UNIVERSIDADE FEDERAL DE MINAS GERAIS
Instituto de Ciências Biológicas
Departamento de Genética, Ecologia e Evolução
Programa de Pós-graduação em Genética

Fernanda Alvarenga Lima Barroso

Aplicação bioterapêutica de *Lactobacillus delbrueckii* CIDCA 133 selvagem e recombinante (pExu:*hsp65*) em modelo murino de mucosite intestinal

Belo Horizonte

2022

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Aplicação bioterapêutica de *Lactobacillus delbrueckii* CIDCA 133 selvagem e recombinante (pExu:*hsp65*) em modelo murino de mucosite intestinal

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Orientador: Prof. Dr. Vasco Azevedo

Coorientador: Dr. Luís Cláudio Lima de Jesus

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ATA DE DEFESA DE TESE

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Fernanda Alvarenga Lima Barroso	

Às quatorze horas do dia **17 de outubro de 2022**, reuniu-se, Auditório Prof. Mário Souza Couto Barbosa - Escola de Veterinária da UFMG, a Comissão Examinadora de Tese, indicada pelo Colegiado do Programa, para julgar, em exame final, o trabalho intitulado: **"Aplicação bioterapêutica de Lactobacillus delbrueckii CIDCA 133 selvagem e recombinante (pExu:hsp65) em modelo murino de mucosite intestinal "**, requisito para obtenção do grau de Doutora em **Genética.** Abrindo a sessão, o Presidente da Comissão, **Vasco Ariston de Carvalho Azevedo**, após dar a conhecer aos presentes o teor das Normas Regulamentares do Trabalho Final, passou a palavra à candidata, para apresentação de seu trabalho. Seguiu-se a arguição pelos Examinadores, com a respectiva defesa da candidata. Logo após, a Comissão se reuniu, sem a presença da candidata e do público, para julgamento e expedição de resultado final. Foram atribuídas as seguintes indicações:

Prof./Pesq.	**Instituição**	**CPF**	**Indicação**
Vasco Ariston de Carvalho Azevedo	UFMG	283.171.225-49	APROVADA
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Jean Guy LeBlanc	CERELA/Argentina	AAD258698	APROVADA
Rodrigo Dias Carvalho	UFBA	080.173.536-00	APROVADA

Pelas indicações, a candidata foi considerada: APROVADA.

O resultado final foi comunicado publicamente à candidata pelo Presidente da Comissão. Nada mais havendo a tratar, o Presidente encerrou a reunião e lavrou a presente ATA, que será assinada por todos os membros participantes da Comissão Examinadora.

Belo Horizonte, 17 de outubro de 2022.

Vasco Ariston de Carvalho Azevedo - Orientador (UFMG)

Luís Cláudio Lima de Jesus - Coorientador (UFMG)

Cristina Stewart Bittencourt Bogsan (USP)

Anderson Miyoshi

Yves Le Loir (INRAE/França)

Jean Guy LeBlanc (CERELA/Argentina)

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UNIVERSIDADE FEDERAL DE MINAS GERAIS
Programa de Pós-Graduação em Genética
Instituto de Ciências Biológicas

FOLHA DE APROVAÇÃO

"Aplicação bioterapêutica de Lactobacillus delbrueckii CIDCA 133 selvagem e recombinante (pExu:hsp65) em modelo murino de mucosite intestinal "

Fernanda Alvarenga Lima Barroso

Tese aprovada pela banca examinadora constituída pelos Professores:

Vasco Ariston de Carvalho Azevedo
UFMG

Luís Cláudio Lima de Jesus
UFMG

Cristina Stewart Bittencourt Bogsan
USP

Anderson Miyoshi
UFMG

Yves Le Loir
INRAE/França

Jean Guy LeBlanc

CERELA/Argentina

Rodrigo Dias Carvalho
UFBA

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Dedico este trabalho a minha menina Maria Flor (47, XX+21) que me ensina todos os dias que aquilo que é considerado um erro para a genética foi na verdade o maior acerto da minha vida, porque o amor não conta cromossomos!

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"O caminho muda, e muda o caminhante. É um caminho incerto, não o caminho errado. Eu, caminhante, quero o trajeto terminado. Mas no caminho, mais importa o durante”.

(Estevão Queiroga)

RESUMO

A mucosite intestinal é uma inflamação da mucosa intestinal que ocorre frequentemente em pacientes sob tratamento com drogas anticâncer, como o 5-Fluorouracil (5-FU). Por ser um sério problema na clínica médica, tem crescido a busca por estratégias terapêuticas que visem minimizar este efeito colateral. Microrganismos probióticos são considerados uma promissora abordagem para esta finalidade, devido às suas propriedades anti-inflamatórias. Como forma de potencializar os efeitos benéficos destes microrganismos, ferramentas de biotecnologia e biologia molecular têm sido desenvolvidas e aprimoradas para a construção de linhagens probióticas recombinantes capazes de produzir e entregar biomoléculas terapêuticas às superfícies de mucosa do hospedeiro. Nesse contexto, a proteína de choque térmico 65 kDa (Hsp65) de *Mycobacterium leprae* produzida por bactérias probióticas vem apresentando boas características imunomoduladoras. Assim, este trabalho avaliou os mecanismos moleculares envolvidos com o efeito enteroprotetor de *Lactobacillus delbrueckii* CIDCA 133 selvagem e recombinante (pExu:*hsp65*) em modelo de mucosite intestinal induzida pelo quimioterápico 5-FU (300mg/Kg). Os principais resultados obtidos revelaram que a administração oral de CIDCA 133 selvagem atenua os danos epiteliais e inflamatórios induzido por 5-FU por meio da prevenção do infiltrado de células inflamatórias, redução da expressão gênica de marcadores envolvidos na ativação da via de sinalização de NF-κB (*Tlr2, Tlr4, Nfkb1, Tnf, Il6, Il12* e *Il1b*) e regulação positiva da expressão da citocina imunoregulatória *Il10.* Além disso, os efeitos benéficos da linhagem podem ser atribuídos à sua capacidade em aumentar a expressão gênica de marcadores envolvidos na barreira epitelial, como a mucina 2 e as proteínas de junção firme (ocludina, claudina 2, zonulina e JAM), colaborando para a preservação da permeabilidade intestinal, e assim melhorando a arquitetura da mucosa e seu funcionamento. Em paralelo, utilizando as técnicas de biologia molecular e melhoramento genético foi construído o vetor de expressão eucariótica pExu:*hsp*65 e a linhagem recombinante rCIDCA 133:HSP65, a qual também foi usada em um ensaio *in vivo* utilizando o mesmo modelo murino de inflamação intestinal. A administração oral da linhagem recombinante potencializou os efeitos de CIDCA 133 selvagem, no qual foi possível observar uma maior preservação do epitélio intestinal destruído por 5-FU por meio da redução da expressão gênica de *Il1b* e *Il6*, e aumento na expressão gênica de mucina 2, e também das proteínas de junção firme claudina 1, claudina 2 e JAM. A entrega da bactéria recombinante também potencializou o número de células caliciformes produtoras de muco, e a redução do infiltrado inflamatório na mucosa e da permeabilidade intestinal. Desta forma, diante dos resultados promissores alcançados, o presente estudo abre caminho para a consolidação da linhagem de *Lactobacillus delbrueckii* CIDCA 133 como probiótica além de demonstrar a capacidade deste microrganismo de entregar de maneira eficiente o vetor de expressão eucariótica pExu:*hsp65,* se apresentando, portanto, como uma estratégia terapêutica promissora para a prevenção e tratamento da mucosite intestinal, e de outras doenças inflamatórias relacionadas ao trato gastrointestinal.

Palavras-chave: 5-Fluorouracil; Inflamação intestinal; Probióticos; Proteína de choque térmico; Proteína recombinante; Imunomodulação; Barreira epitelial

ABSTRACT

Intestinal mucositis is an inflammation of the intestinal mucosa that occurs frequently in patients under treatment with anticancer drugs, such as 5-Fluorouracil (5-FU). Because it is a serious problem in clinical practice, there is a growing search for therapeutic strategies that aim to minimize this side effect. Probiotic microorganisms are considered a promising approach for this purpose, due to their anti-inflammatory capacity. As a way to potentiate the beneficial effects of these microorganisms, biotechnology and molecular biology tools have been developed and improved to build recombinant probiotic strains capable of producing and delivering therapeutic biomolecules to the host mucosal surfaces. In this context, the heat shock protein 65 Kda (Hsp65) of *Mycobacterium leprae* produced by probiotic bacteria has been showing good immunomodulatory characteristics. Thus, this work evaluated the molecular mechanisms involved with the enteroprotective effect of wild and recombinant *Lactobacillus delbrueckii* CIDCA 133 (pExu:*hsp*65) in a model of intestinal mucositis induced by the chemotherapy 5-FU (300mg/Kg). The main results obtained revealed that oral administration of wild-type CIDCA 133 attenuates 5-FU-induced epithelial and inflammatory damage through prevention of inflammatory cell infiltrate, reduced gene expression of markers involved in the activation of NF-κB signaling pathway (Tlr2, Tlr4, Nfkb1, Tnf, Il6, Il12 and Il1b) and positive regulation of the expression of immunoregulatory cytokine Il10. Furthermore, the beneficial effects of the strain can be attributed to its ability to increase gene expression of markers involved in the epithelial barrier, such as mucin 2 and firm junction proteins (occludin, claudin 2, zonulin, and JAM), collaborating to preserve intestinal permeability, and thus improving mucosal architecture and function. In parallel, using molecular biology and genetic improvement techniques, the eukaryotic expression vector pExu:*hsp*65 and the recombinant strain rCIDCA 133:HSP65 were constructed, which was also used in an *in vivo* assay using the same murine model of intestinal inflammation. Oral administration of the recombinant strain potentiated the effects of wild-type CIDCA 133, in which greater preservation of the intestinal epithelium destroyed by 5-FU was observed through reduced gene expression of Il1b and Il6, and increased gene expression of mucin 2, and also of the firm junction proteins claudin 1, claudin 2, and JAM. Delivery of the recombinant bacterium also potentiated the reduction of inflammatory infiltrate in the mucosa, intestinal permeability, and number of mucus-producing calliciform cells. Thus, in view of the promising results achieved, the present study paves the way for the consolidation of the *Lactobacillus delbrueckii* CIDCA 133 strain as a probiotic in addition to demonstrating the ability of this microorganism to efficiently deliver the eukaryotic expression vector pExu:*hsp*65 presenting itself as a promising therapeutic strategy for the prevention and treatment of intestinal mucositis, and other inflammatory diseases related to the gastrointestinal tract.

Keywords: 5-Fluorouracil; Intestinal inflammation; Probiotics; Recombinant protein; Immunomodulation; Epithelial barrier

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LISTA DE ABREVIATURAS

5-FU 5-Fluourouracil
^{99m}Tc Tecnécio-^{99m}
^{99m}Tc-DTPA Ácido dietilenotriaminopentacético marcado com tecnécio-^{99m}
AMPs Peptídeos antimicrobianos (do inglês, *antimicrobial peptides*)
BAL Bactéria do Ácido Láctico
CD Células dendríticas
CEBIO Centro de Bioterismo
CEIs Células epiteliais intestinais
CEUA Comitê de Ética no Uso de Animais
CIDCA 133 *Lactobacillus delbrueckii* subsp. *lactis* CIDCA 133
DMSO Dimetilsulfóxido
DTPA Ácido dietilenotriaminopentaacético (do inglês, *diethylenetriaminepentaacetic acid*)
DSS do inglês, *Dextran Sulfate Sodium*
EPO Peroxidase de Eosinófilos (do inglês, *Eosinophil peroxidase*)
FAO do inglês, *Food and Agriculture Organization*
FDA do inglês, *Food and Drug Administration*
GRAS do inglês, *Generally Recognized As Safe*
Hsps do inglês, *Heat Schok Proteins*
IgA Imunoglobulina A
IL Interleucina
JO Junção de Oclusão
M Molar
MBq Megabecquerel
MET Microscopia eletrônica de transmissão
MHC Complexo maior de histocompatibilidade (do inglês, *major histocompatibility complex*)
MPO Mieloperoxidase
MRS Meio de cultura Man, Rogosa e Sharpe
OPD O-fenilenodiamina
ORF do inglês, *Open Reading Frame*

PAMP Padrões Moleculares Associados a Patógenos (do inglês, *Pathogen-associated molecular pattern*)

PAS Ácido Periódico Combinado de Schiff (do inglês, *Periodic Acid Schiff*)

pExu do inglês, *Extra Chromosomal Unit*

pCMV Promotor do citomegalovírus

PMSF Fluoreto de fenilmetano sulfonil (do inglês, *phenylmethylsulfonyl fluoride*)

QPS do inglês, *Qualified Presumption of Safety*

ROS Espécie Reativa de Oxigênio (do inglês, *reactive oxygen species*)

sIgA Imunoglobulina A secretória

TGI Trato Gastrointestinal

TJs do inglês, *tight junctions*

TLR do inglês, *Toll like Receptor*

TMB 3,3',5,5'-tetrametilbenzidina

TNF Fator de Necrose Tumoral (do inglês, *tumor necrosis factor*)

Treg Linfócito T regulatório (do inglês, *Regulatory T cells*)

WHO do inglês, *World Health Organization*

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PREFÁCIO

Este manuscrito foi organizado e escrito nas seções descritas abaixo, a fim de facilitar a sua apresentação e compreensão:

1 - Introdução Geral: Uma breve introdução apresentando as Bactérias do Ácido Láctico e as novas aplicações biotecnológicas e terapêuticas destes microrganismos.

2 – Revisão Bibliográfica: Será abordado as Bactérias do Ácido Láctico e os microrganismos com perfil probiótico e sua interação com o trato gastrointestinal. Será apresentado também a mucosite intestinal, doença inflamatória escolhida como modelo deste trabalho, e os principais trabalhos já reportados utilizando probióticos, selvagens ou recombiantes, para a terapêutica dessa doença. Esta seção também discutirá as principais ferramentas genéticas já desenvolvidas para a construção de bactérias lácticas recombinantes, visando explorar e potencializar os resultados já obtidos com as linhagens selvagens, e os principais resultados já descritos na literatura utilizando essas cepas em modelos animais. Por fim, um capitulo de livro já publicado será inserido nessa revisão bibliográfica, pontuando as principais características do trato gastrointestinal e sua microbiota, além de apresentar brevemente algumas doenças inflamatórias decorrentes do desequilíbrio da homeostase intestinal e os principais estudos já reportados utilizando bactérias probióticas recombinantes expressando diferentes moléculas para o tratamento ou prevenção destas doenças.

3 - Relevância do Projeto de Tese: A utilização de Bactérias do Ácido Láctico na promoção da saúde humana e animal é algo bem caracterizado e vem sendo cada vez mais explorado diante de novas linhagens identificadas e testadas, além do desenvolvimento de cepas recombinantes com o aprimoramento das técnicas de biologia molecular. Neste contexto, este trabalho se propôs a explorar as propriedades imunomoduladoras associadas à utilização da bactéria *L. delbrueckii* CIDCA 133, selvagem e recombiante, em um modelo de mucosite intestinal induzida pelo quimioterápico 5- Fluorouracil (5-FU), fornecendo bases científicas para a aplicação probiótica deste microrganismo.

4 – Objetivos Da Tese: Avaliar os mecanismos moleculares envolvidos com o efeito enteroprotetor de *Lactobacillus delbrueckii* CIDCA 133 selvagem e recombinante (pExu:*hsp65*) em modelo de mucosite intestinal.

5 - Capítulo I: Apresentado em forma de artigo científico, demonstrará os principais mecanismos moleculares associados com o efeito enteropotetor da linhagem selvagem *L. delbrueckii* CIDCA 133 no modelo de inflamação intestinal induzida por 5-FU.

6 - Capítulo II: Apresentado em forma de artigo científico, descreve a construção da linhagem recombiante *L. delbrueckiii* CIDCA 133 (pExu:*hsp*65), a confirmação da funcionalidade do vetor pExu:*hsp*65 por microscopia confocal e a avaliação do efeito imunomodulador desta linhagem recombinante no modelo murino de mucosite intestinal induzida por 5-FU.

7 - Conclusões Gerais: Sumariza os principais achados desta Tese.

8 - Perspectivas: Descreve as próximas etapas a serem desenvolvidas a partir dos dados obtidos nesta Tese.

9 - Referências: Lista as referências da introdução geral e da revisão bibliográfica usadas para uma melhor fundamentação teórica desta Tese.

Anexos: Lista as principais produções científicas publicadas durante o doutorado.

1 INTRODUÇÃO GERAL

As Bactérias do Ácido Láctico (BAL) compõem um grupo de microrganismos Gram-positivos bastante diversos, que compartilham, dentre outras tantas características, a capacidade de produzir ácido láctico (via homofermentativa) e/ou ácido láctico e outros produtos metabólicos (via heterofermentativa) a partir da metabolização de diferentes açucares, principalmente glicose (Carr *et al.*, 2002; Makarova & Koonin, 2007).

Devido a essa importante característica, esses microrganismos têm sido utilizados há séculos na preparação de alimentos fermentados, seja como culturas iniciadoras ou adjuntas (Motta & Gomes, 2015; Colombo *et al.*, 2018; Cosentino *et al.*, 2018). Além disso, como consequência da acidificação do meio (pH 4,5-3,5) e também da produção de ácidos orgânicos (láctico, acético e propiônico) e bacteriocinas resultantes do processo de fermentação, esses microrganismos são responsáveis por inibir o crescimento de agentes patogênicos e deteriorantes de alimentos fermentados, levando, assim, a uma maior conservação desses produtos e aumentando seu tempo de prateleira (Cosentino *et al.*, 2018).

O consumo de produtos fermentados realizados por algumas linhagens de BAL tem promovido efeitos benéficos ao hospedeiro, incluindo capacidade imunomoduladora. Tal característica está intrinsicamente associada à propriedade probiótica desses microrganismos, cujos efeitos benéficos vêm sendo demonstrado em diferentes modelos de doenças, principalmente aquelas relacionadas ao trato gastrointestinal, incluindo a mucosite intestinal (Oh *et al.*, 2017; de Jesus *et al.*, 2019; Hu *et al.*, 2020).

Além de sua já consolidada utilização na indústria de alimentos, as BAL apresentam também um enorme potencial biotecnológico para serem utilizadas como: (i) “usinas celulares” para a produção de proteínas heterólogas e biomoléculas de interesse biotecnológico e terapêutico (Nouaille *et al.*, 2003; Bermúdez-Humarán *et al*., 2004); e (ii) vetores para a entrega de vacinas de DNA (Chatel *et al*., 2008; Guimarães *et al*., 2006a; Innocentin *et al*., 2009; Tao *et al*., 2011).

Nesse contexto, o crescente avanço no conhecimento genético destes microrganismos aliado ao desenvolvimento das técnicas de biotecnologia e biologia molecular propiciou a construção de importantes vetores de clonagem e também de sistemas de expressão de proteínas heterólogas e de vacinas de DNA para fins terapêuticos e de imunização. Diversos vetores desenvolvidos para utilização na abordagem de vacina de DNA, usando bactérias lácticas como veículos de entrega ao nível da mucosa, utilizam principalmente as bactérias do gênero

Lactococcus (Guimarães *et al.*, 2006b; Guimarães *et al.*, 2009; Tao *et al.*, 2011; Mancha-Agresti *et al.*, 2017; Yagnik *et al.*, 2018).

Visando ampliar a utilização de outras BAL como carreadores de vacina de DNA, novos vetores vêm sendo construídos e aprimorados. Entre estes vetores, destaca-se o pExu (*Extra Chromosomal Unit*) (6854 Kb), construído por nosso grupo de pesquisa (Mancha-Agresti, *et al.*, 2017a), e que apresentou estabilidade para se replicar em diferentes linhagens testadas até o momento, como *E. coli* Top10, *L. lactis* MG1363, e *Lactobacillus delbrueckii* CNRZ327.

Assim, a aplicação de um vetor de amplo espectro como o pExu, combinado com as vantagens da imunização por via das mucosas e o já reconhecido efeito benéfico que muitos microrganismos probióticos possuem, possibilita o desenvolvimento de novas estratégias de terapias gênicas visando o tratamento ou prevenção de diversas doenças inflamatórias, principalmente aquelas que acometem o trato gastrointestinal, como a mucosite intestinal.

2 REVISÃO BIBLIOGRÁFICA

2.1 Mucosite intestinal e 5-Fluorouracil

A mucosite é uma inflamação com consequências clínicas importantes que acomete o trato gastrointestinal (TGI), sendo mais frequentemente associada à cavidade oral e ao intestino delgado (Sonis, 2004; Peterson *et al.*, 2012). A mucosite é um dos efeitos colaterais mais comuns em pacientes submetidos à radioterapia e/ou quimioterapia, e cuja incidência encontra-se em torno de 40 a 80% nos indivíduos submetidos a esses tratamentos (Lalla *et al.*, 2008; Campos *et al.*, 2014).

Nos casos mais leves, a mucosite é caracterizada por presença de lesões atróficas eritematosas nas quais a mucosa ainda permanece intacta, fazendo com que os pacientes apresentem uma sensibilidade aos alimentos semelhante à uma queimação, a qual pode ser gerenciada com relativa facilidade. Por outro lado, nos casos mais severos da doença, os pacientes normalmente apresentam dor intensa, que usualmente requer analgesia medicamentosa, devido às graves lesões ulcerativas que penetram na camada submucosa com exacerbada resposta inflamatória (Sonis, 2004).

A mucosite apresenta como principais sinais e sintomas a diarreia, náuseas, vômitos, dor e distensão abdominal, ulcerações, constipação e má absorção de nutrientes (Lalla *et al.*, 2008; van Vliet *et al.*, 2010), debilitando ainda mais os pacientes que já se encontram imunossuprimidos. Estes fatores estão clinicamente associados à redução da qualidade de vida dos pacientes, além de estarem associados a um pior prognóstico da doença, visto que aumentam o risco de infecções sistêmicas, podendo levar a desnutrição, diminuição das doses ou adiamento/descontinuidade do tratamento e as chances de cura, e assim contribuindo para aumento da letalidade nesse grupo de indivíduos (Van vliet *et al.*, 2010).

Entre os principais agentes quimioterápicos que desencadeiam a mucosite destaca-se o 5-Fluourouracil (5-FU), um antineoplásico da classe das fluopirimidinas que é amplamente utilizado no câncer de colorretal avançado e nas neoplasias malignas de cabeça e pescoço (Giacchetti *et al.*, 2000; Chang *et al.*, 2012; Kim *et al.*, 2015).

O 5-FU é um quimioterápico antimetabólito, análogo da uracila e da timina, diferenciando-se destas por apresentar um átomo de flúor no lugar do átomo de hidrogênio na posição 5 do anel aromático (**Figura 1**) (Longley *et al.*, 2003; Batista *et al.*, 2020).

Figura 1: **Estrutura química do 5-fluorouracil e seus análogos uracila e timina**. Todas as estruturas se diferem no radical presente na 5ª posição do anel aromático (adaptado de Batista *et al*., 2020).

O efeito antineoplásico do 5-FU ocorre por meio da sua conversão em metabólitos secundários (FdUMP, FdUTP e FUTP) que atuam por meio de três diferentes mecanismos de ação, respectivamente, conforme ilustrado na **Figura 2**: (i) inibição da atividade da enzima timidilato sintase, essencial para a síntese e o reparo do DNA; (ii) ligação à estrutura do DNA inibindo a sua síntese e, consequentemente, bloqueando a divisão celular; e (iii) incorporação ao RNA, desencadeando danos à sua composição, resultando em perda funcional e morte celular (Longley *et al*., 2003; Zhang *et al*., 2008; Miura *et al*., 2010).

Figura 2: Metabólitos do 5-fluorouracil e seus alvos moleculares. O 5-FU é metabolizado intracelularmente em FdUMP, que se liga à enzima timidilato sintetase, resultando em uma produção reduzida de dTTP, bloqueando a síntese e o reparo do DNA; FdUTP, que se liga ao DNA inibindo sua duplicação e transcrição; ou FUTP, que se liga ao RNA levando a uma perda de função. Todos os três metabólitos causam danos à célula ao nível genômico, culminando na morte celular (adaptado de Batista *et al*., 2020)

O 5-FU além de atuar sobre as células neoplásicas também atua sobre células normais, principalmente aquelas com elevada frequência de renovação, como as células da medula óssea e as células epiteliais do trato gastrointestinal (Wang *et al*., 2006; Soares *et al*., 2013; Song *et al*., 2016). Em decorrência dessa inespecificidade é que muitos pacientes submetidos ao tratamento com 5-FU apresentam a mucosite intestinal (Sonis, 2004), uma vez que este quimioterápico conduz à apoptose das células intestinais, com subsequente perda da arquitetura da mucosa, com alteração das vilosidades e criptas, alterações na permeabilidade intestinal causando translocação bacteriana, e aumento dos níveis de alguns mediadores inflamatórios importantes, tais como recrutamento de neutrófilos (Sakai *et al*., 2014), produção de citocinas pro-inflamatórias TNF-α, IL-1β, IL-6, Il-17A e IL-22 (Sakai *et al*., 2013) e mediadores de apoptose celular como a caspase 3 (Zhang *et al*., 2018).

O impacto direto que o 5-FU tem sobre o TGI altera a arquitetura do epitélio, e o funcionamento das células e estruturas que o compõem, como os enterócitos (adaptados para a função metabólica e digestiva), células caliciformes (produtoras de muco, cujo principal componente são as mucinas, fornecendo proteção contra invasão de microrganismos patogênicos e antígenos microbianos), e proteinas do complexo Junção de Oclusão (JO) (formada por proteínas denominadas *tight junctions* (TJs) pertencentes à família das claudinas, ocludinas, zonulinas e das moléculas de adesão juncional - JAM), as quais funcionam como uma vedação entre as células epiteliais adjacentes, atuando como barreira a moléculas e microrganismos nocivos). Outras células que merecem destaque são as células de Paneth (células secretoras de peptídeos antimicrobianos), e as células M (*microfold cells*), as células dendríticas, os linfócitos intraepiteliais e células B, os quais medeiam, respectivamente, o transporte, o reconhecimento e apresentação de antígenos às células especializadas do sistema imune, e produzem imunoglobulina A secretória (sIgA) para combater patógenos (Peterson & Artis, 2014; König *et al*., 2016; Turner, 2009; Suzuki, 2013; Tsukita *et al*., 2001; Van Itallie & Anderson, 2006; Camilleri *et al*., 2012; Vancamelbeke & Vermeire, 2017; Salvo Romero *et al*., 2015).

Baseado nas alterações que o 5-FU promove no epitelio intestinal, modelos que expliquem a patobiologia da mucosite foram propostos, sendo um desses modelos descrito por Sonis (2004), que divide a evolução da mucosite em cinco estágios: (i) iniciação, (ii) resposta primária ao dano, (iii) amplificação do sinal, (iv) ulceração e (v) cicatrização (**Figura 3**):

- **(I) INICIAÇÃO**: essa fase tem início rapidamente após a administração da radio ou quimioterapia devido a exposição aos agentes citotóxicos que induzem lesões ao DNA/RNA, levando a formação de espécies reativas de oxigênio (EROS), as quais acabam ocasionando a lesão celular do epitélio basal, endotélio e submucosa (Sonis *et al.*, 2004).
- **(II) RESPOSTA PRIMÁRIA AO DANO**: em decorrência aos danos ocasionados ao DNA/RNA e a produção de EROS descritos anteriormente, ocorre a ativação de diversas vias de transdução de sinal como a via NF-κB (Chang *et al.*, 2012), que transmitem sinais dos receptores na superfície celular para o interior da célula, induzindo a expressão de diversos mediadores inflamatórios, tais como IL-8, TNF-α, COX-2, IL-6, IL-1β e *i*NOS, os quais possuem papéis importantes na toxicidade da mucosa, aumentando a lesão tecidual e induzindo a apoptose celular (Logan *et al.*, 2007; Chang *et al.*, 2012; Li *et al.*, 2017).
- **(III) AMPLIFICAÇÃO DE SINAL**: além de causar danos aos tecidos, a presença de citocinas pró-inflamatórias ativam vias que aumentam a produção de outros mediadores inflamatórios, amplificando o sinal via mecanismo de feedback positivo. Consequentemente, o aumento na produção desses fatores inicia uma cascata de reações inflamatórias que leva à ativação de metaloproteinases de matriz, cuja produção culmina em danos teciduais adicionais, exacerbando a lesão (Sonis, 2004).
- **(IV) ULCERAÇÃO**: Devido à perda da integridade da mucosa há formação de úlceras, e aumento da permeabilidade intestinal, facilitando a translocação bacteriana (Cinausero *et al.*, 2017). Além disso, observa-se o aumento da profundidade das criptas, encurtamento das vilosidades, levando a diminuição da área absortiva e aumento do infiltrado de células como neutrófilos, eosinófilos e macrófagos, que migram para o local da lesão via quimiotaxia, onde reconhecem, fagocitam e destroem as bactérias patogênicas e antígenos exógenos (Sonis, 2004; Soares *et al.*, 2013; Villa & Sonis, 2015).
- **(V) CICATRIZAÇÃO**: após a interrupção do tratamento, ocorre a cicatrização das lesões por meio da proliferação e diferenciação celular (Sonis, 2004).

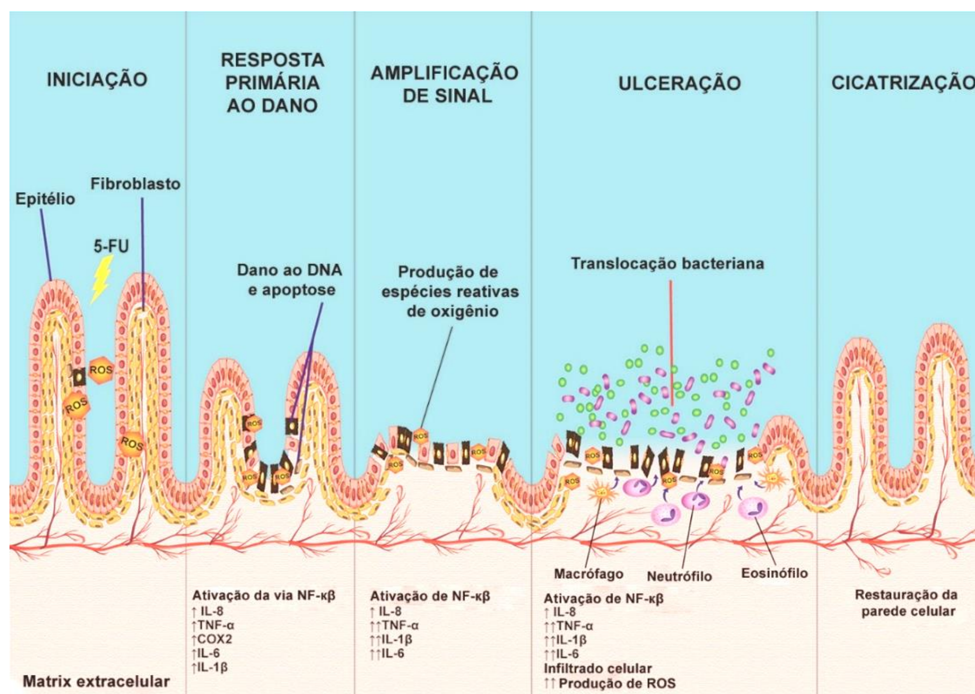


Figura 3: Fases da mucosite intestinal. Fases da mucosite intestinal. A fase de iniciação ocorre no primeiro momento em que a mucosa intestinal é exposta à toxicidade do 5-FU, promovendo danos ao DNA e induzindo a produção de espécies reativas de oxigênio. Consequentemente, várias vias de transdução de sinais são ativadas (resposta ao dano primário), como a via NF-κB, propiciando a indução de vários mediadores pro-inflamatórios (IL-8, TNF-α, COX-2, IL-6 e IL-1β) que desempenham papéis importantes na toxicidade da mucosa. Esses mediadores, por sua vez, causam amplificação do sinal via mecanismo de feedback positivo, ativando vias que aumentam a inflamação e o estresse oxidativo, exacerbando a lesão, e destruindo progressivamente a mucosa, o que leva à fase de ulceração. A cicatrização espontânea da lesão ocorre após interrupção do tratamento, caracterizada por proliferação, e diferenciação celular, e consequentemente restauração da mucosa (adaptado de Batista *et al*., 2020).

Apesar de sua relevância clínica, não existe até o momento um protocolo padrão ouro para o tratamento deste grave efeito colateral (Lalla *et al*., 2014). As opções de tratamento existentes são limitadas e em geral não promovem o reparo da mucosa, sendo utilizados antimicrobianos, antibióticos e analgésicos apenas para aliviar os sintomas provocados pela inflamação. Por esse motivo, estudos que visem descrever e caracterizar os mecanismos envolvidos na progressão dos sintomas ainda são necessários, como também o desenvolvimento de novas terapias que promovam o reparo tecidual sem comprometer os efeitos antineoplásicos do tratamento (Smith *et al*., 2008; Wang *et al*., 2016). Entre essas alternativas terapêuticas, destacam-se o uso de probióticos, devido as propriedades imunomoduladoras que estes microrganismos são capazes de induzir.

2.2 Probióticos como alternativa terapêutica para a mucosite intestinal

O termo probiótico é definido como "*microrganismos vivos que, quando ingeridos em quantidades adequadas, conferem benefícios para a saúde do hospedeiro*" (FAO/WHO, 2001). Para ser efetivamente categorizado como probiótico, o microrganismo além de conferir algum benefício à saúde do hospedeiro, deve cumprir com outros pré-requisitos como: i) não possuir propriedades patogênicas; ii) resistir ao ambiente gastrointestinal, como a acidez, enzimas digestivas e os sais biliares; iii) ser estável, permanecendo viável desde os processos de manipulação, estocagem industrial até o consumo; e iv) ser capaz de modular o sistema imunológico (Turner, 2009; Tripathi & Giri, 2014; Kleerebezem *et al.*, 2019).

Diversos estudos têm demonstrado que os benefícios conferidos à saúde pelo consumo dos probióticos está atrelado principalmente às funções exercidas sobre o trato gastrointestinal (Rehman *et al.*, 2012; Shi *et al.*, 2017). Os principais mecanismos de ação dos probióticos relacionados ao trato gastrointestinal incluem (**Figura 4**):

(i) Exclusão competitiva dos patógenos por meio da competição por nutrientes e sítios de adesão à mucosa, gerando exclusão competitiva de patógenos intestinais como, por exemplo, *Salmonella enterica* Typhi, *Escherichia coli*, *Proteus vulgaris* e *Acinetobacter baumannii* (Collado *et al.*, 2010; Halder *et al.*, 2017; Monteagudo-Mera *et al.*, 2019; Plaza-Diaz *et al.*, 2019);

(ii) Produção de compostos antimicrobianos como as bacteriocinas, e outras substâncias como peróxido de hidrogênio, ácidos orgânicos voláteis, ácido láctico e ácido acético que também podem afetar o crescimento de bactérias potencialmente patogênicas como *Gardnerella vaginalis* (Gaspar *et al.*, 2018), *Listeria monocytogenes* (Liu *et al.*, 2017), *E. coli* e *Staphylococcus aureus* (Yi *et al.*, 2016), entre outros;

(iii) Produção e secreção de ácidos graxos de cadeia curta (Short Chain Fatty Acids-SCFAs) tais como acetato, propionato e butirato, compostos importantes na diferenciação e proliferação celular, podendo também atuar na sinalização de vias imunológicas, induzindo a expressão de marcadores anti-inflamatórios (Batista *et al.*, 2020; Parada Venegas *et al.*, 2019);

(iv) Modulação do sistema imune por meio da interação com as células do epitélio intestinal e células do sistema imune inato e adaptativo associados ao intestino (células dendríticas, monócitos, macrófagos e linfócitos) (Azad *et al.*, 2018),

estimulando a produção de citocinas, células T reguladoras (Tregs) e produção de imunoglobulinas como a IgA secretória (sIgA) (Batista *et al.*, 2020); e

(v) Proteção da barreira intestinal por meio da indução da produção e secreção de mucina pelas células caliciformes, e do aumento da expressão de genes relacionados à composição das proteínas de junção firme (*tight juctions)*, como claudinas, ocludina e zonulina, reduzindo, assim, a permeabilidade intestinal paracelular (Anderson *et al.*, 2010; Ewaschuk *et al.*, 2008; Lépine *et al.*, 2018).

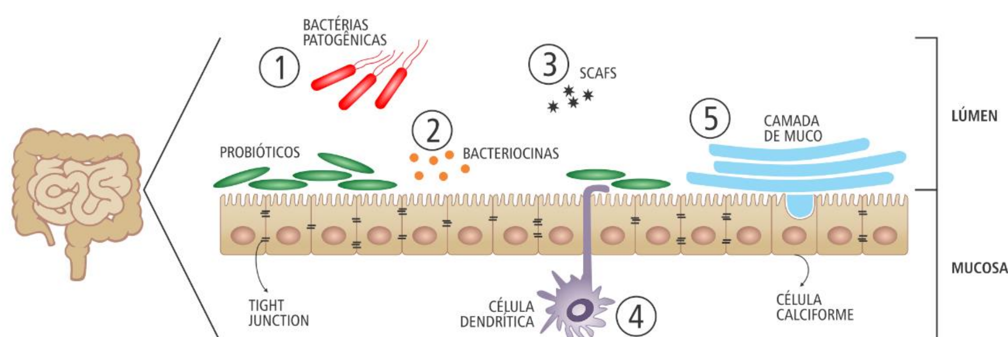


Figura 4: Os principais mecanismos de ação dos probióticos no trato gastrointestinal incluem: 1) exclusão competitiva de patógenos; 2) produção de compostos antimicrobianos; 3) produção e secreção de ácidos graxos de cadeia curta; 4) modulação do sistema imunológico e 5) proteção da barreira intestinal por meio do aumento das proteínas tight junctions, produção de mucinas e IgA secretória.

A progressão e severidade da mucosite está intrinsicamente associada à desregulação da microbiota intestinal. Nesse contexto, a modulação da microbiota por meio da administração oral de bactérias probióticas torna-se uma estratégia terapêutica promissora para atenuar a mucosite induzida pelo agente quimioterápico 5-FU (Li *et al.*, 2017; Van vliet *et al.*, 2010; Von Bültzingslöwen *et al.*, 2003).

Por exemplo, a administração de *Bifidobacterium infantis* (1×10^9 CFU) foi capaz de reduzir os sintomas associados à mucosite intestinal induzida por 5-FU em ratos, levando a uma melhora dos índices relacionados ao peso corporal, a altura das vilosidades, além de reduzir a expressão de NF-κB e fatores pró-inflamatórios (IL-1β e TNF) nos animais (Yuan *et al.*, 2015). O efeito protetor desta mesma linhagem foi investigado também em um modelo sinérgico de tratamento de câncer colorretal utilizando os quimioterápicos 5-FU e Oxaliplatina, sendo observado melhora nos danos da mucosa intestinal, redução da resposta do tipo Th1 e Th17 e aumento da resposta de CD4+ CD25+Foxp3+Tregs (Mi *et al.*, 2017).

