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

Tatiane Melo Preisser

IMUNOMODULAÇÃO DA DIABETES TIPO 1 EM MODELOS MURINOS: EFEITOS DECORRENTES DA ADMINISTRAÇÃO ORAL DE LINHAGENS DE *Lactococcus lactis* CARREADORAS DE PLASMÍDEOS VACINAIS

Belo Horizonte
2021

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Orientador: Prof. Dr. Anderson Miyoshi

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Às quatorze horas do dia **10 de novembro de 2021**, reuniu-se remotamente a Comissão Examinadora de Tese, indicada pelo Colegiado do Programa, para julgar, em exame final, o trabalho intitulado: **"Imunomodulação da diabetes tipo 1 em modelos murinos: efeitos decorrentes da administração oral de linhagens de Lactococcus lactis carreadoras de plasmídeos vacinais"**, requisito para obtenção do grau de Doutora em **Genética.** Abrindo a sessão, o Presidente da Comissão, **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**
Anderson Miyoshi	UFMG	034.357.036-01	APROVADA
Frederico Marianetti Soriani	UFMG	249.486.428-35	APROVADA
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Andréa Queiroz Maranhão	UnB	284.615.683-20	APROVADA
Sérgio Oliveira de Paula	UFV	964.432.276-20	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, 10 de novembro de 2021.

Anderson Miyoshi (UFMG)

Frederico Marianetti Soriani (UFMG)

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Instituto de Ciências Biológicas
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FOLHA DE APROVAÇÃO

"Imunomodulação da diabetes tipo 1 em modelos murinos: efeitos decorrentes da administração oral de linhagens de Lactococcus lactis carreadoras de plasmídeos vacinais"

Tatiane Melo Preisser

Tese aprovada pela banca examinadora constituída pelos Professores:

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"A ciência e a vida cotidiana não podem
e não devem ser separadas."

Rosalind Franklin

RESUMO

A diabetes tipo 1 (T1D) é uma doença autoimune que culmina com a destruição das células beta no pâncreas e, subsequentemente, com a deficiência na produção de insulina. As citocinas desempenham um papel crucial no desenvolvimento da diabetes, orquestrando o recrutamento e a ação das células imunes, não só para destruir as células produtoras de insulina, mas também para preservá-las. Portanto, o objetivo deste estudo foi investigar o efeito da administração oral de linhagens de *Lactococcus lactis* MG1363 FnBPA$^+$ carreando plasmídeos codificadores de IL-4 e IL-10 no modelo de diabetes induzido por estreptozotocina (STZ) e em camundongos diabéticos não-obesos (NOD). Os camundongos induzidos por STZ que receberam, de forma combinada, as linhagens bacterianas portadoras de plasmídeos codificadores de IL-4 e IL-10 apresentaram menor incidência de diabetes e mais ilhotas pancreáticas preservadas do que os camundongos que receberam as linhagens bacterianas individualmente. Já nos camundongos NOD, a administração combinada de *L. lactis* MG1363 FnBPA$^+$ (pValac::*dts::IL-4*) e *L. lactis* MG1363 FnBPA$^+$ (pValac::*IL-10*) resultou em proteção contra a diabetes experimental. Foi demonstrado que a administração das bactérias recombinantes juntas preveniu a hiperglicemia e reduziu a destruição das ilhotas pancreáticas em camundongos NOD. Além disso, níveis aumentados de IL-4 e IL-10 no soro e no tecido pancreático revelou um efeito sistêmico dessa intervenção e também favoreceu um microambiente anti-inflamatório. A redução de IL-12 no pâncreas foi essencial para a regulação da inflamação, resultando em nenhuma incidência de diabetes em camundongos NOD que receberam as bactérias recombinantes. Os níveis normais de sIgA intestinal após a administração a longo prazo das linhagens de *L. lactis* portadoras de plasmídeos codificando IL-4 e IL-10 indicam o desenvolvimento de tolerância oral e corroboram o uso desta ferramenta de entrega via mucosa. Pela primeira vez, as linhagens de *L. lactis* MG1363 FnBPA$^+$ carreando vetores de expressão eucariótica codificando IL-4 e IL-10 são testadas nos principais modelos de T1D: induzido por STZ e de camundongos NOD. Assim sendo, nosso estudo demonstra que essa estratégia inovadora possui um potencial imunomodulador para investigações adicionais em T1D e outras doenças autoimunes.

ABSTRACT

Type 1 diabetes (T1D) is an autoimmune disease that culminates in beta cell destruction in the pancreas and, subsequently, deficiency in insulin production. Cytokines play a crucial role in the development of diabetes, orchestrating the recruitment and action of immune cells, to not only destroy insulin-producing cells but also preserve them. Therefore, the aim of this study was to investigate the effect of orally administered *Lactococcus lactis* MG1363 FnBPA$^+$ strains carrying plasmids encoding IL-4 and IL-10 in the streptozotocin- (STZ-) induced diabetes model and in nonobese diabetic (NOD) mice. The STZ-induced mice that were treated with combined bacterial strains carrying plasmids encoding IL-4 and IL-10 showed lower incidence of diabetes and more preserved pancreatic islets than the mice that received the individual bacterial strains. In NOD mice, combined administration of *L. lactis* MG1363 FnBPA$^+$ (pValac::*dts::IL-4*) and *L. lactis* MG1363 FnBPA$^+$ (pValac::*IL-10*) resulted in protection against experimental diabetes. It was shown that the combined administration of recombinant bacterial prevented hyperglycemia and reduced the pancreatic islets-destruction in NOD mice. In addition, increased levels of IL-4 and IL-10 in serum and pancreatic tissue revealed a systemic effect of this intervention and also favored an anti-inflammatory microenvironment. Reduced concentrations of IL-12 in pancreas were essential to the regulation of inflammation, resulting in no incidence of diabetes in NOD mice that received the recombinant bacteria. Normal levels of intestinal sIgA after long-term administration of the *L. lactis* strains carrying plasmids encoding IL-4 and IL-10 indicate the development of oral tolerance and corroborate the use of this tool of mucosal delivery. For the first time, *L. lactis* MG1363 FnBPA$^+$ strains carrying eukaryotic expression vectors encoding IL-4 and IL-10 are tested in the main models of T1D: STZ-induced and NOD mouse models. Therefore, our study demonstrates this innovative strategy provides immunomodulatory potential for further investigations in T1D and other autoimmune diseases.

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LISTA DE ABREVIATURAS

% – por cento

°C – graus Celsius

± – mais ou menos

ADA – *American Diabetes Association*

ADP – adenosina difosfato

ANOVA – *analysis of variance*

APC – célula apresentadora de antígeno

ATP – adenosina 5'-trifosfato

β – beta

BGH poli-A – sequência sinal de poliadenilação do Hormônio Bovino de Crescimento

BLG – β-lactoglobulina

BSA – *bovine serum albumin*

C – citosina

CD – *Cluster of Differentiation*

CDA – *Canadian Diabetes Association*

cDNA – ácido desoxirribonucleico complementar

CEUA – Comissão de Ética no Uso de Animais

CFU – *Colony Forming Units*

CIBio – Comissão Interna de Biossegurança

Cm – cloranfenicol

d – dia

dL – decilitro

DMSO – dimetilsulfóxido

DNA – ácido desoxirribonucleico

DO_{600} – densidade óptica no comprimento de onda de 600 nanômetros

dts – sequência de endereçamento nuclear

ELISA – *enzyme-linked immunosorbent assay*

Ery – eritromicina

FAO – *Food and Agriculture Organization*

FnBPA – proteína A de ligação à fibronectina

g – grama

G – guanina

GAD – *glutamic acid descarboxylase*

GADA – *glutamic acid descarboxylase autoantibody*

GALT – tecidos linfoides associados a mucosas

Gli – glicose

GLUT2 – proteína transportadora de glicose 2

GRAS – *generally recognized as safe*

GWAS – *genome-wide association study*

h – horas

H_2SO_4 – ácido sulfúrico

HbA1c – hemoglobina glicada

HLA – *human leukocyte antigen system*

HRP – *horseradish peroxidase*

Hsp – *heat shock protein*

i.g. – intragástrica

i.p. – intraperitoneal

i.v. – intravenosa

IAA – *insulin autoantibody*

***idd* –** *insulin-dependent diabetes*

IFN-α – interferon alfa

IFN-ɣ – interferon gama

Ig – imunoglobulina

IgA – imunoglobulina A

IL – interleucina

iNOS – óxido nítrico sintase induzível

IVC – *individually ventilated caging*

KDa – quilodaltons

Kg – quilograma

L – litro

LAB – *lactic acid bacteria*

LPS – lipopolissacarídeo

M – molar

M0 – macrófago *naive*

M2 – macrófago do tipo 2

mAbs – *monoclonal antibodies*

MDA – malonaldeído

mg – miligramas

MHC – *major histocompatibility complex*

mL – mililitro

MLNs – linfonodos mesentéricos

mmol – milimol

µg – micrograma

µL – microlitro

N – normal

n – número de animais utilizados em cada grupo experimental

Na_2CO_3 – carbonato de sódio

Na_2EDTA – ácido etilenodiaminotetracético dissódico

NaCl – cloreto de sódio

NAD – *nicotinamide adenine dinucleotide*

$NaHCO_3$ – bicarbonato de sódio

NK – célula *natural killer*

nm – nanômetros

NO – óxido nítrico

NOD – *non-obese diabetic*

ORF – janela aberta de leitura

***p value* –** p valor

PBS – *phosphate buffered saline*

pCMV – promotor de citomegalovírus

pH – potencial hidrogeniônico

PMSF – fluoreto de fenilmetilsulfonil

pValac – plasmídeo denominado "*Vaccination Using Lactic Acid Bacteria"*

q.s.p. – quantidade suficiente para

rIL-4 – proteína IL-4 recombinante

ROS – espécies reativas de oxigênio

rpm – rotações por minuto

***Saline* –** solução salina 0,9%

SD – desvio padrão

sIgA – imunoglobulina A secretória

STZ – estreptozotocina

SV40 – vírus símio 40

T1D – diabetes tipo 1

TGF-β – *transforming growth factor* β

TGI – trato gastrointestinal

Th – linfócito (ou célula) T auxiliar

TNF-α – fator de necrose tumoral α

Treg – linfócito T regulador (ou células T regulatórias)

UFC – unidade formadora de colônia

UFMG – Universidade Federal de Minas Gerais

VC – *vehicle control*

WHO – *World Health Organization*

ZnT8A – *zinc transporter 8 autoantibody*

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1. INTRODUÇÃO

1.1 Diabetes tipo 1

A diabetes tipo 1 (T1D – do inglês, *Type 1 Diabetes*) é uma doença autoimune crônica que resulta da destruição das células β pancreáticas – responsáveis pela produção de insulina no nosso corpo. Esse processo danoso ocorre pela infiltração de células mononucleares (incluindo linfócitos T e B), monócitos, células dendríticas e células *natural killer* (NK) nas ilhotas pancreáticas, estabelecimento de inflamação no local – denominada insulite – e culmina na destruição seletiva das células β. Com isso, não há produção endógena de insulina, um hormônio essencial para a entrada da glicose do sangue nas células e, subsequentemente, síntese de energia para o funcionamento normal do organismo (Boldison & Wong, 2016; Ilonen *et al.*, 2019). Diante disso, destaca-se a seriedade que deve ser encarada essa doença, a qual impacta diretamente um processo contínuo e vital para a homeostase.

A T1D se apresenta como uma doença heterogênea e complexa que, apesar dos muitos anos de pesquisa dedicados ao entendimento dessa desordem, a precisa etiologia e mecanismos patológicos ainda precisam ser elucidados. Dados evidenciam que a T1D se apresenta como uma das doenças crônicas mais comuns da infância. E, embora seja diagnosticada, na maioria, em crianças entre 5 e 7 anos de idade ou próximo à puberdade, cabe ressaltar que a T1D pode surgir em qualquer idade (Atkinson *et al.*, 2014; Buzzetti *et al.*, 2017). Como as estatísticas sobre novos casos da T1D na vida adulta são insuficientes, ainda não se sabe ao certo quão grande é esse número. Além disso, quando a doença se manifesta na vida adulta, a destruição das células β tende a ser mais lenta do que quando a T1D inicia na infância, o que faz a distinção entre a diabetes tipo 1 e tipo 2 ainda mais desafiadora (Tuomi *et al.*, 2014).

O atlas da *International Diabetes Federation*, documento que reúne os principais dados de diabetes no mundo, havia mostrado que, em 2017, aproximadamente 96 mil crianças e adolescentes com menos de 15 anos eram diagnosticados anualmente com T1D (International Diabetes Federation, 2017). Apenas dois anos depois, esse número subiu para mais de 98 mil crianças e adolescentes dessa mesma faixa etária diagnosticados anualmente com T1D. Esse número pode subir para mais de 128 mil novos casos, quando são considerados adolescentes com até 19 anos de idade. E, infelizmente, as projeções apontam para um aumento de aproximadamente 3% na incidência anual dessa doença, indicando a

importância de se investigar novas alternativas terapêuticas para a T1D (International Diabetes Federation, 2019).

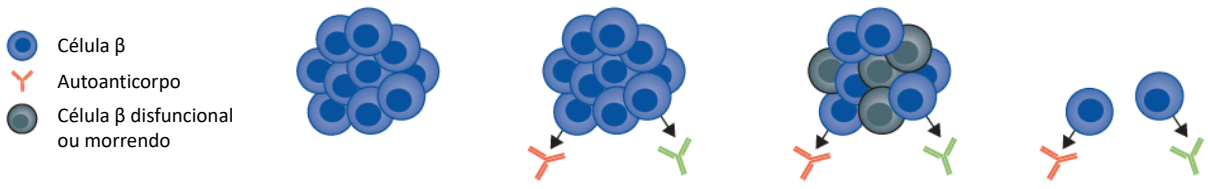
O Brasil é o terceiro país do mundo com o maior número de novos casos de crianças e adolescentes (até 14 anos) diagnosticados com T1D por ano. Nosso país também está na terceira posição do *ranking* de países com maior número de crianças e adolescentes com T1D prevalente, ficando atrás apenas da Índia e Estados Unidos (International Diabetes Federation, 2019).

Os critérios para diagnóstico da diabetes foram estabelecidos por um consenso da WHO (do inglês, *World Health Organization*), *American Diabetes Association* (ADA) e *Canadian Diabetes Association* (CDA), sendo eles: (i) glicemia ≥ 126 mg/dL (7,0 mmol/L) após jejum de oito horas; (ii) glicemia ≥ 200 mg/dL (11,1 mmol/L) medida duas horas após a ingestão de 1,75 g de glicose por kg (dose máxima permitida: 75 g de glicose) pela via oral; (iii) glicemia aleatória ≥ 200 mg/dL (11,1 mmol/L); ou (iv) níveis de HbA1c (hemoglobina glicada) ≥ 6,5%. Porém, a classificação entre diabetes tipo 1 e tipo 2 depende de variáveis na apresentação clínica do diagnóstico, como a idade do paciente, brusquidão da hiperglicemia, presença de cetose, grau de obesidade e necessidade de insulina no momento do diagnóstico (Alam *et al.*, 2014; World Health Organization, 2016). Apesar dos esforços para padronizar o diagnóstico da T1D e, assim, decidir qual a terapia mais adequada e efetiva para cada caso, as causas e a tipologia da doença em questão permanecem incertas. Entre 5 e 15% dos adultos diagnosticados com diabetes tipo 2 podem, na realidade, ter T1D com autoanticorpos circulantes. Isso significa que os números atuais de casos de T1D pode, na verdade, estar bastante subestimado e, mais importante, que muitas vidas podem estar em risco devido ao diagnóstico incorreto (Atkinson *et al.*, 2014). Dessa forma, a busca pela compreensão da T1D por completo, como suas causas, tipologia, progressão, diagnóstico, formas de prevenção (se possível), particularidades e terapias efetivas são de extrema importância, tendo em vista o preocupante prognóstico dos casos dessa doença no cenário global.

Sabe-se que os sintomas clínicos mais comuns apresentados pelos indivíduos que serão diagnosticados com T1D são: polidipsia (sede excessiva), poliúria (aumento da produção de urina) – chegando até a urinar durante o sono noturno, perda de peso repentina, sensação de fome constante, falta de energia e fatiga (Simmons & Michels, 2015; International Diabetes Federation, 2019). Apesar das melhorias na terapia insulínica intensiva e no monitoramento da glicemia alcançadas nas últimas décadas, a maioria dos indivíduos

portadores da T1D não se mantém na faixa recomendada de níveis glicêmicos. Sendo assim, essas pessoas estão sujeitas, por um alto risco, a desenvolver complicações microvasculares (como retinopatias, nefropatias e neuropatias) e, também, macrovasculares (como doenças cardiovasculares) associadas ao diabetes, hipoglicemia severa e depressão (Simmons *et al.*, 2016). Ademais, países onde o acesso à insulina não é facilitado, com distribuição restrita ou irregular desse hormônio, ou quando o serviço de saúde de qualidade não é acessível a todos, os acometidos pela T1D sofrem ainda mais com as complicações em decorrência da doença e também com altos níveis de mortalidade precoce (International Diabetes Federation, 2019).

A T1D pode ser dividida em quatro diferentes fases de desenvolvimento: na fase 0 a doença caracteriza-se pela presença de até 1 autoanticorpo positivo, ausência de disglicemia (alteração da taxa glicêmica) e clinicamente assintomática. Já na fase 1, o indivíduo pode apresentar dois ou mais autoanticorpos, mas ainda mantém 100% da massa de células β pancreáticas. Na fase 2, já é possível verificar, além dos autoanticorpos positivos, o início das alterações na taxa glicêmica, mas a doença ainda permanece como clinicamente assintomática. Aproximadamente 6 meses antes de iniciar a chamada fase 3 da doença é que se inicia a redução significativa na massa de células β. Só então nessa última fase que o indivíduo apresenta os sintomas – quando a hiperglicemia, poliúria, sensação de sede persistente e perda de peso se tornam evidentes. Sabe-se também que o tempo de duração de cada uma dessas fases é extremamente diverso e difícil de estimar entre os acometidos pela T1D. Ademais, na grande maioria dos casos, só quando a doença se torna clinicamente sintomática (fase 3) e 80-90% da massa de células β já está morta é que ocorre o diagnóstico da doença (Figura 1.1) (Dayan *et al.*, 2019).



	Fase 0	Fase 1	Fase 2	Fase 3
Características:	• 0-1 autoanticorpo positivo • Sem disglicemia • Clinicamente assintomático	• ≥2 autoanticorpos positivos • Sem disglicemia • Clinicamente assintomático	• 2 autoanticorpos positivos • Disglicemia • Clinicamente assintomático	• 2 autoanticorpos positivos • Hiperglicemia • Clinicamente sintomático
Massa de célula β:	100%	100%	100% (redução a partir de 6 meses antes da fase 3)	10-20%

Figura 1.1: Fases de desenvolvimento da T1D até o início clínico. Adaptado de: Dayan *et al.*, 2019.

Ao longo dos anos, há uma vasta investigação científica que almeja pontuar e traçar correlação entre possíveis fatores de susceptibilidade à T1D e o desenvolvimento da doença. Muitos estudos conseguiram mapear a influência de polimorfismos genéticos associados à diabetes e a presença de autoanticorpos específicos. As regiões gênicas relacionadas à suscetibilidade a doença em questão foram denominadas *loci idd* (do inglês, *insulin-dependent diabetes*). Estudos de associação genômica ampla (GWAS – do inglês, *Genome-Wide Association Study*) e de sequenciamento já identificaram mais de 60 *loci* associados a susceptibilidade à T1D. Os resultados de GWAS em combinação o conhecimento advindo das "ciências ômicas" indicam que a variação genética tanto das células β pancreáticas quanto das células imunes é fundamental para a mediação do risco à T1D (Bakay *et al.*, 2019). O *locus* do HLA (do inglês, *Human Leukocyte Antigen system*) de classe II, localizado no cromossomo 6, é um dos *loci idd* mais bem estudados na literatura. Os genes HLA codificam proteínas de superfície celular envolvidas na apresentação de antígenos e autotolerância, sendo, assim, genes críticos na regulação da resposta imunológica (Zaccardi *et al.*, 2016). Ademais, a região HLA consiste em uma das mais polimórficas de todo o genoma humano, com 22.436 alelos diferentes até setembro de 2021 (https://www.ebi.ac.uk/ipd/imgt/hla/stats.html - Último acesso: 26 de setembro de 2021), o que dificulta ainda mais as análises de associação. Os haplótipos HLA-DR3-DQ2 e HLA-DR4-DQ8 são indicados como os principais fatores de risco genético à T1D devido a sua associação com o aparecimento de autoanticorpos [como anti-insulina e anti-GAD65 (do inglês, *Glutamic Acid Descarboxylase*)]. Além disso, esses haplótipos parecem determinar a idade em que ocorrerá a soroconversão, que consiste na idade mais precoce que algum autoanticorpo específico é detectado. Logo, isso implica que indivíduos que possuem esses haplótipos de suscetibilidade apresentam um risco aumentado de desenvolver autoanticorpos mais precocemente e, por conseguinte, chegar à fase sintomática da doença mais rapidamente (Nguyen *et al.*, 2013; Wållberg & Cooke, 2013; Li *et al.*, 2014).

Estudos demonstram que a T1D é uma doença que possui vários fatores genéticos de risco associados, porém, apenas esses fatores não são suficientes para o desenvolvimento da doença. Sabe-se também que a taxa de concordância para T1D entre gêmeos monozigóticos é de 30%; o que corrobora a influência dos fatores ambientais no progresso da doença. Um grande aumento na incidência de T1D em crianças de populações geneticamente estáveis é uma das mais fortes evidências para a influência dos fatores ambientais na etiologia da doença. A partir de meados do século XX tem sido observado um aumento no número de

casos de T1D em crianças que apresentam baixo risco de desenvolvimento da doença quando observado fatores genéticos de susceptibilidade (Ilonen *et al.*, 2019). Apesar do intenso esforço das pesquisas, a atuação desses fatores no desenvolvimento da T1D ainda não é bem definida, muito menos um consenso. Diferentes influências ambientais têm sido propostas como possíveis fatores etiológicos da T1D, como: infecções virais (principalmente por enterovírus), composição da microbiota, a introdução de produtos lácteos e alimentos com glúten prematuramente na alimentação do bebê e até eventos gestacionais. No entanto, ainda é necessária a validação desses agentes como possíveis determinantes da doença, através da compreensão de como esses mecanismos influenciariam a autoimunidade ou como uma combinação de eventos ambientais dispararia o aparecimento de autoanticorpos contra células β pancreáticas (Simmons & Michels, 2015; Katsarou *et al.*, 2017).

Os primeiros indícios de autoimunidade nos indivíduos que, posteriormente, desenvolverão a T1D consistem na presença de autoanticorpos. Os mais frequentes são os autoanticorpos que reconhecem a insulina (IAA – do inglês, *Insulin AutoAntibody*), GAD65 (GADA – do inglês, *Glutamic Acid Descarboxylase AutoAntibody*), a tirosina fosfatase relacionada à ilhota IA-2 (IA-2A – do inglês, *Islet Antigen-2 AutoAntibody*) e o transportador de zinco 8 (ZnT8A – do inglês, *Zinc Transporter 8 AutoAntibody*). Os estudos vêm demonstrando que a presença de, pelo menos, dois dos autoanticorpos IAA, GADA e IA-2A poderia ser utilizado como marcador do provável desenvolvimento da T1D, uma vez que 80% das crianças com dois desses autoanticorpos desenvolvem a doença nos próximos 15 anos (Ziegler *et al.*, 2013; Ilonen *et al.*, 2019). Dessa forma, sugere-se que o acompanhamento e a mensuração dos níveis desses autoanticorpos no soro poderia auxiliar na predição da T1D em alguns pacientes, antes mesmo da disglicemia ou dos sintomas clínicos aparecerem (Ling *et al.*, 2018).

Sabe-se que, antes de chegar à fase sintomática, quando geralmente a doença é diagnosticada, uma série de eventos determinantes para o progresso da T1D ocorre (Figura 1.2). O desenvolvimento da diabetes inicia-se com a suscetibilidade genética do indivíduo concomitante a um fator ambiental. No pâncreas, ocorre um aumento de produção de interferon alfa (IFN-α) pelas células β e, subsequentemente, da expressão de MHC (do inglês, *Major Histocompatibility Complex*) de classe I. Isso faz com que as células β sejam expostas ao ataque de linfócitos T CD (grupamento de diferenciação – do inglês, *Cluster of Differentiation*) 8^+ autorreativos com especificidade para autoantígenos pancreáticos. Por

conseguinte, esses autoantígenos liberados pelas células produtoras de insulina são internalizados pelas células apresentadoras de antígenos (APCs – do inglês, *Antigen-Presenting Cells*) residentes e levados para o linfonodo pancreático. Simultaneamente a esses eventos, na periferia, o fator ambiental causou uma mudança no perfil metabólico do indivíduo, criando um ambiente pró-inflamatório que favorece respostas de linfócitos T efetores, em detrimento das respostas de linfócitos T reguladores (Treg). Os autoantígenos das células β pancreáticas apresentados nesse contexto pró-inflamatório, juntamente com linfócitos T $CD4^+$, induzem a diferenciação de linfócitos B em plasmócitos, dando início, assim, à produção de autoanticorpos (soroconversão). Os linfócitos T $CD8^+$ autorreativos também são estimulados a proliferar e migrar para o pâncreas. Há, então, uma segunda onda de morte de células β, decorrente da ação de grânulos citotóxicos, como perforina e granzima, assim como a atuação de citocinas pró-inflamatórias, como IFN-γ (interferon γ) e TNF-α (fator de necrose tumoral α – do inglês, *Tumor Necrosis Factor* α), resultando no comprometimento da produção de insulina – chamado de pseudo-atrofia. Tal fato causa a liberação de novos autoantígenos que são englobados pelas APCs, incluindo os linfócitos B que migraram para o pâncreas, e essas se transportam para o linfonodo pancreático. Isso faz com que surjam novas especificidades de linfócitos T $CD4^+$ e T $CD8^+$ e linfócitos B, em um processo conhecido como espalhamento de epítopo, provocando uma depleção ainda mais severa das células β, tanto na função como na massa. De forma surpreendente, a inflamação autoimune gerada pode estimular a proliferação de células β. Ademais, em alguns casos, os linfócitos Treg podem se sobrepor e atenuar a resposta imune efetora. Assim sendo, o balanço entre a resposta autorreativa destrutiva e a resposta reguladora pode promover um estado transiente de produção de insulina autossuficiente, mesmo após o início clínico da doença – período conhecido como "fase de lua-de-mel". Eventualmente, a resposta autorreativa prevalece, levando à destruição quase que completa das células β produtoras de insulina, o que faz com que, quando a T1D é diagnosticada, apenas 10-30% das células β estão funcionais (Van Belle *et al.*, 2011).

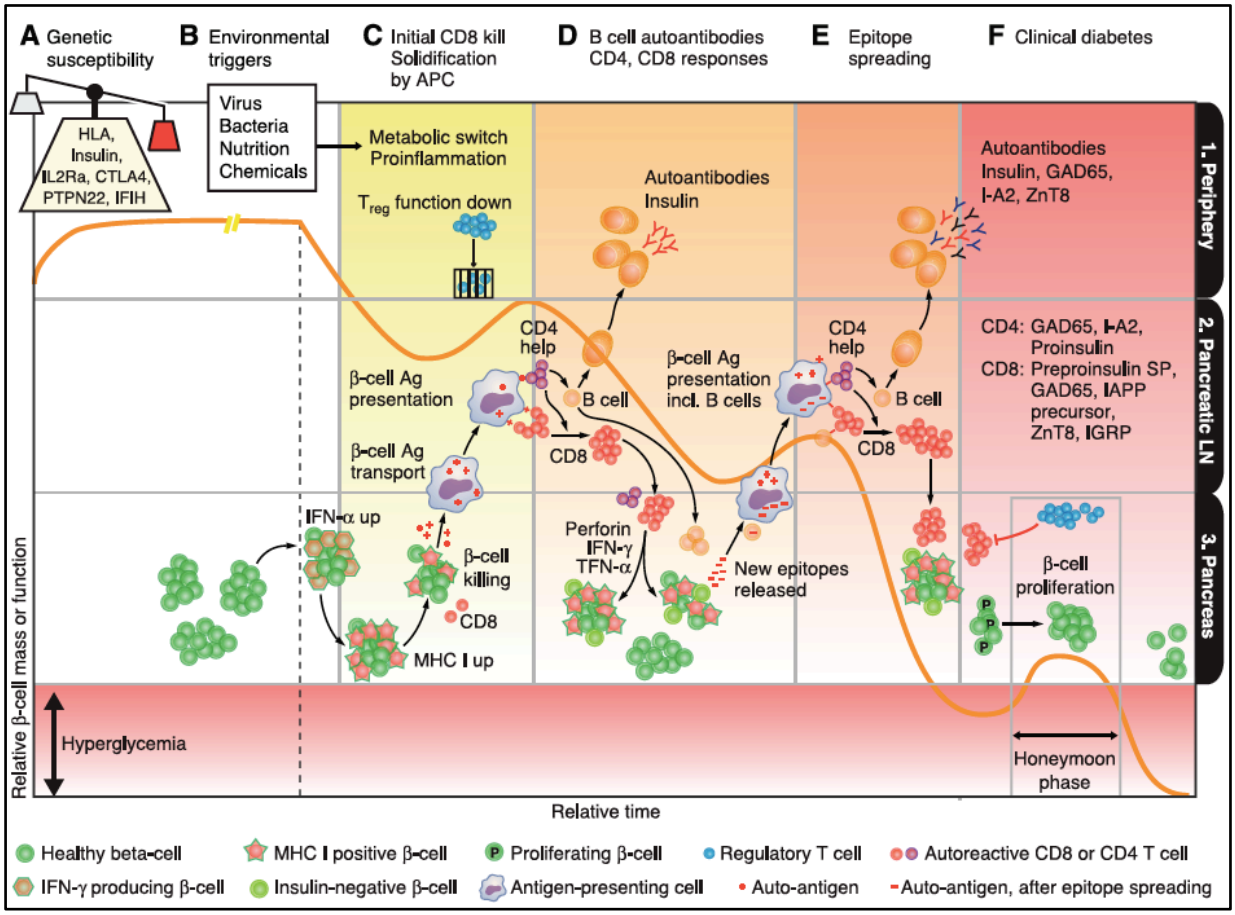


Figura 1.2: Representação esquemática dos possíveis eventos envolvidos no surgimento e desenvolvimento da T1D. Fonte: Van Belle *et al.*, 2011.

Apesar desses eventos propostos, ainda há vários questionamentos que precisam ser elucidados acerca do desenvolvimento da T1D. Diante disso, a utilização de modelos experimentais que mimetizam a doença em camundongos tem sido fundamental para o avanço das pesquisas visando o esclarecimento de todos processos envolvidos na progressão da doença.

1.1.1 Modelo experimental de indução química por estreptozotocina

A estreptozotocina (STZ) é um composto natural, isolado da bactéria encontrada no solo *Streptomyces achromogenes* e apresenta, em sua estrutura química, uma nitrosoureia ligada a um grupo metil e a uma molécula de glicose (Figura 1.3). Esse composto químico caracteriza-se como um agente alquilante, hidrofílico e que possui meia-vida de 5-15 minutos (Rossini *et al.*, 1977). Em meados dos anos 60, foi descoberto que a STZ apresentava uma

toxicidade seletiva para as células β pancreáticas, fazendo com que esse composto fosse foco de estudos para verificar a possibilidade de utilizá-lo como agente indutor da diabetes experimental em camundongos (Mansford & Opie, 1968).

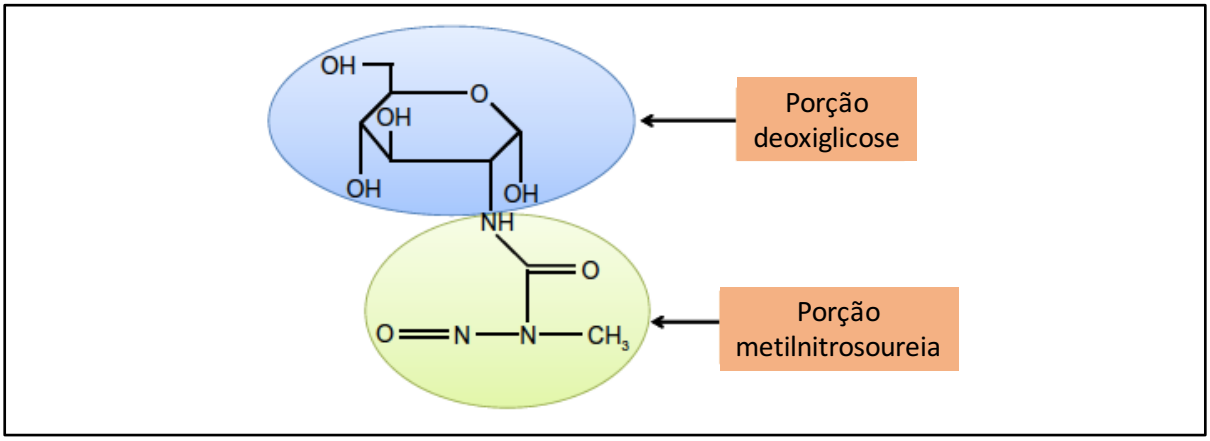


Figura 1.3: Estrutura química da estreptozotocina. Adaptado de: Wu & Yan, 2015.

Assim, em 1976, Like e Rossini criaram um modelo de indução química da T1D experimental em camundongos através de múltiplas doses de STZ. Desde a sua criação, esse modelo foi vastamente utilizado na pesquisa por apresentar semelhanças significativas com a doença em humanos e contribuiu com muitos achados importantes para o entendimento da T1D (Like & Rossini, 1976; Leiter, 1982).

A STZ é particularmente danosa para as células β pancreáticas por ser um análogo citotóxico da glicose que é reconhecido e transportado para o interior dessas células, via proteína transportadora de glicose 2 (GLUT2). Esse receptor é o único transportador de glicose de células mamíferas que reconhece a STZ e a conduz para dentro da célula. Ademais, as células β pancreáticas apresentam altos níveis de expressão de GLUT2, tornando, assim, a toxicidade de STZ sítio-específica (Lenzen, 2008).

Dentro da célula produtora de insulina, o mecanismo de ação da STZ que culmina na morte celular tem sido muito estudado ao longo dos anos. Foram propostas três vias distintas envolvidas:

i) Metilação do DNA (ácido desoxirribonucleico – do inglês, *Deoxyribonucleic Acid*): a adição de grupos metil ao DNA da célula β resulta na ativação da enzima nuclear poli-ADP (adenosina difosfato – do inglês, *Adenosine DiPhosphate*) ribose-sintetase, em uma tentativa de reparo celular, levando à depleção de NAD^+ (do inglês, *Nicotinamide Adenine*

Dinucleotide) e, consequentemente, dos estoques de ATP (adenosina 5'-trifosfato – do inglês, *Adenosine 5'-Triphosphate*), como resultado de uma super-estimulação dos mecanismos de reparo do DNA. O esgotamento das reservas de energia da célula β resulta na inibição da biossíntese e secreção de insulina, culminando com o processo de necrose celular. Apesar do processo de metilação do DNA ser o principal responsável pela morte celular, a metilação de proteínas por STZ também contribui para a toxicidade desse composto às células β pancreáticas (Murata *et al.*, 1999; Pieper *et al.*, 1999; Bolzán & Bianchi, 2002).

ii) Produção de óxido nítrico (NO – do inglês, *Nitric Oxide*): outro mecanismo responsável pela ação diabetogênica do STZ tem sido atribuído a sua capacidade de agir como doador de NO nas células pancreáticas, levando à alquilação do DNA e, como consequência, a morte celular. As células β são particularmente sensíveis aos danos causados pelo NO e também pelos radicais livres devido a essas apresentarem baixos níveis de enzimas que eliminam esses compostos danosos (Spinas, 1999).

iii) Geração de radicais livres: STZ é conhecido por causar aumento nos níveis de malonaldeído (MDA – do inglês, *Malondialdehyde*) e, paralelamente, diminuição da atividade de enzimas antioxidantes, como catalase, glutationa-peroxidase e superóxido dismutase. Essas alterações resultam na suscetibilidade do pâncreas à indução de estresse oxidativo por STZ. Outro envolvimento importante de espécies reativas de oxigênio (ROS – do inglês, *Reactive Oxygen Species*) durante o metabolismo de STZ é a produção de ácido úrico como produto final da degradação de ATP. Essa reação gera ROS, como os radicais hidroxil e superóxido, acelerando o processo de destruição das células β (Eleazu *et al.*, 2013).

A indução química da T1D por múltiplas doses de STZ proporciona o estabelecimento extremamente rápido da doença em camundongos C57BL/6 machos, facilitando, assim, o estudo dessa desordem. Esse modelo experimental exibe fenótipo e infiltrado celular das ilhotas pancreáticas mais parecidos com o que é visto na T1D humana (Müller *et al.*, 2002; Costa *et al.*, 2016). Além disso, já foi demonstrado que a administração de STZ é responsável pelo desenvolvimento de uma resposta humoral com produção de anticorpos e, também, por elicitar uma resposta celular mediada por linfócitos T autorreativos para antígenos das células β (Herold *et al.*, 1995; Horwitz *et al.*, 2002).

1.1.2 Modelo experimental espontâneo: camundongos NOD

Na década de 80, um outro modelo animal para estudo da T1D foi desenvolvido e, desde então, se tornou o principal modelo utilizado nas pesquisas envolvendo a doença em questão. A linhagem de camundongos NOD (do inglês, *Non-Obese Diabetic*) foi desenvolvida, por Makino e colaboradores, a partir de intercruzamentos da linhagem de camundongos propensa à catarata JcI:ICR (Makino *et al.*, 1980; Kikutani & Makino, 1992). Os camundongos NOD desenvolvem a diabetes autoimune espontaneamente devido à combinação genética de alguns *loci* importantes para a doença presente nesses animais – o que pode ser comparado à susceptibilidade genética à T1D em humanos. O *idd1* é o principal *locus* que contribui para o desenvolvimento da doença em questão em camundongos NOD. Múltiplos genes envolvidos com o MHC dessa linhagem, denominados H-2^{g7}, contribuem de forma interativa e culminam com as características-chave da T1D, como o início da insulite e a progressão da doença. Os camundongos NOD são homozigotos para o haplótipo H-2^{g7}, o que contribui para disfunções severas das APCs e, subsequentemente, podem promover o desenvolvimento de linfócitos T autorreativos à antígenos das ilhotas pancreáticas (Driver *et al.*, 2011; Acharjee *et al.*, 2013). Estudos também mostraram que algumas variantes do MHC de classe I contribuem para a progressão da doença em humanos. Camundongos NOD manipulados geneticamente para expressar moléculas do MHC de classe I HLA-A2.1 aceleraram a doença nesse modelo (Marron *et al.*, 2002). Ademais, mais de 40 genes não relacionados diretamente ao MHC também contribuem para predisposição à T1D nos camundongos NOD (Pearson *et al.*, 2016). Dessa forma, a similaridade na susceptibilidade genética é um componente comum e essencial para o desenvolvimento da T1D tanto em humanos quanto em camundongos NOD, o que representa um dos mais fortes argumentos para o uso desse modelo experimental nas pesquisas há quase quatro décadas.

A linhagem de camundongos NOD apresenta outras similaridades importantes com a T1D em humanos, como os subtipos celulares que predominam no infiltrado inflamatório que avança nas ilhotas pancreáticas, sendo eles: linfócitos T $CD8^+$, macrófagos e linfócitos B; a presença de autoanticorpos específicos para antígenos pancreáticos e de linfócitos T autorreativos, o que ratifica a vasta utilização dos camundongos NOD nas pesquisas científicas da T1D (King & Bowe, 2016; Pearson *et al.*, 2016).

A incidência da diabetes autoimune experimental é de 80-90% em camundongos NOD fêmeas e 60% em machos da mesma linhagem (Brito-Casillas *et al.*, 2016). Essa incidência

tende a se manter alta quando os camundongos são mantidos em um ambiente *germ-free* (Chen *et al.*, 2020). Ademais, a idade do aparecimento da T1D experimental costuma variar entre as colônias, mas geralmente ocorre entre 14 a 20 semanas de idade em camundongos NOD fêmeas e um pouco mais tardiamente em machos. Análises histológicas demonstraram que um infiltrado mononuclear pode ser visualizado ao redor das ilhotas pancreáticas (peri-insulite) com 3-4 semanas de idade. Esse infiltrado evolui e invade as ilhotas (processo denominado insulite) nas semanas subsequentes, sendo que a maioria dos animais NOD apresenta insulite severa a partir da 10ª semana de vida (Anderson & Bluestone, 2005; King & Bowe, 2016). Diante de todas as características explicitadas da linhagem de camundongos NOD, diversos estudos utilizando esse modelo experimental apresentaram resultados que contribuíram para o entendimento e também para descobertas importantes acerca da patogênese e desenvolvimento imunológico da T1D em humanos (Jayasimhan *et al.*, 2014).

1.2 As citocinas IL-4 e IL-10 no contexto da diabetes tipo 1

Descoberta em 1982, a interleucina (IL) 4 consiste em uma citocina atraente para o estudo da T1D por ser produzida majoritariamente por células T auxiliares (Th – do inglês, T *helper*) do tipo Th2 ativadas (Jansen *et al.*, 1990; Luzina *et al.*, 2011) e induzir a diferenciação e ativação de macrófagos M0 (macrófagos *naive*) para o perfil M2, os quais são reconhecidos por regular a inflamação (Gordon & Martinez, 2010). Os linfócitos T auxiliares foliculares, que correspondem a uma subpopulação de linfócitos T $CD4^+$ localizados nos centros germinativos dos linfonodos, também produzem altos níveis de IL-4. Mastócitos, basófilos e eosinófilos também podem produzir a citocina em questão (Ho & Miaw, 2016). Além disso, a IL-4 induz a diferenciação de células Th em células Th2, o que resulta na liberação de citocinas desse padrão de resposta imunológica e inibe a diferenciação de células Th1 e Th17 (Opal & DePalo, 2000; Luzina *et al.*, 2012).

Já a IL-10 é reconhecidamente uma citocina com potente atividade anti-inflamatória, o que se opõe ao quadro inflamatório característico da T1D (Moore *et al.*, 2001). Ademais, a IL-10 pode ser produzida por vários subtipos celulares, como monócitos, macrófagos, linfócitos B, Treg, Th2, Th17 e células dendríticas (Tian *et al.*, 2014). A IL-10 atua como um importante regulador imune, capaz de inibir a secreção de citocinas do padrão Th1, como

IFN-γ, e estimular a diferenciação e proliferação de linfócitos B, T e macrófagos (Asadullah, 2003).

Sabendo que as citocinas possuem um papel-chave na regulação imunológica e no desenvolvimento da T1D, vários estudos testaram o potencial terapêutico de IL-4 e IL-10, por diferentes formas de entrega e rotas de imunização, tanto em camundongos NOD como em camundongos submetidos à múltiplas doses de STZ, obtendo resultados promissores (Rabinovitch *et al.*, 1995; Cameron *et al.*, 1997; Müller *et al.*, 2002; Papaccio *et al.*, 2002). Dentre esses trabalhos, destaca-se o de Rapoport e colaboradores que, em 1993, mostraram que a administração de IL-4 recombinante exógena é capaz de reverter a falta de responsividade de linfócitos T periféricos e provenientes do timo em camundongos NOD, culminando na proteção desses contra o desenvolvimento da T1D (Rapoport *et al.*, 1993). No ano seguinte, um estudo mostrou que administração subcutânea de IL-10 humana recombinante atrasou o início da T1D e reduziu significantemente a incidência da doença em camundongos NOD. Além disso, os pesquisadores observaram a prevenção do infiltrado inflamatório nas ilhotas pancreáticas e a produção normal de insulina, indicando que IL-10 suprime a progressão autoimune da T1D, sugerindo um potencial papel terapêutico dessa citocina (Pennline *et al.*, 1994). A administração combinada de plasmídeos codificadores de IL-4 e IL-10 utilizando um carreador polimérico catiônico, injetado pela via intravenosa, também mostrou efeitos significativos na redução da insulite, preservação das ilhotas pancreáticas e prevenção do avanço da T1D em camundongos NOD (Ko *et al.*, 2001).

Em 1999, Wood e colaboradores demonstraram que a administração de STZ suprime a proliferação de linfócitos T do timo em resposta à estimulação com mitógeno *in vitro*. Foi visto, também, que a secreção de IL-4 foi dramaticamente reduzida, enquanto que a secreção de IL-2 e IFN-γ por linfócitos T não foi, significativamente, inibida. Foi, então, adicionado IL-4 à cultura de linfócitos T provenientes de animais que passaram pela administração de STZ. Como resultado, observou-se a restauração da resposta proliferativa das células em cultura. Nos testes *in vivo*, camundongos BALB/c machos foram submetidos a cinco doses diárias e consecutivas de STZ na concentração de 40 mg/kg. Os animais foram pré-tratados com a proteína IL-4 recombinante (rIL-4), pela via subcutânea, por duas semanas antes e duas após a indução química da T1D; o que resultou na reversão do defeito de proliferação de timócitos induzido por STZ e preveniu tanto a destruição da ilhota pancreática como a hiperglicemia (Wood *et al.*, 1999).

Ainda, em outro estudo, foram utilizadas nanomicelas catiônicas, injetadas pela via intramuscular, para entrega de plasmídeos codificadores de IL-4 e IL-10 no modelo de múltiplas doses de STZ em camundongos BALB/c machos. Os resultados mostraram níveis significativamente maiores de IL-4 e IL-10 no soro dos animais que receberam as nanomicelas contendo um único plasmídeo codificador dessas citocinas, assim como níveis reduzidos de glicemia. Na análise histológica, os pâncreas desses animais exibiram um pouco de infiltrado inflamatório nas ilhotas, mas esse era bem menor do que o exibido por animais que tiveram apenas a administração de STZ, indicando um efeito protetor das citocinas IL-4 e IL-10 (Mandke & Singh, 2012).

Dessa forma, diante das características e funções imunológicas conhecidas de IL-4 e IL-10 e dos resultados obtidos em estudos anteriores nos modelos murinos de T1D experimental, essas citocinas figuram como boas candidatas para o estudo dos mecanismos envolvidos nessa desordem, assim como uma nova terapia, visando a manutenção das células β pancreáticas e, consequentemente, a normoglicemia (Waldron-Lynch & Herold, 2011). Entretanto, a estratégia de entrega dessas citocinas é um desafio recorrente nas pesquisas atuais. Nesse cenário, o uso de bactérias como veículos de entrega de DNA emerge como uma alternativa favorável, abrindo novas perspectivas para o estudo de diversas desordens, por meio da imunomodulação.

1.3 Bactérias como veículos de entrega de DNA

Em 1980, estudos realizados por Walter Schaffner demonstraram, pela primeira vez, que as bactérias também são capazes de transferir material genético diretamente para células mamíferas *in vitro*, quando cópias em tandem do genoma do vírus SV40 (vírus símio 40 – do inglês, *Simian Virus 40*) carreadas por *Escherichia coli* foram transferidas, em co-cultura, para células mamíferas. Desde então, a utilização das bactérias como sistema de entrega de DNA tem sido cada vez mais explorado e aprimorado (Schoen *et al.*, 2004; Becker *et al.*, 2008).

Sabe-se que o processo de transferência do DNA plasmidiano para células mamíferas consiste, primeiramente, na entrada da bactéria na célula-alvo por meio de endocitose. A bactéria é, então, mantida em uma vesícula que irá se fundir ao lisossomo, formando o

fagolisossomo, visando a degradação. Nesse ponto, ainda não está completamente elucidado o que exatamente ocorre, mas há duas hipóteses: na primeira, a bactéria é lisada no fagolisossomo e o plasmídeo consegue escapar para o citoplasma ou, na segunda hipótese, a bactéria escapa do compartimento de degradação, sofre lise no citoplasma e libera o plasmídeo. Subsequentemente, esse plasmídeo se desloca para o núcleo, onde a ORF (janela aberta de leitura – do inglês, *Open Reading Frame*) de interesse será expressa (Figura 1.4) (Schoen *et al.*, 2004; Pontes *et al.*, 2011).

