UNIVERSIDADE FEDERAL DE MINAS GERAIS
INSTITUTO DE CIÊNCIAS BIOLÓGICAS
DEPARTAMENTO DE GENÉTICA, ECOLOGIA E EVOLUÇÃO
PROGRAMA DE PÓS-GRADUAÇÃO EM GENÉTICA

VANESSA PECINI DA CUNHA

EFEITOS IMUNOMODULADORES DE UMA LINHAGEM DE *LACTOCOCCUS LACTIS* INVASIVA E PRODUTORA DO ANTÍGENO HSP65 DE *MYCOBACTERIUM LEPRAE* EM MODELOS MURINOS DE COLITE AGUDA E CRÔNICA INDUZIDAS PELO ÁCIDO TRINITROBENZENO SULFÔNICO (TNBS)

BELO HORIZONTE
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Tese apresentada ao Programa de Pós-Graduação em Genética, Departamento de Genética, Ecologia e Evolução, Instituto de Ciências Biológicas da Universidade Federal de Minas Gerais como requisito para a obtenção do título de Doutora em Genética.

Orientador: Prof. Dr. Anderson Miyoshi
Coorientadora: Dra. Vanessa Bastos Pereira

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ATA DA DEFESA DE TESE

Vanessa Pecini da Cunha

Às quatorze horas do dia **30 de outubro de 2020**, reuniu-se, a Comissão Examinadora de Tese, indicada pelo Colegiado do Programa, para julgar, em exame final, o trabalho intitulado: **"Efeitos imunomoduladores de uma linhagem de Lactococcus lactis invasiva e produtora do antígeno Hsp65 de Mycobacterium leprae em modelos murinos de colite aguda e crônica induzidas pelo ácido trinitrobenzeno sulfônico (TNBS)"**, requisito para obtenção do grau de Doutora em **Genética.** Abrindo a sessão, o Presidente da Comissão, **Dr. Anderson Miyoshi**, após dar a conhecer aos presentes o teor das Normas Regulamentares do Trabalho Final, passou a palavra à candidata, para apresentação de seu trabalho. Seguiu-se a arguição pelos Examinadores, com a respectiva defesa da candidata. Logo após, a Comissão se reuniu, sem a presença da candidata e do público, para julgamento e expedição de resultado final. Foram atribuídas as seguintes indicações:

Prof./Pesq.	Instituição	CPF	Indicação
Dr. Anderson Miyoshi	UFMG	03435703601	Aprovada
Dr. Álvaro Cantini Nunes	UFMG	67815960634	Aprovada
Dr. Adriana Abalen Martins Dias	UFMG	544.099.346-00	Aprovada
Dr. Marcelo de Macedo Brigido	Universidade de Brasília	31643140191	Aprovada
Dr. Andréa Queiroz Maranhão	Universidade de Brasília	284615683-20	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, 30 de outubro de 2020.

Dr. Anderson Miyoshi - Orientador _____

Dr. Álvaro Cantini Nunes _____

Dr. Adriana Abalen Martins Dias _____

Dr. Marcelo de Macedo Brigido _____

Dr. Andréa Queiroz Maranhão _____

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“A persistência é o caminho do êxito.”

Charles Chaplin

RESUMO

As doenças inflamatórias intestinais (IBDs) compreendem um conjunto de distúrbios que afetam o trato gastrointestinal. As IBDs abrangem, essencialmente, duas enfermidades: a doença de Crohn (CD) e a colite ulcerativa (UC), que são caracterizadas por uma inflamação crônica e recorrente da mucosa. Além disso, uma das principais complicações associadas à CD é o desenvolvimento de fibrose, resultante do acúmulo excessivo de colágeno nos tecidos intestinais. O tratamento, geralmente, é realizado com aminossalicilatos, corticosteroides, imunomoduladores, terapias biológicas, antibióticos e cirurgia, contudo, está atrelado à vários efeitos adversos. Em vista desse problema, há necessidade de se desenvolver tratamentos mais eficientes aos pacientes com CD. Assim, as proteínas do choque térmico (HSPs) podem ser uma alternativa, haja vista que são consideradas antígenos importantes na regulação das células T efetoras. Logo, este trabalho teve como objetivo avaliar o potencial imunomodulatório da linhagem invasiva e produtora de Hsp65 [*L. lactis* NCDO2118 FnBPA+ (pXYCYT:Hsp65)] em modelos murinos de colite aguda e crônica, como uma estratégia terapêutica alternativa contra a CD experimental. Para tal, o plasmídeo pXYCYT:Hsp65 foi transformado na linhagem *L. lactis* NCDO2118 FnBPA+, resultando na linhagem *L. lactis* NCDO2118 FnBPA+ (pXYCYT:Hsp65). Em seguida, a funcionalidade dessa linhagem foi avaliada, *in vitro*, para a produção de Hsp65 por *Western blotting* e para invasão em células Caco-2. Os resultados demonstraram que a linhagem foi capaz de produzir Hsp65 e invadir eficientemente células eucarióticas. Posteriormente, *in vivo*, a colite experimental foi induzida em camundongos BALB/c pelo ácido trinitrobenzeno sulfônico (TNBS) e o tratamento realizado oralmente com *L. lactis* NCDO2118 FnBPA+ (pXYCYT:Hsp65) por gavagem intragástrica. A administração oral dessa linhagem recombinante foi capaz de atenuar a severidade da colite aguda, por meio, principalmente, da redução dos níveis de IL-12 e IL-17 e aumento dos níveis de IL-10 e de sIgA. Em seguida, essa linhagem também foi capaz de melhorar a colite crônica (inflamação e fibrose), através da regulação das citocinas pró-fibróticas IL-13 e TGF-β e da citocina regulatória IL-10. Finalmente, este trabalho constitui uma abordagem inovadora e promissora para o tratamento alternativo da CD experimental, utilizando a imunidade de mucosas e a imunomodulação para restaurar a homeostase intestinal dessa IBD.

Palavras-chave: Doença de Crohn. TNBS. *Lactococcus lactis*. Hsp65.

ABSTRACT

Inflammatory bowel diseases (IBDs) are a set of disorders that affect the gastrointestinal tract. IBDs essentially comprise two diseases, Crohn's disease (CD) and ulcerative colitis (UC), which are characterized by chronic and recurrent inflammation of the mucosa. In addition, one of the main complications of CD is the development of fibrosis, which results from excessive accumulation of collagen in the intestine layers. The treatment is usually performed with anti-inflammatory drugs, immunosuppressants, antibiotics, biological drugs and surgery, but these treatments are linked to several adverse effects. In view of this problem, there is a need to develop more effective treatments for patients with CD. Thus, heat shock proteins (HSPs) might serve as an alternative treatment because these antigens play important roles in the regulation of effector T cells. Therefore, the present study aimed to evaluate the immunomodulatory potential of the invasive and Hsp65-producing strain [*L. lactis* NCDO2118 FnBPA+ (pXYCYT:Hsp65)] in murine models of acute and chronic colitis as an alternative therapeutic strategy against experimental CD. For this, the pXYCYT:Hsp65 plasmid was transformed into the *L. lactis* NCDO2118 FnBPA+ strain, resulting in the *L. lactis* NCDO2118 FnBPA+ (pXYCYT:Hsp65) strain. Then, the functionality of the strain was evaluated *in vitro* for Hsp65 production by *Western blotting* and for invasion into Caco-2 cells. The results demonstrated that the strain was able to produce Hsp65 and efficiently invade eukaryotic cells. Subsequently, *in vivo*, experimental colitis was induced by trinitrobenzene sulfonic acid (TNBS) in BALB/c mice, and the mice were treated orally with *L. lactis* NCDO2118 FnBPA+ (pXYCYT:Hsp65) via intragastric gavage. Oral administration of the recombinant strain was able to attenuated the severity of acute colitis by mainly reducing IL-12 and IL-17 levels and increasing IL-10 and sIgA levels. Next, this strain was also able ameliorated chronic colitis (inflammation and fibrosis) through regulation of the pro-fibrotic cytokines IL-13 and TGF-β and the regulatory cytokine IL-10. Finally, this work describes an innovative and promising approach for alternative treatment of experimental CD using mucosal immunity and immunomodulation to restore the intestinal homeostasis of this IBD.

Keywords: Crohn's disease. TNBS. *Lactococcus lactis*. Hsp65.

LISTA DE FIGURAS

LISTA DE ABREVIATURAS

15-LOX-1– Enzima oxidativa 15-lipoxigenase-1

APC – Célula apresentadora de antígeno

ATG16L1 – *Autophagy related 16 Like 1*

BCR – Receptor de células B

BGH poli-A – Sequência sinal de poliadenilação do hormônio bovino de crescimento

CARD9 – *Caspase Recruitment Domain Family Member 9*

CCL3 – *Chemokine (C-C motif) ligand 3*

CD – Doença de Crohn

CD4+ – Linfócito T CD4+

CLEC7A – *C-type lectin domain containing 7A*

Cm – Antibiótico cloranfenicol

CMV – Promotor do citomegalovírus

CYT – Citoplasmático

DC – Células dendríticas

DNA – Ácido desoxirribonucleico

DSS – Dextrano sulfato de sódio

EPO – Peroxidase eosinofílica

ESAT-6 – Proteína de 6 kDa secretada por *Mycobacterium tuberculosis*

FDA – Administração americana de alimentos e medicamentos

FnBPA+ – Proteína A de ligação a fibronectina

GABA – Ácido gama-aminobutírico

GATA-3 – Fator de transcrição de células T

GIT – Trato gastrointestinal

GRAS – Geralmente reconhecido como seguro

GWAS – Estudos de associação genômica

HLA – Antígeno leucocitário humano

HSBP – Proteína de ligação ao choque térmico

HSE – Elemento de choque térmico

HSF – Fator de choque térmico

HSP – Proteína do choque térmico

Hsp65 – Proteína do choque térmico de 65 kDa de *Mycobacterium leprae*

IBD – Doença inflamatória intestinal

IFN-γ – Interferon-γ

Ig – Imunoglobulina

IL – Interleucina

ILC – Células linfoides inatas

JAM – *Journal of Applied Microbiology*

LAB – Bactérias do ácido láctico

lHSP – *Large HSP*

LPS – Lipopolissacarídeo

LRRK2 – *Leucine-rich repeat kinase 2*

MG1363 – *Lactococcus lactis* subsp. *cremoris*

MHC II – Complexo principal de histocompatibilidade

MPO – Enzima mieloperoxidase

MUC5AC – Mucina 5AC

MUC6 – Mucina 6

NAG – Enzima N-acetilglicosaminidase

NCDO2118 – *Lactococcus lactis* subsp. *lactis*

NFATp – Fator Nuclear de Células T Ativadas

NF-κB – Fator nuclear Kappa B

NICE – Sistema de expressão controlada por nisina

NK – Células *Natural Killer*

NOD2 – *Nucleotide oligomerization domain-like receptor 2*

Nuc – Nuclease de *Staphyloccocus aureus*

ORF – Quadro de leitura aberto

OVA – Ovalbumina

pValac – Plasmídeo *Vaccination using lactic acid bacteria*

P*xylT* – Promotor induzido por xilose

RBS – Sítio de ligação ao ribossomo

repA – Origem de replicação de *Lactococcus lactis*

repC – Origem de replicação de *Escherichia coli*

RNA – Ácido ribonucleico

SEC – Secretado

sHSP – *Small HSP*

SICE – Sistema de expressão induzido por estresse

sIgA – Imunoglobulina secretória A

SP – Peptídeo sinal

T-bet – Fator de transcrição de células T

TCR – Receptor de células T

TecnoGen – Laboratório de Tecnologia Genética

TGF-β – Fator de transformação do crescimento β

Th – Célula T auxiliar

TLR – Receptor do tipo *Toll*

TNBS – Ácido 2,4,6-trinitrobenzenosulfônico

TNF-α – Fator de necrose tumoral α

Tregs – Células T regulatórias

Tregs Foxp3+ – Células T regulatórias Foxp3+

UC – Colite ulcerativa

Usp45 – Proteína de 45 kDa de *Lactococcus lactis*

XIES – Sistema de expressão induzido por xilose

ZIREX – Sistema de expressão induzido por zinco

SUMÁRIO

1 APRESENTAÇÃO

1.1 DELINEAMENTO DA TESE

Este manuscrito será composto, primeiramente, por uma Revisão de Literatura sobre as doenças inflamatórias intestinais, as proteínas de choque térmico e as bactérias do ácido láctico. Em relação as bactérias lácticas, será apresentado, mais especificamente, a bactéria láctica modelo *Lactococcus lactis*, como veículo para a entrega de novas estratégias terapêuticas às mucosas. Após a Revisão literária, será apresentada as Justificativas para a realização deste trabalho, assim como os Objetivos Gerais e Específicos pretendidos com este estudo.

Na sequência, os Resultados obtidos serão apresentados em duas versões, sendo: a) um artigo científico publicado na revista *Journal of Applied Microbiology* (JAM – Fator de Impacto 2.683); e, b) um artigo científico sob revisão na revista *Scientific Reports* (Fator de Impacto 3.998). Desta forma, as seções de Material e Métodos e Discussão foram suprimidas e as informações relacionadas à essas seções serão apresentadas nas versões dos artigos. O artigo publicado na JAM, descreve a construção da linhagem invasiva e produtora de Hsp65 [*L. lactis* NCDO2118 FnBPA+ (pXYCYT:Hsp65)], bem como a sua funcionalidade *in vitro* e a capacidade anti-inflamatória em modelo murino de colite aguda, induzida quimicamente pelo ácido TNBS. No segundo artigo, será apresentado o potencial anti-inflamatório e antifibrótico dessa linhagem recombinante em modelo de colite crônica, também induzida por TNBS. Esses modelos experimentais mimetizam aspectos fundamentais da doença de Crohn em seres humanos, como: o perfil de resposta imune inflamatória e a fibrose intestinal.

Posteriormente, serão apresentadas as Conclusões, as Perspectivas e as Referências consultadas para a elaboração deste manuscrito. Finalmente, nos Anexos, serão apresentadas três colaborações em artigos científicos e duas participações em capítulos de livros, ambas desenvolvidas e publicadas pela equipe do Laboratório de Tecnologia Genética – TecnoGen – da Universidade Federal de Minas Gerais (UFMG).

2 REVISÃO DE LITERATURA

2.1 DOENÇAS INFLAMATÓRIAS INTESTINAIS

Comumente conhecidas como IBDs, as doenças inflamatórias intestinais referem-se, principalmente, a duas desordens: a doença de Crohn (CD, do inglês, *Crohn's Disease*) e a colite ulcerativa (UC, do inglês, *Ulcerative Colitis*). Ambas as enfermidades se caracterizam por uma inflamação idiopática crônica e recidivante da mucosa do trato gastrointestinal (GIT, do inglês, *Gastrointestinal Tract*) (FOERSCH; WALDNER; NEURATH, 2013).

As IBDs representam um importante problema de saúde pública mundial, ocorrendo de maneira mais drástica em países desenvolvidos (MOLODECKY et al., 2012). Essas doenças, afetam, aproximadamente, 1,5 milhão de pessoas na América do Norte e 3,2 milhões de pessoas na Europa (NG et al., 2017; ANANTHAKRISHNAN; KAPLAN; NG, 2020).

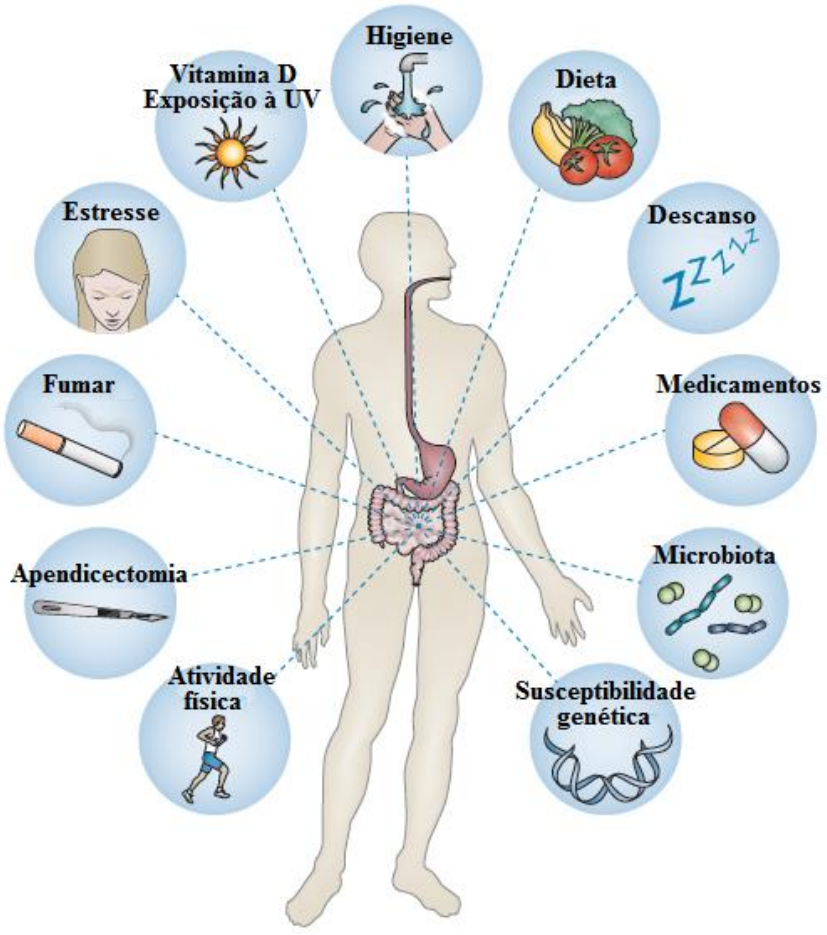
Ademais, em países subdesenvolvidos a incidência e a prevalência são menores, contudo, nas últimas décadas, a prevalência na América Latina, incluindo o Brasil, tem aumentado notavelmente (KAPLAN, 2015; QUARESMA; KAPLAN; KOTZE, 2019). Estudos brasileiros relataram uma prevalência no número de casos por 100.000 habitantes de: 22,6 em Botucatu/São Paulo (VICTORIA; SASSAK; NUNES, 2009); 12,8 no Piauí (PARENTE et al., 2015); 38,2 no Espírito Santo (LIMA MARTINS; VOLPATO; ZAGO-GOMES, 2018); e 52,6 em São Paulo (GASPARINI; SASSAKI; SAAD-HOSSNE, 2018).

Esses estudos também mostraram que as IBDs são mais comuns nas regiões mais desenvolvidas do Brasil, como o Sudeste (VICTORIA; SASSAK; NUNES, 2009; LIMA MARTINS; VOLPATO; ZAGO-GOMES, 2018; GASPARINI; SASSAKI; SAAD-HOSSNE, 2018), em relação às regiões com menor índice de desenvolvimento, como o Nordeste (PARENTE et al., 2015). Além disso, o Sudeste possui um estilo de vida mais semelhante aos países desenvolvidos, com uma dieta ocidental e influências genéticas, já que a população é parcialmente composta por imigrantes europeus e seus descendentes (QUARESMA; KAPLAN; KOTZE, 2019).

Tais distúrbios intestinais, acometem indivíduos jovens, entre 15 e 40 anos (NEURATH, 2014), podendo ter impactos sociais em sua qualidade de vida e econômicos significativos pela ausência ao trabalho (FRÓES et al., 2018; PARRA et al., 2019). As IBDs afetam ambos os sexos, no entanto, alguns estudos relataram uma maior tendência, no número de casos, no sexo feminino (VICTORIA; SASSAK; NUNES, 2009; PARENTE et al., 2015; GASPARINI; SASSAKI; SAAD-HOSSNE, 2018).

Embora a etiopatogenia não esteja totalmente elucidada, acredita-se que vários fatores contribuam para o desencadeamento da inflamação da mucosa intestinal, tais como: a genética, a imunologia, o microbioma e o meio ambiente (Figura 1) (ANANTHAKRISHNAN, 2015). Desse modo, a manifestação clínica das IBDs ocorrem em indivíduos geneticamente predispostos que apresentam uma resposta imunológica anormal à microbiota intestinal após a exposição aos estímulos ambientais (PONDER; LONG, 2013; KAPLAN, 2015).

Figura 1 – As interações entre a genética, a imunologia e a microbiota do hospedeiro, bem como a exposição ambiental levam ao desenvolvimento das IBDs.



Fonte: Adaptado pela autora de acordo com a bibliografia estudada (ANANTHAKRISHNAN, 2015).

No que se refere aos aspectos genéticos, por meio de estudos de associação genômica (GWAS, do inglês, G*enome-Wide Association Studies*) foram identificados mais de 230 alelos de risco associados com as IBDs (TURPIN et al., 2018). Ademais, esses alelos estão localizados em genes que estão envolvidos, mais especificamente, na resposta imune ao microbioma intestinal. Por exemplo, mutações nos genes NOD2, ATG16L1 e LRRK2 diminuem a secreção de peptídeos antimicrobianos pelas células de Paneth; mutações em CLEC7A e CARD9 estão associadas à diminuição da abundância de espécies de *Lactobacillus*, devido ao reconhecimento imune alterado pelas células dendríticas (DCs, do inglês, *Dendritic Cells*) e pelos macrófagos; mutações em NOD2 estão relacionadas também com o aumento da abundância de espécies de *Escherichia* e *Bacteroides vulgatus* e a diminuição das espécies de *Faecalibacterium* (JOSTINS et al., 2012; COHEN et al., 2019). Além disso, uma sinalização prejudicada por ATG16L1 está associada com o aumento da produção das imunoglobulinas IgG e IgA contra a microbiota comensal, resultando assim, na perda da tolerância à microbiota intestinal; e, polimorfismos nos genes de MHC II ou HLA afetam a produção de IgA em resposta à microbiota. Por fim, defeitos na produção de muco, devido a mutações nos genes MUC5AC e MUC6, alteram o microbioma intestinal e aumentam a suscetibilidade à colite (COHEN et al., 2019).

As alterações imunológicas, dos portadores das IBDs, caracterizam-se por uma ativação descontrolada das células imunes intestinais que culminam na natureza progressiva e destrutiva dessas patologias (NEURATH, 2019; RODA et al., 2020). Além disso, a CD é associada a uma resposta de perfil imune Th1 e Th17, mediado, predominantemente, pela secreção das citocinas IFN-γ e TNF-α, enquanto a UC é mediada por células Th2, Th9 e Th17 que produzem IL-5, IL-9 e IL-13 (TOMASELLO et al., 2014; NEURATH, 2017). Enfim, o aumento de citocinas produzidas pelas células T regulatórias (Tregs), como IL-10 e TGF-β, contribuem para melhoria do quadro inflamatório das IBDs (VASOVIC et al., 2016; SCHETT; NEURATH, 2018).