Um estudo em humanos também demonstrou que a administração da formulação probiótica Colon Dophilus^{TM} em 46 pacientes com câncer de colorretal e submetidos ao tratamento com irinotecano levou a uma redução da incidência de diarréia grave e enterocolite. Essa formulação é composta pelos microrganismos *Bifidobacterium breve*, *Bifidobacterium bifidum*, *Bifidobacterium longum*, *Lactobacillus rhamnosus*, *Lactobacillus acidophilus*, *Lactobacillus casei*, *Lactobacillus plantarum*, *Streptococcus thermopilus*, *Lactobacillus brevis* e *Bifidobacterium infantis* enriquecidos com o prebiótico inulina, maltodextrina, estearato de magnésio e ácido ascórbico, mostrando-se seguro e efetivo na redução da gravidade da toxicidade gastrointestinal causada pelo quimioterápico (Mego *et al*., 2015).

Adicionalmente, a administração oral de *L. acidophilus* (16×10^9 UFC/Kg) demonstrou ser capaz de diminuir a lesão intestinal ocasionada por 5-Fluorouracil (5-FU) (450 mg/kg) através da inibição da sinalização da via NF-κB e redução dos níveis das citocinas pró-inflamatórias TNFα, IL1β e da quimiocina CXCL-1 (Justino *et al*., 2015).

Todos estes resultados revelam os efeitos terapêuticos promissores dos probióticos na mucosite intestinal. No entanto, deve-se ressaltar que estes efeitos são espécie e linhagem dependentes (McFarland, *et al*., 2018), o que mostra a necessidade que novos probióticos sejam identificados e seus efeitos e mecanismos devem ser avaliados.

2.3 *Lactobacillus delbrueckii* subsp. *lactis* CIDCA 133: uma promissora linhagem probiótica

Uma linhagem que vem tendo suas propriedades benéficas exploradas é a *Lactobacillus delbrueckii* subsp. *lactis* CIDCA 133. Esta linhagem foi isolada do leite de vaca cru na região de La Plata da Argentina, e pertence à coleção de culturas do Centro de Investigação e Desenvolvimento em Criotecnologia de Alimentos (CIDCA) da Universidade Nacional de La Plata (UNLP), na Argentina. Essa linhagem tem emergido nos últimos anos como uma promissora bactéria probiótica, cujos efeitos benéficos e de segurança vem sendo reportados (Kociubinski *et al.*, 1996; Rolny *et al.*, 2016; De Jesus *et al.*, 2019), ampliando os campos para estudo e aplicações deste microrganismo.

Kociubinski e colaboradores (1999) demonstraram pela primeira vez, em estudos *in vitro,* a capacidade de CIDCA 133 de resistir aos sais biliares. Posteriormente, um estudo de Probiogenômica dessa linhagem relatou a presença de diversos genes codificadores de proteínas associadas à resistência a diferentes estresses (acidez, sais biliares, salinidade, temperatura) (de Jesus *et al.*, 2021), que podem estar relacionados à sua capacidade de sobreviver à passagem

pelo trato gastrointestinal e durante a produção e armazenamento de produtos fermentados derivados dessa linhagem.

CIDCA 133 é capaz também de inibir o crescimento de patógenos envolvidos no processo de deterioração de alimentos e causadores de diarreia sanguinolenta, como *Pseudomonas aeruginosa* (Kociubinski *et al.*, 1996) e *E. coli* enterohemorrágica (EHEC) (Hugo *et al.*, 2008), respectivamente. Adicionalmente, esta linhagem também foi capaz de estimular o sistema imune inato e adaptativo de células eucarióticas infectadas com *Bacillus cereus* e *Citrobacter rodentium* (Hugo *et al.*, 2017; Rolny *et al.*, 2016), demonstrando propriedades imunomodulatórias importantes para controlar infecções por agentes patogênicos.

Aspectos relacionados à segurança do consumo da linhagem também vêm sendo explorados, sendo que não foram observados alterações clínicas, danos inflamatórias ou histopatológicos em animais sadios que consumiram a linhagem (de Jesus *et al.*, 2021b). Além disso, o consumo de CIDCA 133 foi capaz de estimular a expressão gênica de citocinas com perfil anti-inflamatório (como *Il10* e *Tgfb1*), inibir a expressão do gene *Nfkb1* (p105) (de Jesus *et al.*, 2021a), reduzir a expressão gênica do o marcador de stress oxidativo (*nos2*) e aumentar ou não alterar a expressão dos marcadores de barreira epitelial, como as proteínas de junção firme ocludina, claudina 2, e zonulina (de Jesus *et al.*, 2021b).

Efeito anti-inflamatório e enteroprotetor de CIDCA 133 contra danos ocasionados à mucosa intestinal após a administração do quimioterápico 5-FU (300mg/kg) foi previamente demonstrado por nosso grupo de pesquisa, por meio da administração de uma formulação de leite fermentado pela linhagem. Foram observados resultados promissores tanto na recuperação da arquitetura do epitélio com prevenção na degeneração de células caliciformes, quanto na redução da permeabilidade intestinal, secreção de IgA e do infiltrado de células polimorfonucleares (de Jesus *et al.*, 2019). No entanto, não se conhece os mecanismos moleculares associados a esse efeito enteroprotetor.

Assim, diante de todos os resultados obtidos por estudos *in vitro* e *in vivo*, CIDCA 133 tem despertado o interesse do nosso grupo de pesquisa e despontado com resultados imunomoduladores satisfatórios, abrindo espaço para que novos estudos sejam realizados, e explorem o potencial probiótico e biotecnológico dessa linhagem, como por exemplo sua eficácia terapêutica em diferentes modelos de doenças inflamatórias e sua aplicação como potencial linhagem carreadora de moléculas terapêuticas (proteínas recombinantes) usadas em abordagem de terapia gênica.

2.4 Produção de Moléculas Recombinantes para o tratamento da Mucosite Intestinal

Além dos probióticos, novas terapias como o uso de proteínas anti-inflamatórias recombinantes também vendo exploradas em diferentes condições patológicas inflamatórias. No entanto, ao contrário de linhagens probióticas selvagens, poucos estudos exploram o potencial de linhagens probióticas recombinantes para expressão/entrega de moléculas terapêuticas na mucosite intestinal (Carvalho *et al*., 2018; Jacouton *et al*., 2019; Ma *et al*., 2014; Rezende *et al*., 2013).

Um dos poucos estudos é o relatado por Caluwaerts e colaboradores (2010), o qual mostrou que a linhagem recombinante *L. lactis* AG013 secretora do fator trefoil humano 1 (hTFF-1) foi capaz de reduzir a progressão e a severidade da mucosite oral induzida por radiação em hamster. Carvalho e colaboradores (Carvalho *et al*., 2017) utilizando também uma linhagem recombinante demonstraram que a cepa *L. lactis* NZ9000 carreando o sistema de expressão heteróloga induzível por nisina (NICE) para expressão da proteína associada a pancreatite I (PAP) foi capaz de prevenir a mucosite intestinal induzida por 5-FU em modelo murino. Foi observado que o tratamento com essa linhagem foi capaz de preservar a arquitetura das vilosidades, aumentar a atividade das células de Paneth (Carvalho *et al*., 2017), e suprimir o crescimento de *Enterobacteriaceae* durante a inflamação (Carvalho *et al*., 2018).

Diante dos importantes achados relatados nos estudos utilizando linhagens probióticas selvagens ou recombinantes, acreditamos que este é um caminho importante a ser explorado visando à consolidação dos probióticos como alternativa terapêutica para o tratamento desta e outras desordens intestinais.

2.5 Vacinas de DNA

Atrelado aos efeitos benéficos dos probióticos, novos vetores vêm sendo desenvolvidos para a entrega de moléculas de interesse biotecnológico por estes microrganismos. Entre esses vetores, destacam-se os de “vacinas de DNA”, que são usados tanto em abordagens terapêuticas como de vacinação.

Basicamente, a vacina de DNA padrão consiste fundamentalmente em um plasmídeo formado por duas regiões: uma região de replicação procariótica e outra região de expressão eucariótica, responsável por codificar um ou mais antígenos vacinais (Ingolotti *et al*., 2010) (**Figura 5**).

A região de propagação procariótica consiste em uma origem de replicação bacteriana, que permite a propagação e amplificação dos plasmídeos em bactérias e um marcador de

seleção responsável pela estabilidade e manutenção do plasmídeo na célula bacteriana. Geralmente os marcadores de seleção correspondem a genes cujos produtos conferem resistência a antibióticos, ou podem ser marcadores considerados *food-grade* como os genes de complementação auxotrófica ou que conferem imunidade à bacteriocinas, resistência a metais pesados, etc (Ingolotti *et al*., 2010; Landete, 2017).

A região de expressão eucariótica, em contraponto, contém a unidade de transcrição composta por: (i) o promotor, geralmente um promotor viral constitutivo que permite um alto nível de expressão da ORF de interesse em células eucarióticas; (ii) o sítio de múltipla clonagem onde a ORF do gene de interesse é inserido, e (iii) sequências de consenso específicas como a sequência de Kozak, o códon de parada e a cauda Poli (A) (Hobernik & Bros, 2018). O promotor do citomegalovírus (pCMV) é o mais utilizado atualmente sendo considerado bastante forte na promoção da expressão constitutiva do gene de interesse (Kutzler & Weiner, 2008). A presença da sequência de Kozak (ACCATGG) é importante uma vez que se faz necessária no mRNA eucariótico para iniciar a síntese proteica enquanto que, para se garantir a terminação correta da sequência polipeptídica, também é importante inserir um ou mais códons de parada. A adição da sequência sinal de poliadenilação (AAUAAA), por sua vez, é necessária para a correta finalização da transcrição, adição da cauda poli-A e exportação do mRNA do núcleo para o citoplasma, conferindo maior estabilidade ao mRNA (Xu *et al.*, 2002; Kutzler & Weiner, 2008).

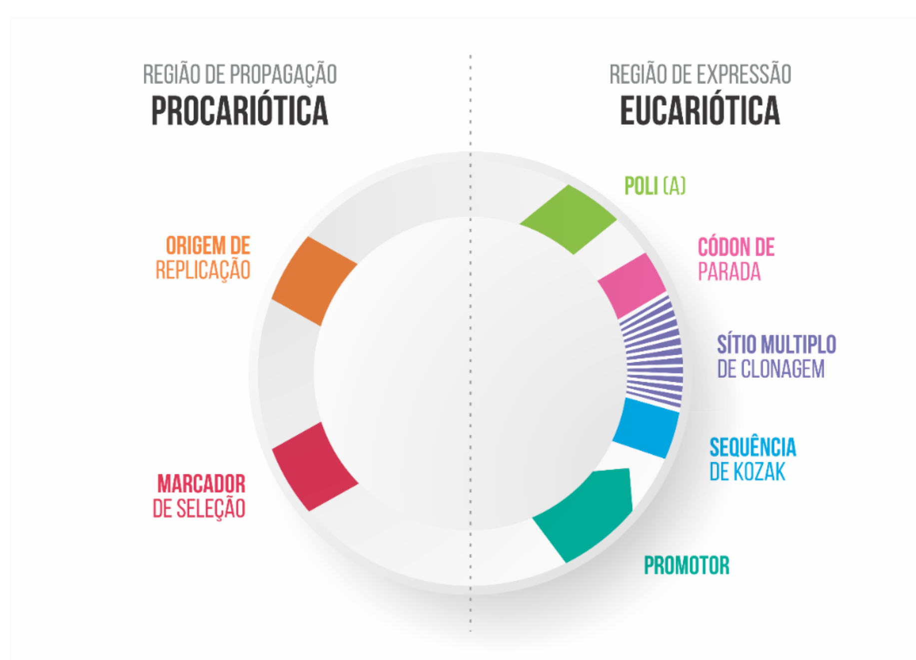


Figura 5: Representação esquemática de um plasmídeo para utilização como vacina de DNA carreada por bactérias. Na parte da esquerda da figura está representada a região de propagação procariótica, responsável pela propagação e manutenção do plasmídeo vacinal na célula bacteriana. Essa região contém uma origem de replicação procariótica e um marcador de seleção. A parte da direita da figura representa a região de expressão eucariótica

composta pelo promotor, a sequência de Kozak, o sítio de clonagem múltipla, o códon de parada e a sequência do sinal de poliadenilação (Poli-A) (Adaptado de Coelho-Rocha *et al.*, 2020).

Para que a vacinação com DNA seja bem-sucedida o plasmídeo vacinal deve atingir o núcleo das células alvo para que a transcrição da ORF (do inglês, *Open Reading Frame*) do gene de interesse ocorra. No entanto, para que isso ocorra é necessário transpor uma série de barreiras que impedem a transferência do DNA exógeno para o seu alvo.

As vacinas de DNA podem ser administradas por vias invasivas como as vias intradérmica, intraperitonial, subcutânea e intramuscular (Cherif *et al.*, 2011; Doria-Rose, 2003), ou por vias não invasivas, como a via de mucosas (Guimarães *et al.*, 2009). A via invasiva é a mais empregada em estudos com vacinas de DNA, sendo a injeção intramuscular a mais comumente utilizada (Kutzler & Weiner, 2008). Muitas vezes, as vacinas administradas por esta via utilizam o DNA "nu", o que requer múltiplas doses e grandes quantidades do plasmídeo para se obter os resultados desejados de imunização, uma vez que o plasmídeo administrado é pobremente distribuído e pode ser rapidamente degradado pelo organismo, inviabilizando o processo de imunização (Babiuk, 2003; Le *et al.*, 2000; Wang *et al.*, 2008). Com o objetivo de otimizar a entrega de vacinas de DNA diretamente no interior das células alvo, diversos métodos de transfecção celular vêm sendo testados, como o uso de bactérias carreadoras destes plasmídeos.

Diversos estudos visando utilizar bactérias como vetores de vacinas de DNA têm sido conduzidos, os quais utilizam principalmente patógenos intracelulares atenuados, como *Salmonella typhi*, *Listeria monocytogenes*, *Shigella flexneri*, *Yersinia enterocolitica* e *Escherichia coli,* para este fim (Schoen *et al.*, 2004; Daudel *et al.*, 2007). No entanto, uma grande preocupação com a questão da segurança do uso de patógenos atenuados é o risco residual associado à possibilidade de reversão à forma do fenótipo patogênico, não sendo totalmente seguros para uso humano, especialmente em crianças e pacientes imunocomprometidos (DUNHAM, 2002). Assim, considerando esse risco, a utilização de bactérias não patogênicas, tais como diversos representantes do grupo Bactérias do Ácido Lático (BAL), é uma alternativa atraente para uso como veículo de entrega de plasmídeos vacinais (Wells & Mercenier, 2008; Wells, 2011), devido algumas linhagens apresentarem nível de segurança conhecido, bem como propriedades imunomoduladoras e benéficas ao hospedeiro.

O mecanismo pelo qual ocorre a translocação intracelular do plasmídeo vacinal das bactérias para o núcleo da célula hospedeira ainda é pouco conhecido. Entre as principais hipóteses sobre seu mecanismo de ação sugere-se que, depois de administradas por exemplo

pela via oral, as bactérias carreadoras da vacina de DNA entram em contato com a superfície intestinal, onde são reconhecidas e fagocitadas pelas células epiteliais intestinais (CEIs), como as células M e os enterócitos, ou pelas células imunes, como as células dendríticas (CD) (Kaiserlian & Etchart, 1999; Weiss & Chakraborty, 2001; Wells, 2011; Mancha-Agresti *et al.*, 2017; Coelho-Rocha *et al.*, 2018). Este reconhecimento possivelmente se dá através dos receptores de reconhecimento de padrões (receptores Toll-like e Nod-like) que podem responder a alguns componentes bacterianos conhecidos como padrões moleculares associados a patógenos (PAMPs), que por sua vez, agem como antígenos naturais após a invasão das células hospedeiras (Barbosa & Rescigno, 2010). Após entrar nas células eucarióticas (enterócitos, células M ou células dendríticas), as bactérias são geralmente englobadas por uma vesícula primária fagolisossômica, levando à lise celular e à liberação do plasmídeo vacinal no citoplasma celular e que pode então migrar para o núcleo através da rede de microtúbulos e suas proteínas motoras associadas. No núcleo, a ORF de interesse será transcrita para subsequente síntese proteica usando a maquinaria celular do próprio hospedeiro (Weiss & Chakraborty, 2001). O antígeno expresso pode ser apresentado pelo MHC de classe I para ativar as células T CD8+, ou também pode ser expresso como proteína extracelular apresentada pelo MHC de classe II para ativar a produção de anticorpos e a resposta da célula auxiliar T CD4+, induzindo assim respostas imunológicas celular e humoral específicas contra o antígeno codificado (de Azevedo *et al*., 2015; Pontes *et al*., 2011) (Figura **6**).

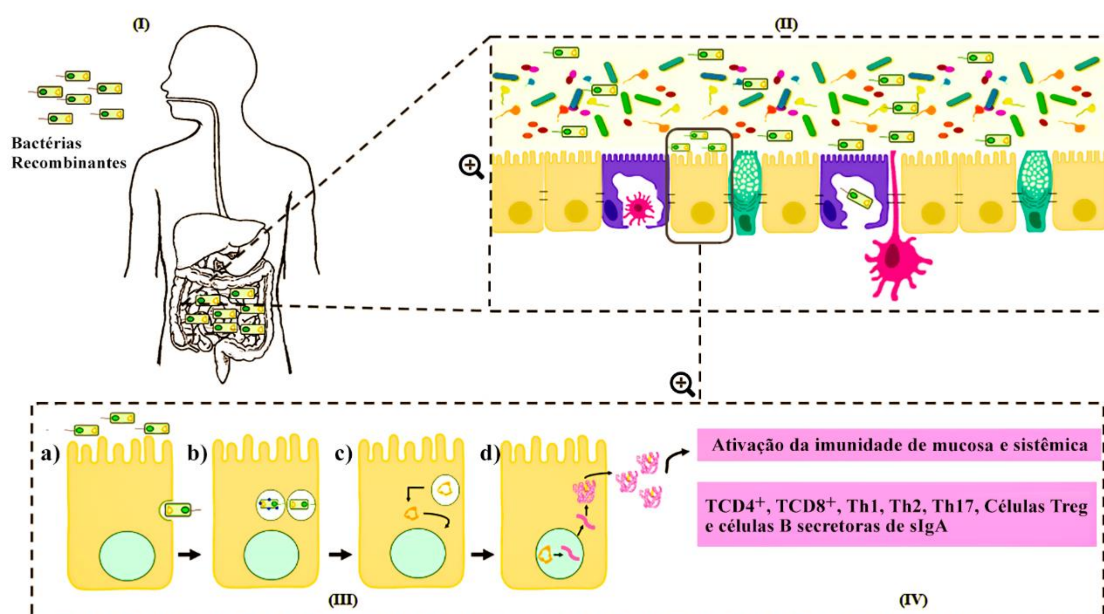


Figura 6: Representação esquemática do mecanismo proposto para o sistema de entrega de vacina de DNA em células de mamíferos usando como veículo de entrega linhagens bacterianas. (I) Administração oral das bactérias recombinantes; (II) na superfície intestinal as bactérias entram em contato com as células da mucosa,

onde são reconhecidas pelas células M (roxo), enterócitos (amarelo) ou células imunes, como as células dendríticas (rosa). (III) As bactérias são absorvidas pelo complexo fagolisossômico e lisadas (IIIa e IIIb); o plasmídeo vacinal escapa da vesícula e atinge o núcleo da célula hospedeira (IIIc). Dentro do núcleo ocorre a transcrição do gene de interesse (IIId) e (IV) a proteína produzida é exposta ao sistema imunológico, induzindo ambas respostas imunes, celular e humoral (Adaptada de (Tavares *et al.*, 2020)).

2.6 Vetor pExu

Diversos vetores têm sido desenvolvidos para utilização na abordagem de vacina de DNA usando bactérias lácticas como veículos de entrega ao nível da mucosa, principalmente bactérias do gênero *Lactococcus* (Guimarães *et al.*, 2006b; Guimarães *et al.*, 2009; Tao *et al.*, 2011; Mancha-Agresti *et al.*, 2017; Yagnik *et al.*, 2018). Visando ampliar a utilização de outras BAL como carreadores de vacina de DNA, nosso grupo construiu um novo vetor denominado pExu (*Extra Chromosomal Unit*) com tamanho de 6854 Kb e que apresenta amplo espectro de utilização (Mancha-Agresti *et al.*, 2017). Este plasmídeo possui uma origem de replicação do tipo teta, o que o torna mais estável tanto estrutural quanto segregacionalmente, e o gene que confere resistência à eritromicina como marcador de seleção. Sua funcionalidade foi avaliada após a clonagem da ORF do gene repórter *egfp (*proteína fluorescente verde*)*, cuja expressão foi observada *in vitro*, após transfecção em cultura de células eucarióticas mamíferas, e também *in vivo*, no intestino delgado de camundongos BALB /c após gavagem com *Lactococcus lactis* MG1363 (pExu:*egfp*) (Mancha-Agresti *et al.*, 2017). Além das propriedades citadas acima, este vetor foi também utilizado com sucesso em diferentes bactérias (*E. coli* Top10*, L. lactis* MG1363, *Lactobacillus delbrueckii* CNRZ327 e *L. delbrueckii* CIDCA 133) e apresentou estabilidade para se replicar em todas as linhagens testadas até o momento.

A aplicabilidade do pExu foi demonstrada por Coelho-Rocha e colaboradores (2018) usando a cepa microencapsulada de *L. lactis* MG1363 transportando o vetor pExu codificando a ORF do gene repórter da proteína fluorescente vermelha mCherry (*L. lactis* pExu:m*Cherry*). A linhagem foi testada em camundongos BALB/c e avaliada em diferentes momentos pós-administração. A expressão de mCherry foi observada em todas as porções do intestino, demonstrando que a entrega do vetor pExu:m*Cherry* por *L. lactis* às células eucarióticas *in vivo* foi eficiente e o plasmídeo foi capaz de atingir o núcleo das células, permitindo a expressão da proteína (Coelho-Rocha *et al*., 2018).

Devido a sua versatilidade, o vetor pExu pode ser usado para a entrega de diferentes moléculas de interesse terapêutico, como por exemplo as proteínas de choque térmico (HSPs), cujos efeitos imunomodularores vêm sendo reportados.

2.7 A proteína do choque térmico Hsp65 de *Mycobacterium leprae*

As proteínas de choque término ou *Heat Schok Proteins* (Hsps) são chaperonas moleculares importantes para a sobrevivência das células procarióticas e eucarióticas. Em particular, elas ajudam proteínas recém-sintetizadas a atingir sua conformação ativa, mediam seu transporte através dos canais de membrana e as protegem contra desnaturação, agregação ou diversos tipos de estresse, por exemplo, estresses oxidativo e térmico (Wallin *et al*., 2002; Saibil, 2013; Niforou *et al*., 2014).

As Hsps são classificadas de acordo com seu peso molecular em kilodaltons (kDa), sendo as mais estudadas a Hsp60, Hsp70 e Hsp90, equivalentes aos pesos de 60, 70 e 90 kDa, respectivamente (Ellis, 1990). Essas proteínas foram altamente conservadas ao longo do processo evolutivo e por isso, são altamente homólogas entre as diferentes espécies, provavelmente por exercerem funções básicas na manutenção da célula e são encontradas em praticamente todos os compartimentos subcelulares, incluindo o núcleo, o citosol, as mitocôndrias e o retículo endoplasmático (Pechmann *et al*., 2013; Niforou *et al*., 2014). Essa alta homologia pode levar a um reconhecimento imunológico cruzado entre elas; os anticorpos e células T contra Hsp60 e Hsp70, bacterianas, por exemplo, também reconhecem Hsp60 e Hsp70, mamíferas e vice-versa (Srivastava, 2002; Tsan & Gao, 2004; van Eden *et al*., 2005).

Em condições normais as Hsps são expressas constitutivamente nas células, enquanto que em condições de estresse, passam a ser super expressas. Normalmente, essas proteínas constituem 5% do total de proteínas intracelulares, mas sob condições adversas passam a representar 15% ou mais (Srivastava, 2002). Dentre essas condições adversas, podemos citar o aumento da temperatura, a deficiência nutricional, a exposição a mediadores pró-inflamatórios (TNF e IFN-γ), o estresse oxidativo, o tratamento com drogas anti-inflamatórias não esteróides, a infecção viral, a radiação ultravioleta, os metais pesados e as neoplasias (Tsan & Gao, 2004; van Eden *et al*., 2005).

Contudo, uma das funções mais importantes das Hsps está relacionada com sua influência na regulação do sistema imune (Nishikawa *et al*., 2008). Estas proteínas podem ativar o sistema imune através de diversos receptores de superfície, como TLR2, TLR4, CD14, CD91 e CD40 (Binder *et al*., 2004). De um modo geral, as Hsps parecem ser importantes candidatas para a indução de células T reguladoras, tornando-se potenciais alternativas para o uso terapêutico da tolerância oral em doenças inflamatórias e autoimunes (Rezende *et al*., 2013).

Assim, a proteína Hsp65 derivada de *Mycobacterium leprae*, proteína homóloga a Hsp60 dos mamíferos, tem sido utilizada com sucesso na prevenção e/ou tratamento de doenças

inflamatórias, em modelos experimentais, como a encefalomielite (Rezende *et al*., 2013), a diabetes tipo 1 (Oliveira, 2014), a colite ulcerativa (Gomes-Santos *et al*., 2017), dentre outras.

Rezende e colaboradores (2013) demonstraram que a administração oral de *L. lactis*, expressando Hsp65, foi capaz de impedir o desenvolvimento de encefalomielite auto-imune experimental (EAE). Os autores observaram um reduzido infiltrado celular inflamatório e ausência de sinais de lesão na medula espinhal. Relataram também uma redução de IL-17 e aumento de IL-10 nos linfonodos mesentéricos e na cultura de célula do baço. Os animais que receberam essa linhagem tiveram também um aumento de Tregs $CD4^+$ $Foxp3^+$ e Tregs $CD4^+$ LAP^+ no baço, linfonodo mesentérico e inguinal e na medula espinhal, sugerindo que essa linhagem seja capaz de estimular a expansão de células T reguladoras envolvidas no controle do desenvolvimento da EAE em camundongos (Rezende *et al*., 2013).

Utilizando a mesma estratégia, Oliveira (2014) verificou que a administração de *L. lactis*, expressando Hsp65, levou a uma expansão de células Tregs $CD4^+$ $Foxp3^+$ e $CD4^+$ LAP^+ no baço e nos linfonodos mesentéricos dos animais imunizados em um modelo animal para diabetes de tipo 1 demonstrando também o envolvimento das células Treg no desenvolvimento e controle da doença.

Adicionalmente, Gomes-Santos e colaboradores (2017) utilizando a mesma linhagem de *L. lactis* expressando Hsp65 observou, no modelo de colite induzida por DSS, uma redução da perda de peso, bem como diminuição da diarreia e do sangramento retal pelos camundongos tratados. A proteção da mucosa foi acompanhada por níveis diminuídos de TNF-α, IL-6, IL-4 e IL-5, aumento de IL-10 no cólon e de células Tregs no baço. Em camundongos $IL\text{-}10^{-/-}$, a administração dessa molécula reduziu os sinais da inflamação e aumentou a frequência de células Tregs $CD4^+$ LAP^+ e os níveis de TGF-β no cólon, no entanto, não foi capaz de reduzir a infiltração inflamatória das células neste órgão, apontando a importância da IL-10 nos mecanismos imunomoduladores induzidos pela linhagem produtora de Hsp65 neste modelo de doença (Gomes-Santos *et al*., 2017).

Mais recentemente, Guerra e colaboradores (2021) demonstraram que a administração oral desta mesma cepa recombinante de *L. lactis* secretora de Hsp65 de *Mycobacterium leprae*, também foi capaz de atenuar os efeitos da inflamação causada pela infecção por *Leishmania braziliensis*, com produção de citocinas anti-inflamatórias e expansão de células T reguladoras nos linfonodos dos camundongos.

Assim, baseado nos achados dos trabalhos acima, no efeito probiótico de CIDCA 133 e na aplicação do vetor pExu para entrega de diferentes moléculas de interesse biotecnológico,

acreditamos que a entrega direcionada do plasmídeo vacinal pExu carreando a ORF de Hsp65 usando a linhagem CIDCA 133 como vetor de entrega se apresenta como uma estratégia para potencializar os resultados positivos já obtidos pela utilização da linhagem selvagem, e surge como alternativa atraente para o desenvolvimento de novas estratégias de prevenção ou tratamentos mais eficazes para a mucosite intestinal.

Como fechamento dessa Revisão Bibliográfica apresentamos o capítulo **Recombinant Probiotics and Microbiota Modulation as a Good Therapy for Diseases Related to the GIT**, publicado em 24 de março de 2019 no livro **“**The Health Benefits of Foods - Current Knowledge and Further Development” da editora IntechOpen (Londres), doi: 10.5772/intechopen.88325, onde os principais aspectos do trato gastrointestinal e sua microbiota são abordados, além de discorrer brevemente sobre algumas doenças inflamatórias intestinais e os principais estudos já reportados utilizando bactérias probióticas selvagens ou recombiantes no tratamento ou prevenção destas doenças.

Chapter

Recombinant Probiotics and Microbiota Modulation as a Good Therapy for Diseases Related to the GIT

Luís Cláudio Lima de Jesus, Fernanda Alvarenga Lima, Nina Dias Coelho-Rocha, Tales Fernando da Silva, Júlia Paz, Vasco Azevedo, Pamela Mancha-Agresti and Mariana Martins Drumond

Abstract

Many diseases that affect the gastrointestinal tract (GIT) have great influence on the quality of life of the majority of patients. Many probiotic strains are being highly studied as a promising candidate due to their beneficial effect reported in the GIT. With the purpose of increasing the beneficial characteristics of some probiotics strains and, consequently, to improve further the reported results, many probiotic strains expressing or encoding different proteins, with anti-inflammatory activities, have been developed. These recombinant strains have been reported as good candidates for the treatment of different pathological conditions, especially colitis and mucositis disease since they have been shown to have positive results and good perspectives for GIT inflammation. Thus, this chapter will first address the aspects of the gastrointestinal tract in humans as well as its microbiota. In a second moment, it will discuss about chronic diseases, mainly the intestinal ones. Finally, it will discuss about probiotics, especially concerning on lactic acid bacteria (LAB), and its action in the prevention and treatment of these diseases. At the final part, we will point out aspects on the development of recombinant strains and the results found in the literature on disease models.

Keywords: *L. lactis*, *Lactobacillus*, DNA vaccine, heterologous protein

1. The human gastrointestinal tract

The human gastrointestinal tract is formed by a complex ecosystem which includes the gastrointestinal epithelium, immune cells, and resident microbiota [1] and comprehends one of the biggest existent interfaces between the host, environmental factors, and antigens in the human body.

The intestine encompasses a broad variety of microorganisms (bacteria, archaea, eukarya, and viruses) [2] from more than 3500 different species [3, 4] that coevolved with the host in a mutually beneficial relationship [5, 6]. The composition and density of bacterial populations in adult individuals differ considerably over the GIT. The area

of the GIT that has highest microorganism abundance is the colon (10^{14}) followed by dental plaque (10^{12}), ileum (10^{11}), saliva (10^{11}), and skin (10^{11}) [7]. However, low concentrations (up to 10^2–10^7 cells/mL) and bacterial diversity are found in the upper GIT (stomach, duodenum, jejunum) [3, 4], since the presence of acid, bile salts, and pancreatic secretions hinders the bacterial colonization [8], so that there is no nutritional competition between the microbiota and the host [9]. Thus, both function and structure of microbial communities are significant and are closely related. However, function could be the more important measure of microbiome health, since bacterial ecology suggests that analogous ecosystems have similar function although they have moderately diverse composition [10, 11].

2. Gut microbiota

The importance and the specific functions that gut microbiota has in human nutrition and health are well settled. The attributed functions can be classified in three classes: metabolic, protective, and trophic [12]. The gene diversity of the microbial community provides a variety of enzymes and biochemical pathways, specific to the host, able to contribute to short-chain fatty acid (SCFA) production by carbohydrate fermentation and production of some vitamins such as K, B12, biotin, folic acid, and pantothenate. These factors added to synthesis of amino acids from ammonia or urea contributing to the metabolic function of the microbiota [13, 14].

The gut microbiota's protective function is related to barrier effect, once the resident bacteria generate a resistance line which avoid pathogens/opportunistic bacteria and maintain normal mucosal function. The activity of some bacteria to secrete antimicrobial substances, such as bacteriocins, is able to inhibit the growth of other bacteria and nutrient competition [15, 16].

Regarding trophic functions of gut microbiota, the interaction between resident microorganisms has influence in differentiation and proliferation of epithelial cells [17], as well as in the development and regulation of the immune system by numerous and varied interactions between microbes, epithelium, and gut lymphoid tissues [18].

It is important to highlight that the interactions between the gut microbiota and the host immune system are required to preserve the gut homeostasis [19–21]. When this relationship is affected, alterations in bacterial function and diversity lead to the imbalance in the composition of the resident microbiota, favoring either the growing of pathogenic bacteria or the decreasing in beneficial bacteria in a process known as dysbiosis [22], which appoint a great threat to gut integrity and is intrinsically related to the development and progression of several diseases, such as inflammatory bowel diseases.

3. Chronic inflammatory diseases

One of the most well-characterized chronic inflammatory diseases that mainly affect the digestive tract is inflammatory bowel disease (IBD), which includes ulcerative colitis (UC) and Crohn's disease (CD). The exact etiology of IBD is still unclear, but the strict relation between genetic and the environmental factors, such as enteric immune dysregulation and alterations in the intestinal microbiome [23, 24], is broadly known. Besides, these diseases generate substantial morbidity and have a high prevalence in developed countries (5 in 1000 individual are affected) they remain to increase in developing nations [25].

Both diseases, UC and CD, present different pathogenesis, symptomatology, inflammatory profiles, and gut microbiota composition. CD is characterized by the irregular transmural inflammation (extending deeply into the submucosal regions) which can

affect any portion of the GIT and often made difficult by strictures, abscesses, and fistulae. On the other hand, the inflammation presented in UC is restricted to the superficial layers of the intestinal mucosa characterized by mucosa erosion and/or ulcer, generally localized in the region of the gut most colonized by bacteria, the colon [26, 27]. In addition, regarding the immune response associated with these diseases, it is possible to relate CD with an increased IL-12, IL-23, IL-27, interferon γ (IFN-γ), and tumor necrosis factor-α (TNF-α) production, all associated with Th1 and Th17 immune responses, different from UC which is correlated with a Th2 immune response, with high levels of IL-5 and transforming growth factor-β (TGF-β) production [28].

4. IBD complications and microbiota manipulation

It is important to highlight that the principal cause of death in IBD patients is colorectal cancer (CRC) [29]. Frequent episodes of inflammatory process in the intestinal mucosa are related to development of this disease, which is the second most frequently identified cancer in females and the third in males.

There are increased evidences that environmental factors such as lifestyle and diet alterations have effect in CRC incidence [30]. This effect has been documented because there is evidence showing an essential relationship between dietary antigens and antigens of commensal bacteria with the regulatory T cells (Tregs), which maintain the immune tolerance and, consequently, reduce the risk of tumorigenesis associated with inflammation [31].

In this context, it was reported that the higher consumption of diet rich in grains and vegetables decreases the incidence of CRC. This effect involves different mechanisms such as the diminution in the fecal transit time due to the increase in the stool bulk, and consequently, it reduces the contact of carcinogen with colon cells and the fermentation of these fibers of colonic components [14, 32]. In addition, significant reduction in concentration of acetate, propionate, and butyrate with increase in fecal pH [33] and the decrease in the number of obligate anaerobe microorganisms have been reported in individuals with colon cancer [34] when compared with healthy people. Thus, intestinal environmental alterations are the keys to evolution toward adenoma and afterward to CRC progression [35].

It has been also reported that up to 30% of patients with UC need surgical management such as the restorative proctocolectomy with ileal pouch-anal anastomosis (IPAA) [36]. This procedure removes the entire colon and rectum while preserving the anal sphincter and, hence, normal bowel function and fecal continence, therefore acting as an internal pelvic place for intestinal contents [37]. Around 50–60% of UC patients with following IPAA develop inflammation in the ileal pouch, generating the condition called "pouchitis." The reported incidence of pouchitis is variable, generally because of the diagnostic criteria that have been used to define this syndrome [38, 39]. In addition, although its pathogenesis is uncertain, the main hypothesis for the mechanism by which the disease occurs is the break in the mucosal barrier generated by dysbiotic microbiome in susceptible patients, generating an unusual mucosal immune activation [40]; still the disease typically responds to antibiotics.

Corresponding to the increased attention given to the role of the intestinal microbiota in a variety of diseases, there has been an intense exploration of potential means to manipulate the intestinal microbiome either by probiotic administration or fecal microbiota transplant (FMT) for therapeutic effect [41].

In this context, a randomized clinical trial based on a 1-week treatment with anaerobically prepared donor FMT, compared with autologous FMT, resulted in a higher probability of remission in 8 weeks for patients with UC, revealing that stool administration from healthy donors to UC or CD patients is an intervention that seeks to restore a healthier balance of gut microbes and control IBD [42]. Data on FMT for

Crohn's disease is rather more limited than for UC, but it has been shown that single standardized FMT resulted in a clinical remission sustained for more than 9 months in CD patients [43]. However, the authors suggest that further studies are needed to enhance the knowledge about the use of stool transplantation for IBD treatment.

Alteration in the gut microbiome composition with increase in some groups of microorganisms, such as *Clostridium* and *Fusobacterium*, was also reported in patients with pouchitis [44, 45]. In this context, literature evidences indicate that the probiotic administration such as VSL#3 is effective in the chronic pouchitis prevention [46]. On the other hand, FMT to pouchitis treatment did not report the same beneficial results. Only three reports with this approach [47–49] exposed that neither clinical remission nor any adequate response was observed in the evaluated patients suggesting that the efficacy of FMT for pouchitis after proctocolectomy is limited [49]. The importance of standardization of this procedure needs to be highlighted to improve its efficacy, since frequency, route of administration (e.g., endoscopy, nasogastric tube, colonoscopy), and the criteria of choice of healthy donor are very important parameters to be considered.

5. Intestinal mucositis

Different chemotherapy regimens such as FOLFOX (5-fluorouracil and oxaliplatin), FOLFIRI (5-fluorouracil and irinotecan), and triple FOLFOXIRI regimen (5-fluorouracil, oxaliplatin, and irinotecan) [50, 51] are adopted for different types of cancer but with a broad range of collateral effects.

Mucositis is the most common side effect in patients undergoing chemotherapy/radiotherapy treatments, which consist in an inflammation and/or ulcers in the gastrointestinal tract [52] with consequent loss of cells from the epithelial barrier of the GIT. Many symptoms are related to gastrointestinal mucositis, such as diarrhea, severe abdominal pain, bleeding, fatigue, malnutrition, dehydration, electrolyte imbalance, and infections, with potential fatal complications which can conduce to reduction or interruption of antitumor treatment [53] and consequently leads to longer hospitalization.

