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FERNANDA RODRIGUES LEITE ROLIM

AVALIAÇÃO DO EFEITO PROBIÓTICO DE *Lactobacillus rhamnosus* EM1107 ADICIONADO EM QUEIJO DE COALHO CAPRINO EM RATOS COM SALMONELOSE

JOÃO PESSOA - PB

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Tese apresentada ao Programa de Pós-Graduação em Ciência e Tecnologia de Alimentos, Centro de Tecnologia, Universidade Federal da Paraíba, em cumprimento aos requisitos para obtenção do título de Doutora em Ciência e Tecnologia de Alimentos.

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Tese aprovada em 30 de setembro de 2019.

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Don't worry about a thing
Cause every little thing
Gonna be all right.

Bob Marley.

RESUMO

Probióticos são microrganismos que promovem benefícios à saúde do hospedeiro quando consumidos em quantidades adequadas. Os alimentos são veículos que protegem as estirpes durante a passagem pelo trato gastroinstestinal, sendo que, os queijos apresentam vantagens como: disponibilidade de nutrientes, pH elevado, maior capacidade de tamponamento e baixo teor de oxigênio. A aplicação de probióticos em queijos tem se expandido e, portanto, esta tese teve como proposta produzir um artigo de revisão a respeito dos conceitos gerais dos probióticos e sobre pesquisas desenvolvidas que utilizaram diferentes estirpes probióticas em tipos variados de queijos, bem como, as avaliações *in vitro* ou *in vivo* utilizadas nesses estudos; e um artigo original que objetivou avaliar o efeito probiótico de *Lactobacillus rhamnosus* EM1107, adicionado ou não em queijo de coalho caprino, em ratos infectados com *Salmonella enterica* por meio da avaliação do perfil de marcadores do extresse oxidativo, da modulação do sitema imune e microbiota intestinal. Foram selecionados ratos Wistar albinos machos com 6 semanas de idade e distribuídos aleatoriamente em cinco tratamentos experimentais: grupo controle negativo (NC): ratos que receberam solução salina e que não foram infectados com salmonelose; grupo controle positivo (PtC): animais que receberam solução salina e foram infectados com salmonelose; grupo do queijo de cabra controle (CCh): animais tratados com queijo sem a cultura probiótica e infectados com salmonelose; grupo do queijo de cabra adicionado de *L. rhamnosus* EM1107 (LrCh): animais tratados com queijo probiótico e infectados com salmonelose e grupo do *L. rhamnosus* (EM1107): ratos que foram tratados com o inóculo da cultura probiótica e que foram infectados com salmonelose. De acordo com os resultados, a administração de EM1107, seja em uma matriz de queijo ou isoladamente, foi capaz de reduzir a colonização por *Salmonella* no primeiro dia após a infecção, no lúmen intestinal. A análise metataxonômica do gene rRNA 16S revelou que o probiótico levou a alterações significativas na microbiota intestinal de ratos pela modulação de bactérias comensais, como as pertencentes à família Lachnospiraceae e gêneros *Blautia*, *Prevotella* e *Lactobacillus*. Os parâmetros imunomoduladores de NF-κB, TNF-α, IL-1β e IFN-γ também foram reduzidos em diferentes dias de infecção, mas EM1107, quando administrado isoladamente, apresentou melhor influência desses parâmentros, além da redução nos níveis de MPO e MDA. Nas análises histopatológicas, uma redução na inflamação tecidual intestinal foi observada em ratos dos grupos EM1107, LrCh e CCh comparados ao grupo PtC. Assim, pode-se concluir que o EM1107, sozinho ou adicionado ao queijo, foi capaz de minimizar os danos teciduais induzidos pela *Salmonella* por meio da modulação da microbiota intestinal, dos parâmentros imunológicos e da redução da inflamação intestinal, a nível histopatológico, dos ratos dos grupos LrCh e EM1107, com ênfase para este último grupo, que obteve melhores efeitos associados às propriedades antioxidantes e imunomoduladoras do que o queijo probiótico, embora o queijo adicionado ou não com estirpe a probiótica pudesse mitigar os danos ao tecido intestinal.

Palavras-chave: Queijo de coalho caprino, microbiota intestinal, probiótico, patógeno, *Lactobacillus rhamnosus*, *Salmonella* Enteritidis.

ABSTRACT

Probiotics are microorganisms that promote host health benefits when consumed in adequate amounts. The food is a vehicle that protects the strains during the passage through the gastrointestinal tract, and the cheeses have advantages such as: availability of nutrients, high pH, higher buffering capacity and low oxygen content. The application of probiotics in cheese has expanded and, therefore, this thesis aimed to produce a review article on the general concepts of probiotics and on researches that used different probiotic strains in various types of cheese, as well as the evaluations. *in vitro* or *in vivo* used in these studies; and an original article that aimed to evaluate the probiotic effect of *Lactobacillus rhamnosus* EM1107, added or not in goat curd cheese, in rats infected with *Salmonella enterica* by evaluating the oxidative excess marker profile, immune system modulation and intestinal microbiota. Male 6-week-old albino Wistar rats were selected and randomly assigned to five experimental treatments: negative control group (NC): rats that received saline and were not infected with salmonellosis; positive control group (PtC): animals that received saline and were infected with salmonellosis; control goat cheese group (CCh): animals treated with cheese without probiotic culture and infected with salmonellosis; *L. rhamnosus* EM1107 (LrCh) goat cheese group added: animals treated with probiotic cheese and infected with salmonellosis and *L. rhamnosus* group (EM1107): rats that were treated with the probiotic inoculum and were infected with salmonellosis. According to the results, administration of EM1107, either in a cheese matrix or isolated, was able to reduce *Salmonella* colonization on the first day after infection in the intestinal lumen. Metataxonomic analysis of the 16S rRNA gene revealed that the probiotic led to significant changes in the intestinal microbiota of rats by modulation of commensal bacteria, such as those belonging to the Lachnospiraceae family and genera *Blautia*, *Prevotella* and *Lactobacillus*. The immunomodulatory parameters of NF-κB, TNF-α, IL-1β and IFN-γ were also reduced on different days of infection, but EM1107, when administered alone, had better influence of these parameters, besides the reduction in MPO and MDA levels. In the histopathological analysis, a reduction in intestinal tissue inflammation was observed in rats of the EM1107, LrCh and CCh groups compared to the PtC group. Thus, it can be concluded that EM1107, alone or added to cheese, was able to minimize *Salmonella*-induced tissue damage by modulating intestinal microbiota, immunological parameters and reducing intestinal inflammation at histopathological level of rats. LrCh and EM1107 groups, with emphasis on the latter group, which had better effects associated with antioxidant and immunomodulatory properties than probiotic cheese, although cheese added or not with probiotic strain could mitigate damage to intestinal tissue.

