Cheese) — *Pf* 129

Bacterial pellets extraction and surface proteins "shaving"

Surface protein proteolysis =shaving: 1 hour-37°C- 300 U of trypsin-Agitation at 180 rpm

Incorporation of bacterial pellets in caecal medium

Cecum content → 10 000 g, 15 min → Supernatant → Recovery

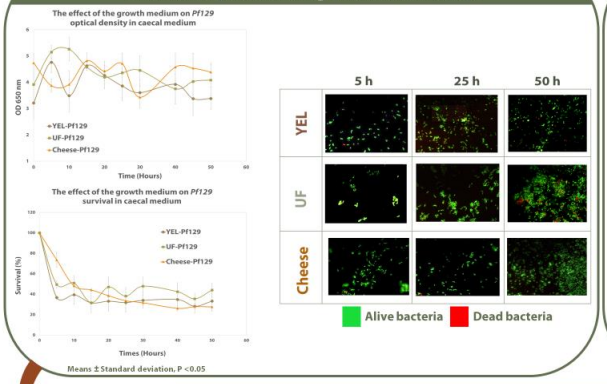
Autoclaving 115°C-15 min → 20 000 g, 30 min → Sterilization by filtration → Caecal medium

Caecal cultures incubation

50 hours — 37°C — Anaerobiosis

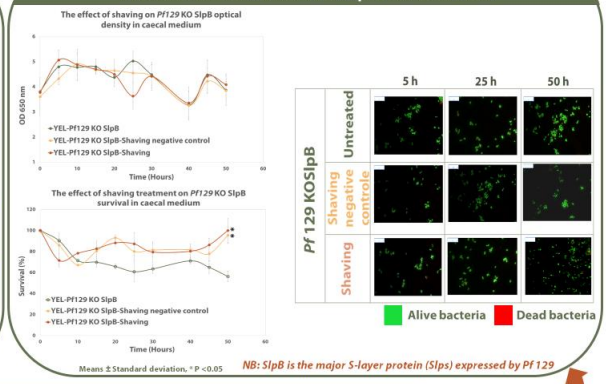
Survival counting — Optical density monitoring — live/dead fluorescence labeling

Pf 129 uses a viable but nonculturable state to adapt to caecal environment , whatever the growth medium of *Pf 129*



Means ± Standard deviation, P <0.05

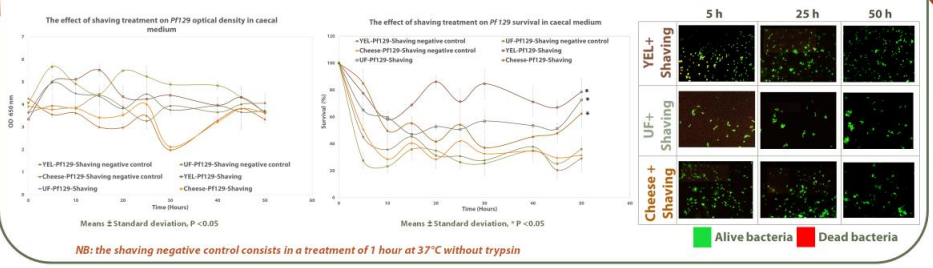
The mutation of SlpB does not change *Pf 129* culturable state but shaving treatment enhances also *Pf* KO SlpB culturable state



Means ± Standard deviation, * P <0.05

NB: SlpB is the major S-layer protein (Slps) expressed by Pf 129

Shaving treatment enhances *Pf* 129 culturable state in caecal medium, whatever the growth medium of *Pf 129*



Means ± Standard deviation, P <0.05

Means ± Standard deviation, * P <0.05

NB: the shaving negative control consists in a treatment of 1 hour at 37°C without trypsin

The surface proteins proteolysis enhances *Pf 129* culturable state and so may be its metabolic activity in caecal medium.
The Slps proteins seem to be not involved directly in *Pf 129* adaptation to caecal environment.
=> A comparative proteomic analysis will address the molecular mechanisms involved in an enhanced culturable state of shaved bacteria.

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THE CHEESE MATRIX MODULATES THE IMMUNOMODULATORY PROPERTIES OF *PROPIONIBACTERIUM FREUDENREICHII* IN PIGLETS

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CONTEXT

Probiotic — *P. freudenreichii* CIRM-BIA 129 = ITGP20 — A 2-in-1 Bacterium

Immunomodulatory proteins (Slps) · Short chain fatty acids (SCFA) · Bifidogenic factors

Cheese starter — Flavor compounds

FONCTIONAL CHEESE → The cheese matrix impacts :

- Production of immunomodulatory metabolites
- Protection of Slps against digestive proteolysis
- Tolerance to digestive stresses

→ Is *P. freudenreichii* (PF) immunomodulatory effect impacted by the cheese matrix?

STRATEGY

Cheese matrix (n=7) · PF-cheese (n=7) · PF-culture (n=7)

→ Daily gavage during 2 weeks with 11 log CFU/jour

→ Animals euthanasia at day 15

- Fecal contents
- Intestinal mesenteric lymph nodes → Mesenteric lymph nodes cells (MLNC)
- Blood → Peripheral blood mononuclear cells (PBMC)

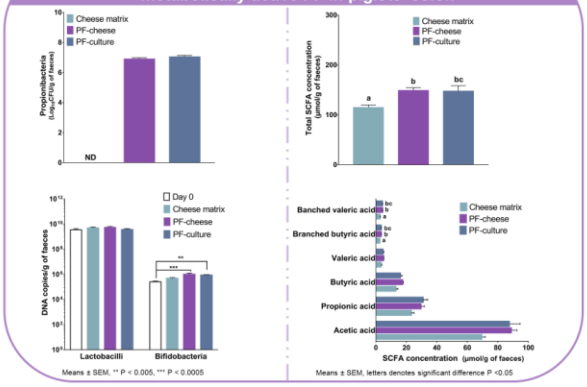
MLNC / PBMC →

- Short fatty acids dosage
  - ✓ Acetic, propionic, butyric and valeric acids
- Lymphocytes T phenotype (Th2, Treg, Th17)
- Cytokine dosage
  - ✓ IL-10, IL-17, TNFα, IFNγ

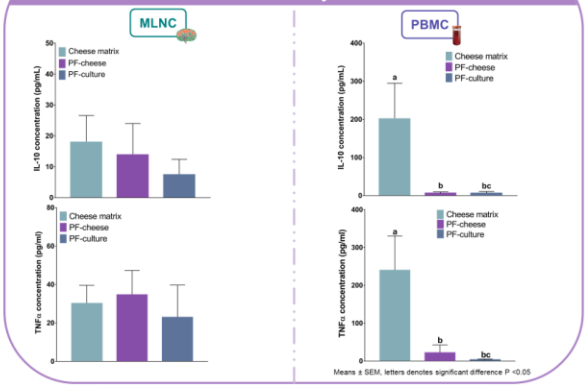
Microbiota modulation
- ✓ Propionibacteria
- ✓ Lactobacilli
- ✓ Bifidobacteria

Ex vivo stimulation of cells
- ✓ Lipopolysaccharides (LPS) -> IL-10, TNFα & IFNγ dosage

