UNIVERSIDADE FEDERAL DE MINAS GERAIS
PROGRAMA DE PÓS-GRADUAÇÃO EM INOVAÇÃO TECNOLÓGICA

Desenvolvimento e avaliação do efeito terapêutico de queijos potencialmente probióticos em modelo murino de colite ulcerativa

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Belo Horizonte
Minas Gerais
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Tese apresentada ao Programa de Pós-Graduação em Inovação Tecnológica da Universidade Federal de Minas Gerais, como requisito parcial à obtenção do Título de Doutora em Inovação Biofarmacêutica.

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Orientador: Prof. Dr. Vasco Ariston de Carvalho Azevedo

Coorientador: Dr. Fillipe Luiz Rosa do Carmo

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ATA DA SESSÃO DE DEFESA DA 14ª TESE DO PROGRAMA DE PÓS-GRADUAÇÃO EM INOVAÇÃO TECNOLÓGICA E BIOFARMACÊUTICA DA UNIVERSIDADE FEDERAL DE MINAS GERAIS, DA DISCENTE BÁRBARA FERNANDES CORDEIRO, Nº DE REGISTRO 2018718449.

Aos 29 (vinte e nove) dias do mês de junho de 2021, às 8 horas e 30 minutos, na plataforma on-line Google Meet, reuniu-se a Comissão Examinadora composta pelos Professores Doutores: Vasco Ariston de Carvalho Azevedo do Programa de Pós-graduação em Inovação Tecnológica e Biofarmacêutica da UFMG (Orientador), Fillipe Luiz Rosa do Carmo do INRAE, STLO, Institut Agro, Agrocampus Ouest Rennes - França (Coorientador), Cristina Stewart Bittencourt Bogsan da Universidade de São Paulo – USP, Yves Le Loir do INRAE, STLO, Institut Agro, Agrocampus Ouest, Rennes - França, Valbert Nascimento Cardoso do Departamento de Análises Clínicas e Toxicológicas da UFMG, Carlos Alberto Tagliati do Programa de Pós-graduação em Inovação Tecnológica e Biofarmacêutica da UFMG e Aristóteles Góes Neto do Departamento de Microbiologia da UFMG para julgamento da Tese de Doutorado em Inovação Tecnológica e Biofarmacêutica - Área de Concentração: Inovação Biofarmacêutica e Biotecnológica da discente Bárbara Fernandes Cordeiro, Tese intitulada: **"Desenvolvimento e avaliação do efeito terapêutico de queijos potencialmente probióticos em modelo murino de colite ulcerativa".** O Presidente da Banca abriu a sessão e apresentou a Comissão Examinadora, bem como esclareceu sobre os procedimentos que regem da defesa pública de tese. Após a exposição oral do trabalho pela discente e arguição pelos membros da Banca Examinadora na ordem registrada acima, com a respectiva defesa da candidata. Finda a arguição, a Banca Examinadora se reuniu, sem a presença da discente e do público, tendo deliberado unanimemente pela sua **APROVAÇÃO**. Nada mais havendo para constar, lavrou-se e fez a leitura pública da presente Ata que segue assinada por mim e pelos membros da Comissão Examinadora e pelo Coordenador do Programa (via Sistema Eletrônico de Informações – SEI). Belo Horizonte, 29 de junho de 2021.

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BAP: Bactérias do Ácido Propiônico

BL: Bactérias Láticas

CD: *Crohn´s Disease*

DAI: *Disease Activity Index*

DSS: *Dextran Sodium Sulfate*

IBD: *Inflammatory Bowel Disease*

IFN: Interferon

IgA: Imunoglobulina A

IFNγ: interferon γ

IG: Indicação Geográfica

IL: Interleucina

INPI: Instituto Nacional de Propriedade Industrial

INRA: *Institut National de la Recherche Agronomique*

***L. casei*:** *Lactobacillus casei*

L. lactis: *Lactococcus Lactis*

LGCM: Laboratório de Genética Celular e Molecular

LIA: Laboratório Internacional Associado

MUC2: Mucina-2

P. freudenreichii: *Propionibacterium freudenreichii*

SCFA: Ácidos graxos de Cadeia Curta

TGI: Trato Gastrointestinal

TNFα: Fator de Necrose Tumoral

UC: *Ulcerative Colitis*

UFC: Unidade Formadora de Colônias

UFMG: Universidade Federal de Minas Gerais

ZO: *Zonula Occlud*

ORGANIZAÇÃO GERAL DA TESE

Nesta seção, será descrita a organização geral do trabalho, bem como o contexto geral em que este projeto está inserido.

Na primeira parte deste manuscrito, foi feita uma revisão dos temas mais importantes para o entendimento global do trabalho. Nesta seção, intitulada “Introdução Geral”, discutimos sobre os alimentos funcionais, principalmente os de base láctea e o atual cenário científico-mercadológico do desenvolvimento desses novos produtos funcionais probióticos. Em seguida, apresentamos os queijos como veículo para a introdução de bactérias probióticas e a importância desse alimento no contexto regional brasileiro, principalmente para o estado de Minas Gerais. Seguidamente, trazemos uma revisão dos principais grupos de bactérias probióticas utilizadas para o desenvolvimento de produtos alimentares, dando enfoque àquelas que foram usadas para a produção dos produtos alimentares deste trabalho, bem como os possíveis mecanismos de ação por meio dos quais estas bactérias exercem seus benefícios no hospedeiro. Por fim, discutimos sobre as doenças inflamatórias intestinais, seus principais sintomas e tratamentos, já que utilizamos a colite ulcerativa como modelo animal para testarmos o potencial probiótico dos queijos desenvolvidos.

Após esta introdução geral, expomos uma justificativa da importância deste trabalho no contexto médico-científico atual. Já no capítulo 1, apresentamos um estudo realizado pelo *Institut National de la Recherche Agronomique* (INRA-França) em colaboração com o Laboratório de Genética Celular e Molecular (LGCM) da Universidade Federal de Minas Gerais (UFMG-Brasil), coordenado pelo Prof. Dr. Vasco Azevedo, como parte do convênio, celebrado pelo Laboratório Internacional Associado (LIA). Esse acordo de parceria entre os laboratórios visa conduzir projetos colaborativos, relacionados às bactérias e doenças inflamatórias na saúde humana e animal, através da formação de estudantes, por meio de cursos e treinamentos especializados na área, além de intercâmbios científicos entre alunos brasileiros e franceses.

Nesse contexto, no ano de 2018, dentro do quadro de umas das colaborações do LIA, o LGCM-UFMG recebeu a aluna de doutorado Houem Rabah da escola de doutorado *Agrocampus Ouest* - França. O estágio no Brasil fazia parte do projeto de pesquisa de doutorado de Houem Rabah intitulado "Vetorização de *Propionibacterium freudenreichii* e suas proteínas imunomoduladas em matriz de queijo durante a passagem pelo trato digestivo". Para finalizar este projeto, foi desenvolvido na França um queijo Emmental a partir de três bactérias probióticas (*Propionibacterium freudenreichii* CIRM-BIA 129, *Lactobacillus delbrueckii* CNRZ327 e *Streptococcus thermophilus* LMD-9), e o potencial terapêutico desse queijo foi investigado em um modelo animal de colite ulcerativa induzido por sulfato de dextrano de sódio (DSS). O LGCM e o grupo de pesquisa coordenado pelo Prof. Dr. Vasco Azevedo, que já possuíam expertise nesse modelo murino de indução da colite ulcerativa, foram responsáveis por todo o delineado experimental do projeto e os ensaios que se sucederam à eutanásia dos animais. Este trabalho rendeu a publicação de um artigo original que será apresentado no capítulo 1 deste manuscrito.

Impulsionados pelos bons resultados alcançados no trabalho do queijo Emmental probiótico, resolvemos trazer a plataforma de desenvolvimento do queijo probiótico para a realidade brasileira, já que o queijo Emmental não é consumido com frequência no Brasil. Desta forma, mesmo que já possuíamos comprovados resultados clínicos nos camundongos, ele não seria um alimento funcional com boa entrada no mercado brasileiro.

Diante desse cenário, o LGCM iniciou uma colaboração com o Laboratório de Processamento de Alimentos, do Instituto Federal do Rio de Janeiro, coordenado pelo Prof. Dr. Adriano Gomes da Cruz, pós-doutor em Tecnologia de Alimentos. O laboratório coordenado por ele é reconhecido por grandes trabalhos no processamento de produtos lácteos como queijos, leites fermentados, sobremesas, e fórmulas infantis, além do desenvolvimento de produtos funcionais probióticos, prebióticos, simbióticos, paraprobióticos e posbióticos. A colaboração entre os dois laboratórios inclui o desenvolvimento de vários alimentos funcionais entre leites

fermentados e queijos probióticos para tratamento de doenças como a colite, mucosite e a hipertensão (os dois últimos trabalhos, em fase de submissão).

Nessas circunstâncias, desenvolvemos um queijo Prato, tipicamente brasileiro, contendo a bactéria probiótica *L. casei* 01 e testamos o seu potencial probiótico no modelo murino de colite ulcerativa induzido por DSS. Entretanto, contrariando as nossas expectativas, o queijo Prato apresentado no capítulo 2 deste trabalho não demonstrou as caraterísticas básicas necessárias para ser considerado um bom alimento funcional.

Em razão dos resultados negativos, estávamos novamente diante do desafio de desenvolver um novo queijo que fosse realmente efetivo para o tratamento e/ou prevenção da colite ulcerativa. Nesse cenário, resolvemos alterar os principais parâmetros do desenvolvimento de um alimento funcional: a matriz do queijo e a bactéria probiótica.

Nesse novo trabalho, também realizado em parceria como o Laboratório de Processamento de Alimentos do Prof. Dr. Adriano Gomes da Cruz, desenvolvemos um queijo Minas Frescal, contendo a bactéria *Lactococcus Lactis* NCDO 2118 e utilizamos um protocolo de tratamento da colite ulcerativa aguda induzida por DSS, descrito por Wirtz et al. (2017). Os resultados desse estudo mostraram que, desta vez, os animais que consumiram o queijo Minas Frescal probiótico apresentavam uma redução na gravidade da colite com uma atenuação dos sinais clínicos da doença e menor inflamação colônica. Somado a isto, o queijo Minas Frescal apresentou também outras características positivas que nos fazem acreditar que o queijo Minas probiótico desenvolvido pelo nosso grupo possuia potencial para ser considerado um bom alimento para o tratamento da colite ulcerativa.

Após a apresentação dos três artigos originais, foi feita uma discussão geral do trabalho, com a apresentação dos principais resultados alcançados em cada capítulo e as conclusões que podemos tirar de cada um deles. Apresentamos, ainda, as perspectivas futuras para finalização desta tese, as referências usadas neste manuscrito e, por fim, todos os trabalhos científicos já publicados nesses anos de

doutorado, os artigos em fase de submissão e as patentes depositadas ao longo desses anos.

RESUMO

Os alimentos funcionais são aqueles produtos que fornecem um benefício à saúde do consumidor, além das já tradicionais alegações nutricionais. Neste trabalho, desenvolvemos três queijos e testamos o efeito do consumo de cada um em modelos murinos de colite ulcerativa induzida por DSS. No capítulo 1, utilizamos o queijo do tipo Emmental, um queijo tipicamente francês, contendo a bactéria probiótica *P. freudenreichii* 129 na prevenção da colite aguda induzida por DSS. Os resultados deste estudo mostraram que os animais tratados preventivamente com o queijo Emmental apresentaram melhoras nos sintomas da colite. Além disso, o consumo do queijo probiótico ocasionou uma redução na secreção de IgA, restaurou a expressão de genes da barreira epitelial e evitou a indução do fator de TNFα, IFNγ e IL-17. No capítulo 2, desenvolvemos um queijo funcional do tipo Prato contendo a bactéria *L. casei* 01 e o testamos em um modelo de prevenção da colite, semelhante ao apresentado no capítulo1. Entretanto, diferentemente dos resultados do consumo do queijo Emmental, o queijo Prato não alcançou os resultados esperados para a prevenção da colite. Diante desse cenário, desenvolvemos um novo queijo do tipo Minas Frescal, contendo a bactéria probiótica *L. lactis* NCDO 2118. Os efeitos terapêuticos do consumo deste queijo foram testados em um modelo murino de tratamento da colite induzida por DSS. Os resultados desse estudo, apresentados no capítulo 3, mostraram que os animais que consumiram o queijo Minas Frescal probiótico tiveram uma redução na gravidade da colite com atenuação dos sinais clínicos da doença. Além disso, a ingestão do queijo restaurou a barreira epitelial do colón e modulou a produção de citocinas pró e anti-inflamatórias. Nosso estudo mostrou ainda que o acréscimo da bactéria probiótica não alterou as características organolépticas do queijo Minas Frescal e teve boa aceitação pelo consumidor. Desta forma, o queijo desenvolvido neste trabalho atendeu aos requisitos mínimos para ser considerado um alimento funcional, abrindo perspectivas para o desenvolvimento de novos alimentos probióticos funcionais para nutrição personalizada no contexto das IBDs.

Palavras-chave: Probiótico; Alimentos Funcionais; Colite; Queijo; Doença inflamatória intestinal; *Propionibacterium*; *Lactobacillus*; *Lactococcus*.

ABSTRACT

Functional foods are those that provide a consumer health benefit, in addition to traditional nutritional allegations. In this work, we developed three kinds of cheese and tested the effect of their consumption on murine models of DSS-induced ulcerative colitis. In chapter 1, we used Emmental cheese, a typically French cheese, containing the probiotic bacterium *P. freudenreichii* 129 in the prevention of acute colitis. The results of this study showed that animals treated preventively with Emmental cheese showed improvements in the symptoms of colitis. In addition, the consumption of probiotic cheese caused a reduction in the secretion of Immunoglobulin A, restored the expression of epithelial barrier genes, and avoided the induction of TNFα, IFNγ, and IL-17. In chapter 2, we developed a functional Prato cheese containing *L. casei* 01 and tested it in a colitis prevention model, similar to that presented in chapter 1. However, unlike the results of consumption of Emmental cheese, the Prato cheese has not achieved the expected results for the prevention of colitis. In this scenario, we developed a new cheese of the Minas Frescal type, containing the probiotic bacteria *L. lactis* NCDO 2118. The therapeutic effects of consuming this cheese were tested in a murine model of treatment of colitis induced by DSS. The results of this study, presented in chapter 3, showed that the animals that consumed the Minas Frescal probiotic cheese had a reduction in the severity of the colitis with attenuation of the clinical signs of the disease. In addition, the consumption of this cheese restored the colon's epithelial barrier and modulated the production of pro and anti-inflammatory cytokines. Our study also showed that the addition of probiotic bacteria did not alter the organoleptic characteristics of Minas Frescal cheese and was well accepted by the consumer. Thus, the cheese developed in this work met the minimum requirements to be considered a functional food, opening new perspectives for the development of new functional probiotic foods for personalized nutrition in the context of IBDs.

Keywords: Probiotic; Functional Foods; Colitis; Cheese; Inflammatory Bowel Disease; *Propionibacterium*; *Lactobacillus*; *Lactococcus*

INTRODUÇÃO GERAL

1. Alimentos probióticos funcionais

Os alimentos funcionais são aqueles alimentos, naturais ou processados, que possuem compostos que fornecem efeitos benéficos, além de efeitos nutricionais adequados, de uma forma que seja relevante para melhorar o estado de saúde e o bem-estar do consumidor (ROLIM et al., 2020). Entre eles, os alimentos funcionais que contêm bactérias probióticas têm ganhado bastante notoriedade, principalmente, devido ao seu potencial terapêutico em combinação com drogas convencionais ou no tratamento de disbiose gastrointestinal e em doenças crônicas, metabólicas e genéticas graves (MITSUOKA, 2014). Esses benefícios acabaram por impulsionar um grande interesse científico-mercadológico para o desenvolvimento de novos produtos funcionais contendo bactérias probióticas (COLOMBO et al., 2018).

Nesse contexto, o mercado global de probióticos vem crescendo continuamente e deve movimentar até 57,2 bilhões de dólares no ano de 2022, com uma taxa de crescimento anual de 7,8% (DIEZ-GUTIÉRREZ et al., 2020), sendo as *Lactobacillus* as bactérias probióticas mais utilizadas para fabricação desses produtos, responsáveis por quase 63,1% do *market share*, seguido pela *Bifidobacterium* e *Streptococcus*, com 27,6% e 4,2%, respectivamente (DIEZ-GUTIÉRREZ et al., 2020). No mesmo sentido, observamos que também houve um substancial crescimento no número de patentes relacionadas aos probióticos, depositadas nos principais repositórios de patentes do mundo **(Figura 1)**.

Atualmente, a maioria dos produtos funcionais probióticos são aqueles de base láctea, principalmente leites fermentados e iogurtes, devido à maior aceitação comercial e por serem um dos principais constituintes da dieta diária da população como um todo (COLOMBO et al., 2018; MAA, 2017). Além disso, os alimentos lácteos têm excelente valor nutricional, já que o leite é uma complexa mistura de proteínas bioativas que são responsáveis por uma ampla gama de atividades biológicas, como atividades anti-hipertensivas, antioxidantes e antidiabéticas, além

de serem fontes de lipídeos, sacarídeos, imunoglobulinas, enzimas, peptídeos antimicrobianos e oligossacarídeos como a lactose (BHAT; BHAT, 2011; CHUGH; KAMAL-ELDIN, 2020).

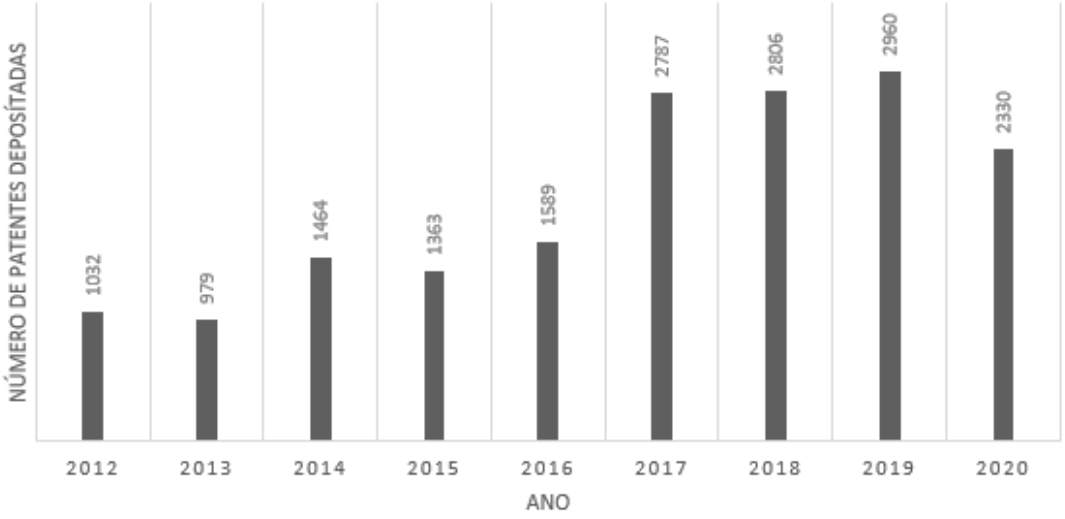

Figura 1: Número de depósito de patentes nos anos de 2012 a 2020. Base de dados Patentscope, utilizando a equação de busca ("functional foods" or "Probiotic* foods" or "functional probiotic foods" or probiotic*). Fonte: Elaborada pela autora.

Os produtos de base láctea são ainda, em sua maioria, excelentes matrizes para proteger as bactérias probióticas durante os processos industriais e durante a passagem pelo trato gastrointestinal (TGI) (CARMO et al., 2017). Essa característica se torna extremamente importante para o sucesso do desenvolvimento de um produto probiótico funcional à medida que, para exercerem os efeitos benéficos no hospedeiro, os microrganismos adicionados aos produtos alimentares devem sobreviver às pressões altamente maléficas às bactérias, como as altas temperaturas e ao pH muito ácido (CORDEIRO et al., 2019a). No Brasil, a legislação regulamenta que, para um produto ter essa alegação de propriedade probiótica funcional, ele deve possuir uma quantidade mínima de bactérias probióticas viáveis e ter demonstrada sua sobrevivência frente às condições do trato digestório humano (BRASIL, 2018). Alguns trabalhos citam recomendações para porção diária de microrganismos viáveis que devem ser ingeridos, sendo o mínimo de 10^8 a 10^9 unidades formadoras de colônias (UFC), o que corresponde ao consumo de 100 g

de produto contendo 10^6 a 10^7 UFC/g (MINELLI; BENINI, 2008; PEREIRA et al., 2018).

A partir desse contexto, fica claro que a escolha da matriz láctea durante o processo de fabricação de um produto probiótico se torna decisiva tanto para o processo de aceitação do consumidor quanto para garantir a viabilidade do microrganismo (PEREIRA et al., 2018). Um bom produto funcional deve, portanto, conciliar os hábitos alimentares, a qualidade organoléptica do produto, seu aporte nutricional e os benefícios gerados pelo acréscimo do ingrediente funcional.

2. Os queijos como alimentos funcionais

O queijo é um concentrado proteico-gorduroso, cuja obtenção é feita mediante a coagulação do leite e a posterior retirada do soro (SILVA, 2005a). Devido a sua extrema versatilidade, os queijos possuem uma grande importância na história da dieta humana (LÓPEZ-EXPÓSITO et al., 2017). O seu alto teor de gordura, proteínas, minerais, lipídeos e vitaminas faz do queijo um alimento altamente nutritivo e rico em energia, sendo consumido em todas as partes do planeta e recomendado para todas as idades (MATERA et al., 2018). Nos últimos anos, a produção de queijo movimentou cerca de 114,1 bilhões de dólares ao ano e o seu valor de mercado aumentou a uma taxa média anual de 1,1% no período entre 2013 e 2019 (GLOBAL TRADE, 2020). Impulsionado pelo aumento da demanda por alimentos mais nutritivos e saudáveis nesses últimos anos, estima-se que o mercado mundial do queijo continuará com um padrão de crescimento para a próxima década, chegando a um volume total de cerca de 31 milhões de toneladas de queijos produzidos até o final de 2030 (GLOBAL TRADE, 2020).

Em especial, no Brasil, o queijo possui uma enorme importância social, econômica e cultural, por isso, é considerado um Patrimônio Cultural Imaterial Brasileiro, concedido pelo Instituto do Patrimônio Histórico e Artístico Nacional (IPHAN, 2008). O queijo está presente na mesa de praticamente todos os brasileiros e é, atualmente, considerado uma das principais *commodities* comercializadas no país (ALVES et al., 2017; MATERA et al., 2018).

Em termos mercadológicos, o estado de Minas Gerais corresponde hoje por mais de 25% de toda a produção de queijos do Brasil (FIGUEIREDO, 2018). Os laticínios do estado são compostos, em sua maior parte, por estabelecimentos de pequeno porte, distribuídos principalmente nas microrregiões do Serro, Canastra, Araxá, Campo das Vertentes, Cerrado, Triângulo Mineiro e Serra do Salitre (MORENO, 2013). Essas regiões possuem mais de 9 mil produtores familiares, com uma produção anual estimada em mais de 30 mil toneladas e gerando mais de 26 mil empregos diretos (OLIVEIRA, 2016). Os queijos produzidos nas regiões do Serro e Canastra, por exemplo, exibem uma importância tão grande no cenário da agroindústria brasileira que receberam, nos anos de 2011 e 2012 respectivamente, a certificação de origem por meio da Indicação Geográfica (IG), concedida pelo Instituto Nacional de Propriedade Industrial (INPI). A IG é uma identidade cultural de reconhecimento da notoriedade, reputação, qualidade e valor intrínseco do produto, distinguindo-o de similares e agregando valor de mercado ao produto produzido naquela região (INPI, 2016).

Essa vasta versatilidade dos queijos permitiu a produção de diversos tipos distintos no país, sendo a maior parte em escala industrial (DINIZ, 2013). Entretanto, apesar da enorme diversidade de novos produtos no mercado interno de queijos, a Muçarela, o queijo Prato, o Requeijão e o queijo Minas Frescal são os tipos mais consumidos pelo brasileiro (SILVA, 2005b, 2005a) A Muçarela e o queijo Prato, por exemplo, representam cerca de 60% das vendas de queijos no Brasil e são os produtos mais populares entre os consumidores de baixa renda (ALVES et al., 2017). Em contrapartida, as variedades com baixa quantidade de gorduras, como é o caso da Ricota e do queijo Minas Frescal, agradam a um segmento de mercado em expansão, composto de consumidores adeptos de um estilo de vida mais saudável (ALVES et al., 2017).

Os queijos são, desta forma, produtos lácteos com uma boa entrada no mercado, e a sua versatilidade de formas, cores, sabores e texturas permitem que o produto se ajuste aos hábitos e à rotina alimentar de qualquer consumidor. Aliado às essas características, o queijo é ainda uma excelente matriz de proteção para microrganismos (ROLIM et al., 2020), em especial, devido às boas condições

anaeróbias criadas pelo conteúdo proteína-gordura da massa do queijo, ao seu alto pH e à baixa acidez que permitem a formação de coacervatos que microencapsulam as bactérias (SILVA et al., 2018c). Esses coacervados reduzem o contato do microrganismo com os ambientes, protegendo-os dos estresses encontrados durante os processos industriais e durante a passagem pelo TGI (LEROY; DE VUYST, 2014). Essas características que permitem o encapsulamento das bactérias probióticas, aliadas ao excelente valor nutricional e boa entrada no mercado consumidor, tornam o queijo um produto lácteo com um excelente potencial para o desenvolvimento de um alimento funcional probiótico.

3. As bactérias probióticas

As principais bactérias probióticas usadas na produção de alimentos fermentados, na produção agrícola, farmacêutica e médica fazem parte do grupo chamado de bactérias láticas (BLs) (BINTSIS, 2018). As BLs incluem microrganismos Gram-positivos, que não formam esporos, são fermentativas, anaeróbias facultativas e obtêm energia através da conversão de açúcares em ácido lático (DABA; ELNAHAS; ELKHATEEB, 2021). As espécies desse grupo podem ser encontradas naturalmente em ambientes ricos em nutrientes, como vegetais e frutas em decomposição, e até mesmo no trato oral, urogenital ou intestinal de mamíferos e outros animais (CARVALHO et al., 2017). As principais bactérias do grupo das BLs, utilizadas como probiótico, incluem algumas espécies dos gêneros *Lactococcus, Lactobacillus* e *Bifidobacterium* (TAVARES et al., 2020).

A importância dessas espécies na fabricação de alimentos funcionais está associada, principalmente, às suas atividades metabólicas seguras, ao seu rápido crescimento e à capacidade de acidificação do ambiente, o que inibe a proliferação de patógenos causadores de deterioração, aumentando, assim, a vida de prateleira dos produtos (BINTSIS, 2018; CORRÊA-OLIVEIRA et al., 2016; ŞANLIER; GÖKCEN; SEZGIN, 2019). Além disso, as BLs possuem pH ótimo de crescimento entre 3,5 e 6,5, o que lhes dá uma boa capacidade de sobrevivência ao pH ácido do estômago, tornando possível a sua manutenção ao longo do TGI (CORDEIRO et al., 2018). As BLs são ainda responsáveis por garantirem o gosto, o aroma e a textura

característica dos produtos fermentados (CANON et al., 2020).

A *Lactobacillus casei* (*L. casei*), por exemplo, é uma das espécies mais importantes do grupo das BLs e são largamente utilizadas na preparação de alimentos lácteos probióticos, como em culturas iniciais para queijos e leites fermentados, como o Yakult e o Actimel, além de serem usadas na produção de carnes e vegetais fermentados (REZAUL et al., 2017). As *L. casei* também podem ser encontradas em toda a extensão do trato gastrointestinal e urogenital humano, desempenhando um importante papel na manutenção da homeostase da microbiota residente (HORI; MATSUDA; OISHI, 2020). Vários estudos já comprovaram que o consumo de linhagens de *L. casei* é capaz de promover benefícios à saúde, sendo, portanto, reconhecidas como bactérias probióticas e podendo ser utilizadas para tratamento ou prevenção de doenças (CARVALHO et al., 2017). Estudos prévios do nosso grupo de pesquisa, por exemplo, demonstrou que o consumo de um leite probiótico fermentado pela linhagem *L. casei* BL23 foi capaz de reduzir os sintomas da mucosite, induzida pelo quimioterápico 5-Fluorouracil (5-FU), através da modulação da barreira intestinal e produção de mucinas (CORDEIRO et al., 2018). Em um outro estudo foi demonstrado que a administração de uma outra linhagem de *L. casei,* chamada de LH23, melhorou significativamente o quadro inflamatório da colite ulcerativa induzida por DSS em camundongo, reduzindo o infiltrado de macrófagos, da secreção de citocinas inflamatórias e da atividade da mieloperoxidase nos camundongos tratados com a LH23 (LIU et al., 2020).

Outra importante espécie de BLs descritas na literatura são as chamadas *Lactococcus lactis*. Elas são comumente isoladas de plantas e de produtos lácteos e foram as primeiras BLs cujo genoma foi completamente sequenciado, por isso, possuem grande importância como ferramentas para manipulação genética (DA SILVA et al., 2019; OLIVEIRA et al., 2017). Elas são bactérias Gram-positivas que não produzem endotoxinas, LPS ou qualquer outro produto metabólico tóxico, sendo, portanto, seguras para o consumo (DE LEBLANC et al., 2015). Apesar de não serem consideradas bactérias comensais, as *L. lactis* conseguem transitar constantemente através do TGI, após a ingestão de produtos lácteos e vegetais fermentados por elas (LAROUTE et al., 2021). Algumas linhagens de *L. lactis* são

ainda produtoras de ácido gama-aminobutírico (GABA), um neurotransmissor que atua modulando o sistema nervoso central, contribuindo para o relaxamento da musculatura lisa e para a redução da pressão arterial (OLIVEIRA et al., 2014). Alguns estudos já atribuíram o consumo de linhagens específicas de *L. lactis* a efeitos probióticos benéficos, como no alívio de sintomas e no tratamento de diversas doenças (BERLEC et al., 2017; COOK; GYSEMANS; MATHIEU, 2018; LIU et al., 2019; LUERCE et al., 2014; NISHITANI et al., 2009).

Outro importante grupo de bactérias utilizadas na indústria para a produção de alimentos lácteos são as chamadas bactérias do ácido propiônico (BAPs). O gênero que representa tal grupo é denominado *Propionibacterium* cuja principal característica é a produção de ácido propiônico durante a fermentação (DO CARMO et al., 2019). As BAPs são actinobactérias, Gram-positivas, em formato de bastonetes pleomórficos, que não esporulam, além de serem anaeróbicas a aerotolerantes (THIERRY et al., 2011). O gênero pode ser dividido de acordo com seu *habitat* natural, podendo ser bactérias propiônicas lácticas ou bactérias propiônicas cutâneas comensais. As propionibacterias cutâneas, por exemplo, são encontradas principalmente na pele e mucosa, sendo responsáveis pelas acnes cutâneas (BARNARD et al., 2020). Já as propionibacteria lácteas, como a *Propionibacterium freudenreichii* (*P. freudenreichii*), são encontradas principalmente em produtos lácteos, como nos queijos, e representam a principal espécie do grupo das bactérias propiônicas (RABAH; ROSA DO CARMO; JAN, 2017).

Nos últimos anos, a *P. freudenreichii* têm sido associada a propriedades probióticas promissoras que incluem, principalmente, a modulação de vários parâmetros da homeostase intestinal em modelos de doenças inflamatórias, além da produção de ácidos graxos de cadeia curta (SCFAs) como o acetato e propionato, e outros metabólitos como vitamina B9 e B12 (CORDEIRO et al., 2018; COUSIN et al., 2012, 2016; DO CARMO et al., 2019; PLÉ et al., 2015). Já foi relatado, por exemplo, que as linhagens do grupo das *P. freudenreichii* são capazes de limitar a gravidade da colite induzida por ácido trinitrobenzenossulfônico (TNBS) e de mucosite intestinal induzida por 5-FU (CORDEIRO et al., 2018; UCHIDA; MOGAMI, 2005), além de infecções por microrganismos patogênicos (PLÉ et al., 2015). Estudos recentes

identificaram que essas propriedades probióticas das *P. freudenreichii* estão diretamente ligadas à presença de proteínas da camada superficial, as chamadas proteínas da camada S (*Surface Layer Proteins*, Slp) (DO CARMO et al., 2019).

4. Mecanismo de ação dos probióticos

Apesar dos benefícios do consumo dessas bactérias já serem bastante explorados pela literatura científica, a forma exata pelas quais essas bactérias interagem e exercem os efeitos benéficos no hospedeiro ainda precisa ser melhor esclarecida, já que esses mecanismos são diversos, heterogêneos e específicos para cada linhagem analisada (PLAZA-DIAZ et al., 2019) **(Figura 2).**

O que já se sabe é que os mecanismos de ação dos probióticos podem envolver a exclusão competitiva de patógenos e a liberação de bacteriocinas, que evitam a colonização do intestino por bactérias patogênicas, c*omo Staphylococcus aureus*, *Salmonella typhimurium* e *Pseudomonas* (MOUSAVI KHANEGHAH et al., 2020). Além disso, linhagens de bactérias probióticas exercem seus benefícios por meio da proteção da barreira epitelial, formada pela camada de células do intestino. Essa barreira epitelial, além de ser uma barreira física contra a entrada de microrganismos do meio externo para o interno, é capaz de ativar uma potente resposta imunológica no hospedeiro (IZCUE; COOMBES; POWRIE, 2009; VAN DER VELDEN et al., 2014). Inúmeros estudos já mostraram que os probióticos têm a capacidade de modular os processos envolvidos na preservação dessa barreira da mucosa, controlando, por exemplo, os genes envolvidos na expressão das chamadas proteínas *tigh junction* (DO CARMO et al., 2019; EWASCHUK et al., 2008; MENNIGEN et al., 2009a; PLÉ et al., 2015; WANG et al., 2018). As *tigh junction* incluem as proteínas transmembrânicas, como claudinas e ocludinas, e proteínas de arcabouço citoplasmático, como as da família das *zonula occluden* (ZO) (LANDY et al., 2016). As proteínas transmembranares medeiam as adesões celulares e vedam os espaços, já as proteínas citoplasmáticas conectam as proteínas transmembranares ao citoesqueleto de actina, mantendo, assim, a forma e a integridade da camada epitelial da mucosa (RODRIGUES et al., 2016).

