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# *Lacticaseibacillus casei* CSL3: imobilização e aplicação em matriz alimentar, influência nos parâmetros bioquímicos, de estresse oxidativo e modulação da resposta inflamatória

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Pelotas, 2022

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## RESUMO

Probióticos são microrganismos, que quando consumidos em concentrações adequadas, conferem benefícios à saúde do hospedeiro. Para serem caracterizados como probióticos os microrganismos devem comprovar sua influência positiva através de testes *in vitro*, *in situ* e *in vivo*. Logo, o objetivo do presente estudo foi avaliar a viabilidade de *Lacticaseibacillus casei* CSL3 imobilizado em pedaços de abacaxi e aplicado em queijo P*etit-suisse*, bem como a ação probiótica *in vitro* e *in vivo* nos parâmetros bioquímicos, de estresse oxidativo, histológicos e modulação da resposta inflamatória. Após imobilizado e inserido em sistema alimentar avaliou-se - *L. casei* CSL3 – quanto sua viabilidade durante o armazenamento do produto, bem como após sua passagem pelo trato gastrointestinal simulado. Foram realizadas análises físico-químicas (pH, acidez, atividade de água, umidade, proteína e sinérese) e sensoriais (índice de aceitação e intenção de compra) para caracterização do produto. A avaliação da citotoxicidade do isolado e produção de óxido nítrico foram realizadas em células (*in vitro*). Após *L. casei* CSL3 ser administrado aos camundongos no período experimental de 30 dias e desafiados contra *Salmonella* Typhimurium e *Escherichia coli* O157:H7, realizaram-se análises dos níveis séricos de glicose, colesterol total, aspartato aminotransferase (AST) e alanina aminotransferase (ALT), triglicerídeos sanguíneo do estresse oxidativo em tecidos e produção de catalase, da colonização do trato gastrointestinal, da histopatologia dos rins, fígados e intestino e da expressão relativa de citocinas pro-inflamatórias. *Lacticaseibacillus casei* CSL3 manteve sua viabilidade superior a 8 log UFC $g^{-1}$, quando imobilizado em pedaços de abacaxi e inserido em queijo *Petit Suisse* durante período de armazenamento e superior a 7 log UFC $g^{-1}$ após passagem pelo TGI simulado. Na avaliação da toxicidade e influência na produção de óxido nítrico, *in vitro*, a bactéria mostrou-se segura para aplicação em concentrações de 8 log UFC $g^{-1}$ e observou-se que com o aumento da concentração, aumentava a produção de óxido nítrico. Nos testes bioquímicos, os resultados comprovam que *L. casei* CSL3 influenciou na redução da concentração de glicose e triglicerídeos séricos e do

estresse oxidativo nos tecidos dos animais. Ao competir contra *Salmonella* Typhimurium e *Escherichia coli* O157H7 o probiótico colonizou o trato gastrointestinal dos camundongos, protegeu tecidos do fígado e intestino e estimulou positivamente a expressão relativa da citocina IL-2, e negativamente de IL-4 e TNF-α. Ao final do estudo pode-se concluir que *L. casei* CSL3, possui características probióticas comprovadas através da manutenção de sua viabilidade quando imobilizado e inserido em matriz alimentar *(in situ)*, bem como pelos parâmetros bioquímicos, histológicos e imunológicos (*in vitro* e *in vivo*).

Palavras-chave: *L. casei* CSL3, probiótico, *Petit Suisse*, parâmetros bioquímicos, sistema imunológico

**ABSTRACT**

Probiotics are microorganisms that, when consumed in adequate concentrations, confer health benefits on the host. To be characterized as probiotics, microorganisms must prove their positive influence through in vitro, in situ and in vivo tests. Therefore, the objective of the present study was to evaluate the viability of *Lacticaseibacillus casei* CSL3 immobilized in pineapple pieces and applied in *Petit-suisse* cheese, as well as the probiotic action *in vitro* and *in vivo* in biochemical, oxidative stress, histological parameters and modulation of inflammatory response. *Lacticaseibacillus casei* CSL3, was immobilized in pineapple pieces and applied in a food system to evaluate the viability during storage of the product, as well as after its passage through the simulated gastrointestinal tract. Physical-chemical (pH, acidity, water activity, moisture, protein and syneresis) and sensory (acceptance index and purchase intent) analyzes were performed to characterize the product. The evaluation of the cytotoxicity of the isolate and production of nitric oxide were carried out in cells (in vitro). After *L. casei* CSL3 was administered to mice in the experimental period of 30 days and challenged with *Salmonella* Typhimurium and *Escherichia coli* O157:H7, analyzes of the serum levels of glucose, total cholesterol, ALT, AST, blood triglycerides of oxidative stress were performed in tissue and catalase production, colonization of the gastrointestinal tract, histopathology of kidneys, livers and intestines and the relative expression of pro-inflammatory cytokines. *Lacticaseibacillus casei* CSL3 maintained its viability higher than 8 log CFU $g^{-1}$ when immobilized in pineapple pieces and inserted into *Petit Suisse* cheese during storage and higher than 7 log CFU $g^{-1}$ after passing through the simulated GIT. Regarding the assessment of toxicity and influence on the production of nitric oxide, in vitro, the bacterium proved to be safe for application at concentrations of 8 log CFU $g^{-1}$ and it was observed that with increasing concentration, the production of nitric oxide increased. In the biochemical tests, the results show that *L. casei* CSL3 influenced the reduction of the concentration of glucose and serum triglycerides and the oxidative stress in the tissues of the animals. By competing with *Salmonella* Typhimurium and *Escherichia coli* O157H7, the probiotic colonized the gastrointestinal tract of mice, protected liver and intestine tissues and positively stimulated the relative expression of the cytokine IL-2, and negatively of IL-4

and TNF-α. At the end of the study, it can be concluded that *L. casei* CSL3 has proven probiotic characteristics through the maintenance of its viability when immobilized and inserted into a food matrix (*in situ*), biochemical, histological and inflammatory parameters (*in vitro* and *in vivo*).

Key-words: *L. casei* CSL3, probiotic, *Petit Suisse*, biochemical parameters, immune system

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## 1 INTRODUÇÃO GERAL

Devido ao crescente interesse da população por cuidados com sua saúde, o mercado global para produção de alimentos probióticos foi estimado em US$ 3.493,42 milhões em 2021 para US$ 6.060,51 milhões em 2028, esperando-se uma taxa de crescimento anual de 8,2% de 2022 a 2028 (JOSHI, 2022). Os probióticos são definidos como microrganismos vivos, que, quando administrados em quantidades adequadas, conferem benefício à saúde do hospedeiro (WHO/FAO, 2001, HILL *et al.,* 2014).

Para ser considerado probiótico, o microrganismo deve atender a determinados requisitos, entre os quais a tolerância ao trânsito gastrointestinal. Este é um critério de seleção funcional, pois ao serem ingeridos, os microrganismos são expostos ao meio gástrico, particularmente ácido, para então na sequência encontrarem altas concentrações de sais biliares na primeira porção do intestino delgado (VINDEROLA *et al*., 2017).

Outro requisito importante é a adesão de probióticos ao epitélio intestinal, que contribuirá para a persistência dos mesmos na superfície da mucosa. A hidrofobicidade da superfície celular bacteriana tem sido usada como preditor, *in vitro*, da interação de bactérias com as células epiteliais intestinais do hospedeiro (NIVOLIEZ *et al*., 2014; ROKANA *et al*., 2018). Além dos estudos de aderência às células, experimentos *in vivo* têm sido utilizados para pesquisar a capacidade imunoestimuladora de isolados potencialmente probióticos (CENCIČ & LANGERHOLC, 2010).

Estudos utilizando animais demonstram que probióticos podem influenciar diretamente a resposta imunosistêmica, assegurando a homeostase da microbiota intestinal que está intimamente ligada ao tratamento de doenças metabólicas (HAN *et al*., 2021; KARAFFOVÁ *et al*., 2021; TOUKAM *et al*., 2021). O aumento da produção de ácidos graxos de cadeia curta (AGCC), regulação do metabolismo dos ácidos biliares e a redução de resistência à insulina são alguns dos diferentes mecanismos utilizados por esses microrganismos para conceder benefícios ao hospedeiro (KANG *et al*., 2022).

Microrganismos probióticos e seus metabólitos sintetizados a partir de processos fermentativos, são frequentemente associados à capacidade

antioxidante. Os radicais livres podem causar danos ao DNA, proteínas, lipídios e outras moléculas celulares, causando a perda gradual de funções fisiológicas e doenças. Arslanova *et al*. (2021), Lee & Kang (2022), Lin *et al*. (2022) dentre outros autores, destacam em seus estudos, utilizando como modelo camundongos, os variados métodos de ação que diferentes linhagens probióticas possuem no estresse oxidativo.

Outra ação que pode inibir a alteração na composição da população microbiana intestinal, é a atividade antimicrobiana. Substâncias como ácidos graxos de cadeia curta, etanol, ácidos orgânicos, diacetil, acetaldeídos, peróxido de hidrogênio e peptídeos (bacteriocinas) são produzidos por muitos probióticos. Entre tais compostos, as bacteriocinas, em particular, estão envolvidas principalmente no aumento da permeabilidade da membrana das células-alvo, o que leva à despolarização do potencial de membrana e, por fim, à morte celular (KAREEM *et al*., 2014; SIMOVA *et al*., 2009).

Pesquisas destacam o uso de bactérias probióticas na competição contra microrganismos patogênicos de importância em alimentos. Considerados os principais agentes etiológicos de origem bacteriana, responsáveis por surtos de doenças transmitidas por alimentos (DTA) no mundo, *Salmonella* spp. e *Escherichia coli* (CDC, 2021) chamam a atenção por possuírem patogenicidades distintas, pois enquanto a primeira atua invadindo as células eucarióticas (ZHANG *et al*., 2019), a segunda possui mecanismos que envolvem a internalização de toxinas no epitélio intestinal (HILL *et al*., 2014[b]; POKHAREL *et al*., 2016).

Em virtude de importantes características benéficas de probióticos, diversos produtos alimentícios contendo essas bactérias foram introduzidos no mercado global nas últimas décadas. Durante o desenvolvimento desses alimentos, as culturas probióticas são adicionadas aos mesmos (KAILASAPATHY, 2013). A presença destes microrganismos no produto alimentício, portanto, não deve afetar adversamente sua qualidade e propriedades sensoriais (MOHAMMADI & MORTAZAVIAN, 2011).

Dentre os alimentos que veiculam probióticos, os produtos lácteos destacam-se, por possuírem uma densa matriz com teor de gordura

relativamente alto, o que pode oferecer proteção adicional às bactérias durante sua passagem pelo trato gastrointestinal (BERGAMINI *et al*., 2005), bem como apresentam uma alta taxa de consumo em todo o mundo (OECD/FAO, 2018).

No entanto, a eficácia dos produtos alimentares probióticos depende do número de células viáveis nos produtos no momento do consumo, recomendado de pelo menos 6 a 7 log de $UFC.g^{-1}$ ou $mL^{-1}$ (PAPADOPOULOU *et al*., 2018). Muitos fatores influenciam a viabilidade de microrganismos probióticos em produtos alimentares durante a produção, processamento e armazenamento. Dentre os fatores estão: pH, acidez, oxigênio molecular, atividade de água, presença de sal e de açúcar; produtos químicos como peróxido de hidrogênio, bacteriocinas, aromatizantes artificiais e agentes corantes; parâmetros de processamento como tratamento térmico, temperatura de incubação, taxa de resfriamento do produto, materiais de embalagem, métodos de armazenamento e escala de produção (TRIPATHI & GIRI, 2014).

Com a finalidade de proteger tais microrganismos das condições adversas impostas pelo processamento do alimento, bem como durante sua passagem pelo trato gastrointestinal, surgem técnicas de imobilização celular. Essas técnicas caracterizam-se pelo confinamento físico em uma determinada região do espaço com a preservação de atividades desejadas. As técnicas de imobilização podem ser divididas em quatro categorias principais, com base no mecanismo físico empregado: aprisionamento em uma matriz porosa, fixação ou adsorção em superfícies sólidas, auto-agregação por floculação (natural) ou com agentes de reticulação e contenção mecânica por barreira (MITROPOULOU *et al.*, 2013).

Entre os possíveis suportes de imobilização, destacam-se os biopolímeros e materiais naturais de pureza e qualidade alimentar, como aqueles que possuem carboidratos não-digeríveis, com a finalidade de investigar sua aplicação na produção de alimentos (MITROPOULOU *et al*., 2013).

As frutas contêm carboidratos não digeríveis, que constituem a base para a imobilização celular. Pesquisas demonstram que alguns tecidos vegetais como maçã, pêra, marmelo (KOURKOUTAS *et al*., 2005;

KOURKOUTAS *et al*., 2006), morango e banana (SIDIRA et al., 2013) possuem capacidade de imobilizar certas bactérias probióticas. A utilização da fruta abacaxi - como suporte para imobilização de bactérias probióticas - torna-se uma alternativa devido aos altos níveis de fibras, das quais 99,2% faz parte da fração insolúvel e 0,8% da fração solúvel (MARTÍNEZ et al., 2012), de vitamina C e de carotenoides, além do fato do Brasil ser um dos maiores produtores e consumidores em nível mundial dessa fruta (BENÍTEZ et al., 2012; SANEWSKI et al., 2018).

Nesse contexto, o presente estudo teve por objetivo avaliar a viabilidade de *Lacticaseibacillus casei* CSL3 imobilizado em pedaços de abacaxi, com aplicação em matriz alimentar, bem como avaliar os efeitos de CSL3 nos parâmetros bioquímicos, de estresse oxidativo, colonização da microbiota intestinal, prevenção de danos aos tecidos e modulação da resposta inflamatória através de testes *in vitro* e *in vivo*.

O microrganismo em estudo *Lacticaseibacillus casei* CSL3 (anteriormente denominado *Lactobacillus casei* CSL3), faz parte da Coleção de Bactérias Probióticas e Culturas Iniciadoras do Laboratório de Microbiologia de Alimentos (UFPel). A bactéria isolada de silagem de colostro bovino (Vitola *et al*., 2018), foi identificada por métodos fenotípicos e moleculares, bem como análises *in vitro* e aplicação *in situ* em manteiga (Bellinazo *et al*., 2019) e iogurte (Ames *et al*., 2021), realizados em estudos anteriores pelo grupo de pesquisa.

Os estudos realizados, estão apresentados na Tese estruturada em quatro capítulos: No Capítulo 1, revisão bibliográfica, encontra-se a abordagem acerca de microrganismos probióticos, aplicações tecnológicas dos mesmos, principalmente do gênero *Lacticaseibacillus*, influência benéfica nos parâmetros bioquímicos e de estresse oxidativo no hospedeiro, estímulo à modulação da resposta inflamatória, bem como a importância de estudos *in vivo*, contemplando o comportamento do probiótico quando inserido em sistemas complexos conectados.

No Capítulo 2, é abordado o estudo envolvendo a imobilização de *L. casei* CSL3 em fruta como suporte, aplicação na produção de queijo tipo *Petit*

*Suisse* e manutenção da viabilidade do microrganismo durante o armazenamento do produto e passagem pelo trânsito gastrointestinal simulado.

No Capítulo 3, é apresentado a primeira parte do estudo *in vivo* onde foram avaliados parâmetros bioquímicos e de estresse oxidativo dos animais tratados com *L. casei* CSL3 e desafiados com *Salmonella* Typhimurium e *Escherichia coli*. E finalmente, no Capítulo 4 é abordada a influência da bactéria em estudo *L. casei* CSL3, no sistema imunológico do hospedeiro, avaliando síntese de óxido nítrico, colonização do trato gastrointestinal, expressão relativa de citocinas pró-inflamatórias e histopatologia, quando em competição contra bactérias patogênicas.

## 2 HIPÓTESES

H1: *Lacticaseibacillus casei* se mantém viável em maiores concentrações quando imobilizado em um suporte;

H2: O isolado *L. casei* CSL3 quando imobilizado e aplicado em sistema alimentar mantém-se viável durante sua passagem pelo trato gastrointestinal simulado, bem como durante a vida útil do produto;

H3: *Lacticaseibacillus casei* CSL3 poderá agir reduzindo os níveis séricos de glicose, colesterol total e triglicerídeos sanguíneo de animais desafiados com *Salmonella* Typhimurium e *Escherichia coli* O157:H7;

H4: *Lacticaseibacillus casei* CSL3 influencia na redução do estresse oxidativo, aumentando a concentração da enzima catalase e restringindo a produção de substâncias reativas ao ácido tiobarbitúrico em tecidos de animais desafiados com *Salmonella* Typhimurium e *Escherichia coli* O157:H7;

H5: O isolado *L. casei* CSL3 tem ação na resposta inflamatória aumentando os níveis de interleucinas, interferons e fator de necrose tumoral de animais desafiados com *Salmonella* Typhimurium e *Escherichia coli* O157:H7.

# 3 OBJETIVOS

## 3.1 Objetivo geral

Avaliar a viabilidade de *Lacticaseibacillus casei* CSL3 imobilizado em pedaços de abacaxi e aplicado em queijo P*etit-suisse*, bem como a ação probiótica *in vitro* e *in vivo* nos parâmetros bioquímicos, de estresse oxidativo, colonização da microbiota intestinal, prevenção de danos aos tecidos e modulação da resposta inflamatória.

## 3.2 Objetivos específicos

- Determinar o melhor suporte para imobilização de *L. casei* CSL3;
- Aplicar *L. casei* CSL3 imobilizado em pedaços de abacaxi, em queijo *Petit-suisse*;
- Avaliar a viabilidade de *L. casei* CSL3 imobilizado durante a vida útil de queijo *Petit-suisse*, armazenado sob refrigeração;
- Determinar os parâmetros físico-químicos, microbiológicos e a aceitação sensorial do queijo *Petit-suisse*;
- Avaliar a viabilidade de *L. casei* CSL3 imobilizado e aplicado no queijo *Petit-suisse*, quando submetido às condições gastrointestinais simuladas;
- Determinar os níveis séricos de glicose, colesterol total, triglicerídeos, creatinina e das enzimas AST e ALT sanguíneos de camundongos tratados com *L. casei* CSL3 desafiados com *S*. Typhimurium e *E. coli* O157:H7;
- Avaliar concentrações de catalase e de substâncias reativas ao ácido tiobarbitúrico (TBARs) em camundongos tratados com *L. casei* CSL3 desafiados com *S*. Typhimurium e *E. coli* O157:H7;
- Determinar, *in vitro,* a viabilidade e a concentração de óxido nítrico produzido por macrófagos RAW tratados com diferentes concentrações de *L. casei* CSL3;
- Quantificar a microbiota das fezes de camundongos, tratados com *L. casei* CSL3 desafiados com *S*. Typhimurium e *E. coli* O157:H7;

- Avaliar a histopatologia do epitélio intestinal de camundongos tratados com *L. casei* CSL3 desafiados com *S*. Typhimurium e *E. coli* O157:H7;
- Mensurar os níveis de IL-2, IL-4 e TNF-α, de camundongos, tratados com *L. casei* CSL3 desafiados com *S*. Typhimurium e *E. coli* O157:H7.

# 4 CAPÍTULO 1: Revisão Bibliográfica

## 4.1 Bactérias probióticas

Nos últimos anos, a crescente demanda, no mercado, por alimentos que tragam benefícios à saúde, vêm chamando atenção por parte das indústrias de alimentos (GVR, 2019). Segundo a Organização Mundial da Saúde, padrões alimentares juntamente a hábitos de vida, constituem fatores de extrema importância para a redução do risco de doenças (WHO/FAO, 2003).

Neste cenário de grande demanda por novas tendências na área de alimentos, surgem os chamados alimentos funcionais. Tal alegação está diretamente relacionada ao papel metabólico ou fisiológico que um nutriente ou um não nutriente tem no crescimento, desenvolvimento e manutenção do organismo humano (BRASIL, 1999). Estima-se que a maior parcela do mercado total de alimentos funcionais é representado por produtos probióticos (GVR, 2019).

O termo probiótico é utilizado para designar microrganismos viáveis que quando consumidos em quantidades suficientes exercem benefícios à saúde do hospedeiro (HILL *et al*., 2014a; WHO/FAO, 2001). Dentre as bactérias que compõe esse grupo, destacam-se linhagens de bactérias ácido-lácticas (BAL) dos gêneros *Enterococcus*, *Lactobacillus*, *Lactococcus* e *Streptococcus* e outros gêneros não pertencentes ao grupo de BAL como *Bifidobacterium* (DORON & SNYDMAN, 2015; INABA et al., 2014; MACHADO & SOCCOL, 2015). O gênero *Lactobacillus* recebeu nova classificação taxonômica, onde 261 espécies avaliadas foram subdivididas em 25 gêneros, dos quais 23 são gêneros novos, e apenas 38 espécies permaneceram classificadas como *Lactobacillus* (ZHENG et al., 2020).

Metabolicamente as BAL são divididas em dois grupos: as homofermentativas, caracterizadas pela produção de ácido lático como principal produto da fermentação e, as heterofermentativas, que além do ácido lático podem produzir metabólitos como etanol, ácido acético e dióxido de carbono (CARR *et al*., 2002). Quanto aos seus produtos oriundos do metabolismo secundário, tem-se entre eles peptídeos com atividade

antimicrobiana (bacteriocinas), assim como exopolissacarídeos e enzimas (MONIKA *et al*., 2017; PATEL *et al*., 2012).

A seleção de microrganismos com potencial probiótico requer uma abordagem sistemática, pois na maioria dos casos, o grande número de isolados leva à necessidade de uma sequência de testes para reduzir progressivamente seu número. Ao final destas etapas, os isolados que apresentarem o maior número de propriedades funcionais bem como a ausência de características patogênica, são selecionados (DE MELO PEREIRA *et al*., 2018). Os testes de triagem pelos quais essas linhagens passam se encontram citados na Tabela 1.

Tabela 1. Triagem utilizada para seleção de linhagens potencialmente probióticas

| **Requesitos** | **Testes** |
|---|---|
| **Avaliação de segurança** | Identificação taxonômica;<br>Ausência de virulência;<br>Produção de enterotoxinas;<br>Atividade hemolítica;<br>Transferência de genes de resistência a antimicrobianos de uso clínico; |
| **Tolerância ao estresse** | Enzimas salivares;<br>Enzimas gástricas;<br>Temperatura corporal;<br>Baixo pH;<br>Suco gástrico;<br>Sais biliares; |
| **Habilidade de adesão** | Ensaio de auto-agregação;<br>Grau de hidrofobicidade;<br>Adesão às células epiteliais de mamíferos; |
| **Atividade antimicrobiana** | Produção de metabólitos antimicrobianos;<br>Competição com organismos patogênicos;<br>Co-agregação com patógenos; |
| **Propriedades funcionais associadas ao hospedeiro** | Anticâncer;<br>Anticolesterolêmico;<br>Antidepressiva;<br>Anti-ansiedade;<br>Anti-obesidade; |

| | |
|---|---|
| | Antidiabética;<br>Imunoestimulação;<br>Secreção de moléculas funcionais; |
| **Propriedades tecnológicas** | Qualidade sensorial;<br>Ensaio de viabilidade celular;<br>Estresse ao processamento de alimentos;<br>Estresse relacionado ao armazenamento; |
| **Caracterização OMICA** | Genômica;<br>Transcriptômica;<br>Proteômica;<br>Metabolômica. |

Fonte: Adaptado pelo autor de DE MELO PEREIRA et al. (2018)

O risco ao introduzir microrganismos viáveis na dieta deve ser avaliado e a conclusão de que "os probióticos possuem *status* GRAS (*Generally Recognised As Safe*)", não deve ser totalmente considerada. Para isso iniciativas de alguns países visa estabelecer critérios para a avaliação de segurança de probióticos. Recomendações comuns incluem registros de histórico de isolamento, identificação taxonômica e ausência de virulência como por exemplo a produção de toxinas (CULLIGAN *et al*., 2009; BRASIL, 2018; BRASIL, 2019).

No momento da ingestão a bactéria deve resistir a presença de enzimas na cavidade oral como amilase e lisozima. Após, essas enfrentam fatores antimicrobianos no estômago, como baixo pH, presença de suco gástrico e pepsina, assim como no intestino como a presença de pancreatina e sais biliares (FONTANA *et al*., 2013).