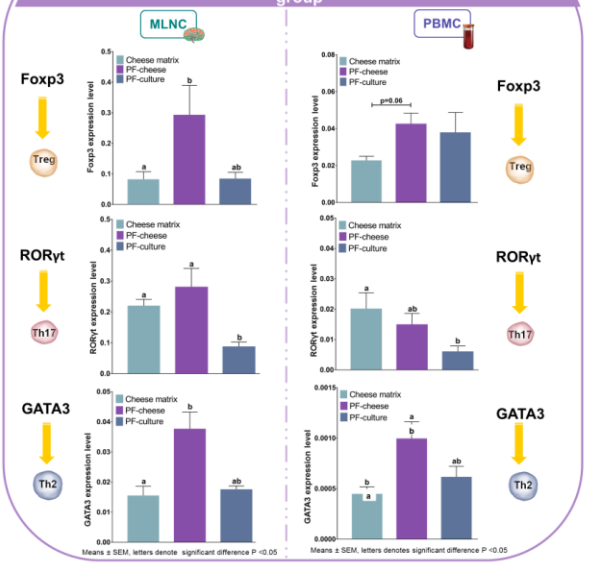
Both PF-culture and PF-cheese delivered the same amount of metabolically active *PF* in piglets' colon



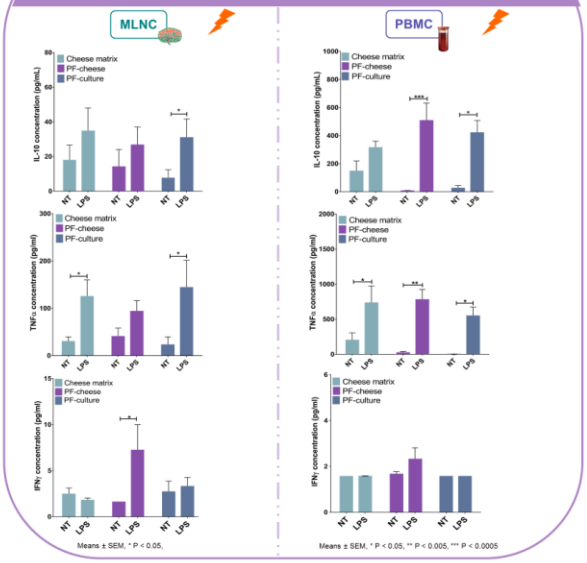
PF ingestion decreased TNFα and IL-10 secretion only in PBMC, whatever the delivery vehicle of *PF*



PF-cheese enhanced Treg and Th2 populations and PF-culture decreased Th17 population, compared to the control group



Ex vivo response of MLNC to LPS stimulation was different according to the delivery vehicle of *PF*



The cheese matrix doesn't enhance the amount of metabolically active PF in piglets colon, however the functional cheese immunomodulates differently the intestinal and the systemic immunity compared to PF-culture:
We suppose it is due to a difference in surface compounds, as absence or presence of immunomodulatory surface layer proteins Slps

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Propionic fermentation by the probiotic *Propionibacterium freudenreichii* to functionalize whey

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Whey
P. freudenreichii
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T lymphocytes phenotype

ABSTRACT

A new probiotic functionalized sweet whey was evaluated. Weaned healthy piglets consumed sweet whey (SW), unmodified or fermented by *P. freudenreichii* CIRM-BIA 129 (PF-SW). Fecal short chain fatty acids amounts remained unchanged. Bifidobacteria were enhanced in the PF-SW group, and so was the expression of T-bet, which orchestrates Th1 differentiation of T lymphocytes, in mesenteric lymph nodes immune cells (MLNC). This was consistent with *ex vivo* increased TNF-α secretion by MLNC in response to lipopolysaccharide (LPS). The consumption of the functionalized whey induced a different response in peripheral blood mononuclear cells (PBMC) to *ex vivo* stimulations, as the inhibition of TNF-α secretion in response to concanavalin A stimulation. Thus, by cultivating a probiotic GRAS bacterium in concentrated whey, prior to spray drying, it is possible to transform this by-product into a functional ingredient. This opens new avenues for the development of functional ingredients through enhanced valorisation of whey.

1. Introduction

Whey, once considered as a by-product of the dairy industry, constitutes a source of high nutritional quality proteins, a main constituent of infant formulas and a food ingredient for weight management (Luhovyy, Akhavan, & Anderson, 2007). It is used to develop functional beverages using emerging technologies (Amaral et al., 2018; Cappato et al., 2018; Guimarães et al., 2018). Whey proteins constitute functional ingredients, rich in essential and branched amino acids and bioactive peptides (Dullius, Goettert, & de Souza, 2018), with reported antioxidant, anticancer, prebiotic and phenylketonuria management properties (Patel, 2015) and with versatile techno-functional abilities, such as interfacial, foaming, emulsifying, and gelation properties (Schong & Famelart, 2017). They can form nanohydrogels, nanofibrils or nanotubes (Ramos et al., 2017) and can encapsulate probiotic bacteria (defined as "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host"(Hill et al., 2014)), prebiotics and synbiotics (Kareb & Aïder, 2018).

We recently developed a simple and straightforward "2-in-1" method to grow and dry probiotic propionic acid bacteria in a food-grade medium. Propionibacteria are cultivated in hyperconcentrated whey, which triggers a remarkable adaptive response of the bacteria, leading to enhanced tolerance towards multiple stresses (Huang, Rabah et al., 2016). The corresponding whey cultures, with a high biomass, are then subjected to spray-drying, which was applied from lab-scale to industrial scale, with limited (or no) loss of viability during the drying process (Huang et al., 2017; Huang, Cauty et al., 2016). Lactobacilli, including *Lactobacillus casei* and *L. acidophilus*, and bifidobacteria, including *Bifidobacterium infantis* and *B. longum*, are the most used probiotic bacteria (Heshmati et al., 2018). We thus adapted this process to lactobacilli and bifidobacteria, with similar results (Huang, Cauty et al., 2016) and unpublished results). This innovation opens new

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perspectives for the development of probiotic formulations.

Probiotics are nowadays recognized as key components of personalized nutrition, for different target populations. They may participate in improving symptoms in the context of liver diseases (Chávez-Tapia et al., 2015), childhood rotavirus infections (Yang, Pei, Qin, & Wei, 2019), lung cancer and related diseases (Sharma, Viswanath, & Park, 2018), oxidative stress (Heshmati et al., 2018; Zhao et al., 2017), Inflammatory bowel diseases (IBD) (Celiberto et al., 2018), as well as irritable bowel syndrome (IBS) (Corbitt, Campagnolo, Staines, & Marshall-Gradisnik, 2018), which constitute emerging troubles in developed countries. Consumption of probiotic bacteria with immunomodulatory properties, in conjunction with usual treatments, allows to prolong remissions between relapses, in the context of IBD (Bibiloni et al., 2005; Mimura et al., 2004). Considering IBS, clinical studies also conclude to the efficacy of selected strains of probiotic bacteria (Ford et al., 2014). Considering new-borns, early colonisation, which is modulated by the quality of ingested milk, is a key determinant of the human gut maturation, including its gut-associated lymphoid tissue. Actually, the balance between Th1 and Th2 responses in the new-borns is skewed towards a Th2 profile (Adkins, Leclerc, & Marshall-Clarke, 2004; Basha, Surendran, & Pichichero, 2014) and they are not able to mount an efficient immune response to a large number of pathogens, as Th1 responses are compromised at several steps (Maródi, Goda, Palicz, & Szabó, 2001). Bifidobacteria, nowadays added to infant formula, improve the Th1 immune response (Wu, Yang, Xu, & Wang, 2016). Accordingly, early colonisation by bifidobacteria prevents atopy (Lee et al., 2018). Another bacterium playing a key protective role, at an early stage of life, is *Propionibacterium freudenreichii*. Indeed, a commensal strain, UF1, was recently shown to mitigate intestinal inflammation *via* an intense increase of Th17 cells (Colliou et al., 2017). Consumption of human breast milk, which attenuates the incidence of necrotizing enterocolitis and atopy, correlates with enrichment of the gut microbiota in actinobacteria, including bifidobacteria (Duranti et al., 2017) and propionibacteria (Colliou et al., 2017). *P. freudenreichii* exhibits strain-dependent immunomodulatory properties (Foligné, Breton, Mater, & Jan, 2013) in accordance with its healing effect in the context of colitis (Foligné et al., 2010), which led to the development of functional cheeses (Plé et al., 2015).