Em relação ao microbioma, sabe-se que em caso de disbiose, o desenvolvimento da inflamação está frequentemente associado com distúrbios quantitativos e qualitativos da biodiversidade microbiana (TOMASELLO et al., 2014; LLOYD-PRICE et al., 2019). Desse modo, pacientes com IBDs, apresentam uma redução da diversidade de filos e gêneros bacterianos, como: *Firmicutes*, *Bacteroidetes*, *Lactobacillus* e *Eubacterium* (HOLD et al., 2014; MENTELLA et al., 2020). E, um aumento da família *Enterobacteriaceae*, assim como: *Enterococcus* e *E. coli* na CD e UC (TURPIN et al., 2018). Finalmente, quanto à composição da microbiota, tem-se notado um aumento das espécies anaeróbias facultativos, como: *E. coli*,

Fusobacterium varium e *Enterococcus faecalis* em relação aos anaeróbios obrigatórios, como: *Clostridium* e *Faecalibacterium prausnitzii* (OKA; SARTOR, 2020).

Os fatores ambientais contribuem para o desenvolvimento das IBDs, como também atuam agravando os seus sintomas. Dentre esses fatores, pode-se citar: o tabagismo, a dieta e os hábitos de higiene na infância (PONDER; LONG, 2013; ANANTHAKRISHNAN, 2015). O tabagismo, por exemplo, aumenta o risco de desenvolver a CD, assim como o risco de cirurgia precoce e recorrência pós-operatória; e a cessação do tabagismo melhora o seu prognóstico (KAPLAN, 2015; ANANTHAKRISHNAN; KAPLAN; NG, 2020). Ainda, no mundo ocidental, as dietas ricas em gorduras saturadas e pobres em fibras estão associadas às IBDs (GREEN et al., 2019). E, a hipótese da higiene, postula que, em sociedades urbanizadas, as crianças têm menos exposição aos microrganismos ambientais, desse modo, maior chance de que infecções, na vida adulta, desencadeiem uma resposta imune anormal no hospedeiro (SELVARATNAM et al., 2019). Essa resposta imunológica desregulada, consequentemente, resultaria no desenvolvimento de uma inflamação no GIT, como a CD (RODA et al., 2020).

2.1.2 Doença de Crohn

A CD tem uma incidência próxima de 12,7 na Europa, 5 na Ásia e no Oriente Médio e 20,2 na América do Norte e, por conseguinte, uma prevalência de 322 na Europa e 219 na América do Norte por 100.000 habitantes (MOLODECKY et al., 2012). O Brasil, infelizmente, não dispõe de dados epidemiológicos consistentes, devido à dificuldade de diagnóstico, bem como da carência de informações confiáveis e unificadas. No entanto, sabe-se por meio de relatos regionais que há um aumento da incidência e da prevalência da CD no país (VICTORIA; SASSAK; NUNES, 2009; LIMA MARTINS; VOLPATO; ZAGO-GOMES, 2018; GASPARINI; SASSAKI; SAAD-HOSSNE, 2018; QUARESMA; KAPLAN; KOTZE, 2019).

A título de exemplo, estudos brasileiros relataram uma incidência e prevalência, respectivamente, de: 3,5 e 5,65 em Botucatu, no centro-oeste de São Paulo (VICTORIA; SASSAK; NUNES, 2009); 5,3 e 14,1 no Espírito Santo (LIMA MARTINS; VOLPATO; ZAGO-GOMES, 2018); 6,14 e 24,3 em São Paulo (GASPARINI; SASSAKI; SAAD-HOSSNE, 2018) por 100.000 habitantes. Ademais, trabalhos também revelaram que essa

enfermidade apresenta uma maior incidência na população branca e parda do que na população amarela e negra (VICTORIA; SASSAK; NUNES, 2009; SCHOFFEN; PRADO, 2011).

A CD é uma síndrome que acomete, mais frequentemente, indivíduos com menos de 30 anos, afetando, adolescentes e adultos (RODA et al., 2020); no entanto, casos em crianças têm sido descritos (SUNDQVIST et al., 2019). Essa doença pode afetar qualquer parte do GIT, não obstante, as regiões mais afetadas do intestino, são: o íleo terminal, o ceco, o cólon e a região perianal (BOUMA; STROBER, 2003). A inflamação é salteada, caracterizando-se pela presença de segmentos de intestino normal com regiões afetadas, e a histologia é transmural, afetando todas as camadas da parede intestinal (NEURATH, 2014).

O sintoma principal dessa doença é a dor abdominal associada à diarreia, febre, fadiga, perda de peso e enfraquecimento devido à dificuldade na absorção de nutrientes (TORRES et al., 2017; BALESTRIERI et al., 2020). Além disso, uma das principais complicações da CD é o desenvolvimento de fibrose, caracterizada pelo acúmulo excessivo de matriz extracelular rica em colágeno nos tecidos do intestino (ROGLER; HAUSMANN, 2017; LIAN et al., 2018). A fibrose intestinal relacionada à CD, ocorre em 30-50% dos pacientes, podendo causar sérios danos, como: estenose, fístula ou abscessos (LI; KUEMMERLE, 2014; CURCIARELLO; DOCENA; MACDONALD, 2017). Ademais, a fibrose é responsável por 75% das ressecções cirúrgicas, na primeira década, após o seu diagnóstico clínico (YUN; KIM; KIM, 2019).

A severidade da doença pode variar de leve à severa e o tratamento médico é realizado a fim de diminuir a inflamação anormal do GIT e também aliviar os sintomas gerais. Apesar disso, o objetivo principal é alcançar a remissão da doença e mantê-la pelo maior tempo possível (AKOBENG et al., 2016). Assim, dentre os medicamentos utilizados para esses propósitos, encontram-se: aminossalicilatos, corticosteroides, imunomoduladores, terapias biológicas e antibióticos (SOBRADO et al., 2016).

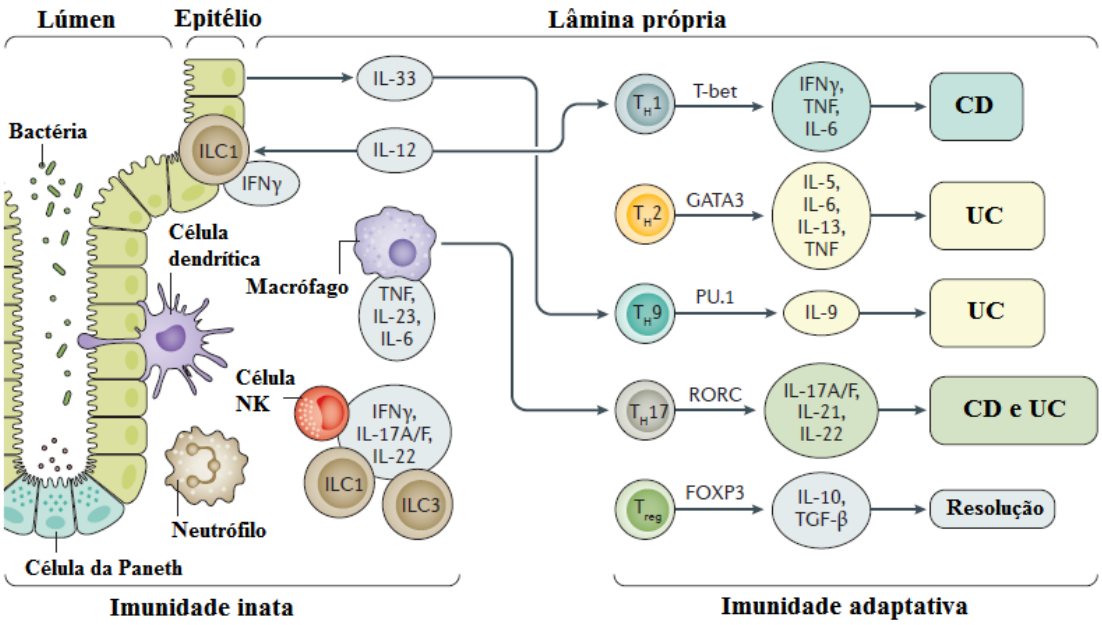
Dentre os aminossalicilatos, a sulfassalazina e a mesalazina atuam no revestimento intestinal e diminuem a inflamação (COWARD et al., 2017). Os corticosteroides, como a predizolona e a budesonida tem ação anti-inflamatória rápida e não devem ser usados cronicamente (YLISAUKKO-OJA et al., 2019). Em meio aos imunomoduladores, a azatioprina, 6-mercaptopurina, metotrexano, ciclosporina e tacrolimus atuam suprimindo a resposta imune do hospedeiro para que não causem uma inflamação contínua (CHANDE;

TSOULIS; MACDONALD, 2013; NEURATH, 2017). Nas terapias biológicas, o infliximabe e o adalimumabe são anticorpos monoclonais, anti-TNF, que suprimem a inflamação intestinal (NEURATH, 2019). E, os antibióticos, como o metronidazol e o ciprofloxacino são usados quando ocorrem infecções com abscessos e fístulas na região anal (NITZAN et al., 2016).

Conforme exposto acima, existe uma infinidade de compostos farmacêuticos disponíveis para o tratamento da CD, todavia, estão associados a sérios efeitos adversos, como por exemplo: dor de cabeça, diarreia e náuseas; o que contribui para reduzir a adesão do paciente e piorar sua condição de saúde (FAKHOURY et al., 2014). Existe, ainda, o tratamento cirúrgico por meio de ressecção ou enteroplastia para os casos em que ocorre complicações, tais como: estenose, abscesso, fístula, hemorragia ou câncer; ou quando é caracterizado a intratabilidade clínica (SOLINA et al., 2016; SAPCI; GORGUN, 2019).

Devido a CD se tratar de uma IBD multifatorial, será abordado a seguir, mais detalhadamente, os aspectos da imunidade inata e adaptativa relacionadas à inflamação intestinal aguda e crônica dessa enfermidade. Dessa forma, uma barreira intestinal defeituosa, associada à disbiose microbiana induz a um acúmulo e a uma ativação de células imunes locais, resultando na secreção de citocinas pró-inflamatórias que substituem os sinais anti-inflamatórios e causam uma inflamação intestinal crônica (Figura 2) (NEURATH, 2019).

Figura 2 – Células imunes e citocinas nas IBDs.



Fonte: Adaptado pela autora de acordo com a bibliografia estudada (NEURATH, 2017).

A inflamação intestinal se inicia com as células apresentadoras de antígenos (APCs, do inglês, *Antigen Presenting Cell*), como: macrófagos e DCs, secretando a interleucina pró-inflamatória IL-12 (EFTYCHI et al., 2019). Inclusive, a IL-12 é considerada a principal citocina da resposta imune Th1 e identificada, na literatura, como sendo a mediadora central da colite humana (STROBER; FUSS, 2011). Posteriormente, a IL-12 leva à ativação das células Th1, que por sua vez, iniciam a secreção de outras citocinas pró-inflamatórias, como: IFN-γ, TNF-α e IL-6 (ATREYA; NEURATH, 2018). Vale ressaltar, ainda, que TNF-α e IL-6 são importantes mediadoras da inflamação crônica na colite (LAWRANCE et al., 2017; NEURATH, 2019).

Além disso, uma resposta de perfil Th17 também tem sido associada com a CD (ATREYA; NEURATH, 2018). Desse modo, durante a progressão inflamatória, o aumento da citocina IL-23, sintetizada por células mielóides e pelos neutrófilos, levam à ativação das células Th17. Além disso, IL-23 também ativa as células linfóides inatas do tipo 3 (ILC3, do inglês, *Type 3 Innate Lymphoid Cells*), que induzem a secreção de IL-17A, IL-17F e IL-22 (NEURATH, 2019). Ademais, a IL-23 causa a inflamação crônica do cólon em resposta ao comprometimento da barreira epitelial intestinal (EFTYCHI et al., 2019).

A IL-13 é uma citocina de perfil imune Th2 com efeitos pleiotrópicos. E, é produzida por uma variedade de tipos celulares, tais como: células Th2 CD4+, células linfóides inatas do tipo 2 (ILC2), eosinófilos, mastócitos, basófilos e células NK (CURCIARELLO; DOCENA; MACDONALD, 2017). Além disso, é uma importante indutora de fibrose em doenças autoimunes crônicas, como a CD (RIEDER; FIOCCHI, 2009; GIUFFRIDA et al., 2019). Nesse sentido, essa interleucina e seu receptor estão super expressos em áreas de fibrose em pacientes com a doença (LEE; KWON; CHO, 2018).

Por outro lado, na resolução da inflamação intestinal, as células Tregs Foxp3+, produzindo IL-10 e TGF-β, estão, comumente, envolvidas (SCHETT; NEURATH, 2018). A IL-10, ponderada como a principal citocina regulatória da inflamação, suprime as respostas imunes exacerbadas da mucosa e mantém a homeostase intestinal e a tolerância à microbiota comensal (VASOVIC et al., 2016); bem como inibe a deposição de colágeno nos tecidos intestinais (LAWRANCE et al., 2017). Já a citocina TGF-β é uma importante imunossupressora, contudo, possui um papel pró-fibrogênico durante a CD (YUN; KIM; KIM, 2019; ISWANDANA et al., 2020).

Enfim, considerando que até o presente momento não há conhecimento exato sobre o agente causal da CD e da necessidade de tratamentos mais eficazes aos seus portadores, faz-se necessário a busca por novas estratégias terapêuticas para essa IBD. Neste contexto, o estudo em modelos animais de colite são ferramentas indispensáveis para a compreensão das alterações histopatológicas e morfológicas do intestino, assim como para o desenvolvimento de novas moléculas terapêuticas (RANDHAWA et al., 2014; WIRTZ et al., 2017).

2.1.3 Modelos murinos de colite induzidas por TNBS

O modelo murino de inflamação intestinal induzida pelo ácido trinitrobenzeno sulfônico (TNBS, do inglês, *2,4,6 Trinitrobenzene Sulfonic Acid*), é usado, frequentemente, para estabelecer a inflamação aguda (ALBUQUERQUE et al., 2020), como também a inflamação crônica (GAO et al., 2020). A inflamação do cólon pode ser realizada em camundongos, como: SJL/J, C57BL/6 e BALB/c, por meio da administração intraretal do TNBS juntamente com etanol (RANDHAWA et al., 2014). O etanol, neste caso, é essencial para quebrar a barreira da mucosa, enquanto o TNBS hapteniza proteínas autólogas ou microbianas do cólon, tornando-as imunogênicas para o sistema imunológico do hospedeiro (WIRTZ et al., 2007; JIMINEZ et al., 2015).

Esse modelo, induz uma inflamação transmural difusa no intestino, caracterizada pelo aumento da infiltração de leucócitos, edema e ulceração (ISIK et al., 2011; SCHULTE et al., 2019). Ademais, pode resultar em colite crônica, com: diarreia grave, perda de peso e prolapso retal (RANDHAWA et al., 2014). A administração desse agente químico, em modelos animais, está associada à ativação, predominantemente, da resposta imune Th1, manifestada pela infiltração de linfócitos T CD4+ para o local inflamado (WIRTZ et al., 2017). Assim, devido a resposta Th1 envolvendo as citocinas efetoras IL-12 e TNF-α, esse modelo foi, especificamente, relacionado com a CD em seres humanos (ANTONIOU et al., 2016; QIN et al., 2019).

Experimentalmente, na colite aguda, a resposta imune inicia-se com um perfil Th1 e aumento das citocinas IL-12, IFN-γ e TNF-α (JONES-HALL; GRISHAM, 2014). De maneira secundária, ocorre o aumento da produção de IL-23, indicando o início da colite crônica (JIMINEZ et al., 2015). No decurso da inflamação, o perfil de citocinas muda para Th17, devido ao aumento das citocinas IL-17 e IL-25 e, finalmente, se altera para uma resposta imune com

predomínio das citocinas IL-13 e TGF-β (JIMINEZ et al., 2015). Além disso, a IL-13 contribui para promover uma fibrose persistente na lâmina própria de camundongos BALB/c com a colite crônica induzida por TNBS (KIESLER; FUSS; STROBER, 2015; GIUFFRIDA et al., 2019).

Desta forma, a CD, por possuir uma condição progressiva, sem cura e que requer cuidados por toda a vida, faz-se necessário a busca por tratamentos mais eficientes e com menos efeitos adversos. Logo, as proteínas do choque térmico (HSPs, do inglês, *Heat Shock Proteins*), consideradas imunorreguladoras, podem ser uma nova alternativa terapêutica a ser investigada com o intuito de manter a homeostase intestinal em modelos experimentais de CD.

2.2 PROTEÍNAS DO CHOQUE TÉRMICO

As HSPs são chaperonas conservadas evolutivamente e importantes para sobrevivência das células procarióticas e eucarióticas (VAN EDEN; BONORINO; VAN DER ZEE, 2013; TUKAJ; KAMINSKI, 2019), pois, são essenciais em diversos processos celulares, tais como: dobragem, redobragem, translocação e degradação de proteínas intracelulares sob condições normais e de estresse (TOMASELLO et al., 2014; ZININGA; RAMATSUI; SHONHAI, 2018).

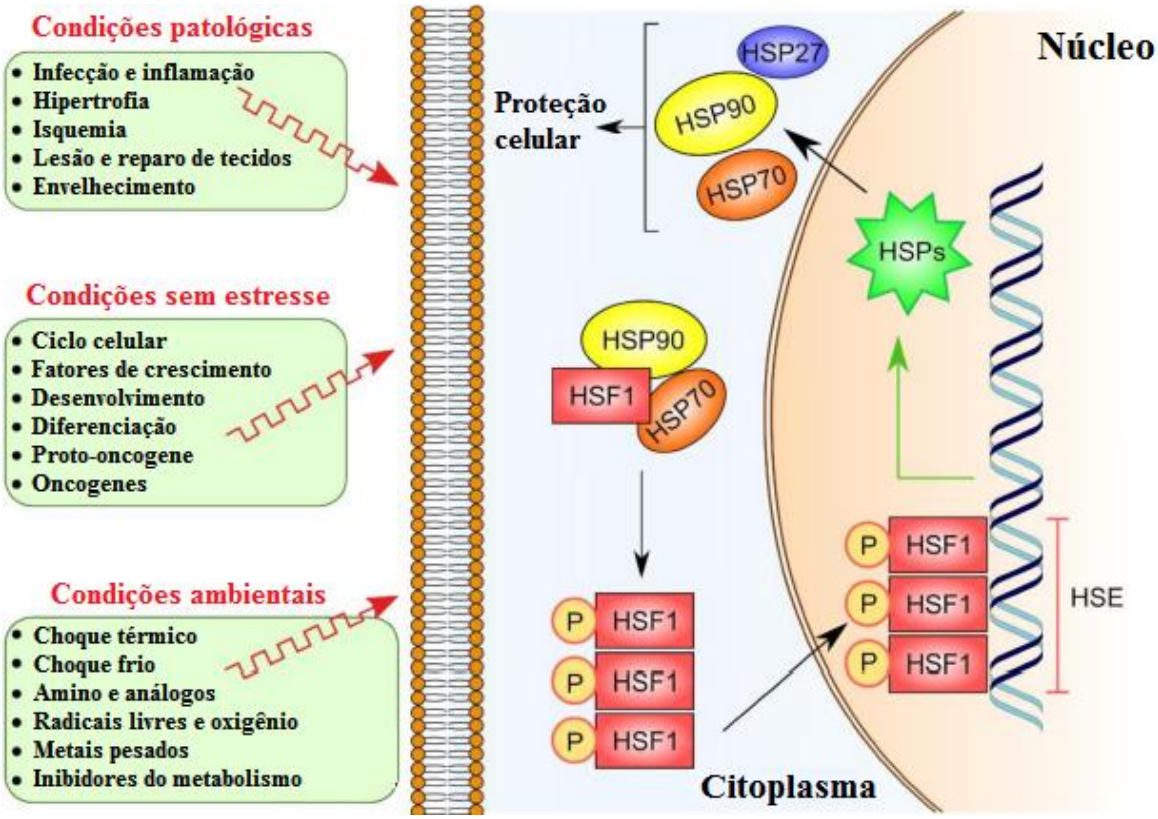
Essas moléculas são classificadas em seis famílias com base em seu peso molecular, como: HSPs pequenas (sHSPs, do inglês, *Small HSPs*), HSP40, HSP60, HSP70, HSP90 e HSPs grandes (lHSPs, do inglês, *Large HSPs*) (FINLAYSON-TRICK et al., 2019). Além disso, são encontradas em uma variedade de locais na célula, incluindo: membrana plasmática, citoplasma, mitocôndria, retículo endoplasmático e núcleo; mas também podem ocorrer extracelularmente (BOLHASSANI; AGI, 2019; CAPPELLO et al., 2019).

As HSPs são altamente homólogas dentre as espécies, em especial, entre bactérias e humanos (VAN EDEN et al., 2019). Assim, devido a esse mimetismo molecular, algumas HSPs mamíferas possuem homólogos microbianos conservados, resultando, em um reconhecimento imunológico cruzado entre elas (ZONNEVELD-HUIJSSOON et al., 2013). Inclusive, HSP60 e HSP70 micobacterianas induzem células Tregs, produtoras de IL-10, em diversos modelos experimentais de doenças inflamatórias autoimunes (VAN EDEN et al., 2017).

Essas chaperoninas são expressas constitutivamente nas células (TUKAJ; KAMINSKI, 2019), contudo, sob condições de estresse passam a ser super expressas (DUBREZ et al., 2020). Dentre essas condições de estresse, pode-se incluir: o aumento da temperatura (febre), a deficiência nutricional, a exposição a mediadores pró-inflamatórios (TNF-α e IFN-γ), o estresse oxidativo, o tratamento com drogas anti-inflamatórias não esteroides, substâncias tóxicas e infecções virais ou bacterianas (VAN EDEN; VAN DER ZEE; PRAKKEN, 2005; MILANI; BASIRNEJAD; BOLHASSANI, 2019).

Condições adversas, como as supracitadas, acarretam em dobramentos incorretos ou agregações proteicas que desencadeiam uma resposta ao estresse e induzem a transcrição dos genes das HSPs (Figura 3) (HOTER; RIZK; NAIM, 2019). Essas proteínas, por sua vez, tentam reestabelecer o equilíbrio entre a síntese, a montagem e a degradação proteica (POCKLEY, 2001; HOTER; RIZK; NAIM, 2019).

Figura 3 – Regulação da transcrição dos genes das HSPs pelo fator de choque térmico 1 (HSF1).