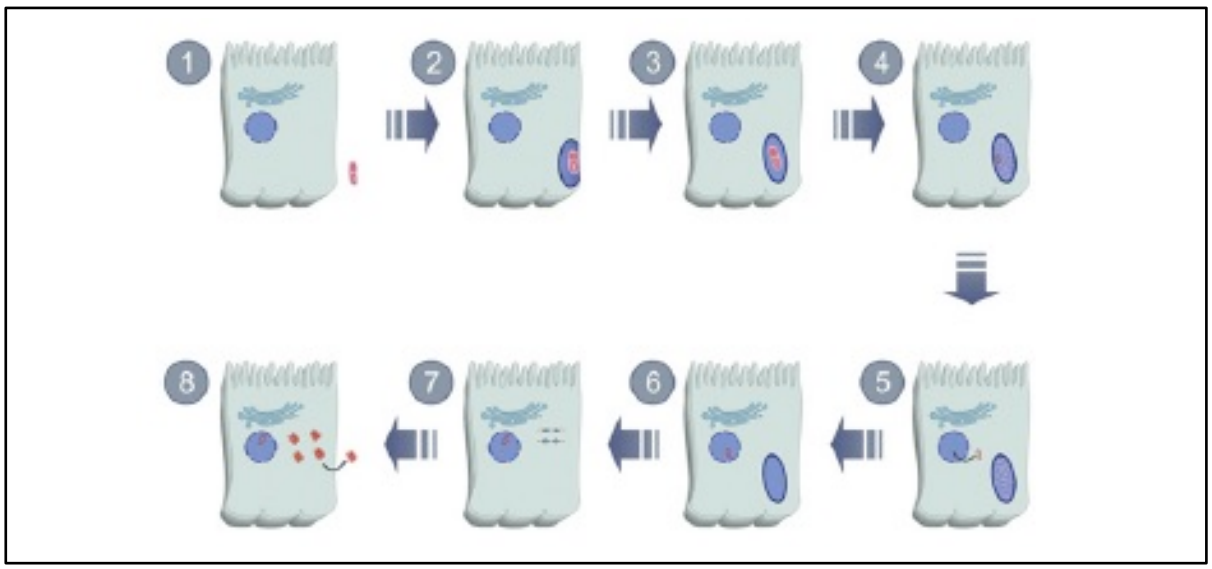


Figura 1.4: Representação esquemática da transferência do DNA plasmidiano carreado por uma bactéria para uma célula mamífera. (1) A célula intestinal e a bactéria com o plasmídeo contendo a ORF de interesse. (2) Entrada da bactéria na célula alvo. (3) Formação do fagolisossomo contendo a bactéria. (4) Lise bacteriana. (5 e 6) Liberação dos plasmídeos no citoplasma e transferência desses para o núcleo da célula hospedeira. (7) Expressão da ORF de interesse dentro do núcleo. (8) Transcrição e tradução da proteína de interesse pela maquinaria da célula eucariótica. **Adaptado de:** Pontes *et al.*, 2011.

A utilização de bactérias como veículos de entrega de vacinas de DNA apresenta muitas vantagens em comparação com outras estratégias de vacinação, como carreadores virais, DNA nu, entrega de purificados ou antígenos proteicos. A primeira vantagem é a entrega da ORF de interesse de maneira "segura" diretamente para a célula mamífera, dado que a bactéria funciona como uma barreira contra possíveis degradações no trato gastrointestinal. Outra vantagem é possibilitar a expressão de antígenos modificados pós-traducionalmente e a apresentação de epítopos restritos conformacionalmente, já que todo o processamento é feito pela maquinaria eucariótica. Além disso, linhagens bacterianas carreadoras são de fácil manipulação, uma vez que, nesse caso, não são necessárias etapas

adicionais de amplificação e purificação do plasmídeo (Schoen *et al.*, 2004). Ademais, a utilização de bactérias vivas por si só já contribui como um adjuvante imunoestimulatório devido às características intrínsecas desse vetor (da Silva *et al.*, 2014).

Uma etapa importante para obter sucesso na vacinação de DNA utilizando bactérias carreadoras é a escolha de uma boa rota de imunização. Nesse contexto, a via de mucosas se destaca das demais por apresentar efeitos secundários reduzidos, ser de fácil administração, ter custos de produção e implementação reduzidos e, principalmente, pela capacidade de estimular tanto respostas imunes locais quanto sistêmicas – o que não ocorre na via parenteral, por exemplo (Schoen *et al.*, 2004; da Silva *et al.*, 2014; Banerjee & Mitragotri, 2017). Para que essas respostas imunológicas ocorram, primeiramente, a bactéria precisa transpor a barreira epitelial, o que pode acontecer de formas distintas: se a bactéria for invasiva, ela pode entrar diretamente pela célula epitelial; pela extensão dos dendritos das células dendríticas entre as junções das células epiteliais e, o que se sucede na maioria dos casos, pela absorção das células M que estão localizadas no epitélio ligado a folículos de tecidos linfoides associados a mucosas (GALT – do inglês, *Gut-Associated Lymphoid Tissues*), como as placas de Peyer [folículo indutivo de mucosa no qual ocorre apresentação de antígenos pelas APCs para os linfócitos e formação da resposta imune, incluindo também a síntese de IgA (imunoglobulina A)] (Figura 1.5). Essas células M, caracterizadas pela borda irregular "em escova" e redução de microvilos, são especializadas em transpor macromoléculas luminais, antígenos particulados e microrganismos comensais e patogênicos pela barreira epitelial. Logo, quando esses chegam na placa de Peyer, eles são capturados pelas APCs, podendo se propagar para o baço, fígado e linfonodos. Após essa ingestão pelas células dendríticas, por exemplo, o DNA plasmidiano é liberado no citosol, chegando até o núcleo, onde é expresso. O antígeno é então reconhecido como endógeno e, assim, apresentado via moléculas MHC de classe I, gerando uma resposta T $CD8^+$ específica. Porém, o antígeno também pode ser produzido por outras células, sendo esse capturado por uma APC para processamento e posterior apresentação via MHC de classe II, o que resulta na ativação de linfócitos Th $CD4^+$ e linfócitos B, sendo que esse último culmina na produção de anticorpos (Schoen *et al.*, 2004; Meeusen, 2011; Lamichhane *et al.*, 2014).

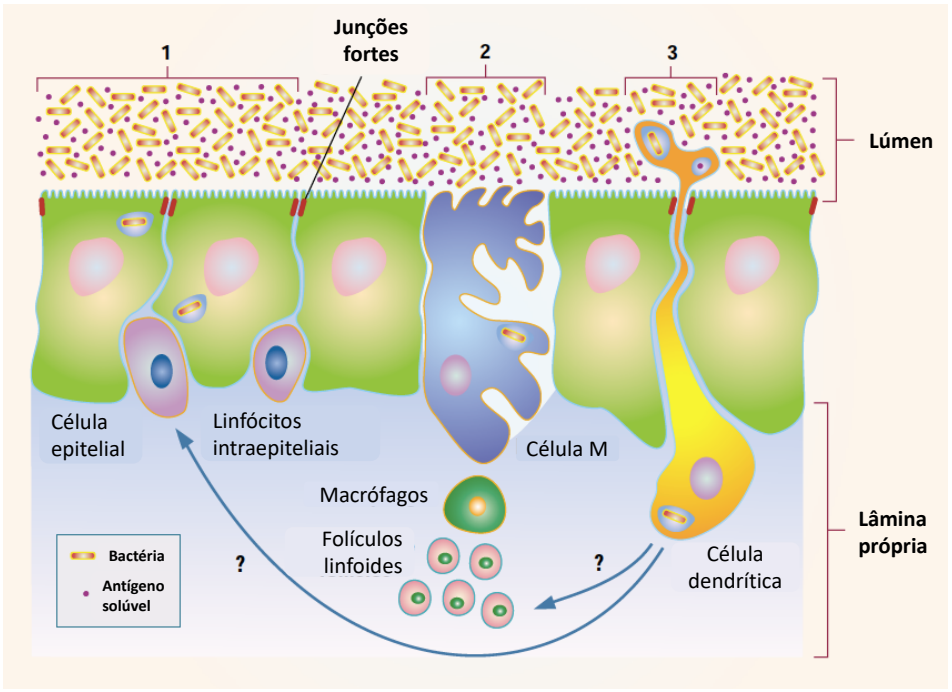


Figura 1.5: Representação esquemática de mecanismos utilizados por bactérias para transpor a barreira epitelial. A superfície intestinal é formada por células epiteliais unidas por junções fortes (também chamadas de *tight juctions*) para impedir a entrada de micróbios e macromoléculas. Entretanto, algumas bactérias invasivas podem entrar diretamente nas células epiteliais (1), enquanto outras utilizam células M, que estão situadas sobre agregados linfocíticos, como as Placas de Peyer, para romper essa barreira (2). Outra rota possível é através da captação das bactérias pelas projeções das células dendríticas que se estendem para o lúmen intestinal (3). **Adaptado de:** Gewirtz & Madara, 2001.

Tendo em vista o êxito da estratégia de vacinação de DNA, também deve-se escolher qual bactéria terá a função carreadora do plasmídeo vacinal/terapêutico. Logo, existem duas alternativas: a utilização de bactérias patogênicas atenuadas ou bactérias não-patogênicas. Ciente de que, com a utilização de patógenos atenuados, existe o risco de reversão do potencial patogênico e restrição do uso em crianças e pacientes imunossuprimidos, surge a predileção pelo uso de bactérias não-patogênicas. Nesse cenário, um grupo de bactérias se destaca como vetores promissores: as bactérias do ácido láctico (LAB – do inglês, *Lactic Acid Bacteria*).

1.3.1 Bactérias do ácido láctico

As LAB formam um grupo de microrganismos que são caracterizados como gram-positivos e não-formadores de esporos. Esse grupamento não reflete uma classificação filogenética, mas um conjunto de bactérias que, além das características em comum supracitadas, apresentam a habilidade de produzir ácido láctico a partir da fermentação de açúcares. Dentre os gêneros bacterianos que pertencem ao grupo das LAB, destacam-se: *Lactococcus, Lactobacillus, Leuconostoc, Pediococcus* e *Streptococcus* (Azevedo & Miyoshi, 2004; Wells & Mercenier, 2008; Pontes *et al.*, 2011; Castillo Martinez *et al.*, 2013).

A fermentação de alimentos através das LAB é uma das mais antigas formas de biopreservação realizada pela humanidade (Soomro *et al.*, 2002). Sabe-se, por exemplo, que essas bactérias são muito utilizadas para a produção de queijo (espécies de *Lactococcus*) e iogurtes (espécies de *Streptococcus* e *Lactobacillus*) (Wells & Mercenier, 2008). Ademais, as LAB também são utilizadas na indústria alimentícia para formação de sabor, preservação, produção de aditivos e suplementos, síntese de bacteriocinas e exopolissacarídeos (Zhu *et al.*, 2009). Dessa forma, ao longo dos anos foi estabelecida uma associação segura entre as LAB e sua ingestão pelos humanos (Wells & Mercenier, 2008; Bermúdez-Humarán *et al.*, 2011).

As LAB que estão presentes na nossa dieta são classificadas como organismos "GRAS" (do inglês, *Generally Recognized As Safe*), considerados seguros para o consumo humano (Pontes *et al.*, 2011). Ademais, dentre as várias propriedades das LAB, salienta-se a sua capacidade probiótica (Pontes *et al.*, 2011). De acordo com a FAO (do inglês, *Food and Agriculture Organization*) e a WHO, probióticos são "microrganismos vivos que, quando administrados em quantidades adequadas, conferem um benefício à saúde do hospedeiro" (Wells & Mercenier, 2008). A utilização das LAB e de seus produtos como probióticos têm demonstrado resultados efetivos na prevenção do desenvolvimento de lesões na mucosa gástrica, no alívio de sintomas alérgicos e auxílio na proteção contra infecções via patógenos, através de efeitos imunomodulatórios na imunidade inata e adaptativa (Tsai *et al.*, 2012).

Além de todas essas aplicações das LAB na indústria alimentícia, essas também são visadas como "biorreatores" para a produção de moléculas de interesse biotecnológico; utilizadas como veículos para síntese e apresentação de antígenos e, ainda, para a entrega de vacinas de DNA. Assim, a utilização de LAB recombinantes para a entrega de compostos

com finalidades terapêuticas e profiláticas tem recebido destaque devido aos resultados promissores. Seguindo essa estratégia, a utilização da LAB modelo *Lactococcus lactis* tem sido vastamente testada como carreadora de vacinas de DNA (Wells & Mercenier, 2008; Pontes *et al.*, 2011).

1.3.1.1 *Lactococcus lactis* como vetor de entrega de vacinas de DNA

Lactococcus lactis é uma bactéria heterofermentativa, mesofílica (com temperatura ideal de crescimento por volta de 30°C), não-invasiva e que não coloniza o trato gastrointestinal (TGI) de homens e animais. Além de ser vastamente utilizada na indústria láctea, *L. lactis* é considerada o organismo modelo das LAB por ter o seu genoma completamente sequenciado, por existirem muitas ferramentas genéticas já desenvolvidas para utilização nessa espécie e, subsequentemente, pela facilidade de ser manipulada geneticamente. Ademais, a linhagem mais utilizada de *L. lactis,* identificada como MG1363, não possui plasmídeo e não produz nenhuma protease extracelular. E, ainda, outra vantagem de se utilizar *L. lactis* como vetor para entrega de proteínas ou vacinas de DNA consiste no perfil de segurança existente, ratificado pelo *status "*GRAS" da mesma (Bermúdez-Humarán *et al.*, 2011; Pontes *et al.*, 2011).

O avanço das pesquisas e, por consequência, uma melhor compreensão sobre *L. lactis*, resultou na expansão do uso desse organismo modelo para novos campos além da indústria alimentícia. Expressão de proteínas heterólogas, transferência gênica, entrega de drogas terapêuticas e utilização como vetores vacinais são exemplos das novas abordagens possíveis de *L. lactis* na atualidade (Bahey-El-Din *et al.*, 2010; Bahey-El-Din & Gahan, 2011).

No contexto da entrega de plasmídeos terapêuticos/vacinais via mucosa, a bactéria *L. lactis* se destaca por, além das características supracitadas, ser uma bactéria gram-positiva e, assim, não possuir lipopolissacarídeos (LPS) endotóxicos; apresentar resistência ao pH ácido do suco gástrico e, logo, ser capaz de sobreviver no TGI; ser não-comensal e, desse modo, apresentar menores chances de provocar efeitos colaterais após longo tempo de ingestão. Ademais, agindo como um vetor, *L. lactis* elicita uma resposta imune no seu hospedeiro, sendo considerada pouco imunogênica – característica considerada vantajosa em comparação com outros vetores bacterianos patogênicos (Bahey-El-Din & Gahan, 2011; Wyszyńska *et al.*, 2015).

Em 2006, foi publicado o primeiro ensaio clínico de fase I utilizando *Lactococcus lactis* recombinante expressando IL-10 em pacientes com doença de Crohn. Esse estudo demonstrou que a estratégia de utilizar LAB geneticamente modificadas em humanos é segura, promissora e constitui uma abordagem realmente possível para utilização na medicina (Braat *et al.*, 2006). Desde então, cada vez mais esforços têm sido feitos por toda a comunidade científica para explorar todo o potencial de *L. lactis* como vetor vacinal via mucosa.

Para comprovar que a linhagem *L. lactis* MG1363 é capaz de entregar os plasmídeos vacinais às células eucarióticas de forma efetiva, Guimarães e colaboradores fizeram vários testes *in vitro*. Após 3 horas de incubação de células da linhagem Caco-2 com *L. lactis* MG1363 contendo um plasmídeo carreador do cassete de expressão eucariótica com cDNA (ácido desoxirribonucleico complementar) de β-lactoglobulina (BLG – do inglês, *β-lactoglobulin*) bovina, foi observada a transferência desse plasmídeo para as células eucarióticas e consequente expressão e secreção da proteína BLG (Guimaraes *et al.*, 2006). Dois anos depois, Chatel e colaboradores comprovaram que, por meio da administração oral dessa mesma linhagem bacteriana em camundongos, células intestinais murinas foram capazes de produzir a proteína BLG em 53% dos animais testados. Ainda que os resultados obtidos foram favoráveis, notou-se que a taxa de transferência de DNA *in vivo* foi baixa. Dessa forma, hipotetiza-se que, pelo menos em parte, esse fato pode ser explicado devido à *L. lactis* não ser uma bactéria patogênica e, assim, não ser capaz de persistir por um tempo maior no TGI e interagir com as células intestinais, dificultando a entrega do plasmídeo (Chatel *et al.*, 2008).

Cientes de que a entrega do plasmídeo vacinal à célula eucariótica consiste em uma etapa fundamental para a sucesso dessa estratégia, foram feitos diversos testes visando amplificar as propriedades de entrega de DNA por *L. lactis*. Uma das tentativas que alcançaram maior efetividade foi a modificação da bactéria carreadora para que essa fosse capaz de expressar uma invasina denominada proteína A de ligação à fibronectina (FnBPA – do inglês, *Fibronectin-Binding Protein A*) de *Staphylococcus aureus*. A FnBPA é uma glicoproteína que medeia a adesão bacteriana ao fibrinogênio, elastina e fibronectina, além de intermediar a captação da bactéria por células hospedeiras não-fagocíticas. Experimentos *in vitro* e *in vivo* comprovaram que as linhagens de *L. lactis* MG1363 FnBPA^{+} exibem maior invasividade que as linhagens selvagens, fazendo, assim, que essas linhagens recombinantes

sejam reconhecidas como vetores de entrega de plasmídeos terapêuticos/vacinais com grande efetividade e potencial (Innocentin *et al.*, 2009; Pontes *et al.*, 2011, 2012).

Com o objetivo de garantir a segurança e eficácia de entrega do plasmídeo e subsequente expressão desse pela célula eucariótica, no ano de 2009, Guimarães e colaboradores construíram um novo plasmídeo de expressão denominado pValac (do inglês, *Vaccination Using Lactic Acid Bacteria*). Esse vetor contém uma região eucariótica composta pelo promotor de citomegalovírus (pCMV), um sítio de clonagem múltipla e uma sequência sinal de poliadenilação do Hormônio Bovino de Crescimento (BGH poli-A), necessária para a expressão gênica pelas células eucarióticas hospedeiras. Enquanto a região procariótica do plasmídeo possui origens de replicação para que esse possa se propagar tanto em *E. coli* quanto em *L. lactis*, além do gene de resistência ao antibiótico cloranfenicol (Cm) para auxiliar na seleção das linhagens recombinantes (Guimarães *et al.*, 2009).

Um fator que poderia prejudicar o êxito da estratégia desenhada para a entrega de plasmídeos terapêuticos/vacinais por *L. lactis* via mucosa é a baixa eficiência de importação do DNA para o núcleo da célula hospedeira. Como consequência, seriam observados níveis reduzidos de expressão gênica, impedindo, assim, de alcançar os objetivos almejados com os experimentos. Visando resolver essa problemática, Souza e colaboradores inseriram a sequência de endereçamento nuclear (*dts* – do inglês, *DNA Nuclear Targeting Sequence*) do vírus SV40 no plasmídeo pValac, criando uma nova versão para esse vetor. Dessa forma, pValac::*dts* tem como objetivo aumentar a exportação do DNA plasmidiano para o núcleo e, por conseguinte, obter maiores taxas de expressão gênica pela célula hospedeira (Souza *et al.*, 2016).

Assim, o uso da linhagem recombinante *L. lactis* MG1363 FnBPA^{+} como veículo de entrega do plasmídeo pValac contendo uma ORF de interesse, como de IL-4 ou IL-10, para a expressão dessas citocinas pelas células da mucosa intestinal poderia representar uma estratégia alternativa para os estudos que visam expandir a compreensão dos mecanismos envolvidos e também da imunomodulação de desordens autoimunes, como a diabetes tipo 1.

1.4 Justificativa de realização de trabalho

O número de casos de T1D é crescente a cada ano e as projeções mostram que o número de acometidos pela doença ainda aumentará em todo o mundo nas próximas décadas (International Diabetes Federation, 2019). Atualmente, apesar de existir o tratamento com a reposição insulínica exógena, sabe-se que o a indivíduo que recebeu o diagnóstico de T1D tem que passar por um processo de mudança e adaptação a novos hábitos na sua rotina, como a aferição frequente da glicemia, aplicação da insulina e atenção aos alimentos consumidos; e costuma apresentar até implicações psicossociais e comportamentais, atribuídas à doença (Winkley, 2006; Delamater *et al.*, 2014).

Ademais, o gasto mundial direto em saúde com diabetes em 2019 foi estimado em US$ 760 bilhões. E as estimativas apontam para um aumento desses gastos nos próximos anos: US$ 825 bilhões até 2030 e US$ 845 bilhões até 2045 (Williams *et al.*, 2020). Um estudo feito pela universidade britânica *King's College* em parceria com a Universidade de Gottingen, na Alemanha, fez um levantamento das despesas com o tratamento médico da diabetes e os impactos na atividade econômica, como a perda de produtividade de trabalhadores e as mortes prematuras decorrentes da doença e complicações decorrentes da mesma em 180 países. A divulgação do estudo mostrou que, no Brasil, os gastos com a diabetes foram de US$ 57,7 bilhões em 2015. Segundo as estimativas mais conservadoras, essas despesas podem subir para US$ 97 bilhões até 2030, podendo chegar a US$ 123 bilhões no pior dos cenários avaliados pelo estudo europeu (Bommer *et al.*, 2018).

Assim, cientes do impacto da T1D na vida dos acometidos pela doença, no núcleo de convivência desses e também na sociedade, pesquisas que buscam o entendimento dessa desordem devem ser fortemente encorajadas. Elucidar os mecanismos envolvidos constituem uma etapa crucial para alcançar uma terapia que traga melhor qualidade de vida a essas pessoas e, possivelmente, até a descoberta de formas efetivas de prevenção da T1D.

Nos estudos que mimetizam experimentalmente a doença, observa-se um perfil de suscetibilidade à T1D associado ao aumento da expressão de citocinas do padrão Th1 em detrimento das citocinas de perfil Th2 (Müller *et al.*, 2002). É reconhecido, também, que as células que produzem IL-4 ou outras citocinas do padrão Th2 são protetivas, sugerindo que uma intervenção capaz de afetar a diferenciação de linfócitos T pode resultar em uma proteção efetiva contra o desenvolvimento da T1D (Wållberg & Cooke, 2013). Já a IL-10 atua

como um importante regulador imune, podendo inibir a secreção de citocinas do padrão Th1, o que também contribui para um cenário protetivo contra a T1D.

Dessa forma, a estratégia escolhida neste estudo foi a utilização de linhagens de *L. lactis* MG1363 FnBPA^{+} portadoras dos vetores de expressão eucariótica pValac::*dts::IL-4* (Figura 1.6-A) e pValac::*IL-10* (Figura 1.6-B) – ambos desenvolvidos pelo nosso grupo de pesquisa, cuja funcionalidade já foi comprovada e o potencial terapêutico atestado em diferentes modelos murinos de inflamação intestinal (Del Carmen *et al.*, 2013; Zurita-Turk *et al.*, 2014; Souza *et al.*, 2016). As linhagens bacterianas recombinantes administradas pela via oral, ao alcançar a mucosa intestinal, são internalizadas pelas células eucarióticas, culminando na síntese dessas citocinas pela maquinaria de transcrição e tradução da célula hospedeira.

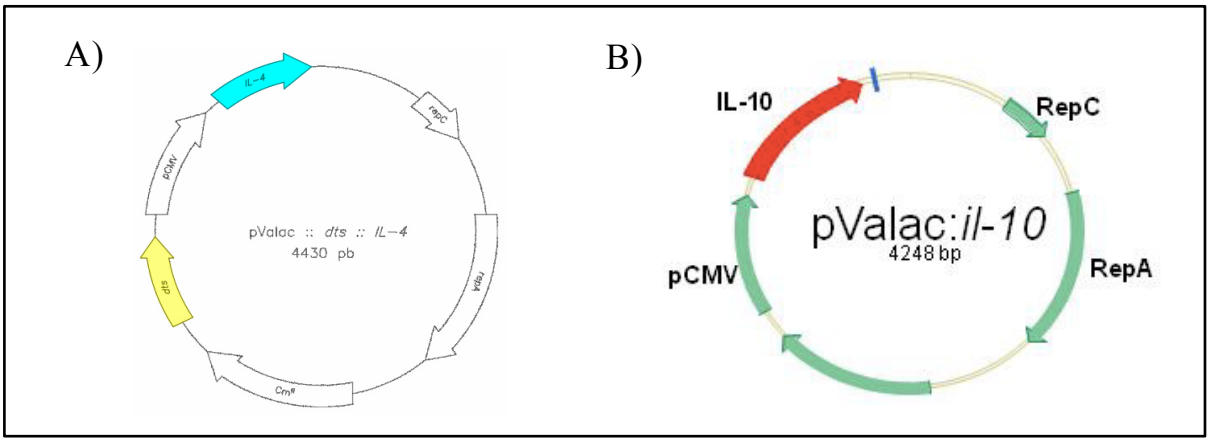


Figura 1.6: Representação esquemática dos plasmídeos (A) pValac::*dts::IL-4* e (B) pValac::*IL-10*. Em (A) observa-se a sequência de endereçamento nuclear DTS (amarelo), o promotor pCMV, a ORF *IL-4* (azul), as origens de replicação eucariótica repC e procariótica repA e a ORF que confere resistência ao antibiótico cloranfenicol (Cm^R). **Fonte:** Souza *et al.*, 2016. Em (B) observa-se o promotor pCMV, as origens de replicação eucariótica repC e procariótica repA e a ORF *IL-10*. **Fonte:** Del Carmen *et al.*, 2013.

Logo, hipotetiza-se que o aumento de IL-4 e IL-10, durante o tempo de ingestão das bactérias, chegue, por meio da circulação esplâncnica, até o principal órgão envolvido no desenvolvimento da T1D – o pâncreas. Ademais, os linfonodos duodenopancreáticos podem ser uma via "mais direta" de ligação entre as bactérias recombinantes amostradas no duodeno e cólon transverso e o local de montagem da resposta imune durante o desenvolvimento da T1D. Sendo assim, o presente trabalho se propôs a avaliar o efeito imunomodulatório provocado pela ingestão das linhagens recombinantes *L. lactis* MG1363 FnBPA^{+}

(pValac::*dts::IL-4*) e *L. lactis* MG1363 FnBPA^{+} (pValac::*IL-10*) nos principais modelos experimentais de T1D: induzido quimicamente por múltiplas doses de STZ e em camundongos NOD, constituindo, assim, uma abordagem alternativa para a compreensão dos mecanismos associados à progressão e desenvolvimento da T1D.

2. OBJETIVOS

2.1 Objetivo geral

Avaliar e comparar os efeitos decorrentes da administração da linhagem invasiva *L. lactis* MG1363 FnBPA$^+$ portadora dos vetores de expressão eucariótica pValac::*dts::IL-4* ou pValac::*IL-10* nos principais modelos murinos de diabetes tipo 1: induzido quimicamente por STZ e em camundongos NOD.

2.2 Objetivos específicos

- Avaliar a incidência da diabetes experimental em camundongos induzidos com STZ e em camundongos NOD, ambos submetidos à administração oral das linhagens recombinantes *L. lactis* MG1363 FnBPA$^+$ (pValac::*dts::IL-4*) e *L. lactis* MG1363 FnBPA$^+$ (pValac::*IL-10*);

- Analisar e comparar os níveis de citocinas no soro e no pâncreas dos camundongos que receberam as linhagens bacterianas geneticamente modificadas e foram submetidos ao modelo espontâneo ou de indução química da T1D experimental;

- Avaliar alterações no cólon intestinal dos camundongos submetidos a múltiplas doses de STZ e a camundongos NOD, que receberam pela via oral, bactérias lácticas codificadoras de IL-4 e IL-10;

- Avaliar a translocação das bactérias *L. lactis* MG1363 FnBPA$^+$ (pValac::*dts::IL-4*) e *L. lactis* MG1363 FnBPA$^+$ (pValac::*IL-10*) administradas oralmente aos camundongos;

- Analisar histologicamente o pâncreas dos camundongos C57BL/6 induzidos por STZ e camundongos NOD, após administração das linhagens recombinantes contendo plasmídeos codificadores de IL-4 e IL-10;

- Avaliar e comparar o potencial imunomodulatório das linhagens recombinantes *L. lactis* MG1363 FnBPA$^+$ (pValac::*dts::IL-4*) e *L. lactis* MG1363 FnBPA$^+$ (pValac::*IL-10*) no modelo de indução química por STZ e no modelo espontâneo de diabetes experimental, com camundongos NOD.

3.MATERIAIS E MÉTODOS

3.1 Linhagens celulares, plasmídeos e condições de cultivo

Foram utilizadas as linhagens *L. lactis* MG1363 FnBPA$^+$, *L. lactis* MG1363 FnBPA$^+$ (pValac::*dts*::*IL-4*) e *L. lactis* MG1363 FnBPA$^+$ (pValac::*IL-10*) (Tabela 3.1), as quais foram cultivadas em caldo M17 ou meio M17 sólido (1,5% de ágar bacteriológico) suplementado com 0,5% de glicose, acrescidos ou não dos antibióticos eritromicina (Ery) (5 μg/mL) e cloranfenicol (Cm) (10 μg/mL), a 30°C por 24-48 horas.

Tabela 3.1: Linhagens bacterianas usadas neste trabalho.

Linhagem	Características	Referência
Lactococcus lactis MG1363 FnBPA$^+$	Linhagem *L. lactis* MG1363 expressando a FnBPA de *S. aureus.*	Que *et al.*, 2001.
Lactococcus lactis MG1363 FnBPA$^+$ (pValac::*dts::IL-4*)	Linhagem *L. lactis* MG1363 FnBPA$^+$ portadora do plasmídeo pValac::*dts::IL-4.*	Souza *et al.*, 2016.
Lactococcus lactis MG1363 FnBPA$^+$ (pValac::*IL-10*)	Linhagem *L. lactis* MG1363 FnBPA$^+$ portadora do plasmídeo pValac::*IL-10.*	Del Carmen *et al.*, 2013.

3.2 Avaliação do potencial imunomodulatório da linhagem *L. lactis* MG1363 FnBPA$^+$ portadora dos vetores pValac::*dts::IL-4* ou pValac::*IL-10* em modelos experimentais de T1D

3.2.1 Animais

Foram utilizados camundongos C57BL/6 machos com 8-9 semanas de idade, fornecidos pelo Biotério Central da Universidade Federal de Minas Gerais (UFMG) e camundongos NOD fêmeas a partir do nascimento, gentilmente fornecidos pela Dra. Ana Maria Caetano de Faria. Todos os animais foram mantidos em micro isoladores no sistema IVC (do inglês, *Individually Ventilated Caging*), com temperatura, umidade, fluxo de ar e ciclo de luz (12 horas no claro e 12 horas no escuro) controlados e com livre acesso a água e ração.

3.2.2 Modelos experimentais de T1D

No modelo de diabetes experimental induzido quimicamente, camundongos C57BL/6 machos receberam injeção intraperitoneal (i.p.) de cinco doses (50 mg/kg) diárias e consecutivas de STZ (Sigma-Aldrich®) dissolvido em tampão citrato de sódio pH 4,5 (0,05 M) (Figura 3.1-A). Essa solução foi injetada em, no máximo, dez minutos após o preparo entre os dias 0 e 4 do cronograma experimental. Os animais foram mantidos em jejum por quatro horas antes e duas horas após a indução da T1D experimental. No dia 14 do cronograma experimental, os animais foram eutanasiados seguindo todas as normas de bioética e bem-estar animal.

Devido às características dos camundongos NOD descritas na seção 1.1.2, esses se caracterizam como um modelo espontâneo da T1D experimental, ao qual não é necessário nenhuma intervenção para indução da doença nos animais. Os camundongos NOD fêmeas foram acompanhados desde o nascimento até a 20ª semana de vida, quando esses foram eutanasiados para o prosseguimento das análises (Figura 3.2-B).

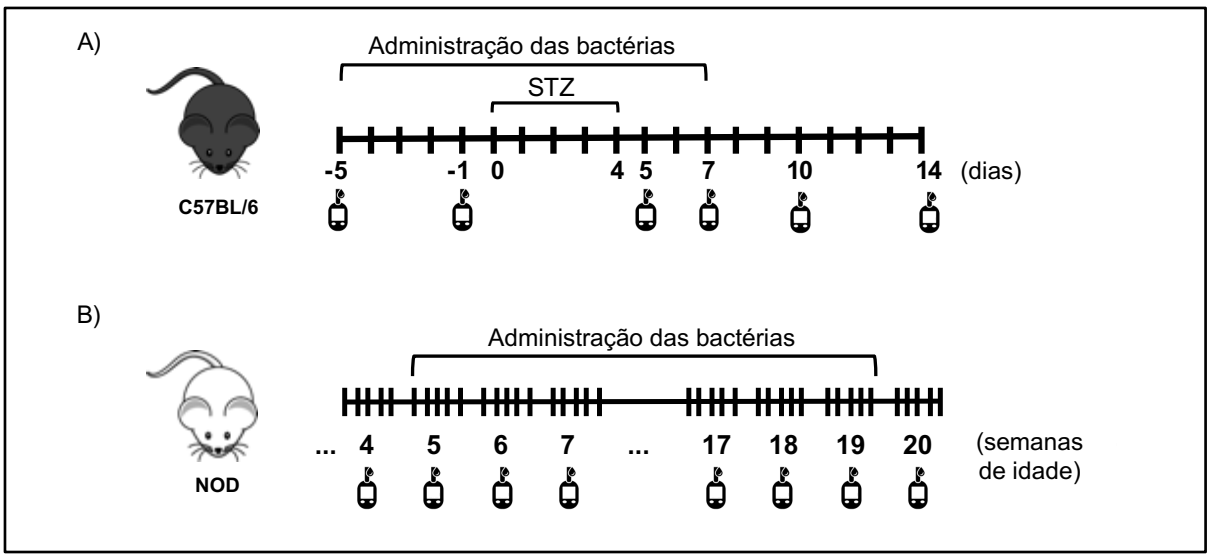


Figura 3.1: Representação esquemática dos cronogramas para os modelos experimentais de T1D induzido por STZ e camundongos NOD. A) A diabetes foi induzida experimentalmente em camundongos C57BL/6 machos por injeção intraperitoneal de tampão citrato de sódio + STZ por 5 dias consecutivos (dia 0 até dia 4). Os animais receberam a administração bacteriana diariamente do dia -5 ao dia 7. As amostras de sangue foram coletadas nos dias -5, -1, 5, 7, 10 e 14 para medição dos níveis de glicemia. Os animais foram eutanizados no dia 14. B) Os camundongos NOD receberam administração intragástrica de suspensões bacterianas 5 dias/semana por 15 semanas consecutivas. Os níveis glicêmicos foram medidos semanalmente durante o experimento. Os animais foram eutanasiados no final da 20ª semana.

3.2.3 Preparo de doses das linhagens bacterianas

O preparo das doses das diferentes linhagens de *L. lactis* foi iniciado com o pré-inóculo de 10 µL da devida linhagem bacteriana em 5 mL de caldo M17 contendo glicose 0,5% (M17-Gli) e suplementado com o antibiótico correspondente. Caso a dose a ser preparada fosse da linhagem *L. lactis* MG1363 FnBPA$^+$, adicionou-se Ery (5 µg/mL); caso fossem as linhagens *L. lactis* MG1363 FnBPA$^+$ (pValac::*dts::IL-4*) ou *L. lactis* MG1363 FnBPA$^+$ (pValac::*IL-10*), adicionou-se Ery (5 µg/mL) e Cm (10 µg/mL). O pré-inóculo foi, então, deixado em estufa a 30°C por 18 horas.

Passado esse tempo, uma alíquota de cada pré-inóculo foi diluída em 150 mL de caldo M17-Gli suplementado com o devido antibiótico de modo que as culturas apresentassem um valor inicial de densidade óptica no comprimento de onda de 600 nanômetros (DO_{600}) de 0,05. Assim, essa cultura foi mantida na estufa a 30°C até atingir uma DO_{600} de 1,0-1,2. Logo após, alíquotas de 1 mL de cada cultura foram estocadas a -80°C, utilizando-se glicerol 80% estéril na proporção de 3:1.

Após o preparo e estocagem das doses, foi feito um teste para verificar a viabilidade das culturas bacterianas presentes nas doses. Dessa maneira, foi feito o plaqueamento da diluição 10^{-6} de uma alíquota de cada cultura em meio M17-Gli, suplementado com o respectivo antibiótico de cada linhagem bacteriana. As placas foram feitas em duplicata e incubadas em estufa a 30°C por 24 horas.

Passado esse intervalo, foi feita uma estimativa da viabilidade de cada uma das linhagens por meio da contagem de unidades formadoras de colônias (UFCs)/mL. A partir desse dado, foi calculado o volume necessário de cada cultura para se obter o número de UFCs estipulado para cada dose ($1x10^9$ UFCs para cada animal).

3.2.4 Administração de doses de *L. lactis*

Nos dias de suplementação bacteriana dos cronogramas experimentais, o volume de cultura a ser utilizado, segundo o cálculo de viabilidade das doses, foi centrifugado a 7.000 rpm (rotações por minuto) por dois minutos, o sobrenadante foi descartado e o precipitado de

bactérias foi suspendido em 100 μL de solução salina 0,9% (9 g NaCl; 1 L água destilada). Após o preparo, as doses foram mantidas resfriadas até o momento da sua administração.

A cada animal, foi administrada uma dose composta por uma suspensão da linhagem bacteriana em questão na concentração de 10^9 UFCs em 100 μL de solução salina 0,9%.

No cronograma experimental de T1D experimental induzida por STZ, do dia -5 ao dia 7, os animais receberam, pela via intragástrica (i.g.), a suplementação bacteriana (vide Figura 3.1-A). Para tanto, os grupos foram divididos de acordo com a indução química da T1D e também pelas doses recebidas: (i), (ii) e (iii) solução salina 0,9%; (iv) *L. lactis* MG1363 FnBPA$^+$; (v) *L. lactis* MG1363 FnBPA$^+$ (pValac::*dts::IL-4*); (vi) *L. lactis* MG1363 FnBPA$^+$ (pValac::*IL-10*); e (vii) *L. lactis* MG1363 FnBPA$^+$ (pValac::*dts::IL-4*) e *L. lactis* MG1363 FnBPA$^+$ (pValac::*IL-10*) (Tabela 3.2).

Tabela 3.2: Divisão dos grupos experimentais quanto a indução química da T1D experimental e a dose administrada aos animais.

	Grupo	**Indução (i.p.)**	**Constituição da dose administrada pela via intragástrica**	**Nº de animais**
Camundongos C57BL/6 não submetidos à indução da diabetes experimental	- (Controle negativo)	Solução salina 0,9%	Solução salina 0,9%	18
	VC (*Vehicle Control*, do inglês, controle do veículo)	Tampão citrato de sódio	Solução salina 0,9%	18
Camundongos C57BL/6 submetidos à indução da diabetes experimental	*Saline*-STZ	STZ + Tampão citrato de sódio	Solução salina 0,9%	18
	F-STZ	STZ + Tampão citrato de sódio	*L. lactis* MG1363 FnBPA$^+$	18
	F-IL-4-STZ	STZ + Tampão citrato de sódio	*L. lactis* MG1363 FnBPA$^+$ (pValac::*dts::IL-4*)	18
	F-IL-10-STZ	STZ + Tampão citrato de sódio	*L. lactis* MG1363 FnBPA$^+$ (pValac::*IL-10*)	18
	F-IL-4/IL-10-STZ	STZ + Tampão citrato de sódio	*L. lactis* MG1363 FnBPA$^+$ (pValac::*dts*::*IL-4*) e *L. lactis* MG1363 FnBPA$^+$ (pValac::*IL-10*)	18

No cronograma experimental do modelo espontâneo de T1D, entre a 5ª e 19ª semana de vida (vide Figura 3.1-B), os camundongos NOD receberam, pela via i.g., solução salina 0,9% ou suplementação bacteriana contendo as linhagens bacterianas *L. lactis* MG1363 FnBPA$^+$ (pValac::*dts*::*IL-4*) e *L. lactis* MG1363 FnBPA$^+$ (pValac::*IL-10*) (Tabela 3.3).

Tabela 3.3: Divisão dos grupos experimentais de acordo com o que foi administrado oralmente aos camundongos NOD.

Grupo	Constituição da dose administrada pela via intragástrica	Nº de animais
Saline-NOD	Solução salina 0,9%	12
F-IL-4/IL-10-NOD	*L. lactis* MG1363 FnBPA$^+$ (pValac::*dts::IL-4*) e *L. lactis* MG1363 FnBPA$^+$ (pValac::*IL-10*)	12

3.2.5 Monitoramento e avaliação da incidência de T1D experimental

Para monitorar os níveis glicêmicos dos animais, foi feita a coleta de uma mínima amostra de sangue periférico, obtido através de um corte transversal na cauda dos animais, sem que fosse necessária a contenção desses. A dosagem foi feita em fitas próprias para o glicosímetro Accu-Chek® Active (Roche). Foram considerados diabéticos os animais que apresentaram duas medidas seguidas de níveis de glicose sanguínea superiores a 200 mg/dL (Costa *et al.*, 2016). No cronograma experimental da indução química da T1D, a glicemia foi aferida nos dias -5, -1, 5, 7, 10 e 14 (vide Figura 3.1-A), enquanto que no cronograma do modelo espontâneo da doença, a glicemia foi aferida uma vez por semana (vide Figura 3.1-B).

3.2.6 Detecção de citocinas e sIgA por ELISA

No dia da eutanásia, os animais foram submetidos a uma sobredose de solução anestésica (composta por Xilazina 2%, Cetamina 0,1 g/mL e PBS 1X), administrada pela via intraperitoneal, visando a anestesia e analgesia dos mesmos. Posteriormente, o soro sanguíneo, pâncreas e cólon intestinal foram coletados para avaliação das respostas

imunológicas induzidas por meio da realização de testes ELISA (do inglês, *Enzyme-Linked Immunosorbent Assay*).

Primeiramente, foi feito o processamento das amostras de pâncreas e cólon para possibilitar a realização dos testes ELISA. Sendo assim, as amostras foram homogeneizadas num tampão especial para a detecção de citocinas [23,4 g NaCl; 500 µL Tween® 20 (Sigma-Aldrich®); 5g BSA (USBiological); 34 mg PMSF (Sigma-Aldrich®); 1 mL DMSO (Sigma-Aldrich®); 44,6 mg cloreto de benzetônio (Sigma-Aldrich®); 372 mg Na_2EDTA; 40 µL aprotinina (10 mg/mL) (Sigma-Aldrich®); PBS 1X q.s.p. 1 L], utilizando o homogeneizador de amostras Omni (Omni International™). O homogenato foi centrifugado a 10.000 rpm, a 4°C, por 10 minutos. Os sobrenadantes foram armazenados a -80°C até o momento da sua utilização.

Já o sangue foi coletado, através da punção da aorta descendente, e processado para posterior análise de citocinas por meio dos testes ELISA. Para isso, as amostras de sangue foram incubadas em estufa à 37°C por, no máximo, 1 hora. Essas foram, então, centrifugadas a 5.000 rpm por 10 minutos, a 4ºC. O soro sanguíneo foi retirado e armazenado a -20°C até o momento da sua utilização.

Os procedimentos dos ELISAs do tipo "sanduíche" (Figura 3.2) foram feitos segundo as instruções dos fabricantes dos *kits* – BD OptEIA™ (BD) e R&D DuoSet (R&D Systems™) – para a dosagem de IL-2, IL-4, IL-10, IL-12 e TGF-β (do inglês – *Transforming Growth Factor* β) no pâncreas e cólon intestinal e também para a dosagem de IL-4 e IL-10 no soro sanguíneo. A leitura de todos os testes ELISA foi feita a 492 nm utilizando a leitora de microplacas POLARIS MA616 (Marconi).

Também foram realizados testes ELISA para detecção de imunoglobulina A secretória (sIgA) do lavado intestinal dos animais. Para essa análise, foi necessário, primeiramente, lavar o lúmen do cólon dos animais com 5 mL de PBS 1X e centrifugar o lavado a 1.200 rpm, a 4°C, por 10 minutos. O sobrenadante foi, então, armazenado a -80°C até o momento da sua utilização.

Os testes ELISA para detecção de sIgA no lavado intestinal foram realizados do seguinte modo: alíquotas de 100 µL do anticorpo de captura (1 mg/mL) – anticorpo de cabra anti-Ig de camundongo [*Goat Anti-Mouse Ig, Human ads*-UNLB (Southern Biotech)] –

diluído em tampão carbonato pH 9,6 (1,86 g Na_2CO_3; 2,93 g $NaHCO_3$; água destilada q.s.p. 1L) numa proporção de 1:4.000 foram adicionadas a cada um dos poços utilizados de placas de 96 poços (Nunc™). As placas foram, então, incubadas em câmara úmida à 4°C *overnight*. Passado esse tempo, os poços foram lavados com solução salina-tween [NaCl 0,15 M; Tween® 20 (Sigma-Aldrich®) 0,05%] três vezes e a eles foram adicionados 200 µL de PBS-caseína. Uma hora depois, os poços foram lavados, novamente, com solução salina-tween três vezes. Depois, foram adicionadas alíquotas de 100 µL do padrão – IgA de camundongo [*Mouse* IgA *Isotype Control* (Southern Biotech)] – em diluição seriada em cada um de oito poços. Além disso, foram adicionados 200 µL de cada um dos lavados e 200 µL de PBS-caseína (branco) em seus respectivos poços. Após uma hora, os poços foram lavados com solução salina-tween três vezes e a eles foram adicionados 100 µL do anticorpo de detecção – anticorpo de cabra anti-IgA de camundongo conjugado com HRP [do inglês, *Goat Anti-Mouse* IgA (*α chain specific*) *Horseradish Peroxidase* (HRP) *conjugate* (Southern Biotech)] diluído em PBS-caseína numa proporção de 1:8.000. Mais uma hora depois, os poços foram lavados com solução salina-tween três vezes. Foram, então, adicionados 100 µL da solução para revelação a cada poço, sendo a placa incubada ao abrigo da luz até que a cor fosse desenvolvida. Nesse momento, a revelação foi interrompida por meio da adição de 20 µL de H_2SO_4 2N a cada um dos poços. A leitura das amostras foi realizada a 492 nm por meio da utilização da leitora de microplacas POLARIS MA616 (Marconi).

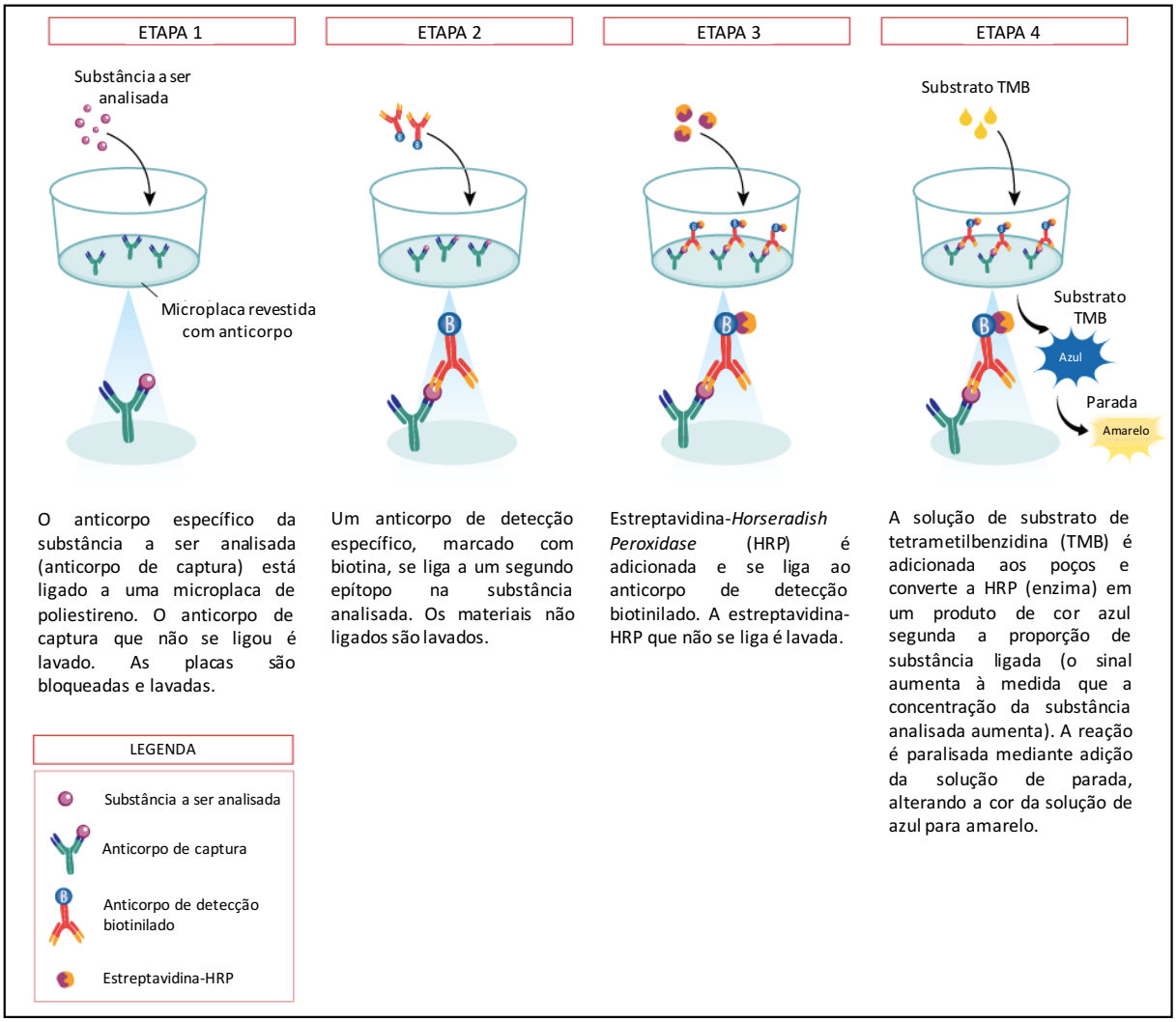


Figura 3.2: Representação esquemática das etapas para realização do ELISA do tipo "sanduíche". Adaptado de: https://www.rndsystems.com/resources/technical/duoset-elisa-development-systems-assay-principle

3.2.7 Análise histológica

Após a eutanásia, os pâncreas de animais dos dois cronogramas experimentais foram retirados e fixados em formalina tamponada a 10%, embebido em parafina e seccionados para análise histológica. Os cortes foram corados com hematoxilina e eosina e pelo menos 15 ilhotas por amostra foram avaliadas e ranqueadas às cegas por um investigador independente, com base na classificação descrita na Tabela 3.4.