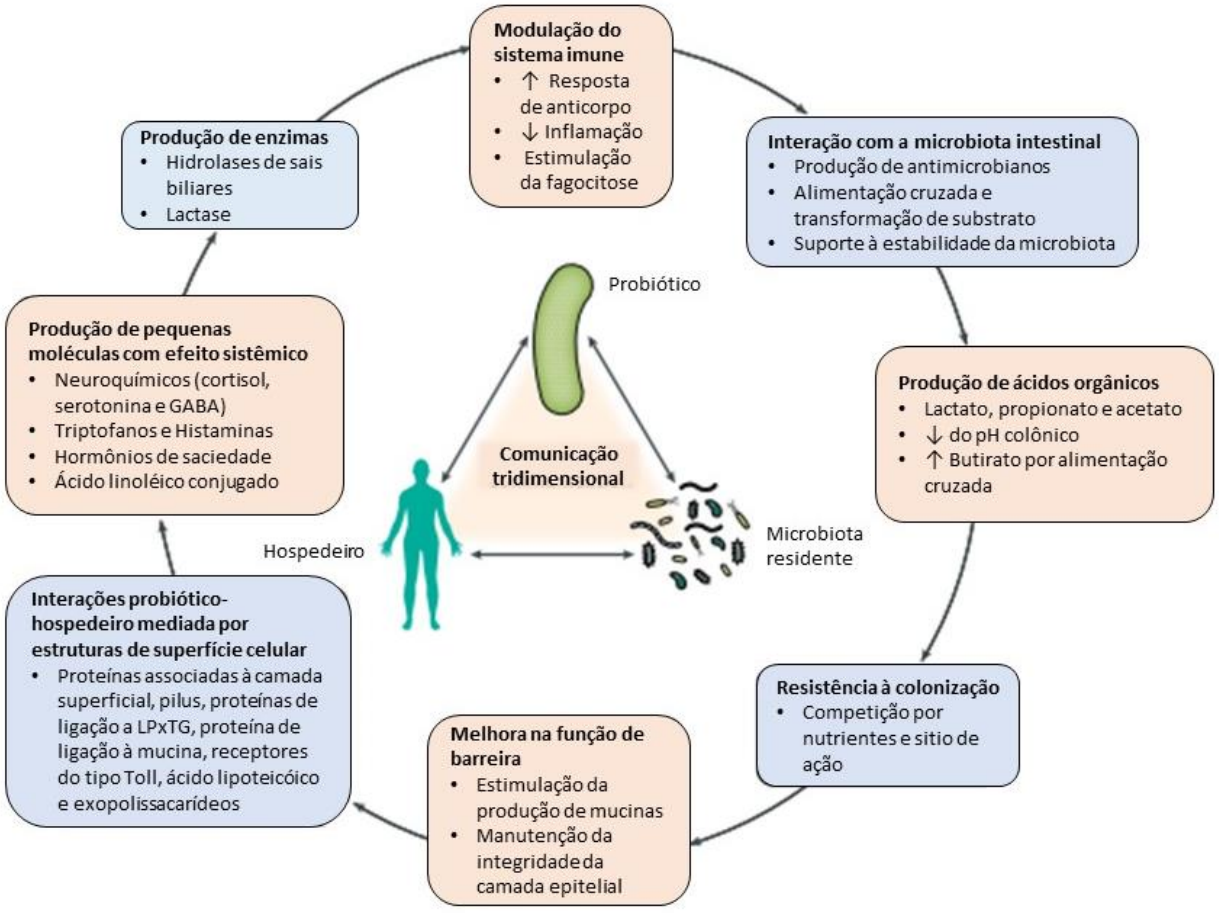

Figura 2: Mecanismo de ação dos probióticos. Fonte: Adaptada de SANDERS et al. (2019)

Além dessas proteínas do arcabouço epitelial, as bactérias probióticas têm a capacidade de induzir a produção de outras proteínas envolvidas na manutenção da homeostase do intestino, como a mucina-2 (MUC2), que é produzida por células especializadas, chamadas de células caliciformes e são as principais constituintes do muco que recobre o intestino (GRONDIN et al., 2020). Esse muco é responsável por impedir a adesão direta de microrganismos ao epitélio e sua translocação para o interior do lúmen, além de serem importantes para a lubrificação e proteção do epitélio intestinal contra substâncias tóxicas vindas do meio exterior (ABRANTES et al., 2020). Além disso, nesse muco encontra-se a imunoglobulina A secretória (sIgA), que é um anticorpo responsável pela exclusão imune de antígenos e microrganismos patogênicos (PABST; CEROVIC; HORNEF, 2016). A sIgA é produzida por células plasmáticas localizadas na lâmina própria da mucosa intestinal

e é lançada ao lúmen, ficando retida no muco. A sIgA interage, então, com os antígenos presentes no interior do TGI, permitindo o seu aprisionamento no muco (MALDONADO GALDEANO et al., 2019). A neutralização desses antígenos acaba por impedir a sua ligação a receptores de superfície celular, gerando uma resposta imune pró-inflamatória mais branda (PABST; SLACK, 2020). Tal mecanismo de exclusão imune realizado pela sIgA é considerado uma das principais respostas de defesa não inflamatórias do sistema imune intestinal (CORTHSY, 2009).

Por fim, os probióticos ainda são capazes de conferir proteção imunológica ao hospedeiro por meio da regulação e estimulação das respostas imunológicas (SANDERS et al., 2019). Essa modulação se dá via respostas do sistema imune do hospedeiro através da interação com células epiteliais, células dendríticas, monócitos, macrófagos, linfócitos e através da modulação na produção de citocinas pró e anti-inflamatórias (BATISTA et al., 2020). Por meio da produção dessas citocinas, os probióticos desencadeiam uma resposta imune que estabelece uma rede de sinais entre as diferentes células imunes (MALDONADO GALDEANO et al., 2019). Tal imunomodulação mediada pelos probióticos é resultado da interação de moléculas conservadas da parede celular desses microrganismos com receptores de reconhecimento do hospedeiro que induzem as vias de sinalização do sistema e a liberação da cascada de citocinas específicas, entre elas a IL-10, que é a citocina mais importante para controlar a homeostase na mucosa intestinal (ASHRAF; SHAH, 2014; CORTHÉSY; GASKINS; MERCENIER, 2007; OELSCHLAEGER, 2010). É importante destacar que a forma como essa modulação irá acontecer dependente da linhagem probiótica consumida (MALDONADO GALDEANO et al., 2019).

5. Doenças Inflamatórias Intestinais

As doenças Inflamatórias Intestinais (IBD, do inglês, *Inflammatory Bowel Disease*) são desordens crônicas, não infecciosas que afetam severamente o TGI, podendo levar ao comprometimento da estrutura e função do intestino (SILVEIRA et al., 2015). As IBDs são marcadas por períodos de remissões e recidivas, e, apesar da etiologia exata da doença ainda não estar bem esclarecida, estudos recentes sugerem que a evolução da doença esteja relacionada à combinação de

predisposição genética do indivíduo, desbalanço imunológico, alterações na composição da microbiota do hospedeiro, acompanhada da influência do meio ambiente (LI et al., 2017) **(Figura 3)**.

A Doença de Crohn (CD do inglês, *Crohn's Disease*) e a Colite ulcerativa (UC, do inglês, *Ulcerative Colitis*) são as formas mais comuns das IBDs (CALDERÓN et al., 2018). Ambas compartilham manifestações clínicas semelhantes, como cólicas abdominais intensas, diarreia, sangramento retal, perda de peso, febre, fraqueza, fadiga e desnutrição (JAKUBCZYK; LESZCZYNSKA; GÓRSKA, 2020). Entretanto, apesar de existirem muitas similaridades entre a CD e a UC, elas se diferem em aspectos importantes (WILHELM; LOVE, 2017). Na CD, por exemplo, ocorre um processo inflamatório descontínuo que pode afetar qualquer porção do TGI, desde a boca até o reto, caracterizando-se por apresentar regiões afetadas, intercaladas por zonas saudáveis e por ser uma inflamação transmural e às vezes granulomatosa (JEENGAR et al., 2017). Já na UC, a mais comum das IBDs e o foco deste trabalho, a inflamação é limitada à porção do cólon e do reto, afetando apenas as camadas mucosa e submucosa desses segmentos (LUERCE et al., 2014; SILVA; PINTO; MATEUS, 2019). Em contrapartida, a presença de edemas, depleção de células caliciformes, alterações na arquitetura do tecido epitelial e presença de ulcerações são algumas das características histopatológicas similares nas duas doenças (MALOY; POWRIE, 2011).

Na última década, as IBDs se transformaram em um desafio de saúde pública à medida que se tornaram uma doença global com incidência acelerada no planeta (NG et al., 2017). Um estudo recente mostrou que o número de indivíduos com IBD aumentou de 3,7 milhões nos anos de 1990 para mais de 6,8 milhões a partir de 2017, um aumento de aproximadamente 83.7% em casos de prevalência global (ALATAB et al., 2020). Além disso, o número total de mortes relacionadas à UC ou a CD teve um aumento de 65%, nesse mesmo período, pulando de 23 mil mortes para mais de 38 mil mortes (ALATAB et al., 2020). É importante ressaltar que essa prevalência pode variar conforme as regiões geográficas. Países desenvolvidos como os do continente Europeu e América do Norte exibem os maiores números (KAPLAN; WINDSOR, 2021). Entretanto, países recém-industrializados na Ásia,

África e América do Sul, incluindo o Brasil, vêm apresentando um aumento importante na incidência das IBDs. Os estudos sugerem que o recente processo de urbanização e industrialização vivenciada por esses países talvez seja o principal fator para essa aceleração, já que mudanças no estilo de vida, hábitos alimentarem ruins, estresse e exposição à poluição são fatores desencadeadores importantes (CALDERÓN et al., 2018).

Figura 3: Principais fatores causadores das doenças inflamatórias intestinais Fonte: Adaptada de JAKUBCZYK; LESZCZYNSKA; GÓRSKA (2020).

6. Tratamentos para colite ulcerativa

Os agentes farmacológicos são a base do tratamento da colite ulcerativa, que buscam a indução e a manutenção da remissão da doença, a prevenção e o tratamento de complicações e o restauro do *status* nutricional do paciente, já que ainda não existe cura definitiva (WILHELM; LOVE, 2017). A escolha da terapia farmacológica é baseada na gravidade e localização da doença, na eficácia do

agente e inclui, principalmente, a administração de anti-inflamatórios, drogas imunossupressoras, antibióticos e intervenção cirúrgica em casos mais graves (PITHADIA; JAIN, 2011).

Tais tratamentos, apesar de melhorarem o quadro inflamatório geral da doença e aliviarem os sintomas, apresentam uma eficácia moderada e possuem efeitos colaterais graves (LAURELL; SJÖBERG, 2017). Os medicamentos da classe dos corticoides, por exemplo, possuem ação anti-inflamatória e imunossupressora, porém, quando prescritos por períodos prolongados, podem acarretar reações adversas como vômitos, alterações de humor, ganho excessivo de peso, hiperglicemia e cefaleia, além de comprometerem todo o sistema imune do paciente, tornando-o mais suscetível a infecções (BERNSTEIN, 2015).

Os antibióticos, por sua vez, são eficazes no tratamento de certas condições ocasionadas pela inflamação colônica, como os abcessos, as fístulas e infecções por microrganismos patogênicos. No entanto, a utilização de antibióticos por longos períodos levanta preocupações quanto à resistência bacteriana e possíveis infecções graves por superbactérias (RAHIMI et al., 2007).

Por fim, a intervenção cirúrgica também é uma alternativa para os casos mais graves da colite, principalmente quando o paciente apresenta obstrução e abscessos, ou quando a doença não responde aos demais tratamentos (TRIANTAFILLIDIS; MERIKAS; GEORGOPOULOS, 2011). Entretanto, a cirurgia, na maioria das vezes, acarreta a diminuição da superfície de absorção e o aumento da velocidade do trânsito intestinal que levam a uma diarreia profusa, desidratação, alterações do equilíbrio eletrolítico e ácido-básico, além de acometimento nutricional (NETO et al., 2011).

A falta de tratamentos eficazes, portanto, fez com que a comunidade médico-científica buscasse por novas terapias para as IBDs, que apresentassem menores efeitos colaterais, maior segurança e uma eficácia mais duradoura. Neste sentido, a administração de bactérias probióticas para o tratamento de distúrbios inflamatórios gastrointestinais já foi extensamente avaliada, apresentando resultados promissores

quanto à sua eficácia na melhora dos sintomas dessas doenças (BERLEC et al., 2017; DE JESUS et al., 2019; LUERCE et al., 2014; MENNIGEN et al., 2009a; SANTOS ROCHA et al., 2014). Ademais, vários estudos têm proposto desenvolvimento de alimentos funcionais contendo probióticos como terapia para o tratamento e prevenção da colite ulcerativa ou como adjuvante, em combinação com as terapias convencionais. Os alimentos funcionais probióticos, desta forma, melhorariam os aspectos clínicos da doença, ao mesmo tempo que conseguiriam fornecer um melhor aporte nutricional aos pacientes (DERIKX; DIELEMAN; HOENTJEN, 2016; LIU et al., 2019; LUERCE et al., 2014; ZHANG et al., 2021).

JUSTIFICATIVA

As doenças inflamatórias intestinais (IBD, do inglês, *Inflammatory Bowel Disease*), entre elas a colite ulcerativa, são desordens crônicas, não infecciosas que afetam severamente o trato gastrointestinal, podendo levar ao comprometimento da estrutura e função do intestino. As IBDs são um desafio à saúde pública à medida que se tornaram uma doença global com incidência acelerada, atingindo cerca de 6,8 milhões de pessoas no planeta. Os agentes farmacológicos são a base do tratamento da colite, entretanto, tais tratamentos, apesar de melhorarem o quadro inflamatório geral da doença, apresentam uma eficácia moderada e possuem efeitos colaterais graves. Por esta razão, tem-se buscado novas terapias para as IBDs, que apresentem menores efeitos colaterais, maior segurança e uma eficácia mais duradoura. Neste sentido, vários estudos têm proposto o desenvolvimento de alimentos funcionais contendo probióticos para o tratamento e prevenção da colite ulcerativa ou como um adjuvante, para serem usados em combinação com as terapias convencionais. Esses alimentos funcionais probióticos têm a capacidade de melhorar os aspectos clínicos da doença, ao mesmo tempo que conseguem fornecer um melhor aporte nutricional aos pacientes.

O desenvolvimento de um produto funcional probiótico, por sua vez, é um desafio enorme para a comunidade médico-científica, já que, para que um alimento funcional seja realmente efetivo, ele deve conciliar os hábitos alimentares naturais do indivíduo, ter um bom aporte nutricional, além de ter a capacidade de gerar efeitos benéficos ao consumidor pelo acréscimo do ingrediente funcional. Nesse contexto, os queijos se tornam um excelente veículo para a incorporação de bactérias probióticas e o desenvolvimento de um alimento funcional, pois são alimentos altamente nutritivos e ricos em energia, além de serem consumidos em todas as partes do planeta, fazendo parte da rotina alimentar da maioria das pessoas, em especial no estado de Minas Gerais que detém 25% de toda a produção de queijos do país. Além disso, os queijos são excelentes matrizes para microrganismos, protegendo-os dos estresses encontrados durante os processos industriais e durante a passagem pelo TGI, mantendo-os vivos e permitindo que exerçam os seus efeitos probióticos.

Neste trabalho de tese, propusemos o desenvolvimento de queijos probióticos funcionais, para nutrição personalizada no contexto da IBDs. Levamos em consideração o tipo de queijo utilizado como veículo, atentando para que fossem variedades de queijos consumidos rotineiramente, além da escolha correta da bactéria probiótica utilizada e a forma com que ele seria consumido. Ao final deste trabalho, desenvolvemos um queijo que atendeu aos critérios básicos para ser considerado um alimento funcional, com potencial promissor para ser utilizado no Brasil em pacientes com colite ulcerativa.

OBJETIVO GERAL

Este trabalho de tese tem como objetivo geral desenvolver queijos probióticos funcionais e investigar o potencial probiótico desses produtos em um modelo de colite ulcerativa induzida por DSS, através da avaliação dos principais sintomas clínicos e morfológicos da colite, assim como da capacidade desse produto em modular a resposta imune do hospedeiro, diminuindo a inflamação na mucosa intestinal dos animais.

CAPÍTULO 1

Desenvolvimento do queijo Emmental para a prevenção de colite ulcerativa

Neste capítulo, apresentaremos o trabalho realizado pelo *Institut National de la Recherche Agronomique* (INRA-França) em colaboração com o Laboratório de Genética Celular e Molecular (LGCM) da Universidade Federal de Minas Gerais (UFMG-Brasil), onde avaliamos o efeito probiótico de um queijo do tipo Emmental contendo a bactéria *P. freudenreichii* CIRM-BIA 129, em modelo preventivo de colite ulcerativa induzida por DSS.

O queijo Emmental é um queijo do tipo duro, caracterizado pela formação de grandes orifícios em sua massa e consumido rotineiramente por grande parte da população francesa (MCSWEENEY; OTTOGALLI; FOX, 2017). O processo tecnológico deste queijo consiste em uma sucessão de estresses abióticos, incluindo cozimento, acidificação, agitação, moldagem, salga e maturação em ambientes com diferentes temperaturas (GAGNAIRE et al., 2015). Em seu processo de amadurecimento, são utilizadas espécies bacterianas do tipo BLs, além de bactérias do grupo das BAPs, em especial, a *P. freudenreichii*, responsáveis por garantirem as características organolépticas e pelo surgimento desses orificios típicos do queijo Emmental.

Estudos anteriores já haviam investigado que as matrizes de queijo eram boas como veículo de entrega para evitar a proteólise digestiva das proteínas de superfícies das *P. freudenreichii* (RABAH et al., 2018). É importante destacar que as propriedades probióticas dessas linhagens estão diretamente ligadas à presença dessas proteínas da camada superficial. Desta forma, uma matriz de proteção que evite a proteólise dessas proteínas de superfície é extremamente importante para manter a capacidade de modulação do sistema imune intestinal pelas *P. freudenreichii* (PLÉ et al., 2015). A bactéria *P. freudenreichii* 129 utilizada neste trabalho foi considerada uma das linhagens mais probióticas do grupo das BAPs

(FOLIGNÉ et al., 2010), e seu potencial terapêutico já foi comprovado em trabalhos anteriores do nosso grupo (DO CARMO et al., 2019; PLÉ et al., 2015).

Esses resultados abriram perspectivas para o desenvolvimento de alimentos funcionais lácteos fermentados por *P. freudenreichii* 129. Neste sentido, decidiu-se investigar se a incorporação da linhagem *P. freudenreichii* 129 a uma matriz de queijo Emmental poderia amenizar os sintomas inflamatórios das IBDs e se a associação dela com outras bactérias como *Lactobacillus delbrueckii* CNRZ327 e *Streptococcus thermophilus* LMD-9, também conhecidamente probióticas, poderia causar um efeito sinérgico sobre a eficácia anti-inflamatória do produto, aumentando o seu potencial probiótico. Os resultados desse trabalho foram publicados na revista *Microorganisms*, em 2020.

Beneficial Propionibacteria within a Probiotic Emmental Cheese: Impact on Dextran Sodium Sulphate-Induced Colitis in Mice

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Abstract: Backgrounds and Aims. Inflammatory Bowel Diseases (IBD), including Ulcerative Colitis (UC), coincide with alterations in the gut microbiota. Consumption of immunomodulatory strains of probiotic bacteria may induce or prolong remission in UC patients. Fermented foods, including cheeses, constitute major vectors for bacteria consumption. New evidences revealed anti-inflammatory effects in selected strains of *Propionibacterium freudenreichii*. We thus hypothesized that consumption of a functional cheese, fermented by such a strain, may exert a positive effect on IBD. Methods. We investigated the impact of cheese fermented by *P. freudenreichii* on gut inflammation. We developed an experimental single-strain cheese solely fermented by a selected immunomodulatory strain of *P. freudenreichii*, CIRM-BIA 129. We moreover produced, in industrial conditions, an Emmental cheese using the same strain, in combination with *Lactobacillus delbrueckii* CNRZ327 and *Streptococcus thermophilus* LMD-9, as starters. Consumption of both cheeses was investigated with respect to prevention of Dextran Sodium Sulphate (DSS)-induced colitis in mice. Results. Consumption of the single-strain experimental cheese, or of the industrial Emmental, both fermented by *P. freudenreichii* CIRM-BIA 129, reduced severity of subsequent DSS-induced colitis, weight loss, disease activity index and histological score. Both treatments, in a preventive way, reduced small Immunoglobulin A (IgA) secretion, restored occludin gene expression and prevented induction of Tumor Necrosis Factor α (TNFα), Interferon γ (IFNγ) and Interleukin-17 (IL-17). Conclusions. A combination of immunomodulatory strains of starter bacteria can be used to manufacture an anti-inflammatory cheese, as revealed in an animal model of colitis. This opens new perspectives for personalized nutrition in the context of IBD.

Keywords: probiotic; colitis; cheese; inflammation; propionibacteria; Emmental; intestine; inflammatory bowel disease

1. Introduction

Functional foods are defined as "ingredients that affect beneficially one or more target functions in the body, beyond adequate nutritional effects, in a way that is relevant to either an improved state of health and well-being and/or reduction of the risk of a disease" [1,2]. The dairy fermented foods, including cheeses, constitute a large part of our daily diet. Several investigations recently showed that specific bacteria strains of starter bacteria, typically employed in dairy fermented foods, can exert probiotic properties such as microbiota modulation, anti-cancerous and anti-inflammatory effects, in a strain-dependent manner. In this perspective, the development of functional dairy fermented foods by using probiotic starter bacteria, may constitute a promising manner to reduce the risk of diseases, which are related to lifestyle and diet. Some lactic acid bacteria and dairy propionibacteria strains were characterized for their immunomodulatory properties, specifically in Inflammatory Bowel Diseases (IBD) [3,4]. IBD, including Ulcerative Colitis (UC) and Crohn's disease, are thought to result from a dysregulated innate and adaptive immune response towards the gut microbiome, in genetically susceptible host [5–7]. Ingested probiotic microorganisms may play a favourable role in the treatment of UC [8,9]. The consumption of specific food-grade microorganisms, selected for their immunomodulatory properties, alone or in combination with conventional drugs, was shown to induce and/or to enhance remission in UC patients [10].

New evidences [11,12] revealed the anti-inflammatory potential of selected strains of *Propionibacterium freudenreichii*, which is a cheese ripening starter routinely used, in association with lactic acid bacteria, in the production of Swiss-type cheese such as Emmental cheese [13]. *P.freudenreichii* contributes to Emmental's characteristic flavour and openings. It produces valuable metabolites with anti-inflammatory properties such as short chain fatty acids, 1.4-dihydroxy-2-naphthoic acid (DHNA), conjugated fatty acids, and surface proteins, which are produced in food matrices such as cheese [3]. Selected strains of *P. freudenreichii* were shown to induce the production of IL-10 in human peripheral blood mononuclear cells [12,14]. This in vivo immunomodulatory property correlates with the ability of these selected strains to protect from Trinitrobenzenesulfonic acid (TNBS)-induced colitis in mice [14]. This anti-inflammatory effect is mediated by specific surface proteins, found only at the surface of specific strains of *P. freudenreichii* [15,16]. A dairy food matrix was shown to protect such immunomodulatory surface proteins from digestive proteolysis [17,18]. Furthermore, consumption of experimental cheese fermented by *P. freudenreichii*, alone or with *Lactobacillus delbrueckii* subsp. *lactis*, protected mice from acute colitis induced by TNBS [19,20]. This led to limited induction of colitis markers such as serum IL-6, serum Amyloid A, colonic myeloperoxidase activity, as well as colonic mRNA expression level of *Il6, Tnfa, Il1b, Il10, Cox2* and *Hmox*. By contrast, colonic mRNA expression level of *Zo1, Pparg* and *Ifng*, repressed by TNBS, were restored by *P. freudenreichii* CIRM-BIA 129 consumption [14,19,20]. In accordance with our previous results, propionibacteria were recently reported to be enriched in the microbiota of infants as a result of breast-feeding, which attenuates the incidence of necrotizing enterocolitis. These authors isolated *Propionibacterium* UF1, closely related to *P. freudenreichii*, from healthy

children and described it as a commensal *Propionibacterium* mitigating intestinal inflammation, via Th17 cell regulation and, regulating neonatal intestinal immunity [11,21].

Similar properties were reported for lactic acid bacteria, which may be used for Emmental cheese manufacturing [22]. Regarding all these data, we hypothesized that selection of specific lactic acid bacteria and dairy propionibacteria strains presenting anti-inflammatory properties could lead to a potentially probiotic Emmental favouring the treatment of IBD [19,20,23,24]. Therefore, the aim of this study was to evaluate the beneficial impact of an Emmental, made using three selected anti-inflammatory strains *P. freudenreichii* CIRM-BIA 129 [20], *Lactobacillus delbrueckii* subsp. *lactis* CNRZ327 [23], and *Streptococcus thermophilus* LMD-9 [25], in the context of Dextran Sodium Sulphate (DSS)-induced colitis in mice.

2. Materials and Methods

2.1. Bacterial Strain

Lactobacillus delbrueckii subsp. *lactis* CNRZ327, *Propionibacterium freudenreichii* CIRM-BIA 129 (equivalent to ITGP20 strain) and *Streptococcus thermophilus* LMD-9, were provided by the international microbiological resource centre CIRM-BIA (Centre International de Ressources Microbiennes, Bactéries d'Intérêt Alimentaire). Lactobacilli were cultured in MRS (de Man, Rogosa, Sharpe) medium (Difco™ Lactobacilli MRS Broth, Difco Laboratories, Becton, Dickinson and Company, Sparks, MD, USA) as described [26]. Thermophilic streptococci in M-17 as described [27]. Dairy propionibacteria in YEL (Yeast Extract Lactate) as described [28]. Except for MRS (Difco Laboratories), all bacterial reagents were from Biokar Diagnostics, Beauvais, France.

2.2. Cheeses Manufacturing for Animal Studies

Two kinds of cheeses were made for animal studies, (1) a single-strain experimental cheese fermented by *P. freudenreichii* only and (2) an industrial Emmental cheese fermented by *Lactobacillus delbrueckii subsp. lactis*, *Streptococcus thermophilus* and *P. freudenreichii*.

(1) The experimental single-strain cheese, solely fermented by *P. freudenreichii* CIRM-BIA 129, was prepared as previously described [19,20]. Briefly, this probiotic strain was grown in a sterilised cow milk, supplemented with milk proteins, milk cream and casein peptone, to generate an experimental pre-cheese reaching 10^9 colony forming units (CFU)/mL, pH 5.5). This was then subjected to coagulation (chy-max® Extra, Chr. Hansen, Hørsholm, Denmark), cutting, heating (10 min, 40 °C), moulding, pressing (2 h, 37 °C), drying and wrapping. All these steps were performed under laminar flow. The biochemical composition of the cheese, determined as described previously [29,30], was: dry matter 58 g/100g, lipids 28 g/100g, proteins 29 g/100g, carbohydrates 0 g/100g, and calcium 840 mg/100g. As a germ-free control, a sterile control cheese matrix was prepared in the same way as the single-strain cheese, but without starter bacteria addition. In that aim, the sterilised cow milk, supplemented with milk proteins, milk cream and casein peptone, was acidified using

Glucono Delta Lactone, prior to the same cheese manufacturing procedure, as described previously [20]. Propionibacteria were enumerated on Yeast-Extract-Lactate-Agar (YELA). Coliforms, mesophilic flora, thermophilic flora, yeast, and molds, all below 10 CFU/g, were also enumerated respectively according to KF, NF ISO 4832, NF ISO 4833, NF V 08–059, and NF V 08–059 method (Table S1).

(2) The probiotic Emmental cheese was manufactured at an industrial scale (a 80 kg cheese wheel) by Entremont Alliance© Company (Malestroit, France) using their production standard process. *Lactobacillus delbrueckii* subsp. *lactis* CNRZ327, *Streptococcus thermophilus* LMD-9 and *P. freudenreichii* CIRM-BIA 129, all 3 provided by CIRM-BIA, were used as starters. Lactobacilli were enumerated by CFU counting on MRS-agar at 42 °C under anaerobiosis [30], streptococci on M17-agar at 42 °C [30] and propionibacteria on lithium-glycerol-agar at 30 °C under anaerobiosis [31] as described previously. To check that the propionibacterial strain recovered after cheese making was identical to that used as a starter, a strain-specific PFGE analysis was applied to isolated colonies as described previously [31–33]. Briefly, chromosomal DNA samples were prepared according to Gautier et al. [32] and digested using *Xba* I. Electrophoresis was run at 14 °C on a 1% agarose gel on a Chef DR II system (Bio-rad, Richmond, UK) with the following parameters: initial time 2s, final time 20s, migration time migration 20h, voltage 6V.cm^{-1} = 200V. For thermophilic lactobacilli, parameters were: enzyme *Asc*I [34], initial time 1s, final time 10s, migration time 16h, Voltage: 6V.cm^{-1} = 200V. For thermophilic streptococci, parameters were: enzyme *Sma*I [35], initial time 2s, final time 20 s, migration time 24h, Voltage: 6V.cm^{-1} = 200V. Gels are presented in the Figures S1–S3. As a routine control at Entremont Alliance© Company, absence of *E. coli*, of coagulase^{+} Staphylococci, of Listeria and of Salmonella was checked using, respectively, the following media: RAPID'E.coli (Sanofi Diagnostics Pasteur, NF (Normes Françaises) ISO (International Organization for Standardization) 16649-2 norm), Baird Parker + RPF (Rabbit Plasma Fibrinogen) (NF ISO 6888-2/A1 norm), RLM (Rapid'L Mono, NF ISO 11290-2) and XLD (Xylose-Lysine-Désoxycholate) + compass Salmonella (NF ISO 6579-1/2017 norm).

2.3. Animals, Feeding Procedure and Dextran Sulfate Sodium Induced Colitis

The experimental set-up of the animal study is depicted in Figure 1. Female C57BL6 mice (8 weeks old,) were obtained from Federal University of Minas Gerais (UFMG–Belo Horizonte, Brazil). The study was approved (11/03/2019) by the Brazilian Ethics Committee on Animal Use (CEUA-UFMG, Brazil, protocol 364/2018). They were randomly divided into groups of six and housed in a controlled environment (with a temperature of 25 °C, a 12 h/12 h light/dark cycle and ad libitum access to food and water). For the *in vivo* experiment, animals were divided into 5 groups of 18 C57BL6 mice. One control group received no DSS (naïve control) and four other groups received DSS. Mice were gavaged daily with 400 mg (per day per animal) of cheese prepared as described above, or with PBS (Phosphate-Buffered Saline). Cheeses were suspended in PBS buffer pH 7.4. Firstly, 400 mg of cheese were resuspended in 500 µL PBS and homogenised with the aid of the IKA T 10 Basic Ultra Turrax homogeniser probe (IKA®-Werke GmbH & Co. KG, Staufen, Germany) for 2–3 min. Mice were fed by intragastric gavage for seven consecutive days: 500 µl of PBS buffer, or 400 mg of the germ-

free dairy matrix, or 400 mg of single-strain cheese or 400 mg of Emmental cheese. This amount of cheese was set to provide 10^9 CFU of *P. freudenreichii*, for the single-strain cheese and the Emmental cheese, as described previously [19,20]. The maximal volume given daily by gavage was set according to the good practice guide to the administration of substances [36]. Then, DSS-colitis was induced by adding 3% dextran sulfate sodium (DSS) (36–50 kDa, CAT 260110, LOT Q5756 MP Biomedicals, Illkirch-Graffenstadenn, France), to the drinking water for 7 days. Among each group of 18 mice, all mice were analysed for weight loss, colon length and DAI (disease activity index) as indicated below. Among each group of 18 mice, 6 mice were randomly selected for histological analysis using the Swiss-roll technic, 6 other mice were randomly selected for RT-PCR (Reverse Transcription Polymerase Chain Reaction) analysis of gene expression, and 6 other mice were randomly selected for ELISA quantification of cytokines.