Keywords: Goat coalho cheese, intestinal microbiota, probiotic, pathogen, *Lactobacillus rhamnosus*, *Salmonella* Enteritidis.

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ALT	Glicemia, alanina aminotransferase
AST	Aspartato aminotransferase
ANOVA	Análise de variância
ANVISA	Agência Nacional de Vigilância Sanitária
BAL	Bactérias Ácido-Láticas
BHI	Brain Heart Infusion
CCh	Grupo controle do queijo
cDNA	Ácido desoxirribonucléico complementar
CEUA	Comissão de Ética no Uso de Animais
CN	Grupo controle negativo
CPt	Grupo controle positivo
CT	Colesterol total
DNA	Ácido desoxirribonucléico
DTNB	Ácido Ditiobisnitrobenzóico
EDTA	Ácido etilenodiaminotetracético
ELISA	*Enzime-linked Immunosorbent Assay*
EFSA	Agência Européia de Segurança Alimentar
EMBRAPA	Empresa Brasileira de Pesquisa Agropecuária
EROs	Espécies reativas de oxigênio
FAO	Food and Agriculture Organization of the United Nations
FIOCRUZ	Fundação Oswaldo Cruz
GADPH	Gliceraldeído 3-fosfato desidrogenase
GALT	Sistema linfático associado ao intestino
GRAS	Geralmente Reconhecido como Seguro
HDL-c	Lipoproteína de alta densidade
H&E	Hematoxilina de Harris e Eosina
HTAB	Brometo de hexadeciltrimetilamônico
IECs	Células Epiteliais Intestinais
IFN-γ	Inteferon gama

IgA	Imunoglobulina A
IL	Interleucina
LDL-c	Lipoproteína de baixa densidade
LEfSe	Análise discriminante linear
LrCh	Grupo do queijo probiótico
MDA	Malondialdeído
MPO	Mieloperoxidase
MRS	Man, Rogosa and Sharpe
MUC	Mucina
NF-κβ	Fator Nuclear Kappa B
NK	Natural Killer
PBS	Tampão fosfato salino
PCR	Reação de Cadeia Polimerase
PCoA	Análise de Coordenadas Principais
PP	Placas de Peyer
QC	Grupo controle do queijo
rRNA	Ácido ribonucleico ribossómico
TCA	Ácido tricloroacético
TG	Triglicerídeos
TGI	Trato Gastrointestinal
TNF-α	Fator de Necrose Tumoral alfa
UFC	Unidade Formadora de Colônia
WHO	World Health Organization

SUMÁRIO

1 INTRODUÇÃO

De acordo com a definição mais recente, probióticos são microrganismos vivos que promovem uma melhoria da saúde do hospedeiro, quando consumidos em quantidades adequadas. Os efeitos benéficos são diversos, porém, é específico para cada estirpe, assim como a dose recomendada de consumo, os quais devem ser determinados individualmente, por meio de estudos, para microrganismo (ZIELINSKA, KOLOHYN-KRAJEWSKA, 2018; FAO/WHO, 2002). Estudos clínicos provaram a eficácia dos probióticos no tratamento de doenças como obesidade, diabetes, doenças alérgicas, Síndrome do Intestino Irritado (SII) e diferentes tipos de câncer, dentre outros (KERRY et al., 2018; MARKOWIAK, ŚLIŻEWSKA, 2017).

Além disso, alguns probióticos atuam na prevenção de doenças relacionadas a bactérias patogênicas, como a *Salmonella enterica* que está entre os principais agentes causadores de doenças transmitidas por alimentos no mundo. Esse patógeno promove uma infecção entérica que pode causar uma grande variedade de doenças, que vão de uma leve gastroenterite à febre tifóide (CASTILLO et al., 2012). A terapêutica usual para o combate desse agente inclui uso de antimicrobianos, porém, a utilização frequente e prolongada de antibióticos não só pode aumentar a resistência aos mesmos, entre os sorovares de *Salmonella*, mas também, altera a flora comensal intestinal do hospedeiro (CARMO et al., 2018; HAMMAD; SHIMAMOTO, 2010).

Assim, o desenvolvimento de estratégias terapêuticas compelmentares, para o tratamento e prevenção de distúrbios gastrintestinais, como a utilização de probióticos, provou ser uma opção eficiente contra essas doenças (ACURCIO et al., 2017) por meio do restabelecimento do equilíbrio da microbiota intestinal. Essa propriedade funcional é substancial, pois a microbiota intestinal tem um papel crucial na saúde e o seu desequilíbrio tem sido associado a doenças (VYAS; RANGANATHAN, 2012). Por conseguinte, torna-se necessário investigar como as mudanças na microbiota em resposta à suplementação de probióticos modulam a função imune inata ou adquirida do hospedeiro que levam à melhora da saúde (MA; SUZUKI; GUAN, 2018).

Para tanto, a utilização de técnicas avançadas, como metagenômica, é fundamental para analisar a modulação promovida pelos probióticos e identificar a microbiota intestinal do hospedeiro para se desenvolver melhores estratégias e

maximizar a eficácia dos probióticos, inclusive, quando inseridos nos alimentos (MA; SUZUKI; GUAN, 2018). Alimentos assumiram maior importância nos últimos anos como diferentes matrizes que podem abrigar esses microrganismos, podendo assim, ser utilizados tanto para fins nutricionais como terapêuticos, e dentre esses produtos, os derivados lácteos são os mais utilizados (TURKMEN; AKAL; ÖZER, 2019; DAS et al., 2013).

Com isso, os queijos têm se destacado por possuírem características como matriz sólida com efeito tampão, alto pH e baixa tensão de oxigênio, que conferem proteção a essas estirpes ao longo do trânsito gastrointestinal (TGI) do hospedeiro (ALMEIDA et al., 2018; ROLIM et al., 2015; PITINO et al., 2012). A utilização do leite de cabra para a fabricação de queijos é uma alternativa ao de vaca, pois, esse possui uma excelente qualidade nutricional com proteínas de alto valor biológico, uma maior digestibilidade e é uma opção para alérgicos ao leite de vaca. Assim, a combinação de leite de cabra e ingredientes funcionais, como as estirpes probióticas, é uma das áreas de pesquisa mais promissoras para a moderna indústria de laticínios caprinos (MUKDSI et al., 2013).