Tabela 3.4: Parâmetros para avaliação do grau de infiltrado inflamatório nas ilhotas de Langerhans. **Adaptado de:** Papaccio, 1993 e Greiner *et al.*, 2014.

Parâmetro	Classificação
Sem sinais visíveis de infiltrado inflamatório nas ilhotas de Langerhans.	Sem insulite
Poucos elementos infiltrados nas ilhotas pancreáticas, com uma distribuição irregular, principalmente na periferia das ilhotas.	Peri-insulite
Infiltração moderada (peri- e intra-ilhota), com < 50% de destruição das ilhotas de Langerhans.	Insulite moderada
Infiltração extensa, com > 50% de destruição das ilhotas de Langerhans.	Insulite severa

3.2.8 Análise de translocação bacteriana

Para verificar a translocação das linhagens bacterianas administradas aos animais submetidos à T1D experimental, camundongos C57BL/6 receberam, pela via intragástrica, 100 µL de suspensão bacteriana com as linhagens *L. lactis* MG1363 FnBPA$^+$ (pValac::*dts::IL-4*) e *L. lactis* MG1363 FnBPA$^+$ (pValac::*IL-10*, em uma dose final de 10^9 UFCs em 100 µL de solução salina 0,9%. O baço, fígado e linfonodos mesentéricos (MLNs) foram coletados sob condições rigorosas de assepsia 2 h, 4 h, 6 h, 12 h, e 24 h após a administração das linhagens bacterianas. Os tecidos foram macerados em 500 µL de solução salina 0,9% e, 100 µL deste homogenado foram distribuídos em placa contendo meio M17 (Sigma-Aldrich) suplementado com ágar, glicose 0,5%, cloranfenicol (10 µg/mL) (Sigma-Aldrich) e eritromicina (5 µg/mL) (Sigma-Aldrich). As placas foram incubadas a 30°C por 24 h.

3.3 Análises estatísticas

Os resultados obtidos a partir de três réplicas experimentais foram analisados em conjunto, primeiramente, pelo programa computacional online *GraphPad QuickCalcs Outlier Calculator* (http://graphpad.com/quickcalcs/Grubbs1.cfm) (GraphPad Software) objetivando excluir as leituras estatisticamente fora da média ($p < 0,05$). Em seguida, com o auxílio do

programa computacional GraphPad Prism 5.0 (GraphPad Software), a análise de variância foi realizada por meio do uso do teste *One-Way* ANOVA e do pós-teste Tukey. Para incidência de diabetes, o teste $\chi 2$ foi utilizado. Para todas as análises, os valores com intervalo de confiança menor que 0,05 ($p < 0,05$) foram considerados estatisticamente significativos.

3.4 Aspectos bioéticos e de biossegurança

3.4.1 Bioéticos

Os procedimentos e as manipulações dos animais foram realizados conforme as normas da Comissão de Ética no Uso de Animais (CEUA) da UFMG, sendo o protocolo do CEUA para uso de animais em experimentação encontrado sob o número 206/2016.

3.4.2 Biossegurança

As devidas precauções de biossegurança foram tomadas de acordo com normas e regulamentos de biossegurança estabelecidos pela Universidade Federal de Minas Gerais [Comissão Interna de Biossegurança (CIBio) da UFMG].

4. RESULTADOS E DISCUSSÃO

4.1 Avaliação da incidência da T1D experimental no modelo induzido quimicamente e no modelo espontâneo da doença

Para avaliação da incidência da T1D, os níveis glicêmicos foram monitorados ao longo de todo o cronograma experimental. Observou-se, então, que a administração das linhagens bacterianas nos grupos induzidos por STZ (grupos F-STZ, F-IL-4-STZ, F-IL-10-STZ e F-IL-4/IL-10-STZ) não conseguiu impedir o aumento da glicemia nos animais após a indução química da T1D. Dessa forma, os níveis de glicose no sangue nesses grupos foram semelhantes aos observados no grupo *Saline*-STZ (Figura 4.1-A). Um aumento progressivo na incidência de diabetes experimental também foi observado nos grupos que receberam injeções de STZ, independentemente da administração das linhagens bacterianas pela via oral (Figura 4.1-B).

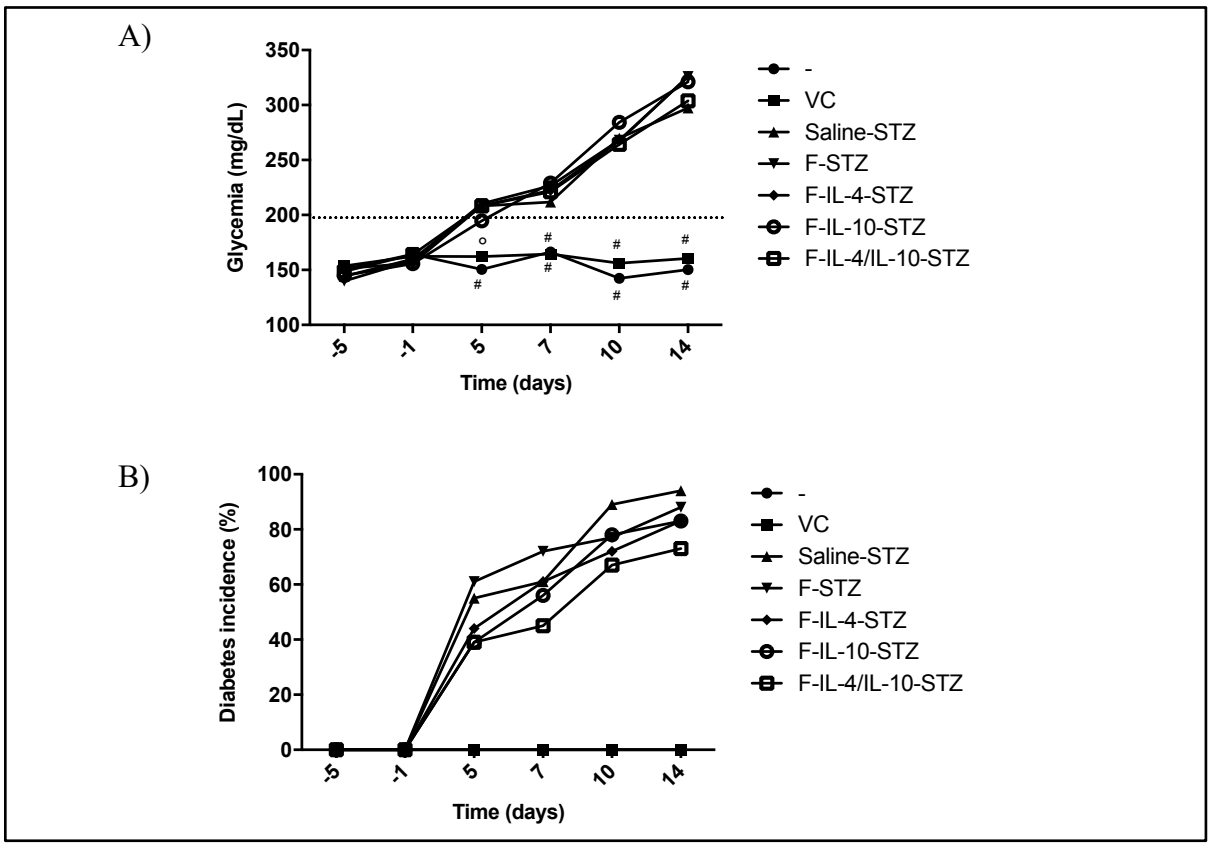


Figura 4.1: Monitoramento da glicemia e incidência da T1D experimental em camundongos C57BL/6 submetidos à indução química por STZ e administração intragástrica de linhagens geneticamente modificadas de *L. lactis*. Camundongos C57BL/6 foram injetados, pela via intraperitoneal, com solução salina 0,9%, solução veicular (tampão citrato de sódio) ou STZ durante cinco dias consecutivos (50 mg/kg/d). A administração bacteriana determinada para cada grupo experimental foi realizada pela via intragástrica do dia -5 ao dia 7. A) Os níveis glicêmicos (mg/dL) foram medidos nos dias -5, -1, 5, 7, 10, e 14. Os dados são mostrados como média ± SD de três experimentos

independentes (n = 18). #: grupo experimental cuja glicemia foi significativamente diferente dos grupos *Saline*-STZ, F-STZ, F-IL-4-STZ, F-IL-10-STZ e F-IL-4/IL-10-STZ; °: grupo experimental cuja glicemia foi significativamente diferente dos grupos *Saline*-STZ, F-STZ, F-IL-4-STZ e F-IL-4/IL-10-STZ ($p \leq 0,05$). B) Análise da incidência de diabetes nos grupos experimentais ao longo do tempo.

A Tabela 4.1 compara a porcentagem de camundongos diabéticos e não-diabéticos entre os grupos experimentais (grupos F-STZ, F-IL-4-STZ, F-IL-10-STZ e F-IL-4/IL-10-STZ) e o grupo controle da doença (grupo *Saline*-STZ) no último dia do cronograma experimental. Ao analisar a distribuição de camundongos diabéticos, verifica-se que todos os grupos apresentaram porcentagens semelhantes. Entretanto, nota-se também que o grupo F-IL-4/IL-10-STZ exibiu a maior porcentagem de camundongos não-diabéticos (37%).

Tabela 4.1: Comparação da porcentagem de camundongos diabéticos e não-diabéticos entre os grupos experimentais e o grupo controle da T1D no dia 14 do cronograma experimental.

	Animais diabéticos (n=76)	**Animais não-diabéticos (n=14)**	***p value***
Saline-STZ	22% (17)	7% (1)	$p = 0,44$
F-STZ	21% (16)	14% (2)	
F-IL-4-STZ	20% (15)	21% (3)	
F-IL-10-STZ	20% (15)	21% (3)	
F-IL-4/IL-10-STZ	17% (13)	37% (5)	

Em contraste com os resultados observados no modelo induzido quimicamente por STZ, os camundongos NOD que receberam pela via oral as linhagens *L. lactis* MG1363 FnBPA$^+$ (pValac::*dts::IL-4*) e *L. lactis* MG1363 FnBPA$^+$ (pValac::*IL-10*) (grupo F-IL-4/IL-10-NOD) apresentaram normoglicemia (níveis glicêmicos inferiores a 200 mg/dL) até o final do cronograma experimental (Figura 4.2-A), indicando que essa intervenção protegeu os camundongos NOD do desenvolvimento espontâneo da T1D previsto nesse modelo. Os resultados também mostraram que a incidência de diabetes aumentou no grupo *Saline*-NOD a partir da 15ª semana, enquanto os animais do grupo F-IL-4/IL-10-NOD permaneceram protegidos contra a diabetes durante todo o período experimental (Figura 4.2-B). A análise comparativa das porcentagens de camundongos diabéticos e não-diabéticos mostrou que a diferença entre os grupos *Saline*-NOD e F-IL-4/IL-10-NOD foi estatisticamente significativa (Tabela 4.2). Na 20ª semana, todos os camundongos diabéticos pertenciam ao grupo *Saline*-

NOD, portanto, o grupo F-IL-4/IL-10-NOD não apresentava nenhum camundongo com glicemia alterada. Além disso, verificou-se que, ao observar os camundongos não-diabéticos, apenas 14% desses animais estavam no grupo *Saline*-NOD, enquanto os 86% restantes pertenciam no grupo F-IL-4/IL-10-NOD. Ademais, a comparação dos níveis glicêmicos e a porcentagem de incidência de diabetes nos grupos de camundongos induzidos por STZ e de camundongos NOD no final de cada cronograma experimental corroboram com as análises anteriores (Tabela 4.3).

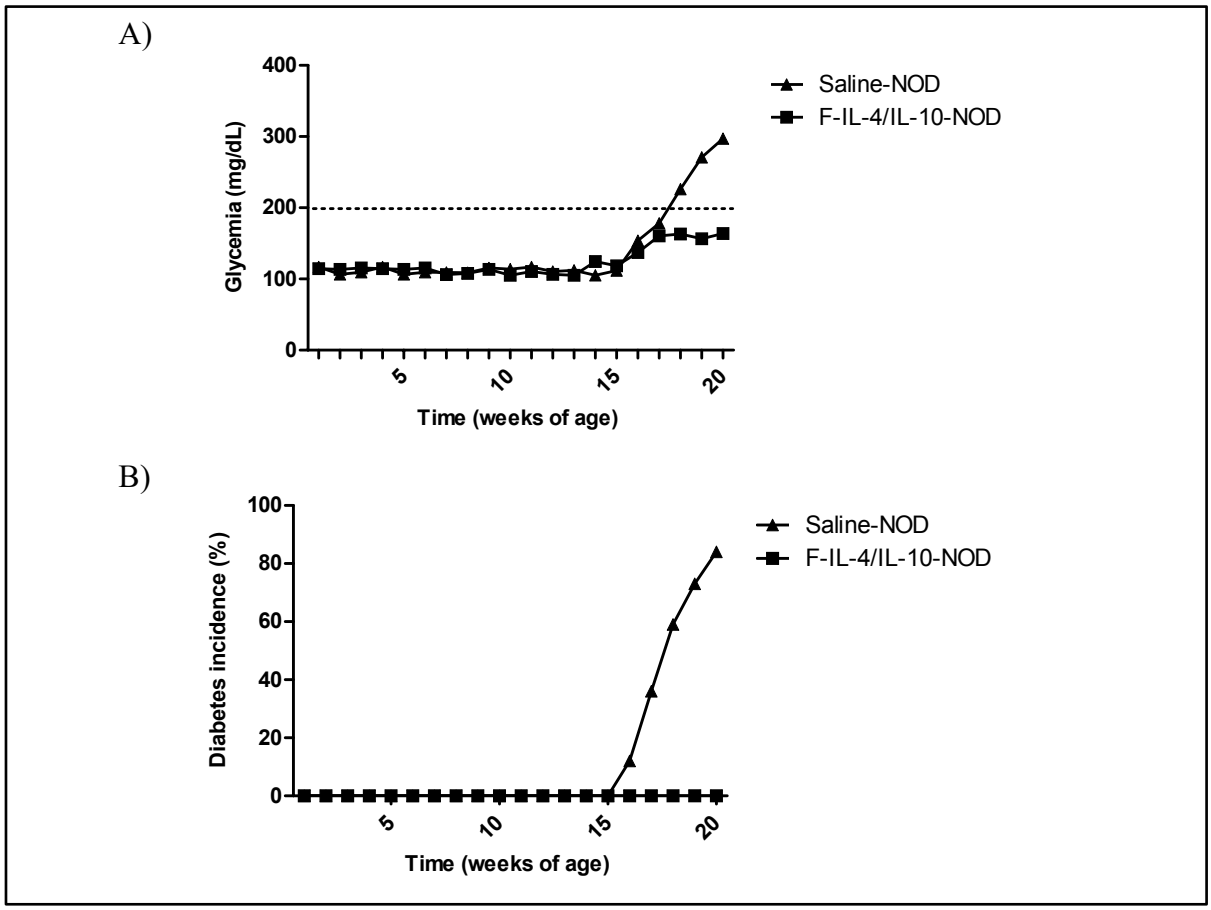


Figura 4.2: Monitoramento da glicemia e incidência da T1D experimental em camundongos NOD com administração intragástrica de linhagens geneticamente modificadas de *L. lactis*. Camundongos NOD receberam uma suspensão de *L. lactis* MG1363 FnBPA$^+$ (pValac::*dts::IL-4*) e *L. lactis* MG1363 FnBPA$^+$ (pValac::*IL-10*) (grupo F-IL-4/IL-10-NOD) ou solução salina 0,9% (grupo *Saline*-NOD) 5 dias/semana entre a 5ª e a 19ª semana do cronograma experimental. A) Os níveis glicêmicos (mg/dL) foram medidos semanalmente. Os dados são mostrados como média ± SD de três experimentos independentes (n = 12). B) Análise da incidência de diabetes nos grupos experimentais ao longo do tempo.

Tabela 4.2: Comparação da porcentagem de camundongos diabéticos e não-diabéticos entre os grupos experimentais de camundongos NOD ao final da 20ª semana do cronograma.

	Animais diabéticos (n=10)	**Animais não-diabéticos (n=14)**	***p value***
Saline-NOD	100% (10)	14% (2)	$p = 0{,}00003$
F-IL-4/IL-10-NOD	0% (0)	86% (12)	

Tabela 4.3: Comparação dos parâmetros clínicos observados no modelo de STZ e nos camundongos NOD.

	Modelo induzido por STZ: dia 14							**Camundongos NOD: 20ª semana**	
	-	VC	*Saline*-STZ	F-STZ	F-IL-4-STZ	F-IL-10-STZ	F-IL-4/IL-10-STZ	*Saline*-NOD	F-IL-4/IL-10-NOD
Glicemia (mg/dL)	150	160	297	326	325	321	303	298	177
Incidência de diabetes (%)	0	0	95	89	84	84	72	84	0

4.2 Avaliação das respostas imunológicas no pâncreas de camundongos que receberam as linhagens *L. lactis* MG1363 FnBPA$^+$ portadoras dos vetores pValac::*dts::IL-4* ou pValac::*IL-10* nos dois modelos experimentais de T1D

Para melhor analisar os efeitos decorrentes da administração das linhagens bacterianas geneticamente modificadas, amostras de pâncreas dos animais submetidos aos dois modelos experimentais de T1D foram processadas e analisadas quanto aos níveis de algumas citocinas.

Ao observar os níveis pancreáticos de IL-2 no modelo induzido por STZ, verificou-se que, com exceção dos controles negativo e do veículo, o grupo que recebeu as linhagens bacterianas portadoras dos vetores vacinais codificadores de IL-4 e IL-10 (grupo F-IL-4/IL-

10-STZ) foi o único que exibiu níveis significativamente maiores de IL-2 do que o grupo controle da doença (grupo *Saline*-STZ) (Figura 4.3-A).

Estudos anteriores relataram que a IL-2 desempenha um papel fundamental no equilíbrio entre imunorregulação e imunossupressão, uma vez que essa citocina é capaz de expandir e ativar células T regulatórias (Tregs); o que, subsequentemente, fornece uma supressão ativa da resposta efetora (Long *et al.*, 2013; Rosenzwajg *et al.*, 2014). Particularmente na T1D, a administração de baixas doses de IL-2 promove a sobrevivência de células Tregs e protege camundongos NOD do desenvolvimento da doença (Tang *et al.*, 2008). Já foi relatado que Tregs de pacientes com diabetes apresentam produção e/ou sinalização deficiente de IL-2 e, se a via de IL-2 for bloqueada, a frequência de Tregs diminui, e o número de indivíduos afetados pela T1D aumenta (Long *et al.*, 2011). Assim, os níveis normais de IL-2 em camundongos induzidos por STZ e que receberam as linhagens bacterianas de *L. lactis* MG1363 FnBPA^{+} portadoras de plasmídeos que codificam IL-4 e IL-10 podem ter contribuído para a ativação de células Treg no pâncreas e atenuado a progressão da insulite, característica determinante da doença.

A citocina TGF-β corresponde a uma citocina pleiotrópica envolvida em respostas imunes supressivas e também inflamatórias. Nos modelos murinos de T1D, já foi demonstrado que TGF-β está associada à indução de células T tolerogênicas, levando à prevenção do desenvolvimento da doença (Sanjabi *et al.*, 2017). Em relação aos níveis pancreáticos de TGF-β e também da IL-10, foi encontrada uma diferença significativa apenas entre os grupos controle negativo e *Saline*-STZ (Figura 4.3-B e C, respectivamente). Já os níveis de IL-4 (Figura 4.3-D) e IL-12 (Figura 4.3-E) foram similares entre as amostras pancreáticas de todos os grupos experimentais. Acredita-se que essas observações sejam devido à indução química por STZ ser um modelo experimental desenvolvido em um curto período de tempo. Dessa forma, para observar alterações significativas nos níveis dessas citocinas seria necessário um tempo experimental mais longo.

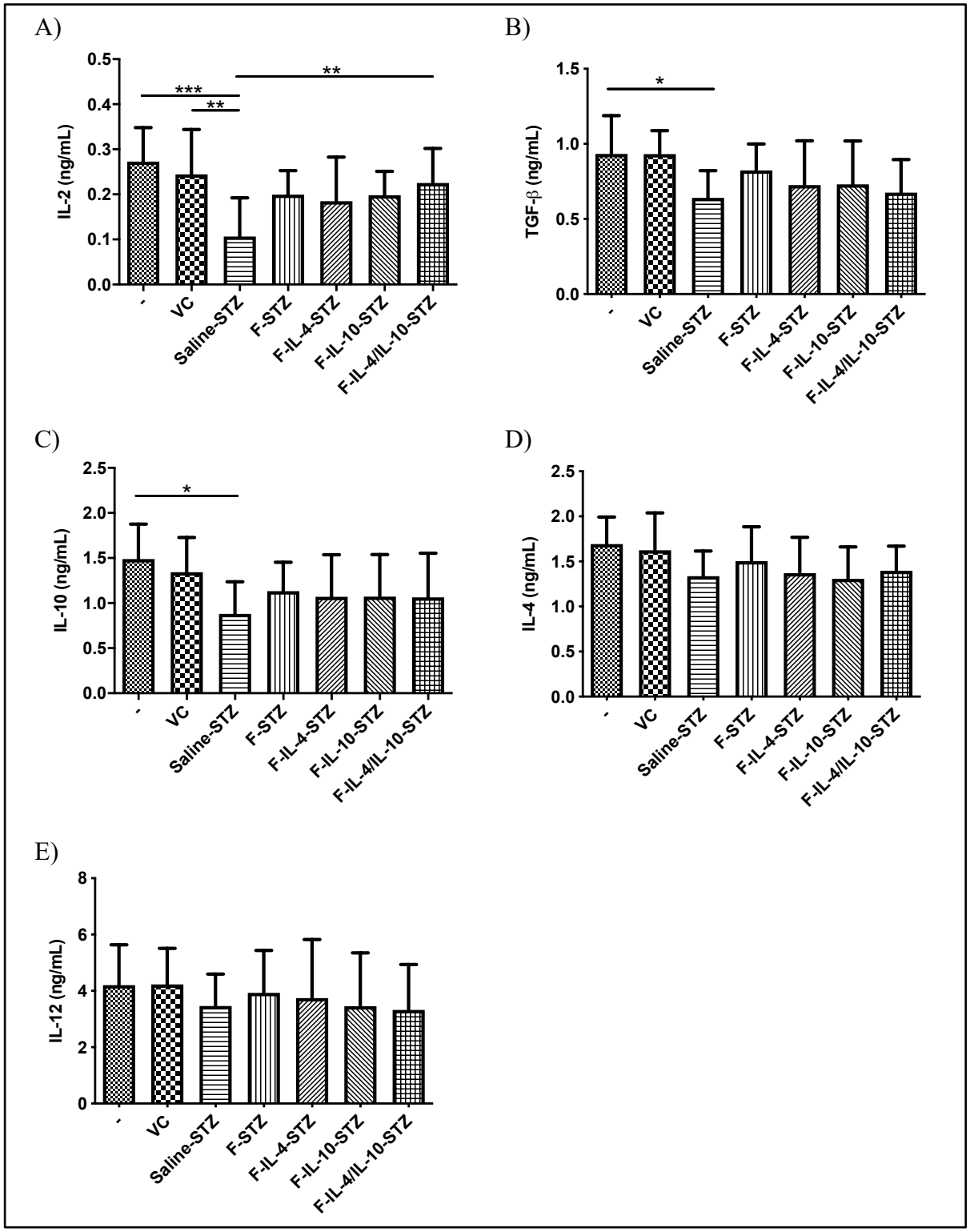


Figura 4.3: Níveis de citocinas pancreáticas de camundongos C57BL/6 induzidos por STZ submetidos à administração intragástrica de linhagens geneticamente modificadas de *L. lactis.* Após o período experimental, os níveis de IL-2, TGF-β, IL-10, IL-4 e IL-12 foram analisados (A-E, respectivamente; n = 18). -: controle negativo; VC: controle do veículo; *Saline:* animais que receberam salina 0,9% i.g.; F: animais que receberam *L. lactis* MG1363 FnBPA$^+$ i.g.; F-IL-4: animais que receberam *L. lactis* MG1363 FnBPA$^+$ (pValac::*dts::IL-4*) i.g.; F-IL-10: animais que receberam *L. lactis* MG1363 FnBPA$^+$ (pValac::*IL-10*) i.g.; F-IL-4/IL-10: animais que receberam *L. lactis* MG1363 FnBPA$^+$

(pValac::*dts::IL-4*) e *L. lactis* MG1363 FnBPA$^+$ (pValac::*IL-10*) i.g.; STZ: camundongos que receberam injeções intraperitoneais de estreptozotocina por cinco dias consecutivos. Os dados são mostrados como média ± SD de três experimentos independentes. *p value*: *p ≤ 0,05; **p ≤ 0,01; ***p ≤ 0,001.

Os níveis das mesmas citocinas pancreáticas também foram mensuradas no modelo espontâneo da T1D experimental. Como discutido anteriormente, a IL-2 é uma citocina pleiotrópica que favorece a expansão e ativação de células Treg tanto em camundongos como em humanos (Grinberg-Bleyer *et al.*, 2010). Uma vez que as células Treg não possuem a capacidade de produzir IL-2, essas células são dependentes da produção parácrina para o seu desenvolvimento e, consequentemente, executar sua função. Já foi demonstrado que quantidades insuficientes de IL-2 no pâncreas foram responsáveis pela baixa sobrevivência de células Treg nesse tecido, o que levaria à quebra da autotolerância e, assim, ao desenvolvimento da T1D (Tang *et al.*, 2008). Estudos também demonstram que a citocina TGF-β desempenha uma função importante na recuperação de ilhotas pancreáticas no modelo espontâneo de diabetes, uma vez que essa citocina é capaz de induzir células Treg na periferia (Park *et al.*, 2008). Entretanto, nos camundongos NOD, verificamos que as citocinas IL-2 (Figura 4.4-A) e TGF-β (Figura 4.4-B) apresentaram níveis similares nos grupos *Saline*-NOD e F-IL-4/IL-10-NOD. Supõe-se que não foi observado diferença significativa dessas citocinas entre os grupos devido ao momento no cronograma experimental em que foram coletadas as amostras.

A citocina IL-10 atua nas ilhotas pancreáticas suprimindo a produção de IFN-γ, IL-1β, IL-12 e TNF-α – citocinas pró-inflamatórias que estimulam a progressão da insulite destrutiva (Xu *et al.*, 2015). Cada vez mais, vários estudos comprovam a importância da IL-10 nas intervenções terapêuticas na T1D. Dentre esses, foi demonstrado que o tratamento sistêmico de camundongos NOD de 4 semanas de idade com altas doses de um vetor viral expressando IL-10 murina resultou no aumento de células Treg CD4$^+$CD25$^+$ e, assim, foi capaz de prevenir o desenvolvimento da T1D (Goudy *et al.*, 2003). No atual estudo, foi observado que os níveis de IL-10 foram significativamente maiores em camundongos NOD que receberam *L. lactis* MG1363 FnBPA$^+$ (pValac::*dts::IL-4*) e *L. lactis* MG1363 FnBPA$^+$ (pValac::*IL-10*) do que no grupo que recebeu solução salina (Figura 4.4-C).

Por se caracterizar como uma citocina do perfil Th2, a IL-4 se torna interessante para utilização em intervenções no modelo experimental de T1D, uma vez que esse consiste em um modelo mediado por respostas pró-inflamatórias Th1. A IL-4 é conhecida por induzir a geração de macrófagos do perfil M2 e já foi demonstrado em camundongos NOD que essas células podem assumir funções regulatórias no desenvolvimento da T1D experimental (Osada *et al.*, 2013). Os animais que receberam linhagens de *L. lactis* contendo vetores vacinais codificadores de IL-4 e IL-10 (grupo F-IL-4/IL-10-NOD) apresentaram níveis significativamente maiores de IL-4 em comparação com o grupo controle (grupo *Saline*-NOD) (Figura 4.4-D). É sugerido que a intervenção proposta neste trabalho resultou na imunomodulação da dinâmica de citocinas no tecido pancreático, alcançando o objetivo de preservação das ilhotas de Langerhans e manutenção dos níveis normais e saudáveis de glicose no sangue.

Sabe-se que a citocina IL-12 é secretada por células dendríticas, macrófagos teciduais residentes e células B em resposta a estímulos associados à defesa do organismo e cicatrização. Essa citocina promove a diferenciação de células T CD4 *naive* em células Th1 produtoras de IFN-γ, estimulando um microambiente de perfil pró-inflamatório (Vignali & Kuchroo, 2012; Teng *et al.*, 2015). No experimento utilizando camundongos NOD, verificou-se que os níveis de IL-12 pancreática estavam significativamente menores em animais que receberam as linhagens bacterianas geneticamente modificadas (grupo F-IL-4/IL-10-NOD), comparado com o grupo que recebeu solução salina pela via intragástrica (grupo *Saline*-NOD) (Figura 4.4-E). Esse resultado indica que a administração combinada de *L. lactis* MG1363 FnBPA$^+$ (pValac::*dts::IL-4*) e *L. lactis* MG1363 FnBPA$^+$ (pValac::*IL-10*) foi capaz de impedir o aumento da IL-12 e, consequentemente, evitar um microambiente pró-inflamatório no local primário do desenvolvimento da T1D – o pâncreas.

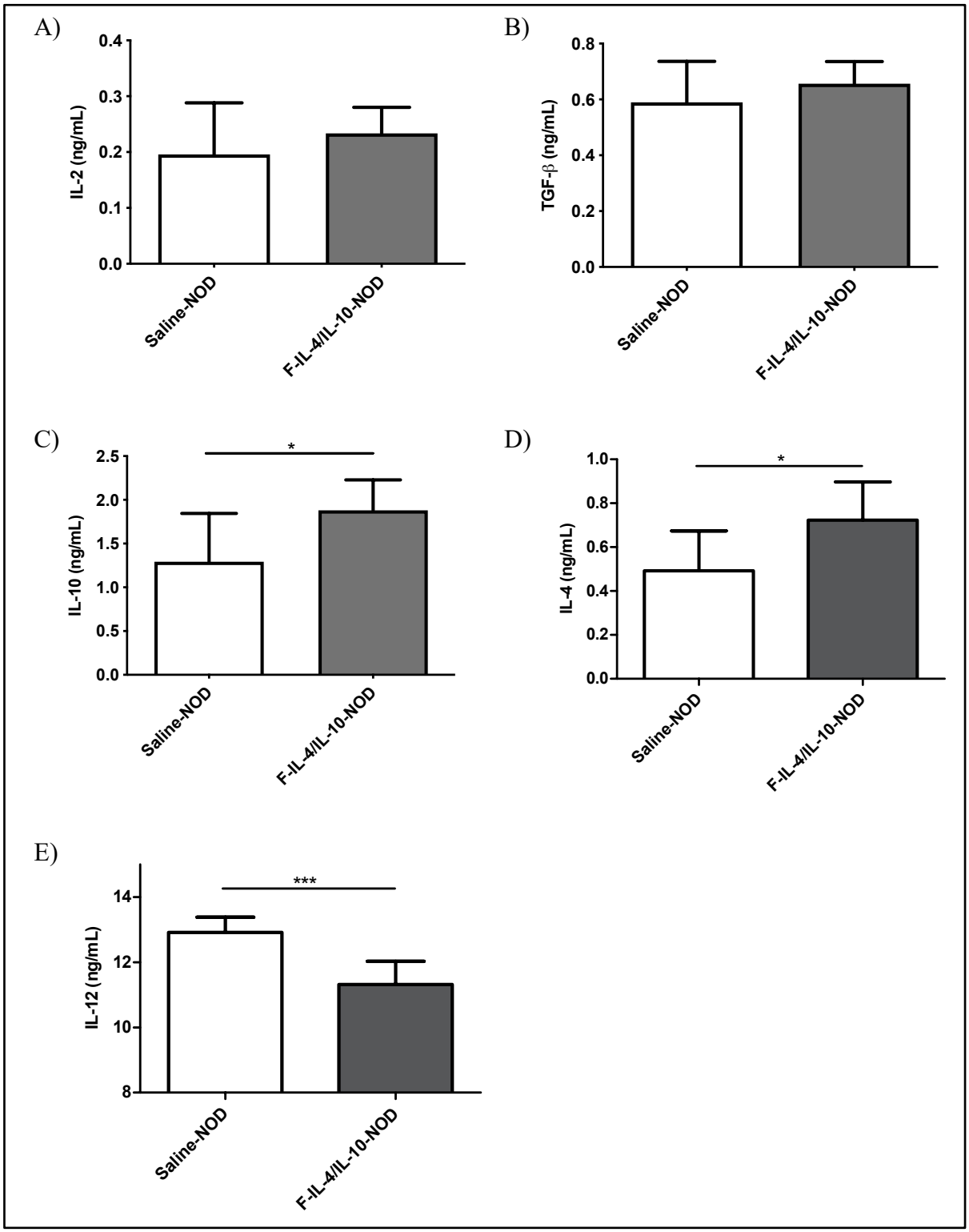


Figura 4.4: Níveis de citocinas pancreáticas de camundongos NOD submetidos à administração intragástrica de linhagens geneticamente modificadas de *L. lactis.* Após o período experimental, os níveis de IL-2, TGF-β, IL-10, IL-4 e IL-12 foram analisados (A-E, respectivamente; n = 12). *Saline:* animais que receberam salina 0,9% i.g.; F-IL-4/IL-10: animais que receberam *L. lactis* MG1363 FnBPA$^+$ (pValac::*dts::IL-4*) e *L. lactis* MG1363 FnBPA$^+$ (pValac::*IL-10*) i.g.; NOD: camundongos diabéticos não-obesos. Os dados são

mostrados como média ± SD de três experimentos independentes. *p value*: *p ≤ 0,05; ***p ≤ 0,001.

4.3 Avaliação das respostas imunológicas no soro sanguíneo de camundongos que receberam linhagens bacterianas recombinantes codificadoras de IL-4 ou IL-10 nos dois modelos experimentais de T1D

Haja vista a importância da IL-4 e IL-10 na estratégia escolhida de imunodulação nos modelos experimentais de T1D, foi feita a mensuração dos níveis sistêmicos dessas citocinas no soro sanguíneo dos animais. No modelo induzido quimicamente por STZ, todos os grupos experimentais apresentaram níveis similares de IL-4 e IL-10 (Figuras 4.5-A e B, respectivamente). Acredita-se que tais resultados seguem em concordância com o perfil das outras análises realizadas nos camundongos que receberam injeções de STZ. A indução química da T1D caracteriza-se como um modelo extremamente rápido, o que acarreta em certa "agressividade" na progressão da doença simulada. Dessa forma, alterações sutis na dinâmica das citocinas são mais difíceis de serem observadas. Por esse motivo, o modelo espontâneo da doença se apresenta como o mais adequado para analisar estratégias imunomodulatórias de intervenção na T1D (King, 2012; Brito-Casillas *et al.*, 2016).

Em camundongos NOD que receberam doses de *L. lactis* MG1363 FnBPA$^+$ (pValac::*dts::IL-4*) e *L. lactis* MG1363 FnBPA$^+$ (pValac::*IL-10*) (grupo F-IL-4/IL-10-NOD) observou-se níveis significativamente maiores de IL-4 (Figura 4.5-C) e de IL-10 (Figura 4.5-D) no soro, quando comparado aos animais que receberam apenas salina pela via intragástrica (grupo *Saline*-NOD). Acredita-se que esses resultados sejam uma consequência direta da administração das linhagens bacterianas portadoras de vetores codificadores de IL-4 e IL-10. Essas observações se mostram como importantes indícios de que as bactérias ingeridas foram internalizadas por células do trato gastrointestinal dos camundongos NOD e as citocinas IL-4 e IL-10 foram sintetizadas pela maquinaria das células eucarióticas. Ademais, a verificação de que os níveis sistêmicos dessas citocinas estão aumentados demonstra que a intervenção aqui proposta é capaz de alcançar todo o organismo através da circulação sanguínea, e não apenas o microambiente de passagem das bactérias.

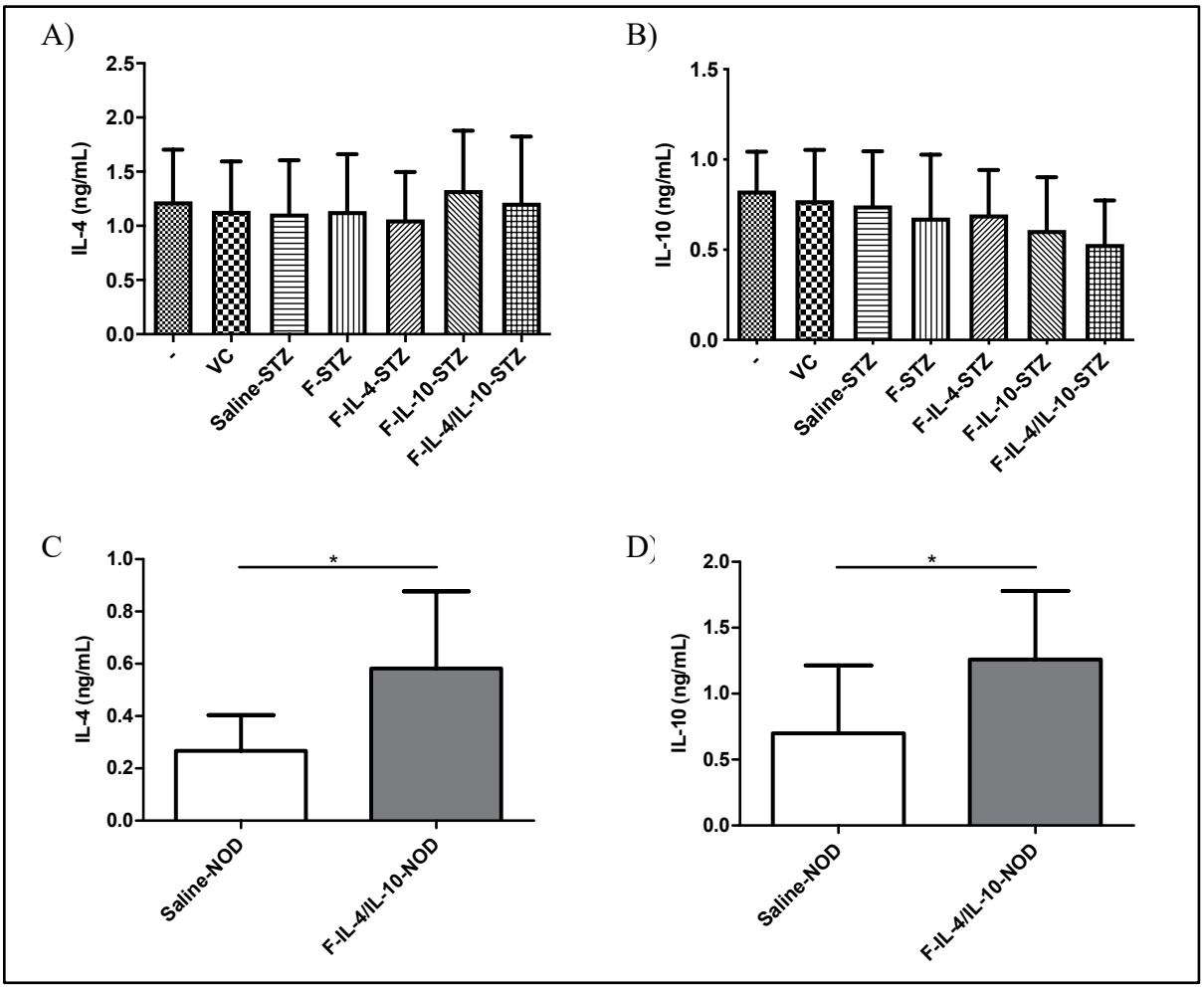


Figura 4.5: Níveis de IL-4 e IL-10 no soro de camundongos C57BL/6 induzidos por STZ e de camundongos NOD, ambos submetidos à administração intragástrica de linhagens geneticamente modificadas de *L. lactis.* Camundongos C57BL/6 com injeção i.p. de salina, tampão citrato de sódio ou STZ + tampão citrato de sódio e administração oral de linhagens bacterianas foram eutanizados no dia 14 do cronograma experimental e o soro coletado para medir os níveis IL-4 (A) e IL-10 (B) (n = 18). Após a administração oral de salina ou das bactérias, o soro dos camundongos NOD foi coletado logo após a eutanásia na 20ª semana do cronograma experimental para medir os níveis de IL-4 (C) e IL-10 (D) (n = 12). -: controle negativo; VC: controle do veículo; *Saline:* animais que receberam salina 0,9% i.g.; F: animais que receberam *L. lactis* MG1363 FnBPA$^+$ i.g.; F-IL-4: animais que receberam *L. lactis* MG1363 FnBPA$^+$ (pValac::*dts::IL-4*) i.g.; F-IL-10: animais que receberam *L. lactis* MG1363 FnBPA$^+$ (pValac::*IL-10*) i.g.; F-IL-4/IL-10: animais que receberam *L. lactis* MG1363 FnBPA$^+$ (pValac::*dts::IL-4*) e *L. lactis* MG1363 FnBPA$^+$ (pValac::*IL-10*) i.g.; STZ: camundongos que receberam injeções intraperitoneais de estreptozotocina por cinco dias consecutivos; NOD: camundongos diabéticos não-obesos. Os dados são mostrados como média ± SD de três experimentos independentes. *p value*: *p ≤ 0,05.

4.4 Avaliação das respostas imunológicas no cólon intestinal de camundongos que receberam as linhagens *L. lactis* MG1363 FnBPA$^+$ (pValac::*dts::IL-4*) e *L. lactis* MG1363 FnBPA$^+$ (pValac::*IL-10*) nos dois modelos experimentais de T1D

Visto que as linhagens bacterianas geneticamente modificadas foram entregues pela via oral, destaca-se a importância de verificar se essas causaram alguma alteração relevante no trato gastrointestinal dos animais. Dessa forma, os níveis de várias citocinas (IL-2, IL-4, IL-10, IL-12 e TGF-β) foram mensurados nos homogenatos de cólon dos camundongos induzidos por STZ e dos camundongos NOD e não foram observadas diferenças significativas nos níveis das citocinas testadas entre os grupos experimentais (dados não mostrados). Dessa forma, verificou-se que a administração das linhagens bacterianas em questão não altera o microambiente das citocinas no cólon intestinal ou, caso isso tenha ocorrido, foi de forma temporária e por um curto período de tempo. Tal observação é de extrema importância, uma vez que a passagem das bactérias pelo trato gastrointestinal foi a rota escolhida para a intervenção nos modelos experimentais de T1D.

Também foi feita a mensuração dos níveis de sIgA pois já é bem estabelecido na literatura que esse anticorpo apresenta propriedades imunomodulatórias e está envolvido não apenas na resposta a patógenos, mas também na manutenção da homeostase entre o trato gastrointestinal e os microrganismos comensais e ingeridos (Mantis *et al.*, 2011; Corthésy, 2013; Salerno-Goncalves *et al.*, 2016). Além disso, a redução nos níveis de sIgA pode estar relacionada a um mau funcionamento das células do sistema imunológico nas placas de Peyer em algumas doenças, tais como a doença celíaca e T1D (Mulder & Mulder-Bos, 2006). No modelo induzido por STZ, notou-se uma diminuição da produção de sIgA em todos os camundongos C57BL/6 que receberam linhagens bacterianas (grupos F-STZ, F-IL-4-STZ, F-IL-10-STZ e F-IL-4/IL-10-STZ) em comparação com os grupos controle negativo e controle do veículo, que receberam apenas solução salina pela via intragástrica (Figura 4.6-A). Esses resultados estão em concordância com os dados de alta incidência de animais diabéticos nos grupos que receberam as linhagens bacterianas geneticamente modificadas. Ademais, o modelo experimental induzido por STZ caracteriza-se pela rápida indução e severo desenvolvimento da T1D, o que pode também ter se somado à administração das bactérias e contribuído para a redução observada dos níveis de sIgA.

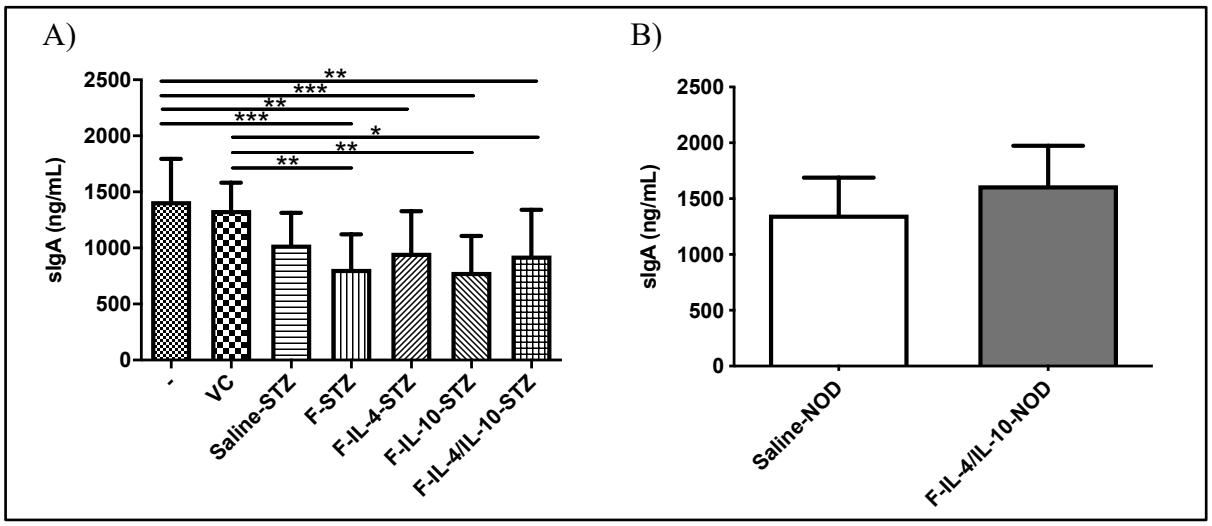


Figura 4.6: Níveis de sIgA no lavado intestinal de camundongos C57BL/6 induzidos por STZ e de camundongos NOD, ambos submetidos à administração intragástrica de linhagens geneticamente modificadas de *L. lactis*. Após o período observacional de cada cronograma experimental, os níveis de IgA secretória foram mensurados nos camundongos C57BL/6 induzidos por STZ (A; n=18) e nos camundongos NOD (B; n = 12). -: controle negativo; VC: controle do veículo; *Saline:* animais que receberam salina 0,9% i.g.; F: animais que receberam *L. lactis* MG1363 FnBPA$^+$ i.g.; F-IL-4: animais que receberam *L. lactis* MG1363 FnBPA$^+$ (pValac::*dts::IL-4*) i.g.; F-IL-10: animais que receberam *L. lactis* MG1363 FnBPA$^+$ (pValac::*IL-10*) i.g.; F-IL-4/IL-10: animais que receberam *L. lactis* MG1363 FnBPA$^+$ (pValac::*dts::IL-4*) e *L. lactis* MG1363 FnBPA$^+$ (pValac::*IL-10*) i.g.; STZ: camundongos que receberam injeções intraperitoneais de estreptozotocina por cinco dias consecutivos; NOD: camundongos diabéticos não-obesos. Os dados são mostrados como média ± SD de três experimentos independentes. *p value*: *p ≤ 0,05; **p ≤ 0,01; ***p ≤ 0,001.

Já no modelo espontâneo da doença foi observado que os camundongos NOD que receberam as bactérias contendo plasmídeos codificadores de IL-4 e IL-10 pela via oral durante 15 semanas consecutivas apresentaram níveis de sIgA semelhantes aos dos camundongos que receberam apenas solução salina. Acredita-se que a ingestão prolongada das bactérias no modelo espontâneo da doença pode ter levado à indução da tolerância oral nos animais, como relatado anteriormente por outros estudos que também administraram linhagens recombinantes de *L. lactis* pela via intragástrica (Robert & Steidler, 2014). Recentemente, nota-se que a utilização da bactéria *L. lactis* modificada como meio de entrega de antígenos de interesse tem sido cada vez mais explorada. Ademais, a manutenção e/ou restabelecimento da tolerância tem se destacado como característica relevante dessa estratégia promissora em modelos experimentais não apenas de T1D, mas até de doenças infecciosas, como a leishmaniose (Cook *et al.*, 2020; Guerra *et al.*, 2021).