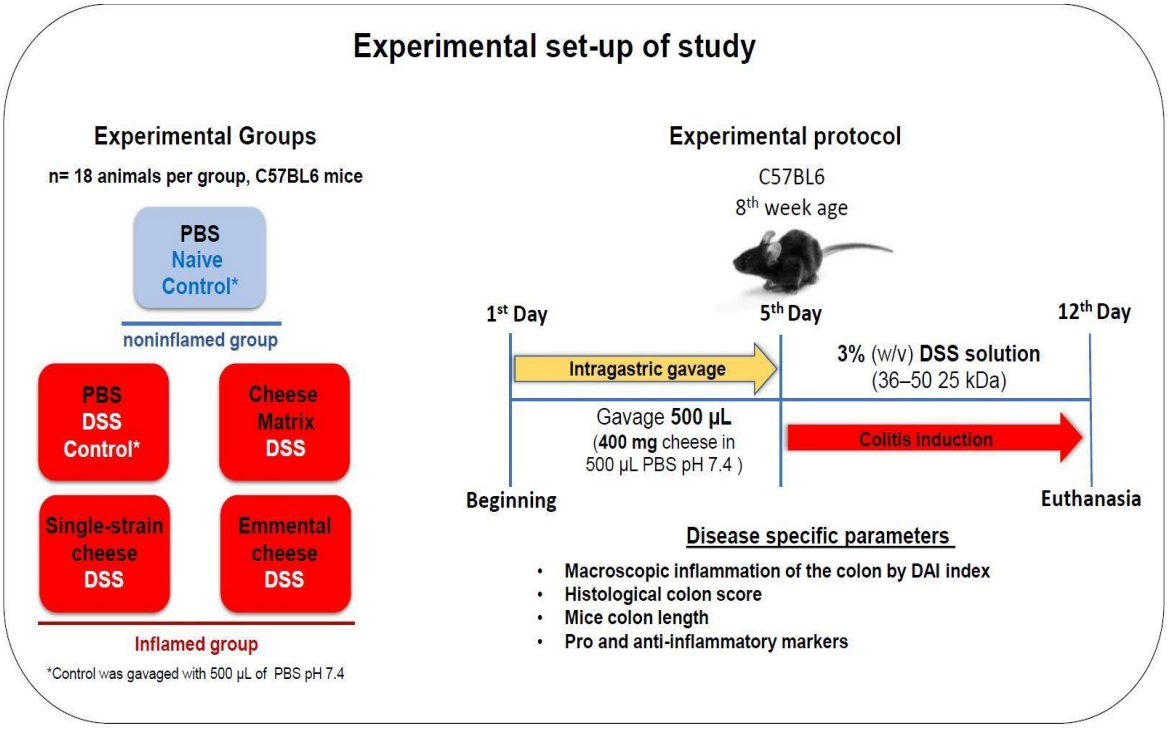

Figure 1. Experimental design of the evaluation of anti-inflammatory effects of a preventive intervention implementing *P. freudenreichii*-fermented cheeses, in the context of DSS-colitis. C57BL6 mice were divided into 5 groups, receiving different pre-treatments for 5 days, prior to induction of colitis. Colitis was then induced using 3% DSS in drinking water for 7 days prior to euthanasia. Different disease parameters were monitored to study the severity of colitis.

As a negative control, for each preventive treatment (germ-free cheese matrix control, single strain *P. freudenreichii* cheese or industrial Emmental cheese), one group constituted of 6 mice was gavaged accordingly and left without DSS prior to euthanasia.

2.4. Assessment of DSS-Induced Colitis

The severity of colitis was assessed for 7 days before animal sacrifice. Weight loss, stool consistency and blood content were assessed. The DAI (disease activity index) score was calculated based on these markers, as previously described [37]. For each parameter, a score was given: absent (0), mild (1), moderate (2) and severe (3). The disease activity index corresponds to the sum of different scores.

2.5. Histology

Histomorphological analyses were conducted as follows. Briefly, the distal portion of the mice colon was collected after the euthanasia and gently washed with PBS. Colon tissue samples were immersed in formaldehyde solution (4% *v*/*v*) for tissue fixation (Sigma-Aldrich, St. Louis, MO, USA). The material was then embedded in paraffin, and a 4µm section of each sample was placed on a glass slide and stained with Hematoxylin-Eosin (Sigma-Aldrich). Slide images from each experimental group were captured (using a 20x objective) on a Spot Insight Color digital camera attached to the Olympus BX-41 Microscope using SPOT® version 3.4 capture software. The score was determined according to McCafferty et al. [38], as the following features: extent of destruction of normal mucosal architecture (0: normal; 1: mild; 2: moderate; and 3: extensive damage), presence and degree of cellular infiltration (0: normal; 1: mild; 2: moderate; and 3: transmural infiltration), extent of muscle thickening (0: normal; 1: mild; 2: moderate; and 3: extensive thickening), presence or absence of crypt abscesses (0: absent; 1: present) and the presence or absence of goblet cell depletion (0: absent; 1: present). Mice were scored blindly by an expert pathologist.

2.6. Quantification of Secretory IgA in the Small Bowel Content

After mice sacrifice, the small bowel was collected. The content was washed using 10 mL of PBS. The intestinal contents were then vortexed and centrifuged (850× *g*, 30 min, 4 °C) as previously described [39]. The pellet was discarded while IgAs were quantified in the supernatant. Measurement of the levels of secretory IgA (sIgA) were determined by Enzyme-Linked Immunosorbent Assay (ELISA) in small bowel intestinal fluids. Microtiter plates Nunc-Immuno Plates, MaxiSorpTM (Nunc, Roskilde, Denmark) were coated with goat antibodies directed against mouse IgA, diluted 1:2000 in coating buffer antibodies (Southern Biotechnology, Birmingham, AL, USA) for 18 h at 4 °C. The plates were washed with saline (NaCl 0.9%) added with Tween 20 (0.05%) (Vetec, Rio de Janeiro, Brazil) and blocked with 200 µL PBS-casein (0.05%) for 1 h at room temperature. Intestinal fluid samples were diluted in PBS-casein (0.25%) and then added to the plate. After incubation for 1 h at room temperature, the wells were washed and biotin-conjugated anti-mouse IgA antibody (Southern Biotechnology Associates, Inc., Birmingham, AL, USA) diluted in PBS-casein (0.25%) (1: 10,000) and incubated

for 1 h at 37 °C. Then, peroxidase-conjugated streptavidin (1:10,000) was added (Southern Biotechnology Associates, Birmingham, AL, USA). After 1 h of incubation, 100 µl of orthophenylenediamine (OPD) (Sigma Aldrich) and H_2O_2 (0.04%) were added to each well. Plates were kept away from light until the coloration developed. The reaction was stopped by addition of 2N H_2SO_4. Reading was performed on a Model 450 Microplate Reader (Bio-Rad, Philadelphia, PA, USA), at 492 nm absorbance. The results were measured in concentration of sIgA (µg) per ml of intestinal fluid, according to the standard curve.

2.7. Gene Expression Analysis in the Distal Colon

The distal colon was cut into 1 cm fragments which were collected and stored in RNAlater at -80 °C until RNA extraction according to [40]. Total RNA was isolated using RNeasy mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Residual DNA was digested by adding RNas-free DNase I (Thermofisher Scientific, Bordeaux, France). Samples were then treated with Turbo DNA free Kit® (Thermofisher Scientific). cDNA for each sample was produced with high capacity cDNA Reverse Transcription kit (Thermofisher Scientific). Quantitative PCR (Polymerase Chain Reaction) was performed using iTaq universal SYBR green supermix (Thermofisher Scientific) and by using gene specific primers for colonic cells (Table S3). Actin and GAPDH genes were used as housekeeping genes. Amplification reactions were performed on an ABI PRISM 7900HT Sequence detection system (Thermofisher Scientific). The amplification cycle consisted of the following steps: 95 °C for 30 s, and 40 cycles of 95 °C for 15 s and 60 °C for 30 s. The results of gene expression of the control group (with no treatment) were used as calibration data. Expression levels are represented as fold changes ($2^{-\Delta\Delta Ct}$), using the means and standard deviation of target genes.

2.8. Tissues Preparation and Cytokines Quantification by ELISA

Colon fragments (100mg for each mouse) were homogenized in 1mL of PBS buffer containing 0.05% tween-20 (Vetec, Rio de Janeiro, Brazil), 0.1 mM phenylmethylsulfonyl fluorid (MP Biomedicals, Solon, Ohio, USA), 0.1 mM benzethonium chloride (Sigma-Aldrich), 10 mM EDTA (ethylenediaminetetraacetic acid) (Synth, Brazil) and 20 KIU aprotinin A (Sigma-Aldrich). Tissues mixtures were centrifuged (3.000× *g*, 10 min) and supernatants were collected for ELISA immunoassays using DuoSet ELISA kit (R&D Systems, Minneapolis, MN, USA). Plates (Nunc®, Sigma-Aldrich) were coated with purified monoclonal antibodies anti IL-10, IL-1β, IL-12p70, IL-17, IFN-γ, TGF-β, TNF-α and IL-6, overnight at 4°C. Plates (Nunc-Immuno Plates, MaxiSorp) were washed by TBS (Tris-buffered saline) and supernatant from homogenized colon tissues were added. Plates then were incubated overnight at 4 °C. After plates washing, biotinylated monoclonal antibodies against different cytokines were added to coated plates and incubated for 2 h at room temperature. The revelation was performed by adding 100 µl/well of a citrate buffer containing Orthophenyldiamine (Sigma-Aldrich) (1 mg/mL) and 0.04% (*v*/*v*) H_2O_2. Then, 2N H_2SO_4 solution was added to stop the reaction. The absorbance was measured at 492 nm using an ELISA reader (Bio-Rad, Philadelphia, PA, USA).

2.9. Statistical Analysis

The protective effect of the different cheeses in the DSS-induced colitis preventions data was analysed using two-way (weight monitoring) and one-way (all other biomarkers monitoring) ANOVA followed by Tukey multiple comparisons test. Statistical significance was set at $p < 0.05$. Statistical analyses were performed in GraphPad Prism version 7.00 for Windows (GraphPad Software, San Diego, CA, USA.). All data were expressed as mean values and standard deviation (SD).

3. Results

3.1. Both Experimental and Industrial Emmental Cheeses Contain P. freudenreichii CIRM-BIA 129

Microbiological analyses showed that P. freudenreichii CIRM-BIA 129 grew up to 1.10^{10} and up to 4.10^{9} CFU/g, in the experimental single-strain cheese and in the industrial Emmental cheese, respectively (Table S1). The experimental single-strain cheese contained P. freudenreichii CIRM-BIA 129 as the only bacterium. In Emmental, dairy propionibacteria constituted the main bacterial population, above 10^{9} CFU/g, while lactobacilli and streptococci were much lower, close to 10^{6} CFU/g. In Emmental, the identity of dairy propionibacteria was checked by Pulsed-field gel electrophoresis (PFGE) (Table S2 and Figure S1). P. freudenreichii CIRM-BIA 129 was the only propionibacterial strain present in the Emmental cheese. When looking at thermophilic lactobacilli, different strains were identified, but L. delbrueckii CNRZ327, used as starter, was predominant (60%). For S. thermophilus, four different strains were found and the starter strain LMD-9 was not the predominant one (Table S2 and Figures S2 and S3). These are non-starter lactic acid bacteria.

3.2. Emmental Cheese Mitigates DSS-Induced Colitis in Mice

In these experiments, we assessed the preventive effect of *P. freudenreichii*, consumed either alone in a single-strain experimental cheese, or together with *S. thermophilus* and *L. delbrueckii* in an industrial Emmental cheese, in the context of DSS-induced colitis in mice. Mice received 400 mg of cheese per day, which corresponds to a dose close to 10^{9} live propionibacteria per day. Figure 1 illustrates the experimental set-up of the experiment. General biomarkers of DSS-induced colitis severity were mitigated, as described below, in the context of colitis. As expected, the different cheeses (germ-free cheese matrix, single-strain cheese or Emmental cheese) failed to modify these biomarkers in healthy mice in the absence of DSS (data not shown).

3.3. Disease Activity Index, Body Weight Loss, Colon Length and Histological Score in DSS-Colitis Mice

DSS-induced colitis caused significant body weight loss in all mice groups, compared to the healthy group (PBS) at days 6 and 7(Figure 2A). However, consumption of the single-strain cheese and of the Emmental cheese attenuated the body weight loss, compared to the PBS-DSS and to the cheese matrix-DSS groups (Figure 2A). Indeed, at day 7, the weight of mice consuming both cheeses were significantly different from that of control colitis mice, while consumption of the placebo cheese matrix failed to limit weight loss. More precisely, Emmental cheese consumption significantly limited weight loss: −5.882% ± 3.275 ($p < 0.05$),

compared to control group DSS: −11.65% ± 5.368 (Figure 2B). By contrast, cheese matrix failed to limit weight loss (Figure 2B). As expected, DSS-induced colitis increased the disease activity index (DAI), which takes into account the body weight loss, the severity of diarrhoea, and the presence of blood in faeces (Figure 3A). The cheese matrix did not reduce the DAI, whereas consumption of the single-strain cheese or of the Emmental cheese significantly reduced the DAI, compared to the PBS-DSS group (Figure 3A). DSS-induced colitis caused colon shortening in all mice groups (Figure 3B). The intake of the cheese matrix, the single-strain cheese or the Emmental cheese did not prevent the colon shortening (Figure 3B). Regarding histological analysis of the mice colon, a variation of the histopathological score, depending on the treatment, was observed. DSS exposure drastically affected the mucosal architecture with ulcerations and extent of muscle thickening of the colon, as well as inflammatory cell infiltration, oedema and goblet cell depletion. Histopathological score was null in control conditions, while it was strongly increased following DSS treatment ($p < 0.0001$) (Figure 4). Consumption of Emmental cheese, prior to colitis induction, significantly reduced ($p < 0.05$) this score (Figure 4), as evidenced by limited destruction of the mucosal architecture and limited degree of cellular infiltration, compared to PBS-DSS control group (Figure 4). Altogether, these results show that the Emmental cheese reduced the severity of DSS-induced colitis.

3.4. Mice Intestinal IgA Secretory Production

Concentration of secretory IgA in the small intestine of all mice groups was quantified using ELISA.The DSS-induced colitis increased significantly the IgA secretion, from 8.63 ± 1.11 to 15.63 ± 1.02 µg/mL (Figure 5 and Supplemental Table S4). Consumption of control cheese matrix did not attenuate IgA secretion, whereas consumption of single-strain cheese or of Emmental cheese reduced it significantly (7.59 ± 1.71 and 5.51 ± 3.15), compared to the PBS-DSS mice group (Figure 5). There was no significant difference, in terms of IgA secretion, between the single-strain cheese and the Emmental cheese (Figure 5).

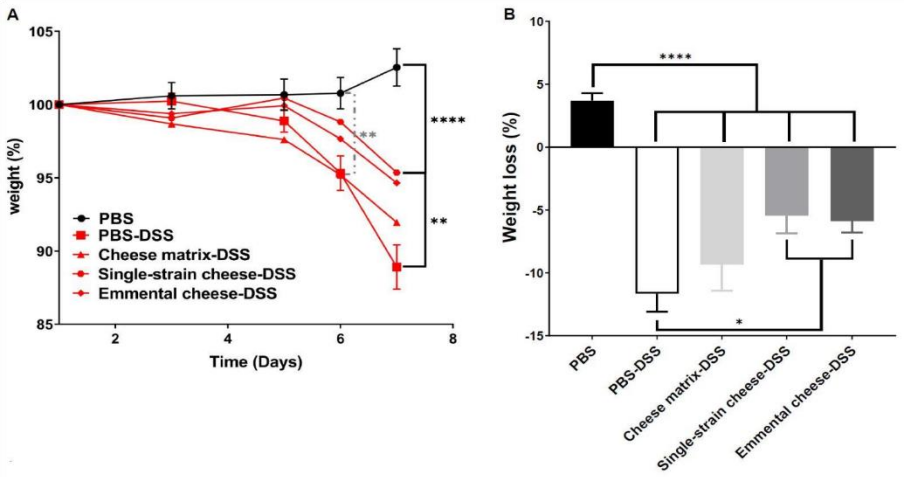

Figure 2. Impact of cheese matrix, single-strain cheese and Emmental cheese on colitis-induced body weight loss. (**A**) Time-course of mice body weight monitoring, and differences across groups. (**B**) Body weight loss observed at the 7th day of DSS colitis induction, and differences across groups. Groups were as follows. PBS: healthy group gavaged using PBS buffer as a sham. PBS-DSS: DSS-treated-colitis control group gavaged using PBS buffer as a sham. Cheese matrix-DSS: DSS-treated group gavaged using a germ-free dairy matrix. Single-strain cheese-DSS: DSS-treated group gavaged using an experimental single-strain cheese containing *P. freudenreichii* CIRM-BIA 129 as a sole bacterium. Emmental-DSS: DSS-treated group gavaged using an industrial Emmental cheese produced using *P. freudenreichii* CIRM-BIA 129 as a ripening starter. The data represent the mean ± SD of 18 mice per group. Multiple comparisons were performed, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ **** $p < 0.0001$.

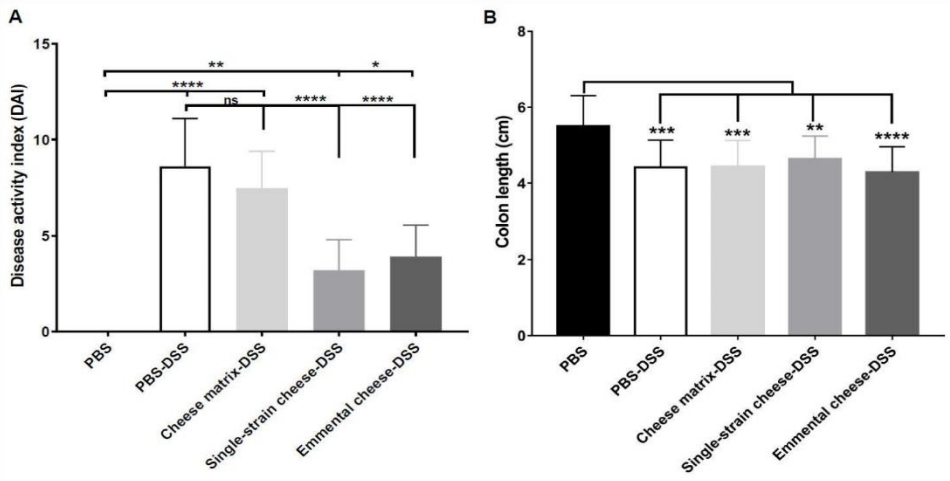

Figure 3. Impact of cheese matrix, single-strain cheese and Emmental cheese on the severity of DSS-induced colitis. Seven days after colitis induction, disease activity index (DAI) (**A**) and Colon length (**B**) were determined. Groups were as follows. PBS: healthy group gavaged using PBS buffer as a sham. PBS-DSS: DSS-treated-colitis control group gavaged using PBS buffer as a sham. Cheese matrix-DSS: DSS-treated group gavaged using a germ-free dairy matrix. Single-strain cheese-DSS: DSS-treated group gavaged using an experimental single-strain cheese containing *P. freudenreichii* CIRM-BIA 129 as a sole bacterium. Emmental-DSS: DSS-treated group gavaged using an industrial Emmental cheese produced using *P. freudenreichii* CIRM-BIA 129 as a ripening starter. The data represent the mean ± SD of 18 mice per group. Multiple comparisons were performed, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ **** $p < 0.0001$.

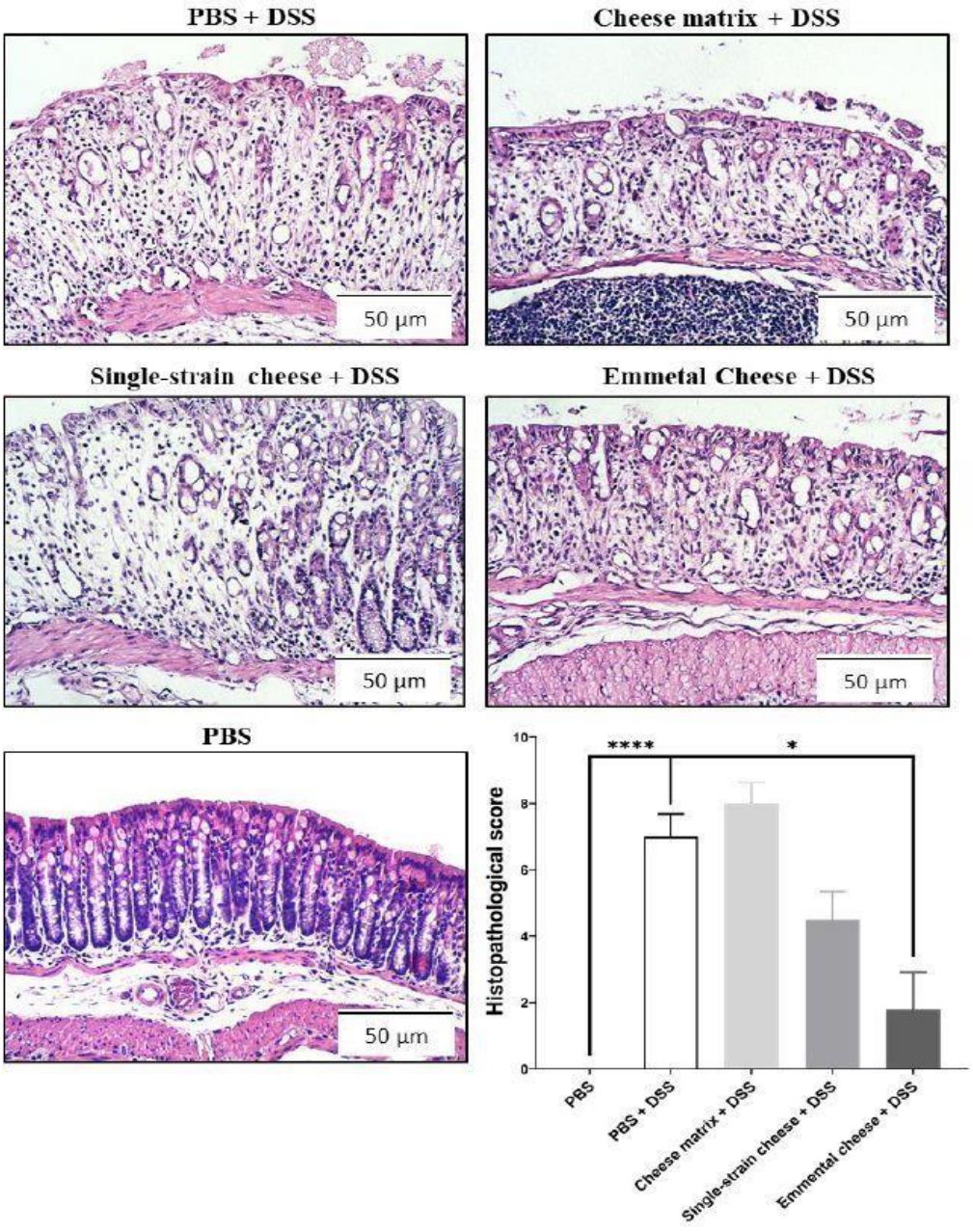

Figure 4. Impact of cheese matrix, single-strain cheese and Emmental cheese on DSS-induced histopathological damages. Representative images of mice colon mucosa sections, stained with haematoxylin, are shown. Image acquisition phase was done with a 20× magnification objective. Scale bar = 50 µm. Histopathological scores were determined. Groups were as follows. PBS: healthy group gavaged using PBS buffer as a sham. PBS-DSS: DSS-treated-colitis control group gavaged using PBS buffer as a sham. Cheese matrix-DSS: DSS-treated group gavaged using a germ-free dairy matrix. Single-strain cheese-DSS: DSS-treated group gavaged using an experimental single-strain cheese containing *P. freudenreichii* CIRM-BIA 129 as a sole bacterium. Emmental-DSS: DSS-treated group gavaged using an industrial Emmental cheese produced using *P. freudenreichii* CIRM-BIA 129 as a ripening starter. The data represent the mean ± SD of 6 mice per group. Multiple comparisons were performed, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ **** $p < 0.0001$.

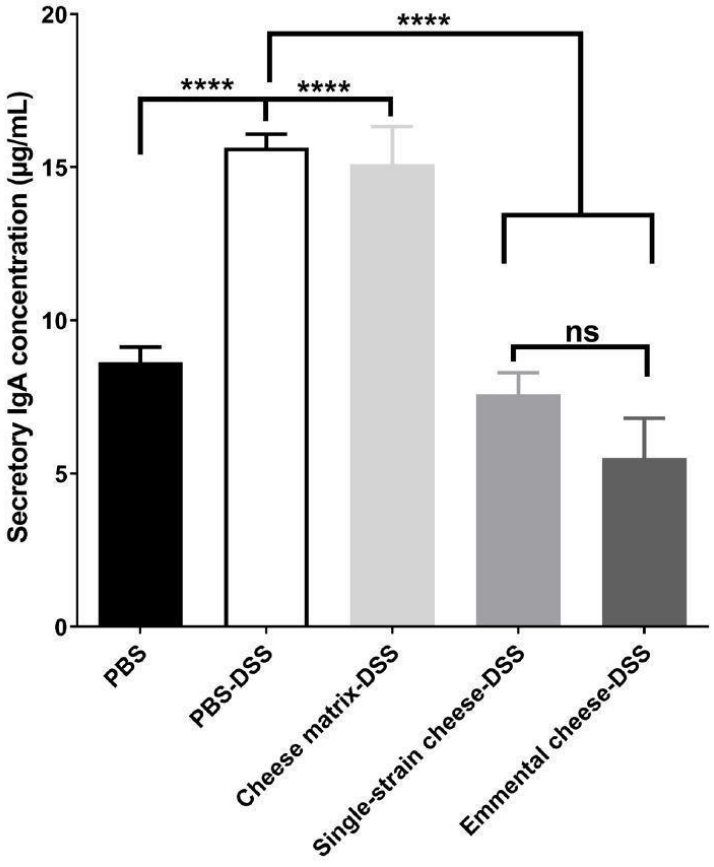

Figure 5. Impact of cheese matrix, single-strain cheese and Emmental cheese intake on small bowel IgA secretion. Secretory IgA concentration in the small bowel content was determined by ELISA quantification. The data represent the mean ± SD of 18 mice per group. Groups were as follows. PBS: healthy group gavaged using PBS buffer as a sham. PBS-DSS: DSS-treated-colitis control group gavaged using PBS buffer as a sham. Cheese matrix-DSS: DSS-treated group gavaged using a germ-free dairy matrix. Single-strain cheese-DSS: DSS-treated group gavaged using an experimental single-strain cheese containing *P. freudenreichii* CIRM-BIA 129 as a sole bacterium. Emmental-DSS: DSS-treated group gavaged using an industrial Emmental cheese produced using *P. freudenreichii* CIRM-BIA 129 as a ripening starter. Multiple comparisons were performed, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ **** $p < 0.0001$.

3.5. Mice Colonic Oxidative Stress and Epithelial Barrier

Consumption of a pure culture of *P. freudenreichii* was previously shown to modulate colic expression of markers of inflammation, of oxidative stress, and of gut epithelial barrier integrity, in TNBS-colitis mice [19,20]. We sought here such modulation, as a result of *P. freudenreichii*-containing cheese, in DSS-colitis mice. The gene expression of different markers of the colonic oxidative stress and epithelial barrier integrity was assessed by quantitative RT-PCR. No significant change was observed regarding expression of genes involved in epithelial barrier integrity, as *claudind1*, *mucin 2*, *zonula occludens1* and *zonula occludens2* genes (data not shown). However, DSS-induced colitis triggered a significant decrease of *occludin* gene expression, which was

prevented by consumption of Emmental cheese, yet not of the cheese matrix (Figure 6A). DSS-induced colitis triggered an oxidative stress in colonic cells as indicated by the induction of nitrite oxide synthase (*iNOS*) gene expression (Figure 6B). All the dairy products tested here, including the cheese matrix, the single-strain experimental cheese, and the Emmental cheese, prevented this *iNOS* induction.

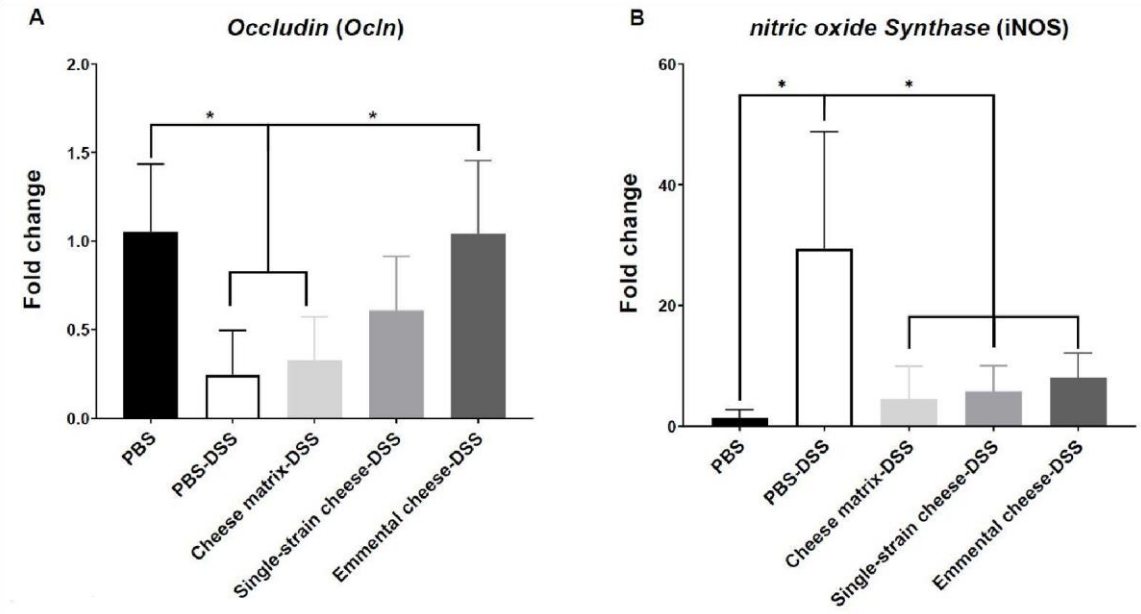

Figure 6. Impact of cheese matrix, single-strain cheese and Emmental cheese on colonic expression of markers of cell barrier and oxidative stress. Colonic mRNA expression levels of (**A**) Ocln and (**B**) iNOS genes were analysed. The data represent the mean ± SD of 6 mice per group. Groups were as follows. PBS: healthy group gavaged using PBS buffer as a sham. PBS-DSS: DSS-treated-colitis control group gavaged using PBS buffer as a sham. Cheese matrix-DSS: DSS-treated group gavaged using a germ-free dairy matrix. Single-strain cheese-DSS: DSS-treated group gavaged using an experimental single-strain cheese containing *P. freudenreichii* CIRM-BIA 129 as a sole bacterium. Emmental-DSS: DSS-treated group gavaged using an industrial Emmental cheese produced using *P. freudenreichii* CIRM-BIA 129 as a ripening starter. Multiple comparisons were performed, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ **** $p < 0.0001$.

3.6. Pro-Inflammatory and Anti-Inflammatory Gene Expression in Mice Colon

Consumption of *P. freudenreichii* was also reported to modulate colic expression of cytokines in mice. We sought such modulation, as a result of *P. freudenreichii*-containing cheese, in DSS-colitis mice. The expression of pro-inflammatory and anti-inflammatory cytokines genes expression in the colonic cells was assessed in all mice groups. No significant modification of expression of *Tgf*β*1* or of *IL21* was observed (data not shown). DSS-induced colitis did not modify *IL10* expression (Figure 7. A). Similarly, consumption of the cheese matrix and of the single-strain cheese did not change *IL10* and *Tgf*β*1* expression (data not shown). However, consumption of Emmental cheese enhanced *IL10* expression in the DSS-induced colitis group, compared to all mice groups (Figure 7A). DSS-induced colitis did not induce significant change in *IL1*β and *Tnf*α expression in colonic cells, compared to the healthy group (Figure 7B,C). However, the single-strain cheese

intake enhanced *IL1*β expression during DSS colitis, compared to the healthy group (Figure 7B). The cheese matrix, the single-strain cheese and the Emmental cheese decreased significantly *Tnf*α expression, compared to the healthy group (Figure 7C). In addition, these three dairy products all attenuated the increase of *Ifn*γ expression triggered by DSS (Figure 7D).