Fonte: Adaptado pela autora de acordo com a bibliografia estudada (HOTER; RIZK; NAIM, 2019).

A regulação da transcrição dos genes das HSPs é mediada pela interação entre os fatores de transcrição, como o fator de choque térmico (HSF, do inglês, *Heat Shock Factor*) e o elemento de choque térmico (HSE, do inglês, *Heat Shock Elements*) nas regiões promotoras dos genes. Assim, em condições normais, o HSF1, o principal fator em vertebrados, está presente no citoplasma como uma molécula monomérica latente que é incapaz de ligar-se ao DNA (POCKLEY, 2001; HOTER; RIZK; NAIM, 2019).

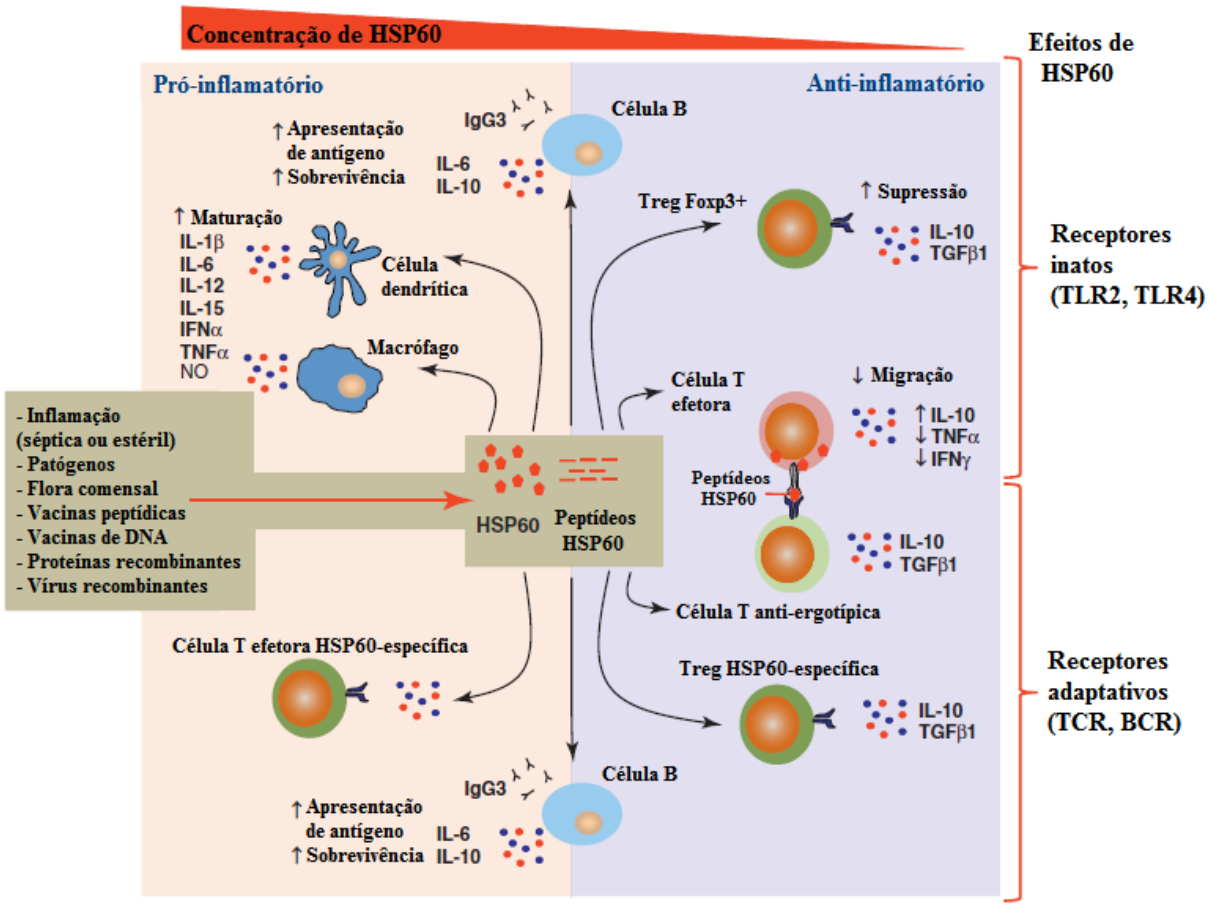
No entanto, em situações de estresse e com o aumento de proteínas não funcionais, HSF1 é fosforilado (P) e trimerizado. Posteriormente, os trímeros translocam-se do citoplasma para o núcleo e se ligam a regiões promotoras, mediando a transcrição dos genes das HSPs. A atividade dos trímeros de HSF é regulada pelas HSPs, como HSP70 e HSP90, e pela proteína de ligação ao choque térmico 1 (HSBP1, do inglês, *Heat Shock Binding Protein* 1) (POCKLEY, 2001; HOTER; RIZK; NAIM, 2019).

Além das funções descritas anteriormente e dada à versatilidade funcional das HSPs, como importantes moléculas antigênicas, essas podem ser utilizadas como agentes terapêuticos para a geração de imunidade protetora, ou ainda, para a regulação de processos inflamatórias (VAN EDEN et al., 2019). Neste contexto, as HSP60 são antígenos imunodominantes capazes de induzir a produção de anticorpos e a ativação de células T (COELHO; FARIA, 2012; COHEN; ZANIN-ZHOROV, 2013; QUINTANA; COHEN, 2011; ZININGA; RAMATSUI; SHONHAI, 2018).

2.2.1 HSP60 e a regulação da inflamação

As proteínas HSP60 são antígenos imunogênicos, que podem ser ativados tanto pelo sistema imune inato (via TLR2 e TLR4) (COHEN; ZANIN-ZHOROV, 2013; MILANI; BASIRNEJAD; BOLHASSANI, 2019), quanto pelo sistema imune adaptativo (via TCR e BCR) (QUINTANA; COHEN, 2011). Além do mais, a concentração local das HSP60 irá determinar se as suas funções serão pró-inflamatórias ou anti-inflamatórias (Figura 4) (QUINTANA; COHEN, 2011; ZININGA; RAMATSUI; SHONHAI, 2018).

Figura 4 – Efeitos imunológicos das HSP60 mediados pelo sistema imune inato e adaptativo.



Fonte: adaptado pela autora de acordo com a bibliografia estudada (QUINTANA; COHEN, 2011).

Assim, em altas concentrações de HSP60, por meio do contato natural ou artificial, a ativação via TLR (principalmente via TLR4) de DCs e macrófagos, promovem uma inflamação por diversos mecanismos, como: maturação de DCs, aumento da apresentação de antígenos e secreção de citocinas pró-inflamatórias, tais como: IL-1β, IL-6, IL-12, IL-15, IFN-γ e TNF-α (QUINTANA; COHEN, 2011). Essa atuação pró-inflamatória ocorre por meio de monócitos (macrófagos e DCs), células B e T efetoras (ZININGA; RAMATSUI; SHONHAI, 2018).

Por outro lado, baixas concentrações de HSP60, favorecem um perfil anti-inflamatório, por meio da sinalização, via TLR2, em células efetoras e Tregs CD4+CD25+ (Foxp3+) (ZANIN-ZHOROV et al., 2003; QUINTANA; COHEN, 2011). O reconhecimento de peptídeos-HSP60 (próprios ou bacterianos) pelas células T HSP60-específicas por meio de seus TCRs podem promover inflamação (por meio de células T efetoras HSP60-específica) ou interromper a inflamação (por meio de células T anti-ergotípicas) (VAN EDEN; VAN DER ZEE; PRAKKEN, 2005; ZININGA; RAMATSUI; SHONHAI, 2018).

Ademais, as HSP60 atuam como co-estimuladoras de células Tregs, pois modulam a expressão de fatores de transcrição envolvidos na diferenciação das células T, diminuindo a expressão dos fatores de Th1, como T-bet, NF-κB e NFATp, e aumentando os de Th2, como o GATA-3 (ZANIN-ZHOROV et al., 2005). Assim, células T ativadas por HSP60 diminuem a secreção de TNF-α e IFN-γ, e aumentam a secreção de citocinas regulatórias, como a IL-10 e TGF-β (VAN EDEN; VAN DER ZEE; PRAKKEN, 2005; ZANIN-ZHOROV et al., 2005; QUINTANA; COHEN, 2011).

As células B também estão envolvidas nos mecanismos de regulação da inflamação envolvendo as proteínas HSP60, podendo, então, mediar tanto efeitos pró-inflamatórios (via apresentação de anticorpos e antígenos), quanto efeitos anti-inflamatórios (via produção de IL-10). Além disso, as células B, são estimuladas a proliferar, a resistir à apoptose e a produzir anticorpos IgG3 (QUINTANA; COHEN, 2011).

Por fim, as HSP60 parecem ser antígenos atraentes para o tratamento das doenças autoimunes crônicas, por proporcionar os efeitos anti-inflamatórios, referidos anteriormente, e também por sua expressão ser regulada no local da inflamação (QUINTANA; COHEN, 2011; VAN EDEN et al., 2017). Consistente com isso, vários estudos demonstraram o potencial imunorregulador dessas proteínas em diversos modelos animais, como por exemplo: artrite e colite (ULMANSKY et al., 2015), diabetes tipo 1 (LU et al., 2016), esclerose múltipla (ZORZELLA-PEZAVENTO et al., 2017) e aterosclerose (HU et al., 2018).

2.3 A BACTÉRIA LÁCTICA MODELO – *Lactococcus lactis*

L. lactis é um importante microrganismo pertencente ao grupo heterogêneo das bactérias do ácido láctico (LAB, do inglês, *Lactic Acid Bacteria*) (WELLS, 2011; SONG et al., 2017). Essas bactérias, secretam esse ácido como um dos produtos fermentativos do metabolismo dos carboidratos (CASTILLO MARTINEZ et al., 2013; WELS et al., 2019). Até o momento, *L. lactis* compreende quatro subespécies, nomeadamente: *L. lactis* subsp. *lactis*, *L. lactis* subsp. *cremoris*, *L. lactis* subsp. *hordniae* e *L. lactis* subsp. *tructae* (KHEMARIYA et al., 2017).

Embora, *L. lactis* esteja frequentemente associada a laticínios, essa bactéria foi isolada originalmente de plantas (SONG et al., 2017). Fenotipicamente, essas bactérias lácticas são classificadas, como: gram-positivas, anaeróbias facultativas, não produtoras de catalase, não

formadoras de esporos, heterofermentativas e imóveis (PONTES et al., 2011; WYSZYŃSKA et al., 2015; OLIVEIRA et al., 2017). Ademais, *L. lactis* é uma espécie mesófila, tendo, portanto, uma temperatura ideal de crescimento em torno de 30°C (PONTES et al., 2011).

L. lactis tem sido utilizada há séculos pelos seres humanos na fermentação de alimentos, especialmente: queijos, iogurtes, chucrutes e similares (SONG et al., 2017). Assim, tornando-se reconhecidamente como segura e detentora do *status* GRAS (do inglês, *Generally Regarded as Safe*) pela FDA (do inglês, *American Food and Drug Administration*) (DE CASTRO et al., 2018). Além de conferir sabor e textura, *L. lactis* por meio da produção do ácido láctico também afere proteção aos alimentos (WELS et al., 2019). Ademais, algumas cepas produzem bacteriocinas, ratificando, ainda mais, o seu papel na indústria alimentícia (KHEMARIYA et al., 2017).

Além da importância econômica na indústria de alimentos, *L. lactis* tornou-se a LAB modelo, quando se trata de novas aplicações biotecnológicas utilizando engenharia genética. Alguns aspectos, tornaram-na um modelo desejável, como: fácil manipulação laboratorial (MIYOSHI et al., 2004; PONTES et al., 2011), genoma pequeno e totalmente sequenciado (BOLOTIN et al., 1999; WEGMANN et al., 2007; OLIVEIRA et al., 2014) e um grande número de ferramentas de clonagem e de expressão gênica desenvolvidas (DE CASTRO et al., 2018; CHO; YIM; SEO, 2020).

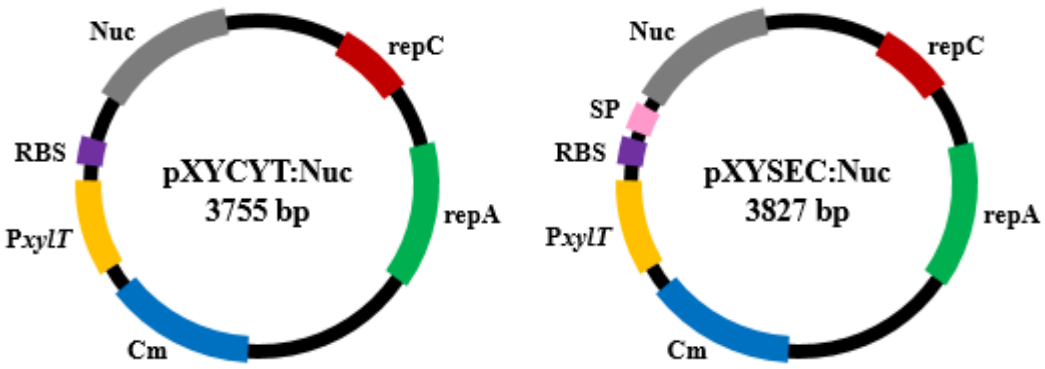
Outras características favorecem a administração de *L. lactis* como vetor para produção de moléculas de interesse médico e para a entrega de vacinas de DNA às mucosas (WELLS; MERCENIER, 2008; WELLS, 2011). Os exemplos, são: baixa imunogenicidade, podendo ser continuamente administradas em programas de imunização (MERCENIER; MÜLLER-ALOUF; GRANGETTE, 2000), resistência ao ambiente ácido estomacal, permitindo com que as bactérias cheguem íntegras à mucosa intestinal (WELLS; MERCENIER, 2008), e não possuir LPS em sua parede celular, eliminando os riscos de choque por endotoxinas (BAHEY-EL-DIN; GAHAN, 2011). Além disso, a administração de *L. lactis* através das mucosas, pode estimular repostas imunológicas tanto sistêmicas quanto de mucosas, portanto, capazes de provocar a produção da imunoglobulina secretória A (sIgA) (WELLS; MERCENIER, 2008).

2.3.2 *Lactococcus lactis* e a expressão de proteínas heterólogas

Diversos sistemas induzíveis foram desenvolvidos para a expressão de proteínas heterólogas em *L. lactis*, como por exemplo: NICE (KUIPERS et al., 1995), XIES (MIYOSHI et al., 2004), ZIREX (MU et al., 2013) e SICE (BENBOUZIANE et al., 2013). Dentre esses sistemas, destaca-se o XIES (do inglês, *Xylose-Inducible Expression System*), que foi construído pelo nosso grupo de pesquisa para expressão de proteínas recombinantes na linhagem *L. lactis* NCDO2118 (*L. lactis* subsp. *lactis*) (MIYOSHI et al., 2004).

O XIES é um vetor de expressão procarioto (Figura 5) (MIYOSHI et al., 2004). Sendo assim, constituído por um plasmídeo, contendo: a) um promotor induzido por xilose (P*xylT*); b) um sistema de endereçamento proteico [citoplasmático (CYT) ou secretado (SEC)], composto pelo sítio de ligação ao ribossomo (RBS, do inglês, *Ribosome-Binding Site*) e/ou peptídeo sinal (SP, do inglês, *Signal Peptide*) da proteína Usp45 de *L. lactis*; c) um sítio de clonagem múltipla; d) duas origens de replicação, sendo repA de *L. lactis* e repC de *E. coli*; e, e) um gene que confere resistência ao antibiótico cloranfenicol (MIYOSHI et al., 2004).

Figura 5 – Representação esquemática do sistema XIES para endereçamento citoplasmático (pXYCYT:Nuc) e secretado (pXYSEC:Nuc) de proteínas.



Fonte: Adaptado pela autora de acordo com a bibliografia estudada (MIYOSHI et al., 2004).

Inicialmente, o sistema XIES foi capaz de: a) produzir níveis elevados da proteína modelo nuclease de *Staphylococcus aureus* (NUC, do inglês, *Nuclease Gene*); b) endereçar corretamente o produto final para o citoplasma ou meio extracelular; e, c) permitir induzir ou reprimir a expressão gênica pela adição de xilose ou glicose, respectivamente. Esse sistema

apresentou, ainda, outras vantagens, como: ser de fácil manipulação, menos dispendioso e, principalmente, seguro para o uso em animais (MIYOSHI et al., 2004).

Posteriormente, outras moléculas também foram clonadas no XIES, pelo nosso grupo de pesquisa, visando ratificar, ainda mais, as provas de conceito envolvidos nesse sistema. Assim, em meio a esses trabalhos científicos, pode-se mencionar a clonagem das seguintes proteínas: Hsp65 – a proteína de choque térmico de 65 kDa de *Mycobacterium leprae* (DE AZEVEDO et al., 2012), IL-10 – a interleucina 10 de *Rattus norvegicus* (MARINHO et al., 2010) e 15-LOX-1 – a enzima oxidativa 15-lipoxigenase-1 (SARAIVA et al., 2015).

No contexto da proteína Hsp65, essa foi empregada na prevenção da encefalomielite e da colite experimental em camundongos C57BL/6 (REZENDE et al., 2013; GOMES-SANTOS et al., 2017). A administração oral de *L. lactis*, secretando Hsp65, foi capaz de impedir o desenvolvimento de encefalomielite, devido à diminuição do escore clínico, bem como a redução da produção de IL-17 e a elevação de IL-10. Ademais, houve um aumento de células Tregs (CD4+Foxp3+ e CD4+LAP+) no baço, linfonodos mesentéricos e inguinal e na medula espinhal (REZENDE et al., 2013). Utilizando a mesma estratégia, contudo, na colite induzida por DSS (do inglês, *Dextran Sulfate Sodium*), constatou-se que os efeitos terapêuticos de *L. lactis* deveu-se à diminuição das citocinas pró-inflamatórias (IFN-γ, IL-6 e TNF-α) e aumento das citocinas anti-inflamatórias (IL-10) no cólon, e a expansão de células Tregs (CD4+Foxp3+ e CD4+LAP+) no baço e nos linfonodos mesentéricos (GOMES-SANTOS et al., 2017).

Nos estudos utilizando a citocina IL-10, tanto secretada quanto citoplasmática, *L. lactis* foi utilizada no tratamento de modelos murinos para asma (MARINHO et al., 2010) e para CD (DEL CARMEN et al., 2011). A administração de IL-10, no modelo de inflamação alérgica das vias aéreas, induzido por ovalbumina (OVA), foi eficiente em suprimir a inflamação pulmonar, por meio da diminuição do número de eosinófilos, atividade da peroxidase de eosinófilos (EPO), do nível de IgE e IgG1 anti-OVA, produção de IL-4 e CCL3 e hipersecreção de muco em comparação com o grupo asmático (MARINHO et al., 2010). Já no tratamento da CD, induzida por TNBS, os camundongos BALB/c que receberam *L. lactis* produtora de IL-10 tiveram uma redução do escore de danos do intestino grosso, assim como uma redução dos níveis de IFN-γ nos fluidos intestinais (DEL CARMEN et al., 2011).

Em relação a enzima 15-LOX-1, essa molécula foi utilizada pelo fato de ser uma candidata potencial à resolução das IBDs devido a suas ações anti-inflamatórias (SARAIVA et al., 2015; CARVALHO et al., 2016). Assim, em modelo animal de IBD, induzida pelo ácido TNBS, a administração de leite fermentado por *L. lactis* produtora de 15-LOX-1 foi eficaz na prevenção dos danos inflamatórios intestinais associados à colite (SARAIVA et al., 2015). Ainda, utilizando a mesma linhagem de *L. lactis*, constatou-se que, esse tratamento foi eficiente na inflamação, induzida por DSS, diminuindo as citocinas pró-inflamatórias, como: IFN-γ e IL-4, e aumentando a citocina regulatória IL-10 (CARVALHO et al., 2016).

Finalmente, também foram descritas propriedades anti-inflamatórias para a linhagem selvagem *L. lactis* NCDO2118 (*L. lactis* subsp. *lactis*) em modelo murino de colite, induzida por DSS (LUERCE et al., 2014). O efeito protetor da linhagem foi relacionado com o aumento dos níveis de IL-10 no cólon e à indução de células Tregs nos linfonodos mesentéricos. De acordo com esse estudo, os efeitos imunomoduladores intrínsecos de *L. lactis* NCDO2118 estavam, possivelmente, relacionadas às proteínas que são secretadas pelas bactérias, como o ácido gama-aminobutírico (GABA, do inglês, *Gamma-Aminobutyric Acid*), no interior do hospedeiro (LUERCE et al., 2014; OLIVEIRA et al., 2017).

2.3.3 *Lactococcus lactis* e a entrega de vacinas de DNA

Como pode-se perceber, a utilização de *L. lactis* para a produção de proteínas heterólogas de interesse médico é, atualmente, uma realidade consolidada. No passado, à medida que novas informações e novos instrumentos genéticos eram gerados, novas possibilidades de uso eram vislumbradas para utilização nestas bactérias modelo. Sendo assim, a utilização de *L. lactis* como um veículo carreador de vacinas vivas de DNA, para células eucarióticas, foi considerada uma fronteira a ser quebrada nesta área científica.

Deste modo, o nosso grupo de pesquisa desenvolveu uma linhagem, geneticamente modificada, de *L. lactis* [*L. lactis* MG1363 (*L. lactis* subsp. *cremoris*)] que, após ter sido transformada com o plasmídeo pOri23–fnbA (FnBPA+), passou a expressar, constitutivamente, a proteína A de ligação a Fibronectina (FnBPA, do inglês, *Fibronectin-Binding Protein A*) de *Staphylococcus aureus* (QUE et al., 2001). Essa transformação, conferiu um caráter de

invasividade para essa linhagem, haja vista que a FnBPA medeia a adesão da bactéria ao tecido hospedeiro, possibilitando sua entrada em células não fagocíticas (LIU et al., 2019).

Depois, em experimentos, *in vitro* e *in vivo*, foi demonstrado que *L. lactis* MG1363 FnBPA+ foi capaz de ser internalizada por células epiteliais humanas, da linhagem Caco-2, de maneira mais eficiente que a linhagem selvagem (INNOCENTIN et al., 2009). Concomitante ao desenvolvimento dessa linhagem invasiva (*L. lactis* MG1363 FnBPA+), o nosso grupo de pesquisa também desenvolveu, ainda em 2009, um novo vetor plasmídico, denominado pValac (do inglês, *Vaccination Using Lactic Acid Bacteria*) (GUIMARÃES et al., 2009).

