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Bruna Maria Salotti de Souza

Avaliação do potencial probiótico de *Lactobacillus casei* e *Lactobacillus fermentum* autóctones e aplicação em leites fermentados com diferentes matrizes

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Dedico

Ao meu marido Uender que, com muito carinho,
apoiou e não mediu esforços para que eu chegasse
até esta etapa na minha vida.

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“Duvida sempre de ti, até que os dados não deixem lugar para dúvidas”.

Louis Pasteur

RESUMO

O objetivo deste estudo foi avaliar as propriedades probióticas das estirpes *Lactobacillus casei* e *Lactobacillus fermentum*, selecionar novas estirpes seguras para o desenvolvimento de produtos fermentados e a partir desta seleção definir uma matriz apropriada para ser fermentada e avaliar o efeito do leite fermentado pelas cepas selecionadas na translocação bacteriana, análises fecais e morfologia intestinal de camundongos BALB/c. Os testes *in vitro* realizados foram auto-agregação, co-agregação, hidrofobicidade, produção de β-galactosidase, sobrevivência ao trato gastrointestinal (TGI) e suscetibilidade a antibióticos. As cepas selecionadas foram adicionalmente testadas pela presença de genes que codificam adesão, agregação e colonização, fatores de virulência, resistência a antibióticos e produção de aminas biogênicas, seguido da avaliação de parâmetros cinéticos acidificantes no leite e sobrevivência das cepas sob condições de GIT simuladas durante refrigeração. A maioria das cepas de ambas as espécies apresentou alta auto-agregação, algumas cepas apresentaram capacidade de co-agregação com outras bactérias ácido láticas (BAL) e/ou patógenos, e ambas as espécies apresentaram valores de hidrofobicidade baixos. Sete cepas de *L. casei* e seis cepas de *L. fermentum* foram produtoras da enzima β-galactosidase e dez cepas sobreviveram bem a simulação de condições estressantes do TGI. Todas as cepas foram resistentes a vancomicina e quase todas as cepas eram resistentes à kanamicina. *L. casei* SJRP38 e *L. fermentum* SJRP43 foram distinguidos entre as outras cepas de BAL pelo seu maior potencial probióticos. Três matrizes foram fermentadas por *L. casei* SJRP38 e *L. fermentum* SJRP43 e os parâmetros cinéticos de acidificação ao longo da fermentação foram realizados. Os animais foram divididos em quatro grupos: controle água (CW), controle do leite (CM), leite fermentado por *L. casei* SJRP38 (FMLC) e leite fermentado por *L. fermentum* SJRP43 (FMLF), ambas fermentações com co-cultura com a cepa comercial de *S. thermophilus* TA040 ao longo do tratamento. Após 14 dias de alimentação, os camundongos BALB/c foram contidos manualmente e individualmente, e a eutanásia foi realizada e colhido sangue, fígado, baço, intestino delgado, intestino grosso e conteúdo intestinal foram removidos para estudos de translocação, morfologia intestinal e microbiologia intestinal. Durante o protocolo experimental, nenhuma atividade notável ou alterações comportamentais foram observadas nos grupos de camundongos. Não houve variação estatística do peso de camundongos BALB/c entre os quatro grupos experimentais, em todos os períodos avaliados. Em geral, a administração de leite fermentado resultou em alterações microbiológicas na composição fecal, levando a uma redução no *Clostridium* spp. e aumento de *Streptococcus* spp., *Lactobacillus* spp. e *Bifidobacterium* spp. A população de *Lactobacillus* spp. e *Bifidobacterium* spp. no intestino delgado e no intestino grosso foram afetados pelo grupo de tratamento. O grupo FMLF apresentou menor população de *Lactobacillus* spp. no intestino delgado e *Bifidobacterium* spp. no intestino grosso. Não houve diferenças significativas entre os outros grupos. Os resultados de incidência de translocação de bactérias intestinais para diferentes tecidos e sangue mostraram que nenhuma bacteremia foi observada nos diferentes grupos experimentais. Não houve diferença estatística quanto à espessura do epitélio duodeno entre os quatro grupos; no entanto, no grupo CW do grupo do jejuno-íleo apresentou a maior altura epitelial, quando comparado aos outros grupos CM, FMLC e FMLF. A altura do

epitélio do cólon também foi maior no grupo CW do que os demais grupos CM, FMLC e FMLF. Quanto à profundidade da cripta, no duodeno houve diferença significativa entre o grupo CM e os demais grupos, que apresentaram maior profundidade de cripta. Na porção jejuno-íleo, a maior profundidade de cripta foi mostrada no grupo CW do que nos outros grupos. Na parte do cólon, não houve variações significativas entre os grupos. A morfometria das vilosidades do intestino delgado mostrou que um menor tamanho das vilosidades do duodeno foi observado no grupo CW, quando comparado aos outros grupos, no entanto, na porção jejuno-íleo não foram observadas diferenças significativas entre os grupos. A avaliação morfométrica das microvilosidades intestinais mostrou que no duodeno, o grupo CM apresentou tamanho de microvilosidades estatisticamente maior do que os outros grupos. Na parcela do jejuno-íleo, apenas os grupos FMLC e FMLF apresentaram diferenças significativas entre si em tamanho de microvilosidades, enquanto na parte do cólon não houve diferenças no tamanho de microvilosidades entre os grupos CW e FMLC e entre os grupos CM e FMLF. Houve uma frequência estatisticamente maior de células caliciformes no duodeno nos grupos CM, FMLC e FMLF, quando comparado ao grupo CW, no entanto, na parcela do jejuno-íleo, a diferença foi restrita entre os grupos CW e CM e a frequência das células caliciformes nos outros grupos foi superior a 40%. Em conclusão, o consumo de leite fermentado com cepas de *L. casei* SJRP38 e *L. fermentum* SJRP43 promoveu benefícios ao intestino do hospedeiro.

Palavras chave: histologia; morfometria; probióticos; trato gastrointestinal.

ABSTRACT

The objective of this study was to evaluate the probiotic properties of the strains *Lactobacillus casei* and *Lactobacillus fermentum*, to select new safe strains for the development of fermented products and from this selection to define an appropriate matrix to be fermented and to evaluate the effect of the fermented milk by the strains selected in the bacterial translocation, fecal analyzes and intestinal morphology of BALB/c mice. The *in vitro* tests performed were self-aggregation, co-aggregation, hydrophobicity, β-galactosidase production, gastrointestinal tract survival (GIT) and antibiotic susceptibility. The selected strains were additionally tested for the presence of genes encoding adhesion, aggregation and colonization, virulence factors, antibiotic resistance and biogenic amine production, followed by the evaluation of kinetic parameters in the milk and survival of the strains under simulated GIT conditions during cooling. Most of the strains of both species presented high self-aggregation, some strains presented co-aggregation capacity with other lactic acid bacteria (BAL) and / or pathogens, and both species showed low hydrophobicity values. Seven strains of *L. casei* and six strains of *L. fermentum* were β-galactosidase enzyme producers and ten strains survived the simulation of stressful conditions of TGI. All strains were resistant to vancomycin and almost all strains were resistant to kanamycin. *L. casei* SJRP38 and *L. fermentum* SJRP43 were distinguished among the other strains of BAL by their greater potential probiotics. Three matrices were fermented by L. casei SJRP38 and L. fermentum SJRP43 and the kinetic parameters of acidification along the fermentation were performed. The animals were divided into four groups: water control (CW), milk control (CM), milk fermented by L. casei SJRP38 (FMLC) and milk fermented by L. fermentum SJRP43 (FMLF), both fermentations with co-culture with the commercial strain of S. thermophilus TA040 throughout the treatment. After 14 days of feeding, the BALB / c mice were contained manually and individually, and euthanasia was performed and harvested blood, liver, spleen, small intestine, large intestine and intestinal contents were removed for translocation studies, intestinal morphology and intestinal microbiology. During the experimental protocol, no remarkable activity or behavioral changes were observed in the groups of mice. There was no statistical variation in the weight of BALB/c mice among the four experimental groups, in all periods evaluated. In general, administration of fermented milk resulted in microbiological changes in fecal composition, leading to a reduction in *Clostridium* spp. and increased *Streptococcus* spp., *Lactobacillus* sp. and *Bifidobacterium* spp. The population of *Lactobacillus* spp. and *Bifidobacterium* spp. in the small intestine and large intestine were affected by the treatment group. The FMLF group presented lower population of *Lactobacillus* spp. in small intestine and *Bifidobacterium* spp. in the large intestine. There were not significative differences among the other groups. Results of incidence of translocation of gut bacteria to different tissues and blood showed that no bacteremia was observed in the different experimental groups. There was no statistical difference regarding the thickness of duodenum epithelium among the four groups; however, in the jejunum-ileum portion CW group presented the highest epithelial height, when compared to the other groups CM, FMLC and FMLF. The height of epithelial colon was also higher in CW group than the other groups CM, FMLC and FMLF. Regarding the crypt depth, in the duodenum there was a significant difference between the CM group and the other groups, which presented greater crypt depth. In the

jejunum-ileum portion the greater crypt depth was shown in the CW group than in the other groups. In the colon portion, there were no significant variations among groups. The morphometry of the small intestinal villi showed that a smaller size of the duodenum villi was observed in the CW group, when compared to the other groups, however, in the jejunum-ileum portion no significant differences among the groups were observed. The morphometrically evaluation of intestinal microvilli showed that in the duodenum, the CM group presented statistically larger microvilli size than the other groups. In the jejunum-ileum portion, only the FMLC and FMLF groups presented significant differences between each other in microvilli size, while in the colon portion, there were no differences in microvilli size between the CW and FMLC groups, and between the CM and FMLF groups. There was a statistically higher frequency of goblet cells in the duodenum in the CM, FMLC and FMLF groups, when compared to the CW group; however, in the jejunum-ileum portion the difference was restricted between the CW and the CM groups and the frequency of goblet cells in the other groups was higher than 40%. In conclusion, the consumption of fermented milk with *L. casei* strains SJRP38 and *L. fermentum* SJRP43 promoted health benefits to the host.

Key-words: gastrointestinal tract; histology; morphometry; probiotics.

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SUMÁRIO

INTRODUÇÃO E JUSTIFICATIVA

O leite e seus derivados há anos fazem parte importante de uma dieta equilibrada para alimentação humana. Dentre os derivados lácteos, se destacam os produtos lácteos fermentados por micro-organismos específicos, responsáveis por efeitos terapêuticos no hospedeiro, denominados de produtos probióticos.

Para exercerem os efeitos benéficos, os micro-organismos probióticos devem manter sua viabilidade no produto, durante a estocagem, até o consumo. Entretanto, muitos fatores, relacionados ao tipo de produto, micro-organismos presentes ou a matriz alimentícia (composição, presença de aditivos, compostos bioativos, entre outros) podem interferir nesta viabilidade, de forma a maximizar ou minimizar seu desempenho funcional.

Estudos realizados *in vivo* têm demonstrado o potencial clínico dos probióticos, com melhora no estado geral do paciente (humano e animal) em muitas doenças. Os probióticos foram caracterizados por suprimir diarreia, aliviar a intolerância à lactose e complicações pós-operatórias, por exibir atividades antimicrobianas, reduzir os sintomas do intestino irritável, reduzir a ocorrência de doença inflamatória do intestino, entre outros efeitos descritos na literatura atual.

Para seleção de novas culturas probióticas é necessária a avaliação de suas propriedades funcionais, tecnológicas e de segurança das cepas, além da falta de patogenicidade, a tolerância às condições do trato gastrointestinal (TGI), a capacidade de aderir à mucosa gastrointestinal e de exclusão competitiva de organismos patogênicos.

Considerando os efeitos benéficos dos micro-organismos probióticos, a literatura atual mostra o aumento do interesse em estudos sobre produtos alimentícios fermentados como uma fonte de novas linhagens de probióticos. Além disso, as cepas isoladas de produtos lácteos fermentados são, provavelmente, os candidatos mais adequados para aplicação em diversos

tipos de alimentos probióticos, uma vez que estão melhor adaptados às condições do meio, portanto, podem ser mais competitivos do que as cepas de probióticos isoladas de outras fontes.

Trinta e oito cepas isoladas de queijo muçarela de búfala foram avaliadas pela produção de substâncias antimicrobianas, obtendo-se resultados promissores, com a obtenção de quatro isolados produtores de bacteriocinas (PAULA et al., 2015). Nesses isolados também foi feita a avaliação das propriedades probióticas. As cepas estudadas apresentaram sobrevivência ao TGI quando inoculadas em meio MRS, leite UAT (ultra alta temperatura) integral e leite UAT desnatado, e obtiveram ótimos resultados de auto-agregação, co-agregação e hidrofobicidade. A produção da enzima β-galactosidase foi observada em três e somente duas cepas apresentaram o gene de adesão à mucosa (EF-Tu). A cepa de *Lb. delbrueckii* subsp. *bulgaricus* não apresentou fatores genéticos de virulência, sendo considerada a cepa de maior potencial probiótico entre as BAL estudadas (JEROMYMO-CENEVIVA, 2013).

Pretende-se estender os estudos do potencial probiótico para dezenove cepas de BAL, identificadas a partir do sequenciamento do gene 16S rRNA (SILVA, 2010) como *Lactibacillus casei* (12 cepas) e *Lactobacillus fermentum* (7 cepas). Estas culturas já foram caracterizadas quanto à capacidade de produção de CO_2 a partir do uso da glicose, habilidade de crescimento em diferentes temperaturas (15, 30 e 45 ^{o}C), pH (4, 5, 7,0 e 9,6) e concentração de NaCl (4, 5, 6 e 10%), além da capacidade de assimilar citrato, sendo que os resultados obtidos demonstraram o potencial benéfico à saúde humana.

Além disso, como os *Lactobacillus* apresentam alto potencial de aplicação na indústria de alimentos, a avaliação das propriedades probióticas é importante, uma vez que podem ser identificadas novas cepas para o desenvolvimento industrial de alimentos funcionais. Espera-se que este conhecimento possa resultar em novas alternativas para o desenvolvimento de produtos inovadores com características sensoriais e tecnológicas adequadas.

ORGANIZAÇÃO DOS CAPÍTULOS

O presente trabalho foi organizado em quatro capítulos para melhor apresentação e entendimento dos assuntos abordados. O “Capítulo I” consiste dos objetivos do trabalho e revisão bibliográfica do tema abordado da tese. O “Capítulo II” foi redigido na forma de artigo científico e submetido à publicação no periódico *Probiotics and Antimicrobial Proteins*, classificado como A2 pelo Qualis/CAPES. Após a análise, os revisores apontaram que o trabalho poderá ser aceito se forem realizadas pequenas alterações. O “Capítulo III” também foi redigido na forma de artigo científico, a ser submetido à publicação em periódico internacional classificado pelo Qualis/CAPES. O “Capítulo IV” trata das conclusões gerais deste trabalho.

CAPÍTULO I

Objetivos e Revisão Bibliográfica

1. OBJETIVOS

1.1. Objetivo Geral

Avaliar as propriedades probióticas de doze cepas de *Lb. casei* e sete cepas de *Lb. fermentum*, selecionar as cepas seguras para aplicar em leites fermentados com diferentes matrizes, selecionar a matriz que proporcionar melhor resistência a passagem pelo trato gastrointestinal (TGI) e avaliar o produto *in vivo*, realizando testes em animais.

1.2. Objetivos Específicos

Avaliar as características de suscetibilidade à antibióticos e o potencial probiótico por meio das propriedades de auto-agregação, co-agregação, hidrofobicidade, produção de β-galactosidase e resistência ao TGI;

Selecionar cepas seguras e com elevado potencial probiótico para realizar testes adicionais de tolerância às condições simuladas do trato gastrointestinal (TGI), produção de β-galactosidase e presença de genes de virulência;

Aplicar a cepa selecionada em leite fermentado com diferentes matrizes, avaliar os parâmetros cinéticos de fermentação e a resistência às condições simuladas do TGI;

Selecionar a matriz que proporcionar maior viabilidade das cepas probióticas e avaliar o produto fermentado *in vivo*, realizando teste em animais.

2. REVISÃO BIBLIOGRÁFICA

2.1. Bactérias ácido-láticas (BAL)

As bactérias ácido-láticas (BAL) são utilizadas há séculos na produção de uma variedade de produtos lácteos fermentados. Além disso, são utilizadas para a conservação de alimentos devido à produção de ácidos orgânicos e metabólitos antimicrobianos, tais como ácido lático, ácido acético, diacetil, peróxido de hidrogênio e bacteriocinas (JERONYMO-CENEVIVA et al., 2014).

Essas BAL compreendem a classe mais representativa de organismos probióticos e desempenham papel importante durante o processo de elaboração dos produtos fermentados, tais como: produção de ácido lático, redução do teor de lactose, melhorias nas características tecnológicas (sensorial, física e química) e melhoria na segurança alimentar (JERONYMO-CENEVIVA et al., 2014).

Apesar de longos anos de pesquisa, o termo "bactéria ácido lático" (BAL) não era representado por eficiente definição até recentemente. Atualmente a definição de consenso considera que BAL são anaeróbias, aerotolerantes, Gram positivas e fermentam uma variedade de açúcares com produção de ácido lático como produto final (SAUER et al., 2017). As BAL compreendem um grupo de micro-organismos com diversas características morfológicas, metabólicas e fisiológicas semelhantes, apresentam a forma de cocos ou bacilos, não formam esporos, são anaeróbias facultativas ou microaerófilas, ácidos tolerantes, e produzem ácido lático como o principal produto da fermentação dos carboidratos. Estes micro-organismos são catalase-negativos (com exceção de alguns *Pediococcus* e *Lactobacillus*, que produzem pseudo-catalase) e se reproduzem por fissão binária (WALSTRA et al., 2006).

A classificação das BAL em diferentes gêneros é baseada na morfologia, multiplicação em diferentes temperaturas, configuração do ácido lático produzido, modo de fermentação da

glicose, capacidade de multiplicação em altas concentrações salinas e tolerância às condições ácidas e básicas. Além disso, a taxonomia das BAL também leva em conta a relação filogenética entre os diferentes micro-organismos desse grupo, elucidada através do sequenciamento do RNA ribossômico (HOLZAPFEL et al., 2001).

As bactérias Gram-positivas são divididas em dois grupos filogenéticos *Firmicutes* e *Actinobacteria*, com base em comparações do conteúdo de guanina + citosina (G + C) da sequência do DNA ribossomal. O grupo *Actinobacteria* compreende bactérias com conteúdo G + C superior a 50%, enquanto que o grupo *Firmicutes* compreende bactérias em que a composição do DNA é de baixo conteúdo do par G + C. Sendo assim, as BAL do gênero *Bifidobacterium* pertencem ao filo *Actinobacteria*, por exibirem elevado conteúdo de G + C e os gêneros *Lactobacillus, Lactococcus, Enterococcus, Streptococcus, Pediococcus* e *Leuconostoc* estão inseridos no filo *Firmicutes*, exibindo baixo conteúdo de G + C (VASILJEVIC, SHAH, 2008; TRIVEDI et al., 2010).

De acordo com a temperatura ótima de desenvolvimento, as BAL são definidas como micro-organismos mesofílicos (20-40 °C) ou termofílicos (40-50 °C). São consideradas bactérias nutricionalmente exigentes, pois precisam de nutrientes específicos, tais como aminoácidos e vitaminas (SALMINEN et al., 2004).

Em função do metabolismo da glicose, as BAL são classificadas como homofermentativas, pela produção de ácido lático como o principal ou único produto da fermentação, tais como *Lactococcus, Pediococcus, Enterococcus, Streptococcus* e alguns *Lactobacillus;* e heterofermentativas, produtoras de igual quantidade de lactato, dióxido de carbono e etanol, além da produção de compostos de *flavor* e aroma, como acetaldeído e diacetil, como *Leuconostoc, Weissela* e alguns *Lactobacillus* (JAYAMANNE; ADAMS, 2006).

As BAL produzem ácidos orgânicos, tais como o ácido lático, acético e ácido propiônico, como produtos finais da fermentação de hidratos de carbono. A produção destes ácidos

orgânicos fracos geralmente limita a multiplicação de bactérias e fungos, incluindo muitos micro-organismos patogênicos e deteriorantes (LEE et al., 1999; GUCHTE et al., 2002; ROSS et al., 2002).

As BAL constituem um grupo altamente heterogêneo, geralmente com status GRAS (*Generally Recognized As Safe*), como ocorre com os micro-organismos dos gêneros *Lactococcus* e *Lactobacillus*; no entanto, bactérias dos gêneros *Streptococcus* e *Enterococcus* possuem algumas espécies que podem ser consideradas patógenos oportunistas (SALMINEN et al., 1998; LEROY, VUYST, 2004).

Atualmente, a União Européia (UE) se concentra muito intensamente em garantir a segurança dos alimentos e especialmente nos perigos químicos e microbiológicos. Perigos microbiológicos não são apenas os micro-organismos patogênicos, mas também podem ser as culturas microbianas iniciadoras (*starter*) adicionadas durante a produção de alimentos.

A taxonomia antiga definia quatro principais gêneros envolvidos em fermentações de alimentos, ou seja, *Lactobacillus, Leuconostoc, Pediococcus* e *Streptococcus* (WESSELS et al., 2004). No entanto, reclassificações alteraram este agrupamento original e o grupo BAL atualmente é composto dos seguintes gêneros: *Aerococcus, Alloiococcus, Carnobacterium, Dolosigranulum, Enterococcus, Globicatella, Lactobacillus, Lactococcus, Lactosphaera, Leuconostoc, Melissococcus, Oenococcus, Pediococcus, Streptococcus, Tetragenococcus, Vagococcus* and *Weisella* (CROWLEY et al., 2013).

Na indústria de alimentos, são utilizadas com frequência bactérias de seis gêneros/espécies: *Lactococcus* (leite), *Lactobacillus* (leite, carne, vegetais e cereais), *Leuconostoc* (vegetais, leite), *Pediococcus* (vegetais, carne), *Oenococcus oeni* (vinho) e *Streptococcus thermophilus* (leite) (INÊS et al., 2008).

A maioria das BAL pertence ao gênero *Lactobacillus*. Estas estão presentes na natureza em ambientes ricos em nutrientes, incluindo silagens, vegetais fermentados e também fazem parte

da microbiota autóctone da cavidade oral, vagina e trato digestório de animais e seres humanos, onde exercem influência benéfica (CHERIGUENE et al., 2007; JERONYMO, 2013).

Os *Lactobacillus* e *Bifidobacterium* estão entre as bactérias com o menor risco para os seres humanos. Nas bacteremias em que os *Lactobacillus* estão envolvidos a frequência observada é de apenas 0,1-0,24%, e em praticamente todos os casos há uma doença grave subjacente, que pré-dispõe o indivíduo a infecções oportunistas (WESSELS et al., 2004; VINDEROLA et al., 2017).

O gênero *Lactobacillus* é composto por micro-organismos heterogêneos quanto às características fenotípicas e genotípicas. Apresentam formato de bastonetes regulares e temperatura ótima de multiplicação entre 30 a 40 °C, são Gram-positivos e não esporulados, sendo acidúricos com pH ótimo entre 5,5 e 6,2, além de atuarem no intestino delgado. São encontrados em muitas fontes vegetais e animais e no TGI e leite humano, mas também em muitos produtos de origem vegetal (ROBINSON, 2002; RAJILIĆ-STOJANOVIĆ, DE VOS, 2014).