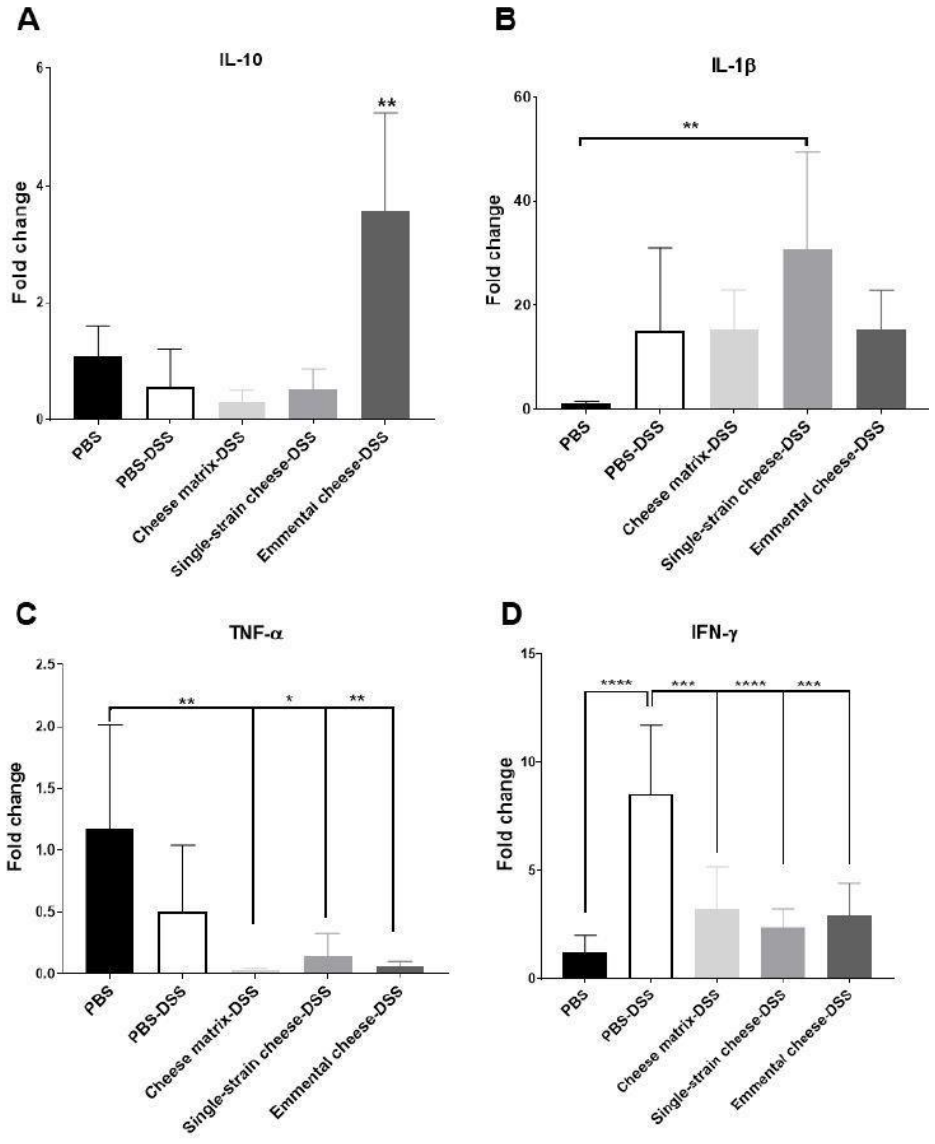

Figure 7. Impact of cheese matrix, single-strain cheese and Emmental cheese on colonic expression of cytokines genes during DSS-induced colitis. Colonic mRNA expression levels of (**A**) IL-10, (**B**) IL-1β, (**C**) TNFα and (**D**) IFNγ were determined. Data represent the mean ± SD of 6 mice per group. Groups were as follows. PBS: healthy group gavaged using PBS buffer as a sham. PBS-DSS: DSS-treated-colitis control group gavaged using PBS buffer as a sham. Cheese matrix-DSS: DSS-treated group gavaged using a germ-free dairy matrix. Single-strain cheese-DSS: DSS-treated group gavaged using an experimental single-strain cheese containing *P. freudenreichii* CIRM-BIA 129 as a sole bacterium. Emmental-DSS: DSS-treated group gavaged using an industrial Emmental cheese produced using *P. freudenreichii* CIRM-BIA 129 as a ripening starter. Multiple comparisons were performed, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ **** $p < 0.0001$.

3.7. Pro-Inflammatory and Anti-Inflammatory Cytokines Concentration in Mice Colon

The concentration of pro-inflammatory and anti-inflammatory cytokines in the colonic tissues was assessed in all mice groups by ELISA quantification (Figure 8.). DSS-induced colitis increased IL-10 secretion, compared to the healthy group (Figure 8A). The cheese matrix intake further enhanced secretion of IL-10 during DSS-induced colitis, compared to the healthy group (Figure 8A). However, fermented cheeses consumption reduced IL-10 induction (Figure 8A). DSS-induced colitis increased TGFβ secretion in the PBS-DSS and the cheese matrix-DSS groups (Figure 8B). The Emmental cheese and the single-strain cheese ingestion did not influence significantly TGFβ secretion, compared to the DSS-PBS and the healthy groups (Figure 8B). Concerning IL-6, its concentration was increased during colitis, while this increase was prevented by Emmental cheese consumption (Figure 8C). Similarly, only Emmental cheese attenuated IFNγ secretion induced by the DSS-induced colitis (Figure 8D). DSS-induced colitis increased IL-17 secretion, compared to the healthy group, which is exacerbated by the cheese matrix intake (Figure 8E). Only Emmental cheese was able ($p < 0.05$) to decrease IL-17 secretion, compared to the PBS-DSS group (Figure 8E). DSS-induced colitis induced a significant increase of TNFα only in the cheese matrix group (Figure 8F). DSS-induced colitis did not induce significant change in IL-12 secretion, compared to the healthy group (Figure 8G). Cheese intake did not either alter significantly IL-12 secretion, compared to healthy group (Figure 8G). Finally, DSS-induced colitis increased IL-1β secretion compared to the healthy group, and this was not attenuated by the Emmental cheese intake (Figure 8H).

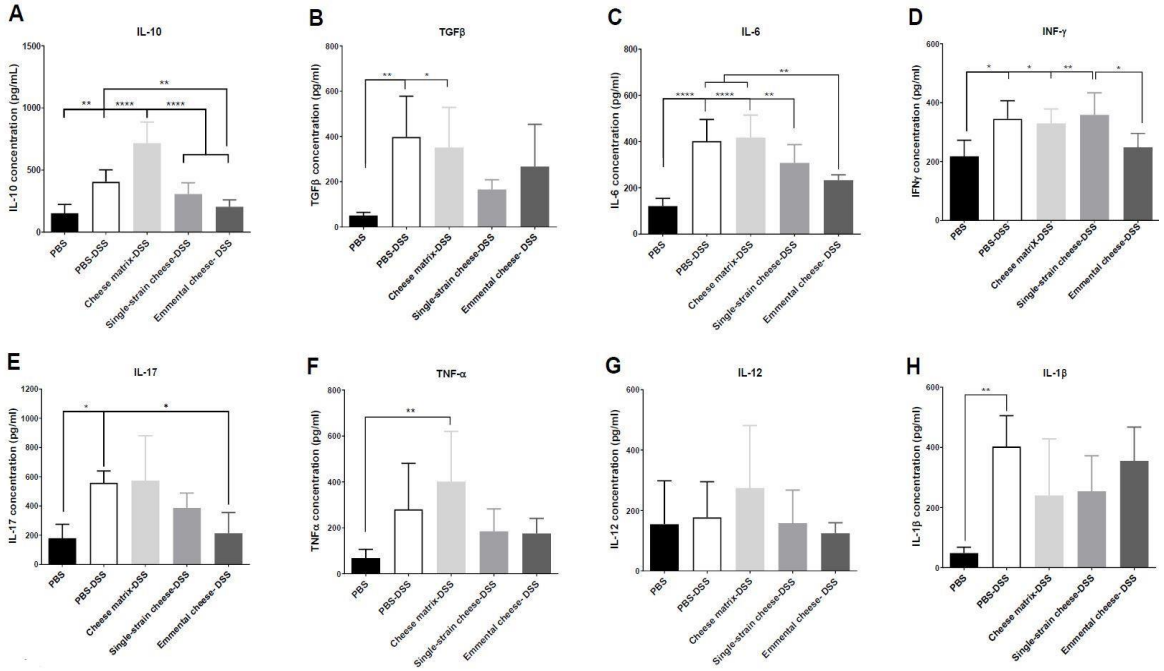

Figure 8. Impact of cheese matrix, single-strain cheese and Emmental cheese on colonic secretion of cytokines during DSS-induced colitis. Cytokines concentration of (**A**) IL-10, (**B**) TGFβ, (**C**) IL-6, (**D**) IFNγ, (**E**) IL-17, (**F**) TNFα, (**G**) IL-12 and (**H**) IL-1β were quantified by ELISA. Data represent the mean ± SD of 6 mice per group. Groups

were as follows. PBS: healthy group gavaged using PBS buffer as a sham. PBS-DSS: DSS-treated-colitis control group gavaged using PBS buffer as a sham. Cheese matrix-DSS: DSS-treated group gavaged using a germ-free dairy matrix. Single-strain cheese-DSS: DSS-treated group gavaged using an experimental single-strain cheese containing *P. freudenreichii* CIRM-BIA 129 as a sole bacterium. Emmental-DSS: DSS-treated group gavaged using an industrial Emmental cheese produced using *P. freudenreichii* CIRM-BIA 129 as a ripening starter. Multiple comparisons were performed, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ **** $p < 0.0001$.

4. Discussion

Previous studies indicated the protective role of the consumption of selected strains of *Propionibacterium freudenreichii*, in the context of TNBS-induced colitis in mice [14,19,20]. In this study, we investigated the protective effect of the consumption of a "probiotic" Emmental cheese, in the context of DSS-induced colitis. This Emmental cheese was manufactured at an industrial scale in a local cheese maker's plant and starter bacteria were selected for their immunomodulatory properties: *P. freudenreichii* CIRM-BIA 129 [20], *L. delbrueckii* subsp. *lactis* CNRZ327 [23] and *S. thermophilus* LMD9 [25]. At the end of ripening, propionibacteria constituted the main flora, above 10^9 CFU.g^{-1}, which is typical of Emmental cheese [41,42], and the strain *P. freudenreichii* CIRM-BIA 129 was the only one detected, as shown by PFGE analysis. Thermophilic streptococci and lactobacilli were much lower, close to 10^6 CFU.g^{-1}, which is also typical, as these bacteria grow up to 10^8 to 10^9 CFU.g^{-1}, during curd fermentation, and then experience massive cell death during Emmental ripening [41,42]. Although *L. delbrueckii subsp. lactis* CNRZ327 was the predominant lactobacillus, they contained the strains added as starters, as well as other strains of thermophilic streptococci and lactobacilli. Indeed, non-starter lactic acid bacteria are usually found in Emmental [41–43].

In this study, Emmental probiotic cheese was compared to a previously described model of experimental single-strain cheese [19], fermented only by *P. freudenreichii* CIRM-BIA 129, and to a germ-free cheese placebo matrix, in a DSS-induced colitis model. No mortality was observed. DSS consumption caused an abrupt weight loss in the control group (DSS). However, the pretreatment with the Emmental cheese prevented it. Both Emmental cheese and single-strain cheese attenuated colitis severity and reduced inflammation, as evidenced by the disease activity index. However, only Emmental cheese was able to decrease the histopathological score compared to control group (DSS). These results are consistent with previous studies dealing with propionibacteria [19,20,23,24]. *P. freudenreichii* CIRM-BIA 129 alone, or in combination with lactic acid bacteria, attenuated TNBS-induced colitis [12,14,19,20]. However, neither of the two fermented cheeses prevented the colon shortening triggered by DSS. Similarly, in a previous study, consumption of *P. freudenreichii* CIRM-BIA 129 in combination with lactic acid bacteria did not prevent colon shortening during TNBS-induced colitis [44]. DSS-induced colitis increased IgA secretion in the small bowel, while both single-strain and Emmental cheeses attenuated this increase. Increased IgA secretion is the expression of ileal barrier disturbance by the inflammatory response. Reduced inflammation, as a result of cheese consumption, thus led to attenuated IgA response, suggesting that DSS-induced colitis would affect not only the large intestine but also the small intestine [45,46]. Accordingly, *P. freudenreichii* CIRM-BIA 129 was

shown to reduced IgA response in the context of 5-fluorouracyl-induced mucositis [47]. Indeed, disruption of the gut barrier integrity is a key step of inflammatory bowel diseases [48]. DSS-induced colitis induced a significant increase of nitric oxide synthase (iNOS) expression in the colon tissues and this increase was attenuated by the three tested dairy products. However, only Emmental cheese restored colonic expression of the *Ocln* gene encoding the transmembrane protein *occludin*, which contributes to intestinal barrier function [49].

Cytokines are major mediators of colitis pathogenesis [5]. We thus assessed expression and concentration of cytokines in the colonic tissues. Results of cytokine expression were partially consistent with cytokine secretion results. DSS-induced colitis increased the secretion of IL-10 and of TGFβ, which is consistent with the observed induction of these cytokines in UC patients [50]. Both single-strain and Emmental cheeses attenuated this increase. However, only Emmental cheese, consumed as a protective pre-treatment, increased IL-10 gene expression in the colonic tissue, compared to all other groups. IL-10 is an anti-inflammatory cytokine which inhibits the production of IL-1β, IL-6, and TNF-α. Its increase has a protective effect towards colitis, only if it is triggered before DSS-colitis induction [51]. *L. delbrueckii subsp. lactis* CNRZ327, as well *P. freudenreichii* CIRM-BIA 129, were previously shown to increase, in animal models, the subset of Treg FOXP3+ cells, which produce a high amount of IL-10 [18,23,47]. Similarly, TGFβ is an anti-inflammatory mediator which is highly produced by mononuclear cells of UC patients [50]. Both single-strain and Emmental cheeses attenuated inflammation, and thus TGFβ secretion, in the context of DSS-colitis in this study.

Colitis increased secretion of IFNγ and of IL-6 and tended to increase the secretion of TNFα. At the expression level, only IFNγ expression was induced by DSS, compared to the healthy group. Consumption of the germ-free cheese matrix did not modulate the expression of TNFα, but decreased that of IFNγ, compared to the PBS-DSS group. However, the cheese matrix did not attenuate the secretion of IFNγ, TNFα and IL-6 triggered by the DSS. Both single-strain and Emmental cheeses attenuated expression of IFNγ gene but only Emmental cheese decreased the IFNγ secretion, compared to the PBS-DSS group. Similarly, Emmental cheese consumption decreased the secretion of IL-6. IFNγ, IL-6 and TNFα are pro-inflammatory cytokines highly secreted in the gut mucosa of UC patients [50]. TNF-α is produced by antigen-presenting cells and by macrophages. It induces the secretion of IL-6 and IFNγ, the typical cytokine of Th1 cells subset. IL-6 and TGFβ secretion can induce Th17 cells, which are involved in IBD pathogenesis [52,53]. Indeed, IL-17, the Th17 cells cytokine, was increased during DSS-induced colitis and tended to be attenuated (p = 0.06) by the Emmental cheese administration only. Taken together, these results show that consumption of the complex Emmental cheese and of the single-strain cheese triggered different mechanisms. Propionic acid bacteria in Emmental cheese are predominant, while lactic acid bacteria undergo massive lysis during cheese ripening [54–56]. Killed lactic acid bacteria were, however, shown to modulate chemically-induced colitis [57–60]. The anti-inflammatory properties of lactic acid bacteria are probably mediated by cell wall components of lysed or dead cells. The interactions between propionic acid and lactic acid bacteria are poorly characterized and may modulate their probiotic properties. As an example, on one hand, the proteolytic activity of lactic acid bacteria

can affect propionic acid bacteria immunomodulatory surface proteome, as well as their ability to produce beneficial metabolites [13,61]. On the other hand, lactic acid bacteria proteolysis may cause liberation of bioactive peptides from caseins, which can participate to the anti-inflammatory property of an Emmental cheese [62,63]. Further studies are needed to decipher interactions, between propionic and lactic acid bacteria, and how they affect their probiotic attributes. These studies will provide screening criteria to choose the most effective lactic and propionic acid bacteria to develop anti-inflammatory functional foods.

As a conclusion, an Emmental cheese, produced in industrial conditions, using well-characterized immunomodulatory starter strains, was able to mitigate the severity of DSS-induced colitis in a mice model. This protective effect seems to result from a synergy between lactic acid and propionic acid bacteria. It opens new perspectives for clinical studies on patients suffering from ulcerative colitis. Further clinical studies, implementing *P. freudenreichii*, should also take into account its interaction with the human gut microbiota. Previous studies reported a bifidogenic effect for propionibacteria consumption [64,65]. The modulation of other potent symbionts, including *Akkermansia*, *Lactobacillus* and *Faecalibacterium* species, should also be evaluated. Conversely, modulation of potent pathobionts, with a pro-inflammatory potential, deserves attention. As an example, sulfate-reducing bacteria were reported to participate to inflammation in experimental colitis [66]. They contribute to homeostasis disruption during intestinal inflammation, by promoting intestinal damage through generation of hydrogen sulfide at high levels [67]. Indeed, colitis correlates with a modified sulfate-reducing bacteria community in mice, exhibiting enhanced production of hydrogen sulphide [68]. This last may in turn affect the gut microbiota and probiotic efficacy [69]. Indeed, some lactobacilli, such as *L. reuteri*, *L. pentosus* and *L. paracasei*, may be extremely sensitive to hydrogen sulphide [70]. Conversely, lactic acid bacteria in the small bowel may provide lactate, which serves as an electron donor, while sulfate serves as an electron acceptor, in the production of hydrogen sulphide, as a toxic product in the small-large intestinal axis [71]. Its inhibitory effect on probiotic bacteria, including lactobacilli and propionibacteria, should thus be taken into account. Complex interactions between probiotic fermented dairy products and the human gut microbiota, as well as its metabolites, will determine their beneficial role in the context of gut inflammation.

Supplementary Materials: Supplementary materials can be found at http://www.mdpi.com/2076-2607/8/3/380/s1. **Figure S1**: PFGE analysis of Propionibacterium freudenreichii clones, **Figure S2:** PFGE analysis of thermophilic streptococci clones, **Figure S3:** PFGE analysis of themophilic lactobacilli clones, **Table S1:** Microbiological analysis of the Emmental cheese, the single-strain cheese and the cheese matrix, **Table S2:** PFGE analysis of different colonies isolated from petri dishes done for *P. freudenreichii*, *Lactobacillus* and *S. thermophilus* enumeration in the Emmental cheese, **Table S3:** Specific primer sequences in order to target murine genes analysed in the study, **Table S4:** Secretory IgA in the different experimental mice groups.

Author Contributions: H.R., F.L.R.d.C., R.D.d.O.C., B.F.C., S.H.d.S., E.R.O., L.L., D.C.C. and A.M.C.F. performed animal experiments. G.G., M.H.-O., H.R. and G.J. performed microbiology and experimental cheese development. Y.L.L., V.A., G.B. and G.J. designed and supervised the study. All the authors participated to the writing of the manuscript. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

Cox	Cyclooxygenase
DAI	Desease Activity Index
DSS	Dextran Sodium Sulphate
Hmox	Heme oxigenase
IFN	Interferon
Ig	Immunoglobulin
IBD	Inflammatory Bowel Disease
Th	T helper
TNF	Tumor Necrosis Factor
UC	Ulcerative Colitis
Zo1	Zonula occludens 1
Pparg	Peroxisome proliferator-activated receptor gamma

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CAPÍTULO 2

Desenvolvimento do queijo Prato contendo *Lactobacillus casei* 01 na prevenção de colite ulcerativa

A fim de desenvolver um queijo probiótico tipicamente brasileiro, decidiu-se por produzir um queijo do tipo Prato contendo a bactéria *Lactobacillus casei* 01 (*L. casei* 01) e testar o seu potencial probiótico em modelo de colite ulcerativa induzido por DSS, semelhante ao utilizado no capítulo 1 deste trabalho.

A escolha do queijo Prato se deu, principalmente, porque estudos anteriores já haviam demonstrado que o queijo do tipo Prato possuía uma boa capacidade de proteger bactérias probióticas durante os processos industriais e um bom desempenho em estudos *in vivo,* na prevenção do desenvolvimento de cálculos renais em modelo de rato (MARTINS et al., 2018; SILVA et al., 2018a, 2018b, 2017). Além disso, o queijo Prato é um queijo gordo, de média umidade e consistência semidura muito consumido no território brasileiro, principalmente pela população de baixa renda (ALVES et al., 2017; BRASIL, 1997). O produto é considerado o mais importante queijo curado do Brasil, representando 20% do total de queijos produzidos no país (SILVA et al., 2018a).

A escolha da linhagem *L. casei* 01 se deu devido a trabalhos anteriores que comprovaram os benefícios do consumo desta bactéria na redução dos sintomas de infecção intestinal por microrganismos patogênicos e pelas suas atividades antioxidantes, bem como demonstrou-se diminuição dos níveis de colesterol e atividade antitumorogênica (GALDEANO; PERDIGO, 2006; SPERRY et al., 2018). Além disso, o consumo da *L. casei* 01 por pacientes com artrite reumatoide acarretou uma melhora na resposta imune e uma consequente redução do processo inflamatório dos pacientes (ALIPOUR et al., 2014). Desta forma, a *L. casei 01* apresentava os atributos necessários para ser incorporada em um alimento funcional.

Os resultados do estudo do efeito probiótico do queijo Prato contendo a bactéria *L. casei* 01 na prevenção da colite ulcerativa induzida por DSS foram publicados na revista *International Dairy Journal*, no ano de 2019.

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Prato cheese containing *Lactobacillus casei* 01 fails to prevent dextran sodium sulphate-induced colitis

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abstract

The role of experimental probiotic Prato cheese containing *Lactobacillus casei* 01 in the prevention of dextran sodium sulphate (DSS)-induced ulcerative colitis in mice was evaluated. For the DSS *in vivo* model, mice were divided into six groups. Groups 1e3 represented noninflamed groups and groups 4e6 received a DSS (2%) solution. Mice from groups 1 and 4 intragastrically received 500 mL of phosphate- buffered saline (control groups), while mice from groups 2 and 5 intragastrically received 500 mL of conventional Prato cheese (control groups). Finally, mice from group 3 and 6 intragastrically received 500 mL of Prato probiotic cheese containing *L. casei* 01 (9.47 log cfu mL ^{1}). Groups treated with probiotic Prato cheese exhibited reduced weight loss caused by the consumption of DSS solution. However, the probiotic cheese failed to reduce the inflammation scores in DSS-induced colitis group. Overall, probiotic Prato cheese was not effective to ameliorate the symptoms of DSS-induced colitis.

1. Introduction

Inflammatory bowel diseases (IBD), including ulcerative colitis (UC) and Crohn's disease (CD), are disorders that severely affect the gastrointestinal tract (GIT), which can lead to irreversible impairment of its structure and function (Schreiber, Nikolaus, & Hampe, 1998). Although IBDs are marked by periods of clinical remission and relapse whose exact aetiology is still not well understood, scientific evidence suggests that they result from abnormal immune responses to the gut microbiota in individuals with genetic predisposition (Santos Rocha et al., 2014). Epidemiological studies have shown that the incidence of IBD has increased considerably throughout the world, thus becoming an important global public health problem (Ananthakrishnan, 2015). According to Kaplan (2015), the reported prevalence of Crohn's disease and ulcerative colitis in Northern America and Europe is approximately 300 per 100,000 inhabitants. However, in countries undergoing robust industrialisation, like South American countries, the incidence, and prevalence of IBDs has increased, mainly due to the drastic changes in eating habits together with the food production system (Ng et al., 2017). Moreover, these changes may alter the host's commensal microbiota and the immune response, in addition to the genetic predisposition of the individuals (Kaplan, 2015).

UC is the most common clinical form of IBD, and the main symptoms include abdominal pain, diarrhoea, rectal bleeding, malaise, and weight loss (Neurath, 2012). The inflammation during UC is limited to the colon and affects only the mucosa and submucosa layers of this segment, with the presence of oedema, goblet cell mucus depletion, changes in tissue architecture and ulcerations (Cho, 2008; Maloy & Powrie, 2011; Tontini, Vecchi, Pastorelli, Neurath, & Neumann, 2015). The treatments of UC are based on the administration of anti-inflammatories, immunosuppressive drugs, antibiotics or surgeries (Pithadia & Jain, 2011). Although such treatments reduce the inflammatory action of the disease and relieve symptoms, they are not curative and can lead to serious side effects in patients (Luerce et al., 2014). In this context, probiotic bacteria have been suggested as promising candidates for the UC

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treatment (Chibbar & Dieleman, 2015; Elsa, Chain, Sokol, Langella, & Bermudez-Humaran, 2017; Hosoya, Ogawa, Sakai, & Kadooka, 2012; Luerce et al., 2014; Santos Rocha et al., 2014).

Probiotics are live microorganisms that, when administered in adequate amounts, confer a health benefit on the host (Hill et al., 2014; WHO, 2002), and several strategies to improve their survival in food products have been reported (Champagne, Gomes da Cruz, & Daga, 2018). Probiotic bacteria have been extensively explored in inflammatory disease models with promising results. The therapeutic effects of probiotics are based on different mechanisms of action, which have been successfully demonstrated in experimental colitis animal models (Abraham & Quigley, 2017).

Among the probiotic microorganisms, *Lactobacillus casei* 01 stands out, a lactic acid bacteria strain that has been associated with health benefits, such as immune system stimulation, antioxidant activity, cholesterol lowering, anticarcinogenic activity and reduction in pathogen infection symptoms (Galdeano & Perdigo, 2006; Sperry et al., 2018). In addition, a clinical trial with rheumatoid arthritis (RA) patients showed an improved immune response in RA patients serum after the supplementation with a daily capsule of probiotic *L. casei* 01 (10^8 cfu), with an increase in anti-inflammatory cytokine IL-10 and, consequently, a reduction of the inflammation process caused by RA (Alipour et al., 2014); thus, *L. casei* 01 strain could be used as RA adjunctive therapy. Studies regarding probiotic lactic acid bacteria and previous finds concerning *L. casei* 01, reinforce the choice of this strain as a good candidate for a "2 in1" probiotic and dairy product.

A great number of potential health benefits have been associated with the regular intake of probiotic-containing products, especially fermented dairy products, including some types of cheese (Carmo et al., 2017). In addition, a single-bacteria cheese (*Propionibacterium freudenreichii*) was able to reduce experimental colitis in mice model (Ple et al., 2015). Strategies to improve the efficacy of the therapeutic effects through the addition of probiotics to food products have been studied, indicating the important role of bacterial viability factor (Champagne et al., 2018; Hill et al., 2014).

Cheese has been recognized as an adequate matrix for protection of probiotics, due to its high fat and protein contents, which can form complex coacervates that enable the microencapsulation of the probiotic bacteria for oral delivery (Chapeau et al., 2017; Cruz, Buriti, Souza, Faria, & Saad, 2009; Montassier et al., 2016), besides the benefits of regular ingestion confirmed by several authors in animal and human clinical models (Lollo et al., 2015; Sperry et al., 2018). Prato cheese is a ripened Brazilian cheese, accounting for 20% of all cheese produced in Brazil (Nepomuceno, Junior, & Costa, 2016). Previous studies have shown a good performance of Prato cheese as a functional food, i.e., a nutritional supplement with the ability to promote physiological benefits to the host, which is consumed as part of a regular diet. In fact, Prato cheese was shown to have the ability to protect the probiotic bacteria (*L. casei* 01) during ripening and storage in previous studies (Silva et al., 2017, 2018a,b), besides the good performance of *in vivo* studies on the prevention of development of renal calculi in rat model (Martins et al., 2018). It is important to perform different clinical trials using probiotics associated to foods which taken part of the normal diet of the a population to provide data that will help improve the understanding of the benefits caused by the probiotic strain as there is a consensus which they are related to the interaction among the probiotic strain and food matrix. In this context, the aim of this present study was to evaluate the role of probiotic Prato cheese made with *L. casei* 01 in the prevention of dextran sodium sulphate (DSS)-induced ulcerative colitis in mice.

2. Material and methods

2.1. Cheese processing

Two types of cheese were produced, as follows: a conventional cheese i. e. Prato cheese (starter culture consisting of *Lactococcus lactis* ssp. *lactis* and *Lc. lactis* ssp. *cremoris* R-704) and a probiotic cheese containing *L. casei* 01 (Chr. Hansen, Valinhos, Brazil), as described by Silva et al. (2018c). The bacteria viability during cheese processing and storage (30 days) was evaluated in a previous study (Silva et al., 2017). The experiment was conducted at the Advanced Centre in Food Technology (NATA), using 120 L of full-fat pasteurised milk (65 C, 30 min). Milk was cooled until 37-35 C, and the lactic acid bacteria starter (*Lc. lactis* ssp. *lactis* and *Lc. lactis* ssp. *cremoris* R-704) was added directly to the milk (1% w/v, 7e8 log cfu g^{-1}) and allowed to stand for 40 min. For the manufacture of probiotic cheese, the *L. casei* 01 were added together with the starter culture directly to the milk (2% w/v, about 7e8 log cfu g^{-1}) and allowed to stand for 40 min. Then, calcium chloride (80 mL per 120 L milk), annatto dye (36 mL per 120 L milk) and coagulant (Ha La 1175, Chr Hansen Industria e Comercio, Sao Paulo, Brazil) were added for milk coagulation within 35e50 min. The optimal curd set point was determined, and the curd was cut into 1 cm cubes and submitted to slow mixing for 15 min. Then, part (30%) of whey was removed, and further heating was carried out by progressively adding hot water at 80 C (25 L) to increase the temperature to 42 C (0.33 C min^{-1}), until reaching the typical consistency of Prato cheese. Then, the curd was placed in rectangular plastic moulds (2 kg) and pressed (0.1 MPa for 15 min; 0.24 MPa for 30 min; and 0.31 MPa for 90 min). Cheeses were kept for 5 h at room temperature and then dried at 12 C for 72 h, vacuum-packed, and stored at 12 C for 25 days.

2.2. Probiotic bacteria and lactic acid bacteria counts

M17 agar (Oxoid Brasil LTDA, Sao Paulo, Brazil) was used to enumerate *Lc. lactis*, incubated at 37 C for 72 h under aerobic conditions. The *L. casei* 01 counts were performed in duplicate using MRS agar (Oxoid Brasil LTDA, Sao Paulo, Brazil) containing vancomycin 0.1% (w/v), and incubated at 37 C for 72 h under anaerobic conditions (Silva et al., 2018a,b). Anaerobic jars (Anaerobac Probac Ltd.®) were used to generate an anaerobic atmosphere, thus ensuring anaerobic conditions.

2.3. Proximate composition, calcium and sodium levels

The proximate composition (moisture, protein, and fat; g 100 g^{-1}) and the mineral contents (Ca and Na) were determined according to the conventional methods (Silva et al., 2018a). Moisture was determined by oven-drying 5 g sample at 100e105 C for 24 h. Protein was determined by the Kjeldahl method, and fat was determined by the Gerber method. Ca and Na levels were determined by atomic absorption spectrometry in an air-acetylene flame using the iCE 3000 series atomic absorption spectrometer (Thermo-Scientific, Hemel Hempstead, Hertfordshire, UK).

2.4. Animal model for ulcerative colitis

Female C57/BL6 inbred mice strain of 8 weeks of age were obtained at Federal University of Minas Gerais (UFMGeBelo Horizonte, Brazil). Mice were kept in a temperature-controlled room with access to water and standard laboratory chow diet ad libitum. The study was approved by the Ethics Committee on Animal Experimentation of the Federal University of Minas Gerais (CEUA-UFMG, Brazil, protocol 340/2017).

2.5. Manufacture of probiotic cheese and conventional Prato cheese for in vivo model

Both cheeses were suspended in phosphate buffered saline (PBS; NaCl 8 g L^{-1}; KCl 0.2 g L^{-1}; NaH_2PO_4 1.44 g L^{-1}; K_2HPO_4 0.24 g L^{-1}; pH 7.4). Briefly, the cheeses were weighed and 250 mg of Prato cheese or probiotic cheese were resuspended in 250 mL PBS buffer pH 7.4 and homogenised with the aid of the IKA T 10 Basic Ultra Turrax homogeniser probe for 2 min. Samples were prepared daily according to this procedure, prior to intragastric gavage. Bacterial viability in cheese solution (Prato cheese or probiotic Prato cheese) was determined by cfu counts, which were performed according to the previously described protocol (section 2.2). Each mouse received 500 mL of cheese solution (Prato cheese or probiotic Prato cheese) by intragastric administration.

2.6. Pretreatment and DSS-induced colitis

The pretreatment with cheese (Prato cheese or probiotic Prato cheese) and colitis induction was performed according to schematic workflow (Fig. 1). The animals had free access to food before and during the DSS colitis induction phase. The intragastric administration of probiotic cheese and conventional Prato cheese solution was performed for 7 days before the beginning of the DSS colitis induction. At day 8, the colitis was chemically induced by administration of 2% (w/v) DSS aqueous solution (36e50 kDa, MP Biomedicals, CAT 260110, LOT Q5756), for 7 days (day 15). For the experimental *in vivo* study, mice were divided into six groups, each containing 5e6 animals per group. Animals from group 1e3 rep- resented the noninflamed group that drank DSS-free water, and consisted of: group 1 receiving intragastrically 500 mL PBS (group PBS); group 2 receiving intragastrically 500 mL conventional Prato cheese suspension (group Prato cheese) and group 3 gavage with 500 mL probiotic Prato cheese suspension containing *L. casei* 01 (group Probiotic cheese). Mice from group 4e6 received DSS (2%) solution as the only source of drinking water to cause colon inflammation, and consisted of: group 4 receiving 500 mL PBS (group PBS þ DSS); group 5 receiving 500 mL conventional Prato cheese suspension (group Prato cheese þ DSS) and finally, group 6 receiving 500 mL probiotic cheese suspension (group Probiotic cheese DSS). All mice were euthanized at day 15 (last day of the experimental). Xylazine (8 mg kg^{-1}) and ketamine (100 mg kg^{-1}) were administered by intraperitoneal route and mice were subsequently euthanised by cervical dislocation.

2.7. Weight monitoring, food intake, and liquid intake

Mice weight was individually measured every day until the end of the experiment (day 15). Weight change was expressed as the percentage of change in weight in relation to the initial body weight. Food intake and liquid intake was carefully measured throughout the experiment.