O pValac foi construído a fim de otimizar os procedimentos de clonagem e transformação e, consequentemente, melhorar as estratégias de entrega de DNA por *L. lactis*. Esse plasmídeo foi formado pela fusão: a) do promotor do citomegalovírus (CMV), garantindo a expressão da ORF de interesse em células eucarióticas; b) de um sítio de clonagem múltipla; c) da sequência sinal de poliadenilação do Hormônio Bovino de Crescimento (BGH poli-A), para estabilizar o transcrito de RNA mensageiro; d) de origens de replicação para permitir a propagação do plasmídeo tanto em *E. coli* quanto em *L. lactis*; e, e) de um gene de resistência ao cloranfenicol, para a seleção das linhagens recombinantes (GUIMARÃES et al., 2009).

Após o desenvolvimento da linhagem recombinante invasiva *L. lactis* MG1363 FnBPA+ contendo o vetor pValac que é capaz de codificar ORFs de interesse em células mamíferas, o nosso grupo de pesquisa construiu e testou a eficácia dessa estratégia em diferentes modelos experimentais. Dentre os trabalhos científicos realizados, destacam-se: IL-10 de *Mus musculus* para o tratamento de colite (ZURITA-TURK et al., 2014), ESAT-6 e Ag85A de *Mycobacterium tuberculosis* para o tratamento de tuberculose (PEREIRA et al., 2015; MANCHA-AGRESTI et al., 2017) e IL-4 de *Mus musculus* para o tratamento da CD (SOUZA et al., 2016).

2.4 JUSTIFICATIVA DE REALIZAÇÃO DESTE TRABALHO

As IBDs compreendem um conjunto de distúrbios que afetam o GIT, cujas taxas de incidência e prevalência vem aumentando globalmente. Dentre as IBDs, a CD é uma síndrome incurável que tem como sintomas a dor abdominal associada à diarreia, febre, fadiga e perda de

peso. Além disso, uma das principais complicações dessa enfermidade é o desenvolvimento de fibrose, na qual se caracteriza pelo acúmulo de colágeno nos tecidos do intestino.

Embora existam formas de remediar a inflamação da CD, não há tratamentos eficazes para impedir o desenvolvimento da fibrose e suas complicações. Neste sentido, as HSPs, antígenos importantes na regulação de células T efetoras, podem ser candidatas promissoras ao desenvolvimento de novas terapias anti-inflamatórias e antifibróticas para a CD.

Ademais, as HSP60 apresentam potencial para diminuição das respostas imunes Th1 e aumento das respostas Th2 e Treg, respostas, essas, necessárias para o controle dessa enfermidade. Inclusive, Hsp65 micobacterianas, produzidas por *L. lactis*, demonstraram efeitos terapêuticos por meio do aumento da secreção de células Tregs (CD4+Foxp3+ e CD4+LAP+).

Assim, objetivando potencializar a entrega de Hsp65 de *M. leprae*, uma proteína homóloga a HSP60 de mamíferos, diretamente aos enterócitos da mucosa intestinal, unificou-se ferramentas biotecnológicas pré-existentes do nosso laboratório, visando uma estratégia terapêutica inovadora contra a CD experimental. Logo, reuniu-se em uma única linhagem de *L. lactis* NCDO2118 a produção de Hsp65 pelo sistema XIES e também o mecanismo de entrega por meio da expressão constitutiva da proteína A de ligação a Fibronectina (FnBPA+).

Cabe salientar, que esse novo sistema utilizou a linhagem selvagem *L. lactis* NCDO2118 (*L. lactis* subsp. *lactis*) que possui propriedades anti-inflamatórias intrínsecas. Isto posto, a união desses instrumentos genéticos proporcionou o desenvolvimento de uma nova linhagem invasiva e produtora de Hsp65 – *L. lactis* NCDO2118 FnBPA+ (pXYCYT:Hsp65) – com propriedades imunomoduladoras.

Por fim, hipotetiza-se que o uso dessa nova linhagem invasiva [*L. lactis* NCDO2118 FnBPA+ (pXYCYT:Hsp65)] para a produção e entrega de Hsp65, em modelos murinos de CD aguda e crônica, induzidas quimicamente pelo ácido TNBS, pode restabelecer a homeostase intestinal e, consequentemente, diminuir os danos decorrentes dessa IBD.

3 OBJETIVOS

3.1 OBJETIVO GERAL

- Avaliar os efeitos imunomodulatórios da linhagem invasiva e produtora de Hsp65 [*L. lactis* NCDO2118 FnBPA+ (pXYCYT:Hsp65)] em modelos murinos de colite aguda e crônica, induzidas por TNBS, visando o desenvolvimento de uma nova estratégia terapêutica para a CD experimental.

3.2 OBJETIVOS ESPECÍFICOS

Na colite aguda:

- Avaliar os aspectos clínicos de saúde animal e os índices de danos macroscópicos;
- Avaliar os índices de danos histopatológicos;
- Avaliar os níveis da enzima N-acetilglicosaminidase (NAG);
- Avaliar os níveis da enzima mieloperoxidase (MPO);
- Avaliar as citocinas pró-inflamatórias: IFN-γ, TNF-α, IL-12, IL-4, IL-6 e IL-17;
- Avaliar as citocinas anti-inflamatórias: TGF-β e IL-10;
- Avaliar os níveis da imunoglobulina secretória A (sIgA).

Na colite crônica:

- Avaliar os aspectos clínicos de saúde animal (peso);
- Avaliar os danos histopatológicos da inflamação (aguda e crônica) e da fibrose;
- Avaliar os níveis da enzima mieloperoxidase (MPO);
- Avaliar as citocinas pró-inflamatórias: IFN-γ, IL-12, IL-6, IL-13, IL-17;
- Avaliar as citocinas anti-inflamatórias: TGF-β e IL-10;
- Avaliar os níveis da imunoglobulina secretória A (sIgA).

4 RESULTADOS

4.1 ARTIGO COMPLETO PUBLICADO

4.1.1 Invasive *Lactococcus lactis* producing mycobacterial Hsp65 ameliorates intestinal inflammation in acute TNBS-induced colitis in mice by increasing the levels of the cytokine IL-10 and secretory IgA - https://doi.org/10.1111/jam.14695

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ORIGINAL ARTICLE

Invasive *Lactococcus lactis* producing mycobacterial Hsp65 ameliorates intestinal inflammation in acute TNBS-induced colitis in mice by increasing the levels of the cytokine IL-10 and secretory IgA

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Keywords
Crohn's disease, Hsp65, IL-10, *Lactococcus lactis*, secretory IgA, TNBS.

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Abstract

Aims: To investigate the anti-inflammatory activity of an invasive and Hp65-producing strain *Lactococcus lactis* NCDO2118 FnBPA+ (pXYCYT:Hsp65) in acute 2,4,6-trinitrobenzene sulphonic acid (TNBS)-induced colitis in mice as an innovative therapeutic strategy against Crohn's disease (CD).
Methods and Results: The pXYCYT:Hsp65 plasmid was transformed into the *L. lactis* NCDO2118 FnBPA+ strain, resulting in the *L. lactis* NCDO2118 FnBPA+ (pXYCYT:Hsp65) strain. Then, the functionality of the strain was evaluated *in vitro* for Hsp65 production by Western blotting and for invasion into Caco-2 cells. The results demonstrated that the strain was able to produce Hsp65 and efficiently invade eukaryotic cells. Subsequently, *in vivo*, the anti-inflammatory capacity of the recombinant strain was evaluated in colitis induced with TNBS in BALB/c mice. Oral administration of the recombinant strain was able to attenuated the severity of colitis by mainly reducing IL-12 and IL-17 levels and increasing IL-10 and secretory immunoglobulin A levels.
Conclusions: The *L. lactis* NCDO2118 FnBPA+ (pXYCYT:Hsp65) strain contributed to a reduction in inflammatory damage in experimental CD.
Significance and Impact of the Study: This study, which used *L. lactis* for the production and delivery of Hsp65, has scientific relevance because it shows the efficacy of this new strategy based on therapeutic protein delivery into mammalian enterocytes.

Introduction

Inflammatory bowel diseases (IBDs) are worldwide public health problem and characterized by chronic and recurrent idiopathic inflammation in the gastrointestinal tract (Foersch *et al.* 2013). These diseases occur most dramatically in developed countries, affecting approximately 1·5 million people in North America and two million people in Europe (Ng *et al.* 2017). Although the aetiopathogenesis is not entirely clear, it is believed that several factors contribute to intestinal mucosal inflammation, such as environment, genetics, the microbiota and patient immunity (Ananthakrishnan 2015).

Among IBDs, ulcerative colitis (UC) and Crohn's disease (CD) are the most well-known pathologies. CD occurs usually between 15 and 40 years, affecting adolescents and adults (Neurath 2014); however, cases in children have been reported (Sundqvist *et al.* 2019). In this disease, the most affected regions of the intestine are the terminal ileum, cecum, colon and perianal region.

Inflammation is characterized by the presence of normal bowel segments with affected regions, and histology has indicated that this inflammation affects all layers of the intestinal wall (Bouma and Strober 2003).

The symptoms of CD are diarrhoea, abdominal cramps, fever and fatigue (Neurath 2014). In addition, affected individuals may present complications such as stenosis, fistulas and colon cancer (Wirtz *et al.* 2017). The severity of the disease varies from mild to severe, and treatment is performed with anti-inflammatory drugs, immunosuppressants, antibiotics, biological drugs and surgery; however, these therapies are linked to several side effects, such as headache and vomiting (Fakhoury *et al.* 2014).

In view of this problem, there is a need to develop more effective treatments for patients with CD. Thus, heat shock proteins (HSPs) can be an interesting alternative since they are immunodominant antigens and may be good candidates for the therapeutic induction of tolerance in chronic inflammatory and autoimmune diseases (Coelho and Faria 2012; van Eden *et al.* 2019).

HSP60, which is activated by TLR-2, is capable of inhibiting T-cell chemotaxis, which, consequently, leads to decreased migration to inflamed sites (Zanin-Zhorov *et al.* 2003). HSP60 acts as a costimulator of Treg cells and modulates the expression of transcription factors involved in T-cell differentiation, decreasing the expression of Th1 factors such as T-bet, NF-κB and NFATp and increasing that of Th2 factors such as GATA-3. Thus, HSP60-activated T cells decrease the secretion of TNF-α and IFN-γ and increase the secretion of IL-10 (Zanin-Zhorov *et al.* 2005).

In this context, our research group developed the xylose-inducible expression system (XIES) for the expression of heterologous proteins in *Lactococcus lactis* NCDO2118 (*L. lactis* subsp. *lactis*) (Miyoshi *et al.* 2004). Subsequently, using the XIES, we constructed an *L. lactis* NCDO2118 strain that expressed a 65-kDa *Mycobacterium leprae* protein (de Azevedo *et al.* 2012).

Additionally, this strain was able to prevent the development of dextran sulphate sodium (DSS)-induced colitis in C57BL/6 mice (Gomes-Santos *et al.* 2017). The therapeutic effects of this strain were due to decreases in the levels of proinflammatory cytokines (IFN-γ, IL-6 and TNF-α), increases in the levels of an anti-inflammatory cytokine (IL-10) in the colon and the expansion of Treg cells ($CD4^+$ $Foxp3^+$ and $CD4^+$ LAP^+) in the spleen and mesenteric lymph nodes (Gomes-Santos *et al.* 2017).

Anti-inflammatory properties were also described for the wild-type strain of *L. lactis* NCDO2118 in a murine model of DSS-induced UC (Luerce *et al.* 2014). The immunomodulatory effects was related to increased IL-10 levels in the colon and the induction of Treg cells in the mesenteric lymph nodes (Luerce *et al.* 2014).

Therefore, aiming to deliver Hsp65 directly into mammalian cell enterocytes, a new recombinant *L. lactis* NCDO2118 strain expressing *Staphylococcus aureus* Fibronectin-binding protein A (FnBPA+) (Que *et al.* 2001) was developed in this study. FnBPA is a glycoprotein that mediates the adhesion of bacteria to host tissue, enabling bacterial entry into nonphagocytic cells (Liu *et al.* 2019).

Thus, here, we hypothesized that the use of the invasive *L. lactis* NCDO2118 FnBPA+ (pXYCYT:Hsp65) strain for the production and delivery of Hsp65 in the cytoplasmic fraction within intestinal mucosa cells in a murine model of CD can restore intestinal homeostasis and thereby decrease the damage resulting from this disease.

Materials and methods

Bacterial strains, plasmids and growth conditions

The strains and plasmids used in this work are listed in Table 1. The *L. lactis* strains were grown in M17 medium (Sigma-Aldrich, São Paulo, Brazil) supplemented with 0·5% glucose (Synth, Diadema, Brazil) at 30°C without shaking. Recombinant *L. lactis* strains were selected by the addition of chloramphenicol (10 μg ml^{-1}) (Sigma-Aldrich) and/or erythromycin (5 μg ml^{-1}) (Sigma-Aldrich).

Construction of the *L. lactis* NCDO2118 FnBPA+ (pXYCYT:Hsp65) strain

Lactococcus lactis NCDO2118 FnBPA+ (pXYCYT:Hsp65) construction was divided into two parts: in the first part, the *L. lactis* MG1363 FnBPA+ strain was used for extraction of the pOri23-fnbA (FnBPA+) plasmid by the alkaline lysis method (Birnboim and Doly 1979; Poquet *et al.* 1998). Then, the pOri23-fnbA (FnBPA+) plasmid was transformed into *L. lactis* NCDO2118, resulting in the *L. lactis* NCDO2118 FnBPA+ strain. In the second part, the pXYCYT:Hsp65 plasmid was transformed into the *L. lactis* FnBPA+ strain, resulting in the *L. lactis* NCDO2118 FnBPA+ (pXYCYT:Hsp65) strain. To confirm the new recombinant strain, primers for the pXIES vector (pXIESF, 5′-CTGGTAATGATTGTTGGCTTG-3′, and pXIESR, 5′-GGTATCGATAAGCTTGATATC-3′) and for part of the fibronectin ORF (FnAF, 5′-CAACACTATTGTGTCCACCG-3′, and FnAR, 5′-TCAGCTATTGATATCGATTA′3′) were used for polymerase chain reaction (PCR), and the products were submitted for sequencing analysis.

Table 1 Bacterial strains and plasmids used in this work

Bacterial strains/ plasmids	Characteristics	Source
Bacterial strains		
Lactococcus lactis NCDO2118	*Lactococcus lactis* subsp. *lactis*—wild type (wt)	Luerce *et al.* (2014)
Lactococcus lactis MG1363 FnBPA+	*Lactococcus lactis* subsp. *cremoris* carrying the plasmid pOri23-fnbA (Ery^r)*	Que *et al.* (2001)
Lactococcus lactis NCDO2118 (pXYCYT:Hsp65)	*Lactococcus lactis* carrying the plasmid pXYCYT:Hsp65 (Cm^r)†	de Azevedo *et al.* (2012)
Lactococcus lactis NCDO2118 FnBPA+	*Lactococcus lactis* carrying the plasmid pOri23-fnbA (Ery^r)*	This work
Lactococcus lactis NCDO2118 FnBPA+ (pXYCYT:Hsp65)	*Lactococcus lactis* carrying the plasmids pOri23-fnbA (Ery^r)* and pXYCYT:Hsp65 (Cm^r)†	This work
Plasmids		
pOri23-fnbA (FnBPA+)	Shuttle vector (fnbA/ori ColE1/ P23/Ery^r)* for constitutive expression of FnBPA	Que *et al.* (2001)
pXYCYT:Hsp65	Cytoplasmic expression vector p*XylT*T:Nuc in which the ORF Nuc was replaced by the ORF Hsp65 of *Mycobacterium leprae*	de Azevedo *et al.* (2012)

*Ery^r: gene that confers resistance to erythromycin.
†Cm^r: gene that confers resistance to chloramphenicol.

Induction of Hsp65 expression, protein extraction and immunodetection

To confirm Hsp65 expression, *L. lactis* NCDO2118 FnBPA+ (pXYCYT:Hsp65) was induced with 2% xylose (Dynamics, São Paulo, Brazil) and 0·5% galactose (Vetec, Duque de Caxias, Brazil). Bacterial cultures were grown until reaching an OD_{600} nm ~2, for approximately 8 h, before performing protein extractions. Then, 2 ml of culture was collected and processed as previously described (Miyoshi *et al.* 2004). The protein extract, containing recombinant Hsp65, was resolved on a 12% polyacrylamide gel (SDS-PAGE) (Laemmli 1970). Hsp65 immunodetection was performed with a polyclonal anti-Hsp65 primary antibody provided by Farmacore Biotecnologia Ltda (Ribeirão Preto, Brazil) and the WesternBreeze Kit—Chromogenic Western Blot Immunodetection (Novex, Waltham, MA) according to the manufacturer's recommendations.

Assays of bacterial invasiveness

An invasion test was performed with human colon adenocarcinoma cells (Caco-2), which were provided by the Laboratory of Experimental Genetics (LGEX) from the Federal University of Minas Gerais (UFMG), as described (Innocentin *et al.* 2009). The Caco-2 cells were grown to approximately 80% confluence in Dulbecco's modified Eagle's medium supplemented with 10% foetal bovine serum. *Lactococcus lactis* strains were cultured (OD_{600} nm ~ 1·5), washed and diluted in 1X phosphate-buffered saline (PBS1X) to achieve a multiplicity of infection of 10^3 bacteria per eukaryotic cell. The following strains were used for coculture: *L. lactis* NCDO2118 (N group; noninvasive/wild-type strain), *L. lactis* NCDO2118 FnBPA+ (pXYCYT:HSP65) (NFX group; invasive strain not expressing Hsp65) and *L. lactis* NCDO2118 FnBPA+ (pXYCYT:HSP65) (NFXi group; invasive strain expressing Hsp65). The plates containing Caco-2 cells and *L. lactis* were incubated for 1 h. After this time, extracellular bacteria were killed with gentamicin (20 μg ml^{-1}) exposure for 2 h, and the Caco-2 cells were lysed with 0·2% Triton X-100. From the cell lysate, 100 μl was plated at dilutions of 10^{-3} to count colony forming units (CFU) per ml.

Animals

Conventional 6- to 7-week-old female BALB/c mice weighing approximately 20 g were obtained from the UFMG Bioterismo Center (CEBIO). The animals were kept in microisolators (six animals/microisolator) housed in ventilated racks, with controlled temperature (22 ± 2°C), humidity (50 ± 10%), air flow (35 exchanges/hour) and light exposure (12-hour light/dark cycle) with free access to water and feed, except on the day of colitis induction, during which the animals underwent a 6-h fast. All animal procedures and manipulations were approved by the Animal Use Ethics Commission (CEUA, Protocol no.: 341/2017) of the UFMG.

Induction of acute 2,4,6-trinitrobenzene sulphonic acid (TNBS)-induced colitis

Colitis was induced as previously described (Souza *et al.* 2016). After fasting, mice were anesthetized with ketamine (100 mg kg^{-1}) (Agener, São Paulo, Brazil) and xylazine (10 mg kg^{-1}) (Ceva, Paulínia, Brazil), and inflammation was induced in each animal by intrarectal administration of 100 μl of a TNBS solution (2 mg per mouse) containing 40 μl of 5% (w/v) TNBS in H_2O (Sigma-Aldrich), 50 μl of absolute ethanol (Synth) and 10 μl of PBS1X. The procedure was performed on the 2nd day of the experimental schedule.

Treatment of acute intestinal inflammation

Treatment of acute TNBS-induced colitis consisted of intragastric gavage with 1×10^9 CFUs of bacteria in 100 µl of 0·9% saline, after reaching an OD_{600} nm ~ 2, for approximately 8 h. Bacterial administration was performed for four consecutive days, from the 1st to the 4th day of the experimental schedule. Thus, for experimental procedures, six experimental groups ($n = 18$ per group) were formed. The animals subjected to TNBS administration were treated with (i) 0·9% saline (C+ group); (ii) *L. lactis* NCDO2118 (N group; noninvasive/wild-type strain); (iii) *L. lactis* NCDO2118 (pXYCYT:Hsp65) (NXi group; noninvasive strain expressing Hsp65); or (iv) *L. lactis* NCDO2118 FnBPA+ (pXYCYT:Hsp65) (NFXi group; invasive strain expressing Hsp65). Additionally, animals that were negative controls for intestinal inflammation (C group) or vehicle controls for TNBS (VC group) received 0·9% saline as treatment.

Clinical and macroscopic assessment of colitis

Mice were evaluated from the 1st to the 5th day of the experimental schedule for clinical health parameters. For this, the presence or absence of diarrhoea, presence or absence rectal bleeding and body weight were observed for each individual (Endharti and Permana 2017). Macroscopic colonic damage was assessed on the 5th day of the experimental schedule, as previously described (Cenac *et al.* 2002). The evaluation was performed during euthanasia by at least three researchers and consisted of evaluation of the presence or absence of nine characteristics related to intestinal inflammation including adhesion, oedema, stenosis, erythema, haemorrhage, ulceration, blood in the stool, mucus and diarrhoea. Each parameter, if observed, was assigned a score point (0–1 point), except for adhesion and erythema, which, depending on their severity or extent, respectively, could receive up to two points (Cenac *et al.* 2002).

Histopathological analysis of acute colitis

For histological scoring, intestines were stained with haematoxylin and eosin (H&E). The analysis of histological findings was performed as previously described (Ameho *et al.* 1997). In this evaluation, the following classification scale was considered: Grade 0, the colon within normal limits; Grade 1, the mucosa and submucosa with mild oedema and an inflammatory infiltrate with some neutrophils, occasional presence of erosions in the mucosa, and intact mucosal muscle; Grade 2, grade 1 changes involving 50% or more of the sample; Grade 3, the mucosa and submucosa with oedema and moderate inflammatory infiltration (predominance of neutrophils), presence of erosions with areas of mucosal ulceration, extending through the mucosal muscle to the submucosa, rare inflammatory cells invading the muscle layers and the absence of necrosis in the muscle layer; Grade 4, grade 3 changes involving 50% or more of the sample; Grade 5, the mucosa presenting extensive ulceration with coagulative necrosis inferiorly marginated by numerous neutrophils and relatively few mononuclear cells, and mucosal necrosis extending to the muscle layer; and Grade 6, grade 5 changes involving 50% or more of the sample.