O interesse por esses agentes domina a indústria de produtos de saúde naturais e, com isso, torna-se necessária a confirmação da eficácia de propriedades promotoras de saúde de novas estirpes selecionadas, que envolve uma gama mais ampla de estudos, desde experiências iniciais *in vitro* até estudos *in vivo* e, por fim, ensaios clínicos em humanos (FAZILAH et al., 2018). Em vista disso, resultados de testes *in vitro* de identificação e caracterização da estirpe, testes de segurança, avaliação quanto às suas propriedades tecnológicas, além de testes de sobrevivência do microrganismo em matriz alimentar e ao longo do TGI simulado, entre outras propriedades, recomendaram *L. rhamnosus* EM1107 como um candidato promissor para ser um probiótico aplicado em queijo de coalho caprino (ROLIM et al., 2015; DOS SANTOS et al., 2015).

Estudos em animais foram o passo seguinte para a investigação das propriedades funcionais da estirpe, como o estudo desenvolvido por Rodrigues et al. (2018), que observaram um efeito protetor de *L. rhamnosus* EM1107, adicionado ou não, em queijo de coalho caprino sob os efeitos danosos promovidos por colite ulcerativa, em animais. Os resultados favoráveis desse estudo, encorajaram o desenvolvimento desta pesquisa que avaliou o efeito probiótico dessa estirpe em modelo de doença infecciosa. Nesse contexto, esta tese teve como proposta produzir uma revisão abordando sobre os

conceitos gerais dos probióticos e sobre pesquisas desenvolvidas que utilizaram diferentes estirpes probióticas em tipos variados de queijos, além de avaliar o efeito probiótico de *L. rhamnosus* EM1107, adicionado ou não em queijo de coalho caprino, em ratos infectados com *Salmonella enterica* serovar Enteritidis.

2 ABORDAGEM METODOLÓGICA

2.1 METODOLOGIA UTILIZADA PARA O ARTIGO DE REVISÃO

O artigo de revisão da presente tese é classificado como revisão do tipo integrativa. Assim, para a elaboração do artigo, utilizou-se o site de buscas “portal de periódicos capes” e as seguintes palavras-chave: probiótico, queijo, alimento funcional, microrganismo e *Lactobacillus* entre os anos de 2014 e 2019 e foram selecionados artigos que desenvolveram experimentos com queijos probióticos dos últimos 10 anos.

2.2 LOCAIS DE EXECUÇÃO DAS ANÁLISES

Os experimentos foram realizados em diferentes laboratórios, de acordo com as análises executadas. A produção dos queijos, os ensaios com os animais e as análises microbiológicas foram realizados no Laboratório de Laticínios CCS/UFPB, Laboratório de Nutrição Experimental CCS/UFPB e Laboratório de Bioquímica e Microbiologia de Alimentos DN/CCS/UFPB, respectivamente. Os ensaios de dosagens bioquímicas e PCR foram realizados no Laboratório de Farmacologia III/UFRN. A avaliação histopatológica foi realizada no Laboratório de Histopatologia/UFRPE. Por fim, o perfil metagenômico do microbioma intestinal foi realizado no Laboratório de Produtos de Origem Animal CCA/UFPB, Areia, PB.

2.3 PROCEDÊNCIA DAS CULTURAS BACTERIANAS E PREPARO DOS INÓCULOS

A cultura probiótica de *Lactobacillus rhamnosus* EM1107 foi obtida da "Coleção de Microorganismos de Interesse para a Agroindústria Tropical" da Embrapa Agroindústria Tropical/Fortaleza, Ceará, Brazil. A cultura de *Salmonella* Enteritidis P125109, mantida pelo Dr. Paul Andrew Barrow, foi obtida da "School of Veterinary Medicine and Science” da “The University of Nottingham”, Inglaterra.

Os inóculos de *L. rhamnosus* foram preparados para os dias de fabricação dos queijos probióticos e, diariamente, para administração aos animais do grupo tratado com

a estirpe isolada. Para os dois casos, a concentração utilizada foi de 9 log UFC/mL, garantindo assim, que, tanto o queijo quanto o grupo tratado com a bactéria isolada, atingissem a faixa de 8 a 9 log UFC/mL ou g no momento da administração aos animais. O inóculo de *S*. Enteritidis foi preparado para o dia da infecção dos grupos dos animais com salmonelose, na concentração de 9 log UFC/mL. Esta concentração foi padronizada por meio de estudos pilotos anteriores, levando-se em consideração a resistência à *Salmonella* dos animais da linhagem utilizada nesse estudo.

Para a preparação dos inóculos, foram feitos repiques em ágar MRS (Sigma-Aldrich, St. Louis MO, EUA), para a estirpe probiótica e em ágar Verde Brilhante (Oxoid), para a *Salmonella*, sendo incubadas a 37 °C/24 h. Em seguida, as estirpes probióticas e patogênicas foram inoculadas em caldo MRS (Sigma-Aldrich) e BHI (Sigma-Aldrich), respectivamente, a 37 °C/18 h. Após esse período, os inóculos foram centrifugados (4500 g, 15 min, 4 °C), lavados duas vezes e ressuspendidos em solução salina estéril (NaCl a 0,85%). Para se chegar à concentração de 9 log UFC/mL, concentrou-se os inóculos, ressuspendendo-os com a quantidade de 50% do volume inicial de solução salina ao final das lavagens.

Os níveis desejados dos inóculos foram verificados por meio da realização de diluições em série (10^{-1} a 10^{-8}) e submetidas à contagem de células viáveis utilizando o método de Miles et al. (1938). *L. rhamnosus* foi inoculado em ágar MRS (Sigma-Aldrich) utilizando-se a técnica da microgota e incubado sob condições anaeróbicas (BD GasPakTM EZ Anaerobe container system, Becton, Dickinson and Company, USA), a 37 °C/48 h; *S*. Enteritidis foi inoculada em ágar BHI (Sigma-Aldrich) utilizando-se a técnica da microgota e incubada sob condições aeróbicas, a 37 °C/24 h. Os resultados foram expressos como o log das unidades formadoras de colônias por mL (log de UFC/mL).