It is thus a main challenge to incorporate these beneficial GRAS bacteria in food, including in infant formulas used when breast-feeding is not possible. Considering that we have developed a straightforward method to grow and dry such probiotics in whey, we thus investigated the probiotic effect of functionalized whey, with a particular focus on immune modulation. We tested a sweet whey functionalized by *P. freudenreichii* CIRM-BIA 129 which was shown recently to exert remarkable healing effects in the context of induced colitis, in accordance with its ability to induce *in vitro* immunomodulatory cytokines in human immune cells. We used piglets, recognized as a valuable human model which were given daily functionalized sweet whey powder or the corresponding sweet whey powder for 14 days. The *in vivo* metabolic activity of *P. freudenreichii* was assessed by determining short chain fatty acids (SCFA) concentration and bifidobacteria population in feces. Basal immune T lymphocyte phenotypes basal cytokine secretion, and responses to inflammatory stimuli or mitogen were tested on isolated peripheral blood mononuclear cells (PBMC) or mesenteric lymph node immune cells (MLNC).

2. Methods

2.1. PF-SW and SW preparation

Sweet whey (SW) powder was purchased from Lactalis ingredients (Mayenne, France). Its gross composition, expressed as w/w%, was: total solids 94.81, total nitrogen 11.81, non-protein nitrogen 3.11, lactose 67.91, ash 6.85, calcium 0.37, magnesium 0.11, sodium 0.59, potassium 2.27, chloride 1.72, phosphate 1.14, citrate 2.26, pH 6.52. *Propionibacterium freudenreichii* (PF) CIRM-BIA 129, alias ITG P20, was provided by the French Dairy Interbranch Organization (CNIEL) maintained and pre-cultured by the CIRM-BIA Biological Resource Center (Centre International de Ressources Microbiennes-Bactéries d'Intérêt Alimentaire, INRA, Rennes, France), using the rich YEL medium (Malik, Reinbold, & Vedamuthu, 1968). The PF-SW powder was prepared as described previously (Huang et al., 2017; Huang, Cauty et al., 2016). SW medium with 20 wt% total solid content was prepared by rehydration of SW powder in deionized water and autoclaved at 100 °C for 30 min. It was inoculated with YEL precultures prior to static incubation, 30 °C, 5 days, to reach a maximal population of 5×10^9 CFU mL^{-1}. This fermented SW was then spray-dried using a pilot-scale Mobile Minor™ spray dryer (GEA Niro A/S, Denmark) at the maximum evaporation rate of 5 kg water h^{-1} as described (Huang, Cauty et al., 2016). The obtained powder, designated PF-SW, contained a viable population of propionibacteria of 10^{10} CFU g^{-1}. It was stored under vacuum at 4 °C before use.

2.2. Animal procedures

The experimental protocol was performed in accordance with recommendations of the French law (2001-464 29/05/01) and EEC (86/609/CEE) for the care and use of laboratory animals. The protocol was approved by the ethical committee on animal experimentation of Rennes (France), under the certificate of authorization to experiment 2017010922379066-V2. Pigs were sacrificed by electronarcosis followed by exsanguination, and every effort was made to minimize animal suffering.

Fourteen 8-week old piglets ((Pietrain × Landrace) × Large White, initial body weight 13.8 ± 0.4 kg) from the experimental herd of INRA (UEPR, France) were housed in individual cages, in two separate rooms to avoid cross-contamination. After 1 week of acclimation to their new environment, piglets were gavaged every morning (between 9.00 and 10.00 am), with 10 g of either PF-SW or SW, resuspended in sterile water, during 14 consecutive days. Thirty minutes after gavage, they were offered a standard pig diet. Food was removed from the cage at 4:00 pm to monitor daily food intake. Animals were fasted from 4:00 pm to 9:00 am but had free access to water. Piglets were weighed three times a week and faecal samples were collected at day 0 and 14. Immediately after collection, faecal samples were either frozen at −20 °C for further qPCR or diluted in ortho-phosphoric acid (50/50 V/V) to stop fermentation. Samples were then stored at −20 °C until short chain fatty acid (SCFA) analysis. At the end of the 14-day treatment period, pigs were sacrificed 30 min after their last meal by electronarcosis and exsanguination. Blood was collected in sterile BD vacutainer®CPT™ tubes (containing sodium heparin as well as Ficoll™Hypaque™ density fluid and a polyester gel barrier) at room temperature. Following centrifugation at 1500 g for 20 min without brake, PBMC were isolated by carefully pipetting the interface above the gel barrier. Additional washing and centrifugation with Hank's balanced saline solution (HBSS), supplemented with 200 UI/mL penicillin and 200 µg/mL streptomycin, resulted in a suspension of concentrated PBMC. After laparotomy, 4 g of mesenteric lymph nodes (MLN) were placed in ice-cold HBSS for mononuclear cell isolation as already described (Ferret-Bernard et al., 2010).

2.3. Quantification of faecal populations of propionibacteria, bifidobacteria and lactobacilli

Propionibacteria, Lactobacilli and Bifidobacteria were quantified in faeces collected at day 0 (before treatment) and at day 14 (end of the treatment). QIAamp DNA Stool Kit was used to extract DNA from faeces samples. Propionibacteria concentrations were measured by qPCR of 5S subunit gene of transcarboxylase as described previously (Hervé, Fondrevez, Cheron, Barloy-Hubler, & Jan, 2007). Briefly, ten-fold dilutions of *P. freudenreichii* CIRM-BIA 129 were prepared in saline

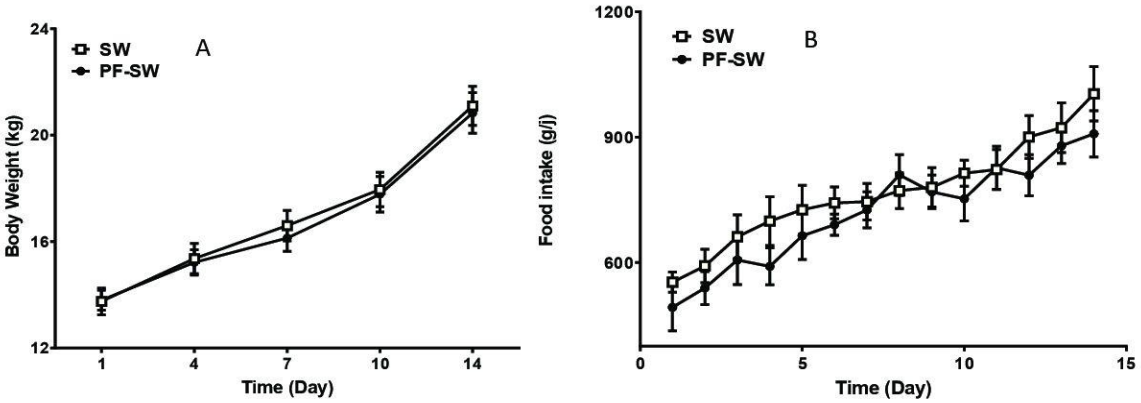

Fig. 1. Growth and food intake. Body weight (A) and food intake (B) were monitored throughout the experiment (d1, d4, d7, d10 and d14). The piglets received, in addition to their feed, either sweet whey (SW), or *P. freudenreichii* CIRM-BIA 129 – fermented sweet whey (PF-SW). Results are represented as means ± SEM (n = 7). $P < 0.05$.