O próximo passo é avaliar se a bactéria possui a capacidade de colonizar as células epiteliais do trato gastrointestinal. Essa adesão é um processo de contato complexo que envolve duas membranas, portanto é dependente do equilíbrio das interações eletroestáticas e forças de *Van der Waals*, e alguns estudos apontam que componentes extracelulares bacterianos e a composição circundante pode influenciar na adesão (BOONAERT & ROUXHET, 2000; JIANG *et al*., 2017; WANG *et al*., 2018).

A capacidade de auto-agregação de certas linhagens permite que a bactéria atinja alta densidade celular no intestino, o que contribui no mecanismo de adesão (PESSOA *et al*., 2017; VITOLA *et al*., 2018), enquanto a hidrofobicidade da superfície celular permite uma maior interação entre as células eucarióticas e o microrganismo (DUARY *et al*., 2011).

Uma vez aderidas ao epitélio, os microrganismos potencialmente probióticos produzem componentes antimicrobianos extracelulares como ácidos orgânicos, enzimas, peróxido de hidrogênio e bacteriocinas. Outros mecanismos de antagonismo incluem competição por nutrientes, co-agregação com patógenos e estimulação do sistema imunológico *(*VERA-PINGITORE *et al*., 2016; VERÓN *et al*., 2017).

## 4.2 Aplicação tecnológica de probióticos

A aplicação de culturas probióticas em diferentes matrizes alimentares sempre representou um desafio para a indústria. Distintas linhagens bacterianas apresentam diferentes sensibilidades quanto a fatores físico-químicos como pH, acidez titulável, oxigênio molecular, atividade de água, presença de sal, açúcar, peróxido de hidrogênio, bacteriocinas, aromatizante artificial e agentes corantes, tratamento térmico, temperatura de incubação, taxa de resfriamento, materiais utilizados nas embalagens e métodos de armazenamento dos produtos (TRIPATHI & GIRI, 2014).

A viabilidade e atividade metabólica dos probióticos são características importantes quando considerada sua aplicação em alimentos, pois essas bactérias precisam manter-se em concentrações adequadas (≥7 log UFC.$g^{-1}$) tanto durante a vida útil do produto, quanto após a passagem pelo trato gastrointestinal (DA CRUZ *et al*., 2010).

A composição dos alimentos pode servir tanto como proteção para os microrganismos probióticos como prejudiciais a estes, por isso torna-se necessário estudar sua compatibilidade com diferentes matrizes (MATTILA-SANDHOLM *et al*., 2002). Os aditivos geralmente utilizados nas indústrias alimentícias incluem aromatizantes naturais ou artificiais, agentes corantes, antimicrobianos como nisina, natamicina, lisozima e nitrito de sódio. Esses

podem afetar drasticamente a viabilidade de bactérias probióticas adicionadas nos produtos (VINDEROLA *et al*., 2002).

Diferentes promotores de crescimento, como glicose, vitaminas, minerais, caseína, hidrolisados de proteína de soro de leite são fortificados em produtos lácteos para aumentar a taxa de multiplicação das espécies. Certos ingredientes como fruto-oligossacarídeos e galacto-oligossacarídeos, considerados prebióticos, podem ter um efeito positivo na retenção da viabilidade dos probióticos em produtos alimentares, durante seu armazenamento (REZA MOHAMMADI *et al*., 2011; NOBAKHTI *et al*., 2009).

O aumento da capacidade tampão do leite, possibilita uma maior viabilidade de probióticos em produtos fermentados lácteos durante o armazenamento. Além disso, a matéria seca da matriz do produto absorve íons de hidrogênio, levando a um aumento nas quantidades de ácidos orgânicos não dissociados (HEYDARI *et al*., 2011).

As matrizes em produtos alimentícios sólidos, como a estrutura do gel nos queijos, sustentam as células probióticas reduzindo sua exposição a fatores prejudiciais (KARIMI *et al*., 2011). O alto teor de gordura presente e ambiente anaeróbico da matriz ajudam a proteger as células probióticas, tanto no produto quanto durante o trânsito gastrointestinal (LEE & SALMINEN, 2009).

O conteúdo de oxigênio e o potencial redox estão entre os fatores importantes que afetam a viabilidade dos probióticos, especialmente durante o período de armazenamento. O oxigênio molecular é prejudicial à sobrevivência e a multiplicação dos probióticos, uma vez que a maioria das espécies é estritamente anaeróbica, podendo isto ocorrer de três maneiras, este ser diretamente tóxico para algumas células, certas culturas produzirem peróxidos tóxicos na presença de oxigênio e radicais livres produzidos pela oxidação de componentes, como por exemplo, gorduras (HOLZAPFEL *et al*., 2001; VUYST, 2000). O nível de oxigênio dentro da embalagem durante o armazenamento dos produtos probióticos deve ser o mais baixo possível, a fim de evitar toxicidade e morte do microrganismo e a consequente perda de funcionalidade deste no produto (MAJID *et al*., 2018).

A viabilidade de bactérias probióticas durante o armazenamento é inversamente relacionada à temperatura de armazenamento *(*GARDINER *et al*., 2000). Os produtos alimentares probióticos que possuem alta atividade de água e consequentemente uma elevada umidade devem preferencialmente ser armazenados a uma temperatura de refrigeração, situada na faixa de 4 ºC - 5 ºC, pois dessa forma mantem-se suas atividades metabólicas reduzidas (MORTAZAVIAN *et al*., 2007).

A sobrevivência dos probióticos durante o armazenamento é consideravelmente afetada pelo pH e acidez dos produtos (MORTAZAVIAN *et al.*, 2010). Um valor de pH muito baixo, causado pelo aumento da concentração de ácidos orgânicos não dissociados em produtos fermentados, aumenta o efeito bactericida destes ácidos. *Lactobacillus* spp. são capazes de se multiplicar e sobreviver em produtos com valores de pH entre 3,7 e 4,3, enquanto espécies de *Bifidobacterium*, por serem menos tolerantes ao ácido, um nível de pH abaixo de 4,6 é prejudicial à sua sobrevivência (BOYLSTON *et al*., 2004; TRIPATHI & GIRI, 2014).

Alimentos fermentados, em particular os de origem láctea, são comumente utilizados como carreadores desses microrganismos. Tais alimentos fornecem uma importante contribuição para a dieta humana em diversos países e esse processo além de servir como uma forma de preservação atribui propriedades sensoriais ao produto (REZAC *et al*., 2018).

Os queijos são derivados lácteos com potencial para a entrega do probiótico em seu sítio de ação no intestino humano, devido as características físicas e químicas desse alimento. Seu elevado pH (≈ 5,0 – 6,5), baixa acidez titulável, capacidade tamponante, consistência sólida, conteúdo lipídico elevado e baixa passagem de oxigênio, podem proteger os microrganismos durante o tempo de armazenamento do produto bem como sua passagem pelo trânsito gastrointestinal (KARIMI *et al*., 2011).

### 4.2.1 Imobilização celular

A imobilização de células probióticas emerge como uma tecnologia que se desenvolveu rapidamente na última década. Sua aplicação possui como

principal intuito a entrega do microrganismo no intestino, garantindo sua viabilidade (PHROMTHEP & LEENANON, 2017).

Define-se imobilização como o aprisionamento celular dentro ou através de uma matriz, enquanto o encapsulamento é o processo de formação de um revestimento contínuo em torno da célula que está totalmente contida dentro da parede da cápsula. Em ambos os casos, deve ser permitida a difusão bidirecional de moléculas, como o fluxo de oxigênio, nutrientes e fatores de crescimento, essencial para o metabolismo celular e a difusão para o exterior de produtos residuais (MITROPOULOU *et al*., 2013).

Naturalmente, muitos microrganismos possuem a capacidade de aderir, produzir polímeros naturais e sobreviver em diferentes tipos de superfícies assim, as células podem multiplicar-se dentro de estruturas naturais como as de exopolissacarídeos (EPS) (DINIC *et al*., 2018).

Os métodos de imobilização podem ser divididos, baseados no mecanismo físico empregado, em quatro categorias principais: aprisionamento dentro de uma matriz porosa (1) devido à penetração de células até que sua mobilidade seja obstruída pela presença de outras células ou pela formação de material poroso *in situ*, fixação ou adsorção em superfícies sólidas de suporte (2) por adsorção física devido a forças eletrostáticas ou por ligação covalente entre a membrana celular e o transportador, autoagregação por floculação (natural) ou por agentes de reticulação induzidos artificialmente (3) e contenção mecânica por trás de uma barreira (4), que pode ser uma membrana microporosa ou uma microcápsula (HOFMAN & THONART, 2002). Tais métodos podem ser observados na Figura 1.

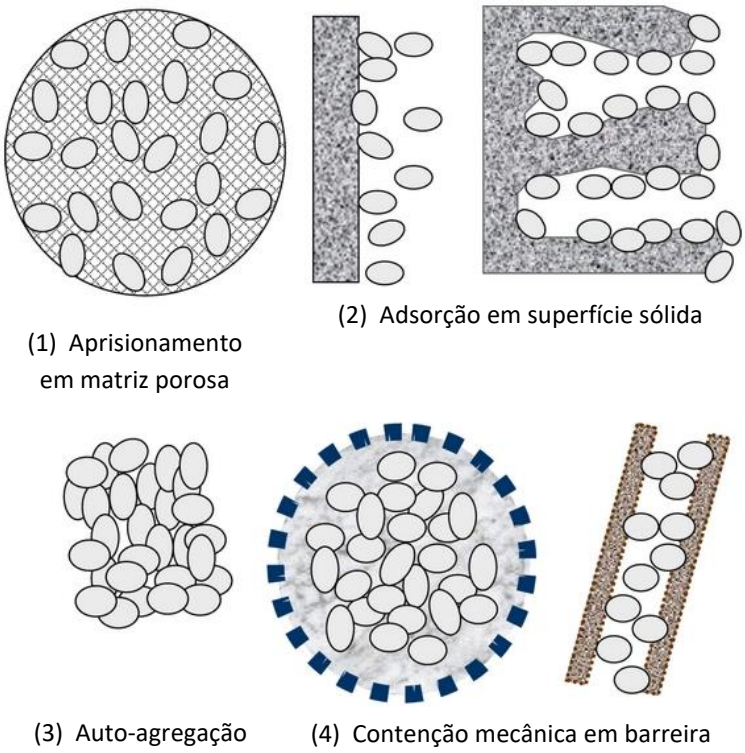


Figura 1. Métodos de imobilização celular (Fonte: MITROPOULOU *et al*., 2013)

No entanto, nem todo tipo de suporte é adequado para a produção de alimentos. O material utilizado como suporte para imobilização celular deve ter estabilidade química, física e biológica durante o processamento e nas condições de reação, ter resistência mecânica suficiente, especialmente para sua utilização em reatores industriais, não ser tóxico tanto para a célula imobilizada quanto para o produto, e possuir alta capacidade de carga. Disponibilidade de material e custo-benefício do processo de imobilização sempre devem ser considerados. Outros critérios, como características físicas (porosidade, inchaço, compressão e comportamento médio das partículas), bem como a possibilidade de crescimento microbiano, biodegradabilidade e solubilidade, são específicos da aplicação e também devem ser levados em consideração (MITROPOULOU *et al*., 2013).

Entre numerosos suportes de imobilização, apenas alguns são considerados adequados para a produção de alimentos. Por exemplo, materiais inorgânicos são geralmente excluídos porque são caracterizados

como inadequados para nutrição humana ou animal. Em vez disso, os biopolímeros e suportes naturais de pureza de qualidade alimentar são preferíveis (MITROPOULOU *et al*., 2013).

Exemplos de pesquisas e aplicações sobre imobilização celular demonstram uma série de vantagens proporcionadas pela técnica, como atividade prolongada e estabilidade das células imobilizadas, uma vez que o suporte de imobilização pode atuar como agente protetor contra alterações físico-químicas (pH, temperatura, sais biliares, etc.) (DOHERTY *et al*., 2010; HEIDEBACH *et al*., 2009[b], 2009[a]; SAUCIER & CHAMPAGNE, 2005; SIDIRA *et al*., 2010), densidades celulares mais altas que levam a maior produtividade e aumentam a absorção e o rendimento do substrato, maior tolerância a alta concentração de substrato e inibição do produto final, redução do risco de contaminação microbiana devido a altas densidades celulares e maior atividade de fermentação, capacidade de fermentação em baixa temperatura e/ou maturação para certos produtos alimentares e redução dos tempos de fermentação/ maturação em determinadas circunstâncias *(*MITROPOULOU *et al*., 2013; OTHMAN *et al*., 2017).

As frutas contêm carboidratos não digeríveis, que constituem a base para a imobilização celular e além de garantirem a entrega do microrganismo no intestino, podem ser utilizadas na produção de alimentos atribuindo aos mesmos caraterísticas sensoriais únicas (KOURKOUTAS *et al*., 2005). Ao serem incorporados em tecidos de origem vegetal, os probióticos fornecem novas categorias de produtos funcionais e novas oportunidades comerciais. No entanto, mais pesquisas são necessárias para a seleção de suportes de imobilização que podem facilitar a entrega direcionada de bactérias probióticas em várias regiões do trato gastrointestinal (KOURKOUTAS *et al.,* 2006; SIDIRA *et al*., 2013).

Entre as frutas consumidas, o abacaxi (*Ananas comosus* (L.) Merrill) destaca-se como uma das mais importantes culturas de frutas tropicais do mundo. De acordo com as estatísticas apresentadas pela Conferência Das Nações Unidas Sobre Comércio e Desenvolvimento, o Brasil encontra-se na

terceira posição dos maiores produtores colhendo cerca de 2.478.178 toneladas por ano (UNCTAD, 2016).

Com relação a sua composição, a porção comestível da fruta contém de 81,2 a 86,2% de umidade, e 13 a 19% de sólidos totais, dos quais sacarose, glicose e frutose são os principais componentes. Os carboidratos representam até 85% do total de sólidos, enquanto as fibras representam 2-3%. Dos ácidos orgânicos, o ácido cítrico é o mais abundante. A polpa possui baixíssimo teor de cinzas, compostos nitrogenados e lipídios (0,1%). De 25 a 30% dos compostos nitrogenados são proteínas. Dentre seus principais minerais estão cálcio, cloro, potássio, fósforo e sódio. Um de seus componentes que chama atenção do mercado mundial são as fibras já exploradas amplamente por diversas indústrias (DE LA *et al*., 2005).

Estudos já demonstraram a eficácia de certas frutas quando utilizadas como suporte para imobilização, por adsorção em superfície, de bactérias probióticas e, quando aplicado em sistema alimentar, esse biocatalizador confere apelo comercial por atribuir novas características sensoriais ao produto (KOURKOUTAS *et al*., 2005; KOURKOUTAS *et al*., 2006; KOURKOUTAS *et al*., 2006; TERPOU *et al*., 2019). Portanto torna-se de interesse avaliar a fruta abacaxi como uma alternativa de suporte para imobilização de microrganismos.

### 4.3 *Lacticaseibacillus casei*

O gênero *Lactobacillus* spp. (Família Lactobacillaceae), compreendia mais de 200 espécies e subespécies (SUN *et al*., 2015). Estas estão presentes em ambientes como alimentos fermentados (lácteos, cárneos e vegetais) (AO *et al*., 2012) e no trato gastrointestinal de humanos/ animais (CASEY *et al*., 2004). São importantes porque muitas espécies de *Lactobacillus* são conhecidas por suas propriedades probióticas (LEBEER *et al*., 2008).

Além disso, devido sua importância biotecnológica e relacionada à saúde, nos últimos anos houve um crescente interesse em explorar a genômica do gênero, que dentre as BAL, é considerado o mais diversificado. Tendo seu genoma variando de 1.23 mil pares de base (Mpb) a 4.91 Mpb (ANBA-MONDOLONI *et al*., 2013), *Lactobacillus* spp. abriga características importantes como ilhas de adaptação ao meio em que se encontra e estruturas

chamadas de unidades autônomas potenciais (UAP) (ALTERMANN & KLAENHAMMER, 2011) .

No entanto em 2020, o gênero *Lactobacillus* passou por uma reclassificação baseada em suas características de filogenia do genoma central, identidade de aminoácidos média dos pares (conservada), genes de assinatura específicos de clado, critérios fisiológicos e a ecologia dos organismos. Esta reclassificação reflete a posição filogenética dos microrganismos e agrupa-os em nichos com propriedades ecológicas e metabólicas compartilhadas. Com base nessa abordagem, de um total de 25 gêneros, 23 propostos foram recém-criados integrando a esse grupo *Lacticaseibacillus*, *Latilactobacillus*, *Liquorilactobacillus*, *Ligilactobacillus*, *Lactiplantibacillus* e *Furfurilactobacillus*. Gêneros pertencentes anteriormente às famílias *Lactobacillaceae* e *Leuconostocaceae*, nesse momento passam a fazer parte da família *Lactobacillaceae*, tendo com isso alterações na descrição da família (ZHENG *et al*., 2020).

Entre as espécies destaca-se *Lacticaseibacillus casei* (anteriormente denominado *Lactobacillus casei*), por possuir um comprimento de genoma total mediano de 3.036 Mpb, 2736 proteínas e um conteúdo de C+G em torno de 46,4%. A ampla distribuição ecológica de *L. casei* reflete uma flexibilidade metabólica que tem impulsionado a aplicação generalizada da espécie nas indústrias de alimentos e farmacêutica; diferentes linhagens são empregadas como culturas iniciadoras na fermentação de leite, como culturas coadjuvantes para acelerar ou intensificar o desenvolvimento do sabor em queijos curados e como probióticos para influenciar beneficamente a saúde (BROADBENT *et al*., 2012).

Recentes pesquisas, evidenciam que o isolamento de linhagens de *L. casei*, potencialmente probiótico, provenientes de diferentes fontes terão ações distintas sob o hospedeiro, podendo comprová-las através de testes *in vitro* e *in vivo*, como a influência do sobrenadante livre de células (SLC) de *Lactobacillus casei* MYSRD 108, isolado de alimento fermentado, contra *Salmonella paratyphi* e biofilme formado pela bactéria (DIVYASHREE *et al*., 2021), *L. casei* BL23 teve ação protetiva em camundongos contra o

desenvolvimento de câncer colorretal, onde reduziu os escores histológicos e os valores do índice proliferativo, além de mostrar efeito imunomodulador (JACOUTON *et al*., 2017), bem como *L. casei* Shirota em conjunto a *Bifidobacterium animalis* atuando sob o sistema imunológico em células Caco-2 infectadas com *E. coli* enteroagregativa (FERREIRA *et al*., 2021).

*Lacticaseibacillus casei* CSL3 (*Lactobacillus casei* CSL3), previamente isolado de silagem de colostro bovino, demonstrou ser seguro para aplicação em alimentos além de possuir características que o tornam candidato em potencial a linhagem probiótica, através de testes *in vitro*. No estudo realizado a bactéria demonstrou capacidade de manter-se viável após trânsito gastrointestinal simulado, alto percentual de auto-agregação/ co-agregação e atividade antagonista contra microrganismos patogênicos de importância alimentar (VITOLA *et al*. 2018).

### 4.4 Probióticos: parâmetros bioquímicos e de estresse oxidativo

No ano de 1998, a Organização Mundial da Saúde (OMS), define síndrome metabólica como fatores que favorecem um conjunto de anormalidades metabólicas e fatores clínicos como resistência à insulina, dislipidemia, hipertensão arterial, obesidade abdominal que quando em conjunto podem acarretar no aumento do risco para o desenvolvimento de doenças cardiovasculares.

Pesquisas realizadas demonstraram que a suplementação de dietas com probióticos fornece efeitos benéficos ao hospedeiro, por meio de intervenções direcionadas ao tratamento de componentes ou complicações da síndrome metabólica através da modulação da microbiota intestinal. O mecanismo de ação dos probióticos que são simulados em modelos *in vivo* e *in vitro* apoiam a hipótese de que os mesmos poderiam reduzir os fatores de risco relacionados à síndrome metabólica (XAVIER-SANTOS *et al*., 2020).

Dois mecanismos distintos estão ligados ao efeito hipocolesterolêmico de probióticos, o primeiro relaciona a assimilação da molécula de colesterol na membrana celular da bactéria, reduzindo assim a absorção de colesterol do trato gastrointestinal, o segundo é responsável por inibir a reabsorção de

ácidos biliares mediante a produção da enzima sal biliar hidrolase (BSH) que catalisam a desconjugação de sais de ácidos biliares (LE & YANG, 2019; LIANG *et al*., 2020).

O controle glicêmico por parte dos probióticos pode estar relacionado à interação de citocinas inflamatórias e anti-inflamatórias que contribuem diretamente para a homeostase da glicose, como IL-6 e TNF-α. Sendo o efeito considerado cepa dependente, ainda há estudos sobre a síntese de ácidos graxos de cadeia curta e butirato, os quais induzem a produção de GLP-1 que seria peptídeo semelhante a glucagon, hormônio com potente ação hipoglicemiante, que se liga a receptores de proteína G com alta afinidade localizadas nas células pancreáticas beta, e estimula a transcrição do gene da insulina *cast* (CASTRO & LUCHESE, 2022; WEI *et al*., 2015).

Estudos comprovam que probióticos podem possuir ação na redução dos níveis das enzimas ALT e AST em pessoas com obesidade e doença hepática gordurosa não-alcoólica (ABDEL MONEM, 2017; FAMOURI *et a*l., 2017). A alanina aminotransferase (ALT) é uma enzima que catalisa a conversão de alanina e α-cetoglutarato em piruvato e glutamato, contribuindo para o metabolismo celular do nitrogênio e a gliconeogênese hepática. Danos ao fígado ou a qualquer órgão causam um rápido aumento no nível de ALT na corrente sanguínea, provando que a medição dessa é um indicador valioso da função hepática. A enzima aspartato aminotransferase (AST) é encontrada em quase todos os tecidos do corpo e determina a troca reversível do grupo amino entre o glutamato e o aspartato. Danos às células no rim, fígado e pâncreas causam níveis anormalmente elevados de AST no soro sanguíneo (YADAV *et al*., 2022).

Entre os benefícios dos probióticos, podem também apresentar funções importantes no controle do estresse oxidativo. O estresse oxidativo pode ser definido como uma condição na qual um excesso de derivados reativos de oxigênio supera as defesas antioxidantes. Embora o aumento do estresse oxidativo seja uma consequência do envelhecimento, este tem sido relacionado ao desenvolvimento de vários distúrbios e patologias metabólicas (DA SILVA & RUDKOWSKA, 2016). Os probióticos podem limitar o estresse oxidativo

através da redução de interleucina 1, fator de necrose tumoral, aumento dos níveis de glutationa, bloqueio da produção de superóxido e radicais hidroxila, estímulo e reforço do sistema imunológico (MOHAMMADI *et al*., 2015).

### 4.5 Probióticos: modulação da resposta imunológica

O corpo humano possui uma variedade de mecanismos utilizados em sua autopreservação. As respostas imunes inatas representam o sistema de alerta precoce, pois são projetadas para impedir que microrganismos patógenos tenham acesso ao corpo e para ajudar a eliminar aqueles que já tiveram acesso (CHAPLIN, 2010). Já a resposta imune adaptativa, tem como base uma resposta específica a um determinado antígeno apresentado pelo microrganismo, caso o mesmo já tenha ultrapassado as barreiras inatas, porém com um retorno mais lento e apresentando um componente de memória (UTHAISANGSOOK *et al*., 2002; WANG *et al*., 2021).

Todavia certos microrganismos patogênicos possuem capacidade de se aderir à superfície do epitélio, modificando sua estrutura para possibilitar a invasão celular ou ainda, aderir com posterior lesão das células epiteliais, ambos com posterior processo inflamatório. Nesse sentido, *Salmonella* spp. e *Escherichia coli* figuram entre os agentes etiológicos responsáveis por surtos de doenças transmitidas por alimentos (DTA) (CDC, 2021).

A patogênese das infecções ocasionadas por *Salmonella* spp. está intimamente ligada à translocação de proteínas bacterianas para o interior da célula eucariótica, onde desempenham diferentes funções. A maior parte destas proteínas, são injetadas na porção líquida do citoplasma da célula do hospedeiro, por meio de dois sistemas de secreção tipo III, onde o primeiro causa um rearranjo no citoesqueleto (actina), formando ondulações na superfície da membrana da célula eucariótica, que levam ao englobamento da bactéria (ENG *et al*., 2015; PRADHAN *et al*., 2020).

