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PROGRAMA DE PÓS-GRADUAÇÃO EM GENÉTICA

Dissertação de Mestrado

IMPACTO DA INATIVAÇÃO DO GENE *slpB* NOS EFEITOS TERAPÊUTICOS DE *Propionibacterium freudenreichii* CIRM-BIA 129 EM MODELO MURINO DE MUCOSITE INDUZIDA POR 5-FLUOROURACIL

Orientado: Sara Heloísa da Silva

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Belo Horizonte
2020

Sara Heloísa da Silva

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Dissertação apresentada ao programa de Pós-Graduação em Genética da Universidade Federal de Minas Gerais como requisito parcial para obtenção do título de Mestre em Genética.

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ATA DA DEFESA DE DISSERTAÇÃO

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Às quatorze horas do dia **31 de março de 2020**, reuniu-se, no Instituto de Ciências Biológicas da UFMG, a Comissão Examinadora de Dissertação, indicada pelo Colegiado do Programa, para julgar, em exame final, o trabalho intitulado: **"Impacto da inativação do gene *slpB* nos efeitos terapêuticos de *Propionibacterium freudenreichii* CIRM-BIA 129 em modelo murino de mucosite induzida por 5-Fluorouracil"**, requisito para obtenção do grau de Mestre em **Genética.** Abrindo a sessão, o Presidente da Comissão, **Dr. Vasco Ariston de Carvalho Azevedo**, após dar a conhecer aos presentes o teor das Normas Regulamentares do Trabalho Final, passou a palavra à candidata, para apresentação de seu trabalho. Seguiu-se a arguição pelos Examinadores, com a respectiva defesa da candidata. Logo após, a Comissão se reuniu, sem a presença da candidata e do público, para julgamento e expedição de resultado final. Foram atribuídas as seguintes indicações:

Prof./Pesq.	Instituição	CPF	Indicação
Dr. Vasco Ariston de Carvalho Azevedo	UFMG	283.171.225-49	Aprovada
Dr. Fillipe Luiz Rosa do Carmo	UFMG	053826516 02	Aprovada.
Dr. Aristóteles Góes Neto	UFMG	544.348.825-20	Aprovada
Dr. Alfonso Gala-Garcia	UFMG	01607877694	Aprovada.
Dra. Ana Cristina Gomes-Santos	UNA	05510870698	APROVADA

Pelas indicações, a candidata foi considerada: Aprovada
O resultado final foi comunicado publicamente à candidata pelo Presidente da Comissão.
Nada mais havendo a tratar, o Presidente encerrou a reunião e lavrou a presente ATA, que será assinada por todos os membros participantes da Comissão Examinadora.
Belo Horizonte, 31 de março de 2020.

Dr. Vasco Ariston de Carvalho Azevedo - Orientador _____

Dr. Fillipe Luiz Rosa do Carmo - Coorientador _____

Dr. Aristóteles Góes Neto _____

Dr. Alfonso Gala-Garcia _____

Dra. Ana Cristina Gomes-Santos _____

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Capítulo 1

LISTA DE ABREVIAÇÔES

5-FU - 5-Fluorouracil

CB 129 - *Propionibacterium freudenreichii* CIRM-BIA 129

CB129Δ*slpB* - *Propionibacterium freudenreichii* CIRM-BIA 129 knockout cromossomal do gene *slpB*

CDs- Células dendríticas

CEUA - Comissão de Ética no Uso de Animais

CFU – Colony forming unit (unidades formadoras de colônia)

GRAS – Generally Recognized As Safe (Geralmente Reconhecida como Segura)

IBD - Inflammatory Bowel disease

ICB – Instituto de Ciências Biológicas

INRA - Institut national de la recherche agronomique

LAB – Lactic acid bacteria (bactérias do ácido lático)

MRS – de Man, Rogosa and Sharpe broth

TGI – Trato gastrointestinal

SCFAS - Short chain fatty acids (ácidos graxos de cadeia curta)

Slaps - S-Layer-Associated Proteins

Slps - S-layer proteins (proteínas da camada S)

SURFING - Starter SURFace against INflammation of the Gut

PBMC - Peripheral Blood Mononuclear Cell (células mononucleares do sangue periférico)

UFMG – Universidade Federal de Minas Gerais

YEL - Yeast Extract Sodium Lactate (meio de cultura)

RESUMO

Propionibacterium freudenreichii CIRM-BIA 129 (*P. freudenreichii* selvagem, WT) é uma bactéria Gram-positiva usanda na maturação de queijo, que atualmente teve seu potencial probiótico investigado. Em estudos anteriores o consumo dessa bactéria provou exercer efeitos imunomoduladores em modelo murino de colite induzida por agente químico. Parte dos efeitos imunomodulatórios dessa linhagem depende das proteínas da camada S (Slp) à quais podem estar envolvidas na persistência no intestino, adesão às células e muco do hospedeiro ou imunomodulação. Precisamente, a inativação do gene para a proteina SlpB na linhagem *P. freudenreichii* WT ressultou na linhagem mutante Δ*slpB* que teve sua capacidade de adesão às células epiteliais *in vitro* reduzida, em comparação a linhagem parental. Contudo, o efeito imunomodulador da proteína SlpB da linhagem *P. Freudenreichii* WT não está claro. Dessa forma, fica evidente elucidar o papel anti-inflamatório dessa proteína *in vitro* e *in vivo*. Em um ensaio *in vitro*, a linhagem selvagem de *P. freudenreichii* reduziu a expressão de citocinas IL-8 ($p < 0,0001$) e TNF-α ($p < 0,0001$) em células HT-29 estimuladas por Lipopolissacarídeo (LPS). Em contrapartida, a linhagem mutante *P. freudenreichii* ΔslpB, absente da proteína SlpB, não foi capaz de reduzir a expressão dessas citocinas. Posteriormente, ambas as linhagens foram investigadas *in vivo* em um modelo de mucosite induzida por 5-fluorouracil (5-FU) em camundongos BALB/c. A mucosite é um efeito colateral comum da quimioterapia com 5-FU, caracterizada por lesão nas mucosas, inflamação, diarréia e perda de peso. A linhagem selvagem WT evitou a perda de peso, reduziu a inflamação e, consequentemente, os escores histopatológicos. Além disso, regulou a expressão de marcadores envolvidos no processo inflamatório, incluindo os genes Claudin-1 (*cld1*, $p < 0,0005$) e IL-17a (Il17a, $p < 0,0001$), bem como os genes IL-12 ($p < 0,0001$) e IL-1β ($p < 0,05$) níveis de citocinas. A linhagem mutante apresentou efeito contrário relativo à expressão do gene *cld1* e nos níveis de IL-12. Este trabalho enfatiza a importância do SlpB na capacidade de *P. freudenreichii* de reduzir a inflamação em um modelo de mucosite. Ele abre perspectivas para o desenvolvimento de produtos probióticos para diminuir os efeitos colaterais da quimioterapia usando bactérias GRAS com propriedades imunomoduladoras de proteínas de superfície.

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

ABSTRACT

Propionibacterium freudenreichii CIRM-BIA 129 (*P. freudenreichii* wild-type, WT) is a Gram-positive bacteria used in cheese maturation, which has currently had its probiotic potential investigated. In previous studies, the consumption of this bacterium proved to exert immunomodulatory effects in a murine model of chemical agent-induced colitis. Part of the immunomodulatory effect of this strain depends on the S layer proteins (Slp) to which may be involved in persistence in the intestine, adhesion to the cells and mucus of the host or immunomodulation. Precisely, the inactivation of SlpB protein gene in the *P. freudenreichii* WT strain resulted in the Δ*slpB* mutant strain that had its ability to adhere to epithelial cells in vitro reduced, compared to the parental strain. However, the immunomodulatory effect of the SlpB protein of the P. Freudenreichii WT strain is not clear. Thus, it is necessary to elucidate the anti-inflammatory role of this protein *in vitro* and *in vivo*. In an in vitro assay, P. freudenreichii WT reduced expression of IL-8 ($p<0.0001$) and TNF-α ($p<0.0001$) cytokines in LPS-stimulated HT-29 cells. *P. freudenreichii* Δ*slpB*, lacking the SlpB protein, failed to do so. Subsequently, both strains were investigated in vivo in a 5-FU-induced mucositis mice model. Mucositis is a common side effect of cytotoxic chemotherapy with 5-FU, characterized by mucosal injury, inflammation, diarrhea, and weight loss. The WT strain prevented weight loss, reduced inflammation, and consequently, histopathological scores. Furthermore, it regulated key markers, including Claudin-1 (cld1, $p<0.0005$) and IL-17a (Il17a, $p<0.0001$) genes, as well as IL-12 ($p<0.0001$) and IL-1β ($p<0.0429$) cytokines levels. Mutant strain displayed opposite regulatory effect on cld1 expression and IL-12 levels. This work emphasizes the importance of SlpB in *P. freudenreichii* ability to reduce mucositis inflammation. It opens perspectives for the development of probiotic products to decrease the side effects of chemotherapy using GRAS bacteria with immunomodulatory surface protein properties.

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

INTRODUÇÃO GERAL

Trato gastrointestinal

O trato gastrointestinal (TGI) humano tem como principais funções a digestão e absorção de alimentos. O TGI se estende da boca ao ânus, e compreende boca, esôfago, estômago, intestino delgado (duodeno, jejuno e íleo), ceco, intestino grosso, cólon e reto. Constitui-se de um tubo oco formado por um tecido subdivido em mucosa, submucosa, muscular e adventícia, estas camadas representam uma barreira física que separa o lúmen intestinal do interior do corpo, como mostrado na figura 1 (GELBERG, 2014). O epitélio da mucosa intestinal é formado por uma única camada de enterócitos os quais são conectadas por junções célula-célula. As junções possuem três principais propósitos: adesão, para manter a integridade do tecido; criação de uma barreira que controla a passagem de patógenos, água e outras moléculas; e sinalização, para receber e transmitir sinais que possam afetar o tecido (CITI, 2018). Além disso, o epitélio intestinal é capaz de transportar Imunoglobulina A (IgA) secretada para o lúmen, que é produzida por células plasmáticas localizadas na lâmina própria. No lúmen a IgA atua como antimicrobiana para impedir que as bactérias ultrapassem a mucosa intestinal, mantendo a homeostase entre o hospedeiro e sua microbiota (HOOPER, 2015).

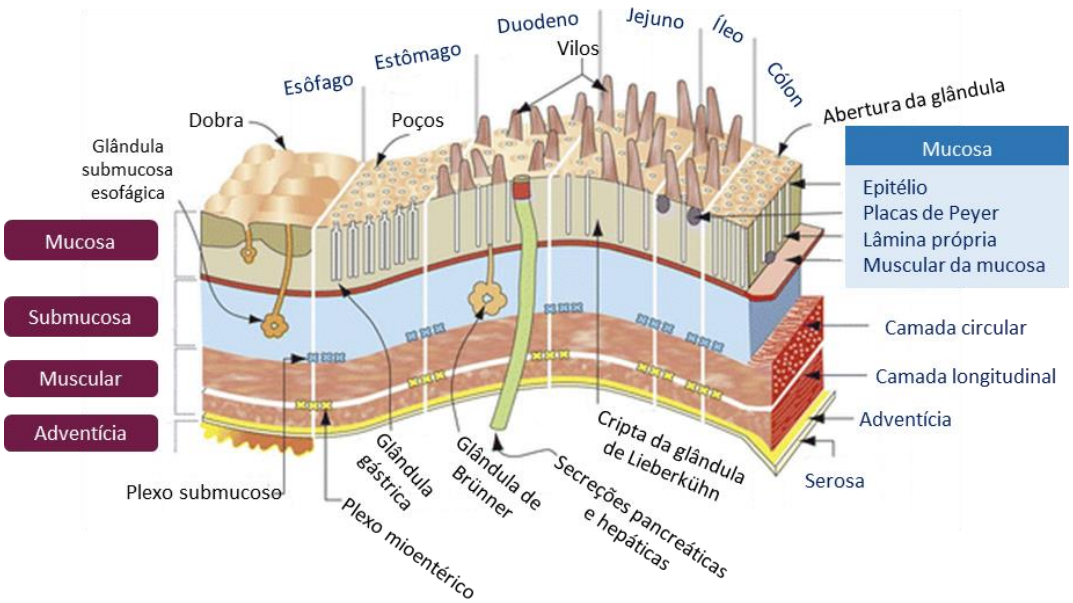


Figura 1: Representação esquemática da organização anotômica e histológica do tubo digestivo. Adaptado de Kierzenbaum, A. L. (2002). *Histology and Cell Biology: An Introduction to Pathology*, Mosby, St. Louis. Zachary and McGavin, *Pathologic Basis of Veterinary Disease,* 5th ed., Copyright © 2012 by Mosby, Inc., uma afiliação de Elsevier Inc.

Além dos enterócitos, o epitélio intestinal possui células caliciformes, estas são responsáveis pela produção de uma camada de muco na superfície epitelial, um dos principais componentes deste muco são as proteínas mucinas, como a MUC2, que são altamente glicosiladas e se unem para formação do muco (Figura 2). Esta camada de muco é subdividida em duas, uma mais externa que é móvel e colonizada por uma grande quantidade de bactérias e uma mais interna que está em contato direto com as células epiteliais e é densamente compactada, nesta última as bactérias são incapazes de penetrar, são impedidas também pela produção de proteínas antibacterianas produzidas pelas células epiteliais (HOOPER, 2015; JOHANSSON et al., 2008).

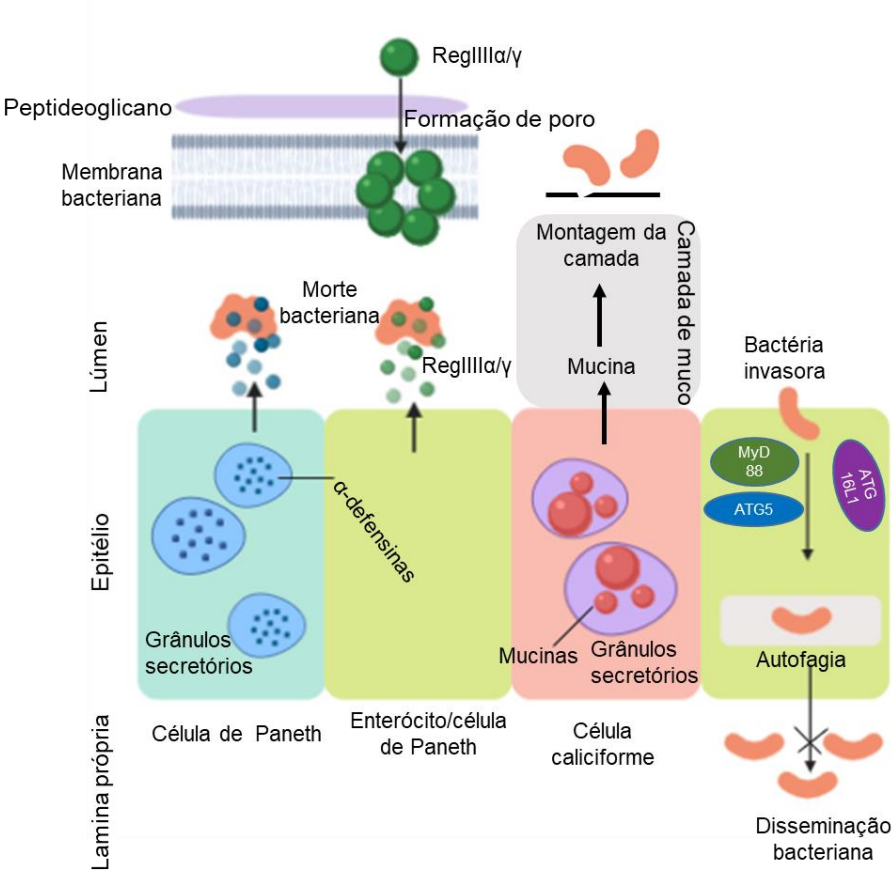


Figura 2: Representação de mecanismos intrínsecos das células epiteliais da defesa imune inata. Adaptado de Hooper L. V., 2015. As células epiteliais são capazes de executar atividades imunológicas. As células de Paneth secretam diversos antimicrobicidas. RegIIIα (humano) e RegIIIγ (camundongo) são antimicrobianos secretadas pelos enterócitos e pelas células de Paneth, eles se ligam ao peptioglicanos das bactérias Gram-positivas e formam um poro em sua membrana, provocando a morte. As células caliciformes secretam proteínas mucinas que se reúnem para formação do muco que imita o contato bacteriano com a superfície epitelial. A autofagia é ativada quando bactérias invadem as células e ocorre pra impedir a disseminação para tecidos profundos. A autofagia depende de sinalizadores, como TLR MYD88 e outros fatores de autofagia, como ATG5 e ATG16L1 (Hooper, 2015).

As células epiteliais produtoras de proteínas antimicrobianas, são chamadas células de Paneth, se localizam na base das criptas de Lieberkuhn e possuem grande quantidade de grânulos contendo microbicidas, como α-defensinas e lectinas tipo-C. Quando estas células recebem sinais microbianos, liberam as proteínas para o lúmen onde estas contribuem para eliminação de patógenos. Além disso, as células de Paneth participam da renovação do tecido epitelial (Figura 2) (HOOPER, 2015).

As células epiteliais se comunicam com células do sistema imune para regular e coordenar respostas imunológicas. Esta comunicação pode ser feita através do transporte de IgA, da participação na apresentação de antígenos e secreção de citocinas. As citocinas são produzidas de acordo com sinais recebidos pelas células epiteliais e assim ativam uma resposta imune adaptativa. Já a apresentação de antígenos ao sistema imune pode ser facilitada por células caliciformes e enterócitos (HOOPER, 2015).

Fica claro que as células epiteliais tem um papel fundamental no sistema imune do hospedeiro. Além disso, existe uma correlação entre os microrganismos que habitam o TGI i.e. microbiota intestinal e a maturação, desenvolvimento e regulação do sistema imunológico (PALM et al., 2015).

Microbiota

O TGI abriga uma grande comunidade de microrganismo, chamada de microbiota, composta por bactérias, vírus, fungos e protozoários, sendo as bactérias mais abundantes. Juntos representam mais de 100 trilhões de microrganismos que codificam mais de três trilhões de genes, os quais influenciam características imunológicas e metabólicas do hospedeiro (VALDES et al., 2018). Nos seres humanos são encontrados 2172 espécies de bactérias, classificadas em 12 filos, sendo Proteobacteria, Firmicutes, Actinobacteria e Bacteroidetes, os mais representativos (THURSBY; JUGE, 2017). As bactérias residentes, ou seja, as que colonoziam o TGI, são importantes para impedir o estabelecimento de bactérias patogênicas, já bactérias transientes, adivindas da alimentação, podem integrar ao TGI temporariamente e assim influenciar na estrutura e função da microbiota intestinal (ZHANG et al., 2016). A microbiota auxilia na maturação e desenvolvimento de células imunológicas, na hematopoese,

na coleta e armazenamento de energia, assim como em uma variedade de funções metabólicas como a fermentação, a produção de vitaminas, butirato e folato, absorção de carboidratos não digeridos (CLEMENTE et al., 2012; PIWOWAREK et al., 2018; ROY; TRINCHIERI, 2017). Em condições normais, a mucosa intestinal gera uma tolerância aos microrganismos comensais permitindo a sua manutenção no interior do lúmen. Porém, fatores como a dieta, o uso de drogas, o estresse ambiental e os fatores genéticos podem alterar a composição normal da microbiota, quebrando o equilíbrio dinâmico entre os microrganismos e o TGI e desregulando a homeostase do organismo, o que é chamado de disbiose e pode causar doenças gastrointestinais, como Colite Ulcerativa, Doença de Crohn. Contudo, estudos recentes mostram que a desregulação da microbiota pode ser contornada pela dieta, o que inclui o consumo de bactérias probióticas (HOLZAPFEL; SCHILLINGER, 2002; MCFARLAND, 2014; PETERSON et al., 2015).