2.8. Colitis macroscopy analysis

The disease activity index (DAI) was determined as described by Murthy et al. (1993) by scoring three major colitis clinical signs: weight loss, diarrhoea, and rectal bleeding. All mice were sacrificed at day 15, and longitudinal abdominal incision was performed to remove the intestine and colon for further analyses. The colon length was measured from the cecum to the final portion of the rectum. The values obtained for each animal were used to calculate the mean of each group.

2.9. Colitis histomorphological analysis

For histomorphological analysis, the distal portion of the mice colon was collected after the euthanasia and washed with PBS. Afterward, tissue samples were immersed in formaldehyde solution (4%) for tissue fixation. The material was embedded in paraffin, and a 4 mm section was placed on a glass slide and stained with haematoxylin and eosin (HE). Slides of each experimental group were photographed (20 magnification objective) using a digital camera (Spot Insight Color) coupled to an optical microscope (Olympus, BX-41, Japan). The histological inflammation score was determined as described by McCafferty et al. (2000), considering the following features: extent of destruction of normal mucosal architecture (0: normal; 1: mild; 2: moderate; and 3: extensive damage), presence and degree of cellular infiltration (0: normal; 1: mild; 2: moderate; and 3: transmural infiltration), extent of muscle thickening (0: normal; 1: mild; 2: moderate; and 3: extensive thickening), presence or absence of crypt abscesses (0: absent; 1: present) and the presence or absence of goblet cell depletion (0: absent; 1: present). The histopathological score was measured by a

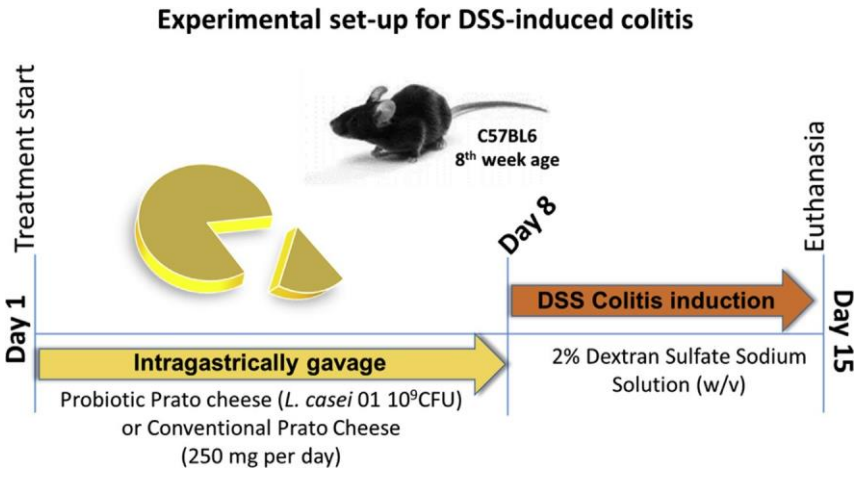

Fig. 1. Experimental protocol of colitis induced in a murine model. C57BL6 mice (n ¼ 6 animals per group) were pre-treated with Prato cheese or probiotic cheese for 7 days.

pathologist, through the evaluation of the histological colon sections.

2.10. Measurement of secretory IgA

To determine the secretory IgA (sIgA), the small bowel intestinal content of mice was collected using PBS, and the sIgA concentration was determined as previously described by Cordeiro et al. (2018). Briefly, intestinal fluid samples from the small bowel were vortexed and centrifuged for 30 min at 850 *g* at 4 C. Then, the supernatant was transferred to NuncMaxiSorp 96 well ELISA plates and used for determining sIgA concentration. The results were expressed as the sIgA concentration (mg) per mL of intestinal fluid, according to the standard curve.

2.11. Relative expression of cytokines in colon

The quantitative gene expression in colon tissue was determined according to Oliveira et al. (2018). After mice euthanasia, small fragments (1 cm) of colon were collected and total RNA was isolated using RNeasy mini kit (Qiagen; Hilden, Germany) according to the manufacturer's protocol. Samples were treated using DNase I to digest residual genomic DNA (Invitrogen; Waltham, MA, USA) and then Turbo DNA-free Kit® (Ambion; Austin, TX, USA) was used for DNA removal according to manufacturer's instructions. Reverse transcription was performed to obtain cDNA of the samples, using High Capacity cDNA Reverse Transcription kit (Applied Biosystems; Foster City, CA, USA). The quantitative PCR (qPCR) was determined using iTaq universal SYBR green supermix (Biorad; Hercules, CA, USA) and gene specific-primers for IL-10, IL-6, *muc2*, Claudin-1 (Cld1), ZO-1, ZO-2 and Occludin, and housekeeping genes for b-actin and GAPDH 23. The amplification cycle consisted of the following steps: 95 C for 30 s, and 40 cycles of 95 C for 15 s and 60 C for 30 s on an ABI PRISM 7900HT Sequence Detection System (Applied Biosystems). The results of gene expression of the control group (with no treatment) were used as calibration data. Results were expressed as a fold-change of expression levels, using the mean and standard deviations of target cytokine expression (2^{-DDCt}).

Colonic Barrier Integrity Index was determined according to Zaylaa et al. (2018), which indicates the strain potential to restore the epithelial barrier function. The index was calculated by combining the % of mRNA relative gene expression of four tight junction proteins (Occludin, Claudin-1, ZO-1, and ZO-2) when compared with the PBS control group, which was considered as 100%.

2.12. Statistical analysis

All analyses were performed in triplicate, and the results were expressed as mean ± standard deviation. Parametric data were analysed using one-way ANOVA followed by Tukey post-test. Statistical analyses were performed in GraphPad Prism version 7.00 for Windows (GraphPad Software, San Diego, CA, USA.). Asterisks represent significant differences between the strains, and were indicated as follows: *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$.

3. Results

3.1. Probiotic and lactic acid bacteria counts, proximate composition, and calcium and sodium contents of probiotic and conventional Prato cheese

Both the starter and probiotic counts remained above 8 log cfu g^{-1} for all cheeses after 60 days of refrigerated storage, with values of 8.12e9.02 and 8.75 log cfu g^{-1} for *Lc. lactis* and *L. casei* 01,

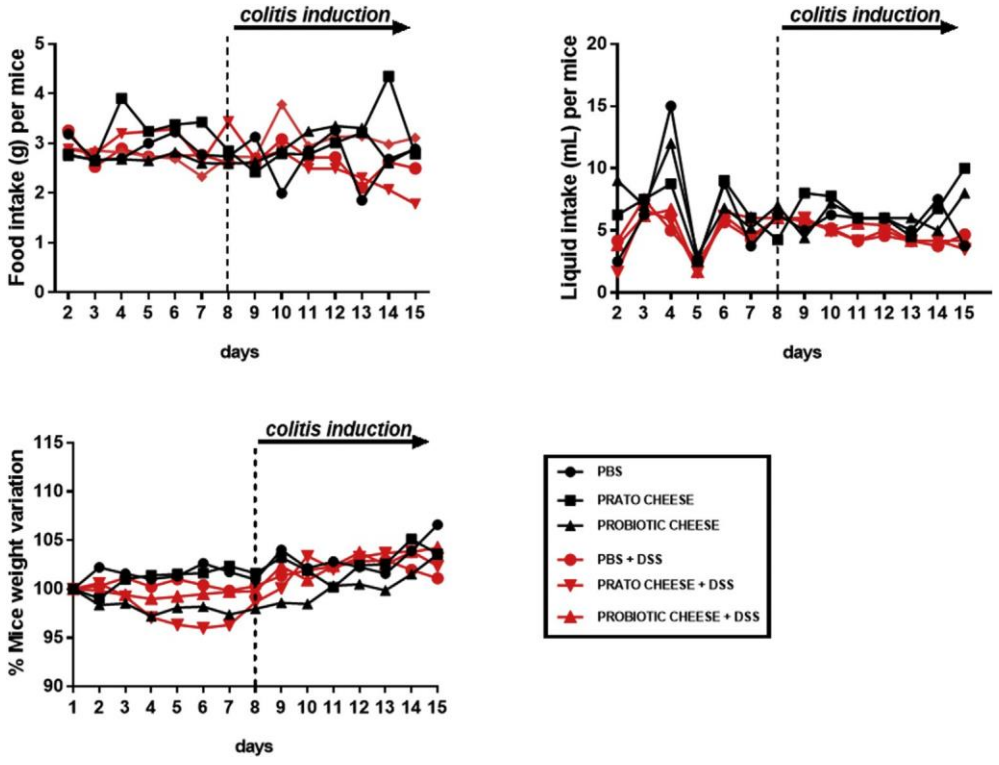

Fig. 2. Food consumption (A), liquid consumption (B) and weight variation (C) observed during experimental procedure: , phosphate buffered saline (PBS); , Prato cheese; , probiotic cheese; PBS þ DSS; , Prato cheese þ DSS; , probiotic cheese þ DSS.

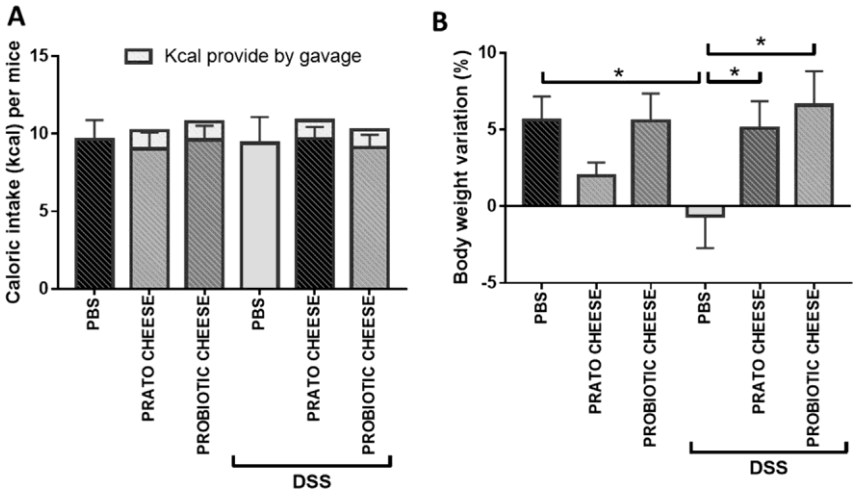

Fig. 3. Caloric intake in kcal per mice (A) during experimental procedure and weight loss (B) observed after beginning colitis induction.

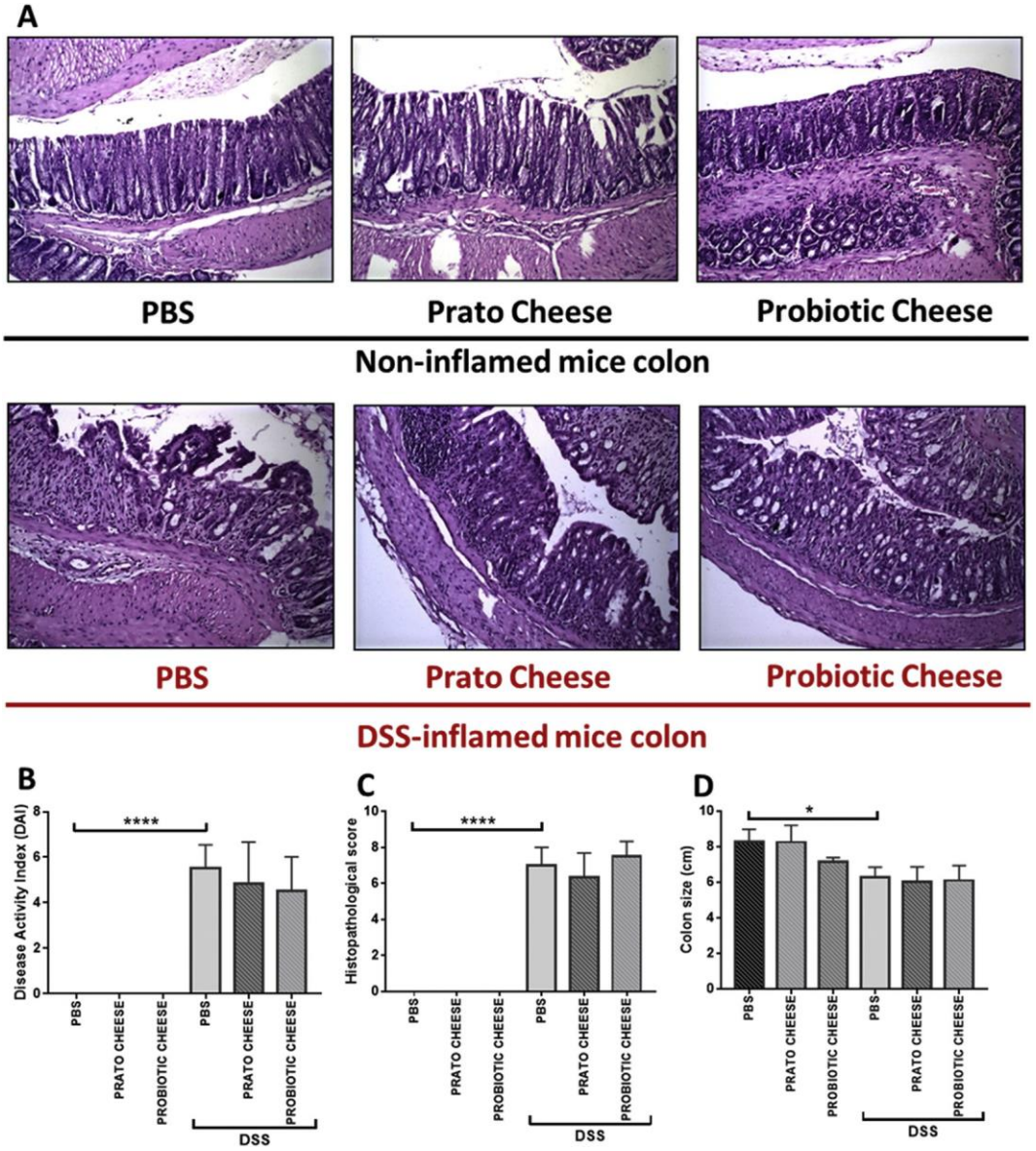

Fig. 4. Representative haematoxylin and eosin-stained images from colon mucosal histopathology (A), (B) disease activity index (DAI) for assessing DSS colitis severity and (C) histopathological score obtained in mice and (D) colon shortening (E).

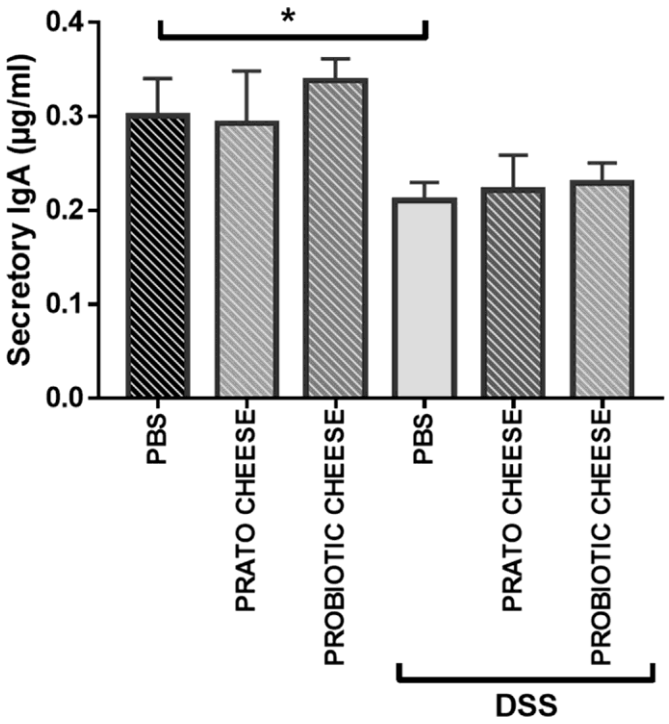

Fig. 5. Secretory immunoglobulin A (sIgA) in the small intestine content of healthy or inflamed mice.

respectively ($p > 0.05$). For intragastric gavage used in clinical trial, the cheese solution was resuspended in PBS pH 7.4 and presented 8.45 log cfu mL ^{1} *Lc. lactis* in Prato cheese and 8.32 log cfu mL ^{1} *Lc. lactis* and 9.47 log cfu mL ^{1} *L. casei* 01 in probiotic cheese.

Regarding the proximate composition, both the Prato cheese and probiotic Prato cheeses presented moisture, fat, and protein levels from 51.5 to 52.6, 36.9 to 38.5, and 29.3 and 27.5% (w/w) respectively ($p > 0.05$). Concerning Total solids, a range from 47.4 to 48.5% (w/w) was observed for Prato cheese and probiotic Prato cheese, respectively. Regarding the mineral content, the calcium and sodium levels ranged from 902.3 to 954.31 mg 100 g ^{1} and 666.7 to 621.9 mg 100 g ^{1} for Prato cheese and probiotic Prato cheese, respectively ($p > 0.05$).

3.2. Food intake, liquid intake, and body weight

The amount of Prato cheese or probiotic cheese administered by intragastric gavage (250 mg) for the pretreatment of DSS-induced colitis corresponds to approximately 8.7% of the daily food intake considering 2.87 ± 0.19 g per mouse. Fig. 2 shows the food intake (Fig. 2A) during experimental procedure, which includes the administration of DSS solution, the liquid intake (Fig. 2B) and the percentage variation in body weight (Fig. 2C) of all animals. No significant differences ($p < 0.05$) were observed for food intake, liquid intake and body weight variation between the groups during 15 days of the experiment. Caloric intake was calculated (Fig. 3A) according to food consumption. Gavage with Prato cheese (Prato cheese or probiotic) accounted for an extra 1.6 kcal to the daily intake, which was 9.40 ± 0.71 kcal per animal per day during the experimental procedure. However, the intragastric gavage did not alter the daily caloric content in each animal. No significant difference was found between groups, including the inflamed or non-inflamed groups. Although the administration of DSS solution led to a weight loss in mice (0.6%), the treatment with cheese containing or not the probiotic bacteria prevented the weight loss of the animals (Fig. 3B), with a weight gain similar to that of the healthy animals (6.5%).

3.3. Disease activity index and microscopic evaluation of inflamed colon

The results showed that the administration of DSS in drinking water induced an acute inflammation in the mice colon (Fig. 4), which was evidenced through the analysis of the major colitis clinical signs (weight loss, diarrhoea, and rectal bleeding), yielding a combined score (DAI) (Fig. 4B). However, the treatment with probiotic cheese was not sufficient to change this clinical condition, as the DAI score was not significantly different ($p > 0.05$) for the mice treated with the cheese made with *L. casei* 01 (4.5 DAI score) when compared with the untreated mice (5.5 DAI score). In addition, the administration of 2% DSS in water was able to alter the morphological structure of the mice colon, with a decrease in colon size (8.3 cm in the control and 6.2 in PBS inflamed mice) (Fig. 4C) and an increase in the histopathological parameters (Fig. 4A, B). This behaviour led to changes in the mucosal architecture of the colon and extent of muscle thickening, as well as inflammatory cell infiltration. However, the pretreatment with the probiotic cheese was not able to prevent the destruction of the intestinal mucosa, as

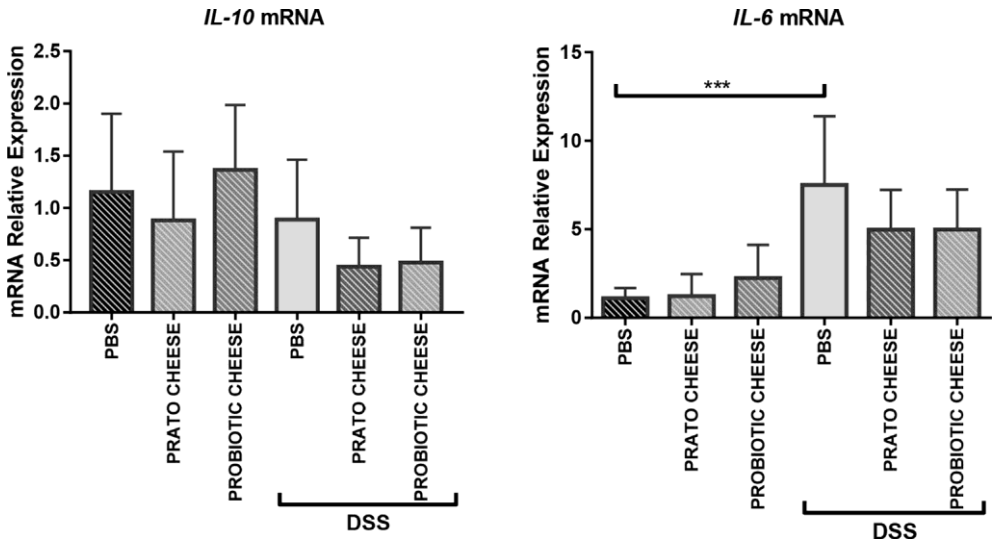

Fig. 6. Relative expression of mRNA of the anti-inflammatory IL-10 and pro-inflammatory IL-6 genes in mice colon.

expected. Therefore, there were no significant differences in the histopathological score and colon size for all inflamed groups.

3.4. Secretory IgA levels in mice small intestine

Fig. 5 represents sIgA levels in the small bowel intestinal fluid, at day 15. The results showed that the administration of probiotic cheese in healthy mice led to an increase in sIgA when compared with both the PBS control and mice treated with Prato cheese, which was not observed in DSS-induced colitis mice with no significant differences ($p > 0.05$) between the inflamed groups.

3.5. IL-10 and IL-6 gene expression in mice colon

To investigate the potential mechanisms of probiotic cheese containing *L. casei* 01 in mice, the anti-inflammatory IL-10 and pro-inflammatory IL-6 gene expression was evaluated. In both healthy

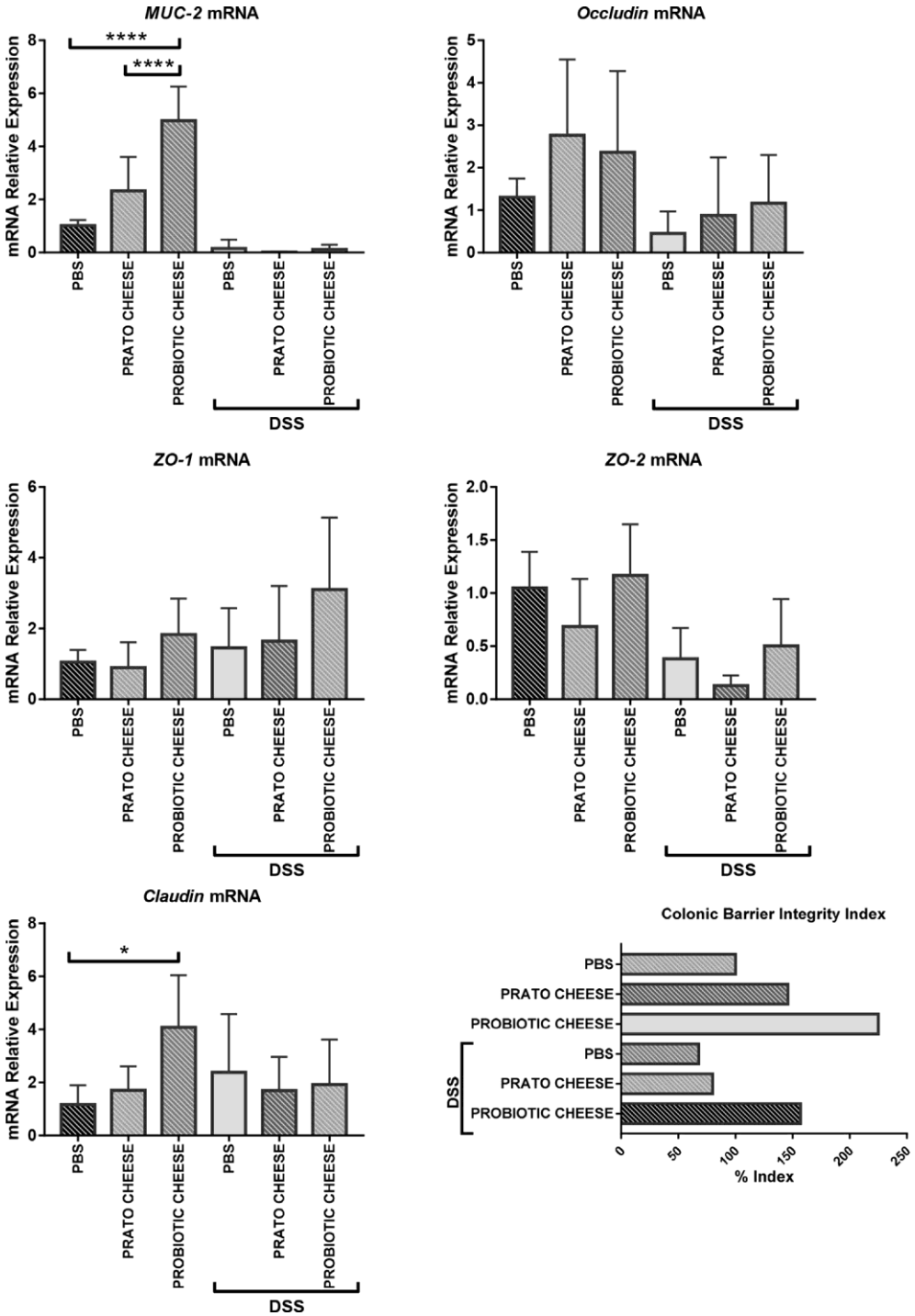

Fig. 7. Relative expression of mRNA of the (A) MUC-2, (B) Occludin (C) ZO-1, (D) ZO-2, (E) Claudin-1, genes in mice colon. Expression levels was monitored by RT-PCR (F) Colonic Barrier Integrity Index was calculated from the combination of mRNA gene expression of tight junction (TJ) proteins (Occludin, Claudin-1, ZO-1 and ZO-2).

mice and mice receiving DSS, no significant difference was observed for the IL-10 gene expression (Fig. 6A), while the IL-6 gene expression was increased in inflamed mice (Fig. 6B). Nevertheless, as previously observed in our other results, the probiotic cheese was not able to alter the inflammatory scenario in mice.

3.6. *Intestinal barrier genes relative expression in mice colon*

The potential of Prato probiotic cheese to modulate genes involved in epithelial barrier integrity in healthy and DSS-induced colitis mice was evaluated by the relative expression of genes encoding tight junction (TJ)-associated proteins (Occludin, ZO-1, ZO-2, and Claudin-1), and the MUC-2 gene expression. No significant differences for the relative expression of the genes Occludin (Fig. 7B), ZO-1 (Fig. 7C), ZO-2 (Fig. 7D) was observed. However, a significant increase was observed in the mRNAs for MUC-2 ($p < 0.0001$) and Claudin-1 ($p < 0.05$), as shown in Fig. 7A, E, respectively, for the healthy mice treated with probiotic Prato cheese when compared with the untreated animals (PBS). Interestingly, the colonic barrier integrity (CBI) index was calculated, and the pretreatment with probiotic Prato cheese showed the highest CBI index in both the healthy mice and the DSS-induced colitis mice (Fig. 7F).

4. Discussion

The incidence of IBDs has considerably increased throughout the world, thus becoming an important global public health problem (Ananthakrishnan, 2015). This increase has been associated with a change in lifestyle that includes the intake of processed foods, usually rich in fat and sugar and poor in fiber, in addition to the intensified and uncontrolled use of antibiotics, especially during childhood (Ananthakrishnan, 2015; Vangay, Ward, Gerber, & Knights, 2015). Current treatments, including anti-inflammatory drugs such as aminosalicylates and corticosteroids, immunosuppressive agents can produce significant side effects and low efficacy of the treatment (Bernstein, 2015; Pithadia & Jain, 2011). Studies have focused on alternative therapies to alleviate the symptoms caused by the disease, including the use of probiotics, which exerts anti-inflammatory effects and has been proposed for the treatment of ulcerative colitis (Carvalho et al., 2017; Rabah, Rosa do Carmo, & Jan, 2017).

Probiotics can act in the host by enhancing the intestinal epithelial barrier, or by modulating the immune response system through their interaction with intestinal cells and regulation of anti and pro-inflammatory cytokines. Moreover, probiotics can adhere to the intestinal mucosa and decrease pathogen adhesion to the host epithelial cells and competitively exclude pathogenic microorganisms. In addition, some probiotics species produce antimicrobial compounds which inhibit pathogenic microorganism proliferation.

These mechanisms are the basis for determining whether the species has a probiotic potential to be used in a disease model (Bermudez-Brito, Plaza-Díaz, Muñoz-Quezada, Gomez-Llorente, & Gil, 2012). The probiotics potential has been studied in IBD murine models (Fujiya, Ueno, & Kohgo, 2014; Santos Rocha et al., 2014), such as the DSS-induced colitis model, which mimics the mucosal injury, ulceration, diarrhoea, impaired mucus epithelial barrier function, and inflammatory cytokine production features routinely observed in human UC (Laroui et al., 2012). The functional foods, including the probiotic cheese, may be an attractive alternative to attenuate the symptoms of IBD, besides presenting an excellent market potential (Al Mijan & Lim, 2018; Silva et al., 2018c).

Colonization of probiotic bacteria in the human gut is associated with beneficial effects on the host, such as by modulating intestinal microbiota (Carmo et al., 2017). Thus, the pretreatment using probiotic inserted in protective matrices shows a good choice as to deliver the anti-inflammatory effects and thereby reduce inflammation caused by IBDs. It is worth emphasising that the therapeutic effects of probiotics foods depend on the ability of the bacteria to survive the industrial process and the storage period (Carmo et al., 2017), as these environments impose a series of bad growth conditions, which can severely affect bacterial viability (Cordeiro et al., 2018). Thus, a food matrix, for example, cheese can be used to maximize the tolerance of bacteria to the stressful environments and to increase their probiotic ability. In this sense, Prato cheese of this study has proven to be a good probiotic protection matrix as it presented the minimum requirements of 8 log cfu g^{-1} counts after storage, being in accordance with the regulatory recommendations for probiotic concentrations to promote beneficial effects (9 log cfu g^{-1} or cfu mL^{-1}) (Brasil, 2007). Concerning Prato cheese, the Brazilian legislation has established moisture and fat in dry matter (FDM) as high fat (>60%) and high moisture (46e54.9%). Moreover, Prato cheese (30 g) should provide approximately 15% of the daily protein intake recommended by law (50 g protein per day). For mineral content, calcium and sodium levels should range from 163 to 226 mg and 60e206 mg per serving (30 g), respectively (Matera et al., 2018). Indeed, the proximate composition of all Prato cheese of this study including moisture, fat, protein, calcium and sodium levels was in accordance with the Brazilian regulation (Brasil, 1997).

Whereas Prato cheese is a good food matrix for bacteria survival, this study investigated the ability of Prato cheese containing *L. casei* 01 strain to prevent the symptoms of chemically induced colitis by DSS in mice. The experimental procedure was based on previous studies (Ple et al., 2016, 2015) which showed that the 5-day pretreatment with probiotic strains in cheese matrices succeeded in alleviating the symptoms of colitis. Weight loss is one of the clinical parameters observed in colitis (Luerce et al., 2014). The present results showed that the administration of DSS caused a weight loss in the control group (PBS), as expected; however, the pretreatment with the Prato cheese and probiotic cheese was able to interfere with weight loss. This result suggests that the Prato cheese, as well as the probiotic Prato cheese, possess nutraceutical compounds, such as vitamins, amino acids, and fatty acids, which may aid in the prevention of DSS-induced weight loss (Larussa, Imeneo, & Luzza, 2017).

In the present study, no reduction on the macroscopic inflammatory disease score and the histological score of colitis was observed for the probiotic-treated mice, with no changes in the colon shortening of the treated groups. Thus, the pretreatment with probiotic Prato cheese had no effect on colonic inflammation in DSS-induced colitis mice model. In accordance with our results, Kennedy and collaborators reported that the probiotic therapy with *Lactobacillus plantarum* species 299 (LP299) failed to alleviate symptoms of colitis (Kennedy, Hoper, Deodhar, Kirk, & Gardiner, 2000). Our findings reinforce that the viability of the strain in a protective matrix, such as cheese, is not the decisive factor for the therapeutic effects of probiotics, thus the probiotic strain must act through multiple mechanisms to attenuate inflammatory processes in the host. Indeed, the therapeutic effect of functional food is dependent on the adequate selection of a probiotic strain that will act effectively in the proposed disease model. However, the delivery matrix (Prato cheese) of the probiotic candidate may enhance the probiotic effect of the strain, as observed in *L. casei* BL23, which is dependent on a dairy matrix to have a significant effect on the DSS-induced mice model (Lee, Yin, Griffey, & Marco, 2015). In this case, the choice of another probiotic strain might yield different results for future studies.

The role of mucus layer and sIgA in gut homeostasis is clear. The intestinal mucus layer provides a barrier limiting bacterial contact with the underlying epithelium, and sIgA reduces the penetration of commensal bacteria by preventing their adhesion to the epithelium (Pabst, Cerovic, & Hornef, 2016). Several studies have shown that the consumption of probiotics is associated with increased sIgA levels since this antibody can limit the penetration of pathogenic bacteria into host tissues through the neutralization of antigens (Malin, Suomalainen, Saxelin, & Isolauri, 1996; O'Sullivan, 2001). Although the immune exclusion or neutralization has been recognized as a key function of SIgA and often attributed as an important component of protective immunity, little is known about the specific details of the process (Stokes, Soothill, & Turner, 1975). This fact is very relevant in DSS-induced colitis models, once DSS is toxic to the intestinal epithelium, promoting increased bacterial translocation (Laroui et al., 2012; Okayasu et al., 1990). In our study, as also observed by Zurita-Turk et al. (2014), there was no significant difference in sIgA levels between the healthy control group and the DSS-induced control group. Interestingly, the treatment with *L. casei* 01 led to an increase in IgA levels in the small intestine of healthy mice, while *L. casei* 01 was not effective to alter the concentrations of this immunoglobulin during the colitis disease.