As espécies pertencentes à ordem *Lactobacillales* são de natureza abundante e adequadas para modulação de microbiota intestinal e incorporação a muitos sistemas alimentares (VINDEROLA et al., 2017). A divisão dos *Lactobacillus* é baseada na característica fermentativa, sendo que os homofermentativos fermentam glicose exclusivamente em ácido lático, como ocorre com *Lb. acidophilus, Lb. gasseri, Lb. crispatus, Lb. johnsonii, Lb. delbruekii, Lb. helveticus* e *Lb. salivarus*. As espécies heterofermentativas, fermentam hexoses em ácido lático, ácido acético e dióxido de carbono, como ocorre com *Lb. brevis, Lb. buchneri, Lb. fermentum* e *Lb. reuteri*. Além disso, é possível caracterizar os *Lactobacillus* em facultativamente heterofermentativos, tais como *Lb. curvatus, Lb. plantarum, Lb. sakei, Lb. casei, Lb. paracasei* e *Lb. rhamnosus* (SALMINEN et al., 2004).

2.2. Bactérias ácido-láticas probióticas

O estímulo ao consumo de BAL surgiu no início do século XX, quando Metchnikoff sugeriu que a ingestão destes micro-organismos vivos, presentes em leite fermentado, aumentava a longevidade do hospedeiro. Metchnikoff (1907) atribuiu os efeitos positivos observados na saúde do hospedeiro a uma redução na população de bactérias deteriorantes e bactérias produtoras de toxinas no trato digestivo, sendo assim, iniciava-se o conceito de bactérias probióticas.

Desde então, o desenvolvimento e consumo de alimentos ou suplementos dietéticos que contêm micro-organismos probióticos têm crescido consideravelmente. Nas últimas décadas, os principais líderes da indústria de alimentos têm explorado o conceito e colocam no mercado uma grande variedade de novos produtos contendo bactérias probióticas, que são oferecidas em nutracêuticos ou em alimentos fermentados, incluindo os produtos lácteos (BURGAIN et al., 2014).

Segundo Saad (2006), os probióticos podem ser definidos como "suplementos microbianos vivos e não patogênicos que influenciam positivamente o organismo e aumentam de maneira significativa o valor nutritivo e terapêutico dos alimentos, através do equilíbrio microbiano intestinal e das funções fisiológicas do trato intestinal humano".

A palavra probiótico provém do grego, que significa "para a vida". Alimentos lácteos, fermentados ou não, incluindo soro lácteo, iogurte, sorvete, sobremesas e mesmo queijos, são veículos comuns de sua administração (SÁNCHEZ et al., 2009).

De acordo com a Agência Nacional de Vigilância Sanitária (ANVISA, 2002), probióticos são "micro-organismos vivos capazes de melhorar o equilíbrio microbiano intestinal produzindo efeitos benéficos à saúde do indivíduo", enquanto a FAO/WHO (2002) define probióticos como "micro-organismos de vida livre, que quando administrados em quantidade adequada,

conferem benefícios à saúde do hospedeiro", porém um novo painel de consenso realizou uma adequação gramatical à definição original da FAO/OMS, e probióticos passou a ser definido como "micro-organismos vivos que, quando administrados em quantidades adequadas, conferem um benefício à saúde do hospedeiro" (HILL et al., 2014).

A incorporação de bactérias probióticas em produtos lácteos fermentados implica na necessidade de manter as células viáveis para o consumo, sendo que a viabilidade celular do probiótico em leites fermentados depende do tipo de produto, dos aditivos químicos (aromatizantes, conservantes, espessantes, estabilizantes, etc.) utilizados na fabricação do produto e das possíveis interações entre as bactérias láticas probióticas (VINDEROLA et al., 2011).

Quando os probióticos são adicionados aos alimentos é necessário o monitoramento das populações desses micro-organismos, pois é comprovado que estes micro-organismos probióticos demonstram suas atividades apenas quando presentes em concentrações adequadas nos alimentos fermentados. A dose terapêutica mínima diária sugerida de probióticos é de 10^6-10^7 unidades formadoras de colónias – UFC (COLOMBO et al., 2014).

Kempka (2008) descreveu uma recomendação de ingestão semanal mínima de 300 a 500 g de produtos lácteos fermentados contendo entre 10^6 a 10^7 UFC/mL, porém de acordo com Agência Nacional de Vigilância Sanitária - ANVISA, a recomendação é de 10^8 a 10^9 UFC de micro-organismos viáveis na porção diária do produto ingerido (BRASIL, 2007a). Ainda, Kimoto-Nira et al. (2009) descreveram que para proporcionar algum efeito ao organismo, as BAL devem atingir entre 10^6 - 10^9 UFC/g no conteúdo intestinal.

Os benefícios atribuídos aos probióticos podem incluir: atenuação de reações alérgicas; aumento da biodisponibilidade de determinados nutrientes, redução da concentração do colesterol sérico, melhora da saúde do trato urogenital, aumento da quantidade de anticorpos IgA circulantes, bloqueio da adesão de bactérias patogênicas aos tecidos epiteliais, melhoria

dos sintomas de câncer no intestino e de doenças inflamatórias intestinais, como a síndrome do intestino irritado e constipação intestinal, além de controle de infecções orais (YAN, POLK, 2008; REIS et al., 2011; CHIANG, PAN, 2012; PERSICHETTI et al., 2014).

Pugliesi et al. (2014) destacaram ainda efeitos benéficos de culturas probióticas que merecem destaque, tais como o estímulo da motilidade intestinal com consequente alívio da constipação intestinal, a melhor absorção de determinados nutrientes, melhor utilização de lactose e alívio dos sintomas de intolerância a esse açúcar, a diminuição dos níveis de colesterol, o efeito anticarcinogênico e o estímulo do sistema imunológico, através do estímulo da produção de anticorpos e da atividade fagocítica contra patógenos intestinais, além da exclusão competitiva e produção de compostos antimicrobianos.

Os efeitos dos probióticos podem ser classificados em três categorias: (1) capacidade de modular a defesa do hospedeiro; (2) efeito direto sobre outros micro-organismos patogênicos, tornando-os importantes na prevenção e restauração do equilíbrio da mucosa intestinal; e (3) efeito baseado na eliminação de produtos resultantes do metabolismo microbiano, como toxinas, o que resulta na desintoxicação do intestino de quem os consomem (OELSCHLAEGE, 2010).

Nas últimas décadas as propriedades nutritivas e terapêuticas dos alimentos funcionais contendo micro-organismos probióticos têm sido foco de muitos estudos. Entretanto, esses efeitos benéficos conferidos pelos probióticos são cepa-específicos e não espécie ou gênero-específico (REIS et al., 2011).

2.3. Aspectos relevantes na seleção de novas cepas probióticas

Vários aspectos, incluindo propriedades funcionais, tecnológicas e de segurança das cepas tem que ser levados em consideração ao selecionar as culturas probióticas. Os critérios de seleção das cepas incluem a falta de patogenicidade, a tolerância às condições do TGI, a

capacidade de aderir à mucosa gastrointestinal e a exclusão competitiva de organismos patogênicos (OUWEHAND et al., 2002).

Para que um micro-organismo seja considerado um probiótico é necessário: sobreviver às adversidades do estômago, resistir a passagem por todo trato digestório, além de ter a capacidade de colonizar o intestino, mesmo que temporariamente, por meio de adesão ao epitélio intestinal (DE MORAES; JACOB, 2006). Ao se aderir ao epitélio intestinal poderá competir com agentes patogênicos e desencadear uma resposta inflamatória e imunológica do hospedeiro, proporcionando alterações favoráveis à microbiota intestinal, inibir o crescimento de bactérias patogênicas, além de promover uma digestão favorável, estimulando função imunológica e aumentando a resistência a infecções (OLIVEIRA, 2007).

A Autoridade Europeia para a Segurança dos Alimentos (EFSA), criada em 2002 para resolver as questões científicas e técnicas cada vez mais importantes e complexas relativas à alimentação e segurança alimentar na União Europeia, estabeleceu diretrizes para realização de testes de segurança para micro-organismos associados a alimentos (FONTANA et al., 2013).

O Comitê Científico da Alimentação Animal da EFSA propôs que para a avaliação de segurança deve seguir quatro etapas: (i) definição da taxonomia do micro-organismo; (ii) coleta de informações suficientes fornecendo a base para o conhecimento do *status* de segurança, incluindo a literatura científica, a história de uso, as aplicações industriais e de dados ecológicos e de intervenção humana; (iii) exclusão da patogenicidade; e (iv) definição da utilização final. Se não houver problemas de segurança para um determinado grupo taxonômico, ou se quaisquer preocupações de segurança foram dissipadas depois da avaliação, a segurança pode ser concedida (SCAN, 2002).

Além disso, outros fatores devem ser considerados na avaliação da segurança dos probióticos: registrar a história do isolamento e a classificação taxonômica dos possíveis probióticos;

controlar a fabricação para eliminar contaminação dos probióticos com outros micro-organismos ou substâncias, além de determinar o estado fisiológico da população consumidora, com especial atenção para uso em populações vulneráveis, incluindo recém-nascidos. Alguns autores ainda propõe que um probiótico deve satisfazer os seguintes critérios: demonstrar seu efeito benéfico sobre o hospedeiro; não ser patogênico, não-tóxico e livre de efeitos secundários adversos significativos; ser capaz de sobreviver às condições do trato gastrointestinal (*in vitro* e *in vivo*); estar presente no produto em uma quantidade adequada de células viáveis para conferir o benefício de saúde; ser compatível com as condições da matriz do produto, de processamento e de armazenamento para manter as propriedades desejadas; além de ser rotulado com exatidão (FONTANA et al., 2013; BERNARDEAU et al., 2008; EFSA, 2016; SHARMA et al., 2014).

A resistência das bactérias probióticas a antibióticos é amplamente discutida por alguns pesquisadores. A resistência a um antibiótico pode ser inerente a uma espécie bacteriana ou gênero (resistência intrínseca) ou adquirida por mutação ou aquisição de genes (TOOMEY et al., 2009).

De acordo com Courvalin (2006), são dois os tipos de resistência bacteriana a antibióticos: intrínseca e adquirida. A resistência intrínseca ou natural está presente em todas as cepas de um determinado gênero ou espécie. Ela define os limites da ação de um antibiótico; é cromossômica e não transferível para outras bactérias. Por outro lado, a resistência adquirida pode ocorrer devido a dois mecanismos distintos: mutação ou aquisição de um gene resistente de outra bactéria por transferência lateral. No primeiro caso, a resistência é não-transferível, e no segundo, o gene adquirido pode ser re-transferido a outra bactéria, tornando-a resistente.

O gênero *Lactobacillus* não é na maioria dos casos patogênico e, portanto, clinicamente menos importante que o gênero *Enterococcus* e *Streptococcus*, mesmo assim é necessário analisar a presença de genes de resistência a antibióticos. Existe uma preocupação de que

essas bactérias não patogênicas atuem como um reservatório de genes de resistência aos antibióticos e possam transferir esses genes para bactérias patogênicas e oportunistas durante a passagem pelo TGI, contribuindo assim, para a transferência desses genes para bactérias presentes no ambiente do TGI, tornando-as multirresistentes (SALYERS et al., 2004; DICKS et al., 2009).

Várias pesquisas já demonstraram ser possível a transferência de genes de resistência de cepas de *Lactobacillus* para cepas de bactérias patogênicas (GEVERS et al., 2003; JACOBSEN et al., 2007), reforçando a necessidade do estudo de segurança das cepas de BAL para a aplicação em alimentos.

Tradicionalmente, uma vez que a segurança é estabelecida, os critérios de seleção mais comumente utilizados incluíram exposição a baixos pH e sais biliares, estudos de adesão ao muco ou linhas celulares como indicadores de "colonização intestinal temporária" (VINDEROLA et al., 2017).

Outro aspecto relevante na seleção de novas cepas é a viabilidade necessária dos probióticos para exercer o efeito benéfico. As condições do TGI influem nesta viabilidade. O pH baixo e a ação antimicrobiana da pepsina podem ser considerados os principais fatores prejudiciais para a viabilidade dos probióticos no estômago. O pH do estômago varia geralmente de 2,5 a 3,5, mas pode ser tão baixo quanto pH 2,0 em maiores taxas de secreção de suco gástrico, ou tão elevado como pH 6,0 ou mais, após a ingestão de alimentos (RANADHEERA et al., 2012). Os alimentos normalmente permanecem no estômago por 2-4 horas, dependendo da natureza física dos alimentos, sendo que os alimentos líquidos geralmente transitam mais rapidamente do que os sólidos (HUANG; ADAMS, 2004).

O duodeno, no início do intestino delgado, também apresenta condições adversas às BAL, em virtude da presença de sais biliares. Concentrações de 0,15-0,3% de sais biliares têm sido

recomendadas como adequadas para seleção de bactéria probiótica para uso em humanos (YANG; ADAMS, 2004).

A fim de proporcionar esse efeito na saúde do hospedeiro, as bactérias probióticas devem sobreviver à passagem pelo trato gastrointestinal, tolerando enzimas gástricas e ácidos biliares. No entanto, a sobrevivência de bactérias probióticas no TGI de humanos ou animais é um processo complexo e envolve a disponibilidade de nutrientes, tipo de dieta, interações com as bactérias autóctones no trato gastrointestinal, além de propriedades de adesão e colonização do epitélio intestinal. Estas propriedades funcionais podem ser influenciadas pelos alimentos transportadores utilizados como carreador do probiótico (HUANG, ADAMS, 2004; RANADHEERA et al., 2010; TODOROV et al., 2011).

A perda intensa da viabilidade dos micro-organismos no trato digestório tem elevado a busca por novas estratégias para a manutenção da viabilidade. Entre elas, a seleção de cepas resistentes às condições do TGI, a adição de uma matriz alimentícia adequada, como o leite ou iogurte, e até mesmo a adição de prebióticos, que atuam estimulando seletivamente a atividade de algumas bactérias do cólon, ou ainda a utilização da microencapsulação dos probióticos, que tem sido sugerido como um método promissor para a sua proteção (SINGH, 2008; VASILJEVIC, SHAH, 2008).

As formulações alimentares com pH próximo do neutro (> 5) e alta capacidade de tamponamento, aumentam o pH do trato gástrico e assim, aumentam a estabilidade dos probióticos (MAINVILLE et al., 2005). Certos componentes na matriz alimentícia podem interagir com os probióticos, alterando o seu desempenho funcional, como por exemplo: o elevado teor de gordura dos queijos pode proteger os probióticos durante a passagem através do TGI (VALERIO et al., 2006). Portanto, aplicar os probióticos em uma matriz alimentar adequada é o melhor meio para maximizar sua eficácia (RANADHEERA et al., 2012). Espirito Santo et al. (2011) relataram ainda que existem evidências de que as matrizes

alimentares desempenham um papel importante nos efeitos benéficos dos probióticos na saúde do hospedeiro.

As bactérias ácido-láticas com perfil probiótico, após sobreviverem às condições adversas do TGI, devem conseguir se aderir às células epiteliais do intestino, formando uma defesa contra a colonização de micro-organismos patogênicos (REID; BURTON, 2002).

A agregação é uma característica importante para a formação de biofilme pelas bactérias probióticas, auxiliando-as na aderência à mucosa intestinal de humanos e animais, enquanto a co-agregação de bactérias ácido láticas com outras bactérias pode ser considerada uma característica positiva, pois é um dos passos para eliminar os micro-organismos patogênicos do trato gastrointestinal (TODOROV; DICKS, 2008). A agregação e co-agregação são específicas de cada cepa e provavelmente envolve proteínas com funções específicas, tais como ligação ao muco, agregação e adesão intracelular (KLEEREBEZEM et al., 2010).

A hidrofobicidade celular também é uma característica importante para a adesão dos probióticos; é uma interação inespecífica entre as cepas bacterianas e as células do hospedeiro. A interação inicial pode ser fraca, muitas vezes reversível, seguidas por processos de adesão mediada por mecanismos mais específicos, envolvendo as proteínas da superfície celular e os ácidos lipoteicóicos. As bactérias com elevada hidrofobicidade geralmente apresentam fortes interações com as células da mucosa do hospedeiro (ROJAS et al., 2002). A hidrofobicidade pode ajudar a célula no processo de adesão, mas não é um pré-requisito para a bactéria conseguir uma forte adesão (DUARY et al., 2012).

Além dos fatores mencionados para a seleção de novas cepas, a produção da enzima β-galactosidase por bactérias ácido-láticas é de grande importância para a saúde do consumidor, pois esta enzima tem a função de hidrolisar a lactose em galactose e glicose. Esta enzima é produzida por alguns micro-organismos na membrana da mucosa do intestino delgado, e indivíduos intolerantes à lactose possuem atividade da β-galactosidase menor do que 10% dos

níveis da infância. Esse declínio faz com que a digestão da lactose seja insuficiente no intestino delgado, caracterizada por um aumento da concentração de glicose no sangue ou da concentração de hidrogênio na respiração após a ingestão de 50 g de lactose; essas condições são designadas como sintomas da má digestão de lactose (SCRIMSHAW; MURRAY, 1988). Por outro lado, as pessoas intolerantes à lactose, cuja produção de β-galactosidase no intestino é baixa ou deficiente, podem consumir leite fermentado por estas cepas, pois estas bactérias irão degradar a lactose, reduzindo ou eliminando os sintomas da intolerância (JERONYMO, 2013).

Em 2008 a EFSA rejeitou todos os pedidos de novos probióticos, o que reflete a necessidade de mais evidências científicas e estudos *in vivo* bem desenhados. Contrariamente ao padrão europeu, o Canadá tem uma lista positiva de espécies que podem ser comercializadas como probióticos (HILL et al., 2014).

2.4. Alimentos Funcionais e Leites Fermentados

O consumo de probióticos em suas diversas formas é comum e aumenta rapidamente, nos Estados Unidos, 3.9 milhões de adultos usaram suplementos probióticos em 2015 com aumento de quatro vezes em relação ao ano de 2007. Além disso os números de vendas sugerem que os probióticos são a categoria de suplementos mais compradas pelos consumidores (BRADLEY et al., 2015; PARKER et al., 2018).

Em resposta ao crescente número de consumidores preocupados na melhora de sua saúde, a demanda pelos alimentos funcionais no mercado tem aumentado e, consequentemente, a indústria alimentícia tem investido no desenvolvimento de uma variedade de novos alimentos funcionais (REIS et al., 2011), pois o efeito de alguns alimentos na saúde dos consumidores é amplamente conhecido (KOMATSU et al., 2008).

A denominação de alimentos funcionais surgiu primariamente no Japão, por volta dos anos 80, referindo-se àqueles alimentos que auxiliavam em funções específicas do corpo, além do valor nutritivo, devido à presença de alguma substância bioativa. A primeira definição de alimentos funcionais foi proposta pela *Foods for Specified Health Use* (FOSHU), como "alimentos que têm efeito específico na saúde devido à sua composição química", e esses alimentos que não devem expor quem os consome, ao risco higiênico ou de saúde (COSTA et al., 2013).

Segundo Akin e Ozcan (2017), alimentos funcionais são caracterizados como alimentos integrais, enriquecidos ou aprimorados que proporcionam benefícios para a saúde, além dos nutrientes essenciais. A descrição aceita para alimentos funcionais é de que são alimentos para os quais podem ser satisfatoriamente demonstrados que afetam beneficamente uma ou mais funções do organismo, além de garantirem efeitos nutricionais adequados, conduzindo a uma melhoria ao estado geral de saúde, bem-estar e redução do risco de doenças (EUROPEAN COMMISSION CONCERTED ACTION ON FUNCTIONAL FOOD E SCIENCE IN EUROPE, 1999).

Não há na legislação brasileira uma definição para alimento funcional, mas sim a alegação de propriedade funcional, alegação de propriedade de saúde e estabelece as diretrizes para sua utilização e as condições de registro para os alimentos com alegação de propriedade funcional e de saúde. Quando empregada a alegação de propriedade funcional, engloba-se o papel metabólico ou fisiológico que o composto tem no crescimento, desenvolvimento, manutenção e outras funções normais do organismo humano, enquanto a alegação de saúde compreende a existência da relação entre o alimento ou ingrediente com doença ou condição relacionada à saúde (BRASIL, 1999).

Produtos lácteos funcionais podem melhorar a saúde e o bem-estar quando consumidos nos níveis recomendados. Os leites fermentados são os produtos selecionados pelas indústrias

alimentícias como veículo de culturas probióticas, sendo considerados comercialmente os principais alimentos que contém estes compostos (SANCHEZ et al, 2009). Os produtos lácteos fermentados possuem componentes biologicamente ativos que há muito tempo são parte importante de uma dieta funcional (AKIN; OZCAN, 2017).

Estudos anteriores relataram que o consumo de leite fermentado oferece vários benefícios para a saúde do hospedeiro, como suas propriedades anti-hipertensivas, hipolipidêmicas antipatogênicas e anti-inflamatórias, que auxiliam os sistemas de defesa contra o estresse oxidativo e o tratamento de doenças cardíacas (RODRÍGUEZ-FIGUEROA et al., 2012; HAMED et al., 2018).

A fermentação é um método de conservação amplamente utilizado desde os primórdios da civilização. Historicamente era realizado pela ausência de métodos de refrigeração ou pasteurização e envolvia a coagulação do leite por micro-organismos autóctones, obtendo-se um produto final com características e propriedades físico-químicas diferentes da matéria-prima (FARIA et al., 2006). Atualmente, os processos de fermentação de alimentos envolvem a seleção criteriosa da matéria prima, ingredientes, aditivos e culturas específicas, e os processos de produção são rigorosamente controlados para a obtenção das características físico-químicas, microbiológicas e sensoriais desejadas no produto.

De acordo com o Regulamento Técnico de Identidade e Qualidade de Leites Fermentados, entende-se por Leites Fermentados os produtos adicionados ou não de outras substâncias alimentícias, obtidas por coagulação e diminuição do pH do leite, ou reconstituído, adicionado ou não de outros produtos lácteos, por fermentação lática mediante ação de cultivos de micro-organismos específicos, incluindo o iogurte, o leite fermentado, o leite acidófilo, kefir e coalhada. Os micro-organismos específicos devem ser viáveis, ativos e abundantes no produto final durante seu prazo de validade (BRASIL, 2007b).

Considera-se que alguns leites fermentados apresentam propriedades terapêuticas por serem elaborados com bactérias ácido-láticas (BAL) probióticas, que incluem *Lactobacillus* e *Bifidobacterium*, cuja origem geralmente é o trato gastrointestinal humano e que apresentam efeitos bioquímicos e biológicos nos nutrientes do leite e terapêuticos no consumidor (FARIA et al., 2006).

A possibilidade de veiculação de micro-organismos vivos em produtos lácteos fermentados, capazes de manter o equilíbrio entre bactérias patogênicas e não patogênicas fez com que as BAL fossem estudadas com relação aos seus efeitos probióticos (SAARELA et al., 2000). Além disso, diversos estudos têm explorado o desenvolvimento de novos produtos inovadores, com a adição de ingredientes funcionais, tais como prebióticos, ácido linoleico conjugado (CLA), peptídeos bioativos, vitaminas e minerais. Estes estudos visam atender a crescente demanda dos consumidores por alimentos saudáveis, saborosos e inovadores.