Probióticos

Os probióticos, que são atualmente definidos como sendo: microrganismos vivos que, quando administrados em quantidades adequadas, conferem benefícios à saúde do hospedeiro (HILL et al., 2014; WHO, 2002), estão presentes na microbiota intestinal, além de também serem encontrados em alimentos, como queijos, leite fermentado e outros. Entre as bactérias com capacidade probiótica utilizadas para a produção desses produtos, as bactérias láticas (BLs) são amplamente estudadas. As BLs incluem um grupo heterogêneo de microrganismos que obtém energia através da conversão de açúcares em ácido lático. Morfologicamente, elas possuem forma de bastonetes e cocos, são Gram-positivas, não formam esporos, são imóveis, anaeróbicas facultativas e estritamente fermentativas. O grupo é composto por 13 diferentes gêneros, sendo que espécies Lactobacillus ssp, Streptococcus ssp, Lactococcus ssp. são as mais amplamente estudas (JAY et al., 2000). Algumas linhagens de BLs como os Lactobacillus, são capazes de modular a resposta inflamatória em modelo animal de Doenças Inflamatórias Intestinais (DII), atuando com potencial terapêutico anti-inflamatório. Os microrganismos probióticos podem ter um papel tanto na modulação das inflamações intestinais levando a estabilização da microbiota intestinal comensal, como na diminuição de populações de bactérias patogênicas que podem acarretar uma resposta inflamatória (CARVALHO et al., 2017b).

Os mecanismos que permitem aos probióticos exercer um efeito benéfico no hospedeiro são classificados principalmente em três categorias: efeitos probióticos metabólicos, normalização da composição da microbiota e interações moleculares entre probiótico e o hospedeiro, como mostrado na figura 3 (SÁNCHEZ et al., 2017; VIEIRA et al., 2013). Além disso, a seleção de cepas probióticas deve levar em consideração critérios que favorecem a ação in situ. Esses critérios incluem robustez a vários estresses abióticos e bióticos, como estresses digestivos, adesão ao epitélio intestinal, persistência no trato digestivo e capacidade de induzir uma resposta imune no hospedeiro (CARVALHO et al., 2017; RABAH, et al., 2017).

As linhagens bacterianas melhor descritas como probióticos, estudadas há muito tempo por seus efeitos benéficos, pertencem aos gêneros Bifidobacterium e Lactobacillus (O'TOOLE et al., 2017). Diante da busca emergente por novos probióticos, pesquisadores têm se esforçado para isolar e caracterizar novas espécies de bactérias que exercem efeitos terapêuticos no hospedeiro. Nesse contexto, as bactérias propiônicas do leite apareceram como probióticos promissores, principalmente por causa de seus efeitos imunomoduladores recentemente revelados (RABAH et al., 2017). O gênero Propionibacterium possui espécies cutâneas e láticas, a figura 4 mostra a distribuição filogenética deste gênero descrita por McDowell, 2013. *Propionibacterium freudenreichii*, que é o foco deste trabalho, é bem distinta das propionibactérias cutâneas, que são patogênicas oportunistas.

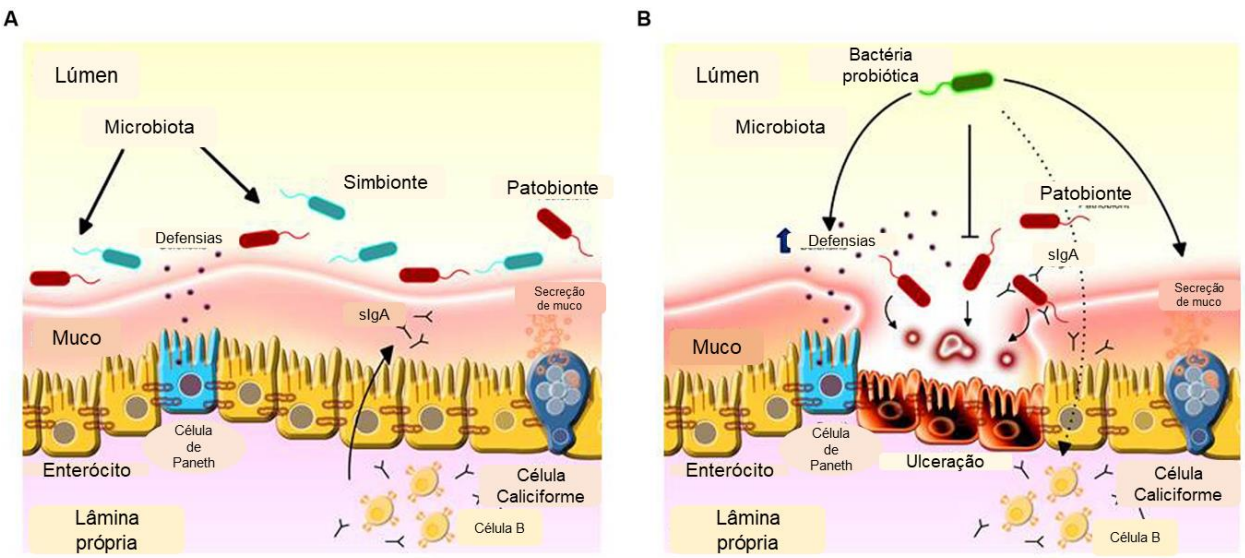
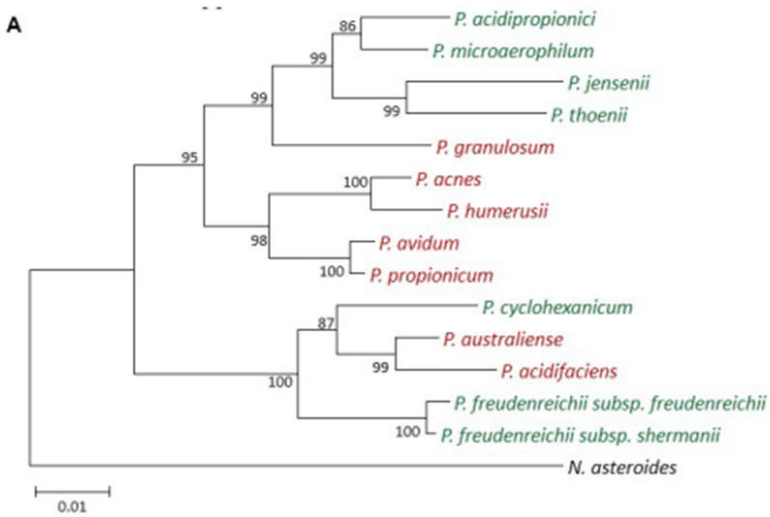


Figura 3: Mecanismos anti-inflamatórios probióticos de LAB na mucosa intestinal. Adaptado de Carvalho et al., 2017. (A) Homeostase intestinal proporcionada pela microbiota saudável, a qual estimula a produção de muco, ativade das células de Paneth e produção de sIgA. (B) Crescimento excessivo de patógenos que degradam a mucina e causam inflamação na mucosa. Probióticos impede o crescimento de patógenos, aumenta a secreção de defensinas, fortifica a estabilidade das junções apertadas, estimula a produção de sIgA, impedindo respostas inflamatórias.



B

Propionibactéria lática (clássica) Não patogênicas	Propionibactéria cutânea Patogênicas oportunistas
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*

Figura 4. (A) Árvore filogenética de evolução mínima de Propionibacteria baseada em sequências 16S rDNA. Adaptado de McDowell, 2013. (B) Repartição das espécies de *Propionibacterium* em dois grupos distintos, espécies láticas são apresentadas em verde e cutâneas em vermelho. Adaptado de Cousin, 2010.

Propionibacteria

A principal espécie de bactérias propiônicas, *Propionibacterium freudenreichii*, foi descrita pela primeira vez por E. von Freudenreich e S. Orla-Jensen. É uma das principais bactérias ingeridas diariamente na dieta francesa, devido à sua presença obrigatória no Emmental, como agente de refino (COUSIN et al., 2011). *P. freudenreichii* é uma bactéria propiônica do leite, uma Actinobactéria Gram-Positiva, caracterizada por uma alta produção de ácido propiônico por uma via de fermentação chamada Wood-Werkman, que envolve o ciclo da transcarboxilase (THIERRY et al., 2011). Cepas selecionadas dessa bactéria demonstraram robustez notável e toleram condições adversas, como tensões no trato digestivo, que incluem ácido estomacal e sais biliares (RABAH et al., 2017). *P. freudenreichii* e *P. acidipropionici* receberam o status "Geralmente Reconhecida como Segura" (GRAS), bem como o de "Presunção qualificada de

segurança" (QPS) (EFSA, 2013; KLINMAN et al., 2010). Além disso, as bactérias propiônicas lácteas são as únicas bactérias GRAS que produzem vitamina B12 de qualidade alimentar em escala industrial (THIERRY et al., 2011). Atualmente, *Propionibacterium* ssp. cada vez mais atraem a atenção por causa de suas propriedades probióticas promissoras. Estas propriedades estão ligadas a atributos versáteis, como a produção de ácidos graxos de cadeia curta (AGCCs), de ácidos graxos conjugados, de proteínas de superfície bioativas, do ácido 1,4-di-hidroxi-2-naftoico (DHNA, o fator de crescimento bifidogênico), de vitamina B12, bem como uma grande capacidade de suportar condições digestivas estressantes, adesão às células epiteliais intestinais e propriedades imunomoduladoras (RABAH et al., 2017).

Recetemente, a linhagem *P. Freudenreichii* CIRM-BIA 129 tiveram suas propriedades probióticas, demonstradas em modelos de camundongos com doença inflamatória intestinal (DII) (LE MARÉCHAL et al., 2015; PLÉ et al., 2015, 2016). Essas doenças constituem um grupo de condições idiopáticas e inflamatórias intestinais, incluindo colite ulcerosa e doença de Crohn (CARVALHO et al., 2017; RABAH et al., 2017). Essas propriedades benéficas de *P. freudenreichii* podem estar associadas a presença de proteínas da camada S. De fato, essas proteínas foram associadas a efeitos probióticos no gênero *Lactobacillus* e algumas medeiam interações importantes com o hospedeiro (HYNÖNEN; PALVA, 2013).

Nesse contexto, uma cepa de *P. freudenreichii*, CIRM-BIA 129, também conhecida como ITG P20, que possui proteínas da camada S, atraiu recentemente atenção por causa de suas propriedades anti-inflamatórias (LE MARÉCHAL et al., 2015; PLÉ et al., 2015, 2016).

Proteínas de superfície

As bactérias gram-positivas, dependendo da espécie, e em algum momento da cepa, podem ser cobertas por uma camada protéica paracristalina externa, não covalentemente ligada à superfície celular, chamada camada superficial ou camada S. As proteínas da camada S foram descritas pela primeira vez em 1953 por Houwink e foram encontradas pela primeira vez em *Spirillum* sp. e, posteriormente, encontradas em muitas bactérias probióticas, como espécies de *Lactobacillus* (HOUWINK, 1953; SLEYTR et al., 2014). Ao longo das décadas, através de uma série de estudos, foi demonstrado que as proteínas da camada S exercem papéis versáteis em funções cruciais de bactérias, como determinação ou manutenção da forma celular, peneira

molecular, atividades enzimáticas, adesão, co agregação, modulação de células imunológicas do intestino, proteção contra estresses ambientais e peptídeos antimicrobianos (HYNÖNEN; PALVA, 2013).

A camada S é encontrada em muitas espécies bacterianas, no entanto, a sequência de proteínas da camada S é altamente variável entre espécies de bactérias (HOUWINK, 1953; SLEYTR et al., 2014). Essas proteínas da camada S têm sido associadas à tolerância ao estresse em condições ambientais adversas, como no trato gastrointestinal (DO CARMO et al., 2017). Além disso, certas proteínas da camada S mediam a interação entre essas bactérias e o hospedeiro através do processo de adesão, consequentemente podem favorecer a entrega de metabólitos benéficos e de compostos nutracêuticos, bem como através da modulação das funções TGI (HYNÖNEN; PALVA, 2013).

Estudos recentes mostraram que a linhagem mais anti-inflamatória de *P. freudenreichii*, a CIRM-BIA 129, exibe cinco proteínas de superfície distintas extraíveis pela guanidina: SlpA, SlpB, SlpE, Internaline like A (Inl A) e Large surface protein A (Lsp UMA). Esse conjunto de proteínas está ligado às propriedades imunomoduladoras de *P. freudenreichii* (DEUTSCH et al., 2017; LE MARÉCHAL et al., 2015). De fato, essas proteínas da camada S demonstraram desempenhar um papel na regulação dos processos inflamatórios nos distúrbios do TGI. Assim, a seleção de bactérias probióticas que possuem proteínas da camada S podem determinar funcionalidades e, portanto, efeitos benéficos de produtos alimentares ou suplementos, em futuras aplicações biotecnológicas. Foi demonstrado que as propionibactérias láticas aderem às células epiteliais intestinais de camundongos *ex vivo* e *in vivo* (ZARATE, 2012), bem como às linhas celulares intestinais humanas *in vitro* (HUANG; ADAMS, 2003; MOUSSAVI; ADAMS, 2010). Além disso, a adesão é um critério-chave na seleção de linhagens, uma vez que é descrita como o passo inicial na colonização do hospedeiro (HAVENAAR; BRINK; VELD, 1992, p. Havenar; PREISING et al., 2010; RIEDEL et al. 2006). A adesão depende de compostos superficiais cruciais, incluindo as proteínas da camada S (LEBEER; VANDERLEYDEN; DE KEERSMAECKER, 2010). A elucidação das moléculas envolvidas nos mecanismos de adesão constitui um passo fundamental no entendimento das interações entre a bactéria e o hospedeiro. Considerando o papel central que as proteínas da camada S podem desempenhar em outras bactérias, investigamos esse processo pouco caracterizado nas

propionibactérias. Nesta seção, objetivamos, assim, decifrar os mecanismos envolvidos na adesão de *P. freudenreichii* às células epiteliais intestinais. Este trabalho abre novas perspectivas para a seleção de deformações adesivas. Por sua vez, isso pode melhorar as etapas iniciais da colonização do hospedeiro, favorecer a interferência probiótica / hospedeiro, permitir a entrega local de compostos nutracêuticos e modular a resposta inflamatória do hospedeiro através de efeitos imunomoduladores.

Relata-se que componentes extracelulares, incluindo proteínas da camada S, são determinantes da ação probiótica e desempenham um papel importante na modulação da adesão de microrganismos às superfícies epiteliais no hospedeiro (JOHANSSON et al., 2011; OTTE; PODOLSKY, 2004). Além da adesão às células hospedeiras, as proteínas da camada S também podem atuar como barreira física contra fatores externos (SLEYTR; MESSNER, 1988). A expressão de certas proteínas da camada S está relacionada à tolerância *in vitro* a condições ambientais extremas, como variações de pH, sais biliares, proteases e condições gastrointestinais simuladas (CHEN et al., 2007; ESLAMI; KERMANSHAHI; ERFAN, 2013; SMIT et al., 2001). Observou-se que a remoção de proteínas da camada S da superfície de *Lactobacillus hilgardii* aumenta sua suscetibilidade a enzimas bacteriolíticas e estresse físico-químico (DOHM et al., 2011).

No estudo de Carmo e colaboradores (2017) demonstraram que a linhagem *P. freudenreichii* CIRM-BIA 129 submetidas a raspagem enzimática com tripsina freudenreichii, perdem as proteínas da camada S e a capacidade de aderir às células HT-29. Além disso, a incubação dessa mesma linhagem, submetida a raspagem enzimática, com proteínas da camada S extraídas com guanidina, da mesma linhagem (*P. freudenreichii* CIRM-BIA 129) restaurou essa adesão. Especificamente, a linhagem mutante para a proteína SlpB, *P. freudenreichii* CIRM-BIA 129Δ*slpB* (CB129Δ*slpB*) exibiu uma adesão diminuída às células epiteliais intestinais HT-29 (DO CARMO et al., 2017b). Estes resultados demonstram a participação da proteína da camada S de *P. freudenreichii* CIRM-BIA 129, principalmente SlpB, na adesão, como foi relatado para a proteína SlpA em *Lactobacillus acidophilus* NCFM (BUCK et al., 2005).

A figura 5A ilustra a comunicação entre os probióticos e o hospedeiro, mediada pelas Células Epiteliais Intestinais (CEI) e as células imune no TGI, esta comunicação é iniciada através do

dos Padrões Moleculares Associados a Patógenos (PMAPs) reconhecidos pelos Receptores de Reconhecimento de Padrões (RRPs). As proteínas da camada S são importantes nas interações entre o TGI e os probióticos, mantendo a homeostase intestinal (DO CARMO et al., 2018a). As proteínas da camada S de linhagens de Lactobacilos são responsáveis por efeitos anti-inflamatórios, estimulando vias que reduzem a inflamação. Por exemplo, o SlpA de *L. helveticus* MIMLh5 reduz a ativação de NF-κB nas células Caco-2 (TAVERNITI et al., 2013). Além disso, proteínas da camada S do probiótico *L. acidophilus* diminuem a secreção de interleucina IL-8 em células Caco-2 estimuladas pelo *S. typhimurium* pró-inflamatório, IL-8 é um importante mediador pró-inflamatório secretado tanto por células intestinais como por macrófagos ativados, que junto com IL-12 leva ao desenvolvimento de células T auxiliares (Th1) (LI et al., 2011Sanchez-Muñoz et al. 2008). Finalmente, SlpA de *L. acidophilus* NCK2187 medeia sinais regulatórios, que por sua vez aliviam a gravidade da colite em um modelo de camundongo (LIGHTFOOT et al., 2015). Em conformidade, a remoção de proteínas da camada S da superfície de *P. freudenreichii* CIRM-BIA 129 suprimiu a indução de citocinas anti-inflamatórias em célunas mononucleares do sangue periférico humanas (PBMCs – do inglês, Peripheral Blood Mononuclear Cells) (FOLIGNÉ et al., 2010). A figura 5B mostra como os probióticos podem reduzir a ativação de NF-kB através do reconhecimento de proteínas da camada S e consequentemente IL-8, limitando a resposta pró-inflamatória.

Além do mais, as proteínas da camada S podem interagir com células dendríticas (CDs) da lâmina própria e dos linfonodos mesentéricos (figura 5A). As CDs medeiam a diferenciação de linfócitos T naive, a depender do estímulo recebido, pode induzir a diferenciação em Th1, Th2, por exemplo. Por fim, a figura 5C mostra que as proteínas da camada S c ontribuem para o efeito anti-inflamatório dos probióticos no TGI, interagindo com células dendríticas por meio dos RRPs, que dão início à diferenciação de células Treg (DO CARMO et al., 2018a)

Diante das observações feitas nos estudos precedentes, a proteína SlpB da linhagem *P. freudenreichii* CIRM-BIA 129 apresenta uma forte correlação nas interações probióticas com o hospdeiro. Desse modo, é interessante investigar o comportamento da bactéria probiótica nocaute para o gene *SlpB*, em um modelo inflamatório de mucosite induzida por 5-Fluorouracil, e assim avaliar o papel desta proteína *in vivo*.