N-acetyl-β-dglucosaminidase (NAG) and myeloperoxidase (MPO) assay

To evaluate cells that participate in the inflammatory process, such as macrophages and neutrophils, the activities of the enzymes NAG and MPO, respectively, were measured. The colon tissues (50 mg) were collected and homogenized in (i) cytokine extraction buffer (23·4 g NaCl; 500 µl Tween-20; 5 g BSA; 34 mg PMSF in 1 ml of DMSO; 44·6 mg benzethonium chloride; 372 mg Na_2EDTA; 40 µl aprotinin (10 mg ml^{-1}) (Sigma-Aldrich); PBS1X q.s.p. 1 l); (ii) buffer I pH 4·7 (0·1 mol l^{-1} NaCl; 0·02 mol l^{-1} Na_3PO_4; 0·015 mol l^{-1} Na_2EDTA); and (iii) 0·2% NaCl and 1·6% NaCl-5% glucose. After, the homogenates were divided in two parts: in the first part, which was tested for NAG, was diluted in 0·1% saline triton and citrate-phosphate buffer pH 4·5 (100 ml 0·1 mol l^{-1} $C_6H_8O_7$; 155 ml 0·1 mol l^{-1} Na_2HPO_4). Then, the substrate 4-nitrophenyl N-acetyl-β-d-glucosaminide (Sigma-Aldrich) added and was incubated at 37°C for 10 min. The reaction was stopped by adding 0·2 mol l^{-1} glycine buffer pH 10·6 (0·8 mol l^{-1} glycine; 0·8 mol l^{-1} NaCl; 0·8 mol l^{-1} NaOH). In the second part, the homogenate which was tested for MPO, was frozen in liquid nitrogen and thawed in water at room temperature three times. Then, it was diluted in buffer II pH 5·4 (0·05 mol l^{-1} Na_3PO_4; 0·5% HETAB) and had the substrate 3,3′,5,5′-tetramethylbenzidine (TMB) (Sigma-Aldrich) added and was incubated at 37°C for 5 min. Afterward, 0·002% H_2O_2 was added and incubated at 37°C for 5 min. The reaction was interrupted by the addition 1 mol l^{-1} H_2SO_4. NAG activity was measured at 405 nm, and MPO activity was measured at 450 nm; both were measured with a POLARIS MA616 (Marconi, São Paulo, Brazil) microplate reader.

Determination of cytokine levels

Cytokines from macerated colonic tissue were measured by a sandwich ELISA. Colon samples (100 mg) were

homogenized in 1 ml of cytokine extraction buffer. After, samples were centrifuged at 6272 ***g*** for 10 min at 4°C, and the supernatant was stored at −80°C until use. Pro- and anti-inflammatory cytokines were evaluated following the manufacturer's instructions; specifically IL-12 levels were measured by means of a BD OptEIATM (BD) kit, and IL-4, IL-6, IL-10, IL-17, IFN- γ, TNF-α and TGF-β levels were measured by means of kits from R&D DuoSet (R&D Systems, Minneapolis, MN). The absorbance was measured at 492 nm with a POLARIS MA616 (Marconi) microplate reader.

Determination of secretory immunoglobulin A levels

Secretory immunoglobulin A (sIgA) levels were measured in intestinal lavage fluid by capture ELISA. Colon was washed with 5 ml of PBS1X at room temperature and the fluid centrifuged at 90 ***g*** for 10 min at 4°C. The supernatant was used to measure sIgA levels using Goat anti-mouse IgG, human ads-UNLB and goat anti-mouse IgA conjugated with horseradish peroxidase (HRP) antibodies (Southern-Biotech, Birmingham, AL) were used in the assay according to the manufacturer's instructions. The absorbance was measured at 492 nm with a POLARIS MA616 (Marconi) microplate reader.

Statistical analysis

The experimental results are presented as the arithmetic mean ± SD. Data were analysed using GraphPad Prism 6·0 (GraphPad Statistical Software) for analysis of variance by one-way ANOVA and Tukey's *post hoc* test. Differences between groups with P values <0·05 were considered statistically significant.

Results

L. lactis NCDO2118 FnBPA+ (pXYCYT:Hsp65) produces *M. leprae* Hsp65 and invades Caco-2 cells

The plasmid pXYCYT:Hsp65 was successfully transformed into the invasive *L. lactis* FnBPA+ strain, resulting in a recombinant strain capable of invading eukaryotic cells and producing Hsp65: *L. lactis* NCDO2118 FnBPA+ (pXYCYT:Hsp65). The confirmation of the *L. lactis* FnBPA+ (pXYCYT:Hsp65) strain was performed by PCR and DNA sequencing of both the FnBPA and Hsp65 ORFs (data not shown).

Then, after confirmation of successful construction, *L. lactis* NCDO2118 FnBPA+ (pXYCYT:Hsp65) underwent induction of gene expression with xylose, and subsequently, the recombinant mycobacterial protein Hsp65 could be identified by Western blotting (data not shown).

Finally, *in vitro* assays using Caco-2 human epithelial cells showed that *L. lactis* NCDO2118 FnBPA+ (pXYCYT:Hsp65) (NFX and NFXi groups) was internalized more efficiently than the wild-type strain *L. lactis* NCDO2118 (N group) ($P < 0·05$) (Fig. 1).

L. lactis NCDO2118 FnBPA+ (pXYCYT:Hsp65) ameliorates clinical health parameters of acute TNBS-induced colitis

To examine whether *L. lactis* FnBPA+ (pXYCYT:Hsp65) ameliorates clinical symptoms of TNBS-induced colitis in mice, clinical health parameters including weight changes and macroscopic scores were evaluated. For the clinical signs, it was noted that compared with the C+ group, which presented diarrhoea and rectal bleeding, the NFXi group had reduced damage caused by acute colitis (data not shown).

Regarding weight, compared to those in the control groups (C− and VC groups), the animals in the C+ group showed a decrease in the body weight percentage ($P < 0·05$). However, when compared with the C+ group, the NFXi group exhibited amelioration of this parameter beginning on the 3rd day of the experimental schedule ($P < 0·05$) (Fig. 2a).

For the macroscopic damage score, compared to those in the C+ group, which presented organ adhesion, erythema, ulceration in the colon, mucus in the stool and

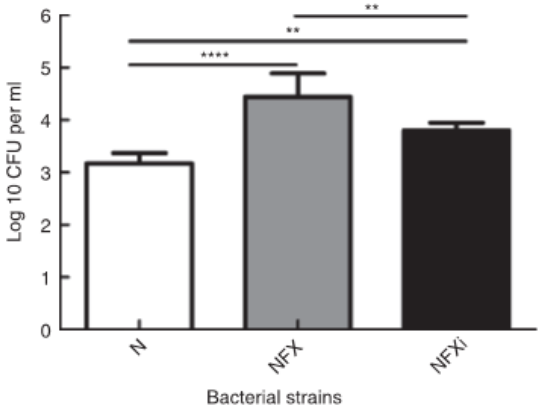

Figure 1 Assays of *Lactococcus lactis* NCDO2118 FnBPA+ (pXYCYT:Hsp65) invasion into Caco-2 cells. Bacterial strains: N: *L. lactis* NCDO2118—noninvasive/wild-type strain (□); NFX: *L. lactis* NCDO2118 FnBPA+ (pXYCYT:Hsp65)—invasive strain not expressing Hsp65 (■); and NFXi: *L. lactis* NCDO2118 FnBPA+ (pXYCYT:Hsp65)—invasive strain expressing Hsp65 (■). Data are shown as the mean ± SD of three independent experiments ($n = 9$). P values: $^{**}P < 0·01$; $^{****}P < 0·0001$.

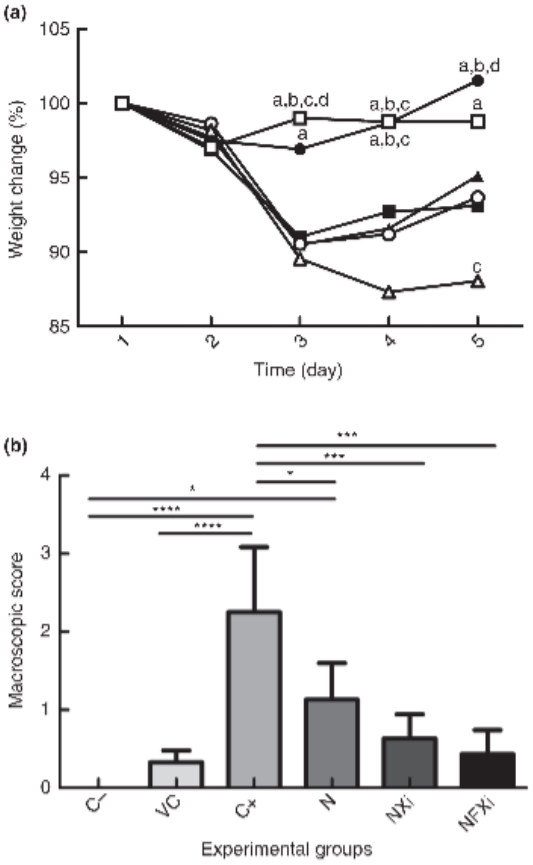

Figure 2 Body weight variations and macroscopic scores of mice with acute TNBS-induced colitis. (a) Percentage of initial body weight of BALB/c mice with or without TNBS administration as a function of time ($n = 18$). Experimental groups: C−: negative control for colitis (□); VC: TNBS vehicle control (●); C+: positive control for colitis (Δ); N: *Lactococcus lactis* NCDO2118 noninvasive/wild-type strain (○); NXi: *L. lactis* NCDO2118 (pXYCYT:Hsp65)—noninvasive strain expressing Hsp65 (▲); and NFXi: *L. lactis* NCDO2118 FnBPA+ (pXYCYT:Hsp65)—invasive strain expressing Hsp65 (■). (a) Experimental group whose body weight percentages were significantly different from the percentages of the C+ group. (b) Experimental group whose body weight percentages were significantly different from the percentages of the N group. (c) Experimental group whose body weight percentages were significantly different from the percentages of the NXi group. (d) Experimental group whose body weight percentages were significantly different from the percentages of the NFXi group. (b) Macroscopic scores of BALB/c mice with or without TNBS administration. Experimental groups: C−: negative control for colitis (□); VC: TNBS vehicle control (▒); C+: positive control for colitis (▒); N: *L. lactis* NCDO2118—noninvasive/wild type strain (▒); NXi: *L. lactis* NCDO2118 (pXYCYT:Hsp65)noninvasive strain expressing Hsp65 (▒); and NFXi: *L. lactis* NCDO2118 FnBPA− (pXYCYT:Hsp65)—invasive strain expressing Hsp65 (■). Data are shown as the mean ± SD of three independent experiments ($n = 18$). P values: $^{*}P < 0.05$; $^{***}P < 0.001$; $^{****}P < 0.0001$.

diarrhoea, the animals in the NFXi group showed a significant decrease in inflammation ($P < 0.05$) (Fig. 2b).

L. lactis NCDO2118 FnBPA+ (pXYCYT:Hsp65) attenuates histopathological changes in the colon

Based on previous data, we investigated whether *L. lactis* FnBPA+ (pXYCYT:Hsp65) alters histological damage in mice with acute TNBS-induced colitis. The C− group did not present intestinal alterations and maintained the histological architecture within the normal range (Fig. 3a). In the mucosa, the glandular crypts presented a continuous epithelium, regular goblet/enterocyte proportion, thin lamina propria and lack of the presence of spaces that could characterize oedema. The mucosal muscle remained continuous and preserved. The thin submucosa and intact muscular and serous layers were also present (Fig. 3b).

However, the C+ and N groups had mucosal and submucosal lesions with oedema and moderate to severe inflammatory infiltration (neutrophil predominance). The presence of erosions with areas of mucosal ulceration extending through the mucosal muscle to the submucosa was observed, as was the presence of rare inflammatory cells invading the muscle layer. Some samples had necrosis that extended to the muscle layer (Fig. 3a,b). The VC group had histological damage with mild lesions, and the NXi and NFXi groups had indicators of histological damage with mild to moderate lesions (Fig. 3a,b). Lesions were marked by the mucosa and submucosa exhibiting mild oedema, an inflammatory infiltrate with some neutrophils, and the punctate presence of mucosal erosions, and the mucosal muscle layer remained intact (Fig. 3b). However, the NFXi group exhibited better histological damage scores than the C− group ($P < 0.05$) (Fig. 3a,b).

L. lactis NCDO2118 FnBPA+ (pXYCYT:Hsp65) decreases inflammatory immune cell recruitment

The activities of NAG and MPO, markers for macrophage and neutrophil influx, respectively, were assessed to detect the capacity of *L. lactis* FnBPA− (pXYCYT:Hsp65) to reduce inflammatory cell infiltration into the colon. In the NAG evaluation, all groups presented similar levels in the measurement of this enzyme, with no significant differences observed (data not shown). Regarding MPO, the C+ group presented higher neutrophil infiltration, differing from all other groups (the C−, VC, N, NXi and NFXi groups) ($P < 0.05$). There was a reduction in MPO activity in the group treated with *L. lactis* FnBPA− (pXYCYT:Hsp65) (the NFXi group) compared with the C+ group ($P < 0.05$) (Fig. 4).

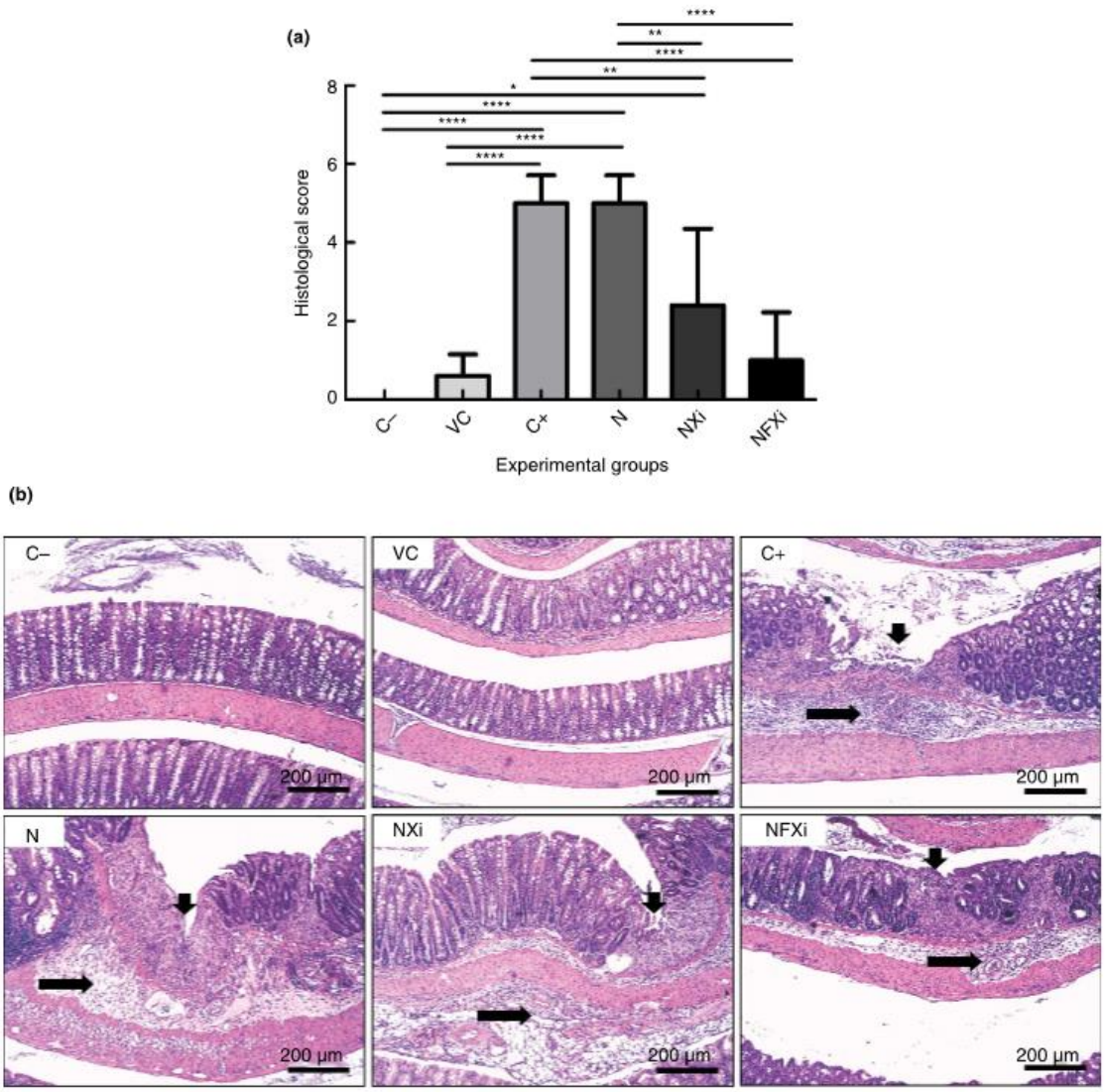

Figure 3 Scores and histological features of the colon of mice with acute TNBS-induced colitis. (a) Histological scores of BALB/c mice with or without TNBS administration. Experimental groups: C−: negative control for colitis (□); VC: TNBS vehicle control (▒); C+: positive control for colitis (▒); N: *Lactococcus lactis* NCDO2118—noninvasive/wild-type strain (■); NXi: *L. lactis* NCDO2118 (pXYCYT:Hsp65) —noninvasive strain expressing Hsp65 (■); and NFXi: *L. lactis* NCDO2118 FnBPA+ (pXYCYT:Hsp65)—invasive strain expressing Hsp65 (■). (b) Histological features of the colon of BALB/c mice subjected to TNBS administration or not. H&E staining. Long arrows indicated inflammatory infiltrates, and short arrows indicate erosion. Data are shown as the mean ± SD of three independent experiments ($n = 6$). *P* values: $^{*}P < 0.05$; $^{**}P < 0.01$; $^{****}P < 0.0001$.

L. lactis NCDO2118 FnBPA+ (pXYCYT:Hsp65) modulates the production of cytokines in the inflamed colon

Next, the effects of oral administration of *L. lactis* FnBPA+ (pXYCYT:Hsp65) on the expression of pro- and anti-inflammatory cytokines were measured. Evaluating the Th1 cytokine profile by measuring IFN-γ levels indicated that there were no significant differences observed among the groups (data not shown). Regarding TNF-α, among the groups that received TNBS, the C+ group had a higher concentration of this cytokine, and the groups treated with *L. lactis* (the NXi and NFXi groups) exhibited lower concentrations of TNF-α in the colon

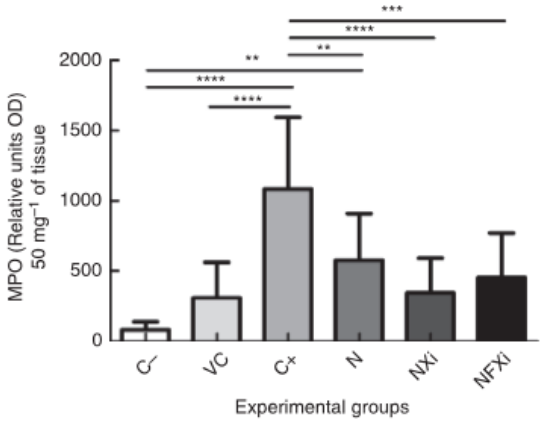

Figure 4 Myeloperoxidase (MPO) levels in BALB/c mice subjected to TNBS administration or not. Experimental groups: C−: negative control for colitis (□); VC: TNBS vehicle control (▒); C+: positive control for colitis (▒); N: *Lactococcus lactis* NCDO2118—noninvasive/wild-type strain (■); NXi: *L. lactis* NCDO2118 (pXYCYT:Hsp65)—noninvasive strain expressing Hsp65 (■); and NFXi: *L. lactis* NCDO2118 FnBPA+ (pXYCYT:Hsp65)—invasive strain expressing Hsp65 (■). Data are shown as the mean ± SD of three independent experiments (n = 12). P values: $^{**}P < 0{\cdot}01$; $^{***}P < 0{\cdot}001$; $^{****}P < 0{\cdot}0001$.

($P < 0{\cdot}05$) (Fig. 5a). For IL-12 measurements, the NFXi group had the lowest concentration of this cytokine and was significantly different from the VC, C+ and N groups ($P < 0{\cdot}05$) (Fig. 5b).

Among Th2 cytokines, IL-4 levels did not differ among the experimental groups (data not shown). For IL-6, among the groups that received TNBS, the C+ group had the highest levels of this cytokine, differing statistically from the NXi and NFXi groups ($P < 0{\cdot}05$) (Fig. 5c). In the measurement of the levels of IL-17, a Th17 cytokine, the C+ and N groups had the highest rates of positive expression of this cytokine in the inflamed colon, and the NFXi group was the only group that exhibited IL-17 production maintained at levels similar to those in the C− group ($P < 0{\cdot}05$) (Fig. 5d).

Finally, cytokines related to a regulatory profile were evaluated, and no differences in TGF-β concentrations were observed (data not shown). Nevertheless, for IL-10 measurements, the C− and NFXi groups presented the highest IL-10 concentrations and differed significantly from the C+ and N groups ($P < 0{\cdot}05$) (Fig. 5e).

Lactococcus lactis NCDO2118 FnBPA+ (pXYCYT:Hsp65) stimulates sIgA production

Finally, the capacity of *L. lactis* FnBPA+ (pXYCYT:Hsp65) to stimulate increased sIgA secretion in acute TNBS-induced colitis was investigated. The C+ and N groups presented lower concentrations of this immunoglobulin than the other groups ($P < 0{\cdot}05$). However, compared to the C+ and N groups, the NFXi group showed an increased sIgA concentration ($P < 0{\cdot}05$) (Fig. 6).

Discussion

In recent years, HSPs have received attention in the scientific community because of their ability to inhibit inflammatory processes. For example oral administration of *M. leprae* Hsp65-producing *L. lactis*, a protein homologous to the mammalian Hsp60 protein, has been shown to induce oral tolerance and promote protection against encephalomyelitis (Rezende *et al.* 2013) and colitis (Gomes-Santos *et al.* 2017).

However, by optimizing Hsp65 delivery to inflamed sites in chronic and autoimmune diseases, the use of an invasive *L. lactis* strain (*L. lactis* NCDO2118 FnBPA+) may enhance the beneficial results of this experimental strategy. Thus, the aim of this study was to evaluate the therapeutic effects of an invasive and Hsp65-producing strain (*L. lactis* NCDO2118 FnBPA+ (pXYCYT:Hsp65)) in intestinal inflammation chemically induced by TNBS.