Furthermore, our findings showed that Prato probiotic cheese was able to stimulate *muc2* gene expression only in healthy animals. One of the components of the epithelial barrier is mucin 2, which is secreted by goblet cells. The *muc2* gene is responsible for encoding the main mucin that composes the intestinal mucus layer, which is important to prevent the direct adhesion of microorganisms to the epithelium and lubricate the intestinal walls (Cordeiro et al., 2018; Niv, 2016). Although Prato cheese containing *L. casei* 01 was able to increase the stimulation of production of mucus in healthy animals, it was not able to alter the production profile of *muc2* in animals with severe colitis. In accordance with our results, Duary, Bhausaheb, Batish, and Grover (2012) also showed that despite the *muc2* gene was overexpressed in healthy animal treated with *L. plantarum* Lp91, it was not able to alter the gene expression in colitis inflammation scenario. It is known that *muc2* knockout mice have a propensity to spontaneously develop colitis (Burger-van Paassen et al., 2011; Johansson et al., 2008; Van der Sluis et al., 2006; Wenzel et al., 2014). Moreover, a decrease in *muc2* expression has been observed in patients with ulcerative colitis (Tytgat, van der Wal, Einerhand, Büller, & Dekker, 1996; Van Klinken, Van der Wal, Einerhand, Buller, & Dekker, 1999).

Similarly, although no significant differences were observed in the TJP genes of the present study, except for Claudin-1, the results of the qualitative index that measures the colonic barrier integrity showed that the pretreatment with probiotic cheese was more effective to alter the barrier integrity when compared with the colitis-induced mice (PBS inflamed group).

The capacity of probiotic strains to improve the intestinal barrier in healthy animals has already been reported by other studies (Bruewer, Samarin, & Nusrat, 2010; Wang et al., 2018). However, further studies should be performed to verify the effect of *L. casei* 01 on the improvement of the mucosal barrier, since it failed to strengthen the gut epithelial integrity against the DSS. In addition, it was observed that the probiotic Prato cheese did not increase *il*10 mRNA levels in the colon of DSS-induced colitis mice.

A previous study has shown that the protective effect of probiotic *L. lactis* NCDO2118 against ulcerative colitis in mice was related to increased IL-10 levels in the colon (Luerce et al., 2014). Furthermore, *L. lactis* NCDO2118 was ineffective in the reduction of *IL6* mRNA levels, as was *Lactobacillus fermentum* CECT5716 in another ulcerative colitis model (Mane et al., 2009). The parameters analysed in this study, secretory IgA, *IL10* and *IL6* mRNA levels, and CBI index are directly connected to the probiotic mechanisms responsible for attenuating the inflammatory process caused by DSS. It is noteworthy that probiotic cocktail VSL# 3 (*L. plantarum* 299v, *Lactobacillus salivarius*, or *Bifidobacterium infantis* 35624), when used in a murine model of DSS-induced colitis, was able to change the composition of the cecal microbiota by increasing the *Bifidobacterium* spp. concentration (Gaudier, Michel, Segain, Cherbut, & Hoebler, 2005). However, VSL # 3 was ineffective in alleviating the inflammatory process caused by the effect of the chemical agent, probably due to the fact it does not enhance the epithelial barrier or increase mucin production (Gaudier et al., 2005). Therefore, the *L. casei* 01 strain delivered in a cheese matrix does not show the ability to change these parameters and failed to alleviate the inflammatory process.

5. Conclusion

Prato cheese has proven to be a good protective matrix to ensure the viability of the *L. casei* 01 strain during storage. Moreover, the continuous consumption of the experimental probiotic Prato cheese was able to interfere with the weight loss in DSS-induced mice. However, the pretreatment with probiotic Prato cheese to stimulate some parameters in healthy mice was not able to alter the parameters in DSS-inflamed mice. It is likely that the pretreatment period was not sufficient to control the disease parameters, as well as the probiotic potential of the *L. casei* 01 strain. Probably, the lack of probiotic potential observed in the present study, based on the property of alleviating the inflammatory process caused by experimental colitis, is due to several factors including the inability of the strain to modulate the immune response to control the pro-inflammatory environment induced by DSS, to improve the epithelial barrier or to modulate the intestinal commensal microbiota communities to withstand intestinal environmental changes caused by DSS. Therefore, further studies are required on ulcerative colitis model aimed to explore the use of cheese as a protective matrix associated with other selected probiotic strains with high anti-inflammatory potential.

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CAPÍTULO 3

Queijo Minas Frescal probiótico no tratamento da colite ulcerativa

Neste capítulo, apresentaremos os resultados do efeito terapêutico do queijo Minas Frescal contendo a bactéria *L. lactis* NCDO 2118, em um modelo murino de colite induzida por DSS.

O queijo Minas Frescal é um queijo branco, macio e fresco, ligeiramente ácido e com uma vida útil de 14-21 dias (ROCHA et al., 2020). Ele é um dos queijos mais consumidos no Brasil e constitui uma importante atividade da indústria de laticínios do país, devido ao alto rendimento e ausência de período de maturação, o que permite um rápido retorno do investimento, diminuindo os custos para o consumidor (CLAROS et al., 2019). Além disso, o queijo Minas Frescal é um queijo leve, com baixa quantidade de gorduras, o que agrada aos consumidores adeptos de um estilo de vida mais saudável, um mercado atualmente em expansão no Brasil (ALVES et al., 2017). Somando a essas características, ressalta-se que a alta atividade hídrica do queijo Minas Frescal, seu pH acima de 5, o baixo teor de sal e a ausência de conservantes sintéticos tornam-o uma excelente matriz de proteção para as bactérias probióticas (SPERRY et al., 2018). A ingestão de queijo Minas Frescal probiótico por sua vez, já se mostrou eficaz em induzir melhorias significativas na hipertensão (LOLLO et al., 2015; SPERRY et al., 2018), na atividade anti-hiperglicêmica (GROM et al., 2020) e na regulação do sistema imunológico (LOLLO et al., 2012).

A escolha da linhagem *L. lactis* NCDO 2118, cujo genoma foi sequenciado por nosso grupo de trabalho (OLIVEIRA et al., 2014), se deu, principalmente, por esta ser uma BL, do grupo das *Lactococcus lactis* subsp. *Lactis*, isolada de ervilhas congeladas e que possui grande versatilidade fisiológica para adaptar-se a ambientes estressantes, o que a torna uma bactéria com excelente potencial para ser incluída em produtos probióticos funcionais (DA SILVA et al., 2019). Além disso,

a *L. lactis* NCDO 2118 possui comprovadas propriedades probióticas exibindo atividades anti-inflamatórias e imunomoduladoras. Um estudo do nosso grupo de pesquisa demonstrou que a *L. lactis* NCDO 2118 foi capaz de melhorar os sinais clínicos da colite ulcerativa, mantendo a integridade da barreira epitelial, reduzindo a produção de citocinas pró-inflamatórias e aumentando a expressão de células T reguladoras (Treg) (LUERCE et al., 2014). Outro estudo do nosso grupo comprovou ainda que a NCDO 2118 é capaz de produzir GABA e de exercer atividade anti-hipertensiva em ratos espontaneamente hipertensos (SARAIVA et al., 2016).

Os dados do efeito terapêutico do queijo Minas Frescal contendo a *L. lactis* NCDO 2118 no tratamento da colite ulcerativa induzida por DSS foram publicados na revista *Frontiers in Microbiology*, no ano de 2021.

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Therapeutic Effects of Probiotic Minas Frescal Cheese on the Attenuation of Ulcerative Colitis in a Murine Model

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Inflammatory bowel diseases (IBDs) constitute disturbances of gastrointestinal tract that cause irreversible changes in the structure and function of tissues. Ulcerative colitis (UC), the most frequent IBD in the population, is characterized by prominent inflammation of the human colon. Functional foods containing probiotic bacteria have been studied as adjuvants to the treatment or prevention of IBDs. The selected probiotic strain *Lactococcus lactis* NCDO 2118 (*L. lactis* NCDO 2118) exhibits immunomodulatory effects, with promising results in UC mouse model induced by dextran sodium sulfate (DSS). Additionally, cheese is a dairy food that presents high nutritional value, besides being a good delivery system that can be used to improve survival and enhance the therapeutic effects of probiotic bacteria in the host. Therefore, this work investigated the probiotic therapeutic effects of an experimental Minas Frescal cheese containing *L. lactis* NCDO 2118 in DSS-induced colitis in mice. During colitis induction, mice that consumed the probiotic cheese exhibited reduced in the severity of colitis, with attenuated weight loss, lower disease activity index, limited shortening of the colon length, and reduced histopathological score. Moreover, probiotic cheese administration increased gene expression of tight junctions' proteins *zo-1*, *zo-2*, *ocln*, and *cln-1* in the colon and increase IL-10 release in the spleen and lymph nodes. In this way, this work demonstrates that consumption of probiotic Minas Frescal cheese, containing *L. lactis* NCDO 2118, prevents the inflammatory process during DSS-induced colitis in mice, opening perspectives for the development of new probiotic functional foods for personalized nutrition in the context of IBD.

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INTRODUCTION

Functional food products are defined as "natural or processed foods containing known or unknown biologically active compounds which provide a clinically proven and documented health benefit for the prevention, control or treatment of chronic diseases when in used in defined, effective and non-toxic amounts" (Rolim et al., 2020). Among them, functional foods containing probiotic bacteria have been proposed for being safe for consumption and have the ability to modulate the responses in the host by cellular components or metabolites produced (Rabah et al., 2017). In this context, there is a wide demand for new functional foods, in particular, foods enriched by the addition of probiotics, driving the food's industry to develop new products proven effective for health (Sperry et al., 2018).

Cheese is one of the most consumed dairy foods and comprises, from the nutritional view, a source with good nutritional value, given high contents of protein, minerals, and vitamins (Matera et al., 2018). Moreover, cheese, especially soft cheese, is an excellent delivery system to introduce probiotics into the gastrointestinal tract (GIT), due to it is anaerobic conditions created by the protein-fat contents, which can form complex coacervates that microencapsulated the probiotic bacteria and also high pH and low acidity present in this kind of the cheese (Silva et al., 2018). These coacervates reduce the contact with a highly acidic gut environment and thereby promote probiotic bacteria survival (Castro et al., 2015). Noteworthy, the Minas Frescal cheese is one of the most consumed cheese in Brazil and constitutes one important activity of the dairy industries, due to the high yield and absence of maturation period, which allows a quick return on investment and, consequently, lowers costs to the consumer (Sperry et al., 2018; Rocha et al., 2020). These features show that Minas Frescal cheese is a good candidate for manufacturing a new probiotic function dairy food.

Lactococcus lactis strain is a Gram-positive lactic acid bacterium (LAB) that exhibit simple metabolism and rapid growth and, due to that, are widely used in food fermentation (Da Silva et al., 2019). More specifically, NCDO 2118, used in this work, is a strain of *L. lactis* subsp. *lactis* isolated from frozen peas (Oliveira et al., 2014) and was previously demonstrated anti-inflammatory and immunomodulatory activities in the treatment of diseases, especially, in inflammatory bowel diseases (IBDs; Luerce et al., 2014). Furthermore, the functional analysis of *L. lactis* NCDO 2118 genome reflected a physiological adaptation ability to environmental changes like industrial processes and transit through the human GIT (Da Silva et al., 2019). These characteristics make *L. lactis* NCDO 2118 an excellent candidate to be introduced in probiotic functional foods.

IBDs, which include ulcerative colitis (UC) and Crohn's disease (CD), are marked by periods of remission and relapse of an inflammation condition in the GIT and have a high prevalence in westernized countries, reaching about 0.5% of these populations (Silva et al., 2019). The etiology of IBD still not well understood, but scientific evidence suggests that that the genetic susceptibility, associated with intestinal microbiota alterations, causing an exacerbated immune response in the host is involved in IBD pathogenesis (Zhang and Li, 2014). UC is the most frequent condition of IBD in the population, affecting the large intestine, also called the colon. UC causes small irritation and ulcers in the colon, pain, diarrhea often with blood in the stool, and weight loss (Cordeiro et al., 2019). Nowadays, studies have shown that consumption of probiotic bacteria has therapeutic effects on UC, which is demonstrated to decrease the colon inflammation in a mouse model as well as in UC patients (Mañé et al., 2009; Luerce et al., 2014; Santos Rocha et al., 2014; Berlec et al., 2017; Jakubczyk et al., 2020; Rabah et al., 2020). Thus, this study hypothesizes that Minas Frescal cheese, made using *L. lactis* NCDO 2118, has a therapeutic effect in dextran sodium sulfate (DSS)-induced colitis mouse model.

MATERIALS AND METHODS

Cheese Processing

The cheese processing was performed in accordance with Grom et al. (2020). Fifty liters of raw milk with 3.2% w/w fat (Núcleo Avançado de Tecnologia de Alimentos) was pasteurized for 15 s at 72°C (Model pro110, Arpifrio, São Paulo, Brazil), cooled to 37°C, and equally divided into two portions of 25 l, each for processing of conventional and probiotic Minas Frescal cheese. Then, 0.2 g/l of calcium chloride (Labsynth, Sao Paulo, Brazil) and 3 g/l of coagulant powder (Halamix power, Chr. Hansen) were added into the milk and maintained in a double- jacketed tank for 40 min to coagulate. After, 0.1 g/l at probiotic culture *L. lactis* NCDO 2118 [7–8 log colony-forming unit (CFU)/g] was added to the probiotic cheese, while no addition of lactic bacteria was performed on conventional cheese. The curd was cut, the cheese whey was removed, and the grains were put in 250-g plastic molds. Dry salting was performed by direct addition of 0.8% w/v NaCl on the cheese surface. Cheeses were packed and stored at 5°C.

Physicochemical Analyses of Conventional and Probiotic Cheese

The proximate composition (moisture, protein, and fat) was evaluated according to the methodology previously described (BRASIL, 2006). To determine the moisture content of cheeses, we oven-dried 5 g of a sample at 100–105°C, for 24 h. Protein quantification and fat levels were determined by the Kjeldahl and Gerber methods, respectively (BRASIL, 2006). All results were expressed as g/100 g.

The content analysis of calcium and sodium levels in both kinds of cheeses were performed by inductively coupled plasma (ICP) optical emission spectrometry (Spectro Analytical Instruments, Kleve, Germany) previously described by Felicio et al. (2016). Sodium and calcium standards were used to obtain the calibration curves. Ten grams of samples was hydrolyzed, for 16 h, using 2 ml of nitric-perchloric acid solution (2:1), at 120 ± 2°C. Samples were heated in a digestion block (Technal, São Paulo, Brazil) to 100 ± 2°C for 1 h and maintained for more,

than 2 h, at 170 ± 2°C. Then, after the samples reach room temperature, we added 2 ml of nitric- perchloric acid and heated them again for a further 4 h at 170 ± 2°C.

To obtain the pH levels of both cheese, we inserted a digital pH meter electrode (Micronal, B-375, Digimed, Piracicaba, São Paulo, Brazil) into the diluted cheese samples as previously described (Silva et al., 2017).

Bioactivity

To measure the bioactive peptides in cheese samples, we evaluate the angiotensin I-converting enzyme inhibitor (ACEI), antioxidant activity assay [2,2-diphenyl-1-picrylhydrazyl (DPPH)], and α-amylase and α-glucosidase inhibition.

The ACEI in probiotic and conventional cheese was determine by spectrophotometric assay, according to Konrad et al. (2014) The ACEI was calculated as follows: ACE inhibitory activity (%) = [(B − A)/ (B − C)] × 100, where A is the absorbance in the presence of ACE and ACE components, B is the absorbance with ACE and without the ACE component, and C is the absorbance without the ACE or ACE component.

The DPPH radical-scavenging method previously described was used to determine the antioxidant activity capacity of cheeses (Lee et al., 2016). For that, 200 µl of 10% cheese sample was mixed with 1 ml of 100 µmol/l of DPPH solution. Besides that, as a positive control, butylated hydroxytoluene at 1 mg/ml concentration was used. After 15 min, the absorbance was measured at 517 nm using a spectrophotometer. The DPPH was calculated as follows: DPPH radical-scavenging activity (%) = [1 − (sample absorbance at 517 nm/control absorbance at 517 nm)] × 100.

The measurement of α-glucosidase and α-amylase inhibitory activities was determined according to Grom et al. (2020). The α-glucosidase inhibitory activity was determined dissolving 100 µl of α-glucosidase (0.2 units/ml) in 100 µl of phosphate buffer (pH 6.8), mixed with 150 µl of water-soluble extracts, and incubated for 20 min at 37°C. Then, 100 µl of 2.5 mM of p-nitrophenyl α-D-glucopyranoside was added to start the reaction. After incubation at 37°C for 20 min, the reaction was stopped, and 80 µl of sodium carbonate solution (0.2 mol/l) was added. The absorbance of *p*-nitrophenol was read at 405 nm using CMax Plus microplate reader (Promega, São Paulo, Brazil).

The α-amylase inhibitory activity was measured, and 100 µl of human salivary α-amylase (20 units/ml) with 100 µl of water-soluble extracts was added and incubated at 37°C for 20 min. Then, 250 µl of starch solution (10 g/l) in phosphate buffer (pH 6.8) was added, and the solution was incubated at 37°C for 5 min. To stop the reaction, 250 µl of dinitrosalicylic reagent (1% 3,5-dinitrosalicylic acid and 12% sodium potassium tartrate in 0.4 M of NaOH) was added and heated at 100°C for 10 min. After that, the sample was cooled at room temperature using a cold-water bath, and then 2.000 µl of distilled water was added to the mixture. The absorbance was performed at 540 nm using a spectrophotometer. The percentage (%) of α-glucosidase and α-amylase inhibition was calculated as described by Grom et al. (2020).

Evaluation of Therapeutic Effects of Minas Frescal Cheese Containing *L. lactis* NCDO 2118 in the Dextran Sodium Sulfate-Induced Colitis Model

Animals

Conventional female C57BL/6 mice of 8 weeks of age, obtained at Universidade Federal de Minas Gerais (UFMG, Belo Horizonte, Brazil), were used in this work. They were housed in plastic cages in a room with controlled temperature (18–23°C), light cycle of 14-h light/10-h dark, relative humidity (40–60%), and *ad libitum* access to food and water. All experimentais procedures realized in this work were approved by the Ethics Committee on Animal Experimentation of the Universidade Federal de Minas Gerais (CEUA-UFMG, Brazil) by protocol no. 364/2018.

Experimental Design and Dextran Sodium Sulfate-Induced Colitis

Prior to intragastric gavage, cheese samples were daily prepared resuspending 250 mg of each cheese, separately in 250 ml of phosphate buffer (pH 7.4; 1:1), and homogenized for 2 min using an IKA T 10 Basic Ultra Turrax homogenizer. Bacterial viability in both cheese solutions was determined by CFU counts. The mice were divided randomly into six main groups, each containing six animals per group (**Table 1**). Groups 1–3 represented the healthy control group (no DSS) that received drinking water from the same source and consisted of group 1 received only water content (Group Naive); group 2 received conventional Minas Frescal cheese (group conventional ch.); and group 3 was given probiotic Minas Frescal cheese containing *L. lactis* NCDO 2118 (group NCDO ch.). All mice from groups 4–6 (experimental groups) received DSS (36–50 kDa, MP Biomedicals, CAT 260110, LOT Q5756), as the only drinking source, prepared to a concentration of 1.7% in filtered drinking water and provided to the animals daily, for 7 days, according to acute colitis model previously described (Wirtz et al., 2017). Animals from group 4 received only drinking water with DSS (group DSS) and no treatment; mice from group 5 were treated with conventional cheese (group DSS + conventional ch.), and group 6 were treated with probiotic Minas Frescal cheese containing *L. lactis* NCDO 2118 (DSS + NCDO ch.). For this experimental procedure, all mice received 0.5 ml of the respective treatments, in a single daily dose, by intragastric gavage, concomitantly with DSS induction (for 7 days). Each animal received approximately 2.5×10^6 CFU/g of probiotic bacteria, per day, according to the results obtained by previous studies (Cordeiro et al., 2019; Rabah et al., 2020) and the adequate amount of bacteria for effect on the colon (Minelli and Benini, 2008). Mice were euthanized on the seventh day. All *in vivo* experiments were done in biological triplicate.

Assessment of Colitis Disease

Mouse body weight was individually measured during all experimental days. Water and food consumption were also recorded daily. The disease activity index (DAI) was determined

TABLE 1 | Experimental groups and the respective treatments.

Healthy control group (consumption of drinking water)		Inflamed groups [consumption of DSS (1.7%) in the drinking water]	
Group	**Treatment**	**Group**	**Treatment**
Naive	H_2O	DSS	H_2O
Conventional ch.	Conventional cheese	DSS + conventional ch.	Conventional cheese
NCDO ch.	Probiotic cheese containing *L. lactis* NCDO 2118	DSS + NCDO ch.	Probiotic cheese containing *L. lactis* NCDO 2118

All groups were gavaged daily, with 0.5 ml of the appropriate treatments, for 7 days.
DSS, dextran sodium sulfate.

on the last experimental day, as described by Cooper et al. (1993). This score measurement three major colitis clinical signs: weight loss, levels of diarrhea, and presence of rectal bleeding. To access the intestine and colon for future assays, a longitudinal abdominal incision was performed in all mice. The colon length of each mouse was individually measured (from the cecum to rectum), and the values obtained were used to indicate the mean of each experimental group, in cm. Then, the distal portion of each colon was collected and washed with phosphate-buffered saline (PBS) for making colonic segment rolls for histomorphological analysis. These rolls were immersed in formaldehyde solution (4%, v/v) for tissue fixation, and after that, they were embedded in paraffin. A section (4 μm) was placed on a glass slide and stained with hematoxylin and eosin (H&E; Marchal Bressenot et al., 2015). Then, the sections were photographed (20× magnification objective) using a digital camera (Spot Insight Color) coupled to an optical microscope (Olympus, BX-41, Japan). The histological inflammation score was determined by a pathologist. To measure the level of histological inflammation in the colon tissue, the score previously described was used (Wirtz et al., 2017). This score considered the following features: tissue damage (0: none; 1: isolated focal epithelial damage; 2: mucosal erosions and ulcerations; 3: extensive damage deep into the bowel wall) and lamina propria inflammatory cell infiltration (0: infrequent; 1: increased, some neutrophils; 2: submucosal presence of inflammatory cell clusters; 3: transmural cell infiltrations). The total score ranging from 0 (no changes) to 6 (widespread cellular infiltrations and extensive tissue damage) was obtained by the sum of these two sub-scores (tissue damage and lamina propria inflammatory cell infiltration). Furthermore, to stain mucus-producing goblet cells, other cuts of the paraffinized colon samples were produced and stained by the Periodic acid- Schiff (PAS; Prisciandaro et al., 2011). Ten random field images from each sample were made using the 40× objective, and then with the use of ImageJ software (version 1.8.0), the intact goblet cells were counted. The total number of goblet cells was expressed as the number of cells per high-power field (hpf; 40×, 108.2 μm^2).

Measurement of Secretory Immunoglobulin A

Secretory immunoglobulin A (sIgA) was determined by linked immunosorbent assay (ELISA), according to Cordeiro et al. (2018). For that, 96-well plates (Nunc-Immuno Plates, MaxiSorp) were coated with anti-IgA antibodies (Southern Biotechnology, Birmingham, AL, United States) and incubated overnight. Plates were washed in salina-Tween (salina with 0.05% of Tween-20; SIGMA Chemical Co) and blocked with 200 μl of PBS-casein (0.05%) for 1 h at room temperature.

Intestinal lavage contents were added, and the plate was serially diluted (1:100) and incubated at room temperature for 1 h. Plates were washed with salina-Tween, and then, biotin-conjugated anti-mouse IgA antibody (Southern Biotechnology; 1:10,000 in PBS-casein) was added. Plates were incubated for 1 h at 37°C, and then, biotinylated monoclonal antibody anti-IgA (BD Biosciences) was added and incubated for 1 h at room temperature. Subsequently, peroxidase-labeled streptavidin (Southern Biotechnology) was added. Plates were washed in salina-Tween and incubated again with 100 μl of *o*-phenylenediamine (OPD; Sigma, St. Louis, MO, United States) and H_2O_2 (0.04%) for 1 h at room temperature. For stop reaction, 20 μl/well of 2 N of H_2SO_4 was added. Reading was performed on Bio-Rad Model 450 Microplate Reader at 492-nm absorbance. The results of total sIgA were measured, according to the standard curve, in a concentration of sIgA (ng) per ml of intestinal fluid.

Measurement of the Activity of Myeloperoxidase

The levels of neutrophil infiltration in the colon tissue were assessed by measurement of myeloperoxidase (MPO) activity, as previously described by Porto et al. (2019). For MPO quantification, a piece of colon tissue (10 mg) was homogenized proportionally in 1.9 ml/100 mg of PBS and centrifuged at 12,000 *g* for 10 min. The supernatant was discarded, and the pellet formed was lysed and centrifuged again. The supernatant formed was discarded again, and the pellet was resuspended proportionally in 1.9 ml/100 mg of 0.5% hexadecyltrimethyl ammonium bromide (HTAB) diluted in PBS. Afterward, were subjected to a freeze-thaw cycle (3×) using liquid nitrogen and then centrifuged at 12,000 *g* at 4°C for 10 min. To realize the enzymatic assay, we added an equal amount of substrate (1.5 mM/l of OPD and 6.6 mM/L of H_2O_2 in 0.075 mM/L of Tris-HCl pH 8.0) to the supernatant. To stop the enzymatic reaction, 50 μl of 1 M of H_2SO_4 was added. The absorbance was read in a spectrophotometer (Spectramax M3, Molecular Devices, LLC, Sunnyvale, CA, United States) at 492 nm. The results were expressed as arbitrary units (AU/mg).

Gene Expression Analysis in the Colon

The quantitative gene expression in colon fragment was determined according to Do Carmo et al. (2019). For that, fragments of 1 cm of the colon were collected, and then, the total RNA was isolated using PureLink RNA Mini Kit (Thermo Fisher Scientific)

according to the manufacturer's protocol. Afterward, DNase I (Invitrogen, Waltham, MA, United States) was used to digest residual genomic DNA of samples, and then Turbo DNA-free Kit (Ambion, Austin, TX, United States) was used for DNA removal following the manufacturer's protocol. RNA quality was checked by agarose gel and NanoDrop® ND-1000 (260/230 ratio). To obtains the sample cDNA, the High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA, United States) was used. Quantitative PCR (qPCR) was determined using iTaq Universal SYBR Green Supermix (Bio-Rad, Hercules, CA, United States) and gene-specific primers, according to Do Carmo et al. (2019), for zonula occludens 1 and 2 (*zo-1* and *zo-2*, respectively), occludin (*ocln*), claudin-1 (*cln-1*), mucin-2 (*MUC-2*), inducible nitric oxide synthase (*iNOS*), and cytokine genes for interleukin-10 (*IL-10*), *IL-17*, *IL-1β*, as well as housekeeping genes encoding β-actin (*actβ*) and GAPDH (*gapdh*). The amplification cycles were performed as follows: 95°C for 30 s and 40 cycles of 95°C for 15 s and 60°C for 30 s on ABI PRISM 7900HT Sequence Detection System (Applied Biosystems). Results were expressed as a fold change of expression levels, using the mean and standard deviations of target expression ($2^{-\Delta\Delta CT}$).

Cell Preparation for Culture and Flow Cytometry

Cell suspension preparation for cytokine analysis and flow cytometry measurements were performed according to Canesso et al. (2018). Firstly, as UC affects the distal portions of the intestine, especially, the colon (Mizoguchi et al., 2020), we extracted the cecal lymph node (which drains the cecum) and the colonic lymph node (which drains the colon) for cell culture (Vieira et al., 2012; Esterházy et al., 2019). As the colon lymph node is very small, we did a pool mixing the two lymph nodes to reach enough cells for cell labeling. After that, the organs were macerated with sterile complete RPMI medium [containing 10% fetal bovine serum (FBS)] using a glass tissue macerator. Them, the organs were centrifuged and resuspended in a complete RPMI medium. For the spleen, cell-culture preparation was necessary to lysate the red blood cells, adding 9 ml of distilled water for 5 s. To stop this lysis process, 1 ml of PBS (10×) was added. The spleen capsule was removed to facilitate the presence of only immune cells. These cells were centrifuged and isolated from medium and then were incubated at 1×10^6 cells per well, for cytokine secretion analyses, and another 1×10^6 cell were incubated with antibodies for flow cytometry.

Cytokine Quantification by ELISA

Cells isolated from spleen and lymph node culture were cultured in 96-well plate (1×10^6 cells/well) in sterile supplemented RPMI 1640 and stimulated or not with 1 mg/ml of anti-CD3 and anti-CD28, according to Canesso et al. (2018). The cells were incubated in an atmosphere of 5% CO_2 for 48 h at 37°C, for measurement of IL-10, IL-17, and IL-1β cytokines, by ELISA, according to the manufacturer's instructions (R&D Systems).

Flow Cytometry Analyses

Isolated cells from the spleen and lymph nodes were washed with PBS and pre-incubated with purified rat anti-mouse CD16/CD32 (Fc Block, clone: 2.4G2, BD Biosciences Pharmingen) for 20 min at 4°C to block FcγRII/III receptors. For surface staining, cells were incubated at 4°C for 30 min with anti- CD45.2 (FITC; clone: 104, BD Biosciences Pharmingen) and anti-CD4 (Pacific Blue, clone: RM4-5, BD Biosciences Pharmingen) fluorochrome-conjugated monoclonal antibodies. For intracellular staining, cells were first permeabilized following the *eBioscience Foxp3 Kit*, according to the manufacturer's instructions, and later incubated with anti-FoxP3 (APC) [Alexa Fluor 647, clone R16-715 (RUO), BD Biosciences Pharmingen], anti-LAP (PerCP- eFluor 710, clone: TW7-16B4, eBioscience), and anti-RORγt (PE; clone: Q31-378, BD Biosciences Pharmingen) fluorochrome- conjugated monoclonal antibodies for 30 min at 4°C. Individual controls (singles) were made containing only one labeled antibody in each tube, and also tubes with fluorescence minus one (FMO) were used. The gating strategy and the FMO controls are based on forward and side scatters, selecting splenocytes as a function of cell size and granularity ($n = 6$). Flow cytometry analysis was performed on a FACSCanto (BD Biosciences, San Jose, CA). The frequency (%) of positive cells and the mean fluorescence intensity were analyzed with the aid of the FlowJo program, version 10.0 (Tree Star, Ashland, OR, United States).

Statistical Analyses

Data were analyzed using one-way ANOVA followed by Tukey post-test and performed in GraphPad Prism version 7.00 for Windows (GraphPad Software, San Diego, CA, United States). The experimental assays were performed in triplicate, and the results were expressed as mean ± standard deviation. Asterisks demonstrated in all figures represent the significant differences between the experimental groups and were indicated as follows: $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$.

RESULTS

Proximate Composition and Mineral Content of Conventional and Probiotic Minas Frescal Cheese

Proximate composition (moisture, protein, fat, and lactose), sodium, calcium contents, and pH values are presented in **Table 2**. Our results showed that the addition of *L. lactis*

TABLE 2 | Proximate composition and mineral contents of conventional and probiotic Minas Frescal cheese.

	Conventional cheese	Probiotic cheese
Moisture	67.2 ± 1.43	68.2 ± 1.55
Proteins	16.2 ± 1.56	17.5 ± 1.34
Fat	14.4 ± 1.11	14.7 ± 1.13
Lactose	2.2 ± 0.3	1.8 ± 0.1
Na	542 mg/kg	549 mg/kg
Ca	312 mg/kg	316 mg/kg
pH	5.52 ± 0.34	5.42 ± 0.21

Values are expressed as mean ± standard deviation. Moisture, protein, fat, and lactose values are expressed in g/100 g cheese, while sodium and potassium values are expressed in mg/kg. pH is dimensionless. Cheese analysis performed in triplicate.

TABLE 3 | Bioactive compounds from conventional and probiotic cheese.

Conventional cheese	Probiotic cheese	
DPPH	22.3 ± 0.3b	43.3 ± 0.65a
ACEI	13.3 ± 0.73b	32.4 ± 1.13a
α-Amylase	19.2 ± 1.45b	28.9 ± 0.99a
α-Glucosidase	10.3 ± 0.91b	16.7 ± 1.12a

Values are expressed in mean ± standard deviation. a–b Different letters in the same row indicate a significant difference ($p < 0.05$). ACEI, DPPH, α-amylase, and α-glucosidase are expressed as a percentage of inhibition (%). Cheese analysis performed in triplicate.

ACEI, angiotensin I-converting enzyme inhibitor; DPPH, 2,2-diphenyl-1-picrylhydrazyl.