2.4 FABRICAÇÃO DO QUEIJO DE COALHO

Para a realização desse estudo, foram produzidos 2 tipos de queijos de coalho: o queijo de coalho probiótico, onde a coagulação foi realizada com adição da enzima coagulante (HA-LA, CHR Hansen, Valinhos, São Paulo, Brasil) e *L. rhamnosus* EM1107 e o queijo de coalho controle, com a adição apenas do coalho para a

coagulação. O leite de cabra para a fabricação dos queijos foi obtido da Cooperativa Capribom® (Monteiro, PB).

O leite foi submetido ao tratamento térmico (90 °C ±2 /15 min) e, após isso, resfriou-se o mesmo a 37 °C para a adição da cultura probiótica (20 mL de ressuspensão da estirpe em solução salina a 9 log UFC/mL para cada litro de leite), no caso do queijo probiótico, e adição de cloreto de cálcio (4 g/10 L de leite) e coalho comercial (seguindo as recomendações do fabricante), seguido de homogeneização e fermentação para formação do coágulo (40-50 min).

Posteriormente, efetuou-se o corte do coágulo, seguido de repouso (5 min) com posteriores mexeduras (3 min) intercaladas com o repouso (3 min) da massa. Em seguida, retirou-se cerca de 50% do volume do soro para aquecimento a 70 °C e retorno do soro à massa (com atenção para que a temperatura da massa não ultrapasse 50 °C), a qual permaneceu em repouso por 8 min. Após esse período, realizou-se a dessoragem e salga da massa (0,8 g de sal/litro de leite utilizado no processamento). Em seguida, realizou-se a enformagem e prensagem dos queijos por 4 horas. Por conseguinte, os queijos foram armazenados assepticamente em embalagens plásticas estéreis e submetidas ao vácuo, seguido do armazenamento refrigerado a 10 °C.

Após a fabricação, os queijos foram analisados quanto à qualidade microbiológica e quanto à contagem total de *Lactobacillus*. Assim, foram realizadas análises para coliformes totais e fecais, *Staphylococcus aureus* coagulase-positivo, *Salmonella spp.* e *Lactobacillus.* Os procedimentos para as análises de controle de qualidade microbiológica foram conduzidos de acordo com a American Public Health Association (2001) e os padrões de qualidade seguiram os limites estabelecidos pelo Regulamento Técnico de Identidade e Qualidade de Queijos (RTIQ) (BRASIL, 1996).

Para a contagem de *Lactobacillus*, pesou-se 10 gramas de queijo macerado assepticamente e transferiu-se para recipiente com 90 mL de água peptonada e demais diluições seriadas (10^{-1} a 10^{-8}). Em seguida, inoculou-se em ágar MRS (Sigma-Aldrich) utilizando-se a técnica de microgota e incubou-se sob condições anaeróbicas (BD GasPakTM EZ Anaerobe container system, Becton, Dickinson and Company, USA), a 37 °C/48 h. Os resultados foram expressos como o log das unidades formadoras de colônias por g (log de UFC/g). Após a realização das análises, verificou-se que os queijos possuíam qualidade de acordo com a legislação e a contagem de *Lactobacillus*

para o queijo probiótico foi de 9 log UFC/mL, enquanto que o queijo controle não apresentou contagem para *Lactobacillus*.

2.5 ENSAIO EXPERIMENTAL

Para a realização do ensaio experimental, utilizou-se ratos Wistar (*Rattus norvegicus*), machos, albinos, com 6 semanas de idade, mantidos em gaiolas, à temperatura ambiente (± 22 °C), com ciclo claro-escuro 12/12 h e acesso à ração (Presence-Purina®) e água *ad libitum*. As operações experimentais e manipulação dos animais de laboratório foram realizadas de acordo as normas estabelecidas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA, Brasil) e foram aprovadas pela Comissão de Ética no Uso de Animais da Universidade Federal da Paraíba (CEUA/UFPB - protocolo n° 021/2016). Os animais foram distribuídos aleatoriamente em 5 grupos:

- Controle negativo (NC): ratos que receberam 1 mL de solução salina e que não foram infectados com salmonelose;
- Controle positivo (PtC): animais que receberam 1 mL de solução salina e infectados com salmonelose;
- Controle do queijo (CCh): animais alimentados com o equivalente a 1 g de queijo sem a cultura probiótica e infectados com salmonelose;
- Queijo probiótico (LrCh): animais alimentados com o equivalente a 1 g de queijo probiótico e infectados com salmonelose;
- Probiótico isolado (EM1107): ratos que foram tratados com 1 mL inóculo da cultura probiótica e que foram infectados com salmonelose.

Os animais tiveram acesso aos respectivos tratamentos durante todos os dias do experimento (24 dias) por gavagem intragástrica. Para os grupos dos queijos, macerou-se o mesmo, sob condições estéreis e adicionou-se água destilada esterilizada (3:1 queijo/água) formando-se uma “solução de queijo” que facilitou a administração do mesmo, via sonda intragástrica. Essa solução foi analisada, 2 vezes por semana, quanto à contagem de EM1107, utilizando-se a mesma metodologia realizada para o queijo (item 2.3), e verificou-se que essa, se manteve em 9 log UFC/mL.