solution and enumerated using YELA medium. One hundred microliters of each dilution was then added to 200 mg of faeces from naïve pig and thoroughly mixed. DNA was then extracted in the same way as unknown samples. A standard curve was generated and results are expressed as log [bacteria] per gram of sample. For lactobacilli and bifidobacteria quantification, 10-fold serial dilutions of target genomic DNA extracted from pure cultures of *Bifidobacterium longum* CIRM-BIA 1336 or *Lactobacillus pentosus* CIRM-BIA 660 were performed (Table S1). The linear equation for the standard curve was then used to interpolate the numbers of copies present in the unknown samples.

2.4. Short chain fatty acid analysis

SCFA were separated on a BP20 (SGE) column and quantified by a flame ionization detector as previously described (Jouany et al., 1981). Isocaproic acid was used as an internal standard. The results are expressed as micromolar per g of faecal matter.

2.5. PBMC and MLNC ex vivo culture

Cells were suspended in complete RPMI 1640 medium (Sigma) supplemented with 10% foetal calf serum (FCS), 100 IU/ml penicillin and 100 mg/ml streptomycin to achieve cell concentration of 5×10^6 cells/ml for PBMC and 10×10^6 cells/ml for MLNC in 96-well flat-bottomed plates. Cells were stimulated for 72 h at 37 °C, under 5% CO_2 atmosphere, in basal condition (complete RPMI alone) or in presence of 200 µg/ml of PF surface layer proteins (Slps). Slps were extracted as previously described (11) and proteins were concentrated by filtration using VivaSpin −10 kDa. Cells were also cultured in presence of 10 µg/ml lipopolysaccharide from *E. coli* 0111:B4 (LPS) or of 0.5 µg/ml Concanavalin A (ConA, sub-optimal concentration) or a combination of Slps + LPS and Slps + ConA. Culture supernatants were harvested and stored at −20 °C until cytokine measurement. Remaining cells were resuspended in FCS 10% DMSO (Hybri-max, Sigma) and stored at −150 °C until mRNA extraction.

2.6. Cytokines quantification

Concentrations of IL-10, IFN-γ and TNF-α were measured in culture supernatants of PBMC and MLNC, using capture sandwich ELISA porcine ELISA kit (R&D Systems, Lille, France) according to the manufacturer's instructions. IL-17 concentration was measured also using capture sandwich ELISA kit (VetSet™, Kingfisher Biotech, USA).

2.7. RT-qPCR

Quantitative PCR was performed to determine T-bet, GATA3, FOXP3 and RORγt mRNA levels in PBMC and MLNC. Primers used for mRNA quantification are listed in Table S1. Total RNA from cells was isolated by Trizol reagent (Invitrogen Ambion), and cDNA was synthesized using a qScript cDNA synthesis kit (Quanta Biosciences). Amplification was performed as previously described (Rabah et al., 2018). The transcripts level of the target genes was normalized to the transcript level of hprt gene (housekeeping gene, see Table S1). These primers were described previously (Delroisse et al., 2008; Hermann-Bank, Skovgaard, Stockmarr, Larsen, & Mølbak, 2013; Hernández et al., 2009; Hervé et al., 2007; Muráni, Murániová, Ponsuksili, Schellander, & Wimmers, 2007; Young, Ibuki, Nakamori, Fan, & Mine, 2012). *Hprt* expression was not affected by the tested matrices. The results are expressed as expression level (ΔCT), in duplicate analysis for each piglets (n = 7) for the three groups.

2.8. Statistical analysis

Growth and food intake data were analysed using 2-way ANOVA testing treatment (PF vs PF-SW) and time effects. Difference in propionibacteria, lactobacilli and bifidobacteria between day 0 and 14 was tested using Mann-Whitney test. For SCFA concentrations, cytokine secretions and gene expressions *t*-test were used. Statistical significance was set at $P < 0.05$. Analysis performed using Graph Pad Prism Software (Prism 7 for Windows). All data are expressed as mean values ± standard errors of the mean.

3. Results

3.1. Growth and food intake

Consumption of PF-SW impacted neither piglet growth (Fig. 1A) nor food intake (Fig. 1B). Animals remained healthy during the 14-day treatment period.

3.2. Faecal bacterium and short-chain fatty acid concentrations

PF concentration was evaluated by qPCR in faeces at day 14. PF level was 7.0 ± 0.7 log cfu/g of faeces in PF-SW pigs. PF was not detected in faeces from SW pigs. Lactobacilli and bifidobacteria concentrations were evaluated in faeces at day 14 and compared to the initial level at day 0. Compared to day 0, consumption of SW or PF-SW for 14 days did not change lactobacilli faecal concentration (Fig. 2A). On the other hand, consumption of PF-SW during 14 days significantly

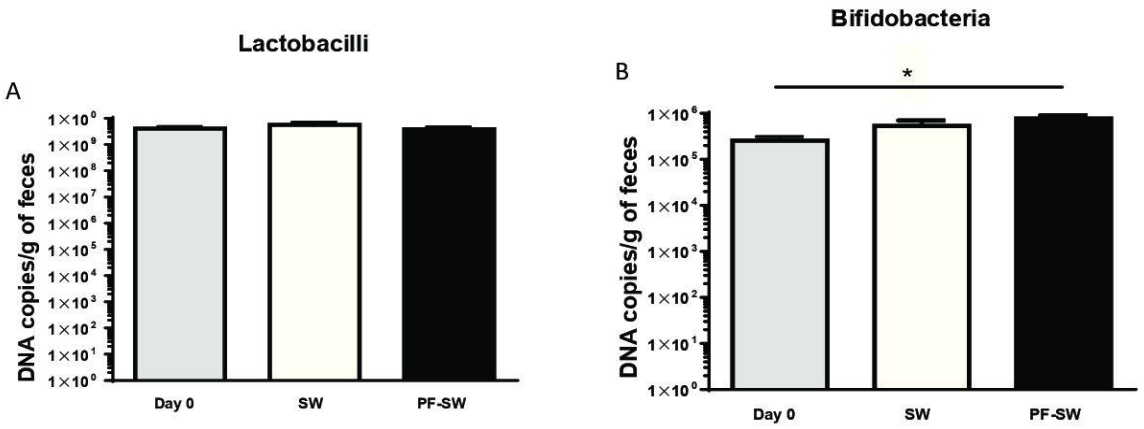

Fig. 2. Lactobacilli and Bifidobacteria faecal concentrations. Lactobacilli (A) and bifidobacteria (B) total population in faeces were assessed at day 14. The piglets received, in addition to their feed, either sweet whey (SW), or *P. freudenreichii* CIRM-BIA 129 - fermented sweet whey (PF-SW). Lactobacilli and bifidobacteria concentrations were determined by qPCR in faeces. Results are represented as means ± SEM (n = 7). * P < 0.005.

increased bifidobacteria faecal concentration compared to day 0 (P = 0.02, Fig. 2B). Consumption of SW did not significantly change bifidobacteria faecal concentration (P = 0.33, Fig. 2B).