4.5 Avaliação da translocação das linhagens *L. lactis* MG1363 FnBPA$^+$ (pValac::*dts::IL-4*) e *L. lactis* MG1363 FnBPA$^+$ (pValac::*IL-10*) administradas pela via intragástrica

Uma vez que as linhagens bacterianas foram administradas oralmente e passaram por todo o trato gastrointestinal dos animais, destaca-se a importância de avaliar se, durante essa passagem, as bactérias se translocaram para outros órgãos. Foi observado que, até 24 h após a gavagem, nenhuma translocação das linhagens *L. lactis* MG1363 FnBPA$^+$ (pValac::*dts::IL*-4) e *L. lactis* MG1363 FnBPA$^+$ (pValac::*IL-10*) foi detectada no baço dos animais (dados não mostrados). Entretanto, notou-se uma pequena quantidade de bactérias translocadas para o fígado 2 h e 4 h após a administração intragástrica das linhagens bacterianas (Figura 4.7-A). Além disso, também verificamos a presença de *L. lactis* MG1363 FnBPA$^+$ (pValac::*dts::IL-4*) e *L. lactis* MG1363 FnBPA$^+$ (pValac::*IL-10*) nos MLNs, principalmente 6 h após a gavagem (Figura 4.7-B). O monitoramento também mostrou que 12 e 24 h após a administração bacteriana pela via oral, não havia mais nenhuma das linhagens geneticamente modificadas de *L. lactis* no fígado e nos MLNs. Em termos de biossegurança, a verificação de que as bactérias se translocam para órgãos extra-gastrointestinais em pequenas quantidades e apenas de forma transitória se apresenta como uma vantagem desta estratégia de intervenção em modelos experimentais de T1D.

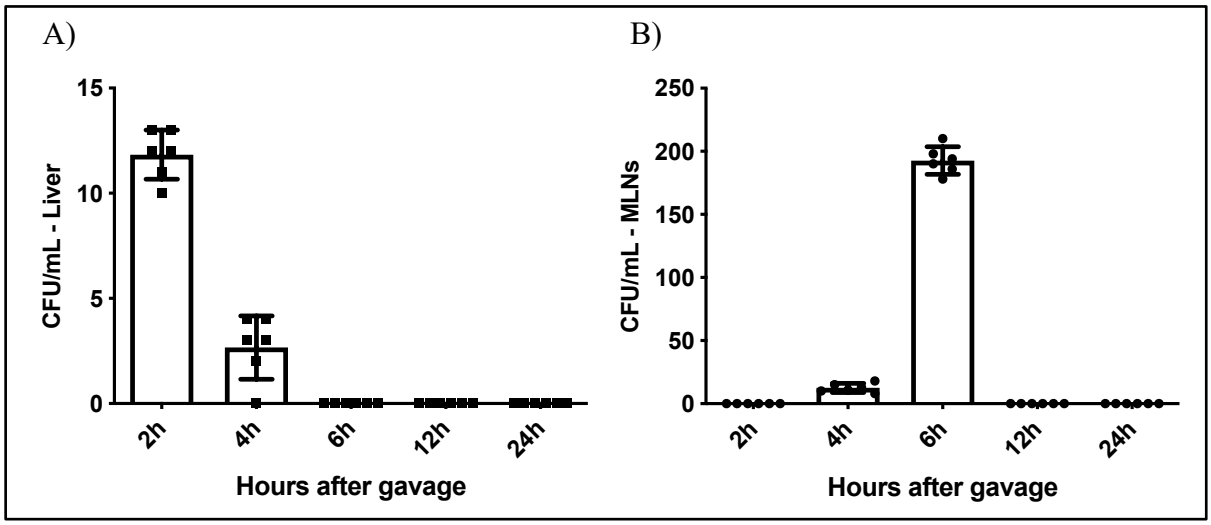


Figura 4.7: Translocação transiente de *L. lactis* MG1363 FnBPA$^+$ (pValac::*dts::IL-4*) e *L. lactis* MG1363 FnBPA$^+$ (pValac::*IL-10*) foi detectada no fígado (*liver,* em inglês) e nos linfonodos mesentéricos (MLNs). Os órgãos foram coletados 2 h, 4 h, 6 h, 12 h e 24 h após a administração das bactérias. Logo, esses foram macerados em solução salina 0,9% e

plaqueados em meio seletivo. Após 24 h de incubação a 30ºC, foi feita a contagem das colônias bacterianas que cresceram do homogenato de fígado (A) e dos linfonodos mesentéricos (B). Os resultados estão apresentados como unidades formadoras de colônia (CFU – do inglês, *Colony Forming Units*) por mL. Os dados são mostrados como média de três experimentos independentes (n = 6).

4.6 Avaliação histológica do pâncreas de camundongos que receberam as linhagens *L. lactis* MG1363 FnBPA$^+$ portadoras dos vetores pValac::*dts::IL-4* ou pValac::*IL-10* nos dois modelos experimentais de T1D

Para investigar mais profundamente os efeitos imunomodulatórios da intervenção experimental planejada para os dois modelos murinos de T1D, foi feita a análise da presença e grau de insulite nas ilhotas pancreáticas dos animais. A análise histológica mostrou que a administração intragástrica de ambas as linhagens bacterianas [*L. lactis* MG1363 FnBPA$^+$ (pValac::*dts::IL-4*) e *L. lactis* MG1363 FnBPA$^+$ (pValac::*IL-10*) (grupo F-IL-4/IL-10-STZ)] nos camundongos induzidos por STZ preservou mais ilhotas pancreáticas sem sinais visíveis de inflamação do que quando essas linhagens foram administradas individualmente (grupos F-IL-4-STZ e F-IL-10-STZ) (Figura 4.8-A). Tal observação se mostra em concordância com a análise prévia dos camundongos diabéticos e não-diabéticos nos grupos experimentais que receberam a indução química por STZ: os animais que receberam a administração combinada das linhagens bacterianas codificadoras de IL-4 e IL-10 (grupo F-IL-4/IL-10-STZ) mostraram melhores resultados do que a administração individual das mesmas linhagens bacterianas (vide seção 4.1).

A avaliação da insulite também revelou que a administração das linhagens *L. lactis* MG1363 FnBPA$^+$ (pValac::*dts::IL-4*) e *L. lactis* MG1363 FnBPA$^+$ (pValac::*IL-10*) aos camundongos NOD foi capaz de impedir a progressão da inflamação na maior parte das ilhotas pancreáticas analisadas durante o período observacional. Os camundongos do grupo F-IL-4/IL-10-NOD apresentaram 60% das ilhotas sem sinais de insulite, enquanto no grupo controle (grupo *Saline*-NOD) apenas 5% das ilhotas pancreáticas não apresentaram sinais de infiltrado inflamatório (Figura 4.8-A). Esses resultados também corroboram a análise de incidência da T1D experimental, em que os camundongos NOD que receberam as bactérias pela via oral se mostraram protegidos da doença em questão (vide seção 4.1). A Figura 4.8-B

mostra fotos representativas do grupo *Saline*-NOD (i-ii) e do grupo F-IL-4/IL-10-NOD (iii-iv), capturadas durante a análise histológica das ilhotas pancreáticas.

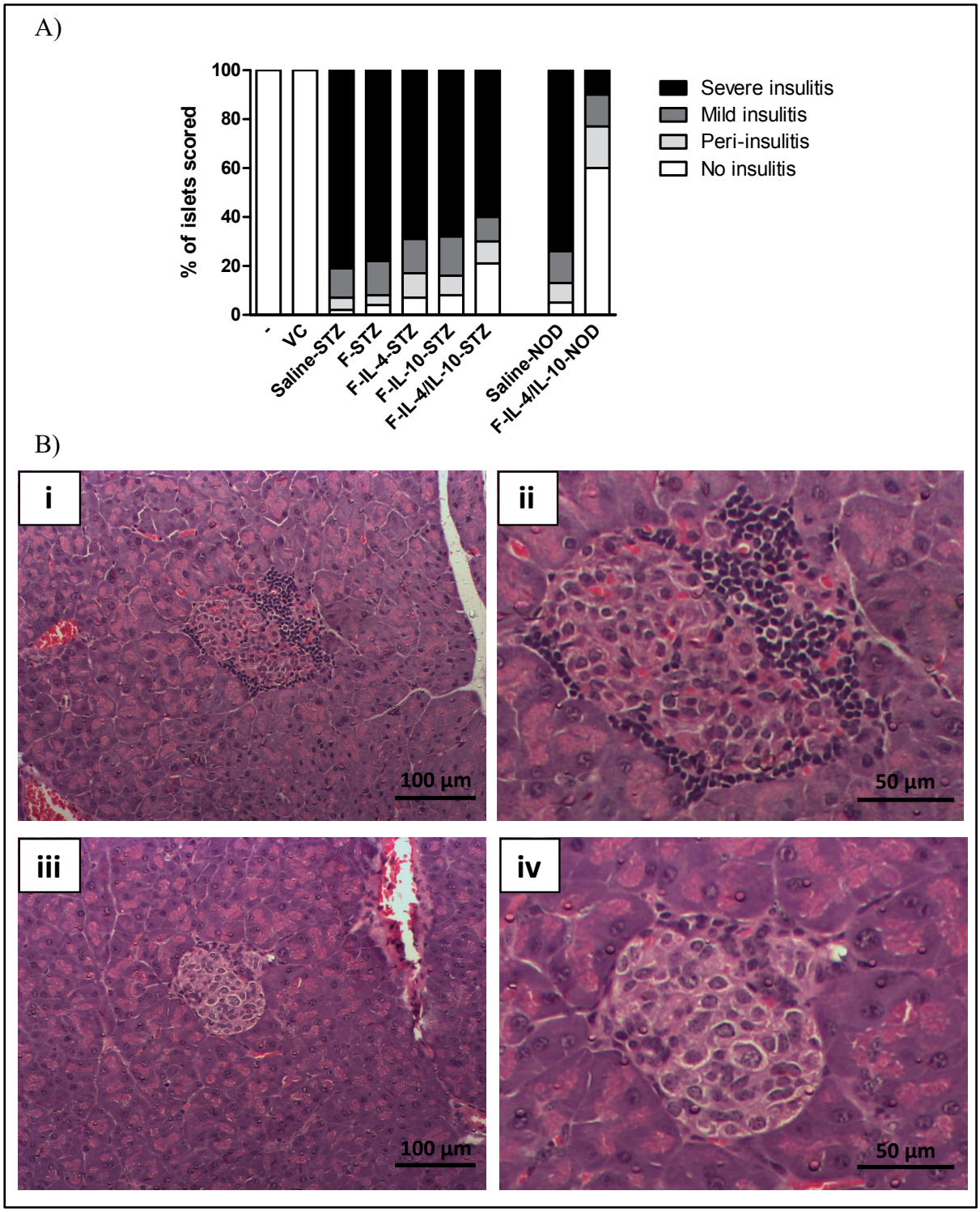


Figura 4.8: Administração combinada de *L. lactis* MG1363 FnBPA$^+$ (pValac::*dts::IL-4*) e *L. lactis* MG1363 FnBPA$^+$ (pValac::*IL-10*) exerce efeitos protetivos nas ilhotas pancreáticas. A) Amostras de pâncreas de camundongos induzidos por STZ e camundongos NOD foram coletadas ao final do período observacional, processadas e ranqueadas às cegas quanto ao grau de infiltrado inflamatório nas ilhotas pancreáticas como: insulite severa,

moderada, peri-insulite ou sem insulite. Pelo menos 15 ilhotas foram avaliadas em cada amostra de pâncreas. Os dados são mostrados como média de três experimentos independentes (n = 6). B) Ilhota pancreática representativa do grupo *Saline*-NOD (i-ii) e do grupo F-IL-4/IL-10-NOD (iii-iv). -: controle negativo; VC: controle do veículo; *Saline:* animais que receberam salina 0,9% i.g.; F: animais que receberam *L. lactis* MG1363 FnBPA$^+$ i.g.; F-IL-4: animais que receberam *L. lactis* MG1363 FnBPA$^+$ (pValac::*dts::IL-4*) i.g.; F-IL-10: animais que receberam *L. lactis* MG1363 FnBPA$^+$ (pValac::*IL-10*) i.g.; F-IL-4/IL-10: animais que receberam *L. lactis* MG1363 FnBPA$^+$ (pValac::*dts::IL-4*) e *L. lactis* MG1363 FnBPA$^+$ (pValac::*IL-10*) i.g.; STZ: camundongos que receberam injeções intraperitoneais de estreptozotocina por cinco dias consecutivos; NOD: camundongos diabéticos não-obesos.

4.7 Discussão geral

A diabetes tipo 1 é uma condição crônica na qual as células pancreáticas são destruídas por células T autorreativas. Apesar da etiologia da doença não ser completamente compreendida, sabe-se que a ausência do fino equilíbrio entre regulação e imunidade é um ponto-chave da T1D (Figura 4.9). A influência genética e de fatores ambientais desencadeiam o predomínio de células T CD4 e CD8 patogênicas, células B patogênicas e células dendríticas ativadas (Bluestone *et al.*, 2010). Portanto, mais estudos que almejam esclarecer a etiologia, como a T1D progride e melhores formas de tratamento são extremamente importantes e necessários.

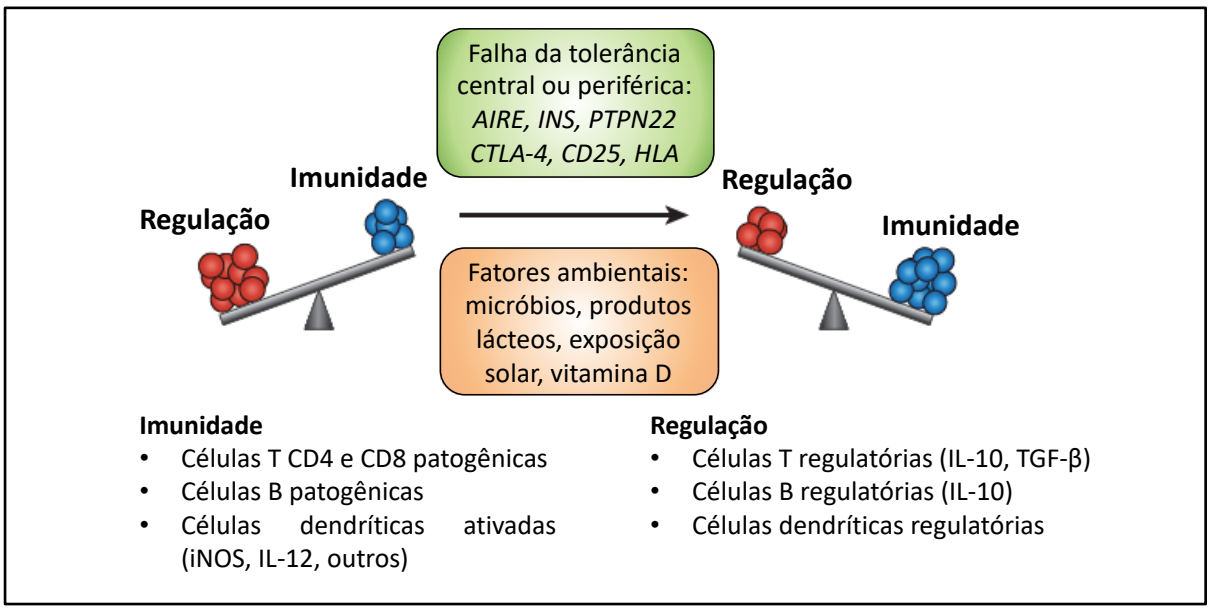


Figura 4.9: Equilíbrio do sistema imunológico é a chave para a compreensão do desenvolvimento da T1D. Essa representação esquemática mostra o delicado equilíbrio entre

regulação e imunidade que podem desencadear a progressão da T1D. Destaca-se também a influência de vários genes que culminam na falha da tolerância central ou periférica (quadro verde) e também os fatores ambientais que alguns estudos sugerem estar relacionados à etiologia da doença (quadro laranja). Os principais tipos de células que afetam o equilíbrio imune localmente são listados juntamente com as citocinas e proteínas reguladoras. iNOS: óxido nítrico sintase induzível. **Adaptado de:** Bluestone *et al.*, 2010.

Estudos iniciais usando múltiplas doses de STZ e camundongos NOD mostraram que as citocinas IL-4 e a IL-10 têm o potencial de mudar o curso natural da T1D, evitando a progressão da insulite e mantendo níveis normais de glicemia (Wood *et al.*, 1999; Cameron *et al.*, 2000; Mandke & Singh, 2012). Entretanto, a rota de administração e a estratégia para entrega dessas citocinas em modelos animais de T1D é um desafio recorrente. Assim, neste trabalho, decidimos utilizar a bactéria láctica *Lactococcus lactis* para entrega de plasmídeos codificadores de citocinas, uma vez que essa é considerada uma ferramenta eficiente para administração via mucosas, entrega de agentes imunoestimulatórios e indução de tolerância oral (Robert & Steidler, 2014). Dessa forma, a administração oral de *L. lactis* MG1363 FnBPA^{+} carreando vetores de expressão eucariótica codificadores de IL-4 e IL-10 (pValac::*dts::IL-4* e pValac::*IL-10*, respectivamente) nos dois modelos murinos mais amplamente utilizados para mimetizar experimentalmente a T1D é relatada neste trabalho pela primeira vez.

No modelo de T1D induzido quimicamente por STZ, decidimos verificar os efeitos das linhagens bacterianas portadoras dos plasmídeos que codificam IL-4 e IL-10 de forma individual e combinada. Observamos que em todos os grupos experimentais que os animais receberam injeções de STZ, esses se tornaram diabéticos. Entretanto, verificou-se que o grupo que recebeu as linhagens bacterianas *L. lactis* MG1363 FnBPA^{+} (pValac::*dts::IL-4*) e *L. lactis* MG1363 FnBPA^{+} (pValac::*IL-10*) conjuntamente (grupo F-IL-4/IL-10-STZ) apresentou o maior número de camundongos protegidos contra a incidência da diabetes experimental.

Ademais, os animais que receberam STZ e as linhagens bacterianas codificadoras de IL-4 e IL-10 pela via oral apresentaram níveis de IL-2 pancreáticos similares aos dos grupos negativo e de controle do veículo e significativamente mais altos que os do grupo *Saline*-STZ. As principais células produtoras de IL-2 são os linfócitos T CD4+ e CD8+ ativados convencionalmente, mas linfócitos T autorreativos também são uma fonte possível de IL-2 (Hartemann & Bourron, 2012). É sugerido que os linfócitos T efetores autorreativos

reconheçam autoantígenos apresentados pelas células dendríticas, produzam IL-2 quando ativados e, então, ativam Tregs específicas. Por fim, essas células Tregs poderiam suprimir os linfócitos T autorreativos, mantendo, assim, a tolerância imune (Malek & Castro, 2010). Além disso, sabemos que células Tregs ativadas pela citocina IL-2 podem suprimir ativamente a destruição das células β no pâncreas, impedindo o desenvolvimento da doença. Dessa forma, avaliando os resultados obtidos nos experimentos com STZ, sugere-se que a administração oral das linhagens *L. lactis* MG1363 FnBPA^{+} (pValac::*dts::IL-4*) e *L. lactis* MG1363 FnBPA^{+} (pValac::*IL-10*) de forma combinada tenha favorecido a expansão e ativação de células Treg e, assim, atenuado a insulite no pâncreas dos animais.

A avaliação dos níveis de sIgA se faz necessária devido às bactérias geneticamente modificadas terem sido administradas pela via oral e também por esse anticorpo estar envolvido na imunomodulação e homeostase do TGI. Ademais, já foi demonstrado que, em doenças imunes como a T1D, a redução dos níveis de sIgA pode ser um indicativo importante de falha dos mecanismos imunológicos considerados "normais" no nosso organismo (Mulder & Mulder-Bos, 2006). Observamos que, no modelo induzido por STZ, grupos que receberam as bactérias durante poucos dias tinham reduzido significativamente os níveis intestinais de sIgA. Entretanto, os camundongos NOD que receberam bactérias por via oral durante 15 semanas apresentaram níveis de sIgA semelhantes aos dos camundongos que não receberam bactérias. Sugere-se que essa diferença nos níveis de sIgA entre os modelos de T1D seja explicada pela ingestão prolongada de bactérias no modelo espontâneo da doença, que pode ter estimulado o desenvolvimento da tolerância oral nos camundongos. Tal situação já foi demonstrada por outros estudos que administraram, pela via oral, linhagens geneticamente modificadas de *L. lactis,* o que reafirma o potencial imunomodulatório dessa estratégia (Robert & Steidler, 2014).

Corroborando as outras análises, os resultados das análises histológicas confirmaram que a administração combinada das linhagens bacterianas codificadoras de IL-4 e IL-10 no modelo de STZ foi mais bem sucedida em atenuar a progressão da insulite do que a administração individual dessas mesmas linhagens. Alguns estudos mostraram que as citocinas IL-4 e IL-10, quando combinadas, induzem um efeito sinérgico capaz de suprimir a imunidade celular (Powrie *et al.*, 1993; Faust *et al.*, 1996). Embora o mecanismo subjacente não seja bem e nem claramente conhecido, sugere-se que as ações conjuntas e distintas dessas citocinas poderiam contribuir para um resultado mais robusto ao final (Ko *et al.*, 2001). Como

esse é o primeiro estudo utilizando a bactéria *L. lactis* geneticamente modificada no modelo de T1D induzido quimicamente por STZ, acredita-se que os resultados até aqui obtidos são importantes de serem discutidos. Além disso, esses resultados nos levaram à investigação dos efeitos da administração combinada das linhagens de *L. lactis* MG1363 FnBPA$^+$ portadoras dos plasmídeos de expressão eucariótica codificadores de IL-4 e IL-10 no modelo espontâneo de T1D, utilizando camundongos NOD.

O modelo de camundongos NOD apresenta semelhanças importantes com a T1D em humanos, o que o torna um modelo valioso para investigações acerca dessa doença. Sabe-se que altos e descontrolados níveis de glicemia estão associados a uma rápida progressão da doença e complicações graves em pacientes com T1D. Assim, o controle da glicemia é um fator importante no contexto da doença. No atual trabalho foi demonstrado que, em camundongos NOD, a administração de *L. lactis* MG1363 FnBPA$^+$ (pValac::*dts::IL-4*) e *L. lactis* MG1363 FnBPA$^+$ (pValac::*IL-10*) pela via oral foi capaz de regular de forma eficaz os níveis de glicemia durante todo o período de observação. Portanto, a estratégia escolhida de utilizar conjuntamente essas bactérias geneticamente modificadas durante 15 semanas resultou na ausência de incidência de T1D experimental em camundongos NOD.

No presente estudo, foi demonstrado também que a administração oral das linhagens bacterianas levou a efeitos sistêmicos. Camundongos NOD que receberam *L. lactis* MG1363 FnBPA$^+$ carreando plasmídeos codificadores de IL-4 e IL-10 apresentaram níveis significativamente aumentados de IL-10 no soro e tecido pancreático. A IL-10 é uma importante citocina anti-inflamatória que pode modular os processos imunes, inibindo a síntese de citocinas pró-inflamatórias e favorecendo um *status* de tolerância (Moore *et al.*, 2001). A IL-10 demonstrou ser ativa em superfícies mucosas, agindo sinergicamente e aumentando a tolerância oral no modelo de camundongos NOD (Slavin *et al.*, 2001). Essa citocina também pode estabilizar a função de células Tregs através da manutenção da expressão de Foxp3 (Murai *et al.*, 2009). Além disso, Takiishi e colaboradores sugerem que a produção de IL-10 pelas células Tregs é o principal mecanismo responsável pela regulação da inflamação alcançada pela terapia baseada em *L. lactis* no modelo de camundongos NOD (Takiishi *et al.*, 2017). Como a T1D está intimamente relacionada à inflamação, portanto, é razoável especular que o aumento dos níveis de IL-10 obtidos no soro e no pâncreas de camundongos NOD que receberam as linhagens de *L. lactis* portadoras dos plasmídeos de expressão eucariótica de IL-4 e IL-10 foi, pelo menos inicialmente, devido à administração

das bactérias e subsequente expressão dessas citocinas. Acredita-se que esse resultado foi crucial para a regulação da inflamação observada, consequente controle da glicemia e ausência de incidência de T1D experimental.

A IL-4 é uma citocina imunomoduladora pleiotrópica produzida e secretada principalmente por células Th2. As intervenções que favorecem o perfil Th2 e a administração de citocinas que contrariam o perfil Th1, como a IL-4 e a IL-10, têm mostrado sucesso na supressão da insulite e na prevenção da T1D (Rapoport *et al.*, 1993; Pennline *et al.*, 1994; Ko *et al.*, 2001). Estudos anteriores mostraram que tanto a administração de IL-4 recombinante, quanto a expressão pancreática de IL-4 foram capazes de proteger os camundongos NOD da diabetes experimental (Rapoport *et al.*, 1993; Mueller *et al.*, 1996). Além disso, Li e colaboradores também revelaram que a alteração na síntese de citocinas nas células T, tendenciando para a produção de IL-4, pode contribuir para a proteção da doença em camundongos NOD (Li *et al.*, 2019). De acordo com os resultados relatados no atual trabalho, a administração oral de *L. lactis* MG1363 FnBPA^{+} (pValac::*dts::IL-4*) e *L. lactis* MG1363 FnBPA^{+} (pValac::*IL-10*) mostrou resultados promissores, revelando um aumento dos níveis de IL-4 no soro e no pâncreas de camundongos NOD, apontando para o perfil Th2 e, assim, protetivo, no contexto da T1D.

Além disso, tanto a IL-4 quanto a IL-10 parecem suprimir a produção da citocina pró-inflamatória IL-12 por células apresentadoras de antígenos, diminuindo, assim, a diferenciação das células Th1 (Levings & Schrader, 1999; Maynard & Weaver, 2008). As evidências acumuladas indicam que a IL-12 é uma citocina-chave que induz diferenciação de células Th1, levando à aceleração da diabetes experimental em camundongos NOD (Trembleau *et al.*, 1995, 2003; Adorini, 2001). Além disso, Nitta e colaboradores relataram que a IL-12 produzida localmente por células infiltrantes no pâncreas desempenha um papel patológico no desenvolvimento da T1D (Nitta *et al.*, 2001). Portanto, sugerimos que a redução significativa na concentração de IL-12 pancreática observada em camundongos NOD que receberam ambas as linhagens bacterianas geneticamente modificadas contribuiu para a regulação da inflamação, não favorecendo a diferenciação de células Th1 e nem a produção de citocinas pró-inflamatórias.

Evidências mais claras do efeito protetor da administração combinada de *L. lactis* MG1363 FnBPA^{+} (pValac::*dts::IL-4*) e *L. lactis* MG1363 FnBPA^{+} (pValac::*IL-10*) foram obtidas através da análise histológica. Corroborando a normoglicemia apresentada pelos

camundongos NOD que receberam administração conjunta das linhagens bacterianas, a maioria das ilhotas pancreáticas foi preservada, sem sinais de insulite. Coletivamente, nossos dados indicam que a preservação das ilhotas pancreáticas, que é resultante da redução da destruição autoimune, foi desencadeada pelo aumento dos níveis de IL-4 e IL-10 e pela diminuição dos níveis de IL-12. Assim sendo, essa alteração no perfil de citocinas no ambiente pancreático possivelmente alterou os subtipos de células T que ali predominavam, favorecendo a proliferação de células Treg e contribuindo para a proteção da doença.

Linhagens de *L. lactis* geneticamente modificadas têm sido utilizadas como uma ferramenta versátil para imunoterapia em várias condições inflamatórias, como a T1D (Cook *et al.*, 2018). Uma vez que essas bactérias podem sobreviver ao trato gastrointestinal e são utilizadas para a entrega de plasmídeos e proteínas às células presentes na mucosa, avaliamos a translocação bacteriana para alguns órgãos extra-gastrointestinais. Estudos anteriores mostraram que a translocação bacteriana pode ocorrer com frequência, mesmo em condições saudáveis (Berg, 1999; Vaishnavi, 2013; Nagpal & Yadav, 2017). *L. lactis* geneticamente modificada carreando plasmídeos codificadores de IL-4 e IL-10 foram detectados principalmente nos MLNs, que são conhecidos por serem o local-chave para a tolerância oral. Sabe-se que as células Tregs induzidas proliferam nos MLNs e são posteriormente liberadas na circulação para modular a destruição autoimune de células β pancreáticas de forma dependente de IL-10 (Macpherson & Smith, 2006; Worbs *et al.*, 2006; Cook *et al.*, 2018). Bresson e colaboradores demonstraram que a expansão das células Tregs que produzem IL-4 e IL-10 foi determinante para suprimir a resposta autorreativa em modelos animais de T1D (Bresson, 2006). Estudos promissores usando *L. lactis* geneticamente modificado como ferramenta de entrega de antígenos e compostos bioativos revelaram que a indução de tolerância oral e proliferação de células Tregs foram capazes de reverter a diabetes de início recente em camundongos NOD (Takiishi *et al.*, 2012, 2017). Portanto, nós hipotetizamos que a administração de *L. lactis* MG1363 FnBPA^{+} (pValac::*dts::IL-4*) e *L. lactis* MG1363 FnBPA^{+} (pValac::*IL-10*) foi capaz de proteger contra a destruição das células β devido à expansão das células Tregs produtoras de IL-4 e IL-10 no pâncreas. Além disso, nenhuma das linhagens de bactérias geneticamente modificadas foi detectada nos MLNs, baço e fígado após 12 h da administração oral, indicando que essas linhagenss são de fato transitórias, o que consiste em um atributo relevante no contexto das práticas de biossegurança.

Em suma, o efeito protetor contra a T1D experimental desencadeado pela administração oral das linhagens *L. lactis* MG1363 FnBPA$^+$ portadoras dos plasmídeos pValac::*dts::IL-4* e pValac::*IL-10* verificado nos camundongos NOD não foi observado no modelo induzido por STZ. Essa diferença observada nos resultados quando utiliza-se os dois principais modelos experimentais de T1D já foi relatada em outros estudos (Yasuda *et al.*, 2009; da Rosa *et al.*, 2013; Pavlovic *et al.*, 2018), e pode estar associada com as características distintas desses modelos murinos. O modelo de indução por STZ é caracterizado pela rápida destruição das células produtoras de insulina no pâncreas, por meio de um processo que envolve a variação dinâmica das células imunes (Lukic *et al.*, 1998; Yaochite *et al.*, 2013; Goyal *et al.*, 2016). Como o STZ é capaz de induzir a diabetes mesmo na ausência de células B e T, a interpretação dos resultados requer prudência quando as análises correlacionam diretamente com o desenvolvimento da T1D em humanos (Reddy *et al.*, 1995; King, 2012). Nesse contexto, o modelo experimental espontâneo, utilizando os camundongos NOD, integra o componente genético e também as implicações do processo imunopatológico ao longo da progressão da doença, o que o torna um modelo mais adequado para testar terapias que visam modular a resposta autoimune (King, 2012; Brito-Casillas *et al.*, 2016).

Vale pontuar também as limitações do nosso estudo, uma vez que os experimentos científicos são feitos de forma controlada e limitada com relação a uma série de variáveis. Não conseguimos utilizar todos os grupos experimentais controle que seriam desejáveis nos experimentos envolvendo camundongos NOD, dado que a aquisição e manutenção desses animais são extremamente onerosas. Entretanto, as análises e discussões realizadas com todos os grupos experimentais testados no modelo induzido quimicamente por STZ nos mostraram que deveríamos concentrar esforços na administração combinada das linhagens bacterianas portadoras dos plasmídeos codificadores de citocinas. Além disso, essas limitações não invalidam as nossas descobertas e discussões geradas sobre a administração combinada de *L. lactis* MG1363 FnBPA$^+$ carreando os plasmídeos de expressão eucariótica codificadores de IL-4 e IL-10 em camundongos NOD.

5. CONCLUSÃO

Este é o primeiro trabalho a administrar oralmente linhagens geneticamente modificadas de *L. lactis* portadoras de plasmídeos de expressão eucariótica que codificam as citocinas IL-4 e IL-10 nos dois principais modelos experimentais de T1D: induzido por STZ e em camundongos NOD. Dessa forma, este se apresenta como um estudo inicial, que fornece novas e relevantes observações sobre a imunomodulação nos modelos murinos de T1D. Verificou-se que a administração combinada de linhagens de *L. lactis* MG1363 FnBPA$^+$ carreando os plasmídeos de expressão eucariótica pValac::*dts::IL-4* e pValac::*IL-10* é capaz de amenizar a progressão inicial da insulite no modelo de T1D induzido por STZ, através do aumento dos níveis de IL-2 no tecido pancreático. Também foi demonstrado que, em camundongos NOD, as linhagens de *L. lactis* MG1363 FnBPA$^+$ portadora dos vetores vacinais codificadores de IL-4 e IL-10, quando administradas oralmente, podem reduzir de forma efetiva a incidência da T1D e controlar os níveis glicêmicos. Além disso, essa intervenção combinada foi capaz de prevenir a progressão da insulite, devido ao aumento dos níveis de IL-4 e IL-10 – conhecidas pela supressão do *milieu* pró-inflamatório – no pâncreas e soro e redução dos níveis da citocina pró-inflamatória IL-12 no tecido pancreático. Por fim, a sinalização dinâmica das citocinas desencadeada pelas linhagens *L. lactis* MG1363 FnBPA$^+$ (pValac::*dts::IL-4*) e *L. lactis* MG1363 FnBPA$^+$ (pValac::*IL-10*) exerceu um papel crucial na imunomodulação da doença. Logo, novas investigações envolvendo moléculas imunoestimulatórias devem ser incentivadas para compreendermos essa doença em sua totalidade e, assim, alcançarmos terapias mais eficazes e até, possivelmente, a cura e a prevenção da T1D.

6. PERSPECTIVAS

A intervenção realizada no atual trabalho tem como intuito principal a análise dos efeitos decorrentes de linhagens de *L. lactis* portadoras de plasmídeos vacinais codificadores de IL-4 e IL-10 administradas previamente à manifestação clínica da T1D. Dessa forma, a avaliação da imunomodulação da doença foi priorizada para auxiliar no entendimento da dinâmica dos componentes-chave do sistema imunológico ao longo do desenvolvimento da doença – mimetizada pelos modelos animais.

Como perspectiva deste trabalho, visamos agora nos aproximar da pesquisa translacional. Estudos prévios mostraram que indivíduos que possuem parentes de primeiro e segundo grau afetados pela T1D parecem se atentar mais aos primeiros sintomas da doença, porém, nesses casos, a doença se manifesta mais cedo e de forma mais agressiva (Bizzarri *et al.*, 2015). Entretanto, a maior parte dos casos de T1D ocorre em indivíduos que não possuem histórico familiar da doença, o que dificulta ainda mais o diagnóstico precoce (Maahs *et al.*, 2010). Sendo assim, a maioria das pessoas afetadas pela doença geralmente só iniciam a busca por um diagnóstico quando notam algum sinal clínico – como excesso de urina e sede e também níveis glicêmicos alterados. Logo, esse momento pode ser visto como um ponto crucial para o início de uma intervenção terapêutica visando impedir o desenvolvimento e/ou estabelecimento da doença.

Ao observar as imunoterapias para a T1D que passaram por testes clínicos, o uso de anticorpos monoclonais (mAbs – do inglês, *monoclonal Antibodies*) anti-CD3 se destaca não apenas por ser uma das abordagens imunológicas mais bem estudadas para a doença em questão, mas também por conseguir alterar o curso da T1D por muitos anos, dependendo da dose e momento de administração (Skyler, 2013). O CD3 consiste em um complexo proteico encontrado na superfície celular de linfócitos T que age na transdução de sinais vindos do receptor de antígenos e, assim, inicia-se uma cascata de eventos que culminam na ativação desses linfócitos. Dessa forma, os mAbs anti-CD3 são capazes de impedir a lise dos alvos pelos linfócitos T (Kaufman & Herold, 2009). Após a análise dos resultados da utilização de mAbs anti-CD3 em modelos pré-clínicos de T1D, foi sugerido que essa imunoterapia induz imunotolerância, possivelmente mediada por células Tregs, ao invés de supressão imune crônica (Chatenoud *et al.*, 1994; Herold & Taylor, 2003; Kaufman & Herold, 2009).

Nos testes clínicos envolvendo mAbs anti-CD3 em pacientes com T1D de início recente, os pesquisadores obtiveram um certo grau de eficácia (como menor necessidade de insulina e melhor controle da glicemia). Entretanto, esses mAbs não conseguiram atingir os

endpoints primários dos testes, como uma mudança significativa no quadro clínico (Keymeulen *et al.*, 2005, 2010) . Logo, a conclusão obtida foi que os mAbs anti-CD3 podem ser uma intervenção importante para o alcance da imunorregulação da T1D, entretanto, como uma intervenção única, esses não conseguem alcançar os efeitos esperados. Devido à complexidade imunológica da doença, a comunidade científica propõe a utilização combinada de agentes que sejam capazes de modular o sistema imune dentro do cenário da T1D (Cook *et al.*, 2018).

Para propor uma nova intervenção, é extremamente importante o estudo detalhado e cuidadoso de citocinas e antígenos envolvidos na destruição das células β por linfócitos T autorreativos, além da escolha dos agentes moduladores, rota de administração e momento que esses serão realizados. Nesse contexto, em 1990, Elias e colaboradores descobriram que, em camundongos NOD, um dos antígenos que marca as células β reage de maneira cruzada com a proteína de choque térmico de 65-kDa (Hsp-65 – do inglês, *Heat shock protein*) de *Mycobacterium tuberculosis*. Assim, a destruição das células β – característica da T1D – foi ligada ao aumento espontâneo de linfócitos T anti-Hsp65. Esses linfócitos específicos também mostraram habilidade para causar insulite e hiperglicemia em camundongos NOD jovens (Elias *et al.*, 1990). Adicionalmente, no modelo de diabetes induzido por STZ, uma vacina de DNA contendo o gene Hsp65 de *Mycobacterium* injetada intramuscularmente mostrou ser capaz de proteger os animais contra a insulite (Santos *et al.*, 2009). Já a administração oral de uma linhagem recombinante de *L. lactis* NZ9000 expressando Hsp65 e o peptídeo P277 derivado de Hsp60 humana repetido em tandem também mostrou reduzir a incidência da T1D no modelo de camundongos NOD (Ma *et al.*, 2014). Ma e colaboradores acreditam que parte da prevenção obtida nesse estudo de 2014 seja devida à redução dos níveis de IFN-γ e aumento dos níveis da citocina anti-inflamatória IL-10 em esplenócitos.

Como mostrado nos resultados do atual trabalho, a IL-10 é uma citocina relevante no panorama da T1D, a qual é apta a modular os processos imunológicos e que apresenta propriedades anti-inflamatórias que podem ser utilizadas na tentativa de prevenir a progressão da doença. Em 2014, Robert e colaboradores utilizaram linhagens de *L. lactis* administradas pela via oral para entregar o autoantígeno GAD-65 e IL-10, em combinação com anticorpos anti-CD3, em camundongos NOD com início recente de diabetes. Como resultado, eles observaram a preservação da massa de células β e também a restauração da normoglicemia (Robert *et al.*, 2014).

Diante disso, a proposta de perspectiva deste trabalho é utilizar camundongos NOD com início recente de diabetes, o que será atestado pela medição da glicosúria e da glicemia, realizadas três vezes por semana. Quando o animal apresentar duas medições consecutivas de glicemia que excedam 200 mg/dL, esse será considerado o início recente da doença e assim, iniciaremos as intervenções (esse será considerado o dia 0). A partir do dia 0, os camundongos receberão, pela via intravenosa (i.v.), 2,5µg/dia de mAb anti-CD3 (clone 145–2C11; Bio X Cell, West Lebanon, NH) por cinco dias consecutivos (vide Figura 6.1). Concomitantemente, pela via oral, os animais receberão doses contendo 2 x 10^9 unidades formadoras de colônias (UFCs) de cada uma das linhagens bacterianas: *L. lactis* NCDO2118 FnBPA$^+$ (pXYCYT:*Hsp65*) (linhagem capaz de produzir a proteína Hsp65 na forma citoplasmática) e *L. lactis* MG1363 FnBPA$^+$ (pValac::*IL-10*) (ambas as linhagens construídas pelo nosso grupo de pesquisa), cinco vezes por semana, durante seis semanas consecutivas.

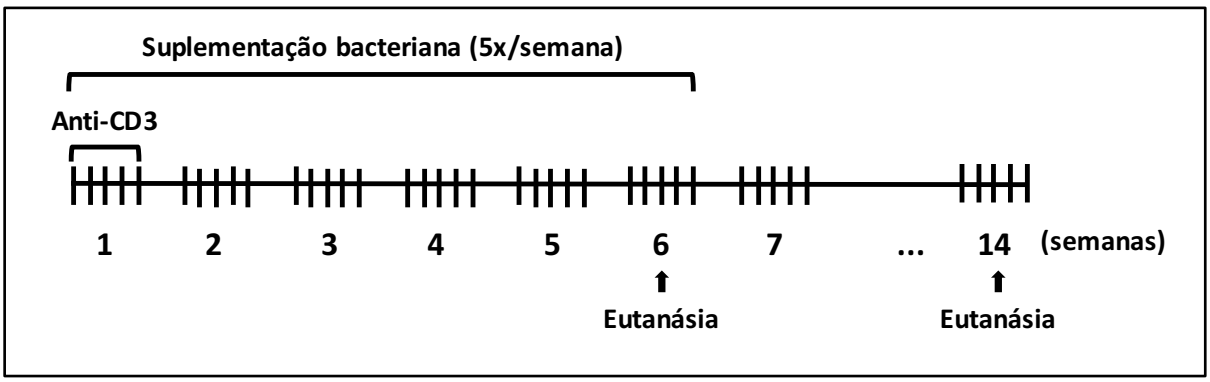


Figura 6.1: Representação esquemática do cronograma experimental em camundongos NOD com início recente de diabetes. Após duas medições consecutivas de glicemia que excedam 200 mg/dL, será iniciada a contagem da primeira semana dos experimentos. Então, os camundongos NOD receberão administração i.v. de 2,5µg/dia de mAb anti-CD3 por cinco dias consecutivos. A administração i.g. das linhagens bacterianas ocorrerá cinco vezes por semana durante seis semanas consecutivas. Uma parcela dos animais será eutanasiada ao final da 6ª semana dos experimentos, enquanto a outra parte será eutanasiada ao final da 14ª semana.

Os grupos experimentais serão os seguintes: animais que receberão apenas salina 0,9% pela via oral (não-tratados); animais que receberão as duas linhagens bacterianas i.g. (LF-Hsp65 + LF-IL-10); animais que receberão apenas os mAbs anti-CD3 i.v. (anti-CD3); animais que receberão mAbs anti-CD3 i.v. e apenas a linhagem bacteriana produtora de Hsp65 i.g. (anti-CD3 + LF-Hsp65); animais que receberão mAbs anti-CD3 i.v. e apenas a linhagem bacteriana com plasmídeo codificador de IL-10 i.g (anti-CD3 + LF-IL-10); e animais que

receberão mAbs anti-CD3 i.v. e as duas linhagens bacterianas i.g (anti-CD3 + LF-Hsp65 + LF-IL-10).

A mensuração da glicemia, da glicosúria e o acompanhamento do peso dos animais serão feitos três vezes por semana. A remissão da T1D será definida como a ausência da glicosúria e o retorno à normoglicemia. Parte dos animais será eutanasiada imediatamente após o término da intervenção (seis semanas após o início do tratamento) e a outra parte dos animais será eutanasiada quatorze semanas após o início do tratamento. O pâncreas, soro sanguíneo, linfonodos, cólon intestinal e baço dos animais serão retirados para análise e delineamento do imunofenótipo de cada grupo experimental.

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8. ANEXOS

ANEXO 1:

"*Lactococcus lactis* as a DNA vaccine delivery system"

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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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INTRODUCTION

Vaccination stimulates the development of immune responses to avoid infection and protect the individual from establishing a disease. Many diseases like tetanus, diphtheria, polio and hepatitis A and B have decreased significantly with the advent of vaccines (Roush et al., 2007). However, emerging infectious diseases have become challenges to be faced by the vaccinology field and new strategies must be developed to produce more efficient vaccines to protect the population worldwide. Within this scenario, DNA vaccines emerge as good alternatives to invest time and efforts, as they may be a way to overcome these new obstacles. DNA vaccines consist of eukaryotic expression plasmids that have the coding sequence of an antigen of interest inserted into their multiple cloning sites. Although different administration routes exist, the goal of these vaccines is that the plasmid DNA reaches the host cell nucleus so that it can be transcribed, translated and expressed by the eukaryotic cell and, in this way, elicit the expected immune responses (Pereira et al., 2014).

The first findings that reported about the potential of vaccines occurred in 1990, when Wolf and colleagues injected a DNA plasmid containing the coding sequence of β-galactosidase and verified the expression of this protein in quadriceps muscle sections of mice (Wolf et al., 1990). In 1992, after injecting a plasmid encoding the human growth hormone (hGH) into mice´s skin, Tang and colleagues observed the production of specific antibodies against the protein, indicating that the injected DNA was expressed and elicited a specific humoral immune response (Tang et al., 1992). Subsequently, in 1993, two independent research groups vaccinated mice with DNA plasmids encoding different viral proteins and showed success when challenging against lethal influenza (Fynan et al., 1993; Ulmer et al., 1993). These studies were fundamental to understand how DNA could be used for vaccination and thus, protect against a pathogen or an antigen. To date, the scientific community has been striving for the use of DNA vaccines to become a reality around the world.

Since the discovery that DNA vaccines could be valuable tools to generate immune responses, four DNA vaccines have been approved for veterinarian use (Kutzler & Weiner, 2008). In 2005, two prophylactic vaccines against infection with the West Nile Virus in horses and the Hematopoietic Necrosis Virus in salmon were licensed in The United States and Canada, respectively. Following, in 2008, a therapeutic vaccine against fetal loss, which expressed the growth hormone releasing hormone (GHRH), was licensed for use in swine in Australia and in 2010 a therapeutic vaccine to treat oral melanoma in dogs was licensed in the United States. The success of these approved DNA vaccines showed perspectives of an innovative platform to prevent and treat emerging diseases.

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STRUCTURE OF DNA VACCINES

DNA vaccines are bacterial-derived plasmids essentially composed of two distinguishable backbones: one for prokaryotic propagation and the other for eukaryotic expression (Liu, 2003). The prokaryotic propagation backbone consists of an origin of replication, which allows the plasmid to multiply during cell division and thus, to be passed on to daughter cells. Additionally, to ensure that all bacterial cells are carrying the plasmid of interest, DNA vaccines have a prokaryotic selectable marker generally consisting of a resistance gene to a specific antibiotic. The eukaryotic expression backbone comprises a transcriptional unit that allows for antigen expression in eukaryotic cells. A constitutive or inducible promoter has to be carefully chosen according to the desired expression, as, for example, to enhance protein expression and immunogenicity, strong viral promoters, such as the cytomegalovirus (CMV) and Simian Virus 40 (SV40) promoters, are good options. For correct recognition of messenger RNA (mRNA) and initiation of the translation process by the eukaryotic machinery, a consensus sequence, called Kozak sequence, is present after the promoter and includes the start codon (ACCATGG). Following is the coding sequence of the antigen of interest, which is transcribed, translated and, if necessary, post-translationally modified by the eukaryotic host cell. At the end of the gene sequence, there is the stop codon, which follows the correct reading frame to accurately end translation. Finally, the backbone presents the polyadenylation sequence (poly A), which stabilizes the mRNA and ensures that it is exported from the nucleus to the cytoplasm of eukaryotic cells without degradation.

Over the years, strategies aiming at increasing the effectiveness of DNA vaccines have been developed. Because prokaryotes prefer codons that differ from those more frequently used in mammalian cells, in order to achieve optimal expression by the host cells, the coding sequence of the antigen of interest can be adapted for codons with more available corresponding transfer RNA (tRNA) (Wang et al., 2006). Strategies to enhance non-specific and antigen-specific responses and stimulatory cytokines are also encouraged, and include the use of immunostimulatory sequences, co-injection of immunomodulatory plasmids or use of adjuvants (Bode et al., 2011; Marc et al., 2015). Moreover, to increase the number of plasmids that reach the host cell nucleus and, consequently, are expressed, DNA nuclear targeting sequences (DTS) can be added to the eukaryotic backbone of DNA vaccines (Miller & Dean, 2009).

CHARACTERISTICS OF DNA VACCINES

DNA vaccination is a strategy designed to comply with efficient and effective immunization. Clinical trials have shown bottlenecks that need to be improved as well as

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the advantages of DNA vaccines, highlighting the potential of this innovative vaccination platform (Marc et al., 2015). At first, a recurrent concern was whether plasmids could integrate into the mammalian genome and its consequent unknown implications. However, the probability of plasmid integration into the host genome could be considered negligible, making vaccination with DNA safe (Glenting & Wessels 2005). The time spent to develop and produce DNA vaccines is short, compared to other platforms such as egg-based protein vaccines, and can rapidly be manufactured in large-scale to meet global demands (Moss 2009). In addition, the production of DNA vaccines is inexpensive compared to conventional vaccines, making this vaccination strategy even more attractive. Due to their composition, DNA vaccines are thermally stable, allowing for easy storage and shipping, making it possible to reach locations that do not have the appropriate refrigerated structure storage that some vaccines require (Ghanem et al., 2013).