Após a invasão, a destruição das células M e dos enterócitos permite que o microrganismo entre em contato com os macrófagos residentes no tecido, então é secretada uma proteína que induz à apoptose da célula. Esta etapa é essencial para a sobrevivência bacteriana, já que o recrutamento

adicional de fagócitos facilita seu espalhamento sistêmico. O segundo sistema de secreção tipo III secreta proteínas efetoras que permitem a sobrevivência e multiplicação desta bactéria nos macrófagos (ENG *et al.,* 2015; PRADHAN *et al*., 2020)

A diversidade patogênica de *E. coli* compreende pelo menos cinco categorias que causam infecções intestinais por diferentes mecanismos, estas são chamadas de diarreiogênicas. *Escherichia coli* do sorotipo O157:H7 é reconhecido como patógeno associado a surtos de diarreia sanguinolenta onde sua virulência dependerá da presença de elementos genéticos móveis (RAHAL *et al*., 2012).

Grande parte das cepas de *E. coli* O157:H7 produzem uma gama de proteína de membrana externa como intimina (codificada pelo gene *eae*), determinante genético da formação de lesões de fixação (A/E), mecanismo central na patogênese da *E. coli* enteropatogênica (EPEC) e que ainda não apresentam o gene responsável pela produção de shiga-toxina (*stx*). Ferdous *et al*. (2015) relata em sua pesquisa que cepas stx - negativas podem ser progenitores de *E. coli* produtora de shiga-toxina preparados para adquirir um ou mais fagos conversores de Stx.

Nos últimos anos, estudos apontam que certas espécies probióticas são efetivas na prevenção de doenças intestinais no corpo humano, uma vez que possuem a capacidade de se aderir a diferentes tipos de células, o que é considerada como uma das características essenciais para o microrganismo fornecer benefícios ao hospedeiro. A adesão às células epiteliais intestinais presentes nos tecidos do trato gastrointestinal pode levar a colonização deste por linhagens probióticas (YOUSEFI *et al*., 2019; JAVANSHIR *et al*., 2021; PAVELJŠEK *et al*., 2021).

Diversas linhagens, bem como seus metabólitos, podem exercer distintas atividades imunomoduladoras em diferentes condições patológicas, como doença alérgica, colite, artrite reumatoide, câncer colorretal, depressão, ansiedade, entre outras. O chamado *crosstalk* (comunicação cruzada) entre as células imunes inatas, células imunes adaptativas e a microbiota intestinal

controlam o equilíbrio entre a tolerância imunológica e a inflamação (MEZOUAR *et al*., 2018; CIANCI *et al*., 2018).

No sistema imune inato, células dendríticas (CD) e epiteliais são geralmente as primeiras a entrarem em contato com os microrganismos residentes no intestino e seus produtos metabólicos. As células dendríticas, uma das principais células apresentadoras de antígeno, ocupam os tecidos linfóides associados ao intestino (GALT), ou estão distribuídas por toda a lâmina própria do intestino e quando em contato com microrganismos, as mesmas atuam como “sensores”, ativando diferentes vias de sinalização que resultam em modificações de seus fenótipos e secreção de citocinas (SCHIAVI *et al*., 2015).

Certas linhagens probióticas ainda podem induzir à formação da enzima heme oxigenase em CD, que catalisa a degradação do grupo heme (importante para o transporte de oxigênio), produzindo biliverdina, ferro ferroso e monóxido de carbono, resultando em células T reguladoras da mucosa (Treg) dentro do GALT. As células Treg são componentes importantes da tolerância imunológica. Essa tolerância é definida como a não-resposta a um determinado antígeno (Ag), induzida pela exposição prévia a este (KARIMI *et al*., 2012; WONG *et al*., 2016).

A imunorregulação de CD também está associada a componentes da parede celular bacteriana. Por exemplo, o polissacarídeo capsular A, presente no microrganismo, interage com TLR-2 (*Toll Like Receptor*) presente nas DC plasmocitoides, que reconhece padrões moleculares. São então enviadas respostas através da limitação da expressão de citocinas pró-inflamatórias e estimulação da secreção de interleucina 10 (IL-10) por linfócitos T CD4 + de células T que intercedem na resposta anti-inflamatória (DASGUPTA *et al*., 2014).

Enquanto a apresentação de antígenos bacterianos filamentosos segmentados por DC intestinais, dependente do complexo principal de histocompatibilidade (MHCII), sendo este crucial para a indução de células Th17, a administração de probióticos pode estimular DC modulando as

populações bacterianas no intestino (FUENTES *et al*., 2008; GOTO *et al*., 2014).

As células epiteliais são conhecidas por sua importante função absortiva. Essas protegem o hospedeiro de microrganismos patogênicos e agentes tóxicos, formando uma barreira mucosa. Existe uma relação complexa entre a barreira mucosa e as células imunes subjacentes da lâmina própria e das placas de Peyer, bem como com o conteúdo luminal (HABIL *et al*., 2014).

As bactérias probióticas podem reforçar as defesas da barreira mucosa, induzindo peptídeos antimicrobianos, como a β-defensina-2 humana (hBD-2) de amplo espectro produzidos por células epiteliais, células de Paneth, neutrófilos e macrófagos, que contribuem para a resposta imune inata (HABIL *et al*., 2014). Outro mecanismo influenciado pelos probióticos que é essencial para a manutenção da barreira epitelial é a suprarregulação da autofagia de células epiteliais intestinais, onde através de uma via catabólica, evolutivamente conservada, o conteúdo citoplasmático (proteínas e organelas) é envolto por membranas duplas e fundido com lisossomas para degradação (RONG LIN *et al*., 2014).

Além disso, tais microrganismos podem promover regulação positiva da expressão do gene *Muc2*, responsável pela produção de mucina que desempenha um papel como a primeira linha de defesa intestinal contra infecção e lesão (WANG *et al*., 2014). Após a infecção, os probióticos podem suprir a produção de citocinas e quimiocinas pró-inflamatórias, que normalmente são secretadas por células epiteliais intestinais e ativam uma resposta imune eficiente (BOONMA *et al*., 2014; LI *et al*., 2013).

Quando em contato com o sistema imune adaptativo, os antígenos lipídicos probióticos influenciam as células *natural killer* (NK) (GUI *et al*., 2020). Seu efeito benéfico contra certas doenças, é atribuído à sua capacidade de aumentar o número de células Tregs e regular negativamente certas citocinas pró-inflamatórias (KIM *et al*., 2014; LIU *et al*., 2010). A modulação da resposta das células T, pode se dar através de seus metabólitos, como ácidos graxos de cadeia curta, além de ativar o receptor acoplado à proteína G43 (ATARASHI *et al*., 2013). Sabe-se que certas linhagens regulam negativamente a expressão e

a produção de TNF-α e lNFγ (OWAGA *et al*., 2015) e podem ainda polarizar a resposta imune para Th1 ao invés de Th2 (TANABE, 2013).

Os probióticos podem aumentar o número de células positivas no emplastro de Peyer e na lâmina própria, levando a uma indução da produção de imunoglobulina A (IgA) que desempenha um papel essencial na proteção da mucosa do hospedeiro contra os agentes patogénicos das mucosas. A IgA inibe que a bactéria se ligue às células epiteliais e neutraliza as toxinas (SAKAI *et al*., 2014). Embora os linfócitos B sejam responsáveis pela secreção de anticorpos específicos e pela resposta humoral, eles podem regular negativamente a imunidade produzindo IL-10 durante doenças autoimunes e infecciosas, ou ainda aumentar o número de células B de memória IgG e IgC total de IgG em resposta a certas vacinas (ROSSER *et al*., 2014) .

Uma das importantes ações desses microrganismos no sistema imunológico é sua influência no estímulo ou repressão da secreção de citocinas por parte das células, onde os mesmos podem ser categorizados como imunoestimuladores e imunorreguladores. As citocinas são produzidas por diferentes tipos de células imunes, principalmente subconjuntos de linfócitos T e podem ser classificadas em três grupos principais, citocinas Th1 (TNF, INF-γ, lL-12, IL-2, etc.), citocinas Th2 (IL-4, IL-5, IL-6, IL-13) e citocinas reguladoras (IL-10, TGF- β) (KEMGANG *et al*., 2014).

A interleucina 2 (IL-2) é uma proteína que estimula a proliferação de células T, aumenta a atividade citotóxica das células natural killer e desencadeia a liberação de citocinas pró-inflamatórias. As células B ativadas por IL-2 geram anticorpos IgM secretores em vez de associados à membrana, e os macrófagos ganham maturidade e produzem o fator de crescimento transformador-β (TGF-β) (WATERS *et al*., 2018).

Já a interleucina 4 (IL-4) é uma citocina que funciona como um potente regulador da imunidade secretada principalmente por mastócitos, células Th2, eosinófilos e basófilos. O efeito da sinalização de IL-4 é mediado através da cadeia alfa do receptor de IL-4 (IL-4Rα). Ao se ligar ao seu ligante, IL-4Rα se dimeriza com a cadeia gama comum para produzir o complexo de sinalização tipo 1 localizado principalmente nas células hematopoiéticas. O complexo de

sinalização tipo 1 é crítico para o desvio de Th2 das células T e o desenvolvimento de macrófagos ativados alternativamente (GADANI *et al*., 2013).

Uma das citocinas pró-inflamatórias primárias e mais potentes, o TNF-α é capaz de promover a proliferação de várias células ou sinalizar a apoptose, desempenhando um papel central na inflamação e na imunidade. As vias de sinalização celular que estimulam a produção de TNF-α podem ocorrer pela presença de substâncias na parede celular de microrganismos como lipopolissacarídeos (LPS) de bactérias Gram-negativas e ácido lipoteicóico (LTA) e peptidoglicanos de bactérias Gram-positivas, podendo levar a uma resposta inflamatória sistêmica. A inibição ou a indução da expressão de TNF-α por probióticos pode fornecer efeitos imunossupressores ou imunoestimulantes, respectivamente (VINCENZI *et al*., 2021).

### 4.6 Estudos *in vivo*: probióticos

Um critério chave para a seleção de linhagens potencialmente probióticas, está na capacidade de tolerância a estresses como resistência aos processos de fabricação industrial ou passagem simulada *in vitro* pelo trânsito gastrointestinal (VINDEROLA *et al*., 2017).

Uma vez estabelecida a segurança do isolado, através de avaliação *in vitro*, os critérios de seleção usualmente aplicados são constituídos pela simulação a resistência gástrica, com a exposição a pH ácido/ sais biliares e simulação da colonização intestinal a partir da aderência do microrganismo a linhagens celulares. No entanto, testes *in vitro* utilizam-se de condições ideais em laboratório e, portanto, fornecem informações muitas vezes diversas do entendimento da funcionalidade do probiótico, em sistema complexo, como preditor de efeitos *in vivo* (PAPADIMITRIOU *et al*., 2015).

Alguns exemplos dessas diferenças são demonstrados na simulação do trânsito gastrointestinal, onde, enquanto nos ensaios *in vivo* espera-se um gradiente de células estressadas em diferentes níveis no intestino delgado, nos ensaios *in vitro* todas as células microbianas são expostas ao mesmo pH pelo mesmo período de tempo. Com relação a capacidade de aderência do

probiótico à superfície celular intestinal, o uso de linhagens Caco-2 e HT-29 como modelos *in vitro* para estudo da capacidade de adesão não ocorrem em condições completamente similares quando avaliados em modelos *in vivo*, já que intestino grosso e delgado possuem composição de açúcar diferente na superfície celular ( VINDEROLA *et al*., 2017).

Microrganismos probióticos podem diferir em sua capacidade de desencadear sinais efetores em termos de células epiteliais imunocompetentes e intestinais (TAVERNITI *et al*., 2022). A prevenção de infecções por patógenos através da administração de probióticos pode ser mediada por mecanismos imunes e/ou não imunes. Isolados que exibem atividade inibitória *in vitro* contra patógenos são usados em modelo já estabelecido de infecção murina (ABDO *et al*., 2019; MAHOOTI *et al*., 2019). Ensaios *in vivo*, utilizando como modelo camundongo aplicados no estudo dos mecanismos subjacentes à patogênese e capacidade do isolado na prevenção ou tratamento de infecções (WANG *et al*., 2019; MULAW *et al*., 2020; SONG *et al*., 2020)

O uso de camundongos gnotobióticos no estudo da interação probiótico-hospedeiro, permite ultrapassar a barreira da complexidade do ambiente intestinal humano e combinar atividades metabólicas com a microbiota intestinal de composição conhecida (GEIRNAERT *et a*l., 2015; KIM *et al*., 2021)

No entanto, se um isolado apresentar potenciais benefícios à saúde do hospedeiro em ensaios i*n vitro*, então torna-se válida a realização de ensaios biológicos e funcionais *in vivo*, para confirmação dessas características, já que testes *in vitro* funcionam como uma triagem preliminar para redução do número de isolados submetidos à estudos mais complexos.

## CAPÍTULO 2

# *Lactobacillus casei CSL3:* Evaluation of supports for cell immobilization, viability during storage in *Petit Suisse* cheese and passage through gastrointestinal transit *in vitro*

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## Abstract

The present study predicted an evaluation of supports for immobilization of *Lactobacillus casei* CSL3, as well as its viability during storage in *Petit Suisse* cheese and passage through the GIT *in vitro*. In order to choose the appropriate support, immobilizations were performed with three dehydrated fruits: pineapple, guava and kiwi. In these fruits, the concentration of the microorganism was evaluated for a period of 43 days under refrigeration. *Petit Suisse* cheese was prepared and divided into two portions: C – *L. casei* CSL3 in its free state – and T1 – *L. casei CSL3* immobilized on pineapple (biocatalyst). Physicochemical (pH, acidity, water activity, moisture, protein and syneresis), microbiological (probiotic viability, simulated GIT, sanitary and hygienic aspects) and sensory analyses were performed. It could be observed that the pineapple maintained a higher concentration of bacteria at the end of storage time, thus being the chosen support. When evaluated for the production of lactic acid, the immobilization maintained higher catalytic activity. Regarding the viability of the probiotic during storage time and its simulated GIT, there were no differences, which leads us to assume that by participating in the fermentation, the immobilization maintained a greater stability of bacteria.


**Keywords:** lactic acid bacteria; biocatalyst; immobilization; pineapple; food matrix

## 5.1 Introduction

In recent years, the interest in food that brings benefits to consumers has been increasingly explored by the industry. Belonging to the functional food group, probiotics are characterized as viable microorganisms. When administered in sufficient quantities, (of at least $10^6$–$10^7$ CFU/g) they influence health promotion (WHO/FAO, 2002).

To be considered probiotic, a strain must cross a series of barriers imposed by the gastrointestinal tract such as the presence of enzymes, stomach juice and the resident microbiota, among other factors (Franz & Holzapfel, 2011).

The bacterium of the present study – *L. casei* CSL3 – was isolated from bovine colostrum silage and evaluated for its probiotic potential *in vitro*, demonstrating potential for auto-aggregation, co-aggregation, tolerance to the passage through the simulated gastrointestinal tract and antagonistic activity against some pathogens of importance in foods. Besides these characteristics, the bacterium did not show virulence factors: this would allow its future application in food (Vitola et al., 2018).

Studies have shown that, when applied to food, probiotic concentration will depend on storage conditions, characteristics of the food matrix and the processing through which it will pass. Some foods are considered to be promising vehicles for the delivery of the microorganism, such as dairy products. They represent one of the largest segments of the market among probiotic foods (Boylston et al., 2004). However, there are no reports on the

addition of potentially probiotic isolates of *Lactobacillus casei* from dairy sources in *Petit Suisse* cheeses.

Research converges towards the use of immobilization of probiotic bacteria in order to maintain viability during storage time (Kourkoutas, Kanellaki, et al., 2006) and their catalytic activities (Freeman & Lilly, 1998), as well as its protection from extremely adverse conditions of the gastrointestinal tract (Terpou et al., 2017).

The development of cellular immobilization (CI) techniques combined with the use of new materials as supports has been researched with the purpose of creating new products. Examples are probiotic frozen yogurt fortified with sea buckthorn berries (Terpou et al., 2019), cheese with probiotic lactobacilli immobilized on casein (Dimitrellou et al., 2017), probiotic yogurt with pieces of fresh or dehydrated apple and wheat grains (Bosnea et al., 2017).

Therefore, the aim of the present study was to evaluate supports for immobilization of potentially probiotic *L. casei* CSL3, its viability during storage in *Petit Suisse* cheese and its passage through gastrointestinal transit *in vitro*.

## 5.2 Materials and methods

### 5.2.1 Materials

The experiment employed pineapple (*Ananas comosus*), guava (*Psidium guajava*) and kiwi (*Actinidia deliciosa*) in dehydrated pieces – purchased in bulk from local market –, pasteurized milk (Da Fazenda®), calcium chloride (0.25 g/L, Synth, São Paulo, Brazil), starter culture (1% vol/vol, *Streptococcus thermophilus* TH-4®, Chr. Hansen, Valinhos, São Paulo, Brazil), *Lactobacillus*

*casei* CSL3 isolated by Vitola et al. (2018), rennet (Ha-La®, Valinhos, São Paulo, Brazil) and sterilized milk cream (20% wt/wt fat, CCGL®, Cruz Alta, Brazil).

### 5.2.2 Experimental design

A two-factorial scheme involving formulation and refrigerated storage time was used for choice of the immobilization support. The experiment (*Petit Suisse* cheese) was entirely causalized with three experimental replicates. Another two-factorial scheme involving formulation and refrigerated storage time was used to assess water activity, moisture, protein, pH, acidity and viability of probiotic bacteria. Finally, three-factorial schemes were used to assess syneresis and gastrointestinal transit. Syneresis was assessed according to formulation, refrigerated storage time and applied centrifugal force, whereas gastrointestinal transit was evaluated according to formulation, refrigerated storage time and conditions of the gastrointestinal tract.

### 5.2.3 Culture growth and cell immobilization

In order to evaluate the most suitable support for immobilization of probiotic bacteria, three dehydrated fruits were used. *L. casei* CSL3 was grown on de Man, Rogosa and Sharpe (MRS) broth (Acumedia®, Lansing, USA), for 18 h at 37 °C, after being centrifuged at 4.165 *g* for 10 min at 20 °C.

Cell immobilization on dehydrated pineapple, guava and kiwi pieces (≈ 1.0 $cm^3$ cubes) was carried out as described previously in Kourkoutas et al. (2005) with modifications. In brief, fruit pieces (≈ 50 g) were placed under

ultraviolet light for complete sterilization, being then introduced into 200 mL of MRS broth together with 1 g of moist *pellet* containing a concentration of 10 log CFU/mL of *L. casei* CSL3. The fermentation occurred at 37 °C without agitation, for 48 h.

Thereafter, the broth was drained and the fruits were washed three times with phosphate buffered saline (PBS). *Lactobacillus casei* CSL3 were counted in the broth after fermentation and in the fruits, stored under refrigeration (4 °C), for 43 days.

After choosing the support, the methodology was repeated in greater proportions with modifications, using 500 g of fruit pieces (≈ 0.5 $cm^3$ cubes), 8 g of moist *pellet* (≈ 10 log CFU/mL) and 2 L of MRS broth, maintaining the same fermentation conditions previously mentioned. The size of the pieces was reduced with the intention of increasing the contact surface, thus allowing a larger number of *L. casei* CSL3 cells to adhere.

### 5.2.4 Probiotic *Petit Suisse* cheese production

The base mass was prepared according to the methodology described by Cardarelli et al. (2008), with modifications proposed by Esmerino et al. (2013). Pasteurized milk was heated to 45 °C, being then added *S. thermophilus* by direct inoculation, followed by homogenization. The containers with the inoculated milk were maintained at 43 °C until pH reached 6.3 to 6.5, and then rennet was added. The mixture was homogenized and maintained at 43 °C, until a curd formed with a pH between 5.6 and 5.8.

The curd was cut into cubes, which were placed in sterile cloth sacks to drain off the whey at a temperature of 10 °C for 12 h. After drainage, the base mass was separated into two portions based on the *Petit Suisse* Cheese Identity and Quality Standard (BRAZIL, 2000), which allows the addition of up to 30% non-dairy ingredients. Hence, the control treatment (C) received 5% sucrose, 5% cream and 0,7% (w/w) of moist *pellet* (≈ 10 log CFU/g) free *L. casei* CSL3, whereas treatment 1 (T1) received 5% sucrose, 5% cream and 20% biocatalyst containing immobilized *L. casei* CSL3.

### 5.2.5 Physicochemical analysis

Determination of pH (AOAC 981.12/90) and total titratable acidity (AOAC 497.05) were performed every seven days during the experiment period (8 weeks), while water activity (AOAC 978.18), moisture (AOAC 925-09) and proteins (AOAC 920-87) were examined in the first and last weeks. All analyses followed the methodology described by the Association of Official Analytical Chemists (AOAC).

### 5.2.6 Determination of forced syneresis

In order to evaluate the forced syneresis of processed *Petit Suisse* cheese, the methodology described by Wolfschoon-Pombo et al. (2018) was followed.

In the first step, aliquots of each sample (45 g ± 0.05 g) were centrifuged under conditions of 3,000 x *g* for 10 min at 10 °C. An increase to 9,000 x *g* and

15,000 x *g* was set for the next two steps with the same retention time and temperature. After each step, the serum obtained was separated and weighed.

### 5.2.7 Microbiological analyses

#### 5.2.7.1 Viability of probiotic bacteria

In order to monitor the viability of the *L. casei* CSL3, the analyses were performed in all eight weeks of storage. Samples of *Petit Suisse* cheese (25 g) were homogenized in 225 mL of 0.1% peptone water (Acumedia, Brazil), followed by serial dilutions. MRS agar (KASVI, Brazil) was used for the counting of probiotic bacteria, supplemented with 0.3% bovine bile (Sigma-Aldrich®, Missouri, EUA), according to protocol described by Vinderola & Reinheimer (1999). The plates were incubated at 37 °C for 48 h in anaerobic jars.

#### 5.2.7.2 Simulated gastrointestinal tract

The *in vitro* simulation was continuous (as happens during actual digestion). The elements reproduced in the simulation were the enzymes, their concentration, the period of time and the intensities of stirring in all steps (in order to simulate the peristaltic movements). This simulation followed the method described by Madureira et al. (2011), except for the mouth step. Samples of treatments C and T1 were collected in the first and eighth week of storage time and the viable numbers of *L. casei* were verified by counting on MRS agar.

### 5.2.8 Scanning Electron Microscopy (SEM)

The samples of *L. casei* CSL3 immobilized on pineapple before and after its passage through simulated gastrointestinal tract were examined in a scanning electron microscope (SEM), Jeol, JSM - 6610LV, with EDS microwave (USA), at the Southern Electron Microscopy Center located at the Federal University of Rio Grande (FURG), Brazil.

### 5.2.9 Sensory acceptance

The study was submitted to the Research Ethics Committee belonging to the Faculty of Medicine of the Federal University of Pelotas (UFPel), being approved and registered under no. 66106917.0.0000.5317. The *Petit Suisse* cheese formulated with *L. casei* CSL3 immobilized on pieces of pineapple showed absence of thermotolerant coliforms (45 °C), coagulase-positive staphylococci, *Salmonella* spp. and *Listeria monocytogenes* (ANVISA, 2001).

The sensory analysis was performed in a laboratory containing individual booths in standard conditions of light and temperature (23 °C). A total of 100 individuals participated in this analysis. After being informed of every detail regarding the scope of the research, they evaluated the sample containing *L. casei* CSL3 immobilized in pineapple.

The samples (~25 g) were served in disposable plastic cups and evaluated according to the degree of liking of odor, taste, consistency, appearance and global acceptance using a 9-point hedonic scale (ISO 11036, 2014). The acceptance index was calculated out of the average score for overall aspect.

### 5.2.10 Statistical analysis

Analyses related to immobilization, as well as those related to *Petit Suisse* cheese, were performed in duplicate with three biological replications, totaling six results for each sample. The data obtained were analyzed for normality by the Shapiro-Wilk test; homoscedasticity was analyzed by the Hartley test. Subsequently, the data were submitted to an analysis of variance (ANOVA) through the F-test ($p \leq 0.05$). Finding statistical significance, we used the T-test to compare treatment factors with two levels and Tukey's test to three levels. A confidence interval at 95% probability was applied for eight-level refrigerated storage time when significant according to the F-test ($p \leq 0.05$).