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CAPÍTULO II

Lactobacillus casei and *Lactobacillus fermentum* strains isolated from mozzarella cheese: probiotic potential, safety, acidifying kinetic parameters and viability under gastrointestinal tract conditions

Lactobacillus casei and *Lactobacillus fermentum* strains isolated from mozzarella cheese: probiotic potential, safety, acidifying kinetic parameters and viability under gastrointestinal tract conditions

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ABSTRACT

The objective of this study was to evaluate the probiotic properties of *Lactobacillus casei* and *Lactobacillus fermentum* strains, as well as to select novel and safe strains for future development of functional fermented products. The *in vitro* auto-aggregation, co-aggregation, hydrophobicity, β-galactosidase production, survival to gastrointestinal tract (GIT) and antibiotic susceptibility were evaluated. The selected strains were additionally tested by the presence of genes encoding adhesion, aggregation and colonization, virulence factors, antibiotic resistance and biogenic amines production, followed by the evaluation of acidifying kinetic parameters in milk, and survival of the strains under simulated GIT conditions during refrigerated storage of fermented milk. Most strains of both species showed high auto-aggregation; some strains showed co-aggregation ability with other lactic acid bacteria (LAB) and/or pathogens, and both species showed low hydrophobicity values. Seven *Lact. casei* and six *Lact. fermentum* strains produced β-galactosidase enzyme and ten strains survived well the simulation of the GIT stressful conditions evaluated *in vitro*. All strains were resistant to vancomycin and almost all the strains were resistant to kanamycin. *Lact. casei* SJRP38 and *Lact. fermentum* SJRP43 were distinguished among the other LAB strains by their higher probiotic potential. *Lact. fermentum* SJRP43 presented fewer genes related to virulence factors and antibiotic resistance, and needed more time to reach the maximum acidification rate (V_{max}). The other kinetic parameters were similar. Both strains survived well (> 8 $\log_{10}$ CFU/mL) to the GIT simulated conditions when incorporated in fermented milk. Therefore, these strains presented promising properties for further applications in fermented functional products.

Keywords: beneficial effects; intestinal microorganisms; lactic acid bacteria; probiotic microorganisms.

Introduction

Probiotic products are most commonly consumed worldwide in the form of yogurt or other fermented dairy products, although they are found and administrated in many different forms, including a wide variety of products (probiotic fermented milk, probiotic cheese, probiotic non-dairy fermented products, probiotic ice cream, supplements, and other products). Their claimed health effects reinforce the importance of commercial development of these products [1].

A large number of lactic acid bacteria (LAB) strains are well characterized and currently marketed as probiotics, and the best studied strains belong to the genera *Lactobacillus* and *Bifidobacterium*, especially *B. animalis* subsp. *lactis* and *Lact. acidophilus* strains [2]. According to the World Health Organization and Brazilian legislation, probiotics are 'live micro-organisms that, when administrated in adequate amounts, offer health benefits to the host' [3, 4], by improving the gut health and wellbeing in humans and animals.

In addition to improving gut health, probiotics may play a beneficial role in several medical conditions, including lactose intolerance, cancer, allergies, hepatic diseases, *Helicobacter pylori* infections, urinary tract infections and hyperlipidemia [5, 6]. Furthermore, other beneficial health effects have been attributed to probiotic fermented milk consumption, including reduction of total cholesterol, improvement of immune system by increasing the resistance to infection, and beneficial avoidance of oxidative stress during exhausting physical exercises [7, 8].

The human colonic microbiota is a complex ecosystem harboring several groups of bacteria, dominated by obligate anaerobes which promote normal intestinal functions, and offers to the host protection against infections. Nevertheless, a disturbance in the composition of this complex population of microorganisms can predispose towards gastrointestinal tract (GIT) disorders and intestinal dysfunctions.

Several aspects, including functional, technological and safety properties of the strains have to be taken into account when selecting probiotic cultures. Selection criteria of strains include lack of pathogenicity and antibiotic resistance, tolerance to GIT conditions, ability to adhere to the gastrointestinal mucosa and competitive exclusion of pathogenic organisms [9]. Although there are many commercial strains available in the market, it is desirable to characterize novel strains, which could present unique properties.

In the light of the importance of characterizing new probiotic strains, the aims of this study were to investigate the probiotic potential of LAB strains, and to select novel safety strains, for the future development of functional fermented products.

Materials and Methods

Lactic Acid Bacteria Strains

Nineteen mesophilic LAB strains from the cultures collection of the Laboratory of Milk and Dairy Products, Institute of Biosciences, Languages and Exact Sciences, São Paulo State University (IBILCE/UNESP, São José do Rio Preto, SP, Brazil) were used, as follows: *Lact. casei* (SJRP38, SJRP39, SJRP48, SJRP135, SJRP136, SJRP143, SJRP144, SJRP147, SJRP148, SJRP150, SJRP151 and SJRP166) and *Lact. fermentum* (SJRP32, SJRP40, SJRP41, SJRP43, SJRP46, SJRP60 and SJRP164), previously isolated from the processing of water-buffalo mozzarella cheese, identified by 16S rRNA gene sequencing, and characterized by their growth at various temperatures, pH and salt concentration, assimilation of citrate, and production of CO_2 from glucose [10].

The strains of *Lact. casei* and *Lact. fermentum* were cultured in Man-Rogosa-Sharpe - MRS broth (Difco, Becton Dickinson Co., Sparks, MD, USA), stored at -80 °C, in the presence of 20% sterile glycerol (v/v) as a cryoprotector. For revitalization, the isolated LAB strains were inoculated (2%, v/v) in 10 mL MRS broth and incubated aerobically for 24 h at 37 °C [11].

Susceptibility to Antibiotics

The LAB strains susceptibility to antibiotics was evaluated according to Charteris et al. [12] by the disc diffusion test. The antibiotics were chosen according to the list proposed by the European Food Safety Authority [13].

LAB cultures were activated in the MRS and incubated for 18 - 24 h to obtain 7 - 8 $\log_{10}$ CFU/mL; 10 µL were spread on the surface of the MRS medium (Difco), supplemented with 1% agar, to facilitate the diffusion of the evaluated substances. The antibiotic discs (Oxoid Ltd., Basingstoke, UK) were manually placed on the surface and plates were incubated for 24 h aerobically. The susceptibility to the antibiotics was expressed in millimeters of the

inhibition zones and the susceptibility of isolates was scored as resistant, moderately susceptible and susceptible, according to the cut-off values proposed by Charteris et al. [12]. The assay was repeated on three independent occasions and in triplicate each time (n = 9).

Auto-aggregation and Co-aggregation Determination

The auto-aggregation and co-aggregation tests were performed according to Todorov et al. [14]. The strains were activated in MRS broth for 24 h at 37 °C and harvested by centrifugation (7000 x *g* for 10 min at 20 °C), washed twice and diluted in sterile 0.85% saline solution. The cell suspension (1 mL) with optical density adjusted at 660 nm (OD_{660nm}) = 0.3 was transferred to a 2-mL sterile Eppendorf tube and the samples were incubated aerobically at 4 °C, 37 °C and 42 °C for 60 min. These temperatures were chosen to evaluate the behavior of the strains in the refrigerated storage temperature (4 °C), the body temperature (37 °C) and the incubation temperature of fermented milk (37 °C and 42 °C). The cell suspension was harvested by centrifugation (300 x *g* for 2 min at 20 °C) and the OD_{660nm} of the supernatant was determined. The auto-aggregation was determined using the following equation [14]: % auto-aggregation = $[(OD_0 - OD_{60})/OD_0] \times 100$, wherein OD_0 refers to initial OD and OD_{60} refers to the OD value determined after 60 min.

The experimental protocol for the study of co-aggregation was the same used for auto-aggregation. The LAB strains and the indicating microorganisms *Lact. helveticus* SJRP191 and *Ent. faecium* SJRP20 were cultivated in 10 mL MRS broth and the strains of *Listeria monocytogenes* ATCC 15313 and *Listeria innocua* ATCC 33090 in BHI at 30 °C. The cells were harvested by centrifugation (7000 x *g* for 10 min at 20 °C) after 24 h growth, washed twice in sterile saline solution 0.85% to obtain the OD_{660nm} = 0.3. The degree of co-aggregation was determined by absorbance readings of paired studied cultures and co-aggregation partners' suspensions (500 µL LAB cells in combination with 500 µL wof the indicating microorganism cells) in a sterile plastic cuvette. The cell suspension of each LAB strain and the co-aggregation partner were incubated aerobically for 60 min at 37 °C. Cells were harvested (300 x *g* for 2 min at 20 °C) and the OD_{660nm} of the supernatant was determined. The co-aggregation was determined using the following equation [14]: % co-aggregation = $[(OD_0 - OD_{60})/OD_0] \times 100$, wherein OD_0 refers to initial OD and OD_{60} refers to the OD value measured after 60 min. All tests were carried out in triplicate, in three different moments (n = 9).

Cell Surface Hydrophobicity

The ability of the LAB cell surface to adhere to hydrophobic compounds was evaluated according to the method reported by Todorov and Dicks [15]. The LAB strains were grown aerobically in MRS broth at 37 °C for 18 h. The cells were centrifuged (6700 x *g* for 6 min at 4 °C), washed twice in phosphate buffer 0.1 M, suspended in the same solution, and the OD_{580nm} was measured. Cell suspension (1.5 mL) was added to 1.5 mL *n*-hexadecane (Sigma Aldrich, St. Louis, MO, USA) and vortexed for 2 min. The aqueous and organic phases were allowed to separate at room temperature for 30 min. Following, an aliquot of 1 mL of the aqueous phase was removed to determine the OD_{580nm}. The percentage of hydrophobicity was determined by the equation: % hydrophobicity = $[(OD_0 - OD_{30})/OD_0] \times 100$, wherein: OD_0 refers to initial OD and OD_{30} refers to the OD value measured after 30 min. The tests were carried out in triplicate, in three different moments (n = 9).

Study of β-galactosidase Production

The β-galactosidase enzyme activity was determined using paper discs impregnated with *o*-nitrophenol-β-D-galactopyranose (ONPG Disks, Sigma Aldrich), according to the manufacturer´s instructions. The LAB strains were activated at 37 °C for 18 h, then, streaked in Petri dishes with MRS agar, and incubated aerobically at 30 °C for 48 h. In addition, one colony of each strain was added in tubes containing ONPG discs and 100 µL sterile 0.85% saline solution. The tubes were incubated at 37 °C, and observed at an interval of 1 h, for up to 6 h. The color transition to yellow by the release of the chromogenic compound *o*-nitrophenol indicates a positive result for the production of β-galactosidase [16]. The test was performed in three independent experiments in duplicate (n = 6).

Tolerance to Simulated GIT Conditions

The simulated GIT conditions - the gastric and enteric phases - were prepared according to Bautista-Gallego et al. [17], with slight modifications. Simulated gastric juice was prepared in a buffer solution with NaCl (2.05 g/L) (Synth, Diadema, SP, Brazil), KH_2PO_4 (0.6 g/L) (Synth, Diadema, SP, Brazil), $CaCl_2$ (0.11 g/L) (Dinâmica, Diadema, SP, Brazil) and KCl

(0.37 g/L) (Dinâmica, Diadema, SP, Brazil). The pH was adjusted to 2.0 with 1 M NaCl (Synth, Diadema, SP, Brazil). Simulated enteric juice was prepared using Na_2HPO_4 (Synth, Diadema, SP, Brazil) heptahydrated (50.81g/L) and NaCl (8.5 g/L). The pH was adjusted to 8.0 with anhydrous KH_2PO_4. The solutions were autoclaved at 121 °C for 15 min and stored until use.

The LAB strains were grown in MRS broth, harvested by centrifugation (10000 x *g* for 10 min at 4 °C) and pellets washed in phosphate-buffered saline (PBS) solution (0.1 M, pH 7.2). Then, the LAB cells were re-suspended in the synthetic gastric juice to obtain 8 - 9 $\log_{10}$ CFU/mL. Before use, pepsin from porcine gastric mucosa (P7000; 3 g/L, Sigma Aldrich) was added in this solution, and the cells were incubated at 37 °C for 120 min in a water bath. Harvested LAB cells from the gastric digestion step were serial diluted in PBS and the enumeration of LAB strains was performed by plating serial dilution on the surface of MRS agar (Difco), at times 0 (T_0), and 120 (T_{120}) min of analysis.

To simulate the small intestine conditions, simulated enteric juice was used. Before use, pancreatin from porcine pancreas (P3292; 1 g/L, Sigma Aldrich) and bile salts (70168; 3 g/L, Sigma Aldrich) were added. The harvested LAB cells from the gastric phase were washed in PBS solution, and re-suspended in the same volume of the simulated enteric juice. Then, the samples were incubated for 360 min at 37 °C in a water bath. The count was realized as previously described (T_{360}). All results were presented as $\log_{10}$ CFU/g. All solutions of enzymes were freshly prepared and filter-sterilized using a 0.22 μm membrane filter (Merck Milipore Ltd., Cork, Ireland) prior to each use.

Selection of LAB Strains for Additional Tests

The *Lact. casei* and *Lact. fermentum* strains which presented the highest probiotic potential in the preliminary tests, were submitted to additional tests to check their safety, adhesion properties and ability to ferment milk.

Presence of Genes Encoding Adhesion, Aggregation and Colonization, and Virulence Factors, Antibiotic Resistance and Biogenic Amines in *Lact. casei* and *Lact. fermentum* Strains

The selected strains of *Lact. casei* and *Lact. fermentum* were tested for the presence of genes encoding adhesion, aggregation and colonization [18, 19], virulence, antibiotic resistance and decarboxylase of amino acid genes [20 - 34]. DNA was extracted using the QIAgen DNeasy Blood & Tissue Kit (Qiagen, Hilden, NW, DE), followed by DNA concentration estimation using the NanoDrop2000 Spectrophotometer (Thermo Scientific, Waltham, MA, USA). PCRs were performed according to the references listed in Tables 1 and 2, and the amplified products were separated by electrophoresis in 0.8 to 2.0% (w/v) agarose gels in 0.5 x TAE buffer. The gels were stained in 0.5 TAE buffer containing 0.5 μg/mL ethidium bromide (Sigma Aldrich).

Behavior of *Lact. casei* and *Lact. fermentum* Strains in Milk

The strains of *Lact. casei* SJRP38 and *Lact. fermentum* SJRP43 were activated in MRS broth for 48 h at 37 °C and harvested by centrifugation (6000 x *g* for 6 min at 4 °C), washed twice and diluted in sterile 0.85% saline, and harvested by centrifugation under the same condition. Then, the LAB cells were suspended in 10 mL of sterile reconstituted skim milk powder (RSMP - Molico®, Nestlé, Araçatuba, SP, Brazil). The *Strep. thermophilus* TA040 (Chr. Hansen, Hørsholm, DK) culture (0.015 g) was suspended, as recommended by the manufacturer, in 50 mL sterile RSMP and activated at 42 °C for 30 min.

The skim milk powder (Nestlé) was reconstituted in water to achieve 12% total solids (w/v), added of 7% (w/v) sucrose, as the amount usually commercially applied, and heat treated at 90 °C for 10 min in a thermal processor (Thermomix, Cloyes-sur-le-Loir, France). The RSMP was dispensed into sterile 500 mL bottles into a laminar flow chamber and cooled to 42 °C in an ice-water bath. Then, 1% of the inoculated RSMP (8 $\log_{10}$ CFU/mL) were added according to the treatments, in two separate independent experiments: Lc, composed of *Lact. casei* SJRP38 in co-culture with the commercial strain of *Strep. thermophilus* TA040, and Lf, composed of *Lact. fermentum* SJRP43 in co-culture with the commercial strain of *Strep. thermophilus* TA040. After inoculation, the bottles were transferred to a water bath which was connected to a CINAC system (Cynetique d'acidificacion, Alliance Instruments, Frepillon,

FR), which allows continuous measurement and recording of pH values, as well as the evaluation of the kinetics of acidification throughout the run. The fermentations were performed at 42 °C to a pH of 4.6, then the fermented milk was cooled to 15 °C and the gels were broken using a perforated stainless-steel disc with up and down movements for approximately 1 min. The product was placed in sterile plastic cups and stored at 4 °C for 28 days for analysis. The maximum acidification rate (V_{max}) was calculated as the temporal variation of pH (dpH/dt) and expressed in 10^{-3} units of pH/min. During the fermentation process, the following kinetic parameters were evaluated: t_{max} - time in hours to reach V_{max}, pH $_{Vmax}$ - pH in V_{max}, t $_{pH5.5}$ - time in hours to reach pH 5.5, t $_{pH5.0}$ - time in hours to reach pH 5.0, and t $_{pH4.6}$ - time in hours to reach pH 4.6.

Survival of LAB Cultures in Fermented Milk under Simulated Gastrointestinal Conditions

The viability of *Lact. casei* SJRP38 and *Lact. fermentum* SJRP43 under simulated GIT conditions was evaluated on the day the fermented milk was produced (0) and on the 14th and 28th days of refrigerated storage, as previously described. The simulated gastric and enteric phases were prepared according to Bautista-Gallego et al. [17] with minor modifications.

Strep. thermophilus colonies were enumerated in M17 agar (Himedia, Mumbai, MH, IN), whereas those of *Lactobacillus* sp. were carried out in MRS agar (Acumedia, Lansing, MI, USA) with a bile solution added (0.15%) (Sigma Aldrich). Plates of *Strep. thermophilus* and *Lactobacillus* sp. were incubated under anaerobic conditions at 42 °C for 48 h. Viability was expressed as $\log_{10}$ CFU/mL fermented milk.

Statistical Analyses

Statistical analyses were performed using the software Statistica 7.0 (StatSoft, Inc., 2004, Tulsa, OK, USA). One-way ANOVA followed by Tukey's test was applied to detect significant differences ($P \leq 0.05$). The treatments were compared among the strains from the same species for auto-aggregation, co-aggregation capacity, cell surface hydrophobicity, and *in vitro* viability of *Lactobacillus* sp. under simulated GIT conditions. The treatments Lc and Lf were compared in terms of kinetic parameters of acidification. For the viability of LAB

strains in fermented milk under simulated GIT conditions during storage, the same species and treatment were compared during the assay; the samples of the same treatment under the same GIT phase conditions were compared during the storage time.

Results and Discussion

Susceptibility to Antibiotics

All LAB strains were susceptible to ampicillin (AMP), erythromycin (E), clindamycin (DA), tetracycline (TE) and chloramphenicol (C). However, all LAB strains were resistant to vancomycin (VA). The percentage of *Lact. casei* resistant to kanamycin (K) was considerable (85%), and only *Lact. casei* SJRP38 and SJRP136 showed moderate susceptibility (MS). In the case of *Lact. fermentum*, 29% of the strains were resistant to kanamycin and the others were moderately susceptible. Eighty percent of LAB strains were susceptible to streptomycin (S) (Table 3).

Although all the LAB strains presented resistance to vancomycin, this is not a concern because this resistance is intrinsic and codified by chromosomic genes. Therefore, it will not be transferable to pathogens. It occurs due to the presence of operons encoding enzymes: (i) synthesis of low affinity precursors in which the C-terminal D-Ala is transferred by a D-lactate (D-lac), or by a D-serine (D-Ser), thereby modifying the connection target of vancomycin, and (ii) elimination of high affinity precursors which are normally produced by the host, thereby removing the connection target of vancomycin [35].

The occurrence of antibiotic resistance by LAB strains is reported in the literature. All *Lact. casei* strains evaluated by Birri et al. [36] were resistant to vancomycin and susceptible to erythromycin, chloramphenicol and tetracycline. Jeronymo-Ceneviva et al. [37] analyzed LAB strains isolated from cheese and also obtained 100% of strains resistant to vancomycin. Nevertheless, all the *Lact. delbrueckii* subsp. *bulgaricus* strains were susceptible to vancomycin and gentamycin, whereas the other strains, including *Lact. rhamnosus* GG evaluated by Casarotti et al. [38] were resistant. Taking these reports into consideration, this resistance is acceptable.

Auto-aggregation and Co-aggregation Properties

Most strains of both species showed high auto-aggregation, ranging from 60.97 to 96.18%, and the best results were observed for *Lact. casei* SJRP166 and *Lact. fermentum* SJRP32 (Table 4). The aggregation was shown to be specific for each strain and may vary in the same taxonomic group [14]. The capacity of adhere to the intestinal mucous surface makes it a crucial feature in the selection of a probiotic culture. The intestine colonization by probiotic strains can generate beneficial biological responses: they can influence the immune system and increase the competition with pathogens in the intestine and prevent their elimination from the GIT by peristalsis [15].

As expected, the highest values were observed at 4 °C and 37 °C, since the LAB in this study are mesophilic, noting that the values were relatively lower at 42 °C (Table 4). Similar results were observed in a study evaluating the auto-aggregation of other LAB strains, such as *Lact. paracasei* ST284BZ (99%) and *Lact. pentosaceus* ST712BZ (67%) [15].

The probiotic LAB, after surviving the adverse conditions of GIT, should in general be able to adhere to intestinal epithelial cells, forming a defense against the colonization of pathogenic micro-organisms. The adhesion of pathogens to the mucosa is inhibited by an adsorption competition to the mucus or the epithelial cells; in some cases, it may be inhibited by the production of hydrogen peroxide or bacteriocins [39, 40].

Aggregation is an important feature for biofilm formation by probiotic bacteria, assisting them in adherence to intestinal mucosa of both humans and animals. Additionally, the co-aggregation between LAB and other bacteria, such as *L. monocytogenes*, is a positive feature, because it is one of the steps to eliminate pathogenic microorganisms from the GIT [15].

In this study, a high co-aggregation with indicating microorganisms was identified. The highest results obtained for co-aggregation were observed with both *Ent. faecium* SJRP20 and *Lact. helveticus* SJRP191, which can facilitate the presence of these strains in the human GIT. However, lower results of co-aggregation with *L. innocua* ATCC 33090 were identified, 64.71% and 75.85% on average for *Lact. casei* and *Lact. fermentum*, respectively (Table 4).

The co-aggregation of different LAB strains (*Lact. curvatus* ET30, *Lact. curvatus* ET31, *Lact. delbrueckii* ET32, and *Lact. fermentum* ET35, isolated from salmon) with *E. faecalis* ATCC 19443 ranged from 10 to 20%, showing that co-aggregation is strain-specific. Lower results were obtained by Todorov et al. [41]. In another study, *Ent. faecium* SJRP20 (30.32%), *Lact.*

delbrueckii subsp. *bulgaricus* SJRP49 (33.22%), *Ent. faecium* SJRP65 (39.65%) and *Ent. durans* SJRP68 (24.24%) presented low co-aggregation capacity with *L. monocytogenes; Enterococcus* sp. SJRP101 (25.21%) and *Ent. faecium* SJRP69 (34.06%) also presented low capacity to associate with *L. innocua* [42].

Cell Surface Hydrophobicity

In general, LAB strains presented low values of hydrophobicity; *Lact. casei* SJRP39, SJRP144 and SPRP147 and *Lact. fermentum* SJRP60 and SJRP164 showed the highest values of cell surface hydrophobicity (around 60%). The hydrophobicity of other *Lact. casei* strains ranged between 9.66 to 69.36 %, while the other *Lact. fermentum* strains varied from 0.30 to 68.81 % (Table 4).

Bacteria with high percentage of hydrophobicity typically have high capacity of adherence to the intestinal mucosa cells [14]. The cell surface hydrophobicity is one of the physicochemical characteristics which can facilitate the first contact between the microorganisms and the host's intestinal wall cells [43]. Furthermore, the hydrophobicity of the bacterial surface can affect the adhesion capacity and auto-aggregation of bacteria to different surfaces [44]. The hydrophobicity is related to the access of probiotic bacteria into the mucosa; however, it is not a prerequisite for a strong adhesion to cells [45].

Other authors evaluated LAB strains from various fermented products and found similar hydrophobicity results. However, the results were extremely variable (5-47%), and only three strains showed high hydrophobicity, varying from 38 to 66% [46]. Authors who studied the hydrophobic potential of LAB strains found high levels for *Lact. plantarum* ST16Pa (68.7%) and *Lact. fermentum* (78.9%). Other studied LAB strains showed smaller results of hydrophobicity, such as *Lact. delbrueckii* (43.7%) and *Ped. acidilactici* (51.3%) [47, 41]. Other strains isolated from the same origin (water-buffalo mozzarella cheese), presented high levels of hydrophobicity: 64.9% for *Lact. casei* SJRP35, 66.7% for *Leuc. citreum* SJRP44, 68.9% for *Lact. delbrueckii* subsp. *bulgaricus* SJRP57 and 72.2% for *Leuc. mesenteroides* subsp. *mesenteroides* SJRP58 [37].