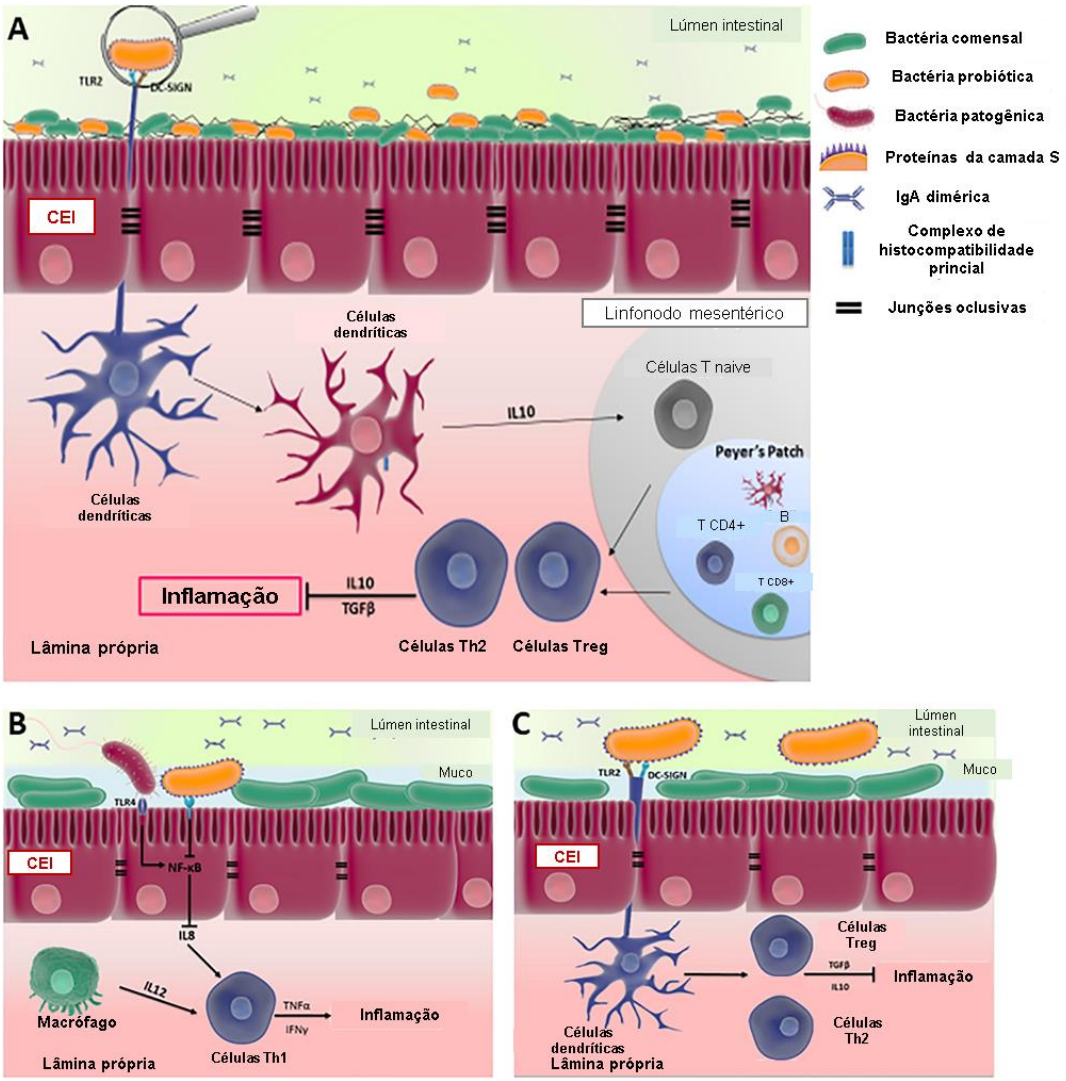


Figura 5. Comunicação entre bactérias probiotióticas e o hospedeiro, mediada por CEIs e células imunes, dentro dos tecidos linfóides associados à mucosa. (A) Visao geral da interação CDs com bactérias probióticas, que inicia uma tolerância ao induzir uma resposta anti-inflamatória Treg/Th2, enquanto a interação de CDs com pátogenos induz uma resposta pró-inflamatória Th1/Th17. (B) As proteínas da camada Sinibem a resposta pró-inflamatória das CEs, reduzindo NF-kB que é induzido por patógenos. (C) Proteínas da camada S são reconhecidas pelas CDs via DC-SING e TLR2, induzindo uma resposta de tolerância no linfonodo mesentérico. Esta representação esquemática é baseada principalmente em estudos *in vitro*. Adapatado de Do Carmo 2018.

Mucosite

A mucosite é caracterizada pela inflamação e danos nas células epiteliais do TGI, e é um dos efeitos colaterais mais graves do tratamento de câncer de cabeça e pescoço com radioterápicos e quimioterápicos, podendo causar bacteremia, desnutrição e uso de analségicos, o que leva ao prolongamento hospitalar do paciente, muitas vezes sendo necessário interromper o tratamento

do câncer devido à gravidade da doença. Os pacientes acometidos apresentam sintomas como, dor abdominal, diarréia grave, náuseas, vômitos e inchaços (VAN VLIET et al., 2010).

A radioterapia destrói as células cancerígenas, mas também prejudica a mucosa saudável, ocasionando desde lesões eritematosas até ulcerações purulentas na mucosa (LÔBO; MARTINS, 2009). Os quimioterápicos 5-Fluouracil (5- FU) e Irinotecano, os mais comuns utilizados em neoplasias de cabeça e pescoço (SANTOS et al., 2011), também causam danos graves a mucosa do TGI (LONGLEY et al., 2003). A associação da radioterapia com a quimioterapia durante o tratamento está diretamente relacionada a intensidade, incidência e duração da mucosite (SANTOS et al., 2011). O quimioterápico 5-FU, por exemplo, tem a capacidade de inibir a replicação do DNA e ao integrar ao RNA compromete vários processos celulares dependentes de proteínas (ABURJAILE et al., 2019). Além disso, a mucosite induzida pelo tratamento com 5-FU causa severa perda de peso, encurtamento das vilosidades intestinais e inflamação da mucosa, juntamente com maior predisposição para infecções secundárias locais e sistêmicas (CARVALHO et al., 2017b). Estima-se que 60 a 100% de pacientes fazendo de quimioterápicos desenvolvem mucosite (ABURJAILE et al., 2019).

Atualmente tratamentos utilizados para mucosite são anestésicos locais, analgésicos e antibióticos. Os anestésicos têm efeito de duração curta, além de dimimuir o paladar e o fluxo de saliva, o que ocorre na má ingestão de alimentos. O uso de antibióticos de amplo aspectro, também podem ocasionar alteração no paladar, dor abdominal e alterar a coloração dos dentes, além do mais, seu uso pode levar a um quadro de disbiose (ABURJAILE et al., 2019). Enquanto isso, os tratamentos disponíveis para a mucosite são pouco eficazes e proporcionam efeitos colaterais graves, e alguns estudos propuseram o uso de cepas bacterianas probióticas, como candidatos promissores ao tratamento da mucosite, uma vez que estes organismos podem levar a uma homeostase intestinal, através de seus mediadores anti-inflamatórios, e assim reduzir os danos causados pela mucosite (CARVALHO et al., 2017b).

Neste contexto, o probiótico *Propionibacterium freudenreichii* representa um bom alvo, uma vez que possui proteínas na camada S. Em estudo com camundongos BALB/c, *P. freudenreichii* foi capaz de prevenir a colite ulcerativa induzida por ácido trinitrobenzenossulfônico (TNBS- do inglês, trinitrobenzene sulfonic acid) de maneira linhagem dependente, reduzindo, por

exemplo, a perda de peso dos animais e o escore histopatológico (FOLIGNÉ et al., 2010). Sendo assim, torna-se relevante correlacionar o papel da proteína SLpB da camada S no modelo de mucosite, e assim compreender um pouco mais sobre os mecanismos anti-inflamatórios de *P. freudenreichii*.

JUSTIFICATIVA

As Neoplasias de Cabeça e Pescoço (NCP) aparecem em terceiro lugar como a causa mais comum de óbito por câncer no mundo (GALBIATTI et al., 2013), devido ao seu diagnóstico tardio. No Brasil estima-se aproximadamente 13.470 novos casos de câncer de cavidade oral por 100 mil habitantes (LÔBO; MARTINS, 2009). Durante o tratamento com quimioterápicos há o aparecimento de diversos efeitos colaterais como dermatite, xerostomia, disgeusia, disfagia, náuseas, vômitos e a mucosite gastrointestinal (ALBUQUERQUE et al., 2007; LÔBO et al., 2009). A mucosite, uma inflamação grave do trato GI da mucosa, afeta 80% dos pacientes em tratamento oncológico com base em quimioterapia e radiofármacos (CARVALHO et al., 2017b). Existem tratamentos para a mucosite, mas, além de gastos excessivos para os sistemas de saúde, eles apenas fornecem remissões entre recaídas ou causam efeitos colaterais que aumentam ainda mais os custos do tratamento.Dessa forma, existe um esforço ascendente nos grupos de pesquisa relativos ao uso de cepas probióticas para aliviar os sintomas da mucosite em modelos animais (CARVALHO et al., 2017b).

As bactérias probióticas do gênero Lactobacillus podem aliviar os sintomas de doenças inflamatórias (BIBILONI et al., 2005; GHOURI et al., 2014; SOOD et al., 2009) e recentemente foram descobertas propriedades anti-inflamatórias em cepas específicas usadas no processo de fermentação, como *Propionibacterium freudenreichii,* (FOLIGNÉ et al., 2010, 2013) *Lactobacillus delbrueckii,* (ROCHA et al., 2012; SANTOS ROCHA et al., 2014), *L. helveticus* (RONG et al., 2015; YAMASHITA et al., 2014) e *S. thermophilus* (MÉNARD et al., 2005; RODRÍGUEZ et al., 2010). Entretanto, o mecanismo desse efeito probiótico permanece desconhecido. Neste contexto, as proteínas da camada S, desempenham papéis fundamentais, como exemplo, estão evolvidas no processo de adesão bacteriana que é essencial para colonização, além de auxiliar na comunicação como hospedeiro, podendo contribuir para respostas anti-inflamatórias (DO CARMO et al., 2018a).

A principal esperança nesse contexto reside na perspectiva de potencializar tratamentos da mucosite, prolongar a remissão, melhorar a qualidade de vida e limitar os custos do tratamento. No entanto, são necessários mais estudos em modelos animais para determinar com precisão o

papel das bactérias e suas proteínas da camada S na modulação da inflamação, bem como os mecanismos envolvidos nesses efeitos terapêuticos.

OBJETIVO

Investigar o papel da proteina de superfície SlpB da linhagem *Propionibacterium freudenreichii* CIRM-BIA 129 em um modelo murino de mucosite induzida por 5-Fluorouracil.

CAPÍTULO 1

Probiotic *Propionibacterium freudenreichii* requires SlpB protein to mitigate mucositis induced by chemotherapy

Os resultados obtidos neste trabalho, bem como os materiais e métodos utilizados, serão apresentados em formato de artigo publicado, de acordo com as normas do Programa de Pós-raduação em Genética. O artigo foi publicado em 31 de dezembro de 2019, no periódico científico Oncotarget.

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Probiotic *Propionibacterium freudenreichii* requires SlpB protein to mitigate mucositis induced by chemotherapy

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ABSTRACT

Propionibacterium freudenreichii **CIRM-BIA 129 (*P. freudenreichii* wild type, WT) is a probiotic bacterium, which exerts immunomodulatory effects. This strain possesses extractable surface proteins, including SlpB, which are involved in anti- inflammatory effect and in adhesion to epithelial cells. We decided to investigate the impact of *slpB* gene mutation on immunomodulation *in vitro* and *in vivo*. In an *in vitro* assay, *P. freudenreichii* WT reduced expression of IL-8 ($p<0.0001$) and TNF-α ($p<0.0001$) cytokines in LPS-stimulated HT-29 cells. *P. freudenreichii* Δ*slpB*, lacking the SlpB protein, failed to do so. Subsequently, both strains were investigated *in vivo* in a 5-FU-induced mucositis mice model. Mucositis is a common side effect of cytotoxic chemotherapy with 5-FU, characterized by mucosal injury, inflammation, diarrhea, and weight loss. The WT strain prevented weight loss, reduced inflammation and consequently histopathological scores. Furthermore, it regulated key markers, including Claudin-1 (*cld1*, $p<0.0005$) and IL-17a (*Il17a*, $p<0.0001$) genes, as well as IL-12 ($p<0.0001$) and IL-1β ($p<0.0429$) cytokines levels. Mutant strain displayed opposite regulatory effect on *cld1* expression and on IL-12 levels. This work emphasizes the importance of SlpB in *P. freudenreichii* ability to reduce mucositis inflammation. It opens perspectives for the development of probiotic products to decrease side effects of chemotherapy using GRAS bacteria with immunomodulatory surface protein properties.**

INTRODUCTION

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

Probiotic effects of *P. freudenreichii* also include modulating the gut microbiota and the gut immune system [2]. In 2012, Cousin and collaborators demonstrated that dairy propionibacteria induce production of the regulatory cytokine IL-10 *ex vivo* in porcine colonic mucosa explants, and decrease production of proinflammatory cytokines, such as IL-8 and tumor necrosis factor-α (TNFα), in the gut mucosa of piglets after lipopolysaccharides (LPS) stimulation [4].

P. freudenreichii strains, isolated or associated with other probiotic bacteria, have also been shown to attenuate colitis induced by trinitrobenzene sulfonic acid (TNBS), in BALB/c mice [5]. *P. freudenreichii* was also reported to reduce intestinal and systemic proinflammatory alterations, caused by a high-fat diet, in a mice model [6]. Moreover, dairy propionibacteria strains may alleviate symptoms and stabilize the intestinal microbiota in patients with irritable bowel syndrome [7]. Altogether, these studies attracted attention on *P. freudenreichii* as a promising probiotic to potentiate the treatment of inflammatory diseases [1].

P. freudenreichii strain ITGP20, equivalent to CIRM-BIA 129 (*P. freudenreichii* wild type, WT), was used for the development of two experimental cheeses, one single-strain, and one in association with *Lactobacillus delbrueckii* subsp *lactis* CNRZ327. Both cheeses gave promising results and alleviated TNBS- induced colitis in mice [8, 9]. *P. freudenreichii* anti- inflammatory effect was further shown to depend on specific extractable surface proteins [10]. A surface proteomic analysis of *P. freudenreichii* extractable surface proteins identified three surface-exposed ones, designated SlpA, SlpB and SlpE [10]. Interestingly, the extraction of surface proteins from *P. freudenreichii* WT by guanidine hydrochloride suppresses its ability to induce anti-inflammatory cytokines in human PBMCs [10]. Moreover, in *P. freudenreichii* WT, Carmo and collaborators confirmed that the surface protein SlpB is involved in adhesion to cultured human intestinal epithelial cells HT-29 [11], and mutation of the *slpB* gene caused drastic changes in surface properties [12]. In this context, the great probiotic potential of *P. freudenreichii* in the context of inflammatory bowel diseases [8, 9], and the presence of a characterized extractable surface protein SlpB with immunomodulatory activity [13] led us to challenge this bacterium in another animal model involving inflammation: chemotherapy-induced mucositis [14].

Mucositis is a severe inflammation that affects the Alimentary Tract (AT) of individuals undergoing cancer treatment based on radiotherapy or chemotherapy, such as 5-Flourouracil (5-FU) [15]. Disease is characterized by pathological changes in the small bowel. This includes the presence of degenerative enterocytes, leukocyte infiltrate in the lamina propria, increased mucus production and degeneration of goblet cells, atrophy of villi, hypoplasia and apoptosis of intestinal crypts [16–18]. The side effects are characterized by mucosal injury, inflammation, diarrhea, and weight loss. The currently available treatments of mucositis (cryotherapy, growth factors, anti-inflammatory and antimicrobial agents) are poorly effective and may not be well tolerated. In this context, some studies have proposed the use of probiotic bacterial strains, as promising candidates in the treatment or prevention of inflammatory conditions such as mucositis [14, 19]. Clinical studies indicate a positive effect of selected lactobacilli in patients with mucositis [20, 21], while nothing is known about the effect of probiotic propionibacteria. Accordingly, the MASCC/ISOO (Association of Supportive Care in Cancer/International Society of Oral Oncology) clinical practice guidelines for the management of mucositis secondary to cancer therapy [22] recently added new guidelines, including one suggestion for probiotic agents containing *Lactobacillus* species for the prevention of chemotherapy and radiation- induced diarrhea in patients with pelvic malignancy as an adjuvant treatment. This comes in addition with the previous guidelines in favor of amifostine, octreotide, sucralfate enemas and sulfasalazine.

The aim of this study is to evaluate the probiotic ability of *P. freudenreichii* CIRM-BIA 129 to protect mice against inflammatory mucositis damages induced by 5-FU, and to further investigate the impact of *slpB* gene mutation on such a protection.

RESULTS

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

We investigated the anti-inflammatory potential of *P. freudenreichii* WT, and the impact of the mutation of the *slpB* gene on this potential. HT-29 cells, both in

the presence and in the absence of proinflammatory Lipopolysaccharide (LPS) from *E. coli*, were exposed to both strains, WT and mutant. We monitored changes in the relative expression of genes involved in the inflammatory process (Figure 6).

P. freudenreichii WT induced expression of *il10* (Figure 1A), with significant differences, ($p < 0.0001$), compared to control non-treated cells. The mutant *P.freudenreichii* Δ*slpB* failed to induce *il10* expression, by contrast with the WT strain. LPS did not change *il10* expression, with or without co-stimulation with

P. freudenreichii WT or *P. freudenreichii* Δ*slpB*. LPS strongly induced *il8* (Figure 6B). This induction was inhibited by the presence of *P. freudenreichii* WT, with a significant difference with LPS alone ($p < 0.0001$), yet not by the mutant *P. freudenreichii* Δ*slpB*. As a control,

P. freudenreichii WT did not induce *il8* expression, while the mutant *P. freudenreichii* Δ*slpB* did, when comparedto

P. freudenreichii WT or to untreated control. Accordingly, both LPS and mutant *P. freudenreichii* Δ*slpB* induced *ifna* ($p<0.001$), while *P. freudenreichii* WT did not (Figure 1C). After LPS stimulus, *ifna* expression was higher in the presence of the mutant than in the presence of the WT ($p<0.01$). The pro-inflammatory *tnfa* was induced by LPS, yet not by *P. freudenreichii*, neither WT, nor mutant (Figure 6D). The WT repressed LPS-mediated induction of *tnfa*, while the mutant did not.

We then monitored expression of TLR2, TLR4 and TLR9 receptors genes. LPS *per se* had no effect on *tlr2* expression (Figure 7A). Contrastingly, *P. freudenreichii* WT induced expression of *tlr2*, in comparison with untreated cells ($p < 0.01$). LPS completely suppressed this *tlr2* induction ($p < 0.01$). The mutant *P. freudenreichii* Δ*slpB* had no significant effect on *tlr2* expression.

Concerning *tlr4* receptor gene expression (Figure 7B), it was induced by LPS, compared to the untreated control ($p<0.05$). The mutant strain also triggered *tlr4* expression significantly, compared to control ($p<0.0021$), while *P. freudenreichii* WT did not. This WT strain repressed LPS-induced expression of *tlr4* ($p<0.0001$), while the mutant strain lost this ability.

Neither LPS, nor *P. freudenreichii* WT modified *tlr9* expression significantly, compared to the control (Figure 7C). In control conditions, *tlr9* expression was significantly lower in the presence of the mutant than in the presence of the WT. In LPS-inflamed cells, the opposite was observed with a higher expression in the presence of the mutant than in the presence of the WT ($p<0.001$).