This pathology occurs due to cytotoxic effects of anticancer drugs/radiotherapy that cause damage at the DNA of stem cell (epithelial cell progenitors) with intense oxidative stress and consequent cell death. This apoptotic process is exacerbated affecting the absorption by shortening the villi structure of enterocytes and causing the loss of epithelial barrier with an invasion of inflammatory cells (neutrophils, eosinophils, and macrophages) leading to an increased production of inflammatory mediators at the mucosal area with consequent epithelial erosion and ulceration. The progressive destruction of mucosal integrity causes the rupture of the *tight junctions* proteins, leading to an increase in the intestinal permeability with subsequent penetration of commensal microbiota to the submucosal layer generating bacteria translocation which exacerbates the inflammatory process and intensifies the symptoms [53–57]. Besides, the intestinal microbiota composition is also modified by the chemotherapeutic drugs and radiotherapy action [54, 58, 59] resulting in dysbiosis. After the end of treatment, recovery and restoration of the GIT structure occur [60].

6. Metabolic syndrome

Besides IBD and mucositis, it has been reported that intestinal microbiota has an intrinsic effect on metabolism, potentially contributing to several features of the pathophysiology of metabolic syndrome [61, 62]. The metabolic syndrome is an accumulation of various risk factors (glucose intolerance, hyperinsulinemia,

hypertension, as well as dyslipidemia) which can often be associated with insulin resistance, hypertension with abdominal fat accumulation, and obesity [63–65].

The etiology of metabolic syndrome is not well-defined; however there are evident characteristics and life habits that could contribute to its development such as unbalanced diet, smoking, lack of physical activity, and the genetic predisposition [66]. These factors directly increase the risk of cardiovascular disease and chronic diseases as type 2 diabetes mellitus and obesity, and the interaction between components of both the clinical and biological phenotypes of the syndrome contributes to the development of a pro-inflammatory state [67].

The inflammatory process observed in MS is directly associated with increased oxidative stress. The reactive oxygen species (ROS) are capable of mediating symptoms of diabetes mellitus, such as insulin resistance and decrease in insulin secretion, and attend as precursors for the formation of LDLox (oxidized low-density lipoproteins), responsible for a large part of the development of atherosclerotic lesions, and the increase in circulating cholesterol fractions and glucose [68, 69]. In addition, chronic diseases are directly related to changes in the intestinal microbiome [70, 71], and they are also associated with elevated circulating levels of pro-inflammatory cytokines such as TNF and IL-6 [72].

The probiotic use in attenuating symptoms of different inflammatory diseases is widely reported in the literature. Among the commercial probiotics studied for treatment of these diseases, only a few products have been extensively tested in clinical trials in patients with MS, in order to demonstrate an effective result on weight loss, lipid metabolism, and reduction of inflammatory markers.

Studies performed with *Lactobacillus* strains have shown the ability of these probiotics in reducing the lipid accumulation in adipose tissues, as well as in inducing the subexpression of lipogenic genes [73, 74]. Animals that received diets with high concentrations of lipids and then treated with *L. gasseri* SBT2050 had shown lower intestinal permeability and bacterial translocation, as well as reduction of inflammatory parameters, suggesting that this strain improves the intestinal barrier function [75–78]. In addition, *L gasseri* BRN17 was studied to treat animals with MS caused by the carbohydrate-rich diet consumption. This strain reduced the accumulation of adipose tissue in mice, and it has a beneficial effect on weight loss [79–81]. Another important approach with associated probiotics (*Bifidobacterium*, *Lactobacillus*, and *S. thermophilus*) for treatment of overweight patients has shown an improvement in lipid profile, as well as insulin sensitivity [82]. Besides, recently Hsieh e collaborators [83] demonstrated that administration of live *Lactobacillus reuteri* ADR-1 and killed *Lactobacillus reuteri* ADR-3 strain ameliorated type 2 diabetes mellitus in a clinical trial. The results indicated that the consumption of ADR-1 displayed a reduction effect on serum glycated hemoglobin (HbA1c), triglyceride, and cholesterol levels. On the other hand, the intake of ADR-3 showed a beneficial effect on blood pressure reduction. Besides, a reduction in the levels of pro-inflammatory cytokines (IL-1β), increase in antioxidant enzyme (superoxide dismutase), and the changes in intestinal microflora composition (increase in intestinal level of *Lactobacillus* spp. and *Bifidobacterium* spp. and decrease in *Bacteroidetes*) were observed. Thus, these strategies highlight the beneficial and potential effect of interventions targeting gut microbiota modulation by the use of probiotic strains to treat components or complications of metabolic syndrome.

7. Functional foods

The human being for more than 4000 years has been consuming fermented products, by the fermentation process. At the beginning this practice was done

to preserve foods from either physical, chemical, or microbial alterations. The microorganisms participating in this process are the lactic acid bacteria, extensively widespread in nature and also belong to the GIT communities, able to convert the sugar in lactic acid as well as produce other metabolites which contribute to food modifications, either sensorial or nutritional value. Thus, the terminology "functional food" was attributed to food with health benefits to the consumer including nutritional and physiological function [84–86].

During the fermentation, these bacteria can contribute to improving the digestion of nutrients (lactose, proteins, small peptides, and polysaccharides); providing essential micronutrients (vitamins) as well as bioactive compounds (metabolites) with potential health benefits to the host, such as prevention against enteric inflammation [87, 88]; providing antimicrobial, antihypertensive, hypocholesterolemic, immunomodulatory, antioxidant, and anticancer effects [46, 85, 89–92]; showing ability to regulate the immunity; and, consequently, improving host quality of life [93].

Therefore, the gut communities and the microbial-derived molecules present in the gut lumen have been strongly influenced, either qualitatively or quantitatively, by consumption of dairy products [94] such as yogurts, cheeses, and fermented milk, among other fermented products using probiotic bacteria. Thus, the microbiota manipulation by functional food, probiotics, and prebiotics are evaluated as a beneficial option for treatment of GIT diseases [95].

8. Lactic acid bacteria: the largest group of probiotic bacteria

There is a constant interaction between the host and the bowel commensal bacterial community in order to maintain the homeostasis [3, 96–98]. However, when this mutualist relationship is compromised, the intestinal microbiota may cause and/or contribute to either the establishment or the progression of inflammatory diseases [96–99]. In this context, the search for therapeutic strategies that minimize the development and progression of pathologies caused directly and indirectly by the unbalance of the commensal microbiota has grown. The consumption of probiotic bacteria is one of these strategies, as they present several effects, such as ability to improve the intestinal barrier, stimulate the systemic and mucosal immune system, regulate the composition of the intestinal microbiota, and provide essential micronutrients (such as vitamins and SCFAs) and other bioactive compounds (metabolites) with potential health benefits for the host [100–103].

Probiotics are defined as "live microorganisms that offer host health benefits when administered in adequate amounts" [104, 105]. The majority of the studied probiotics belongs to the group of lactic acid bacteria. However, other microorganisms with probiotic properties also deserve attention, such as yeasts (*Saccharomyces* spp.) and bacteria of the genus *Bifidobacterium* and *Faecalibacterium*, among others [106–108].

LAB, which include, mainly, species from the genus *Lactobacillus*, *Leuconostoc*, *Lactococcus*, *Pediococcus*, and *Streptococcus*, constitute a group of Gram-positive, anaerobic or aerotolerant, nonspore-forming, nonmobile, and highly low pH-tolerant microorganisms. However, the main characteristic of this group is its ability to produce lactic acid as the final product of the fermentation of carbohydrates [109–111].

9. Probiotic effects in gastrointestinal inflammation

LAB are often present in the human gut but also can be introduced by the ingestion of fermented foods, such as yogurt and other fermented milk products and fermented cured meat by-products [103], having the generally recognized as

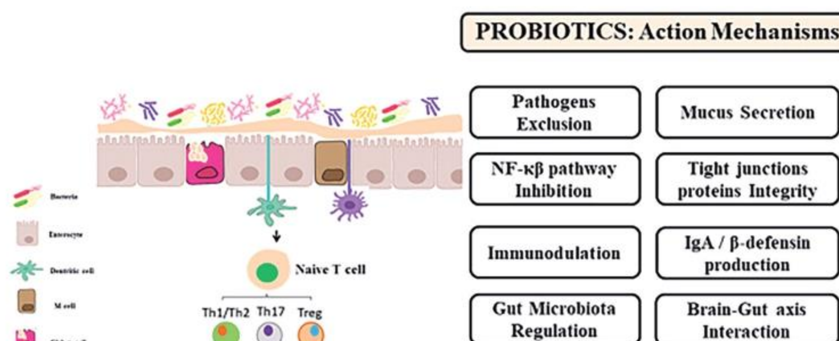


Figure 1.
A schematic diagram about potential action mechanisms of probiotic bacteria.

safe (GRAS) status by the Food and Drug Administration (FDA). *Lactobacillus* spp., *Streptococcus* spp., and *Lactococcus* spp. are the major LAB species with probiotic effects, and they have been used in therapeutic applications for treatment and prevention of various intestinal disorders [112, 113].

Scientific evidence reveals that the mechanisms by which probiotic bacteria ameliorate inflammatory bowel damage are heterogeneous, strain specific, and dependent on the number of available bacteria. Thus, administration of probiotic bacteria, specially LAB, improves intestinal inflammatory responses by (i) modulation and normalization of perturbed intestinal microbial communities; (ii) competitive exclusion of pathogens such as *Staphylococcus aureus* and *Salmonella typhimurium*, among others; (iii) bacteriocin and SCFA production; (iv) enzymatic activities related to metabolization of a number of carcinogens and other toxic substances; (v) adhesion to mucosal cells, cell antagonism, and mucin production; (vi) intestinal permeability reduction by tight junctions protein modulation (e.g., zonulin, claudin, occludin, junctional adhesion molecule); (vii) modulation of the immune system by stimulating Tregs cells, IgA production by B cells, and NF-kβ signaling pathway inhibition; and (viii) interaction with the brain-gut axis via the generation of bacterial metabolites (**Figure 1**) [103, 114–118].

10. Recombinant LAB probiotics

In order to potentialize the beneficial effects of probiotic strains, research has been conducted over the last decades, based on genetic engineering techniques, especially those related to DNA manipulation. Thus, modern methods of genetic engineering open the new opportunities to design and create genetically modified probiotic strains with the desired characteristics or to exclusively target a specific pathogen or toxin to be used either as a vaccine or for drug delivery [119, 120]. Since most of the probiotic strains are part of the LAB group, most of the genetic manipulation studies are carried out with species that belong to this group, such as *Lactococcus* and *Lactobacillus* genera. Consequently, recombinant probiotics have been created for mucosal delivery of therapeutic and/or prophylactic molecules comprising DNA, peptides, single-chain variable fragments, cytokines, enzymes, and allergens [121, 122], leading to the concept of "biodrug" for the prevention and treatment of various diseases [123]. Thus, researches have emphasized the use of species of these genera in two different approaches: the first as producers of heterologous protein and the second as vehicle for delivery of DNA vaccines [124].

10.1.3 Stress-inducible controlled expression system (SICE)

More recently, the stress-inducible controlled expression system was developed using the *L. lactis* groESL promoter [134]. This system induces expression of proteins of interest via stress stimuli such as those found in the GIT (e.g., bile salt, acid pH, antimicrobial peptide, and heat shock proteins) [134, 144]. This system does not require the induction of bacterial culture or the presence of regulatory genes, being a good alternative in the delivery and production of therapeutic proteins at mucosal surfaces.

10.2 LAB as a live vehicle to deliver DNA vaccine plasmids to eukaryotic cells

Among the available approaches to stimulate efficient mucosal responses, the use of bacterial system for DNA delivery and its expression using the eukaryotic cell machinery have been extensively explored. Unlike the production of heterologous protein, in which the bacterium is responsible for the synthesis of the protein of interest, in the DNA vaccine platform, the bacteria only act as a delivery vehicle for prophylactic and therapeutic purposes [109, 145].

New vectors had been developed to approach the DNA vaccine using LAB as live delivery vehicles [146, 147, 148–150]. These vectors present a series of common characteristics such as the presence of a eukaryotic promoter, which allows protein expression by eukaryotic cells; a prokaryotic region, which has a selection marker (usually antibiotic resistance); a multiple cloning site, where the open reading frame (ORF) of interest will be inserted; and a prokaryotic origin of replication, which ensures that the plasmid replicates only in prokaryotic cells [151]. Some molecules (IL-10, IL-4, and HSP65) have been cloned in these vectors to evaluate their effect, especially as a treatment approach in diseases related to the bowel [152, 153], as well as reporters (GFP and Cherry) which allowed the understanding of this platform in the mammalian body [148, 154]. Although further studies need to be conducted in order to elucidate whether the cloning of ORFs of interest in these vectors is really effective pointing to disease prevention and treatment, this approach is undoubtedly an important tool for the development of new techniques with potential in the medical clinic.

11. Next-generation recombinants: using CRISPR-Cas system

Among the different techniques used to construct recombinant LAB strains, the most recent is associated with the use of the clustered regularly interspaced short palindromic repeats (CRISPR)-Cas system, based on the use of a system present in several bacterial strains that works as part of the adaptive immune system of bacteria and archaea against the presence of external DNA, such as plasmids and bacteriophages [155–159].

Although this system has been studied for more than 30 years [160], it was only in 2013 that the first experiments were carried out emphasizing its use as a tool for genome editing [161, 162]. Evaluating the CRISPR databases, it is possible to observe that about 46% of all bacterial genomes presents the CRISPR-Cas system, and this percentage reaches approximately 63% of the sequenced *Lactobacillus* genomes [163]. The natural presence of this system in most of the LAB strains expands the possibilities of genetic manipulation of microorganisms of this group, including probiotic ones [164].

The first gene editing experiment in LAB based on the CRISPR-Cas system was conducted by Oh and van Pijkeren [165] where they were able to edit three different

10.1 LAB as producers of heterologous protein

Many studies are carried out with *Lactococcus lactis* due to its economic importance in the production of cheese and its easy growth and manipulation. In addition, it was the first species of LAB to have its genome completely sequenced, which allowed a greater understanding of its genetic and physiological mechanisms, aiding in the development of technological packages for its genetic manipulation in a laboratory environment [124–128].

There are several ways to make LAB produce heterologous proteins, and the most used form is through the insertion of a plasmid into its cytoplasm. Plasmids are elements of extrachromosomal DNA that are naturally found in prokaryotes. With the advent of the recombinant DNA technique, these elements have been manipulated to act as molecular vehicles that allow the production of proteins of interest by the bacterium [129].

The first heterologous protein production system based on plasmid insertion in LAB was developed for *L. lactis*. These systems included both inducible and constitutive promoters, which ensure efficient expression of the antigen of interest under different conditions [130, 131]. Although it is possible to choose the type of promoter to be used in the vector, the vast majority of expression vectors present inducible promoters that allow controlled expression of the protein of interest by protecting against aggregation and protein degradation in the bacterial cytoplasm. On the other hand, these vectors present safety issues that need to be analyzed since it is necessary to introduce chemical compounds into the culture medium to induce protein expression prior to animal administration [132–134].

With the improvement of cloning and expression techniques, several production systems were developed, specifically for LAB, allowing the production of different molecules of interest, including pathogen antigens, by a large number of LAB species [135–139]. The most commonly used regulation systems in LAB are the following:

10.1.1 Nisin-controlled gene expression (NICE)

Among the heterologous production systems, the most widely studied is the nisin-controlled gene expression system. This system is based on the expression of three genes (nisA, nisF, and nisR) that are involved in the production and regulation of the antimicrobial peptide nisin, which is naturally secreted by different strains of *L. lactis*. In this system the membrane-located histidine kinase NisK senses the signal inducer nisin and autophosphorylates and then transfers the phosphorous group to the intracellular response regulator protein NisR which acts as a transcription activator of nisA/nisF and induces gene expression under pNis promoter. Depending on the presence or absence of the corresponding targeting signals, the protein is either expressed into the cytoplasm or the cell envelope or secreted into the external medium [140]. Thus, it has already been successfully used for the expression of different proteins of medical and biotechnological interest [141, 142].

10.1.2 Xylose-inducible expression system (XIES)

In 2004, Miyoshi and colleagues [143] developed the xylose-inducible expression system whose promoter is the xylose permease gene (pxylT) found in *L. lactis* NCDO2118. This system produces either cytoplasmic or secreted proteins being activated in the presence of xylose and strongly repressed in the presence of glucose, fructose, or mannose [143].

regions of the genome, with efficiency up to 100% in the selected clones. After this pioneering work, few others were published focusing on LAB gene editing [166–168].

Therefore, the use of this technology is presented as a widely viable strategy to be applied in LAB, enabling the development of food-grade recombinant strains in order to allow their future use in the clinic [169].

12. Use of recombinant LAB to treat GIT-related disorders

The use of recombinant *L. lactis* strains, as well as others recombinant LAB strains, using different systems has shown promising results in many studies as an alternative therapy to treat, especially, GIT inflammation and other diseases (**Table 1**).

To arrive at mucosa in sufficient quantities to exert their therapeutic effects, many LAB strains must survive, during their passage through the GIT, stressor factors such as pH, temperature, bile salt concentration, and the presence of antimicrobial peptides [170–172]. In this context, an interest approach was recently developed by Coelho-Rocha and colleagues [154] using an encapsulated recombinant strain (*L. lactis* pExu:*mcherry*) and tested it through the GIT at different times post-administration. They have shown that the microencapsulation process is an effective method to improve DNA delivery, guaranteeing a greater number of viable bacteria able to reach different sections of the bowel [154].

The use of recombinant probiotics to improve therapeutic approaches has been widely studied using different systems with different molecules. As IBDs are a serious clinical topic, many strategies have been tested trying to improve previous results found with wild type strains.

L. lactis MG1363 strain carrying the pTREX1 vector expressing the mouse IL-27 protected mice against the inflammatory effects of dextran sulfate sodium (DSS)-induced colitis. This recombinant strain was able to reduce disease activity scores and pathology features of the large and small bowels and also led to reduced levels of inflammatory cytokines IL-1β, TNF-α, and IFN-γ in colonic tissue. In addition, reduction in the number of $CD4^+$ and $IL\text{-}17^+$ T cells in gut-associated lymphoid tissue and increase in IL-10 production were observed [173].

Besides, it was also demonstrated in a DSS-induced colitis mouse model that the oral administration of *L. lactis* NZ900 strain harboring the NICE system expressing either the anti-inflammatory cytokine IL-10, TGF-β1, secretory leukocyte protease inhibitor (SLPI), or elafin was able to ameliorate some clinical parameters in inflamed mice. Even though it was possible to observe the reduction of weight loss and diarrhea, microscopic colonic damage scores, colon thickness, and myeloperoxidase (MPO) activity, the authors reported that treatments with recombinant *L. lactis* strain delivering either SLPI or elafin were more efficient to reduce signs of colitis than treatments with anti-inflammatory cytokines. Altogether these recombinant strains display anti-inflammatory effects in inflamed mice [174].

Approaches using the invasive *L. lactis* MG1363 $FnBPA^+$, by expressing the FnBpA protein at their surface and carrying the pValac eukaryotic expression vector coding either the IL-10 cytokine [r*L. lactis* $FnPBA^+$ (pValac:*il-10*)] or the IL-4 cytokine [r*L. lactis* $FnPBA^+$ (pValac:*il-4*)] in DSS or trinitrobenzenesulfonic acid (TNBS)-induced acute model of colitis, respectively, were also investigated. The administration of *L. lactis* $FnPBA^+$ (pValac:*il-10*) recombinant strain was capable to reduce the intestinal inflammation by increasing IL-10 levels and sIgA production, accompanied by decreasing IL-6, as well as the restoration of intestinal architecture of mice colon [153]. Besides, the engineered *L. lactis* $FnPBA^+$ (pValac:*il-4*) was able to slump the level of pro-inflammatory cytokine (IL-12, IL-6) and myeloperoxidase activity and increase levels of IL-4 and IL-10, consequently decreasing the colitis harshness [153].

Microorganism	Gene	Expression System	Inflamation Condition	Anti-Inflamatory Properties	References
L. lactis MG1363	Mouse IL-10	SICE	Mouse model of DNBS-induced colitis	Restoration of intestinal architecture; IgA production and IL-6 reduction; Reduced tissue damage	[134]
L. lactis MG1363	Mouse IL-10 and IL-4	pValac vector	Mouse model of DSS/TNBS-induced colitis	Decreased IL-6, IL-12 and MPO activity Reduced tissue damage	[152-153]
L. lactis NZ9000	Mouse TGF-β1; IL-10 and leukocyte protease inhibitor Human Elafin	NICE	Mouse model of DSS-induced colitis	Reduced tissue damage Decreased pro-inflammatory cytokines	[174]
L. lactis NCDO 2118	Human 15-lipoxygenase-1	XIES	Mouse model of DSS-induced colitis	Reduced tissue damage	[175]
L. lactis NCDO 2118	*M. leprae* Hsp65 protein	XIES	Mouse model of DSS-induced colitis	Restoration of intestinal architecture CD4+Foxp3+ and CD4+LAP+ regulatory T cells production	[176]
B. bifidum BS42	Mouse IL-10	BEST	Mouse model of DNBS-induced colitis	Reduced tissue damage	[177]
L. casei BL23	Superoxide dismutase A from *L.lactis* MG1363 Catalase from *L.plantarum* ATCC	pLEM415 vector	Mouse model of TNBS-induced Crohn´s disease	Reduced tissue damage Reduced microbial translocation Increase IL-10/ INF-γ reduction	[180]
S. thermophilus CLR807	Superoxide dismutase A from *L.lactis* MG1363 Catalase from *L.plantarum* ATCC	pIL253 vector	Mouse model of TNBS-induced colitis	Reduced tissue damage Reduced microbial translocation IL-17 reduction	[181]
L.lactis AG013	Human Trefoil Factor 1 (Htff-1)	ThyA native promoter of *L.lactis*	Hamster model of radiation-induced oral mucositis	Reduced clicnical scores of oral mucositis	[186]
L. lactis NZ9000	Human pancreatitis associated protein (Reg3A)	NICE	Mouse model of 5-FU-induced intestinal mucositis	Microbiota Regulation Villus architecture preservation Increased Paneth cells activity	[185, 187]

Microorganism	Gene	Expression System	Inflamation Condition	Anti-Inflamatory Properties	References
L.lactis NCDO2118	*M. leprae* Hsp65 protein	XIES	Mice model of experimental encephalomyelitis	Increased CD4+Foxp3+ regulatory T cells Reduced encephalytogenic CD4+ T cells	[184]
L.lactis MG1363	Mouse IL-17	SICE	Mice model HPV-induced cancer	Reduced tumor size Induced IL-6 and IL-17 secretion	[182]
L.lactis NZ9000	*M. leprae* Hsp65 protein and peptide derived of human Hsp60 protein	NICE	Mice model of diabetes type 1	Reduction of insulitis Inhibition of T cell proliferation	[183]

Table 1.
Protein with anti-inflammatory properties produced in different strains of bacteria.

The human 15-lipoxygenase-1-producing *L. lactis* NCDO2118 harboring the xylose-inducible expression system (pXylt:CYT:*15-LOX-1*) was also effective in attenuating the symptoms of DSS-induced colitis in a murine model [175]. Its oral administration improved the body weight, decreased pro-inflammatory cytokines (IFN-γ and IL-4) while increasing the anti-inflammatory cytokine IL-10, and, consequently, ameliorated the macroscopic damage scores associated with the inflammation.

The oral pretreatment with genetically modified *L. lactis* NCDO2118 able to secrete HSP65 protein from *Mycobacterium leprae*, using XIES system (pXylt:SEC:*hsp65*), prevented DSS-induced colitis in C57BL/6 mice [176]. This protection was associated with reduced pro-inflammatory cytokines, such as IFN-γ, IL-6, and TNF-α; it also increased IL-10 production in colonic tissue and expansion of $CD4^{+}FoxP3^{+}$ and $CD4^{+}$ latency-associated peptide (LAP^{+}) regulatory T cells in the spleen and mesenteric lymph nodes. Besides, the authors showed that this effect was dependent on IL-10 and toll-like receptor 2 (TLR-2) [176].

Although *L. lactis* represents an excellent candidate for a live mucosal vector delivery system, other bacteria have also been explored as promising live vehicles for molecule expression with therapeutic properties, such as *Lactobacillus*, *Bifidobacterium*, and *Streptococcus*. In this context, Mauras et al. [177] using the new *Bifidobacteria* Expression SysTem (BEST) allowing the production of IL-10 in *Bifidobacterium bifidum* BS42(pBESTExp4:*il-10* and pBESTBL1181:*il-10*) demonstrated that the use of these recombinant strains in a DNBS-induced colitis model showed its ability to decrease local inflammation and confirmed therefore its potential for delivery of therapeutic molecules in the colon.

It is well known that IBD is associated with oxidative stress by the increase in concentration of reactive oxygen species in the GIT and impaired antioxidant defenses [178, 179]. In this context, it has been shown that some probiotic LAB strains may play a protective role in IBD by expressing antioxidant enzymes such as superoxide dismutase (SOD) and catalase (CAT) [180, 181].

LeBlanc et al. and Del Carmen et al. [180, 181] showed, respectively, that *L. casei* BL23 and *S. thermophilus* CRL807 transformed with two different plasmids (pLEM415:*mnkat*; pLEM415:*sodA*) (pIL253:*sodA* and pIL253:*mnkat*) harboring

the genes encoding catalase (CAT) or superoxide dismutase (SOD) antioxidant enzymes exhibited anti-inflammatory activities in a mouse model of Crohn's and colitis disease induced by trinitrobenzenesulfonic acid (TNBS). The authors observed a reduction in weight loss, fewer liver microbial translocation, lower macroscopic and microscopic damage scores, and modulation of the IFN-γ/IL-10 [180] and IL-10/IL-17 [181] cytokine production in the large intestines of mice treated with either CAT- or SOD-producing lactobacilli/streptococci.

The stress-inducible controlled expression (SICE) system represented by *L. lactis* MG1363 strain harboring the pLB333 plasmid was developed to avoid the external induction of culture before the host administration [134]. Several interesting molecules were cloned in this system such as IL-10 [134] and IL-17 [182], and the effect of *L. lactis* secreting them was evaluated in mice models. *L. lactis* (pSICE:*il-10*) was tested in a DNBS-induced colitis mice model, resulting in a significant reduction in colitis parameters with improvement in weight loss and a decrease in macroscopic scores [134]. The intranasal administration with *L. lactis* secreting IL-17A (pSICE:*il-17*), in a mice model of human papilloma virus (HPV)-induced cancer, was able to reduce tumor size and induce IL-6 and IL-17 secretion in reactivated splenocytes from mice challenged with the tumoral cell line [182]. Both works confirmed the potential use of *L. lactis* harboring the SICE system to deliver interesting molecules either to colitis or colon cancer patients [134, 182].

Although many studies have focused on the use of recombinant bacteria for the treatment of IBDs, as was previously discussed, the use of recombinant probiotic strains expressing/delivering therapeutic molecules has been explored for treatment or prevention of other diseases such as mucositis, cancer, obesity, multiple sclerosis, and diabetes [182–185].

An in vivo study reported by Caluwaerts et al. [186] showed that recombinant *L. lactis* AG013 secreting human trefoil factor 1(hTFF-1) was able to reduce the severity and course of radiation-induced oral mucositis. Carvalho et al. [187] also demonstrated that a recombinant strain of *L. lactis* NZ9000 using the inducible NICE system to express the human pancreatitis-associated protein (PAP) was able to prevent 5-FU-induced intestinal mucositis in a murine model. It was observed that this protein preserved villous architecture, increased Paneth cell activity [187], and suppressed the growth of *Enterobacteriaceae* during inflammation [185].

It also has been shown that oral administration of a recombinant *L. lactis* NCDO2118 strain (pXylT:SEC:*hsp65*) prevented the development of experimental autoimmune encephalomyelitis (EAE) in C57BL/6 mice [184]. Mice fed daily with this recombinant strain increased the number of natural and inducible $CD4^+FoxP3^+$ and $CD4^+$ latency-associated peptide (LAP^+) regulatory T cells in the spleen, inguinal and mesenteric lymph nodes, as well as in the spinal cord. In addition, a reduction in the recruitment of encephalitogenic $CD4^+$ T cells to the spinal cord was observed, which decreased IgG response against HSP65 and induced an anti-inflammatory cytokine profile (IL-17 reduction and IL-10 increase) during EAE development.

The oral administration of recombinant *L. lactis* expressing HSP65 and tandemly repeated P277 (pCYT:*HSP65-6P277*) was also analyzed in a model of type 1 diabetes mellitus (DM1) [183]. The authors observed that oral administration of recombinant *L. Lactis* resulted in the prevention of hyperglycemia, improved glucose tolerance and reduced insulitis, and induced HSP65- and P277-specific T-cell immunotolerance, as well as antigen-specific proliferation of splenocytes, demonstrating to be an effective therapeutic approach in preventing DM1 [183].

Another study using the *E. coli* Nissle 1917 strain engineered to secrete N-acylphosphatidylethanolamines (NAPEs) (pDEST-At1g78690 expression

plasmid) demonstrated that this strain was able to reduce the obesity of mice fed with a high-fat diet when added to drinking water. N-acyl phosphatidylethanolamines are precursors to the N-acylethanolamine (NAE) family of lipids, which are synthesized in the small intestine in response to feeding and reducing food intake and obesity. Mice that received modified bacteria had dramatically lower food intake, adiposity, insulin resistance, and hepatosteatosis than mice receiving standard water or control bacteria [188]. In addition, it was observed that changes on intestinal microbiota significantly decreased the abundance of *Firmicutes* and increased the abundance of *Proteobacteria*. Thus, these results provide evidence of the potential efficacy of this approach to inhibit the development of metabolic disorders and related diseases.

13. Conclusion

Currently the association between disease progression, especially chronic inflammatory diseases, and intestinal dysbiosis has been more frequently observed. As a clinical strategy, the use of probiotic bacteria, which naturally benefit the host, has been increasingly used on the treatment of diseases related to the GIT. In view of the good results obtained with this approach, researchers have sought through bacterial genetic modification to increase the beneficial potential of probiotics, either through their use for heterologous protein production or as a vehicle for vaccinal plasmid delivery, by developing recombinant bacterial strains and by testing their action in different disease models. And while there are still a number of questions that need to be answered about the use of genetically modified organisms for health care, especially in human, the use of these strains has proven to be a potentially effective therapeutic alternative, so much so that clinical trials using recombinant lineages have already been authorized and conducted in humans.

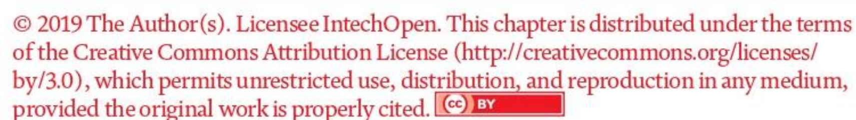
Author details

Luís Cláudio Lima de Jesus[1], Fernanda Alvarenga Lima[1], Nina Dias Coelho-Rocha[1], Tales Fernando da Silva[1], Júlia Paz[1], Vasco Azevedo[1], Pamela Mancha-Agresti[1]* and Mariana Martins Drumond[1,2]

1 Laboratório de Genética Celular e Molecular (LGCM), Instituto de Ciências Biológicas, Departamento de Genética, Ecologia e Evolução, Universidade Federal de Minas Gerais (UFMG), Belo Horizonte, Minas Gerais, Brazil

2 Departamento de Ciências Biológicas, Centro Federal de Educação Tecnológica de Minas Gerais (CEFET/MG), Belo Horizonte, Minas Gerais, Brazil

*Address all correspondence to: p.mancha.agresti@gmail.com



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3 RELEVÂNCIA DA TESE

A utilização de microrganismos probióticos têm ganhado bastante notoriedade nas últimas décadas, não apenas pela sua importância na indústria alimentícia, mas também devido ao seu potencial terapêutico principalmente no tratamento de disbiose gastrointestinal e em doenças crônicas, metabólicas e genéticas graves (Mitsuoka, 2014).

As principais bactérias com perfil probiótico utilizadas para este fim fazem parte do grupo das bactérias do ácido láctico (Bintsis, 2018). Visando potencializar os efeitos probióticos dessas linhagens selvagens, o crescente avanço da biotecnologia e o desenvolvimento de várias técnicas de biologia molecular têm permitido a construção de diferentes linhagens recombinantes de BAL com propriedades anti-inflamatórias. Exemplos bem relatados incluem a construção de linhagens recombinantes de *L. plantarum* (Han *et al*., 2006), *S. thermophilus* (Del Carmen *et al*., 2014), *L. casei* BL23 (Leblanc *et al*., 2011; Watterlot *et al*., 2010) e *L. lactis* (De Moreno De Leblanc *et al*., 2008) capazes de expressar moléculas anti-inflamatórias em diversos modelos animais de doenças inflamatórias (De Moreno De Leblanc *et al*., 2015; Wang *et al*., 2016).

Dentre essas moléculas, as proteínas de choque térmico (do inglês, Heat shock proteins – Hsps), em especial a proteína Hsp65 derivada de *M. Leprae*, tem recebido grande destaque pelo nosso grupo de pesquisa, relatando resultados positivos da utilização desta proteína sobre a imunomodulação nos modelos experimentais de colite (Gomes-Santos *et al*., 2017), encefalomielite autoimune experimental (Rezende *et al*., 2013), artrite (Gusmao-Silva *et al*., 2020) e no modelo de inflamação decorrente da infecção por *Leishmania braziliensis* (Guerra *et al*., 2021).

Embora ainda haja diversas questões quanto a biossegurança no uso de bactérias geneticamente modificadas, as enormes vantagens associadas à essas novas abordagens vêm fortalecendo seu uso nas pesquisas atuais e colocando-as como estratégias terapêuticas promissoras para uso em humanos no futuro.

Assim, a construção da linhagem recombinante CIDCA 133 (rCIDCA 133:Hsp65) e o estudo da capacidade imunomoduladora das linhagens, recombinante e selvagem, em modelo murino de mucosite intestinal tem impacto imediato no campo da vacinologia e terapêutica, e poderá contribuir para a melhor compreensão da imunidade das superfícies de mucosas associadas à utilização de bactérias lácticas com potencial probiótico, além de fornecer embasamentos científicos sobre a eficácia e segurança da utilização de bactérias lácticas recombinantes como carreadores de vacinas de DNA.

4 OBJETIVOS

4.1 Objetivo Geral

Avaliar os mecanismos moleculares envolvidos com o efeito enteroprotetor de *Lactobacillus delbrueckii* CIDCA 133 selvagem e recombinante (pExu:*hsp65*) em modelo de mucosite intestinal.

4.2 Objetivos específicos

- Avaliar os mecanismos moleculares envolvidos com o efeito enteroprotetor de CIDCA 133.
- Construir e avaliar a funcionalidade do vetor pExu:*hsp65* em células eucarióticas.
- Avaliar o efeito terapêutico do vetor pExu:*hsp65* carreado por CIDCA 133 em modelo de mucosite intestinal.
- Avaliar os mecanismos associados ao efeito terapêutico do vetor pExu:*hsp65* carreado por CIDCA 133 em modelo de mucosite intestinal.

5 CAPÍTULO I: *Lactobacillus delbrueckii* CIDCA 133 Ameliorates Chemotherapy-Induced Mucositis by Modulating Epithelial Barrier and TLR2/4/Myd88/NF-κB Signaling Pathways

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Devido aos efeitos anti-inflamatórios reportados, o consumo de probióticos tem sido considerado uma opção terapêutica promissora para melhorar os danos inflamatórios e epiteliais induzidos pela administração de agentes quimioterápicos que causam mucosite intestinal, como o 5-Fluorouracil. A linhagem *Lactobacillus delbrueckii* subsp. *lactis* CIDCA 133 tem emergido como uma promissora bactéria probiótica. No entanto, esta linhagem teve suas propriedades probióticas descritas unicamente por meio de estudos *in vitro*. Nosso grupo de pesquisa foi pioneiro na carateriração *in vivo* da probiose desta linhagem, usando uma formulação de leite fermentado em um modelo de inflamação intestinal induzida por 5-FU. Os resultados promissores demonstraram que o consumo de CIDCA 133 melhorou a arquitetura do epitélio intestinal aletrada por 5-FU, reduzindo a permeabilidade intestinal, o infiltrado de células inflamatórias na mucosa e redução da secreção de IgA. No entanto, não se conhece por quais mecanismos moleculares esta linhagem é capaz de melhorar a mucosite intestinal induzida por 5-FU. Assim, o objetivo deste capitulo foi avaliar se a modulação de marcadores inflamatórios e de barreira epitelial estariam associados ao efeito enteroprotetor de CIDCA 133. O estudo foi realizado em camundongos BALB/c que consumiram CIDCA 133 ($5x10^7$ CFU/mL) por 13 dias, sendo que no décimo dia os animais foram inflamados com uma única dose do quimioterápico 5-FU (300 mg/kg). A modulação de marcadores inflamatórios e da barreira epitelial envolvidos no efeito enteroprotetor da linhagem foi investigado por RT-qPCR. Os resultados obtidos reforçam que o consumo de CIDCA 133 melhora os danos à mucosa intestinal de camundongos inflamados com 5-FU, sendo que a melhora na arquitetura do epitélio intestinal e seu funcionamento envolveu a modulação da ativação da via de sinalização inflamatória NF-κB, com subsequente redução na expressão gênica de citocinas pró-inflamatórias (*Il6* e *Il1b*), e aumento na expressão da citocina imunoregulatória Il10 e de marcadores envolvidos na função de barreira epitelial como a mucina 2, e as proteínas de junção firme: Claudina 1, JAM, e zonulina, sendo, portanto, estes os principais mecanismos moleculares associados ao efeito enteroprotetor da linhagem na mucosite intestinal.

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Lactobacillus delbrueckii CIDCA 133 Ameliorates Chemotherapy-Induced Mucositis by Modulating Epithelial Barrier and TLR2/4/Myd88/NF-κB Signaling Pathway

Fernanda Alvarenga Lima Barroso[1], Luís Cláudio Lima de Jesus[1], Tales Fernando da Silva[1], Viviane Lima Batista[1], Juliana Laguna[1], Nina Dias Coelho-Rocha[1], Kátia Duarte Vital[2], Simone Odília Antunes Fernandes[2], Valbert Nascimento Cardoso[2], Enio Ferreira[3], Flaviano Santos Martins[4], Mariana Martins Drumond[1,5], Pamela Mancha-Agresti[1], Alexander Birbrair[3], Debmalya Barh[1,6]* and Vasco Azevedo[1]*

[1] Departamento de Genética, Ecologia e Evolução, Universidade Federal de Minas Gerais, Belo Horizonte, Brazil, [2] Departamento de Análises Clínicas e Toxicológicas, Universidade Federal de Minas Gerais, Belo Horizonte, Brazil, [3] Departamento de Patologia Geral, Universidade Federal de Minas Gerais, Belo Horizonte, Brazil, [4] Departamento de Microbiologia, Universidade Federal de Minas Gerais, Belo Horizonte, Brazil, [5] Departamento de Ciências Biológicas, Centro Federal de Educação Tecnológica de Minas Gerais, Belo Horizonte, Brazil, [6] Centre for Genomics and Applied Gene Technology, Institute of Integrative Omics and Applied Biotechnology (IIOAB), Purba Medinipur, India

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***Correspondence:**
Debmalya Barh
dr.barh@gmail.com
Vasco Azevedo
vasco@ufmg.br;
vascoariston@gmail.com

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Intestinal mucositis promoted by the use of anticancer drugs is characterized by ulcerative inflammation of the intestinal mucosa, a debilitating side effect in cancer patients undergoing treatment. Probiotics are a potential therapeutic option to alleviate intestinal mucositis due to their effects on epithelial barrier integrity and anti-inflammatory modulation. This study investigated the health-promoting impact of *Lactobacillus delbrueckii* CIDCA 133 in modulating inflammatory and epithelial barrier markers to protect the intestinal mucosa from 5-fluorouracil-induced epithelial damage. *L. delbrueckii* CIDCA 133 consumption ameliorated small intestine shortening, inflammatory cell infiltration, intestinal permeability, villus atrophy, and goblet cell count, improving the intestinal mucosa architecture and its function in treated mice. Upregulation of *Muc2*, *Cldn1*, *Hp*, *F11r,* and *Il10,* and downregulation of markers involved in NF-κB signaling pathway activation (*Tlr2, Tlr4, Nfkb1, Il6,* and *Il1b*) were observed at the mRNA level. This work suggests a beneficial role of *L. delbrueckii* strain CIDCA 133 on intestinal damage induced by 5-FU chemotherapy through modulation of inflammatory pathways and improvement of epithelial barrier function.