β-galactosidase Enzyme Activity

Most *Lact. casei* strains (SJRP38, SJRP39, SJRP48, SJRP135, SJRP143 and SJRP144) are β-galactosidase positive, while all *Lact. fermentum* strains are β-galactosidase producers, except for the SJRP60 strain.

The ability of microorganisms to hydrolyze milk lactose into galactose and glucose by the β-galactosidase enzyme is an important feature from the technological standpoint and, at the same time, it can represent a beneficial property for the consumers' health. Hydrolysis of lactose confers taste, texture and nutritional value to milk and dairy products. β-galactosidase has been described in different organisms, such as bacteria, yeasts and molds. Besides their technological importance, the pure enzymes or the viable β-galactosidase producer-microorganisms have been used to alleviate lactose intolerance [48]. This intolerance is worldwide spread among adult populations and has been successfully treated by the incorporation of microorganisms, mainly lactobacilli or bifidobacteria in dairy products. They act as a source of β-galactosidase for the intraintestinal hydrolysis of lactose or the modulation of colonic microbiota. In this sense, many studies have focused on the metabolism of lactose by potentially probiotic microorganisms [49].

The lactose intolerant people, whose production of β-galactosidase in the gut is insufficient or absent, may benefit from the consumption of milk fermented by *Lact. casei* SJRP38, SJRP39, SJRP48, SJRP135, SJRP143, SJRP144 and *Lact. fermentum* SJRP32, SJRP40, SJRP43, SJRP46 and SJRP164, since these bacteria will degrade lactose, thus reducing or eliminating the symptoms of intolerance. Similar results are described in the literature for *Lact. casei, Lact. plantarum, Lact. brevis* and *Lact. parabuchneri* [50], as well as for *Leuc. mesenteroides* subsp. *mesenteroides* SJRP55 [51].

***In vitro* Tolerance to Simulated GIT Conditions**

All *Lact. fermentum* strains and six *Lact. casei* strains (SJRP38, SJRP135, SJRP144, SJRP148, SJRP150 and SJRP166) survived (population above 4 $\log_{10}$ CFU/mL) up to 360 min when exposed to the simulated GIT conditions analysis (Table 5), which is equivalent to the passage of bacteria through the gastrointestinal tract of humans. A significant reduction of at least 2 $\log_{10}$ CFU/mL at the end of the enteric phase for the *Lact. casei* SJRP38, SJRP135, SJRP144, SJRP148, SJRP150 and SJRP166 strains was observed, although the number of

viable bacteria remained high (Table 5). All *Lact. casei* strains showed population higher than 6 $\log_{10}$ CFU/mL for 120 min at pH 2.0. On the other hand, *Lact. fermentum* strains were less affected by the gastric phase and their population at the end of this phase was superior to 8 $\log_{10}$ CFU/mL.

Ent. faecium SJRP20 and *Ent. faecium* SJRP65 strains evaluated by Nascimento [42] showed similar results, with reduction of 2 $\log_{10}$ CFU/mL between the initial and enteric phases. Positive results are also described in the literature: *Lact. rhamnosus* GG was resistant to the GIT conditions [15] and *Lact. casei* and *Bifidobacterium infantis* showed 100% survival under GIT conditions when the strains were in the presence of milk protein [12].

According to Wu et al. [52], in response to acid stress in the passage through the GIT, LAB employ various mechanisms to overcome damage, including maintenance of intracellular pH and cell membrane functionality. In addition, in the human body, the probiotic bacteria must be able to withstand 0.3 to 0.4% bile during the entire GIT passage. This toxic component damages the bacterial cell membrane and modifies its permeability [53, 54].

The therapeutic effect of probiotic bacteria towards the host is related with their concentration in the intestine lumen, whose value must be, at least 7 $\log_{10}$ CFU/g of fecal content. Studies of the survival of probiotic bacteria are of great importance for the selection and development of new probiotic products and also to improve the understanding of a possible mechanism involving the beneficial effects of these microorganisms. Several tests have been carried out to increase the survival of LAB in the GIT environment, including the selection of strains adapted to stressful conditions, such as pH changes and various bile concentrations. Additionally, some food matrices, such as yogurt buffered with a mixture of milk proteins, administration of probiotic strains with milk or milk proteins and prebiotics, and probiotic protection as microencapsulation techniques have been used to increase the probiotic viability [55].

Selection of *Lact. casei* and *Lact. fermentum* and Additional Tests

For the selection of safe *Lact. casei* and *Lact. fermentum* strains with improved probiotic potential, all previous results were taken into account. *Lact. casei* SJRP38 was considered a strain with better features for future application in functional products because it showed resistance only to vancomycin, which is an intrinsic characteristic of *Lactobacillus* sp.; it

showed high auto-aggregation, especially at 37 °C (> 80%), good co-aggregation (> 60%) and hydrophobicity (close to 40%). It also produced β-galactosidase enzyme and survived well the stressful conditions of GIT, showing 7.30 $\log_{10}$ CFU/mL at the end of the simulation. *Lact. fermentum* SJRP43 also showed resistance only to vancomycin, high auto-aggregation (> 90%) at three different temperatures (4 °C, 37 °C and 42 °C), high co-aggregation (> 70%) and regular hydrophobicity. It also produced β-galactosidase enzyme and the population after the simulation of GIT was 6.55 $\log_{10}$ CFU/mL. These two strains were submitted to additional tests.

Presence of Genes Encoding Adhesion, Aggregation and Colonization, Virulence Factors, Antibiotic Resistance and Biogenic Amines

The presence of *EF 1249-fbp* gene, related to encoding fibrinogen binding protein (adhesion proteins), was detected in the genome of *Lact. fermentum* SJRP43 (Table 1). Such characteristic increases the chance for potential adhesion to intestinal mucosa. A very relevant characteristic of beneficial probiotic bacteria is to adhere to the intestinal mucosa, to modulate the immune system and to competitively exclude pathogens [56].

Both *Lact. casei* SJRP38 and *Lact. fermentum* SJRP43 strains presented low incidence of virulence factors. They were positive for the *efaA*, *brb(B)*, *erm(B)*, and *ant(4')-Ia* genes which are related to the endocarditis antigen, bacitracin, erythromycin and aminoglycoside resistance, respectively. The e*faA* gene was also detected in *Enterococci* and *Lactococci* strains [57]. The *erm(B)* gene, which encodes for erythromycin resistance was positive for genotypic characteristic of both strains; however, this gene is silent because it was not phenotypically expressed (Table 3). The presence of the *erm(B)* gene was also found by Guerrero-Ramos et al. [58].

Lact. casei SJRP38 was also positive for the *aac(6')-Ii, aph(2'')-Ic, aph(3')-III-a* genes, which encode for aminoglycoside resistance; the *erm(C)* gene, which encodes for erythromycin resistance, and the *cpd* gene, which is related to sex pheromones and chemotactic for human leukocytes, besides facilitating conjugation. The aminoglycoside resistance is due to the drug inactivation by cellular aminoglycoside-modifying enzymes [59]. In this study, although genes encoding aminoglycoside resistance were detected, both strains were considered fully or moderately susceptible to aminoglycosides; *Lact. casei* SJRP38 was

susceptible to streptomycin and moderately susceptible to gentamycin and kanamycin; and *Lact. fermentum* SJRP43 was susceptible to gentamycin and streptomycin, and moderately susceptible to kanamycin (Table 3).

Neither strains presented genes related to the production of biogenic amines (*hdc1*, *hdc2*, *tdc* or *odc*) (Table 2). This result is very relevant because biogenic amines are formed as the result of bacterial decarboxylation of amino acids and can be toxic to consumers [60]. Conversely, the presence of these genes has been reported for *Lactobacillus* sp. [37, 38] and *Enterococcus* sp. [57].

Acidifying Kinetic Parameters of Fermented Milk

The strains were evaluated for their ability to fermented milk production in co-culture with the commercial strain *Strep. thermophilus* TA040. The time to reach the maximum acidification rate (V_{max}) was higher when *Lact. fermentum* SJRP43 was used, and the other kinetic parameters, time to reach pH 5.0 and pH 4.6 (end of fermentation) were similar in the samples fermented by *Lact. casei* SJRP38 and *Lact. fermentum* SJRP43 (Table 6).

The acidifying kinetic parameters of different LAB strains have been reported in the literature: V_{max} ranged from 15.9 to 18.9 x 10^{-3} upH/min for milk fermented by *Lact. bulgaricus*, *Lact. acidophilus*, *Lact. rhamnosus* and *B. animalis* subsp. *lactis* BB-12 with co-cultures of *Strep. thermophilus*. When milk was supplemented with 4 g inulin/100 g, there was an increase in the time to reach V_{max}, which ranged from 17.0 to 19.0 x 10^{-3} upH/min [61]. The influence of different combinations of cultures, *Lact. acidophilus* La-5 and *B. animalis* subsp. *lactis* BB-12 as pure cultures or in co-culture with *Strep. thermophilus*, on the kinetic parameters of acidification of fermented milk was evaluated by Casarotti et al. [62]. *Lact. acidophilus* La-5 in pure culture showed the lowest V_{max} values (5.28 x 10^{-3} upH/min), whereas the pure *Strep. thermophilus* culture showed the highest V_{max} values (22.18 x 10^{-3} upH/min).

Many other factors may affect the acidifying kinetic parameters of fermented milk products, such as the LAB or probiotic strains used, the food matrix, and the milk supplementation. Different milk bases, supplemented with quinoa flour at three different concentrations (1 g, 2 g and 3 g/100 g) and quinoa-free milk as control were evaluated. The control treatment (milk without quinoa flour) had V_{max} significantly higher (23.40 x 10^{-3} upH/min) than in the milk

samples that received the flour in different proportions (17.27 to 20.08 x 10^{-3} upH/min), demonstrating the effect of food matrix on the kinetic parameters of acidification [63].

The viability and functional activity of probiotic strains during the products' shelf life were also affected by many conditions, including probiotic strains used, pH, the presence of hydrogen peroxide and dissolved oxygen, the concentration of metabolites such as lactic acid, acetic acid, storage medium, storage temperature and the nature of the added ingredients [64]. Additionally, there is evidence that food matrices play an important role in the beneficial effects of probiotics on the host's health [65]. Ranadheera et al. [66] reported that plain yogurts could maintain a higher level of *Lact. acidophillus* during shelf life than yogurts added of a fruit mixture. The formulation of the product can be manipulated to aid the efficacy of the probiotics growth and survival in food products, since these may be affected by fat content, protein concentration and types, sugars and pH.

LAB Viability in Fermented Milk under Simulated GIT Conditions During Storage

Both strains survived well (> 8 $\log_{10}$ CFU/mL) to the GIT simulated conditions when incorporated in fermented milk during all refrigerated storage time (Table 7). For the autochthonous *Lact. casei* SJRP38 and *Lact. fermentum* SJRP43, this result is very important because it shows that fermented milk protected the *Lactobacillus* spp. cells, when compared to the *Lactobacillus* spp. cells grown on MRS and evaluated under the GIT simulated conditions (Table 5), in which there was a decrease of at least 2 $\log_{10}$ cycles in the viability throughout the assay. Additionally, these data are much higher than those reported for other strains inoculated in milk.

Four LAB strains (*Leuconostoc citreum, Leuc. mesenteroides, Lact. delbrueckii* subsp. *bulgaricus* and *Lact. casei*) were used to ferment whole and skimmed milk. The viability of LAB strains in fermented milk varied from 3.29 to 4.18 $\log_{10}$ CFU/mL after the simulated GIT conditions [37]. The viability of LAB strains in other dairy matrices was also evaluated under the gastrointestinal stress. The viability of *Lact. rhamnosus* GG in synbiotic Amazonian palm berry (açai, *Euterpe oleracea* Mart.) ice cream was stable (around 9 $\log_{10}$ CFU/g) during up to 112 days of storage. The ice cream improved *Lact. rhamnosus* GG survival to simulated GIT, despite the reduction of at least 5 $\log_{10}$ cycles of the bacteria after the gastrointestinal simulation in all storage periods assessed [67]. These reports show that viability of LAB

strains under harsh conditions which simulate GIT stress depends on the food matrix and it is strain dependent.

Conclusion

Considering all the analyses, the *Lact. casei* SJRP38 and *Lact. fermentum* SJRP43 strains were considered safe and with high probiotic potential. The milk fermented with these autochthonous cultures in co-culture of *Strep. thermophillus* had good performance in the passage through the GIT during the 28-day storage period. Therefore, these strains presented promising properties for further applications in fermented functional products.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

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Lactobacillus casei and *Lactobacillus fermentum* strains isolated from mozzarella cheese: probiotic potential, safety, acidifying kinetic parameters and viability under gastrointestinal tract conditions

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Table 1: Presence of genes implicated in adhesion, aggregation and colonization in LAB strains.

Gene	Encoded factor	*Lact. casei* SJRP38	*Lact. fermentum* SJRP43	References
EF 1249-fbp	fibrinogen binding protein	-	+	Fortina et al. [18]
F 2380-maz	membrane-associated zinc metalloprotease	-	-	Fortina et al. [18]
EF 2662-cbp	choline binding protein	-	-	Fortina et al. [18]
Ef-Tu	elongation factor	-	-	Ramiah et al. [19]
*map*A	adhesion proteins	-	-	Ramiah et al. [19]
Mub	adhesion proteins	-	-	Ramiah et al. [19]
prgB	surface protein	-	-	Fortina et al. [18]

+ Means presence and – means absence of genes.

Table 2: Presence of genes implicated in virulence factors and antibiotic resistance in LAB strains.

Gene	Encoded factor	*Lact. casei* SJRP38	*Lact. fermentum* SJRP43	References
Antibiotic resistance				
aac(6')-Ii	aminoglycoside resistance	+	-	Costa et al. [23]
aac(6')-Ie-aph(2")-Ia	aminoglycoside resistance	-	-	Fortina et al. [18]
ant(4')-Ia	aminoglycoside resistance	+	+	Fortina et al. [18]
aph(2")-Ib	aminoglycoside resistance	-	-	Fortina et al. [18]
aph(2")-Ic	aminoglycoside resistance	+	-	Fortina et al. [18]
aph(2")-Id	aminoglycoside resistance	-	-	Fortina et al. [18]
aph(3')-III-a	aminoglycoside resistance	+	-	Fortina et al. [18]
bcr(B)	bacitracin resistance	+	+	Manson et al. [29]
bcr(D)	bacitracin resistance	-	-	Manson et al. [29]
bcr(R)	bacitracin resistance	-	-	Manson et al. [29]
cat A(pIP501)	chloramphenicol resistance	-	-	Aarestrup et al. [21]
erm(A)	erythromycin resistance	-	-	Sutcliffe et al. [33]
erm(B)	erythromycin resistance	+	+	Sutcliffe et al. [33]
erm(B)	erythromycin resistance	-	-	Gevers et al. [27]
erm(C)	erythromycin resistance	+	-	Sutcliffe et al. [33]
Int	transposon integrase gene	-	-	Gevers et al. [27]
int-Tn	tetracycline resistance	-	-	Fortina et al. [18]
tet(K)	tetracycline resistance	-	-	Aarestrup et al. [20]
tet(L)	tetracycline resistance	-	-	Aarestrup et al. [20]
tet(M)	tetracycline resistance	-	-	Aarestrup et al. [20]
tet(O)	tetracycline resistance	-	-	Aarestrup et al. [20]
tet(S)	tetracycline resistance	-	-	Aarestrup et al. [21]
vanA	vancomycin resistance	-	-	Martín-Platero et al. [30]
vanB	vancomycin resistance	-	-	Martín-Platero et al. [30]
vanC1	vancomycin resistance	-	-	Miele et al. [31]
vanC1	vancomycin resistance	-	-	Dutka-Malen et al. [25]
vanC2, vanC3	vancomycin resistance	-	-	Dutka-Malen et al. [25]
vatE	streptogramin resistance	-	-	Robredo et al. [32]

Table 2: Continuation

Gene	Encoded factor	*Lact. casei* SJRP38	*Lact. fermentum* SJRP43	References
Virulence				
ace	adhesion of collagen	-	-	Martín-Platero et al. [30]
*asa*1	aggregation substance	-	-	Vankerckhoven et al. [34]
ccf	sex pheromones, chemotactic for human leukocytes; facilitate conjugation	-	-	Eaton and Gasson [26]
cob	sex pheromones, chemotactic for human leukocytes; facilitate conjugation	-	-	Eaton and Gasson [26]
cpd	sex pheromones, chemotactic for human leukocytes; facilitate conjugation	+	-	Eaton and Gasson [26]
cylA	Cytolysin	-	-	Vankerckhoven et al. [34]
efaA	endocarditis antigen	+	+	Martín-Platero et al. [30]
esp	enterococcal surface protein	-	-	Vankerckhoven et al. [34]
*fsr*A	Gelatinase	-	-	Lopes et al. [28]
*fsr*B	Gelatinase	-	-	Lopes et al. [28]
*fsr*C	Gelatinase	-	-	Lopes et al. [28]
*gel*E	Gelatinase	-	-	Vankerckhoven et al. [34]
hyl	Hyaluronidase	-	-	Vankerckhoven et al. [34]
mur-2	virulance of *Enterococcus* sp.	-	-	Arias et al. [22]
*spr*E	serine protease	-	-	Lopes et al. [28]
Biogenic amines				
*hdc*1	histidine decarboxylase	-	-	de Las Rivas et al. [24]
*hdc*2	histidine decarboxylase	-	-	de Las Rivas et al. [24]
odc	ornithine decarboxylase	-	-	de Las Rivas et al. [24]
tdc	thyrosine decarboxylase	-	-	de Las Rivas et al. [24]

+ Means presence and – means absence of genes.

Table 3: Profile of antibiotics susceptibility[a] of LAB strains. The concentrations of antibiotics are expressed in µg per disc (µg/disc).

	Strains	AMP (10 µg/disc)	VA (30 µg/disc)	CN (10 µg/disc)	K (30 µg/disc)	S (300 µg/disc)	E (15 µg/disc)	DA (2 µg/disc)	TE (30 µg/disc)	C (30 µg/disc)
Lact. casei	SJRP38	S	R	MS	MS	S	S	S	S	S
	SJRP39	S	R	MS	R	S	S	S	S	S
	SJRP48	S	R	MS	R	S	S	S	S	S
	SJRP135	S	R	MS	R	S	S	S	S	S
	SJRP136	S	R	MS	MS	S	S	S	S	S
	SJRP143	S	R	MS	R	S	S	S	S	S
	SJRP144	S	R	MS	R	MS	S	S	S	S
	SJRP147	S	R	MS	R	S	S	S	S	S
	SJRP148	S	R	MS	R	MS	S	S	S	S
	SJRP150	S	R	MS	R	MS	S	S	S	S
	SJRP151	S	R	MS	R	S	S	S	S	S
	SJRP166	S	R	MS	R	S	S	S	S	S
Lact. fermentum	SJRP32	S	R	MS	MS	S	S	S	S	S
	SJRP40	S	R	MS	R	S	S	S	S	S
	SJRP41	S	R	MS	MS	S	S	S	S	S
	SJRP43	S	R	S	MS	S	S	S	S	S
	SJRP46	S	R	MS	R	S	S	S	S	S
	SJRP60	S	R	MS	MS	S	S	S	S	S
	SJRP164	S	R	S	MS	S	S	S	S	S
Susceptible strains		100%	0%	10%	0%	80%	100%	100%	100%	100%

AMP = Ampicillin; VA =Vancomycin; CN = Gentamycin; K = Kanamycin; S = Streptomycin; E = Erytromycin; DA = Clindamycin; TE =Tetracycline; C = Chloramphenicol.

[a] Inhibition zones were measured in millimeters, and the susceptibility of the strains was scored as S = Susceptible; MS = Moderately Susceptible; R = Resistant, according to the cut-off values proposed by Charteris et al. [11].

Table 4: Auto-aggregation (%), co-aggregation capacity (%) and cell surface hydrophobicity (%) of *Lact. casei* and *Lact. fermentum* strains.