To this end, we first evaluated the functionality of *L. lactis* NCDO2118 FnBPA+ (pXYCYT:Hsp65). This strain was able to efficiently produce the recombinant protein Hsp65 and invade eukaryotic cells. Subsequently, its anti-inflammatory effect was evaluated in TNBS-induced colitis. This model is widely used because it resembles the immunological and histopathological aspects of human IBDs (Wirtz *et al.* 2017). Additionally, due to the Th1 immune response profile involving the effector cytokines IL-12 and TNF-α, this model is specifically related to CD in humans (Qin *et al.* 2019).

The protective effect of *L. lactis* NCDO2118 FnBPA+ (pXYCYT:Hsp65) in acute colitis is reported here for the first time. Although some evaluated parameters did not differ significantly among the different treatments with *L. lactis*, it was noteworthy that compared with the C+ group, the NFXi group presented a decrease in the severity of inflammation as indicated by reduced body weight loss and decreases in macroscopic and histological scores. The evaluation of these parameters was important because colitis caused by intrarectal administration of TNBS causes abrupt weight loss, rectal prolapse and transmural inflammation (Kiesler *et al.* 2015; Zarzecki *et al.* 2017).

It is noteworthy that compared with C+ group, the invasive and Hp65-producing strain (*L. lactis* NCDO2118 FnBPA+ (pXYCYT:Hsp65)) was also able to decrease the activity of the enzyme MPO, which is present in neutrophil azurophil granules. This is a relevant result since

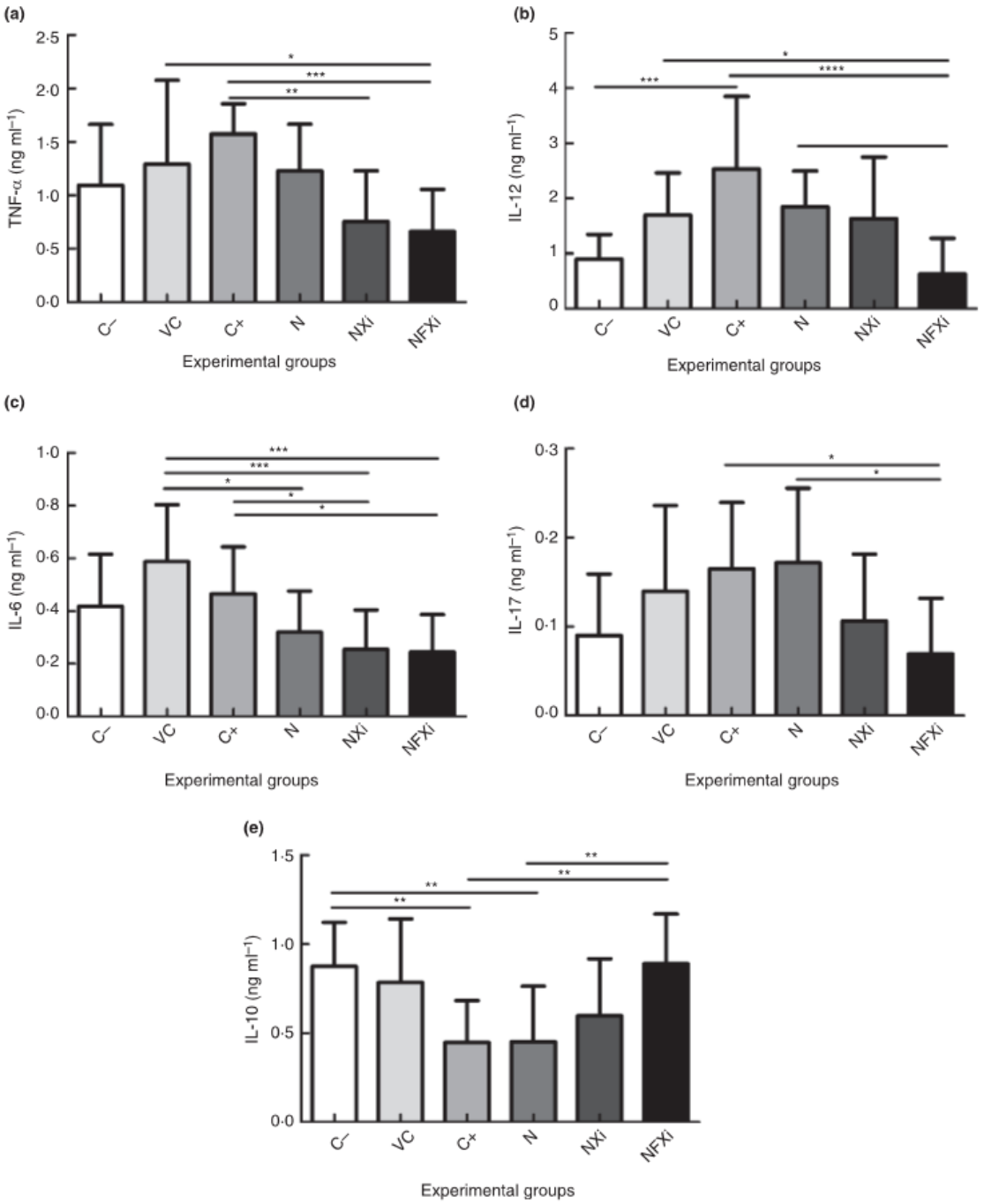

Figure 5 Cytokine levels in BALB/c mice subjected to TNBS administration or not. (a) Tumour necrosis factor α (TNF-α) levels. (b) Interleukin-12 (IL-12) levels. (c) Interleukin-6 (IL-6) levels. (d) Interleukin-17 (IL-17) levels. (e) Interleukin-10 (IL-10) levels. Experimental groups: C−: negative control for colitis (□); VC: TNBS vehicle control (▒); C+: positive control for colitis (▒); N: *Lactococcus lactis* NCDO2118—noninvasive/wild-type strain (▒); NXi: *L. lactis* NCDO2118 (pXYCYT:Hsp65)—noninvasive strain expressing Hsp65 (▒); and NFXi: *L. lactis* NCDO2118 FnBPA+ (pXYCYT:Hsp65)—invasive strain expressing Hsp65 (■). Data are shown as the mean ± SD of three independent experiments (n = 12). P values: $^{*}P < 0{\cdot}05$; $^{**}P < 0{\cdot}01$; $^{***}P < 0{\cdot}001$; $^{****}P < 0{\cdot}0001$.

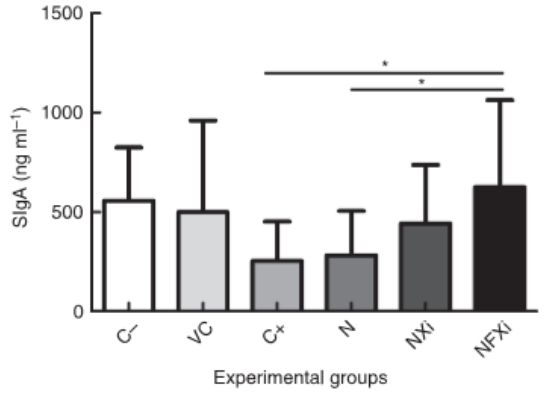

Figure 6 sIgA levels in BALB/c mice subjected to TNBS administration or not. Experimental groups: C−: negative control for colitis (□); VC: TNBS vehicle control (▒); C+: positive control for colitis (▒); N: *Lactococcus lactis* NCDO2118—noninvasive/wild-type strain (■); NXi: *L. lactis* NCDO2118 (pXYCYT:Hsp65)—noninvasive strain expressing Hsp65 (■); and NFXi: *L. lactis* NCDO2118 FnBPA+ (pXYCYT:Hsp65)—invasive strain expressing Hsp65 (■). Data are shown as the mean ± SD of three independent experiments ($n = 18$). *P* value: $^{*}P < 0{\cdot}05$.

neutrophil infiltration is a predominant feature in human IBDs and in the acute TNBS-induced colitis model (Endharti and Permana 2017).

In agreement with previously reported results, *L. lactis* NCDO2118 FnBPA+ (pXYCYT:Hsp65) showed promising results in decreasing the levels of the proinflammatory cytokines IL-12 and IL-17 and increasing the levels of the anti-inflammatory cytokine IL-10 and immunoglobulin sIgA. Regarding IL-12, the data presented showed that the NFXi group presented the lowest concentration of this cytokine during acute colitis. This is an essential effect because IL-12 is a major Th1 cytokine and has been identified as the central mediator of human and TNBS-induced colitis (Strober and Fuss 2011). The results found for the NFXi group agree with the literature, as HSP60 proteins contribute to the reduction in Th1-cell-mediated inflammatory immune responses (Cohen and Zanin-Zhorov 2013).

IL-17 produced by Th17 cells is an interleukin that plays a critical role in the development of inflammation. IL-17 levels are increased in the serum and intestinal tissue of IBDs carriers (Jiang *et al.* 2014). This cytokine favours the production of proinflammatory factors such as TNF-α, IL-6 and IL-1β, demonstrating its function in the amplification and localization of inflammation, as well as in the fibrotic process during CD (Moldoveanu *et al.* 2015). The reduction in the IL-17 concentration in NFXi group animals contributed to the amelioration of inflammation, as it did not favour the production of the proinflammatory cytokines TNF-α and IL-6. Given the results indicating reductions in the levels of the proinflammatory interleukins IL-12 and IL-17, it can be inferred that *L. lactis* NCDO2118 FnBPA+ (pXYCYT:Hsp65) influenced the reductions in the production of these cytokines.

Another relevant result was the increased IL-10 secretion found in the NFXi group. This cytokine plays an anti-inflammatory role and is crucial in controlling immune responses (Zigmond *et al.* 2014). IL-10 is produced by Treg cells and other cells, such as activated macrophages and dendritic cells (Saraiva and O'Garra 2010). IL10 locus polymorphisms pose risks for the development of IBDs, including UC and DC (Franke *et al.* 2008; Franke *et al.* 2010). IL-10- and IL-10R-deficient mice and humans exhibit severe intestinal inflammation, indicating that IL-10-IL-10R signalling plays regulatory roles in tissue homeostasis and IBD prevention (Ip *et al.* 2017).

Additionally, in the evaluation of IL-10, the C+ and N groups presented the lowest concentrations of this cytokine, corroborating the histological findings indicating that both groups presented the highest rates of intestinal inflammation. The C− and NFXi groups had the highest IL-10 concentrations and differed significantly from the C+ and N groups. IL-10 modulates the production of inflammatory mediators by neutrophils, monocytes and macrophages, thereby limiting the secretion of proinflammatory cytokines, such as TNF-α, IL-6 and IL-12 (Alessandri *et al.* 2013). Further corroborating these results, mice treated with Hsp65 produced by *L. lactis* had increased levels of IL-10 in the inflamed colon, which have been shown to be crucial for Hsp65-mediated immunoregulation in DSS-induced colitis (Gomes-Santos *et al.* 2017).

The protective effect of *L. lactis* NCDO2118 FnBPA+ (pXYCYT:Hsp65) was also demonstrated by the induction of an sIgA increase in the NFXi group, as sIgA helped to maintain epithelial barrier function, which could prevent more micro-organisms from entering the inflamed site and exacerbating TNBS-induced colitis. IgA is the most abundant antibody class in the intestines of humans and other mammals (Mantis *et al.* 2011). It is known to be the first line of defence in protecting the intestinal epithelium against enteric toxins and pathogenic micro-organisms, thus contributing to intestinal homeostasis (Pabst and Slack 2020). The results of this study suggested that *L. lactis* NCDO2118 FnBPA+ (pXYCYT:Hsp65) exerted an immunoregulatory effect on acute inflammation, possibly due to the increases in the levels of the regulatory cytokine IL-10 and secretory immunoglobulin sIgA.

In conclusion, the results obtained in this study confirmed the hypothesis that the invasive and Hsp65-

producing strain studied (*L. lactis* NCDO2118 FnBPA+ (pXYCYT:Hsp65)) plays an immunomodulatory role in inflammatory responses in the murine model of CD chemically induced with TNBS. In addition, we demonstrated here that *L. lactis* NCDO2118 FnBPA+ (pXYCYT:Hsp65) was able to attenuate the severity of intestinal inflammation, primarily by decreasing the levels of the proinflammatory cytokines IL-12 and IL-17 and increasing the concentrations of the regulatory cytokine IL-10 and secretory immunoglobulin sIgA. Last, this work describes an innovative and promising approach for alternative treatment of CD using mucosal immunity and immunomodulation to restore intestinal homeostasis.

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Conflicts of Interest

The authors declare that they have no conflicts of interest.

Author contributions

V.P.C. planned and executed the experiments and wrote the manuscript; T.M.P. and V.B.P. planned and executed the experiments; M.P.S. assisted the experiments; D.C.C.M. performed the histopathological analyses; A.M. supervised the experiments and contributed to the writing of the manuscript. All authors read and approved the final manuscript.

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4.2 ARTIGO COMPLETO SUBMETIDO À PUBLICAÇÃO

4.2.1 Mycobacterial Hsp65 antigen delivered by invasive *Lactococcus lactis* reduces intestinal inflammation and fibrosis in TNBS-induced chronic colitis model throught regulation of the cytokines IL-13 and TGF-β

Mycobacterial Hsp65 Antigen Delivered by Invasive *Lactococcus lactis* Reduces Intestinal Inflammation and Fibrosis in TNBS-Induced Chronic Colitis Model Through Regulation of the Cytokines IL-13 and TGF-β

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Abbreviations: BSA, *bovine serum albumin;* CD, Crohn's disease; CEUA, Animal Use Ethics Commission; CFU, colony forming unit; DMSO, dimethyl sulfoxide; DSS, dextran sulfate sodium; ELISA, enzyme-linked immunosorbent assay; FnBPA, fibronectin-

binding protein A; H&E, hematoxylin and eosin; HETAB, hexadecyltrimethylammonium bromide; HRP, horseradish peroxidase; HSP, heat shock protein; IBD, inflammatory bowel disease; MPO, myeloperoxidase; ORF, open reading frame; PBS, phosphate-buffered saline; *PMSF*, phenylmethylsulfonyl fluoride; SD, standard deviation; sIgA, secretory immunoglobulin A; TLR2, Toll-like receptor 2; TMB, 3,3′,5,5′-tetramethylbenzidine; TNBS, 4,6-trinitrobenzene sulfonic acid; UC, ulcerative colitis; XIES, xylose-inducible expression system.

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Abstract: The intragastric administration of invasive *Lactococcus lactis*-producing mycobacterial Hsp65 ameliorates the severity of inflammation and intestinal fibrosis damage in experimental Crohn's disease (CD) mainly by reducing the pro-fibrotic cytokines IL-13 and TGF-β and increasing the regulatory cytokine IL-10.

Background: Intestinal fibrosis associated with Crohn's disease (CD), which a common and serious complication of inflammatory bowel diseases (IBDs), is usually treated with anti-inflammatory drugs, immunosuppressants, antibiotics, biological drugs and surgery, but these treatments are linked to several adverse effects. In this context, heat shock proteins (HSPs) might serve as an alternative treatment because these antigens play important roles in the regulation of effector T cells. We thus evaluated the anti-inflammatory and antifibrotic capacities of an invasive and Hsp65-producing strain – *Lactococcus lactis* NCDO2118 FnBPA+ (pXYCYT:Hsp65) – in chronic intestinal inflammation to assess its potential as an alternative therapeutic strategy against fibrotic CD.

Methods: Experimental colitis was induced by 2,4,6-trinitrobenzene sulfonic acid (TNBS) in BALB/c mice, and the mice were treated orally with *L. lactis* NCDO2118 FnBPA+ (pXYCYT:Hsp65) via intragastric gavage. After immunization, the histological scores of inflammation and fibrosis, the activity of myeloperoxidase (MPO), the cytokine profile and the level of secretory sIgA (sIgA) were evaluated.

Results: The oral administration of *L. lactis* NCDO2118 FnBPA+ (pXYCYT:Hsp65) significantly attenuated the severity of inflammation and intestinal fibrosis in mice ($P < 0.05$) compared with the damage observed in the animals belonging to the other groups. These results are mainly justified by reductions in the levels of the pro-fibrotic cytokines IL-13 and TGF-β and increases in the concentration of the regulatory cytokine IL-10.
Conclusions: The *L. lactis* NCDO2118 FnBPA+ (pXYCYT:Hsp65) strain contributed to reductions in the severity of inflammatory damage in chronic experimental CD, and these findings confirm the effectiveness of this new antifibrotic strategy based on the delivery of therapeutic proteins to inside cells of the host intestinal mucosa.

Key words: Mycobacterial Hsp65, *Lactococcus lactis*, chronic models, intestinal fibrosis, trinitrobenzene sulfonic acid

INTRODUCTION

Inflammatory bowel diseases (IBDs) are a set of disorders that affect the gastrointestinal tract, and their incidence and prevalence rates have been increasing globally in the 21st century.[1] IBDs essentially comprise two diseases, Crohn's disease (CD) and ulcerative colitis (UC), which are characterized by chronic and recurrent inflammation of the mucosa.[2]

CD is an incurable syndrome whose main symptom is abdominal pain associated with diarrhea, fever, fatigue, weight loss and weakness due to difficulty in absorbing nutrients.[3,4] In addition, one of the main complications of CD is the development of fibrosis, which results from excessive accumulation of extracellular matrix rich in collagen in the intestine layers, particularly the submucosa and muscle.[5,6]

Intestinal fibrosis related to CD occurs in approximately 30-50% of patients and can cause serious damage, such as stenosis, fistula or abscesses.[7,8] Additionally, the fibrotic complications in CD are responsible for approximately 75% of surgical resections in the first decade after the clinical diagnosis.[9,10]

Although therapies that modulate CD inflammation are currently available, there are no effective treatments to prevent or reverse fibrosis that affects carriers of the disease. In this sense, heat shock proteins (HSPs), which are important antigens in regulating effector T cells during inflammation,[11,12] can be promising candidates for the development of new antifibrogenic therapies.

Consistent with this notion, HSP60 modulates immune responses, and this modulation leads to the downregulation of Th1 responses and the upregulation of Th2 and Treg responses,[13,14] which are necessary effects for the control of CD. Additionally, the interaction between HSP60 and T cells leads to increased production and secretion of the anti-inflammatory cytokine IL-10 and reduced levels of proinflammatory cytokines, such as IFN-γ and TNF-α.[15]

In this context, our research group constructed a strain of *Lactococcus lactis* NCDO2118 that expresses *Mycobacterium leprae* Hsp65 protein[16] through the xylose-inducible expression system (XIES).[17] We have also demonstrated that this recombinant strain is able to completely prevent colitis in a murine model.[18]

Furthermore, oral treatment with Hsp65-secreting *L. lactis* NCDO2118 ameliorates intestinal inflammation induced by the chemical agent dextran sulfate sodium (DSS) through the induction of CD4+Foxp3+ and CD4+LAP+ Treg cells and via IL-10- and Toll-like receptor 2 (TLR2)-dependent pathways.[18] The wild-type strain (*L. lactis* subsp. *lactis* NCDO2118) also exhibits anti-inflammatory and immunomodulatory properties in an inflamed colon when administered during the remission period of DSS-induced colitis.[19]

More recently, our group developed and evaluated a new recombinant strain of *L. lactis* NCDO2118 that expresses both *Staphylococcus aureus* fibronectin-binding protein A (FnBPA+) and *M. leprae* Hsp65 – *L. lactis* NCDO2118 FnBPA+ (pXYCYT:Hsp65) – in acute colitis.[20] This experimental strategy efficiently delivers Hsp65 to the site affected by the disease and is capable of reducing the severity of inflammatory damage caused by TNBS-induced colitis in mice. These effects are related to reduced levels of IL-12 and IL-17 and increased levels of IL-10 and secretory immunoglobulin A (sIgA).[20]

Therefore, the present study aimed to evaluate the anti-inflammatory and antifibrotic capacities of this invasive and Hsp65-producing strain [*L. lactis* NCDO2118 FnBPA+ (pXYCYT:Hsp65)] in the prevention of experimental chronic colitis chemically induced by TNBS in a murine model.

MATERIAL AND METHODS

Bacterial Strains, Growth Conditions and Induction of Hsp65 Expression

The invasive *L. lactis* FnBPA+ (pXYCYT:Hsp65) strain[20] was grown at 30°C without agitation in M17 medium (Sigma-Aldrich, São Paulo, Brazil) supplemented with 0.5% glucose, chloramphenicol (10 µg/mL) (Sigma-Aldrich, São Paulo, Brazil) and erythromycin (5 µg/mL) (Sigma-Aldrich, São Paulo, Brazil). To induce expression of the *M. leprae* Hsp65 ORF, the recombinant *L. lactis* NCDO2118 FnBPA+ (pXYCYT:Hsp65) strain was grown with 2% xylose (Dinâmica, Indaiatuba, Brazil) and 0.5% galactose (Vetec, Duque de Caxias, Brazil) for approximately 8 hours (until the cells reached an $OD_{600\ nm}$ of ~ 2).[17,20]

Mice

BALB/c female mice (aged 6-7 weeks) were obtained from the Central Bioterium of the Federal University of Minas Gerais (UFMG, Belo Horizonte, Brazil). The mice were maintained in mini-isolators housed in ventilated racks with controlled conditions (temperature of 22 ± 2°C, 50 ± 10% humidity, air flow of 35 exchanges/hour and 12-hour light/12-hour dark cycle)[20] and were given free access to water and rodent food.

Induction of Chronic TNBS Colitis

Chronic intestinal inflammation was induced by 2,4,6-trinitrobenzene sulfonic acid [TNBS, 5% (wt/vol) in H_2O (Sigma-Aldrich, cat. n°. p2297, São Paulo, Brazil)] using a previously described technique.[21] After a 6-hour fasting period, the animals were anesthetized through an intraperitoneal injection of 100 mg/kg ketamine (Agener, São Paulo, Brazil) and 10 mg/kg xylazine (Ceva, Paulínia, Brazil). On day 1, the mice were presensitized by adding 150 µL of 1% TNBS solution dissolved in acetone/olive oil (4:1 v/v) to their shaved dorsal skin. After 7 days, chronic inflammation was induced by the weekly (on days 8, 15, 22, 29, 36 and 43) intrarectal (i.r.) administration of 100 µL of increasing concentrations of TNBS in 50% ethanol [0.75%, 1.5% and 2.5% TNBS (wt/vol)]. The mouse body weight was recorded weekly. On day 54, all the mice were sacrificed under anesthesia. A schematic of the experimental protocol used for the induction of chronic colitis is shown in Figure 1.

Treatment of Chronic Intestinal Inflammation

For the treatment of chronic TNBS-induced colitis, 100 µL of the *L. lactis* FnBPA+ (pXYCYT:Hsp65) strain was administered by intragastric gavage (i.g.). Starting on the

day after the intrarectal administration of TNBS solution, the mice received oral administrations of a bacterial suspension of invasive *L. lactis* FnBPA+ (pXYCYT:Hsp65) [containing 1 x 10^9 colony forming units (CFUs) in 0.9% saline] once daily for 4 consecutive days, and these administrations were performed for 6 consecutive weeks (Fig. 1).