The ability to elicit humoral and cell-mediated immune responses is one of the main advantages of DNA vaccines. While conventional protein-based vaccines commonly only induce antibody production, this new vaccination strategy can stimulate B and T cells, inducing antibody production and cellular responses, supporting their potential to confer protection against different pathogens (Li et al., 2012). The use of genetic material avoids manipulation of deadly infectious microorganisms and results in more safety during the production of DNA vaccines. Additionally, the formulation of DNA vaccines allows for the insertion of distinct antigen-coding sequences into the same plasmid, resulting in a broader immune response. Finally, DNA vaccines can be administered through different routes, increasing the range of strategies to reach the desired immunization process (Reyes-Sandoval & Ertl, 2001; Laddy & Weiner, 2006).

MUCOSAL DNA VACCINES

The respiratory, gastrointestinal and urogenital tracts are covered by mucosal surfaces, which are large tissues protected by a highly specialized innate and adaptive immune system. As the majority of infections affect or start at mucosal surfaces, the mucosal immune system can prevent infectious agents from attaching, colonizing, penetrating and replicating, blocking microbial toxins in the mucosal epithelium (Holmgren & Czerkinsky, 2005).

The preferred mucosal routes for vaccination are the vaginal (Kanazawa et al., 2008), intranasal (Brave et al., 2008) and oral mucosas (Chatel et al., 2008). However, the oral route is the most commonly used because the gastrointestinal tract has the largest surface area in the body, with more than 200m^2 of mucous membrane (Andrew et al., 2011). Thus, the gut-associated lymphoid tissue (GALT) is the largest immune compartment in the

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body, containing the highest number of immune cells (Schroeter & Klaenhammer, 2009; Mowat & Agace, 2014).

The GALT has two prominent non-encapsulated structures, which are the induction sites of immune responses, known as Peyer's patches (PPs), and isolated lymphoid follicles. Additionally, the effector sites include lymphocytes, dendritic cells (DCs) and macrophages that are also dispersed throughout the epithelial layer and lamina propria (Brandtzaeg et al., 2008; Wells, 2011; Mowat & Agace, 2014).

The GALT is characterized by an overlying follicle-associated epithelium (FAE), with low levels of digestive enzymes and less pronounced villi, and is infiltrated by a large number of immune cells. The most notable feature of the FAE is the presence of Microfold cells (M cells), which are specialized for the uptake and transport of particulate antigens from the lumen into an underlying DC-rich subepithelial dome (SED) region, where they can be presented to the adaptative immune cells. PPs, on the other hand, are macroscopic lymphoid follicles located on the antimesenteric side of the small intestine. They are composed of numerous B cell lymphoid follicles, which are flanked by smaller T cell areas. The mesenteric lymph nodes (LM) drain all these sites and connect the mucosal and the systemic immune systems (Mowat, 2003; Wells, 2011; Mowat & Agace, 2014).

The efficacy of oral DNA vaccines depends therefore on whether the administered agents can survive the gastric and intestinal environments, which include pH-induced degradation, enzymes and diffusion across the mucus layers, and whether their residence time in the intestine is sufficient for interaction with and endocytosis by target cells. Therefore, oral administration of vaccines often requires delivery systems that protect against enzymatic degradation and elimination in the gastrointestinal tract, maintaining their high bioavailability (Chadwick et al., 2009). However, results of experimental approaches have proven difficulty to induce strong intestinal immune responses with a single oral administration of naked DNA vaccines and development of effective mucosal delivery systems remains therefore an active area of research.

BACTERIA AS A VEHICLE TOR MUCOSAL DELIVERY OF DNA VACCINES

Initially, DNA vaccines consisted in direct immunization with naked plasmid in tissues, which internalized and expressed the immunogenic antigen (Liu et al., 2011). However, to reach significant levels of cellular transfection, very high doses of plasmids and multiple boosts were required (Van Drunen Littel-van den Hurk et al., 2000; Le et al., 2000; Babiuk et al., 2003), mainly due to high DNA degradation and low efficacy to overcome membrane permeability and cellular uptake, resulting in poor gene expression and low immunity

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(Miller & Dean, 2009). This showed that potent transfection methods had to be developed for the success of DNA vaccines.

In this context, the use of bacteria to transfer vaccine plasmids to mammalian host cells showed to be a promising strategy. In 1980, Schaffner was the first to observe the transfer of DNA from bacterial to mammalian cells, when tandem copies of the SV40 genome, carried by Eacherichia (*E.*) coli strains, were transferred to co-cultured mammalian cells (Schaffner, 1980). Since then, many bacterial transfer systems have been developed and improved to transfer plasmid DNA to eukaryotic host cells.

This approach has several advantages, such as (i) protection of the DNA vaccine from physical elimination and enzymatic digestion, (ii) targeting of the inductive sites of the body, allowing stimulation of the immune system and (iii) appropriate stimulation of the innate immune system to generate effective adaptive immunity (Holmgren & Czerkinsky, 2005).

Moreover, bacterial carriers are easy to manufacture and able to maintain the plasmids with high copy numbers. Circumventing the need to co-administrate plasmid selection markers, required for vaccination with naked DNA, stable replication of plasmids can be further improved by the introduction of bacterial genes essential for survival within their host (Pilgrim et al., 2003). Additionally, in contrast to immunization with naked plasmid DNA, no further plasmid purification steps are needed, reducing costs and labor (Schoen et al., 2004; Becker et al., 2008). Furthermore, the World Health Organization (WHO) recommends the use of mucosal vaccines because of economic, logistic and security reasons (Holmgren & Czerkinsky, 2005; Wells, 2011).

IMMUNOLOGICAL ASPECTS OF BACTERIAL DNA DELIVERY TO THE ORAL MUCOSA

After oral administration, bacteria carrying plasmids are recognized by immune cells and intestinal epithelial cells (IECs) coating mucosal surfaces. Some attenuated pathogenic bacteria can invade IECs by the expression of virulence factors (invasins) to deliver DNA vaccines while other bacteria can be sampled by intestinal lamina propria DCs or by specialized epithelial M cells overlying PPs (Weiss & Chakraborty, 2001).

After bacterial delivery, innate immune receptors expressed by IECs and DCs, such as pattern recognition receptors likeToll-like and Nod-like receptors, can respond to some bacterial components, which serve as natural antigens after host cell invasion (Barbosa & Rescigno, 2010). Bacterial recognition by the immune system modulates the innate immune response thus, promoting a robust and lasting adaptive response (Hoebe et al., 2004).

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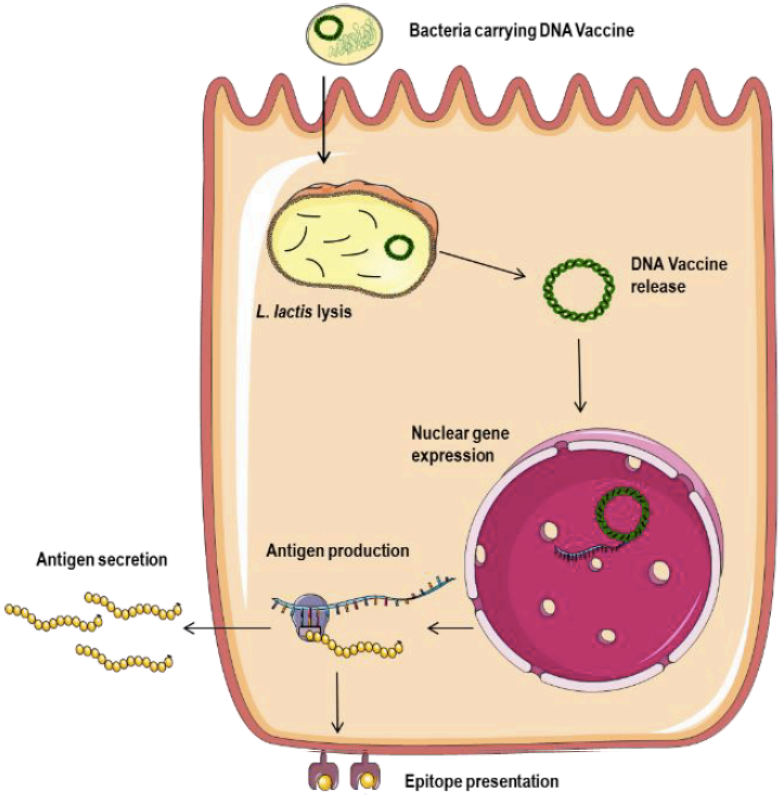


Figure 1. Representation of bacteria-mediated DNA vaccine delivery into intestinal cells. Bacteria enter the host cells and are lysed. The DNA plasmids are released in the cytoplasm and transferred into the nucleus, where expression of the open reading frame (ORF) of interest occurs. After protein synthesis by the host cell machinery, the antigen is processed for immune presentation or for secretion to the extracellular medium, eliciting specific prophylactic immune responses.

Once inside mammalian cells a primary vesicle, the phagosome, envolves the bacteria. This vesicle fuses to a lysosomal compartment where bacterial lysis occurs releasing the DNA plasmids that then reach the host cytosol. The DNA vaccines then migrate to the cell nucleus, where the gene of interest is transcribed for subsequent cytoplasmic protein synthesis by the host cellular machinery (Grillot-Courvalin et al., 1999; Schoen et al., 2004). Exogenous antigens are presented on the surface of epithelial cells or secreted to the extracellular medium, where they can be transported by antigen presenting cells (APCs), especially DCs present in lymphoid follicles, to the nearest mesenteric lymph nodes and be presented to naïve T cells, resulting in the induction of specific cellular and humoral immune responses (Figure 1).

The induction of mucosal immunity, in which antigen-stimulated T cells migrate to the systemic blood and then head to other mucosal surfaces, occurs thus, demonstrating the importance of mucosal immunization to protect against pathogens that infect through this

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route (Brandtzaeg et al., 2008; Mowat & Agace, 2014). Furthermore, plasma cells primed in the mucosa are also attracted to these sites resulting in secretory IgA (sIgA) in the mucosal tissues around the body (Barbosa & Rescigno, 2010). Consequently, effector T lymphocytes and sIgA-producing plasma cells are able to protect the mucosal surfaces. In addition, effector memory cells are also induced, accumulating preferentially in non-lymphoid tissues, especially in the intestinal mucosa (Sallusto et al., 1999).

LACTOCOCCUS LACTIS AS DNA DELIVERY SYSTEM

The non-pathogenic and food-grade bacteria *Lactococcus* (*Lc.*) *lactis* is an attractive alternative for mucosal delivery of DNA vaccines. *Lc. lactis* is the lactic acid bacteria (LAB) model, is very easy to manipulate and has a large number of genetic tools developed (De Vos & Simons, 1994; Bolotin et al., 2001). Additionally, as these bacteria have been used for food fermentation and preservation for centuries, the Food and Drug Administration (FDA) considers *Lc. lactis* a generally regarded as safe (GRAS) microorganism for human consumption (Van de Guchte et al., 2001).

Therefore, the use of *Lc. lactis* for mucosal delivery of DNA vaccines can be justified by the fact that this bacteria (i) does not contain endotoxins, such as lipopolysaccharides (LPS), on their cell wall, eliminating the risk of anaphylaxis and can be therefore delivered safely at high doses in immunization programs, (ii) can be easily engineered for DNA delivery (Mercenier et al., 2000; Miyoshi et al., 2004; Wells & Mercenier, 2008; Brondyk, 2009), and (iii) its administration induces high production of antigen-specific sIgA (Neutra & Kozlowski, 2006).

USE OF WILD TYPE *LACTOCOCCUS LACTIS* AS A VEHICLE FOR DNA DELIVERY

The use of *Lc. lactis* as a potential DNA vaccine delivery system has been successfully show (Pontes et al., 2011; Chatel et al., 2008). Firstly, in 2006, Guimarães and colleagues used a wild type (wt) *Lc. lactis* strain harboring a DNA plasmid coding for bovine ß-lactoglobulin (BLG) to deliver DNA into mammalian cells. Production and secretion of BLG were observed in the intestinal epithelial cell line Caco-2 after its co-culture with the recombinant *Lc. lactis* strain, demonstrating that this bacterium could efficiently deliver a fully functional plasmid into epithelial cells *in vitro* (Guimarães et al., 2006). Then, Chatel and colleagues (2008) confirmed the ability of wt *Lc. lactis* to transfer DNA to mice IECs *in vivo*. This strain demonstrated its ability to translocate the

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intestinal membrane, as BLG cDNA was detected in the enterocytes of mice immunized with *Lc. lactis* carrying the same eukaryotic expression plasmid (Chatel et al., 2008).

Although these studies successfully demonstrated that *Lc. lactis* can be used as a DNA delivery system, different strategies have been developed to increase the ratio of gene transfer and therefore boost the immune response. One of these strategies includes chemical treatments to weaken bacterial cell walls: glycine-treated *Lc. lactis* NZ3900 showed a higher transfer frequency of a eukaryotic expression plasmid encoding for the red fluorescent protein (RFP) to Caco-2 cells (Tao et al., 2011).

Another approach was a new plasmid (pExu) constructed for DNA delivery using *Lc. lactis*, with a theta origin of replication and the pCMV expression cassette. An *in vitro* assay showed that 15.8% of CHO cells were able to express the *enhanced green fluorescent protein* (eGFP) protein after pExu:egfp transfection. Additionaly, *Lc. lactis* MG1363 (pExu:egfp) were administered by gavage to Balb/C mice and the eGFP protein was detected in mice enterocytes (Mancha-Agresti et al., 2016). Also using wt *Lc. lactis* for DNA delivery, a different research group showed that this strain efficiently delivered the pPERDBY reporter plasmid encoding the eGFP to Caco-2 cells *in vitro* (Yagnik et al., 2016).

Furthermore, as described in the following section, a very interesting approach based on the use of recombinant invasive *Lc. lactis* strains have already been successfully demonstrated both *in vitro* and *in vivo*.

RECOMBINANT INVASIVE *LACTOCOCCUS LACTIS* AS DNA DELIVERY VEHICLES

Pathogenic bacteria have refined strategies to overcome host defenses and to interact with the host's immune system. To explore this concept and to increase the ability of lactococci to deliver DNA vaccines, some *Lc. lactis* strains expressing invasins have been constructed and shown to improve DNA delivery (Guimarães et al., 2005; Sleator & Hill, 2006). One such strategy engineered *Lc. lactis* to express InlA from *Listeria monocytogenes*. InlA is a cell wall-anchored protein that binds to the extracellular receptor E-cadherin (Gaillard et al., 1991; Mengaud et al., 1996). The recombinant lactococci (*Lc. lactis* InlA+), efficiently displaying the cell wall-anchored form of InlA, showed invasion rates in Caco-2 cells that were approximately 100-fold higher than those of wt lactococci. *In vivo* the invasive InlA+ strain also proved its capacity to enter epithelial Caco-2 cells with subsequent GFP expression after oral administration to guinea pigs and delivery of a functional eukaryotic plasmid (Guimarães et al., 2005).

To optimize the use of *Lc. lactis* as a DNA delivery system, a new plasmid (pValac - Vaccination using lactic acid bacteria) of 3,742 bp was constructed. The pValac plasmid

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consists of the cytomegalovirus promoter (pCMV) and polyadenylation sequences from the bovine Growth Hormone (BGH), which allows for the transcription of the ORF of interest with a PoliA tail that is essential to stabilize the mRNA transcript in eukaryotic cells. The pValac also contains origins of replication for its propagation in both *E. coli* (OriC) and *Lc. lactis* (OriA), and a chloramphenicol resistance gene for selection of strains harboring the specific plasmid. To evaluate its functionality, the gfp ORF was cloned into the pValac (pValac:gfp) and fluorescence was observed after PK15 cells transfection. Moreover, the *Lc. lactis* InlA+ strain invaded and delivered the pValac:gfp to Caco-2 cells (Guimarães et al., 2009).

However, the use of the *Lc. lactis* InlA+ strain presented a major bottleneck: InlA cannot bind to murine E-cadherin receptors, preventing *in vivo* experiments in these animals and limiting its study only in guinea pigs, which may be difficult to handle and/or expensive (Wollert et al., 2007). In this context and in order to increase the delivery of DNA to mammalian cells (Innocentin et al., 2009), a new recombinant *Lc. lactis* strain, expressing Fibronectin Biding Protein A (FnBPA) of *Staphylococcus aureus*, and that could be used in mice was constructed (Que et al., 2001). FnBPA is a virulence factor, which mediates bacterial adhesion to the host tissue and its entry into host cells (Sinha et al., 1999). FnBPA showed increased interaction and entrance of L. lactis in IECs and thus levels of DNA vaccine delivery. *Lc. lactis* FnBPA+ and *Lc. lactis* InlA+ presented comparable *in vitro* internalization rates by Caco-2 cells. After co-incubated of *Lc. lactis* FnBPA+ with Caco-2 cells, the pValac:gfp plasmid was more efficiently delivered than by non-invasive strains (Innocentin et al., 2009). Additionally, this strain was successfully used to deliver DNA vaccines encoding the BLG allergen and GFP *in vivo*, showing to be more effective than the wt strain (Pontes et al., 2012).

Another interesting work was performed using the mutated form of Internalin A (mInlA), which can bind to murine E-cadherin. mInlA was successfully expressed at the surface of *Lc. lactis* and showed higher invasion rates into Caco-2 cells and consequently higher delivery levels of BLG-coding plasmid when compared with the wt strain. Furthermore, *in vivo* studies demonstrated that *Lc. lactis* mInlA+ tended to increase the number of mice producing BLG (De Azevedo et al., 2012).

All these engineered strains to be used as DNA vaccine delivery systems represent promising tools for mucosal immunization and the strategy to use *Lc. lactis* to deliver prophylactics plasmids has proved to be relevant and possible. Our research group is currently working on the validation of this strategy in the development of DNA vaccines against tuberculosis, a re-emerging infectious disease that deserves great attention in current researches. This approach could therefore allow the use of *Lc. lactis* as efficient DNA vaccine delivery vehicles in a near future.

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LACTOCOCCUS LACTIS AS A DNA VACCINE DELIVERY SYSTEM AGAINST TUBERCULOSIS DISEASE

Tuberculosis (TB), which is caused by *Mycobacterium tuberculosis*, is an infectious disease that affects one third of the global population in its latent form. This, in turn, makes TB one of the main social, economic and public health problems worldwide (Lugo & Bewley, 2008).

The only available vaccine for clinical use, the BCG (Bacillus Calmette-Guérin), has a variable efficacy in adolescents and adults, requiring the development of more effective and economically viable vaccines.

In this context, *Lc. lactis* appears as an attractive alternative for a TB DNA vaccine delivery system, because it elicits an immune response as efficient as the other mucosal bacterial DNA delivery systems (Table 1). Moreover, *Lc. lactis* does not present risk in future clinical use when compared to attenuated pathogenic bacteria.

Table 1. Bacterial mucosal DNA vaccines studies against TB

DNA Vaccine	Administration strategy	Immune Response									Protection
		IFN-γ	TNF-α	IL-17	IL-2	IL-4	IL-10	IgG1	Ig2a	sIgA	
Lc. lactis DNA delivery system											
pValac:Ag85A (Mancha-Agresti 2014)	Intranasal *Lc. lactis* FnBPA+	Yes	Yes	No	No	No	Yes	No	No	Yes	-
pValac:ESAT-6 (Pereira et al., 2015)	Intra-gastric *Lc. lactis* FnBPA+	Yes	No	No	-	No	No	No	No	Yes	-
pValac:ESAT6/Ag85A (Prósperi 2016)	Intra-gastric *Lc. lactis* FnBPA+	Yes	Yes	Yes	-	No	No	No	No	Yes	-
BCG + pValac:ESAT-6 (Pereira et al., 2017)	BCG + Intra-gastric *Lc. lactis* FnBPA+	Yes	Yes	Yes	-	No	No	No	No	No	-
Other bacterial DNA delivery system											
pCMVβ::Ag85A (Parida et al., 2005)	Intranasal *Salmonella typhimurium* ΔaroA SL7207	Yes	-	-	Yes	-	-	-	-	-	Yes
pcDNA_htpX or pcDNA_fbpA (Brun et al., 2008)	Intranasal *E. coli* BM2710/pGB2_inv-hly	Yes	-	-	Yes	-	-	-	-	-	Yes
pVax-ESAT6-Ag85B (Wang et al., 2009)	BCG + Intra-gastric *S. typhimurium* ΔaroA SL7207	Yes	-	-	-	-	-	Yes	Yes	Yes	Yes
pORT-mpt64 (Huang et al., 2010)	Intra-gastric RecA+ *S. typhimurium* SLDAPD	Yes	-	-	-	-	-	-	-	-	Yes

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Thus, our research group developed new strategies for DNA vaccination against TB using the invasive *Lc. lactis* FnBPA+ strain (Innocentin et al.,, 2009) and the pValac vaccinal plasmid (Guimarães et al., 2009), in which the coding sequences of the immunodominant antigens ESAT-6 and/or Ag85A were inserted. Mancha-Agresti and colleagues constructed a DNA vaccine encoding the Ag85A antigen (pValac:Ag85A) which was intranasally administrated to mice by L. lactis FnBPA+, showing a significant increase in the production of IFN-γ and TNF-α by splenocytes and of sIgA in the bronchoalveolar lavage (Mancha-Agresti et al., 2017).

Then, Pereira and colleagues reported a significant increase in the production of IFN-γ by splenocytes and of specific sIgA in the colons of mice orally immunized with the *Lc. lactis* FnBPA^{+} (pValac:ESAT-6) strain (Pereira et al., 2015). Additionally, the administration of this strain enhanced the immune responses after vaccination with BCG, with promising results in relation to IFN-γ, IL-17, IL-6 and TNF-α levels in the spleen of the immunized mice (Pereira et al., 2017).

Finally in 2016, Prósperi developed a *Lc. lactis* strain carrying a multigenic DNA vaccine in which the coding sequences of ESAT-6 and Ag85A were fused [*Lc. lactis* FnBPA^{+} (pValac::e6ag85a)]. After oral administration of this strain to mice, INF-γ, TNF-α and IL-17 levels increased significantly and it was also observed activation of specific cytotoxic T lymphocytes. Additionally, this strategy elicited an antigen-specific mucosal immune response shown by a significant increase in sIgA production in the colons of mice (Prósperi, 2016).

CONCLUSION

The applicability and efficacy of DNA vaccines carried by L. lactis strains, administered by mucosal routes, have been successfully established, generating a specific and broad immune response. Therefore, this immunization strategy can be used in research and development of DNA vaccines against other pathogens, with applications in the fields of immunization and immunomodulation.

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ANEXO 2:

“Recombinant *Lactococcus lactis* carrying IL-4 and IL-10 coding vectors protects against Type 1 Diabetes in NOD mice and attenuates insulitis in the STZ-induced model”

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Research Article

Recombinant *Lactococcus lactis* Carrying IL-4 and IL-10 Coding Vectors Protects against Type 1 Diabetes in NOD Mice and Attenuates Insulitis in the STZ-Induced Model

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Type 1 diabetes (T1D) is an autoimmune disease that culminates in beta cell destruction in the pancreas and, subsequently, deficiency in insulin production. Cytokines play a crucial role in the development of diabetes, orchestrating the recruitment and action of immune cells, to not only destroy insulin-producing cells but also preserve them. Therefore, the aim of this study was to investigate the effect of orally administered *Lactococcus lactis* MG1363 FnBPA$^+$ strains carrying plasmids encoding IL-4 and IL-10 in the streptozotocin- (STZ-) induced diabetes model and in nonobese diabetic (NOD) mice. The STZ-induced mice that were treated with combined bacterial strains carrying plasmids encoding IL-4 and IL-10 showed lower incidence of diabetes and more preserved pancreatic islets than the mice that received the individual bacterial strains. Combined administration of *L. lactis* MG1363 FnBPA$^+$ (pValac::dts::*IL-4*) and *L. lactis* MG1363 FnBPA$^+$ (pValac::*IL-10*) resulted in protection against diabetes in NOD mice. It was shown that the combined treatment with recombinant bacterial by oral route prevented hyperglycemia and reduced the pancreatic islets-destruction in NOD mice. In addition, increased levels of IL-4 and IL-10 in serum and pancreatic tissue revealed a systemic effect of the treatment and also favored an anti-inflammatory microenvironment. Reduced concentrations of IL-12 in pancreas were essential to the regulation of inflammation, resulting in no incidence of diabetes in treated NOD mice. Normal levels of intestinal sIgA after long-term treatment with the *L. lactis* strains carrying plasmids encoding IL-4 and IL-10 indicate the development of oral tolerance and corroborate the use of this potent tool of mucosal delivery. For the first time, *L. lactis* MG1363 FnBPA$^+$ strains carrying eukaryotic expression vectors encoding IL-4 and IL-10 are tested in STZ-induced and NOD mouse models. Therefore, our study demonstrates this innovative strategy provides immunomodulatory potential for further investigations in T1D and other autoimmune diseases.

1. Introduction

Type 1 diabetes (T1D) is a chronic immune-mediated disease characterized by destruction of β cells, the insulin-producing cells in the pancreas. Trafficking of autoreactive T lymphocytes to the pancreatic islets and the resulting inflammatory environment culminates in disease establishment in at-risk individuals [1]. Approximately 19 million people have T1D worldwide, and this number has increased over the years [2]. After diagnosis, lifelong administration of exogenous insulin is required, and this is the only therapy currently available. Moreover, these individuals have to learn how to manage their condition and be aware of the possible disease-related complications.

To study the pathogenesis and immunophysiology of T1D, some murine models have been developed over the

Table 1: Bacterial strains used in this work.

Strain	Characteristics	Source
Lactococcus lactis MG1363 FnBPA$^+$	*L. lactis* MG1363 strain expressing *S. aureus* FnBPA	Que et al. [33]
Lactococcus lactis MG1363 FnBPA$^+$ (pValac::dts::*IL-4*)	*L. lactis* MG1363 FnBPA$^+$ strain carrying the pValac::dts::*IL-4* plasmid	Souza et al. [32]
Lactococcus lactis MG1363 FnBPA$^+$ (pValac::*IL-10*)	*L. lactis* MG1363 FnBPA$^+$ strain carrying the pValac::*IL-10* plasmid	Zurita-Turk et al. [31]

FnBPA: fibronectin-binding protein A; IL-4: interleukin 4; IL-10: interleukin 10; dts: DNA nuclear targeting sequence.

years, each one sharing different mechanisms of the human disease. Chemical induction of T1D through multiple doses of streptozotocin (STZ) is an immune-mediated T1D model discovered by Like and Rossini [3–5]. The STZ-induced model exhibits a phenotype and cellular infiltration similar to those observed in human disease, with a humoral response and autoreactive T cells against pancreatic antigens [6–8]. Another animal model, nonobese diabetic (NOD) mice are an inbred strain developed by Makino et al. from Jcl:ICR mice and one of the most commonly used models in T1D research [9, 10]. NOD mice spontaneously develop autoimmune diabetes and exhibit important features observed in human T1D, such as genetic susceptibility, diabetes-specific autoantibodies, and lymphocytic infiltration of pancreatic islets [11–13].

Cytokines play a key role in connecting the risk factors that contribute to T1D development and trigger the destructive action of T cells in pancreatic islets [14, 15]. In this context, studies investigating the potential of interleukin- (IL-) 4- and IL-10-mediated immunomodulation of T1D through individual or combined administration have shown that this is a new field to be explored [16–19]. In 2012, Mandke and Singh injected cationic nanomicelles intramuscularly to deliver a plasmid encoding IL-4 and IL-10 before STZ induction of T1D, resulting in protection against insulitis. However, entry of the delivery system into the muscle caused the infiltration of a large number of macrophages and lymphocytes at the site of injection [20].

The recurrent problem of biocompatibility and an undesirable immune response during the delivery process encouraged us to use *Lactococcus lactis* as the vehicle for mucosal delivery of plasmids encoding IL-4 and IL-10. This lactic acid bacteria also have been handled as a genetic tool due to some advantages, as do not colonize the tract gastrointestinal, present safe use in humans, and be able to induce tolerance successfully [21, 22]. *L. lactis* bacteria have been engineered to express a variety of molecules, including an invasin called fibronectin-binding protein A (FnBPA) (*L. lactis* MG1363 FnBPA$^+$ strain) to more efficiently deliver the eukaryotic expression plasmids encoding IL-4 and IL-10 [23, 24]. Thus, administration of *L. lactis* MG1363 FnBPA$^+$ (pValac::dts::*IL-4*) and *L. lactis* MG1363 FnBPA$^+$ (pValac::*IL-10*) allows an increased rate of internalization and plasmid transference to eukaryotic cells, which will be able to express IL-4 and IL-10. Systematic administration of these cytokines was shown to be a successful approach in studies involving T1D [25–27]. Moreover, it has already been shown that immune responses triggered by intestinal stimuli can modulate the development of T1D, although the underlying mechanisms remain unclear [28, 29]. Thus, the oral delivery becomes an attractive route to administer the treatment, since the internalization of bacteria by mammalian host cells and subsequent expression of the plasmids are crucial steps in our strategy.

In the present study, we examined the effect of oral administration of *L. lactis* MG1363 FnBPA$^+$ (pValac::dts::*IL-4*) and *L. lactis* MG1363 FnBPA$^+$ (pValac::*IL-10*) in the two most extensively used animal models of T1D, the STZ-induced model, and NOD mice. The combined treatment of these bacterial strains for the first time is an interesting approach, since individual administration has already been shown to be able to reduce inflammation in intestinal inflammation models by our research group [30–32]. Therefore, we hypothesized that combined treatment with *L. lactis* MG1363 FnBPA$^+$ strains carrying eukaryotic expression plasmids encoding IL-4 and IL-10 could result in beneficial effects on T1D.

2. Materials and Methods

2.1. Bacterial Strains and Growth Conditions. The strains used in this work are listed in Table 1. *L. lactis* MG1363 FnBPA$^+$, *L. lactis* MG1363 FnBPA$^+$ (pValac::dts::*IL-4*), and *L. lactis* MG1363 FnBPA$^+$ (pValac::*IL-10*) were grown in M17 medium (Sigma-Aldrich) supplemented with 0.5% glucose with or without chloramphenicol (10 μg/mL) (Sigma-Aldrich) and erythromycin (5 μg/mL) (Sigma-Aldrich) at 30°C.

2.2. Animals. Male C57BL/6 mice, aged 8-9 weeks, were obtained from Biotério Central of Federal University of Minas Gerais (UFMG; Belo Horizonte, MG/Brazil). Female NOD mice were kindly donated by Dr. Ana Maria Caetano de Faria from UFMG. The animals were kept in microisolators on ventilated shelves with air filtration under a 12 h light-dark cycle with free access to standard mouse chow and water. All animal procedures were approved by the Ethics Committee on Animal Use (CEUA, Protocol no: 206/2016) of UFMG/Brazil.

2.3. Experimental Design and Diabetes Monitoring. C57BL/6 mice were subjected to a 4 h fast and were then injected intraperitoneally (i.p.) with 50 mg/kg/day STZ (Sigma-Aldrich) for 5 consecutive days (days 0-4) (Figure 1(a)). STZ was diluted in 0.1 M sodium citrate buffer, pH 4.5, and injected into the mice immediately after preparation. Subsequently, the animals were subjected to a 2 h fast. The vehicle control

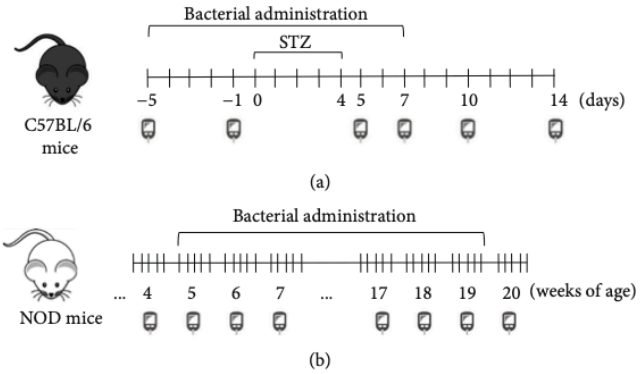


FIGURE 1: Experimental designs of the STZ-induced and NOD mouse models. (a) Diabetes was induced in male C57BL/6 mice by intraperitoneal injection of STZ + sodium citrate buffer for 5 consecutive days (days 0–4). Animals received bacterial administration daily from day -5 to day 7. Blood samples were collected on days -5, -1, 5, 7, 10, and 14 to measure glycemia. Animals were euthanized on day 14 of the experiment. (b) NOD mice received intragastric administration of bacterial suspensions 5 days/week for 15 consecutive weeks. Glycemic levels were measured weekly during the experiment. Animals were euthanized at the end of the 20th week.

group received only sodium citrate buffer i.p. From day -5 until day 7, the animals were intragastrically (i.g.) administered 100 μL of the corresponding *L. lactis* strain at a final dose of 1×10^9 colony forming units (CFU) in 0.9% saline.

NOD mice were i.g. administered 100 μL of bacterial suspension at a final dose of 1×10^9 CFU in 0.9% saline 5 times/week for 15 consecutive weeks (Figure 1(b)).

Blood samples were collected from the tail vein of non-fasted mice on days -5, -1, 5, 7, 10, and 14 in the STZ-induced model (Figure 1(a)) and once a week during the experiments using the NOD mouse model (Figure 1(b)). Glucose levels were determined using a Accu-Chek Active glucometer system (Roche), and mice were considered diabetic when glucose levels were ≥200 mg/dL during two consecutive measurements.

2.4. Study Groups. C57BL/6 mice were allocated into groups that received 0.9% saline i.g., and by intraperitoneal route, the animals received 0.9% saline (negative control group, –) or sodium citrate buffer (vehicle control (VC) group) and groups that received the STZ injections i.p. and, by gavage, administration of 0.9% saline (saline-STZ group), *L. lactis* MG1363 FnBPA$^+$ (F-STZ group), *L. lactis* MG1363 FnBPA$^+$ (pValac::dts::*IL-4*) (F-IL-4-STZ group), *L. lactis* MG1363 FnBPA$^+$ (pValac::*IL-10*) (F-IL-10-STZ group), *L. lactis* MG1363 FnBPA$^+$ (pValac::dts::*IL-4*), and *L. lactis* MG1363 FnBPA$^+$ (pValac::*IL-10*) (F-IL-4/IL-10-STZ group).

NOD mice were distributed into two groups, according to the constitution of dose i.g.: 0.9% saline (saline-NOD group) and *L. lactis* MG1363 FnBPA$^+$ (pValac::dts::*IL-4*), and *L. lactis* MG1363 FnBPA$^+$ (pValac::*IL-10*) (F-IL-4/IL-10-NOD group).

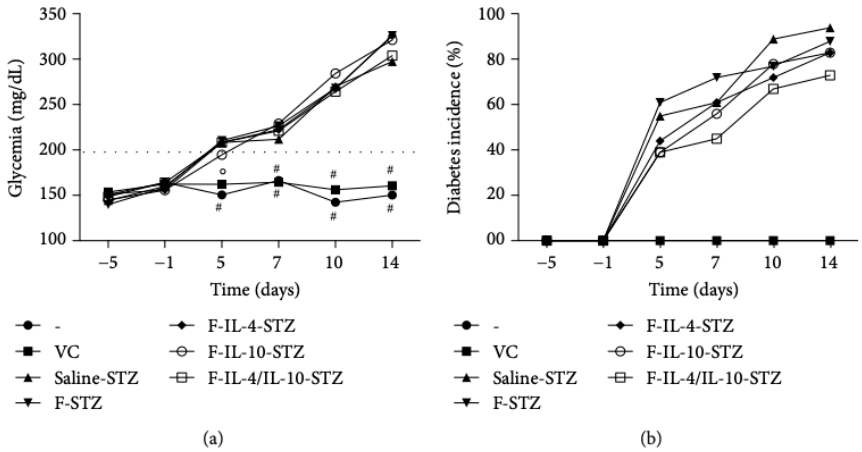
2.5. Cytokine Detection. The blood, the pancreas, and the colon were harvested immediately after euthanasia. Blood samples were incubated at 37°C for 40 minutes and then centrifuged at 5,000 rpm for 10 minutes at 4°C. The serum was collected and stored at -20°C until use. Pancreatic and colon tissues were homogenized in 1 mL buffer (23.4 g NaCl; 500 μL Tween 20; 5 g BSA; 34 mg PMSF; 1 mL DMSO; 44.6 mg BC; 372 mg Na_2EDTA; and 40 μL aprotinin (10 mg/mL) (Sigma-Aldrich); PBS 1× q.s.p. 1 L) per 100 mg of tissue. Then, the samples were centrifuged at 10,000 rpm for 10 minutes at 4°C, and the supernatant was stored at -80°C until use.

The concentrations of IL-4 and IL-10 were measured in serum, and the levels of IL-2, IL-4, IL-10, IL-12, and transforming growth factor β (TGF-β) in pancreatic and colon homogenates were determined using BD OptEIA (BD) and R&D DuoSet (R&D Systems) ELISA kits, according to the manufacturers' instructions.

2.6. Secretory IgA Detection. The concentration of secretory immunoglobulin A (sIgA) in intestinal lavage fluid was measured by capture ELISA using goat anti-mouse Ig, human ads-UNLB (Southern Biotech), and goat anti-mouse IgA (α chain specific) horseradish peroxidase (HRP) conjugate (Southern Biotech), as recommended by the manufacturer.

2.7. Bacterial Translocation. C57BL/6 mice received 100 μL of bacterial suspension (*L. lactis* MG1363 FnBPA$^+$ (pValac::dts::*IL-4*) and *L. lactis* MG1363 FnBPA$^+$ (pValac::*IL-10*)) at a final dose of 1×10^9 CFU in 0.9% saline by oral gavage. The spleen, liver, and mesenteric lymph nodes (MLNs) were collected under strict aseptic conditions 2 h, 4 h, 6 h, 12 h, and 24 h after bacterial administration. The tissues were macerated in 500 μL of 0.9% saline, and 100 μL of this homogenate was plated in M17 medium (Sigma-Aldrich) supplemented with agar, 0.5% glucose, chloramphenicol (10 μg/mL) (Sigma-Aldrich), and erythromycin (5 μg/mL) (Sigma-Aldrich). The plates were incubated at 30°C for 24 h.

2.8. Histological Analysis. After euthanasia, the pancreas was removed, fixed in 10% buffered formalin, embedded in paraffin, and sectioned. Five-μm sections were stained with hematoxylin and eosin (HE), and at least 15 islets per pancreatic sample were evaluated and scored for infiltration progression



(a) (b)

	Diabetic mice ($n = 76$)	Nondiabetic mice ($n = 14$)	P
Saline-STZ	22 % (17)	7 % (1)	
F-STZ	21 % (16)	14 % (2)	
F-IL-4-STZ	20 % (15)	21 % (3)	$P = 0.44$
F-IL-10-STZ	20 % (15)	21 % (3)	
F-IL-4/IL-10-STZ	17 % (13)	37 % (5)	

(c)

Figure 2: Clinical effects of genetically modified *L. lactis* strains encoding IL-4 and IL-10 in the STZ-induced diabetes model. C57BL/6 mice were injected with 0.9% saline, vehicle solution, or STZ for five consecutive days (50 mg/kg/d). Specific bacterial administration was performed daily by the intragastric route from day -5 to day 7. (a) Glycemic levels (mg/dL) were measured at days -5, -1, 5, 7, 10, and 14. The data are shown as the mean ± SD of three independent experiments ($n = 18$). #: experimental group whose glycemia was significantly different from the saline-STZ, F-STZ, F-IL-4-STZ, F-IL-10-STZ, and F-IL-4/IL-10-STZ groups; °: experimental group whose glycemia was significantly different from the saline-STZ, F-STZ, F-IL-4-STZ, and F-IL-4/IL-10-STZ groups. (b) Diabetes incidence over time was analyzed, and (c) the percentage of diabetic and nondiabetic mice between the experimental groups and the disease control group at day 14 was compared. p value: $p \leq 0.05$.

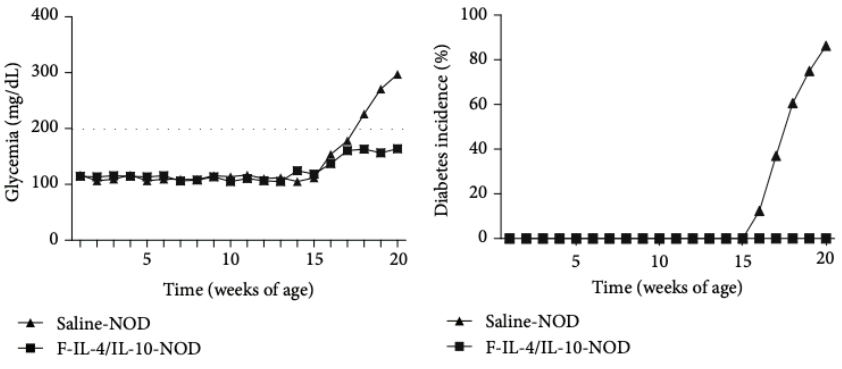
(by an independent investigator in blinded fashion), as follows: no insulitis: no visible signs of infiltration; peri-insulitis: few infiltrating elements with patchy distribution primarily at the periphery of the islet; mild insulitis: mild infiltration (peri- and intraislet) with less than 50% of the islet infiltrated by the immune elements; and severe insulitis: extensive inflammation with more than 50% of the islet infiltrated.

2.9. Statistical Analysis. The GraphPad Prism software, version 5.0 (GraphPad), was used for statistical analysis, and the results are presented as the mean ± standard deviation (SD). One-way ANOVA followed by Tukey's post hoc test was used for the analysis of variance. For diabetes incidence, the χ^2 test was used. p values less than 0.05 ($p < 0.05$) were considered statistically significant.

3. Results

3.1. Effect of Genetically Modified L. lactis on Clinical Manifestations in T1D Models. Glycemic levels were analyzed, and oral bacterial treatment of STZ-induced groups (F-STZ, F-IL-4-STZ, F-IL-10-STZ, and F-IL-4/IL-10-STZ groups) did not alter the increases in glycemic levels at any time after T1D induction; blood glucose levels in these groups were similar to that in the saline-STZ group (Figure 2(a)). A progressive increase in diabetes incidence was also observed in groups that received STZ injections, regardless of oral treatment administration (Figure 2(b)). Figure 2(c) shows the comparison of the percentage of diabetic and nondiabetic mice between the experimental groups (F-STZ, F-IL-4-STZ, F-IL-10-STZ, and F-IL-4/IL-10-STZ groups) and the disease control group (saline-STZ group) at the final time point. The distribution of diabetic mice was analyzed, and by day 14, all groups showed similar percentages. However, the F-IL-4/IL-10-STZ group exhibited the highest percentage of nondiabetic mice (37%).

In contrast to the results observed in the STZ model, NOD mice that received oral treatment with *L. lactis* MG1363 FnBPA$^+$ (pValac::dts::*IL-4*) and *L. lactis* MG1363 FnBPA$^+$ (pValac::*IL-10*) (F-IL-4/IL-10-NOD group) showed normoglycemia (glycemic levels less than 200 mg/dL) until the end of the experimental period (Figure 3(a)) and treatment protected NOD mice from T1D development. Analysis showed that diabetes incidence increased in the saline-NOD group from week 15 onwards, while mice in the F-IL-4/IL-10-NOD group remained protected against diabetes



	Diabetic mice ($n = 10$)	Nondiabetic mice ($n = 14$)	P
Saline-NOD	100 % (10)	14 % (2)	$P = 0.00003$
F-IL-4/IL-10-NOD	0 % (0)	86 % (12)	

(c)

Figure 3: Clinical effects of genetically modified *L. lactis* strains encoding IL-4 and IL-10 in the NOD mouse model. NOD mice were i.g. administered *L. lactis* MG1363 FnBPA$^+$ (pValac::dts::*IL-4*) and *L. lactis* MG1363 FnBPA$^+$ (pValac::*IL-10*) suspension (F-IL-4/IL-10-NOD group) or 0.9% saline (saline-NOD group) 5 days/week from week 5 to week 19. (a) Glycemic levels (mg/dL) were measured once a week from weeks 1 to 20. The data are shown as the mean ± SD of three independent experiments ($n = 12$). (b) Diabetes incidence over time was analyzed, and (c) the percentage of diabetic and nondiabetic mice between the NOD mouse groups at the final time point was compared. p value: $p \leq 0.05$.

Table 2: Comparison of clinical parameters observed in STZ model mice and NOD mice.

			STZ model: day 14					NOD mice: week 20	
	—	VC	Saline-STZ	F-STZ	F-IL-4-STZ	F-IL-10-STZ	F-IL-4/IL-10-STZ	Saline-NOD	F-IL-4/IL-10-NOD
Glycemia (mg/dL)	150	160	297	326	325	321	303	298	177
Diabetes incidence (%)	0	0	95	89	84	84	72	84	0

during the entire experimental period (Figure 3(b)). The percentages of diabetic and nondiabetic mice per group, among all NOD mice, were statistically significant, as shown in Figure 3(c). By 20th week, all diabetic mice belonged to the saline-NOD group, and there were no diabetic mice in the F-IL-4/IL-10-NOD group. In addition, analysis of the nondiabetic mice showed that only 14% of animals were in the saline-NOD group, while the remaining 86% were in the F-IL-4/IL-10-NOD group. The comparison of glycemia and the percentage of diabetes incidence in groups of STZ-induced mice and NOD mice at the final time point (Table 2) corroborates the previous analysis.

3.2. Cytokine Levels in the Serum and Pancreas Are Differentially Modulated in STZ-Induced and NOD Mouse Models of Diabetes. Cytokine profiles in serum and the pancreas were investigated in both T1D models, and in the STZ-induced model, all groups presented similar serum levels of IL-4 and IL-10 (Figures 4(a) and 4(b), respectively). In contrast, NOD mice treated with *L. lactis* MG1363 FnBPA$^+$ (pValac::dts::*IL-4*) and *L. lactis* MG1363 FnBPA$^+$ (pValac::*IL-10*) exhibited significantly higher serum production of IL-4 (Figure 4(c)) and IL-10 (Figure 4(d)) than the saline-NOD group.

Pancreatic levels of IL-2 were analyzed, and other than the negative and vehicle controls in the STZ model, F-IL-4/IL-10-STZ was the only group that exhibited significantly higher IL-2 levels than the saline-STZ group (Figure 5(a)). Regarding TGF-β and IL-10 levels, a significant difference was found only between the negative and saline-STZ control groups (Figures 5(b) and 5(c), respectively). IL-4 (Figure 5(d)) and IL-12 (Figure 5(e)) levels were similar among all pancreatic samples.