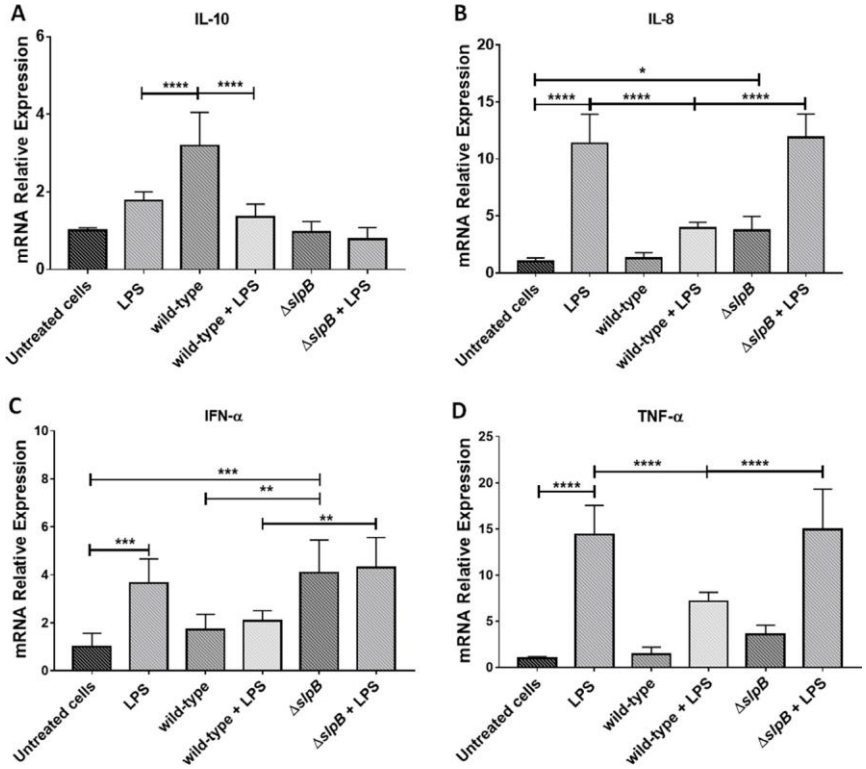

Figure 6: *Propionibacterium freudenreichii ΔslpB* mutant strain induces expression of pro-inflammatory cytokines in HT-29 cells. Relative expression of cytokine genes encoding IL-10 **(A),** IL-8 **(B)**, TNF-α **(C)** and IFN-α **(D)**, in HT-29 cells, stimulated by lipopolysaccharides (LPS), *P. freudenreichii* 129 WT, P. *freudenreichii 129*Δ*slpB*, or combinations thereof, was monitored by RT-PCR. Each cell treatment was done on 3 independent cultures (biological triplicates). Each quantification was done in triplicate (technical triplicates). The means and standard deviations are thus calculated from 9 values. Asterisks represent statistically significant differences between strains and were indicated as follows: *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$, and ****$p < 0.0001$.

Regarding tight junction gene *zo1* (Figure 7D) we did not find any significant differences between controls untreated cells and stimulated cells. In LPS-inflamed cells, *muc2* (Figure 7E) was more expressed in the presence of the mutant than in the presence of the WT ($p<0.0005$), while none of these strains affected its expression in control conditions.

We then extracted surface extractable proteins from *P. freudenreichii* WT using guanidine hydrochloride and purified the SlpB protein following diafiltration and size-exclusion chromatography of this extract. The purified protein was then used to stimulate cultivated HT-29 cells. As shown in Supplementary Figure 1, the purified SlpB protein induced *il10* gene expression in HT-29 cells ($p<0.0005$).

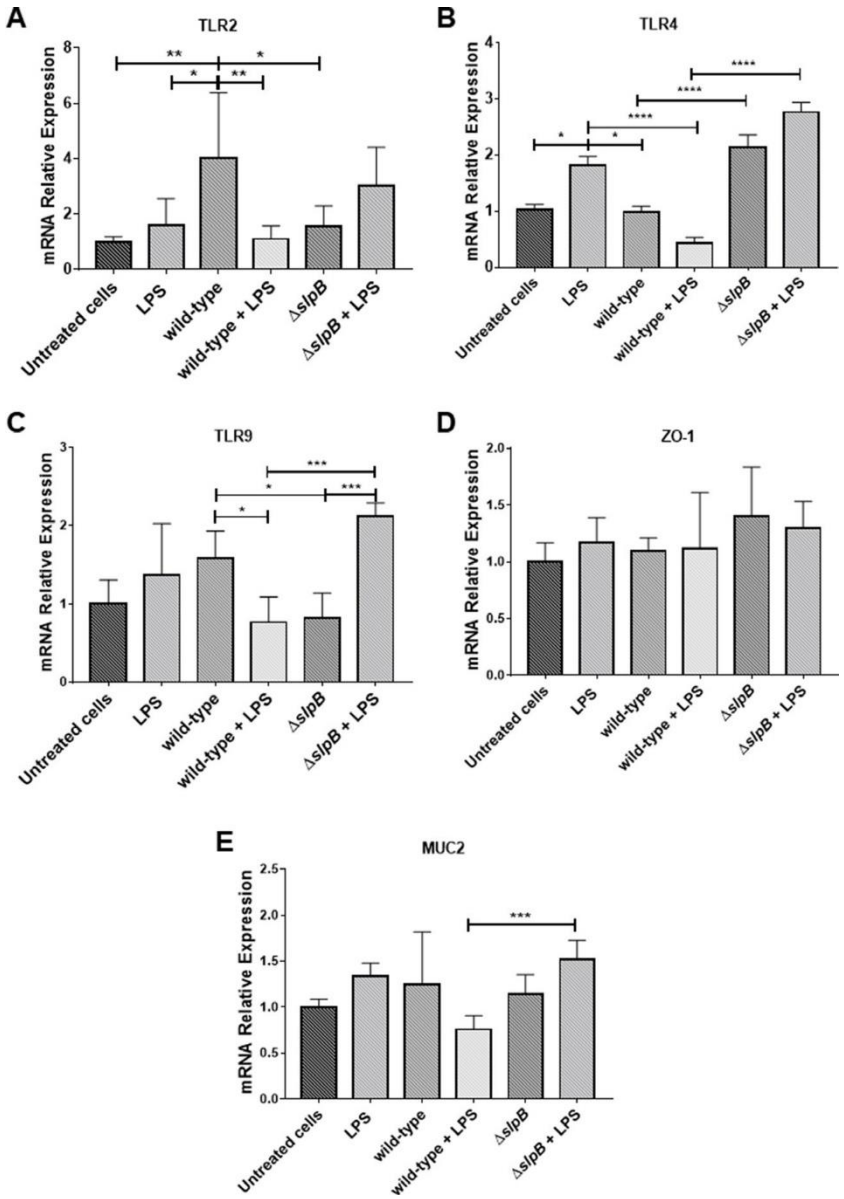

Figure 7: *Propionibacterium freudenreichii* WT strain modulates *in vitro* expression of in Toll-like receptors (TLRs) in HT-29 cells. Relative expression of genes encoding TLR2 **(A)**, TLR4 **(B)**, TLR9 **(C)**, ZO1 **(D)** and of MUC2 **(E)**, in HT-29 cells stimulated by lipopolysaccharides (LPS), *P. freudenreichii 129 WT*, *P. freudenreichii 129ΔslpB*, or combinations thereof, was monitored by RT-PCR. Each cell treatment was done on 3 independent cultures (biological triplicates). Each quantification was done in triplicate (technical triplicates). The means and standard deviations are thus calculated from 9 values. Asterisks represent statistically significant differences between strains and were indicated as follows: *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$, and ****$p < 0.0001$.

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

To further evaluate the protective role of probiotic administration in the context of mucositis, the effect of *P. freudenreichii* on the weight loss of mice after 5-FU administration was studied. The weight of mice belonging to the 8 experimental groups (see Supplementary Figure 2), was monitored before and after 5-FU administration (Figure 8). The body weight in grams as reported in Supplementary Figure 8. No weight difference was observed between groups before 5-FU injection. However, a reduction in weight was clearly observed after 5-FU injection ($p<0.0001$), when compared to untreated groups (Figure 3A). In this last group, *P. freudenreichii* WT strain consumption significantly limited weight loss: 13%±1.15 ($p<0.001$), compared to group receiving water: 20.94%±3.21 (Figure 8B). By contrast, the mutant *P. freudenreichii* Δ*slpB* failed to limit weight loss (19.34%±2.58), compared to *P. freudenreichii* WT group, $p<0.05$).

Although mucositis may affect the whole digestive tract, we examined damages at the ileal level for all mice, as a well-established readout. Regarding histopathological analysis, control groups injected with saline showed no significant difference in ileum mucosal pattern, whether they consumed water, YEL culture medium, or a YEL culture of *P. freudenreichii* WT (Figure 9A). However, consumption of the mutant strain *P. freudenreichii* Δ*slpB* increased histopathological score (Figure 4A), leading to epithelium flattening, areas of erosion and ulceration in the ileum mucosa (Figure 9B). Moreover, submucosa and muscular layer were thicker than in the other control groups (water, YEL and *P. freudenreichii* WT). In the submucosa layer, vessels were dilated, and edema intense. Some areas presented focal hemorrhage in the muscular layer. Furthermore, infiltration by immune cells, polymorphonuclear and mononuclear cells, was observed. These damages are further evidenced in Supplementary Figure 9.

Mucositis histopathological score translates the clear changes in the morphological structure of the ileum. This includes the intensity of cells infiltrate in the *lamina propria*, changes in mucosal architecture and presence of ulceration. In 5-FU-treated groups consuming water and YEL, this corresponded to increased submucosa and muscular layer, villi shortening, epithelium flattening, increased number of inflammatory cells, with diffuse mononuclear polymorphonuclear inflammatory infiltrate in the *lamina propria* (Figure 9B), when compared to the healthy ileum. Consumption of *P. freudenreichii* WT significantly reduced histopathological scores, compared to control 5-FU-treated groups (water and YEL) $p<0.001$ (Figure 9A). This corresponded to a reduction in infiltration, in ulceration and in alterations of the intestinal mucosa (Figure 9B, *P. freudenreichii* WT). The mutant *P.freudenreichii* Δ*slpB*, by contrast, failed to alleviate the tissue damages caused by 5-FU (Figure 9B).

In addition, we measured the height of the villi and the depth of the crypts (Figure 10). There was no significant difference between the control groups injected with saline. The 5-FU-treated groups showed a reduction in villus height. Consumption of *P. freudenreichii* WT partially restored this height ($p>0.0001$), compared to groups receiving either water or *P. freudenreichii* Δ*slpB* (Figure 10A). No significant difference was observed between the 5-FU-treated or non-5-FU-treated groups in terms of crypt depth (Figure 10B).

None of the treatments had a significant effect on the granular density of Paneth cells, in the absence of 5-FU. In the context of 5-FU-mucosistis, granular density was reduced. While the mutant strain had no effect on this reduction, both YEL and *P. freudenreichii* WT limited this reduction (Figure 10D).

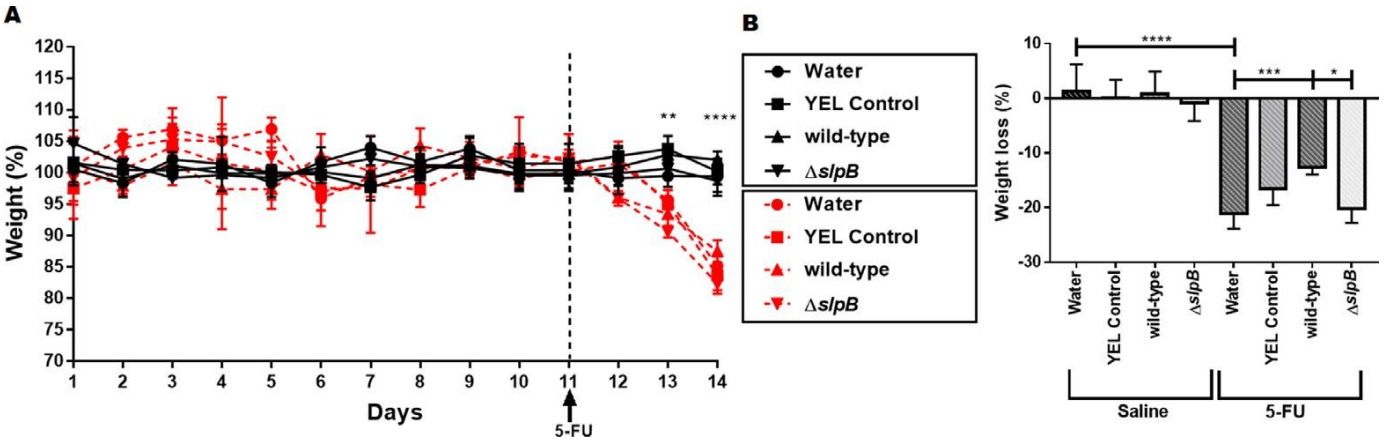

Figure 8: *Propionibacterium freudenreichii* WT strain prevents weight loss in 5-FU-treated mice. (A) Time-course of body weight for mice receiving YEL culture medium YEL (YEL control), the probiotic strain *P. freudenreichi*i 129 WT (wild-type), or the mutant strain *P. freudenreichi*i (Δ*slpB*). Black lines correspond to the groups injected with saline *i.p.* and red lines to the groups injected with 5-FU *i.p.* **(B)** Weight loss observed after 5-FU injection and differences across groups. The means and standard deviations are calculated from daily weighing of 18 animals per group (Three independent replicates with 6 animals per group). Asterisks represent statistically significant differences as follows: * $p<0.05$; ** $p<0.01$; and *** $p<0.001$.

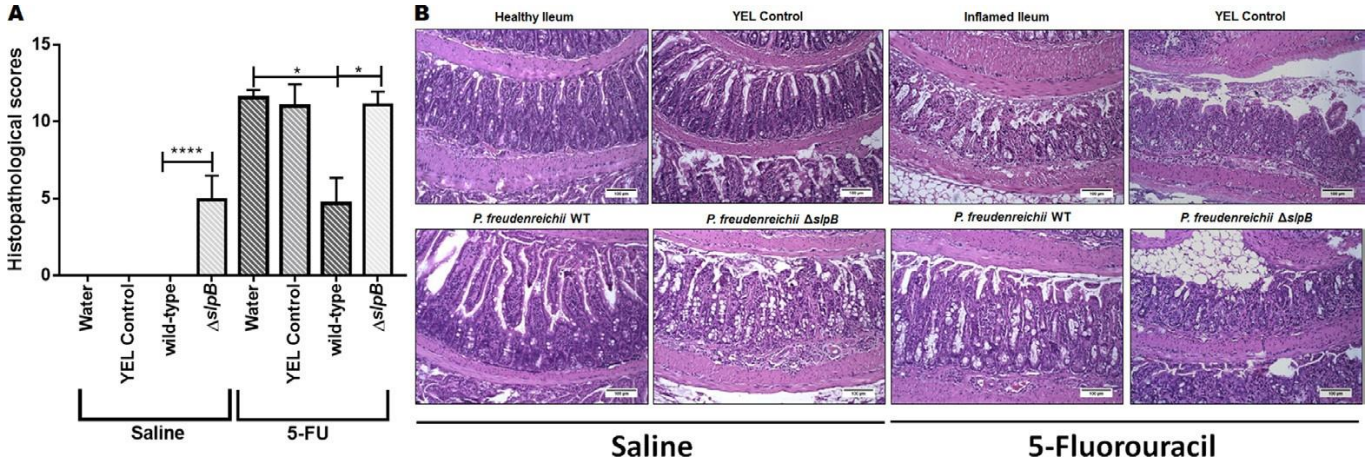

Figure 9: *Propionibacterium freudenreichii* WT strain alleviates mucosal damage in the ileum of 5-FU-treated mice while mutant strain *P. freudenreichii* Δ*slpB* causes inflammation in healthy mice. (A) Histopathological score obtained in healthy and 5-FU-treated mice. The means and standard deviations are calculated from ileum section of 18 animals per group (Three independent replicates with 6 animals per group). Asterisks represent statistically significant differences as follows: * p <0.05; ** p <0.01; *** p <0.001; **** p <0.0001. and **(B)** Representative images of H&E-staining of mice ileal mucosa, demonstrating histopathology. The image acquisition was done with objective magnification at 20x. Scale bar=100μm.

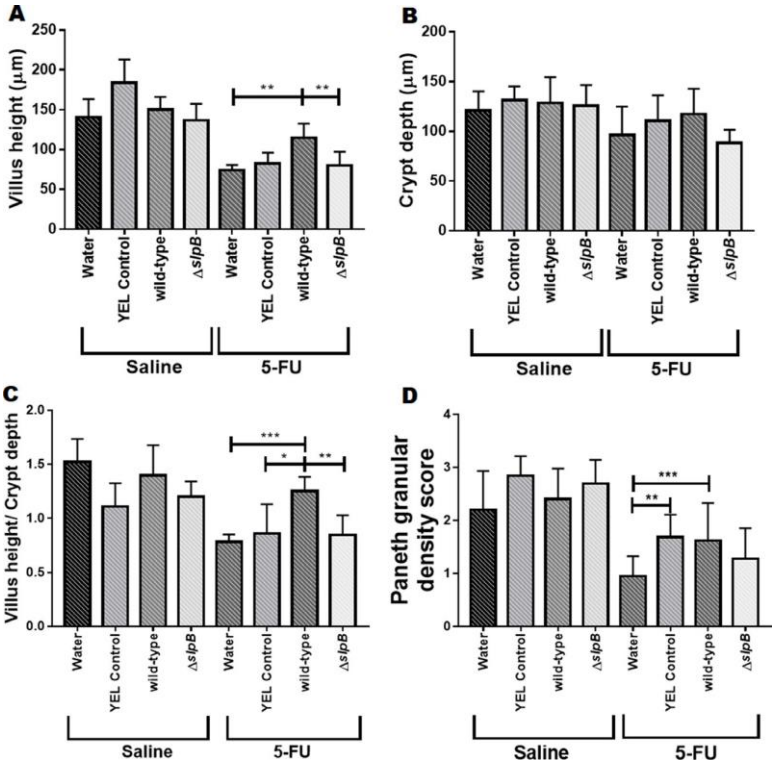

Figure 10: *Propionibacterium freudenreichii* WT strain protects villus architecture and Paneth cells secretory granules density during 5-FU-induced mucositis. Morphometric analysis of villus height **(A)**, crypt depth **(B)** and ratio villus height/crypt depth **(C)** of mice treated with culture media YEL (control), probiotic strain *P. freudenreichii WT* and mutant strain P. *freudenreichii ΔslpB* or without treatment (water) following 5-FU or saline administration. Microscopic morphometric analysis of Paneth cell secretory granules. **(D)** of mice treated with culture media YEL (control), probiotic strain *P. freudenreichii* WT and mutant strain *P. freudenreichii ΔslpB* or without treatment (water) following 5-FU or saline administration. Values were obtained using objective magnification at 40x by measuring ten random images of the ileum of mice. The means and standard deviations are calculated from ileum section of 18 animals per group (Three independent replicates with 6 animals per group). Asterisks represent statistically significant differences as follows: * p <0.05; ** p <0.01; *** p <0.001; **** p <0.0001.