The experimental design included the following four groups (6 mice/group), which were established based on previous results from our research group[20]: i) negative control of chronic colitis (C- group), the mice received 0.9% saline (i.g.) and 0.9% saline (i.r.); ii) positive control of chronic colitis (C+ group), the mice received 0.9% saline (i.g) and TNBS (i.r.); iii) NFX group, the mice were treated with invasive *L. lactis* FnBPA+ (pXYCYT:Hsp65) strain not expressing Hsp65 (i.g.) and TNBS (i.r.); and iv) NFXi group, the mice were treated with invasive *L. lactis* FnBPA+ (pXYCYT:Hsp65) strain expressing Hsp65 (i.g.) and TNBS (i.r.).

Histopathological Evaluation of Intestinal Inflammation and Fibrosis

Each animal was given a histological score to describe the severity of chronic colitis as previously described.[22] Hematoxylin and eosin (H&E)-stained and Gomori-stained colon samples were used to obtain the histological scores of inflammation and fibrosis, respectively.

H&E-stained samples from each layer (mucosa, submucosa, muscle and serosa) of the colon wall were used to score (range of 0 to 4, where 4 indicates the highest severity) the severity of acute and chronic inflammation. The acute and chronic scores from each layer were summed to derive an overall inflammatory score for each mouse, and the maximum possible score was 32. The acute inflammatory score was based on hemorrhage, edema, polymorphonuclear leukocyte infiltration and necrosis, whereas the chronic inflammatory score was based on the detected number of mononuclear cells (lymphocytes and macrophages).

The Gomori-stained samples were assigned a score ranging from 0 to 5 based on the increase in collagen deposition: a score of 0 represents no increase, and scores of 1-5 represent progressive increases in collagen deposition in different layers of the colon wall. A score of 5 represents the most severe fibrosis and indicates increases in collagen throughout all layers, from the mucosa to the serosa. The fibrosis score was multiplied by 1-4 to reflect the extent (0-100%) of the section that exhibits fibrosis.

Assessment of Myeloperoxidase (MPO) Activity

To assess the degree of chronic inflammation, the activity of the enzyme myeloperoxidase (MPO) in colon samples was measured. Colon tissues (50 mg) were collected and homogenized in i) cytokine extraction buffer [23.4 g of NaCl, 500 µL of Tween-20, 5 g of BSA, 34 mg of PMSF, 1 mL of DMSO, 44.6 mg of benzethonium chloride, 372 mg of Na_2EDTA, and 40 µL of 10 mg/mL aprotinin (Sigma-Aldrich, São Paulo, Brazil) in 1 L of q.s.p. PBS1X]; ii) buffer I (pH 4.7; 0.1 M NaCl, 0.02 M Na_3PO_4, and 0.015 M Na_2EDTA); and iii) 0.2% NaCl and 1.6% NaCl-5% glucose. The homogenate was then divided into two parts, and one of the parts was diluted in buffer II (pH 5.4; 0.05 M Na_3PO_4 and 0.5% HETAB). The homogenate was then subjected to three cycles of freezing in liquid nitrogen and thawing in water at room temperature. The substrate 3,3′,5,5′-tetramethylbenzidine (TMB) (Sigma Aldrich, São Paulo, Brazil) was then added, and the mixture was incubated at 37°C for 5 minutes. Subsequently, and 0.002% H_2O_2 was added, and the mixture was incubated for more than 5 minutes at 37°C. The reaction was interrupted by the addition of 1 M H_2SO_4. The color intensity was evaluated using a microplate reader (POLARIS MA616 – Marconi, Piracicaba, Brazil) at 450 nm.

Determination of the Colon Cytokine Profile

The concentrations of pro- and anti-inflammatory cytokines in colon homogenates (100 mg of tissue/1 mL of cytokine extraction buffer) were quantified using sandwich ELISA kits [BD (BD Biosciences, San Jose, CA, USA) and R&D (R&D Systems, Minneapolis, MN, USA)] in accordance with the manufacturers' instructions. The measured cytokines were IFN-γ, IL-12, IL-6, IL-13, IL-17, TGF-β and IL-10, and the absorbances were measured using a spectrophotometer (POLARIS MA616 – Marconi, Piracicaba, Brazil) at 492 nm.

Secretory IgA (sIgA) Assay

The sIgA levels in intestinal lavage were determined using a capture ELISA. The colon was washed with 5 mL of PBS1X, and the fluid was centrifuged at 1,200 rpm and 4°C for 10 minutes. The sIgA levels in the supernatant were measured using goat anti-mouse IgG, human ads-UNLB and goat anti-mouse IgA conjugated with horseradish

peroxidase (HRP) antibodies (Southern Biotech, Birmingham, AL, USA) according to the manufacturer's instructions. The absorbance (492 nm) was measured using a POLARIS MA616 – Marconi instrument (Piracicaba, Brazil).

Statistical Analysis

The results obtained from the assessment of TNBS-induced chronic colitis are presented as the means ± standard deviations (SDs) from three experimental replicates (n = 18). All the data were analyzed by one-way analysis of variance (one-way ANOVA) followed by Tukey's posttest using GraphPad Prism version 6.0 (GraphPad Software, San Diego, CA, USA). The values were considered statistically significant if $P < 0.05$.

ETHICAL CONSIDERATIONS

All the experiments and handling of the mice were performed in accordance with the ethical principles for animal experimentation adopted by the Animal Use Ethics Commission (CEUA, registration number 341/2017) of the Federal University of Minas Gerais (UFMG, Belo Horizonte, Brazil).

RESULTS

Effects of *L. lactis* NCDO2118 FnBPA+ (pXYCYT:Hsp65) in Clinical Health Parameters of Mice with TNBS-Induced Chronic Colitis

In this study, to examine the severity of lesions caused by intrarectal TNBS administration, the body weight and histological scores were evaluated. As shown in Figure 2, the animals subjected to TNBS administration (the NFX and NFXi groups) had lower body weights ($P < 0.05$) than those in the C- group at weeks 4 and 5. However, at weeks 6 and 7, all the mice that received TNBS (the C+, NFX and NFXi groups) presented significant weight loss ($P < 0.05$) compared with those in the C- group. No significant differences were observed among all the groups at weeks 1, 2, 3 and 8.

The histological observations revealed that the mice belonging to the C- group presented neither intestinal inflammation nor fibrosis ($P < 0.05$; Figs. 3A, 4A), and H&E and Gomori staining showed that that the morphology was normal (Figs. 3B, 4B). The mucosa was intact, as demonstrated by the presence of goblet cells and the absence of

erosion and inflammatory infiltrate. The submucosa was intact, as revealed by a basal collagen level and the absence of hyperemia, hemorrhage or inflammatory infiltrate. In addition, no evidence of mucosal muscle injury was observed. The muscular layer itself was intact, with no inflammatory infiltrate, collagen deposition or areas of necrosis. The serosa was thin and did not exhibit any pathological elements (Figs. 3B, 4B).

All the animals with TNBS-induced chronic colitis (with the exception of those belonging to the NFXi group) presented similar symptoms of intestinal inflammation and fibrosis (Figs. 3A, 4A). H&E and Gomori staining showed pathological changes in all the layers (Figs. 3B, 4B). The mucous layer contained areas of erosion and inflammatory infiltrate. The submucosal layer presented hyperemic areas with mild hemorrhage and inflammatory infiltrate with polymorphonuclear and mononuclear leukocytes at degrees ranging from severe to very severe. The muscular layer itself showed areas of necrosis and inflammatory infiltrate, and the serous layer was marked by inflammatory infiltrate (Fig. 3B). Fibrosis was present in all the layers and was predominant in the submucosal layer (Fig. 4B).

The NFXi group exhibited significantly reduced intestinal inflammation ($P < 0.05$) compared with the animals belonging to the C- and NFX groups (Fig. 3A). Additionally, this group presented significantly reduced fibrosis ($P < 0.05$) compared with the other groups (C+ and NFX groups) that were subjected to TNBS administration (Fig. 4A). Thus, H&E and Gomori staining showed that the NFXi group presented lesions characterized as mild based on the intensity of acute inflammatory infiltrate, hyperemia, erosion, necrosis and fibrosis (Figs. 3B, 4B).

Effects of *L. lactis* NCDO2118 FnBPA+ (pXYCYT:Hsp65) on Inflammatory Markers in Mice with TNBS-Induced Colitis

To investigate the effect of MPO activity, which serves as a biomarker for the influx of neutrophils to the inflammatory site, we assessed the ability of *L. lactis* NCDO2118 FnBPA+ (pXYCYT:Hsp65) to decrease inflammatory cell infiltration in colon samples. No difference in MPO activity was observed among the studied groups (data not shown).

Effects of *L. lactis* NCDO2118 FnBPA+ (pXYCYT:Hsp65) on the Secretion of Pro- and Anti-Inflammatory Cytokines in Mice with TNBS-Induced Colitis

To assess whether *L. lactis* NCDO2118 FnBPA+ (pXYCYT:Hsp65) can modulate the cytokine levels in chronic colitis, the levels of IFN-γ, IL-12, IL-6, IL-13, IL-17, TGF-β and IL-10 in colonic tissues were measured. The levels of IFN-γ and IL-12, which are cytokines related to the Th1 immune profile, did not differ among the experimental groups (data not shown).

In contrast, the evaluation of Th2 profile-related cytokines revealed that the NFXi group presented significantly lower IL-6 levels ($P < 0.05$) than those in the C+ group (Fig. 5A) and significantly lower IL-13 levels ($P < 0.05$) than those in the NFX group (Fig. 5B).

The assessment of the IL-17 levels showed that the animals in the NFXi group had significantly lower IL-17 levels ($P < 0.05$) than the animals in the C+ and NFX groups (Fig. 5C). The measurement of anti-inflammatory cytokines revealed that the NFXi group presented significantly decreased TGF-β levels ($P < 0.05$; Fig. 5D) and significantly increased IL-10 levels ($P < 0.05$; Fig. 5E) than those in the NFX group.

Effects of *L. lactis* NCDO2118 FnBPA+ (pXYCYT:Hsp65) on Secretory IgA (sIgA) in Chronic Colitis

The levels of sIgA in intestinal lavage were then determined to investigate whether the oral administration of *L. lactis* NCDO2118 FnBPA+ (pXYCYT:Hsp65) could alter the production of sIgA in the colon. No differences in the sIgA concentrations were observed among the groups (data not shown).

DISCUSSION

CD, a multifactorial IBD for which there is no cure, is characterized by chronic inflammation of the digestive system wall,[23,10] and intestinal fibrosis is a recurrent complication of CD that can lead to serious damage, such as intestinal obstruction.[8] Therefore, in the present study, we used a mouse model of chronic colitis to evaluate the anti-inflammatory and antifibrotic potential of an invasive and Hsp65-producing strain [*L. lactis* NCDO2118 FnBPA+ (pXYCYT:Hsp65)]. Mycobacterial Hsp65 protein produced by *L. lactis* NCDO2118 has been described as a molecule with therapeutic potential in C57BL/6 mice with DSS-induced intestinal inflammation[18] and in BALB/c mice with TNBS-induced colitis.[20]

Initially, we adopted a model of chronic colitis chemically induced by TNBS because the onset of inflammation is immediate, the procedure is relatively simple, and the induction produces a chronic inflammatory lesion associated with intestinal tissue fibrosis.[21,24] Additionally, this model allows us to monitor important animal health parameters, such as the body weight and histological features of acute and chronic inflammation and fibrosis.

The analysis of the body weight showed that all the animals that received TNBS (C+, NFX and NFXi groups) exhibited weight loss compared with those in the C- group. This finding was obtained because this model of chronic colitis promotes transmural inflammation in the animals, which is followed by severe diarrhea, weight loss and rectal prolapse.[25] In addition, at week 8 in the experimental schedule, when the administrations of intrarectal TNBS were completed, all the animals belonging to these three groups exhibited some recovery, and their body weights were nearly equal to that of the C- group. These results might be explained by the decreased ability to maintain TNBS-induced colitis in mice older than 8 weeks.[26]

The analysis of the histological scores of inflammation and fibrosis revealed that the animals in the NFXi group exhibited a significant decrease in the severity of colitis compared with those in the C+ and NFX groups, which emphasized the therapeutic potential of the *L. lactis* NCDO2118 FnBPA+ (pXYCYT:Hsp65) strain. In a similar study, we showed that this new recombinant strain can attenuate the severity of intestinal inflammation in acute TNBS-induced colitis by increasing the levels of the anti-inflammatory cytokine IL-10 and immunoglobulin sIgA.[20]

We measured MPO as an inflammatory biomarker of colitis because an increased concentration of MPO indicates the infiltration of activated neutrophils into inflamed tissue and suggests an exacerbation of inflammation in the colon.[27] In the present study, significant differences could not be detected among the groups, even though the release of MPO is common in both acute and chronic inflammation.[28]

In addition, no significant differences in the proinflammatory cytokines IFN-γ and IL-12 were found among the groups, and this finding was most likely due to the fact that these cytokines were assessed on day 54 of the experimental schedule, which is after the peak in cytokine production. Consistent with our observation, the literature reports that these cytokines (IFN-γ and IL-12) participate in the acute process of intestinal

inflammation induced by TNBS that their peak production thus occurs around day 7 in BALB/c mice.[24]

In the inflamed mucosa of patients with IBDs, the interleukins IL-6 and IL-13, which are cytokines related to the Th2 profile, are commonly found at increased levels.[2,29] The assessment of the IL-6 levels performed in this study showed that the NFXi group presented reduced levels of this proinflammatory cytokine compared with the C+ group. IL-6 has been identified as an important interleukin that mediates chronic inflammation in colitis,[30,31] whereas IL-13 is a potent inducer of fibrosis in chronic autoimmune diseases, including CD.[32,8] In this study, significant differences in the IL-13 levels were found between the NFXi and NFX groups. These results corroborate the histological findings of fibrosis, which revealed that the mice in the NFXi group presented less collagen deposition in the intestinal submucosa.

Because TNBS-induced colitis is characterized by a Th1/Th17 immune profile,[33] the levels of the cytokine IL-17 were measured. The IL-17 results showed a reduction in this proinflammatory cytokine in the NFXi group compared with those the C+ and NFX groups. This reduction is important for achieving decreases in histological changes related to both acute and chronic inflammation and fibrosis because IL-17 exerts profibrotic functions in the intestinal mucosa.[8,34]

The anti-inflammatory cytokines TGF-β and IL-10 were also measured. Although TGF-β is considered an anti-inflammatory cytokine,[35] no significant increase in the level of this cytokine was found in the present study in the NFXi group. Nonetheless, high concentrations of TGF-β, together with IL-13, can contribute to the increase in tissue fibrosis observed in IBDs,[32] and previous studies have shown the profibrogenic role of TGF-β in chronic colitis.[9,30,36,37] Here, the decreases in TGF-β observed in the NFXi group compared with the levels in the C+ and NFX groups, which are associated with the above-mentioned decreases in IL-13, might have contributed to the observed reductions in the intestinal inflammation and fibrosis scores.

Our analysis of IL-10, which is the main regulatory cytokine for inflammation, showed a significant increase in the NFXi group compared with the NFX group. This result possibly explains the inflammation scores and decreased histological features of the NFXi group. Notably, IL-10 is an influential interleukin that can modulate pro- and anti-inflammatory cytokines,[38] and IL-10 can also suppress exacerbated mucosal

immune responses, maintain intestinal homeostasis and tolerance to commensal microbiota[39] and inhibit collagen deposition in the host intestine.[30]

The intestinal sIgA levels were also measured, but no significant differences were found among the groups. In mammals, particularly mice and humans, IgA is the most abundant antibody. sIgA can protect the intestinal mucosa against toxins and infections.[40] Thus, *L. lactis* NCDO2118 FnBPA+ (pXYCYT:Hsp65) did not affect the increased sIgA levels in the chronic colitis model.

CONCLUSIONS

In conclusion, the results demonstrate that the oral administration of *L. lactis* FnBPA+ (pXYCYT:Hsp65) effectively improves various health parameters of animals with chronic colitis. Notably, the effectiveness of this new experimental strategy can be explained by the observed reductions in the severity of inflammation and intestinal fibrosis through decreases in IL-13 and TGF-β and increases in IL-10. Therefore, this strategy of delivering therapeutic proteins to mammalian cells should be considered an alternative approach for maintaining the pro- and anti-inflammatory balance of the gastrointestinal tract in individuals affected by fibrotic CD.

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FIGURES AND LEGENDS

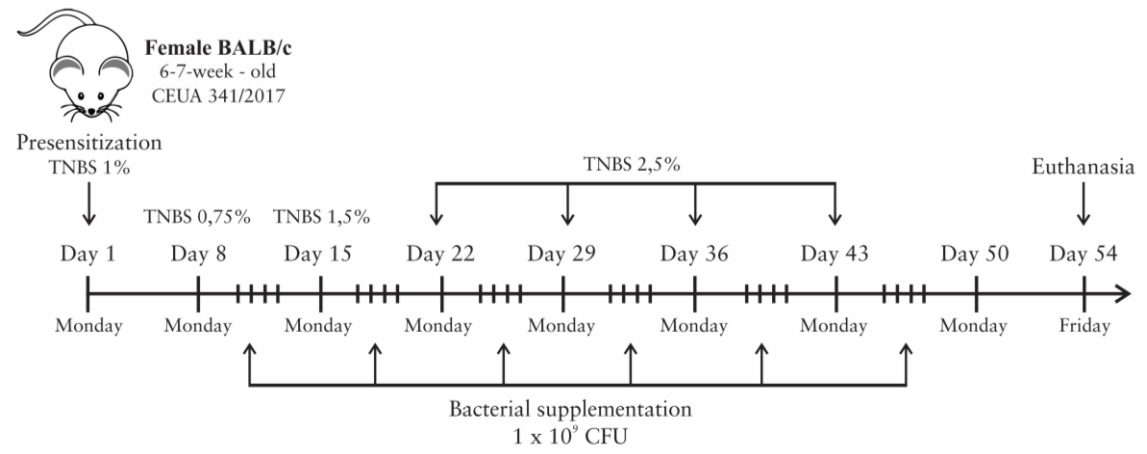


FIGURE 1. Representation of the experimental design for 2,4,6-trinitrobenzene sulfonic acid (TNBS)-induced chronic colitis. Intestinal inflammation was induced by TNBS in BALB/c mice. On day 1, the animals were presensitized with 1% TNBS. After 7 days, chronic colitis was induced by weekly (on days 8, 15, 22, 29, 36 and 43) intrarectal administration of solutions with increasing concentrations of TNBS in 50% ethanol [0.75%, 1.5% and 2.5% TNBS (wt/vol)]. The mice received four intragastric administrations of bacterial suspension containing 1 x 10^9 CFUs per week for 6 consecutive weeks. On day 54, all the mice were euthanized.

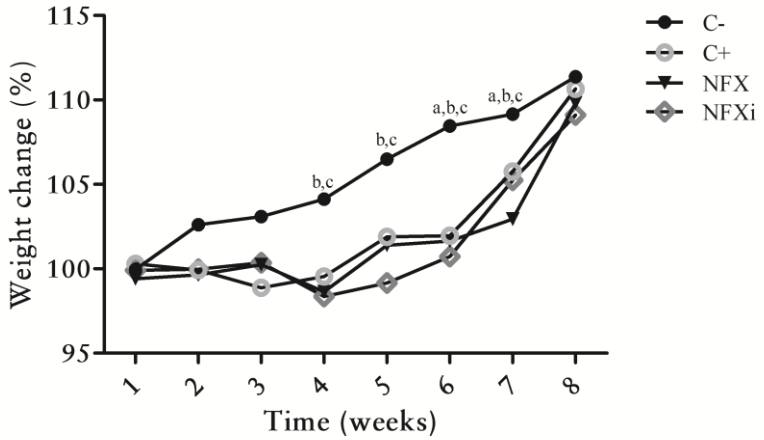
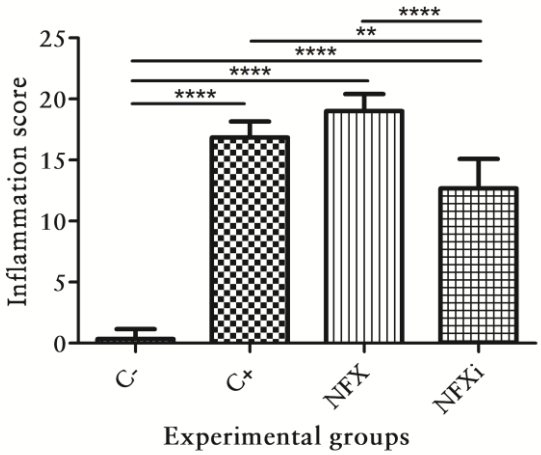


FIGURE 2. Percentage of the initial body weight of BALB/c mice with or without TNBS administration as a function of time. a, Experimental group whose body weight percentages were significantly different from those of the C+ group. b, Experimental group whose body weight percentages were significantly different from those of the NFX group. c, Experimental group whose body weight percentages were significantly different from those of the NFXi group. The data are shown as the means ± SDs from three independent experiments (n = 18). *P* value: $^{*}P < 0.05$.