The levels of pancreatic cytokines in NOD mice were measured, and we found that IL-2 (Figure 5(f)) and TGF-β (Figure 5(g)) levels were similar in both groups. On the other hand, IL-10 production was significantly higher in NOD mice that received *L. lactis* MG1363 FnBPA$^+$ (pValac::dts::*IL-4*) and *L. lactis* MG1363 FnBPA$^+$ (pValac::*IL-10*) than in the saline-NOD group (Figure 5(h)). Furthermore, the F-IL-4/IL-10-NOD group also had significantly increased production of IL-4 in comparison to that in the saline-NOD group (Figure 5(i)). And the level of pancreatic IL-12 was significantly lower in NOD mice treated with bacterial

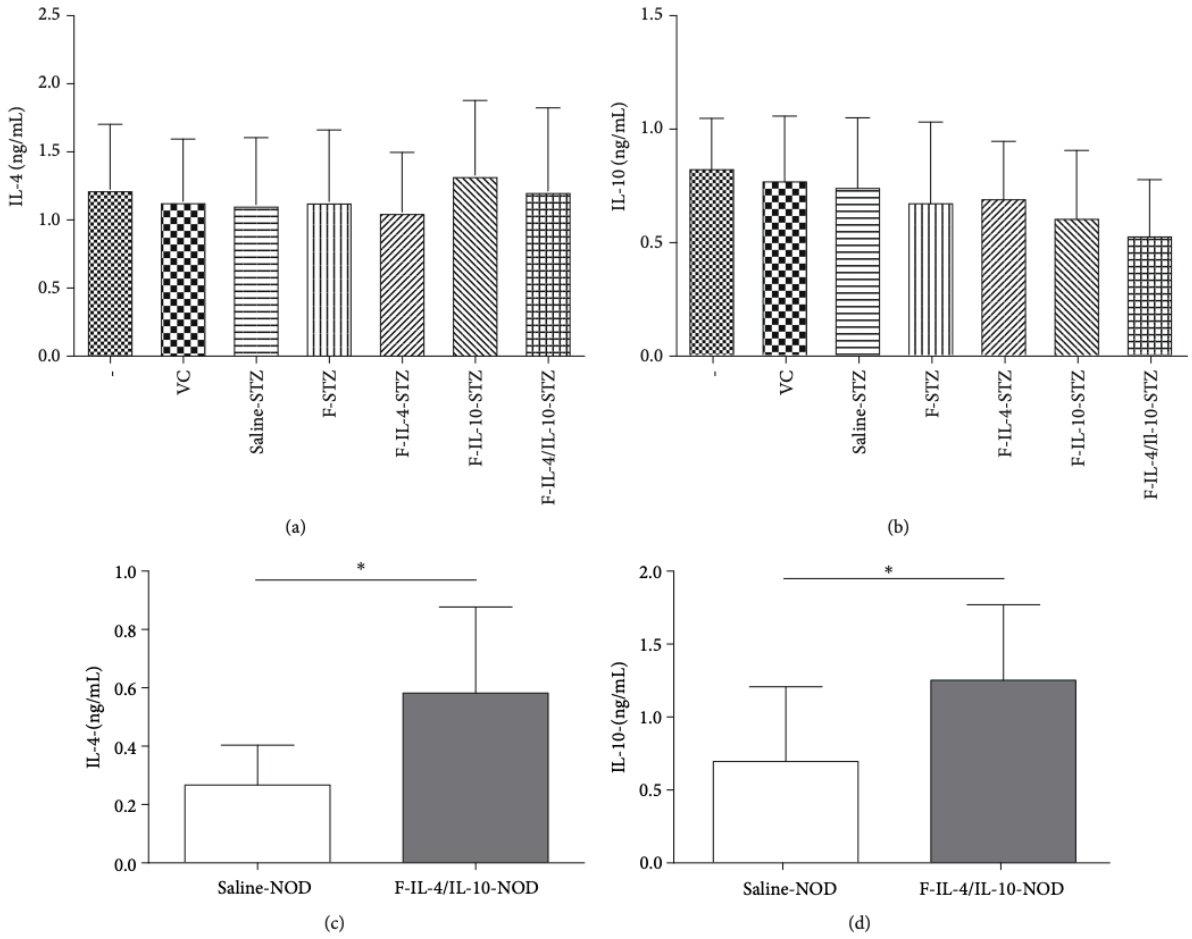


FIGURE 4: Serum levels of IL-4 and IL-10 from STZ-induced C57BL/6 mice and NOD mice, which were both treated with oral bacterial administration. Saline, vehicle, or STZ-injected C57BL/6 mice were euthanized at the end of the experiment, and serum was collected to measure IL-4 and IL-10 levels ((a, b), respectively, $n = 18$). After saline or oral bacterial administration, serum was collected from NOD mice after euthanasia to measure IL-4 and IL-10 levels ((c, d), respectively, $n = 12$). –: negative control; VC: vehicle control; F: mice that received *L. lactis* MG1363 FnBPA$^+$ i.g.; F-IL-4: mice that received *L. lactis* MG1363 FnBPA$^+$ (pValac::dts::*IL-4*) i.g.; F-IL-10: mice that received *L. lactis* MG1363 FnBPA$^+$ (pValac::*IL-10*) i.g.; F-IL-4/IL-10: mice that received *L. lactis* MG1363 FnBPA$^+$ (pValac::dts::*IL-4*) and *L. lactis* MG1363 FnBPA$^+$ (pValac::*IL-10*) i.g.; STZ: mice that received intraperitoneal injections of streptozotocin for five consecutive days; NOD: nonobese diabetic mice. The data are shown as the mean ± SD of three independent experiments. p value: $^*p \leq 0.05$.

strains (F-IL-4/IL-10-NOD group) than in control mice (Figure 5(j)).

3.3. Changes in sIgA Levels but Not Cytokine Production Were Detected in the Intestinal Colon Environment. The levels of several cytokines (IL-2, IL-4, IL-10, IL-12, and TGF-β) in the colon homogenates from STZ-induced and NOD mouse models were measured and did not show significant differences among the groups (data not shown). However, there was decreased production of sIgA in all C57BL/6 mice that received bacterial strains (F-STZ, F-IL-4-STZ, F-IL-10-STZ, and F-IL-4/IL-10-STZ groups) in comparison to that of groups that received only oral saline (negative and VC groups) (Figure 6(a)). Interestingly, as shown in Figure 6(b), the saline-NOD and F-IL-4/IL-10-NOD groups exhibited similar levels of sIgA.

3.4. Detection of Orally Administered Bacterial Strains in the Liver and Mesenteric Lymph Nodes Was Transient. Translocation of the orally administered bacterial strains (*L. lactis* MG1363 FnBPA$^+$ (pValac::dts::*IL-4*) and *L. lactis* MG1363 FnBPA$^+$ (pValac::*IL-10*)) was not detected in the spleen (data not shown) at 24 h after gavage. However, we observed a minute quantity of bacterial translocation in the liver at 2 h and 4 h after oral administration of the bacteria (Figure 7(a)). Additionally, we also verified the presence of *L. lactis* MG1363 FnBPA$^+$ (pValac::dts::*IL-4*) and *L. lactis* MG1363 FnBPA$^+$ (pValac::*IL-10*) in MLNs, primarily at 6 h

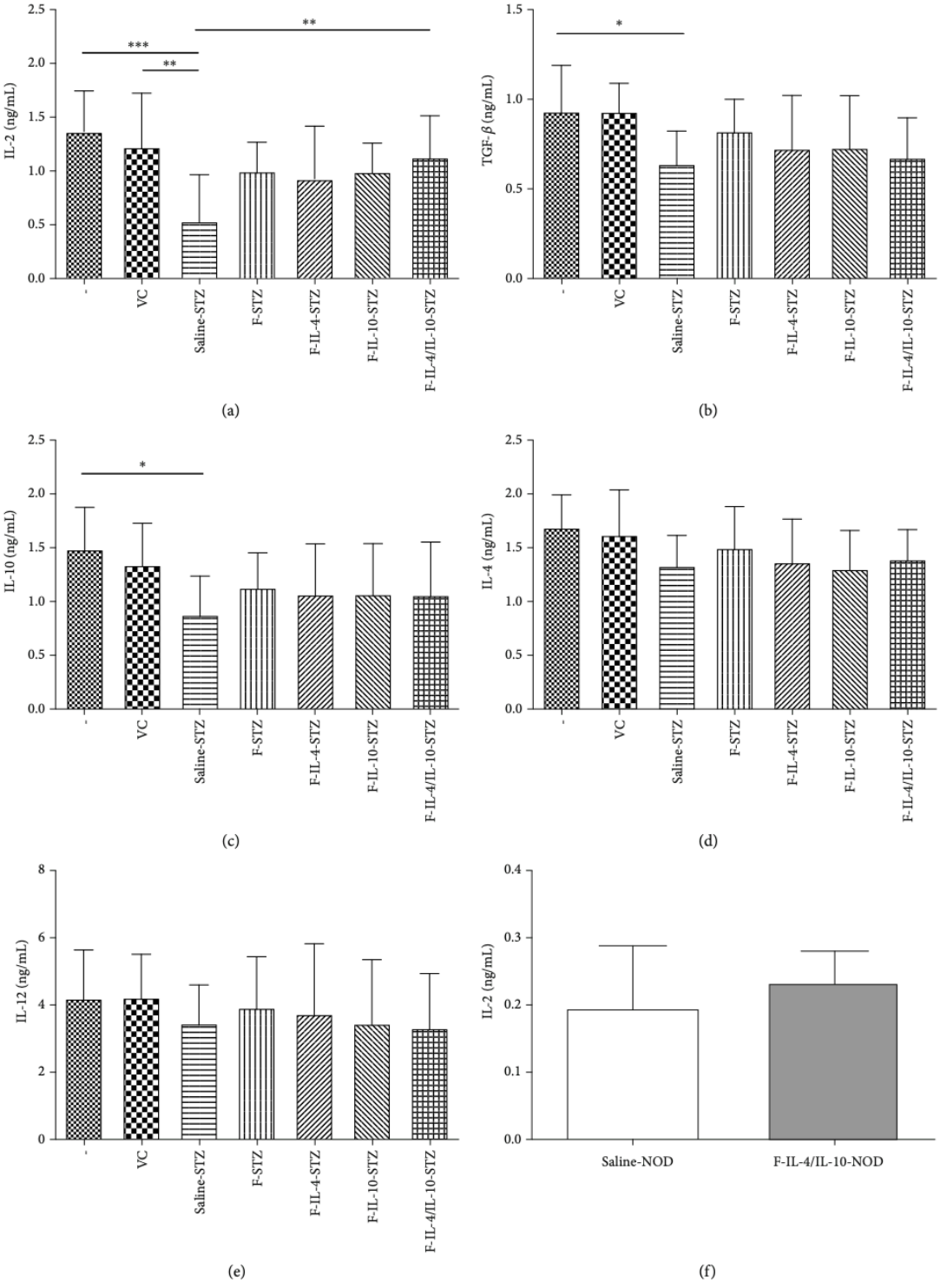


Figure 5: Continued.

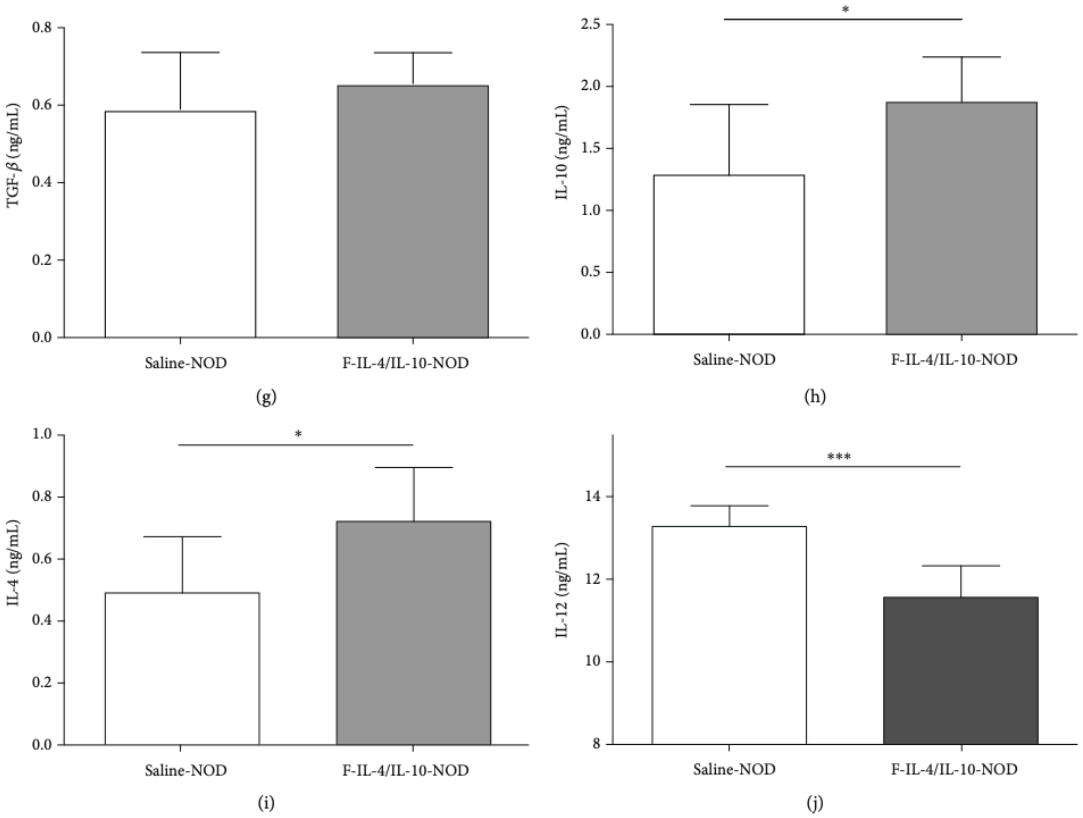


FIGURE 5: Pancreatic cytokine levels of STZ-induced C57BL/6 mice and NOD mice, which were both treated with oral bacterial administration. After the experimental period, the levels of IL-2, TGF-β, IL-10, IL-4, and IL-12 were analyzed in pancreatic homogenates from STZ model mice ((a–e), respectively; $n = 18$) and NOD mice ((f–j), respectively; $n = 12$). –: negative control; VC: vehicle control; F: mice that received *L. lactis* MG1363 FnBPA$^+$ i.g.; F-IL-4: mice that received *L. lactis* MG1363 FnBPA$^+$ (pValac::dts::*IL-4*) i.g.; F-IL-10: mice that received *L. lactis* MG1363 FnBPA$^+$ (pValac::*IL-10*) i.g.; F-IL-4/IL-10: mice that received *L. lactis* MG1363 FnBPA$^+$ (pValac::dts::*IL-4*) and *L. lactis* MG1363 FnBPA$^+$ (pValac::*IL-10*) i.g.; STZ: mice that received intraperitoneal injections of streptozotocin for five consecutive days; NOD: nonobese diabetic mice. The data are shown as the mean ± SD of three independent experiments. p value: $^*p \le 0.05$; $^{**}p \le 0.01$; $^{***}p \le 0.001$.

after oral gavage (Figure 7(b)). At 12 h and 24 h after bacterial administration, none of the genetically modified strains of *L. lactis* were found in the liver or MLNs.

3.5. Insulitis Progression Was Prevented by Combined Treatment with L. lactis Carrying Plasmids Encoding IL-4 and IL-10. Histological analysis demonstrated that treatment with both bacterial strains (*L. lactis* MG1363 FnBPA$^+$ (pValac::dts::*IL-4*) and *L. lactis* MG1363 FnBPA$^+$ (pValac::*IL-10*); (F-IL-4/IL-10-STZ group)) in STZ-induced mouse model preserved more pancreatic islets without visible signs of inflammation than when these strains were administered individually (F-IL-4-STZ and F-IL-10-STZ groups) (Figure 8(a)). As shown in Figures 8(a) and 8(b), insulitis scores also revealed that NOD mice treated with the combined administration of the bacterial strains encoding IL-4 and IL-10 successfully prevented inflammation progression in pancreatic islets during the observational period. Analysis demonstrated that mice from the F-IL-4/IL-10-NOD group presented 60% of the islets without signs of insulitis, compared with 5% in the saline-NOD control group.

4. Discussion

T1D is a chronic condition in which pancreatic β cells are destroyed by autoreactive T cells. After diagnosis, patients have to adapt their lives in an attempt to reach wellness mainly because the available treatment, insulin therapy, is laborious and requires constant care and attention. Therefore, more studies investigating T1D development and improved treatments are absolutely needed.

Early studies using multiple doses of STZ and NOD mice have shown that IL-4 and IL-10 have the potential to change the natural course of T1D by impairing insulitis and maintaining normal levels of glycemia [20, 34, 35]. However, the route of administration and delivery strategy of these cytokines in animal models of T1D is a concerning challenge. Thus, we decided to use the recombinant *L. lactis*, since it is

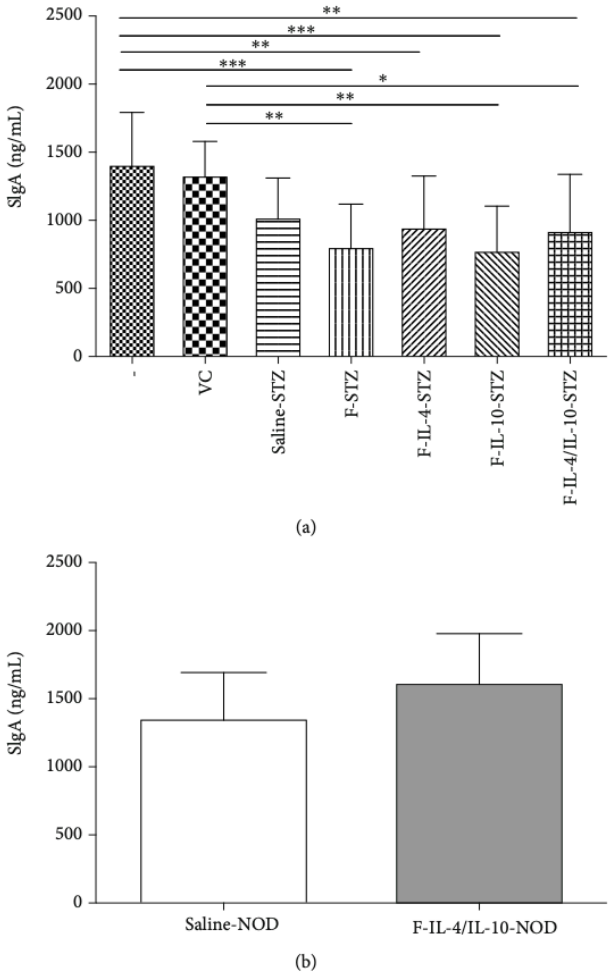


FIGURE 6: sIgA levels in intestinal lavage fluid from STZ-induced C57BL/6 mice and NOD mice, which were both treated with oral bacterial administration. Secretory IgA levels from (a) STZ-induced C57BL/6 mice ($n = 18$) and (b) NOD mice ($n = 12$). –: negative control; VC: vehicle control; F: mice that received *L. lactis* MG1363 FnBPA$^+$ i.g.; F-IL-4: mice that received *L. lactis* MG1363 FnBPA$^+$ (pValac::dts::*IL-4*) i.g.; F-IL-10: mice that received *L. lactis* MG1363 FnBPA$^+$ (pValac::*IL-10*) i.g.; F-IL-4/IL-10: mice that received *L. lactis* MG1363 FnBPA$^+$ (pValac::dts::*IL-4*) and *L. lactis* MG1363 FnBPA$^+$ (pValac::*IL-10*) i.g.; STZ: mice that received intraperitoneal injections of streptozotocin for five consecutive days; NOD: nonobese diabetic mice. The data are shown as the mean ± SD of three independent experiments. p value: $^*p \le 0.05$; $^{**}p \le 0.01$; $^{***}p \le 0.001$.

considered an ideal tool to mucosal delivery and oral tolerance induction [22]. Oral administration of *L. lactis* MG1363 FnBPA$^+$ strains carrying eukaryotic expression vectors encoding IL-4 and IL-10 (pValac::dts::*IL-4* and pValac::*IL-10*, respectively) to treat the two most extensively used animal models of T1D is reported here for the first time.

In the STZ-induced T1D model, we decided to verify the effects of the bacterial strains carrying the plasmids encoding IL-4 and IL-10 individually and combined. Despite all groups that received STZ injections became diabetic, it was noteworthy that the F-IL-4/IL-10-STZ group presented the highest number of mice protected against diabetes incidence.

Moreover, STZ-injected mice orally administered both bacterial strains encoding IL-4 and IL-10 showed pancreatic IL-2 levels similar to those of the negative and vehicle control groups and were significantly higher than the saline-STZ group. Previous studies reported that IL-2 plays a key role in the balance between immunoregulation and immunosuppression, since this cytokine is able to expand and activate regulatory T cells (Tregs), which subsequently provide active suppression of the effector response [36, 37]. Particularly in T1D, low doses of IL-2 can prevent the development of the disease [38]. It has already been reported that Tregs from patients with diabetes exhibit deficient IL-2 production and/or signaling, and if the IL-2 pathway is blocked, the frequency of Tregs decreases, and the number of individuals affected by T1D increases [39]. Thus, the normal levels of IL-2 in STZ-injected mice treated with both bacterial strains

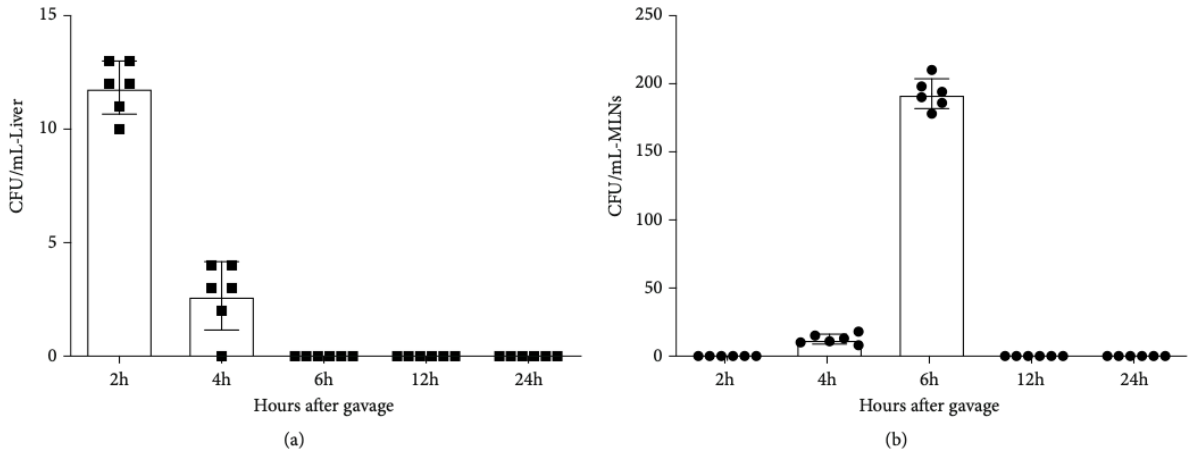


FIGURE 7: Transient translocation of *L. lactis* MG1363 FnBPA$^+$ (pValac::dts::*IL-4*) and *L. lactis* MG1363 FnBPA$^+$ (pValac::*IL-10*) was detected in the liver and MLNs. The organs were collected 2 h, 4 h, 6 h, 12 h, and 24 h after bacterial administration, macerated in 0.9% saline, and plated on selective medium. After 24 h of incubation at 30°C, the bacterial colonies in the plates corresponding to the liver (a) and MLNs (b) were counted. All results are presented as colony forming units (CFU) per mL. The data are shown as the mean of three independent experiments ($n = 6$).

carrying plasmids encoding IL-4 and IL-10 may have contributed to Treg activation in the pancreas and attenuated insulitis progression.

The evaluation of sIgA levels is important because it is already well established that this antibody exhibits immunomodulatory properties and is involved in not only responding to pathogens but also maintaining homeostasis between the gastrointestinal tract and commensal and ingested microorganisms [40–42]. In addition, low sIgA production may be related to a malfunction in immune cells in Peyer's patches in some immune-mediated diseases, such as celiac disease and T1D [43]. In the STZ-induced model, we noted that groups treated with the bacteria during few days had significantly reduced sIgA levels in the gut. However, the NOD mice that received bacteria orally for 15 weeks presented sIgA levels similar to those mice that did not receive bacteria. We believe this difference in sIgA levels between the T1D models can be explained by the long-term ingestion of bacteria in the NOD mouse model, which may have induced oral tolerance in mice, as previously reported by other studies using recombinant *L. lactis* by oral route [22].

Corroborating the others analysis, histological findings confirmed that combined treatment with the bacterial strains encoding IL-4 and IL-10 was more successful in attenuate insulitis progression than the individual administration of these bacterial strains. Results indicating more pancreatic islets with no signs of inflammatory infiltrates suggest a beneficial effect resulting from the combined bacterial strains. Some studies have shown that IL-4 and IL-10 induce a synergistic effect to suppress cellular immunity [44, 45]. Although the underlying mechanism is not well known, it is suggested that the combined and distinct actions of these cytokines could contribute to an enhanced outcome [26]. Since this is the first study using genetically modified *L. lactis* in the STZ-induced T1D model, these results were important and led to the investigation of the effects of the combined administration of *L. lactis* MG1363 FnBPA$^+$ strains carrying the IL-4 and IL-10-coding plasmids in the NOD mouse model.

It is commonly known that the high and uncontrolled levels of glycemia are associated with a rapid progression of disease and more severe complications in patients with T1D. Using the NOD mouse model, we demonstrated that oral administered *L. lactis* MG1363 FnBPA$^+$ (pValac::dts::*IL-4*) and *L. lactis* MG1363 FnBPA$^+$ (pValac::*IL-10*) are able to regulate glycemia levels effectively throughout the observational period. Therefore, the oral treatment with these genetically modified bacteria during 15 weeks resulted in the absence of T1D incidence in NOD mice.

In the present study, we demonstrated that oral administration of the bacterial strains led to systemic effects. NOD mice treated with *L. lactis* MG1363 FnBPA$^+$ encoding IL-4 and IL-10 by oral route presented significantly increased levels of IL-10 in serum and pancreatic tissue. IL-10 is a major anti-inflammatory cytokine that can modulate immune processes, inhibiting the synthesis of proinflammatory cytokines and favoring a tolerant status [46]. IL-10 has been shown to be active at mucosal surfaces, acting synergistically and enhancing oral tolerance in NOD mouse model [47]. IL-10 can also stabilize Treg function through maintenance of the Foxp3 expression [48]. Moreover, Takiishi et al. suggest that the production of IL-10 by Treg cells is the major mechanism responsible by regulation of inflammation reached by *L. lactis*-based therapy in the NOD mouse model [49]. T1D is related to inflammation, and hence, it is reasonable to speculate that increased levels of IL-10 obtained in serum and pancreas of NOD mice treated with the genetically modified *L. lactis* encoding IL-4 and IL-10 was at least initially due to the bacteria administration and subsequent expression of these cytokines. We suggest this

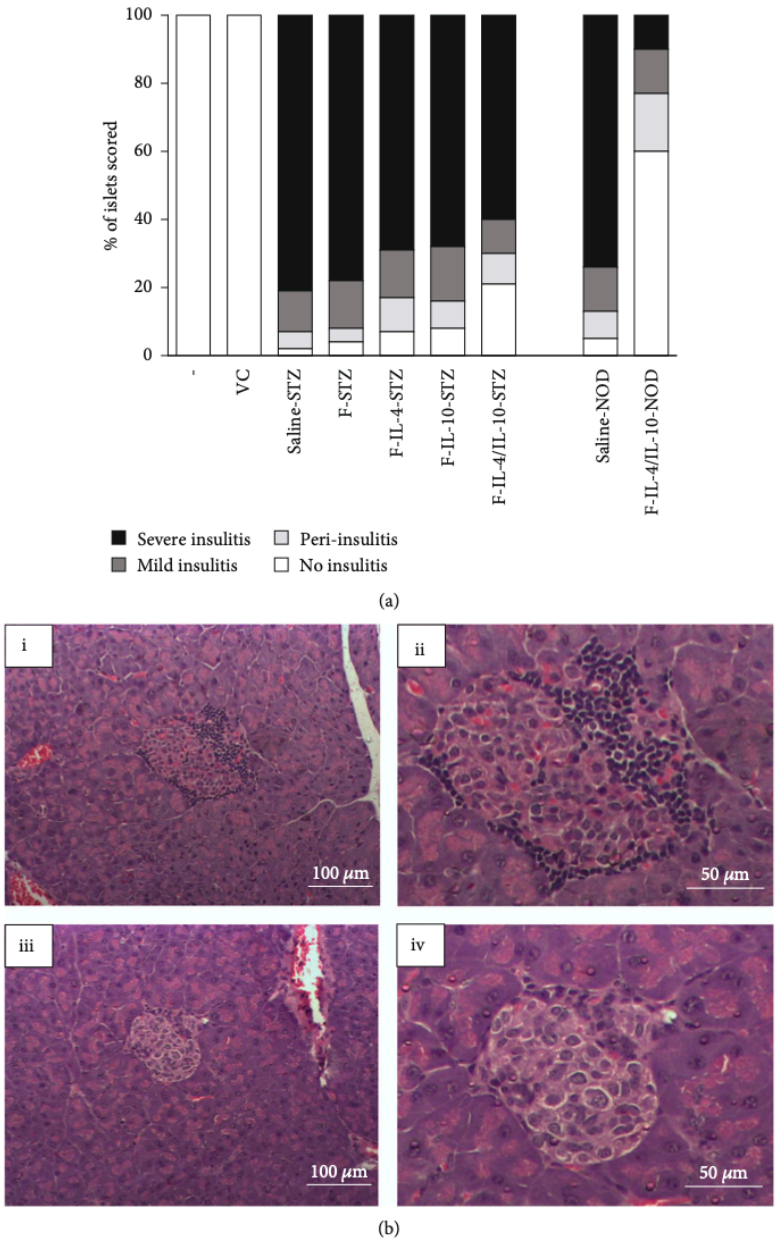


FIGURE 8: Combined administration of *L. lactis* MG1363 FnBPA$^+$ (pValac::dts::*IL-4*) and *L. lactis* MG1363 FnBPA$^+$ (pValac::*IL-10*) exerts protective effects on pancreatic islets. (a) Insulitis scoring was performed in a blind manner on paraffin-embedded pancreatic sections of STZ-induced mice and NOD mice at the end of experimental period. At least 15 islets were evaluated in each pancreatic sample. The data are shown as the mean of three independent experiments ($n = 6$). (b) A representative pancreatic islet from the saline-NOD group (i-ii) and F-IL-4/IL-10-NOD group (iii-iv) is shown.

result was crucial for regulation of inflammation observed, probably through Treg cells, and consequent control of glycemia and no incidence of T1D.

IL-4 is a pleiotropic immunomodulatory cytokine produced and secreted primarily by Th2 cells. Approaches favoring Th2 activity and administration of cytokines that downregulate Th1 activities, such as IL-4 and IL-10, have shown success in suppress insulitis and prevent diabetes [17, 18, 26]. Previous studies showed that both administration of recombinant IL-4 and pancreatic expression of IL-4 were able to protect NOD mice from diabetes [17, 50]. Furthermore, Li et al. also revealed that alteration in cytokine

production in T cells, skewing to IL-4 production may contribute to disease protection in NOD mice [51]. In agreement with previously reported results, oral treatment with *L. lactis* MG1363 FnBPA$^+$ (pValac::dts::*IL-4*) and *L. lactis* MG1363 FnBPA$^+$ (pValac::*IL-10*) showed promising results indicating increased levels of IL-4 in serum and pancreas of NOD mice, further corroborating the beneficial role of the strategy tested here for the prevention of T1D.

Additionally, both IL-4 and IL-10 appear to suppress the production of the proinflammatory cytokine IL-12 by antigen-presenting cells (APCs), thereby downregulating the differentiation of Th1 cells [52, 53]. Accumulating evidence indicates that IL-12 is a key cytokine which induces the differentiation of Th1 cells leading to diabetes acceleration in NOD mice [54–56]. Additionally, Nitta et al. reported that IL-12 locally produced by pancreatic-infiltrating cells plays a pathologic role in the development of T1D [57]. Therefore, we suggest the significant reduction observed in the pancreatic concentration of IL-12 of NOD mice treated with both bacterial strains contributed to the regulation of inflammation, not favoring the differentiation of Th1 cells nor the production of proinflammatory cytokines.

More clear evidence of the protective effect of the combined administration of *L. lactis* MG1363 FnBPA$^+$ (pValac::dts::*IL-4*) and *L. lactis* MG1363 FnBPA$^+$ (pValac::*IL-10*) was obtained by the histological analysis. Corroborating the normoglycemia presented by NOD mice that received oral treatment with both bacterial strains, the majority of pancreatic islets was preserved, without signs of insulitis. Collectively, our data indicate the preservation of pancreatic islets is a result of the reduced autoimmune destruction, triggered by the increased levels of IL-4 and IL-10 and the decreased levels of IL-12. Consequently, this alteration in cytokine production in pancreatic environment possibly altered the T cells subsets, skewing to Treg cell proliferation, which may have contributed to the disease protection.

Genetically modified *L. lactis* have been used as a versatile tool for immunotherapy in several inflammatory conditions, such as T1D [21]. Since these bacterial strains can survive the entire gastrointestinal tract and are used for delivery of plasmids and proteins to mucosal cells, we assessed bacterial translocation to some extragastrointestinal organs. Previous studies have shown that bacterial translocation can occur on a frequent basis even in healthy conditions [58–60]. Genetically modified *L. lactis* encoding IL-4 and IL-10 were detected mostly in MLNs, which are known to be the key site for oral tolerance. The induced Treg cells proliferate in MLNs and are subsequently released in circulation to modulate pancreatic beta cell autoimmune destruction in a IL-10-dependent manner [21, 61, 62]. Bresson et al. demonstrated the expansion of Treg cells producing IL-4 and IL-10 was determinant to suppress autoreactive response in animal models of T1D [63]. Promising studies using genetically modified *L. lactis* as a delivery tool of antigens and bioactive compounds revealed the induction of oral tolerance and proliferation of Treg cells were able to revert new-onset diabetes in NOD mice [49, 64]. Thus, we hypothesized the oral treatment with *L. lactis* MG1363 FnBPA$^+$ (pValac::dts::*IL-4*) and *L. lactis* MG1363 FnBPA$^+$ (pValac::*IL-10*) was able to protect against beta-cell destruction due to expansion of Treg cells producing IL-4 and IL-10 in pancreas. Additionally, neither genetically modified bacteria was detected in the MLNs, spleen, and liver from 12 h after oral administration, indicating that these strains are indeed transient, which is a relevant attribute in the context of biosafety practices.

Altogether, the protective effect against T1D triggered by the oral administration of *L. lactis* MG1363 FnBPA$^+$ strains carrying pValac::dts::*IL-4* and pValac::*IL-10* verified in NOD mice was not observed in the STZ-induced model. This difference between the two main T1D models has already been reported in other studies [65–67], and it may be associated with the distinct characteristics of these models. The STZ model is characterized by rapid destruction of insulin-producing cells in the pancreas through a process involving dynamic variation of immune cells [68–70]. Since STZ is able to induce diabetes even in the absence of B and T cells, interpretation of the results requires prudence when the analysis is correlated with human development of T1D [71, 72]. In this context, NOD mice integrate the genetic component and the implications of the immunopathological process, which makes this a suitable model for testing therapies that aim to modulate the autoimmune response [72, 73].

Regarding the limitations of our study, we could not provide all the desirable experimental control groups with NOD mice, since the acquisition and maintenance of these animals are costly. However, the assessments performed with all groups in the STZ-induced model showed that we should focus efforts on the combined administration of bacterial strains carrying the cytokine-coding plasmids. Additionally, these limitations do not invalidate our valuable findings on combined administration of *L. lactis* MG1363 FnBPA$^+$ strains carrying the eukaryotic expression plasmids encoding IL-4 and IL-10 in the NOD mouse model.

In conclusion, the results of our study demonstrate that the combined administration of *L. lactis* MG1363 FnBPA$^+$ strains carrying the eukaryotic expression plasmids pValac::dts::*IL-4* and pValac::*IL-10* attenuated the initial progression of insulitis in the STZ-induced model by increasing levels of IL-2 in pancreatic tissue and protected NOD mice against T1D in the observational period. Our findings suggest the dynamic cytokine signaling triggered by the oral treatment with the genetically modified bacteria plays a crucial role in the immunomodulation of the disease in the NOD mouse model. Our results showed the oral treatment with *L. lactis* MG1363 FnBPA$^+$ strains encoding IL-4 and IL-10 can effectively reduce the incidence of T1D and control the glycemia levels in NOD mice. Additionally, we demonstrated this combined treatment was able to prevent insulitis progression, primarily by increasing the levels of IL-4 and IL-10, known by suppressing the proinflammatory cytokine milieu, in pancreas and serum and decreasing the levels of the proinflammatory cytokine IL-12 in pancreas. This is the first study to administer orally the combined genetically modified *L. lactis* strains carrying eukaryotic expression plasmids encoding IL-4 and IL-10 in the STZ-induced and in the NOD mouse models. In summary, our study preliminarily

provides novel and relevant observations, enhancing the potential of this innovative approach as part of an alternative therapy to T1D.

Data Availability

The data used to support the findings of this study are included within the article.

Ethical Approval

All applicable international, national, and institutional guidelines for the care and use of animals were followed. The planning, conduct, and management of animals, including biosecurity policies and practices, were submitted to and approved by the Ethics Committee on Animal Use (CEUA, Protocol no: 206/2016) of UFMG/Brazil.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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ANEXO 3:

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

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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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Background

Inflammatory bowel diseases (IBD), including Crohn's disease (CD) and ulcerative colitis (UC), are intestinal disorders characterized by chronic inflammation in the gastrointestinal tract (GIT), and although sharing clinical and pathological features, they differ in their histological aspect and cytokine profiles [1]. The exact etiology and pathogenesis of these diseases are unclear despite much research in the last decades, but it is nowadays generally accepted that they are caused by deregulation of the mucosal immune system towards the native intestinal microbiota in genetically susceptible individuals, resulting in an inappropriate and excessive activation of the intestinal immune system [2]. As no unique and defined causal agent is responsible for the development of these diseases, current treatment proposals, including anti-inflammatory drugs, immunosuppressants and antibiotics, only improve the patient's quality of life while presenting serious side effects and no cure, revealing that better, cheaper and longer lasting treatments are necessary [3].

IL-10-deficient mice (IL-$10^{-/-}$) not raised in a defined specific-pathogen-free (SPF) environment develop a spontaneous gut inflammation, called enterocolitis, that is characterized by weight loss and anemia at the age of 4–6 weeks, lethal up to the age of 3 months [4], and is most severe in the colon, involving the small intestine to a lesser extent. The typical inflammatory lesions of this disease are discontinuous and transmural, and include epithelial hyperplasia, crypt abscesses, ulcers, mucin depletion and bowel wall thickening with infiltration of lymphocytes, plasma cells, macrophages, eosinophils and neutrophils [4]; its development is mediated by CD4+ T cells and an uncontrolled Th1 response [5].

Interleukin-10 (IL-10) is one of the most important anti-inflammatory cytokines in shaping mucosal immune responses in the gut [4, 6]. This cytokine is produced by regulatory T cells, epithelial cells, macrophages, dendritic cells and B1 cells [7] and therefore presents many important properties, including stimulation of B-cell differentiation and immunoglobulin secretion [8] and suppression of macrophage activation to inhibit inflammatory cytokines production. IL-10 is a good therapeutic candidate against IBD due to its immunosuppressive activity and central role in downregulating inflammatory cascades [9, 10]; systemic and oral treatments with recombinant human IL-10 were first tested in CD patients but presented important drawbacks, such as short half-life and extreme sensitivity of the gastrointestinal tract [11–14]. Steidler et al. developed an IL-10-producing *Lactococcus lactis* (*L. lactis*) strain [15] and although initial studies showed promising results, no statistically significant difference in mucosal healing between patients receiving the recombinant strains and a placebo were observed [16].

To overcome IL-10's sensitivity and survival in the gastrointestinal tract, our research group used a novel strategy based on the use of the eukaryotic DNA expression vector pValac, constructed by ourselves in 2009, and a *L. lactis* strain to deliver and trigger DNA expression by epithelial cells of the host. Basically, after oral administration of the bacterial strain carrying the plasmid of interest, these enter target cells, bacteria suffer lysis and plasmids are liberated to the cytoplasm and transferred into the cell's nucleus. Subsequently, the cell's machinery is responsible for the expression of the ORF of interest, translation and protein synthesis [17]. Advantages of this strategy include the targeting of mucosal immunity, simple technology and low cost as well as safe use of *L. lactis*, as this strain does not colonize the gastrointestinal tract and transits it between 2 and 3 days, not becoming part of the normal gut flora.

The constructed *L. lactis* strain carrying the therapeutic pValac:*il-10* plasmid delivers it directly to the host's cells. This strategy does not only ensure a more effective and direct delivery of the therapeutic plasmid but consequently a higher and more efficient IL-10 in situ production. This strategy successfully showed its anti-inflammatory effect and intestinal inflammation prevention in two induced inflammatory mouse models: TNBS (2,4,6-Trinitrobenzenesulfonic acid) and DSS (Dextran Sodium Sulphate) [18, 19], showing to be innovative and promising for the therapeutic IBD treatment.

In this line, the aim of the present work was to evaluate the *L. lactis* MG1363 FnBPA+ (pValac:*il-10*) strain as therapy in IL-$10^{-/-}$ mice and evaluate its capacity to diminish and/or prevent the onset of enterocolitis.

Results

Evaluation of the animals' weight

The animals' weight was assessed throughout the 6 weeks of experimentation, starting at the age of 2 weeks. All experimental groups presented an initial similar weight gain, according to the normal growth of these animals and development of the disease (Fig. 1). However, IL-$10^{-/-}$ mice from the KO and FnBPA groups showed an overall lower weight gain after 2 weeks of experimentation, when compared with the healthy control and the pValac:*il-10* groups. On the other hand, IL-$10^{-/-}$ mice from the pValac:*il-10* group showed rapid weight gain which equaled that of the healthy control group after the 6 weeks of experimentation. Moreover, this group also showed statistically significant differences with the KO and FnBPA groups in weeks 4, 5 and 6 ($p < 0.001$).

Macroscopic and histologic evaluation

The healthy control group presented neither macroscopic nor histologic intestinal lesions (Figs. 2 and 3a)

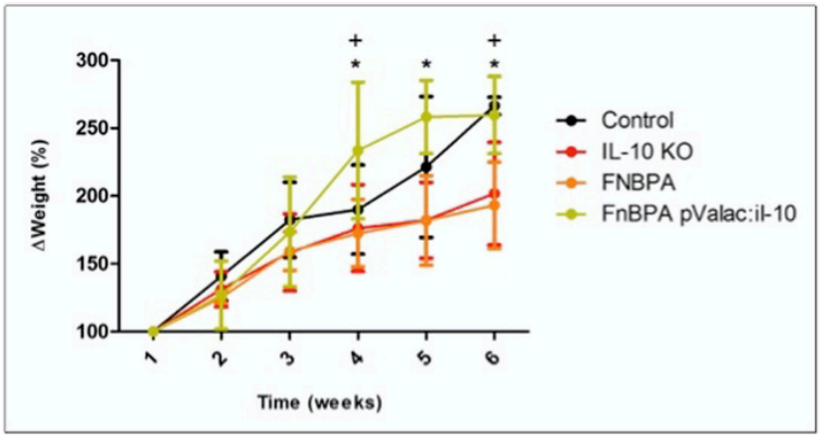


Fig. 1 Corporal weight variation (%) of mice along the 6 weeks of experimentation. Healthy control group: negative control of intestinal inflammation, KO: IL-10$^{-/-}$ mice - positive control of intestinal inflammation; FnBPA: IL-10$^{-/-}$ mice that received the *L. lactis* MG1363 FnBPA+ strain; pValac:*il-10*: IL-10$^{-/-}$ mice that received the *L. lactis* MG1363 (pValac:*il-10*) strain. +: Experimental group (control) whose percentage of corporal weight is statistically different from those of group pValac:*il-10* in week 4 and groups KO and FnBPA in week 6. *: Experimental group (pValac:*il-10*) whose corporal weight percentage is statistically different from those of groups KO and FnBPA in weeks 4, 5 and 6 ($p < 0.001$). The data is presented as the mean ± SD

and is not shown in Fig. 2 as its macroscopic score was 0. Hematoxylin-eosin (HE) staining of samples of the ascending colon (AC) showed that this group presented a normal histological architecture with an intact mucosa, thin submucosal and serosal layers, muscular layer thickness compatible with the analyzed segment and normal proportion of goblet cells. No signs of degenerative, vascular, inflammatory or pathological cell proliferation processes were observed (Fig. 3a).

All IL-10$^{-/-}$ mice, except those from the pValac:*il-10* group, presented similar macroscopic and histologic damage scores regarding the samples of their AC (Figs. 2 and

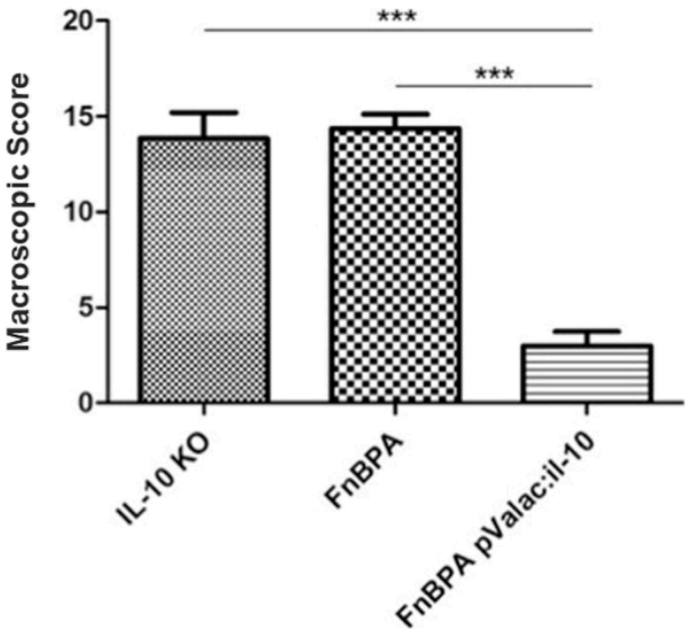


Fig. 2 Macroscopic score of the different groups. Bars represent the mean $N = 9 \pm$ SD. Asterisks represent statistical significance (***$p < 0.0001$)

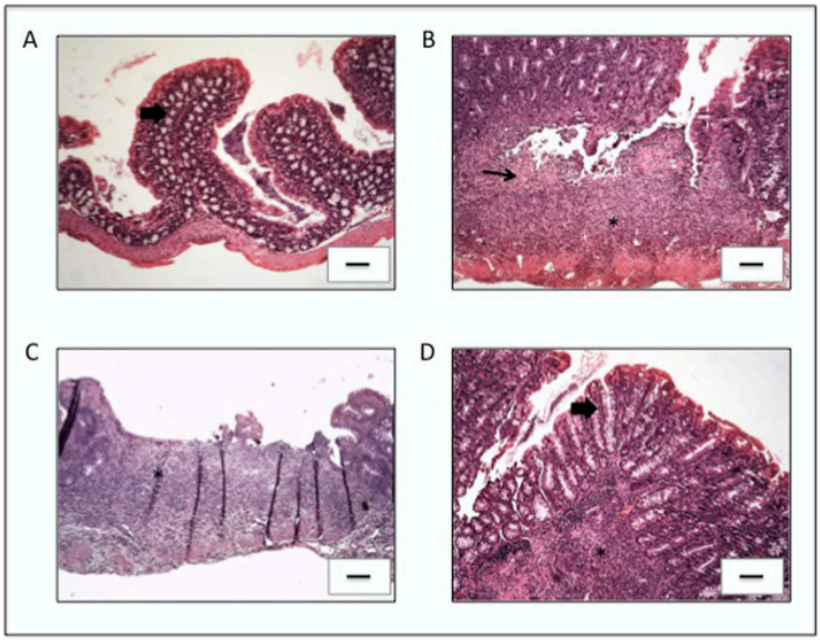


Fig. 3 Histopathology of changes in the AC of all experimental groups. **a** Control group; **b** KO group; **c** FnBPA group and **d** pVala:*il-10* group. Presence of inflammatory infiltrate (asterisks) and mucosal lesion (black thin arrow) can be observed in varied degrees of intensity. The thick arrow highlights the preserved mucosal architecture with the presence of goblet cells. The strains were obtained from the tissue of AC stained with HE. The bar in each image represents 100 μm

3b-c). It was possible to observe a compromised histological architecture with moderate inflammatory infiltrate in the mucosa and presence of mononuclear cells. Areas of erosion and reduction of goblet cells were also observed, as well as moderate inflammatory infiltrate in the submucosal layer and oedema. No evident alterations were observed in the muscular and the serosa layer (Fig. 3b and c). These results show that the disease strongly affected the AC region.

The pValac:*il-10* group, however, showed that administration of *L. lactis* MG1363 FnBPA+ (pValac:*il-10*) to IL-10$^{-/-}$ mice was capable of strongly attenuating the pathology's development, presenting a much lower macroscopic damage score and statistically significant differences with the KO and FnBPA groups (Fig. 2). Furthermore, the pValac:*il-10* group also showed significant improvement of histological patterns: the mucosa of the AC of this group presented light inflammation in less than 40% of animals, with no inflammatory alteration in the rest, no present signals of erosion nor depletion of goblet cells and no inflammatory infiltrate in the submucosal layer (Fig. 3d).

After evaluation of the histopathological damage of AC samples, the histological score was performed, and as expected, the IL-10$^{-/-}$ control and *L. lactis* MG1363 FnBPA+ groups showed statistically significant differences with the *L. lactis* MG1363 FnBPA+ (pValac:*il-10*) group (Fig. 4).

HE staining of samples of the descending colon (DC) of the healthy control group showed that all fragments of this group presented patterns of normality, with normal histological architecture and integral mucosa without erosion and with presence of goblet cells in the adequate proportion. The muscular layer thickness observed, thin submucosal and serosal layers, were compatible with the analyzed segment and no signs of degenerative or pathological cell proliferation processes were observed. Some few animals presented a light inflammatory infiltrate in the region of the mucosa and oedema in the submucosa (Fig. 5a).

IL-10$^{-/-}$ animals of the KO and FnBPA groups presented a compromised histological architecture. HE staining of DC samples of these groups showed moderate inflammatory infiltrate in the mucosa, marked by mononuclear cells; however, no areas of erosion, ulceration or with goblet cells reduction were observed. Moderate oedema and light infiltrate were observed in the submucosa region, with no evident alteration in the muscular and serosa layers (Fig. 5b and c). These results show that the disease also strongly affects the descendant region of the colon.