Propionibacterium freudenreichii WT, yet not the *P. freudenreichii* Δ*slpB* mutant, prevents 5-FU-induced gut permeability

Intestinal permeability was evaluated following oral gavage of mice with radiolabeled diethylenetriaminepentaacetate (^{99m}Tc-DTPA) and subsequent quantification of radioactivity in the blood. There was no effect of the different treatments on permeability in control conditions (Figure 11, saline). However, as expected, 5-FU injection significantly increased intestinal permeability, as indicated by ^{99m}Tc-DTPA amounts in the mice blood, compared to the control groups (Figure 11, 5-FU water control group). However, the consumption of *P. freudenreichii* WT strain (5-FU wild- type group) significantly prevented ($P < 0.01$) the 5-FU- induced increase in intestinal permeability. By contrast, consumption of the *P. freudenreichii* mutant strain (5-FU Δ*slpB* group) failed to prevent this induced permeability ($p<0.2175$).

Propionibacterium freudenreichii Δ*slpB* mutant, yet not the WT strain, induces Th17 cells production in mice spleen

T-cell subpopulation was evaluated in mice spleen cells by using flow cytometry. As shown in Figure 12, consumption of the Δ*slpB* mutant strain increased significantly the frequency of both CD4+ ROR-γt+ T (Figure 12A) and CD4+FOXP3+ T (Figure 12B) cells subset in the mice spleen, when compared to water control groups ($p>0.01$). By contrast, consumption of the WT *P. freudenreichii* strain did not exerted significant effect on neither of these cell subsets in control conditions ($p<0.3602$ CD4+ ROR-γt+ T and $p<0.1613$ CD4+FOXP3+). In 5-FU-treated mice, consumption of the Δ*slpB* mutant resulted in a significant increase in CD4+FOXP3+ and CD4+ ROR-γt+ T cells ($p<0.0001$ and $p<0.0367$ respectively). However, the frequency of CD4+ ROR-γt+ T cells (Figure 12A) was different between WT and Δ*slpB* groups only in 5-FU-treated mice ($p<0.0023$), suggesting a boosting effect of Δ*slpB* strain upon inflammatory stimulation.

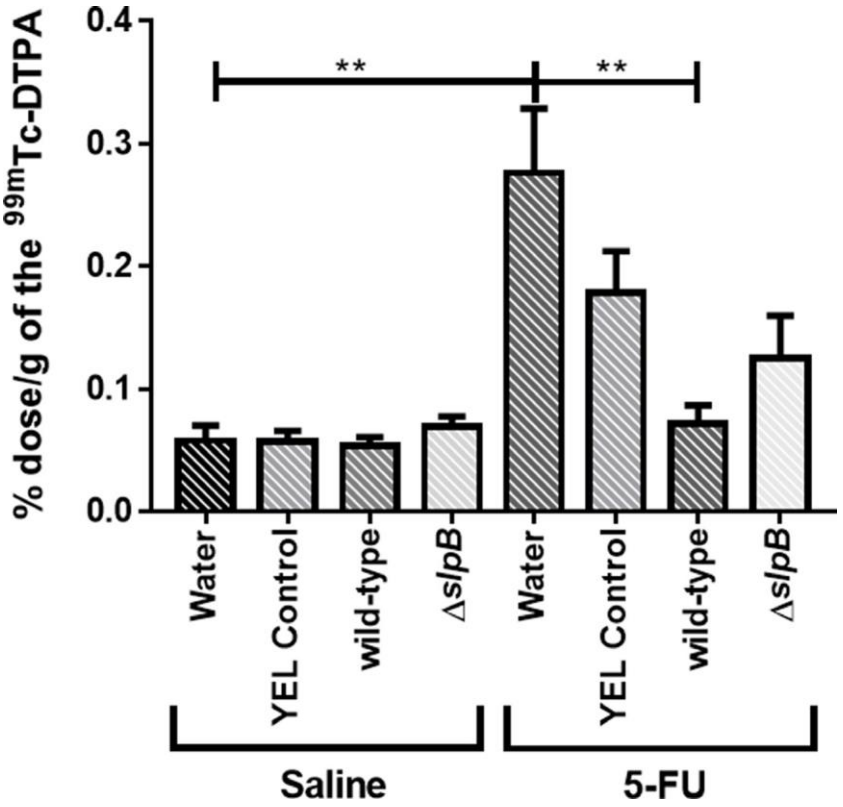

Figure 11: *Propionibacterium freudenreichii* WT consumption decreases intestinal permeability in 5-FU-treated mice. Intestinal permeability was measured 72 h after induction of mucositis by radioactivity determination of technetium-99 m (99mTc- DTPA) in mice blood. The means and standard deviations were calculated from one independent experiment for each of the five mice per group. Asterisks represent statistically significant differences between strains and were indicated as follows: *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$, and ****$p < 0.0001$.

Propionibacterium freudenreichii reduces secretory IgA production

Concentration of secretory IgA (SIgA) in the small intestine of mice, 5-FU-treated and non-5-FU-treated, was measured (Figure 13). Injection of 5-FU increased SIgA, in comparison with untreated mice. This induction was totally suppressed by consumption of both strains. Indeed, both *P. freudenreichii* WT and *P. freudenreichii* Δ*slpB* decreased the amount of SIgA in the 5-FU-treated and non-5-FU-treated groups.

Propionibacterium freudenreichii WT and ΔslpB mutant strains differentially modulate gene expression in the mice ileum

In healthy mice (injected with saline) and in mice injected with 5-FU, no significant difference was found regarding expression of *muc2* gene (Figure 14A). Consumption of *P. freudenreichii* WT significantly increased cld1 gene expression levels in mice injected with 5-FU, compared to water ($p<0.001$), YEL ($p<0.05$) or the mutant *P. freudenreichii* Δ*slpB* ($p<0.001$) (Figure 14B).

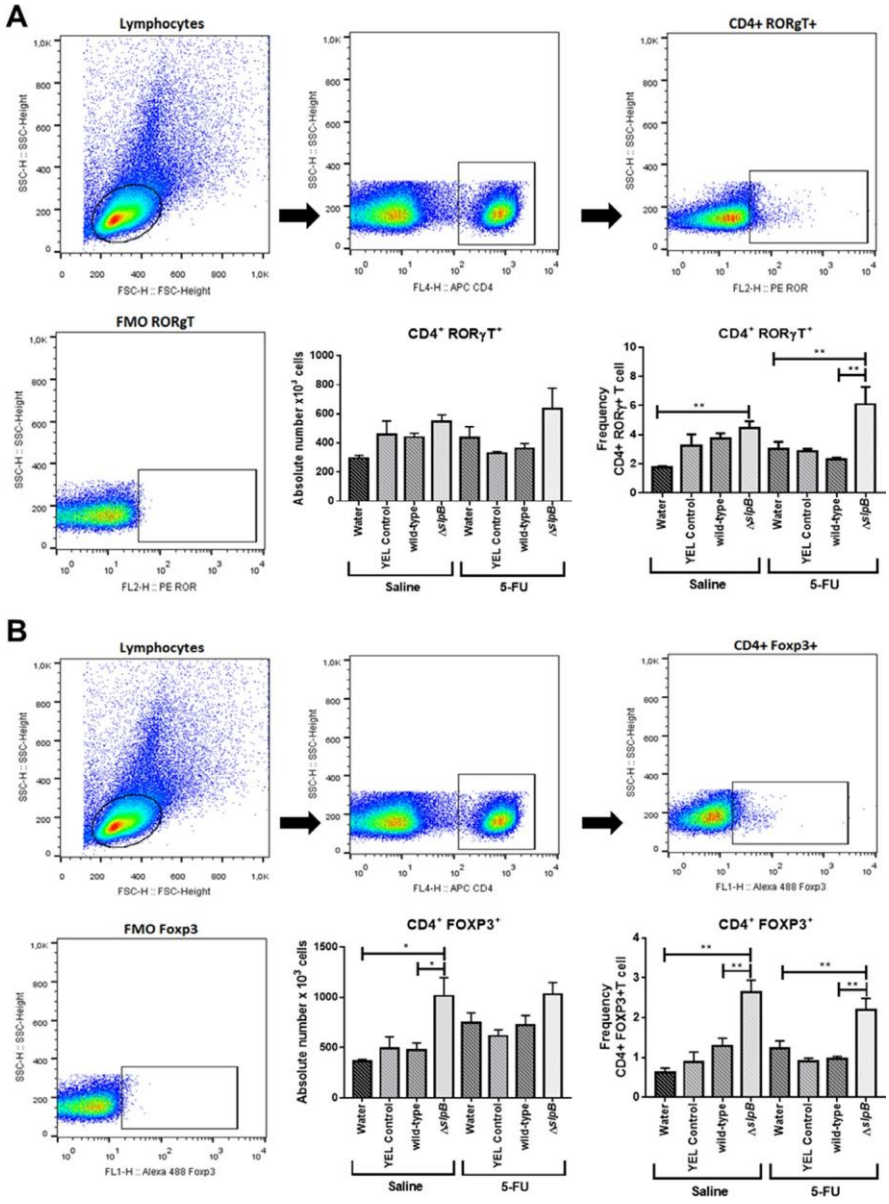

Figure 12: *Propionibacterium freudenreichii ΔslpB* mutant strain induces T lymphocyte production in mice spleen after 5-FU-induced mucositis. T cells were isolated from mice spleen, and the frequencies of **(A)** CD4+Foxp3+ and **(B)** CD4+ RORγt+ T cells, as frequency (percentage) of CD4+ T cells, were assessed by flow cytometry. The first presented plot represents the gating strategy, based on forward and side scatter, selecting splenocytes as a function of cell size and granularity. Among these, the second presented plot shows gating based on anti-CD4 labeling, selecting T cells. Then, the third presented plot shows representative gated of populations of RORγT **(A)** and FOXP3 **(B)** positive T cells. The fluorescence minus one (FMO) control is shown in the fourth plot. The means and standard deviations were calculated from one independent experiment for each of the five mice per group. Asterisks represent statistically significant differences between strains and were indicated as follows: *p < 0.05; **p < 0.01; ***p < 0.001, and ****p < 0.0001.

Consumption of *P. freudenreichii* Δ*slpB* failed to increase *cld1* gene expression. Expression levels of *zo1* only showed significant differences ($p<0.001$) between YEL and *P. freudenreichii* WT in heathy mice (saline) (Figure 14C). Expression of Occludin (*ocln*) was monitored and no significant difference was found (Figure 14D). Expression of *iNOS* (inducible nitric oxide synthase) was poorly affected, except a trend towards enhanced expression as a result of consumption of the mutant strain, compared to groups receiving water ($p<0.05$) and *P. freudenreichii* WT ($p<0.05$), in healthy mice (Saline) (Figure 14E). In addition, consumption of the mutant strain induced expression of *Il17* in healthy mice ($p<0.0001$) (Figure 14F). In 5-FU- treated mice receiving water, 5-FU triggered a significant induction of *Il17*, which was mitigated by consumption of YEL medium YEL ($p<0.01$) or of the *P.* freudenreichii WT culture ($p<0.01$).

Propionibacterium freudenreichii WT and ΔslpB mutant strains differentially modulate cytokines production in the mice ileum

Cytokines were quantified by ELISA in the intestinal mucosa of the 5-FU-treated (5-FU) and non-5-FU-treated groups (Saline) (Figure 15). In mucositis conditions (5-FU), the disease drastically induced all the measured cytokines. Consumption of *P. freudenreichii* WT enhanced the ileal IL-10 concentration in healthy mice ($p<0.05$). However, no significant effect on IL-10 levels was found in mucositis mice (Figure 15A). IL-12 was enhanced by 5-FU and this 5-FU-induction of IL12 was prevented by consumption of *P. freudenreichii* WT ($p<0.001$), when compared to the YEL medium control, while the mutant failed to do so (Figure 15B). None of the treatments affected IL-1β concentration in control healthy mice. In mucositis mice, 5-FU caused an increase in IL-1β concentration. Consumption of *P. freudenreichii* WT however reduced this 5-FU-induction of IL-1β in the context of mucositis ($p<0.01$, Figure 15C). Finally, consumption of *P. freudenreichii* WT significantly enhanced the ratio of IL-10 to IL-12 ($p<0.01$), while the mutant failed to do so (Figure 15D).

DISCUSSION

The probiotic potential of *Propionibacterium freudenreichii* is based on both the release of beneficial metabolites [1] and on key surface proteins responsible for interactions with the host [12, 13, 23–24]. S-layer

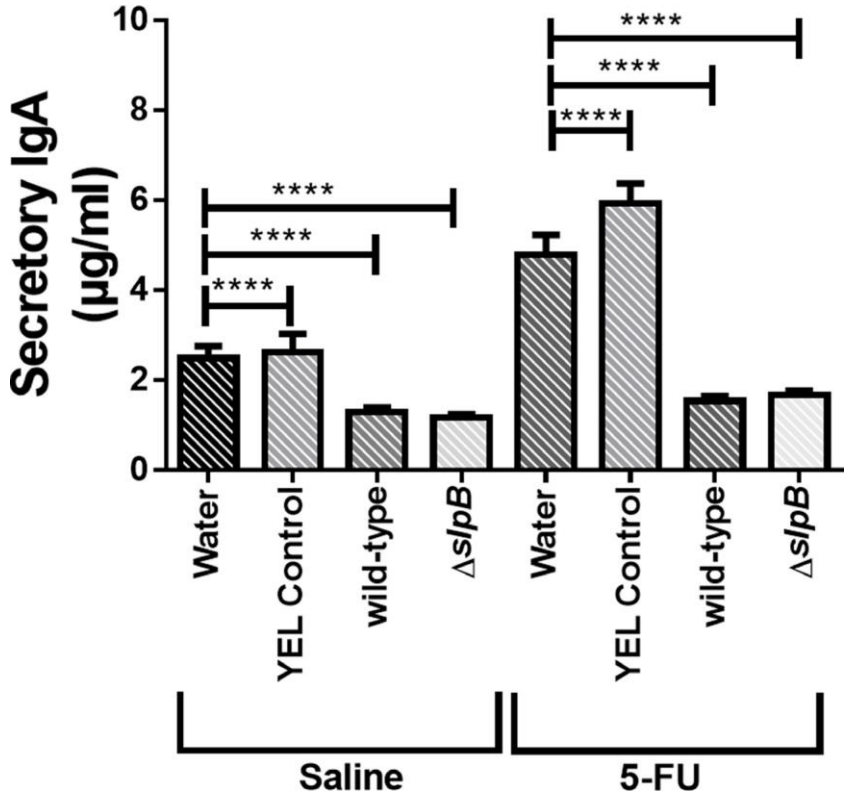

Figure 13: Secretory immunoglobulin A (IgA) in intestinal small bowel content. Quantification of immunoglobulin A secretion (sIgA) in the small intestine of healthy or 5-FU-treated mice. The means and standard deviations are calculated from ileum section of 18 animals per group (Three independent replicates with 6 animals per group). Asterisks represent statistically significant differences as follows: * $p<0.05$; ** $p<0.01$; *** $p<0.001$; **** $p<0.000$.

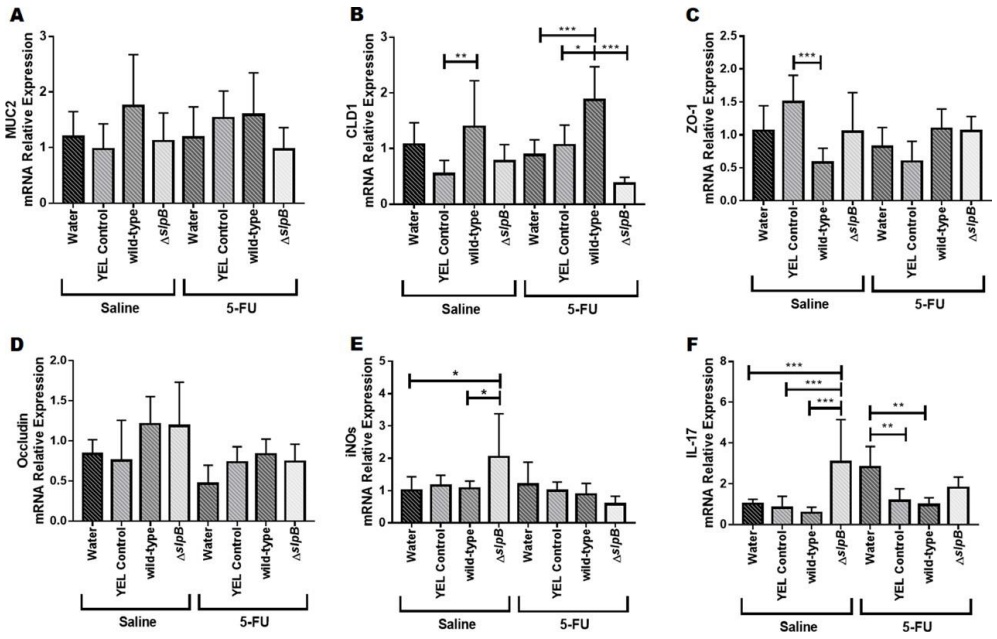

Figure 14: *Propionibacterium freudenreichii* ΔslpB induces expression of IL-17 and of inducible NOS (iNOS) in healthy mice. mRNA relative expression of genes **(A)** *muc2,* **(B)** *cld1*, **(C)** *zo1,* **(D)** *ocln,* **(E)** *iNOS,* and **(F)** *Il17* in mice treated with culture media YEL (control), probiotic strain *P. freudenreichii* WT and mutant strain *P. freudenreichii ΔslpB* or without treatment (water) following 5-FU or saline administration. Expression levels was monitored by RT-PCR. The means and standard deviations are calculated from 6 animals per group from 3 independent replicates and each quantification was done in triplicate (technical triplicates). Asterisks represent statistically significant differences between strains and were indicated as follows: *p < 0.05; **p < 0.01; ***p < 0.001, and ****p < 0.0001.

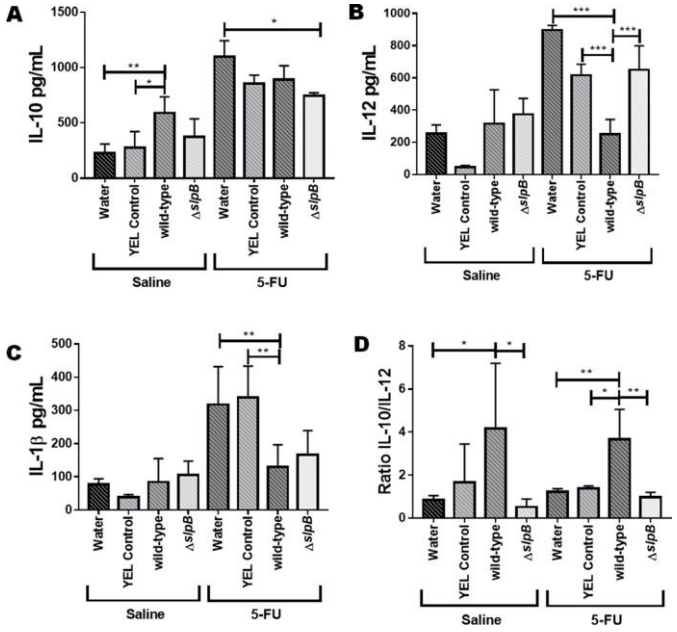

Figure 15: *Propionibacterium freudenreichii* WT strain reduces the pro-inflammatory cytokine IL-12 production during 5-FU-induced mucositis. The secreted levels of **(A)** IL-10, **(B)** IL-12, **(C)** IL-1β, and **(D)** IL-10/IL-12 ratio were determined in the supernatant of homogenized mice ileum tissue using ELISA. Mice consumed water, YEL culture medium (YEL control), a YEL culture of the probiotic strain *P. freudenreichii* WT (wild-type) or a YEL culture of the mutant strain *(ΔslpB).* The means and standard deviations are calculated from 6 animals per group from 3 independent replicates and each quantification was done in triplicate (technical triplicates). Asterisks represent statistically significant differences between strains and were indicated as follows: *p < 0.05; **p < 0.01; ***p < 0.001, and ****p < 0.000

proteins form a non-covalently anchored surface-exposed proteinaceous network [25, 26]. They are involved in various processes, such as mediation of the cross-talk with the host [23], which includes immunomodulation
[10] and adhesion to host cells in *P. freudenreichii* [11]. Immunomodulation and adhesion, two processes tightly linked [27], require S-layer-associated proteins in *Lactobacilus salivarius* REN [28] and in *Lactobacillus acidophilus* NCFM [29, 30]. In *P.freudenreichii* WT, adhesion to intestinal human cells requires SlpB [11]. Moreover, mutation of the *slpB* gene caused pleotropic effects, impairing surface properties, adhesion and stress tolerance [12]. We therefore investigated the impact of this mutation in the context of human intestinal epithelial cells inflammation.