NCDO 2118 did not affect significantly ($p > 0.05$) the proximate composition and mineral content of Minas Frescal cheese, compared with conventional cheese. Overall, probiotic cheese presented 68.2 ± 1.55, 17.5 ± 1.34, and 14.7 ± 1.13 (g/100 g) of moisture, protein, and fat, respectively, while conventional cheese presented 67.2 ± 1.43, 16.2 ± 1.56, and 14.4 ± 1.11, respectively. Regarding lactose amount, conventional cheese shows 2.2 ± 0.3, while probiotic cheese presented 1.8 ± 0.1 g/100 g. Regarding Na and Ca values, probiotic Minas Frescal cheese exhibited 549 and 316 mg/ kg, respectively; meanwhile, conventional cheese presented similar values, 542 mg/kg of sodium and 312 mg/kg of calcium. pH values also did not present differences in both kinds of cheese, retaining a range of 5.

Lactococcus lactis NCDO 2118 on Cheese Enhances the Production of Bioactive Compounds

Table 3 shows the evaluation of bioactive compounds produced by conventional and probiotic cheese. Our results demonstrated that the antioxidant potential (DPPH), ACE inhibitory activity (ACEI), α-amylase, and α-glucosidase on the probiotic cheese, containing L. *lactis NCDO* 2118, presented increased values and were significantly different ($p < 0.05$) compared with conventional cheese. Regarding DPPH inhibition, we observed that values ranged from 22.3 ± 0.3% (convention cheese) to 43.3 ± 0.65% (probiotic cheese). Furthermore, probiotic cheese presented 32.4 ± 1.13% of ACEI, while the conventional cheese presents only 13.3 ± 0.73%. Likewise, probiotic cheese presented the highest values of α-amylase and α-glucosidase (28.9 ± 0.99% and 16.7 ± 1.12%, respectively), while conventional cheese presented 19.2 ± 1.45% and 10.3 ± 0.91%, respectively.

Treatment of Probiotic Cheese Did Not Alter the Liquid and Food Consumption or Caloric Intake of Mice

Figure 1 shows the liquid consumption (**Figure 1A**), the total food consumption (**Figure 1B**), and the caloric intake per mice (**Figure 1C**) during the experimental procedure. We observed a decrease in liquid intake on groups of mice that consumed water solution containing 1.7% of DSS over the experimental day, exhibiting the lowest consumption on the seventh day (3.3 ± 0.45 ml/animal). On the other hand this consumption remains stable in groups receiving only drinking water (6.3 ± 0.441 ml/animal per day; $p < 0.001$). No differences were observed in liquid consumption in mice treated with conventional or probiotic cheese ($p > 0.05$) in health or unhealthy mice. Mice of all experimental groups consumed, on average, the same amount of food (3 g/animal per day), with no statistical difference between any experimental groups studied. Concerning caloric intake (**Figure 1C**), no difference between experimental groups has found by the intragastric gavage with conventional or probiotic cheese. Thus, both kinds of cheese did not alter the daily caloric content.

Probiotic Minas Frescal Cheese Reduced the Weight Loss in Dextran Sodium Sulfate-Induced Ulcerative Colitis Mice

The consumption of probiotic cheese, containing *L. lactis* NCDO 2118 strain, was challenged in the DSS-induced colitis model. Mouse weight loss, monitored during the DSS administration, showed that the animals receiving DSS exhibited body weight loss starting from the third day, after DSS consumption (**Figure 1D**). Otherwise, mice from all healthy control groups presented a significant weight gain throughout the experiment days ($p < 0.0001$). **Figure 1E** shows that treatment with probiotic cheese has a protective effect on colitis-induced body weight loss. Mice from the DSS group that did not receive any treatment showed a marked weight loss (−8.08 ± 2.09%); however, mice treated with the probiotic cheese showed significant improvement in body weight (+0.23 ± 0.80%, $p < 0.0001$). Body weight variation was also statistically different ($p < 0.05$) between DSS + conventional cheese (−4.5 ± 5.9%) and DSS + NCDO cheese (+0.23 ± 0.80%).

Probiotic Minas Frescal Cheese Alleviated Clinical and Macroscopic Signs of Colitis Disease

The shortening of colon length (**Figure 1F**) and the DAI (**Figure 1G**) were analyzed to verify major colitis macroscopic and clinical symptoms. Our results showed that the administration of DSS causes a pronounced shortening in the colon length (4.2 ± 1.12 cm) when compared with the naive group (7.0 ± 1.15 cm, $p < 0.0001$). However, the treatment with probiotic cheese, containing *L. lactis* NCDO 2118, prevents the shortening of the colon (6.2 ± 0.99 cm), being statistically different ($p < 0.0001$) when compared with the DSS group and results in a similar length to healthy animals ($p > 0.05$). Regarding DAI analyses, we observed that the administration of DSS was able to increase significantly (8.6 ± 2.1, $p < 0.0001$) the DAI score, compared with healthy groups (0.06 ± 0.2). Nevertheless, the intake of probiotic cheese was able to decrease significantly the DAI score (4.3 ± 1.8) compared with the DSS group ($p < 0.0001$) or with DSS + conventional cheese group (6.6 ± 3.3, $p < 0.05$).

Colon Mucosal Damages Were Reduced in Mice Treated with Probiotic Minas Frescal Cheese

Figures 1H, 2 reveal the impact of DSS administration and the effect of the probiotic treatment on the morphological

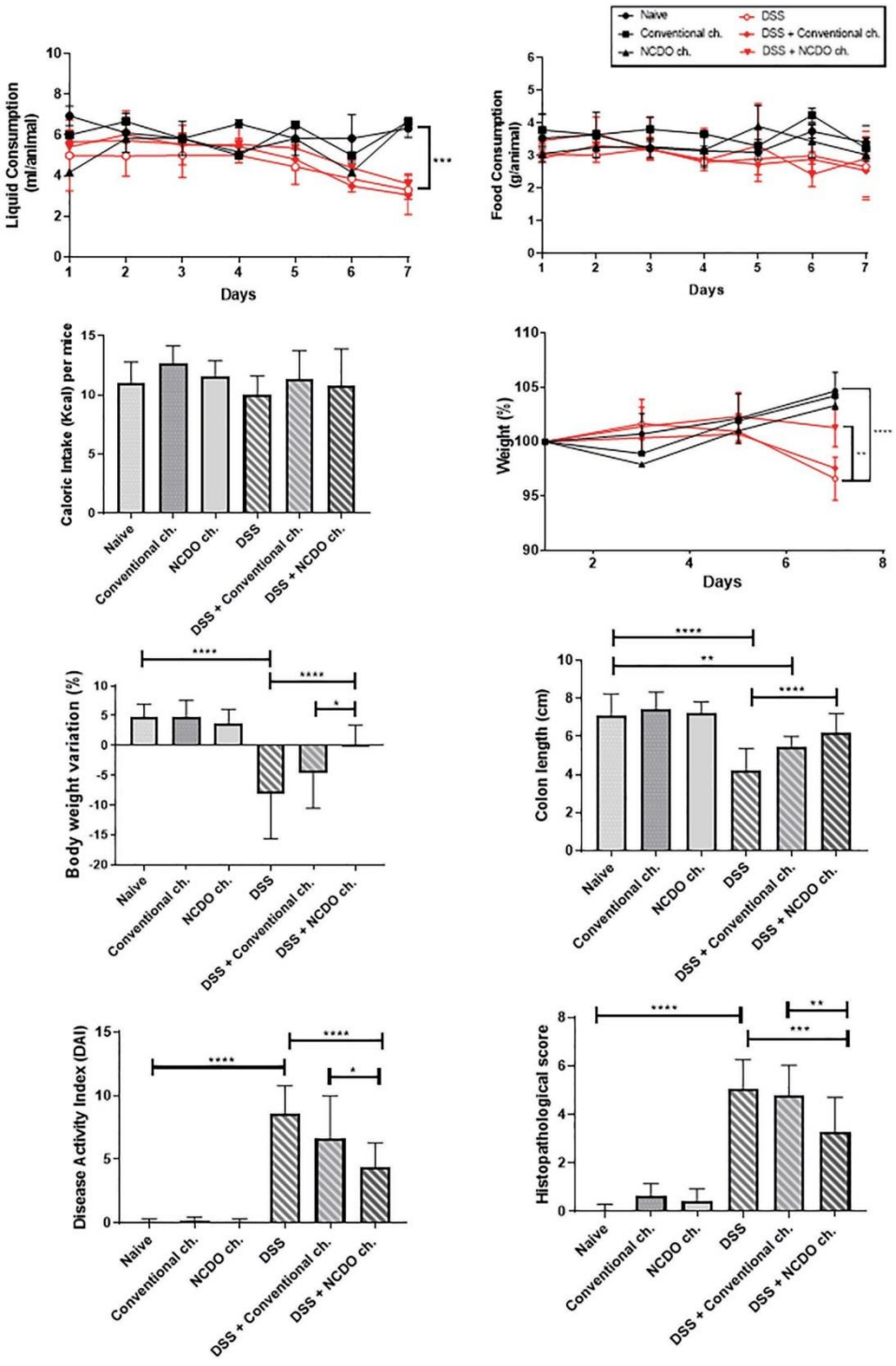

FIGURE 1 | Impact of treatment with probiotic cheese on mice. **(A)** Liquid intake, **(B)** food consumption, and **(C)** caloric intake of mice across the different experimental groups. **(D)** Time course of mouse body weight monitoring during the seven experimental days. **(E)** Body weight loss observed on the seventh day of dextran sodium sulfate (DSS) colitis induction, and differences across the groups. **(F)** Changes in mouse colon length. **(G)** Disease activity index (DAI), a composite measure of weight loss, stool consistency, and presence of blood in stool. **(H)** Histopathological score obtained in mice. Values indicate the mean ± standard deviation. The data represent the mean ± SD ($n = 6$). Asterisks represent statistically significant differences, as follows: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$

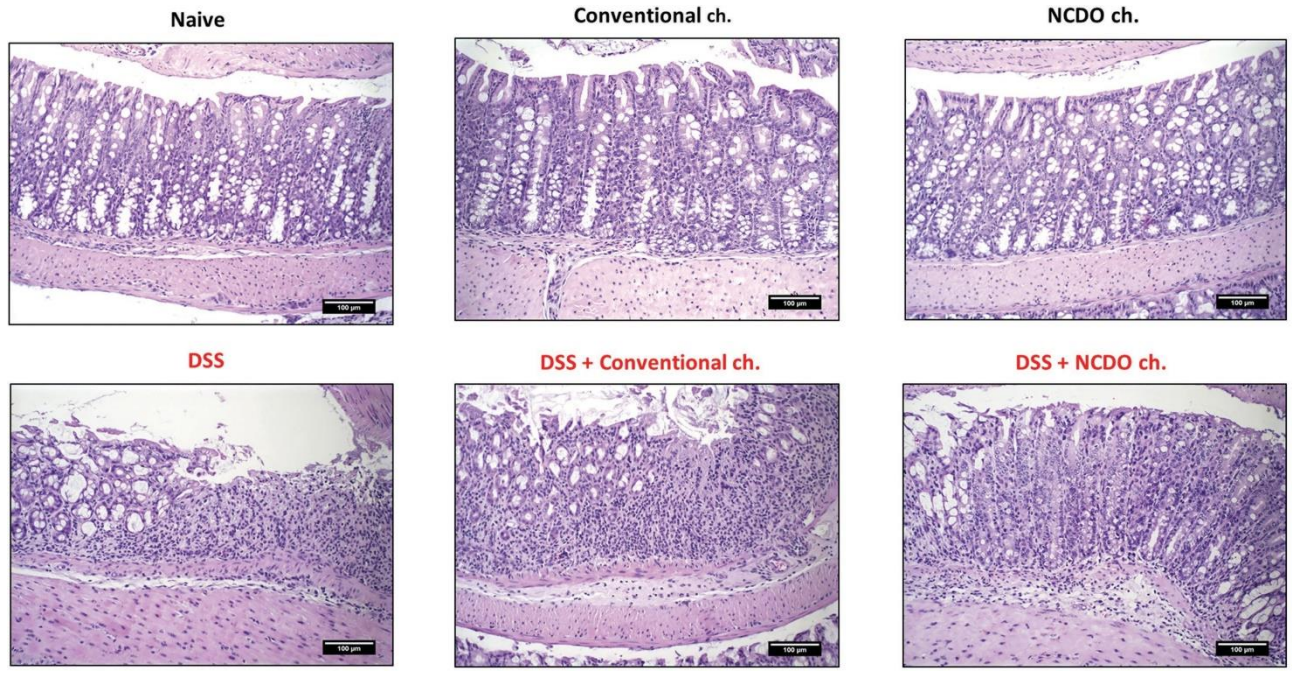

FIGURE 2 | Effect of probiotic cheese administration on the dextran sodium sulfate (DSS)-induced histopathological damages. Representative H&E staining of colon mucosa sections. Image acquisition was done with a 20× magnification objective. Scale bar = 100 μm (n = 6).

structure of the mouse colon. Histopathological score (**Figure 1H**) and histological slide analysis (H&E staining, **Figure 2**) show that mice subjected to DSS consumption presented alterations in the morphological architecture of the colon, with extensive damage deep into the tissue, erosions and ulcerations in the colon of some mice, and increased inflammatory cell infiltration. However, consumption of probiotic cheese in DSS colitis mice was able to ameliorate these mucosal damages. Mice from healthy control groups showed a null histological score, while the DSS group presented a score on average of 5.0 ± 1.1 (p < 0.0001). Consumption of probiotic cheese, in turn, decreases the score to 3.2 ± 1.4, being statistically different to the DSS group (p < 0.001) and DSS + conventional cheese group (4.8 ± 1.2, p < 0.01).

Treatment with Probiotic Minas Frescal Cheese Prevented Degeneration of Goblet Cells and Improved Secretory IgA Production

The administration of DSS provokes a substantial decrease in the number of goblet cells in the colon tissue (56.4 ± 25 goblet cell/hpf) when compared with the naive group (103 ± 27.5, p < 0.01, **Figures 3A,B**). Nonetheless, consumption of probiotic Minas Frescal cheese was still able to prevent this degeneration of goblet cells (101 ± 32.2 goblet cell/hpf), when compared with the DSS group (p < 0.01). Interestingly, the consumption of probiotic cheese provokes an improvement in the number of intact goblet cells, and also in the healthy control group (143.9 ± 16.3 goblet cell/hpf). **Figure 3C** shows levels of sIgA in the small intestine of mice. Our results showed that consumption of probiotic Minas Frescal cheese was able to increase the levels of sIgA (2,800.4 ng/ml) when compared with the naive group (1,750.3 ng/ml) and the DSS group (1,579.7 ng/ml).

Probiotic Cheese Reduced the Inflammatory Cell Infiltration

In this work, we assessed the presence of colon neutrophil infiltrates by detecting its specific MPO enzymes (**Figure 3D**). Our results showed that mice in the DSS group had an inflammatory infiltrate with a very high level of neutrophils (0.0248, 0.008, p < 0.001) when compared with the naive group (0.0109 ± 0.003). However, when mice were treated with probiotic cheese, we found a significant reduction of these cells (0.0137, ± 0.003, p < 0.05), showing MPO levels very similar to those found in healthy animals (p > 0.05). Interestingly, we observed that conventional Minas Frescal cheese also presented reduced values of MPO (0.0148), being statistically different from those of the DSS (p < 0.05) group and similar to those of the DSS + NCDO group.

Probiotic Minas Frescal Cheese Modulated Gene Expression in the Mice Colon

In this work, we sought to evaluable the colonic mRNA expression levels of epithelial barrier genes (*zo-1*, *zo-2*, *ocln*, and *cln-1*), production of mucin gene (*MUC-2*), colonic oxidative

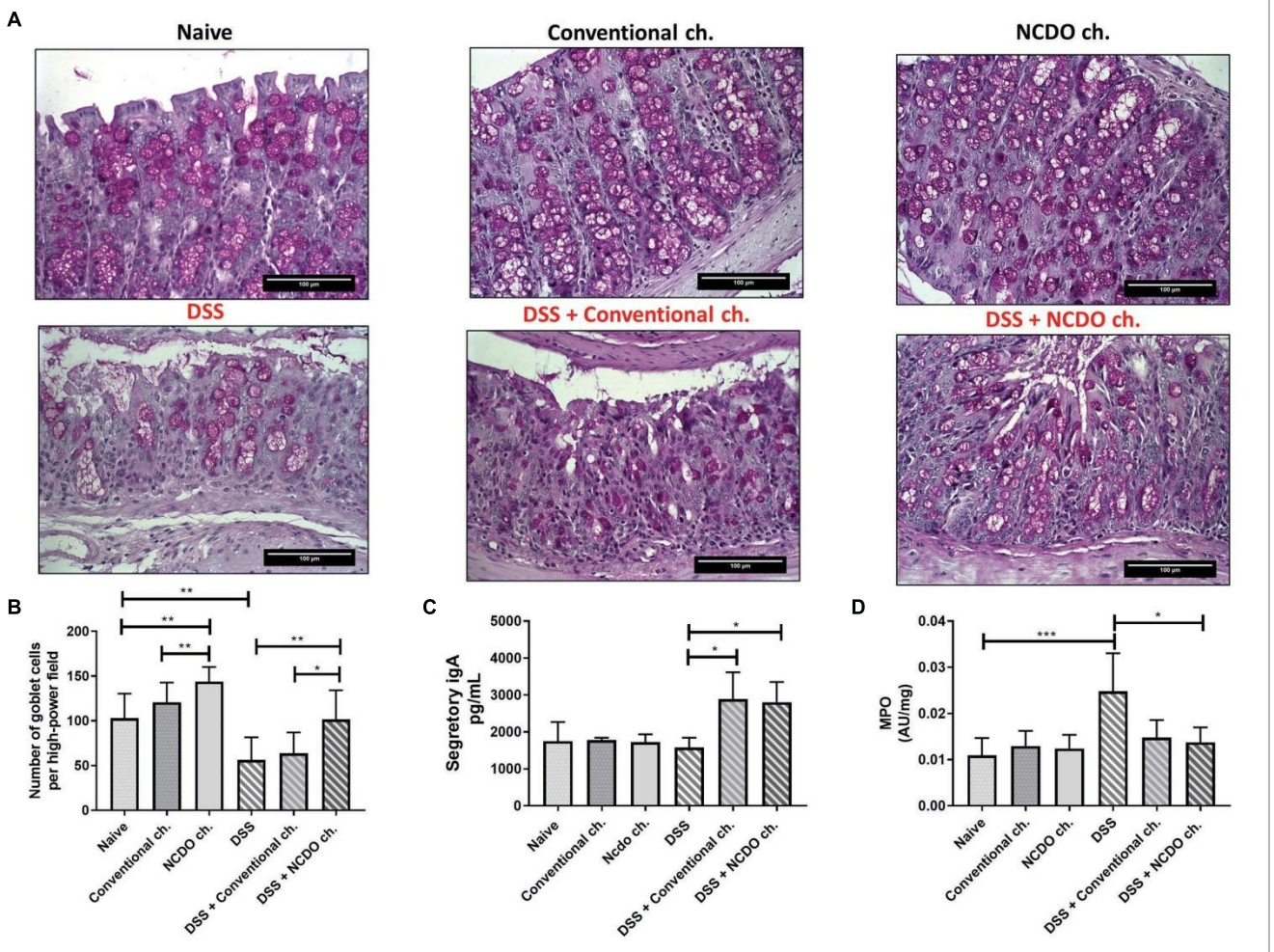

FIGURE 3 | Effect of probiotic cheese administration on goblet cells, secretory immunoglobulin A (sIgA) levels, and the enzyme activity of myeloperoxidase (MPO). **(A)** Representative Periodic acid-Schiff (PAS) staining of colon mucosa sections. **(B)** Number of goblet cells, given by counting intact cells in 10 random field images on mouse colon. **(C)** Quantification of sIgA in the small intestine. **(D)** Levels of MPO activity that indirectly determines the neutrophil concentration in 10 mg of colon tissue. Values indicate the mean ± standard deviation ($n = 6$). Image acquisition was done with a 20× magnification objective. Scale bar = 100 μm ($n = 6$). Asterisks represent statistically significant differences: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

stress (*iNOS*; **Figure 4**), and cytokine gene expression (*IL-10*, *IL-1β*, and *IL-17*; **Figures 5A**–**C**). Our results showed that the consumption of conventional or probiotic cheese in healthy control groups (naive, conventional ch., and NCDO ch. groups) was not able to alter the expression of the genes evaluated ($p > 0.05$). The intake of DSS in drinking water also did not alter the expression of *zo-1*, *zo-2*, *ocln*, *cln-1*, *MUC-2*, *IL-10*, and *IL-17* genes, when compared with the naive group. On the other hand, we observed an increase in the expression of *iNOS* and *IL-1β* genes, when compared with the DSS group and naive group ($p < 0.0001$). Interestingly, we observed that the consumption of probiotic cheese in unhealthy mice induced an increase in the expression of *zo-1*, *zo-2*, *ocln*, and cln-*1* genes of epithelial barrier, compared with the DSS and naive groups, while the expression of *iNOS* and *IL-1β* genes was decreased in animals treated with probiotic cheese. Also, we observed that *MUC-2* gene expression tended to increase in animals treated with probiotic cheese but was not statically different from DSS ($p > 0.05$).

Probiotic Cheese Modulated Cytokine Production in Mice

To clarify the potential mechanisms by which probiotic cheese exerts its beneficial effects, we evaluated the cytokine profiles in the spleen and lymph nodes of mice (**Figure 5**). Our data showed that oral administration of probiotic cheese increased the levels of the anti-inflammatory cytokine IL-10 in the spleen (329.4 pg/ ml, **Figure 5D**) and in the lymph nodes (24.9 pg/ml, **Figure 5G**), when compared with the DSS group (233.6 and 4.24 pg/ml, respectively) and DSS + conventional ch. (161.4 and 3.73 pg/ml, respectively). The intake of DSS led to increased cytokine IL-1β in the spleen (243.7 pg./ml, **Figure 5E**) and IL-17 in the lymph nodes (163.9 pg./ml, **Figure 5I**), when compared with the naïve group (123.8 and 44.4 pg./ml). On the other hand, consumption

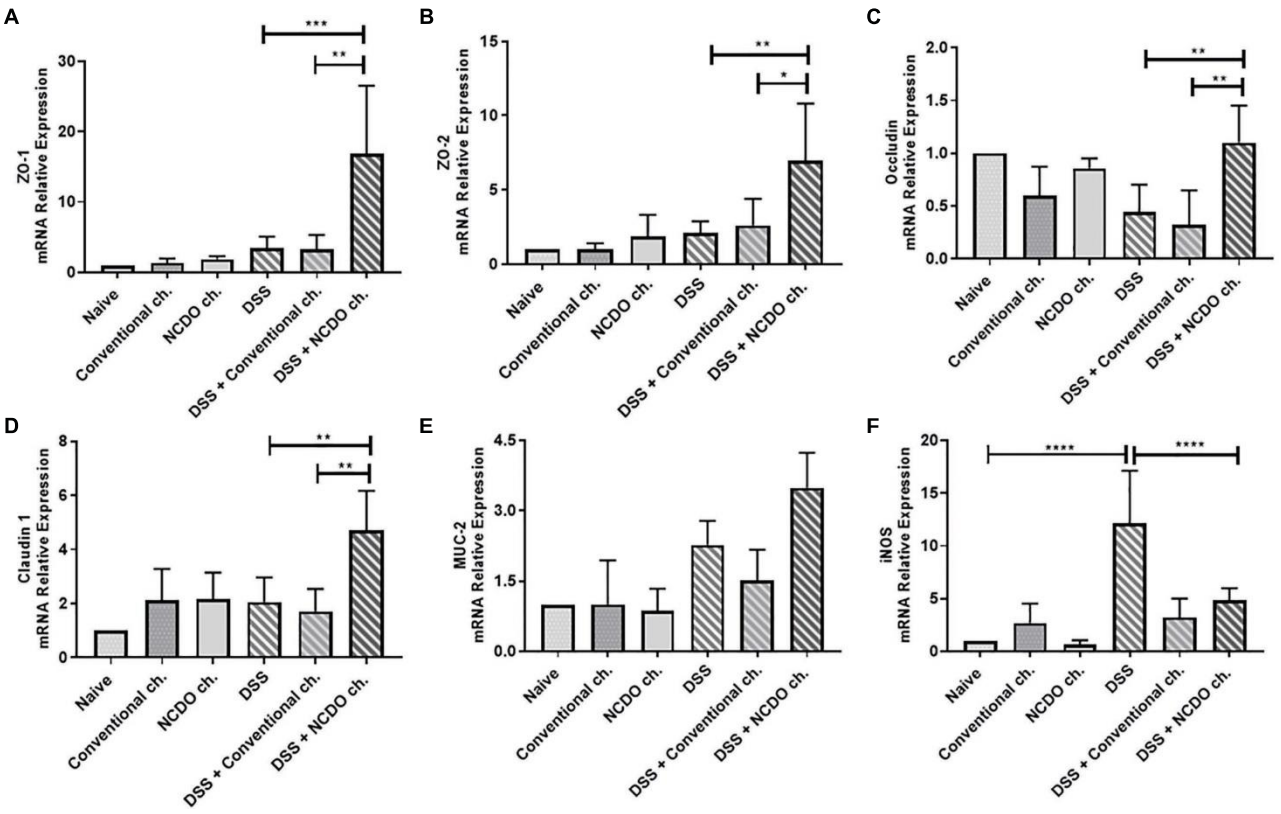

FIGURE 4 | Impact of probiotic cheese on colonic expression markers of cell barrier and oxidative stress. Colonic mRNA expression levels of **(A)** *zo-1*, **(B)** *zo-2*, **(C)** *ocln*, **(D)** *cln-1*, **(E)** *MUC-2*, and **(F)** *iNOS* genes analyzed by qRT-PCR. The data represent the mean ± SD ($n = 6$). Asterisks represent statistically significant differences: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

of probiotic cheese in the DSS mice group was able to maintain IL-1β and IL-17 levels of production similar to healthy animal levels (140.9 and 41.53 pg/ml, respectively).

Probiotic Bacteria, *L. lactis* NCDO 2118, Did Not Alter the Frequency of Tregs in the Spleen and Lymph Nodes

T-cell subpopulation (Treg $CD4^+Foxp3^+$, $CD4^+LAP^+$, and $CD4^+Ror\gamma T^+$) was evaluated in mice spleen and lymph nodes by using flow cytometry (**Figure 6**). The probiotic cheese, containing *L. lactis* NCDO 2118, did not change the percentage of Tregs on the spleen and lymph nodes in both healthy and inflamed mice. No statistical differences were found between the DSS and naive groups for all cells analyzed here.

DISCUSSION

The importance of diet in human health has been described by various scientific evidence; therefore, the development of new food products with health-giving additives and medical benefits is a pressing need (Domínguez Díaz et al., 2020). In this context, probiotic functional foods have been proposed, due to the proven therapeutic benefits of probiotic bacteria by the consumers (Carmo et al., 2017). In this work, we developed a new probiotic Minas Frescal cheese for the treatment of UC.

The addition of certain bacteria to cheese can contribute to altering glycolysis, proteolysis, and lipolysis processes that change the proximate composition and mineral contents of cheese and modify the organoleptic properties of the final product (Cárdenas et al., 2014). Thus, it was necessary to investigate whether the Minas Frescal cheese manufactured with *L. lactis* NCDO 2118 altered the centesimal composition and bioactive compounds of the cheese, as well as whether Minas Frescal cheese was a good matrix to maintain the viability of this probiotic strain. It is worth emphasizing that the beneficial effects of foods containing probiotics strains depend on the ability of these bacteria to survive to industrial process after passing through the GIT, which imposes unfavorable bacterial conditions and can affect probiotic potential (Cordeiro et al., 2019). In this sense, the regulatory agencies around the world recommended that for a probiotic product to be able to exercise its benefits, there must be a viable amount of probiotic bacteria of between 10^6 and 10^7 CFU/g (Castro et al., 2015). In this work, we observed that after manufacturing of Minas Frescal cheese, *L. lactis* NCDO 2118 presented 10^7 CFU/g of viable cells counts, according to the recommendation, and this reinforces that Minas Frescal cheese

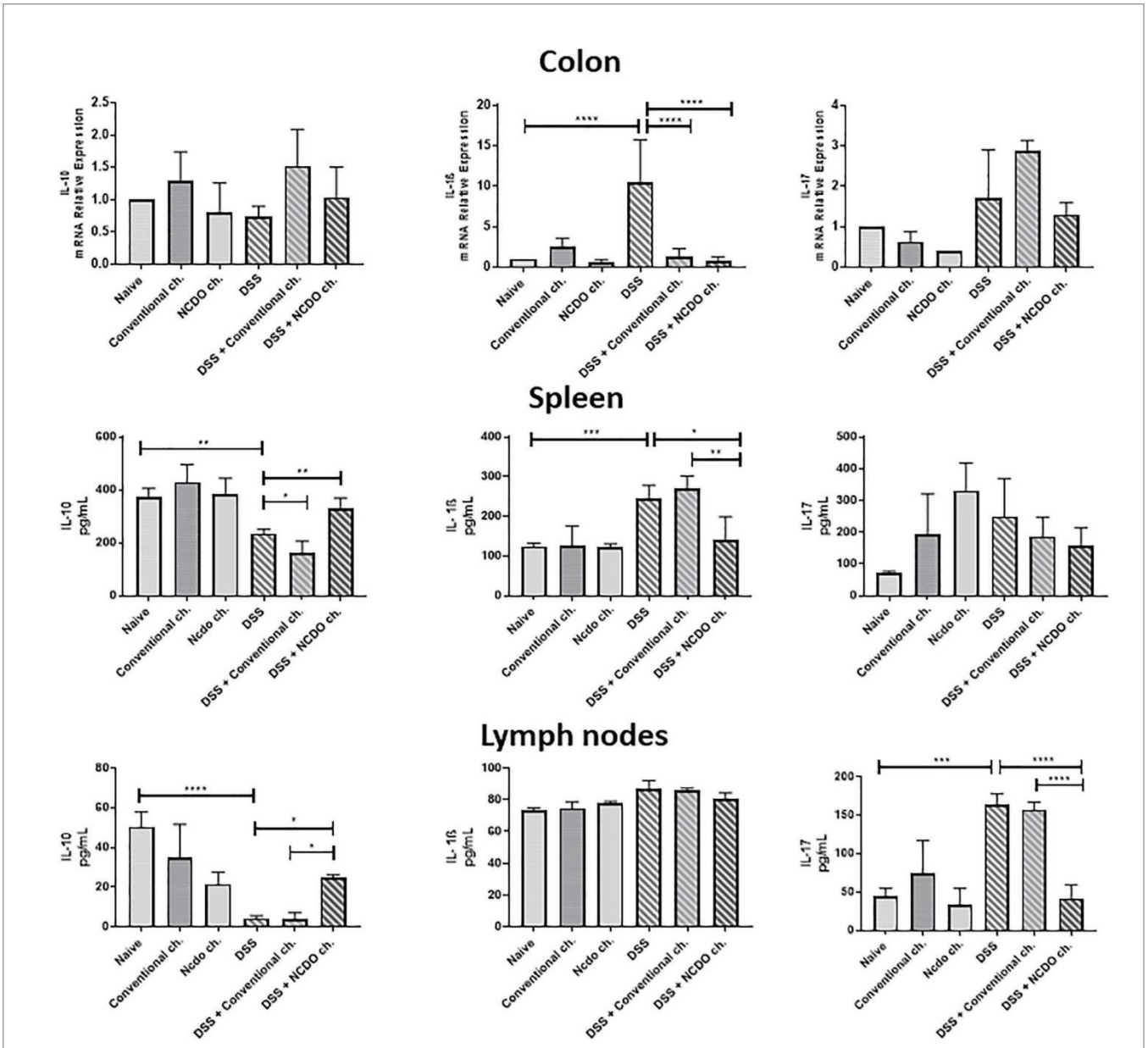

FIGURE 5 | Colonic expression of cytokines genes and immunomodulatory effect of probiotic cheese in dextran sodium sulfate (DSS) colitis mice. Colonic mRNA expression levels of **(A)** *IL-10*, **(B)** *IL-1β*, and **(C)** *IL-17* genes analyzed by qRT-PCR. Enzyme-linked immunosorbent assay (ELISA) of **(D,G)** IL-10, **(E,H)** IL-1β, and **(F,I)** IL-17 cytokines in spleen and lymph node cell culture supernatant, respectively. The data represent the mean ± SD ($n = 6$). Asterisks represent statistically significant differences: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

is a good delivery system to maintain the viability of this probiotic bacteria through manufacturing processes. Previous studies showed that soft cheeses, like Minas Frescal cheese, are a good protective matrix for bacteria (Hosoya et al., 2012; Lollo et al., 2012, 2015; Sperry et al., 2018). In addition, our work demonstrated that *L. lactis* NCDO 2118 added on Minas Frescal cheese did not alter the proximal composition parameters evaluated (moisture, protein, fat, and lactose), as well the sodium and calcium contents, and pH values of the cheese. The probiotic cheese developed in this work still maintained the specifications recommended by the Brazilian legislation law established for moisture (>55%) and fat (25–44.9%) in Minas Frescal cheese dry matter (FDM; Matera et al., 2018).