## 5.3 Results and Discussion

### 5.3.1 Cellular immobilization on different supports

It can be observed in Figure 2 that, during the 43 days of storage, there was a drop in the concentration of immobilized *L. casei* CSL3 in the three fruits. Although the same bacterial concentration was applied to each of them, it can be noted that they immobilized different amounts of *L. casei* CSL3, as shown at time zero. This fact may be related to the undigested carbohydrate content present in each one.

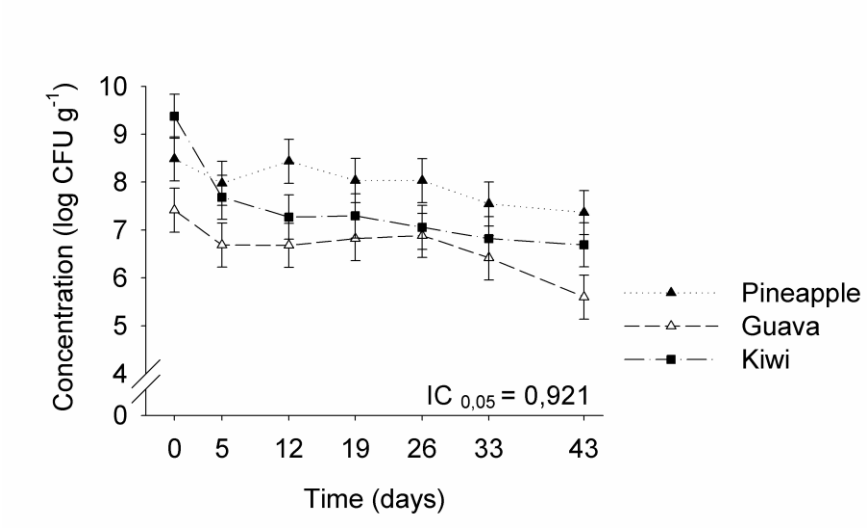



Figure 2. Concentration of *L. casei* CSL3 immobilized on pineapple, guava and kiwi pieces during refrigerated storage time. Vertical bars indicate confidence intervals. Differences were considered significant when there was no overlap between vertical bars.

Research has shown that a suitable support for the immobilization of microorganisms must maintain cell viability, be easy to handle, be available and have a good cost-benefit analysis (Mitropoulou et al., 2013).

At the end of the 43rd day, it was decided to continue the research using pineapple as a support for immobilization, since it maintained a viability of 7.37 log CFU/mL, a higher number when compared to kiwi (6.69 log CFU/mL) and guava (5.60 log CFU/mL).

### 5.3.2 *Petit Suisse* cheese evaluation

#### 5.3.2.1 Determination of pH and acidity

The ANOVA referring to *Petit Suisse* pH showed that there was no interaction between the treatment factors (p = 0.380), with significant effect only

for time ($p < 0.05$). Therefore, the free or immobilized probiotic bacterium does not affect the pH of the product (Figure 3 A).

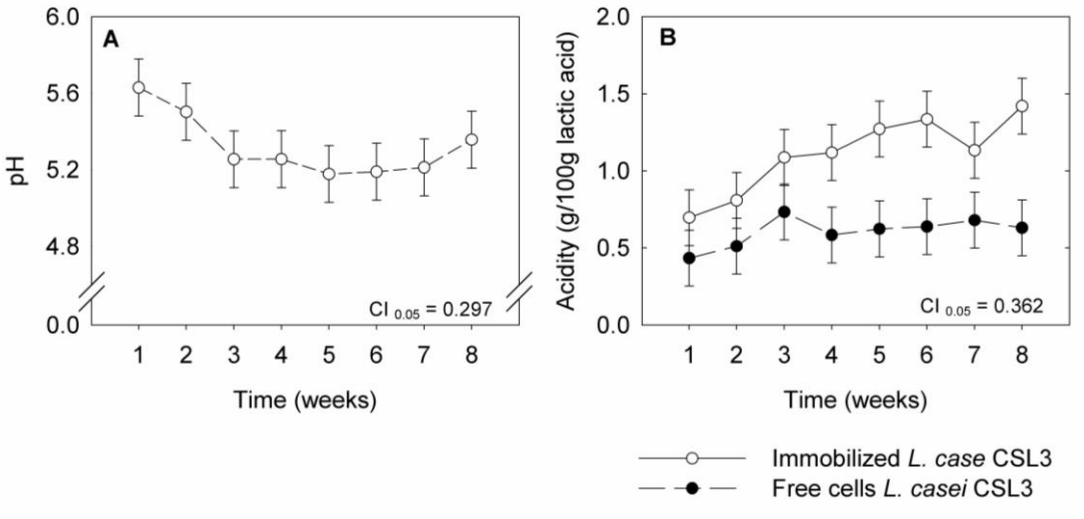



Figure 3. Analysis of pH (A) and acidity (B) in *Petit Suisse* during eight weeks of refrigerated storage. Vertical bars indicate confidence intervals. Differences were considered significant when there was no overlap between vertical bars.

Pereira et al. (2016) evaluated probiotic *Petit Suisse* cheese with jabuticaba extract and observed a decrease in pH after 28 days of storage, ranging from 4.91 to 4.44. It was demonstrated that the presence of probiotic bacteria possibly influenced the production of organic acids by secondary metabolism or the action of the enzyme system.

Regarding acidity, there was interaction between treatment factors ($p = 0.0119$), indicating that formulations and time affect the acidity of the product. Based on the confidence interval obtained from the interaction of treatment factors, it could be observed that there was a difference between the formulations from the 4$^{th}$ week of storage. The treatment with immobilized *L.*

*casei* CSL3 (Figure 3 B) showed higher acidity, whereas the formulation with free *L. casei* CSL3 analyzed over time showed that there is no significant variation in acidity during its storage (Figure 3 B).

The gradual increase in the formation of lactic acid by the immobilized microorganism suggests that, over time, the immobilization maintains a high metabolic activity of the bacterium when compared to its free state (Kourkoutas et al., 2005; Mitropoulou et al., 2013).

Also, it is noted that the production of lactic acid has little influence on the drop in pH. In fact, this drop can be explained by its weak organic acid classification, which has a dissociation finding (pKa) equivalent to 3.86. Therefore, it will be in its form undissociated (Giannuzzi & Zaritzky, 1996).

In a study by Deolindo et al. (2019), the *Petit Suisse* cheese obtained a variation in the acidity percentage from 1.64% to 1.11%: over time, the acidity of the cheese decreased as pH values increased. This behavior was not observed in the present study – in fact, there was an increase in acidity level over the weeks (0.39% - 1.83%), which resulted in the reduction of pH values for both formulations.

#### 5.3.2.2 Determination of forced syneresis

Forced syneresis by centrifugation is determined by a gradual increase in the *g*-force and the weight of the liquid released between steps. An ANOVA showed that there was a triple interaction ($p = 0.0053$) between formulation, centrifugal force and time. A comparison between centrifugal forces showed

that cheese with free and immobilized *L. casei* CSL3 had similar behaviors, since increased centrifugal force resulted in lower syneresis (Table 2).

Table 2. Syneresis percentage of *Petit-suisse* cheese containing *L. casei* CSL3 free and immobilized under different forces during storage time.

| Time | 3,000 g | | 9,000 g | | 15,000 g | |
|---|---|---|---|---|---|---|
| (weeks) | F | I | F | I | F | I |
| 1 | 28.06±7.47$^{aA}$ | 24.04±4.46$^{aA}$ | 11.74±2.22$^{bA}$ | 12.11±1.6$^{bA}$ | 4.44±2.1$^{cA}$ | 6.33±0.9$^{cA}$ |
| 2 | 29.19±5.66$^{aA}$ | 29.23±8.27$^{aA}$ | 10.17±1.23$^{bA}$ | 9.30±1.88$^{bA}$ | 4.56±0.9$^{cA}$ | 6.53±3.4$^{bA}$ |
| 3 | 25.84±5.84$^{aA}$ | 33.09±3.60$^{aA}$ | 12.38±1.21$^{bA}$ | 10.18±1.5$^{bB}$ | 5.33±1.0$^{cA}$ | 5.16±2.03$^{cA}$ |
| 4 | 30.82±2.68$^{aB}$ | 40.09±4.97$^{aA}$ | 10.41±1.21$^{bA}$ | 9.21±2.21$^{bA}$ | 3.04±1.7$^{cA}$ | 3.53±1.29$^{cA}$ |
| 5 | 31.35±4.48$^{aA}$ | 36.19±0.64$^{aA}$ | 10.44±0.73$^{bA}$ | 8.28±2.41$^{bA}$ | 4.50±1.4$^{cA}$ | 4.10±0.75$^{cA}$ |
| 6 | 28.64±4.11$^{aA}$ | 30.53±2.14$^{aA}$ | 12.90±1.51$^{bA}$ | 10.79±1.8$^{bA}$ | 4.31±2.1$^{cA}$ | 5.04±1.76$^{cA}$ |
| 7 | 21.29±5.38$^{aA}$ | 27.76±1.91$^{aA}$ | 11.11±5.54$^{bA}$ | 12.66±1.4$^{bA}$ | 5.20±2.9$^{bA}$ | 5.25±1.76$^{cA}$ |
| 8 | 25.05±6.10$^{aA}$ | 33.00±11.13$^{aA}$ | 15.01±2.18$^{bA}$ | 10.85±1.1$^{bB}$ | 6.83±1.9$^{cA}$ | 6.75±1.06$^{bA}$ |

F: free *L. casei* CSL3; I: immobilized *L. casei* CSL3

Different lower case letters on the same line indicate difference by Tukey test ($p < 0.05$) comparing the three levels of centrifugal forces (3,000 g, 9,000 g, 15,000g) setting formulation and time treatment factors. Different upper case letters in the same line indicate difference by T-test ($p < 0.05$) when comparing cheese with free and immobilized *L. casei* CSL3, setting centrifugal force and time. Mean ± standard deviation (n = 6)

The comparison between cheeses with free and immobilized *L. casei* CSL3 showed that there was a difference in the 3$^{rd}$ and 8$^{th}$ weeks when applied force of 9,000 *g*, and in the 4$^{th}$ week when applied force of 3,000 *g*. However, there was no difference between the formulations (free and immobilized *L. casei* CSL3) when applied force of 15,000 *g*.

Regarding the comparison over storage time, it could be observed that the treatments submitted to centrifugal force of 3,000 *g* presented variations in syneresis (Figure 4). The formulations subjected to 9,000 *g* and 15,000 *g* did not change syneresis over the storage time (Figure 4), showing that the serum

release in the first stage (3,000 *g*) was higher than in the other stages. This is because a large part of the whey had already been released. A similar result occurred in a study with cream cheese samples without the addition of stabilizers (Wolfschoon-Pombo et al., 2018).

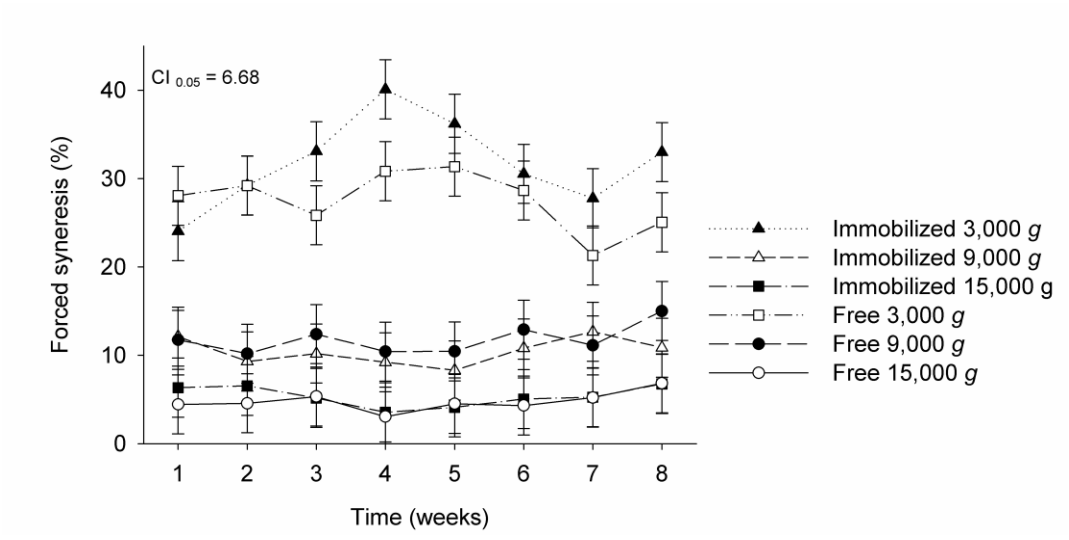



Figure 4. Percentages of forced syneresis under different rotations for *Petit Suisse* cheeses containing free and immobilized *L. casei* CSL3. Vertical bars indicate confidence intervals. Differences were considered significant when there was no overlap between vertical bars.

Cheese containing the free bacteria under centrifugation of 3,000 *g* presented lower syneresis at the 7$^{th}$ week of storage, compared to 1$^{st}$, 2$^{nd}$, 4$^{th}$, 5$^{th}$ and 6$^{th}$ weeks. The 8$^{th}$ week showed intermediate syneresis values and did not differ from any other week (Figure 4). In cheese containing the immobilized bacteria, when applied a centrifugal force of 3,000 *g* there was increase in syneresis from 1$^{st}$ to 3$^{rd}$ week and from 3$^{rd}$ to 4$^{th}$ week. There was reduction in syneresis from 4$^{th}$ to 6$^{th}$ week and from 7$^{th}$ to 5$^{th}$ week.

Cheese containing immobilized *L. casei* CSL3 maintained a higher percentage of syneresis (3,000 *g*) when compared to free *L. casei* CSL3 at the end of storage time. This fact can be attributed to the increase in the acidity rate (Lucey, 2001), which makes the elastic component lower, thus having a less compact matrix that will release the whey more easily (Ningtyas et al., 2017).

The addition of the biocatalyst could influence this percentage, because during the immobilization process, the dehydrated pineapple remains in contact with the broth culture medium, causing the fruit to absorb and retain this liquid through osmotic equilibrium (A. K. Yadav & Singh, 2014).

#### 5.3.2.3 Moisture and protein water activity ($a_w$)

According to the Identity and Quality Technical Regulation established in Normative Instruction nº 53 (Brasil, 2000), in order to receive the definition of *Petit Suisse*, a cheese must have a moisture not lower than 55.0% and a minimum protein content of 6.0%.

An ANOVA for moisture analysis indicated that only the formulations had significant effect ($p = 0.0005$), showing that the refrigerated storage time did not affect humidity. *Petit Suisse* with immobilized *L. casei* CSL3 showed a higher percentage of humidity (69.29%) compared to *Petit Suisse* containing free bacteria (66.42%).

The addition of pineapple pieces in treatment one (T1) had direct influence on moisture content, keeping it higher when compared to the control group with free bacteria (C). Research working on the development of this type of cheese demonstrates moisture percentages ranging from 63.0% to 76.0%

(Prudencio et al., 2008; Matias et al., 2014; Pereira et al., 2016). Also, it is suggested that protein concentration, coagulum cut size and coagulation temperature may directly interfere in the moisture of a content in its base mass (Panthi et al., 2019).

There was no significant effect of treatment factors on protein content (formulations x refrigerated storage time). The average protein value of *Petit Suisse* was 13.00% ± 1.23.

When producing *Petit Suisse* cheese with different raw materials, such as bovine milk, sour cream together with soy and soy milk, Matias et al., (2014) found that protein values ranged from 10.75% (sour cream and soy) to 17.10% (bovine milk). Considering this range, it is clear that the protein value found in this study (13.00%) is intermediate, which suggests that protein content is influenced by various factors: the milk used in the formulation, dairy animal species, breed, age and diet, along with lactation phase, parity (number of births), farming system, physical environment and season (Dominguez-Salas et al., 2019).

Regarding the percentage of proteins, it is common understanding that for cheese making, an essential step is coagulation. This process is simply the modification of the micellar structure of caseins, which represent 80% of the total proteins present in milk (Q. Li & Zhao, 2019).

The water activity (aw) makes it possible to assess the availability of free water in the food, that is available for reactions and has direct influence on microbial development and viability when in food products, since most of these bacteria keep their active metabolism at $a_w$ above 0.920.

Comparison between formulations (p = 0.0408) indicated that there is no difference in water activity at the 8th week (Table 3). However, there was a difference in the comparison between 1st and 8th weeks for both formulations (Table 3).

Table 3. Analysis of water activity in *Petit Suisse* containing immobilized and free *L. casei* CSL3

| Time | Immobilized *L. casei* CSL3 | Free *L. casei* CSL3 |
|---|---|---|
| 1st week | 0.973±0.002$^{aB}$ | 0.972±0.002$^{aB}$ |
| 8th week | 0.976±0.001$^{aA}$ | 0.978±0.001$^{aA}$ |

Different lower case letters on the same line indicate difference (p <0.05) by the T-test comparing the different formulations, setting the time. Different upper case letters in the same column indicate difference (p <0.05) by T-test for storage time, fixing formulation. Mean ± standard deviation (n = 6)

The water-holding capacity in acidified milk gels is determined by the microstructure of the protein network. If the water binding is not sufficient, it will not remain attached to the micelle during storage, and consequently the free water content will increase (Mortensen et al., 2010) .

### 5.3.2.4 Microbiological analyses

#### 5.3.2.4.1 Viability of *L. casei* CSL3

For cheeses containing immobilized and free bacteria, it was verified that there was a significant effect only for formulation (p = 0.022), this being respectively 8.78 log CFU/g and 8.99 log CFU/g. The refrigerated storage time did not influence the counts.

Dimitrellou et al. (2014) evaluated *L. casei* ATCC 393, free and immobilized, as starter cultures in the production of probiotic cheese. In this study, it was observed that there was no difference between the viability of the

bacteria in both treatments over the storage period, which corroborates results of the present study. This fact can be attributed to the protection of the microorganism in the protein matrix that forms the cheese. Thus, both formulations maintained the viability of probiotic bacteria at appropriate concentrations (≥ $10^6$ CFU/g) to confer the proposed beneficial effect (WHO/FAO, 2006).

In the current experiment, the cheese was supplemented with the probiotic, showing that, when it does not participate in fermentation, the bacteria are independent of the support. However, it is assumed that by acting in this process as a biocatalyst, the immobilized microorganism will receive the protection that will keep it stable, as proved by Kourkoutas et al. (2005).

**5.3.2.4.2 Simulated gastrointestinal transit (GIT) condition**

There is significant effect for time ($p < 0.0001$) and formulation ($p = 0.0123$) and interaction between time and formulation ($p = 0.0181$). Thus, gastrointestinal transit conditions did not affect probiotic viability, either in its free or immobilized form. Cheese containing free bacteria, when analyzed in the 1st week of refrigerated storage, showed higher GIT counts than cheese containing immobilized bacteria. However, at their 8th week of storage the counts for free or immobilized bacteria during GIT did not differ (Table 4).

Table 4. *Petit Suisse* containing immobilized and free *L. casei* CSL3 subjected to GIT during refrigerated storage time (log CFU/g).

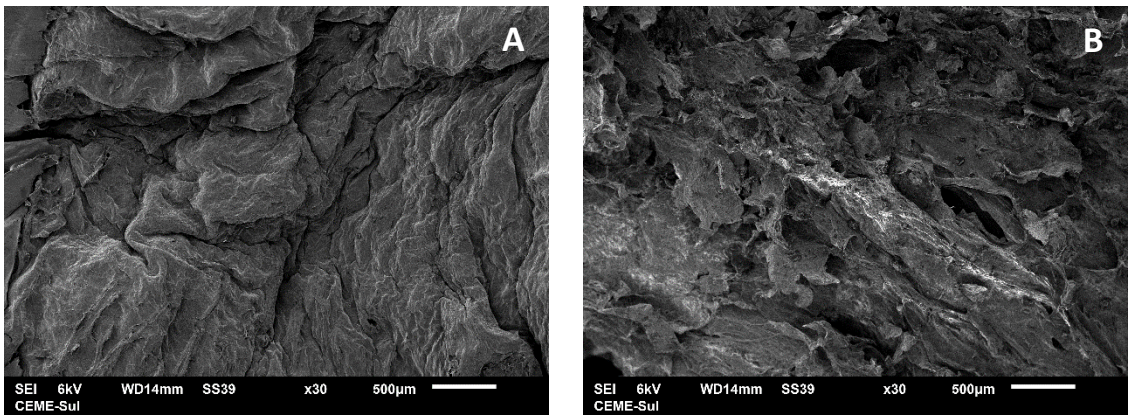
| **Time** | **Immobilized *L. casei* CSL3** | **Free *L. casei* CSL3** |
|---|---|---|
| 1st week | $7.02 \pm 0.54^{bB}$ | $7.54 \pm 0.71^{aA}$ |
| 8th week | $7.70 \pm 0.40^{aA}$ | $7.73 \pm 0.27^{aA}$ |

Different lower case letters on the same line indicate difference ($p < 0.05$) by the T-test comparing the different formulations, setting the time. Different upper case letters in the same

column indicate difference ($p < 0.05$) by T-test for storage time, fixing formulation. Mean ± standard deviation (n = 6).

Terpou et al. (2019) immobilized *L. casei* CSL3 ATCC 393 in buckthorn berries and observed that, at the end of simulated gastrointestinal transit, the yogurt containing the immobilized bacteria remained in concentration 7.47 log CFU/g while the free bacteria remained at a concentration of one logarithmic cycle less. In comparison to this study, we observed that there was no difference in the 8$^{th}$ week of storage for both formulations, which may suggest that the chosen food matrix protected the probiotic bacteria.

It is noted that, after passage through the gastrointestinal transit, the plant tissue was damaged (Figure5 B) compared to the image prior to the GIT (Figure 5 A). *L. casei* CSL3 (Figure 5 C), when finding a hostile environment with high acidity and presence of distinct enzymes, changed its morphology from bacilli to a coccoid form (Figure 5 D).



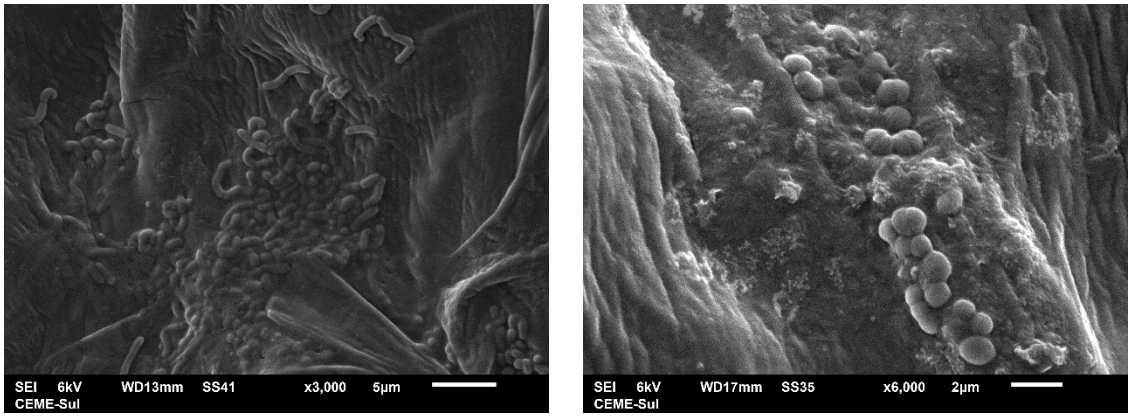



Figure 5. Scanning electron microscopy (SEM) micrographs of pineapple after freeze-drying; (A) Pineapple surface before GIT passage; (B) Pineapple surface after GIT passage; (C) *L. casei* CSL3 immobilized on pineapple surface before GIT passage; (D) *L. casei* CSL3 immobilized on pineapple surface after GIT passage.

Changes in the morphology of bacteria when exposed to adverse situations have been observed in previous studies (Nishino et al., 2018; Sanhueza et al., 2015). These adverse situations can be the presence of acids, other bacteria or methodologies that subject them to a certain level of stress.

### 5.3.2.5 Sensory acceptance

Figure 6 shows the results obtained from the sensory analysis of *Petit Suisse* cheese containing *L. casei* CSL3 immobilized on pineapple pieces.