The present report confirms a key role of SlpB in *P. freudenreichii* probiotic potential. In HT-29 cells, *P. freudenreichii* WT has the ability to induce the release of IL-10, and to reduce that of IL-8 [31]. The ability to induce IL-10 plays a crucial role in the prevention of damage during inflammatory processes [32, 33]. *P.freudenreichii* Δ*slpB* strain was shown here to lose this ability to induce IL-10, probably because 1) of the major modifications of the cell surface properties [12] and 2) of reduced adhesion [11]. Accordingly, purified *P. freudenreichii* SlpB protein induced IL-10 expression in HT-29 cells. Indeed, strains of *P. freudenreichii* which express SlpB induce IL-10 in PBMCs, while those which produce high amounts of SlpA fail to do so [13].

The ability to limit induction of IL-8 is also important in the anti-inflammatory effect, because IL-8 triggers the recruitment of neutrophils in addition to further pro-inflammatory signals in the *lamina propria* [34, 35]. SlpB may also be responsible for the ability to down-regulate IL-8, an anti-inflammatory property shared by several probiotics [36]. Interestingly, the mutant strain, devoid of SlpB, lost the ability to regulate the expression of IL-8, which may decrease their anti-inflammatory potential. This is also observed concerning TNF-α, a pro- inflammatory cytokine, which controls the production of another inflammatory mediator. *P. freudenreichii* WT repressed TNF-α expression in LPS-stimulated HT-29, as reported for other probiotic bacteria or their culture supernatants [37, 38]. Again, the mutant strain *P. freudenreichii* Δ*slpB* was unable to inhibit induction of proinflammatory cytokines.

Extractable surface proteins, including Slps, were already shown to mediate immunomodulation in other probiotic bacteria, including *L. helveticus* MIMLh5 [39],
L. acidophilus ATCC4356 [40], *L. acidophilus* NCFM
[41] and *L. acidophilus* NCK2187 [42]. Other extractable surface proteins are also involved in *L. acidophilus* NCFM and mutation of the corresponding genes can drastically affect immunomodulatory properties [43, 44]. Slps of *L. helveticus* NS8 decreased IL12 induction by LPS in mouse macrophage cell line RAW264.7 [45]. Contrastingly, *L. helveticus* MIMLh5 and its SlpA stimulated the innate immune system by inducing proinflammatory mediators such as TNFα and cyclooxygenase 2 (COX-2) in the human macrophage cell line U937 via TLR2 recognition [39]. Similarly, *L. brevis* Slps induce TNFα in monocyte- derived dendritic cells (moDC) [46].

Toll-like receptors participate in the host cells pro-inflammatory response and play a key role in the regulation of the balance between the Th1 and Th2 type of response [47]. Probiotics' anti-inflammatory effects may include modulation of Toll-like receptors (TLRs) [48, 49]. Probiotic bacteria may indeed modulate TLRs in a strain- dependent manner. *P. freudenreichii* WT was shown here to enhance TLR2 expression in HT-29 cells, suggesting enhanced reaction towards bacterial LTA, while the mutant failed to do so. In the presence of LPS, no significant effect of *P. freudenreichii*, neither WT nor mutant, was observed regarding TLR2 and TLR9 expression. The pivotal role of the SlpB protein is most evident when monitoring expression of the *tlr4* gene. This expression was shown here to be enhanced by LPS stimulation as previously reported [50]. This induction was totally suppressed by the *P. freudenreichii* wild-type strain, showing another key probiotic ability to impair a pro-inflammatory response machinery. By contrast, LPS-induction of the *tlr4* gene was not repressed by the mutant strain.

Probiotic bacteria may modulate TLRs in a strain- dependent manner. As an example, both *L. plantarum* BFE 1685 and *L. rhamnosus* GG up-regulate TLR2 and TLR9 expression in HT-29 cells [51]. *L. paracasei* F19 strongly induces TLR2 and *E. Coli* K4-induced TLR4 [52]. *L. rhamnosus* GG limits the inflammatory response of porcine intestinal epithelial cells exposed to LPS, by modulating TLR expressions and inhibiting MAPK and NF-κB signaling [53]. By contrast, *Lactobacillus rhamnosus* LGG decreases the expression of TLR2 and TLR-9 in HT-29 cells exposed to *Salmonella* or ot LPS [51, 53]. Each probiotic strain has its specific properties and may specifically modulate expression of pro and anti-inflammatory cytokines and receptors in HIECs.

Considering the above-mentioned *in vitro* regulation of key cytokines and receptors by *P.freudenreichii* WT, expressing SlpB, we decided to exploit such immunomodulatory properties, and to address the importance of SlpB, in a preclinical relevant mucositis model. Mucositis is an inflammatory disease that significantly affects cancer patients undergoing antineoplastic chemotherapy such as 5-fluorouracil (5- FU). Available treatments for mucositis have limitations and probiotics are considered in this context [19].

P. freudenreichii WT was able here to reduce the tissue damages caused by 5-FU, to preserve villi height, to limit *lamina propria* infiltration by inflammatory cells and weight loss, in agreement with other studies showing the efficacy of other probiotics [54–56]. We observed such damages at the ileal level, although mucositis may

affect the whole digestive tract, as it is a well-established mucositis readout in mice [54–56]. The observed decrease in SIgA levels can be correlated with the protected integrity of the epithelial barrier and consequently protection against pathogens [57–59]. However, mutation of the slpB gene did not change the effect of propionibacteria on sIgA levels. Levels of SIgA increased, when inflammatory stimuli threatened the integrity of the mucosa [58]. They decreased in the group treated with *P. freudenreichii* WT, suggesting that inflammation was contained.

To further evaluate systemic inflammation, we analyzed CD4+T cells expressing FOXP3+ and RORγt+ in mice spleens. In accordance with histology and with cytokines modulation, we observed an amplification of the immune process caused by Δ*slpB* mutant strain, with increased frequencies of both CD4+Foxp3+ and CD4+ROR-γt+ cells in spleens of healthy and 5-FU- treated mice. Regulatory T cells (Tregs) can suppress a wide range of immune cells and play a key role in the maintenance of homeostasis [60], as well as in physiological and pathological immune responses [61]. In our study, the increase in frequency of Tregs might be a compensatory mechanism to counterbalance the cells and mediators of inflammation regarding innate and adaptive cells caused by Δ*slpB* mutant strain.

It is known that CD4+ ROR-γt+ cells are involved in the pathology of inflammatory bowel diseases such as ulcerative colitis and Crohn's disease [62, 63]. It is plausible that, after Δ*slpB* mutant strain consumption, naïve T cells start expressing RORγt, polarizing towards a proinflammatory response via the Th17/IL-17A pathway. IL-17A can modulate the activation and recruitment of neutrophils in the ileum, which is in accordance with the increased histological score and the enhanced expression of the *Il-17a* gene [64]. In addition, the mutant strain enhanced a subpopulation of CD4+Foxp3+ Treg cells. Regulatory CD4+ T cells expressing Foxp3 are very abundant throughout the intestinal mucosa and their expansion seems to be a homeostatic default mechanism triggered to control the pro-inflammatory Th17 effector cell response triggered by Δ*slpB* mutant strain, as already described in humans [65, 66].

Mucositis induced by 5-FU is linked to an inflammatory process and significantly alters intestinal permeability [67]. We report here that consumption of *Propionibacterium freudenreichii* WT prevented this alteration, a potential that may prevent the exposition of the host to intestinal toxins and bacteria caused by intestinal permeabilization and thus to systemic inflammation [68]. Induced mucositis is accordingly linked to a reduction in the expression of the Claudin-1 gene [69]. Indeed, the structure of tight junctions is an essential factor of the integrity of the epithelial barrier [70, 71]. No significant effect of the different treatments was observed, regarding the expression of genes encoding ZO-1 and Muc2 proteins. However, treatment with probiotic *P. freudenreichii* WT increased *cld1* gene expression in the 5-FU-treated group. Claudin-1 is involved in tight junctions formation and in epithelial cells intercellular adhesion [72]. *P. freudenreichii* WT consumption reduced ileal level of IL-12, which was elevated in the mucositis model [73]. Healthy mice (without 5-FU) consuming *P.freudenreichii* WT (group 1) exhibited enhanced levels of immunomodulatory IL-10, a marker of anti-inflammatory effect also reported for *Lactobacillus acidophilus* [74]. They exhibited a higher IL-10/IL-12 ratio, which was proposed as an anti-inflammatory probiotic effect marker [75]. Moreover, this ratio was also increased in mice with mucositis consuming *P. freudenreichii* (group 7). It is plausible that IL-10 played a key role in *P. freudenreichii* WT containing the inflammatory process driven by 5-FU, given the importance of this cytokine in gut homeostasis. Mice receiving *P. freudenreichii* Δ*slpB* exhibited a histopathological score different from those receiving the probiotic strain *P. freudenreichii* WT, but closer to that of mucositis control groups. The mutant lost the ability to maintain architectural integrity of the ileum mucosa. In addition to losing its anti-inflammatory capacity, the mutant strain induced inflammation in the ileum of healthy mice (not receiving 5-FU), in accordance with its inefficacy to protect from mucositis. It failed to reduce the abrupt weight loss caused by 5-FU, in contrast with the probiotic strain. The *cld1* expression, decreased in mucositis, was restored by consumption of the probiotic *P. freudenreichii* WT, but not by the mutant, in accordance with its inability to restore gut permeability. Accordingly, extractable surface proteins are associated with the induction of the expression of tight junctions gene encoding Claudin-1, Occludin, JAM-1, and ZO-1 by other probiotics [23]. Moreover, consumption of the mutant increased ileal expression of iNOS, inducible nitric oxide synthase, an enzyme responsible for the generation of cytotoxic and immunoregulatory free radical NO, which is linked to inflammatory processes [76]. Its expression is triggered by IL-17, a pro-inflammatory T cell cytokine [77]. The ileum contains a great number of IL-17 producing cells [78]. Thus, the induction of both iNOS and IL-17 by the mutant may contribute to the onset of inflammation in healthy mice and to its inability to alleviate mucositis induced by 5-FU.

MATERIALS AND METHODS

Bacterial strains and culture conditions

The wild-type strain *P. freudenreichii* strain ITGP20, equivalent to CIRM-BIA 129 (*P. freudenreichii* WT), was provided by the CNIEL (Centre National Interprofessionnel de l'Economie Laitière) and maintained by the CIRM-BIA (International Centre for Microbial Resources – Food Associated Bacteria). This strain, as well as the genetically modified *P. freudenreichii* Δ*slpB*

strain (*P. freudenreichii* Δ*slpB*) [11], were grown at 30°C in Yeast Extract Lactate (YEL) broth [79]. For the *P. freudenreichii* Δ*slpB* mutant, YEL culture media were supplemented with chloramphenicol (10 μg.mL^{-1}). The growth of *P. freudenreichii* was monitored by measuring the optical density at 650 nm (OD_{650nm}), as well as by counting colony-forming units (CFUs) in YEL containing 1.5% agar, according to Malik and collaborators [79]. Propionibacteria were then used for animal feeding, or harvested in stationary phase (76 h, 2 x 10^9 CFU.mL^{-1}, determined by plate counts) by centrifugation (8,000 × g, 10 min, 4°C) and washed in PBS, prior to surface protein extraction, or to HT-29 cells challenging.

Purification of SlpB proteins

A one-litre culture of *P. freudenreichii* strain ITGP20 was prepared as indicated above. Propionibacteria, washed in PBS, were centrifuged (8,000 × g, 10 min, 4°C) and resuspended in 5 M Guanidine hydrochloride (Sigma- Aldrich, St. Louis, MO, USA). After 15 min of incubation, 50°C, bacteria were removed by centrifugation (8,000 × g, 10 min, 20°C). The resulting Guanidine hydrochloride extract contains surface extractable proteins as previously described [10]. The Guanidine hydrochloride extract was concentrated, with elimination of molecules below 30 kDa, by diafiltration using vivaspin 20 30,000 MWCO cells (Sartorius, Stonehouse, Gloucestershire, United Kingdom) and following the provided instructions. The concentrated proteins were washed and recovered in PBS buffer using the same diafiltration cells. The resulting extract was separated by FPLC size-exclusion chromatography. An ÄKTA Purifier 10 system (Amersham Biosciences, Uppsala, Sweden) operating at 0.5 mL/min, equipped with a Superdex 75 10/300 column and a Monitor UV- 900 detector operating at 280 nm was used. PBS buffer was used as a mobile phase. The chromatogram and corresponding electrophoretic analysis are shown in Supplementary Figure 1 and Supplementary Figure 5. SlpB, eluted at 11 mL, was then used to stimulate HT-29 cells as described below.

HT-29 cell challenging

HT-29 cells were routinely grown in T-25 flasks in complete medium DMEMc containing (Dominique Dutscher, Brumath, France) 10% (v/v) fetal calf serum (PAN-Biotech GmbH, Aidenbach, Germany), 100 U/mL penicillin and 100 μg/mL streptomycin sulphate) at 37°C with 5% CO_2. Trypsin (0.05%)/EDTA (0.2%) (Gibco, Saint Aubin, France) was used to release adherent cells for subculturing. For the experiment, 10^5 cells were seeded in 12-well plates (1 ml of medium per well) and the growth medium was changed every 2 days. HT-29 cells were grown until complete confluence, 1.10^6 cells per well in 1 ml volume. Prior to challenging cells, complete medium was replaced with antibiotic-free medium for 3 hours. HT- 29 cells were subjected to the different treatments: 7h with 100 ng/mL of Lipopolysaccharide (LPS from E. coli 0111: B4, Sigma), or 7h with *P. freudenreichii* WT, or 7h with *P. freudenreichii* Δ*slpB*. In parallel, cells were also subjected to co-treatments for 7 h: LPS in combination with *P. freudenreichii* WT or *P. freudenreichii* Δ*slpB*, MOI 10 (1 x 10^7 CFU per well in 1 ml volume). The percentage of HT- 29 cells viability after the different stimulation conditions was checked by trypan blue staining and the cells viability was not affected.

HT-29 cell 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). Real-time PCR reactions were set up in CFX96 real- time system (Bio-Rad, Marne la Coquette, France). Each PCR reaction was performed in a 16 μL reaction mixture containing 5 μL SYBR Green PCR Master Mix (Biorad), 5 μL of properly diluted cDNA (350 ng of cDNA for all genes), 3 μL mixture of each primer (*actβ* and *gapdh* as housekeeping genes, and *il8*, *tnfa*, *il10*, *ifna*, *muc2*, *zo1*, *tlr9*, *tlr4* and *tlr2*) 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 using the following program: 3 minutes at 95°C and 40 cycles of 2 steps consisting of 5 seconds at 95°C and 30 seconds at 60°C. The relative quantification of the mRNA levels of the target genes was determined using CFX Manager Software. The transcript level was normalized to the transcript level of housekeeping genes encoding β-actin (*actβ*) and GAPDH (*gapdh*). 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). The sequences of primers used in this study are listed in Table 1. We followed key genes previously reported to translate immunomodulatory response to probiotics in LPS-stimulated intestinal epithelial cells [31, 37, 51, 53]. Each cell treatment was done on 3 independent cultures (biological triplicates). Each quantification was done in triplicate (technical triplicates). The means and standard deviations are thus calculated from 9 values.

Evaluation of probiotic properties of *P. freudenreichii* WT and *P. freudenreichii* Δ*slpB* to prevent mucositis

Animals

Conventional female BALB/c mice, between 6 and 8 weeks of age, were obtained at Federal University of Minas Gerais (UFMG–Belo Horizonte, Brazil). These

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

GENE	PRIMER	SEQUENCE (5′→3′)	PRODUCT SIZE (bp)	Reference
ActB	β-actinF	TGG CTG GGT GTT GAA GGT CT	238	
	β-actinR	AGC ACG GCA TCG TCA CCA ACT		
Gapdh	GapdhF	CAA CGA CCA CTT TGT CAA GC	140	
	GapdhR	TTC CTC TTG TGC TCT TGC TG		
Il8	IL-8F	TGG CTC TCT TGG CAG CCT TC	238	
	IL-8R	TGC ACC CAG TTT TCC TTG GG		
Tnfa	TNFαF	AGC CCA TGT TGT AGC AAA CC	134	[36]
	TNFαR	TGA GGT ACA GGC CCT CTG AT		
Il10	IL-10F	AAA GAA GGC ATG CAC AGC TC	132	
	IL-10R	AAG CAT GTT AGG CAG GTT GC		
Ifna	IFNαF	CTG AAA CCA TCC CTG TCC TC	147	
	IFNαR	CAC AGG CTT CCA GGT CAT TC		
Muc2	MUC2F	CAG CAC CGA TTG CTG AGT TG	140	
	MUC2R	GCT GGT CAT CTC AAT GGC AG		
Zo1	ZO1F	GAA TGA TGG TTG GTA TGG TGC G	191	[91]
	ZO1R	TCA GAA GTG TGT CTA CTG TCC G		
TLR9	TLR9F	GAG CGC AGT GGC AGA CTG GGT G	132	
	TLR9R	CAC AGG TTC TCA AAG AGG GT		
TLR2	TLR2F	GCA GAA GCG CTG GGG AAT GG	300	[50]
	TLR2R	GGA TGC CTA CTG GGT GGA GAA		
TLR4	TLR4F	GGT GGA AGT TGA ACG AAT GG	182	
	TLR4R	CCA GCA AGA AGC ATCAGG TG		

Gene identification, forward and reverse oligonucleotide sequences 5'–3', product sizeand reference.

Mice were kept in a temperature-controlled room with *ad libitum* access to water and standard chow diet. The study was approved by the Ethics Committee on Animal Experimentation of the Federal University of Minas Gerais (CEUA-UFMG, Brazil, protocol 366).

Ethics statement

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

Experimental set-up

The experimental set-up is illustrated in Supplementary Figure 1. BALB/c mice were randomly divided into eight groups (6 mice per group). Animals were fed daily orally by 5 mL of water (groups 1 and 5); 5 mL of YEL culture medium (groups 2 and 6) or 5 mL of YEL containg 10^9 CFU mL^{-1} of either *P. freudenreichii* WT (groups 3 and 7) or *P. freudenreichii* Δ*slpB* (groups 4 and 8) for 10 days. On the eleventh day, the consumption of culture medium and bacteria were discontinued and then all groups received only water. Mucositis was induced on the 11th day of the experimental procedure by a single intraperitoneal injection of 5-fluororacil (300 mg/ kg) for groups 5 to 8. An injection of saline (NaCl 0.9%) was used as a control for groups 1 to 4 [57]. Mice were euthanized on the 14th day. *In vivo* assays were performed in biological triplicate.