Keywords: 5-FU-induced mucositis, probiotics, *Lactobacillus delbrueckii*, anti-inflammatory cytokines, intestinal permeability, tight junction proteins

INTRODUCTION

Intestinal mucositis is a commonly reported side effect in oncology patients undergoing chemoradiotherapy (Sonis, 2004; Kim et al., 2018) due to non-selective anticancer drugs (Miura et al., 2010; Crombie and Longo, 2016). 5-Fluorouracil (5-FU), an antimetabolite analog to uracil, is one of the most frequently used antitumor drugs that cause mucositis, with an incidence higher than 40% of cancer cases even if treated with the standard dose (10-15 mg/kg for 3-4 days intravenously). 5-FU exerts cytotoxic effects by inhibiting thymidylate synthase (TS) and incorporating its metabolites into DNA/RNA, leading to cell death and apoptosis (Longley et al., 2003; Miura et al., 2010). However, due to its non-selectivity, besides affecting neoplastic cells, this drug also promotes damage to healthy cells (Miura et al., 2010; Crombie and Longo, 2016).

Mucositis caused by 5-FU affects the small bowel mucosa, characterized by intense inflammation, dysbiosis, alteration in intestinal epithelium architecture such as villus atrophy, and loss of epithelial barrier integrity due to tight junction protein disruption and goblet cell degeneration. Additionally, it subsequently increases intestinal permeability and reduces mucin secretion (Sonis, 2004; van Vliet et al., 2010; Song et al., 2013). Except for palliative therapy, there is no known treatment option to prevent chemotherapy-induced intestinal mucositis (Batista et al., 2020).

Probiotic strains, such as *Lactobacillus* spp., mainly belong to the Lactic Acid Bacteria group (LAB), and they have been investigated as an alternative therapeutic approach against intestinal mucositis (Batista et al., 2020). Several studies have shown the efficacy of various LABs in preventing intestinal mucositis by regulating the microbiota in dysbiosis (Chang et al., 2018), improving the inflammatory process, modulating oxidative stress (Justino et al., 2015; Hu et al., 2020; Quaresma et al., 2020), and protecting the epithelial barrier by maintaining the integrity of tight junction proteins and goblet cells (Yeung et al., 2015; Barroso et al., 2021). Despite these beneficial effects, the use of these inactivated microorganisms has also been highlighted (Jin et al., 2020; Nakai et al., 2021; Trindade et al., 2021), to minimize possible risks of bacteria translocation producing systemic infections, especially in premature infants or immune-compromised patients (Doron and Snydman, 2015; Zawistowska-Rojek and Tyski, 2018). In addition, it has been demonstrated that many of the effects (such as immunomodulation and microbiota regulation) of inactivated probiotics are similar to their metabolically active form, suggesting that these effects can be attributed to cell wall proteins of these microorganisms that interact with host cells during their passage through the gastrointestinal tract (GIT) (Hsieh et al., 2016; Aiba et al., 2017; Jin et al., 2020; Nobre et al., 2022).

Among the *Lactobacillus* genus, *Lactobacillus delbrueckii* subsp. *lactis* CIDCA 133, a strain isolated from raw cow's milk, has been related to probiotic properties. This includes its ability to inhibit the growth of spoilage bacteria, such as *Escherichia coli* and *Pseudomonas aeruginosa* (Kociubinski et al., 1999; Hugo et al., 2008), and to stimulate dendritic cells and murine macrophages infected with pathogenic *Bacillus cereus* or *Citrobacter rodentium* (Rolny et al., 2016; Hugo et al., 2017). One of our pioneering studies showed that a fermented milk formulation containing *L. delbrueckii* CIDCA 133 could attenuate antineoplastic 5-FU-induced intestinal epithelial tissue disruption by inhibiting degeneration of goblet cells, intestinal permeability, and inflammatory cell infiltration (de Jesus et al., 2019). In our other studies, we reported that no adverse effect was observed in healthy BALB/c mice after CIDCA 133 consumption, but its consumption stimulated the mucosal immune system and epithelial barrier by upregulating the gene expression of tight junction protein occludin (*Ocln*) (de Jesus et al., 2021a), anti-inflammatory cytokines (*Il10* and *Tgfb1*) and mucin 2 (*Muc2),* and inhibiting the proinflammatory transcription factor *Nfkb1* (p105) (de Jesus et al., 2021b). In this context, this study investigated whether the modulation of inflammatory and epithelial barrier biomarkers by *Lactobacillus delbrueckii* CIDCA 133 would be associated with its beneficial effects against 5-FU chemotherapy-induced intestinal mucositis.

MATERIALS AND METHODS

Bacterial Strain Growth Conditions

The bacteria *Lactobacillus delbrueckii* CIDCA 133 belongs to CIDCA 133 center (Centro de Investigación y Desarrollo en Criotecnología de Alimentos, Universidad Nacional de La Plata, Argentina). The strain was grown on MRS broth (Man, Rogosa and Sharpe) (Kasvi, São José dos Pinhais, Brazil) for 18 h at 37°C. CIDCA 133 (5×10^7 CFU/mL) dose was determined by growth curves using counting colony formation-unit (CFU) (de Jesus et al., 2021b).

Animals and Ethics Statement

Conventional BALB/c mice (male, weight 20-24 g, six weeks old) were obtained from the Federal University of Minas Gerais (UFMG, Belo Horizonte, Brazil). Mice were kept in polycarbonate-ventilated cages in a controlled room with a temperature of 25 ± 2°C under a 12 h light/dark cycle, a standard chow diet, and *ad libitum* access to water for 24 h before experiments. All procedures were promptly approved by the Animal Experimentation Ethics Committee (CEUA-UFMG, number 66/2021) and were done according to the Brazilian Society of Sciences in Laboratory Animals (SBCAL) guidelines.

Experimental Design

Mice were grouped into four experimental groups ($n = 6$): negative control (NC), probiotic (CIDCA 133), inflamed (5-FU), and inflamed and treated with probiotic (5-FU + CIDCA 133). Mice consumed MRS broth (NC and 5-FU group) or CIDCA 133 (5×10^7 CFU/mL) (CIDCA 133 and 5-FU + CIDCA 133 group) by continuous feeding for 13 days. Bottles were changed every 24 h. Mice (5-FU and 5-FU + CIDCA 133 group) were inflamed intraperitoneally (*i.p.*) on the 10th day with a single injection of 5-Fluorouracil (300 mg/kg) (Fauldfluor® , Libbs, São Paulo, Brazil) (de Jesus et al., 2019). Control groups (NC and CIDCA 133) received saline solution (NaCl 0.9%) injection. Seventy-two

hours after mucositis induction, the animals were euthanized by anesthesia deepening [xylazine (16 mg/kg) and ketamine (80 mg/kg)] (Ceva, São Paulo, Brazil). Liquid and feed intake and body weight were evaluated daily before euthanasia. Blood, thymus, spleen, and ileum sections were collected for analysis.

Histological Analysis

After euthanasia, the entire small intestine length was measured. Ileum sections were washed with PBS 0.1 M, rolled up, and fixed in a 10% buffered formaldehyde solution (Labsynth, São Paulo, Brazil). This material was embedded in paraffin, sectioned at 4 μm thick, stained with hematoxylin and eosin (HE) for scoring and morphometric analysis, and periodic acid-Schiff (PAS) for goblet cells count.

The histological inflammation score was determined as previously described by Soares et al. (2008). For morphometric analysis, ten field images of the ileum of each animal were captured using a BX41 optical microscope (Olympus, Tokyo, Japan). Twenty villus heights and crypt depth (magnification of 200 ×) and ten field/slides of goblet cells count (magnification 400 ×) were measured. These analyses were performed using *ImageJ* 1.51j.8 software (NIH, Bethesda, MD, United States).

Inflammatory Cell Infiltration

Neutrophil and eosinophil recruitment in the intestinal mucosa was performed by myeloperoxidase (MPO) and eosinophil peroxidase (EPO) enzyme activities, respectively (Barroso et al., 2021). For this purpose, mice ileum sections were homogenized, centrifuged, and lysed by hypotonic solution with three cycles of freezing and thawing in liquid nitrogen. After centrifugation, the supernatant was used to quantify enzyme activities (colorimetric assay). The assay was read at an absorbance of 492 nm (EPO) and 450 nm (MPO) on a microplate spectrophotometer (Spectramax M3, Molecular Devices, LLC, Sunnyvale, CA, United States). The results are expressed as MPO or EPO arbitrary units (AU)/mg of tissue.

Mice Ileum Relative Gene Expression Analysis

Total RNA Isolation

Mice ileum (1.0 cm) was collected and stored in RNAlater (Invitrogen, Carlsbad, CA, United States) at −20°C to preserve the samples. Total RNA isolation was carried out using an RNeasy Mini Kit (QIAGEN, Hilden, Germany), and residual DNA was degraded by DNAse I from the TURBO DNA-free™ Kit (Invitrogen, Carlsbad, CA, United States) according to the kit protocol. Complementary deoxyribonucleic acid (cDNA) synthesis was performed according to the manual instructions of the Applied Biosystems High-Capacity cDNA Reverse Transcription kit (Thermo Fisher, Waltham, MA, United States).

Quantitative PCR (qPCR)

qPCR was performed using PowerUp™ SYBR® Green Master Mix (Thermo Fisher, Waltham, MA, United States) and the gene-specific primers for expression of Toll-like receptor 2 and 4 (*Tlr2, Tlr4*), myeloid differentiation primary response gene 88 (*Myd88*), nuclear factor NF-kappa-B p105 subunit (*Nfkb1),* tumor necrosis factor (*Tnf*), interleukin 1 beta (*Il1b*), interleukin 6 (*Il6*), interleukin 10 (*Il10*), mucin 2 (*Muc2*), claudin 1 and 2 (*Cldn1, Cldn2*), junctional adhesion molecule 1 (*F11r*), zonulin (*Hp*) and occludin (*Ocln*). The glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) gene was used as an endogenous reference (**Table 1**; Giulietti et al., 2001; Song et al., 2013; Volynets et al., 2016; Zheng et al., 2017; Chang et al., 2020). Amplification cycles (initial denaturation at 95°C for 10 min, 95°C for 15 seg, annealing/extension at 60°C for 1 min, 40 cycles followed by a dissociation stage for recording the melting curve) were performed using Applied Biosystems 7900HT Fast Real-Time PCR System. The expression levels were presented as fold change using the $2^{-\Delta\Delta CT}$ method. Data are representative of two independent experiments.

Intestinal Permeability Evaluation

Mice received 0.1 mL of DTPA (diethylenetriaminepentaacetic acid) via gavage, labeled with 18.5 MBq 99mtechnetium

TABLE 1 | Quantitative polymerase chain reaction (qPCR) primers used in this study.

Gene	Primer Forward	Primer Reverse	References
Gapdh	TCACCACCATGGAGAAGGC	GCTAAGCAGTTGGTGGTGCA	Giulietti et al., 2001
Tlr2	ACAATAGAGGGAGACGCCTTT	AGTGTCTGGTAAGGATTTCCCAT	Chang et al., 2020
Tlr4	ATGGCATGGCTTACACCACC	GAGGCCATTTTTGTCTCCACA	Chang et al., 2020
Myd88	ATCGCTGTTCTTGAACCCTCG	CTCACGGTCTAACAAGGCCAG	Chang et al., 2020
Nfkb1 (p105)	GTGGAGGCATGTTCGGTAGTG	TCTTGGCACAATCTTTAGGGC	Zheng et al., 2017
Il6	GAGGATACCACTCCCAACAGACC	AAGTGCATCATCGTTGTTCATACA	Giulietti et al., 2001
Il10	GGTTGCCAAGCCTTATCGGA	ACCTGCTCCACTGCCTTGCT	Giulietti et al., 2001
Tnf	ACGTGGAACTGGCAGAAGAG	CTCCTCCACTTGGTGGTTTG	Song et al., 2013
Il1b	CTCCATGAGCTTTGTACAAGG	TGCTGATGTACCAGTTGGGG	Song et al., 2013
Muc2	GATGGCACCTACCTCGTTGT	GTCCTGGCACTTGTTGGAAT	Volynets et al., 2016
Cldn1	TCCTTGCTGAATCTGAACA	AGCCATCCACATCTTCTG	Volynets et al., 2016
Cldn2	GTCATCGCCCATCAGAAGAT	ACTGTTGGACAGGGAACCAG	Volynets et al., 2016
Ocln	ACTCCTCCAATGGACAAGTG	CCCCACCTGTCGTGTAGTCT	Volynets et al., 2016
Hp	CCACCTCTGTCCAGCTCTTC	CACCGGAGTGATGGTTTTCT	Volynets et al., 2016
F11r	CACCTTCTCATCCAGTGGCATC	CTCCACAGCATCCATGTGTGC	Volynets et al., 2016

on the last experimental day (de Barros et al., 2018). After four hours, total blood was collected without any stabilizer and measured the radioactivity level in an automated gamma radiation counter (PerkinElmer Wallac Wizard 1470-020 Gamma Counter, Waltham, MA, United States). Permeability was calculated by the percentage of DTPA administered dose per gram of blood (% ID/g), as follows: counts per min = (blood *cpm/cpm* of the administered dose) × 100.

Organ Index

To evaluate the toxic effects of 5-FU on lymphopoietic organs, splenic and thymus indices were carried out as previously described by Ren et al. (2019), as following: organ index = (weight of organ/body weight of the mice) × 100.

Statistical Analysis

The data normality was performed using the Shapiro-Wilk test. Data were evaluated by one-way ANOVA followed by Tukey's *post hoc* test (parametric data) or by the Kruskal-Wallis test and post-tested by Dunn's test (non-parametric data). The Mann-Whitney test analyzed food and liquid intake before and after 5-FU-inflammation induction. Spearman test was used for correlation analysis. All data were performed using GraphPad Prism 8.0 software (GraphPad Software, San Diego, CA, United States.), with a *p-value* < 0.05. The results are presented as mean ± standard deviation.

RESULTS

Lactobacillus delbrueckii CIDCA 133 Not Stop Weight Loss, or Liquid and Food Intake in 5-FU-Treated Mice

No abnormal clinical behavior or mortality was observed in mice before and after 5-FU inflammation induction. Body weight loss was also evaluated to check whether treatment with CIDCA 133 could relieve the symptoms of 5-FU-induced inflammation. The body weight variation in inflamed mice was observed 24 hours after the administration of the antineoplastic 5-FU (**Figure 1A**) when compared to control groups, with mice in the 5-FU group showing significant body weight loss (13.43 ± 2.76%) compared to the NC group (1.47 ± 3.41%) ($p < 0.0001$) (**Figure 1B**). CIDCA 133 treatment (5-FU + CIDCA 133 group) was unable to mitigate 5-FU-induced body weight loss (13.54 ± 1.07%) ($p = 0.998$) (**Figure 1B**).

Furthermore, the time course of the mice's liquid and food intake was also evaluated (**Figures 1C,E**). As expected, after 10th the 5-FU group showed a significant reduction in the average food (2.33 ± 0.40 g) (**Figure 1D**) and liquid (3.79 ± 0.42 mL) (**Figure 1F**) consumption compared to the NC group (food intake: 4.03 ± 0.61 g; liquid intake: 9.81 ± 0.21 mL) ($p < 0.01$). CIDCA 133 consumption (5-FU + CIDCA 133 group) did not improve these parameters (food intake: 2.83 ± 0.38 g; liquid intake: 3.47 ± 0.63 mL) ($p = 0.49$) (**Figures 1D,F**).

Lactobacillus delbrueckii CIDCA 133 Reduces 5-FU-Induced Inflammatory Cell Infiltration

The inflammatory infiltrates in the intestinal mucosa are one of the most common characteristics of 5-FU inflammation induction. In this study, we assessed the presence of ileum neutrophil and eosinophil infiltrates by detecting specific myeloperoxidase (MPO) and eosinophil peroxidase (EPO) enzyme activities, respectively.

5-FU group showed an increased level of MPO (1.74 ± 0.57 AU/mg) (**Figure 2A**) and EPO (1.15 ± 0.19 AU/mg) (**Figure 2B**) activity when compared to the NC group (MPO: 0.34 ± 0.18 AU/mg; EPO: 0.32 ± 0.09 AU/mg) ($p < 0.0001$). However, a reduction in neutrophil and eosinophil recruitment after CIDCA 133 (5-FU + CIDCA 133 group) administration (MPO: 0.73 ± 0.23 AU/mg; EPO: 0.45 ± 0.21 AU/mg) ($p < 0.001$) (**Figures 2A,B**) was observed.

Regarding the toxic effects of 5-FU on lymphopoietic organs, administration of 5-FU (5-FU group) induced thymus (0.04 ± 0.008) and spleen (0.12 ± 0.009) atrophy compared to the NC group (thymus: 0.12 ± 0.03; spleen: 0.24 ± 0.01) ($p < 0.001$). However, CIDCA 133 consumption (5-FU + CIDCA 133 group) was not able to ameliorate this toxic effect (thymus: 0.03 ± 0.005, $p = 0.89$; spleen: 0.13 ± 0.005, $p = 0.84$).

Lactobacillus delbrueckii CIDCA 133 Mitigates Intestinal Mucosa Damage Induced by 5-FU

Regarding histopathological analysis, mice inflamed with 5-FU (5-FU) showed significant alterations in mucosal integrity, such as edema, villus shortening, crypt necrosis, goblet cell number reduction, and intense polymorphonuclear cells infiltration into the lamina propria and villi, compared to the NC group (**Figure 3A**). These results were correlated with the respective histopathological scores (**Figure 3B**).

The inflamed mice treated with CIDCA 133 (5-FU + CIDCA 133 group) showed a decrease in 5-FU-induced mucosal damage. Villus height preservation (**Figure 3C**), an increased villus height to crypt depth ratio (**Figure 3E**), and a reduction in inflammatory infiltration in the villus and lamina propria were observed histologically in the 5-FU + CIDCA 133 group compared to the 5-FU group. However, no significant difference in crypt depth was observed across groups (**Figure 3D**), and CIDCA 133 strain consumption (5-FU + CIDCA 133 group) did not improve the histological score (**Figure 3B**).

These above findings can be associated to preservation of intestine length. 5-FU administration (5-FU group) also reduced the small intestine length (52.75 ± 2.27 cm) compared to the control group (NC) (56.33 ± 0.93 cm) ($p < 0.001$). However, treatment with CIDCA 133 (5-FU + CIDCA 133 group) prevented this shortening (55.00 ± 1.45 cm) at similar levels to the NC group ($p < 0.05$) (**Figure 3F**).

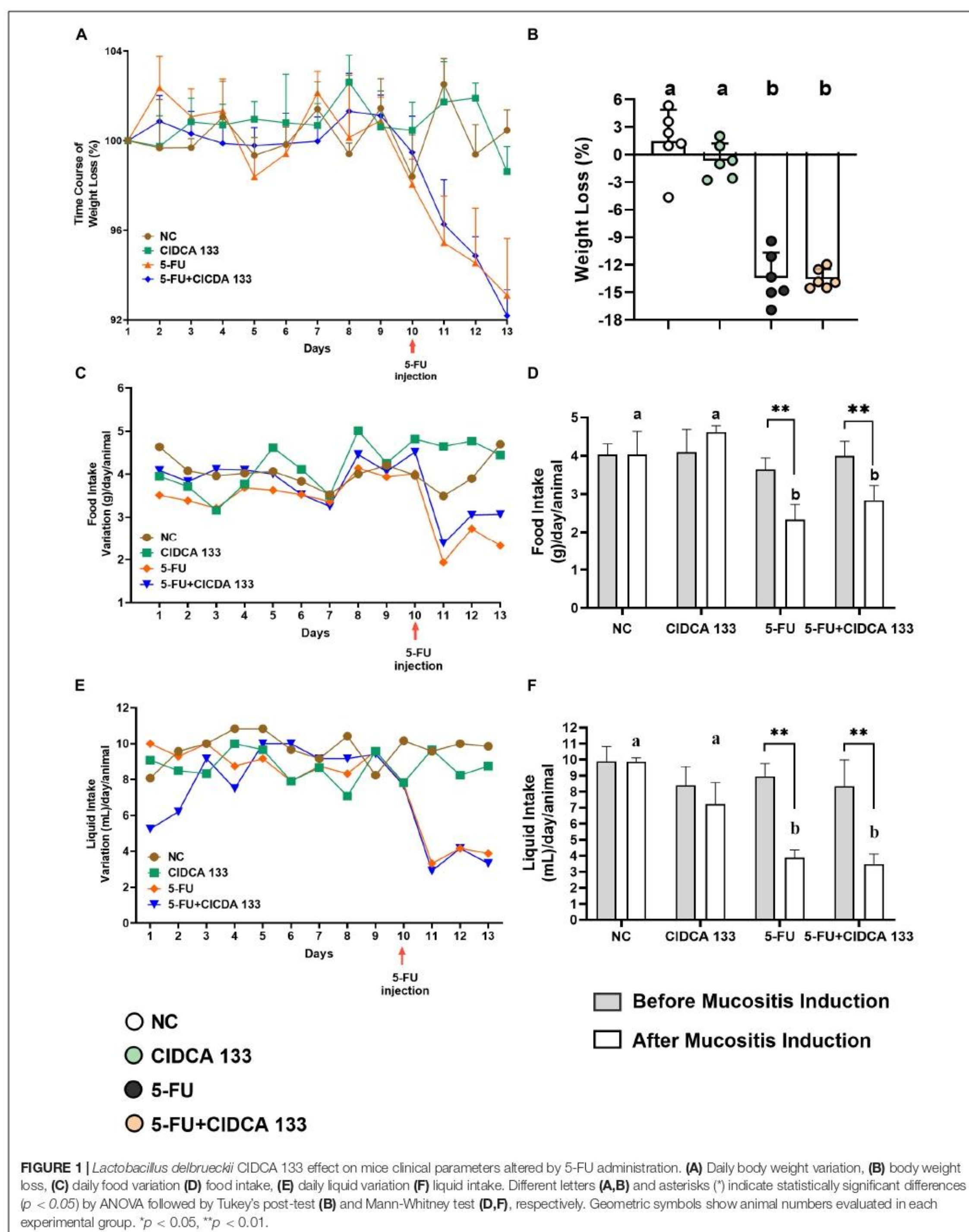

FIGURE 1 | *Lactobacillus delbrueckii* CIDCA 133 effect on mice clinical parameters altered by 5-FU administration. **(A)** Daily body weight variation, **(B)** body weight loss, **(C)** daily food variation **(D)** food intake, **(E)** daily liquid variation **(F)** liquid intake. Different letters **(A,B)** and asterisks (*) indicate statistically significant differences ($p < 0.05$) by ANOVA followed by Tukey's post-test **(B)** and Mann-Whitney test **(D,F)**, respectively. Geometric symbols show animal numbers evaluated in each experimental group. *$p < 0.05$, **$p < 0.01$.

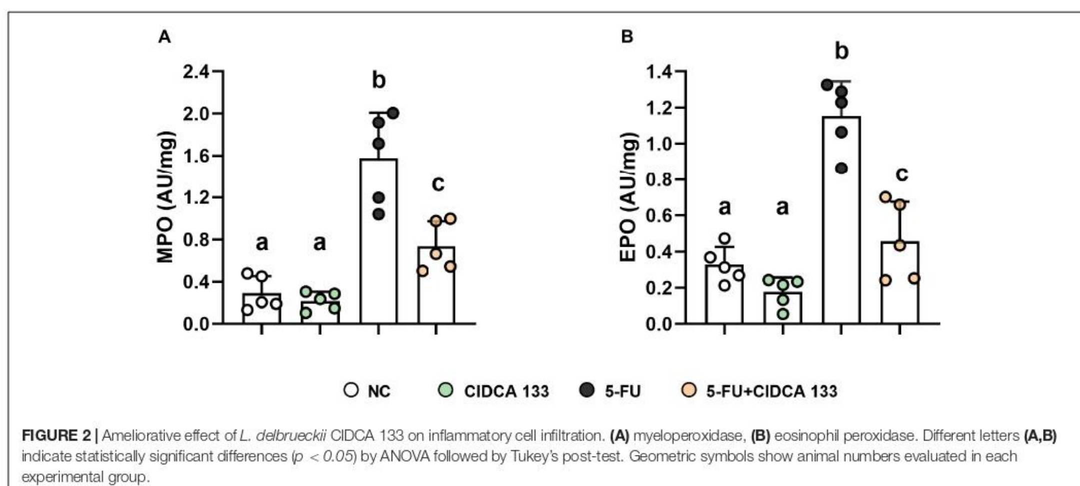

FIGURE 2 | Ameliorative effect of *L. delbrueckii* CIDCA 133 on inflammatory cell infiltration. **(A)** myeloperoxidase, **(B)** eosinophil peroxidase. Different letters **(A,B)** indicate statistically significant differences ($p < 0.05$) by ANOVA followed by Tukey's post-test. Geometric symbols show animal numbers evaluated in each experimental group.

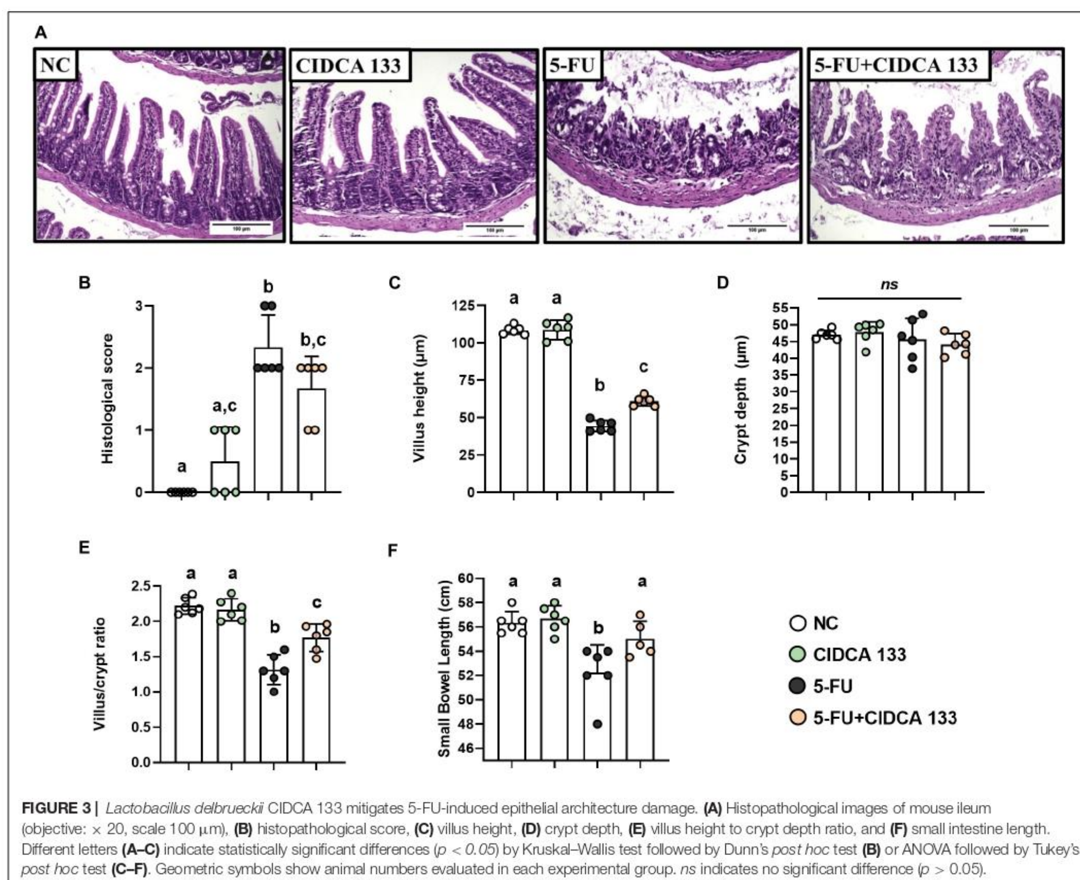

FIGURE 3 | *Lactobacillus delbrueckii* CIDCA 133 mitigates 5-FU-induced epithelial architecture damage. **(A)** Histopathological images of mouse ileum (objective: × 20, scale 100 μm), **(B)** histopathological score, **(C)** villus height, **(D)** crypt depth, **(E)** villus height to crypt depth ratio, and **(F)** small intestine length. Different letters **(A–C)** indicate statistically significant differences ($p < 0.05$) by Kruskal–Wallis test followed by Dunn's *post hoc* test **(B)** or ANOVA followed by Tukey's *post hoc* test **(C–F)**. Geometric symbols show animal numbers evaluated in each experimental group. *ns* indicates no significant difference ($p > 0.05$).

Lactobacillus delbrueckii CIDCA 133 Modulates Gene Expression of the TLR2/4/Myd88/NF-κB Signaling Pathway

Mice in the 5-FU group displayed significantly increased mRNA expression of *Tlr2* (2.99 ± 0.64; $p < 0.0001$) (**Figure 4A**), *Tlr4* (1.90 ± 0.52; $p < 0.001$) (**Figure 4B**), *Myd88* (3.33 ± 1.14; $p < 0.0001$) (**Figure 4C**), and *Nfkb1* (2.80 ± 0.88; $p < 0.0001$) (**Figure 4D**) compared to the NC group. Compared to the 5-FU-treated group, the mRNA expression profiles of *Tlr2* (1.50 ± 0.33; $p < 0.0001$) (**Figure 4A**), *Tlr4* (1.19 ± 0.37; $p < 0.01$) (**Figure 4B**), *Myd88* (1.36 ± 0.62; $p < 0.001$) (**Figure 4C**), and *Nfkb1* (p105) (1.64 ± 0.30; $p < 0.01$) (**Figure 4D**) were significantly reduced after CIDCA 133 consumption (5-FU + CIDCA 133).

Lactobacillus delbrueckii CIDCA 133 Downregulates Proinflammatory Cytokines and Upregulates *Il10* Gene Expression

Mice in the 5-FU group exhibited a significant increased mRNA expression of *Il6* (7.57 ± 1.63; $p < 0.0001$) (**Figure 5A**), *Il1b* (3.26 ± 0.83; $p < 0.0001$) (**Figure 5B**) and *Tnf* (1.82 ± 0.52; $p < 0.05$) (**Figure 5D**) and a downregulation of *Il10* (0.37 ± 0.15; $p < 0.01$) (**Figure 5C**) compared to the NC group. After CIDCA 133 consumption (5-FU + CIDCA 133 group), downregulation of *Il6* (4.21 ± 1.29; $p < 0.001$) (**Figure 5A**) and *Il1b* (1.191 ± 0.68; $p < 0.001$) (**Figure 5B**) and upregulation of *Il10* (0.95 ± 0.24; $p < 0.01$) (**Figure 5C**) mRNA expression were observed compared to the 5-FU group. No difference in *Tnf* (1.84 ± 0.49; $p = 0.99$) (**Figure 5D**) gene expression was observed between the 5-FU + CIDCA 133 group and the 5-FU group.

Lactobacillus delbrueckii CIDCA 133 Reduces 5-FU-Induced Goblet Cell Depletion and Upregulates *Muc2* Gene Expression

Significant goblet cell number reduction was also observed in the 5-FU-treated (5-FU group) ileum-inflamed mice (14.25 ± 3.68 cell/field) compared to the NC group (32.99 ± 1.91 cell/field) ($p < 0.0001$), but treatment with CIDCA 133 (5-FU + CIDCA 133) reduced the loss of goblet cells (21.84 ± 2.24 cell/field) ($p < 0.05$) (**Figure 6A**). A downregulation of *Muc2* gene expression was also exhibited in the inflamed ileum of the 5-FU mice (5-FU group) (0.28 ± 0.22) ($p < 0.001$) and its upregulation after treatment with CIDCA 133 (5-FU + CIDCA 133 group) (1.48 ± 0.40) ($p < 0.0001$) (**Figure 6B**) was observed. No positive correlation was observed between villus' goblet cells count and *Muc2* gene expression among inflamed (5-FU group) (r = -0.1000, p = 0.9500) (**Figure 6C**) and treated mice (5-FU + CIDCA 133 group) (r = 0.6000, p = 0.3500) (**Figure 6D**).

Lactobacillus delbrueckii CIDCA 133 Ameliorates 5-FU-Induced Increased Intestinal Permeability and Upregulates Tight Junction Gene Expression

Intestinal permeability was evaluated by measuring the radioactivity uptake in the blood after oral administration of radiolabeled diethylenetriamine-pentaacetate (^{99m}Tc-DTPA). As expected, 5-FU administration (5-FU group) significantly increased intestinal permeability in treated mice (0.21 ± 0.01% ID/g) compared to the NC group (0.02 ± 0.00% ID/g) ($p < 0.0001$). However, treatment with CIDCA 133 (5-FU + CIDCA 133 group) significantly reduced 5-FU-induced intestinal permeability (0.04 ± 0.01% ID/g; $p < 0.0001$) (**Figure 7A**).

Gene expression of tight junction proteins was also measured. Mice inflamed with 5-FU (5-FU group) exhibited a significant reduced mRNA expression of tight junction proteins *Cldn1* (0.56 ± 0.17; $p < 0.05$) (**Figure 7B**), *Fr11* (0.54 ± 0.16; $p < 0.05$) (**Figure 7C**), and *Hp* (0.47 ± 0.20; $p < 0.01$) (**Figure 7D**) compared to the NC group. The mRNA expression of *Cldn1* (1.17 ± 0.13), *Fr11* (0.99 ± 0.20) and *Hp* (1.19 ± 0.21) was significantly upregulated after CIDCA 133 consumption (5-FU + CIDCA 133 group) compared to the 5-FU group ($p < 0.01$). No differences in *Cldn2* (1.20 ± 0.27) (**Figure 7E**) and *Ocln* (1.29 ± 0.44) (**Figure 7F**) gene expression were observed across the groups ($p > 0.05$).

DISCUSSION

5-Fluorouracil (5-FU) is a commonly used antineoplastic drug that shows intestinal mucositis as one of its main side effects. This gastrointestinal disorder is characterized by inflammation and ulceration of the intestinal epithelium (Sonis, 2004) and is a limiting factor in oncology therapy success.

Probiotic consumption has been considered a promising therapeutic option to ameliorate the inflammatory and epithelial damage induced by 5-FU administration due to their reported anti-inflammatory effects (Batista et al., 2020; Cristofori et al., 2021). The protective effect of *L. delbrueckii* CIDCA 133 on 5-FU-induced epithelial damage was previously demonstrated using a fermented milk formulation (de Jesus et al., 2019). In this study, we investigated whether the beneficial effect of *L. delbrueckii* CIDCA 133 on damage caused by 5-FU-induced intestinal mucosal would be associated with the ability of this probiotic strain to regulate the gene expression of inflammatory and epithelial barrier markers.

Due to 5-FU cytotoxicity, severe epithelial damage has been reported as one of the most critical features of mucositis pathobiology. This damage includes epithelial architecture, mucosal barrier integrity alteration, goblet cell depletion, villus shortening, intestinal permeability, and increased polymorphonuclear cell infiltration. All these parameters were evaluated in this study to assess the ameliorative and anti-inflammatory activity of the CIDCA 133 strain. Although CIDCA 133 treatment did not improve the histological score, the

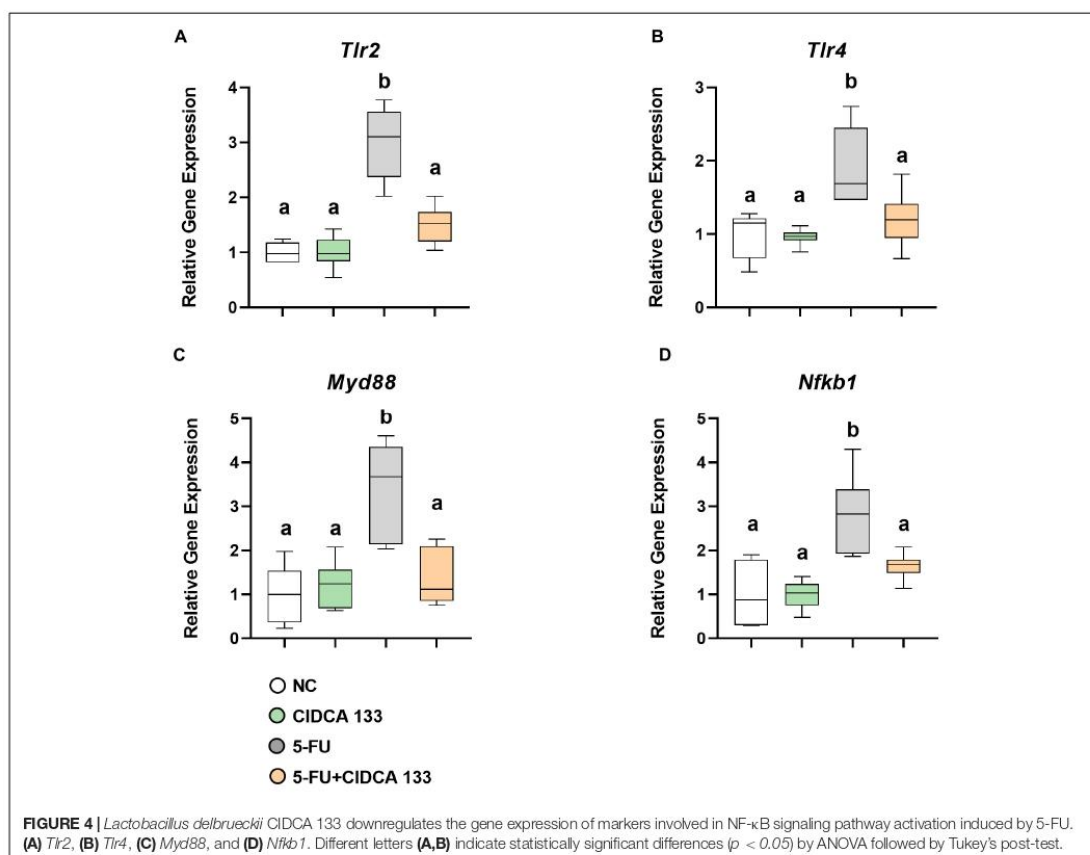

FIGURE 4 | *Lactobacillus delbrueckii* CIDCA 133 downregulates the gene expression of markers involved in NF-κB signaling pathway activation induced by 5-FU. **(A)** *Tlr2*, **(B)** *Tlr4*, **(C)** *Myd88*, and **(D)** *Nfkb1*. Different letters **(A,B)** indicate statistically significant differences ($p < 0.05$) by ANOVA followed by Tukey's post-test.

strain was able to reduce the tissue damage and preserve villus height induced by 5-FU, corroborating with previous studies that had demonstrated the protective effect of other probiotics in the intestinal architecture of inflamed mice with 5-FU (Yeung et al., 2015; Kato et al., 2017; Oh et al., 2018).