Cheeses are recognized not only for their high nutritional value but also for the production of bioactive peptides, from casein hydrolyzed by proteases and peptidases (López-Expósito et al., 2017). Some of these peptides can resist gastrointestinal digestion, responsible for biological activities such as antihypertensive, antioxidant, and antidiabetic activities (Livney, 2010). However, the introduction of some probiotic bacteria

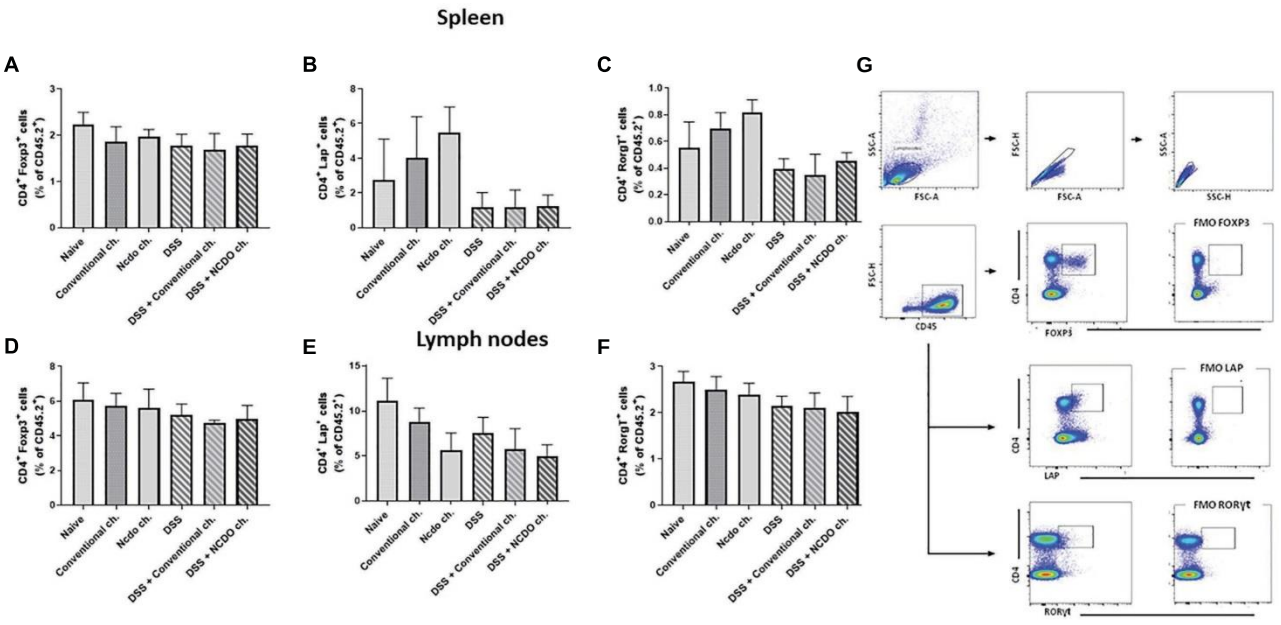

FIGURE 6 | Evaluation of T lymphocyte production in spleen and lymph nodes after induced colitis. T cells were isolated from mouse spleen and lymph node, and the frequencies of $CD4^+Foxp3^+$ **(A, D)**, $CD4^+RORγt+$ T cells **(B,E)**, and $CD4^+Lap+$ T **(C,F)** cells as frequency (%) of $CD4^+$ T cells were assessed by flow cytometry. **(G)** Plots with the gating strategy based on forward and side scatters, selecting splenocytes as a function of cell size and granularity (n = 6).

i.e., LAB, in this dairy product can increase the production of bioactive peptides (Smacchi and Gobbetti, 2000; Ayyash et al., 2018). Sperry et al. (2018) suggested that *Lactobacillus casei* 01 can generate high levels of antihypertensive (ACE-I) and antioxidant peptides (DPPH) in Minas Frescal cheese. In this sense, our probiotic Minas Frescal cheese, with *L. lactis* NCDO 2118, also induced an increased amount of antihypertensive (ACEI), antioxidant (DPPH), and antidiabetic activities (α-amylase and α-glucosidase) when compared with conventional Minas Frescal cheese. Interestingly, it is recognized that oxidative stress (OS) is one of the factors involved in the onset of IBD symptoms (Moura et al., 2015); therefore, we suggest that the increase in DPPH levels in probiotic Minas Frescal Cheese could help to ameliorate inflammation conditions in UC mice.

Considering the cheese properties presented, we decided to exploit the therapeutic effect of the consumption of the probiotic Minas Frescal cheese, containing *L. lactis* NCDO 2118, in the context of DSS-induced colitis in mice.

The UC disease symptoms include weight loss, tummy pain, recurring diarrhea with blood in the stool, and malaise (Zhang and Li, 2014). The inflammation reaches the mucosa and submucosa layers of the colon section, with the presence of edema, significant depletion of goblet cells, and changes in tissue architecture and ulcerations (Cordeiro et al., 2019). UC treatments are based on the control of the symptoms and administration of anti-inflammatories and antibiotics, immunosuppressive drugs, and surgeries in severe cases. However, none of these treatments are curative and instead provoke serious collateral effects in UC patients (Chibbar and Dieleman, 2015). In this context, functional probiotic foods have been suggested to be used alone or in combination with conventional drugs and act like adjuvant therapy to enhance remission in UC patients (Rabah et al., 2020). Regarding the mice weight loss triggered by DSS administration, we observed that the treatment with the probiotic Minas Frescal cheese, for seven experimental days, was able to prevent weight loss in mice. It is important to clarify that no differences in food consumption or caloric intake were observed in all groups analyzed, which suggested that this weight gain is linked to probiotic cheese administration. As in previous studies, consumption of probiotic bacteria in a dairy food was able to prevent weight loss in inflammatory disorder mouse models (Santos Rocha et al., 2014; Plé et al., 2016; Cordeiro et al., 2018). In this work, we observed that unhealthy mice treated with probiotic Minas Frescal cheese exhibited attenuated clinical and macroscopic signs of colitis disease. This is mainly demonstrated by a decrease in DAI, hence, less diarrhea and rectal bleeding as well as the prevention of colon shortening triggered by DSS action. Similarly, Luerce et al. (2014) demonstrated that *L. lactis* NCDO 2118 improved the clinical signs of colitis by reducing the macroscopic inflammatory score of the disease, also seen by Rabah et. al (2020) who observed a reduction in the signs of UC induced by DSS by the consumption of a probiotic Emmental cheese.

Pathological assessment of UC is evidenced by extensive architectural damage of colon tissue, with erosions and ulcerations and depletion of the mucosal surface. Moreover, there was an increase in inflammatory cell infiltration in the *lamina propria*, i.e., neutrophil infiltrates, and also depletion of goblet cells

(Jeengar et al., 2017). The activity of MPO is an indicator of this extent of neutrophil infiltrates in the mucosa (Ivanovska et al., 2017). Nevertheless, our results showed that the administration of probiotic Minas Frescal cheese protects the colon mucosa from DSS injury, marked by a decrease in the histological score and also a decrease in MPO levels. Besides that, we observed that probiotic cheese was able to decrease the expression of *iNOS* gene. This gene encodes the enzyme responsible for the generation of cytotoxic and immunoregulatory free radical NO, which is related to several inflammatory processes (Sakthivel and Guruvayoorappan, 2013). These results together demonstrated that there is less inflammation in the colon tissue of DSS mice treated with probiotic cheese.

Probiotic Minas Frescal cheese, with *L. lactis* NCDO 2118, was also able to preserve the number of intact goblet cells in the colon mucosa. These cells are responsible for producing the mucus that covers the intestinal mucosa. Considering that this mucus contains high levels of sIgA, we suggested that the increased sIgA levels, observed in the intestinal content of mice, were driven by the maintenance of the number of goblet cells due to the consumption of probiotic cheese. Precisely, mucus production by goblet cells and increased levels of sIgA were reported to be some of the mechanisms of probiotic action in the host (Rogier et al., 2014). Interestingly, dairy milk can significantly induce the host response to pathogens, enhance the integrity of the mucus layer (Tong et al., 2020), and increase secretory IgA in the small and large intestines (Schofield and Palm, 2018). In our previous works, we verified that dairy milk matrices, including cheese matrix, can increase IgA secretion (Cordeiro et al., 2018 Rabah et al., 2020), while the milk matrix shows an increase in the number of goblet cells (Cordeiro et al., 2018). Thus, this would explain the increases in the levels of sIgA and goblet cells found in the group treated with our probiotic cheese. On the other hand, to uncover the exact mechanisms, the expression of intestinal immune-related gene and sIgA levels needs to be better explored. Besides that, it is recognized that the presence of the mucus in the gut prevents the adhesion of microorganisms to the mucosa and their translocation into the lumen (Grondin et al., 2020). Moreover, the mucus is important for the lubrication and protection of the intestinal epithelium from toxic substances coming from the external environment, such as DSS (Abrantes et al., 2020). Thus, in mouse colon inflammation caused by DSS intake, it is common to observe a decrease in goblet cell number, but it can be restored by the consumption of probiotic bacteria (Rodrigues et al., 2018; Zhang et al., 2018; Abrantes et al., 2020). It is important to highlight that MUC-2 is the major glycoprotein constituent of intestinal mucus and is secreted primarily by goblet cells (Perez-Vilar, 2007). Interestingly, we also observed an increase in *MUC-2* gene expression in mice treated with probiotic cheese, corroborating with the observed increased production of the mucus in these mouse groups. As seen in a previous study, probiotic strain can stimulate *MUC-2* expression in intestinal goblet cells and mitigate acute colitis in a mouse model (Ma et al., 2020). Moreover, our findings showed that probiotic cheese administration also increased the gene expression of *zo-1*, *zo-2*, *ocln*, and *cln-1*. These genes are responsible for the expression of tight junction proteins that maintaining the epithelial barrier and control cellular permeability (Landy et al., 2016).

The host's cytokine-mediated immune response plays a pivotal role during the development of acute colitis (Ko and Auyeung, 2014). Probiotic bacteria have a great ability to promote increased levels of anti-inflammatory cytokines and also to lead to a decrease in the production of pro-inflammatory cytokines (Carvalho et al., 2017). In IBD, IL-17 and IL-1β cytokines are related to the extensive lymphocyte, plasma cell, and macrophage infiltration into the tissue (Melgar et al., 2005; Shen and Durum, 2017). The decrease in the transcriptional colonic levels of IL-1β and the secretion of IL-1β and IL-17 (spleen and lymph node, respectively) by the consumption of probiotic cheese in the disease DSS group can be mediated by the action of IL-10 secretion (spleen and lymph node). IL-10 is the most important cytokine to control homeostasis in the intestinal mucosa (Sun et al., 2018), and probiotic bacteria, mainly LAB, are known to be able to increase IL-10 levels in the gut (Maldonado Galdeano et al., 2019). Interestingly, low transcriptional levels of IL-1β on the DSS groups treated with cheese (conventional or probiotic cheese) were observed. It is plausible to say that the downregulation of IL-1β can be associated with milk components in the cheese matrix, as noted by Kanwar et al. (2016). In addition, we can observe a systemic effect on the increase in IL-10 and decrease in IL-1β in the spleen of animals treated with probiotic cheese. In the local effects (lymph nodes), we see a decrease in IL-17 and an increase in IL-10, which corroborates with the low transcriptional colonic levels of IL-1β. This suggests that the effect of probiotic cheese may be associated with a decrease of pro-inflammatory Th1 and Th17 cytokines that can be linked to the enhanced production of IL-10 in the lymph nodes and spleen, as previously reported (Santos Rocha et al., 2014).

$Foxp3^+$ Tregs are the subgroup of $CD4^+CD25^+$ T cells that have the capacity to inhibit the reactive effects of T cells by producing cytokine transforming growth factor β1 (TGF-β1) and IL-10 (Maldonado Galdeano et al., 2019). $CD4^+$ T cells expressing $FOXP3^+$, LAP^+, and RORγt+ as analyzed in mouse spleens and lymph nodes (cecum and colon) show that treatment with probiotic cheese did not change regulatory T cell populations. Our work suggests that $Foxp3^+$ Tregs are not responsible directly for the therapeutic effects of probiotic bacteria, despite increased levels of IL-10 (lymph nodes and spleen). It is plausible that the therapeutic effects of probiotic Minas Frescal cheese did not act *via* the adaptive immunity. However, IL-10 staining in regulatory T cells populations can be conducted to elucidate this hypothesis. Precisely, to confirm these results, it is necessary for other experiments to be able to indicate if the release of IL-10 is by innate immune cells such as macrophages and dendritic cells.

CONCLUSION

We demonstrated that Minas Frescal cheese containing the well-characterized probiotic bacteria *L. lactis NCDO* 2118 was able to alleviate the severity of DSS-induced colitis in

a mice model, limiting histopathological damages, restoring intestinal barrier by increased expression of gene related to tight junction protein, and modulating the cytokine production in mice. Probiotic Minas Frescal cheese was also able to prevent the degeneration of goblet cells and to reduce the inflammatory cell infiltration in the colon mucosa. Moreover, experimental probiotic cheese investigated in this work was able to produce high levels of bioactive peptides with antihypertensive, antioxidant, and antidiabetic activities. These results, together, open new perspectives for the development of probiotic functional foods for use in combination with conventional drugs or for use as an adjuvant therapy to enhance remission in UC patients.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by Ethics Committee on Animal Experimentation of the Universidade Federal de Minas Gerais (CEUA-UFMG, Brazil, protocol 364/2018).

AUTHOR CONTRIBUTIONS

BC, VA, and FC conceived and designed the experiments. JA, LL, and AF performed and analyzed immunomodulatory experiments. MB was a major contributor to animal experimentation. EF performed, analyzed, and interpreted the histological analysis from colon slides. BC, GB, AG-G, and FC wrote the original draft. GJ and YL gave scientific advice. JG, RS, RR, MS, MF, EE, and AG-G manufactured the cheeses and performed centesimal and mineral composition. All authors contributed to data interpretation, drafted the manuscript, critically revised the manuscript, and approved its final version

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DISCUSSÃO GERAL

Os alimentos funcionais são aqueles produtos que fornecem um benefício à saúde do consumidor, além das já tradicionais alegações nutricionais (MITSUOKA, 2014). Entre eles, os alimentos que contêm bactérias probióticas são, hoje, uma das tendências mais importantes no mercado dos alimentos funcionais, já que representam uma alternativa para a prevenção e o tratamento de diversas doenças (NADELMAN et al., 2019). Os dados mercadológicos dos últimos anos indicam que o setor dos alimentos funcionais probióticos tem crescido continuamente, impulsionando o surgimento de novos produtos para o consumidor (DIEZ-GUTIÉRREZ et al., 2020). Os dados de análise patentária, que são poderosas ferramentas de informações tecnológicas e de inteligência competitiva do mercado, nos confirmam que há, de fato, no campo dos probióticos, um enorme esforço científico para o desenvolvimento de tecnologias mais inovadoras para a exploração mercadológica (REIS et al., 2016).

O desenvolvimento de um produto probiótico funcional que gere um impacto positivo no mercado consumidor envolve, entretanto, a escolha correta da bactéria, da matriz de proteção onde ela será inserida e da forma que ele será consumido. Estes pontos são cruciais tanto para o processo de aceitação do consumidor quanto para a garantia de que o alimento consiga, de fato, gerar algum benefício à saúde (PEREIRA et al., 2018).

Neste sentido, para a execução deste trabalho, foram selecionados três tipos distintos de queijo, o Emmental, o Prato e o queijo Minas Frescal, na tentativa de desenvolver produtos probióticos funcionais para terapia e prevenção de doenças entéricas. Esses queijos foram selecionados por serem produtos lácteos consumidos rotineiramente na França e no Brasil, sendo, então, de fácil inserção no mercado consumidor de cada um desses países (ALVES et al., 2017). Além disso, estudos prévios já haviam mostrado que essas variedades de queijos eram bons veículos para bactérias probióticas, mantendo-as vivas durante os processos de fabricação dos produtos e durante a passagem pelo TGI (GROM et al., 2020; LOLLO et al., 2012, 2015; PLÉ et al., 2015; SILVA et al., 2018c).

No capítulo 1 deste trabalho, utilizamos o queijo Emmental contendo a bactéria probiótica *P. freudenreichii* 129 na prevenção da colite aguda, sendo que os animais consumiram o queijo de forma preventiva (7 dias antes), e, em seguida, a doença era induzida através do consumo do DSS, por mais 7 dias. Esse modelo animal de colite ulcerativa utilizando o DSS vem sendo amplamente utilizado devido à facilidade na indução da doença, rapidez, simplicidade, reprodutibilidade, uniformidade e controlabilidade (EICHELE; KHARBANDA, 2017). A versatilidade de tal modelo possibilita modificações nas doses e ciclos de tratamento, permitindo modelar as formas agudas, recorrentes e crônicas da inflamação intestinal (WIRTZ et al., 2017).

Os animais tratados preventivamente com os queijos Emmental apresentaram menor perda de peso, índice de atividade da doença e menor escore histológico. Além disso, o consumo do queijo probiótico ocasionou uma redução na secreção de Imunoglobulina A (IgA), restaurou a expressão do gene ocludina e evitou a indução do fator de necrose tumoral α (TNFα), interferon γ (IFNγ) e interleucina-17 (IL-17). **(Figura 4).** Esses resultados demonstraram, portanto, que o consumo do queijo probiótico foi capaz de prevenir o aparecimento de sintomas graves da inflamação intestinal característica da doença. Esse efeito ocorreu, possivelmente, devido à capacidade da *P. freudenreichii* 129 em persistir no TGI do animal, permitindo o aparecimento dos efeitos anti-inflamatórios mesmo após o fim do consumo da bactéria, ou devido à capacidade dessa linhagem em secretar fatores bifidogênicos e outros compostos nutracêuticos que podem modular a microbiota comensal e restaurar a disbiose desencadeadora da colite ulcerativa (RABAH; ROSA DO CARMO; JAN, 2017). Estudos anteriores já haviam demonstrado que a *P. freudenreichii* 129 era capaz de persistir no TGI prevenindo a colite (PLÉ et al., 2015). Outros estudos comprovaram que linhagens de *P. freudenreichii* são capazes de produzirem fatores bifidogênicos, como o ácido 1,4-di-hidroxi-2-naftoico (DHNA) e 2-amino-3-carboxi-1,4-naftoquinona (ACNQ), que afetam de forma positiva a microbiota do indivíduo (OKADA et al., 2006). Esses dados do queijo Emmental nos mostram que ele é um queijo eficaz para melhorar os sintomas da colite, sendo, portanto, um alimento funcional com boas perspectivas para ser usado na prevenção dessa doença.

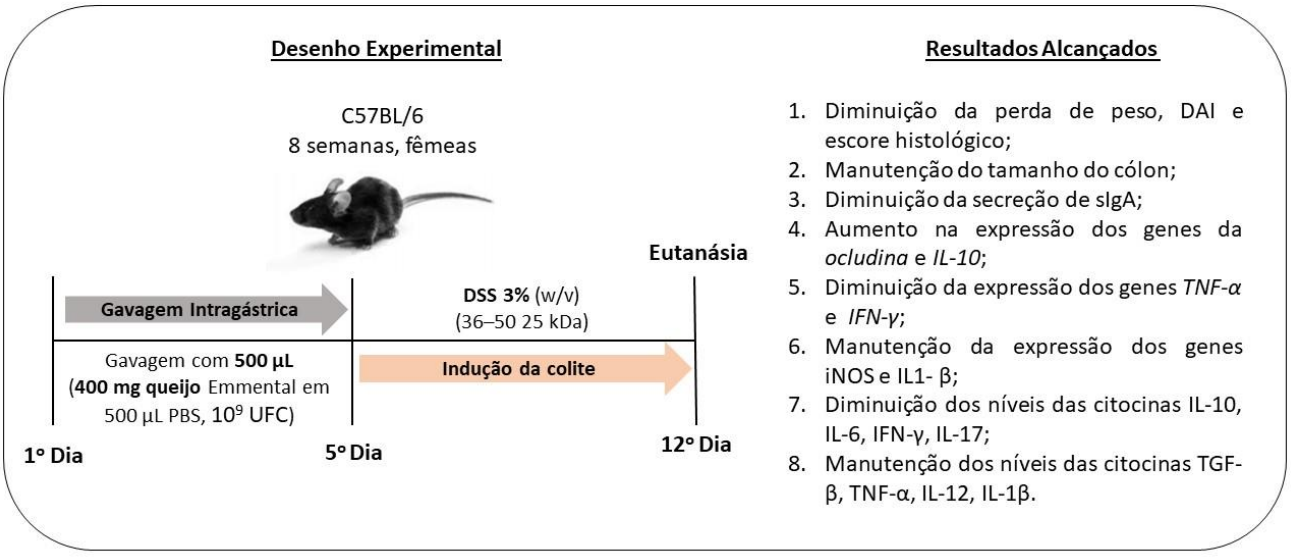

Figura 4: Desenho representativo dos principais resultados obtidos pelo consumo de queijo Emmental na atenuação da colite ulcerativa induzida por DSS, em camundongos

No capítulo 2 deste trabalho, desenvolvemos um queijo Prato, tipicamente brasileiro, contendo a bactéria *L. casei* 01, e testamos o seu efeito em modelo animal semelhante ao utilizado no estudo anterior do queijo Emmental. Entretanto, apesar dos bons resultados anteriores apresentados, tanto pelo uso do queijo Prato como matriz de proteção, quanto pelo potencial probiótico da *L. casei* 01, o queijo Prato desenvolvido neste trabalho não alcançou os resultados esperados para a prevenção da colite. Podemos observar que os dados clínicos e histomorfológicos como perda de peso, DAI e escore inflamatório não apresentaram diferenças significativas quando comparávamos os animais doentes tratados e não tratados com o queijo probiótico. Parâmetros como secreção de igA e a expressão de genes ligados a citocinas anti-inflamatórias (IL-10) e pró-infamatórias (IL-6), também não apresentaram diferenças estatísticas entre os grupos experimentais observados **(Figura 5)**. Esses resultados nos permitiram inferir que a *L. casei* 01, possivelmente, não tem a capacidade de persistir no intestino por tempo suficiente para exercer um efeito preventivo no camundongo. Apesar de já ter sido associada a resultados benéficos (GALDEANO; PERDIGO, 2006; SPERRY et al., 2018), o consumo da *L. casei* 01 nem sempre representou uma atividade probiótica positiva em outros

modelos de doenças inflamatórias e infecções (CORDEIRO et al., 2019b; NADELMAN et al., 2019).

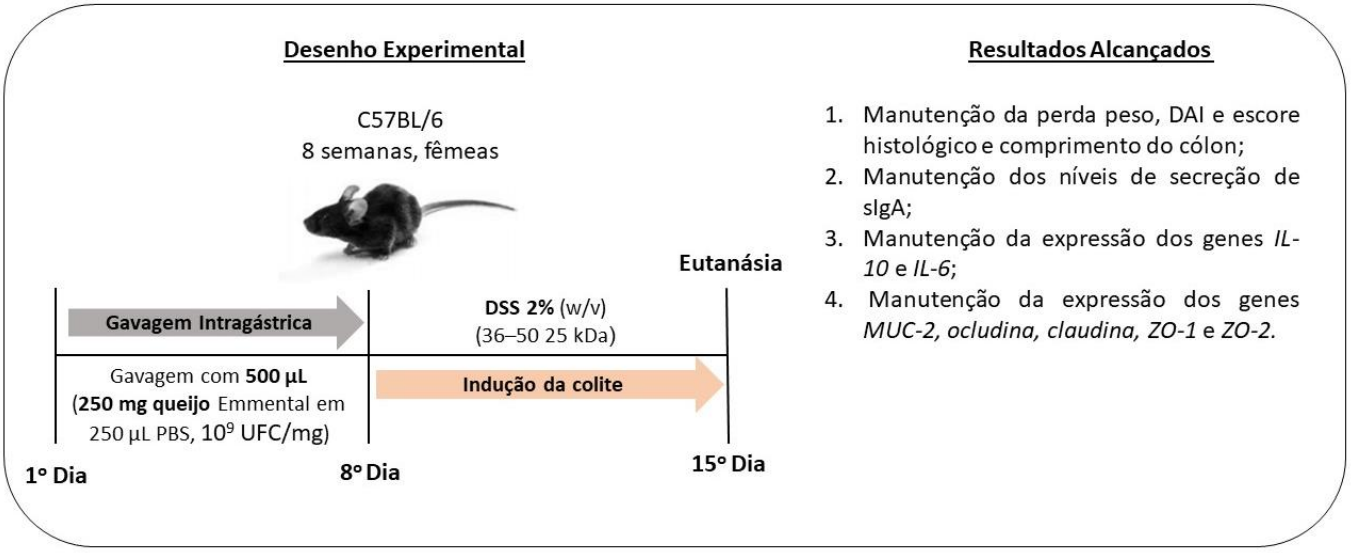

Figura 5: Desenho esquemático demonstrando os principais resultados alcançados pelo consumo do queijo Prato contento *L. casei* 01 na prevenção da colite ulcerativa

Diante dos resultados negativos apresentados pelo consumo do queijo Prato, buscamos desenvolver um outro queijo na tentativa de se obter um produto que fosse realmente efetivo. Para tanto, produzimos um queijo, agora do tipo Minas Frescal, que, assim como o Prato, tem uma boa entrada no mercado brasileiro (ALVES et al., 2017). Além disso, escolhemos a linhagem *L. Lactis* NCDO 2118, que possui comprovado potencial probiótico em modelo murino de colite ulcerativa (DA SILVA et al., 2019; LUERCE et al., 2014). Decidimos, também, por alterar o protocolo de prevenção da colite para um protocolo de tratamento, considerando que buscávamos desenvolver um produto para terapia adjuvante e que fosse usado durante as recidivas da doença.

Os resultados deste estudo mostraram que os animais que consumiram o queijo Minas Frescal probiótico tiveram uma redução na gravidade da colite, apresentando atenuação dos sinais clínicos da doença como menor perda de peso, fezes menos diarreicas e sanguinolentas, um encurtamento mais limitado do cólon e pontuação histopatológica reduzida **(Figura 6)**. Além disso, a ingestão do queijo

restaurou a barreira epitelial do colón pelo aumento da expressão dos genes *claudina*, *ocludina*, *ZO-1* e *ZO-2* e do gene *MUC-2*; modulou a produção de citocinas, como a IL-10, IL-17 e IL-1β, sugerindo que o efeito do consumo do queijo probiótico pode estar associado a uma diminuição das citocinas pró-inflamatórias do tipo Th1 e Th17, que possivelmente estão ligadas à produção aumentada de IL-10 nos tecidos; à prevenção da degeneração das células caliciformes produtoras de muco que protegem a mucosa do intestino e à diminuição da infiltração de neutrófilos no tecido, indicando um quadro inflamatório mais reduzido.

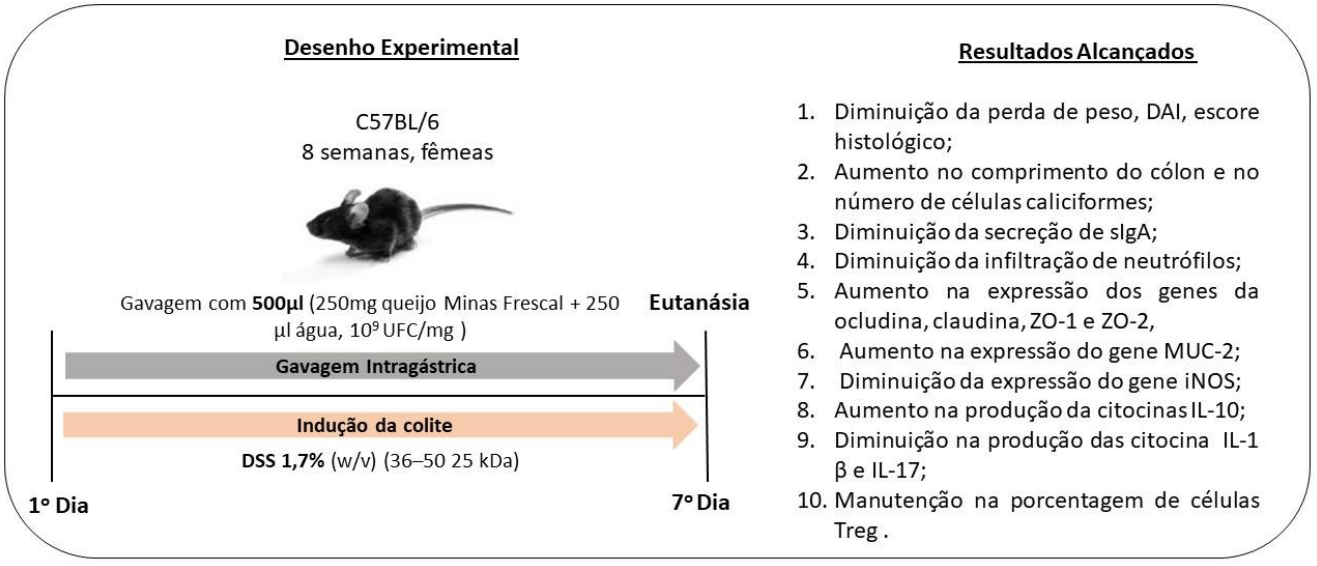

Figura 6: Desenho esquemático apresentando os principais resultados obtidos pelo consumo de queijo Minas Frescal contendo a *L. lactis* NCDO 2118

O consumo do queijo probiótico experimental investigado no capítulo 3 também foi capaz de produzir altos níveis de peptídeos bioativos, com atividades anti-hipertensivas, antioxidantes e antidiabéticas. Em contrapartida, a adição da *L. lactis* NCDO 2118 não alterou os parâmetros de composição centesimal do queijo (umidade, proteína, gordura e lactose), bem como os teores de sódio e cálcio e valores de pH, mantendo as características organolépticas típicas da variedade Minas Frescal. Por fim, uma análise sensorial do queijo probiótico desenvolvido neste trabalho indicou que o produto teve uma boa aceitação pelos provadores,

apresentando uma nota geral estatisticamente maior que a nota dada ao queijo Minas Frescal convencional **(Tabela 1).**

Tabela 1: Aceitação do consumidor ao queijo Minas Frescal probiótico

QUEIJO	APARÊNCIA	AROMA	SABOR	TEXTURA	ASPECTO GERAL
Minas Frescal convencional	7.4 (1.5)	6.51 (1.45)	6.91 (1.11)[b]	6.02 (1.34)[b]	6.92 (1.34)[b]
Minas Frescal probiótico	7.8 (1.21)	6.2 (1.89)	7.53 (1.34) [a]	7.12 (0.92)[a]	7.78 (1.77)[a]

*Os valores representam médias e desvio padrão. Valores médios de 80 consumidores (45 mulheres, 35 homens, idade entre 17– 63 anos), recrutados aleatoriamente na Universidade Federal Fluminense (UFF). Os consumidores foram solicitados a usar uma escala hedônica híbrida de 9 pontos (1 = não gostou imensamente, 5 = não gostei nem desgostei e 9 = gostei imensamente) para avaliar o grau de aprovação dos queijos no que diz respeito a aparência, aroma, sabor, textura e aspecto geral. O estudo foi aprovado pelo Comitê de Ética em Pesquisa com Seres Humanos da Universidade Federal Fluminense.
[A, B] Letras diferentes na mesma coluna demonstram diferenças estatísticas entre os grupos (p <0,05).

Interessantemente, esses resultados refletem uma semelhança com os resultados exibidos no capítulo 1, quando os animais consumiram o queijo Emmental probiótico. Esses dados nos sugerem que os efeitos terapêuticos de um alimento funcional contendo essas bactérias probióticas na atenuação da colite ulcerativa induzida por DSS estejam ligados à proteção da barreira epitelial, pelo aumento da expressão de proteínas de barreira e pela regulação do sistema imune inato, principalmente pelo aumento da produção de citocinas anti-inflamatórias. Por sua vez, o consumo do queijo Prato contendo a *L. casei* 01 não foi capaz de gerar repercussão nesses parâmetros, como foi demonstrado no capítulo 2, e, desta forma, o consumo deste queijo não causou melhoras significativas nos sinais clínicos da colite. Tais resultados corroboram com dados já publicados na literatura (ALIPOUR et al., 2014; GOMES-SANTOS et al., 2012; LUERCE et al., 2014; MALDONADO GALDEANO et al., 2019; MENNIGEN et al., 2009b; SANTOS ROCHA et al., 2014; VAN DER SLUIS et al., 2006).

Por fim, o trabalho apresentado no capítulo 3 demonstrou que o consumo do queijo Minas Frescal probiótico, contendo *L. lactis* NCDO 2118, atenua o processo inflamatório durante a colite induzida por DSS em camundongo e possui uma boa aceitação do consumidor e uma aplicação industrial, apresentando um bom valor mercadológico e podendo, portanto, ser produzido em larga escala. Essas

características, em conjunto, abrem boas perspectivas para o desenvolvimento de um novo alimento probiótico funcional para nutrição personalizada no contexto das IBDs.

PERSPECTIVAS

1. Testar o efeito probiótico do queijo Minas Frescal contendo a *L. lactis* NCDO 2118 em modelo de prevenção da colite ulcerativa induzida por DSS e avaliar seus efeitos clínicos, histomorfológicos e imunológicos;

2. Testar o efeito probiótico do queijo Minas Frescal contendo a *L. lactis* NCDO 2118 em outros modelos de doenças, como mucosite induzida por quimioterápicos, hipertensão e câncer colorretal, induzido por Azoximetano, abrindo o leque de uso do produto probiótico;

3. Criar estratégias de proteção para a tecnologia desenvolvida, seja por meio de patente ou *Know-How*;

4. Produzir o queijo em escala-piloto;

5. Criar um modelo de negócio para o queijo probiótico e buscar pela transferência da tecnologia para empresas interessadas no desenvolvimento do produto em larga escala.

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APÊNDICE

PRODUÇÕES ACADÊMICAS

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