SCFA concentrations were determined at day 14 in faecal samples. Compared to SW, PF-SW consumption did not modify SCFA concentration (Fig. 3).

3.3. MLNC phenotype and cytokine secretion

MLNC from PF-SW and SW pigs were isolated after 14 days of treatment. Cell phenotype was assessed by measuring transcription factor mRNA levels by qPCR: T-bet (Th1), GATA3 (Th2), FOXP3 (Treg) and RORγt (Th17). T-bet mRNA level was 2.7-fold greater in PF-SW than SW pig MLNC (P = 0.007, Fig. 4B). The other transcription level factor was not altered by PF-SW consumption.

Basal secretions of TNF-α, IFN-γ, IL-10 and IL-17 by MLNC after 72 h of culture were not different between SW- and PF-SW piglets (Suppl. Fig. 1).

LPS induced TNF-α secretion in MLNC from PF-SW pigs (P = 0.006, Fig. 5A) but not SW ones (P = 0.54, Fig. 5A). Addition of Slps, the immunomodulatory surface protein of PF to the culture media significantly reduced LPS-induced TNF-α secretion by MLNC in PF-SW pigs (P = 0.02, Fig. 5A). MLNC from both PF-SW and SW pigs secreted TNF-α in response to a sub-optimal dose of ConA (P = 0.003 and 0.002, respectively, Fig. 5B). Con A induced-TNF-α secretion was inhibited when Slps were added to the culture media in both pig groups (P = 0.03 and 0.02, respectively, Fig. 5B). LPS did not increase significantly IL-10 secretion in MLNC from neither PF-SW nor SW pigs (Fig. 5C). However, when Slps were added to the culture media, IL-10 secretion was significantly increased (P = 0.0001 and 0.0002 for PF-SW and SW pigs respectively, Fig. 5C), with no difference between the two pig groups. ConA did not induce significant IL-10 secretion, either alone (P = 0.09 and 0.20 for PF-SW and SW pigs, respectively, Fig. 5D) or in combination with Slps (P = 0.06 and 0.22 for PF-SW and SW pigs, respectively, Fig. 5D). Neither LPS nor ConA stimulation elicited IFN-γ secretion by MLNC, irrespective of pig treatment (Suppl Fig. 2).

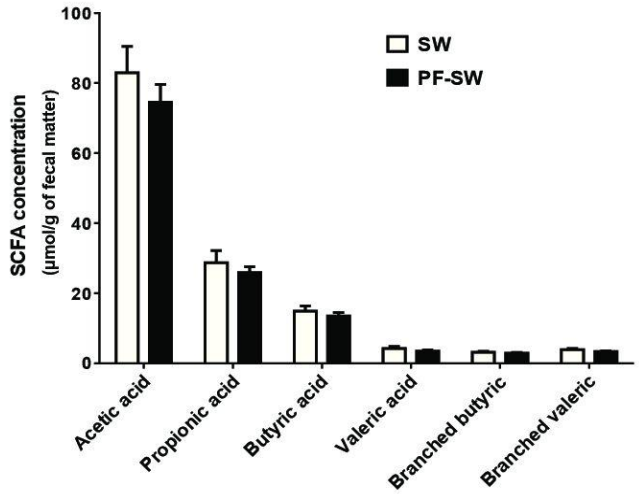

Fig. 3. Faecal SCFA concentrations. Analysis of short chain fatty acids (SCFA) concentration in faeces contents at day 14. The piglets received, in addition to their feed, either sweet whey (SW), or *P. freudenreichii* CIRM-BIA 129 - fermented sweet whey (PF-SW). The total SCFA concentration, the concentration of each SCFA type (acetic, propionic, butyric and valeric acids), and the concentration of branched short chain fatty acids were measured by gas-phase chromatography. Results are represented as means ± SEM (n = 7).

3.4. PBMC phenotype and cytokine secretion

PBMC from PF-SW and SW pigs were isolated after 14 days of treatment. PBMC phenotype, assessed by transcription factor mRNA levels, was not different between PF-SW and SW pigs (Fig. 6). PBMC basal TNF-α, IFN-γ, IL-10 and IL-17 secretion after 72 h of culture was not different between pigs (Suppl. Fig. 3).

TNF-α secretion by PBMC was significantly increased by LPS stimulation in both PF-SW and SW pigs (P = 0.02 and 0.02, respectively, Fig. 7A). Addition of Slps to the culture media decreased PBMC LPS-induced TNF-α secretion in SW pigs (P = 0.05, Fig. 7A) but not PF-SW ones (P = 0.45, Fig. 7A). ConA significantly increased TNF-α secretion by PBMC in SW pigs (P = 0.003, Fig. 7B). This effect was prevented by addition of Slps to the culture media (P = 0.04, Fig. 7B). However, TNF-α secretion by PBMC from PF-SW pigs was not increased by ConA stimulation (P = 0.25, Fig. 7B). Neither LPS nor ConA elicited IL-10 secretion by PBMC, irrespective of pig treatment (Fig. 7C and D). However, Slp addition to the culture media induced IL-10 secretion in response to LPS or ConA in PBMC from PF-SW pigs (P = 0.0003 and 0.0006, respectively, Fig. 7C and D). Neither LPS nor ConA stimulation elicited IFN-γ secretion by PBMC, irrespective of pig treatment (Suppl Fig. 2).

4. Discussion

Fermented food products, whether traditional or innovative, are increasingly attracting attention of consumers and stakeholders.