It is essential to highlight that in addition to the damage reported at the ileal level, we also observed that 5-FU affected the whole digestive tract and other organs' functions. This finding can be demonstrated by shortening the small intestine length after administering the antineoplastic agent. 5-FU promoted intestinal shortening, and mucosal destruction led to reduced nutrient absorption, which might correlate with body weight loss observed during mucositis (Vieira et al., 2012; Maioli et al., 2014; Kato et al., 2017; de Barros et al., 2018).

Our study shows that consumption of CIDCA 133 reduced the shortening of the small intestine but did not improve food and hydric intake, and body weight loss. Similar results were reported by Maioli et al. (2014) and Antunes et al. (2016) in animals with mucositis induced by 5-FU. The researchers observed that their experimental treatment did not influence food intake and weight loss. These results reinforce how intestinal mucositis, as a side effect of 5-FU administration, may become a severe problem in chemotherapy. In addition, unlike what was observed, the beneficial effect of milk fermented by CIDCA 133 on body weight loss and histopathological score in inflamed mice with 5-FU has been previously reported by us (de Jesus et al., 2019), demonstrating that dairy formulation with this probiotic seems to be more promising in the clinical manifestations of 5-FU administration-derived mucositis. One possible explanation for this outcome is that dairy formulations possess nutraceutical compounds (*e.g.,* amino acid, fibers) as well as probiotic-derived bioactive metabolites (*e.g.,* SCFA) (Galdino et al., 2018; Trindade et al., 2018), making them more nutritious and beneficial than MRS culture broth. Furthermore, despite improving the probiotic's inflammatory parameters, the positive effects on liquid and food intake, and body weight may be more prominent with the intestinal healing progresses, ameliorating the gastrointestinal dysmotility (Soares et al., 2008), increasing the area of nutrient absorption, and consequently improving the mice's clinical status (de Jesus et al., 2019). It is also interesting to point out that although probiotics demonstrate multiple beneficial effects, a single strain does not seem to be clinically sufficient, so it is

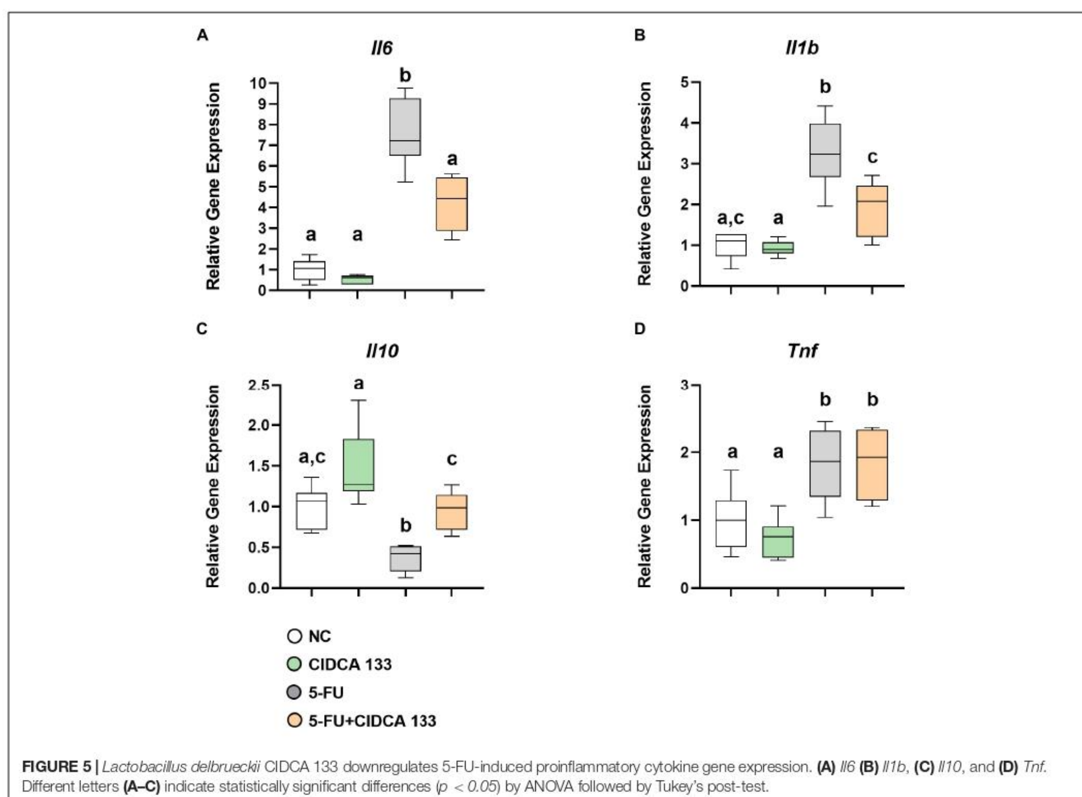

FIGURE 5 | *Lactobacillus delbrueckii* CIDCA 133 downregulates 5-FU-induced proinflammatory cytokine gene expression. **(A)** *Il6* **(B)** *Il1b*, **(C)** *Il10*, and **(D)** *Tnf*. Different letters **(A–C)** indicate statistically significant differences ($p < 0.05$) by ANOVA followed by Tukey's post-test.

proposed that the combination of several probiotic strains may be most effective (Prisciandaro et al., 2011a; Yeung et al., 2015).

The production of several inflammatory markers, such as proinflammatory cytokines (IL-6, TNF-α, IL-1β, IFN-γ, CXCL1), via exacerbated activation of the TLR2/4/Myd88/NF-κB signaling pathway, is another feature caused by the administration of 5-FU (Chang et al., 2012; Li et al., 2017; Justino et al., 2020). Probiotics can modulate the host immune response through their interaction with intestinal epithelial cells via Toll-like receptors (TLRs) and regulate the balance between the T helper type 1 and 2 (Th1 and Th2) responses induced by NF-κB activation (Kawai and Akira, 2007; Mukherjee et al., 2016). Therefore, we also investigated the effects of CIDCA 133 consumption and its impact on the gene expression associated with immune system activation. We observed a decrease in the relative gene expression of *Tlr2, Tlr4, Myd88,* and *Nfkb1* after CIDCA 133 strain consumption by inflamed mice, while the mRNA expression of proinflammatory cytokines (*Il6* and *Il1b*) and regulatory cytokine *Il10* were down- and upregulated, respectively. Regarding TNF cytokine, its gene expression was upregulated after 5-FU administration, and transcript levels were maintained after consumption of CIDCA 133. TNF is a key factor in inflammatory diseases. However, it has been demonstrated that epithelial-derived TNF-α can stimulate epithelial cell proliferation, a mechanism used by probiotics to promote intestinal epithelial barrier regeneration and improve the innate immune response (Pagnini et al., 2010; Giorgetti et al., 2015). This property may be a mechanism used by CIDCA 133 in its beneficial effect against 5-FU epithelial damage.

The balance of pro-and anti-inflammatory cytokines is fundamental to control the exacerbation of intestinal damage in 5-FU-induced mucositis. Upregulation of *Il10* in mice inflamed with 5-FU and treated with CIDCA 133 evidenced the anti-inflammatory effects that this strain can provide. This finding corroborates de Jesus et al. (2021b), who showed that CIDCA 133 could modulate inflammatory responses possibly by controlling NF-κB signaling pathway activation through upregulation of immunoregulatory molecules such as *Il10* and *Tgfb1* to maintain intestinal homeostasis.

Our results are supported by other studies in which administration of probiotics, such as *Saccharomyces boulardii* CNCM I-745 (Justino et al., 2020), *Lactobacillus acidophilus* (Justino et al., 2015), or *Lacticaseibacillus casei* (Yeung et al., 2015), also improved the inflammatory and functional aspects of intestinal inflammation induced by 5-FU through the modulation of the expression of *Tlr2, Tlr4, Myd88, Nf-κB*, and

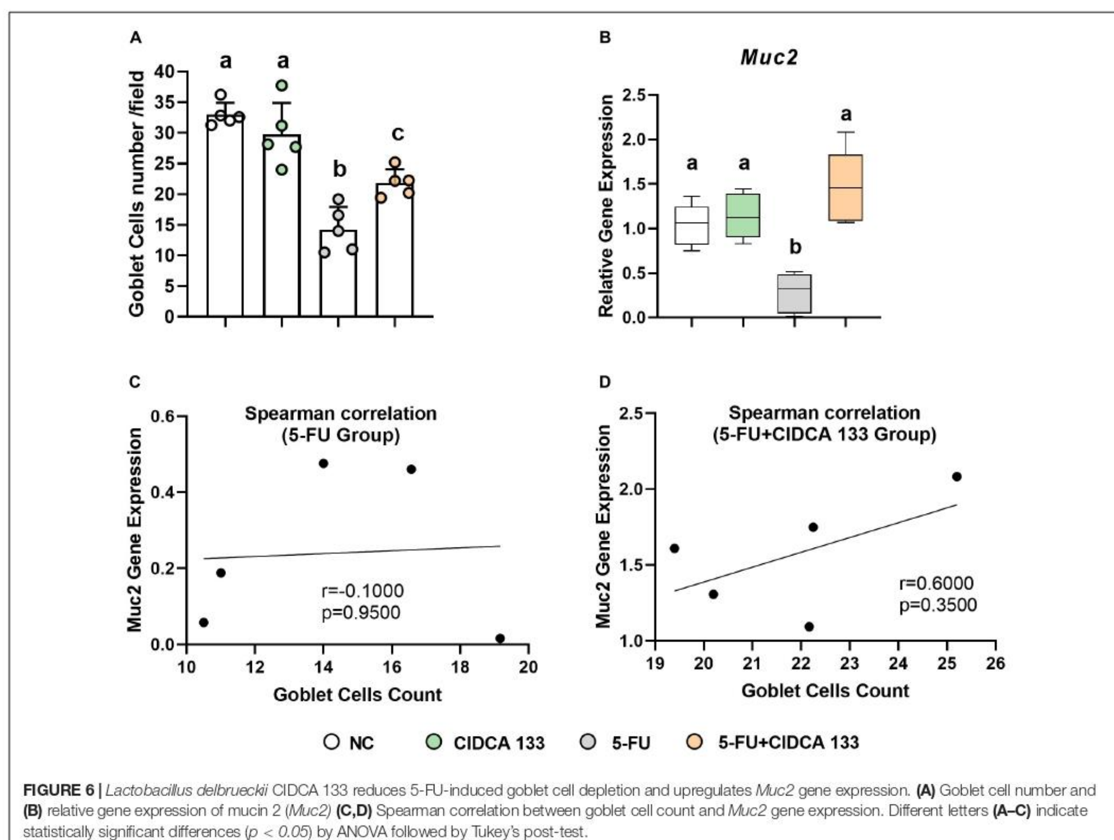

FIGURE 6 | *Lactobacillus delbrueckii* CIDCA 133 reduces 5-FU-induced goblet cell depletion and upregulates *Muc2* gene expression. **(A)** Goblet cell number and **(B)** relative gene expression of mucin 2 (*Muc2*) **(C,D)** Spearman correlation between goblet cell count and *Muc2* gene expression. Different letters **(A–C)** indicate statistically significant differences ($p < 0.05$) by ANOVA followed by Tukey's post-test.

cytokines *Il6, Il1b, Il10,* and *Ifng*. It should be noted that not all probiotics showed anti-inflammatory effects in 5-FU-induced epithelial damage (Prisciandaro et al., 2011b; Maioli et al., 2014). Despite sharing similar probiotic features (Plaza-Diaz et al., 2019), the mechanisms used in a 5-FU inflammation context may be different from one strain/specie to another, and this may be related to the probiotic dose used, the antineoplastic agents type and experimental protocols for inducing mucositis, as well as the biotic form as beneficial microorganisms are administrated (viable, inactivated or their secreted products) (Chang et al., 2012; Yeung et al., 2015; Batista et al., 2020; Wu et al., 2021).

The intestinal mucosa destruction and production of proinflammatory cytokines induced 5-FU cytotoxicity, resulting in the recruitment of eosinophils and neutrophils into the lamina propria. This study shows increased activities of MPO and EPO in 5-FU-inflamed mice, which was reduced after administration of CIDCA 133. The beneficial effect of this strain on the recruitment of eosinophils and neutrophils to the intestinal mucosa reduced the oxidative stress and production of proinflammatory cytokines induced by these cells. Consequently, it prevented the exacerbation of tissue damage and the development of ulcers. These findings are consistent with other studies that also observed beneficial effects of different probiotic microorganisms in decreasing inflammatory cell infiltrate induced by 5-FU administration, such as *Bifidobacterium sp.* (Kato et al., 2017; Quaresma et al., 2020), *Lactobacillus acidophilus* (Oh et al., 2018) *Lacticaseibacillus rhamnosus* (Trindade et al., 2021) and *Rhodotorula mucilaginosa* (Coutinho et al., 2021).

Polymorphonuclear cell infiltration is also associated with an increase in intestinal permeability (IP), probably as a result of reactive oxygen species (ROS) production by these cells, villous atrophy, and reduced expression of tight junction proteins (van Vliet et al., 2010; Li et al., 2017). In this study, IP was studied by measuring blood radioactivity after oral intake of ^{99m}Tc-DTPA, a hydrophilic macromolecule that rarely crosses the intestinal barrier under healthy conditions, making it a good radiotracer marker for measuring IP (Andrade et al., 2015). CIDCA 133 consumption was able to prevent intestinal permeability induced by 5-FU significantly. The permeability was decreased due to upregulation of tight junctions (*Cldn1, F11r,* and *Hp*) and *Muc2* gene expression, reduction in mucus-producing goblet cell number loss, and villus preservation by the strain. These results agree with other studies that also showed beneficial effects of probiotics in attenuating 5-FU-induced intestinal permeability

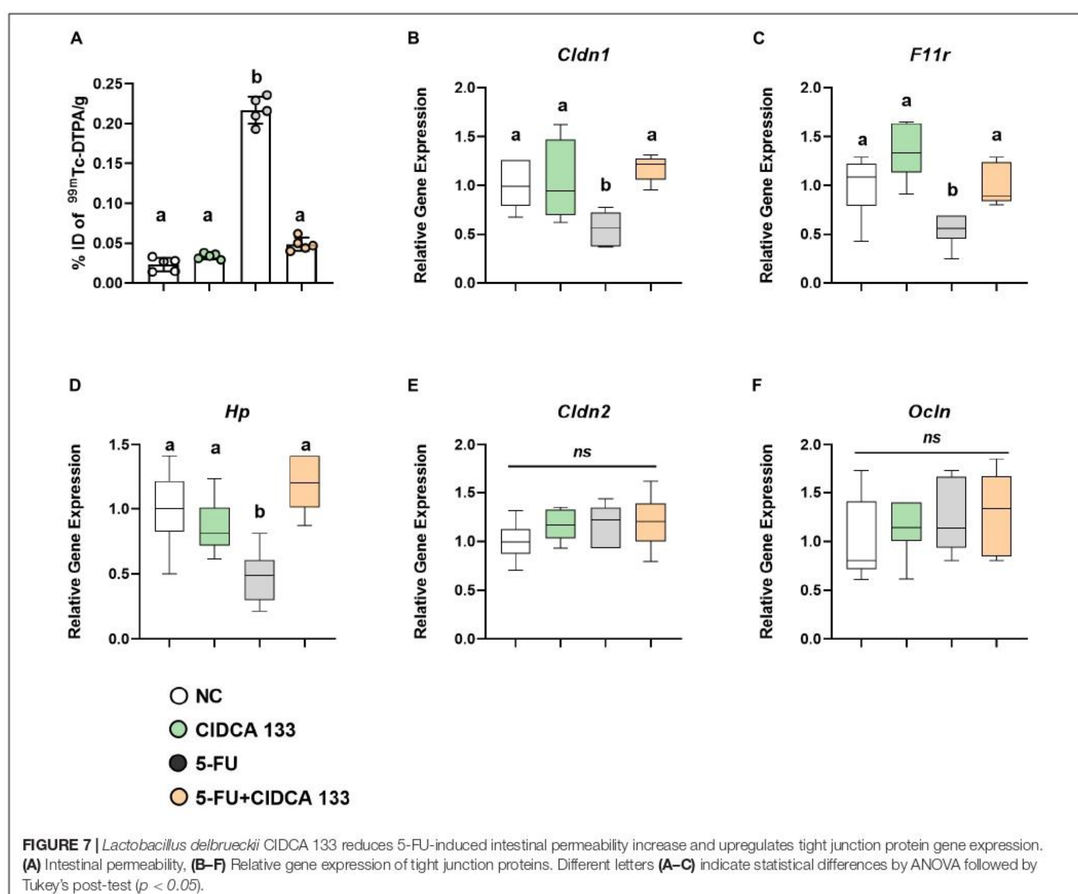

FIGURE 7 | *Lactobacillus delbrueckii* CIDCA 133 reduces 5-FU-induced intestinal permeability increase and upregulates tight junction protein gene expression. **(A)** Intestinal permeability, **(B–F)** Relative gene expression of tight junction proteins. Different letters **(A–C)** indicate statistical differences by ANOVA followed by Tukey's post-test ($p < 0.05$).

(Tang et al., 2017; Trindade et al., 2018; Porto et al., 2019) and goblet cell number loss (Yeung et al., 2015; Kato et al., 2017; de Jesus et al., 2019).

Reactive oxygen species production by inflammatory cells promotes intestinal cell apoptosis (Soares et al., 2008). On the basis of this information, we suggest CIDCA 133 can maintain the maturation of mucus-producing goblet cells and tight junctions gene expression by reducing apoptosis of epithelial cells resulting from inflammatory pathways activation, via direct contact of its bacterial cells components (*e.g.*, surface or extracellular proteins, SCFA) with intestinal cells receptors (*e.g.*, TLR2/TLR4; GPR43) (Karczewski et al., 2010; van Vliet et al., 2010; Wang et al., 2018; Liu et al., 2020; Rose et al., 2021). This process would reduce the inflammatory cells infiltrate and their oxidative stress generated on the intestinal mucosa, thus preserving the function and structure of intestinal epithelial components, such as tight-junction proteins, goblet cells, and enterocytes. Furthermore, our results showed that although there was no difference between the groups related to crypt depth, there was an improvement in the crypt-villus ratio after consumption of CIDCA 133, demonstrating that possibly villus height improvement may be associated with the migration of enterocytes, produced by the crypt stem cells, to repair the intestinal epithelium by replacing the dead cells (Yeung et al., 2015). This, therefore, reflects the increase in the number of goblet cells present in the villi, as observed in our study.

Stringer et al. (2009) suggested that rapid mucin secretion in the small intestine due to 5-FU administration occurs primarily in the crypts, mainly caused by enteric neurotransmitters acting on epithelial cells, including goblet cells (Stringer et al., 2009). Furthermore, studies demonstrate that the maturation of goblet cells results from epithelial activation of TLR2/TLR4 (Podolsky et al., 2009; Dheer et al., 2016).On the other hand, TLR activation is also attributed to dysbiotic commensal microbiota (Li et al., 2017). Based on this information, we suggest that CIDCA 133 can upregulated mucin 2 as a mechanism to control the dysbiotic microbiota and maintain intestinal homeostasis. Altogether, these above mechanisms may lead to translocation

inhibition of pathogenic microorganisms and toxins into the lamina propria, thereby attenuating an exacerbated inflammatory response (Sonis, 2004; Krishna Rao and Samak, 2013; Maioli et al., 2014), and, thus, reinforcing the importance of this strain in maintaining the epithelial barrier, as previously demonstrated by de Jesus et al. (2019) and de Jesus et al. (2021a).

The ameliorative effect of CIDCA 133 has already been demonstrated by our research group using a dairy formulation, which so far has been shown to be the best matrix for the beneficial effects of CIDCA 133 when compared to some results of our work. However, our findings showed the main molecular pathways used by this probiotic strain to promote its beneficial effects, which have not been reported in previous studies.

CONCLUSION

Our results reinforce that *Lactobacillus delbrue*ckii CIDCA 133 strain consumption improved mucosal damage in mice undergoing chemotherapy with 5-FU, demonstrating to be a promising adjuvant therapeutic strategy to attenuate 5-FU-induced intestinal mucositis. Furthermore, our work suggests that this strain can re-establish intestinal mucosa homeostasis involving different molecular mechanisms through modulation of NF-κB activation, proinflammatory cytokines, and tight junction proteins expression. Therefore, more studies will be performed to fully explain the mechanisms used for this strain on intestinal inflammation context, reinforcing its use as a probiotic.

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 Local Animal Experimental Ethics Committee of the Federal University of Minas Gerais (CEUA-UFMG).

AUTHOR CONTRIBUTIONS

FB, PM-A, and LJ: conceptualization. FB, LJ, VB, MD, KV, NC-R, FM, SF, and VC: methodology. LJ, FB, and EF: formal analysis and investigation. FB, LJ, JL, and TS: writing-original draft preparation. AB, DB, and VA: writing-review and editing. All authors read and approved the final manuscript.

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6 CAPÍTULO II: Intake of *Lactobacillus delbrueckii* (pExu: *hsp*65) Prevents the Inflammation and the Disorganization of the Intestinal Mucosa in a Mouse Model of Mucositis.

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Os efeitos benéficos atribuídos ao consumo de BAL com perfil probiótico tem chamado a atenção da comunidade cientifica para a utilização desses microrganismos em diferentes abordagens biotecnológicas. A fim de potencializar estes efeitos benéficos, pesquisas baseadas em técnicas de engenharia genética têm sido realizadas, visando um amplo espectro de aplicações dessas bactérias. Diversas proteínas de interesse biotecnológico (como moléculas anti-inflamatórias) já foram produzidos por BAL por meio da engenharia genética. Algumas novas aplicações desses microrganismos incluem as vacinas de mucosa, no qual estas bactérias atuam como veículos para a entrega de plasmídeos vacinais/terapêuticos com cassetes de expressão eucariótica, onde a expressão da proteína de interesse é realizada pelas próprias células do hospedeiro. Uma das moléculas produzidas por engenharia genética usando BAL é a proteína Hsp65 de *M. leprae*, que cada vez mais vem sendo ratificada como uma estratégia eficaz na prevenção de modelos experimentais de IBDs, como a colite ulcerativa. Embora muitos estudos com proteínas recombinantes sejam voltados para as IBDs, poucos estudos exploram o potencial de linhagens probióticas recombinantes para expressão/entrega de moléculas terapêuticas na mucosite intestinal. Diante disso, no trabalho apresentado neste capítulo foi realizado a construção do vetor pExu:*hsp*65 e da linhagem recombinante *Lactobacillus delbrueckii subsp. lactis* CIDCA 133 (pExu:*hsp*65), denominada aqui de rCIDCA 133:HSP65, e avaliado o efeito enteroprotetor dessa linhagem recombinante no modelo de mucosite intestinal induzida por 5-FU. Para realizar o estudo, camundongos BALB/c foram inflamados com uma única dose do quimioterápico 5-FU (300 mg/kg) e tratados com rCIDCA 133:HSP65 (10^7 CFU/mL) durante 13 dias. Os resultados obtidos demonstraram que o tratamento com rCIDCA 133:HSP65 melhorou os danos inflamatórios e histopatológicos ocasionados à mucosa intestinal pela administração de 5-FU por meio da modulação de marcadores inflamatórios e de barreira epitelial. Além disso, foi observado que os efeitos da linhagem selvagem foram potencializados com a produção de Hsp65. Essa abordagem revelou resultados promissores da utilização desta linhagem recombinante, abrindo caminho para a validação desta abordagem como alternativa terapêutica promissora para as doenças inflamatórias intestinais.

microorganisms

Article

Intake of *Lactobacillus delbrueckii* (pExu:*hsp65*) Prevents the Inflammation and the Disorganization of the Intestinal Mucosa in a Mouse Model of Mucositis

Fernanda Alvarenga Lima Barroso [1,†], Luís Cláudio Lima de Jesus [1,†], Camila Prosperi de Castro [1], Viviane Lima Batista [1], Ênio Ferreira [2], Renata Salgado Fernandes [3], André Luís Branco de Barros [3], Sophie Yvette Leclerq [4], Vasco Azevedo [1], Pamela Mancha-Agresti [1,5,*] and Mariana Martins Drumond [1,6,*]

[1] Laboratório de Genética Celular e Molecular (LGCM), Departamento de—Genética, Ecologia e Evolução, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais (UFMG), Belo Horizonte 31270-901, Brazil; fernanda_alima@hotmail.com (F.A.L.B.); lc.luiis@yahoo.com.br (L.C.L.d.J.); camilaprosperic@gmail.com (C.P.d.C.); vivianelimabio@gmail.com (V.L.B.); vasco@icb.ufmg.br (V.A.)
[2] Departamento de Patologia Geral, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Belo Horizonte 31270-901, Brazil; enioferreira@icb.ufmg.br
[3] Departamento de Análises Clínicas e Toxicológicas, Faculdade de Farmácia, Campus da UFMG, Universidade Federal de Minas Gerais, Cidade Universitária, Belo Horizonte 31270-901, Brazil; renatasalgadof@yahoo.com.br (R.S.F.); brancodebarros@yahoo.com.br (A.L.B.d.B.)
[4] Laboratório de Inovação Biotecnológica, Fundação Ezequiel Dias (FUNED), Belo Horizonte 30510-010, Brazil; sodris2003@gmail.com
[5] Faculdade de Minas-Faminas-BH, Medicina, Belo Horizonte 31744-007, Brazil
[6] Centro Federal de Educação Tecnológica de Minas Gerais (CEFET/MG), Departamento de Ciências Biológicas, Belo Horizonte 31421-169, Brazil
* Correspondence: p.mancha.agresti@gmail.com (P.M.-A.); mmdrumond@gmail.com (M.M.D.); Tel.: +55-31-99817-5004 (P.M.-A.); +55-31-99222-2761 (M.M.D.)
† These authors contributed equally to this work.

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Abstract: 5-Fluorouracil (5-FU) is an antineoplastic drug that causes, as a side effect, intestinal mucositis, acute inflammation in the small bowel. The Heat Shock Protein (Hsp) are highly expressed in inflammatory conditions, developing an important role in immune modulation. Thus, they are potential candidates for the treatment of inflammatory diseases. In the mucositis mouse model, the present study aimed to evaluate the beneficial effect of oral administration of milk fermented by *Lactobacillus delbrueckii* CIDCA 133 (pExu:*hsp65*), a recombinant strain. This approach showed increased levels of sIgA in the intestinal fluid, reducing inflammatory infiltrate and intestinal permeability. Additionally, the histological score was improved. Protection was associated with a reduction in the gene expression of pro-inflammatory cytokines such as Tnf, Il6, Il12, and Il1b, and an increase in Il10, Muc2, and claudin 1 (Cldn1) and 2 (Cldn2) gene expression in ileum tissue. These findings are corroborated with the increased number of goblet cells, the electronic microscopy images, and the reduction of intestinal permeability. The administration of milk fermented by this recombinant probiotic strain was also able to reverse the high levels of gene expression of Tlrs caused by the 5-FU. Thus, the rCIDCA 133:Hsp65 strain was revealed to be a promising preventive strategy for small bowel inflammation.

Keywords: recombinant probiotics; DNA delivery; intestinal mucositis; inflammation; technetium-99m; bacterial translocation; gene expression

1. Introduction

Radio- and chemotherapy and the combination of both are widely used for cancer treatment. 5-Fluorouracil (5-FU) is one of the main chemotherapeutic drugs used to treat several types of cancer. This drug is responsible for several adverse effects, such as mucositis, an inflammatory process that affects the entire digestive tract, causing abdominal

pain, nausea, and diarrhea, which is the main limiting factor for the continuity and, consequently, efficacy of cancer treatment [1–3].

Many strategies have been studied, such as the administration of amino acids [4,5], vitamins [6], antioxidants [7], fatty acids [8,9], and, last but not least, the administration of probiotics, mainly lactic acid bacteria (LAB), such as lactobacilli [10,11], to alleviate the mucositis symptoms. The protective effects of probiotics on the intestinal barrier have been related to their influence on innate and adaptive immunity. Their ability to regulate Toll-like receptors (Tlrs) [12] and customize the composition and activity of the gut microbiota leads them to show many immune and non-immune protective mechanisms [13].

The treatment of intestinal diseases, such as colitis and mucositis, with wild-type probiotics has been reported to show promising results [14–17]. The probiotics display promising results, as demonstrated in different animal models; however, positive results are more limited when treating human inflammatory diseases. For that reason, more studies should be performed.

Thereby, based on these experiences and along with increasing the beneficial characteristics of probiotics, studies on many strains that express or encode different proteins with anti-inflammatory activities as promising candidates for the treatment of other pathological conditions, especially inflammatory bowel disorders, have been performed [15,18–22]. In this context, studies have been developed using the microbial 65 kDa heat shock protein (Hsp65) of *Mycobacterium leprae* (homolog to mammalian Hsp60) in many different animal disease models, to evaluate either prevention or treatment. For instance, to evaluate the effect of this protein in tuberculosis disease, mice [23] and calves [24] were used. Mouse models were used to study colitis [25], encephalomyelitis [26], lupus [27], and atherosclerosis [28], among others, with excellent outcomes, thus demonstrating the relevance of the Hsp65 protein as a good candidate for treatment and therapeutic uses.

Hsp proteins constitute about 5% of all intracellular proteins of prokaryotic and eukaryotic organisms, with high structural homology between bacterial and mammalian Hsps [29]. They participate in protein folding, degradation of misfolded protein, acting as intracellular chaperones, avoiding undesirable protein aggregation during folding and subunit assemblage [30,31], and provide their clearance and recycling [32,33]. The Hsps are ubiquitous antigens expressed as housekeeping proteins [34] and are up-regulated in inflamed tissues, responsible for responding to stressor agents, such as toxins, oxidative injury, inflammatory processes, and infections, leading the reestablishment of homeostasis [35–37].

Hsps, also known as stress proteins, are considered to be conserved proteins during evolution, present in all living organisms. These proteins play an essential role in molecular chaperones, interacting either with proteins tagged for degradation or with foreign polypeptide aggregation [38]. Hsps are up-regulated when cells face stressful situations [39–41], such as an infectious disease, presenting immunoregulatory activities [42], and also participate in cell survival signaling pathways [43]. Additionally, these molecules are considered natural adjuvants since they stimulate internalization by scavenger receptors and even the presentation of antigens through Major histocompatibility complex (MHC) molecules, inducing the production of chemokines, pro-inflammatory cytokines, as well as the production of nitric oxide by macrophages and dendritic cells [42,44,45].

The precise role of Hsps in Inflammatory Bowel Diseases (IBDs) is still not clear. Miao and colleagues [46] correlated the severity of the disease with elevated levels of Hsp70 in human patients with ulcerative colitis. In patients with IBD, autoantibodies were detected to Hsp60 and Hsp70, linking the disease pathogenesis to the cross-reactivity between Hsps from eukaryotic and prokaryotic organisms [47].

Recombinant *Lactococcus lactis* NCDO2118, producing Hsp65 protein, was developed by our research group [48]. This recombinant strain showed promising results when it was tested in colitis [25] and experimental autoimmune encephalomyelitis (EAE) [26] mouse models. Thus, the immunomodulatory and anti-inflammatory action of the Hsps molecules was shown.

Regarding the mechanisms associated with the beneficial effects of Hsp65, Gomes-Santos et al. [25] used the engineered *L. lactis* mentioned above, associated with the protection of the gut with increased Il10 levels in the colon and an expansion, in spleen and mesenteric lymph nodes, of $CD4^{+}Foxp3^{+}$ and $CD4^{+}LAP^{+}$ regulatory T cells, possibly in a dependent effect on Tlr2 and Il10. They also showed a reduction in pro-inflammatory cytokines and maybe the same role could be observed in mucositis since both are injuries of the intestinal tract.

Based on these promising results for heterologous protein production by *L. lactis*, our research group developed a broad-range plasmid called pExu [49] to be used as a DNA vaccine vector. In this approach, the host cells are in charge of protein production, with LAB as the delivery vehicle.

Thus, based on the beneficial action of Hsp65 protein in different diseases, and considering the protective effect of milk fermented by the *L. delbrueckii* CIDCA 133 strain in an intestinal mucositis mouse model previously reported by De Jesus et al. [17], the present study aims to investigate the therapeutic/protective and immunomodulatory effect of recombinant *L. delbrueckii* CIDCA 133 (pExu:*hsp65*) on the harmful effects of 5-FU in the intestinal epithelium.

2. Materials and Methods

2.1. Bacterial Strains and Plasmids

Escherichia coli (*E. coli*) Top10 (Invitrogen) were grown aerobically in Luria–Bertani medium (LB) (Acumedia Lansing, MI, USA) at 37 °C with vigorous shaking. *Lactobacillus delbrueckii* subsp. *Lactis* CIDCA 133, belonging to the culture collection of the Centro de Investigación y Desarrollo en Criotecnología de Alimentos (CIDCA, Facultad de Ciencias Exactas, Universidad Nacional de La Plata, Argentina), were grown in de Man, Rogosa, and Sharpe (MRS) broth (Kasvi, São José dos Pinhais, Brazil) at 37 °C for 16 h in microaerobiosis. When necessary, this medium was supplemented with 2.5 µg/mL of erythromycin (Sigma-Aldrich, St Louis, MO, USA) for the growth of recombinant strains, *L. delbrueckii* CIDCA 133 (pExu:empty), and *L. delbrueckii* CIDCA 133 (pExu:*hsp65*).

2.2. DNA Vaccine Construction: Recombinant L. delbrueckii CIDCA 133 (pExu:hsp65)

To construct the DNA vaccine, the functional pVax:hsp65 plasmid [50] was digested with 10 units of BamHI (Biolabs, England) and 20 units of NotI (Invitrogen, Carlsbad, CA, USA) restriction enzymes. A 3.3 Kb fragment corresponding to the *Mycobaterium leprae* hsp65 gene was obtained with cytomegalovirus (CMV) intron A. The same restriction enzymes were used to digest the empty pExu plasmid [49]. The insert and the digested vector were purified using a commercial kit (IllustraTM GFXTM PCR DNA, GE Healthcare, Chicago, IL, USA). The ligation was performed with T4 DNA ligase (Invitrogen, Carlsbad, CA, USA) for 16 h at 4 °C. After this time, pExu:*hsp65* construction was established by transformation into *E. coli* Top10 by electroporation (1800 V, 200 Ω resistance and 25 µF capacitance pulse in a 0.2 cm cuvette), using the Gene Pulser Xcell™ Electroporation System (Bio-Rad, Richmond, CA, USA). The transformants were plated in a solid medium supplemented with 500 µg/mL of erythromycin to select resistant colonies.

Plasmids from *E. coli* (pExu:*hsp65*) were isolated by alkaline lysis as described by Green and Sambrook [51], and this construction was confirmed by enzymatic digestion (NotI and BamHI). The gene expression was confirmed using a Chinese hamster ovarian cell line [Flp-In™-CHO (Invitrogen, Carlsbad, CA, USA)] (CRL 12023) from ATCC and Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) as described by Coelho-Rocha et al. [52]. Briefly, eukaryotic cells were transfected with 4 µg of sterile pExu:*gfp* (positive control); pExu:*hsp65*; pExu:empty [49]; or any plasmid for the negative control. The immunohistochemical reaction checked the eukaryotic cells' protein expression. Thereby, forty-eight hours post-transfection, the transfected eukaryotic cells were fixed with 4% (*w*/*v*) of paraformaldehyde (Sigma-Aldrich, St. Louis, MO, USA) in phosphate-buffered saline (PBS, 0.1 M) for 15 min and permeabilized with 0.1% (*w*/*v*) Triton ×100 (Vetec, Rio de Janeiro, Brazil) in PBS

for 10 min. The cells were incubated for one hour with monoclonal anti-Hsp65 (D17J) (Invitrogen, Carlsbad, CA, USA), diluted 1/50 in 1% bovine serum albumin (BSA; Sigma-Aldrich, St. Louis, MO, USA) at room temperature. The cells were washed with 0.1 M PBS three times and then incubated with the secondary antibody, goat anti-mouse IgG (H + L) Alexa Fluor® 488 (Life Technologies, Carlsbad, CA, USA, 4 μg/mL, diluted 1/500 in PBS/BSA 1%) for 1 h in reduced light conditions. At the same time, 4,6′-diamidino-2-phenylindole (DAPI, Invitrogen, Carlsbad, CA, USA, 2 μg/mL, diluted 1/300) was used for cell nucleus staining. Samples were mounted and the images were captured using a Zeiss LSM 510 META inverted confocal laser-scanning microscope and collected and analyzed using Zeiss LSM Image Browser software. Duplicate transfection assays were performed.

The pExu:*hsp65* functional plasmid was stabilized into *L. delbrueckii* CIDCA133 to develop the recombinant strain, *L. delbrueckii* CIDCA133 (pExu:*hsp65*). To this end, electrocompetent *L. delbrueckii* were transformed by electroporation [2500 V, 200 Ω resistance, and 25 μF capacitance pulse in a 0.2 cm cuvette, Gene Pulser Xcell™ Electroporation Systems (Bio-Rad, Richmond, CA, USA)] with 1 μg/mL of pExu:*hsp65* plasmid. The transformants were plated on MRS (Kasvi, São José dos Pinhais, Brazil) agar (1.5%, Acumedia®, Indaiatuba, Brazil) plates supplemented with 2.5 μg/mL of erythromycin for 48 h at 37 °C. Plasmids from *L. delbrueckii* CIDCA 133 (pExu:empty and pExu:*hsp65*) were isolated by glass beads (Sigma Aldrich, St. Louis, MO, USA) using Precellys 24 Homogenizer® (1 cycle, 6500 rpm, 15 s) (Bertin Technologies, Montigny Le Bretonneux, France). After that, the alkaline lysis protocol and enzymatic digestion were carried out.

2.3. Dairy Formulation

Fifty microliters of bacteria culture were inoculated into 15 mL of milk medium (12% skimmed milk (*w*/*v*) (Itambé, Belo Horizonte, Brazil), 2% glucose (*w*/*v*) (Labsynth, São Paulo, Brazil), 1.2% (*w*/*v*) yeast extract (Kasvi, São José dos Pinhais, Brazil) with 2.5 μg/mL of erythromycin) for 16 h at 37 °C without shaking to prepare the fermented milk. After that, the culture was diluted 100 times in sterilized milk medium and then administered ad libitum to the mice. To avoid bacterial decantation and clogging, this process was done every 12 h. This beverage and standard chow diet were administered ad libitum for 13 days. The colony-forming units (CFUs) (viable bacteria) administrated to the mice were analyzed by the pour plate method (MRS agar medium with erythromycin, when required), after incubation for 16 h at 37 °C. The CFUs calculated were 3.5×10^6 CFU/mL for *L. delbrueckii* (pExu:*hsp65*), and 5×10^6 for *L. delbrueckii* (pExu:empty).