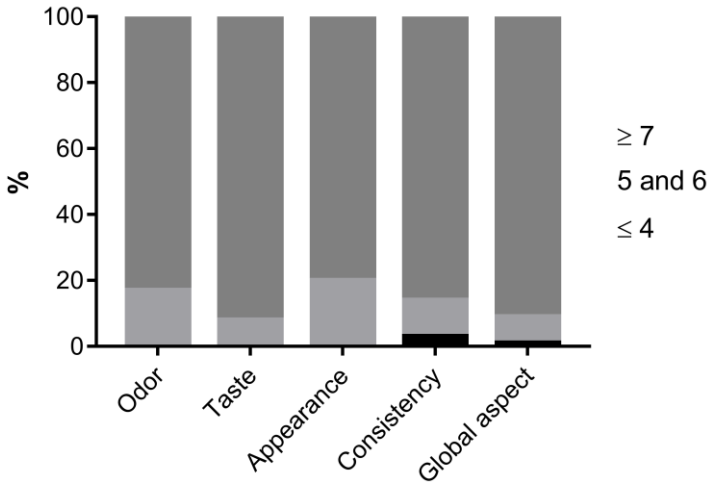



Figure 6. Sensory acceptance indices of *Petit Suisse* cheese with *L. casei* CSL3 immobilized on pineapple pieces.

The 100 evaluators participated in the analysis of the following attributes: odor, taste, appearance, consistency and overall appearance. All attributes had average evaluation rates above 70%, being these 83%, 92%, 80%, 86% and 91% respectively. Concerning sensorial properties, a product is considered acceptable as long as it obtains an index equal to or greater than 70%. The assessed sample had an acceptability index of 91%, which was calculated out of the average for overall aspect. Thus, it was considered to have market potential.

## 5.4 Conclusion

At the end of the study, it was possible to conclude that the most appropriate support for immobilization of *L. casei* CSL3 was pineapple, as it maintained viability at higher concentrations.

It is noteworthy that the immobilization of the bacteria allowed the maintenance of the synthesis of lactic acid in higher concentrations when compared to their free state. There were no significant differences when assessing the viability of the probiotic for both free and immobilized states. The matrix chosen for microorganism supplementation protected the bacteria during its passage through the gastrointestinal transit.

This suggests that, when used as a biocatalyst, the *L. casei* CSL3 set immobilized on pineapple chunks will have a better response in the production of lactic acid, which will aid in the fermentation process.

Also, in the sensorial evaluation of the cheese with *L. casei* CSL3 immobilized in pineapple, all attributes obtained scores higher than 7, thus predicting the acceptance of the product.

Finally, it is important to mention that another step of this research is in progress, in order to assess future commercial application of *L. casei* CSL3: *in vivo* studies are being carried out with the purpose of proving the probiotic potential of the bacterium using an animal model.

## CAPÍTULO 3

# Effect of oral administration of *Lacticaseibacillus* casei CSL3 and challenge with *Salmonella* Typhimurium and *Escherichia coli* O157: H7 on biochemical and oxidative stress parameters in *Swiss* albino mice

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## Abstract

Probiotics are microorganisms that, when consumed in certain contexts, can offer health benefits such as reducing blood glucose and cholesterol levels, acting against food-borne pathogens and even reducing oxidative stress. This study aimed to evaluate biochemical parameters in blood plasma and oxidative stress in tissues of healthy mice treated with *L. casei* CSL3 and challenged with *E. coli* O157:H7 and *S*. Typhimurium. Determinations were performed on the levels of alanine transaminase (ALT) and aspartate aminotransferase (AST) enzymes, creatine, total cholesterol, triglycerides, glucose and thiobarbituric acid reactive substances (TBARs) in tissues (oxidative stress). At the end of the treatments, *Lacticaseibacillus casei* CSL3 had reduced blood glucose levels, had exerted an influence on the production of the catalase enzyme in the kidney, increasing its synthesis and, finally, had influenced the treatments in which the bacterium was challenged with the pathogens. In these, there was a decrease in the concentration of substances reactive to thiobarbituric acid, which suggests that there was a reduction in the oxidative stress generated by the challenge. Therefore, it is concluded that *L. casei* CSL3 positively influenced the blood glucose and triglycerides concentrations as well as the response to oxidative stress.


**Keywords:** glucose, cholesterol, oxidative stress, probiotic, enzymes.

## 6.1 Introduction

Probiotics are live microorganisms that, when administered in adequate amounts, confer a health benefit to the host (Hill et al., 2014b; WHO/FAO, 2001). Belonging to the human and animal microbiome, the *Lactobacillus* genus stands out as being the most widely used probiotic, as well as the new genera from the *Lactobacillus* (e.g. *Lacticaseibacillus)*. Taxonomically, these bacteria belong to the Domain Bacteria, phylum Firmicutes, class Bacilli, order Lactobacillales, family Lactobacillaceae, and these genera are composed of more than 261 species. They are Gram-positive, facultative anaerobes, catalase-negative and non-spore-forming (Kleerebezem & Vaughan, 2009, Zheng et al., 2020).

In order to ensure the survival, adaptation and colonization of the gastrointestinal tract, *Lactobacillus* spp. develops a stress tolerance system, through the coordination, expression or suppression of genes, which maintain the integrity of the cell envelope, repair, protect and export macromolecules. This facilitates interactions with the host and directly contributes to the nutritional, physiological, microbiological and immunological effects (Sengupta et al., 2013; Vandenplas et al., 2015).

It is known that when applied under different circumstances, *Lactobacillus* spp. demonstrate benefits considered significant of the host, and with the advancement of science these fields of applications continue to expand (Zhang et al., 2018). Studies demonstrate that some strains of *Lactobacillus* spp. can influence the reduction of the cholesterol level by assimilating it in the

intestine (Tomaro-Duchesneau et al., 2014), reducing blood glucose levels (da Costa et al., 2018) and also acting as a potential antioxidant (Lee et al., 2016).

The *Lactobacillus* genus has a wide natural habitat, and its high degree of genetic diversity as well as its complex phylogeny influence its metabolism. Therefore, it is of paramount importance to study new isolates in order to observe their influence when applied in a complex system such as *in vivo* experimentation (Wittouck et al., 2019).

As it has been characterized with probiotic potential through *in vitro* and *in situ* tests, the isolate *Lacticaseibacillus casei* CSL3 (formerly named *Lactobacillus casei* CSL3)**,** from bovine colostrum silage, has proved to be safe, not synthesizing gelatinase, DNase, or hemolytic toxins and showing sensitivity to most antimicrobials tested in clinical use. It is noteworthy that the resistance presented to vancomycin and sulfanilamide was considered intrinsic through the plasmid profile. As for the probiotic potential, the isolate showed high levels of self-aggregation and co-aggregation, resistance to passage through the simulated gastrointestinal transit and antibacterial activity against strains of *Listeria monocytogenes*, *Staphylococcus aureus*, *Escherichia coli* and *Salmonella* Typhimurium (Vitola et al., 2018).

The intestine can be considered the main site of action of the most important bacterial pathogens transmitted through food, and in conditions of metabolic syndromes such as diabetes and obesity there is an aggravation of the pathogenicity through these bacteria. A strategy considered current and relevant is the consumption of probiotics, either as supplements or through food products (Mousavi Khaneghah et al., 2020). Studies reveal that at low

concentrations, polyunsaturated fatty acids and short-chain fatty acids such as butyrate, synthesized by probiotics can reduce the pathogenicity of *E. coli* by altering the genes that encode chromosomal pathogenicity and supporting the creation of lesions in the mucosal epithelium. This, in turn, prevents the transcription of virulence genes, responsible for intestinal colonization, by *L. monocytogenes* (Sun et al., 2012) and negatively regulates the expression of virulent genes of *S. Typhimurium* (Peng & Biswas, 2017) .

When *L. casei* CSL3 was applied in a food matrix to evaluate the behavior of the bacteria during the shelf life of the product, the isolate remained viable at concentrations above 8 log CFU. $g^{-1}$. When subjected to continuous simulated gastrointestinal transit it maintained concentrations above 7 log CFU. $g^{-1}$ (Vitola et al., 2020).

Therefore, it is necessary to continue the study by evaluating how *L. casei* CSL3, which is potentially probiotic, acts when administered orally to an animal model. The research in question aims to evaluate biochemical parameters in blood plasma and oxidative stress in tissues of healthy mice treated with *L. casei* CSL3 and challenged with *E. coli* O157:H7 and *S*. Typhimurium.

## 6.2 Materials and methods

### 6.2.1 Microorganisms

The bacterium *Lacticaseibacillus casei* CSL3 used in the present study was previously isolated from bovine colostrum silage and characterized as potentially probiotic by Vitola et al. ( 2018).

*Salmonella* Typhimurium ATCC 14028 and *Escherichia coli* O157:H7 NCTC 12900 were provided by the Food Microbiology Laboratory of the Federal University of Pelotas (RS/ Brazil).

### 6.2.2 Experimental design

The experiment was completely randomized. The analyses of glucose, triglycerides, and cholesterol were arranged in a 6 x 3 two-factorial scheme (treatment and time), while creatine was arranged in a 6 x 2 scheme (treatment and time). The other analyses (AST enzyme, ALT enzyme, kidney TBARs, liver TBARs, brain TBARs, kidney catalase, liver catalase and brain catalase) were arranged in a unifactorial scheme.

### 6.2.3 Bacterial Growth Conditions

The isolate was cultured in MRS broth (deMan, Rogosa and Sharp, Himedia, Mumbai, India) at 37 °C for 18 h, after which an aliquot of 1% was removed and transferred to a shot containing 100 mL of fresh MRS broth, incubating under the same conditions mentioned above. Finally, an aliquot of 1% of the broth containing cultured *L. casei* CSL3 was transferred to 500 mL of fresh MRS broth, maintaining the same growth conditions.

At the end, the MRS broth containing the bacteria was centrifuged at 4,165 *g* for 10 min at 20 °C, the pellet was washed three times with PBS buffer to remove any broth residue used and this was resuspended in the same buffer, stored under refrigeration (temperature 6-8 °C). Counts were performed on the

0, 15th and 30th day of the experiment to assess the maintenance of the isolate concentration.

### 6.2.4 Animal model

In the *in vivo* experiments, 60 female *Mus musculus* mice of the Swiss albino strain with 21 ± 5 days of age were used. After a week of acclimatization, kept under a controlled temperature of 23 ± 4 °C, 12h light/ dark lighting cycle, with feed (without addition of antibiotics and antifungals) and water ad libitum, the animals were divided into 6 groups, with 10 mice per treatment, during the 30 days of the experimental period.

The control group (C) received, by gavage, 300 µL of phosphate buffered saline (PBS); the LC group received 300 µL of *L. casei* CSL3 in a concentration of 8 log CFU. $mL^{-1}$ for the 30 days of the experimental period; the ST group received 300 µL of *S*. Typhimurium in a concentration of 6 log CFU. $mL^{-1}$ on the fifteenth day of the experimental period; the EC group received 300 µL of *E. coli* in a concentration of 6 log CFU. $mL^{-1}$ on the fifteenth day of the experimental period; the LCS group received 300 µL of *L. casei* CSL3 in a concentration of 8 log CFU.$mL^{-1}$ for the 30 days of the experimental period and, on the fifteenth day, it was challenged with *S*. Typhimurium in a concentration of 6 log CFU. $mL^{-1}$; and the LCEC group received 300 µL of *L. casei* CSL3 in a concentration of 8 log CFU. $mL^{-1}$ for the 30 days of the experimental period and, on the fifteenth day, it was challenged with *E. coli* in a concentration of 6 log CFU. $mL^{-1}$ Euthanasia of the animals was performed through anesthetic overdose followed by cardiac puncture.

The study was approved and registered under no. CEEA 5381 by the Ethics Committee on Animal Experimentation (CEEA - Federal University of Pelotas (UFPel). The CEEA/UFPel agreement is approved by the Brazilian National Council for Animal Experimentation Control (CONCEA).

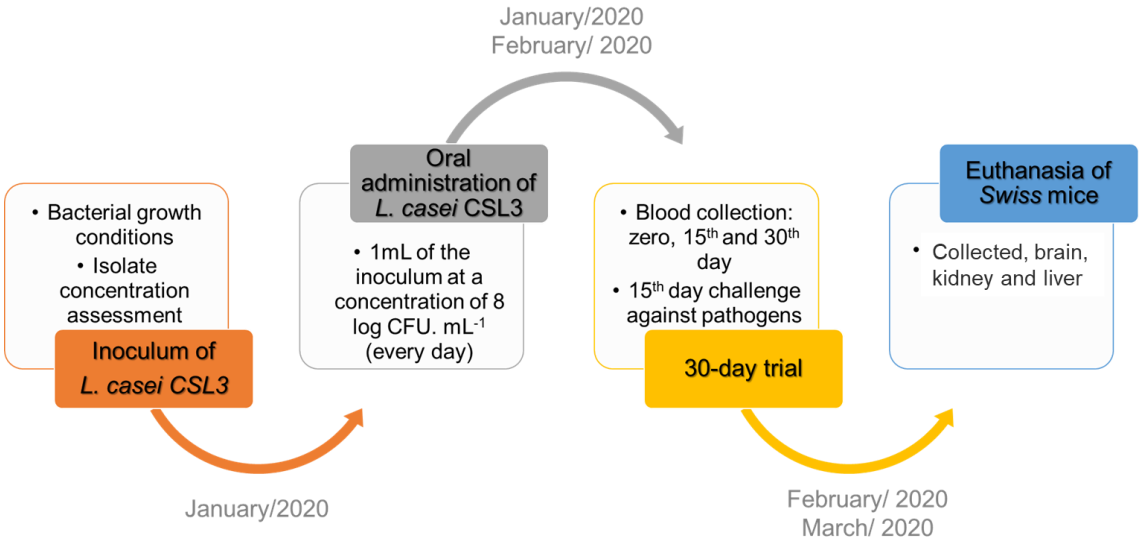


Figure 7. Timeline of the *in vivo* experimental period

### 6.2.4 Determination of serum biochemistry parameters

To determine the levels of AST (aspartate aminotransferase), ALT (alanine aminotransferase), creatinine, total cholesterol, triglycerides and glucose, blood samples from the mice were collected through the facial vein at zero, 15 and 30 days of the experiment.

In summary, 500 µL of blood was deposited in microtubes without anticoagulant, centrifuged (Eppendorf®, Hamburg, Germany) at 200 rpm for 5 minutes, at room temperature (25 ºC), after which only the supernatants were collected. The kits used for the tests were of standardized LabTeste® diagnosis with readings taken on a spectrophotometer (SpectraMaxÒ 190, Molecular

Devices, California USA) at 340 nm (AST/ALT), 546 nm (creatinine), 500 nm (total cholesterol), 505 nm (triglycerides) and 505 nm (glucose).

### 6.2.5 Evaluation of oxidative stress in tissues

On day 31 of the experiment, mice were euthanized by cervical dislocation, and the liver, brain and kidneys were removed aseptically and stored at -70 ºC. For sample preparation, tissues were weighed (0.5 mg) and mixed with TRIS buffer solution with volumes corresponding to five times the weights of the brain and ten times the weights of the livers and kidneys. Homogenization took place by means of a mechanical stirrer, with subsequent centrifugation at 1000 rpm for 10 minutes at room temperature (25 ºC). The supernatant was collected and used in the following methodologies.

To analyze the levels of thiobarbituric acid reactive substances (TBARS), 100 µL of supernatant, obtained above, was mixed with 100 µL of sodium dodecyl sulfate (SDS) solution and 4 mL of color reagent under boiled water for one hour. and then cooled with ice. The mixture was centrifuged at 1600 × *g* at 4 °C for 10 minutes and then the absorbance value was read in a spectrophotometer at 535 nm.

The analysis of the activity of the catalase enzyme consisted of reading in a spectrophotometer under ultraviolet light (UV) of the supernatant in the presence of high concentrations of the hydrogen peroxide solution (30Mm), observing its variations at 240 nm. (Aebi, 1984; Hadwan, 2018).

### 6.2.6 Statistical analysis

The data obtained were analyzed in STATISTICA® 6.0 software, for normality by the Shapiro-Wilk test, for homoscedasticity by the Hartley test and, when data with nonparametric behavior were found, we proceeded with the removal of outliers aiming to obtain data from parametric behavior. Outliers were identified by plotting studentized external residuals (RStudent) against predicted values (variable Y) and by evaluating the graph of Cook's Distance. From the RStudent plots, values out of the range −2 to 2 were considered outliers, and their corresponding observations were removed from the database (Barnett & Lewis, 1994; Rousseeuw & Leroy, 1987). Subsequently, the data were submitted to analysis of variance through the F test ($p \leq 0.05$).

Finding statistical significance, groups with six levels (C, LC, ST, EC, LCS and LCEC) were compared by the Waller-Duncan test ($p < 0.05$), while a confidence interval at 95% probability was used to compare time with three levels (0, 15 and 30 days) and T test ($p < 0.05$) for two levels (0 and 30 days).

## 6.3 Results

### 6.3.1 Determination of blood serum biochemistry parameters

Analysis of variance for the two-factorial scheme showed an interaction between treatment factors for the dependent variables glucose ($p = 0.0005$) and triglycerides ($p = 0.0001$). The comparison of groups can be seen in Table 5. There were no significant differences ($p > 0.05$) regarding cholesterol content, AST (aspartate aminotransferase) and ALT (alanine aminotransferase) between treatment factors (groups and time), maintaining, at the end of the 30 days of

treatment, averages of 113.10 mg. $dL^{-1}$, 3.88 IU. $L^{-1}$ and 8.55 IU. $L^{-1}$, respectively.

From Table 5 it is observed that the glucose concentrations, on days 0 and 15 of the experiment, do not differ significantly between treatments, remaining in the ranges of 119.87 mg. $dL^{-1}$ to 137.71 mg. $dL^{-1}$ and 114.45 mg. $dL^{-1}$ to 133.36 mg. $dL^{-1}$, respectively. However, at 30 days, the groups that received *L. casei* CSL3 (LC) and *S*. Typhimurium (S) had lower glucose concentrations than the control group, the group that received *E. coli* (EC), and the groups *L. casei* + *S*. Typhimurium (LCS) and *L. casei* + *E. coli* (LCEC).

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Table 5. Concentration of glucose and triglycerides in the blood of animals throughout the experimental period treated with *L. casei* CSL3, *S*. Typhimurium and *E. coli*.

| Marker | Period (days) | Groups | | | | | |
|---|---|---|---|---|---|---|---|
| | | C | LC | S | EC | LCS | LCEC |
| Glucose (mg. $dL^{-1}$) | 0 | 133.49±27.26$^{A}$ | 129.51±18.55$^{A}$ | 137.71±21.35$^{A}$ | 119.87±22.97$^{A}$ | 123.65±28.95$^{A}$ | 125.95±21.72$^{A}$ |
| | 15 | 114.45±31.52$^{A}$ | 133.36±21.41$^{A}$ | 118.14±29.49$^{A}$ | 123.58±22.00$^{A}$ | 119.68±29.59$^{A}$ | 115.21±28.28$^{A}$ |
| | 30 | 134.83±31.19$^{B}$ | 107.79±24,34$^{C}$ | 101.67±19.79$^{C}$ | 133.65±20.33$^{B}$ | 115.21±28.28$^{BC}$ | 162.07±31.28$^{A}$ |
| Triglycerides (mg. $dL^{-1}$) | 0 | 208.83±35.67$^{AB}$ | 209.80±36.28$^{AB}$ | 202.25±29.48$^{AB}$ | 232.01±31.03$^{A}$ | 177.03±46.33$^{B}$ | 231.22±37.72$^{A}$ |
| | 15 | 212.52±32.95$^{A}$ | 204.63±23.56$^{A}$ | 190.05±44.46$^{A}$ | 201.84±29.99$^{A}$ | 194.76±34.68$^{A}$ | 231.22±37.72$^{A}$ |
| | 30 | 284.96±35.61$^{A}$ | 205.06±43.24$^{B}$ | 183.20±24.48$^{BC}$ | 170.71±26.32$^{C}$ | 177.30±34.12$^{BC}$ | 184.65±33.98$^{BC}$ |

Means ± standard deviation followed by different capital letters on the line indicate a significant difference by the Waller-Duncan test ($p \leq 0.05$).
C: control group; LC: *L. casei* CSL3 group; S: *S.* Typhimurium group; EC: *E. coli* group; LCST: *L. casei* CSL3 against *S.* Typhimurium group; LCEC: *L. casei* CSL3 against *E. coli* group.

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Figure 8 A, the behavior of glucose levels in the blood of the treated animals can be analyzed, comparing these in terms of the elapsed time of treatment.

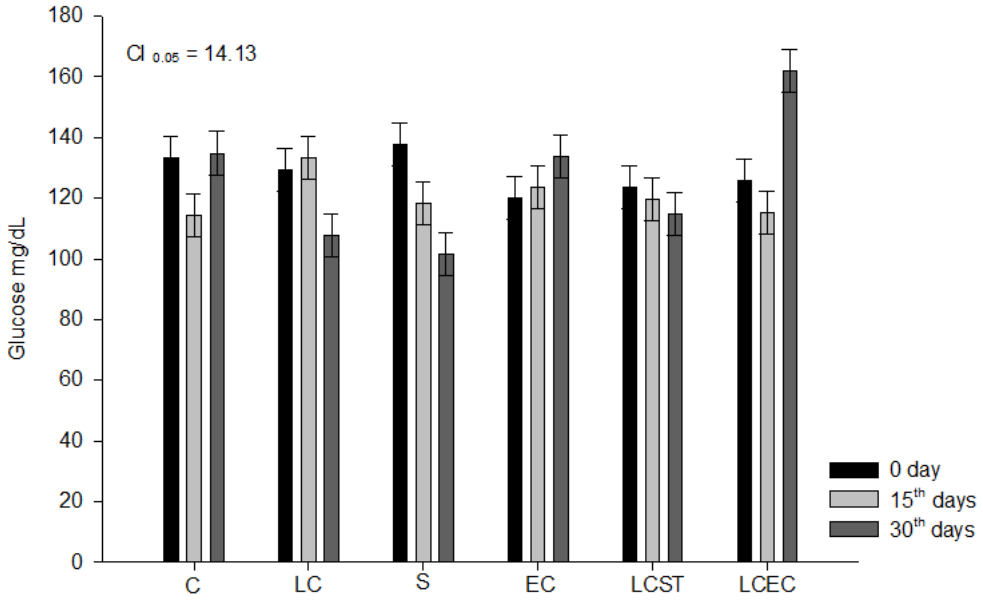


Figure 8 A. Blood glucose concentration during the experimental period of animals treated with *L. casei* CSL3, *S*. Typhimurium and *E. coli.*
C: control group; LC: *L. casei* CSL3 group; S: *S.* Typhimurium group; EC: *E. coli* group; LCST: *L. casei* CSL3 against *S.* Typhimurium group; LCEC: *L. casei* CSL3 against *E. coli* group.

It is noted that the blood glucose concentration of both the control treatment and the treatments that received *E. coli* remained higher in relation to the other treatments. On the other hand, the treatment that received *L. casei* CSL3 and the treatment that received *S.* Tyhimurium maintained the lowest glucose concentrations, not differing significantly from each other.

The concentrations of triglycerides in blood plasma, on day 0 of treatment, the groups that received *E. coli* (EC and LCEC) maintained means higher than the other groups, at 232.01 mg. $dL^{-1}$ and 231.22 mg. $dL^{-1}$, respectively, and that the groups control (C), *L. casei* CSL3 (LC) and *S*. Typhimurium (ST) did not differ from each other. At 15 days, there were no

significant differences between treatments and, at 30 days, the control group (C) maintained a higher concentration than the other groups (284.96 mg.dL$^{-1}$), and the group that received *E. coli* (EC) had the lowest average, which is 170.71 mg.dL$^{-1}$.