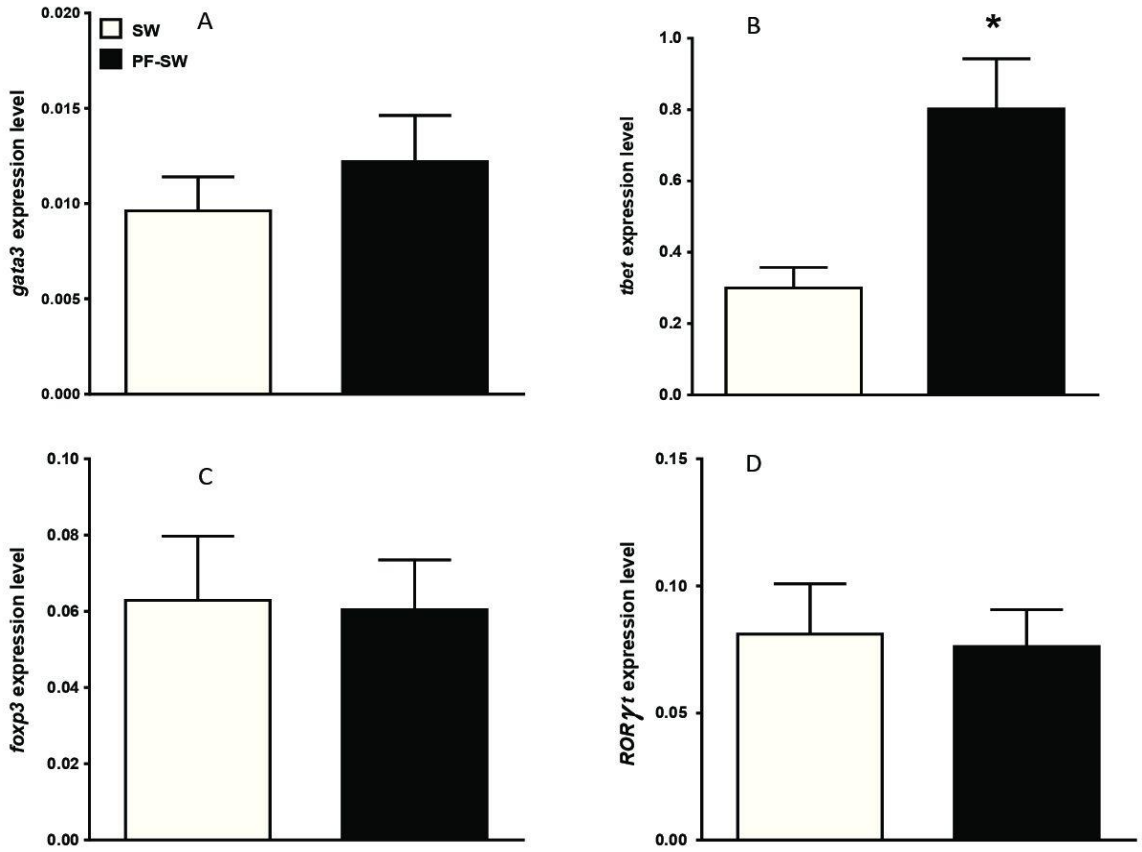

Fig. 4. MLN-phenotype. Basal expression of Tbet, GATA3, FOXP3 and RORγt were analyzed in mesenteric lymph nodes cells (MLNC) from the two piglet groups. The piglets received, in addition to their feed, either sweet whey (SW), or *P. freudenreichii* CIRM-BIA 129 - fermented sweet whey (PF-SW). The level of expression was determined by RT-PCR.

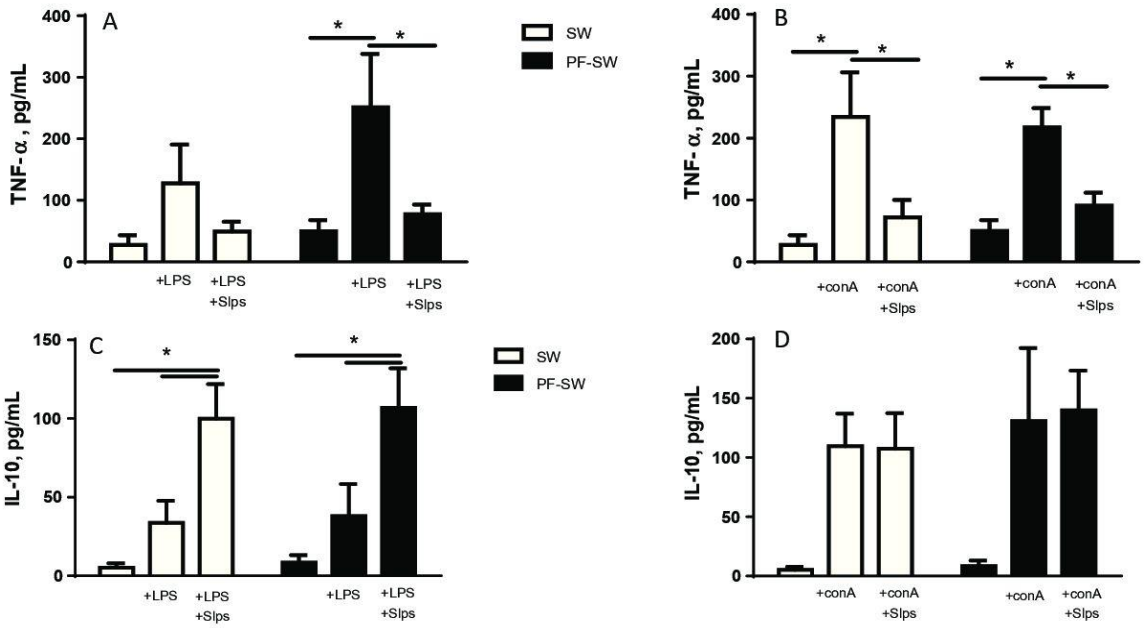

Fig. 5. MLN TNF-α and IL-10 secretion in response to LPS and Concanavalin A. Secretion of IL-10 and TNFα of stimulated mesenteric lymph nodes cells (MLNC) from the two piglet groups. The piglets received, in addition to their feed, either sweet whey (SW), or *P. freudenreichii* CIRM-BIA 129 - fermented sweet whey (PF-SW). Cells were stimulated *ex vivo* with Lipopolysaccharides (LPS) or Concanavalin A (ConA), alone or in combination with extracted P. freudenreichii' surface proteins (Slps). Cytokine concentrations were measured by ELISA and represented as means ± SEM (n = 7). * $P < 0.05$.

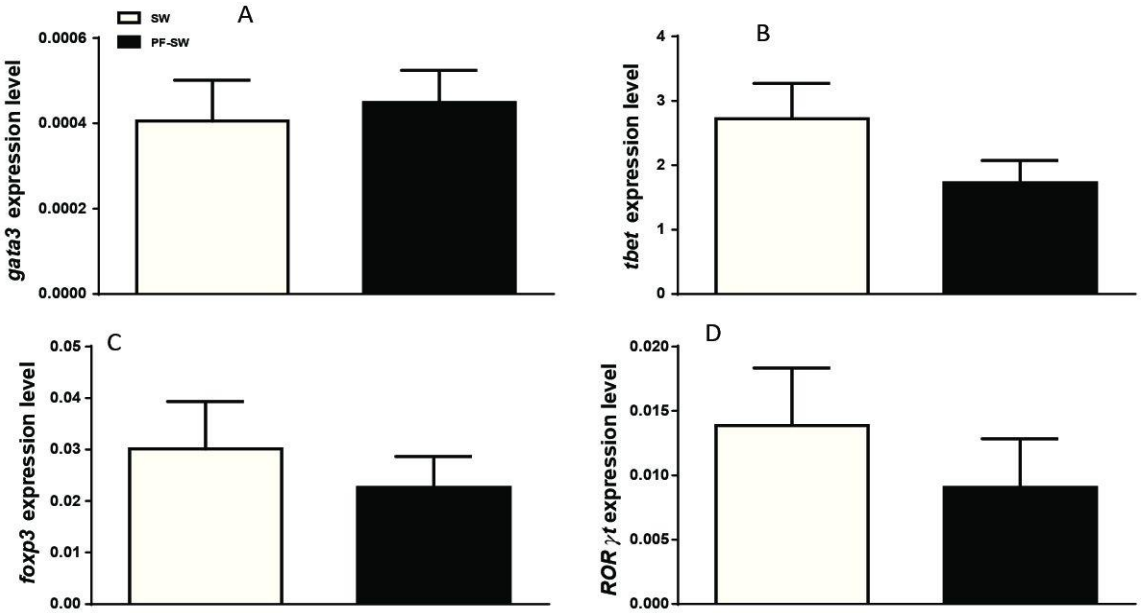

Fig. 6. PBMC phenotype. Basal expression of Tbet, GATA3, FOXP3 and RORγt were analyzed in peripheral blood mononuclear cells (PBMC) from the two piglet groups. The piglets received, in addition to their feed, either sweet whey (SW), or *P. freudenreichii* CIRM-BIA 129 - fermented sweet whey (PF- SW). The level of expression was determined by RT-PCR.