Strains		Auto-aggregation (%)			Co-aggregation with (%)				Hydrophobicity (%)
		4 °C	37 °C	42 °C	*Lact. helveticus* SJRP191	*E. faecium* SJRP 20	*L. innocua* ATCC 33090	*L. monocytogenes* ATCC 15313	
Lact. Casei	SJRP38	$72.44^{a} \pm 0.23$	$87.21^{a} \pm 2.84$	$76.38^{a} \pm 0.77$	$66.97^{b} \pm 0.83$	$67.73^{b} \pm 4.93$	$72.71^{ab} \pm 0.45$	$63.52^{a} \pm 2.87$	$41.04^{abc} \pm 1.58$
	SJRP39	$85.19^{a} \pm 0.24$	$81.32^{a} \pm 0.95$	$77.62^{a} \pm 3.25$	$80.47^{a} \pm 0.78$	$86.92^{a} \pm 0.91$	$80.52^{a} \pm 0.45$	$81.07^{a} \pm 0.88$	$69.36^{a} \pm 0.04$
	SJRP48	$83.13^{a} \pm 5.49$	$87.27^{a} \pm 0.31$	$88.64^{a} \pm 5.39$	$82.86^{a} \pm 0.76$	$85.31^{a} \pm 0.34$	$76.99^{ab} \pm 0.33$	$60.84^{a} \pm 0.33$	$13.69^{bc} \pm 1.78$
	SJRP135	$82.32^{a} \pm 4.96$	$78.00^{a} \pm 0.47$	$79.60^{a} \pm 1.67$	$66.87^{b} \pm 0.87$	$84.37^{a} \pm 0.32$	$70.19^{ab} \pm 0.67$	$69.39^{a} \pm 0.87$	$39.50^{abc} \pm 2.53$
	SJRP136	$76.52^{a} \pm 2.07$	$78.57^{a} \pm 3.45$	$76.04^{a} \pm 1.35$	$69.04^{ab} \pm 0.84$	$75.40^{ab} \pm 0.43$	$63.61^{b} \pm 0.64$	$50.88^{b} \pm 0.45$	$50.48^{abc} \pm 4.14$
	SJRP143	$80.50^{a} \pm 0.51$	$78.82^{a} \pm 5.12$	$76.33^{a} \pm 0.84$	$61.25^{b} \pm 0.67$	$70.77^{ab} \pm 0.32$	$65.57^{b} \pm 0.32$	$55.88^{ab} \pm 0.67$	$9.66^{c} \pm 0.47$
	SJRP144	$91.29^{a} \pm 0.46$	$85.64^{a} \pm 1.60$	$83.43^{a} \pm 7.74$	$75.52^{ab} \pm 0.84$	$65.23^{a} \pm 0.24$	$52.24^{b} \pm 0.87$	$58.82^{ab} \pm 0.56$	$61.09^{abc} \pm 0.83$
	SJRP147	$81.70^{a} \pm 4.21$	$75.92^{a} \pm 3.85$	$82.84^{a} \pm 4.23$	$63.69^{b} \pm 0.66$	$65.16^{a} \pm 0.30$	$55.95^{b} \pm 0.88$	$59.08^{a} \pm 0.34$	$66.07^{ab} \pm 5.41$
	SJRP148	$79.63^{a} \pm 2.79$	$94.17^{a} \pm 5.15$	$74.93^{a} \pm 0.82$	$60.11^{b} \pm 0.72$	$70.11^{ab} \pm 0.64$	$65.64^{b} \pm 0.23$	$54.76^{ab} \pm 1.03$	$24.66^{abc} \pm 3.41$
	SJRP150	$93.14^{a} \pm 0.55$	$85.97^{a} \pm 0.12$	$86.01^{a} \pm 2.48$	$67.73^{b} \pm 0.44$	$63.24^{b} \pm 0.34$	$60.13^{b} \pm 1.02$	$60.71^{a} \pm 0.66$	$22.33^{abc} \pm 6.42$
	SJRP151	$83.22^{a} \pm 3.58$	$80.66^{a} \pm 2.09$	$60.97^{a} \pm 0.80$	$56.75^{b} \pm 0.32$	$65.08^{b} \pm 0.31$	$61.78^{b} \pm 0.91$	$61.68^{a} \pm 0.45$	$16.49^{abc} \pm 4.72$
	SJRP166	$87.15^{a} \pm 0.05$	$93.88^{a} \pm 0.60$	$96.66^{a} \pm 2.19$	$74.55^{ab} \pm 0.37$	$62.07^{b} \pm 0.65$	$51.16^{b} \pm 0.33$	$66.35^{a} \pm 0.49$	$54.85^{abc} \pm 2.90$
Lact. fermentum	SJRP32	$94.04^{a} \pm 2.45$	$96.18^{a} \pm 0.38$	$95.69^{a} \pm 1.26$	$80.53^{a} \pm 0.09$	$83.16^{a} \pm 2.18$	$79.71^{ab} \pm 0.81$	$75.63^{a} \pm 6.69$	$6.67^{b} \pm 0.20$
	SJRP40	$78.01^{a} \pm 1.86$	$79.69^{a} \pm 9.46$	$72.67^{a} \pm 0.32$	$79.40^{a} \pm 2.90$	$78.69^{a} \pm 1.72$	$77.55^{ab} \pm 0.90$	$74.00^{a} \pm 3.10$	$5.84^{b} \pm 1.16$
	SJRP41	$84.88^{a} \pm 2.49$	$90.06^{a} \pm 1.49$	$87.21^{a} \pm 1.06$	$81.40^{a} \pm 0.78$	$70.99^{a} \pm 1.21$	$79.50^{ab} \pm 0.21$	$67.56^{a} \pm 4.69$	$11.06^{b} \pm 2.31$
	SJRP43	$92.31^{a} \pm 1.98$	$91.99^{a} \pm 1.30$	$93.66^{a} \pm 0.93$	$77.00^{a} \pm 1.91$	$81.39^{a} \pm 1.07$	$73.12^{ab} \pm 5.53$	$73.59^{a} \pm 4.54$	$52.78^{a} \pm 0.36$
	SJRP46	$80.58^{a} \pm 1.78$	$80.57^{a} \pm 6.11$	$80.86^{a} \pm 6.59$	$73.53^{a} \pm 1.25$	$79.26^{a} \pm 1.02$	$82.40^{a} \pm 4.77$	$65.01^{a} \pm 2.85$	$0.30^{b} \pm 0.02$
	SJRP60	$90.73^{a} \pm 1.79$	$93.65^{a} \pm 1.57$	$91.54^{a} \pm 2.19$	$75.29^{a} \pm 5.08$	$76.06^{a} \pm 3.28$	$75.24^{ab} \pm 2.11$	$65.01^{a} \pm 1.04$	$61.92^{a} \pm 0.24$
	SJRP164	$92.60^{a} \pm 0.98$	$85.34^{a} \pm 4.44$	$89.69^{a} \pm 1.80$	$74.72^{a} \pm 1.24$	$72.62^{a} \pm 5.87$	$63.41^{b} \pm 0.85$	$62.99^{a} \pm 6.95$	$68.81^{a} \pm 1.82$

Different letters in the same column for the same species denote significant difference ($p < 0.05$). The results are expressed as mean ± standard deviation (SD) ($n = 3$).

Table 5: Viability (log_{10} CFU/mL) of *Lactobacillus sp.* after simulated gastrointestinal tract (GIT) conditions.

Strains		T_0	Gastric Phase T_{120}	Enteric Phase T_{360}
Lact. casei	SJRP38	$9.00^{Aabc} \pm 0.47$	$8.00^{ABa} \pm 0.26$	$7.30^{Ba} \pm 0.07$
	SJRP39	$8.17^{Aabcd} \pm 0.51$	$7.25^{Ba} \pm 0.05$	$<4.00^{Cd} \pm 0.00$
	SJRP48	$7.00^{Ad} \pm 1.00$	$6.17^{Aa} \pm 0.37$	$<4.00^{Bd} \pm 0.00$
	SJRP135	$7.69^{Abcd} \pm 0.07$	$6.30^{ABa} \pm 1.91$	$4.69^{Bc} \pm 0.61$
	SJRP136	$8.00^{Aabcd} \pm 0.97$	$7.47^{Aa} \pm 0.85$	$<4.00^{Bd} \pm 0.00$
	SJRP143	$7.36^{Acd} \pm 0.87$	$6.00^{Ba} \pm 0.20$	$<4.00^{Cd} \pm 0.00$
	SJRP144	$9.17^{Aab} \pm 0.06$	$7.30^{ABa} \pm 1.41$	$5.53^{Bbc} \pm 0.53$
	SJRP147	$9.69^{Aa} \pm 0.57$	$8.30^{Ba} \pm 0.28$	$<4.00^{Cd} \pm 0.00$
	SJRP148	$9.60^{Aa} \pm 0.07$	$7.77^{Ba} \pm 1.00$	$6.07^{Cb} \pm 0.26$
	SJRP150	$8.00^{Aabcd} \pm 0.54$	$7.39^{Aa} \pm 0.34$	$5.51^{Bbc} \pm 0.62$
	SJRP151	$9.30^{Aab} \pm 0.29$	$7.00^{Ba} \pm 0.44$	$<4.00^{Cd} \pm 0.00$
	SJRP166	$9.07^{Aabc} \pm 0.07$	$7.43^{Ba} \pm 0.38$	$6.11^{Cb} \pm 0.26$
Lact. fermentum	SJRP32	$8.67^{Aa} \pm 0.31$	$8.56^{Ba} \pm 0.22$	$4.00^{Cc} \pm 0.17$
	SJRP40	$8.65^{Aa} \pm 0.58$	$8.00^{Ab} \pm 0.35$	$3.80^{Bc} \pm 0.00$
	SJRP41	$8.64^{Aa} \pm 1.23$	$8.07^{Ab} \pm 0.17$	$5.55^{Bb} \pm 0.81$
	SJRP43	$8.47^{Aa} \pm 0.38$	$8.38^{Ab} \pm 0.26$	$6.55^{Bab} \pm 0.50$
	SJRP46	$8.77^{Aa} \pm 0.29$	$8.30^{ABb} \pm 0.24$	$7.47^{Ba} \pm 0.50$
	SJRP60	$9.30^{Aa} \pm 0.27$	$8.47^{Ab} \pm 0.26$	$7.47^{Ba} \pm 0.50$
	SJRP164	$9.47^{Aa} \pm 0.42$	$8.69^{Ab} \pm 0.25$	$6.30^{Bab} \pm 0.36$

T_0 = viability at the beginning of the assay, T_{120} = viability after simulation of gastric conditions (pH 2.0), T_{360} = viability after simulation of enteric conditions (pH 8.0).
Different lower-case letters in the same column denote significant difference ($p < 0.05$) among strains. Different capital letters on the same row denote significant difference ($p < 0.05$) during the assay. The results are expressed as mean ± SD (n = 3).

Table 6: Kinetic parameters of acidification of reconstituted skim milk powder (RSMP) with 7% sucrose.

Treatments	V_{max} (10^{-3} upH/min)	t_{max} (h)	pH_{Vmax}	$t_{pH\ 5,5}$ (h)	$t_{pH5,0}$ (h)	$t_{pH4,6}$ (h)
Lc	$23.39^{a} \pm 0.04$	$2.03^{a} \pm 0.11$	$5.70^{a} \pm 0.23$	$2.25^{a} \pm 0.09$	$3.06^{a} \pm 0.21$	$5.78^{a} \pm 0.04$
Lf	$25.53^{b} \pm 0.16$	$1.96^{a} \pm 0.09$	$5.86^{a} \pm 0.19$	$2.18^{a} \pm 0.12$	$3.25^{a} \pm 0.16$	$5.86^{a} \pm 0.11$

Lc (composed of *Lact. casei* SJRP38 in co-culture with the commercial *Strep. thermophilus* TA040 strain) and Lf (composed of *Lact. fermentum* SJRP43 in co-culture with the commercial *Strep. thermophilus* TA040 strain). V_{max} = maximum acidification rate, t_{max} = time in hours to reach V_{max}, pH_{Vmax} = pH in V_{max}, $t_{pH5.5}$ = time in hours to reach pH 5.5, $t_{pH5.0}$ = time in hours to reach pH 5.0, and $t_{pH4.6}$ = time in hours to reach pH 4.6. Different letters in the same column denote significant difference ($p < 0.05$). The results are expressed as mean ± SD (n = 3).

Table 7: Population (log_{10} CFU/mL) of *Lact. casei* SJRP38 and *Lact. fermentum* SJRP43 in fermented milk on the production day (0), 14th and 28th days of refrigerated storage, during assay simulating the gastrointestinal tract (GIT) conditions.

Treatments	Strains	GIT Phase	0	14	28
Lc	*Lact. casei*	T_0	$8.57^{Aa} \pm 0.31$	$8.92^{Aa} \pm 0.34$	$8.34^{Aa} \pm 0.15$
		T_{120}	$8.37^{Aa} \pm 0.34$	$8.84^{Aa} \pm 0.04$	$8.72^{Aa} \pm 0.39$
		T_{360}	$8.51^{Aa} \pm 0.04$	$9.55^{Aa} \pm 0.20$	$8.65^{Aa} \pm 0.30$
	St. thermophilus	T_0	$9.55^{Aa} \pm 0,20$	$9.49^{Aa} \pm 0.17$	$8.73^{Aa} \pm 0.06$
		T_{120}	$9.23^{Aa} \pm 0,32$	$9.07^{Aa} \pm 0.13$	$8.96^{Aa} \pm 0.28$
		T_{360}	$9.20^{Aa} \pm 0.28$	$9.14^{Aa} \pm 0.22$	$8.86^{Aa} \pm 0.27$
Lf	*Lact. fermentum*	T_0	$8.59^{Aa} \pm 0.26$	$9.30^{Aa} \pm 0.18$	$8.99^{Aa} \pm 0.26$
		T_{120}	$7.63^{Aa} \pm 0.54$	$8.26^{Aa} \pm 0.48$	$8.09^{Ba} \pm 0.30$
		T_{360}	$7.72^{Aa} \pm 0.75$	$8.55^{Aa} \pm 0.40$	$8.42^{ABa} \pm 0.34$
	St. thermophillus	T_0	$9.61^{Aa} \pm 0.11$	$9.39^{Aa} \pm 0.11$	$9.31^{Aa} \pm 0.22$
		T_{120}	$9.26^{Aa} \pm 0.12$	$8.95^{Ba} \pm 0.07$	$8.98^{Aa} \pm 0.19$
		T_{360}	$9.19^{Aa} \pm 0.16$	$9.07^{ABa} \pm 0.23$	$9.10^{Aa} \pm 0.42$

Lc (composed of *Lact. casei* SJRP38 in co-culture with the commercial *St. thermophilus* TA040 strain) and Lf (composed of *Lact. fermentum* SJRP43 in co-culture with the commercial *St. thermophilus* TA040 strain), T_0 = viability at the beginning of the assay, T_{120} = viability after simulation of gastric conditions (pH 2.0), T_{360} = viability after simulation of enteric conditions (pH 8.0). Different capital letters in the same column, for the same species and treatment, denote significant difference ($p < 0.05$) during the assay. Different lower-case letters on the same row denote significant difference ($p < 0.05$) during the storage. Results are expressed as mean ± SD (n = 3).

CAPÍTULO III

Lactobacillus casei SJRP38 and *Lactobacillus fermentum* SJRP43 increased the frequency of goblet cells and villus height in the duodenum in BALB/c mice

ABSTRACT

The objective of this study was to select an appropriate matrix to be fermented by *L. casei* SJRP38 and *L. fermentum* SJRP43 and to evaluate the effect of both strains on translocation, fecal analyses and intestinal morphology of healthy BALB/c mice. The matrix I was composed of 12% total solids reconstituted skim milk powder (RSMP) + 7% sucrose + 1% ready-mix vanilla flavor, and matrix II was composed of 12% total solids RSMP + 7% sucrose + 1% ready-mix vanilla flavor + 5% linseed. The matrices were fermented by both strains separately and resistance to the gastrointestinal tract and kinetic parameters of acidification throughout the run were evaluated. Comparing the two matrices, the addition of linseed in MII affected the fermentation process, and both strains took longer to reach pH 4.6. Additionally, the viability of LAB strains in MI fermented milk was higher under simulated TGI conditions during storage. Then, the matrix I was selected for the animal test. The animals were divided into four groups: water control (CW), milk control (CM), milk fermented by *L. casei* SJRP38 (FMLC) and fermented milk by *L. fermentum* SJRP43 (FMLF), in co-culture culture with *S. thermophilus* TA040. There was no statistical variation in the weight of BALB/c mice among the four experimental groups. In general, administration of fermented milk resulted in microbiological changes in fecal composition, with a decrease in *Clostridium* spp. and an increase in *Streptococcus* spp., *Lactobacillus* spp. and *Bifidobacterium* spp. The populations of *Lactobacillus* spp. and *Bifidobacterium* spp. in both small and large intestines were affected by the treatment group. The FMLF group had a lower population of *Lactobacillus* spp. in the small intestine and *Bifidobacterium* spp. in the large intestine. Regarding the translocation of intestinal bacteria, there was no bacteremia in the different experimental groups. Compared to the other groups, the CW group had a greater thickness of epithelium in the jejunum-ileum and colon portions; the depth of the crypts was greater in the duodenum region for the CM group and in the jejunum-ileum portion for the CW group. The colon showed no significant variation. The morphometry of the small intestine villi showed a larger size in the CM, FMLC and FMLF groups and no significant variation in the jejunum-ileum portion. The CM group presented larger sized intestinal microvilli than the other groups in the duodenum portion; in jejunum-ileum portion, only the FMLC and FMLF groups presented significant differences between them, whereas in the colon part there were no differences between the CW and FMLC groups and between the CM and FMLF groups. There was a statistically higher frequency of goblet cells in the duodenum for the CM, FMLC and FMLF groups; however, in the jejunum-ileum portion, the difference

was restricted to the CW and CM groups. In conclusion, the consumption of fermented milk by *L. casei* SJRP38 and *L. fermentum* SJRP43 strains brought about beneficial effects in the intestines of BALB/c mice.

Key-words: gastrointestinal tract; histology; intestinal morphometry; probiotics.

1. INTRODUCTION

The intestine is considered the largest organ of the human body, about seven to nine meters long, has a surface area of 30 m^2, that harbors up to 100 trillion micro-organisms, representing at least 1000 different species of known bacteria (LI; ZHOU, 2016), it is full of crypts and villi and has its own nervous system (enteric nervous system), which consists of approximately 100 million of neurons, capable of controlling the gastrointestinal tract (GIT), even if the connections with the central nervous system are interrupted (BRINGEL, 2017).

GIT is described as a "forgotten organ" even in the face of the accumulation of evidence revealing the importance of the intestinal microbiota in health promotion (AL ASMAKH; ZADJALI, 2015). For a long time, it was considered an organ of digestion, absorption and excretion, but in the face of so many discoveries, the intestine was classified as "second brain", a new organ within the human body (BRINGEL, 2017; SANCHEZ et al., 2017). The metabolic function also recognized as a function of GIT depends on the type of substrate available to be fermented by this microbiota (AGUIRRE et al., 2014).

The GIT of mammals hosts different microorganisms that co-exist and interact with each other and with the host and form the well-known intestinal microbiota, composed of a great diversity of microorganisms in different anatomical areas and contains four main bacterial phyla: *Proteobacteria*, *Bacteroidetes*, *Actinobacteria* and *Firmicutes* (XU et al., 2015; DOUZANDEH-MOBARREZ, KARIMINIK, 2017; SANCHEZ et al., 2017).

The intestinal microbiota is a key factor in maintaining homeostasis, with functions that affect virtually every organ of the body, such as regulation of bone mass, brain development and behavior, liver function and aspects of adipose tissues and the cardiovascular system (AL ASMAKH et al., 2012; SJÖGREN et al., 2012; KASAHARA et al., 2017; NOVINCE et al., 2017).

The intestinal microbiota is still essential for the metabolism of nutrients, defense of opportunistic pathogens, development of the immune system and regulation of intestinal barrier function. The specific balance of intestinal microbial diversity differs among individuals according to variations in sanitation, social behaviors, age, and genetics and can be disrupted by a number of factors, such as antimicrobial drugs, vaccination, and changes in diet (XU et al., 2015).

One strategy used by the host to maintain its homeostatic relationship with the microbiota is to minimize contact between microorganisms and the epithelial cell surface, limiting tissue inflammation and microbial translocation. GIT, which hosts the highest density of microorganisms, is secreted by the combined action of epithelial cells, mucus, immunoglobulin A (IgA), antimicrobial peptides and immune cells. The production of intestinal mucus provides a primary shield, limiting contact between microbiota and host tissue and avoiding microbial translocation (MCGUCKIN et al., 2011, BELKAID, HARRISON, 2017).

Bacterial translocation is a recommended indicator of toxicity as it is the first step in the pathogenesis process for many opportunistic tensions. The intestinal lumen is lined by epithelial cells responsible for the absorption of water and nutrients that during transplantation effectively prevent the translocation of intraluminal bacteria, but physiological and pathological stimuli may alter this permeability (WANG et al., 2015).

One strategy to maintain the GIT homeostasis and prevent pathogenic bacteria translocation is through the probiotic supplementation. Probiotics work by altering the composition or metabolic activity of the intestinal microbiota and are known to play crucial roles in maintaining the GIT microbial ecosystem (OHLSSON et al., 2014; PENG et al., 2015). Currently scientific evidence highlights the important role that probiotics can play in the digestive system by promoting the relief of clinical signs of diseases such as: infectious diarrhea, antibiotic-associated diarrhea, inflammatory bowel disease, irritable bowel syndrome, *Helicobacter pylori* infection and lactose intolerance (RITCHIE, ROMANUK, 2012; GARCÍA et al., 2017; SÁNCHEZ et al., 2017).

Since 2002, probiotics have been defined as "living microorganisms that, when administered in adequate amounts, confer benefits to host health" (FAO/OMS, 2002), but in 2014 by a new consensus panel the original definition of FAO/WHO has undergone a grammatical modification, defining probiotics as "living microorganisms that, when given in adequate quantities, confer a benefit to host health" (HILL et al. 2014).

The survival of probiotic bacteria in fermented foods is influenced by the physical characteristics of the food matrix that affect bacterial metabolism (FLORENCE et al., 2016). Probiotics are found and administered in different ways, including a wide variety of supplements and functional foods, but are commonly consumed worldwide in the form of yogurt or other fermented dairy products such as fermented milks (PARKER et al., 2018).

Bacterial interactions with milk components play a key role in the protective effect, which assists their survival during exposure under adverse conditions of GIT (BURGAIN et al., 2014). In addition, Aguirre et al. (2016) reported that variable nutrient loads and dietary nutritional ingredients influence the composition and production of metabolites by the intestinal microbiota, confirming the claim that diet is an important factor governing the structure and formation of the intestinal microbiota (MUEGGE et al., 2011; VILLAMIL et al.,

2017). According to Hardy et al. (2013), regular ingestion of probiotic microorganisms is a widely studied approach to harnessing the health benefits conferred by microorganisms that colonize the GIT of the healthy human host.

Historically strains of probiotic bacteria for use in humans were selected mainly among species of the genus *Lactobacillus* and *Bifidobacterium*, commonly present in the GIT and mucosal surfaces of healthy humans or in spontaneously fermented foods (VINDEROLA et al., 2017). The new European Union food law (EU 2015/2283) currently imposes specific requirements for new probiotics in order to ensure a high level of consumer health protection (EFSA, 2016).

New probiotics should clearly demonstrate safety and health benefits (KUMAR et al., 2015). Previous studies such as resistance to antibiotics, resistance genes, aggregation, co-aggregation, hydrophobicity, β-galactosidase enzyme production and resistance to GIT were carried out in the strains of *Lactobacillus casei* SJRP38 and *Lactobacillus fermentum* SJRP43, showing promising results (SALOTTI-SOUZA, 2016). According to Vinderola et al. (2017), there is a need to conduct more robust selection of probiotic potentials and focus on well-defined *in vitro* and *in vivo* studies to document human health benefits.

In this scenario, this study aimed to select an appropriate matrix to be fermented by *L. casei* SJRP38 and *L. fermentum* SJRP43 and evaluate the effect of *L. casei* SJRP38 and *L. fermentum* SJRP43 fermented milk on translocation, fecal analyzes and intestinal morphology of healthy BALB/c mice.

2. MATERIALS AND METHODS

2.1 Lactic acid bacteria (LAB) cultures

The strains of *L. casei* SJRP38 and *L. fermentum* SJRP43 were previously isolated from Mozzarella cheese, identified by 16S rRNA gene sequencing (SILVA, 2015), and

characterized by their safety, technological and probiotic potencial (SALOTTI-SOUZA, 2016). The strains were activated in MRS broth for 48 h at 37 °C and harvested by centrifugation (6000 x *g* for 6 min at 4 °C), washed twice and diluted in sterile 0.85% saline, and harvested by centrifugation in the same condition. Then, the LAB cells were suspended in 10 mL of sterile reconstituted skimmed milk powder (RSMP, Molico®, Nestlé, Araçatuba, SP, Brazil). The *Streptococcus thermophilus* TA040 (Chr. Hansen, Horsholm, DK) culture (0.015 g) was suspended, as recommended by the manufacturer, in 50 mL of sterile RSMP and activated at 42 °C for 30 min.

2.2 Food matrix test and fermented milk production

Two different matrices of fermented milk were previously tested for the *in vivo* assay. The matrix I was composed of 12 % TS RSMP (Molico®, Nestlé), added of 7% sugar (Guarani®, Olímpia, SP, Brazil) and 1% ready-mix vanilla flavor (DOREMUS®, Guarulhos, SP, Brazil), which contains preservative, thickener, and flavorings; the matrix II was composed of 12 % TS RSMP (Molico®, Nestlé), added with 7% sugar (Guarani®, Olímpia, SP, Brazil) and 5% linseed (*Linum usitatissimum L.*).

The mixtures were heat-treated at 90 °C for 10 min on a thermal processor (Thermomix, Cloyes-sur-le-Loir, France), dispensed into 500 mL sterile flasks in a laminar flow chamber and cooled to 42 °C in an ice-water bath. Then, 1% of the inoculated RSMP (8 $\log_{10}$ CFU/mL) were added according to the treatments, in two separate independent experiments: FMLC, composed of *L. casei* SJRP38 in co-culture with the commercial strain of *S. thermophilus* TA040, and FMLF, composed of *L. fermentum* SJRP43 in co-culture with the commercial strain of *Strep. thermophilus* TA040. After inoculation, the flasks were transferred to a water bath (42 °C) and connected to a CINAC system (Cynetique d'acidificacion, Alliance Instruments, Frepillon, FR). This equipment allows continuous

measurement and recording of pH values, as well as the evaluation of the kinetic parameters of acidification throughout the run. The maximum acidification rate (V_{max}) was calculated as the temporal variation of pH (dpH/dt) and expressed in 10^{-3} units of pH/min. During the fermentation process, the following kinetic parameters were evaluated: t_{max} - time in hours to reach V_{max}, pH $_{Vmax}$ - pH in V_{max}, t $_{pH5.5}$ - time in hours to reach pH 5.5, t $_{pH5.0}$ - time in hours to reach pH 5.0, and t $_{pH4.6}$ - time in hours to reach pH 4.6. The fermentations were performed until the 4.6 pH value was reached, then the fermented milk was cooled to 15 °C in an ice-water bath and the gels were broken using a perforated stainless-steel disc with up and down movements for approximately 1 min. Considering the kinetic parameters of the different matrices of fermented milk, one matrix was selected and produced the day before the animal tests, placed in sterile glass bottles and stored at 4 °C for 14 days to feed the animals.