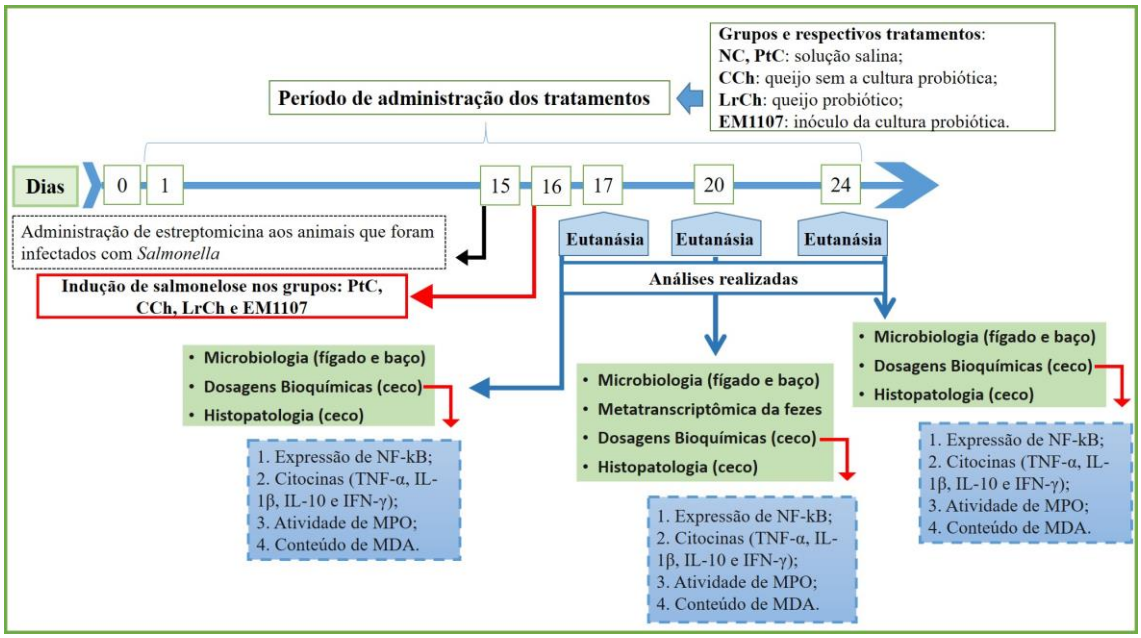
No 15° dia de experimento, administrou-se, via intragástrica, um pré-tratamento com 20 mg de estreptomicina (Sigma-Aldrich) ressuspendida em água destilada, para os grupos de animais que seriam infectados com *Salmonella* e, forneceu-se água destilada para os animais do grupo NC (BARTHEL et al., 2003). Após 24 horas do tratamento com estreptomicina, infectou-se os animais com 1 mL de 9 log UFC de *S.* Enteritidis P125109 e forneceu-se solução salina para os animais do grupo NC, por gavagem intragástrica.

Trinta minutos antes da infecção, foi administrado aos animais 100 µL de solução estéril de bicarbonato de sódio a 10% dissolvido em solução salina (NaCl a 0,85%), por gavagem intragástrica e, para os animais do grupo controle negativo, foi fornecida a mesma quantidade de solução salina (NaCl a 0,85%). A utilização de bicarbonato de sódio antes da indução da infecção promove o aumento do pH do estômago dos animais, contribuindo para a manutenção da viabilidade da estirpe patogênica, pois a *Salmonella* é sensível ao baixo pH do estômago e, com isso, melhora-se o seu potencial infeccioso (TENNANT et al., 2008).

Os animais foram privados de água e ração 4 horas antes e 2 horas depois da administração de P125109 (MOREAU et al., 2016). Após esse período, os animais voltaram a receber água e alimento *ad libitum*. Os animais foram eutanasiados no 1°, 4° e 8° dias, após a infecção, para verificação, no decorrer dos dias, de como o probiótico iria superar os efeitos nocivos de *Salmonella*. As amostras dos órgãos dos animais e conteúdo cecal foram coletadas para a realização de análises posteriores (Figura 1).

A necessidade da utilização da estreptomicina e da indução da infecção com uma alta dose de P125109 se baseia no fato de que os ratos são modelos animais demasiadamente resistentes à *Salmonella* que pode ser devido à microbiota intestinal autóctone desses animais. Com isso, a administração de um antibiótico antes da infecção promove o rompimento dessa microbiota permitindo que a *Salmonella* induza a inflamação intestinal (BORTON et al., 2017).

Figura 1. Desenho experimental com apresentação dos grupos de animais utilizados com respectivos tratamentos e ordem cronológica dos principais procedimentos utilizados no estudo e análises realizadas de acordo com o dia de eutanásia. Legenda: controle negativo (NC); controle positivo (PtC); queijo controle (CCh); queijo probiótico (LrCh); *L. rhamnosus* EM1107(EM1107); Fator de Necrose Tumoral alfa (TNF-α); Interleucina 1 beta (IL-1β); Interleucina 10 (IL-10); Interferon gama (IFN-γ); Mieloperoxidase (MPO); Malondialdeído (MDA).



2.6 ANÁLISE MICROBIOLÓGICA

Amostras do conteúdo cecal foram coletadas, assepticamente, dos animais eutanasiados no primeiro dia após a infecção para verificação da instalação da doença. Uma fração do fígado e baço também foram coletadas, sob condições assépticas, nos 3 dias de eutanásias dos animais, para verificação de translocação bacteriana (KUMAR et al., 2013). Após a pesagem das amostras, realizou-se a homogeneização e diluição proporcionalmente ao peso, seguindo-se com as diluições seriadas (10^{-1} a 10^{-4}), em solução salina (NaCl a 0,85%) e subsequente utilização da técnica de microgota. A incubação foi realizada em ágar verde brilhante (Oxoid) com adição de ácido nalidíxico (Sigma-Aldrich) (100 µL/mL), sob condições aeróbicas a 37 °C/24 h. Os resultados foram expressos como o log das unidades formadoras de colônias por g (log de UFC/g).

A primeira diluição (10^{-1}) das amostras também foi incubada sob condições aeróbicas a 37 °C/24 h, para que, em caso de amostras nas quais os resultados não foram detectáveis pela técnica da microgota, transferiu-se 1 mL da primeira diluição (10^{-1}) para o caldo rappaport vassiliadis R10 (Accumedia) e incubou-se sob condições aeróbicas a 37 °C/24 h. Em seguida, transferiu-se uma alça para o ágar verde brilhante (Oxoid) com adição de ácido nalidíxico (Sigma-Aldrich) (100 µL/mL), sob condições aeróbicas a 37 °C/24 h. Esses resultados foram qualitativos sendo expressos como positivo/ negativo para P125109.

2.7 ANÁLISE DE SEQUENCIAMENTO MICROBIANO POR 16S rRNA

2.7.1 Coleta das amostras

Amostras de fezes dos animais foram coletadas no quarto dia após a infecção (20° dia de experimento), antes da eutanásia. Essas amostras foram coletadas individualmente e acondicionadas diretamente em eppendorfs livres de DNAse e RNAse e estocadas a -80 °C.

2.7.2 Extração de DNA

Aproximadamente 200 mg de fezes foram destinadas para a extração de DNA utilizando-se o Kit FastDNASoil (MP Biomedicals, USA). A concentração e a qualidade do DNA extraído foram determinadas utilizando Nanodrop (ND-1000, NanoDrop Technologies, EUA). O DNA das fezes foi selecionado por grupo de tratamento.