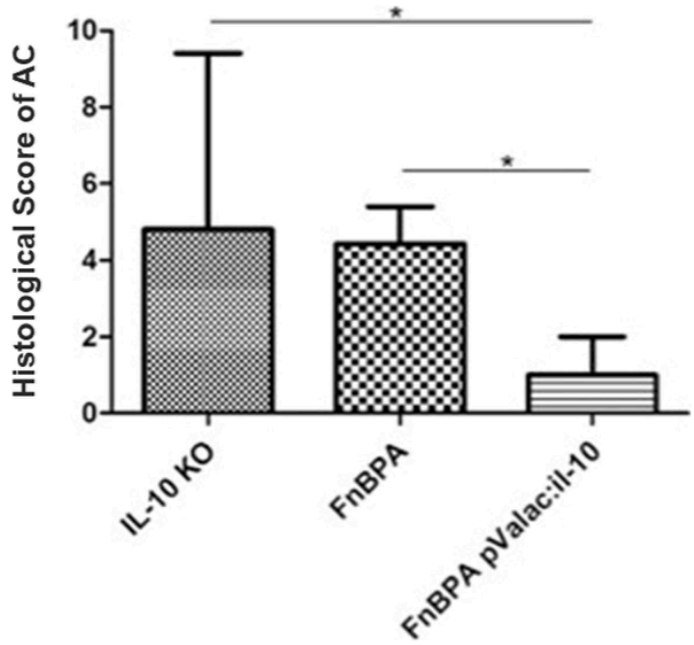


Fig. 4 Histological score of AC samples of all groups. Bars represent the mean $N = 9 \pm SD$. Asterisks represent statistical significance ($*p < 0.05$)

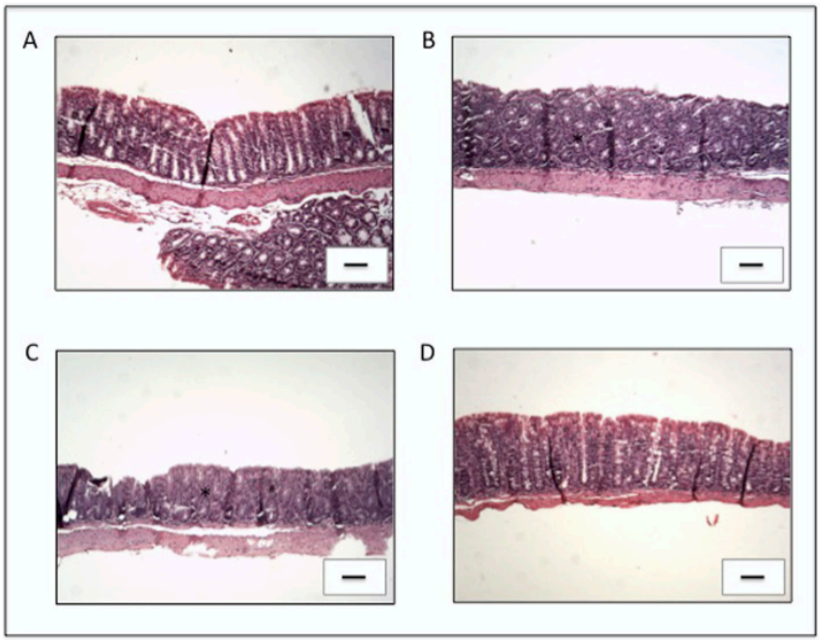


Fig. 5 Histopathology of changes in the DC of all experimental groups. **a** Control group; **b** KO group; **c** FnBPA group and **d** pVala:*il-10* group. Presence of inflammatory infiltrate (asterisks) can be observed in varied degrees of intensity. The strains were obtained from the tissue of DC stained with HE. The bar in each image represents 100 μm

IL-10$^{-/-}$ animals that received the *L. lactis* MG1363 FnBPA+ (pValac:*il-10*) strain showed an attenuated pathologic pattern, with light inflammatory infiltrate in the mucosa and no observed alteration in the epithelium. Moreover, alterations in the submucosa (oedema and infiltrate) were considered light or even absent in some animals (Fig. 5d).

Again, after analysis of histopathological damages of DC samples, the histological score of all groups was realized and showed, as expected, that the KO and FnBPA groups showed major histological damage scores than the *L. lactis* MG1363 FnBPA+ (pValac:*il-10*) group (Fig. 6).

Administration of *L. lactis* MG1363 FnBPA+ (pValac:*il-10*) results in IL-10 production in all evaluated tissues

All IL-10$^{-/-}$ mice from the groups that did not receive any treatment (KO and FnBPA) did not produce IL-10 (Fig. 7). This in turn resulted in statistically significant differences ($p < 0.0006$) with the control group in the small intestine and spleen (Fig. 7c-d) and with the pValac:*il-10* group in all evaluated tissues. As expected, *L. lactis* MG1363 FnBPA+ (pValac:*il-10*) administration to IL-10$^{-/-}$ mice led to IL-10 production in all evaluated tissues. It was quite surprising to observe that IL-10 levels in the control group were lower than in the pValac:*il-10* group, especially in the AC and spleen (Fig. 7a and d), where these were even statistically significant ($p < 0.05$ and $p < 0.001$, respectively).

Administration of the pValac:*il-10* plasmid tends to alter the production of IL-6 and IFN-γ cytokines

L. lactis MG1363 FnBPA+ (pValac:*il-10*) administration to IL-10$^{-/-}$ mice resulted in lower IL-6 production levels in the AC when compared to both the KO and FnBPA groups; however, these were not statistically significant (Fig. 8a). In the small intestine, although not being statistically significant, IL-6 levels of the pValac:*il-10* were lower than in the KO group but slightly higher than in the FnBPA group (Fig. 8b).

In relation to IFN-γ production, the pValac:*il-10* group presented lower levels both in the AC and small intestine with no statistically significant difference with the other groups (Fig. 9). Again, regarding lower IL-6 levels in the pValac:*il-10* group, administration of the *L. lactis* MG1363 FnBPA+ (pValac:*il-10*) strain to IL-10$^{-/-}$ mice tended to reduce IFN-γ production levels.

No bacterial translocation was observed after oral administration

No bacterial translocation was detected in the spleen and liver up to 24 h following oral administration of bacterial strains (Table 1).

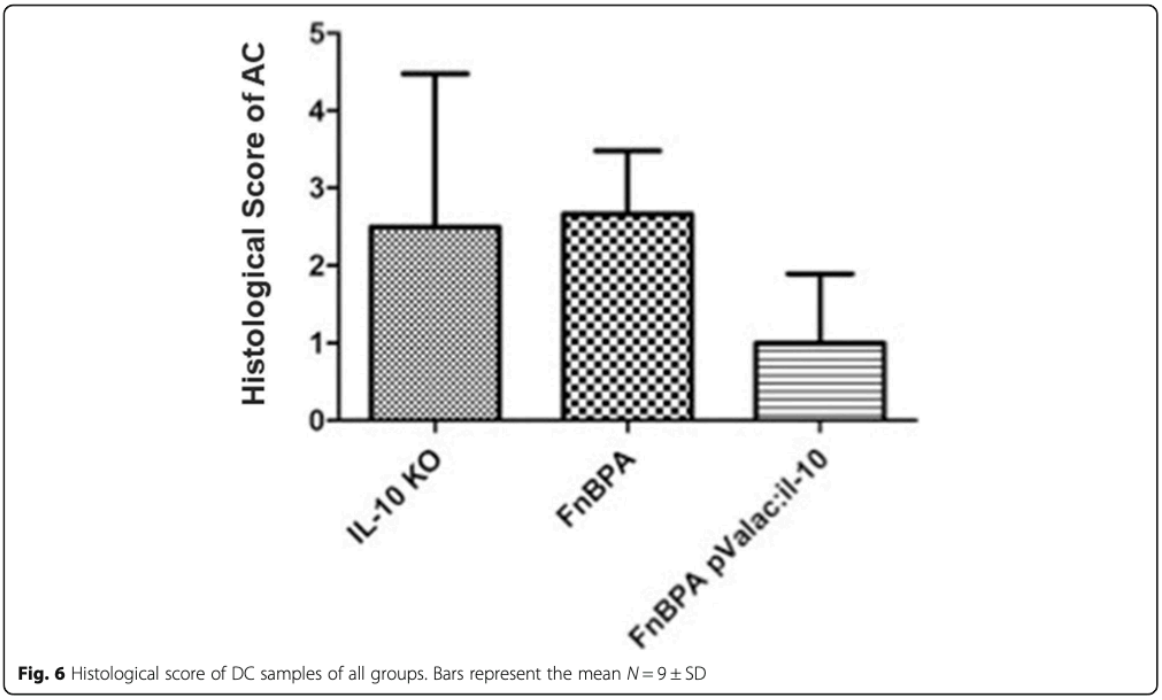


Fig. 6 Histological score of DC samples of all groups. Bars represent the mean $N = 9 \pm SD$

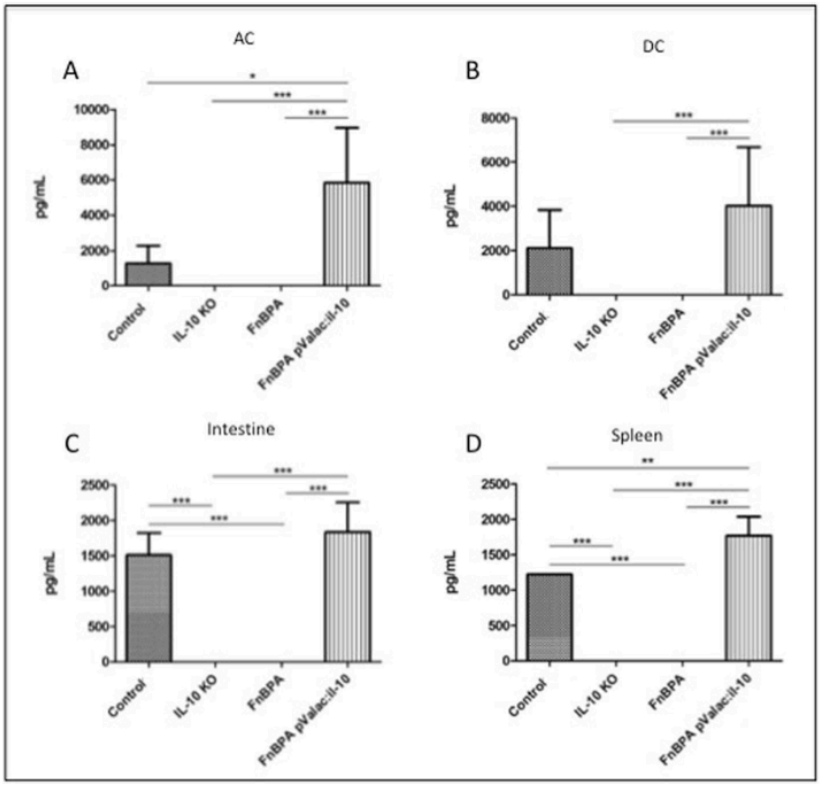


Fig. 7 Administration of the pValac:*il-10* plasmid resulted in IL-10 production by IL-10$^{-/-}$ mice in all evaluated tissues. Levels of IL-10 in (**a**) AC, (**b**) DC, (**c**) small intestine and (**d**) spleen of control, KO, FnBPA and pValac:*il-10* groups. Bars represent the mean $N = 9 \pm$ SD. The asterisks represent statistical significance (*$p < 0.05$, **$p < 0.001$ or ***$p < 0.0006$)

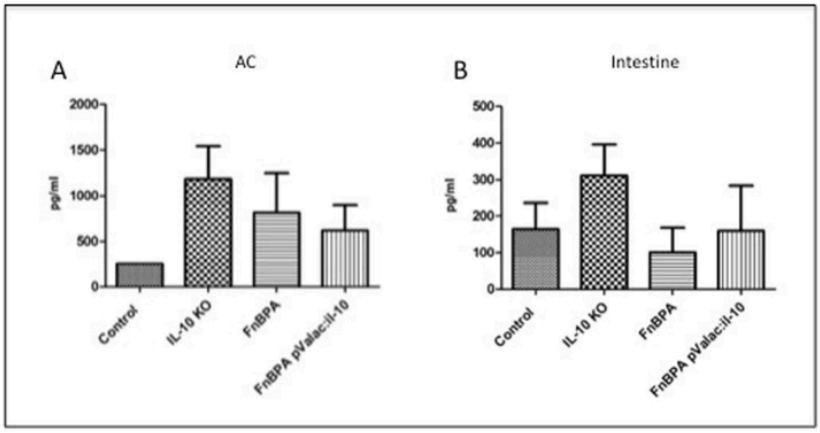


Fig. 8 Administration of the pValac:*il-10* plasmid modulates the production of IL-6 in all tissues evaluated. Levels of IL-6 in (**a**) AC and (**b**) small intestine of control, KO, FnBPA and pValac:*il-10* groups. Bars represent the mean $N = 9 \pm$ SD

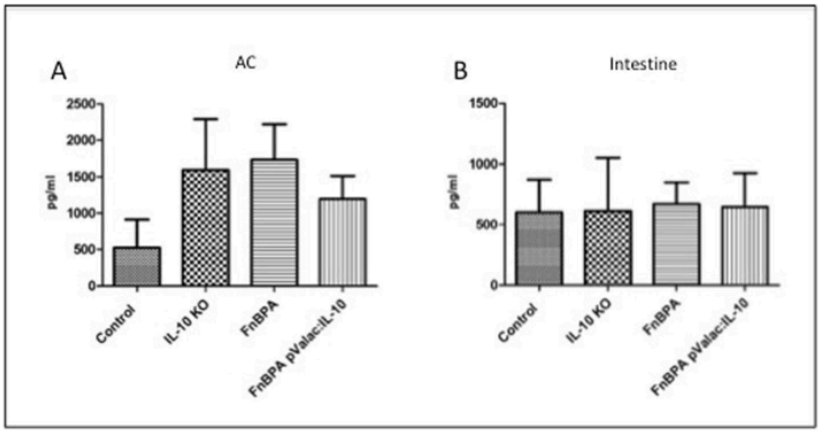


Fig. 9 Effect of the pValac:*il-10* plasmid administration in IFN-γ production. Levels of IFN-γ in (**a**) AC and (**b**) small intestine of control, KO, FnBPA and pValac:*il-10* groups. Bars represent the mean $N = 9 \pm SD$

Discussion

Much research in the last decades has focused in unveiling the etiology and pathogenesis of IBDs to develop therapies for their treatment, as no cure for them exists. Many strategies have based their research on the use of IL-10 due to its anti-inflammatory properties and key role in the development of the disease, as evidenced by IL-$10^{-/-}$ mice, which develop enterocolitis, a severe disease in the colon characterized by weight loss, anemia and strong inflammatory lesions, apparent already at 4 weeks of age and lethal up to the age of 3 months, when maintained under conventional conditions [4].

The research and development of therapies using IL-10 are based on its delivery directly at the intestinal mucosa, as its correct administration and targeting to the sites of inflammation has been the major bottleneck for successful results. These strategies have included the use of polymer-based microparticles [20] and the use of bacterial strains capable of secreting human IL-10 [21–23].

As a new and innovative approach for IL-10 production at inflammation sites, our research group developed a new strategy that involved the use of a *L. lactis* strain expressing FnBPA [17, 24], that has the capacity to efficiently internalize and trigger recombinant DNA expression by human epithelial cells, as delivery vehicle of a eukaryotic expression vector coding for IL-10, pValac:*il-10*, directly to the host's cells in the GIT for recombinant IL-10 in situ production. This strategy does not only ensure a more effective and direct delivery of the therapeutic plasmid but consequently a higher and more efficient IL-10 production. This strategy showed to be capable of preventing inflammation in the TNBS-and DSS-induced mouse models [18, 19].

Table 1 Bacterial translocation

	Time after gavage				
Bacterial Translocation	2 h	4 h	6 h	12 h	24 h
Liver	0	0	0	0	0
Spleen	0	0	0	0	0

In this research, 2 weeks old mice received the pValac:*il-10* plasmid during six consecutive weeks to evaluate its therapeutic effect. Macroscopic and histological analysis showed that non-treated mice presented an overall lower weight gain during this period while those under treatment showed a rapid weight gain equaling that of the healthy control group. This growth retardation in IL-$10^{-/-}$ mice could be explained by the severe lesions in the GIT that led to disturbed nutrient absorption, contrary to the animals that received the therapy and consequently produced IL-10. Regarding the inflammatory damage in the AC and DC, mice from the KO and FnBPA groups presented higher macroscopic damage scores and important lesions in the colon while IL-$10^{-/-}$ mice that received the pValac:*il-10* plasmid showed an attenuated pathology's development and as such a much lower macroscopic damage scores, showing that IL-10 administration does prevent a worse onset of inflammation and therefore disease development.

Other important parameters measured during this period were the levels of pro-inflammatory and anti-inflammatory cytokines. The normal gastrointestinal inflammatory response is tightly regulated, and the balance of pro-inflammatory and anti-inflammatory cytokines produced by CD4+ Th1 and Th2 cells is an important part of this regulatory process [25]. Intestinal inflammation in IL-$10^{-/-}$ mice is characterized by dysregulation of the immune system with regulatory T cells incapable of developing or functionally impaired in the absence of

IL-10, leading to activation of Th1 cells and overproduction of pro-inflammatory cytokines resulting in chronic inflammation [5]. However, administration of the pValac:*il-10* plasmid led to IL-10 production by mice naturally incapable of producing this cytokine, which, as a consequence, lowered the disease development when compared to IL-10$^{-/-}$ mice that did not receive any treatment. As already mentioned, IL-10 is essential to control intestinal immune responses. Moreover, some evidence suggests that IL-10 can inhibit the translocation of nuclear factor-kB, inhibiting the immediate-early pro-inflammatory response [26] and downregulate acute inflammatory responses.

This IL-10 production altered the production of pro-inflammatory cytokines, such as IL-6, which presented lower levels in all tissues of treated mice. IL-6 is produced by T and B lymphocytes, which infiltrate inflammatory lesions in IL-10$^{-/-}$ mice, and which presents high levels during IBDs, therefore playing a functional role in their pathogenesis [4]. Moreover, in IL-10$^{-/-}$ mice, IL-6 can also be considered an inflammatory mediator responsible for maintaining/increasing the intestinal inflammation [5] and its levels were therefore measured. On the other hand, IFN-γ, identified as a major mediator initiating colitis in IL-10$^{-/-}$ neonates [5, 27], showed increased levels in IL-10$^{-/-}$ mice as a result of failed regulatory T cells production or even functionality in the absence of IL-10, suggesting that Th1 cells are activated very early in the disease process [27]. A higher IFN-γ production is related to a more severe colitis [28].

To confirm that the genetically modified *L. lactis* strain is indeed transient through the gastrointestinal tract and does not colonize it, bacterial translocation to the spleen and liver was evaluated. No bacterial translocation was observed in these organs at any of the evaluated times (2 h, 4 h, 6 h, 12 h and 24 h) after oral administration. This is a particularly important safety parameter regarding human use.

The work here presented represents the last step in validating this DNA delivery strategy using the *L. lactis* MG1363 bacterial strain. When first constructing the pValac:*il-10* plasmid, this was tested for its transfection efficiency and subsequent IL-10 production and secretion by eukaryotic cells through ELISA, confocal microscopy and FACS [18]. Following, this *L. lactis* MG1363 FnBPA+ (pValac:*il-10*) strain was tested in two animal models to evaluate not only the success of the strategy in delivering the pValac*il-10* plasmid and following in situ IL-10 production and secretion by the animals cells, but also its immunomodulatory potential in diminishing the onset and development of intestinal inflammation due to increased IL-10 production, when compared with the control groups. In 2013, del Carmen and co-workers demonstrated that the severity of TNBS-induced intestinal inflammation decreased in mice after administration of this strain as a result of an increase in IL-10 levels and decrease in IFN-γ and IL-17 levels, when compared to the not treated groups [18]. Later, Zurita-Turk et al. demonstrated that administration of this *L. lactis* strain resulted in decreased severity of DSS-induced intestinal inflammation, probably also due to increased IL-10 levels and decreased IL-6 levels [19]. These studies showed that direct IL-10 production at inflammation sites produced an anti-inflammatory environment, as well as downregulated Th1-and Th17-mediated inflammation, capable of diminishing the onset and severity of the disease.

Thus, the main interest when testing this bacterial strain in the IL-10$^{-/-}$ model was to evaluate if animals would produce IL-10 and consequently diminish the development of the intestinal inflammation and as such, validate the efficiency of this DNA delivery strategy. The results here presented are very exciting, as they demonstrate that our strategy leads to IL-10 production by mice that are naturally not able to, as well as higher production when compared to healthy mice capable of normally producing this cytokine, however, at a lower rate.

However, this current study presents one research limitation: FACS analyses were not performed. FACS analyses would have brought better knowledge regarding the mechanism of pValac:*il-10* delivery and uptake/invasion into eukaryotic cells, as well as which are the IL-10-producing cell populations. From the obtained results it can be stated that the proposed strategy was successful, as the pValac:*il-10* plasmid was delivered to eukaryotic cells in IL-10$^{-/-}$ mice and subsequent IL-10 production was responsible diminish the development of intestinal inflammation. Nevertheless, the exact mechanism and which cells populations were involved, were not elucidated. In this context, future studies are required to answer these current research limitations.

Conclusions

To evaluate the therapeutic capacity in preventing the onset of spontaneous enterocolitis in IL-10$^{-/-}$ mice, *L. lactis* MG1363 FnBPA+ (pValac:*il-10*) was administered to 2-week old IL-10$^{-/-}$ mice for six consecutive weeks. One feature characterizing the development of this disease is low weight gain, and mice receiving our therapy showed normal weight gain along the 6 weeks of treatment, comparable to the healthy control group. Moreover, these animals also showed overall lower macroscopic and histological damages, both in the AC and DC, when compared to not treated IL-10$^{-/-}$ mice, showing capability in attenuating the pathology's development and improving histological patterns. Moreover, tendency to lower IL-6 and IFN- γ levels were observed in the AC and small intestine, probably due to IL-10 production.

All these results show that administration of the *L. lactis* MG1363 FnBPA+ strain carrying the therapeutic pValac:*il-10* plasmid attenuated the development of spontaneous intestinal inflammation in IL-$10^{-/-}$ mice by maintaining an anti-inflammatory environment in the GIT. Thus, administration of this strain led to IL-10 production by these animals and in this way diminished the onset of inflammation. These results, together with those presented in the TNBS- and DSS-induced inflammatory models, strengthen the potential of this strategy in the therapeutic treatment of IBD.

Methods

Bacterial strains, growth conditions and plasmid

The bacterial strains and plasmid used in this work are listed in Table 2. *L. lactis* strains were grown in M17 medium (Difco, Sparks, MD, USA) supplemented with 0.5% glucose (GM17) at 30 °C without shaking and bacterial selection was performed by addition of 10 μg/mL of chloramphenicol (Cm) and/or 5 μg/mL of erythromycin (Ery). *L. lactis* cultures were grown until an OD_{600} of 1.0–1.2 and stocked in glycerol 80% (1:4); on animal feedings day, doses were centrifuged to eliminate any traces of antibiotic and medium and resuspended in 1 mL of saline solution (0.15 M NaCl).

Animals

To evaluate the therapeutic capacity of the *L. lactis* MG1363 FnBPA+ (pValac:*il-10*) strain in diminishing and/or preventing the onset of enterocolitis, wild-type and IL-$10^{-/-}$ mice on a 129Sv/Ev genetic background were used, as these are the most susceptible to colitis development with homogeneous clinical manifestation [5]. As these animals develop a spontaneous and gradual intestinal inflammation when not maintained in SPF conditions, mice were, for experimental procedures, maintained in collective cages in conventional conditions in an environmentally controlled room with a 12-h light/dark cycle and free access to water and food.

For experimental procedures, experimental groups were adjusted in relation to the animal's weight at the age of 2 weeks and consisted of: i) healthy control group, ii) IL-$10^{-/-}$ control group (KO group) and the IL-$10^{-/-}$ groups that received the iii) *L. lactis* MG1363 FnBPA+ strain (FnBPA group) or the iv) *L. lactis* MG1363 FnBPA+ (pValac:*il-10*) strain (pValac:*il-10* group). As the disease is already apparent at the age of 4 weeks, the in vivo assays started with 2-week old mice that received intragastrically, over six consecutive weeks, 100 μL of the corresponding bacterial strain as suspension at a dose of 2×10^9 CFU/100 μL or 100 μL of 0.9% saline solution (control groups) per day. After 6 weeks (at the age of 8 weeks), all mice were euthanized by overdose of a ketamine (100 mg/kg)/xylazine (10 mg/kg) mixture. The total n of this experimental procedure was 9.

Mice were kindly donated by Dr. Ana Maria Caetano de Faria from the Federal University of Minas Gerais (UFMG - Belo Horizonte, Brazil). All animal procedures and manipulations were approved by the Ethics and Research Committee on Animal Experiments (CEUA) of the Biological Institute of the Universidade Federal de Minas Gerais (UFMG - Belo Horizonte, Brazil) with protocol number 66/2011.

Macroscopic and histologic assessment of enterocolitis

Animals' weight was rigorously evaluated during the 6 weeks of experimental procedure to assess the macroscopic damage of the disease. After this time, as already described in the previous section, animals were euthanized by overdose of a ketamine/xylazine mixture and the colons were excised to assess colonic inflammation using a previously defined scoring system that evaluates the following features: presence or absence of adhesions, strictures and diarrhea (loose, watery stool), and bowel wall thickness (mm). As enterocolitis can affect the colon in different degrees, all colon samples were divided in ascendant and descendant regions for separate evaluation.

All colon samples were fixed in formalin and processed for histologic analysis. HE stained sections were blindly scored based on a semiquantitative scoring system previously described [30] that grades the following features: destruction extent of normal mucosal architecture (0: normal; 1: mild, 2: moderate and 3: extensive damage), presence and degree of cellular infiltration (0: normal; 1: mild, 2: moderate and 3: transmural infiltration), extent of muscle thickening (0: normal; 1: mild, 2: moderate and 3: extensive thickening), presence (1) or absence (0) of crypt abscesses and presence (1) or absence (0) of goblet cell depletion. Scores for each

Table 2 Bacterial strains and plasmid

Bacterial strain	**Characteristics**	**Font**
Lactococcus lactis MG1363 FnBPA+	*L. lactis* MG1363 strain expressing FnBPA of *S. aureus*	[29]
Lactococcus lactis MG1363 FnBPA+ (pValac:*il-10*)	*L. lactis* MG1363 strain expressing FnBPA of *S. aureus* carrying the pValac:*il-10* plasmid	[18]
Plasmid	**Characteristics**	
pValac:*il-10*	(pCMV/CmR/RepA/RepC/IL-10)	[18]

feature were summed up to a maximum possible score of 11.

Tissue preparation and cytokine assay

Samples of AC and DC, small intestine and spleen were weighed and homogenized in phosphate-buffered saline (PBS) containing 0.05% (v/v) Tween-20, 0.1 mM phenylmethylsulphonyl fluoride, 0.1 mM benzethonium chloride, 10 mM EDTA and 20 KIU Aprotinin A using a tissue homogenizer (1 mL/0.1 g) for cytokine assays. Tissue fragments were then homogenized and centrifuged for 10 min at 600 g and 4 °C and supernatants were collected for cytokine assay, as previously described [31]. Briefly, 96-well plates (Nunc) were coated with purified monoclonal antibodies reactive with cytokines IL-6, IL-10 and IFN-γ (BD-Pharmingen) overnight at 4 °C. Wells were washed the next day, supernatants were added and plates incubated overnight at 4 °C. Following, biotinylated monoclonal antibodies against cytokines were added and plates were incubated for 2 h at room temperature. Color reactions were developed at room temperature with 100 μL/well of orthophenylenediamine (OPD) (1 mg/mL) (Sigma, St. Louis, MO, USA), 0.04% H_2O_2 substrate in sodium citrate buffer. Reactions were interrupted by addition of 20 μL/well of 2 N H_2SO_4 and absorbance was measured at 492 nm by an ELISA microplate reader (Bio-Rad). Results were expressed as concentration (pg/mL), according to the standard curve.

Bacterial translocation

IL-$10^{-/-}$ mice received 100 μL of *L. lactis* MG1363 FnBPA+ (pValac:*il-10*) intragastrically at a dose of 2×10^9 CFU/100 μL. Spleen and liver were collected under strict aseptic conditions 2 h, 4 h, 6 h, 12 h and 24 h after bacterial administration. Tissues were macerated in 500 μL of 0.9% saline and 100 μL of this homogenate was plated in M17 medium (Sigma-Aldrich) supplemented with agar, 0.5% glucose, chloramphenicol (10 μg/mL) (Sigma-Aldrich) and erythromycin (5 μg/mL) (Sigma-Aldrich). Plates were incubated at 30 °C for 24 h.

Statistical analysis

Statistical analyses were performed using the GraphPad Prism 5.0 software (San Diego, CA, USA). Student's t-test or analysis of variance (ANOVA) followed by a Tukey comparison post-hoc test were used to assess the significance of differences among groups. Means were considered statistically different when $p < 0.05$ and all results were expressed as mean ± standard deviation (SD).

Abbreviations

AC: Ascending colon; ANOVA: Analysis of variance; CD: Crohn's disease; Cm: Chloramphenicol; DC: Descending colon; DSS: Dextran Sodium Sulphate; Ery: Erythromycin; GIT: Gastrointestinal tract; HE: Hematoxylin-eosin; IBD: Inflammatory bowel diseases; IL-10: Interleukin-10; IL-$10^{-/-}$: IL-10-deficient mouse model; *L. lactis*: *Lactococcus lactis*; OPD: Orthophenylenediamine; PBS: Phosphate-buffered saline; SD: Standard deviation; SPF: Specific-pathogen-free; TNBS: 2,4,6-Trinitrobenzenesulfonic acid; UC: Ulcerative colitis; UFMG: Universidade Federal de Minas Gerais

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Authors' contributions

MZT conceived the study, performed the research, collected, analyzed and interpreted the data and wrote the paper. BMS, CPC, VBP, VPC and TMP participated with the realization of the experiments and contributed to the scientific discussion. AMCF kindly provided the mice for animal experimentation and contributed to the scientific discussion. DCCM carried out all histological analyses and contributed to the scientific discussion. AM conceived the study, participated in its design and coordination, helped to draft the manuscript and gave final approval for publication. All authors read and approved the final manuscript. MZT and AM share co-senior authorship.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

All animal procedures and manipulations were approved by the Ethics and Research Committee on Animal Experiments (CEUA) of the Biological Institute of the Universidade Federal de Minas Gerais (UFMG - Belo Horizonte, Brazil) with protocol number 66/2011.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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ANEXO 4:

“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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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 $m1^{-1}$) (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

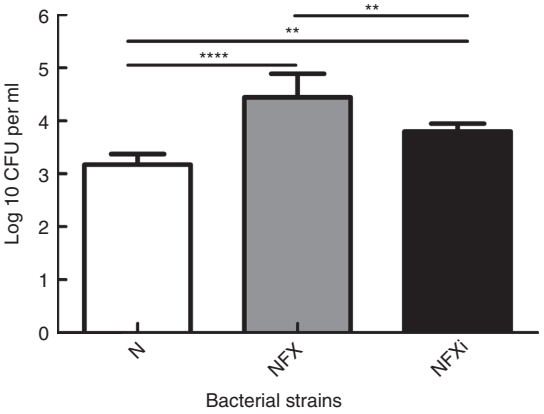


Figure 1 Assays of *Lactococcus lactis* NCDO2118 FnBPA+ (pXYCYT:Hsp65) invasion into Caco-2 cells. Bacterial strains: N: *L. lactis* NCDO2118—noninvasive/wild-type strain (□); NFX: *L. lactis* NCDO2118 FnBPA+ (pXYCYT:Hsp65)—invasive strain not expressing Hsp65 (■); and NFXi: *L. lactis* NCDO2118 FnBPA+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

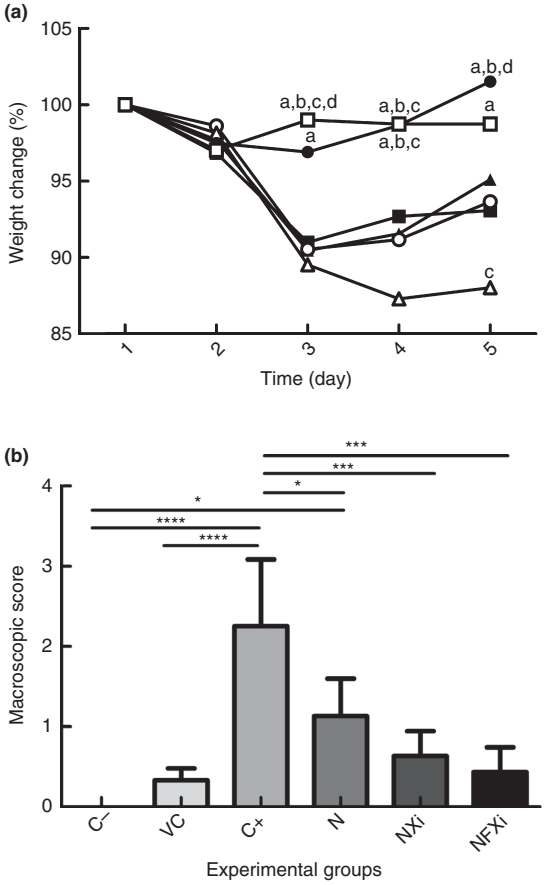


Figure 2 Body weight variations and macroscopic scores of mice with acute TNBS-induced colitis. (a) Percentage of initial body weight of BALB/c mice with or without TNBS administration as a function of time (n = 18). Experimental groups: C−: negative control for colitis (□); VC: TNBS vehicle control (●); 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: $^{*}P < 0{\cdot}05$; $^{***}P < 0{\cdot}001$; $^{****}P < 0{\cdot}0001$.

diarrhoea, the animals in the NFXi group showed a significant decrease in inflammation ($P < 0{\cdot}05$) (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 ($P < 0{\cdot}05$) (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) ($P < 0{\cdot}05$). 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 ($P < 0{\cdot}05$) (Fig. 4).

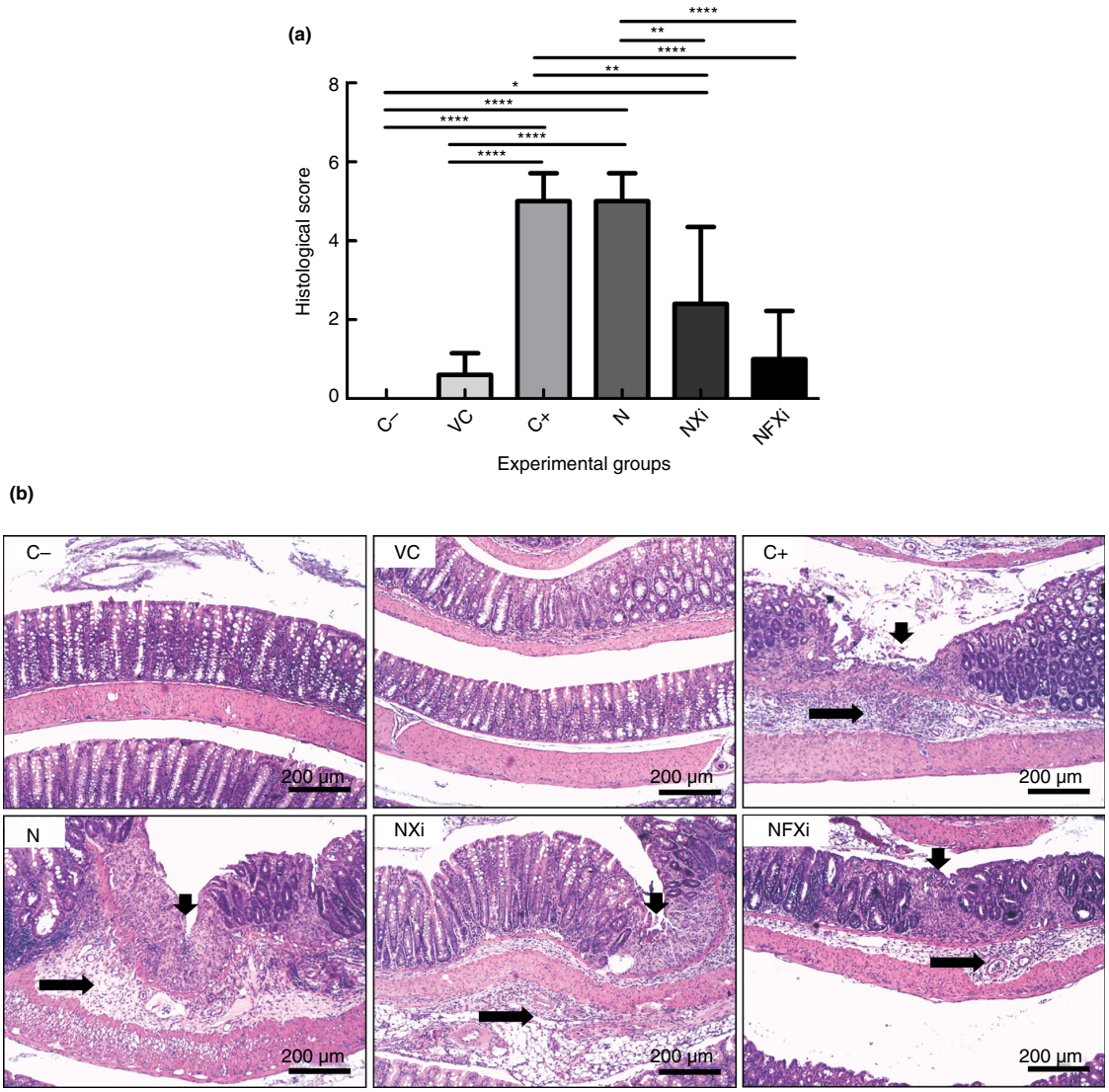


Figure 3 Scores and histological features of the colon of mice with acute TNBS-induced colitis. (a) Histological scores of BALB/c mice with or without TNBS administration. Experimental groups: C−: negative control for colitis (□); VC: TNBS vehicle control (▒); C+: positive control for colitis (▒); N: *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: $^{*}P < 0{\cdot}05$; $^{**}P < 0{\cdot}01$; $^{****}P < 0{\cdot}0001$.

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

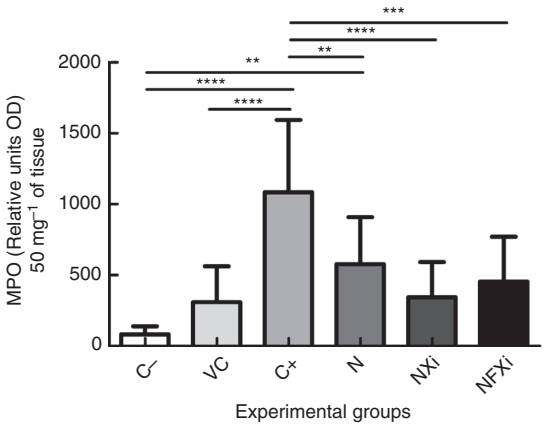


Figure 4 Myeloperoxidase (MPO) levels in BALB/c mice subjected to TNBS administration or not. Experimental groups: C−: negative control for colitis (□); VC: TNBS vehicle control (▒); C+: positive control for colitis (▒); N: *Lactococcus lactis* NCDO2118—noninvasive/wild-type strain (■); NXi: *L. lactis* 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

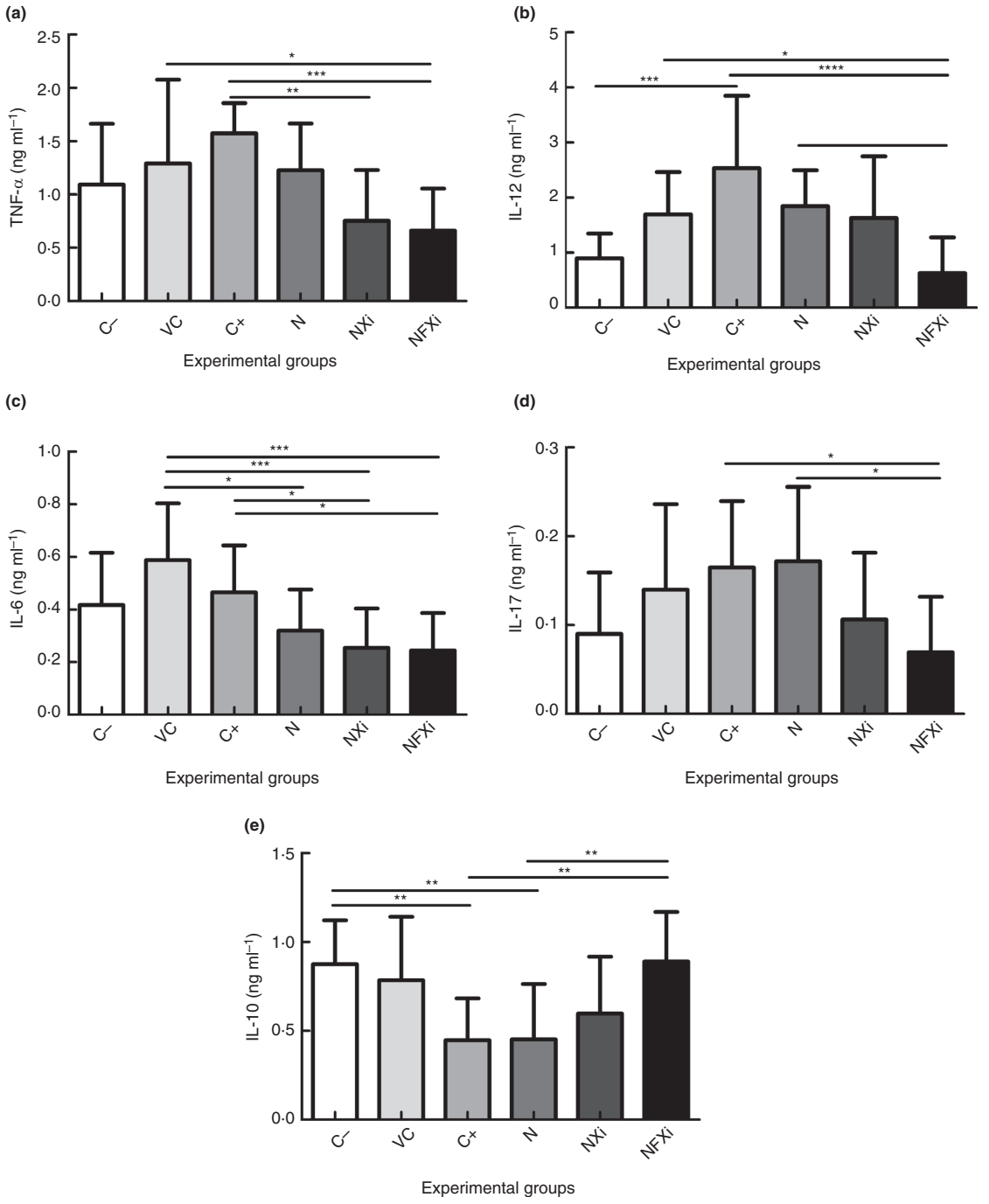


Figure 5 Cytokine levels in BALB/c mice subjected to TNBS administration or not. (a) Tumour necrosis factor α (TNF-α) levels. (b) Interleukin-12 (IL-12) levels. (c) Interleukin-6 (IL-6) levels. (d) Interleukin-17 (IL-17) levels. (e) Interleukin-10 (IL-10) levels. Experimental groups: C−: negative control 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: $^{*}P < 0{\cdot}05$; $^{**}P < 0{\cdot}01$; $^{***}P < 0{\cdot}001$; $^{****}P < 0{\cdot}0001$.

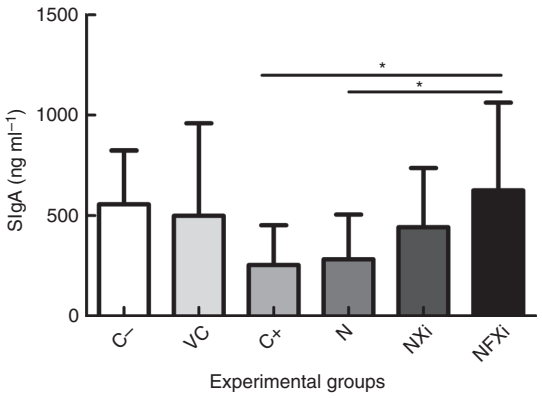


Figure 6 sIgA levels in BALB/c mice subjected to TNBS administration or not. Experimental groups: C−: negative control for colitis (□); VC: TNBS vehicle control (▒); C+: positive control for colitis (▒); N: *Lactococcus lactis* NCDO2118—noninvasive/wild-type strain (▒); NXi: *L. lactis* NCDO2118 (pXYCYT:Hsp65)—noninvasive 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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ANEXO 5:

“Mycobacterial Hsp65 antigen delivered by invasive *Lactococcus lactis* reduces intestinal inflammation and fibrosis in TNBS-induced chronic colitis model”

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Mycobacterial Hsp65 antigen delivered by invasive *Lactococcus lactis* reduces intestinal inflammation and fibrosis in TNBS-induced chronic colitis model

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Intestinal fibrosis associated with Crohn's disease (CD), which a common and serious complication of inflammatory bowel diseases. 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. 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. The oral administration of this strain significantly attenuated the severity of inflammation and intestinal fibrosis in mice ($p < 0.05$). 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. 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.

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 twenty-first 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.

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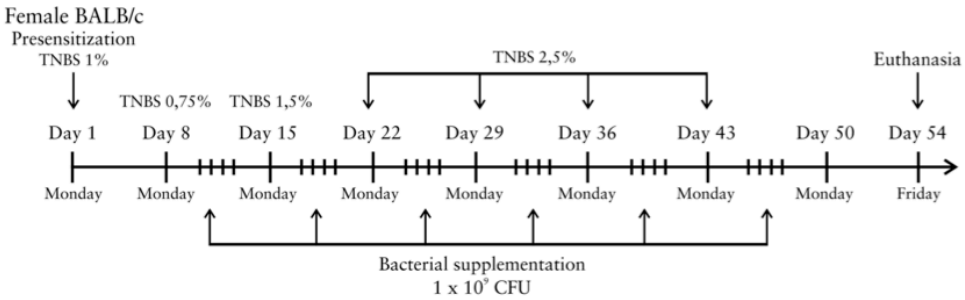


Figure 1. Representation of the experimental design for 2,4,6-trinitrobenzene sulfonic acid (TNBS)-induced chronic colitis. Intestinal inflammation was induced by TNBS in BALB/c μwas induced by weekly (on days 8, 15, 22, 29, 36 and 43) intrarectal administration of solutions with increasing concentrations 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 1×10^9 CFUs per week for 6 consecutive weeks. On day 54, all the mice were euthanized.

Consistent with this notion, HSP60 modulates immune responses, and this modulation leads to the down-regulation 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 h (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-h light/12-h 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. no. p2297, São Paulo, Brazil)] using a previously described technique[21]. After a 6-h 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 Fig. 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 1×10^9 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]: (1) negative control of chronic colitis (C− group), the mice received 0.9% saline (i.g.) and 0.9% saline (i.r.); (2) positive control of chronic colitis (C+ group), the mice received 0.9% saline (i.g) and TNBS (i.r.); (3) NFX group, the mice were treated with invasive *L. lactis* FnBPA+ (pXYCYT:Hsp65) strain not expressing Hsp65 (i.g.) and TNBS (i.r.); and (4) 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 0–4, 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)[22].

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[22].

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 (1) 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]; (2) buffer I (pH 4.7; 0.1 M NaCl, 0.02 M Na_3PO_4, and 0.015 M Na_2EDTA); and (3) 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 min. Subsequently, and 0.002% H_2O_2 was added, and the mixture was incubated for more than 5 min 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 1200 rpm and 4 °C for 10 min. 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).

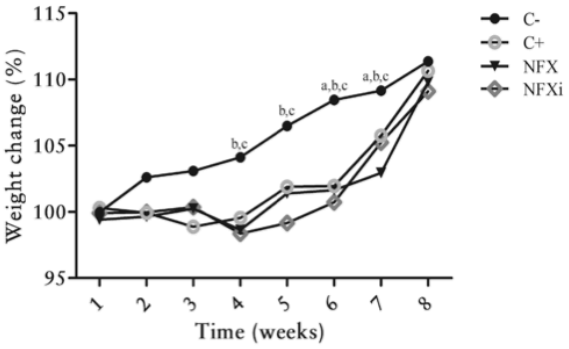


Figure 2. Percentage of the initial body weight of BALB/c mice with or without TNBS administration as a function of time. **a** Experimental group whose body weight percentages were significantly different from those of the C+ group. **b** Experimental group whose body weight percentages were significantly 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: $^{*}p < 0.05$.

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 Fig. 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.

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.

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).