2.4. Mouse Handling and Experimental Design

Conventional BALB/c male, 6 weeks old, weighing 21–24 g, were provided by the animal facility of the Centro de Bioterismo (CEBIO) of the Institute of Biological Sciences, at the Federal University of Minas Gerais (Belo Horizonte, Minas Gerais, Brazil). The animals were kept in polycarbonate open boxes under controlled conditions: temperature around 22 ± 2 °C, 12 h light/dark cycle, humidity of 55 ± 10%, water 24 h before experiments, and standard chow diet available *ad libitum*. All procedures were done in compliance with the Brazilian Society of Sciences in Laboratory Animals (SBCAL) guidelines and were approved by the Animal Experimentation Ethics Committee (CEUA-UFMG, Protocol n° 66/2019, 27 May 2019). The protocol complied with the guidelines recommended by the Institute of Laboratory Animal Resources for the care and use of laboratory animals.

Mice were randomly divided into four different experimental groups (n = 8/group): negative control (CTL); CIDCA 133 (pExu:empty), hereafter called rCIDCA 133; positive control of mucositis (MUC); and CIDCA 133 (pExu:*hsp65*), hereafter rCIDCA133:Hsp65. Animals were orally fed daily either with non-fermented milk supplemented with erythromycin (2.5 μg/mL) (CTL and MUC) or with fermented milk by recombinant strains rCIDCA 133 or rCIDCA133:Hsp65 containing erythromycin (2.5 μg/mL) over 13 days. On the 10th day, all groups except the CTL group received a single intraperitoneal (i.p) injection of 5-FU (300 mg/kg) (Fauldfluor®, Libbs, São Paulo, Brazil) to induce mucositis, following the same

protocol of our previous report [17]. The control group (CTL) received saline solution (i.p). Seventy-two hours after administration, all animals were anesthetized with a ketamine (80 mg/kg) and xylazine (16 mg/kg) mixture (Agener União, Embu-Guaçu, Brazil) and euthanized. The ileum section of the small intestine and the blood of mice were collected for analysis. Furthermore, bodyweight and milk and feed intake were assessed daily.

2.5. Intestinal Permeability Evaluation

The intestinal permeability (IP) can assess gut mucosa integrity. A 0.1 mL (18.5 MBq) sample of diethylenetriamine penta-acetic acid (DTPA) solution labeled with technetium-99m (99mTc-DTPA) was administrated by gavage to the mice on the 13th day of treatment, as previously reported [53]. Four hours later, the mice were anesthetized as previously described. Approximately 200 µL blood was collected, weighed, and the radioactivity level in the blood was determined in an automated gamma counter (PerkinElmer Wallac Wizard 1470-020 Gamma Counter, Waltham, MA, USA). A standard dosage containing the same injected amount was counted simultaneously in a separate tube, which was defined as 100% radioactivity. The results were expressed as the percentage of injected dose per gram (%ID/g) of blood: counts per min = (cpm of blood/cpm of the administered dose) $\times$ 100. where cpm represents the count of radioactivity per minute.

2.6. Bacterial Translocation Study

Bacterial translocation (BT) was evaluated using radiolabeled *E. coli*, as previously reported [54]. Briefly, a culture of *E. coli* ATCC® 10536™ was grown overnight in soybean casein medium agar. Then the grown cells were transferred to 10 mL of sterile 0.9% (*w*/*v*) NaCl solution. Two milliliters of bacterial solution (10^8 CFU/mL) were incubated with 1 mL of stannous chloride solution (580 mM, pH 7.0) at 37 °C for 10 min. After incubation, 99mTc (37.0 MBq) was added, and the mixture was kept at 37 °C for 10 min. The tubes were then centrifuged at 3000$\times$ g for 25 min, and 100 mL of either supernatant and resuspended precipitate in saline were used to measure the radioactivity using a dose calibrator (Capintec CRCw-15R Dose Calibrator; CAPINTEC, Inc., Ramsey, NJ, USA). This procedure was done in triplicate. The percent of 99mTc incorporated into the bacterial cells was determined using the following equation: % labeled bacteria = (cpm of precipitate/(cpm of precipitate + cpm of supernatant)) $\times$ 100. Then, the suspension of 99mTc—*E. coli* (10^8CFU/mL) was used for the bacterial translocation study. To this end, 0.1 mL (1.8 MBq) of 99mTc—*E. coli* were administrated by gavage to all groups on the 13th day, 4 h before euthanasia. After this time, the animals were i.p. anesthetized as described before, the blood sample was collected, and then mice were sacrificed by cervical dislocation. Following the mesenteric lymph nodes (MLNs), blood, liver, spleen, heart, kidneys, and lungs were removed, weighed, and the level of radioactivity present in each organ was measured using an automated gamma counter (PerkinElmer Wallac Wizard 1470-020 Gamma Counter; PerkinElmer, Waltham, MA, USA). The results were expressed as counts per minute (cpm)/g of tissue.

2.7. Histological, Morphological, and Goblet Cell Analyses

Histological assays were done to analyze the architecture of the intestinal mucosa. After euthanasia, the small intestine was removed, and its length was measured. Then the ileum section was washed with PBS 0.1 M, rolled up, and placed into a histological cassette and immersed in a 10% neutral buffered formalin (NBF) solution [100 mL formaldehyde 37–40%, (Synth, São Paulo, Brazil); 4 g/L HPO4Na, (Synth, São Paulo, Brazil); and 6.5 g/L H_2PO_4Na (Vetec, Rio de Janeiro, Brazil)] for 24 h. From a paraffin block containing the samples, 4 µm thick slices were cut, mounted on glass slides, and stained with hematoxylin and eosin (H&E) or periodic acid–Schiff (PAS). To measure the depth of crypts, villus height, goblet cell number, and polymorphonuclear cell infiltrate, the selected image (H&E and PAS) was captured by a BX41 optical microscope (Olympus, Tokyo, Japan) and analyzed using ImageJ 1.51j.8 software (NIH, Bethesda, MD, USA). Image acquisition was performed

with a 20× magnification objective and 20 villi and 20 crypts in random fields of each mouse, images were analyzed, and the ratio of villus height/crypt depth from the intestinal epithelium was found. The study of goblet cells was performed in PAS-stained slides, where 10 field/slides were counted. Histological examination was performed using a blind score based on a system described previously by Soares et al. [55] to obtain the histological score.

2.8. Leukocyte Count

Blood samples were collected from the axial plexus, and the total number of leucocytes was measured by an automatic hematological counter (Bio-2900 Vet, Bioeasy, EUA). Results were expressed as the number of leukocytes per µL of the sample.

2.9. Enzyme Assay: Intestinal Myeloperoxidase (MPO) and Eosinophil Peroxidase (EPO) Activity

The MPO and EPO enzyme activities properly evaluate the neutrophil and eosinophil infiltration, respectively, in the intestinal mucosa [56]. These enzymes' activities were assessed in homogenate ileum tissue described previously by De Jesus et al. [17]. Briefly, 100 mg of tissue were homogenized in 1.9 mL PBS 0.1 M (pH 7.4) using a tissue homogenizer (MA1102 model, Marconi, São Paulo, Brazil). The homogenate was centrifuged (3000× *g* for 10 min), then the pellets were subjected to hypotonic lysis (1.5 mL of 0.2% NaCl). The osmolarity was restored with 1.5 mL of NaCl 1.6% solution supplemented with 5% glucose. Then, samples were centrifuged (3000× *g* for 10 min), and the pellet was resuspended in 0.5% hexadecyltrimethylammonium bromide (HTAB, Sigma-Aldrich, St. Louis, MO, USA) in phosphate buffer. The tissue suspension was homogenized, freeze-thawed three times in liquid nitrogen, and centrifuged for 15 min at 3000× *g*. The resulting supernatant was used in the colorimetric assay to measure the EPO and MPO activities. For EPO assessment, 75 µL of supernatant were added to 75 µL of 1.5 mM O-phenylenediamine (OPD, Sigma-Aldrich, St. Louis, MO, USA), diluted in 0.075 mM Tris–HCl and 6.6 mM H_2O_2 (Synth) and incubated at 37 °C for 30 min. For MPO quantification, 25 µL of supernatant were added to 25 µL of 1.6 mM 3,3,5,5′-Tetramethylbenzidine (TMB, Sigma-Aldrich, St. Louis, MO, USA) in dimethyl sulfoxide (DMSO, Sigma-Aldrich, St. Louis, MO, USA). After the addition of 100 µL 0.5 mM H_2O_2, the solution was incubated at 37 °C for 5 min. Fifty microliters of 1 M H_2SO_4 were added to stop both reactions. Absorbance was measured at 492 nm (EPO) and 450 nm (MPO) on a microplate spectrophotometer (Bio-Rad 450 model, Bio-Rad Laboratories, Hercules, CA, USA). Results were expressed as MPO or EPO arbitrary units (based on absorbance)/100 mg of tissue.

2.10. Intestinal Secretory IgA (sIgA)

An enzyme-linked immunosorbent assay (ELISA) was used to measure the sIgA levels in the small intestine, as described by Martins et al. [57]. To this end, the bowel contents were removed, weighed, and flushed out (PBS 0.1 M (pH 7.2) supplemented with aprotinin (1 µM, Sigma-Aldrich, St. Louis, MO, USA), leupeptin (25 µM, Sigma-Aldrich, St. Louis, MO, USA), pepstatin (1 µM, Sigma-Aldrich, St. Louis, MO, USA), and phenylmethanesulfonyl fluoride (PMSF, 1 mM, Sigma-Aldrich, St. Louis, MO, USA)). Samples were centrifuged (2000 rpm for 30 min at 4 °C), and the supernatant was collected to find the immunoglobulin dosage. Microtiter plates (Nunc-Immuno Plates, MaxiSorp) were coated with goat anti-mouse IgA antibody (M-8769, Sigma-Aldrich, St. Louis, MO, USA) in coat buffer (1 M Na_2CO_3; 0.1 M $NaHCO_3$; pH 9.6) for 18 h at 4 °C, washed (0.1 M PBS + 0.05% Tween 20), and blocked (1% albumin in PBS-Tween 20). Afterward, pre-diluted intestinal fluids (1:1000–0.1 M PBS-Tween 20) were incubated for 1 h. The plates were then washed, and a biotin-conjugated anti-mouse IgA antibody (dil. 1:1000, A4789, Sigma-Aldrich, St. Louis, MO, USA) was added and incubated for 1 h. Finally, 100 µL/well of OPD (1 mg/mL) and 0.04% H_2O_2 substrates were added and set for 10 min. The reaction was stopped with 20 µL/well of 1 M H_2SO_4 (Sigma-Aldrich, St. Louis, MO, USA). The absorbance was determined at 492 nm using a microplate reader (Bio-Rad model 450, Bio-Rad Laboratories, Hercules, CA, USA). The immunoglobulin concentration was

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determined using a purified mouse IgA standard (M-8769, Sigma-Aldrich, St. Louis, MO, USA). The concentration of sIgA was expressed in μg/mL of intestinal content.

2.11. Transmission Electron Microscopy (MET)

An ileum section (1.5 cm) was fixed in modified Karnovsky solution [(2.5% glutaraldehyde, paraformaldehyde 2.5%, in PBS 0.1 M (pH = 7.2)] at 4 °C for 24 h. Then the solution was removed and substituted with PBS 0.1 M (pH = 7.2), the samples were post-fixed/block stained in 2% osmium tetroxide (OsO4) in PBS 0.1 M for 2 h at room temperature, and 2% uranyl acetate in H_2O at 4 °C was added and incubated overnight. The fixed tissues were desiderated with ethanol (PA, LabSynth, São Paulo, Brazil) and acetone (PA, LabSynth, São Paulo, Brazil) and embedded in epoxy resin. Semi-thin cuts were done (approximately 300 nm) using a glass razor. The specific sections were chosen in these samples, and ultra-thin cuts (approximately 60 nm) using diamond razors were performed. These sections were placed on a Cu screen (300 mesh) contrasted with lead citrate stains. The images were examined under transmission electron microscopy (TEM; Tecnai G2-1, SpiritBiotwin EIF, 120 kV).

2.12. RNA Extraction and Real-Time RT-qPCR of Ileum Section

Total RNA was extracted from 30 mg of ileum tissue, previously stored in 500 μL of RNA later solution (Invitrogen, Carlsbad, CA, USA) to preserve the samples, using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) in compliance with the manufacturer's guidelines. The RNA concentrations were determined spectrophotometrically using a NanoDrop 2000 (Thermo Scientific, Waltham, MA, USA), considering absorbance ratios of 280/260 and 260/230 nm. The quality of RNA samples was evaluated in agarose electrophoresis gel (1.5%). The extracted RNA was treated for 15 min at room temperature with DNAse I (Invitrogen, Carlsbad, CA, USA). After that, the enzyme was deactivated (10 min 65 °C with 25 mM EDTA). One microgram of total RNA was reverse transcribed using MultiScrib reverse transcriptase (Thermo Fisher, Waltham, MA, USA) in compliance with its guidelines, forming complementary deoxyribonucleic acid (cDNA) using the following parameters: 25 °C for 10 min, 37 °C for 120 min, 85 °C for 5 min.

Quantitative reverse transcription PCR (RT-qPCR) was performed using Applied Biosystems Power SYBR Green PCR master mix (Thermo Fisher, Waltham, MA, USA) and gene-specific primers for Tnf, Il1b, Il6, Il12, Il10, Muc2, claudin 1, 2, and 5, junctional adhesion molecule 1 (F11r), zonulin, occludin, Tlr2, Tlr4, nitric oxide synthase 2 (Nos2), and Myd88 (Table 1). Transcripts were all normalized using Actb and Gapdh [58] housekeeping genes. The experimental approach was optimized by adjusting the primers' concentrations (5, 10, and 15 pmol) to test for optimal specificity and efficiency. The purity of PCR products was verified by melting curves and gel electrophoresis. The PCR cycle parameters were as follows: initial denaturation at 95 °C for 10 min, 95 °C for 15 s, annealing/extension at 60 °C for 1 min, 40 cycles, followed by a dissociation stage for recording the melting curve. Results were shown graphically as fold changes in gene expression, using the means and standard deviations of target gene expression by Hellemans et al. [59]. Data were analyzed according to the relative expression using the $2^{-\Delta\Delta CT}$ method. Data are representative of two independent experiments.

2.13. Statistical Analysis

The Shapiro–Wilk test assessed data normality. Normal data (body weight loss, small intestine length, sIgA levels, MPO activity, leukocyte count, intestinal permeability, bacterial translocation, villus height to crypt depth ratio, histological score, number of goblet cells, RT-qPCR) were evaluated by analysis of variance (ANOVA) followed by the Bonferroni post hoc test (parametric distribution). Non-normal data (non-parametric distribution) (food and milk intake, EPO activity) were evaluated by the Kruskal–Wallis test followed by Dunn's post hoc test. A Mann–Whitney test was performed to compare

food and milk intake before and after mucositis induction. All data were analyzed using GraphPad Prism 7.0 software, and $p < 0.05$ was considered statistically significant.

Table 1. Primers used in qPCR.

Gene	Foward Primer	Reverse Primer	Reference
Actb	GCTGAGAGGGAAATCGTGCGTG	CCAGGGAGGAAGAGGATGCGG	[60]
Gapdh	TCACCACCATGGAGAAGGC	GCTAAGCAGTTGGTGGTGCA	[58]
Il6	GAGGATACCACTCCCAACAGACC	AAGTGCATCATCGTTGTTCATACA	[58]
Il10	GGTTGCCAAGCCTTATCGGA	ACCTGCTCCACTGCCTTGCT	[58]
Il12p40	GGAAGCACGGCAGCAGAATA	AACTTGAGGGAGAAGTAGGAATGG	[58]
Tnf	ACGTGGAACTGGCAGAAGAG	CTCCTCCACTTGGTGGTTTG	[61]
Il1b	CTCCATGAGCTTTGTACAAGG	TGCTGATGTACCAGTTGGGG	[61]
Muc2	GATGGCACCTACCTCGTTGT	GTCCTGGCACTTGTTGGAAT	[60]
Myd88	ATCGCTGTTCTTGAACCCTCG	CTCACGGTCTAACAAGGCCAG	[62]
Tlr2	ACAATAGAGGGAGACGCCTTT	AGTGTCTGGTAAGGATTTCCCAT	[62]
Tlr4	ATGGCATGGCTTACACCACC	GAGGCCATTTTTGTCTCCACA	[62]
Nos2	CAGCTGGGCTGTACAAACCTT	CATTGGAAGTGAAGCGTTTCG	[58]
Cldn1	TCCTTGCTGAATCTGAACA	AGCCATCCACATCTTCTG	[60]
Cldn2	GTCATCGCCCATCAGAAGAT	ACTGTTGGACAGGGAACCAG	[60]
Cldn5	GCTCTCAGAGTCCGTTGACC	CTGCCCTTTCAGGTTAGCAG	[60]
Occludin	ACTCCTCCAATGGACAAGTG	CCCCACCTGTCGTGTAGTCT	[60]
Zonulin	CCACCTCTGTCCAGCTCTTC	CACCGGAGTGATGGTTTTCT	[60]
F11r	CACCTTCTCATCCAGTGGCATC	CTCCACAGCATCCATGTGTGC	[60]

3. Results

3.1. Eukaryotic Cells Can Express Hsp65 Protein

Enzymatic digestion of the new construction, pExu:*hsp65*, confirmed the successful cloning of the Hsp65 sequence in the pExu vector. The immunohistochemical images revealed a precise localization of Hsp protein expression in the cytoplasm of transfected eukaryotic cells with the pExu:*hsp65* plasmid (Figure 1). Thus, the confocal assay confirmed plasmid pExu:*hsp65* functionality. The construction of recombinant strain *L. delbrueckii* CIDCA 133 (pExu:*hsp65*) was confirmed by plating on an MRS/erythromycin plate and plasmid extraction.

3.2. rCIDCA 133:Hsp65 Prevented Small Intestine Shortening and Decrease in Weight Loss

Intestinal shortening was observed in inflamed animals, the MUC group (~46 $\pm$ 1.80 cm), as expected. Statistical difference was observed ($p < 0.001$) when the MUC group was compared with non-treated animals (CTL group) (~54 $\pm$ 1.34 cm). It was possible to observe that treatments with either recombinant strains (rCIDCA 133 and rCIDCA 133:Hsp65) were able to attenuate the small intestine shortening (~50 $\pm$ 0.93 and ~52.5 $\pm$ 1.34 cm, respectively) ($p < 0.001$). However, intestinal shortening prevention was significantly stronger in animals that received the rCIDCA 133:Hsp65 strain, reaching similar levels to the negative control group (Figure 2A).

The time course of the mice's weight was another parameter that was evaluated. The bodyweight loss of mice of the rCIDCA 133:Hsp65 group was significantly lower

(approximately 4%) than those in the MUC group (about 5.5%) ($p < 0.01$) (Figure 2B). No mortality was observed during the experiment.

Total milk and food intake were similar in all analyzed groups before the induction of mucositis (4 mL and 3 g/day/animal, respectively). There were no significant statistical differences between the experimental groups. After induction of intestinal mucositis, the MUC group showed a reduction in food and milk intake (1.063 ± 0.76 g and 1.208 ± 0.07 mL) when compared with the negative control (2.969 ± 0.25 g and 3.531 ± 0.77 mL) ($p < 0.01$). Nevertheless, the administration of rCIDCA 133 (1.469 ± 0.70 g and 2.094 ± 0.87 mL) and rCIDCA 133:Hsp65 (1.531 ± 0.55 g and 2.750 ± 0.90 mL) treatments was not able to modify these parameters (Figure 2C,D).

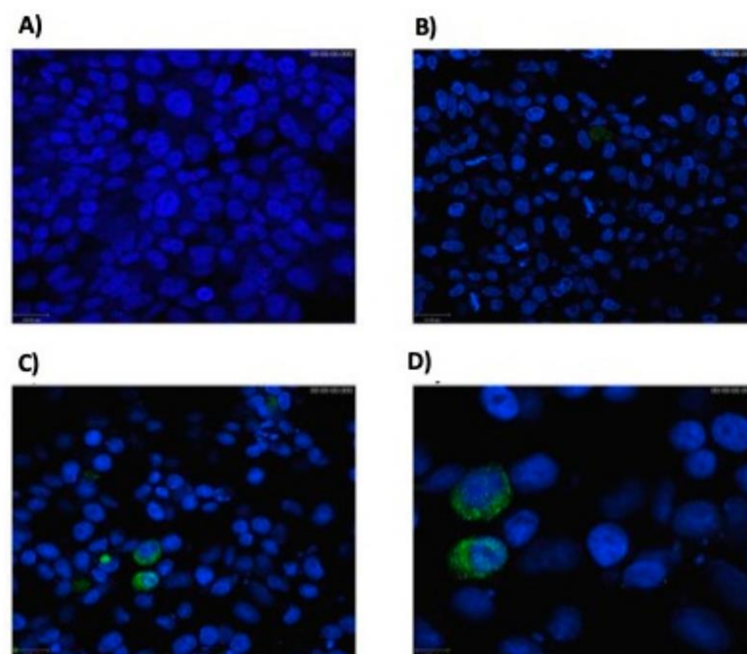

Figure 1. Hsp65 expression in transfected eukaryotic cells. Confocal microscopy: (**A**) negative control: non-transfected Chinese Hamster Ovarian cell line (CHO) cells; (**B**) negative control: non-transfected eukaryotic cells labeled with primary (Mab anti-Hsp65) and secondary (goat anti-mouse IgG (H + L)) antibodies labeled with Alexa 488. (**C**,**D**) CHO cells labeled with primary (Mb_HSP65) and secondary (goat anti-mouse IgG (H + L)) antibodies. In green, the Hsp65 protein is expressed in the cytoplasm of eukaryotic cells. 2D images (**A**–**D**) were acquired in both depths (z-stack) using a Zeiss LSM 510 META inverted confocal laser 1358 scanning microscope with 40× or 60× objective.

3.3. rCIDCA 133:Hsp65 Treatment Reduced Ileum Inflammatory Infiltrate and Increased sIgA Levels

The activity of myeloperoxidase (MPO) and eosinophil peroxidase (EPO) was measured in ileum cell lysates to verify whether treatment with rCIDCA 133:Hsp65 could reduce the infiltration of inflammatory cells, like neutrophils and eosinophils, in the intestinal mucosal layer. As exposed in Figure 3A,B, the MUC group (positive control) significantly presented an increase in MPO and EPO enzymes activities, thus showing an increase in neutrophil and eosinophil recruitment (2.453 ± 0.17 U/mg for MPO; 1.561 ± 0.07 U/mg for EPO) ($p < 0.001$) due to the inflammatory processes. Animals treated with rCIDCA

133:Hsp65 showed a reduction in the activity of both enzymes (1.623 ± 0.22 U/mg for MPO, ($p < 0.001$); 0.434 ± 0.04 U/mg for EPO, $p < 0.01$).

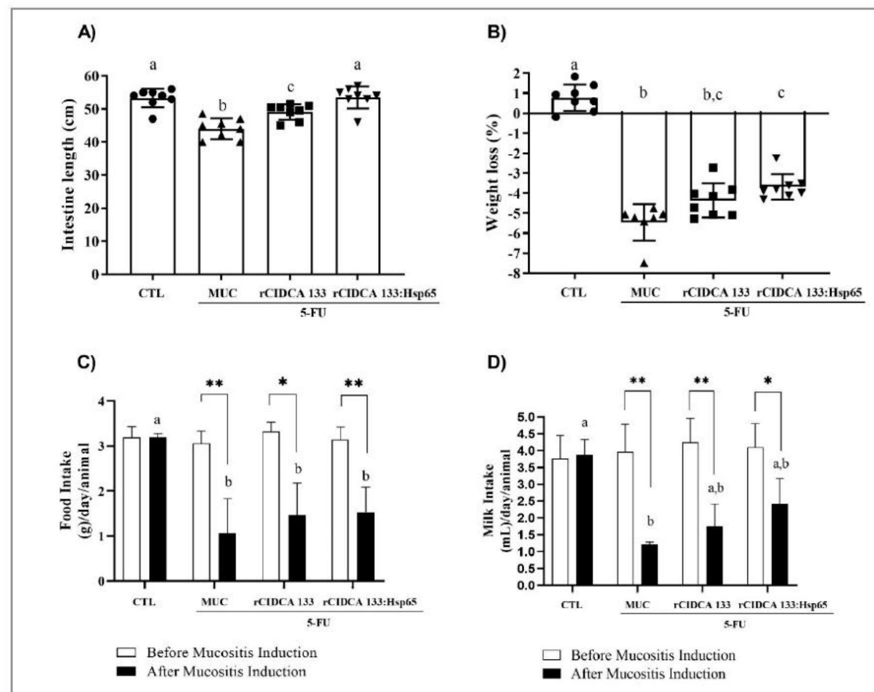

Figure 2. Small intestine length, bodyweight, food and milk intake analysis: (**A**) intestine length, (**B**) bodyweight variation (**C**) food intake, and (**D**) milk intake. (**A**,**B**) ANOVA followed by the Bonferroni post hoc test and (**C**,**D**) Kruskal–Wallis test followed by Dunn's post hoc test. Different letters (a, b, and c) indicate statistically significant differences between groups ($p < 0.05$). The symbols (*) (**) show a statistically significant difference ($p < 0.05$) ($p < 0.01$), respectively, between rCIDCA 133, MUC (positive control), and rCIDCA 133:Hsp65 groups before and after mucositis induction by an unpaired Student's *t*-test (**C**,**D**). Geometric symbols show to the number of animals evaluated in each experimental group. • refers to the animals of CTL group, ▲ refers to the animals of MUC group, ■ refers to the animals of rCIDCA 133 group and ▼ refers to the animals of rCIDCA 133:Hsp65 group.

A significant reduction in the number of blood leukocytes/µL was observed after 5-FU administration in the MUC group (0.775 ± 0.11 cells × 103/µL) when compared to the negative control (CTL) (3.638 ± 0.46 cells × 103/µL) ($p < 0.001$), as expected. However, the reduction of total leukocyte blood rate induced by chemotherapy was minimized after treatment with either rCIDCA 133 (1.440 ± 0.30 cells × 103/µL) ($p < 0.01$) or rCIDCA 133:Hsp65 (1.814 ± 0.60 cells × 103/µL) ($p < 0.001$). No statistical difference was observed between treatments with both recombinant strains (Figure 3C).

Levels of secretory IgA (sIgA) were also investigated. The results showed that the sIgA levels in the intestinal fluid of the MUC group were significantly increased (3517.2 ± 131.8 µg/mL), and a statistical difference was observed when this group was compared with the negative control (2199.4 ± 330.4 µg/mL) ($p < 0.001$). The treatment with rCIDCA 133 showed significantly reduced levels of sIgA (2905.8 ± 242.6 µg/mL); ($p < 0.001$) in the intestinal fluid of the animals that had received chemotherapy. However, mice treated with rCIDCA 133:Hsp65 were able to increase immunoglobulin levels (3988.2 ± 100.5 µg/mL) significantly, higher than in the MUC group ($p < 0.01$) (Figure 3D).

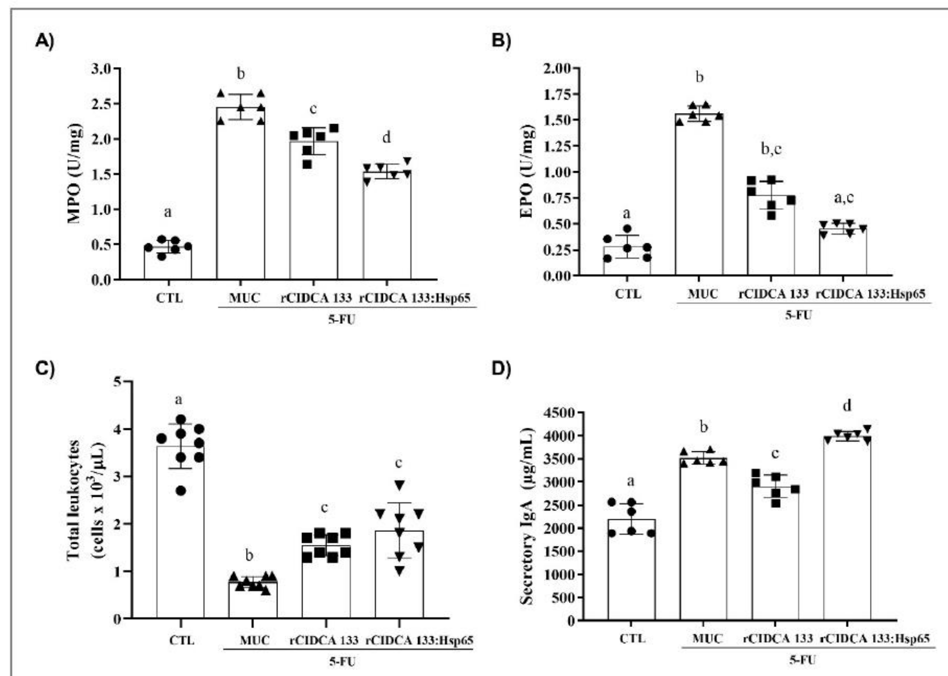

Figure 3. Effect of rCIDCA 133:Hsp65 on inflammatory parameters and epithelial barrier of animals inflamed with 5-fluorouracil (5-FU): (**A**) Intestinal Myeloperoxidase (MPO) and (**B**) Eosinophil Peroxidase (EPO) activity, (**C**) intestinal permeability (%ID/g of 99mTc-DTPA), and (**D**) levels of Intestinal Secretory IgA (sIgA) (µg/mL). Mice received intraperitoneal 5-FU (300 mg/kg) (MUC, rCIDCA 133, and rCIDCA 133:Hsp65 groups) or 0.9% saline solution (CTL group) and were treated with non-fermented milk or recombinant *L. delbrueckii* CIDCA 133-fermented milk ($n = 6$ animals per group). Different letters indicate statistically significant differences ($p < 0.05$) by ANOVA followed by the Bonferroni post hoc test (**A**,**C**,**D**) and Kruskal–Wallis test followed by Dunn's post hoc test (**B**). Geometric symbols show to the number of animals evaluated in each experimental group. • refers to the animals of CTL group, ▲ refers to the animals of MUC group, ■ refers to the animals of rCIDCA 133 group and ▼ refers to the animals of rCIDCA 133:Hsp65 group.

3.4. Reduction in Intestinal Permeability after Treatment with rCIDCA 133:Hsp65

Alteration of mucosal permeability is another side effect of 5-FU treatment. The intestinal permeability was evaluated by measuring radioactivity diffusion in the blood following oral administration of 99mTc-DTPA 72 h after the 5-FU injection. Intestinal permeability was significantly increased in the MUC group (0.258 ± 0.024% ID/g). On the other hand, the CTL group showed lower permeability (0.0187 ± 0.008% ID/g) ($p < 0.001$), as expected. Interestingly, the oral administration with rCIDCA 133:Hsp65 promoted a significant reduction in this parameter (0.0363 ± 0.015% ID/g), reaching similar levels to the negative control (Figure 4A). The same effect was observed in mice treated with the rCIDCA 133 strain. However, the levels of radioactivity were higher than in the rCIDCA 133:Hsp65 group (0.0663 ± 0.026% ID/g and 0.0363 ± 0.015% ID/g, respectively).

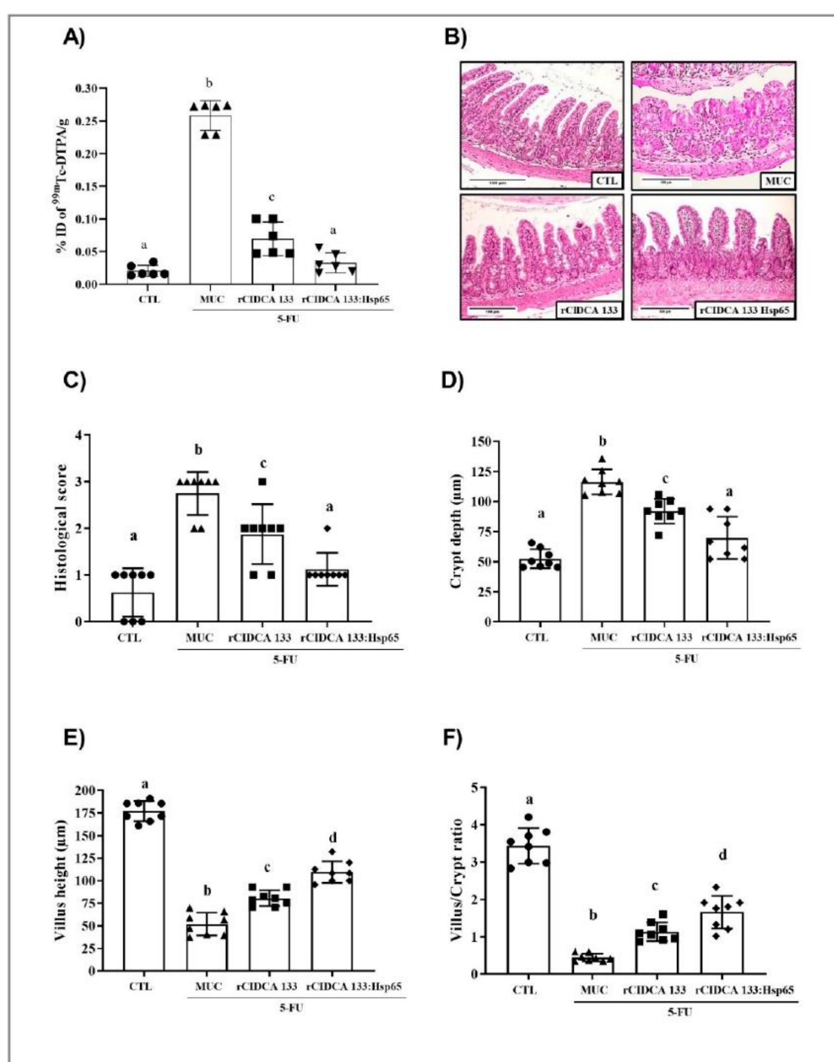

Figure 4. Histopathological and morphometric analysis, intestinal permeability, and evaluation of the relative gene expression of tight junction proteins: (**A**) intestinal permeability (%ID/g of 99mTc-DTPA), (**B**) mucosal histopathology, (**C**) histopathological scores of the ileum of animals (objective: ×20, scale 100 µm), (**D**) morphometrical analysis of crypt depth, (**E**) villus height, (**F**) villus height to crypt depth ratio. Mice received intraperitoneal 5-FU (300 mg/kg) (MUC, rCIDCA 133, and rCIDCA 133:Hsp65 groups) or saline solution (CTL group). They were treated with non-fermented milk supplemented with erythromycin 2.5% (CTL and MUC) or recombinant *L. delbrueckii* CIDCA 133 (rCIDCA133 and rCIDCA133:Hsp65)-fermented milk supplemented with erythromycin 2.5% (n = 8 animals per group). Different letters (a–d) indicate statistically significant differences ($p < 0.05$) by ANOVA followed by the Bonferroni post hoc test. Geometric symbols show to the number of animals evaluated in each experimental group. • refers to the animals of CTL group, ▲ refers to the animals of MUC group, ■ refers to the animals of rCIDCA 133 group and ▼ refers to the animals of rCIDCA 133:Hsp65 group.

3.5. *rCIDCA 133:Hsp65 Reduced Both Mucosal Damage and Degeneration of Goblet Cells in 5-FU-Induced Intestinal Mucositis*

Mice inflamed by 5-FU (MUC group) demonstrated significant alteration in mucosal integrity, such as villus shortening, increased crypt depth, and intense inflammatory cell infiltrate in villi and in lamina propria and submucosa, with ulceration, edema, and vacuolization. Figure 4B shows these findings, which were also confirmed by the scoring system (score 3) [55] (Figure 4C). The rCIDCA 133:Hsp65 and rCIDCA 133 treatments were able to alleviate 5-FU-induced intestinal mucosal damage. The histology assays showed a decreased inflammatory infiltrate, and in the crypt depth and villus height, it was significantly restored (Figure 4D,E). Consequently, the villus height to crypt depth ratio was increased, and histological scores were significantly decreased (1 and 2, respectively) (Figure 4C,F), revealing that both recombinant strains can improve the mucosal preservation in the inflamed mice. However, the treatment with the rCIDCA 133:Hsp65 strain was able to potentiate the decrease in inflammation. A significant decline in goblet cells (12.07± 5.16 cell/field) was observed in the MUC group, as expected. Both recombinant strains studied were able to improve the loss of goblet cell numbers. This protection was significantly higher (36.07 ± 6.64 cell/field) in mice orally treated with rCIDCA 133:Hsp65 than in those which received the rCIDCA 133 strain (26.33 ± 1.98 cell/field) ($p < 0.01$) (Figure 5A,B). On the other hand, the down-regulation of the gene expression of Muc2 mucin (0.17 ± 0.05) after 5-FU administration (MUC group) was observed, with this expression being significantly different from the control groups (Muc2: CTL = 1.10 ± 0.14; rCIDCA 133 = 0.46 ± 0.02) ($p < 0.05$). Animals treated with CIDCA 133:Hsp65 showed an up-regulated expression of the Muc2 gene (0.76 ± 0.17) ($p < 0.05$, Figure 5C).

3.6. *Tight Junctions Exhibit Up-Regulation after Oral Administration with Recombinant Strains*

The results of the gene expression of tight junction proteins, such as Cldn1 (0.15 ± 0.02), Cldn2 (0.06 ± 0.07), Cldn5 (0.26 ± 0.09), occludin (0.14 ± 0.06), zonulin (0.35 ± 0.07), and F11r (0.25 ± 0.14), showed an important gene suppression in ileum tissue in the MUC group, as expected. Both recombinant strains studied, rCIDCA 133 and rCIDCA 133:Hsp65, were able to up-regulate the gene expression of Cldn1 (0.68 ± 0.15; 0.36 ± 0.10) Cldn2 (0.32 ± 0.11; 0.67 ± 0.14), occludin (0.40 ± 0.07; 0.43 ± 0.13), zonulin (0.65 ± 0.16; 0.70 ± 0.09), and F11r (0.71 ± 0.12; 1.07 ± 0.08) ($p < 005$). Animal treatment with rCIDCA 133:Hsp65 strains showed up-regulation in the gene expression of Cldn1 and 2, and F11r (Figure 6A–F).

The ultrastructural examination of ileum mucosa displayed a reduction in the number of microvilli and a greater distance between them. It was possible to observe a higher vacuole number, deficiency of occlusion junctions, and ample open space in adherent junctions and desmosomes compared with control mice. The ultrastructural analyses for either recombinant treatment corroborate the findings of relative gene expression showing, at least in part, that these parameters were ameliorated in animals that received both recombinant strains (Figure 6G).