In Figure 8 B it is possible to evaluate the levels of serum triglycerides in each treatment comparing the times of the experiment

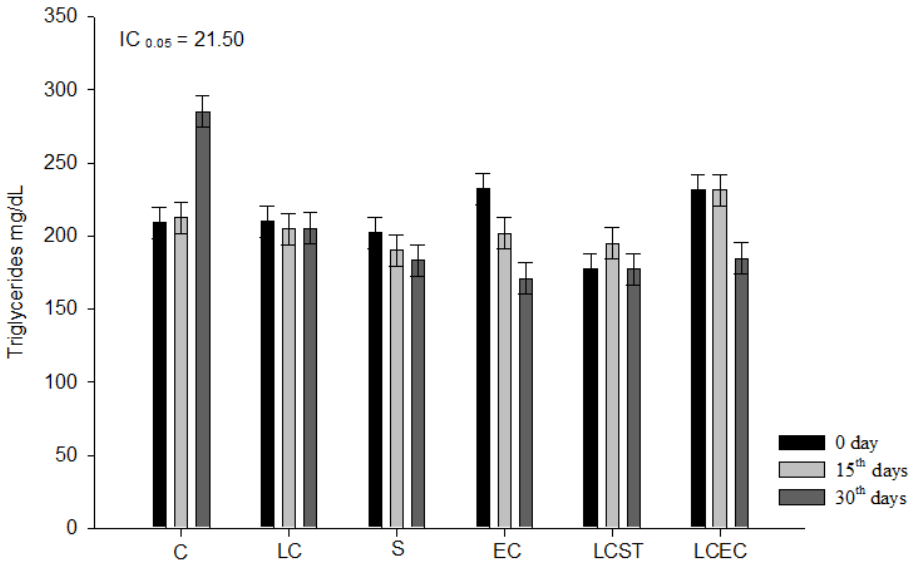


Figure 8 B. Blood triglyceride concentration during the experimental period of animals treated with *L. casei* CSL3, *S*. Typhimurium and *E. coli*.
C: control group; LC: *L. casei* CSL3 group; S: *S.* Typhimurium group; EC: *E. coli* group; LCST: *L. casei* CSL3 against *S.* Typhimurium group; LCEC: *L. casei* CSL3 against *E. coli* group.

In the first week of treatment, it is possible to notice that the groups that received E. coli maintained higher triglyceride concentrations when compared to the other groups. At the end of the treatment time, only the control group increased the concentration of serum triglycerides and the groups that received *L. casei* CSL3, *S*. Typhimurium and *E. coli* O157:H7 maintained reduced values when compared to the control. It is assumed that while potentially probiotic *L.*

*casei* CSL3 acts in the reduction of triglyceride levels by regulating the transcription of certain genes responsible for the reception, oxidation and transport of these lipids, other pathogens favor these to maintain their survival in the host, multiplication and systemic spread.

There was no significant difference between treatments for serum creatine levels, as there was for the time variable. At the end of the thirty days of treatment, there was a reduction in the concentration of creatine in the blood for all treatments, maintaining averages below 0.4 mg. $dL^{-1}$.

### 6.3.2 Evaluation of oxidative stress in tissues

Analysis of variance for the unifactorial scheme indicated a significant difference for the dependent variables kidney catalase ($p = 0.0001$) and kidney TBARS ($p = 0.0168$), which can be seen in Table 6. The other dependent variables (liver catalase, brain catalase, liver TBARS and brain TBARS), were not significant ($p > 0.05$).

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Table 6. Concentration of kidney catalase and kidney TBARS of animals treated with *L. casei* CSL3, *S*. Typhimurium and *E. coli*.

| Market | Groups | | | | | |
|---|---|---|---|---|---|---|
| | C | LC | S | EC | LCS | LCEC |
| Kidney catalase ($UCATmg^{-1}$) | 15.12±20.00$^{B}$ | 70.62±20.68$^{A}$ | 9.65±14.22$^{B}$ | 18.79±20.08$^{B}$ | 8.00±12.61$^{B}$ | 12.99±17.47$^{B}$ |
| Kidney TBARS (nmol $MDA.g^{-1}$) | 979.45±283.33$^{A}$ $^{BC}$ | 1,089.73±282.52$^{AB}$ | 1,122.11±156.69$^{A}$ | 1,128.52±167.35$^{A}$ | 836.86±210.08$^{BC}$ | 859.82±102.91$^{C}$ |

Means ± standard deviation followed by different capital letters on the line indicate a significant difference by the Waller-Duncan test (p≤0.05).
C: control group; LC: *L. casei* CSL3 group; S: *S.* Typhimurium group; EC: *E. coli* group; LCST: *L. casei* CSL3 against *S.* Typhimurium group; LCEC: *L. casei* CSL3 against *E. coli* group

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Table 6 shows the concentrations of the enzyme catalase in the tissues of the kidneys, where it can be seen that there were no significant differences between the control groups (C), the groups that received *S*. Typhimurium (S), *E. coli* (EC), *L. casei* CSL3 and *S*. Typhimurium (LCS) and *L. casei* CSL3 and *E. coli* (LCEC), maintaining averages between 8.00 UCAT.mg$^{-1}$ and 18.79 UCAT.mg$^{-1}$. The group that received only *L. casei* CSL3, potentially probiotic, presented an enzyme concentration higher than the other groups, with a value of 70.62 UCAT.mg$^{-1}$.

Thiobarbituric acid reactive substances (TBARS) are formed as a by-product of lipid peroxidation. With regard to these substances present in kidney tissues, Table 6 shows there were no significant differences between the control groups, *L. casei* CSL3, *S*. Typhimurium and *E. coli*. In the groups that received administration of *S*. Typhimurium (ST) (1,122.11 nmol MDA.g$^{-1}$) and *E. coli* (EC) (1,128.52 nmol MDA.g$^{-1}$, respectively) the concentration of TBARS remained higher than in the groups that received *L. casei* CSL3 and *S*. Typhimurium (LCS) (836.86 nmol MDA.g$^{-1}$) and *L. casei* CSL3 and *E. coli* (LCEC) (859.82 nmol MDA.g$^{-1}$).

## 6.4 Discussion

### 6.4.1 Determination of serum biochemistry parameters

In a study conducted by da Costa et al. (2018), in which they evaluated the influence of *L. plantarum*, derived from tropical fruits, on glucose and triglyceride levels in Wistar rats, the authors observed that one of the strains tested, number 49, significantly reduced (11.51%) the level of glucose in blood

plasma (91.25 mg. $dL^{-1}$ to 80.75 mg.$dL^{-1}$) when compared to the control. The latter maintained the same level (92.25 mg. $dL^{-1}$) during the experimental period (28 days). However, as for triglycerides, it was noticed that both strains (numbers 49 and 201) had no effect, maintaining mean concentrations of 99.50 mg. $dL^{-1}$ and 99.25 mg. $dL^{-1}$, respectively. Results were similar to those found in the present study, where the administration of CSL3 reduced blood plasma glucose levels by 16.78%, but with regard to triglyceride concentration, it did not have a significant effect.

Eslami et al. (2016) evaluated the effect of *L. delbrueckii* PTCC1057 on glucose levels in diabetic mice and proved that the probiotic positively influenced these levels, falling from 297 mg.$dL^{-1}$ (first week of the experiment) to 156 mg.$dL^{-1}$ (fifth week of the experiment). The animals in the control group in which diabetes was induced remained with initial and final means of 205 mg.$dL^{-1}$ and 446 mg.$dL^{-1}$, respectively.

It is known that the α-glucosidase enzyme, located in the small intestine, acts on the hydrolysis of complex carbohydrates, releasing glucose molecules (Hansawasdi et al., 2001). When evaluating the potential of different species of *Lactobacillus* spp. to suppress α-glucosidase activity and inhibit dipeptidyl peptidase IV through the hypoglycemic agent DPP-IV, Zeng et al. (2016) observed that of the 21 potentially probiotic isolates, seven showed greater inhibition of DPP-IV and inhibitory activity of α-glucosidase.

Another study on the influence of bacteria on glucose levels demonstrated an underlying molecular mechanism that negatively regulates the expression of the GSK3β gene, responsible for the synthesis of the glycogen

synthase kinase 3 beta enzyme, genes involved in hepatic gluconeogenesis, and positively regulates the expression of genes related to the phosphoinositide 3 kinase (PI3K)/ protein kinase B (AKT) pathway. AMPK or Adenosine Monophosphate Activated Protein Kinase is an enzyme that also acts as an important regulator as it has the ability to reduce the glucose level by stimulating glucose uptake (Jang et al., 2019).

Importantly, bacterial components such as proteins and exopolysaccharides (Chen et al., 2020) as well as specific metabolites (Das & Goyal, 2015; McNelis et al., 2015; Priyadarshini et al., 2015) can also act by regulating the metabolism of glucose.

Zavišić et al. (2022), suggest that the potential mechanisms underlying probiotics for changes in blood glucose levels and their tolerance include influencing the release of glucagon-like peptide-1 and peptide YY, resulting in increased insulin and decreased secretion. of glucagon, hormones responsible for regulating blood glucose levels.

Regarding the reduction of blood glucose levels in the treatment that received *S*. Typhimurium, this fact may be related to the need for glucose by the pathogen to maintain its intracellular replication and survival in macrophages. This survival during murine infections facilitates their systemic spread from Peyer's patches to the liver and spleen (Bowden et al., 2009).

*Escherichia coli* O157:H7 infection operates through a gradient of symptoms, during which pancreatic islet injuries can occur. The loss of β-cell mass may limit the increase in insulin production in response to any potential future reductions in insulin sensitivity. This fact could justify the glycemic

increase of the animals that received the treatment containing *E. coli* (Suri et al., 2009).

Roselli et al. (2017) studied the impacts of supplementation with a food-derived microbial community on obesity-associated inflammation and on the composition of the intestinal microbiota, and reported that triglyceride levels were reduced when *L. delbrueckii*, *L. fermentum* and *Leuconostoc lactis* isolated from "Mozzarella di Bufala Campana" (147.12 $mg.dL^{-1}$) and a commercial probiotic strain of *L. rhamnosus* (163.21 $mg.dL^{-1}$) were administered, compared to a control (316.98 $mg.dL^{-1}$) that received only the high-fat diet and PBS buffer solution.

When probiotic microorganisms adhere to the intestine, they are capable of fermenting non-digestible carbohydrates from food, increasing the concentration of short chain fatty acids (SCFAs). SCFAs act to redirect plasma cholesterol to the liver (St-Onge et al., 2000). Furthermore, probiotics can also synthesize bile acids through the deconjugation of bile salts in the small intestine, thus preventing the production of micelles that act to absorb cholesterol in the intestine (Ahn et al., 2003; De Boever & Verstraete, 1999; Doncheva et al., 2002).

Potentially probiotic microorganisms can act on the serum levels of triglycerides through the positive regulation of apolipoprotein AV (ApoA-V) that plays an important role in the concentration of triglycerides, peroxisome proliferator-activated receptor α (PPARα) that regulates the oxidation and transport of acids fatty acids, and the farnesoid X receptor (FXR) functioning as a bile acid receptor (Choi et al., 2016).

Tazi et al., (2018) justify in their study that *E. coli* is able to affect the cell cycle and metabolism, playing a fundamental role in modulating the absorption and metabolism of lipids. *E. coli* inhibits lipid secretion *in vitro*, while decreasing circulating levels of chylomicrons *in vivo* under normal diet conditions in mice. Therefore, the pathogen is capable of leading to enterocyte deprivation of carbohydrate energy sources and increased levels of dietary fat absorption and FA beta-oxidation to support mitochondrial metabolism and energy generation.

Creatine is produced by the liver, kidneys and pancreas and then transported to the muscles, where it is broken down through catalysis by the enzyme creatine kinase. Creatinine is a substance derived from the degradation of phosphorylated creatine (or phosphocreatine) and is an important parameter in the evaluation of renal function (Kreider & Stout, 2021).

Creatinine degradation studies have shown that creatinine diffuses into the intestinal tract, where creatininase and creatinine deaminase activity is induced, leading to the breakdown of a subset of the body's creatinine pool and partial recycling of creatinine. Creatininase activity has been demonstrated in several bacterial species that will influence the ability to eliminate creatinine, which may result in reductions in its plasma levels (Lempert, 2019).

With the information mentioned above, it is possible to affirm that the influence of probiotics on health is considered strain-dependent, since each isolate or standard strain can act on different metabolic routes, to generate a common result. This fact can be explained by the genetic diversity found in each bacterial genome.

Lukjancenko et al. (2012) found in their research comparing complete genomes of different genera, usually considered probiotics (*Bifidobacterium*, *Lactobacillus*, *Lacticaseibacillus*, *Lactococcus*, *Leuconostoc*, *Enterococcus*, and *Streptococcus* ), that the central genome shared by all genera resulted in just 63 core gene families out of a pan-genome of 37,053 gene families. Devi & Halami (2019) also point out that within the same genus and bacterial species there are also genetic differences.

### 6.4.2 Evaluation of oxidative stress in tissues

According to the literature, free radicals produced during aerobic respiration cause cumulative oxidative damage in the human body, with the main product being the superoxide anion ($O_2^-$), which undergoes the action of the enzyme superoxide dismutase (SOD), catalyzing its conversion to hydrogen peroxide ($H_2O_2$), which is converted to water by catalase (CAT) and glutathione peroxidase (GPx) (Harman, 1956). This action protects the body's cell against oxidative stress.

Renal tubule cells are rich in mitochondria because reabsorption of solutes requires energy. Such organelles are the biggest producers of oxygen radicals, which makes kidney cells especially vulnerable to oxidative stress and damage (Gyurászová et al., 2020).

Catalase is one of the most important antioxidant enzymes that breaks down $H_2O_2$ into water and oxygen, and is also responsible for the oxidation of low molecular weight alcohols and nitrites in the presence of $H_2O_2$. This enzyme plays a key role in regulating the cellular level of hydrogen peroxide, and its

catabolism protects cells, such as pancreatic β, from oxidative attack and damage by the compound (Nandi et al., 2019).

Although most microorganisms considered to be probiotics are catalase negative, research shows the presence in certain species such as *L. casei* of genes encoding Mn-catalase or pseudocatalase, which would explain their greater activity when compared to other treatments (Averina et al., 2021; Peacock & Hassan, 2021).

Kleniewska et al. (2016), Rajpal & Kansal (2009), Shen et al. (2014) and Yadav et al. (2008) demonstrated significant increases in the activity of the enzyme catalase in blood plasma, liver and kidney, when potentially probiotic bacteria are administered. These results corroborate the present study, where an increase in enzymatic activity can also be observed, and it is of paramount importance to emphasize that females are more prone to this increase in activity when compared to males. This can be explained by the different composition and levels of hormones, since testosterone has pro-oxidant properties, while estrogens have antioxidant effects (Schröder et al., 1996).

Oxidative stress is involved in the pathophysiology of certain diseases related to inadequate diet and lifestyle, such as renal impairment ranging from acute renal failure to chronic renal failure. Thus, increased levels of malondialdehyde (a highly toxic molecule), and its by-product, thiobarbituric acid reactive substances (TBARS), generated through lipid peroxidation, have been reported to be associated with kidney damage (Rodrigo & Bosco, 2006).

When evaluating the excretion rate of TBARS, Punaro et al. (2014) observed that animals where diabetes was induced had higher concentrations

when compared to the group in which diabetes was induced and in which the administration of kefir, fermented milk containing a mixture of lactic acid bacteria and beneficial yeast, took place.

It can be seen in the present study that when *L. casei* CSL3 was administered alone, it did not interfere in TBARS concentrations, but in animals continuously treated with *L. casei* CSL3 challenged with *S*. Typhimurium and *E. coli*, the probiotic influenced the levels of thiobarbituric acid reactive substance, reducing these when compared to treatments that only received the pathogens.

The mechanisms involved in the antioxidant activity of probiotic species may be related to the elimination of reactive oxygen species (ROS), chelating metals, increasing levels of antioxidant enzymes and modulating the microbiota (Feng & Wang, 2020).

Malondialdehyde (MDA) is a by-product produced by free radical-mediated lipid peroxidation, and its level is considered a good biomarker of oxidative stress. A high level of MDA is a result of imbalances in the redox state. Chorawala et al., (2021) showed that probiotics can resist LPS-induced oxidative stress, reducing MDA content. It is worth mentioning that certain pathogenic bacteria can make use of reactive oxygen species to maintain their metabolism and prevalence. *Salmonella* Typhimurium uses ROS derived from host phagocytes during intestinal inflammation, allowing it to outrun the native microbiota (Bäumler & Sperandio, 2016; Winter et al., 2010). *Escherichia coli* synthetizes sulfide, which when colliding with oxygen, in the intestinal lumen, generates $H_2O_2$ through the direct reaction. Under such circumstances, this

bacterium uses $H_2O_2$ as a terminal oxidant for respiration (Khademian & Imlay, 2017).

## 6.5 Conclusion

The probiotic bacterium *L. casei* CSL3 exerted a beneficial influence by reducing blood serum glucose levels, by increasing the concentration of the enzyme catalase in the kidneys of animals treated with the probiotic and by reducing the concentration of thiobarbituric acid reactive substances in the treatments containing the pathogens and *L. casei* CSL3.

However, it is necessary to proceed with the investigation of the potential of this bacterium under the modulation of the immune system, since, as previously mentioned, these microorganisms can have one or more benefits when administered to the host.

# CAPÍTULO 4

## Action of *Lacticaseibacillus casei* CSL3, isolated from bovine colostrum silage, in the prevention of infections caused by *Salmonella* Typhimurium and *Escherichia coli* O157: H7 in mice

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## Abstract

Probiotic bacteria have been associated with countless health benefits. Among these benefits are competition with pathogens for nutrients and site of action and the stimulation of the host's immune system. The present study aimed to evaluate the influence of *L. casei* CSL3 on MTT assay, nitric oxide determination (*in vitro*), intestinal colonization by lactic acid bacteria, histopathological analysis of kidney, liver and intestine tissues and, finally, the relative expression of the genes responsible for the synthesis of IL-2, IL-4 and TNF-α (*in vivo*) in mice challenged with *S.* Typhimurium and *E. coli*. It was possible to observe that concentrations equal to or less than 8 log CFU.mL$^{-1}$ are not considered toxic for the cell, that the production of nitric oxide increases with the increase of the concentration of the probiotic. Furthermore, when administered to mice, *L. casei* CSL3 increases the concentration of lactic acid bacteria and lactic acid bacteria resistant to bile in the feces, and that it is able to reduce the concentrations of *S.* Typhimurium and *E. coli,* when challenged. Damage to the liver and small intestine by pathogenic bacteria was noted, and *L. casei* CSL3 increased the relative expression of IL-2 and reduced IL-4 and TNF-α. Therefore, it can be concluded that the studied bacterium, *L. casei* CSL3, managed to colonize the gastrointestinal tract of treated animals, exerted a positive effect on the immune response of mice through nitric oxide synthesis and influenced the relative expression of pro-inflammatory cytokine genes.


**Keywords:** probiotic; immune system; challenge; pathogenic bacteria.

## 7.1 Introduction

Food-borne diseases are caused by contamination by bacteria, viruses, parasites or chemicals, such as heavy metals, and can occur at any stage of the production, delivery and consumption chain. Their symptoms present as gastrointestinal problems, although they can also produce neurological and immunological symptoms. This growing public health problem influences socioeconomic impact and contributes significantly to the global burden of disease and mortality (WHO, 2020).

According to the Centers for Disease Control and Prevention (CDC) (2020), between the years 2017-2019 the main etiological agents of foodborne diseases were *Salmonella* spp. representing 53.06% and *Escherichia coli* representing 20.40%, which totals more than 60% of the total cases that occurred in that period.

Having a distinct pathogenicity mechanism, *Salmonella enterica* serovar Typhimurium stands out for being a bacterium that is widely linked to foodborne diseases (Andino & Hanning, 2015).

When ingested, *Salmonella* activates a response to acid tolerance, which maintains its intracellular pH above the extracellular environment (Foster & Hall, 1991). Upon reaching its site of action in the small intestine, *S*. Typhimurium crosses the mucous layer and adheres to the epithelial cells. After adhesion, the invasion process occurs, mediated by signaling pathways from the host cell, leading to the interruption of the normal brush border and inducing the subsequent formation of membrane ruffles that surround the bacteria in vesicles

called *Salmonella* containing vacuoles (SCV), where they can survive, multiply and facilitate their dissemination (Andino & Hanning, 2015; Gut et al., 2018).

Like *Salmonella* spp., *Escherichia coli* also is a bacillus, Gram-negative, facultative anaerobic pathogen and is responsible for significant numbers of gastroenteritis cases. Belonging to the EHEC pathotype, serotype O157:H7 causes symptoms ranging from mild diarrhea and hemorrhagic colitis to the potentially fatal hemolytic-uremic syndrome (HUS) (Robins-Browne et al., 2016).

The inflammatory process of this strain is directly related to the lesion known as attaching effacing (A/E) and the production of shiga toxins ($Stx_1$ and $Stx_2$) (Ho et al., 2013). When the bacterium binds and interacts with the intestinal mucosa, histopathological changes are produced in the epithelium (Adamu et al., 2015). The production of the toxin ($AB_5$) binding to the globotriaosylceramide receptor (Gb3) found in Paneth cells in the intestinal mucosa and on the surface of renal epithelial cells, where N-glucosidase prevents protein synthesis, this leads to necrosis and cell death (Rahal et al., 2012; Saeedi et al., 2017).

As the intestine is considered the main site of action of these bacterial pathogens of food importance, the consumption of probiotic microorganisms emerges as a strategy to prevent these from triggering diseases. Research has shown that probiotics, mainly strains of lactic acid bacteria (LAB), can modulate the microbiota of the human gastrointestinal tract by inhibiting the development of opportunistic bacteria (Esaiassen et al., 2018; Jessie Lau & Chye, 2018; Lievin Moal, 2016).

The activity of probiotic strains can be correlated with the production of antimicrobial substances, such as acids, peptides and hydrogen peroxide, which can act to inhibit protein synthesis, interfering with DNA replication and transcription. In addition, probiotics can significantly reduce the invasion of pathogens, competing for host cell receptors, competing for nutrients, or even altering the gene expression responsible for the colonization of enteric bacterial pathogens (Eş et al., 2018).

As a possible candidate to combat pathogens of importance in food, the bacterium *Lacticaseibacillus casei* CSL3 has been characterized as potentially probiotic through *in vitro* (Vitola et al., 2018) and *in situ* (Vitola et al., 2020) tests. During its investigation it maintained its viability when submitted to gastrointestinal transit simulation, had the ability to adhere to epithelial cells and demonstrated antagonistic activity against bacteria such as *S.* Typhimurium, *E. coli*, *L. monocytogenes* and *S. aureus*.

Therefore, this study aimed to evaluate the influence of *Lacticaseibacillus casei* CSL3 on cytotoxicity and nitric oxide synthesis *in vitro*, and on colonization in the gastrointestinal histopathology and on the relative expression of pro-inflammatory cytokines *in vivo*, as well as its action in the challenge with Salmonella Typhimurium and *Escherichia coli* O157: H7.

## 7.2 Materials and methods

### 7.2.1 Microorganisms

Potentially probiotic bacterium *Lacticaseibacillus casei* CSL3 used in the present study was previously isolated from bovine colostrum silage by Vitola et al. (2018).

*Salmonella* Typhimurium ATCC 14028 and *Escherichia coli* O157:H7 NCTC 12900 were provided by the Food Microbiology Laboratory of the Federal University of Pelotas (RS/ Brazil).

### 7.2.2 Experimental design

A one-factor scheme involving the production of nitric oxide and the number of colony forming units of the probiotic bacteria was used to choose the concentration that was given to mice. The intestinal colonization was entirely casualized with three experimental replications. Another two-factor scheme involving treatments (C, LC, ST, EC, LCS and LCEC) and time of probiotic administration was used to assess colonization of the gastrointestinal tract and competition against pathogens.

### 7.2.3 Cell culture

For *in vitro* experiments, the murine macrophage cell line RAW 264.7 (ATCC, TIB-71) was provided by the Virology and Immunology Laboratory of the Federal University of Pelotas (RS/ Brazil). The cells were grown in cell culture bottles containing Minimum Essential Medium (MEM) (Gibco Laboratories, Grand Island, NY) supplemented with 2 % fetal bovine serum (FBS) (Gibco Laboratories, Grand Island, NY), together with antibiotics penicillin G (100 U. $mL^{-1}$) and streptomycin (100 µg.$mL^{-1}$). The incubation maintained a

relative temperature of 37 °C and a controlled atmosphere containing 5% $CO_2$ (Jaffar et al., 2018).