Histological analysis

For histomorphological analysis, the distal portion of the mice ileum was collected after the euthanasia and washed with PBS. Afterwards, rolls were prepared and immersed in formaldehyde solution (4%, v/v) for tissue fixation. This material was embedded in paraffin, and a 4 μm section of samples were placed on a glass slide and stained with hematoxylin and eosin (HE). Histological inflammation score was determined as described by MacPherson & Pfeiffer [80], measuring three major histological changes in mucositis disease: (i) intensity of the infiltrate of mononuclear and polymorphonuclear cells in the *lamina propria*, (ii) presence of ulceration and erosion and (iii) alterations in mucosal architecture. The score was given according to the severity of the lesion in the tissues: absent (0), mild (1), moderate (2) and severe (3). For morphometric analysis, ten images of the ileum of each animal were randomly captured and analyzed using ImageJ software (version 1.8.0). Granular density of Paneth cells was determined by measuring the intracellular area occupied by secretory granules [57]. Villi height and crypt depth were measured vertically from the tip of villi to the base of the adjacent crypt. Villus height/crypt depth ratio from the intestinal epithelium was also measured [57].

Measurement of secretory IgA

For measurement of secretory IgA (sIgA), the small bowel of all euthanized mice were washed using PBS. These materials were vortexed, and centrifuged for 30 min at 850 g at 4° C. Afterwards, the supernatant was transferred to a test tube and used for tested by enzyme-linked immunosorbent assay (ELISA) for IgA concentration as previously described by [57]. The results were expressed as the concentration of sIgA (μg/ml) in intestinal fluid, according to the standard curve.

Flow cytometry analyses of spleen cell subsets

The method used was previously described by Rocha et al [81]. An amount of 1×10^6 cells were isolated from spleen and resuspended in PBS-BSA-NaN_3, pH 7.4 (PBS buffer containing 0.2% BSA (Bovine Serum Albumin) and 0.1% NaN_3). Then, cell surface antigens were labeled with CD4 Monoclonal Antibody (GK1.5), APC (eBioscience) for 30 min at 4°C. Subsequently, for intracellular staining, cells were fixed and permeabilized with Fixation/ Permeabilization working solution (eBioscience) for one hour on ice prior to incubation with Alexa Fluor® 488 Rat Anti-Mouse Foxp3 (BD Pharmingen™) or PE Mouse anti- Mouse RORγt (BD Pharmingen™) for 30 min at 4°C. The cells were then washed in PBS-BSA-NaN_3 (centrifuged at 1200× g for 5 min at 4°C), and resuspended in 200 μL of the same buffer containing 1% v/v paraformaldehyde. Finally, cells were analyzed using a FACS Calibur cytometer (Becton Dickinson, East Rutherford, NJ, USA) and data was analyzed using the FlowJo software (Tree Star, Ashland, OR, USA). At least 10,000 events were counted for each sample. The gating strategy is based on forward and side scatter, then on anti-CD4 labeling, selecting T cells, then on the selection of RORγT and of FOXP3 (B) positive T cells. Dead cells were excluded by size and lymphocytes were gated using FSC/SSC analysis. Doublets were excluded using FSC channel (height x width). FMO was used to set the threshold for labelling superior to isotype control. CD4+ T cells were then gated as previously described [82, 83]. Among these, either RORγT+ e or FOXP3+ cells were selected.

Intestinal permeability

On the last experimental day, after 72 hours of mucositis induction, all animals received 0.1 mL diethylenetriaminepentaacetate acid (DTPA), labelled with 18,5 MBq of ^{99m}technetium, by gavage. Four hours later, the blood was collected, placed in appropriate tubes for radioactive determination and weighing [67]. Results were calculated as percentage of dose per g of blood, by the following equation:

% dose/g blood = (cpm in g of blood/cpm dose of standard) × 100
cpm (counts of radioactivity per minute) [68].

Intestinal tissue preparation and cytokine quantification by ELISA

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

Relative expression of cytokines in mice ileum

Mice ileum total RNA isolation

Quantitative expression of genes in ileum tissue was measured according to Oliveira and collaborators [84]. First, small fragments (1 cm approximately) of ileum were collected and stored in RNAlater (Ambion, Austin, USA) at −80 °C until RNA extraction. Total RNA was isolated using an RNeasy mini kit (Qiagen, Hilden, Germany) according to the manufacturer's recommended protocol. Residual genomic DNA was digested and removed using DNase I (Invitrogen, Waltham, MA, USA) treatment. Samples were then treated with Turbo DNA-free Kit® (Ambion), according to manufacturer's instruction, for DNA removal. cDNA of each sample was produced with High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, USA), according to its manual instructions.

Mice ileum gene expression analysis by qRT-PCR

Quantitative PCR (qPCR) was performed using iTaq universal SYBR green supermix (Biorad, Hercules, CA, USA) and gene specific-primers for *muc2*, Claudin-1 (*cld1*), *Tjp1*, Occludin (*ocln*), *iNOS* and *il-17a* [85–88] as well as housekeeping genes encoding β-actin (*actβ*) and GAPDH (*gapdh*) [85]. Amplification reactions were performed in a final volume of 10 µl, using 5 µl of SYBR green supermix and 10 ng of cDNA. The amplification program consisted of the following steps: 95°C for 30 sec, and 40 cycles of 95°C for 15 sec and 60°C for 30 sec on an ABI PRISM 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA). Expression levels in control group (with no treatment) were used as calibration data. Results are shown graphically as fold changes in gene expression, using the means and standard deviations of target cytokine expression amount ($2^{-\Delta\Delta Ct}$) according to Hellemans, Mortier, De Paepe, Speleman, and Vandesompele (2007) [89]. We monitored expression of key genes previously reported to translate severity of intestinal damages in mice [9, 86–88].

Statistical analyses

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

CONCLUSIONS

This study confirmed the anti-inflammatory effects of *P. freudenreichii* strain ITGP20, equivalent to CIRM-BIA 129. In the context of induced mucositis, this probiotic reduced inflammation, limited histopathological damages, and restored intestinal permeability. This is important in the context of chemotherapy-induced mucositis, to prevent possible translocation of pathogens and systemic inflammation and infection. This work moreover demonstrated, by *in vitro* and *in vivo* approaches, that the mutation of the extractable surface protein *slpB* gene affects directly the probiotic effects of
P. freudenreichii. This is mainly evidenced by the fact that
P. freudenreichii Δ*slpB* loses its ability to regulate pro-inflammatory cytokines in LPS stimulated HT-29 cells, and to alleviate 5-FU induced mucositis. This opens new perspectives for exploring S-layer proteins as possible adjuvants in the treatment of mucositis. Understanding the mechanism responsible for this protective effects deepens the knowledge of Propionibacteria immunomodulatory properties. It opens new perspectives for the utilization of this strain, or of extracted SlpB, in order to alleviate the inflammatory process of mucositis. The clinical guidelines for the management of mucositis recently added a suggestion for the use of probiotics.

P. freudenreichii, to our knowledge, received the GRAS status for its use in cheese, for a healthy population. The safety of its consumption by cancer patients with compromised immunity and mucosal barrier should be investigated in this aim. Moreover, metagenomic studies should address the impact on the structure and activity of the gut microbiota, given that selected *P. freudenreichii* strains produce bifidogenic factors and other nutraceutical compounds that may modulate the commensal microbiota.

Abbreviations

DC-SIGNR1: dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin; HIEC: human intestinal epithelial cell; LPS: lipopolysaccharides; PBMC: peripheral blood mononuclear cell; RT-PCR: reverse transcription-polymerase chain reaction; SLAP: S-layer associated protein; SLH: S-layer homology domain; Slp: surface-layer protein; TLR: toll-like receptor.

Author contributions

FLRdC performed all the experimental design and had a major contribution to the whole experimentation, to data analysis and interpretation, and to the writing of the manuscript. BFC, SHdS, BS, AF, LL, JdLA, CCF, MIAQ,
ERO and SHdCS were major contributors to animal experimentation, cytokines measurement, quantitative PCR, flow cytometry analysis and performed, analyzed and interpreted the secretory IgA. EF and NMR performed, analyzed and interpreted the morphometric analysis and histological analysis from ileum slides. HR performed *in vitro* analysis and data interpretation. AMCF, ACN, YLL, GJ and VA contributed to data interpretation and to writing the manuscript. RMP, SOAF and VNC were involved in permeability assays. VG and MD in protein purification and in RT-Q-PCR. GJ and VA have equally contributed to the supervision of the work.

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CONFLICTS OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest. We declare no competing interest, no conflict of interest, neither financial, nor non-financial.

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CONCLUSÃO GERAL E PERSPECTIVAS

As linhagens de *Proprionibacterium freudenreichii* que são utilizadas na produção do queijo Emental, tem seu potencial probiótico relacionado à produção de metabólitos benéficos, como ácidos graxos de cadeia curta, fatores bifidogênicos e vitaminas, B12, B9 e K2 (RABAH et al., 2017) . Recentemente a linhagem *P. freudenreichii* 129 foi reconhecida como uma linhagem promissora no contexto da inflamação (PLÉ et al., 2015, 2016). Seus efeitos benéficos são dependentes das proteínas de superfície envolvidas na interação com o hospedeiro (DEUTSCH et al., 2017; DO CARMO et al., 2017, 2018b).

As proteínas da camada S estão aderidas à parede celular das bactérias de forma não covalente (GERBINO et al., 2015; SLEYTR et al., 2014) e estão envolvidas na virulência, em efeitos imunomudulatórios, transportes de moléculas, proteção contra ambientes estressantes (DO CARMO et al., 2018a). Em linhagens probióticas tanto as protéinas da camada S como outras proteínas de superfíe extraíveis podem mediar a comuninação com o hospedeiro e levar a efeitos benéficos (DO CARMO et al., 2018a). As proteínas extraíveis de *P. freudenreichii* estão envolvidas na imunomodulação (LE MARÉCHAL et al., 2015) e na adesão às células do hospedeiro (DO CARMO et al., 2017), esta habilidade está relacionada com mecanismos moleculares, como adesão às células epiteliais (PREISING et al., 2010). Precisamente, a adesão *in vitro à*s células epiteliais da linhagem *P. freudenreichii* 129 está associada a presença da proteína de superfície SlpB, ao qual foi confirmado pelo nocaute para o gene *SlpB* (DO CARMO et al., 2017). Além disso, a mutação do gene afetou as propriedades de superfície (hidrofobicidade e potencial zeta) e tolerância a ambientes estressantes simulando o trato gastrointestinal (pH ácido e sal biliar) (DO CARMO et al., 2018b).

P. freudenreichii WT é capaz de aumentar os níveis de IL-10 e reduzir IL-8 (RABAH et al., 2018), IL-10 previne danos durante o processo inflamatório (DENNING et al., 2000; JARRY et al., 2008), enquanto IL-8 desemcedeia o recrutamento de neutrófilos para a lâmina própria (KUCHARZIK et al., 2005; SINGER et al., 2004). Neste trabalho mostramos que a linhagem *P. freudenreichii* Δ*slpB* perdeu a capacidade de induzir a produção de IL-10, provavelmente pela alteração nas propriedades da superfície que ocasionou na redução da capacidade de adesão (DO CARMO et al., 2017, 2018b) e também pela ausência da proteína SlpB. *P. freudenreichii* WT reprimiu a expressão de TNF-α em células HT-29 estimuladas por LPS, uma citocina pró-

inflamatória, o mesmo é observado em outras linhagens probióticas (DUARY et al., 2014; MÉNARD et al., 2004), mas a linhagem *P. freudenreichii* Δ*slpB* perdeu esta habilidade, o que pode estar ligado a ausência da proteína. A proteína SlpB isolada mostrou aqui ser capaz de induzir a expressão de IL-10 em células HT-29, de fato proteínas extraíveis demonstraram ser capazes de mediar a imunomodulação em outras bactérias probióticas, como em *Lactobacillus helveticus* MILh5 que reduz a ativação de NF-κB nas células Caco-2 via SlpA (TAVERNITI et al., 2013).

Os receptores Toll-like (TLRs) podem ser modulados pela interação do hospedeiro com a microbiota, o que leva à participação de processos inflamatórios (MOGENSEN, 2009; PAOLILLO et al., 2009). Em um estudo, *L. plantarum* BFE 1685 e *L. rhamnosus* GG regulam positivamente a expressão de TLR2 e TLR9 em células HT-29, enquanto o patógeno *S. typhimurium* não foi capaz (VIZOSO PINTO et al., 2009). Neste trabalho foi mostrado que *P. freudenreichii* WT aumenta a expressão de TLR2 em células HT-29, o que sugere uma reação aprimorada em relação ao ácido lipoteicóico (LTA- do inglês, Lipoteichoic acid) bacteriano, enquanto a linhagem mutante não o fez. Já nas células estimuladas por LPS, as linhagens selvagens e mutantes não foram capazes de alterar a expressão de TLR2 e TLR9. Em outras linhagens probióticas, como *L. rhamnosus* LGG a expressão de TLR2 e TLR9 foi reduzida em células HT-29 estimuladas com LPS ou Salmonella (GAO et al., 2017; VIZOSO et al., 2009). Mostramos que *P. freudenreichii* WT é capaz de reduzir a resposta inflamatória induzida por LPS. Com base nos resultados analisados em células HT-29, tornou-se necessário avaliar a mutação do gene *SlpB* em um modelo de inflamação *in vivo* e assim averiguar se a capacidade probiótica de *P. freudenreichii* WT seria perdida.

Utilizamos o modelo de mucosite induzido por 5-Fluorouracil (5-FU) em camundongos Balb/c. A mucosite é uma doença inflamatória que afeta o trato gastrointestinal de pacientes em tratamento de câncer de cabeça e pescoço utilizando quimioterápicos, como o 5-FU. Na mucosite, a mucosa intestinal é prejudica, onde se observa, encurtamento das vilosidades, presença de ulcerações entre outros danos (SONIS, 2004). Os tratamentos disponíveis para mucosite, no entanto estes são de curta duração e causam efeitos colaterais nos pacientes, o que pode acarretar na interrupção do tratamento de câncer (CARVALHO et al., 2017b). A linhagem *P. freudenreichii* 129 WT demonstrou vários efeitos benéficos em animais tratados com 5-FU, reduziu os danos teciduais, preservou a altura das vilosidades, com isso, diminuiu os escores

histopatológicos. A infiltração de células inflamatórias na lâmina própria foi reduzida, além disso os animais que consumiram *P. freudenreichii* WT tiveram a perda de peso atenuada, o que está em concordância com outros trabalhos que mostram que probióticos são capazes de regular esses parâmetros (BOWEN et al., 2007; TRINDADE et al., 2018). A imunoglobulina A secretada (IgAs) é um mecanismo de defesa do organismo no intestino delgado e seus níveis foram aumentados na mucosite. IgAs está relacionada com a integridade da mucosa e defesa contra patógenos (CARVALHO et al., 2017a; SCHMUCKER
et al., 2003), aqui mostramos que os níveis de IgAs foram reduzidos em animais que consumiram *P. freudenreichii* WT em relação à aqueles que receberam água, no entanto a mutação do gene *SlpB* não alterou o efeito de probionibactéria nos níveis de IgAs. Em um estudo, o nível de IgAs aumentou quanto a barreira intestinal foi ameaçada por estímulos inflamatórios, o que está em acordo com o observado neste trabalho, sugerindo que inflamação foi contida (59)

Para aprofundar no processo inflamatório, avaliamos células T CD4+ que expressam FOXP3+ e RORyt+ no baço de camundongos e sabe-se que CD4+ ROR-yt então envolvidas na patologia de doenças como colite ulcerativa e doença de Crohn (MONTELEONE et al., 2017; WEI et al., 2017). As células Tregs podem suprimir células imune e manter a homeostase (SAKAGUCHI et al., 2009) ao avaliar as citocinas e a histologia, observamos que a linhagem mutante amplifica o processo inflamatório com frequências aumentadas de CD4 + Foxp3 + e CD4 + ROR-γt + no baço dos animais saudáveis e tratados com 5-FU, o que pode ser um mecanismo para contrabalancear células e mediadores inflamatórios em relação às células inatas e adaptativas causadas pela cepa mutante Δ*slpB*. Sugere-se que após o consumo da linhagem mutante ΔslpB, as células T naive expressaram RORyt+ e polarizaram em direção a uma resposta pró-inflamatória via Th17/IL-17. A citocina IL-17 modula a ativação e recrutamento de neutrófilos, o que condiz com o aumento do escore histológico e de IL-17ª (POWELL et al., 2017). A linhagem mutante também aumentou a população de CD4+ FOXP3+, as células T CD4+ FOXP3+ parecem controlar a resposta pró-inflamatória Th17 desencadeada pela mutante ΔslpB (JUNG et al., 2017; VOO et al., 2009).

Ao avaliar a permeabilidade intestinal, foi observado que *P. freudenreichii* WT evitou que alteração na permeabilidade, o que pode impedir a exposição a toxinas e bactérias intestinais, e consequentemente, uma inflamação sistêmica (GALDINO et al., 2018). A integridade da

barreira epitelial está relacionada com as proteínas de junção, como a claudina 1, que tem seus níveis reduzidos na mucosite (WARDILL et al., 2014), o tratamento com *P. freudenreichii* WT aumentou a expressão deste gene, *Cld*1, nos animais tratados com 5-FU. Em relação aos genes *zo*-1 e *muc2*, não foram observadas nenhuma diferença entre os tratamentos. A mucosite eleva os níveis de IL-12 no íleo e a linhagem *P. freudenreichii* WT foi capaz de reduzir o nível de IL-12. Os animais saudáveis que consumiram *P. freudenreichii* WT mostraram níveis aumentados de IL-10 que é um marcador anti-inflamatório, e também um aumento na razão IL-10/IL-12, que é proposto como um marcador anti-inflamatório em probióticos (FOLIGNE et al., 2007). Possivelmente a IL-10 conteve o processo inflamatório causado pelo 5-FU.

Os animais que consumiram *P. freudenreichii* ΔslpB exibiram um escore histopatológico mais parecido com os animais controle da mucosite, a linhagem mutante perdeu a capacidade de manter a integridade da mucosa do íleo. Além de perder sua capacidade anti-inflamatoria, o mutante foi capaz de induzir uma inflamação no íleo dos animais saudáveis. Não foi capaz de atenuar a perda de peso ocasionada pelo 5-FU, bem como não foi capaz de restaurar os níveis de Cld, o que levou à alteração na permeabilidade intestinal. O que indica que as proteínas de superfície extraíveis estão associadas à expressão de genes que codificam moléculas participantes da construção das zônulas de oclusão, como Claudina-1, Ocludina, JAM-1 e ZO-1, o que já foi observado em linhagem probioticas (DO CARMO et al., 2018a). O mutante Δ*slpB* aumentou a expressão de óxido nítrico sintase induzível (iNOS) que está ligado a processos inflamatórios, sua expressão é desencadeada por IL-17, uma citocina pró-inflamatória (MILJKOVIC et al., 2004; SUSCHEK et al., 2005). A indução tanto de *inos* quanto de *Il-17* pelo mutante pode ter contribuídos para a inflamação observada em animais saudáveis e sua incapacidade de reduzir os danos provocados pela mucosite.

Em resumo, este trabalho mostrou o papel fundamental da proteína de superfície extraível, SlpB, de *P. freudenreichii*. Tanto *in vivo* quanto *in vitro* foi demonstrado que esta proteína é essencial para manter o potencial probiótico da linhagem, tendo papel na imunomudalação e na manutenção da barreira epitelial. Como perspectivas, pretendemos avaliar os mecanismos envolvidos na interação da proteína SlpB com as células utilizando macrófagos e células dendríticas, o que permitirá compreender como a linhagem mutante se tornou pró-inflamatória. Além disso, pretendemos avaliar, por meio da metagenônima, se a bactéria probiotica é capaz de modular a microbiota intestinal de animais saudáveis e doentes.