2.3. Survival of LAB cultures in fermented milk under simulated gastrointestinal conditions

The viability of *Lb. casei* SJRP38 and *Lb. fermentum* SJRP43 under simulated GIT conditions was evaluated on the day of fermented milk production (0) and on the 14th and 28th day of refrigerated storage, as described previously. The simulated gastric and enteric phases were prepared according to Bautista-Gallego et al. [16] with minor modifications.

S. thermophilus colonies were enumerated in M17 agar (Himedia, Mumbai, MH, IN), whereas those of *Lactobacillus* spp. were carried out in MRS agar (Acumedia, Lansing, MI, USA) with a bile solution added (0.15%) (Sigma-Aldrich). Plates of *Strep. thermophilus* and *Lactobacillus* spp. were incubated under anaerobic conditions at 42 °C for 48 h. Viability was expressed as $\log_{10}$ CFU/mL of fermented milk.

2.4 Animals and protocol design

The BALB/c mice (*Mus musculus*) used in this experiment came from the Center for Bioterio and Animal Experimentation (CBEA-UFU, Uberlândia, MG, Brazil) and kept in the Noth Paulista University Center Bioterio (UNORP, São José do Rio Preto, SP, Brazil) in polyethylene boxes, with substrate of shavings, under controlled conditions of average temperature of 25 ºC. The animal protocols were pre-approved by the Ethics Committee for the Use of Animals of UNESP (Protocol number 170/2017 - CEUA), and all experiments comply with the current laws of Brazil.

A total of 30 male mice, six to eight weeks old, male and 20 to 25 g in weight were used. The treatments started after 7 days of acclimatization. All the animals were fed *ad libitum* with conventional balanced rodent diet (23% proteins, 13% vitamins mix, 10% total minerals, 5% raw fiber, 4% ethereal extract, 1.3% Ca, 0.8% P) (Presence®, Paulínia, SP, Brazil), water, milk or fermented milk, according to the group for 14 days, and maintained in a room with 12 light/dark cycle. The animals' weight was measured before the feeding and weekly.

The experimental protocol contained four experimental groups: (i) Water Control (CW, n = 7): the animals received animal feed and filtered water throughout the treatment; (ii) Milk Control (CM, n = 7): the animals received animal feed and milk throughout treatment; (iii) Fermented Milk (FMLC, n = 8): the animals received animal feed and fermented milk by *L. casei* SJRP38 in co-culture with the commercial strain of *S. thermophilus* TA040 throughout treatment; (iv) Fermented Milk (FMLF, n = 8): the animals received animal feed and fermented milk by *L. fermentum* SJRP43 in co-culture with the commercial strain of *S. thermophilus* TA040 throughout treatment. Throughout the experiment, the activity, behavior, and general health of the animals were observed daily.

After 14 days of feeding, BALB/c mice were contained manually and individually, and euthanasia was performed with the combination of xylazine 0.25 mL/animal (Ceva®, São Paulo, Brazil) and ketamine 0.25 mL/animal (Ceva®, São Paulo, Brazil) intraperitoneal administration, using sterile insulin syringe and 13 x 0.45 mm needle. The administration performed in the right lower abdominal quadrant of the animal and in dorsal decubitus positioning. After confirmation of death, blood, liver, spleen, small intestine, large intestine and intestinal contents were removed for translocation studies, intestinal morphology and intestinal microbiology.

2.5 Fecal Microbiology

Two grams of stool sample of each mice group were collected two days before the euthanasia. The collection was done using anatomical sterilized tweezers and glass bottle after cleaning the boxes that were already without the animals. The number of colony forming units log CFU/mL of *Clostridium* spp., *Streptococcus* spp., *Lactobacillus* spp and *Bifidobacterium* spp. was determined using pour plate method and serial dilutions of samples (2 g) in sterile 0.85% saline solution on selective culture media, according to described by Bianchi et al. (2014).

Numbers of total aerobic counts was determined by plating on Standard Methods agar (Acumedia, Indaiatuba, SP, Brazil) and incubation at 37 °C/48 h. MRS agar (Difco, Becton Dickinson Co., Sparks, MD, USA) and incubation at 37 °C/48 h, anaerobically, was used to determine the number of lactobacilli. *Streptococcus* spp. was enumerated aerobically using M17 agar (Difco) and incubation at 37 °C/48 h. *Bifidobacterium* spp. was enumerated anaerobically using formulated BIM-25 medium (RCA agar, nalidixic acid, polymyxin B sulfate, kanamycin sulfate, iodoacetic acid and 2,3,5 – triphenyltetrazolium chloride) and incubation at 37 °C/72 h. RCA and incubation at 37 °C/48 h, anaerobically, was used to

determine the population of *Clostridium* spp. Anaerobic incubation of plates was performed in jars with an atmosphere adjusted by Anaerobac (Probac, São Paulo, Brazil).

2.6 Microbiology of the small intestine and large intestine

Sections of small and large intestine were obtained, and both were washed separately with 3 mL of 0.85% saline solution. The enumeration of *Lactobacillus* spp. and *Bifidobacterium* spp. was performed as mentioned previously.

2.7 Bacterial Translocation

The bacterial translocation was analyzed according to Lara-Villoslada et al. (2007), in the blood, liver and spleen. Blood was collected by cardiac puncture EDTA-containing micro tubes (Vacuplast®, Cotia, SP, Brasil) in sterile conditions. Fifty μL of blood were cultured on MRS (Difco) and BHI agar (Difco) and incubated at 37 °C/48 h in anaerobic or aerobic conditions, respectively. The tissue samples were homogenized in buffered peptone water (1 g/mL) and 100 μL of the resulting homogenates were grown in MRS and BHI agar, as mentioned previously. After 48 h of incubation, the colony-forming units (CFU) of each organ were counted, and the results were expressed as the incidence of translocation (number of animals that detected bacterial development/total number of animals, in percentage). Positive growth in agar was defined by the presence of even a single colony of any microorganisms.

2.8 Histological Sections

After the euthanasia procedure, a portion of approximately 1 cm of the duodenum, jejunum-ileum and ascending colon were collected, fixed in 4% paraformaldehyde (in Sörensen phosphate buffer pH 7.2, 0.1M), washed in water, dehydrated in ethanol, clarified in

xylol and then included in Paraplast (Histosec, Merk, Darmstadt, Germany). The fragments were sectioned at 3-5 μm and stained by Hematoxylin-Eosin (HE) (Merk) and Periodic Acid Schiff's (PAS) (Merk). The histological sections were analyzed in a photomicroscope Olympus BX60 l (Olympus, Tokyo, Japan), and the images were digitized in the software DP-BSW v3.1 (Olympus) and in the virtual blade system BX 61VS (Olympus).

Morphometric analysis of villus height and microvilli, intestinal crypts depth and thickness of the epithelium were performed to evaluate the possible morphological changes resulting from the treatments of the different groups. Two hundred measurements per group in randomized fields were realized; in HE stained sections the 100x magnification for villi and crypt measurements and 1000x magnification for epithelium and microvilli measurements were used.

The frequency of goblet cells was performed through the stereological analysis, based on the M130 test proposed by Weibel (1963). A hundred and thirty points in random fields were collected, using 200x magnification of PAS stained sample, considering only the points belonging to the tissue, so that the frequency of PAS positive cells was relative to the points belonging to the tissue of each field.

2.9 Statistical Analysis

Differences between proportions (bacterial translocation incidence) were analyzed with the chi-squared test with statistical significance set at $p \leq 0.05$. The univariate ANOVA followed by the Tukey test was applied to detect significant differences ($p \leq 0.05$) among the kinetic parameters, weights of the mice in the different groups and different feeding days, fecal microbiology, and microbiology of the small intestine and large intestine. All data obtained in the histology were submitted to statistical analysis, performed in the software Graphpad Prism 5 (Graphpad Software Inc. 1992-2007), using non-parametric test of

Kruskal-Wallis followed by the Dunn test. The results were presented in terms of the mean and standard deviation and the values of $p \leq 0.05$ were considered statistically significant.

3. RESULTS AND DISCUSSION

3.1 Kinetic Parameters of the Food Matrix

The strains were evaluated for their ability to fermented milk production in co-culture with the commercial strain *S. thermophilus* TA040. Using the matrix I (RSMP + 7% sucrose + 1% ready-mix vanilla flavor), the time to reach the maximum acidification rate (V_{max}) was higher when *L. fermentum* SJRP43 (21.52×10^{-3} upH/min) was used, and the other kinetic parameters, time to reach pH 5.0 and time to reach pH 4.6 (end of fermentation) were similar in the fermented milk by *L. casei* SJRP38 and *L. fermentum* SJRP43 (Table 1). Using the matrix II (RSMP + 7% sucrose + 1% ready-mix vanilla flavor + 5% linseed), the time to reach V_{max} was higher when *L. casei* SJRP38 (14.26×10^{-3} upH/min) was used, however, the time to reach pH 5.5, pH 5.0 and pH 4.6 (end of fermentation) was longer when *L. fermentum* SJRP43 was used. Comparing the two matrices, the addition of linseed in MII affected the fermentation process, and both strains took longer to reach pH 4.6.

These differences in kinetic parameters should be related to the metabolim of each *Lactobacillus* sp., such as tolerance to different pH, temperature, sugar concentration, production of enzymes. In previous study (SALOTTI-SOUZA, 2016), milk (without sugar or additives) fermented by *L. fermentum* SJRP43 and *L. casei* SJRP38 presented higher V_{max} (25.53×10^{-3} upH/min and 23.29, respectively) and both strains were β-galactosidase positive.

V_{max} tends to decrease with increasing total solids content because milk with lower total solids has less storage capacity, which can be understood as a decrease in pH higher for the same amount of acid produced (VARGHESE, MISHRA, 2008; BEZERRA et al., 2012).

Casarotti et al. (2014), investigated the effect of supplementing fermented milk with *Streptococcus thermophilus, Lactobacillus acidophilus* La-5 and *Bifidobacterium animalis* spp. *lactis* BB-12 and quinoa flour in increasing concentrations (0, 1 and 3.0 g/100 g) and obtained a higher V_{max} in the control group without addition of quinoa flour and the higher the flour concentration the lower the values of V_{max}. Nevertheless, the addition of quinoa may help probiotics to survive GIT passage, increasing their resistance to gastric and enteric juices. In the present work the results were similar, since the addition of linseed also decreased the ability of the microorganism to acidify milk.

Table 1: Kinetic parameters of acidification of the reconstituted skim milk powder (RSMP) in different matrices, fermented by *Lactobacillus* spp. strains.

Matrix and Treatments	V_{max} (10^{-3} upH/min)	t_{max} (h)	pH_{Vmax}	$t_{pH\ 5,5}$ (h)	$t_{pH5,0}$ (h)	$t_{pH4,6}$ (h)
M I						
Lc	$20.22^{a} \pm 0.22$	$2.43^{a} \pm 0.01$	$5.69^{a} \pm 0.02$	$2.60^{a} \pm 0.14$	$3.56^{a} \pm 0.15$	$6.75^{a} \pm 0.10$
Lf	$21.52^{b} \pm 0.33$	$2.30^{a} \pm 0.05$	$5.74^{a} \pm 0.01$	$2.50^{a} \pm 0.07$	$3.45^{a} \pm 0.08$	$5.86^{b} \pm 0.08$
M II						
Lc	$14.26^{a} \pm 0.12$	$2.43^{a} \pm 0.06$	$5.75^{a} \pm 0.03$	$2.76^{a} \pm 0.15$	$4.18^{a} \pm 0.16$	$8.83^{a} \pm 0.06$
Lf	$11.37^{b} \pm 0.09$	$2.06^{a} \pm 0.11$	$5.51^{a} \pm 0.02$	$2.08^{a} \pm 0.12$	$3.18^{a} \pm 0.08$	$6.23^{b} \pm 0.07$

MI (RSMP + 7% sucrose + 1% ready-mix vanilla flavor); MII (RSMP + 7% sucrose + 1% ready-mix vanilla flavor + 5% linseed); Lc (composed of *L. casei* SJRP38 in co-culture with the commercial strain of *St. thermophilus* TA040) and Lf (compound of *L. fermentum* SJRP43 in co-culture with the commercial strain of *St. thermophilus* TA040). V_{max} = maximum acidification rate, t_{max} = time in hours to reach V_{max}, pH_{Vmax} = pH in V_{max}, $t_{pH5.5}$ = time in hours to reach pH 5.5, $t_{pH5.0}$ = time in hours to reach pH 5.0, and $t_{pH4.6}$ = time in hours to reach pH 4.6. Different letters in the same column denote significant difference ($p < 0.05$). The results are expressed as mean ± SD (n = 3).

3.2 Viability the LAB in fermented milk under simulated TGI conditions during storage

Both strains survived well (> 8 $\log_{10}$ CFU/mL) the simulated GIT conditions when incorporated in the MI fermented milk during all refrigerated storage time (Table 2); however, in the MII fermented milk, both strains decreased up to 2 $\log_{10}$ CFU/mL throughout the storage.

Table 2: Population (log_{10} CFU/mL) of *Lb. casei* SJRP38 and *Lb. fermentum* SJRP43 in fermented milk in matrix I and matrix II on the production day (0) and on the 14th and 28th day of refrigerated storage, during assay simulating the gastrointestinal tract (GIT) conditions.

Treatment	Strains	GIT Phase	0		14		28	
			Matrix I	Matriz II	Matrix I	Matriz II	Matrix I	Matriz II
Lc	*Lact. casei*	T_0	$8.57^{Aa} \pm 0.31$	$7.67^{ABa} \pm 0.44$	$8.92^{Aa} \pm 0.34$	$8.32^{Aa} \pm 0.50$	$8.34^{Aa} \pm 0.15$	$8.59^{Aa} \pm 0.65$
		T_{120}	$8.37^{Aa} \pm 0.34$	$9.14^{Aa} \pm 0.17$	$8.84^{Aa} \pm 0.04$	$8.46^{Aa} \pm 0.41$	$8.72^{Aa} \pm 0.39$	$8.21^{Aa} \pm 0.15$
		T_{360}	$8.51^{Aa} \pm 0.04$	$6.68^{ABb} \pm 0.24$	$9.55^{Aa} \pm 0.20$	$7.31^{Aa} \pm 0.33$	$8.65^{Aa} \pm 0.30$	$5.47^{Bb} \pm 0.29$
	St. thermophilus	T_0	$9.55^{Aa} \pm 0,20$	$8.77^{Aa} \pm 0.47$	$9.49^{Aa} \pm 0.17$	$8.37^{Aa} \pm 0.29$	$8.73^{Aa} \pm 0.06$	$9.34^{Aa} \pm 0.22$
		T_{120}	$9.23^{Aa} \pm 0,32$	$9.30^{Aa} \pm 0.09$	$9.07^{Aa} \pm 0.13$	$8.79^{Aa} \pm 0.31$	$8.96^{Aa} \pm 0.28$	$8.76^{Aa} \pm 0.19$
		T_{360}	$9.20^{Aa} \pm 0.28$	$7.12^{Ba} \pm 0.24$	$9.14^{Aa} \pm 0.22$	$8.04^{Aa} \pm 0.74$	$8.86^{Aa} \pm 0.27$	$7.01^{Aa} \pm 0.22$
Lf	*Lact. fermentum*	T_0	$8.59^{Aa} \pm 0.26$	$8.62^{Aa} \pm 0.35$	$9.30^{Aa} \pm 0.18$	$8.83^{Aa} \pm 0.36$	$8.99^{Aa} \pm 0.26$	$7.55^{Aa} \pm 0.08$
		T_{120}	$7.63^{Aa} \pm 0.54$	$8.40^{Aa} \pm 0.27$	$8.26^{Aa} \pm 0.48$	$8.00^{Aa} \pm 0.24$	$8.09^{Ba} \pm 0.30$	$7.19^{Aa} \pm 0.23$
		T_{360}	$7.72^{Aa} \pm 0.75$	$6.36^{ABa} \pm 0.37$	$8.55^{Aa} \pm 0.40$	$6.42^{ABa} \pm 0.54$	$8.42^{ABa} \pm 0.34$	$5.82^{Ba} \pm 0.27$
	St. thermophillus	T_0	$9.61^{Aa} \pm 0.11$	$9.32^{Aa} \pm 0.15$	$9.39^{Aa} \pm 0.11$	$9.02^{Aa} \pm 0.51$	$9.31^{Aa} \pm 0.22$	$8.93^{Aa} \pm 0.63$
		T_{120}	$9.26^{Aa} \pm 0.12$	$8.06^{Aa} \pm 0.10$	$8.95^{Ba} \pm 0.07$	$8.75^{Aa} \pm 0.26$	$8.98^{Aa} \pm 0.19$	$8.42^{Aa} \pm 0.38$
		T_{360}	$9.19^{Aa} \pm 0.16$	$5.56^{Ba} \pm 0.50$	$9.07^{ABa} \pm 0.23$	$7.40^{ABa} \pm 0.33$	$9.10^{Aa} \pm 0.42$	$7.04^{ABa} \pm 0.75$

Lc (composed of *Lact. casei* SJRP38 in co-culture with the commercial strain of *St. thermophilus* TA040) and Lf (composed of *Lact. fermentum* SJRP43 in co-culture with the commercial strain of *St. thermophilus* TA040), T_0 = viability at the initial of the assay, T_{120} = viability after simulation the gastric conditions (pH 2.0), T_{360} = viability after the simulation of enteric conditions (pH 8.0). Different capital letters in the same column, for the same species and treatment, denote significant difference ($p < 0.05$) during the assay. Different lower-case letter in the same line denote significant difference ($p < 0.05$) during the storage for the same matrix. Results are expressed as mean ± SD (n = 3).

The protective effect of linseed on the tolerance of probiotic strains under simulated GIT conditions was not significant from a microbiological point of view, because, in general, the probiotic counts in matrix II were up to 3 $\log_{10}$ CFU/mL lower than that in matrix I. Similar result was found by Casarotti et al. (2014) in the evaluation of fermented milk with different concentrations of quinoa flour under GIT conditions. The was a reduction of 1 $\log_{10}$ CFU/mL in fermented milk that received quinoa.

3.3 Activity, behavior, and general health of the animals

During the experimental protocol, no notable activity or behavioral changes were observed in the mice groups and no disease or death occurred, and there was no difference in appearance between the animals in the treatment and control groups except for the excitation of the groups CM, FMLC and FMLF in the moments of milk exchange.

3.4 Body Weight Profile of Animals

There was no statistical variation of weight of BALB/c mice among the four experimental groups, in all periods evaluated (Table 2). This result is similar to that reported by Lara-Villoslada et al. (2007), which reported that oral administration of *L. salivarius* CECT5713 to mice had no adverse effects on mouse body weight or food intake.

However, Bogsan et al. (2014) did not identify statistical variation only throughout the experiment in the water control (CW) and milk control (CM) groups when evaluated the weight of BALB/c mice using the two controls (CW and CM) and three other groups: fermented milk *Bifidobacterium lactis* HN019 (FBM), unfermented milk (UFBM) and fermented milk with *Bifidobacterium lactis* HN019 heat treated (FBMHT) for 14 days. The authors also indicated a trend line of increasing weight gain in fermented milk samples with and without heat treatment (FBM and FBMHT), but a decreasing trend line in unfermented

milk (UFBM), which was attributed to a decrease in nutrient absorption capacity due to possible destruction of the intestinal epithelium.

Table 3: Variations in the body weight of the BALB/c mice in grams prior the feeding (D0), and during feeding period: on day 7 (D7) and 14 (D14).

DAYS	WC	CM	FMLC	FMLF
D_0	$24.58^{Aa} \pm 0.57$	$24.26^{Aa} \pm 0.95$	$23.58^{Aa} \pm 0.64$	$24.36^{Aa} \pm 1.75$
D_7	$25.83^{Aa} \pm 0.26$	$23.82^{Aa} \pm 0.19$	$22.71^{Aa} \pm 2.35$	$23.64^{Aa} \pm 2.79$
D_{14}	$26.54^{Aa} \pm 0.22$	$25.20^{Aa} \pm 0.33$	$24.55^{Aa} \pm 0.59$	$25.25^{Aa} \pm 2.47$

CW - water control group, CM - milk control group, FMLC - milk fermented by *L. casei* SJRP38 group, and FMLF - milk fermented by *L. fermentum* SJRP43 group. Different upper-case letters on the same line indicate a significant difference ($p < 0.05$) in the different treatments. Different lowercase letters in the same column indicate a significant difference ($p < 0.05$). Results are expressed as mean ± SD.

3.5 Fecal Microbiology

In general, administration of fermented milk resulted in microbiological changes in fecal composition, leading to a reduction in *Clostridium* spp. and increase of *Streptococcus* sp., *Lactobacillus* sp. and *Bifidobacterium* sp. (Table 3). Additionally, the *Lactobacillus* strain used to produce the fermented milk did not affect the fecal composition. This treatment has a beneficial effect on the intestinal microbial balance and functional health in humans and animals (TIAN et al., 2014). The efficacy of probiotic is attributed to its potential immunomodulatory effects, i.e. its role in maintaining stability of the microbial ecosystem through restoration of the resident microbiota (FORSYTHE; BIENENSTOCK, 2010).

Although *Bifidobacterium* spp. was not administered in the present study, the fermented milk favored a statistically significant increase in the population of this general in feces. This effect can be attributed due to the metabolic activity of the *Lactobacillus* strains administered in FMLC and FMLF groups in the intestine, which may induce changes in the concentration of short chain fatty acids or substrates that favor the multiplication of other bacterial groups (SIERRA et al., 2010). Additionally, the *Bifidobacterium* spp. count in the

colon can increase up to 2.00 $\log_{10}$ CFU/g after the ingestion of a probiotic-containing dietary supplement (Tamime, 2014), which is similar to the observed in the present study.

Zavisic et al. (2012) administered probiotic strains of *Lactobacillus plantarum* G1 and *Lactobacillus casei* G3 in 6- to 8-week-old Wistar rats. The microbiology analysis of the stool revealed the identification of *Clostridium* sp. and *Lactobacillus* sp. which is similar to the results obtained in the present study for all analyzed groups.

Probiotic therapy was also tested in patients positive for *Helicobacter pylori* infection. The treatment was divided into three groups: group A, did not receive probiotic therapy; group B received probiotic therapy with Bioflor® twice daily for days 15 to 28 days; group C received probiotic therapy with Bioflor® twice a day for days 1 to 14 days and for 71 days. All patients were prohibited from consuming other probiotics and the pacients' feces samples were collected on days 0, 15 and 71. During all therapy of the three groups showed high counts of mesophilic aerobes, the counts of *Lactobacillus* spp. were similar in all three groups regardless of the treatment phase (approximately 5.70 $\log_{10}$ CFU/g). The population of *Bifidobacterium* spp. during treatment were high, approximately 8.20 $\log_{10}$ CFU/g, which is different from the present study, since the higher population was approximately 5.6 $\log_{10}$ CFU/g. In all three groups, 71 days after the start of the test all positive changes in the microbiota disappeared, showing the need for continuous consumption of beneficial crops (WANG et al., 2017).