### 7.2.4 MTT assay

The cytotoxicity of *L. casei* CSL3 under RAW 264.7 cells was evaluated by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide method. Briefly, the RAW 264.7 strain with a density of 1.2 x $10^6$ cells/ well was treated with different concentrations of the potentially probiotic bacteria (2 log CFU.$mL^{-1}$ to 9 log CFU.$mL^{-1}$) for 24h. Afterwards, the supernatants were discarded and the MTT solution was added followed by incubation in the dark at 37 °C for 4 h. The formazan crystals present in the cells were dissolved in 50 µL/well of ethyl alcohol for 10 min followed by the absorbance reading $OD_{540}$ nm (Mohanty et al., 2019).

### 7.2.5 Nitric oxide (NO) determination

The levels of NO of the supernatants extracted in the previous methodology (4.2.4) were determined by the Griess reaction. One hundred microliters of culture supernatant were mixed with 100 µL of Griess reagent for 5 min at room temperature and absorbance was measured at $DO_{570}$ nm. Nitrite concentrations were calculated based on a standard curve prepared using different concentrations of sodium nitrite (Sigma, San Luis, Missouri, EUA).

### 7.2.6 Growth conditions *L. casei* CSL3

The isolate was cultured in MRS broth (De Man, Rogosa and Sharp, Himedia, Mumbai, India) at 37 °C for 18 h, after which an aliquot of 1% was removed and transferred to a shot containing 100 mL of fresh MRS broth, incubating under the same conditions mentioned above. Finally, an aliquot of 1% the broth containing cultured *L. casei* CSL3 was transferred to 500 mL of fresh MRS broth, maintaining the same growth conditions.

At the end, the MRS broth containing the bacteria was centrifuged at 4,165 *g* for 10 min at 20 °C; the pellet was washed three times with PBS buffer to remove any broth residue used and this was resuspended in the same buffer, stored under refrigeration (temperature 6-8 °C). Counts were performed on the 0, 15$^{th}$ and 30$^{th}$ dats of the experiment to assess the maintenance of the isolate concentration.

### 7.2.6 *In vivo* model

The animal experiment was submitted and approved by the Ethics Committee on Animal Experimentation (CEEA, Authorization n° CEEA 5381) of the Federal University of Pelotas (UFPel). The CEEA/UFPel agreement is approved by the Brazilian National Council for Animal Experimentation Control (CONCEA).

Sixty female *Mus musculus* mice of the *Swiss* albino strain with 21 ± 5 days of age were used. After a week of acclimatization, kept under controlled temperature of 23 ± 4 °C, 12 h light/ dark lighting cycle, with feed (without addition antibiotics and antifungals) and water *ad libitum*, the animals were divided into 6 groups.

The control group (C) received, by gavage, 300 µL of phosphate buffered saline (PBS) for the 30 days of the experimental period; the LCC group received 300 µL of *L. casei* CSL3 in a concentration of 8 log CFU.mL$^{-1}$ for the 30 days of the experimental period; the SC group received 300 µL of *S.* Typhimurium in a concentration of 6 log CFU.mL$^{-1}$ on the fifteenth day of the experimental period; the ECC group received 300 µL of *E. coli* in a concentration of 6 log CFU.mL$^{-1}$ on the fifteenth day of the experimental period; the LCS group received 300 µL of *L. casei* CSL3 in a concentration of 8 log CFU.mL$^{-1}$ for the 30 days of the experimental period and, on the fifteenth day, it was challenged with *S.* Typhimurium in a concentration of 6 log CFU.mL$^{-1}$, and the LCEC group received 300 µL of *L. casei* CSL3 in a concentration of 8 log CFU.mL$^{-1}$, for the 30 days of the experimental period, and on the fifteenth day it was challenged with *S.* Typhimurium in a concentration of 6 log CFU. mL$^{-1}$. Euthanasia of the animals was performed through anesthetic overdose followed by cardiac puncture.

### 7.2.7 Intestinal colonization assay with lactic acid bacteria

About 100 mg of fresh feces were collected and diluted in 1 mL of sterile PBS. The samples were kept on ice until processing. Serial decimal dilutions were performed followed by spread onto MRS agar, supplemented with 0.3% bile salts for counting total lactic acid bacteria and bile-resistant lactic acid bacteria, incubated under anaerobic conditions for 72 h at 37 °C. For the counting of *S*. Typhimurium and *E. coli* O157:H7, Xylose-lysine-deoxy-cholate

agar (XLD) and MacConkey sorbitol agar were incubated for 24 h at 37 ºC, respectively (Nambiar et al., 2018).

### 7.2.8 Histopathological analysis

Samples of kidney, liver and small intestine were extracted from the mice, during euthanasia and preserved in 10% neutral buffered formaldehyde for histopathological examination. The tissue was dehydrated in a graded series of alcohol and stained with hematoxylin and eosin for conventional morphological evaluation. The tissues were embedded in paraffin and cut to a thickness of 5 mm. Histological evaluations were performed using a light microscope (Olympus BX43, Tokyo, Japan) (Sainte-Marie, 1962).

### 7.2.10 IL2, IL4 and TNF gene expression

Splen were collected, under aseptic conditions, in Hanks balanced solution. The spleens were crushed by passing through a 40 µm cell filter (Falcon, Corning Inc., Corning, NY). The cells were precipitated by the addition of lysis buffer 1 and 2. After centrifugation at 2000 rpm for 7 min, the cells were washed with Hanks' solution, resuspended in RPMI medium supplemented with fetal bovine serum (10%), penicillin (100 U. $mL^{-1}$), streptomycin (100 mg. $mL^{-1}$) and amphotericin B. The cultivation was carried out in 24-well plates with a flat bottom incubated at 37 °C under a controlled atmosphere with 5% $CO_2$ (Song et al., 2016).

The collected cells were stimulated with the same culture of *L. casei* CSL3, in the concentration referring to 8 log CFU. $mL^{-1}$ administered to the animals.

The extraction of RNA from splenocytes occurred according to the protocol established for TRIzol ™ Reagent (Invitrogen, USA). The quantification of the samples was carried out in Nanovue (ng. $\mu L^{-1}$) through the absorbance at 260 nm, which provides the total nucleic acid content, and at 280 nm, which determines the purity of the sample.

The synthesis of cDNA was performed in the Real Time Workstation using the kit “High-Capacity cDNA Reverse Transcription" (Applied Biosystem, USA). For the real-time PCR reaction, 0.25 µL of primer F, 0.25 µL of primer R, 3.5 µL of water, 5.0 µL of Syber and 1.0 µ of cDNA were used. The sequences of the used primers, as well as the reference, are shown in Table 7.

Table 7. Primers used in the evaluation of IL2, IL4 and TNF gene expression, extracted from mice splenocyte

| Gene | Polarity | Primer sequence (5'-3) | Reference |
|---|---|---|---|
| IL-2 | Sense | TTGTGCTCCTTGTCAACAGC | DE ÁVILA (2016) |
| | Antisense | CTGGGGAGTTTCAGGTTCCT | |
| IL-4 | Sense | CCAAGGTGCTTCGCATATT | |
| | Antisense | ATCGAAAAGCCCGAAAGAGT | |
| TNF | Sense | TTTGAATTCCCTGGGTGAGAA | |
| | Antisense | ACAGGGGAGAAATCGATGACA | |
| GAPDH | Sense | AACGACCCCTTCTCATTGAC | |
| | Antisense | TCCACGACATACTCAGCAC | |
| B-actin | Sense | AGAGGGAAATCGTGCGTGAC | |

| | |
|---|---|
| Antisense | CAATAGTGATGACCTGGCCGT |

### 7.2.11 Statistical analysis

The data obtained were analyzed in STATISTICA® 6.0 software, were analysis of variance was applied in the tests of nitric oxide production (*in vitro*) and colonization of the gastrointestinal tract (*in vivo*). When there was a significant difference between treatments, the Bonferroni test was applied, and when there was a significant difference for time, a confidence interval (CI) was applied.

## 7.3 Results and discussion

### 7.3.1 Cell viability assay (MTT) and nitric oxide synthesis

After 24 h in contact with different concentrations of *L. casei* CSL3 (2 log CFU.mL$^{-1}$ to 10 log CFU.mL$^{-1}$), it was observed that concentrations up to 8 log CFU.mL$^{-1}$ maintained cell viability of 98%. Oh et al. (2018) evaluated the probiotic and anti-inflammatory potential of *Lactobacillus rhamnosus* 4B15 and *Lactobacillus gasseri* 4M13, where they observed that up to the 8 log CFU.mL$^{-1}$ concentration did not affect the cell viability of RAW 264.7 macrophages, as in the present study. Therefore, it is estimated that reaching its site of action at this concentration, *L. casei* CSL3 will not influence cell viability.

NO is known as an inflammatory mediator, and its production in phagocytic cells beneficially helps in the host's defense against pathogenic microorganisms, parasites and tumor cells (C. Kang et al., 2019). Figure 10 shows that, with the increase in the concentration of *L. casei* CSL3, there is a

greater synthesis of NO by the macrophages, with a significant difference, ranging from 25 µM to 36 µM.

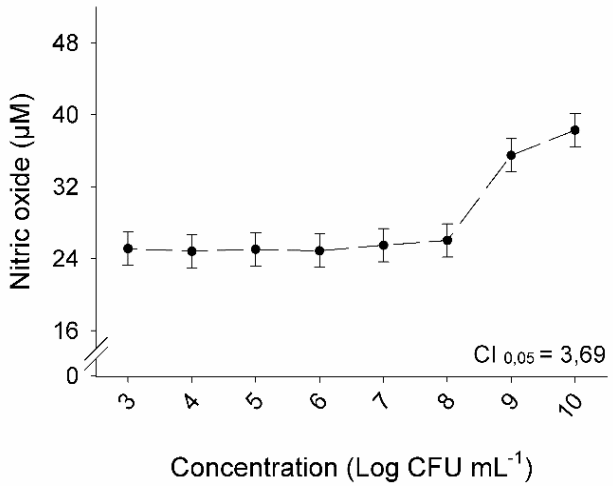



Figure 10. Relationship between the production of nitric oxide (µM) and the concentration of *L. casei* CSL3

When compared to studies that evaluated production by lactic acid bacteria isolated from fermented foods (C. Kang et al., 2019), there is less influence on NO production, with a synthesis varying between 5 µM and 20 µM. When evaluating the immunomodulatory capacity of *Lactobacillus* spp. probiotics against *Campylobacter jejuni* in chicken macrophages, Taha-Abdelaziz et al. (2019) observed a significant increase in NO production when compared to untreated cells.

The mechanisms by which NO acts as an antimicrobial involve the interaction of free radical and reactive nitrogen intermediates with reactive oxygen intermediates such as hydrogen peroxide ($H_2O_2$) and superoxide ($O_2^-$). These intermediates can interact with DNA, react with proteins or even inactivate the metabolism of enzymes (Jones et al., 2010).

### 7.3.2 Intestinal colonization assay with lactic acid bacteria

Table 8 illustrates the viability of total lactic acid bacteria both in the control treatment (C) and in the one that received *L. casei* CSL3 (LCC). Over five weeks, the concentration of LAB remained stable, and was significantly higher in the second group. At the end of the fifth week, there was a reduction in the viability of LAB in the LCC group, maintaining a concentration above 9 log CFU.mL$^{-1}$.

Table 8. Viability of lactic acid bacteria (LAB) and bile-resistant lactic acid bacteria (LABBR) (log CFU.mL$^{-1}$) in the fecal content of animals treated during the experimental period

| Time (weeks) | C | LCC | LCS | LCEC |
|---|---|---|---|---|
| | LAB | | | |
| 1 | 10.09 ± 0.87$^{b}$ | 11.01 ± 0.01$^{a}$ | 9.32 ± 0.23$^{c}$ | 9.61 ± 0.04$^{c}$ |
| 2 | 10.25 ± 0.08$^{b}$ | 10.78 ± 0.14$^{a}$ | 9.36 ± 0.05$^{c}$ | 10.37 ± 0.04$^{b}$ |
| 3 | 9.17 ± 0.14$^{b}$ | 10.61 ± 0.11$^{a}$ | 8.71 ± 0.05$^{c}$ | 10.55 ± 0.09$^{a}$ |
| 4 | 10.05 ± 0.05$^{b}$ | 10.61 ± 0.04$^{a}$ | 8.51 ± 0.23$^{c}$ | 10.15 ± 0.02$^{b}$ |
| 5 | 10.62 ± 0.06$^{a}$ | 9.93 ± 0.21$^{b}$ | 10.01 ± 0.02$^{b}$ | 9.01 ± 0.01$^{c}$ |
| | LABBR | | | |
| 1 | 9.24 ± 0.12$^{c}$ | 10.83 ± 0.36$^{a}$ | 9.50 ± 0.09$^{b}$ | 9.61 ± 0.04$^{b}$ |
| 2 | 10.61 ± 0.07$^{a}$ | 10.60 ± 0.18$^{a}$ | 9.25 ± 0.22$^{b}$ | 10.52 ± 0.09$^{a}$ |
| 3 | 8.90 ± 0.04$^{c}$ | 10.40 ± 0.15$^{a}$ | 8.99 ± 0.04$^{b}$ | 10.23 ± 0.17$^{a}$ |
| 4 | 9.85 ± 0.08$^{b}$ | 10.73 ± 0.03$^{a}$ | 9.04 ± 0.26$^{c}$ | 10.16 ± 0.11$^{b}$ |
| 5 | 10.69 ± 0.13$^{a}$ | 9.70 ± 0.20$^{b}$ | 9.65 ± 0.09$^{b}$ | 9.06 ± 0.11$^{c}$ |

C: control group; LCC: *L. casei* CSL3 group; LCS: *L. casei* CSL3 against *S.* Typhimurium group; LCEC: *L. casei* CSL3 against *E. coli* group; LAB: lactic acid bacteria; LABBR: lactic acid bacteria bile resistance.
Means ± standard deviation followed by different letters on the same line indicate a significant difference using the Bonferroni test ($p \leq 0.05$) when comparing the groups.

As for the concentration of bile-resistant lactic acid bacteria, in the control treatment there was an increase in the concentration from the first to the fifth week. In the group in which the potentially probiotic bacteria were administered, viability remained stable from the first to the fifth weeks, with a reduction in the last week, but maintaining concentrations above 9 log CFU.mL$^{-1}$.

In Figure 11, it can be seen from the time variable that both for the control groups and for the groups that were challenged against *S*. Typhimurium at the end of the fifth week of treatment, the LAB and LABBR counts remained higher when compared to the first weeks. For the groups containing the probiotic and the challenge against *E. coli*, there were significant reductions in the population of LAB and LABBR.

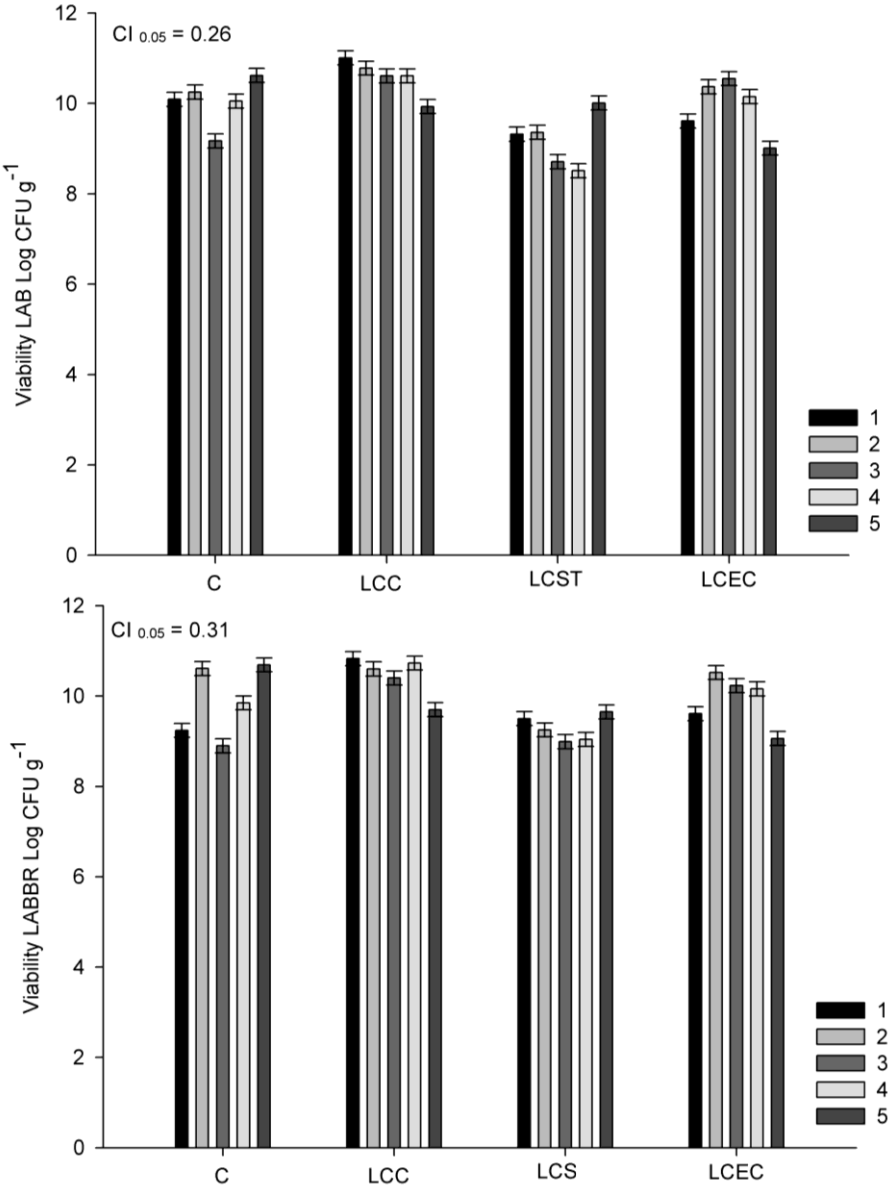



Figure 11. Viability of LAB and LABR in mouse feces comparing the same treatment over the experimental period (weeks)

C: control group; LCC: *L. casei* CSL3 group; LCS: *L. casei* CSL3 against *S.* Typhimurium group; LCEC: *L. casei* CSL3 against *E. coli* group; LAB: lactic acid bacteria; LABBR: lactic acid bacteria bile resistance.

Nambiar et al. (2018) evaluated the effect on the fecal microbiota of mice of supplementing encapsulated *Lactobacillus plantarum* HM47 (8 log CFU.mL$^{-1}$). They observed an increase in the concentration of total lactic acid bacteria in all groups at the end of the 28 days of evaluation, especially those that received encapsulated *L. plantarum* (9.38 log CFU.g$^{-1}$) and encapsulated *L. plantarum* inserted in food matrix (9.76 log CFU.g$^{-1}$). As for *Enterobacteriaceae* viability, it is possible to notice a reduction of approximately one logarithmic cycle in the groups that received LAB.

It is evident that in the third week of treatment, when the pathogens were administered, the LCS group showed a reduction in the total LAB concentration and bile-resistant LAB, while the LCEC group showed an increase in the viability of both. This result corroborates that found by Maia et al. (2001) who, when studying the effect of probiotics challenged with *S*. Typhimurium in mice, noted a reduction in the population of *Lactobacillus* spp. from the faeces.

*Salmonella* Typhimurium employs specialized transporters to acquire essential metals, one of the most important being iron. Such bacteria synthesize and export high-affinity iron chelators of small molecules, called siderophores. To successfully infect the host *S*. Typhimurium must overcome iron limitation. The reduction of LAB and LABBR in the third week after the administration of the pathogen may be related to the competition of these microorganisms for iron, which would limit the metabolism of the microbiota present in the intestine (Deriu et al., 2013).

Wang et al., (2020) reports in his study that strains of *L. casei* can inhibit the colonization of *E. coli* O157:H7 in mice, increasing the expression of MUC2 that will directly affect the adhesion of the pathogen to intestinal epithelial cells, accelerating the process of excretion of *E. coli*, which would explain the increase in LAB and LABBR from the third week onwards, since at higher bacterial concentrations, the expression of mucins in the intestine would be higher. Table 9 shows the viability of *Salmonella* spp. in the faeces of animals where the bacteria were administered and in the control. It is possible to observe that there were no counts of pathogenic bacteria in the control group. However, in the STC group, where *S*. Typhimurium was administered in the third week, its viability is verified in the fourth and fifth week, showing an increasing behavior. In the group that received *L. casei* CSL3 during the 5 weeks of treatment and in the third week was challenged with *S*. Typhimurium, concentrations remained above 5 log CFU.mL$^{-1}$ in the third and fourth week. It is of paramount importance to emphasize that at the end of the fifth week *Salmonella* spp. did not remain viable.

Table 9. Viability of *Salmonella* spp. (log CFU.mL$^{-1}$) in the fecal content of animals treated during the experimental period

| Time (weeks) | C | ST | LCS |
|---|---|---|---|
| 1 | 0.00 ± 0.00 | 0.00 ± 0.00 | 0.00 ± 0.00 |
| 2 | 0.00 ± 0.00 | 0.00 ± 0.00 | 0.00 ± 0.00 |
| 3 | 0.00 ± 0.00$^{b}$ | 0.00 ± 0.00$^{b}$ | 5.61 ± 014$^{a}$ |
| 4 | 0.00 ± 0.00$^{c}$ | 4.55 ± 0.28$^{b}$ | 5.99 ± 0.18$^{a}$ |
| 5 | 0.00 ± 0.00$^{b}$ | 6,09 ± 0.57$^{a}$ | 0.00 ± 0.00$^{b}$ |

C: control group; ST: *S*. Typhimurium group; LCS: *L. casei* CSL3 challenge with *S.* Typhimurium group.
Means ± standard deviation followed by different letters on the same line indicate a significant difference using the Bonferroni test (p ≤ 0.05) when comparing the groups.

It is noted that the concentration of *S*. Typhimurium in the feces of the treatment containing only the pathogen increased from the fourth to the fifth week, in contrast, when constant administration of the probiotic was maintained, this concentration was reduced in the same time interval (Figure 12).

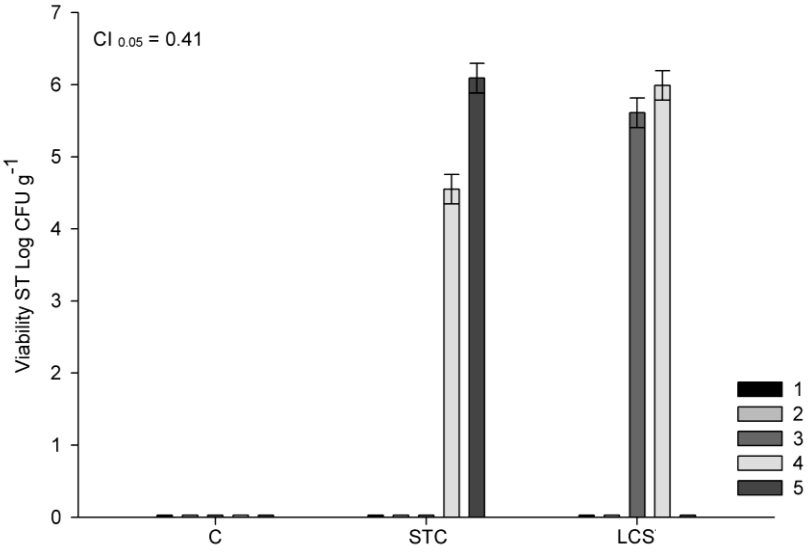



Figure 12. Viability of *Salmonella* spp. (ST) in the fecal content of mice comparing the same treatment over the experimental period (weeks).
C: control group; ST: *S*. Typhimurium group; LCS: *L. casei* CSL3 challenge with *S.* Typhimurium group.

When evaluating the efficacy of probiotic fermented milk against *Salmonella enterica* in mice, Kemgang et al. (2016) observed that the LAB count in the feces of the probiotic group increased 20 days post-infection. The mean values of *Salmonella enterica* counts for the group that received LAB were less than 6 log $CFU.g^{-1}$, while in the control group they remained approximately between 4 log $CFU.g^{-1}$ and 7 log $CFU.g^{-1}$ 20 days after infection.

Studies demonstrate that the survival and replication of *S.* Typhimurium in hemophagocytic macrophages may help to establish a persistent infection.

The pathological damage that results from the continued activation of macrophages at some stage of the infection outweighs the immediate risk that is posed by residual and persistent bacteria, and the immune response is likely to shut down, allowing its persistence (Monack, 2012).