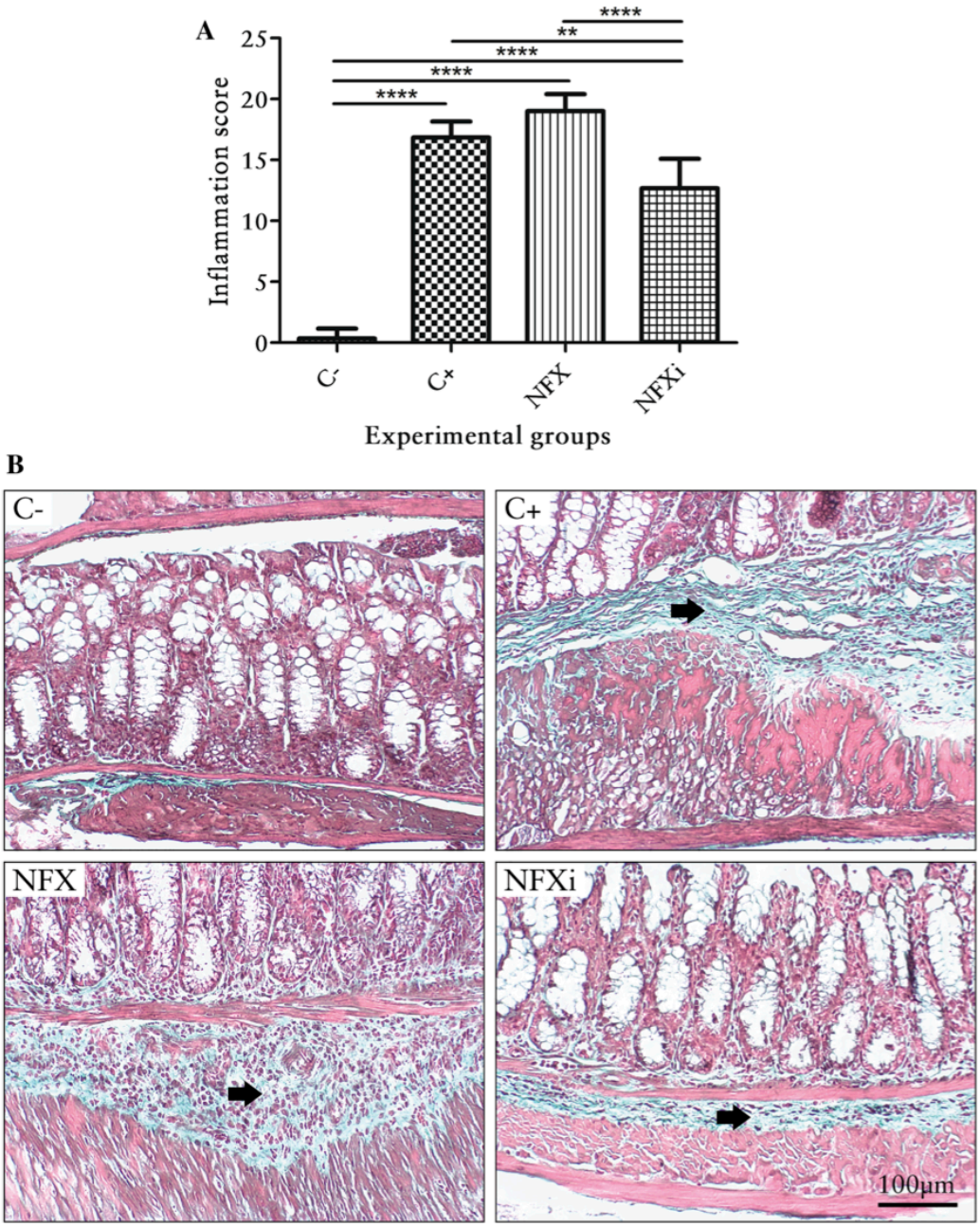


Figure 3. Inflammation scores and histological features of the colon of mice with chronic TNBS-induced colitis. (**A**) Inflammation scores of BALB/c mice with or without TNBS administration. (**B**) Histological features of the colon of BALB/c mice with or without TNBS administration (H&E staining). 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: $^{**}p < 0.01$; $^{****}p < 0.0001$.

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.

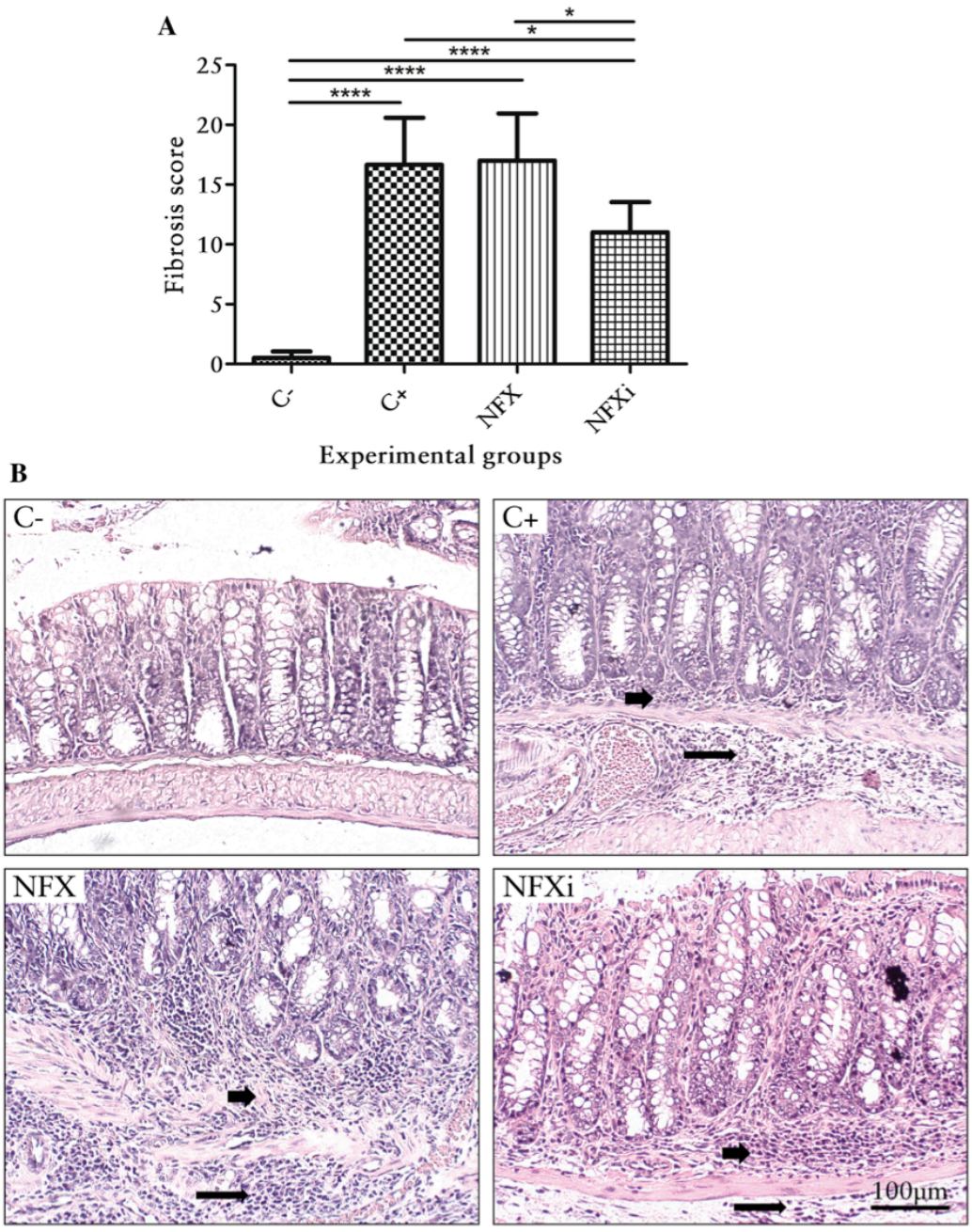


Figure 4. Fibrosis scores and histological features of the colon of mice with chronic TNBS-induced colitis. (**A**) Fibrosis scores of BALB/c mice with or without TNBS administration. (**B**) Histological features of the colon of BALB/c mice with or without TNBS administration (Gomori staining). The 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: $^{*}p < 0.05$; $^{****}p < 0.0001$.

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.

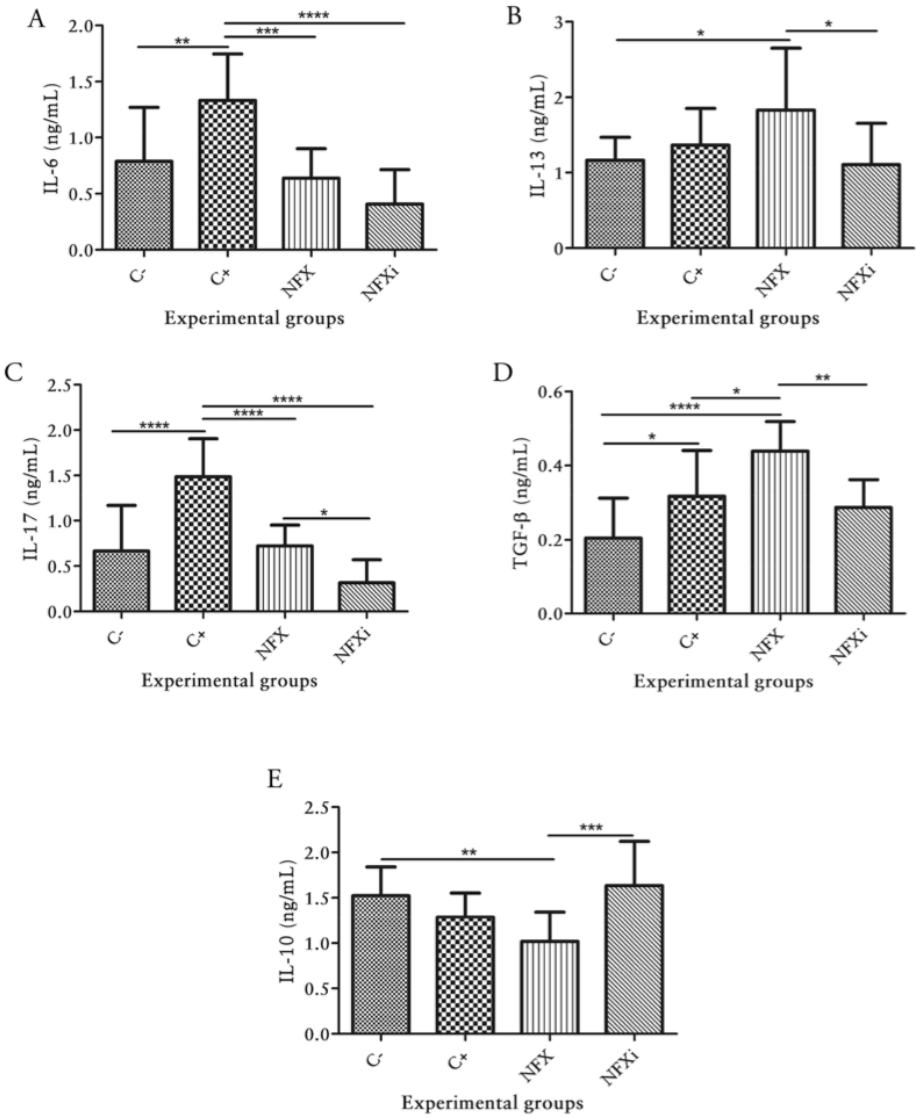


Figure 5. Cytokine levels in BALB/c mice with or without TNBS administration. The concentration of (**A**) Interleukin-6 (IL-6), (**B**) Interleukin-13 (IL-13), (**C**) Interleukin-17 (IL-17), (**D**) Transforming growth factor-(TGF-β) and (**E**) Interleukin-10 (IL-10), were determined in colon homogenates using ELISA. 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.

Discussion

CD, a multifactorial IBD for which there is no cure, is characterized by chronic inflammation of the digestive system wall[10,23], 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[8,32]. 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 infection[40]. Thus, *L. lactis* NCDO2118 FnBPA+ (pXYCYT:Hsp65) did not affect the increased sIgA levels in the chronic colitis model.

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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Author contributions

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

Competing interests

The authors declare no competing interests.

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ANEXO 6:

"*Lactococcus lactis* FNBPA+ (pValac:e6ag85a) induces cellular and humoral immune responses after oral immunization of mice"

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Lactococcus lactis FNBPA$^+$ (pValac:*e6ag85a*) Induces Cellular and Humoral Immune Responses After Oral Immunization of Mice

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The development of a new vaccine strategy against tuberculosis is urgently needed and has been greatly encouraged by the scientific community worldwide. In this work, we constructed a lactococcal DNA vaccine based on the fusion of two *Mycobacterium tuberculosis* antigens, ESAT-6 and Ag85A, and examined its immunogenicity. The coding sequences of the *ESAT-6* and *Ag85A* genes were fused and cloned into the eukaryotic expression pValac vector, and the functionality of the vector was confirmed *in vitro*. Then, *L. lactis* FnBPA$^+$ (pValac:*e6ag85a*) was obtained and used for oral immunization of mice. This strain induced significant increases in IFN-γ, TNF-α, and IL-17 cytokines in stimulated splenocyte cultures, and significant production of antigen-specific sIgA was observed in the colonic tissues of immunized mice. We demonstrated that *L. lactis* FnBPA$^+$ (pValac:*e6ag85a*) generated a cellular and humoral immune response after oral immunization of mice. The strategy developed in this work may represent an interesting DNA mucosal vaccine candidate against tuberculosis, using the fusion of two highly immunogenic antigens delivered by safe lactic acid bacteria.

Keywords: lactic acid bacteria, *Lactococcus lactis*, DNA vaccine, tuberculosis, ESAT6/Ag85A

INTRODUCTION

Tuberculosis (TB), an infectious disease caused by *Mycobacterium tuberculosis*, remains a serious public health problem worldwide, especially in developing countries. The only vaccine available for clinical use is Bacillus Calmette-Guérin (BCG) and, although BCG is widely used, its efficacy against pulmonary TB is controversial (Kaufmann et al., 2015). Hence, the investigation of strategies for the development of more effective and economically viable vaccines against tuberculosis is necessary and has been

greatly encouraged. Over the last few years, the international scientific community has made a substantial effort to search for more effective vaccine alternatives to BCG or to increase its immunogenicity. These alternatives include vaccines based on genetically modified mycobacteria lineages, subunit vaccines and DNA vaccines (Kaufmann et al., 2014). DNA vaccines have the advantages of T cell activation and antibody production without the possible limitations of immunization with live pathogenic microorganisms (Kutzler and Weiner, 2008).

The proteins secreted by *M. tuberculosis* are considered attractive vaccine candidate antigens (Andersen et al., 1991). Among these proteins, two are prominent: ESAT-6 and Ag85A. 6-kDa Early Secreted Antigenic Target (ESAT-6) is an immunodominant antigen secreted in the early stages of infection and is present in pathogenic *M. tuberculosis* but absent in BCG (Pym et al., 2002). Ag85A belongs to the antigen 85 complex (Ag85) and is considered a highly immunogenic virulence factor (Dietrich et al., 2006). Studies have revealed that DNA vaccines using these proteins can increase the T helper cell type 1 (Th1) response, which is critical in protection against tuberculosis (Romano et al., 2006; Xu et al., 2008).

Currently, despite the various available DNA vaccine delivery methods, bacterial mucosal immunization has emerged as a good strategy (Schoen et al., 2004). However, many of these microorganisms are attenuated enteropathogenic bacteria, and the potential risk of reversion may difficult their use (Dunham, 2002). Fortunately, this risk can be circumvented by the use of non-pathogenic bacteria, such as *Lactococcus lactis*.

Lactococcus lactis, considered the model lactic acid bacteria, is classified as Generally Recognized As Safe (GRAS) and has been used for production and delivery of antigens and cytokines to mucosal surfaces for a long time (Wells, 2011). The potential of this microorganism as a vehicle for the delivery of DNA vaccines has been investigated and good results were observed (Del Carmen et al., 2013; Pereira et al., 2015; Souza et al., 2016; Mancha-Agresti et al., 2017; Zurita-Turk et al., 2020). These results were achieved by the use of the pValac vector (Guimarães et al., 2009) and the recombinant strain *L. lactis* FnBPA$^+$ that expresses fibronectin binding protein A (FnBPA) of *Staphylococcus aureus,* an invasin involved in adhesion and invasion of eukaryotic cells (Que et al., 2001). *In vitro* and *in vivo* assays demonstrated the invasiveness of *L. lactis* FnBPA$^+$ and its ability to deliver the pValac vector to eukaryotic cells (Innocentin et al., 2009; Pontes et al., 2012; Almeida et al., 2014).

Thus, our aim was to construct *L. lactis* FnBPA$^+$ harboring the pValac vector containing the fusion ORF *ESAT6-Ag85A* of *M. tuberculosis* and use this system for oral immunization of mice. Moreover, we aimed to evaluate the immune response generated by this vaccine strategy.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, and Growth Conditions

The plasmid and bacterial strains used are listed in **Table 1**. The pValac plasmid was constructed by our group in 2009 (Guimarães et al., 2009). The *L. lactis* FnBPA$^+$ lineage was developed by Que et al. (2001), who kindly gave it to the group of Dr. Philippe Langella from INRAe—France. Dr. Philippe Langella passed it on to us and, currently, both the plasmid and the lineage are part of our microbiological collection.

TABLE 1 | Bacterial strains and plasmids.

Bacterial	Characteristics	References
Escherichia coli TG1	*E. coli* K-12-derived strain	Lucigen
Escherichia coli TG1 (pValac:*gfp*)	*E. coli* TG1 carrying the pValac:*gfp*[a] plasmid	Guimarães et al., 2009
Escherichia coli TG1 (pValac:*e6ag85a*)	*E. coli* TG1 carrying the pValac:*e6ag85a* plasmid	This work
Lactococcus lactis FnBPA$^+$	*L. lactis* MG1363 strain expressing FnBPA[b] of *Staphylococcus aureus*	Que et al., 2001
Lactococcus lactis FnBPA$^+$ (pValac:*e6ag85a*)	*L. lactis* MG1363 strain expressing FnBPA of *S. aureus* carrying the pValac:*e6ag85a* plasmid	This work
Plasmid	**Characteristics**	**References**
pValac:*gfp*	Eukaryotic expression vector (pCMV[c] /Cm(r)[d] /RepA and RepC[e]) containing the coding sequence for GFP	Guimarães et al., 2009
pOri23-*fnbA*	*L. lactis-E. coli* shuttle vector containing the coding sequence for FnBPA (Ery^r)[f]	Que et al., 2001
pValac:*e6ag85a*	pValac vector containing the coding sequence for fusion protein ESAT-6/Ag85A of *Mycobacterium tuberculosis*	This work

[a]*gfp, green fluorescent protein;* [b]*FnBPA, fibronectin binding protein;* [c]*pCMV, cytomegalovirus promoter;* [d]*Cm^r, chloramphenicol resistance gene;* [e]*RepA and RepC, replication origins;* [f]*Ery^r, erythromycin resistance gene.*

Escherichia coli TG1 was grown in LB broth (Acumedia, San Bernardino, United States) at 37°C with shaking. *Lactococcus lactis* FnBPA$^+$ was grown at 30°C without shaking in M17 broth (Sigma-Aldrich, Darmstadt, Germany) enriched with 0.5% glucose (GM17). Recombinant bacteria were selected by addition of the following antibiotics: for *L. lactis* FnBPA$^+$, erythromycin at 5 μg/ml; for *L. lactis* FnBPA$^+$ (pValac*:e6ag85a*), erythromycin at 5 μg/ml and chloramphenicol at 10 μg/ml; and for *E. coli* (pValac*:e6ag85a*), chloramphenicol at 10 μg/ml.

Plasmids from *E. coli* and *L. lactis* were isolated as described by Green and Sambrook (2012), with the following modification: for *L. lactis*, TE-LYS (25% sucrose, 1 mmol EDTA, 50 mmol Tris–HCl, pH 8, lysozyme 10 mg/ml) was added to the samples for 1 h at 37°C before the addition of lysis solution.

DNA Vaccine Construction

The *ESAT-6* and the *Ag85A* coding sequences were amplified by PCR using Pfx Platinum® High-Fidelity DNA Polymerase (Life Technologies, Carlsbad, United States) and the genomic DNA of *M. tuberculosis* H37Rv strain (ATCC 27294) as a template. The oligonucleotides used for *ESAT-6* were as follows: 5′ -

CGGGATCC**CCACCATG**GAGCAGCAGTGGAATTTCGCG-3′ (forward) containing the *Bam*HI restriction site (underlined) and the customized Kozak sequence (in bold) and 5′ -CTAGTCTAGATGCGAACATCCCAGTGACG-3′ (reverse) containing the *Xba*I restriction site (underlined). The reverse primer was designed without a stop codon. The oligonucleotides used for *Ag85A* were as follows: 5′ -CTAGTCTAGAATGCAGCTTGTTGACAGGGTTC-3′ (forward) containing the *Xba*I restriction site (underlined) and 5′ CGGAATTCCTAGGCGCCCTGGGGCGC-3′ (reverse) containing the *Eco*RI restriction site (underlined).

PCR products were purified from agarose gels using illustra™ GFX™ PCR DNA and Gel Band Purification kit (GE Healthcare, Chicago, United States) and individually digested with *Xba*I. Then, fragments were ligated using T4 DNA ligase (Life Technologies, Carlsbad, United States). The fusion fragment *ESAT6-Ag85A* was digested with *Bam*HI and *Eco*RI enzymes and inserted into the corresponding sites of the eukaryotic expression vector pValac (Guimarães et al., 2009), which was previously cleaved with the same restriction enzymes.

The pValac:*e6ag85a* plasmid (**Figure 1**) was transformed into *E. coli* TG1, producing the *E. coli* TG1 (pValac:*e6ag85a)* strain. The insert integrity was confirmed by DNA sequence analysis, using a BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, Foster City, United States) and ABI3130 sequencing equipment (Applied Biosystems, Foster City, United States). Finally, pValac:*e6ag85a* was transformed by electroporation (Langella et al., 1993) into *L. lactis* FnBPA$^+$, producing the *L. lactis* FnBPA$^+$ (pValac:*e6ag85a*) strain.

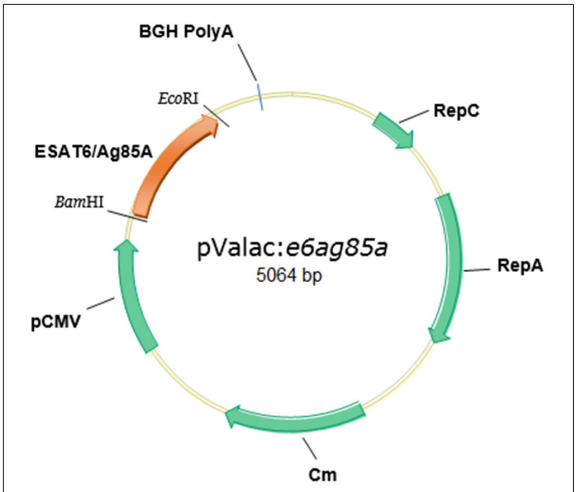


FIGURE 1 | Structure of the pValac:*e6ag85a* vector. Tick marks indicate *Bam*HI and *Eco*RI restriction sites and BGH polyadenylation region (polyA). Arrows indicate cytomegalovirus promoter (pCMV), ORF of the ESAT6/Ag85A fusion protein, replication origin of *E. coli* (RepC), *L. lactis* (RepA), and chloramphenicol resistance gene (Cm).

Cell Transfection and Protein Expression Assays

The pValac:*e6ag85a* vector was transfected into the Chinese hamster ovarian cell line (Flp-In™-CHO; Life Technologies, Carlsbad, United States) to evaluate its functionality by analyzing the expression of the protein by confocal microscopy and flow cytometry. CHO cells were cultured in F12 Ham media (Gibco, Dublin, Ireland) supplemented with 10% fetal bovine serum, 1% L-glutamine, zeocin (100 ng/ml) and 2.5% HEPES [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid]. The cells were transfected with 4 μg of pValac:*e6ag85a* vector using Lipofectamine™ 2000 transfection reagent (Life Technologies, Carlsbad, United States) as described by the supplier. Cells that received the pValac:*gfp* vector served as the positive control; cells that did not receive plasmids served as the negative control. DNA plasmids used in transfection were prepared using Qiagen® Plasmid Midi kit (Qiagen, Hilden, Germany), according to the manufacturer's instruction.

Forty-eight hours post transfection, pValac:*e6ag85a*-transfected and control cells were fixed with 4% paraformaldehyde (Sigma-Aldrich, Darmstadt, Germany) for 15 min and permeabilized with 0.1% Triton X-100 (Sigma-Aldrich, Darmstadt, Germany) for 10 min. Then, the cells were incubated for 2 h with a specific mouse monoclonal immunoglobulin G1 (IgG1) anti-ESAT-6 antibody 1 mg/ml, diluted 1/1000 in PBS/BSA 1% (Abcam ab26246; Abcam, Cambridge, United Kingdom). Next, the cells were incubated with goat anti-mouse IgG (H+L) Alexa Fluor® 488 (4 μg/ml, diluted 1/500 in PBS/BSA 1%; Life Technologies, Carlsbad, United States) for 1 h in darkness. Nuclear labeling was performed concurrently with secondary antibody labeling by incubation with the fluorochrome DAPI 2 μg/ml (Life Technologies, Carlsbad, United States), diluted 1:300 in PBS/BSA 1%. Samples were mounted and images were captured using a Zeiss LSM 510 META inverted confocal laser-scanning microscope (Zeiss, Oberkochen, Germany). Images were collected and analyzed using Zeiss LSM Image Browser software (Zeiss, Oberkochen, Germany).

For flow cytometry analysis, 1×10^6 pValac:*e6ag85a*-transfected and control cells (non-transfected cells) were fixed and permeabilized using a Mouse Foxp3 Buffer Set kit (BD Pharmingen™, San Jose, United States). The cells were then incubated for 30 min with mouse monoclonal IgG1 anti-ESAT-6 antibody Abcam ab26246 diluted 1/1000 in PBS/BSA 1% or with mouse monoclonal anti-Ag85A antibody (H-2b haplotype mice—Mab DT-17/4, source: Professor Kris Huygens, Pasteur Institute of Brussels, Belgium) diluted 1/50 in PBS/BSA 1%. Next, the cells were incubated with secondary goat anti-mouse IgG (H+L) Alexa Fluor® 488 diluted 1/500 in PBS/BSA 1% for 30 min. Finally, the cells were centrifuged and suspended in PBS, and quantification of ESAT-6- and Ag85A-producing CHO cells was performed by BD Accuri™ C6 Flow Cytometer equipment (BD Biosciences, San Jose, United States). The acquired data were analyzed using FCAP array software (BD Biosciences, San Jose, United States).

Animals and Ethical Approval

Conventional female BALB/c mice (6–7 weeks old) were used for the immunization assays. Mice were obtained from

the Central Bioterium of Federal University of Minas Gerais (UFMG, Belo Horizonte, Brazil). They were maintained in mini-isolators housed in ventilated racks with controlled conditions (temperature of 22 ± 2°C, humidity 50 ± 10%, air flow 35 exchanges/hour and light exposure 12-h light/dark cycle) and free access to water and rodent food. 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 66/11) of Federal University of Minas Gerais (UFMG, Belo Horizonte, Brazil).

Immunization

BALB/c mice were randomly divided into the following experimental groups: negative control (saline 0.9%) (-), *L. lactis* FnBPA$^+$ (invasive *L. lactis* strain, negative control) (FN) and *L. lactis* FnBPA$^+$ (pValac:*e6ag85a*) (FEA). Each animal was orally immunized by gavage with 1×10^8 CFU in a final volume of 100 μl of saline 0.9%. The immunizations were performed at three different time points (days 1, 15, and 29) for three consecutive days at each time point. On day 43, all animals were anesthetized by intraperitoneal injection with a ketamine (60 mg/kg) and xylazine (8 mg/kg) cocktail and then euthanized. Two individual experiments were performed for each individual protocol (six animals/group).

Cytokine Measurement

Cytokine levels in the splenocyte supernatants were measured using a commercially available enzyme-linked immunosorbent assay (ELISA) kit (R&D DuoSet kit; R&D Systems™, Minneapolis, United States) according to the manufacturer's instructions to characterize the cellular immune response profile. Briefly, spleens were aseptically removed on the day of sacrifice and macerated; red blood cells were lysed. A total of 1×10^6 splenocytes from each immunized animal were plated (96-well microtiter plates) in complete RPMI-1640 (Sigma-Aldrich, Darmstadt, Germany) medium [10% fetal bovine serum (Gibco, Dublin, Ireland), sodium pyruvate 1 mmol, non-essential amino acids 1 mmol (Gibco, Dublin, Ireland), gentamicin 25 μg/ml (Gibco, Dublin, Ireland), and L-glutamine 2 mmol (LGC Biotecnologia, Cotia, Brazil)]. These cells were submitted to treatment with only RPMI (non-stimulated cells; negative control) or with RPMI containing 5 μg/ml of rESAT-6 (Pereira et al., 2015) and then incubated at 37°C in 5% CO_2. As a positive control, one sample of each experimental group was stimulated with RPMI containing Concanavalin A (ConA—Sigma-Aldrich, Darmstadt, Germany) 16 μg/ml (data not shown).

Cell supernatants were collected to measure the levels of the cytokines interferon-gamma (INF-γ), tumor necrosis factor alpha (TNF-α), interleukin 17 (IL-17), 10 (IL-10), and 4 (IL-4) by sandwich ELISA using the described kit, after 60 hours of stimulation. Absorbance was measured at 492 nm using an Expert Plus Microplate Reader (Biochrom Asys, Cambridge, United Kingdom). Results for all cytokines were calculated by subtracting the basal values of cytokines from cells that received only RPMI medium from the values of cytokines from rESAT-6-stimulated cells.

Characterization of the Humoral Immune Response Pattern

The humoral immune response of immunized animals was evaluated by measuring mucosal anti-ESAT-6 sIgA levels in macerated mouse colons. For this, colons were macerated in buffer solution containing anti-proteases in a ratio of 1 ml of solution to 100 mg of tissue. The IKA T10 Basic Homogenizer workcenter (IKA® Brasil, Campinas, Brazil) was employed for this purpose. The homogenates were centrifuged, the supernatant was collected and the measurement of sIgA was performed by indirect ELISA as follows. Recombinant ESAT-6 (5 μg/ml) was used to coat microliter plates overnight (Pereira et al., 2015). Then, samples without dilution were added and incubated for 1 h. Next, horseradish peroxidase (HRP)-conjugated goat anti-mouse antibodies (Sigma-Aldrich, Darmstadt, Germany) diluted in PBS/casein (1/8000) were added and incubated for 2 h. Finally, orthophenylenediamine (OPD—1 mg/ml; Sigma Aldrich, Darmstadt, Germany) was used for color development as an indicator. Absorbance was measured at 492 nm using an ELISA Expert Plus Microplate Reader (Biochrom Asys, Cambridge, United Kingdom).

Statistical Analysis

Statistical analyses were performed using one-way analysis of variance (ANOVA) followed by Bonferroni post-test using GraphPad Prism 5.0 software (San Diego, CA, United States). The data are expressed as the mean ± standard error of the mean (SEM); $p < 0.05$, $p < 0.01$, and $p < 0.001$ were considered statistically significant.

RESULTS

Construction of the Recombinant Strain *L. lactis* FnBPA$^+$ (pValac:*e6ag85a*)

The fused ORF *ESAT6-Ag85A* (1,322 bp) (Gen Bank number *ESAT-6*: 886209; *Ag85A*: 886132) was directionally cloned into the pValac vector. pValac comprises the cytomegalovirus promoter (pCMV), the polyadenylation sequence of bovine growth hormone (BGH) (unit of eukaryotic expression), the origins of replication for both *E. coli* and *L. lactis*, and the chloramphenicol (Cm) resistance gene (prokaryotic region). The final pValac:*e6ag85a* construct (**Figure 1**) was confirmed by molecular biology methods such as PCR, enzymatic digestion, and sequencing (data not shown). Recombinant *L. lactis* FnBPA$^+$ (pValac:*e6ag85a*) was constructed by transformation of the invasive *L. lactis* FnBPA$^+$ strain with the pValac:*e6ag85a* plasmid.

Eukaryotic Cells Transfected With the Plasmid pValac:*e6ag85a* Can Express ESAT6-Ag85A Protein

The functionality of pValac:*e6ag85a* was confirmed by confocal microscopy and flow cytometry. In confocal microscopy analysis, pValac:*e6ag85a*-transfected CHO cells labeled with a specific mouse anti-ESAT-6 monoclonal antibody and secondary antibody conjugated to Alexa 488 showed green fluorescence

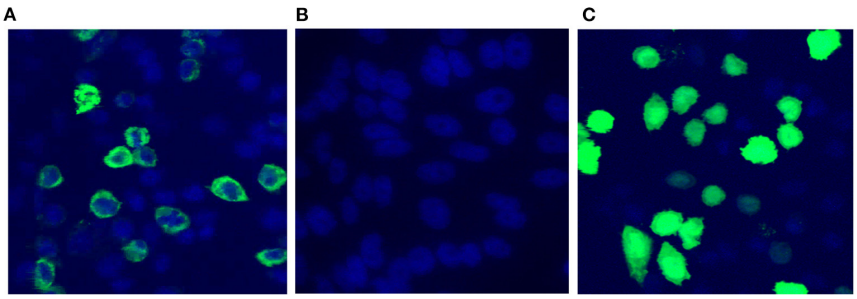


FIGURE 2 | Confirmation of pValac:*e6ag85a* functionality. Confocal Microscopy. **(A)** CHO cells transfected with pValac:*e6ag85a* labeled with anti-ESAT-6 primary antibody, Alexa 488 secondary antibody and DAPI; **(B)** Untransfected CHO cells labeled with anti-ESAT-6 primary antibody, Alexa 488 secondary antibody and DAPI (negative control); **(C)** CHO cells transfected with pValac:*gfp* labeled with DAPI (positive control). Images obtained using a Zeiss LSM 510 META inverted confocal laser-scanning microscope with a 63X objective.

in the cytoplasm, thus confirming the expression of ESAT-6 protein (**Figure 2A**). Protein expression was not detected in untransfected CHO cells labeled with the same primary and secondary antibodies (negative control) (**Figure 2B**). Cells transfected with the pValac:*gfp* plasmid were assayed as a positive control, which showed specific green fluorescence protein (GFP) expression (**Figure 2C**).

In flow cytometry, cells transfected with the plasmid pValac:*e6ag85a* and labeled with the antibody against ESAT-6 (**Figure 3A**) or Ag85A (**Figure 3C**) antigens showed protein expression in 6.3 and 5.6% of the analyzed events, respectively. Indeed, no expression was observed in non-transfected cells (**Figures 3B,D**). Taken together, these results confirmed the functionality of the pValac:*e6ag85a* vector.

Oral Immunization With *L. lactis* FnBPA$^+$ (pValac:*e6ag85a*) Can Induce IFN-γ, TNF-α, and IL-17 Cytokines

To determine the cellular immune response pattern after mouse immunization, we measured the levels of IFN-γ, TNF-α, IL-17, IL-4, and IL-10 cytokines in the supernatant of rESAT-6-stimulated splenocytes. Compared with control mice immunized with *L. lactis* FnBPA$^+$ (FN) or saline (-), mice immunized with *L. lactis* FnBPA$^+$ (pValac:*e6ag85a*) (FEA) exhibited a significant increase in IFN-γ and TNF- α levels (**Figures 4A,B**).

Regarding IL-17, mice immunized with *L. lactis* FnBPA$^+$ (pValac:*e6ag85a*) showed significantly higher levels of this cytokine than did mice immunized with *L. lactis* FnBPA$^+$ or saline (**Figure 4C**). The other cytokines measured, IL-4 and IL-10, which are specific to the Th2 immune response, were not detected in the supernatants of the splenocytes culture.

Increased sIgA Is Detected After Immunization With *L. lactis* FnBPA$^+$ (pValac:*e6ag85a*)

The principal immunoglobulin in mucosa immunity is secretory IgA (sIgA). The results obtained in this study showed that anti-ESAT-6 sIgA levels in the macerated colons of mice immunized with *L. lactis* FnBPA$^+$ (pValac:*e6ag85a*) were significantly higher than those observed in the colons of mice immunized with *L. lactis* FnBPA$^+$ or saline (**Figure 5**). Indeed, these results demonstrated that administration of the *L. lactis* FnBPA$^+$ (pValac:*e6ag85a*) strain induced a mucosal immune response *in vivo*.

DISCUSSION

The use of *Lactococcus lactis* FnBPA$^+$ for mucosal DNA delivery is a promising strategy for immunization. The main mucosae exploited for immunization with DNA vaccines are intranasal (Dou et al., 2012) and oral (Wang et al., 2009; Pereira et al., 2015, 2017). Perhaps the most advantageous is the oral route, considering that the gastrointestinal tract contains more cells of the immune system than any other tissue, representing the largest immunological compartment of the organism (Mowat and Agace, 2014). Thus, the development of DNA vaccines suitable for oral immunization, aiming at reaching the intestinal mucosa and, consequently, their inherent immunological advantages, is particularly interesting.

Recently, using the same vaccination strategy employed in this work but exploring only the role of ESAT-6 as a candidate antigen, Pereira et al. reported a significant increase in IFN-γ production in mice orally immunized with *L. lactis* FnBPA$^+$ (pValac:*esat6*). However, no differences were observed in the expression of other equally important cytokines, such as TNF-α, in the immune response against *M. tuberculosis* (Pereira et al., 2015).

Moreover, Mancha-Agresti et al. (2017) developed the *L. lactis* FnBPA$^+$ (pValac:*ag85a*) DNA vaccine, which encodes the Ag85A antigen. Following intranasal administration of the vaccine to mice, they found a significant increase in production of IFN-γ and TNF-α by the animals, similar to the present study, but with smaller magnitudes. In addition, the increase in IL-17, which

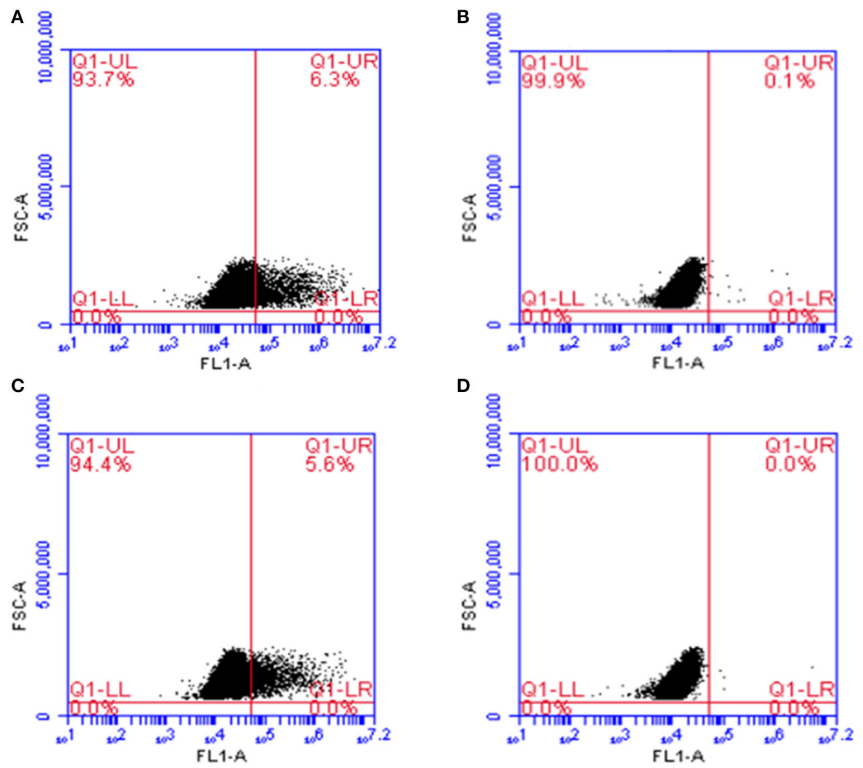


FIGURE 3 | Expression of ESAT-6 and Ag85A proteins by CHO cells transfected with the pValac:*e6ag85a* plasmid. Flow Cytometry. **(A)** Transfected cells labeled with primary anti-ESAT-6 and secondary Alexa 488 antibodies; **(B)** Non-transfected cells labeled with primary anti-ESAT-6 and secondary Alexa 488 antibodies; **(C)** Transfected cells labeled with primary anti-Ag85A and secondary Alexa 488 antibodies; **(D)** Non-transfected cells labeled with primary anti-Ag85A and secondary Alexa 488 antibodies. Images obtained using *FCAP array software* (BD Biosciences).

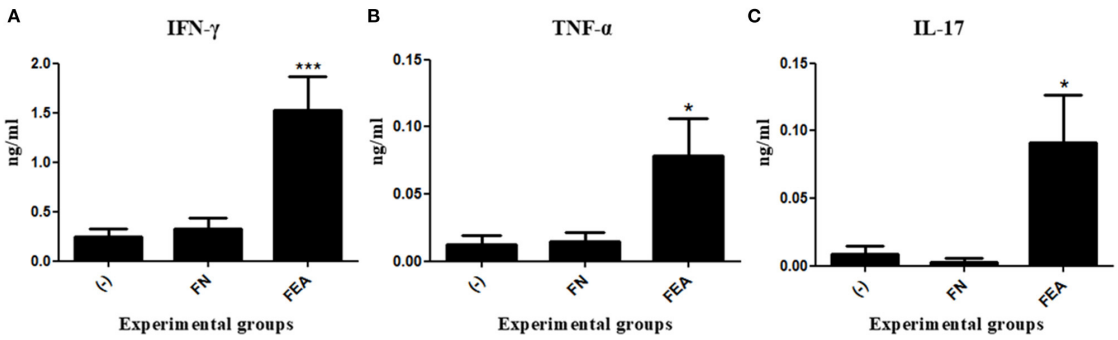


FIGURE 4 | Production of cytokines after oral immunization of mice. Levels of **(A)** IFN-γ, **(B)** TNF-α, and **(C)** IL-17 in the supernatant of splenocyte cultures stimulated with rESAT-6 and analyzed by ELISA. Experimental groups: Saline (-; negative control), *Lactococcus lactis* FnBPA$^+$ (FN; negative control), and *Lactococcus lactis* FnBPA$^+$ (pValac:*e6ag85a*) (FEA). Data are shown as the mean ± SEM of two independent experiments ($n = 12$). *$P < 0.05$ or ***$P < 0.001$.

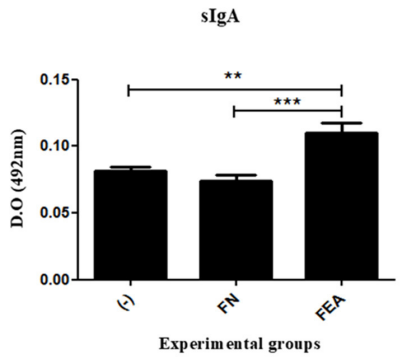


FIGURE 5 | Production of sIgA after oral immunization of mice. Levels of anti-ESAT6 sIgA in the colonic tissues of mice orally immunized with *L. lactis* FnBPA$^+$ (pValac:*e6ag85a*) and analyzed by ELISA. Experimental groups: Saline (-; negative control), *Lactococcus lactis* FnBPA$^+$ (FN; negative control), and *Lactococcus lactis* FnBPA$^+$ (pValac:*e6ag85a*) (FEA). Data are shown as the mean ± SEM of two independent experiments ($n = 12$). $^{**}P < 0.01$ or $^{***}P < 0.001$.

is important in the early stages of TB control, was not verified (Mancha-Agresti et al., 2017).

In this study, we used the coding sequence of these two highly immunogenic proteins in a construct in which they were fused and cloned into the pValac vector. The pValac:*e6ag85a* functionality was confirmed, and this plasmid was transformed into the invasive *L. lactis* FnBPA$^+$ strain. *L. lactis* FnBPA$^+$ (pValac:*e6ag85a*) was used to immunize BALB/c mice by the oral route, and cellular and humoral immune responses were evaluated.

Concerning the cellular immune response, compared with the control groups, the group immunized with the *L. lactis* FnBPA$^+$ (pValac:*e6ag85a*) strain showed increased IFN-γ, TNF-α, and IL-17 levels. IFN-γ is considered the most relevant cytokine in the immune response against *M. tuberculosis* and is mainly secreted in response to the activation of naive CD4$^+$ T cells in Th1-type effector cells. Th1 cells represent a primordial defense against intracellular microorganisms, such as *M. tuberculosis* (Lyadova and Panteleev, 2015). In a previous study, mice deficient in IFN-γ died in the early stages of *M. tuberculosis* infection and presented a high mycobacterial burden in their bodies (Cooper et al., 1993). Similar effects have been observed in humans who have defective IFN-γ signaling pathways, which results in severe infections caused by this microorganism (van de Vosse et al., 2004).

TNF-α, in turn, is a potent activator of macrophages, acting in synergy with IFN-γ in the induction of its antimicrobial activity (Mootoo et al., 2009). Knock-out mice for TNF-α developed a lethal *M. tuberculosis* infection (Jacobs et al., 2000). In humans, the importance of TNF-α has been demonstrated notably by the increased risk of reactivation of latent TB in patients with chronic inflammatory diseases who underwent anti-TNF-α therapies (Feldmann and Maini, 2001; Harris and Keane, 2010).

The IL-17 cytokine, secreted by Th17 cells, acts on the stimulation of the granulocytic lineage, the recruitment of neutrophils to sites of infection and the induction of pro-inflammatory molecules secretion (Lyadova and Panteleev, 2015). In the human bronchial epithelium, Th17 cells induce the expression of antimicrobial mucins and peptides, which together with neutrophil activity, provide mechanisms to combat *M. tuberculosis* (Kao et al., 2004; Tsai et al., 2013). Low levels of IL-17 in TB patient serum is related to high mortality (Lyadova and Panteleev, 2015).

Taken together, these findings support the concept that the generation of Th1 and Th17 cellular immune response profiles with production of their signature cytokines is necessary for TB defense; these patterns were observed in this work. When comparing the results of the present study with those obtained by Mancha-Agresti et al. (2017) and Pereira et al. (2015), we believe that the results obtained here are due to a greater amount of target epitopes coexisting in the same vaccine plasmid, which generated stronger immune responses.

Regarding the mucosal immune response generated after vaccination with *L. lactis* FnBPA$^+$ (pValac:*e6ag85a*), there was a significant difference between the group that received the DNA vaccine and the control groups. Among the functions of this immunoglobulin, the neutralization of antigens and pathogens in the extracellular environment, with the consequent inhibition of mucosal colonization (Corthésy, 2013), is notable. Furthermore, IgA knock-out mice were more susceptible to infection, which was evidenced by the high mycobacterial load found in the lungs of these animals (Williams et al., 2004; Rodriguez et al., 2005). Thus, a vaccine that induces antigen-specific sIgA responses is particularly desirable for protective immunity, especially for diseases in which the mucosa is their primary site of induction, as is the case of tuberculosis.

In other studies that adopted the approach of multigenic vaccines, results similar to those of this study were obtained. Wang et al. constructed a DNA vaccine encoding a fusion protein composed of ESAT-6 and Ag85B antigens (belonging to the same Ag85A family), which was orally delivered to mice through an attenuated strain of *Salmonella typhimurium*. The animals immunized with this vaccine showed high production of antigen-specific IFN-γ as well as increased levels of sIgA and were protected from a *M. tuberculosis* challenge (Wang et al., 2009). Although interesting, this strategy used pathogenic bacteria as a DNA delivery vector; despite attenuation of the pathogenicity, the risks of reversion to its wild phenotype were not eliminated, which may restrict its use.

Dou et al. tested a DNA vaccine encoding the Ag85A/ESAT-6 fusion protein in association with the pro-inflammatory cytokine IL-21. They used a prime boost scheme, with naked DNA intranasally administered first, followed by BCG as the booster. The researchers observed that vaccinated animals had elevated levels of IFN-γ, measured in the supernatant of the splenocyte culture, as well as activation of the cytotoxic activity of NK cells and increased levels of sIgA in the bronchoalveolar lavage. This vaccine also demonstrated protection against a challenge with *M. tuberculosis* (Dou et al., 2012). However, naked DNA delivery may expose the plasmids to a nuclease-rich environment, which may eventually degrade them, rendering the process unfeasible.

Our vaccine strategy can, then, overcome the abovementioned obstacles since it uses bacteria with a recognized safety profile as a delivery vector for the DNA vaccine. In this way, the plasmid is protected against degradation by nucleases, which may lower the required dose for administration. In addition, the oral route explored in this work allows the DNA vaccine to induce a mucosal immune response, reaching the intestinal mucosa, which is considered the body's largest immune compartment (Mowat and Agace, 2014). In turn, mucosal immunization is extremely important for defense against pathogens that access the host organism through this route, as is the case of *M. tuberculosis*.

In conclusion, *L. lactis* FnBPA$^+$ (pValac:*e6ag85a*) was functional after oral immunization and presented features indicative of its potential protective capacity; these results have encouraged us to perform challenge trials with *M. tuberculosis* in the future.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/supplementary material.

ETHICS STATEMENT

The animal study was reviewed and approved by Animal Use Ethics Commission (CEUA, Registration Number 66/11) of Federal University of Minas Gerais (UFMG, Belo Horizonte, Brazil).

AUTHOR CONTRIBUTIONS

CC contributed to experimental work, data analysis, manuscript writing, and editing. BS contributed to experimental work, manuscript writing, and revision. PM-A contributed to experimental work and data analysis. VP, MZ-T, TP, VC, JS, and SL contributed to experimental work. VA contributed to conception and design of the study. AM contributed to conception and design of the study and manuscript revision. All authors contributed to the article and approved the submitted version.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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