A



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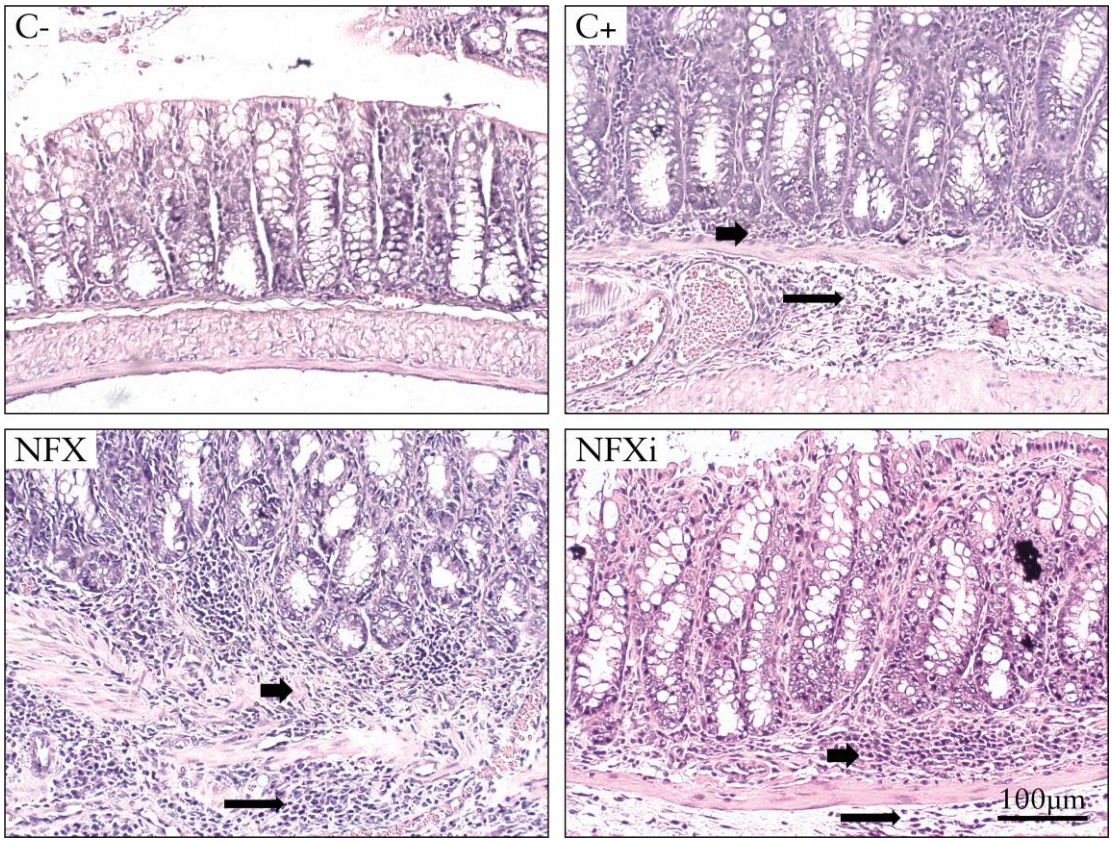
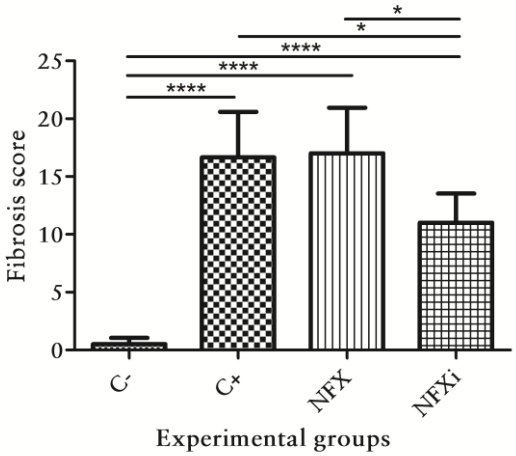


FIGURE 3. Inflammation scores and histological features of the colon of mice with chronic TNBS-induced colitis. A, Inflammation scores of BALB/c mice with or without TNBS administration. B, Histological features of the colon of BALB/c mice with or without TNBS administration (H&E staining). The short arrows point to the inflammatory infiltrate in the intestinal mucosa layer, and the long arrows indicate the inflammatory infiltrate in the intestinal submucosal layer. The data are shown as the means ± SDs from three independent experiments (n = 6). *P* values: $^{**}P < 0.01$; $^{****}P < 0.0001$.

A



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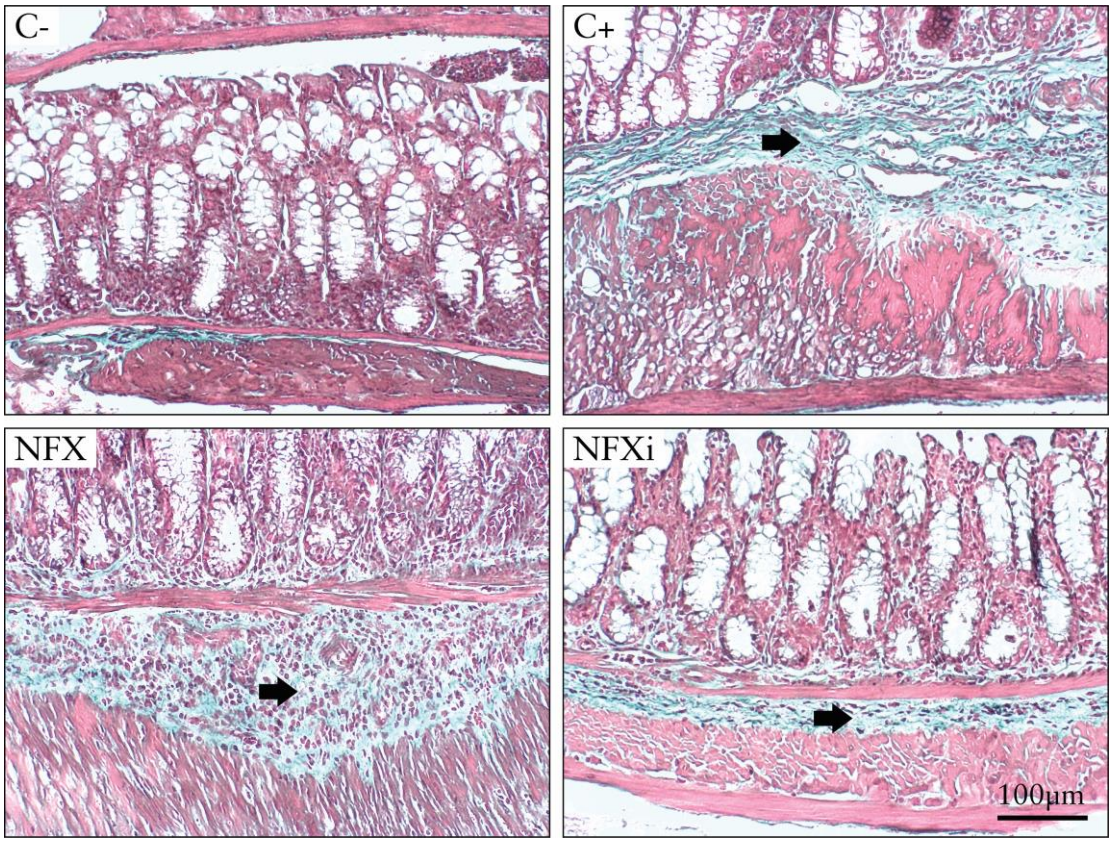

FIGURE 4. Fibrosis scores and histological features of the colon of mice with chronic TNBS-induced colitis. A, Fibrosis scores of BALB/c mice with or without TNBS administration. B, Histological features of the colon of BALB/c mice with or without TNBS administration (Gomori staining). The short arrows indicate fibrosis, as highlighted by the presence of collagen in the intestinal submucosal layer. The data are shown as the means ± SDs from three independent experiments (n = 6). *P* values: $^{*}P < 0.05$; $^{****}P < 0.0001$.

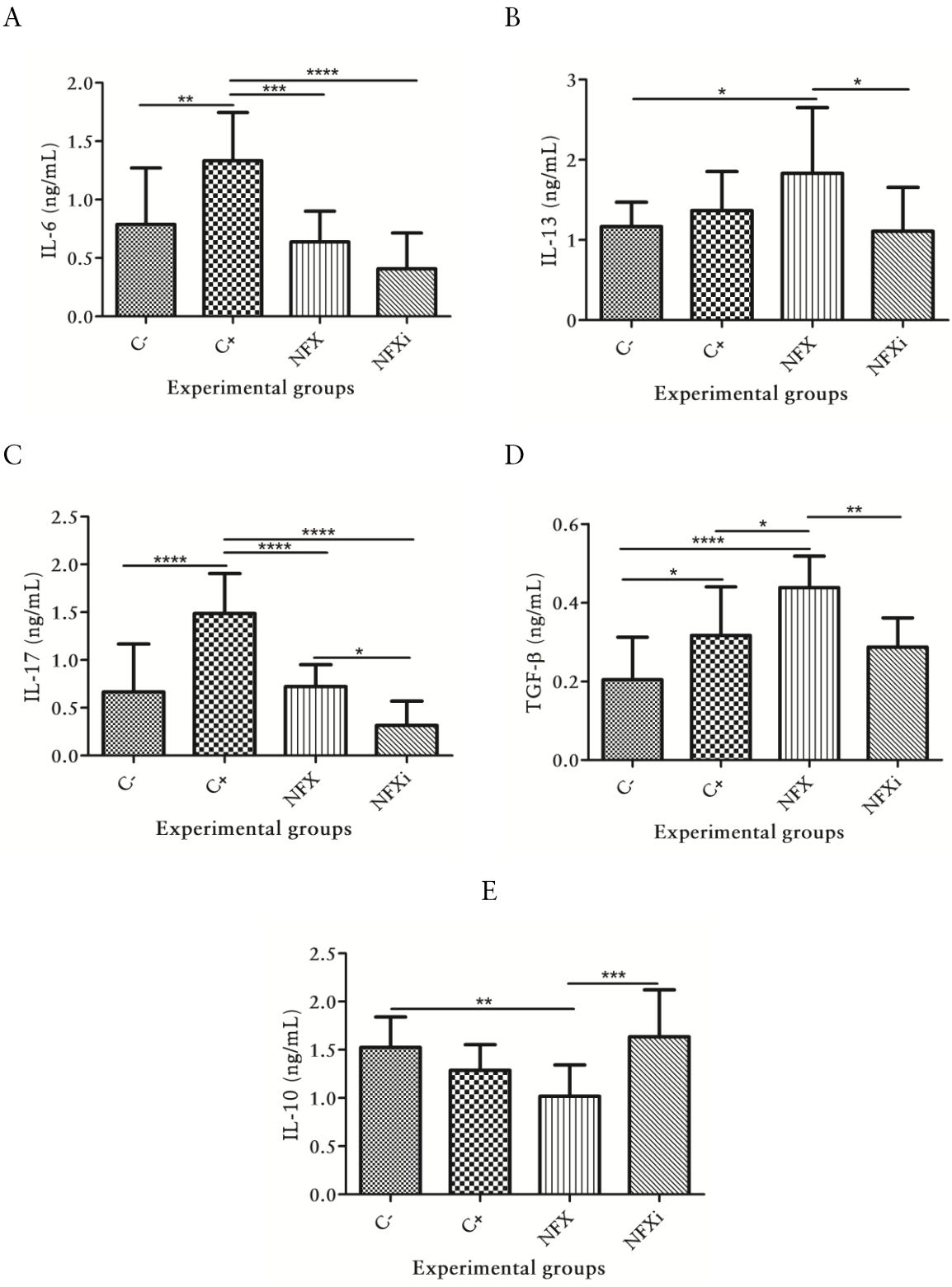

FIGURE 5. Cytokine levels in BALB/c mice with or without TNBS administration. A, Interleukin-6 (IL-6) levels. B, Interleukin-13 (IL-13) levels. C, Interleukin-17 (IL-17) levels. D, Transforming growth factor-β (TGF-β) levels. E, Interleukin-10 (IL-10) levels. The data are shown as the means ± SDs from three independent experiments (n = 12). *P* values: $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$.

5 CONCLUSÕES

Em conclusão, os resultados desses estudos comprovaram que a administração oral de *L. lactis* FnBPA+ (pXYCYT:Hsp65) foi eficaz na melhoria dos parâmetros de saúde animal nos modelos murinos de colite aguda e crônica da CD. É importante salientar que a eficácia dessa nova estratégia experimental, se justificou, na colite aguda, pela capacidade de atenuar a severidade da inflamação, por meio, principalmente, da diminuição das citocinas IL-12 e IL-17 e aumento de IL-10 e sIgA. E, na colite crônica, pela diminuição da gravidade da inflamação (aguda e crônica) e da fibrose intestinal, através da redução de IL-13 e TGF-β e aumento de IL-10. Finalmente, essa estratégia de entrega de proteínas terapêuticas às células mamíferas (enterócitos) deve ser considerada como uma alternativa para a manutenção do equilíbrio pró e anti-inflamatório do GIT em indivíduos acometidos por essa IBD.

6 PERSPECTIVAS

Atualmente, todos os experimentos desenvolvidos nessa tese foram finalizados. No entanto, esse trabalho abriu como perspectiva o desenvolvimento de um novo estudo utilizando a linhagem invasiva e produtora de Hsp65 de *M. leprae* [*L. lactis* NCDO2118 FnBPA+ (pXYCYT:Hsp65)] em modelo murino de colite espontânea.

Assim, propõe-se a utilização de camundongos deficientes para a IL-10 (IL-$10^{-/-}$), que desenvolvem a colite espontaneamente, visando elucidar o papel dessa interleucina na atividade imunomoduladora da supracitada linhagem bacteriana. Pois, foi constatado que a IL-10, tanto na colite aguda como na crônica, induzida por TNBS, estava aumentada.

Com o intuito de esclarecer, ainda mais, o papel da IL-10 na atividade anti-inflamatória de *L. lactis* NCDO2118 FnBPA+ (pXYCYT:Hsp65), pode-se avaliar o perfil de macrófagos M1 e M2. Uma vez que os macrófagos M2 são os maiores responsáveis pelo restabelecimento dos níveis da IL-10 no cólon inflamado de camundongos.

Desse modo, em modelo murino de colite espontânea, pode-se avaliar aspectos convencionais da inflamação, como: os parâmetros de saúde animal, os índices de danos macroscópicos e histopatológicos e as citocinas pró e anti-inflamatórias. Por fim, as quimiocinas pró-inflamatórias e as alterações da microbiota também podem ser investigadas.

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ANEXOS

PARTICIPAÇÃO EM ARTIGOS PUBLICADOS

1. Attenuation of intestinal inflammation in IL-10 deficient mice by a plasmid carrying *Lactococcus lactis* strain - https://doi.org/10.1186/s12896-020-00631-0

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RESEARCH ARTICLE Open Access

Attenuation of intestinal inflammation in IL-10 deficient mice by a plasmid carrying *Lactococcus lactis* strain

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Abstract

Background: Inflammatory bowel diseases (IBD) are intestinal disorders characterized by inflammation in the gastrointestinal tract (GIT) and to date, no efficient treatments exist. Interleukin-10 (IL-10), one of the most important anti-inflammatory cytokines of the immune response, has been under study due to its potential for IBD therapy; however, systemic treatments lead to undesirable side effects and oral administration is limited due to its quick degradation. To avoid these bottlenecks, we previously engineered an invasive *Lactococcus lactis* (*L. lactis*) strain capable of delivering, directly to host cells, a eukaryotic DNA expression vector coding for IL-10 of *Mus musculus* (pValac:*il-10*) that diminished inflammation in two induced mouse models of intestinal inflammation. Thus, the aim of this study was to analyze its therapeutic effect in the IL-10-deficient mouse model (IL-$10^{-/-}$) that spontaneously and gradually develops an inflammation that modifies the immune system and resembles Crohn's disease (CD) in humans, and evaluate if it would also diminish and/or prevent the onset of this disease.

Results: Oral administration of *L. lactis* MG1363 FnBPA+ (pValac:*il-10*) to IL-$10^{-/-}$ mice not only led to IL-10 production by these, but consequently also diminished the severe development of the disease, with animals showing lower macroscopic scores and histological damages, increased IL-10 levels and tendency to lower pro-inflammatory cytokine levels.

Conclusions: The results of this study, together with the previously published ones using this DNA delivery-based strategy, show that it is capable of creating and maintaining an anti-inflammatory environment in the GIT and thus effectively diminish the onset of inflammation in various mouse models.

Keywords: Inflammatory bowel diseases, Interleukin-10, *Lactococcus lactis*, pValac:*il-10*, IL-10-deficient mice

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2. *Lactococcus lactis* carrying a DNA vaccine coding for the ESAT-6 antigen increases IL-17 cytokine secretion and boosts the BCG vaccine immune response - https://doi.org/10.1111/jam.13449

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ORIGINAL ARTICLE

Lactococcus lactis carrying a DNA vaccine coding for the *ESAT-6* antigen increases IL-17 cytokine secretion and boosts the BCG vaccine immune response

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Keywords
BCG, DNA vaccine, ESAT-6, *Lactococcus lactis*, tuberculosis.

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Abstract

Aims: A regimen utilizing Bacille Calmette—Guerin (BCG) and another vaccine system as a booster may represent a promising strategy for the development of an efficient tuberculosis vaccine for adults. In a previous work, we confirmed the ability of *Lactococcus lactis* fibronectin-binding protein A (FnBPA+) (pValac:*ESAT-6*), a live mucosal DNA vaccine, to produce a specific immune response in mice after oral immunization. In this study, we examined the immunogenicity of this strain as a booster for the BCG vaccine in mice.
Methods and Results: After immunization, cytokine and immunoglobulin profiles were measured. The BCG prime *L. lactis* FnBPA+ (pValac:*ESAT-6*) boost group was the most responsive group, with a significant increase in splenic pro-inflammatory cytokines IL-17, IFN-γ, IL-6 and TNF-α compared with the negative control.
Conclusions: Based on the results obtained here, we demonstrated that *L. lactis* FnBPA+ (pValac:*ESAT-6*) was able to increase the BCG vaccine general immune response.
Significance and Impact of the Study: This work is of great scientific and social importance because it represents the first step towards the development of a booster to the BCG vaccine using *L. lactis* as a DNA delivery system.

Introduction

Tuberculosis (TB) remains a serious threat to human health in the world. The *Mycobacterium bovis* Bacille Calmette—Guerin (BCG), the only available vaccine against TB, exhibits unstable protective effects against adult pulmonary TB (WHO 2012). Therefore, more effective vaccines against adult TB are needed. Thus, the vaccination regimen presented may represent a practical and valid intervention for the control of TB.

The use of promising immunodominant antigens, such as ESAT-6 (6-kDa early-secreted antigenic target) in *Mycobacterium tuberculosis*, offers a strategy to improve the immunogenicity and protective efficacy of BCG vaccine (Das Gupta *et al.* 1998; Dhar *et al.* 2000). ESAT-6 is a secretory protein present in pathogenic *M. tuberculosis* and absent in the BCG vaccine (Pym *et al.* 2002). The prime—boost vaccination system has already been successfully tested in several studies, in which the use of ESAT-6 and other mycobacterial antigens encoding DNA vaccine in combination with BCG induced a stronger immune response and was more protective than either vaccine alone (Fan *et al.* 2007; Wang *et al.* 2009; Dey *et al.* 2010; Lu *et al.* 2011; Cervantes-Villagrana *et al.* 2013; Tan *et al.* 2014; Ji *et al.* 2016).

The use of *Lactococcus lactis* for mucosal DNA delivery is a promising strategy. *Lactococcus lactis* is the model lactic acid bacteria, is generally regarded as safe (GRAS status), it does not colonize the intestinal tract, preventing the development of tolerance, and resists the stomach acid environment, being a good alternative compared with attenuated pathogens for oral DNA immunization.

The ability of *L. lactis* to deliver DNA into mammalian cells was first demonstrated using native *L. lactis* strains

3. *Lactococcus lactis* carrying the pValac eukaryotic expression vector coding for IL-4 reduces chemically-induced intestinal inflammation by increasing the levels of IL-10-producing regulatory cells - https://doi.org/10.1186/s12934-016-0548-x

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Microbial Cell Factories

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Lactococcus lactis carrying the pValac eukaryotic expression vector coding for IL-4 reduces chemically-induced intestinal inflammation by increasing the levels of IL-10-producing regulatory cells

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Abstract

Background: Inflammatory bowel diseases are characterized by chronic intestinal inflammation that leads to severe destruction of the intestinal mucosa. Therefore, the understanding of their aetiology as well as the development of new medicines is an important step for the treatment of such diseases. Consequently, the development of *Lactococcus lactis* strains capable of delivering a eukaryotic expression vector encoding the interleukin 4 (IL-4) of *Mus musculus* would represent a new strategy for the elaboration of a more effective alternative therapy against Crohn's disease.

Results: The murine *IL-4* ORF was cloned into the eukaryotic expression vector pValac::dts. The resulting plasmid—pValac::dts::*IL-4*—was transfected into CHO cells so that its functionality could be evaluated in vitro. With fluorescent confocal microscopy, flow cytometry and ELISA, it was observed that pValac::dts::*IL-4*-transfected cells produced IL-4, while non-transfected cells and cells transfected with the empty vector did not. Then, pValac::dts::*IL-4* was inserted into *L. lactis* MG1363 FnBPA$^+$ in order to evaluate the therapeutic potential of the recombinant strain against TNBS-induced colitis. Intragastric administration of *L. lactis* MG1363 FnBPA$^+$ (pValac::dts::*IL-4*) was able to decrease the severity of colitis, with animals showing decreased levels of IL-12, IL-6 and MPO activity; and increased levels of IL-4 and IL-10. Finally, LP-isolated cells from mice administered TNBS were immunophenotyped so that the main IL-4 and IL-10 producers were identified. Mice administered the recombinant strain presented significantly higher percentages of F4/80$^+$MHCII$^+$Ly6C$^-$IL-4$^+$, F4/80$^+$MHCII$^+$Ly6C$^-$IL-10$^+$, F4/80$^+$MHCII$^+$Ly6C$^-$CD206$^+$CD124$^+$IL-10$^+$ and CD4$^+$Foxp3$^+$IL10$^+$ cells compared to the other groups.

Conclusions: This study shows that *L. lactis* MG1363 FnBPA$^+$ (pValac::dts::*IL-4*) is a good candidate to maintain the anti-inflammatory and proinflammatory balance in the gastrointestinal tract, increasing the levels of IL-10-secreting regulatory cells and, thus, demonstrating the effectiveness of this novel DNA delivery-based strategy.

Keywords: *Lactococcus lactis*, Crohn's disease, Interleukin 4, Interleukin 10, Regulatory cells

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Chapter 10

LACTOCOCCUS LACTIS AS A DNA VACCINE DELIVERY SYSTEM

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ABSTRACT

DNA vaccines, which consist of plasmids encoding antigens of interest, emerge as novel options to protect hosts against new emerging infectious diseases. As most pathogens affect or initiate their infection at mucosal surfaces, this is a strategic route to induce specific immune responses. However, to make it possible, many environmental barriers must be surpassed. In this context, the use of bacteria, like Lactococcus lactis, as vehicles to deliver vaccine plasmids by the mucosal route is a promising strategy. Native Lc. lactis was used for this purpose and, aiming to make the plasmid delivery more efficient, invasive recombinant Lc. lactis strains were constructed and tested, thus showing to be able to deliver DNA vaccines more efficiently than the wild type strain. Therefore, in summary, this chapter presents a general view of DNA vaccines and the use of bacteria, especially Lc. lactis, to deliver them to intestinal eukaryotic cells, representing a new strategy for the tuberculosis control.

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