*Escherichia coli* O157:H7 was administered to ECC and LCEC treatments, and in Table 10 its viability in the feces of treated animals can be seen during the five weeks of the experimental period. It can be seen that in the two weeks (4$^{th}$ week and 5$^{th}$ week) in which there were *E. coli* counts, that it remained at lower concentrations in the treatment containing the probiotic bacteria, approximately 2 log CFU.g$^{-1}$ and 1.3 log CFU.g$^{-1}$ difference, respectively.

Table 10. Viability of *Escherichia coli* O157:H7 (log CFU.mL$^{-1}$) in the fecal content of animals treated during the experimental period

| Time (weeks) | C | ECC | LCEC |
|---|---|---|---|
| 1 | 0.00 ± 0.00 | 0.00 ± 0.00 | 0.00 ± 0 |
| 2 | 0.00 ± 0.00 | 0.00 ± 0.00 | 0.00 ± 0 |
| 3 | 0.00 ± 0.00 | 0.00 ± 0.00 | 0.00 ± 0 |
| 4 | 0.00 ± 0.00$^{c}$ | 6.31 ± 0.27$^{a}$ | 4.48 ± 0.17$^{b}$ |
| 5 | 0.00 ± 0.00$^{c}$ | 3.51 ± 0.17$^{a}$ | 2.16 ± 0.11$^{b}$ |

C: control group; ECC: *E. coli* group; LCEC: *L. casei* CSL3 challenged with *E. coli* group.
Means ± standard deviation followed by different letters on the same line indicate a significant difference using the Bonferroni test ($p \leq 0.05$) when comparing the groups.

In Figure 13, it is possible to observe a drop in the concentration of *E. coli* from the fourth to the fifth week of treatment, both for the group containing only the pathogen, and for the group in which the probiotic bacterium was challenged against the pathogen.

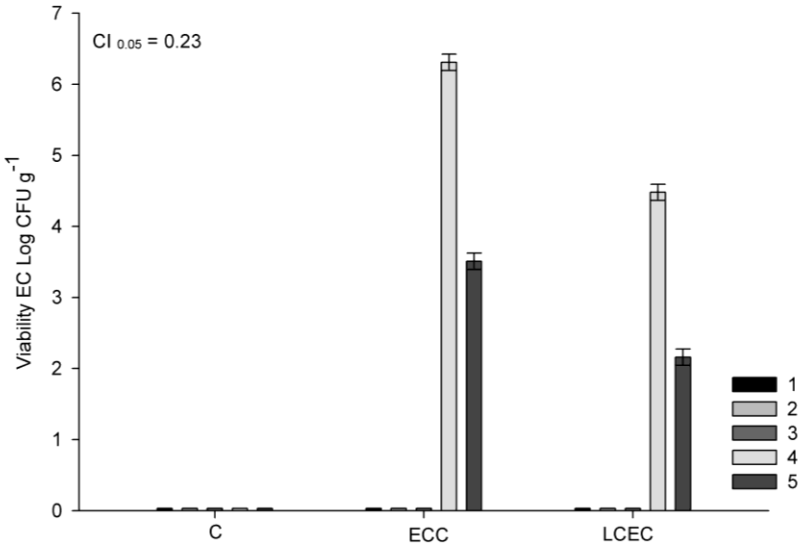



Figure 13. Viability of *Escherichia coli* (EC) in the fecal content of mice comparing the same treatment over the experimental period (weeks)
C: control group; ECC: *E. coli* group; LCEC: *L. casei* CSL3 challenged with *E. coli* group

When evaluating the protection that *Enterococcus faecium* provided to challenged mice against enterotoxigenic *E. coli*, Shao et al. (2022) reported that the number of *E. coli* was reduced at rates of approximately 20 %, inversely proportional to what occurred with the concentration of LAB, which increased at values close to 5%.

### 7.3.3 Histopathological analysis

Microscopic examination of the tissue referring to the kidney revealed that only the group with *S.* Typhimurium administered showed tissue damage in 10% of the animals tested, which is characterized as hydropic tubular degeneration (cellular edema or vacuolar degeneration, due to the accumulation of water and electrolytes inside cells), the first manifestation of cellular damage.

Regarding the tissue analysis of the liver, in the groups that received *S.* Typhimurium and *E. coli*, it was observed that in the first, 50% of the animals showed inflammation, congestion (accumulation of tissue blood) and/or swelling and congestion in the tissues, while in the second group 40% of the animals showed damage by swelling and congestion. When compared to the groups in which there was a challenge with the action of *L. casei* CSL3, it was noted that 70% of the animals belonging to the *L. casei* CSL3 and *Salmonella* spp. did not present any tissue injury, with a 20% reduction in damage when compared to the group that received only the pathogen.

With regard to tissues from the intestine, the group in which *S.* Typhimurium and *E. coli* were administered, 60% and 40% of the animals, respectively, presented mucosal inflammation. These data, when compared to the groups that had the competition by *L. casei* CSL3, potentially probiotic, are promising since in the group containing *L. casei* CSL3 and *S.* Typhimurium there was a 20% reduction in the animals with inflammation in the intestinal mucosa, and a 10% reduction in the *L. casei* CSL3 and *E. coli* groups.

When evaluating the protective effect of *L. rhamnosus* S1K3 (MTCC5957), supplemented in fermented milk, when challenged with *Salmonella enterica*, in the intestinal mucosa, Kemgang et al. (2016) reported the harmful effects of the pathogen on the villi structure of the control group after infection with severe intestinal inflammation. In contrast, the group containing the probiotic reduced the damage caused by *S. enterica,* keeping the villi undamaged.

Ruqin Lin et al. (2017) evaluated the immunization of mice by *L. acidophilus* (LA-ET) by challenging them with *E. coli* O157:H7, and observed that the lactic acid bacterium inhibited the occurrence of A/E lesions by EHEC O157:H7 cells, in addition to inducing higher levels of specific mucosa and an increase in the production of interferon-γ and lL-4 and IL-10, associated with mixed T cell responses (Th1/ Th2).

It is observed from the aforementioned studies that certain strains of lactic acid bacteria are able to positively influence the host through competition with pathogenic bacteria. The mode of action of each LAB will depend on which antagonist strategies will be used against such microorganisms.

Kemgang et al. (2016) noticed the production of peptides with antimicrobial activity, the ability of *L. rhamnosus* S1K3 to adhere to intestinal cells and the influence on the induction of TLRs transcription in Peyer's patches, an increase in IgA and IL-4 and a reduction in TGF-β, actions that contributed together to reduce the viability of *S. enterica*, not allowing colonization by the pathogen.

It is known that *L. casei* CSL3 has antagonist activity against pathogens (*Listeria monocytogenes*, *Escherichia coli*, *Salmonella* sp. and *Staphylococcus aureus*) and has a high rate of self-aggregation and co-aggregation (Vitola et al., 2018), which influence competition for nutrients and site of action in the host. The responses to the histological tests suggest that such abilities had a direct action on the reduction of the percentages of organs harmfully affected by the studied pathogens. With this, it becomes necessary to evaluate the humoral

response, to verify which other attributes of *L. casei* CSL3 may have had an influence on this reduction.

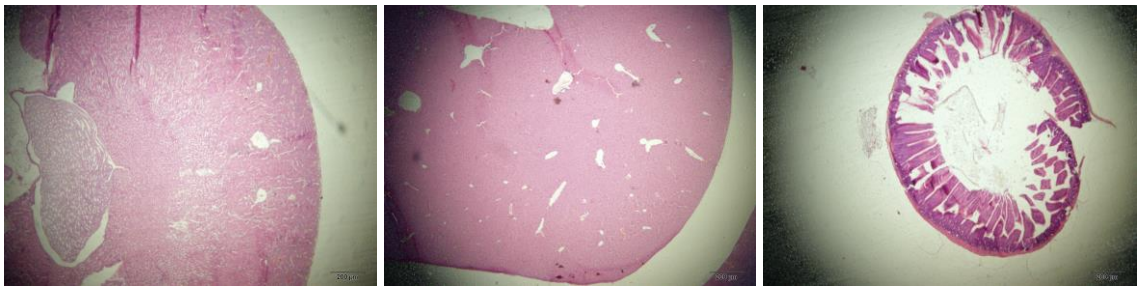


Figure 14 A: Kidney, liver and small intestine of the control group without change (microscopic increase in 4x).

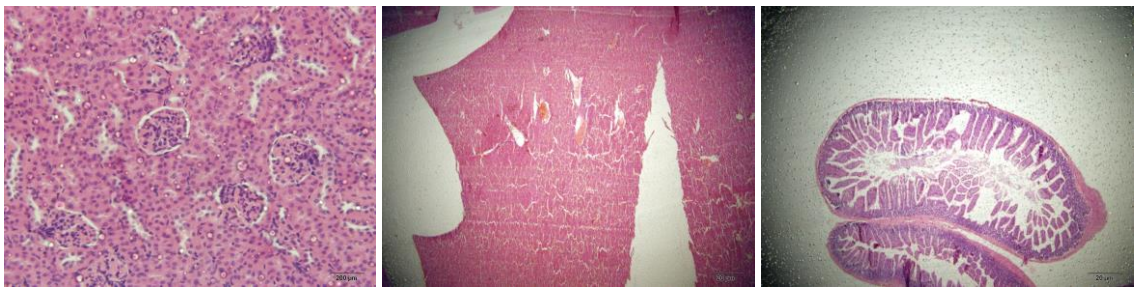
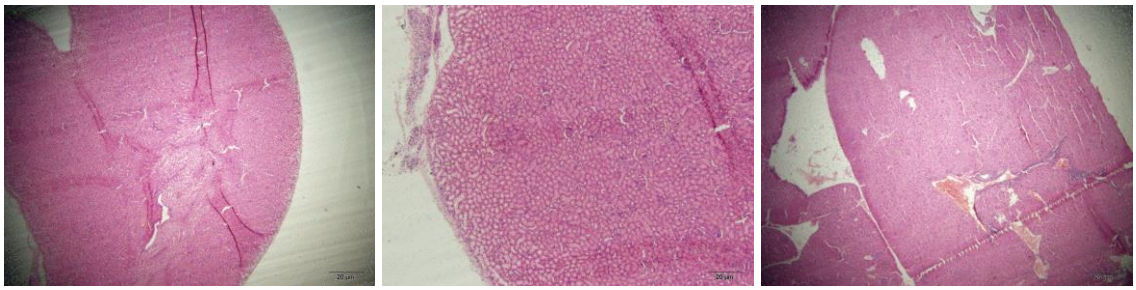


Figure 14 B: Kidney, liver and small intestine unaltered in the group treated with *L. casei* CSL3 (microscopic increase in 4x)



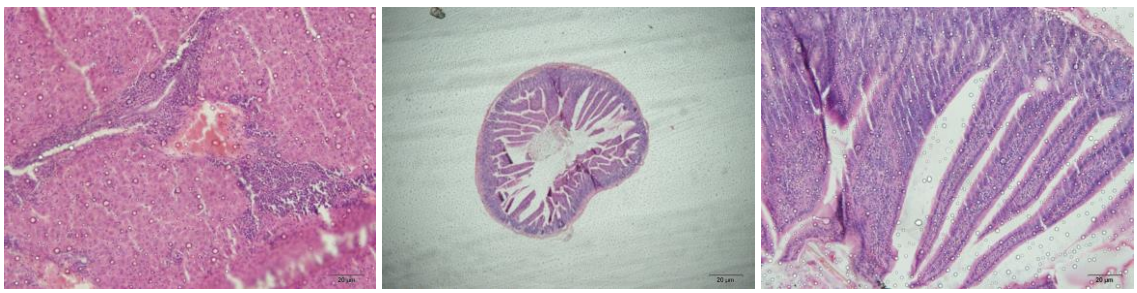


Figure 14 C: Kidney showing hydropic tubular degeneration (microscopic increase in 4x and 20x), liver showing inflammation and congestion (microscopic increase in 4x and 20x) and small intestine presenting inflammation in the mucosa (microscopic increase in 4x and 20x) of the group treated with *S*. Typhimurium.

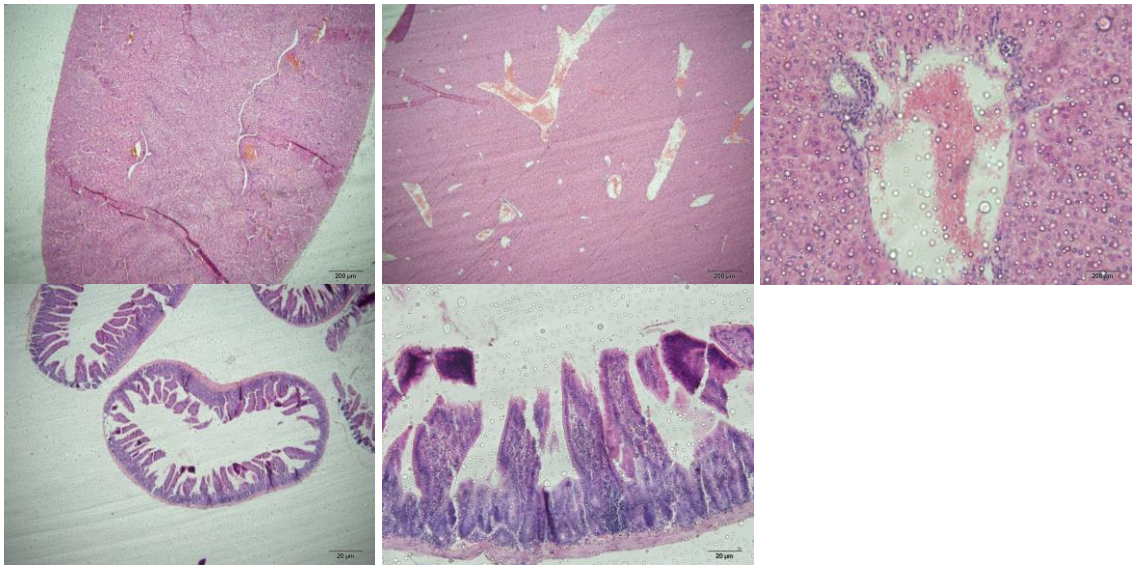


Figure 14 F: Unchanged kidney, liver showing swelling and congestion (microscopic increase in 4x and 20x) and small intestine showing mucosal inflammation (microscopic increase in 4x and 20x) in the *E. coli* O157:H7 treated group.

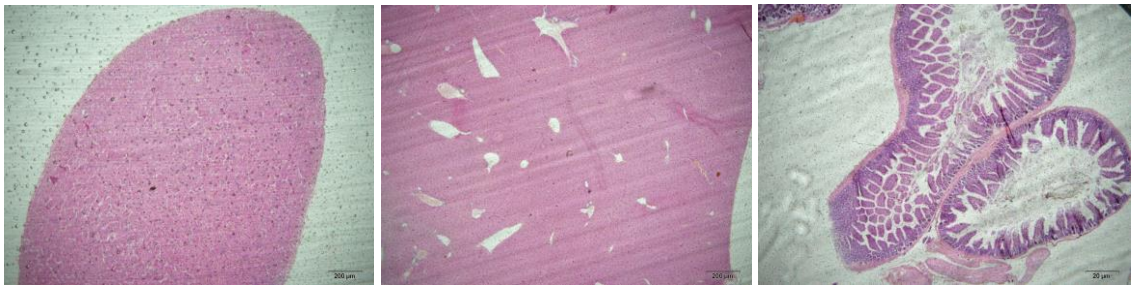


Figure 14 G: A: Kidney, liver and intestine control group without alteration (microscopic increase in 4x) in the group treated with *L. casei* CSL3 challenged with *S*. Typhimurium.

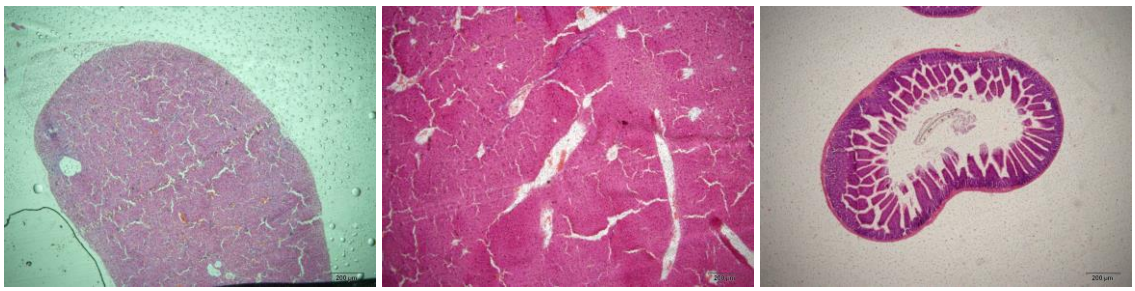


Figure 14 H: Kidney, liver and intestine control group without alteration (microscopic increase in 4x) in the group treated with *L. casei* CSL3 challenged with *E. coli* O157:H7.

### 7.3.4 IL2, IL4 and TNF gene expression

In Figures 15, the relative transcripts of interleukin 2 (IL-2), interleukin 4 (IL-4) and tumor necrosis factor alpha (TNF-α) can be identified in splenocytes that received *in vitro* stimulation, for each treatment tested.

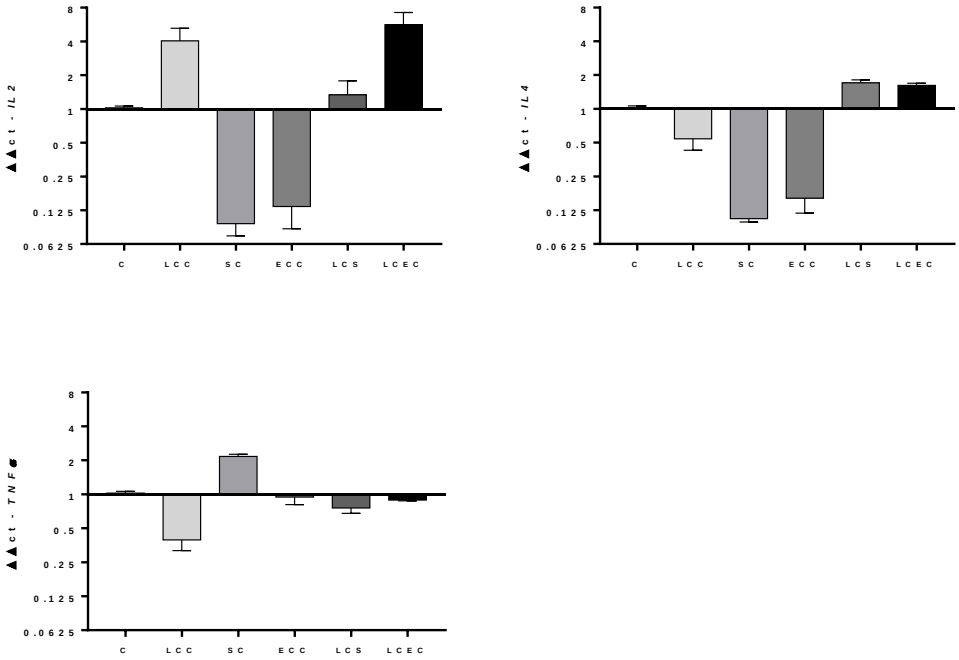



Figure 15. Relative transcription of IL-2, IL-4 and TNF-α, in splenocytes that received *in vitro* stimulation of *L. casei* CSL3, for each treatment. C: control group; LCC: *L. casei* CSL3 group; LCS: *L. casei* CSL3 against *S.* Typhimurium group; LCEC: *L. casei* CSL3 against *E. coli* group.

When comparing relative transcription of interleukins and tumor necrosis factor of control group and probiotic group it is possible to notice with respect to IL-2 that there is an increase of more than 4 fold to approximately, and in those that received the probiotic and were challenged with the pathogenic bacteria *S*. Typhimurium and *E. coli* there is an increase of more than 1 fold to approximately and 4 fold to approximately, respectively.

For the groups that received only the pathogens, it was noted that the stimulus to the relative transcription of IL-2 was lower, with > 0.125 for both bacteria.

Interleukin-2 is responsible for inducing the maturation of B lymphocytes and T cells. This is considered a protein that regulates the activities of white blood cells (leukocytes, often lymphocytes) that are responsible for immunity. Therefore, the increase in this, influenced by *L. casei* CSL3, means a faster immune response on the part of the host, helping to eliminate the agents that cause infections.

Regarding the comparison for the relative transcription of interleukin 4, it was possible to notice in the treatments that received the probiotic bacteria a relative expression of approximately 0.5 fold) while the control maintained a relative expression of approximately 1 fold. When *L. casei* CSL3 was challenged whit *S*. Typhimurium and *E. coli* this relative expression has increased to values greater than 1 fold. For the treatments that received only the pathogens, this relative expression remained at values below 0.25 fold.

Interleukin 4 plays a central role in determining the phenotype of naive CD4+ T cells, promoting their differentiation into IL-4 type 2 (Th2)-producing helper T cells.(Yoshimoto, 2018)Sharma et al. (2014) evaluated the cytokine profile of mice fed with fermented milk supplemented with probiotic *L. fermentum*. They noted, as in the present study, a considerable decrease in interleukin 4 levels in the group fed with the probiotic.

Jain et al. (2010) when evaluating the production of specific cytokines in the supernatant of splenocytes collected from mice fed with probiotic Dahi (curd) could observe an increase in IFN - γ and IL2 in Th1 cells and a reduction in IL4 and IL6 in Th2 cells.

Tumor necrosis factor alpha is able to inhibit and eliminate tumor cells, and can also stimulate the inflammatory response of other cytokines.

Comparing the control group with the group that received the probiotic bacteria, a reduction in the relative transcription of TNF-α maintaining an average lower than 0.5 fold.to. For groups where the probiotic was challenged against *S*. Typhimurium and *E. coli* values less than 1 fold are noted. It is noteworthy that the relative transcription of tumor necrosis factor for the treatment that received only *S*. Typhimurium increased approximately 2 fold.

When evaluating the immune response of mice treated with different probiotics, Liu et al. (2021) observed the blocking of the activation of the TLR4/NF-κB signaling pathway and, therefore, the production of pro-inflammatory cytokines (TNF-α), whereas *S. aureus*, a bacterium considered pathogenic, was significantly and positively correlated with the expression of TNF-α.

## 7.5 Conclusion

*Lacticaseibacillus casei* CSL3 proved to be safe for administration in *in vivo* treatments, as well as having a positive influence on the modulation of the inflammatory response of the hosts. This took place through the reduction in the concentration of pathogenic bacteria in the animal feces, reduction in organ damage in treatments containing the probiotic and an increase in the synthesis of nitric oxide and relative expression of essential interleukins in the fight against the pathological agent.

Therefore, it is important to continue studies with *L. casei* CSL3, exploring other benefits that the bacterium can cause in different types of treatments, from diseases to competition with other pathogenic microorganisms.

## CONSIDERAÇÕES FINAIS

A busca por microrganismos probióticos isolados de novas fontes com características específicas, vêm crescendo ao longo dos anos. As diferenças na constituição dessas fontes como disponibilidade de carbono, nitrogênio e água, valores de pH, bem como a sobrevivência em condições do meio externo como a temperatura, são os principais fatores que conduzem às distintas características apresentadas por esses isolados autóctones.

Ao final dos estudos realizados, pode-se inferir que *L. casei* CSL3, possui capacidade de manter-se viável, quando imobilizado em suportes orgânicos, como pedaços de abacaxi e o biocatalizador ao ser inserido em queijo *Petit Suisse* como matriz alimentar, favoreceu a manutenção da viabilidade durante a passagem pelo trânsito gastrintestinal simulado. Ao ser submetida para avaliações *in vitro* e *in vivo*, a bactéria em estudo, demonstrou possuir características probióticas, reduzindo a concentração de glicose e triglicerídeos no sangue, bem como o estresse oxidativo nos tecidos. Quando em competição contra patógenos de importância alimentar, *L. casei* CSL3 atuou na colonização do trato gastrointestinal, estimulou a expressão relativa de citocinas pro-inflamatórias, e protegeu os tecidos do fígado e intestino das alterações ocasionadas por *Salmonella* Typhimurium e *Escherichia coli* O157:H7.

Portanto, sugere-se que seria interessante dar prosseguimento a estudos com *L. casei* CSL3 para o maior entendimento de outras propriedades benéficas que essa bactéria isolada de silagem de colostro bovino, pode proporcionar ao hospedeiro.

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