3.7. *rCIDCA 133:Hsp65 Reduces Gene Expression of Pro-Inflammatory Molecules and Upregulates the IL10 Expression in Ileum of Inflamed Animals*

The qPCR results reveal that the relative mRNA expression of Tnf (7.28 ± 0.42), Il1b (2.3 ± 0.49), Il6 (1.47 ± 0.34), and Il12 (3.01 ± 0.52) were up-regulated in animals from the MUC group, contrary to those exhibited in the CTL group: Tnf (1.0 ± 0.74) ($p < 0.0001$), Il1b (1.0 ± 0.14) ($p < 0.0001$), Il6 (1.0 ± 0.10) ($p < 0.05$), Il12 (1.0 ± 0.17) ($p < 0.0001$). Oral treatment with either rCIDCA 133 and rCIDCA 133:Hsp65 was able to suppress Tnf (1.94 ± 0.86; 1.48 ± 0.13, respectively) ($p < 0.0001$), Il1b (1.04 ± 0.26; 0.40 ± 0.11, respectively) ($p < 0.0001$), Il6 (1.05 ± 0.18; 0.22 ± 0.07, respectively) ($p < 0.05$; $p < 0.0001$), and also Il12 (1.07 ± 0.27; 1.56 ± 0.75, respectively) ($p < 0.0001$; $p < 0.001$). It was observed that levels of the anti-inflammatory Il10 cytokine were reduced (0.08 ± 0.03) ($p < 0.05$) after the administration of 5-FU (MUC group) in relation to the CTL group (1.0 ± 0.27) ($p < 0.0001$). Both recombinant strains studied, rCIDCA 133 and rCIDCA 133:Hsp65,

were able to up-regulate Il10 (0.71 ± 0.16; 0.77 ± 0.20, respectively) ($p < 0.001$) expression (Figure 7A–E).

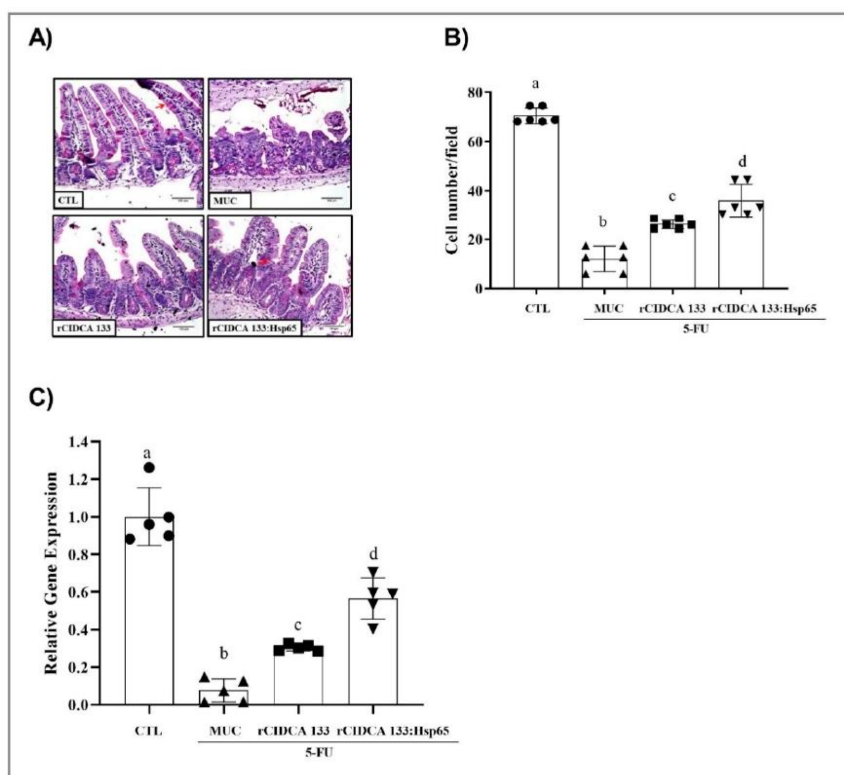

Figure 5. Assessment of goblet cell integrity and evaluation of the relative gene expression of Muc2 mucin: (**A**) representative photomicrographs from ileum section stained with Periodic Acid–Schiff (PAS), the arrows show the goblet cells (objective: ×20, scale 100 µm), (**B**) number of goblet cells/field obtained for experimental groups, and (**C**) level of relative mRNA expression of Muc2 mucin. Mice received intraperitoneal 5-FU (300 mg/kg) (MUC, rCIDCA 133, and rCIDCA 133:Hsp65 groups) or saline solution (CTL group). They were treated with non-fermented milk supplemented with erythromycin 2,5% (CTL and MUC) or recombinant *L. delbrueckii* CIDCA 133 (rCIDCA133 and rCIDCA133:Hsp65)-fermented milk supplemented with erythromycin 2,5% ($n = 6$ animals per group). Different letters (a–d) indicate statistically significant differences ($p < 0.05$) by ANOVA followed by the Bonferroni post hoc test (**B**,**C**). Geometric symbols show to the number of animals evaluated in each experimental group. • refers to the animals of CTL group, ▲ refers to the animals of MUC group, ■ refers to the animals of rCIDCA 133 group and ▼ refers to the animals of rCIDCA 133:Hsp65 group.

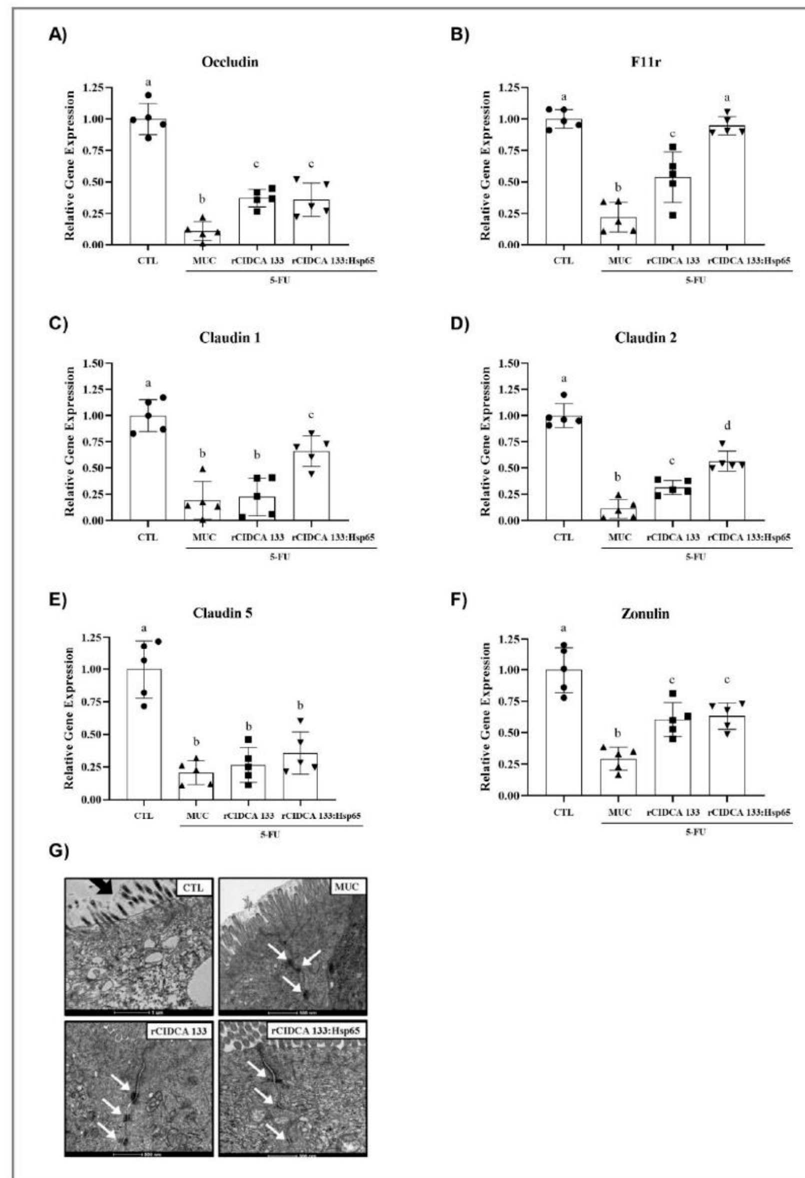

Figure 6. Level of relative mRNA expression of (**A–F**) tight junction proteins (occludin, F11r, claudin 1, claudin 2, claudin 5, and zonulin) determined by qPCR from animal ileum. Different letters (a–d) indicate statistically significant differences ($p < 0.05$) by ANOVA followed by the Bonferroni post hoc test (**A–F**). Geometric symbols show to the number of animals evaluated in each experimental group. • refers to the animals of CTL group, ▲ refers to the animals of MUC group, ■ refers to the animals of rCIDCA 133 group and ▼ refers to the animals of rCIDCA 133:Hsp65 group. (**G**) Evaluation of cell junctions by transmission electron microscopy: dark arrow highlights the reduction in the number and length of microvilli of the MUC group cells compared to the other groups. The white arrows highlight desmosomes' presence at the lateral junctions between the epithelial cells in the CTL (scale = 1 μm), MUC, rCIDCA133, and rCIDCA 133:Hsp65 (scale = 500 nm) groups.

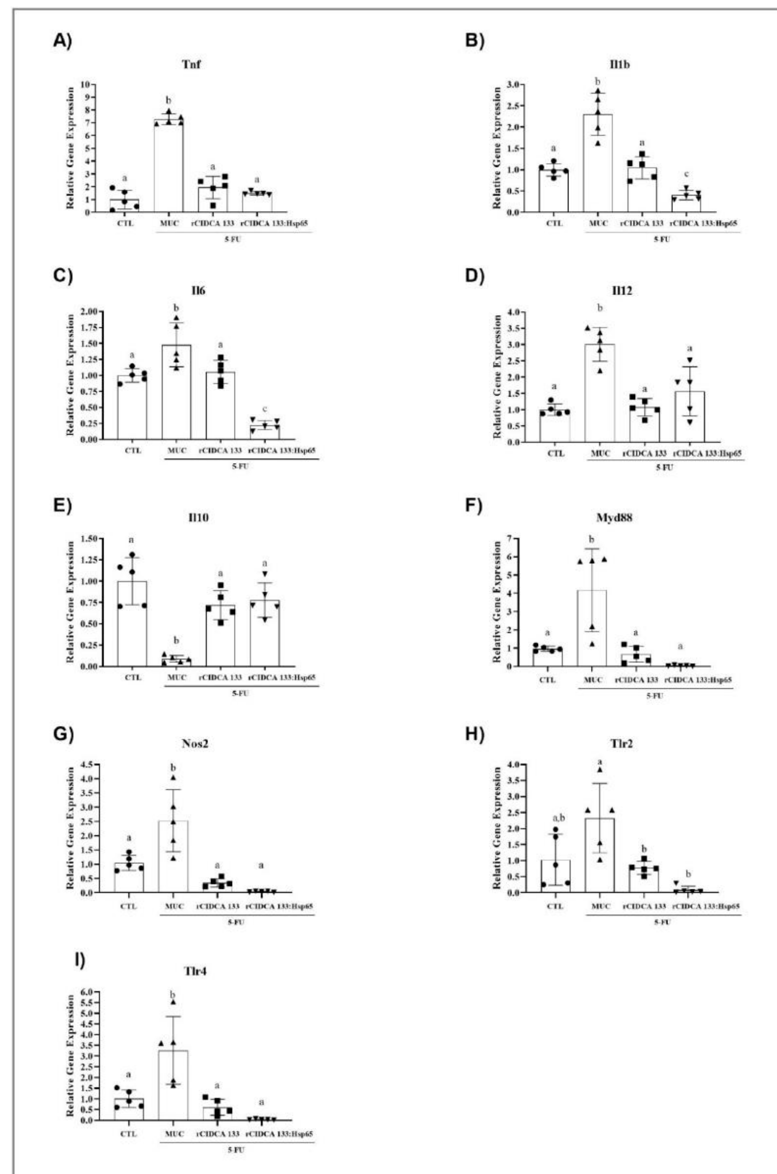

Figure 7. Relative gene expression of (**A–I**) Tnf, Il1b, Il6, Il12, Il10, Myd88, Nos2, Toll-like receptors 2 and 4. Mice received intraperitoneal 5-FU (300 mg/kg) (MUC, rCIDCA 133, and rCIDCA 133:Hsp65 groups) or saline solution (CTL group). They were treated with non-fermented milk supplemented with erythromycin 2.5% (CTL and MUC) or recombinant *L. delbrueckii* CIDCA 133 (rCIDCA133 and rCIDCA133:Hsp65)-fermented milk supplemented with erythromycin 2.5% (n = 5 animals per group). Different letters indicate statistically significant differences ($p < 0.05$) by ANOVA followed by the Bonferroni post hoc test. Geometric symbols show to the number of animals evaluated in each experimental group. • refers to the animals of CTL group, ▲ refers to the animals of MUC group, ■ refers to the animals of rCIDCA 133 group and ▼ refers to the animals of rCIDCA 133:Hsp65 group.

3.8. Treatment with Recombinant Strains of Lactobacillus CIDCA 133 Reduces MYD88, NOS2, TLR2, and TLR4 Gene Expression

The MUC group showed a significant increase in mRNA expression of Myd88 (4.16 $\pm$ 2.26), Nos2 (2.52 $\pm$ 1.090), Tlr2 (2.32 $\pm$ 1.08), and Tlr4 (3.26 $\pm$ 1.58), when compared with the CTL group: Myd88 (1.00 $\pm$ 0.14) ($p < 0.001$), Nos2 (1.0 $\pm$ 0.26) ($p < 0.001$), Tlr2 (1.0 $\pm$ 0.79) ($p < 0.01$), and Tlr4 (1.0 $\pm$ 0.40) ($p < 0.001$). The treatment with the recombinant strains (rCIDCA 133 and rCIDCA 133:Hsp65) resulted in decreased Myd88 (0.65 $\pm$ 0.43; 0.03 $\pm$ 0.02) ($p < 0.001$; $p < 0.0001$), Nos2 (0.34 $\pm$ 0.14; 0.13 $\pm$ 0.02) ($p < 0.0001$), Tlr2 (0.77 $\pm$ 0.20; 0.08 $\pm$ 0.11) ($p < 0.05$; $p < 0.0001$), and Tlr4 (0.60 $\pm$ 0.37; 0.03 $\pm$ 0.01) ($p < 0.0001$) gene expression. There were no statistical differences when these two recombinant groups were compared (Figure 7F–I).

3.9. rCIDCA 133:Hsp65 Was Not Able to Reduce the Bacterial Translocation

Bacterial translocation was evaluated 72 h after the induction of mucositis (Figure 8). Physiological levels of radiolabeled bacteria were detected in all organs, and blood was analyzed from the control group (CTL). This pattern was maintained in animals from the rCIDCA 133 group, except for liver and mesenteric lymph nodes, which were significantly different ($p < 0.05$). Increased amounts of 99mTc–*E. coli* in the blood and all organs were observed in the 5-FU group, as expected. On the other hand, when organs of the rCIDCA: Hsp65 group were analyzed, it was possible to observe a high increment in radiolabeled bacteria. Only heart and kidney showed bacterial translocation similar to physiological levels ($p < 0.05$).

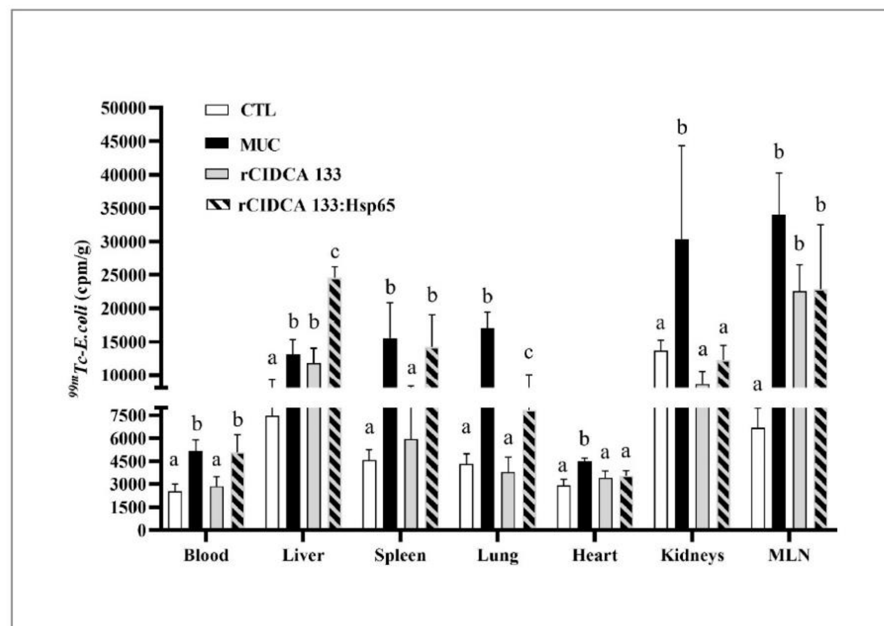

Figure 8. Biodistribution of 99mTc-*Escherichia coli* in animals with intestinal mucositis and treated with rCIDCA 133:Hsp65. cpm, counts per minute; MLN, mesenteric lymph node. Different letters indicate statistically significant differences ($p < 0.05$) by ANOVA followed by the Bonferroni post hoc test.

4. Discussion

The Hsp65 protein has been intensely studied in different experimental models of inflammation. Studies were performed in a murine model of allergic airway inflammation and hyperresponsiveness to ovalbumin (OVA) (mice sensitized with OVA by i.p. injection and then challenged with OVA by inhalation) and reported that the intramuscular administration of Hsp65 from *M. leprae* before sensitization and challenge was able to prevent the development of these diseases. They reported reduced production of Il4 and Il5. They reported increased Il10 and interferon-gamma (Ifng) in bronchoalveolar lavage fluid [63], with this attenuation effect being caused by Hsp65, acting on the modulation of dendritic cell function, as well as $CD4^+$ Th1 cytokine production [64]. Other reports using oral pretreatment with recombinant *L. lactis*, which produces and secretes the Hsp65 protein (*L. lactis* (XIES:hsp65)) [48], showed promissory results: (i) an immunoregulatory effect exhibited by the reduction of pro-inflammatory cytokines (Ifng, Il6 and Tnf), as well as an increase in Il10 in colonic tissues and the expansion of $CD4^+Foxp3^+$ and $CD4^+LAP^+$ regulatory T cells (Treg) in spleen and mesenteric lymph nodes, were able to prevent the Dextran Sulfate Sodium (DSS)-induced colitis in C57BL/6 mice [25] and (ii) prevention of the development of experimental autoimmune encephalomyelitis (EAE) in C57BL/6 mice by reduced inflammatory cell infiltrate and the absence of injury signs in the spinal cord with reduced Il17 and increased levels of Il10 in mesenteric lymph nodes and spleen cell cultures, and also an increase in natural and inducible regulatory T (Treg) cells [26]. Thus, all these findings indicate that Hsp65 from *M. leprae* has a potential therapeutic effect in many inflammation mouse models, and similar effects are shown in this study.

Consistent with this, we decided to develop a DNA vaccine using the pExu vector [49] for the host intestinal cells' local production of Hsp65 protein. After confirming the pExu:*hsp65* vector's functionality in eukaryotic cells, the plasmid was transformed in the *L. delbrueckii* CIDCA 133 strain. This strain was isolated from raw cow milk [65] and has probiotic characteristics described as high resistance to acid pH, bile salt, and entero-hemorrhagic microorganisms [65–67]. It can decrease harmful bacterial enzymatic activities [68] and resist antimicrobial peptides derived from enterocytes and human β-defensins [69,70]. This strain's capacity to stimulate phagocytosis by the induction of reactive oxygen species (ROS and NO) and promote the expression of surface markers related to antigen presentation in the in vitro test was described [71]. All these highlighted characteristics have encouraged us to test this strain in the mucositis inflammation model.

The protective effect of fermented milk by wild-type *Lactobacillus delbrueckii* subsp. *lactis* CIDCA 133 strain was previously tested for the first time by our research group in a mucositis mouse model [17]. In this experiment, mice received fermented milk for 13 days, and on the 10th day, they received the 5-FU drug to induce the mucositis. Animals treated with this strain presented a reduction in intestinal permeability values after the administration of 99mTc-DTPA by gavage. These animals also exhibited a preserved villus/crypt ratio, consequently showing a preserved epithelial architecture, with a significant amount of goblet cells and a reduction in inflammatory infiltrate. Altogether, this has shown the beneficial effect of this probiotic strain in intestinal inflammation, specifically in intestinal mucositis.

Therefore, combining the excellent reported characteristics of this probiotic strain with the benefits of the Hsp65 protein, we investigate the oral treatment capacity with recombinant *L. delbrueckii* CIDCA 133 (pExu:*hsp65*)-fermented milk in a mucositis mouse model.

In this work, mice who received treatment for 13 days with recombinant *L. delbrueckii* CIDCA 133 (pExu:*hsp65*)-fermented milk could avoid the intestinal shortening, reaching a length similar to the negative control. This parameter is very relevant to correlate the nutrient (food and liquid) uptake and the weight loss percentage, and consequently, the state of the animals' malnutrition. Thus, it was possible to attribute the lower weight loss to the group treated with *L. delbrueckii* CIDCA 133 (pExu:*hsp65*). According to a previous report, this effect was due to this bacteria's probiotic effect on the bowel's length [17].

Severe epithelial damage to the intestinal mucosa, especially in the jejunum and ileum sections, was related to mucositis pathobiology. Epithelial architecture and integrity damage with active migration of polymorphonuclear leukocytes (PMNs) stimulated by increased adhesion molecules from nuclear factor kappa B (NF-kB), through the intestinal villus and crypts, are typical signs of mucositis 1. Thus, neutrophil infiltration has been described in several studies that assessed mucositis [17,53,72–75]. The beneficial effect of fermented milk by *L. delbrueckii* rCIDCA 133:Hsp65 on attenuating the intestinal inflammation was demonstrated by the decrease in MPO activity, the histological score with lower inflammatory infiltrate, and higher villus/crypt ratio. Additionally, the restoration of the epithelium's architecture was shown in this work, and the low histological score of these animals was the same as the negative control. Altogether, this denotes the beneficial effect of this treatment.

The beneficial effect of the treatment with *L. delbrueckii* rCIDCA 133:Hsp65 could also be observed in the increased number of goblet cells and the relative expression of the Muc2 gene. Mucins are secreted by the goblet cells and create a layer that protects the epithelium against bacterial penetration [76]. Thus, the higher number of these cells and also the level of expression of Muc2compared to the MUC group reinforce the positive effect of the recombinant treatment.

Secretory IgA is a primary immunologic component of the mucosal surface's extrinsic protective mechanism [77]. Our group's previous reports observed increased sIgA levels in the small bowel of inflamed groups [17,22,78]. This event is related to the intestinal inflammatory process of a host's defense mechanism [79]. However, other authors report a decrease in sIgA levels in inflamed groups [53,80], attributed to malnutrition generated by 5-FU reducing the sIgA responses and Gut-Associated Lymphoid Tissue (GALT) lymphocyte numbers. Following our previous reports, high levels of sIgA were observed in animals that did not receive any recombinant CIDCA 133 strain (MUC group), even more than in animals that received rCIDCA133:Hsp65.

On the other hand, the rCIDCA133 group manifested decreasing levels of this immunoglobulin. This immunoglobulin plays an essential role in different immune system functions and existing IgA antibodies with high and low affinity. Those with high affinity protect intestinal mucosal surfaces against invasion by pathogenic microorganisms, while low-affinity antibodies confine commensal bacteria to the intestinal lumen [81].

In healthy conditions, a small number of bacteria from the gut microbiota can translocate, and are killed during this passage or in the MLNs, a phenomenon that contributes to the maturation and maintenance of a competent gastrointestinal immune system [82–84]. In agreement with this affirmation, the CTL group showed a basal count of radioactivity in the blood and organs. Interestingly, animals treated with rCIDCA 133 only showed higher values of uptake of 99mTc-*E. coli* in MLNs and liver and this event could be explained by gut injury or barrier failure contributing to the translocation of bacteria from the gut as MLNs are the first structures to receive the gut bacteria which, by portal circulation, reach the liver. This translocation pattern was demonstrated in other experimental models, which showed that viable bacteria could be found in the portal circulation, in high amounts, even before their appearance in intestinal lymph [85].

As the damage could be solved partially by rCIDCA 133, the other organs' translocation was the same as in the negative control, which presented the basal count of radioactivity.

Extremely high values of radioactivity were observed in the liver of animals that received rCIDCA133:Hsp65. These results could be attributed to the existence of the gut–liver axis, which is one of the most significant relations between gut microbiota and extra-intestinal organs, representing an extremely close functional and bidirectional communication between these two organs [86]. Damage in the intestinal barrier automatically exposes the liver to bacterial components, such as pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs), which could damage the organ by induction of an immune response, with the release of pro-inflammatory cytokines [87]. As the Hsp65 molecule has a dual role, inflammatory and regulatory, the liver's high inflammation

due to the closer relationship with the gut was not enough to block the dissemination of 99mTc-*E. coli* to the rest of the body. Additionally, as is often reported, the 5-FU cancer treatment generates a disruption of the indigenous microbiota's ecological balance and damage of the gut mucosal barrier by all the events involved in mucositis [88,89], which could lead to dysbiosis [90–92] with epithelium damage. The enteric venous system to the portal vein and the enteric lymphatic drainage are routes that could contribute to delivering bacteria or bacterial compounds from the gut to the circulation [93]. BT was the only parameter for which animals treated with rCIDCA133:Hsp65 did not show satisfactory results. These results can be explained, at least in part, by the fact that Hsps are closely related to apoptosis and ROS generation in human inflammatory diseases [94–96], and it is also known that oxidative stress metabolites can increase epithelial permeability [97]. Even though the treatment with recombinant bacteria has shown a reduction in the permeability, mucosal atrophy, mostly that associated with luminal nutrient deprivation, can be observed in mucositis, has been suggested as a predisposing factor for bacterial translocation [98]. Berg et al. [99] showed that immunosuppression associated with intestinal bacteria overgrowth promoted bacterial translocation in animals with histologically normal bowels. However, further studies must be performed to elucidate if this hypothesis can be applied to this specific mucositis model.

Moreover, two pathways could contribute to gastrointestinal permeability, the transcellular passage of molecules through the enterocytes using channels and membrane pumps, through the apical and basolateral membranes [100,101]. The paracellular route is regulated by tight junction proteins situated between adjacent enterocytes' apical lateral membranes, holding epithelial cells together. Their conservation and preservation are essential for cellular polarity and the intestinal epithelium's epithelial barrier function [93,102]. It is also known that tight junctions prevent the transepithelial movement of lipopolysaccharides and other macromolecules and bacteria [103].

The intestinal permeability was studied either by blood radioactivity after the oral intake of 99mTc-DTPA or by relative mRNA expression of tight junction proteins. It was shown that these two approaches are related because an improvement in this parameter (IP) was observed. The percentage of doses/g of 99mTc-DTPA was significantly lower in animals treated with recombinant strains. Treatment with the rCIDCA133:Hsp65 strain showed similar values to the negative control. Our results are in agreement with other reports where permeability was measured after probiotic treatment [17,53]. All the tight junction proteins studied, except claudin 5, significantly increased the mRNA expression with recombinant treatment. When the mRNA expression of tight junctions in the ileum section was analyzed, an increase was observed. However, the gene expression of claudin 5 did not show increased mRNA expression.

However, the group treated with the milk fermented by rCIDCA133:Hsp65 showed a higher expression in F11r, claudin 1, and claudin 2, although occludin and zonulin did not show a difference between these two treatments, demonstrating that both recombinants strains were able to increase these relative tight junction expressions. The qPCR results were reinforced by transmission electronic microscopy analyses showing improved organization of zonula occludens, zonula adherents, desmosomes, and gap junctions in animals treated with the recombinant strains when compared to the ones of the positive control (MUC group). Altogether, these results show the relevant findings related to the intestinal permeability improvement after the fermented milk administration. These results agree with other reports showing the importance of probiotics in tight junction expression and, consequently, intestinal permeability [104–106].

There is a closer relation between intestinal tight junctions and cytokines' role under pathophysiological conditions. The disfunction of tight junctions mediated by cytokines leads to immune activation and tissue inflammation [107,108].

The exposition to cytotoxic therapy, such as 5-FU, leads to direct damage to cellular DNA, generating cell injury and death in both basal epithelium and submucosal cells and, consequently, increasing reactive oxygen species (ROS) [109]. They cause more severe

damage to the involved tissues with macrophage stimulation and also activate the cascade of inflammatory pathways and transcription factors, such as NFKB 1 [110], which facilitates both the up-regulation of Il1b [111] and also the synthesis of pro-inflammatory molecules such as Tnf, Il6, cyclooxygenase-2 (Ptgs2), and Il12, among others [112,113].

In this study, the anti-inflammatory effect provided by both recombinant strains was shown. Milk fermented by both recombinant strains can attenuate the relative expression of the pro-inflammatory cytokines studied (Il1b, Il6, Il12, and Tnf), evidencing the anti-inflammatory effect that these strains provide. The milk fermented by rCIDCA 133:Hsp65 was more effective in decreasing Il1b and Il6, leading to similar levels in the non-inflamed animals. The importance of these findings is due to the significant role in inflammation of both cytokines [114], participating as mediators in the course of intestinal apoptosis after 5-FU chemotherapy [115], and also causing an increase in intestinal permeability. Il1b and Tnf participate in amplifying the severity of chemotherapy-induced intestinal mucositis [1]. Tnf is relevant because it induces the activation and the recruitment of PMN cells, causing damage and intestinal barrier dysfunction, increasing the intestinal permeability, and, consequently, levels of pathogenic bacteria from the intestinal lumen [116,117]. Il6 production is a hallmark of many human chronic inflammatory states, including mucositis due to its pro-inflammatory properties [118,119]. It has a high level of participation in the inflammatory Tlr4-mediated pathway [120]. The basal levels of Tlr4 are fundamental to control physiological states and intestinal homeostasis [121]. Tlr4 knockout mice showed the importance of this pattern recognition receptor (PRR) in signaling through Myd88 of bacterial translocation [122]. This information could explain our results regarding the high level of translocation observed in animals treated with rCIDCA:Hsp65 since they presented much lower levels, even lower than the negative control, of Tlr4 and also Myd88 mRNA expression. However, further experiments should be performed to elucidate the mechanisms of this complex signaling pathway.

As 5-FU generates a dysbiosis in the bowel, disruption of the mucosal barrier upon injury to intestinal epithelial cells causes the exhibition of Tlr ligands by commensal bacteria to Tlr expressed in many bowel cells [123]. Thus, due to the significant role of the microbiota in intestinal inflammation, the active participation of Tlr in bacterial product recognition and its importance in inflammation induction encourages us to test the in vivo effect of Tlr ligation by commensal-derived products. Other essential intracellular adapters, such as Myd88 and Nod2, were also investigated. Tlrs (except Tlr3) stimulate the cells through Myd88, which mediates the early immune response to pathogens, leading to Nfκb translocation to the nucleus and, consequently, gene expression of encoded pro-inflammatory cytokines and chemotactic cytokines (chemokines) [124,125]. The microbial product also can be recognized by a family of intracellular signaling proteins, called Nod. They can detect microbial ligands in the cytosol. These cytosolic proteins signal Nfkb and MAPK, followed by the induction of numerous genes involved in the inflammatory process, thus triggering host innate immune responses [126–128]. Nod2 recognizes muramyl dipeptide on degraded bacterial cell wall peptidoglycan and can therefore respond to invading Gram-negative and Gram-positive bacteria.

Our results follow those of other authors who describe Tlrs as the major sensors of the innate immune system which recognize highly preserved motifs of microorganisms [129], and are related to the activation of signaling cascades, such as Nfkb, associated with inflammatory responses through the production of pro-inflammatory cytokines [130,131]. Additionally, an increase in the Tlr2 and Tlr4 levels was observed in animals that received 5-FU, and the administration of probiotics was able to reverse these increases [132], as we could demonstrate. This analysis shows, at least, a potential relationship between these parameters. However, mRNA expression cannot affirm that different circulant cytokines' different concentrations will be found due to the possible post-translational modification.

The Il10 cytokine is an important immunoregulatory molecule required to maintain immune homeostasis in the gut and contributes to declining inflammatory responses by down-regulating pro-inflammatory cytokine production at the site of tissue damage [133].

It is the most well-researched cytokine in inflammatory bowel disease (IBD), and its active form is secreted by different types of immune cells, such as Treg, monocytes, and macrophages [134,135]. Studies support the hypothesis that Il10 can attenuate Tnf receptor expression [136,137], which can be related to the results found in this study. In contrast, treatments with both recombinant strains were able to highly improve Il10 expression, with a reduction of Tnf, Il1b, and Il6 expression levels. These results are also supported by histological, MPO, and EPO analyses.

The results together show that animals undergoing induction chemotherapy with 5-FU and pretreated with *L. delbrueckii* CIDCA 133 (pExu:*hsp65*) experienced a reduced magnitude of damage to the mucosal architecture of the small intestine and decreased intestinal permeability, highlighting the importance that this probiotic recombinant strain has in reestablishing the number of goblet cells. Thus, the recombinant probiotic *L. delbrueckii* CIDCA 133 (pExu:*hsp65*) consumption could be an excellent alternative to ameliorating the intestinal damage caused by 5-FU in a mouse model.

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7 CONCLUSÕES GERAIS

Os resultados alcançados por este trabalho até o presente momento nos permitem concluir que a administração oral da linhagem *Lactobacillus delbrueckii* CIDCA 133 conferiu benefícios funcionais aos animais com mucosite intestinal induzida por 5-FU, sendo capaz de diminuir a perda de peso dos animais inflamados, reduzir o infiltrado inflamatório e os danos na mucosa intestinal ocasionaodos pelo agente quimioterápico. Estes achados foram intrinsicamente associados à propriedade probiótica da linhagem em inibir a via inflamatória NF-κB e a expressão gênica de citocinas de perfil pró-inflamatório, além de aumentar a expressão gênica da interleucina imunomodulatória IL10 e das proteínas de junções claudina 1, zonulina e a molécula de junção de aderência 1, envolvidas na função de barreira epitelial.

Por outro lado, o plasmídeo pExu:*hsp*65 mostrou-se funcional em células eucarióticas, uma vez que a expressão da proteína de interesse foi verificada, *in vitro*, pela técnica de imunocitoquímica após transfecção das células. Foi possível observar também que a linhagem CIDCA 133 foi eficiente na entrega do pExu:*hsp*65 no modelo animal *in vivo*, sendo a linhagem recombinante utilizada com sucesso no modelo experimental de mucosite intestinal induzida por 5-FU. O tratamento com CIDCA 133 recombinante (rCIDCA 133:HSP65) também foi capaz de atenuar os danos inflamatórios e histopatológicos ocasionados por 5-FU. No entanto, é necessário ratificar que a linhagem recombinante mostrou-se mais eficiente em melhorar a mucosite, uma vez que potencializou a redução do comprimento intestinal, dos níveis de infiltrado de neutrófilos, e da expressão gênica de marcadores pro-inflamatórios, como as interleucinas Il1b e Il6. A entrega da bactéria recombinante também potencializou a função de barreira epitelial via redução da permeabilidade intestinal, preservação das células caliciformes produtoras de muco, e aumento na expressão gênica de mucina 2, e também das proteínas de junção firme claudina 1, claudina 2 e da molécula de adesão juncional (F11r).

Portanto, os achados do nosso trabalho mostram que *L. delbrueckii* CIDCA 133 selvagem e recombinante melhoram a mucosite intestinal induzida por 5-FU, demonstrando serem estratégias terapêuticas promissoras direcionadas ao tratamento de doenças inflamatórias intestinais.

8 PERSPECTIVAS

Diante dos resultados promissores alcançados após a administração oral de das linhagens de CIDCA 133, selvagem e recombinantes, no modelo de mucosite intestinal induzida por 5-FU em camundongos, temos como perspectivas o desenvolvimento de novos estudos com estas linhagens, como:

- Avaliar a modulação da microbiota intestinal por meio de análises de metagenômica das fezes;
- Testar o potencial de CIDCA 133 na formulação de alimentos funcionais, como por exemplo na preparação de queijo Minas Frescal, e avaliar o efeito probiótico do mesmo em modelos de inflamação;
- Estudar a capacidade imunomoduladora e anti-inflamatória de CIDCA 133 selvagem e rCIDCA 133:HSP65 em outros modelos de doenças inflamatórias que acomete o TGI, como por exemplo o modelo murino de colite induzida por DSS (Dextran sulfato de sódio) e salmonelose.

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ANEXOS

As principais publicações e atividades realizadas durante o período do doutorado (2018-2022) estão listadas na tabela abaixo:

Anexos		Quantidade	Situação
Créditos concluídos		34	Integralizados
Artigo científico			
	1° autor	02	Publicados
	Co-autor	05	Publicados
Capítulo de livro			
	1° autor	01	Publicado
		01	Aceito para publicação
	Co-autor	02	Publicados
Participação em eventos			
	Nacional (participante)	01	Concluído
	Nacional (palestrante)	01	Previsto para novembro (2022)
	Internacional	01	Concluído
Cursos			
	Mini curso	02	Concluído
	Curta duração	02	Concluído
Organização de eventos		01	Concluído

Imprimir currículo

Fernanda Alvarenga Lima Barroso

Endereço para acessar este CV: http://lattes.cnpq.br/8125531547156737

Última atualização do currículo em 20/09/2022

Resumo informado pelo autor

Bacharel em Ciências Biológicas pela Universidade Federal de Minas Gerais (2010) e Mestre em Genética (ênfase em Genética Molecular de Microrganismos e Biotecnologia) pela UFMG (2012) e atualmente doutoranda do mesmo programa pela mesma universidade. Experiência na área de Genética, com ênfase em Genética Molecular e de Microrganismos, atuando principalmente em projetos de pesquisa voltados para o desenvolvimento de novos sistemas de expressão gênica e novas utilizações biotecnológicas das Bactérias do Ácido Láctico.