A placebo-controlled cross-over study was conducted to evaluate the effect of a mixture of probiotic bacteria in humans, which demonstrated that after treatment there was a significant increase in *Lactobacillus* spp. (6.03 $\log_{10}$ CFU/g) whereas in the placebo group the count remained as before (5.61 $\log_{10}$ CFU/g), while the *Bifidobacterium* spp. count remained the same regardless of the treatment received (7.00 $\log_{10}$ CFU/g). The study also evaluated the

presence of *Clostridium* spp. in human feces and found a lower result than the present study in the group treated with probiotic mixture (2.31 $\log_{10}$ CFU/g) (ROESSLER et al., 2012).

Table 4: Microbiology of feces of BALB/c mice analyzed after 12 days of treatment of the different experimental groups.

Microorganisms	CW	CM	FMLC	FMLF
Total Aerobic	$8.70^{a} \pm 0.08$	$8.55^{a} \pm 0.35$	$8.57^{a} \pm 0.13$	$8.46^{a} \pm 0.09$
Clostridium spp.	$6.49^{a} \pm 0.69$	$7.44^{a} \pm 0.47$	$5.22^{b} \pm 0.63$	$5.68^{b} \pm 0.03$
Streptococcus spp.	$4.15^{c} \pm 0.21$	$5.24^{b} \pm 0.33$	$6.60^{a} \pm 0.42$	$6.59^{a} \pm 0.45$
Lactobacillus spp.	$5.50^{b} \pm 0.38$	$5.74^{b} \pm 0.37$	$6.39^{a} \pm 0.12$	$6.64^{a} \pm 0.18$
Bifidobacterium spp.	$3.89^{c} \pm 0.59$	$4.78^{b} \pm 0.01$	$5.57^{a} \pm 0.38$	$5.32^{a} \pm 0.10$

CW - water control group, CM - milk control group, FMLC - milk fermented by *L. casei* SJRP38 group, and FMLF - milk fermented by *L. fermentum* SJRP43 group. Different lowercase letters in the same column indicate a significant difference ($p < 0.05$). Results are expressed as mean ± SD ($n = 3$).

3.6. Microbiology of the small intestine and large intestine

The population of *Lactobacillus* spp. and *Bifidobacterium* spp. in the small intestine and large intestine were affected by the treatment group. The FMLF group presented lower population of *Lactobacillus* spp. in small intestine and *Bifidobacterium* spp. in the large intestine. There were not significative differences among the other groups (Table 4).

The large intestine is a nutritionally enriched anaerobic microenvironment, where conditions are such that facultative anaerobes are able to multiply easily, and the number of anaerobic organisms can be reduced due to the production of reactive oxygen species (ROS), which can be related to lower population of *Bifidobacterium* spp. in the large intestine (MAITY et al., 2012).

The fact that both LAB counts in the intestine are larger than in the stool count may be related to the fact that these bacteria are colonizing the intestinal microbiota (TAMIME, 2014). Salotti-Souza (2016) found that *L. casei* SJRP38 and *L. fermentum* SJRP43 showed high auto-aggregation ability at 37 °C (87.21 ± 2.84% and 91.99 ± 1.30% respectively) and high cell surface hydrophobicity (41.04 ± 1.58% and 52.78 ± 1.16%, respectively. These characteristics are related to high capacity of adherence to cells of the intestinal mucosa

(TODOROV et al., 2011). The cell surface hydrophobicity of the bacterial surface can affect the capacity of adhesion and auto-aggregation of bacteria to different surfaces, and it is one of the physicochemical characteristics which can facilitate the first contact between the microorganisms and the cells of the host intestinal wall (SÁNCHEZ-ORTIZ et al., 2015). Additionally, the presence of facultative anaerobic bacteria in the intestine can provide a low redox potential, due to the use of oxygen traces that spread to the intestinal lumen and allows the colonization of *Bifidobacterium* spp. (TAMIME, 2014).

It is estimated the presence of the genus *Lactobacillus* spp. as it constitutes 6% of the total number of cells in the human duodenum and approximately 0.3% of all colon bacteria, *Bifidobacterium* spp. are described by composing approximately 25% of all intestinal microbiota (NISTAL et al., 2016; ALMONACID et al., 2017).

The increase in the number of *Lactobacillus* spp. in the intestinal microbiota, produces useful metabolites that are responsible for improving intestinal morphology and epithelial cell proliferation (XU et al., 2018). Furthermore, the remarkable variation in intestinal abundance of *Lactobacillus* spp. among healthy individuals indicates that this genus or some species may be useful intestinal biomarkers (HEENEY et al., 2018).

Table 5: Population (log_{10} CFU/g) of *Lactobacillus* spp. and *Bifidobacterium* spp. in the small and large intestine of different groups.

Microorganism	Intestine	CW	CM	FMLC	FMLF
Lactobacillus spp.	Small	$7.97^{a} \pm 0.49$	$7.56^{a} \pm 0.68$	$8.08^{a} \pm 0.34$	$6.36^{b} \pm 1.31$
	Large	$7.80^{a} \pm 0.84$	$7.85^{a} \pm 0.35$	$7.50^{a} \pm 0.89$	$7.22^{a} \pm 0.52$
Bifidobacterium spp.	Small	$7.97^{a} \pm 0.38$	$6.56^{b} \pm 0.55$	$7.94^{a} \pm 0.36$	$7.21^{a} \pm 0.86$
	Large	$7.03^{a} \pm 0.95$	$7.65^{a} \pm 0.83$	$7.92^{a} \pm 0.19$	$6.41^{b} \pm 0.62$

CW - water control group, CM - milk control group, FMLC - milk fermented by *L. casei* SJRP38 group, and FMLF - milk fermented by *L. fermentum* SJRP43 group. Different lowercase letters in the same line, for the same treatment, indicate a significant difference ($p < 0.05$) during the test. Results are expressed as mean ± SD (n = 7 treatment CW and CM, n = 8 treatment FMLC and FMLF).

The colonization of probiotic bacteria is not necessary to have an ecological impact, but it is necessary that the microorganism reaches sufficient metabolic activity for an interaction with the resident members of the microbiota (VALTER et al., 2018). Successful intestinal invasion involves four stages: (a) The microorganism needs to be introduced in sufficient numbers and actively. (b) Habitat filters will be selected for microbes that have the necessary traits to overcome them, while the host selects specifically for symbionts by a variety of mechanisms (epithelial capture). (c) Competition must occur with resident members to access resources to grow and persist in an ecological niche. (d) Successful occupation of niches can result in metabolic activities or competitive, antagonistic or mutualistic interactions that affect the composition or function of the resident community (MALLON et al., 2015).

3.7. Bacterial Translocation

Results of incidence of translocation of gut bacteria to different tissues and blood (Table 5) showed that no bacteremia was observed in the different experimental groups, even the FMLC and FMLF group which received high doses of *Lactobacillus* sp. The bacteremia is characterized by the presence of viable microorganisms in the bloodstream, responsible for serious complications, especially in immune-compromised patients (RAMPELOTTO et al., 2015).

Another important evaluation (Table 5) is that there was a difference in the incidence of liver translocation in relation to the spleen and blood in the four treatments evaluated, including the groups defined as control (CW and CM). We also observed no signs of bacteremia and/or organ infections; however, there were translocations in the liver. These results are because most of the intestinal venous blood reaches the liver through the portal vein and because of this unique blood supply system; the liver is vulnerable to exposure to translocated bacterial products, so the liver represents the first organ in the body that finds not

only the nutrients from the diet, but also other molecules capable of translocating from the intestinal lumen to the bloodstream (SEKI; SCHNABL, 2012).

In other study, *Enterococcus faecium* 2C were analyzed for the incidence of bacterial translocation after 21 days of consumption. The mice showed no signs of bacteremia and/or organ infections, which imply that high doses of the test probiotic orally administered do not increase bacterial translocation either to blood or to spleen or liver KHALKHALI; MOJGANI, 2018).

Table 6: Incidence of bacterial translocation to the blood, liver and spleen in the different treatments offered for 14 days to the BALB/c mice.

Assesments	CW	CM	FMLC	FMLF
Blood				
MRS Incidence	0	0	0	0
MRS Population (log_{10} CFU/g)	absent	absent	absent	absent
BHI Incidence	0	0	0	0
BHI Population (log_{10} CFU/g)	absent	absent	absent	absent
Liver				
MRS Incidence	57.1%	100.0%	25.0%	50.0%
MRS Population (log_{10} CFU/g)	0.47 - 1.60	0.04 - 1.65	0.45 - 0,69	0.47 - 1.32
BHI Incidence	57.1%	85.7%	50.0%	37.5%
BHI Population (log_{10} CFU/g)	0.47 - 1.47	1.30 - 1.83	0.04 - 0.84	0.47 - 1.32
Spleen				
MRS Incidence	28.6%	0	0	0
MRS Population (log_{10} CFU/g)	1.23 - 1.47	absent	absent	absent
BHI Incidence	14.3%	14.3%	0	0
BHI Population (log_{10} CFU/g)	2.03	0.04	absent	absent

CW - water control group, CM - milk control group, FMLC - milk fermented by *L. casei* SJRP38 group, and FMLF - milk fermented by *L. fermentum* SJRP43 group. Population numbers represent the range of bacteria expressed as colony forming units per organ. MRS = de Man, Rogosa, Sharpe agar medium; BHI = brain heart infusion agar.

Lara-Villoslada et al. (2007) administered orally different doses (3 groups) of *Lactobacillus salivarius* CECT5713 in BALB/c mice and two control groups which received the vehicle alone. The authors obtained bacterial cells in the liver and spleen in all groups, including the control group, with amounts reaching 900 CFU/mL, suggesting that bacterial translocation was not associated with the treatment received.

3.8. Histological Sections

The histological slides of duodenum, jejunum-ileum and colon, stained by HE, are shown in Figure 1 (100x magnification) and Figure 2 (1000x magnification). There was no statistical difference regarding the thickness of duodenum epithelium (Figure 3) among the four groups (CW, CM, FMLC and FMLF); however, in the jejunum-ileum portion CW group presented the highest epithelial height (36.96 μm), when compared to the other groups CM, FMLC and FMLF (32.10 μm, 32.17 μm and 32.87 μm respectively). The height of epithelial colon was also higher in CW group (37.67 μm) than the other groups CM, FMLC and FMLF (34.29 μm, 31.85 μm and 34.71 μm respectively).

The GIT barrier consists of a physical barrier consisting of the vascular endothelium, the epithelial cell lining, the mucosal layer and an internal immune barrier (BISCHOFF et al., 2014; CELI et al., 2017). For a long time, the intestinal epithelium was characterized as a protective barrier against bacterial infections, but new studies have revealed the structural and functional complexity of the intestinal epithelium as a whole. Studies on germ-free animals have led to the discovery of the expression of antimicrobial proteins dependent on microorganisms and bacterial activation of autophagy of epithelial cells (BENJAMIN et al., 2013; HOPPER et al., 2015).

Epithelial cells of the small intestine migrate continuously along the axis of the crypts-villi and are exfoliated at the tips of the villi, which leads to a permanent renewal of the

intestinal epithelium, this mechanism can protect the intestinal epithelium from bacterial adhesion and translocation by pathogens invasive (ZHANG et al., 2017).

The increase in the thickness of the epithelium may be related to the fact that some groups of enteric bacteria induce the rapid generation of reactive oxygen species (ROS) inside the host cells as occurs in the presence of *Lactobacillus* spp. (JONES et al., 2013, JONES, NEISH et al., 2017, PÉREZ et al., 2017). The CW group of the present study, in results already demonstrated, presented high counts of this microorganism in the small and large intestine, which may be related to the increased thickness of the epithelium identified in the morphometry.

The smaller thickness of the epithelium of the CM, FMLC and FMLF groups were not expected, considering that among the factors described in the literature that may reduce the thickness of the epithelium are: the thermal stress (LIU et al., 2016), through mechanisms that can include oxidative stress and diet rich in fat and sugars (BISCHOFF et al., 2014; CELI et al., 2017).

In the duodenum there was a significant difference between the CM group (49.70 μm) and the other groups, which presented greater crypt depth, while in the jejunum-ileum portion the greater crypt depth was shown in the CW group (62.35 μm) than in the other groups CM, FMLC and FMLF (48.09 μm, 51.60 μm and 48.64 μm respectively). In the colon portion, there were no significant variations among groups (Figure 4).

Similar results were found by Fomenky et al. (2017), which evaluated the effects of feeding on 48 calves from 2 to 7 days of age for 96 days. The researchers divided the animals into four groups: control group (CTRL), SCB group fed with *Saccharomyces cerevisae boularddi* CNCMI-1079, LA group fed *Lactobacillus acidophillus* BT1386 and a last group receiving a mixture of antibiotics (ATB). In the evaluation of colon histomorphology, it was possible to identify an increase in crypt depth in the CTRL group and in the LA group.

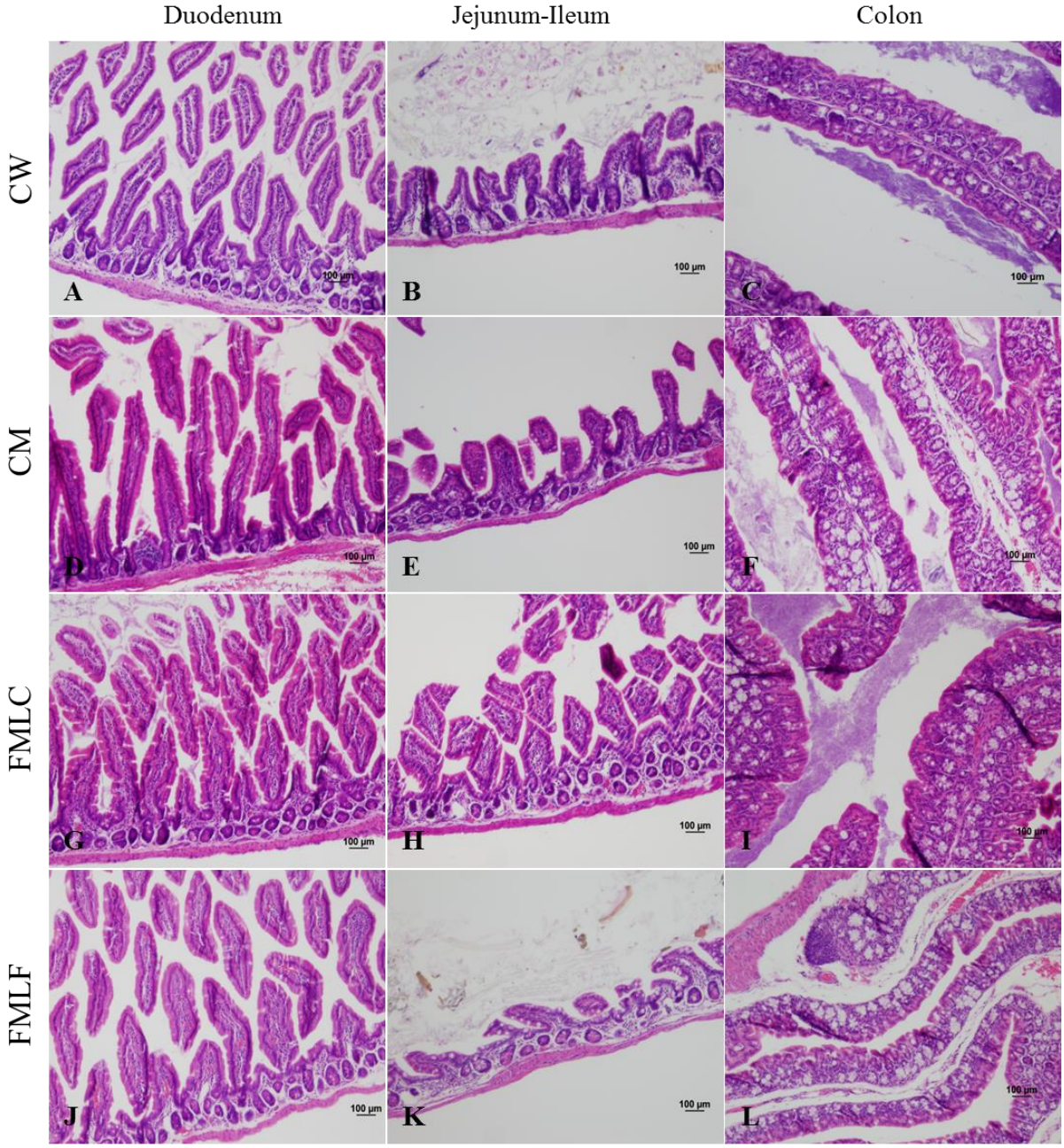

Figure 1: Histological sections of the duodenum, jejunum-ileum and BALB/c colon stained with hematoxylin-eosin (HE) and 100x magnification for the groups: water control (CW: figures A, B and C), milk control (CM: figures D, E and F), milk fermented by *L. casei* SJRP38 (FMLC: figures G, H and I) and milk fermented by *L. fermentum* SJRP43 (FMLF: figures J, K and L).

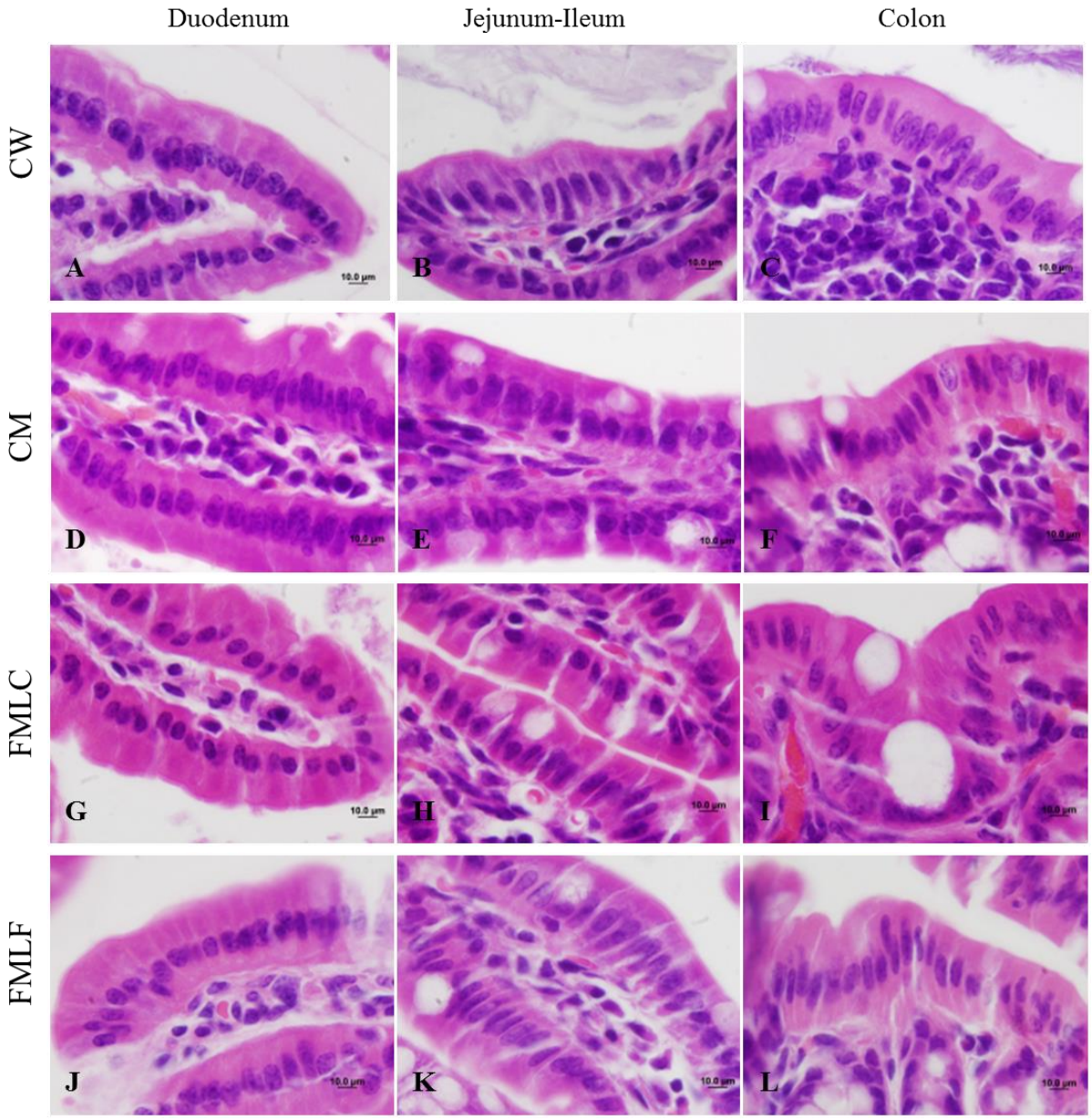

Figure 2: Histological sections of the duodenum, jejunum-ileum and BALB/c colon stained with hematoxylin-eosin (HE) and 1000x magnification for the groups: water control (CW: figures A, B and C), milk control (CM: figures D, E and F), fermented milk by *L. casei* SJRP38 (FMLC: figures G, H and I) and fermented milk by *L. fermentum* SJRP43 (FMLF: figures J, K and L).

At the site of nutrient absorption in the intestine, the presence of villi and microvilli allow the host to maximize absorption by increasing the surface of the epithelium layer (CELI et al., 2017).

The morphometry of the intestinal villi was restricted to the small intestine, because they were not present in the large intestine. A larger size of the duodenum villi was observed in the CM, FMLC and FMLF groups when compared to the CW group, however, in the jejunum-ileum portion no significant differences among the groups were observed (Figure 5).

The morphometrically evaluation of intestinal microvilli (Figure 6), shows that in the duodenum, the CM group presented statistically larger microvilli size than the other groups (3.82 µm). In the jejunum-ileum portion, only the FMLC and FMLF groups presented significant differences between each other in microvilli size, while in the colon portion, there were no differences in microvilli size between the CW and FMLC groups, and between the CM and FMLF groups.

The intestinal microbiota positively influences intestinal mucosal homeostasis, increasing intestinal barrier function, as well as the proliferation and survival of intestinal cells, small intestinal villi of non-germ rats have impaired angiogenesis and slower cell turnover rates epithelial (STAPPENBECK et al., 2002; JONES, NEISH, 2017).

The morphometric evaluation of the use of probiotic bacteria supplementation in fish showed that the crypt depth and epithelial thickness were not affected, but there was an increase of approximately 27% in the height of intestinal villi (ASADUZZAMAN et al., 2018).

Xu et al. (2018) evaluated the effect of the addition of organic acids (OA) and essential oils (EO) separately and together (OA + EO) in the feeding of 210 weaned piglets and observed an increase in the count of *Lactobacillus* spp. in the feces of the three groups, even these were not added in the diet, in addition they observed that in the groups OA and EO there was an increase in the height of the intestinal villi and increase in the depth of the crypts in the duodenum and jejunum. In the present study the improvement of the intestinal morphology was also verified in the CM, FMLC and FMLF groups.