O DNA genômico foi extraído por meio de Kit, seguindo as recomendações do fabricante (DNeasy® PowerSoil® Kit Qiagen, Hilden, Germany). A concentração de DNA foi determinada por spectrofotômetro de microvolume (Colibri) e Qubit® dsDNA HS (Qubit 3 Fluorometer, Invitrogen, San Diego, CA, USA). A integridade do DNA foi avaliada por eletroforese (gel de agarose 1%).

2.7.3 Preparação e sequenciamento das bibliotecas

As bibliotecas de DNA foram preparadas utilizando o kit Nextera XT sample Index Kit (Illumina) com indexação multiplex de acordo com as recomendações do fabricante. Posteriormente, os fragmentos de DNA foram purificados com o reagente Agencourt AMPure XP (Beckman).

O sequenciamento paired-end foi realizado no sequenciador Illumina Miseq utilizando o kit V2 (Illumina Inc., San Diego, CA, EUA) com 2 x 250 bp, de acordo com o protocolo HMP (CAPORASO et al., 2001). O kit Phix (Illumina) a 15% de concentração de corrida foi utilizado como controle de sequenciamento. Resumidamente, as regiões hipervariáveis V3 e V4 do gene rRNA 16s foram usadas para amplificação de PCR utilizando primers (16S Amplicon PCR Forward Primer = *5' TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG; 16S Amplicon PCR Reverse Primer = 5' GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATC).*

2.7.4 Análise de dados de amplicon de genes 16S rRNA e estudos ecológicos

Os procedimentos seguiram sequências fastq brutas, desmultiplexadas e com extremidade final sendo analisadas pela plataforma QIIME 2 v.19.7 (Bolyen et al., 2018). Posteriormente, eles foram mesclados, selecionados através dos tamanhos mínimo e máximo (200 ~ 500 pb), PHRED Score maior que (Q>20) e finalmente subrreplicados para gerar uma tabela OTU (Opetarional Taxonomic Unit) utilizando-se o programa VSEARCH (Rognes et al., 2016). As sequências quiméricas foram filtradas utilizando-se UCHIME (Edgar et al., 2011). A clusterização foi definida como um padrão de 99% de similaridade da região do centróide em relação ao método De Novo. As sequências foram alinhadas usando mafft (Katoh et al., 2002) para construir uma árvore filogenética por fasttree2 (PRICE et al., 2010) que serviu de entrada para a interpretação de estudos ecológicos. O número de sequências por amostra foi normalizado para 14900 leituras.

A diversidade alfa foi estimada pelos índices de Chao1 e Shannon e a diversidade beta foi estimada pelo escalonamento multidimensional clássico calculado com a matriz de distância Unifrac não ponderada (LOZUPONE; KNIGHT, 2005), representada em uma Análise de Coordenadas Principais (PCoA). A abundância relativa

foi avaliada usando o pacote phyloseq v.1.8.2 (MCMURDIE; HOLMES, 2013) do software R v.3.5.7. A classificação taxonômica foi atribuída pelo método Naïve Bayes, à direita do banco de dados SILVA v. 132, para regiões V3-V4 com 99% de similaridade de OTUs (QUAST et al., 2013).

O tamanho do efeito da análise discriminante linear (LEfSe) foi realizado de acordo com Segata et al. (2011), a fim de identificar as OTUs putativas associadas às diferenças entre os grupos de tratamento.

2.8 EXPRESSÃO DO GENE POR PCR (RT-PCR)

A expressão de NF-kB (factor nuclear kappa B) foi obtida por meio da análise de PCR. Para tanto, as amostras de tecido cecal foram armazenadas em tubos livres de RNAse estéreis. O RNA total das amostras do ceco foi isolado utilizando 1000 µL de Trizol (Invitrogen®) com auxílio de um homogenizador de tecidose. Para cada 1 mL de Trizol, foram adicionados 200 µL de clorofórmio e levados para centrifugação a fim de romper as células do tecido e extrair o material genético. Após a trituração procedeu-se a extração de RNA usando Kit SV Total RNA isolation system (Promega Corporation®), conforme especificações do fabricante.

Todas as amostras do RNA foram quantificadas por espectrofotometria usando o equipamento Thermo Scientific NanoDrop™ 2000 (leitura a 260 nm) e a qualidade estimada pelo cálculo da razão 260/280nm. Foi realizada a síntese de fita simples de cDNA - reação de transcrição reversa a partir de 2 µg de RNA total em uma reação usando o Kit High-Capacity cDNA Reverse Transcription (Applied Biosystems, USA) de acordo com instruções do fabricante, e equipamento Step One Plus thermocycler (condições 25 ºC/10 min; 37 ºC/120 min; 85 ºC/4 min; 4º ∞).

A amplificação e detecção por PCR em tempo real foram realizadas em placas de 96 poços no equipamento StepOne Real Time PCR (Applied Biosystems, 95 °C por 5 min, 40 ciclos de 30 s a 95 °C, 30 s a 52-60 °C (baseada no alvo) e 60 s a 72 °C), utilizando fluoróforo SYBR Green PCR Master Mix (Applied Biosystems) e o iniciador (Forward-Reverse/ Fw-Rv) de NF-kB (Fw: CACAAAGGCAAGAGTCCAGA; Rv: ACTGTCCTTGGTGCTGCTGAATG). O gene utilizado como normalizador dos experimentos foi ogliceraldeído-3-fosfato desidrogenase (GAPDH- FW:

CCATCACCATCTTCCAGGAG; RV: CCTGCTTCACCACCTTCTTG). A molécula do fluoróforo se liga à dupla fita de cDNA a cada ciclo de amplificação.

A variação quantitativa relativa da dobra em comparação com o controle foi calculada usando o método Ct comparativo, onde Ct é o número do ciclo no qual a fluorescência primeiro excede o limiar. Os valores de Ct de cada amostra foram obtidos subtraindo os valores de GADPH Ct do valor Ct do gene alvo. Uma quantificação relativa de RNA foi calculada utilizando o método ΔΔCt. A especificidade dos produtos de PCR resultantes foi confirmada por curvas de Melt.

2.9 DOSAGENS BIOQUÍMICAS

2.9.1 Dosagem de citocinas

Para dosagem de citocinas, amostras do ceco foram coletadas, pesadas e picadas em uma placa arrefecida com gelo e acondicionadas em um tubo com 10 mmol/L de tampão de fosfatode sódio (pH 7,4) (1:5 p/v). Os tubos foram colocados em banho-maria com agitação (37 °C) durante 20 min e centrifugados a 9000 g durante 30 s a 4 °C; os sobrenadantes foram congelados a -80 °C até o ensaio. A concentração de TNF-α, IL-1β, IL-10 e IFN-γ foi mensurada com BD OptEIA Elisa (BD Bioscience) de acordo com instruções do fabricante. Os resultados foram expressos em ng/ g de tecido.