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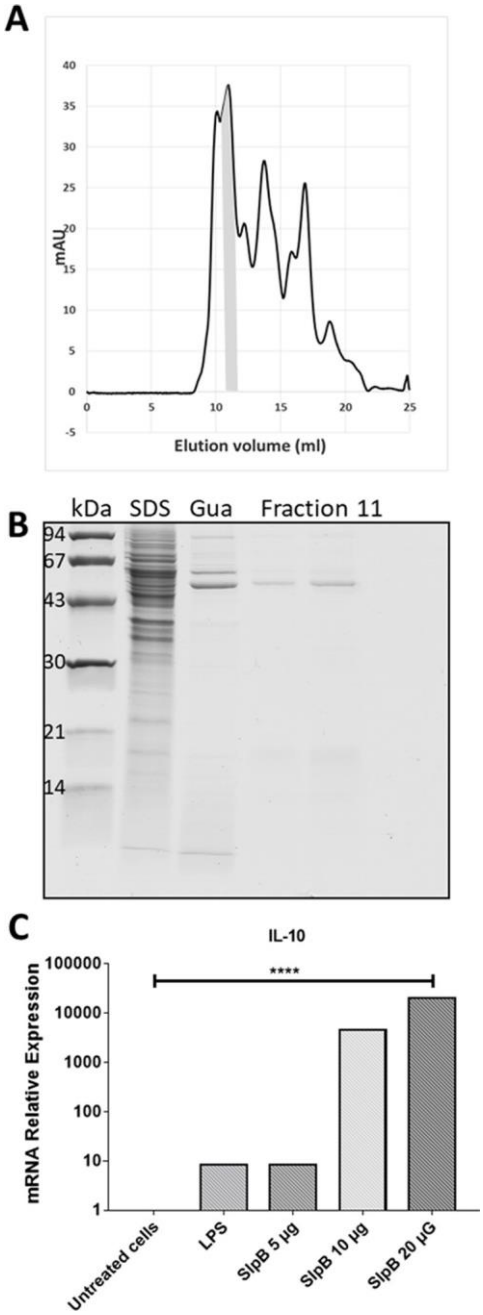
ANEXO 1 – MATERIAL SUPLEMENTAR

www.oncotarget.com Oncotarget, Supplementary Materials

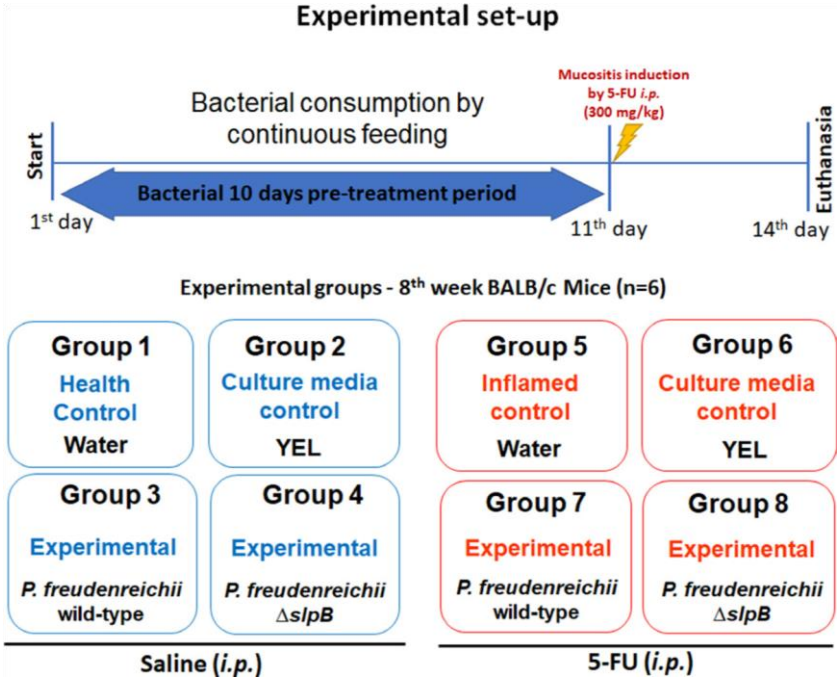
Probiotic *Propionibacterium freudenreichii* requires SlpB protein to mitigate mucositis induced by chemotherapy

SUPPLEMENTARY MATERIALS

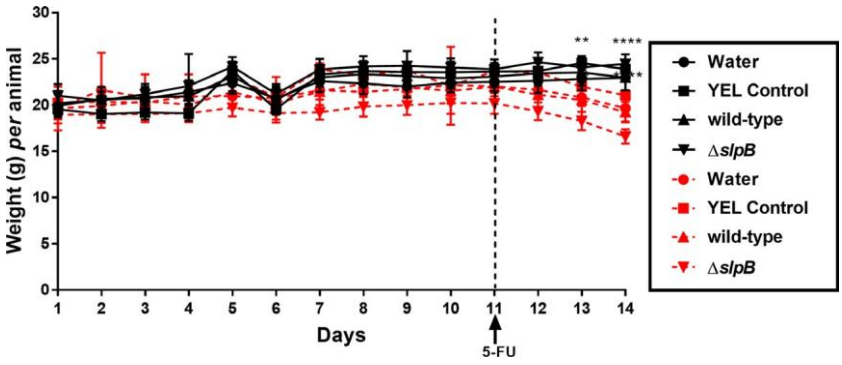


Supplementary Figure 1: Purified SlpB protein induces expression of IL-10 in HT-29 cells. A surface protein Guanidine extract was subjected to size-exclusion chromatography and the chromatogram is shown **(A).** SDS-

PAGE was used to analyze the protein content of a whole-cell protein SDS extract, of the Guanidine extract, and of purfified SlpB in fraction 11 **(B)**. SlpB was used to treat HT-29 cells and expression of *il10* was monitored after 7 h of treatment with different doses **(C)**. ***: $p < 0.001$.

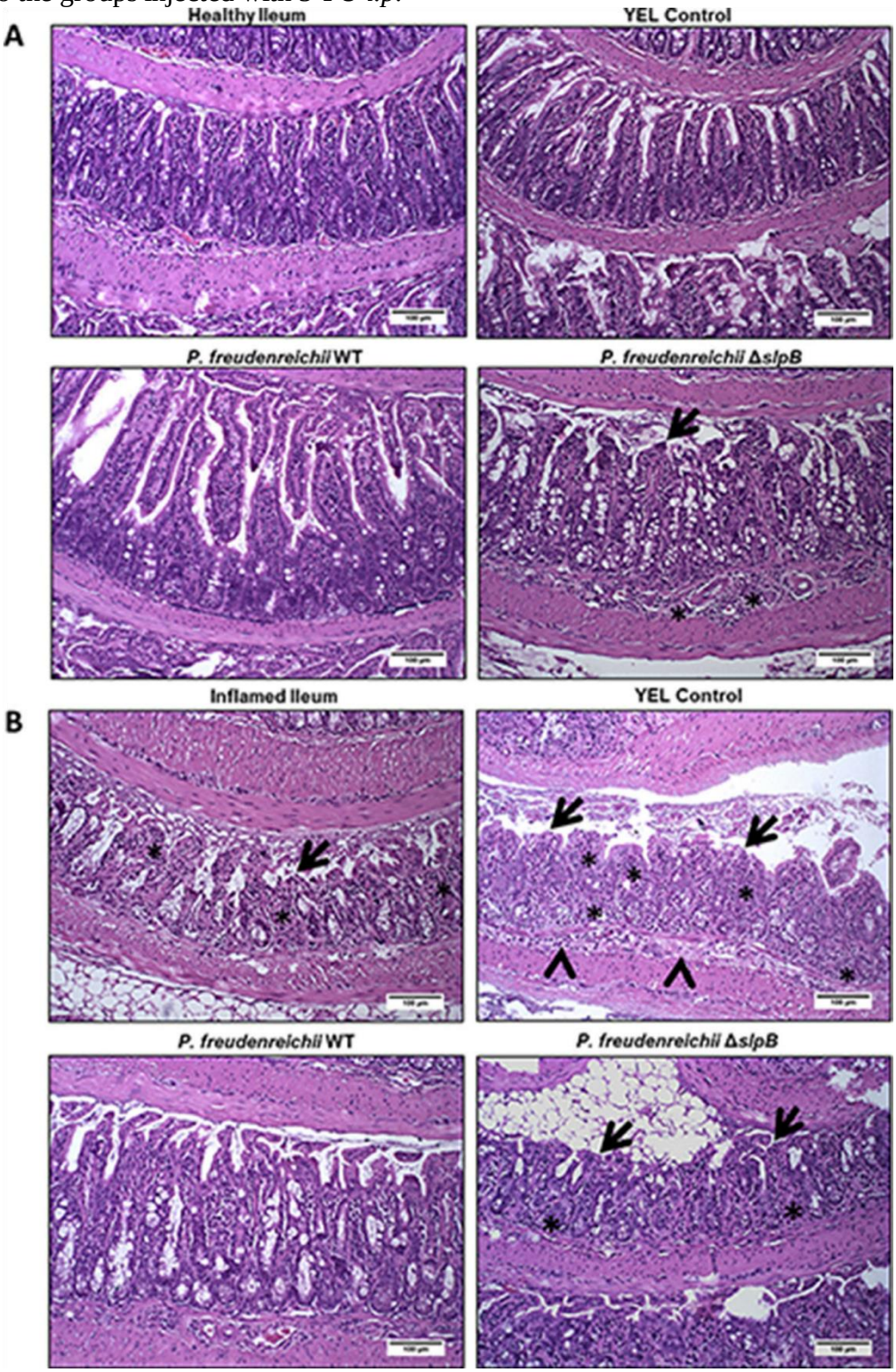


Supplementary Figure 2: Experimental set-up of the animal study. Mice were divided into 8 groups of 6 mice**.** They were given different treatments, as indicated, during 10 days, prior to mucositis induction. Then groups 1 to 4 were injected with a saline solution, while groups 5 to 8 were injected with a solution of 5-FU.



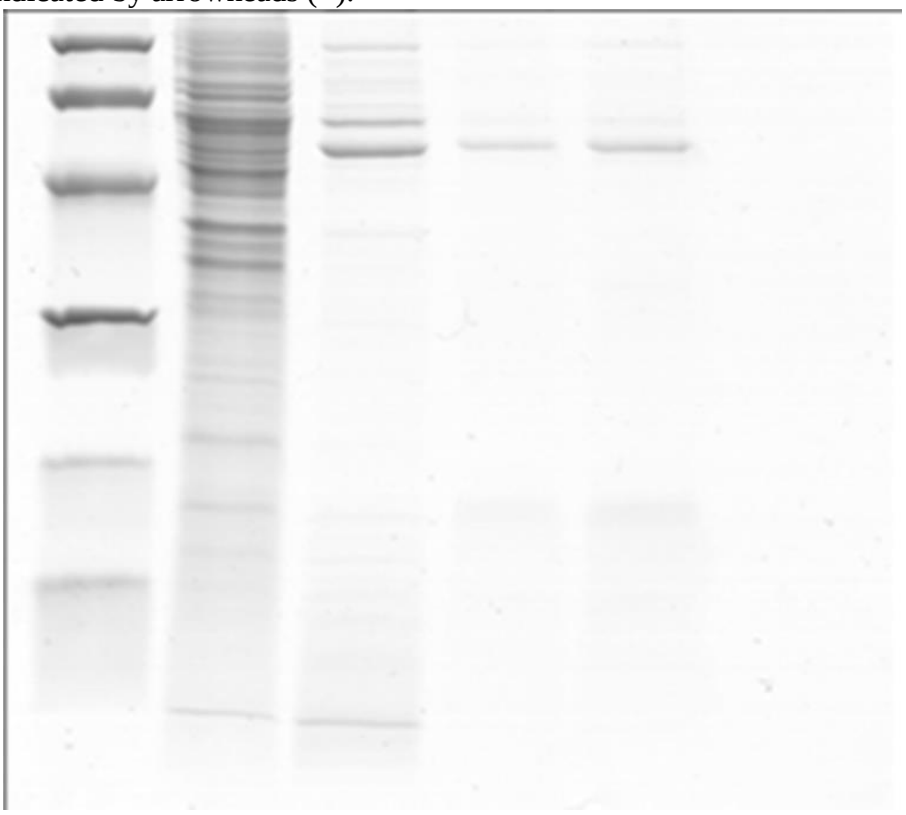
Supplementary Figure 3: Time-course of body weight in grams. Mice receiving YEL culture medium YEL (YEL control), the probiotic strain *P. freudenreichii* 129 WT (wild-type), or the mutant strain *P.*

freudenreichii (Δ*slpB*). Black lines correspond to the groups injected with saline *i.p.* and red lines to the groups injected with 5-FU *i.p.*



Supplementary Figure 4: Representative images of H&E-stained sections of mice ileal mucosa, demonstrating histopathology. (A) Non inflamed groups and **(B)** 5-FU inflamed groups**.** The image acquisition was done with objective magnification at 20x. Scale bar=100µm. Cellular

infiltration was indicated by asterisks (*), Epithelial erosion was indicated by arrows (→), Edema was indicated by arrowheads (>).



Supplementary Figure 5: Uncropped gel from Supplementary Figure 2.

ANEXO 2 – PRODUÇÃO CIENTÍFICA

Artigos Publicados

RABAH, H.; DO CARMO, F.L.R.; CARVALHO, R.D.O.; CORDEIRO, B.F.; DA SILVA, S.H.; OLIVEIRA, E.R.; LEMOS, L.; CARA, D.C.; FARIA, A.M.C.; GARRIC, G.; HAREL-OGER, M.; LE LOIR, Y.; AZEVEDO, V.; BOUGUEN, G.; JAN, G. **Beneficial Propionibacteria within a Probiotic Emmental Cheese: Impact on Dextran Sodium Sulphate-Induced Colitis in Mice.** Microorganisms 2020, 8, 380.

CORDEIRO, BÁRBARA F.; LEMOS, LUISA; OLIVEIRA, EMILIANO R.; **SILVA, SARA H.**; SAVASSI, BRUNA; FIGUEIROA, ALESSANDRA ; FARIA, ANA MARIA C.; FERREIRA, ENIO ; ESMERINO, ERICK A. ; ROCHA, RAMON S. ; FREITAS, MÔNICA Q. ; SILVA, MARCIA C. ; CRUZ, ADRIANO G. ; CARMO, FILLIPE LUIZ R. ; AZEVEDO, VASCO . **Prato cheese containing *Lactobacillus casei* 01 fails to prevent dextran sodium sulphate-induced colitis.** International Dairy Journal, v. 1, p. 104551, 2019.

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DO CARMO, FILLIPE L. R.; RABAH, H.; CARVALHO, R. D. O.; GAUCHER, F.; CORDEIRO, B. F.; **SILVA, S. H.;** LOIR, Y. L.; AZEVEDO, V.; JAN, G. **Extractable**

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

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Patente

AZEVEDO, V.; DO CARMO, FILLIPE L. R.; CORDEIRO, B. F.; CARVALHO, R. D. O.; SAVASSI, B. M. ; OLIVEIRA, E. R. ; **SILVA, S. H.** . Composição Para Tratamento Ou Prevenção Da Mucosite E Uso. 2019, Brasil. Patente: Privilégio de Inovação. Número do registro: BR1020190056142, título: "Composição Para Tratamento Ou Prevenção Da Mucosite E Uso" Instituição de registro: INPI - Instituto Nacional da Propriedade Industrial. Depósito: 22/03/201.

CONSIDERAÇÕES FINAIS

O presente projeto foi desenvolvido no Laboratório de Genética Celular e Molecular (LGCM) do Departamento de Ciências Biológicas da UFMG. O LGCM, liderado pelo Professor Dr. Vasco Azevedo, que tem como missão compartilhar e disseminar conhecimento, contribuindo para a formação de estudantes e pesquisadores.

A pesquisa no LGCM é voltada para a área de Genética, com ênfase em Genética Molecular e de Microrganismos, atuando principalmente nos seguintes temas: Genômica; transcriptômica; proteômica; análises funcionais de genes; biotecnologia e ensaios pré-clinicos. Pontualmente o LGCM possui grande experiência em trabalhos que envolvam modelos murinos de inflamação intestinal e o uso de bactérias probióticas. Nesse escopo, ao longo da iniciação científica e do mestrado, foram desenvolvidas habilidade em todos âmbitos, *in silico*, *in vitro* e *in vivo*.

Referente às habilidades *in silico*

Na bioinformática os genomas sequenciados são analisados através da montagem e anotação utilizando uma série de programas e plataformas, como Blast e UniProt que são ferramentas que comparam sequências de aminoácidos onde também é possíver encontrar a função de diversas proteínas. O Artemis é um navegador de genoma que permite sua visualização e anotação e como complemento tem o CLC genomics, onde é possível detectar e comparar variantes genéticas.

Referente às habilidades *in vitro*

Os trabalhos, *in vitro*, tem início na realização de procotocos básicos, como preparação de meios de cultura e cultivo de bactérias. Seguidos de trabalhos que envolvem probióticos; um passo importante na caracterização de novas linhagens benéficas são os ensaios de estresse, *in vitro*, como ao pH, sais biliares e temperatura, visando simular a passagem pelo TGI. Ao modificar uma linhagem geneticamente, além de outras etapas, tem-se a clonagem e posteriormente a expressão de proteínas recombinantes em bactérias Gram-postivas. Para tanto, são executados protocolos como, preparação de bactérias eletrocompetentes e quimiocompetentes, eletrotransformação e transformação por choque térmico, extração de DNA plasmidial, genômico, RNA e proteínas, digestão enzimática, eletroforese em gel de

agarose e poliacrilamida e PCR convencional. Outra técnica bastante utilizada é o PCR em Tempo Real, para a quantificação dos níveis de expressão gênica, através do mRNA.

Referente às habilidades *in vivo*

Trabalhamos com diferentes linhagens de animais como camundongos Balb/C e C57BL/6 e ratos *Wistar* selvagens e uma linhagem hipertensa. Partindo desde a alimentação e manutenção no biotério até o momento da eutanásia, a qual é feita por aprofundamento de anestésicos via intraperitoneal seguido de deslocamento cervical, no caso dos camundongos, já nos ratos, após o aprofundamento de anestésicos é utilizada uma guilhotina. Após a eutanásia é necessário a retirada de tecidos para realização dos ensaios, assim, o trato gastrointestinal é removido do estômago ao reto, seguido da coleta de fluido intestinal para quantificação de IgA por meio de ELISA, e regiões para dosagem de citocinas utilizando também a técnica de ELISA, quantificação do nível de infiltração de neutrófilos e eosinófilos, por meio dos ensaios de mieloperoxidase (MPO) e peroxidase (EPO), e análises histológicas onde é possível avaliar a arquitetura do tecido. O baço é dissecado para análise da população de célula T, por meio da citometria de fluxo. Além da dissecação dessas regiões, também são utilizadas orgãos mais sensíveis, por exemplo, o cérebro e suas regiões específicas, como córtex pré-frontal, hipotálamo, tálamo e hipocampo. Durante os períodos experimentais, por vezes, os animais precisam ingerir o tratamento através de gavagen intragástrica e dos medicamentos por via intraperitoneal, por exemplo o 5-Fu.

Por fim, todas essas habilidades adquiridas foram usadas nas produções científicas citadas no item anterior. É importante ressaltar também, a redação de textos científicos, como resumos de trabalhos para participação em eventos e escrita de artigos.