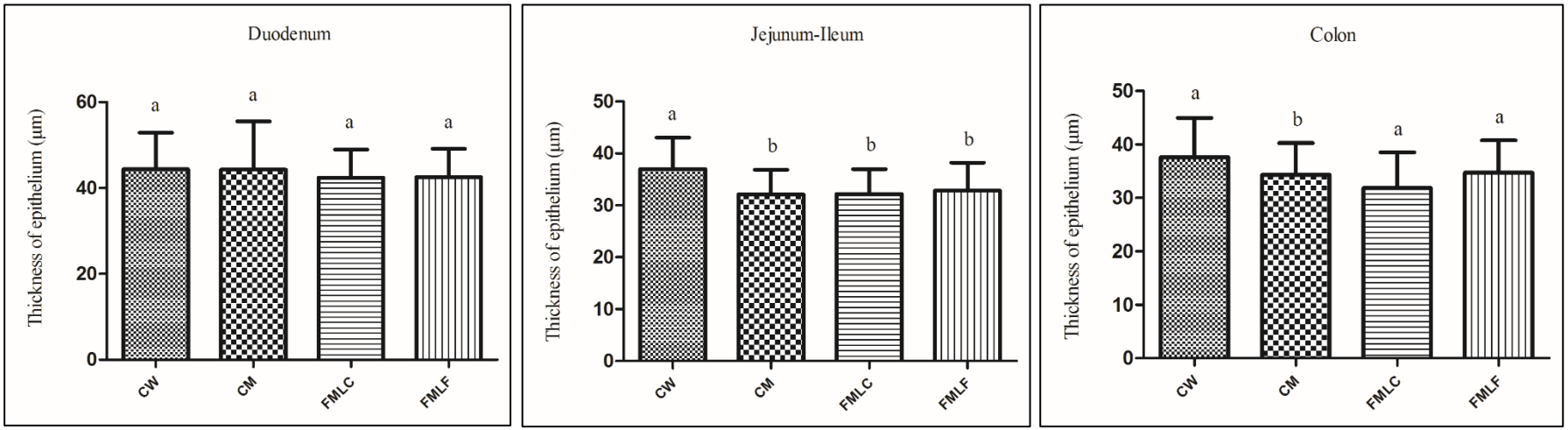

Figure 3: Morphometric analysis of the thickness epithelium of the duodenum, jejunum-ileum and colon in the different groups: CW – water control, CM - milk control, FMLC - fermented milk by *L. casei* SJRP38, and FMLF - fermented milk by *L. fermentum* SJRP43. Different lowercase letters indicate significant difference ($p < 0.05$). The results are expressed as mean ± SD ($n = 200$).

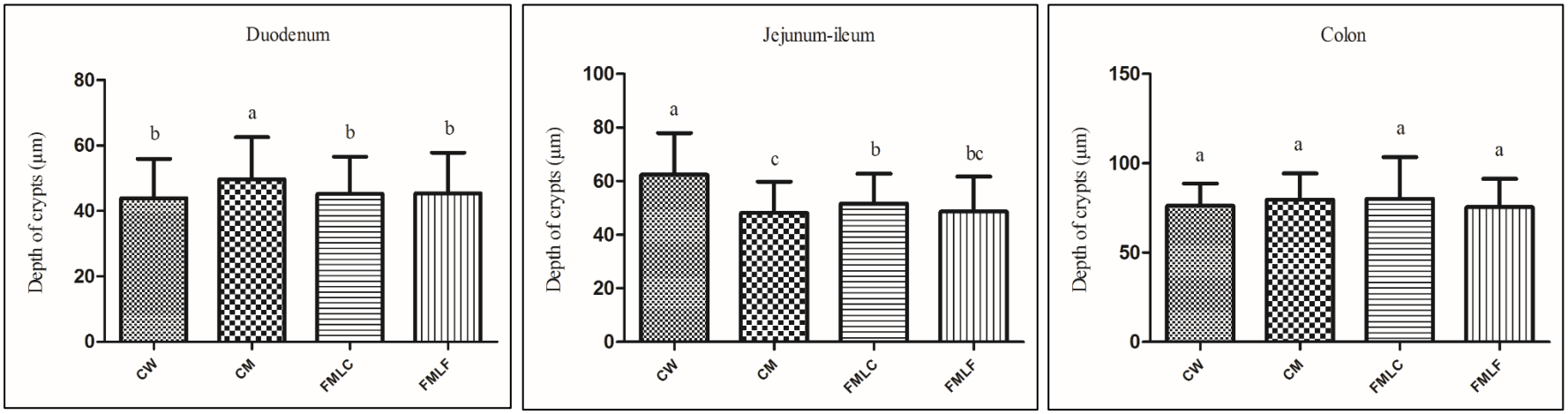

Figure 4: Morphometric analysis of the crypts depth of the duodenum, jejunum-ileum and colon, in the different groups: CW - water control, CM - milk control, FMLC - fermented milk by *L. casei* SJRP38, and FMLF - fermented milk by *L. fermentum* SJRP43. Different lowercase letters indicate significant difference ($p < 0.05$). The results are expressed as mean ± SD ($n = 200$).

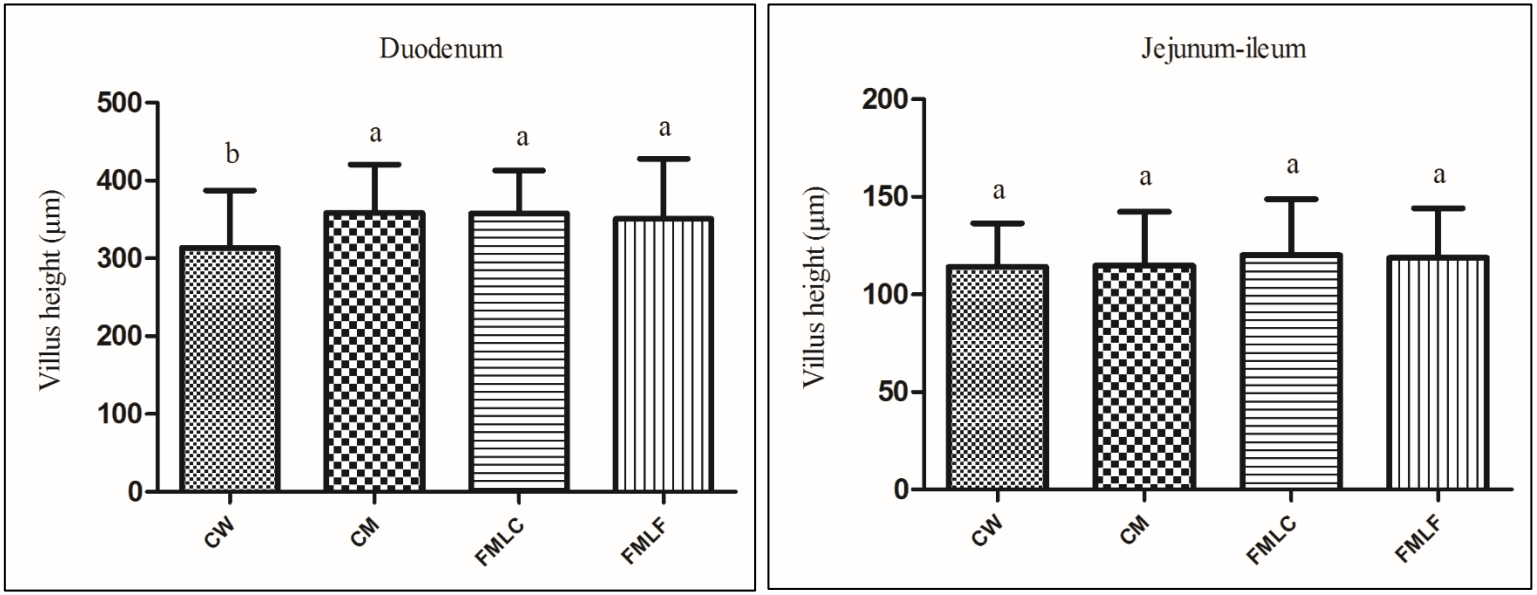

Figure 5: Morphometric analysis of the villi height of the duodenum and jejunum-ileum, in the different groups: CW - water control, CM – milk control, FMLC - fermented milk by *L. casei* SJRP38, and FMLF - fermented milk by *L. fermentum* SJRP43. Different lowercase letters indicate significant difference ($p < 0.05$). The results are expressed as mean ± SD ($n = 200$).

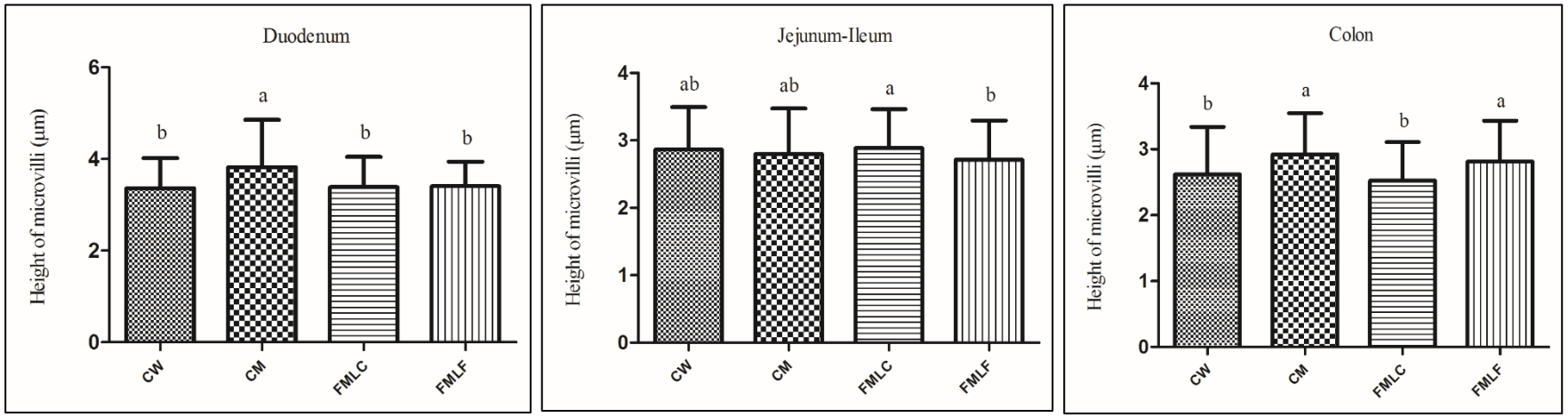

Figure 6: Morphometric analysis of the height of the intestinal microvilli of the duodenum, jejunum-ileum and colon, in the different groups: CW - water control, CM - milk control, FMLC - fermented milk by *L. casei* SJRP38, and FMLF - fermented milk by *L. fermentum* SJRP43. Different lowercase letters indicate significant difference ($p < 0.05$). The results are expressed as mean ± SD ($n = 200$).

There was a statistically higher frequency of goblet cells in the duodenum in the CM (26.28%), FMLC (20.38%) and FMLF (20.38%) groups, when compared to the CW group (10.04%); however, in the jejunum-ileum portion the difference was restricted between the CW (31.91%) and the CM (50.99%) groups. Despite there were not statistical differences among the CM, FMLC, and FMLF groups, the frequency of goblet cells was higher than 40% (Figure 7). The histological sections of the duodenum, jejunum-ileum and colon of BALB/c, stained by periodic acid Schiff's (PAS) are shown in Figure 8. Goblet cells were highlighted in pink (PAS positive).

Goblet cells participate in the absorption and presentation of the antigen to the underlying immune cells, which previously was considered to be a function exclusively of intestinal M cells (HOPPER, 2015). Goblet cells are found intercalated within the epithelial monolayer and are responsible for secretion of the intestinal mucus (mucin glycoproteins), which provides a primary shield that limits the contact between the microbiota and the host tissue. In goblet cells, the preformed mucins are stored in secretory granules, which guarantee the rapid implantation of a protective extracellular matrix (MA et al., 2017; BELKAID, HARRISON, 2017). Mucin genes (MUC in humans and Muc in mice) predominantly found in the normal adult intestine is MUC2 (HASNAIN et al., 2013).

The mucus barrier in the intestine consists of two layers: a loose outer layer that can be penetrated by the commensal microbiota and an adherent inner layer that excludes the commensal microbiota from direct contact with the epithelium (HANSSON; JOHANSSON, 2010), the presence of any defect in this inner layer of mucus will allow pathogenic bacteria to reach the epithelium in large quantities and trigger immune responses of the host (JOHANSSON; HANSSON 2014).

The mucus barrier varies in thickness along the length of the GIT and has a positive correlation with the number of commensal bacteria found at each site, and the higher amount

of goblet cells, the better is the intestinal health (HASNAIN et al., 2013). The microorganisms of the intestinal microbiota can alter the intestinal barrier and make it more or less permeable; moreover, some species of probiotics are able to reduce intestinal permeability, modifying several components, among them the production of mucins (REIS et al., 2017).

Some studies are available in the literature which evaluated the interaction of the nutrition or diet and the presence of goblet cells in the GIT. Palomar et al. (2017) analyzed the effect of probiotic *Lactobacillus* strains (*L. casei* CRL 431, *Lactobacillus paracasei* CNCMI-1518 and a mixture of probiotic fermented milk (PFM) – *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus*) in an experimental model of stress induced by food restriction and mobility in BALB/c mice, two results were interesting: the use of PFM recovered the architecture and length of the intestinal villi which were reduced in size in the other groups and normalized the number of goblet cells that had had an excessive increase in the other groups.

Garg et al. (2017) used *Lactobacillus reuteri* LR6 as an adjuvant to combat the malnutrition of albino mice, all groups received a semi-synthetic diet, but with a variation in casein percentage (16%, 8%, 4% and 2%). The group with malnutrition had a reduced number of villi in the jejunum portion, in addition to a lower number of goblet cells when compared to the control group (16% casein) and this alteration occurs due to the disorders provided in the intestinal microbiota by malnutrition.

Benoit et al. (2015) evaluated the influence of the amount of lipid in the diet on the magnitude of low grade inflammation. The animals were divided into three groups: (i) low fat in the diet, with no fat added (LPD), (ii) 20% milk fat (mHFD) and (iii) 45% fat milk (vHFD). There was a 35% increase in the amount of goblet cells in the duodenum in the vHFD group and these cells were enriched by MUC2. None of the three groups analyzed developed low-grade inflammation.

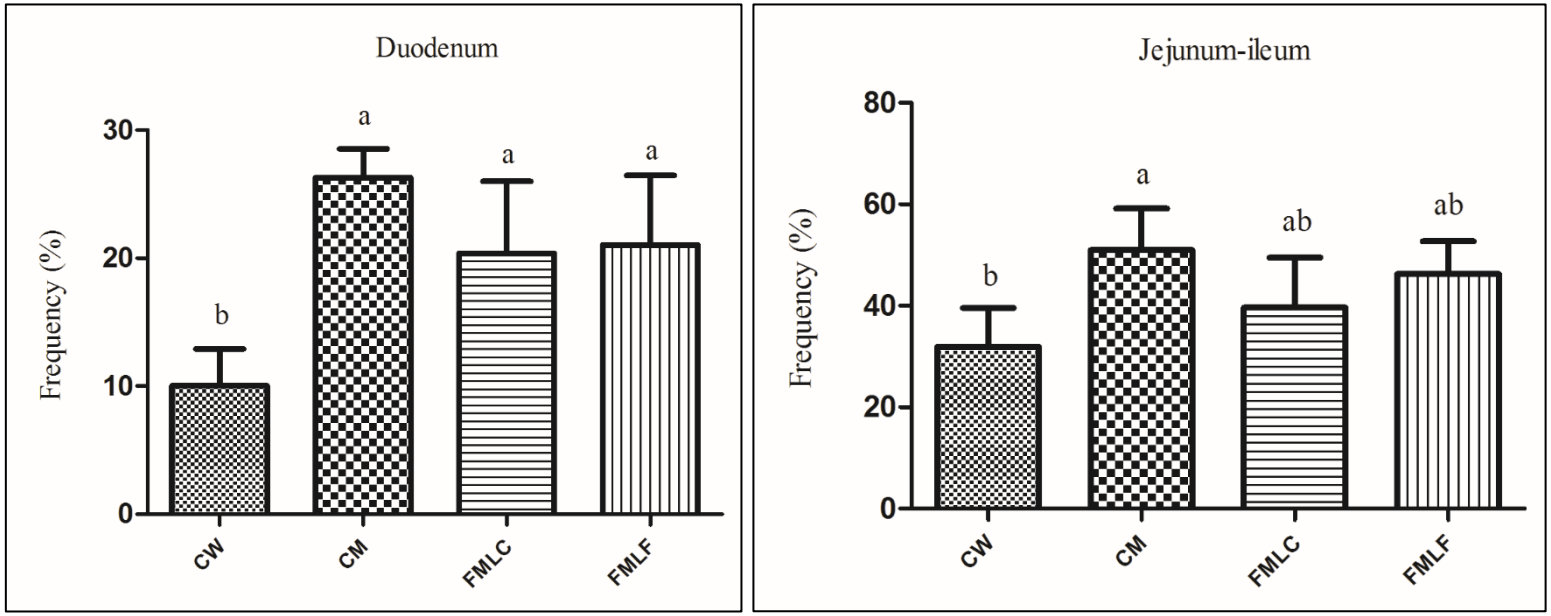

Figure 7: Frequency (%) of goblet cells in the duodenum and jejunum-ileum portions, in the different groups: CW - water control, CM – milk control, FMLC - fermented milk by *L. casei* SJRP38, and FMLF - fermented milk by *L. fermentum* SJRP43. Different lowercase letters indicate significant difference ($p < 0.05$). Results are expressed as mean ± SD ($n = 200$).

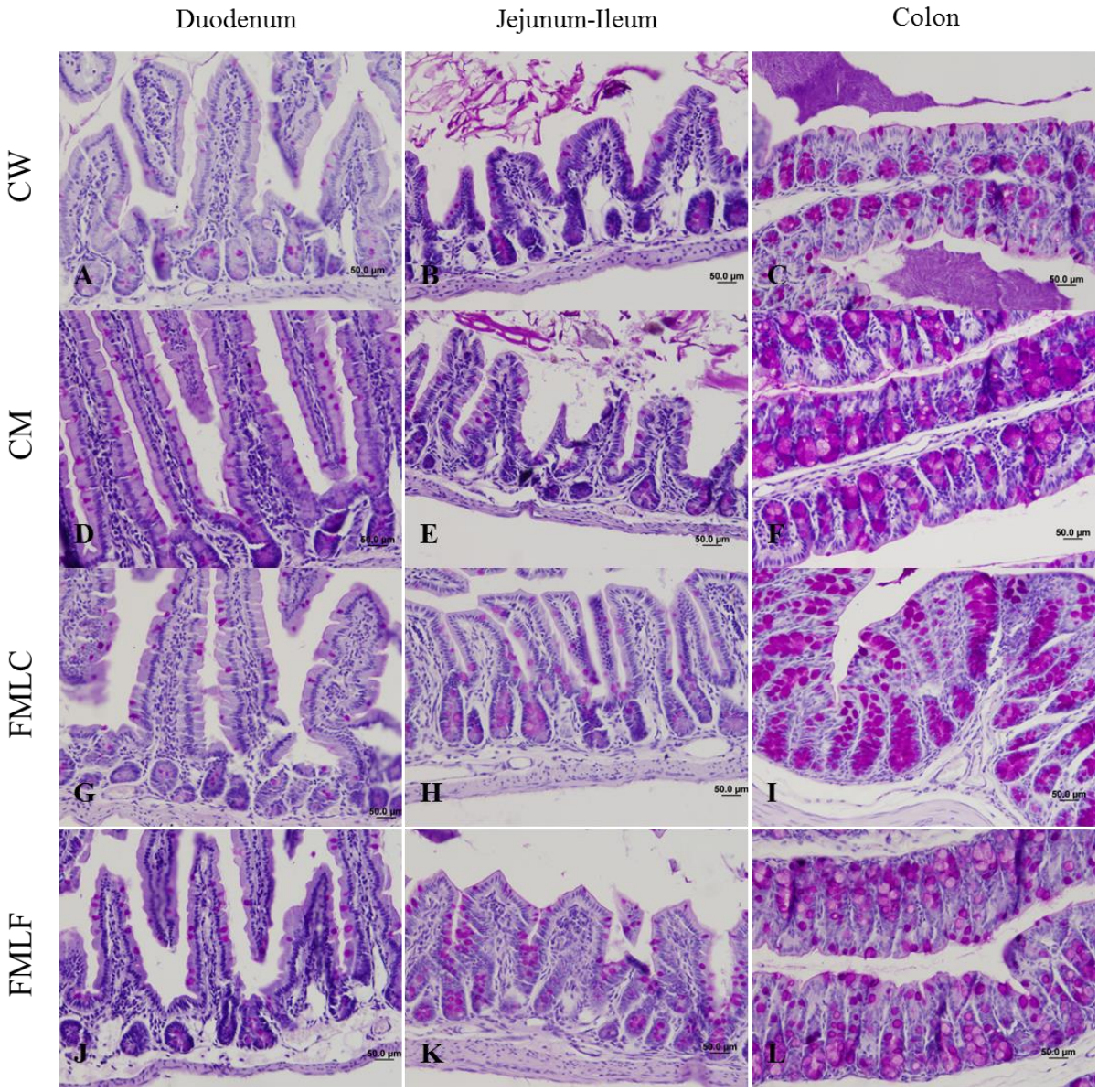

Figure 8: Histological sections of the duodenum, jejunum-ileum and colon of BALB/c, stained by periodic acid Schiff's (PAS), increased by 100x for the groups: water control (CW, Figures 7A, B and C), milk control (CM, Figures 7D, E and F), fermented milk by *L. casei* SJRP38 (FMLC, 7G, H and I), fermented milk by *L. fermentum* SJRP43 (FMLF, Figure 7 J, K and L). Goblet cells were highlighted in pink (PAS positive).

Mice treated with milk and fermented milk may have increased goblet cells related to the presence of adipokines, which are hormones secreted by adipose tissue (adiponectin and leptin) present in milk and in the bloodstream (YAMADA et al., 2012; GODOY-MATOS et al., 2014; SINGH et al., 2014). In vitro and in vivo studies have shown that adiponectin enhances the differentiation of chalice cells and production of MUC2, while leptin increases the synthesis and secretion of intestinal mucin (SAXENA et al., 2006; PLAISANCIE et al., 2006).

According to Benoit et al. (2015), the increase in goblet cells may also be associated with the μ-opioid pathway, because opioid peptides present in dietary proteins, such as milk, induce mucin secretion and overexpression of genes in goblet cells through μ-opioid activation. Likewise, the β-casein peptide f (94-123), which includes the opioid sequence neocasomorphin f (114-119), has been shown to provide increased release of mucin (ZOGHBI et al., 2006; FERNÁNDEZ-TOMÉ et al., 2017). According to Plaisancie et al., (2013), oral administration of the β-casein peptide in rat pups increased the number of goblet cells throughout the small intestine.

4. CONCLUSION

Considering all results, the autochthonous strains of *L. casei* SJRP38 and *L. fermentum* SJRP43 grew better in the MI matrix (RSMP + 7% sucrose + 1% ready-mix vanilla flavor) and presented positive results for the intestinal health of the BALB/c mice.

The animal assay showed that the population of *Lactobacillus* spp. and *Bidobacterium* spp. was higher than 6.0 $\log_{10}$ CFU/g and absence of bacteremia due to the low incidence of bacterial translocation. The ingestion of milk fermented by *L. casei* SJRP38 and *L. fermentum* SJRP43 promoted an increase in the height of the intestinal villi and in the frequency of goblet cells in the duodenum portion. Additionally, the consumption of fermented milk with

L. casei SJRP38 and L. fermentum SJRP43 strains brought about beneficial effects in the intestines of BALB/c mice.

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CAPÍTULO VI

Conclusões Gerais

CONCLUSÕES GERAIS

As cepas *Lactobacillus casei* SJRP38 e *Lactobacillus fermentum* SJRP43 foram consideradas seguras e com alto potencial probióticos, além de apresentarem boa performance na passagem através do trato gastrointestinal durante o período de armazenamento de 28 dias, quando aplicadas em leite com co-cultura de *Streptococcus thermophilus*.

Ambas as cepas autóctones de *L. casei* SJRP38 e *L. fermentum* SJRP43 cresceram melhor na matriz MI (RSMP + 7% de sacarose + 1% de mistura sabor de baunilha), e apresentaram resultados positivos para a saúde intestinal dos camundongos BALB/c pelas avaliações histológicas e morfométricas.