2.9.2 Determinação da atividade de mieloperoxidade (MPO)

A mensuração da atividade de mieloperoxidase do tecido cecal foi realizada pelo método de Krawisz et al. (1984). Uma tira do tecido cecal foi colocada em microtubo de 2,0 mL e levada ao congelamento a uma temperatura de -80 °C. Para se iniciar a determinação da enzima MPO, a tira do tecido foi descongelada e posteriormente pesada. Mediante o peso foi calculada a quantidade do tampão brometo de hexadeciltrimetilamônico (HTAB) para cada amostra na proporção de 1:20 (p/v). Foi adicionada a cada amostra 1/3 do volume total do tampão HTAB e recortado o tecido com uma tesoura, em meio resfriado.

O material fragmentado foi triturado e homogeneizado a frio, sob ação de um triturador (Ultra Stirrer modelo: Ultra 80) e a ele adicionado o restante do tampão HTAB. O homogenato obtido foi submetido à ação de um sonicador (Limp Sonic)

durante 1 minuto e, logo em seguida, a um triplo processo de congelamento-descongelamento durante 1-2 dias. Após o último descongelamento, o homogenato foi centrifugado a 8282 rpm por 5 minutos a 4 °C. Em uma microplaca de 96 poços, foi adicionado 100 µL do sobrenadante de cada amostra e 150 µL do reativo de coloração a cada poço da placa e, logo em seguida, foi determinada a absorbância em um comprimento de onda de 450 nm, através de leitor de microplacas (Polaris), nos tempos 0 e 3 minutos, a 37 °C.

A atividade da enzima MPO foi calculada por interpolação em uma curva padrão, realizada com MPO procedente de neutrófilos humanos e com a peroxidase de rábano. Uma unidade de MPO (U) foi considerada como aquela que degrada 1 nmol/min de peróxido de hidrogênio a 25 °C. Os resultados foram expressos em U/g de tecido.

2.9.3 Determinação do conteúdo de malondialdeído (MDA)

A determinação do conteúdo de MDA foi realizada pelo método descrito por Esterbauer e Cheeseman (1990). Após fragmentação do ceco, uma tira do tecido foi separada para esta análise, colocada em microtubo de 2,0 mL e levada ao congelamento a uma temperatura de -80 °C. Para se iniciar a determinação do MDA, a tira do tecido foi descongelada e posteriormente pesada. Mediante o peso, foi calculada a quantidade de tampão Tris HCl para cada amostra, utilizando-se a proporção de 1:5 (p/v).

O tecido foi picado com uma tesoura, em meio resfriado. O material fragmentado foi triturado e homogeneizado a frio, sob ação de um triturador (Ultra Stirrer modelo: Ultra 80), e a ele adicionado o tampão Tris HCl. O homogenato obtido foi centrifugado a 4950 rpm por 10 minutos a 4 °C e 300 µL sobrenadante foi transferido para um eppendorf, sendo adicionados 750 µL do reativo cromogênico e 225 µL de ácido clorídrico (HCl - 37%).

Em seguida, o material foi colocado em banho-maria com agitação a 45 °C, durante 40 minutos e, posteriormente, levado à uma centrifugação a 4950 rpm durante 5 minutos a 4 °C. Por conseguinte, transferiu-se 300 µL do sobrenadante para microplaca de 96 poços, em duplicata, e levado para leitor de microplacas (Polaris) a um comprimento de onda de 586 nm. O conteúdo de MDA foi calculado através de interpolação em curva padrão com o 1,1,3,3 – tetraetoxipropano, o qual é hidrolisado

durante o passo de incubação com HCl a 45 °C, gerando o MDA. Os resultados foram expressos em nmol/g tecido.

2.10 AVALIAÇÃO HISTOPATOLÓGICA

Fragmentos do ceco foram colhidos dos mesmos animais usados para os estudos bioquímicos. As amostras foram fixadas durante 24 h em formol a 10% e, em seguida, processadas de acordo com a técnica histológica de rotina. Para a inclusão do material em blocos foi utilizada a parafina e os cortes foram realizados com 5 µm de espessura, utilizando-se micrótomo rotativo manual. A coloração dos cortes foi realizada com a técnica (montados entre lâmina e lamínula com resina sintética (Entellan-Merck) de Hematoxilina de Harris e Eosina) e avaliou-se os achados morfológicos das amostras.

2.11 ANÁLISE ESTATÍSTICA

Os resultados da análise microbiológica do conteúdo cecal, das dosagens bioquímicas e PCR foram expressos como média ± erro padrão das médias. As diferenças entre médias foram submetidas à análise de variância (ANOVA), seguido do teste de Tukey. O nível de significância foi estabelecido em $p < 0,05$ para todos os testes, utilizando o programa estatístico Graph Pad Prism 5.0 (GraphPad Software Inc., San Diego, CA, EUA).

As diferenças no índice de alfa diversidade foram avaliadas pelo teste não-paramétrico de Kruskall-Wallis em pares ($p < 00,5$). As dissimilaridades da beta diversidade entre os tratamentos foram avaliadas pelo teste Permutational Multivariate Analysis (PERMANOVA) (ANDERSON, 2001).

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3 RESULTADOS

Os resultados obtidos nesta Tese estão apresentados em forma Artigos Científicos submetidos em periódicos de alto impacto na área de Ciência e Tecnologia de Alimentos.

Artigo de revisão: submetido ao periódico **Trends in Food Science & Technology**, de qualis A1 e fator de impacto: 8.519.

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ARTIGO DE REVISÃO

CHEESE AS FOOD MATRIXES FOR PROBIOTICS: *in vitro* and *in vivo* tests

CHEESE AS FOOD MATRIXES FOR PROBIOTICS: *in vitro* and *in vivo* tests

Abstract

Background: Probiotics are microorganisms that promote beneficial health effects but to do so must survive passage through the gastrointestinal tract, then adhere and colonize in the intestinal epithelium. Foods are vehicles that protect these strains during this passage, and cheeses have advantages such as: availability of nutrients, high pH, higher buffering capacity and low oxygen content. Thus, several studies have elaborated different types of probiotic cheeses and for evaluation of these products *in vitro* evaluations are performed of the viability of the microorganisms during processing, maturation, shelf life of the product, under simulated gastrointestinal conditions, modifications of the physical-chemical properties of food matrices promoted by these microorganisms and their influence on the sensory characteristics, as well as extending the shelf life of the product through inhibition of pathogenic and/or deteriorating bacteria, among others, in addition to *in vivo* tests to verify the functional properties of cheeses in animals and clinical trials in humans to prove the previous analyses.