Transformation of foods by naturally occurring bacteria is becoming more and more popular. It enhances the nutritional properties through the synthesis of vitamins, bioactive peptides, SCFA and polysaccharides (Septembre-Malaterre, Remize, & Poucheret, 2018). Food fermentation also constitutes a way to provide high amounts of beneficial bacteria, the so-called probiotics. Indeed, using appropriate starters, with both technological and probiotic abilities, allows functionalising the final product. We applied this strategy to whey fermented by a selected strain of *Propionibacterium freudenreichii*.

Propionibacterium freudenreichii is a beneficial bacterium and constitutes an archetypal example of "2-in1" bacterium. Indeed, it is traditionally used to elaborate fermented products including Swiss-type cheeses (Thierry et al., 2011). It is also recognized as probiotic with both metabolic and immunomodulatory beneficial effects (Rabah, Rosa do Carmo, & Jan, 2017). Indeed, *P. freudenreichii* revealed remarkable immunomodulatory properties with the ability to induce the modulatory cytokine IL-10, both in immune and in epithelial cells (Foligné et al., 2010; Rabah et al., 2018). This anti-inflammatory effect is highly

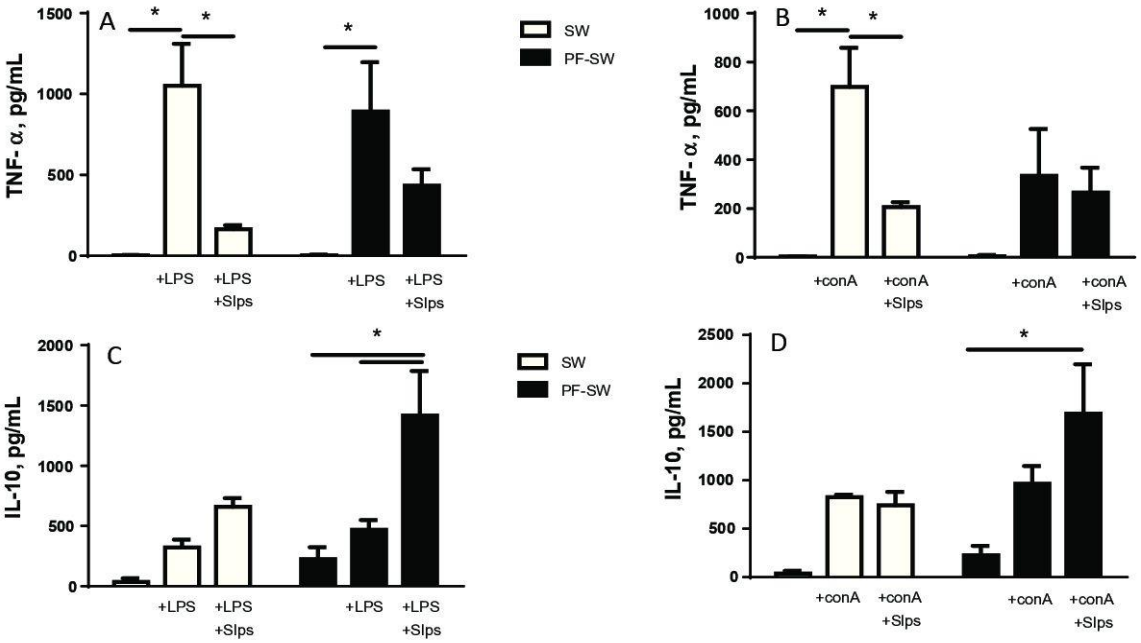

Fig. 7. PBMC TNF-α and IL-10 secretion in response to LPS and Concanavalin A. Secretion of IL-10 and TNFα of stimulated peripheral blood mononuclear cells (PBMC) from the two piglet groups. The piglets received, in addition to their feed, either sweet whey (SW), or *P. freudenreichii* CIRM-BIA 129 - fermented sweet whey (PF-SW). Cells were stimulated *ex vivo* with Lipopolysaccharides (LPS) or Concanavalin A (ConA), alone or in combination with extracted *P. freudenreichii*' surface proteins (Slps). Cytokine concentrations were measured by ELISA and represented as means ± SEM (n = 7). * $P < 0.05$.

strain-dependent (Foligné et al., 2013) and strains, which induce IL-10, protect mice from induced colitis (Foligné et al., 2010). Slps, which mediate immunomodulation (Deutsch et al., 2017; Le Marechal et al., 2015), are expressed within an experimental cheese, which also protects from induced colitis (Plé et al., 2015, 2016). Thus, selected strains of *P. freudenreichii* may play a central role in the development of functionalized food for tailored nutrition. This includes functional food products for inflammatory bowel diseases, based on the ability to attenuate inflammation *via* modulation of cytokines. This includes ingredients for infant formula, where selected bacteria such as bifidobacteria (Duranti et al., 2017) and propionibacteria (Colliou et al., 2017) may attenuate the incidence of necrotizing enterocolitis and of atopy, as does breast feeding. Indeed, infant formulas are currently scrutinized for their biological effects, as they do not afford the same maturation of the human gut microbiota and immunity, as compared to maternal milk (Lewis, Richard, Larsen, & Field, 2017).

We have developed whey powders containing probiotic propionibacteria by using an innovative "2-in-1" method to both grow and dry them in sweet whey (Huang, Cauty et al., 2016). They contain high amounts of live propionibacteria. However, the probiotic effects of these functionalised powders remained unknown. Herein, we challenged functionalised whey, fermented by the selected anti-inflammatory *Propionibacterium freudenreichii* CIRM-BIA 129 strain, in a model of healthy piglets.

Using an animal model close to Human, we demonstrated that consumption of spray-dried *P. freudenreichii*–fermented sweet whey during 14 days enhances bifidobacteria faecal population. Numerous studies accordingly demonstrated that *P. freudenreichii* has the ability to enhance bifiodobacteria growth (Bouglé, Roland, Lebeurrier, & Arhan, 1999; Colliou et al., 2017; Hojo et al., 2002), due to the *in situ* production of bifidogenic metabolites (Isawa et al., 2002; Nagata et al., 2010; Yamazaki, Kano, Ikeda, Isawa, & Kaneko, 1999). However, the consumption of functionalized sweet whey did not enhance here short chain fatty acids in pigs' faeces, compared to control sweet whey. Nevertheless, whey consumption was already shown to enhance short chain fatty acids in rats (Wronkowska et al., 2017). This enhancement, in both groups, may have surpassed previously observed modulation of SCFAs by ingested propionibacteria (Cousin et al., 2012; Lan et al., 2007). We also demonstrated that consumption of *P. freudenreichii*–-fermented sweet modifies intestinal and systemic immune responses to immunostimulating stimuli. Indeed, compared to MLNC from SW-pigs, MLNC from PF-SW pigs harboured a Th1 phenotype characterized by increased expression of Tbet mRNA and TNF-α secretion in response to LPS, suggesting better control of bacterial infection. At the systemic level, PBMC from PF-SW pigs harboured an anti-inflammatory phenotype with lower TNF-α secretion in response to ConA, a non-specific lymphocyte mitogen, and higher secretion of IL-10 in response to LPS and ConA in presence of the Slps compared to SW-pig PBMC. We noticed basal cytokine secretions in MLNC were not consistent with T lymphocytes phenotypes in MLNC investigated by qPCR. We supposed that the basal cytokine secretion is a global response of T, B cells and innate immune cells (macrophages and dendritic cells), not only T lymphocytes. This may explain these discrepancies. Previous studies showed the ability of Slps from *P. freudenreichii* to bind to C-type lectins receptors of monocytes and to modulate the immune system of mice (Colliou et al., 2017). In the case of *P. freudenreichii* CIRM-BIA 129, more investigations are needed to show the existence of similar mechanism or not.