(Texto informado pelo autor)

Nome civil

Nome Fernanda Alvarenga Lima Barroso

Dados pessoais

Nascimento 03/01/1988 - Ipatinga/MG - Brasil

CPF 089.379.056-71

Formação acadêmica/titulação

2018 Doutorado em Genética.
Universidade Federal de Minas Gerais, UFMG, Belo Horizonte, Brasil
Título: Construção e avaliação funcional do plasmídeo vacinal pExu:hsp65 e estudo do efeito terapêutico do leite fermentado por Lactobacillus delbrueckii subsp. lactis CIDCA 133 (pExu:hsp65) em modelo murino de mucosite intestinal.
Orientador: Vasco Ariston de Carvalho Azevedo

2010 - 2012 Mestrado em Genética.
Universidade Federal de Minas Gerais, UFMG, Belo Horizonte, Brasil
Título: Construção e avaliação de um plasmídeo terapêutico para expressão da IL-10 de M. musculus em células mamíferas, utilizando como veículo carreador linhagens de L. lactis invasivas: desenvolvimento de estratégia alternativa para o tratamento das IBDs, Ano de obtenção: 2012
Orientador: Anderson Miyoshi
Co-orientador: Sophie Leclercq e Vasco Azevedo
Bolsista do(a): Conselho Nacional de Desenvolvimento Científico e Tecnológico

2006 - 2010 Graduação em Ciências Biológicas.
Universidade Federal de Minas Gerais, UFMG, Belo Horizonte, Brasil
Título: Clonagem, expressão e endereçamento protéico em Lactococcus lactis da Enterotoxina B de Staphylococcus aureus
Orientador: Anderson Miyoshi

Formação complementar

2019 - 2019 Curso de curta duração em Escuela de Doctorado de Verano 2019. (Carga horária: 40h).
Universidade de Chile, UCHILE, Chile

2018 - 2018 Curso de curta duração em Mini curso RNAs não codificantes. (Carga horária: 4h).
Universidade Federal de Minas Gerais, UFMG, Belo Horizonte, Brasil

2018 - 2018 Curso de curta duração em Biossegurança em Ambiente de Saúde. (Carga horária: 20h).
Universidade Federal de Minas Gerais, UFMG, Belo Horizonte, Brasil

2018 - 2018 Curso de curta duração em Mini curso de Genética Forense. (Carga horária: 4h).
Universidade Federal de Minas Gerais, UFMG, Belo Horizonte, Brasil

2009 - 2009 Curso de curta duração em Biotecnologia aplicada para a produção industrial. (Carga horária: 3h).
Sociedade Brasileira de Genética, SBG, Ribeirao Preto, Brasil

2009 - 2009 Extensão universitária em Biossegurança em OGM:da produção à comercialização. (Carga horária: 80h).
CIBio Instituto de Ciências Biológcas/UFMG, CIBIO, Brasil

2008 - 2008 Curso de curta duração em Curso de Perícia Criminal (Toxicologia Forense). (Carga horária: 8h).
Integra Cursos e Eventos, INC, Brasil

2007 - 2007 Curso de curta duração em Transformação Genética de Milho e Sorgo. (Carga horária: 40h).
Núcleo de Biologia Aplicada/ Embrapa Milho e Sorgo, NBA, Brasil

Atuação profissional

1. Uniclon Biotecnologia - UNICLON

Vínculo institucional

2012 - 2013 Vínculo: Bolsista , Enquadramento funcional: Bolsista, Regime: Dedicação exclusiva

2. Universidade Federal de Minas Gerais - UFMG

Vínculo institucional

2016 - Atual Vínculo: Servidor público , Enquadramento funcional: Técnico de Laboratório - Biologia , Carga horária: 30, Regime: Parcial

2007 - 2012 Vínculo: Estagiário , Enquadramento funcional: Estagiário , Carga horária: 40, Regime: Integral

Atividades

08/2010 - Atual Pesquisa e Desenvolvimento, Instituto de Ciências Biológicas

Linhas de pesquisa:
Novas utilizações biotecnológicas das bactérias lácticas

3. Embrapa Milho e Sorgo - CNPMS

Vínculo institucional

2007 - 2007 Vínculo: Estagiário , Enquadramento funcional: Estagiário , Carga horária: 40, Regime: Dedicação exclusiva
Outras informações:
Estágio de complementação educacional na área de Biologia Molecular

Atividades

02/2007 - 02/2007 Estágio, Núcleo de Biologia Aplicada

Estágio:
Atividade: Transformação de milho via biobalística / Orientadora: Dra. Andrea Almeida Carneiro

4. Colégio Batista Mineiro - CBM

Vínculo institucional

2006 - 2006 Vínculo: Estagiário , Enquadramento funcional: Estagiário , Carga horária: 20, Regime: Parcial

Atividades

02/2006 - 12/2006 Ensino médio

Especificação:
Monitoria de Ciências e Biologia

Linhas de pesquisa

1. Novas utilizações biotecnológicas das bactérias lácticas

Objetivos:Lactococcus lactis, a mais bem caracterizada bactéria láctica (BL), é um microrganismo Gram-positivo amplamente utilizado na indústria alimentícia para a produção e preservação de produtos lácteos fermentados. Desde o início dos anos 90, a despeito de sua utilização industrial, vários grupos de pesquisa voltaram-se para o potencial uso de L. lactis como uma usina celular para a produção de proteínas recombinantes. Hoje, através do desenvolvimento de inúmeras ferramentas genéticas, como sistemas de expressão gênica, estas bactérias são utilizadas não só industrialmente mas também em laboratório para a produção de proteínas heterólogas de interesse biotecnológico como enzimas e antígenos. Neste contexto, esta linha de pesquisa propõe-se a explorar alguns dos instrumentos genéticos disponíveis para uso nas BL, bem como desenvolver novos e alternativos sistemas de expressão que visam implementar ainda mais a utilização destas bactérias.

Produção

Produção bibliográfica

Artigos completos publicados em periódicos

1. doi> COELHO-ROCHA, NINA DIAS; DE JESUS, LUÍS CLÁUDIO LIMA; **BARROSO, FERNANDA ALVARENGA LIMA**; DA SILVA, TALES FERNANDO; FERREIRA, ENIO; GONÇALVES, JOSÉ EDUARDO; DOS SANTOS MARTINS, FLAVIANO; DE OLIVEIRA CARVALHO, RODRIGO DIAS; BARH, DEBMALYA; AZEVEDO, VASCO ARISTON DE CARVALHO
Evaluation of Probiotic Properties of Novel Brazilian Lactiplantibacillus plantarum Strains. Probiotics and Antimicrobial Proteins. JCR, v.14, p.1 - , 2022.

2. doi> **BARROSO, FERNANDA ALVARENGA LIMA**; DE JESUS, LUÍS CLÁUDIO LIMA; DA SILVA, TALES FERNANDO; BATISTA, VIVIANE LIMA; LAGUNA, JULIANA; COELHO-ROCHA, NINA DIAS; VITAL, KÁTIA DUARTE; FERNANDES, SIMONE ODÍLIA ANTUNES; CARDOSO, VALBERT NASCIMENTO; FERREIRA, ENIO; MARTINS, FLAVIANO SANTOS; DRUMOND, MARIANA MARTINS; Mancha-Agresti, Pamela; BIRBRAIR, ALEXANDER; BARH, DEBMALYA; AZEVEDO, VASCO
Lactobacillus delbrueckii CIDCA 133 Ameliorates Chemotherapy-Induced Mucositis by Modulating Epithelial Barrier and TLR2/4/Myd88/NF-κB Signaling Pathway. Frontiers in Microbiology. JCR, v.13, p.1 - 14, 2022.

3. doi> BATISTA, VIVIANE LIMA; DE JESUS, LUÍS CLÁUDIO LIMA; TAVARES, LAÍSA MACEDO; **Barroso, Fernanda Lima Alvarenga**; FERNANDES, LUCAS JORGE DA SILVA; FREITAS, ANDRIA DOS SANTOS; AMERICO, MONIQUE FERRARY; DRUMOND, MARIANA MARTINS; Mancha-Agresti, Pamela; FERREIRA, ENIO; LAGUNA, JULIANA GUIMARÃES; ALCANTARA, LUIZ CARLOS JÚNIOR; AZEVEDO, VASCO
Paraprobiotics and Postbiotics of Lactobacillus delbrueckii CIDCA 133 Mitigate 5-FU-Induced Intestinal Inflammation. Microorganisms. JCR, v.10, p.1418 - , 2022.

4. doi> **BARROSO, FERNANDA ALVARENGA LIMA**; DE JESUS, LUÍS CLÁUDIO LIMA; DE CASTRO, CAMILA PROSPERI; BATISTA, VIVIANE LIMA; FERREIRA, ÊNIO; FERNANDES, RENATA SALGADO; DE BARROS, ANDRÉ LUÍS BRANCO; LECLERQ, SOPHIE YVETTE; AZEVEDO, VASCO; Mancha-Agresti, Pamela; DRUMOND, MARIANA MARTINS
Intake of Lactobacillus delbrueckii (pExu:hsp65) Prevents the Inflammation and the Disorganization of the Intestinal Mucosa in a Mouse Model of Mucositis. Microorganisms. JCR, v.9, p.107 - , 2021.

5. doi> DE JESUS, LUÍS CLÁUDIO LIMA; DE JESUS SOUSA, THIAGO; COELHO-ROCHA, NINA DIAS;

PROFETA, RODRIGO; **BARROSO, FERNANDA ALVARENGA LIMA**; DRUMOND, MARIANA MARTINS; Mancha-Agresti, Pamela; FERREIRA, ÊNIO; BRENIG, BERTRAM; ABURJAILE, FLÁVIA FIGUEIRA; AZEVEDO, VASCO
Safety Evaluation of Lactobacillus delbrueckii subsp. lactis CIDCA 133: a Health-Promoting Bacteria. Probiotics and Antimicrobial Proteins. JCR, v.13, p.1 - , 2021.

6. doi> TAVARES, LAÍSA M.; DE JESUS, LUÍS C. L.; DA SILVA, TALES F.; **BARROSO, FERNANDA A. L.**; BATISTA, VIVIANE L.; COELHO-ROCHA, NINA D.; AZEVEDO, VASCO; DRUMOND, MARIANA M.; Mancha-Agresti, Pamela
Novel Strategies for Efficient Production and Delivery of Live Biotherapeutics and Biotechnological Uses of Lactococcus lactis: The Lactic Acid Bacterium Model. FRONTIERS IN BIOENGINEERING AND BIOTECHNOLOGY. JCR, v.8, p.1 - , 2020.

7. doi> BATISTA, VIVIANE LIMA; DA SILVA, TALES FERNANDO; DE JESUS, LUÍS CLÁUDIO LIMA; COELHO-ROCHA, NINA DIAS; **BARROSO, FERNANDA ALVARENGA LIMA**; TAVARES, LAISA MACEDO; AZEVEDO, VASCO; Mancha-Agresti, Pamela; DRUMOND, MARIANA MARTINS
Probiotics, Prebiotics, Synbiotics, and Paraprobiotics as a Therapeutic Alternative for Intestinal Mucositis. Frontiers in Microbiology. JCR, v.11, p.2246 - , 2020.

8. doi> PEREIRA, VANESSA BASTOS; ZURITA-TURK, MERITXELL; SARAIVA, TESSÁLIA DINIZ LUERCE; DE CASTRO, CAMILA PRÓSPERI; SOUZA, BIANCA MENDES; MANCHA AGRESTI, PAMELA; **LIMA, FERNANDA Alvarenga**; PFEIFFER, VANESSA NATHALIE; AZEVEDO, MARCELA SANTIAGO PACHECO; ROCHA, CLARISSA SANTOS; PONTES, DANIELA SANTOS; AZEVEDO, VASCO; MIYOSHI, ANDERSON
DNA Vaccines Approach: From Concepts to Applications. WORLD JOURNAL OF VACCINES. , v.04, p.50 - 71, 2014.

9. doi> DEL CARMEN, S.; ZURITA-TURK, M.; **LIMA, F. ALVARENGA**; DOS SANTOS, J.S. COELHO; LECLERCQ, S.Y.; CHATEL, J.-M.; AZEVEDO, V.; DE MORENO DE LEBLANC, A.; MIYOSHI, A.; LEBLANC, J.G.
A Novel Interleukin-10 Dna Mucosal Delivery System Attenuates Intestinal Inflammation in a Mouse Model. European Journal of Inflammation. JCR, v.11, p.641 - 654, 2013.

10. doi> LEBLANC, JEAN; CARMEN, SILVINA; TURK, MERITXELL; **LIMA, FERNANDA**; PONTES, DANIELA; MIYOSHI, ANDERSON; AZEVEDO, VASCO; DE LEBLANC, ALEJANDRA
Mechanisms Involved in the Anti-Inflammatory Properties of Native and Genetically Engineered Lactic Acid Bacteria. Anti-Infective Agents. , v.11, p.59 - 69, 2012.

Capítulos de livros publicados

1. AMERICO, M. F.; Andria dos Santos Freitas; Tales Fernando da Silva; Luís Lima de Jesus; **Fernanda Alvarenga Lima Barroso**; Rodrigo Dias de Oliveira Carvalho; Vasco Ariston De Carvalho
Uso de Bactérias Lácticas como Vetores de Entrega de Vacinas Recombinates. In: Uso de Bactérias Lácticas como Vetores de Entrega de Vacinas Recombinates..1, 2022, v.1, p. 102-120.

2. Cláudio Lima de Jesus, Luís; **Alvarenga Lima, Fernanda**; Dias Coelho-Rocha, Nina; Fernando da Silva, Tales; Paz, Júlia; AZEVEDO, VASCO; Mancha-Agresti, Pamela; Martins Drumond, Mariana
Recombinant Probiotics and Microbiota Modulation as a Good Therapy for Diseases Related to the GIT In: The Health Benefits of Foods - Current Knowledge and Further Development.1 ed.: IntechOpen, 2020, p. 1-30.

3. **Fernanda Alvarenga Lima**; Luís Cláudio Lima de Jesus; Viviane Lima Batista; Laísa Macedo Tavares; Rafael Assis; Mariana Martins Drumond; AZEVEDO, V.; MANCHA-AGREST, P.
The Gastrointestinal Disease to the Microbiota Treatment: From the Present to the Future In: The Gastrointestinal Disease to the Microbiota Treatment: From the Present to the Future.01 ed.Las Vegas: Open Access eBooks, 2019, v.2, p. 1-32.

4. Vanessa Bastos Pereira; Marcela Santiago Pacheco Azevedo; Saraiva, T. D. L. S; Bianca Mendes Souza; Meritxell Zurita Turk; Camila Prósperi De Castro; Pamela Mancha Agresti; **Fernanda Alvarenga Lima**; AZEVEDO, V.; Daniela Santos Pontes ; MIYOSHI, A.
Use of bacteria in DNA vaccine delivery In: http://www.formatex.info/microbiology4/vol3/1993-2003.pdf.1 ed.Espanha: Formatex, 2013, v.3, p. 1993-2003.

5. Jean Guy LeBlanc; Silvina del Carmen; **Fernanda Alvarenga Lima**; Meritxell Zurita Turk; MIYOSHI, A.; AZEVEDO, V.; Alejandra de Moreno de LeBlanc
Prospective Uses of Genetically Engineered Lactic Acid Bacteria for the Prevention of Inflammatory Bowel Diseases In: Prospective Uses of Genetically Engineered Lactic Acid Bacteria for the Prevention of Inflammatory Bowel Diseases.1 ed.Londres: IntechOpen, 2012, v.1, p. 223-238.

Trabalhos publicados em anais de eventos (resumo)

1. ZURITA-TURK, M.; **LIMA, F. A.**; DEL CARMEN, S.; Santos, J. S. C dos; PROSPERI, C.C; MANCHA-AGREST, P.; PEREIRA, V. B.; AZEVEDO, V.; LECLERCQ, S.Y.; DE MORENO DE LEBLANC, A.; LEBLANC, J.G.; MIYOSHI, A.
Anti inflamatory effect of a fibronectin-binding protein expressing Lactococcus lactis containing a eukaryotic DNA vector coding for interleukin 10 using a murine model of Crohn´s disease. In: XXXVII Congress of Brazilian Society of Immunology, 2012, Campos do Jordão.
XXXVII Congress of Brazilian Society of Immunology, 2012, Campos do Jordão, Brasil. , 2012.

2. MANCHA-AGREST, P.; PROSPERI, C.C; Santos, J. S. C dos; **LIMA, F. A.**; ZURITA-TURK, M.; PEREIRA, V. B.; Saraiva, T. D. L. S; AZEVEDO, VASCO; LECLERCQ, S.Y.; MIYOSHI, A.
Desenvolvimento de uma nova estratégia vacinal contra tuberculose utilizando Lactococus Lactis invasivas como veículos para a entrega do plasmídeo vacinal codificando o antígeno Ag85A de Mycobacterium Tuberculosis a células mamíferas In: XXI Congresso Latinoamericano de Microbiología, 2012, Santos.
XXI Congresso Latinoamericano de Microbiología, 2012, Santos Brasil. , 2012.

3. PROSPERI, C.C; Santos, J. S. C dos; MANCHA-AGREST, P.; **LIMA, F. A.**; ZURITA-TURK, M.; Saraiva, T. D. L. S; PEREIRA, V. B.; AZEVEDO, V.; LECLERCQ, S.Y.; MIYOSHI, A.
Otimização de um plasmídeo vacinal, a ser veiculado por linhagens invasivas de Lactococcus lactis, por introdução da Sequência de Endereçamento Nuclear do vírus SV40 In: XXI Congresso Latinoamericano de Microbiología, 2012, Santos.
XXI Congresso Latinoamericano de Microbiología, 2012, Santos Brasil. , 2012.

4. **LIMA, F. A.**; ZURITA-TURK, M.; COSTA, K. M.; PROSPERI, C.C; MANCHA-AGREST, P.; PEREIRA, V. B.; Saraiva, T. D. L. S; PFEIFFER, V. N.; Le Loir, Y.; AZEVEDO, V.; MIYOSHI, A.
Clonagem, expressão e endereçamento protéico da enterotoxina B de Staphylococcus aureus em Lactococcus lactis In: 26 Congresso de Microbiologia, 2011, Foz do Iguaçu.
26 Congresso de Microbiologia. , 2011.

5. MANCHA-AGREST, P.; SANTOS, J.; PFEIFFER, V. N.; PEREIRA, V. B.; **LIMA, F. A.**; ZURITA-TURK, M.; PROSPERI, C.C; Saraiva, T. D. L. S; AZEVEDO, V.; LECLERCQ, S. Y.; MIYOSHI, A.
Construção e avaliação funcional do plasmídeo vacinal pValac:Ag85A: Desenvolvimento de uma nova estratégia vacinal contra a tuberculose. In: 26 Congresso de Microbiologia, 2011, Foz do Iguaçu.
26 Congresso de Microbiologia. , 2011.

6. PEREIRA, V. B.; MANCHA-AGREST, P.; SANTOS, J.; Saraiva, T. D. L. S; ZURITA-TURK, M.; PROSPERI, C.C; **LIMA, F. A.**; AZEVEDO, V.; LECLERCQ, S. Y.; MIYOSHI, A.
Construction and functional evaluation of a DNA vaccine for expression of the antigen ESAT-6 of Mycobacterium tuberculosis in mammalian cells, using an invasive lactic bacterium as a carrier vehicle. In: 26 Congresso de Microbiologia, 2011, Foz do Iguaçu.
26 Congresso de Microbiologia. , 2011.

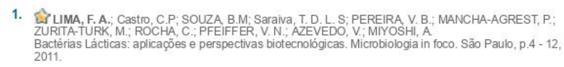
7. ZURITA-TURK, M.; **LIMA, F. A.**; SANTOS, J.; PROSPERI, C.C; MANCHA-AGREST, P.; Saraiva, T. D. L. S; PEREIRA, V. B.; PFEIFFER, V. N.; AZEVEDO, V.; LECLERCQ, S. Y.; MIYOSHI, A.
Desenvolvimento de uma nova estratégia para tratamento de doenças inflamatórias intestinais utilizando Lactococcus lactis invasivas para entrega, em células mamíferas, de um plasmídeo terapêutico codificando a Interleucina-10 (IL-10) de Mus musculus. In: 26 Congresso de Microbiologia, 2011, Foz do Iguaçu.
26 Congresso de Microbiologia. , 2011.

8. PROSPERI, C.C; SANTOS, J.; **LIMA, F. A.**; ZURITA-TURK, M.; MANCHA-AGREST, P.; PEREIRA, V. B.; PFEIFFER, V. N.; Saraiva, T. D. L. S; AZEVEDO, V.; LECLERCQ, S. Y.; MIYOSHI, A.

Otimização de instrumentos genéticos para utilização de Lactococcus lactis como veículos carreadores de vacinas gênicas. In: 26 Congresso de Microbiologia, 2011, Foz do Iguaçu.
26 Congresso de Microbiologia. , 2011.

9. **LIMA, F. A.**; AZEVEDO, V.; MIYOSHI, A.
Expressão heteróloga e endereçamento protéico em Lactococcus lactis da proteína hipotética Rv1419p de Mycobacterium tuberculosis: desenvolvimento de vacina alternativa contra a tuberculose experimental In: 55º Congresso Brasileiro de Genética, 2009, Águas de Lindóia.
Anais do 55º Congresso Brasileiro de Genética. , 2009.

Artigos em revistas (Magazine)



1. **LIMA, F. A.**; Castro, C.P; SOUZA, B.M; Saraiva, T. D. L. S; PEREIRA, V. B.; MANCHA-AGREST, P.; ZURITA-TURK, M.; ROCHA, C.; PFEIFFER, V. N.; AZEVEDO, V.; MIYOSHI, A.
Bactérias Lácticas: aplicações e perspectivas biotecnológicas. Microbiologia in foco. São Paulo, p.4 - 12, 2011.

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Contribuições Científicas (2018-2022)

frontiers in Microbiology

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

Viviane Lima Batista[1], Tales Fernando da Silva[1], Luís Cláudio Lima de Jesus[1], Nina Dias Coelho-Rocha[1], Fernanda Alvarenga Lima Barroso[1], Laisa Macedo Tavares[1], Vasco Azevedo[1], Pamela Mancha-Agresti[1,2] and Mariana Martins Drumond[1,3]**

[1] Laboratório de Genética Celular e Molecular (LGCM), Departamento de Biologia Geral, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais (UFMG), Belo Horizonte, Brazil, [2] Faculdade de Minas, FAMINAS-BH, Belo Horizonte, Brazil, [3] Centro Federal de Educação Tecnológica de Minas Gerais (CEFET/MG), Departamento de Ciências Biológicas, Belo Horizonte, Brazil

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Konstantinos Papadimitriou, University of Peloponnese, Greece

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Jia Yin, Hunan Normal University, China

Correspondence:
Pamela Mancha-Agresti
p.mancha.agresti@gmail.com
Mariana Martins Drumond
mmdrumond@gmail.com

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

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

INTRODUCTION

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

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

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

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REVIEW
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Novel Strategies for Efficient Production and Delivery of Live Biotherapeutics and Biotechnological Uses of *Lactococcus lactis*: The Lactic Acid Bacterium Model

Laísa M. Tavares[1], Luís C. L. de Jesus[1], Tales F. da Silva[1], Fernanda A. L. Barroso[1], Viviane L. Batista[1], Nina D. Coelho-Rocha[1], Vasco Azevedo[1], Mariana M. Drumond[1,2] and Pamela Mancha-Agresti[1,3]**

[1] Laboratory of Cellular and Molecular Genetics, Federal University of Minas Gerais, Belo Horizonte, Brazil, [2] Departamento de Ciências Biológicas, Centro Federal de Educação Tecnológica de Minas Gerais, Belo Horizonte, Brazil, [3] FAMINAS - BH, Belo Horizonte, Brazil

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Portugal

***Correspondence:**
Pamela Mancha-Agresti
p.mancha.agresti@gmail.com
Mariana M. Drumond
mmdrumond@gmail.com

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Lactic acid bacteria (LAB) are traditionally used in fermentation and food preservation processes and are recognized as safe for consumption. Recently, they have attracted attention due to their health-promoting properties; many species are already widely used as probiotics for treatment or prevention of various medical conditions, including inflammatory bowel diseases, infections, and autoimmune disorders. Some LAB, especially *Lactococcus lactis,* have been engineered as live vehicles for delivery of DNA vaccines and for production of therapeutic biomolecules. Here, we summarize work on engineering of LAB, with emphasis on the model LAB, *L. lactis*. We review the various expression systems for the production of heterologous proteins in *Lactococcus* spp. and its use as a live delivery system of DNA vaccines and for expression of biotherapeutics using the eukaryotic cell machinery. We have included examples of molecules produced by these expression platforms and their application in clinical disorders. We also present the CRISPR-Cas approach as a novel methodology for the development and optimization of food-grade expression of useful substances, and detail methods to improve DNA delivery by LAB to the gastrointestinal tract. Finally, we discuss perspectives for the development of medical applications of recombinant LABs involving animal model studies and human clinical trials, and we touch on the main safety issues that need to be taken into account so that bioengineered versions of these generally recognized as safe organisms will be considered acceptable for medical use.

Keywords: ***Lactococcus lactis*, genetic engineering, biotherapeutic molecules, mucosal immunity, safe for consumption**

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Safety Evaluation of *Lactobacillus delbrueckii* subsp. *lactis* CIDCA 133: a Health-Promoting Bacteria

Luís Cláudio Lima de Jesus[1] · Thiago de Jesus Sousa[1] · Nina Dias Coelho-Rocha[1] · Rodrigo Profeta[1] · Fernanda Alvarenga Lima Barroso[1] · Mariana Martins Drumond[2] · Pamela Mancha-Agresti[3] · Ênio Ferreira[4] · Bertram Brenig[5] · Flávia Figueira Aburjaile[1] · Vasco Azevedo[1]

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Abstract
Lactobacillus delbrueckii subsp. *lactis* CIDCA is a new potential probiotic strain whose molecular basis attributed to the host's benefit has been reported. This study investigated the safety aspects of *Lactobacillus delbrueckii* subsp. *lactis* CIDCA 133 based on whole-genome sequence and phenotypic analysis to avoid future questions about the harmful effects of this strain consumption. Genomic analysis showed that *L. delbrueckii* subsp. *lactis* CIDCA 133 harbors virulence, harmful metabolites, and antimicrobial resistance-associated genes. However, none of these genetic elements is flanked or located within prophage regions and plasmid sequence. At a phenotypic level, it was observed *L. delbrueckii* subsp. *lactis* CIDCA 133 antimicrobial resistance to aminoglycosides streptomycin and gentamicin antibiotics, but no hemolytic and mucin degradation activity was exhibited by strain. Furthermore, no adverse effects were observed regarding mice clinical and histopathological analysis after the strain consumption (5×10^7 CFU/mL). Overall, these findings reveal the safety of *Lactobacillus delbrueckii* subsp. *lactis* CIDCA 133 for consumption and future probiotic applications.

Keywords Genomic analysis · *Lactobacillus delbrueckii* · Epithelial barrier · CRISPR-Cas system · Safety assessment

Introduction

Lactobacillus is one of the main microorganisms of the lactic acid bacteria (LAB) group with high importance in the dairy industry [1, 2] and a broad spectrum of beneficial effects, mainly focused on gastrointestinal tract (GIT) diseases treatment [3, 4], diabetes [5], and foodborne pathogens control [6], among others. A new taxonomic classification was proposed for the genus *Lactobacillus*, with *Lactobacillus delbrueckii* being the type species [7]. Among the species, *Lactobacillus delbrueckii* subsp. *lactis* CIDCA 133 is a new potential probiotic strain isolated from raw cow's milk in La Plata, Argentina [8].

The beneficial effects of *L. delbrueckii* subsp. *lactis* CIDCA 133 have been reported, such as its ability to inhibit the growth of pathogenic and spoilage bacteria (e.g., *Pseudomonas aeruginosa* and *Escherichia coli*) [8, 9], to tolerate stress conditions, as bile salts [10] and acidity [11], and survive to enterocyte-derived antimicrobial molecules such as β-defensins [12, 13]. Furthermore, this strain stimulated immune cells like dendritic cells and murine macrophage infected with *Bacillus cereus* and *Citrobacter rodentium* [14, 15] to control the infection generated by these pathogens. It was also reported that *L. delbrueckii* subsp. *lactis* CIDCA 133 protected the mice's intestinal mucosa from inflammatory and histopathological damage caused by chemotherapy [3]. The molecular basis attributed to stress tolerance,

Luís Cláudio Lima de Jesus, Thiago de Jesus Sousa, Flávia Figueira Aburjaile, and Vasco Azevedo contributed equally to this work.

✉ Vasco Azevedo
vasco@icb.ufmg.br

[1] Departamento de Genética, Ecologia e Evolução, Universidade Federal de Minas Gerais, Belo Horizonte, Minas Gerais, Brazil

[2] Departamento de Ciências Biológicas, Centro Federal de Educação Tecnológica de Minas Gerais, Belo Horizonte, Minas Gerais, Brazil

[3] Faculdade de Minas-Faminas-BH, Medicina, Belo Horizonte, Minas Gerais, Brazil

[4] Departamento de Patologia Geral, Universidade Federal de Minas Gerais, Belo Horizonte, Minas Gerais, Brazil

[5] Institute of Veterinary Medicine, University of Göttingen, Göttingen, Germany

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microorganisms

Article

Paraprobiotics and Postbiotics of *Lactobacillus delbrueckii* CIDCA 133 Mitigate 5-FU-Induced Intestinal Inflammation

Viviane Lima Batista [1,†], Luís Cláudio Lima De Jesus [1,†], Laísa Macedo Tavares [1], Fernanda Lima Alvarenga Barroso [1], Lucas Jorge da Silva Fernandes [1], Andria dos Santos Freitas [1], Monique Ferrary Americo [1], Mariana Martins Drumond [2], Pamela Mancha-Agresti [2], Enio Ferreira [3], Juliana Guimarães Laguna [1], Luiz Carlos Júnior Alcantara [1,4,*] and Vasco Azevedo [1,*]

1 Department of Genetics, Ecology and Evolution, Federal University of Minas Gerais, Belo Horizonte 31270-901, Brazil; vivianelimabio@gmail.com (V.L.B.); lc.luiis@yahoo.com.br (L.C.L.D.J.); macedolaisa@gmail.com (L.M.T.); fernanda_alima@hotmail.com (F.L.A.B.); lucazjorge@hotmail.com (L.J.d.S.F.); andria.sfreitas@gmail.com (A.d.S.F.); moniquefamerico@gmail.com (M.F.A.); jujulaguna@gmail.com (J.G.L.)
2 Department of Biological Sciences, Federal Center for Technological Education of Minas Gerais, Belo Horizonte 30421-169, Brazil; mmdrumond@gmail.com (M.M.D.); p.mancha.agresti@gmail.com (P.M.-A.)
3 Department of General Pathology, Federal University of Minas Gerais, Belo Horizonte 31270-901, Brazil; enioferreira@icb.ufmg.br
4 Flavivirus Laboratory, Oswaldo Cruz Institute, Oswaldo Cruz Foundation, Rio de Janeiro 21040-900, Brazil
* Correspondence: luiz.alcantara@ioc.fiocruz.br (L.C.J.A.); vasco@icb.ufmg.br (V.A.)
† These authors contributed equally to this work.

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Abstract: Intestinal mucositis is a commonly reported side effect in oncology practice. Probiotics are considered an excellent alternative therapeutic approach to this debilitating condition; however, there are safety questions regarding the viable consumption of probiotics in clinical practice due to the risks of systemic infections, especially in immune-compromised patients. The use of heat-killed or cell-free supernatants derived from probiotic strains has been evaluated to minimize these adverse effects. Thus, this work evaluated the anti-inflammatory properties of paraprobiotics (heat-killed) and postbiotics (cell-free supernatant) of the probiotic *Lactobacillus delbrueckii* CIDCA 133 strain in a mouse model of 5-Fluorouracil drug-induced mucositis. Administration of paraprobiotics and postbiotics reduced the neutrophil cells infiltrating into the small intestinal mucosa and ameliorated the intestinal epithelium architecture damaged by 5-FU. These ameliorative effects were associated with a downregulation of inflammatory markers (*Tlr2*, *Nfkb1*, *Il12*, *Il17a*, *Il1b*, *Tnf*), and upregulation of immunoregulatory *Il10* cytokine and the epithelial barrier markers *Ocln*, *Cldn1*, *2*, *5*, *Hp* and *Muc2*. Thus, heat-killed *L. delbrueckii* CIDCA 133 and supernatants derived from this strain were shown to be effective in reducing 5-FU-induced inflammatory damage, demonstrating them to be an alternative approach to the problems arising from the use of live beneficial microorganisms in clinical practice.

Keywords: mucositis; chemotherapy; intestinal damage; heat-killed bacteria; cell-free supernatant; immunomodulation; epithelial barrier markers

1. Introduction

Mucositis is characterized as a gastrointestinal (GIT) mucosal inflammation that frequently occurs in patients with cancer disease submitted to radiotherapy [1,2] and/or chemotherapy treatments (5-Fluorouracil, irinotecan, oxaliplatin, etc.) [3,4]. This debilitating condition is associated with pain and difficulty eating, leading to malnutrition, increasing the infection risk, alteration in the patient's clinical status, and therapy delays [5–7].

Many strategies have been investigated to prevent and/or treat mucositis [5,8]. According to the pathobiology phases described by Sonis [3], the chemotherapy-induced dysbiotic intestinal microbiota has an essential role in the progression and severity of mucositis [9].

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Evaluation of Probiotic Properties of Novel Brazilian *Lactiplantibacillus plantarum* Strains

Nina Dias Coelho-Rocha[1] · Luís Cláudio Lima de Jesus[1] · Fernanda Alvarenga Lima Barroso[1] · Tales Fernando da Silva[1] · Enio Ferreira[2] · José Eduardo Gonçalves[3] · Flaviano dos Santos Martins[4] · Rodrigo Dias de Oliveira Carvalho[5] · Debmalya Barh[6] · Vasco Ariston de Carvalho Azevedo[1]

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Abstract
Beneficial effects of *Lactiplantibacillus plantarum* strains have been widely reported. Knowing that the effects of probiotic bacteria are strain-dependent, this study aimed to characterize the probiotic properties and investigate the gastrointestinal protective effects of nine novel *L. plantarum* strains isolated from Bahia, Brazil. The probiotic functionality was first evaluated in vitro by characterizing bile salt and acidic tolerance, antibacterial activity, and adhesion to Caco-2 cells. Antibiotic resistance profile, mucin degradation, and hemolytic activity assays were also performed to evaluate safety features. In vivo analyses were conducted to investigate the anti-inflammatory effects of the strains on a mouse model of 5-Fluorouracil-induced mucositis. Our results suggest that the used *L. plantarum* strains have good tolerance to bile salts and low pH and can inhibit commonly gastrointestinal pathogens. Lp2 and Lpl1 strains also exhibited high adhesion rates to Caco-2 cells (13.64 and 9.05%, respectively). Phenotypical resistance to aminoglycosides, vancomycin, and tetracycline was observed for most strains. No strain showed hemolytic or mucolytic activity. Seven strains had a protective effect against histopathological and inflammatory damage induced by 5-FU. Gene expression analysis of inflammatory markers showed that five strains upregulated interleukin 10 (*Il10*), while four downregulated both interleukin 6 (*Il6*) and interleukin 1b (*Il1b*). Additionally, all strains reduced eosinophilic and neutrophilic infiltration; however, they could not prevent weight loss or reduced liquid/food intake. Altogether, our study suggests these Brazilian *L. plantarum* strains present good probiotic characteristics and safety levels for future applications and can be therapeutically adjuvant alternatives to prevent/treat intestinal mucositis.

Keywords Lactic acid bacteria · Probiotic potential · Stress tolerance · Antimicrobial activity · Anti-inflammatory effect · Intestinal mucositis

Introduction

Probiotics are "live microorganisms which, when administered in adequate amounts, confer a health benefit on the host" [1]. Currently, the use of these microorganisms for human consumption has received increasing attention due to the discovery of new scientific evidence regarding their functional properties and beneficial effects on the host [2]. Most probiotic strains belong to the Lactic Acid Bacteria (LAB) group [3]. Among them, *Lactiplantibacillus*

✉ Vasco Ariston de Carvalho Azevedo
vascoariston@gmail.com

[1] Department of Genetics, Ecology, and Evolution, Biological Sciences Institute, Federal University of Minas Gerais, Minas Gerais, Belo Horizonte 31270-901, Brazil

[2] Department of General Pathology, Biological Sciences Institute, Federal University of Minas Gerais, Minas Gerais, Belo Horizonte 31270-901, Brazil

[3] Department of Pharmaceutic Products, Pharmacy Faculty, Federal University of Minas Gerais, Minas Gerais, Belo Horizonte 31270-901, Brazil

[4] Department of Microbiology, Biological Sciences Institute, Federal University of Minas Gerais, Minas Gerais, Belo Horizonte 31270-901, Brazil

[5] Department of Biochemistry and Biophysics, Institute of Health Sciences, Federal University of Bahia, Salvador, Bahia 40110-909, Brazil

[6] Institute of Integrative Omics and Applied Biotechnology (IIOAB), West Bengal, Nonakuri, Purba Medinipur 721172, India

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CAPÍTULO 06

Uso De Bactérias Lácticas Como Vetores De Entrega De Vacinas Recombinantes

Monique Ferrary Américo [1]; Andria dos Santos Freitas [2];
Tales Fernando da Silva [2]; Luís Lima de Jesus [2]; Fernanda Alvarenga Lima [2];
Rodrigo Dias de Oliveira Carvalho [3]; Vasco Ariston de Carvalho Azevedo [4]

[1] *Mestranda em Genética. Programa de Pós-Graduação em Genética. Universidade Federal de Minas Gerais – UFMG*
[2] *Doutorando(a) em Genética. Programa de Pós-Graduação em Genética. Universidade Federal de Minas Gerais – UFMG*
[3] *Professor Visitante no Departamento de Bioquímica e Biofísica. Universidade Federal da Bahia - UFBA*
[4] *Professor Titular no Departamento de Genética Ecologia e Evolução. Universidade Federal de Minas Gerais – UFMG*

RESUMO

A vacinologia tem avançado rapidamente apesar de ser um campo relativamente novo da Ciência. Entre as várias estratégias que vem sendo utilizadas para o controle de doenças, o uso da tecnologia do DNA recombinante tem trazido perspectivas animadoras para o desenvolvimento de vacinas mais seguras e mais eficazes, incluindo para novos patógenos emergentes. Neste contexto, destaca-se o uso de bactérias lácticas como vetores e adjuvantes. Este capítulo contém uma revisão descritiva sobre os mais relevantes tópicos envolvendo vacinas recombinantes, incluindo a história da arte, mecanismos, estratégias imunizantes e avanços recentes obtidos em estudos de prova de conceito para o desenvolvimento de novas vacinas.

Palavras-chave: Bactérias Lácticas. Biotecnologia. Vacinas de DNA. Imunologia de mucosas.

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eBook on Inflammatory Bowel Disease

Chapter 2

The Gastrointestinal Disease to the Microbiota Treatment: From the Present to the Future

Fernanda Alvarenga Lima[1], Luís Cláudio Lima de Jesus[1], Viviane Lima Batista[1], Laísa Macedo Tavares[1], Rafael Assis[1], Mariana Martins Drumond[1,2], Vasco Azevedo[1], Pamela Mancha-Agresti[1*]

[1]*Laboratório de Genética Celular e Molecular (LGCM), Instituto de Ciências Biológicas, Departamento de Biologia Geral, Universidade Federal de Minas Gerais (UFMG), Belo Horizonte, Minas Gerais, Brazil.*

[2]*Centro Federal de Educação Tecnológica de Minas Gerais (CEFET/MG), Departamento de Ciências Biológicas, Belo Horizonte, Minas Gerais, Brazil.*

[*]***Correspondence to: Pamela Mancha-Agresti,*** *Laboratório de Genética Celular e Molecular (LGCM), Instituto de Ciências Biológicas, Departamento de Biologia Geral, Universidade Federal de Minas Gerais (UFMG), Belo Horizonte, Minas Gerais, Brazil.*

Email: p.mancha.agresti@gmail.com

1. Introduction

Chronic diseases, especially the inflammatory ones, share several characteristics to each other. In this chapter we will first discuss the main characteristics of inflammatory bowel diseases, afterwards the Metabolic Syndrome and associated diseases and, finally, intestinal mucositis that although is not a disease itlself, produces an inflammatory process with serious problems related to the discontinuation of cancer treatment. Second, we will discuss the effects of probiotics on the control, prevention or attenuation of the symptoms of these diseases and, finally, we will discuss about the changes that these diseases cause in the microbiota, emphasizing the methods and results of the researches involving the microbiota and the next steps of scientific research to elucidate the mechanisms involved in the control of inflammatory processes through its manipulation.