As a conclusion, for the probiotic research field, our work indicates that propionibacteria-fermented whey may participate in the prevention and/or the treatment of chronic inflammatory disorders, which have a growing incidence. We show here that this new product enhances intestinal populations of actinobacteria known to modulate the mucosal immune response: bifidobacteria (Wu et al., 2016) and propionibacteria (Colliou et al., 2017). Accordingly, it modulates both local and peripheral immune responses. From the industrial application field point, this opens new avenues for the development of functional ingredients and for the valorisation of whey. By cultivating a probiotic GRAS bacterium in concentrated whey, prior to spray drying, it is possible to transform this by-product into a functional ingredient. Functionalized sweet whey may find a probiotic application in the field of infant formula, in which immunomodulating actinobacteria are desired. It may also find an application as functional food supplement for athletes undergoing training-related stress (Pane, Amoruso, Deidda, Graziano, Allesina, & Mogna, 2018). Furthermore, such functionalized sweet whey combines both probiotics (propionibacteria) and bioactive compounds that they produce (vitamins, SCFAs, bifidogenic compounds), the so-called probioactives, improving the functionality of the fermented product (Champagne, Gomes da Cruz, & Daga, 2018).

Ethics statement

The experimental protocol was performed in accordance with recommendations of the French law (2001-464 29/05/01) and EEC (86/609/CEE) for the care and use of laboratory animals. The protocol was approved by the ethical committee on animal experimentation of Rennes (France), under the certificate of authorization to experiment 2017010922379066-V2. Pigs were sacrificed by electronarcosis followed by exsanguination, and every effort was made to minimize animal suffering.

Conflict of interest

HR is the recipient of a doctoral fellowship from The French association Brittany Food Biotechnology (Bba), the Brittany Region and The National Association of Research and Technology (CIFRE n° 2015/0747). Authors declared no conflict of interest.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jff.2018.11.043.

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Annexe 5 : publications et communications scientifiques en collaboration

❖ Articles scientifiques publiées dans des revues à comité de lecture

Do Carmo FLR**, Rabah H**, Huang S, Gaucher F, Deplanche M, Dutertre S, Jardin J, Le Loir Y, Azevedo V, Jan G. *Propionibacterium freudenreichii* Surface Protein SlpB is involved in adhesion to Intestinal HT-29 Cells. Front Microbiol. 2017; 8.

Huang S, **Rabah H**, Jardin J, Briard-Bion V, Parayre S, Maillard M-B, et al. Hyperconcentrated Sweet Whey, a New Culture Medium That Enhances *Propionibacterium freudenreichii* stress tolerance. Appl Environ Microbiol. 2016 Jul 15; 82(15):4641–51.

Huang S, Méjean S, **Rabah H**, Dolivet A, Le Loir Y, Dong Chen X, Jan G, Jeantet R, Schuck P; 2016; Double use of concentrated sweet whey for growth and spray drying of probiotics: Towards maximal viability in pilot scale spray dryer. Journal of Food Engineering, 196 (2017), pp. 11-17.

❖ Chapitre de livre

Do Carmo FLR, **Rabah H**, Fernandes Cordeiro B, Da Silva SH, Jan G, Azevedo V, Carvalho Dias R. Applications of Probiotic Bacteria and Dairy Foods in Health. Current Research in Microbiology, Open Access eBooks 919 North Market Street Suite 425 Wilmington, DE 19801, 2017.

UNIVERSITE ECOLOGIE
BRETAGNE GEOSCIENCES
LOIRE AGRONOMIE ALIMENTATION

AGRO CAMPUS OUEST

Titre : La vectorisation de *Propionibacterium freudenreichii* et de ses protéines immunomodulatrices par la matrice fromagère vers le tube digestif

Mots clés : *Propionibacterium freudenreichii*, protéines S-layer, vecteur probiotique, matrice fromagère, immunomodulation, colite

Résumé : *Propionibacterium freudenreichii* CIRM-BIA 129 ***(Pf)*** est un levain fromager et détient plusieurs potentialités probiotiques, dont l'immunomodulation. Cette propriété résulte de la production de divers métabolites bénéfiques et de la présence de protéines de surface S-layer (Slps). Cette propriété « 2-en-1 », c'est-à-dire à la fois fermentaire et probiotique, fait de ***Pf*** un bon candidat pour développer des fromages fonctionnels, afin de prévenir les maladies inflammatoires intestinales. L'objectif de cette thèse était d'étudier l'impact de la matrice fromagère sur les propriétés immunomodulatrices de ***Pf***, via ses protéines Slps, par comparaison à une culture sur ultrafiltrat de lait (**UF**). Les études conduites *in vitro* suggèrent que la vectorisation par le fromage module la viabilité de *Pf* lors de la digestion ainsi que dans le côlon. Nous avons montré aussi que la matrice fromagère limite la protéolyse des protéines Slps lors de la digestion gastrique et duodénale. L'étude *in vivo* a montré que les deux matrices de vectorisation, le fromage et l'**UF**, présentent des effets anit-inflammatoires différents. Finalement, un emmental a été fabriqué en sélectionnant des souches immunomodulatrices de bactéries lactiques et propioniques. Son ingestion a protégé des souris d'une colite chimiquement induite. Ce travail montre que les propriétés immunomodulatrices de ***Pf*** sont matrice-dépendantes, tout comme elles sont souche-dépendantes. Il souligne de plus le potentiel offert par l'utilisation d'un fromage de type emmental probiotique comme un levier pour prévenir les maladies inflammatoires intestinales.

Title: The delivery of *Propionibacterium freudenreichii* CIRM-BIA 129 and of its immunomodulatory proteins by the cheese matrix to the digestive tract

Keywords: *Propionibacterium freudenreichii*, S-layer proteins, probiotic delivery vehicle, cheese matrix, Immunomodulation, colitis

Abstract: *Propionibacterium freudenreichii* CIRM-BIA 129 (***Pf***) is a cheese starter and has several probiotic potentialities, including immunomodulation. This property results from the production of various beneficial metabolites, and from the presence of S-layer surface proteins (Slps). The "2-in-1" property, i.e. both fermentative and probiotic properties, makes ***Pf*** a suitable candidate to develop functional cheeses, in order to prevent inflammatory bowel disease. The aim of this thesis was to study the impact of the cheese matrix on the immunomodulatory properties of ***Pf***, mediated by its Slps proteins, compared to a culture on milk ultrafiltrate (**MUF**). *In vitro* studies suggest that ***Pf*** viability is affected by the delivery vehicle, cheese matrix or MUF during digestion as well as in the colon. We also showed that the cheese matrix limits Slps proteins proteolysis during gastric and duodenal digestion The *in vivo* study showed that the two delivery vehicles, cheese and **MUF**, display different antiinflammatory effects. Finally, an industrial-scale Emmental cheese was manufactured using selected immunomodulatory strains of lactic and propionic acid bacteria. It also highlights the potential of using Emmental probiotic cheese as a lever to prevent inflammatory bowel disease.