Scope and Approach: This review aims to approach probiotic characteristics by emphasizing research related to the use of cheeses as matrices to carry these strains and *in vitro* or *in vivo* evaluations of these products.

Key Findings and Conclusion: The application of different probiotics in the most varied types of cheeses has been expanded due to the favorable characteristics of cheeses as carriers of these microorganisms, as well as the sensory effects of these strains in food matrices. Well-conducted *in vitro* and *in vivo* tests are mandatory for validating these products according to regulatory institutions in order for the development of new probiotic cheeses to be efficient.

Keywords: functional food, cheese, microorganism, probiotic, *Lactobacillus*.

1. Introduction

The accumulation of evidence-based information on the relationship between diet and risks of chronic non-communicable diseases has led to considering the health impacts of foods. There has consequently been a greater search for people to live healthier lives. These changes in the lifestyle of the population together with the increase in their life expectancy were the main driving force causing significant changes in the food technology area leading to a growing development of functional foods by the food industry (Turkmen, Akal, & Özer, 2019). Thus, a growth in the functional products market has an estimated expansion of around USD 255.10 billion by 2024 (Grand View Research, 57 Inc., 2016).

These products are defined as natural or processed foods containing known or unknown biologically active compounds which provide a clinically proven and documented health benefit for the prevention, control or treatment of chronic diseases when in defined, effective and non-toxic amounts (Martirosyan, & Singh, 2015). The functional food market is dominated by carotenoids, dietary fibers, fatty acids, minerals, vitamins, prebiotics, probiotics and symbiotics (Turkmen, Akal, & Özer, 2019). Among them, probiotics can be defined as living microorganisms that can act as technological agents improving the characteristics of a food matrix, and can act as therapeutic agents promoting beneficial effects to individuals who ingest them when consumed in adequate quantities (Hill et al., 2014; FAO/WHO, 2002).

The supply of probiotics has been extensively studied, and as a result different ways of delivering these microorganisms to the intestinal system have been developed, often being of pharmaceutical origin such as tablets, capsules and sachets, or are inserted into food (Govender et al., 2013; Coman et al., 2012; Granato et al., 2010). The latter form may be a strategy to facilitate consumption, since when the beneficial microbial culture is added to the food it will be provided daily to the target population without changing the consumer's routine (Miyazima et al., 2017). Dairy products are the most widely used matrices for conveying these microorganisms and cheeses supplemented with probiotics can be an alternative for dairy industries to diversify their products in an increasingly competitive consumer market (Dantas et al., 2016). Many studies have shown success in producing different types of cheeses with the most varied

probiotic strains. Therefore, a careful investigation of the interaction of different probiotics and food components should be considered when developing this type of functional product (Aljewicz, & Cichosz, 2017; Cuffia et al., 2017; Song et al., 2017).

This investigation includes *in vitro* analyses of probiotic cheeses such as an evaluation of the viability of the strains during processing, maturation, shelf life of the product, and under simulated gastrointestinal conditions, as well as analyses of the changes in the physical and chemical properties of food matrices promoted by these microorganisms and their influence on the sensory characteristics and the extension of the shelf life of the product through inhibition of pathogenic and/or deteriorating bacteria, among other analyses. Next, *in vivo* tests were performed to investigate the functional effects of probiotic cheeses in animals, and finally clinical trials in humans to confirm the results found in previous analyses. Thus, the present review was elaborated to address characteristics related to probiotics, emphasizing studies related to the use of cheeses as food matrices and the *in vitro* and *in vivo* assays used for elaborating and evaluating the functional properties of these products.

2. General aspects, selection criteria, health claims and mechanisms of action of probiotics

There has been growing scientific and public interest in the role of microorganisms in maintaining general health and in the prevention and treatment of diseases in recent years. Today, more than ever, consumers are familiar with the term "good" or "useful" bacteria and the expectation that they can help in treating or preventing various diseases (FDA, 2018). *Lactobacillus* and *Bifidobacterium* are the most frequently used genera of microorganisms as probiotics and are part of the heterogeneous group of lactic acid bacteria (LAB) which contemplate the genera *Lactobacillus*, *Bifidobacterium*, *Bacillus* and *Enterococcus* (Zielinska, & Kolohyn-Krajewska, 2018; Fontana et al., 2013; Saad et al., 2013). However, other genera and species of bacteria and even yeast may be used, including *Saccharomyces cerevisae*, *Saccharomyces bourlardii*, *Escherichia coli* estirpe Nissle, and *Bacillus subtilis*, among others (Saad et al., 2013).

The origin of these microorganisms is variable, for example *Lactobacillus* and *Bifidobacterium* are usually found as commensals in the human gastrointestinal tract (GIT). Therefore, the intestinal microbiota of healthy volunteers could be a good source of these bacteria (Pereira et al., 2018; Zielinska, & Kolohyn-Krajewska, 2018). Probiotics can also be isolated from other cavities of the human body such as the oral and vaginal cavities, as well as from colostrum or human milk, milk from other animal species and their derivatives, and from other fermented products of animal origin such as meat, in addition to products of plant origin such as fruits and other plants (Torres-Maravilla et al., 2016; Jiang et al., 2016; Garcia et al., 2016; Vasilev et al., 2015; Santos et al., 2015; Douillard et al., 2013). Thus, the origin of microorganisms from human GIT is no longer a criterion indicated as being essential for a strain to be used, so much so that more and more scientific evidence indicates new unconventional sources of isolation very frequently (Zielinska, & Kolohyn-Krajewska, 2018; Rodrigues et al., 2018; Santos et al., 2015).

The selection of microorganisms from these various sources requires a systematic approach, because the large number of isolated strains initially leads to the need for a sequence of tests to progressively reduce the number of probiotic candidates (Pereira et al., 2018). These tests are based on already established selection criteria that should consider safety, as well as technological and functional aspects (Markowiak, & Śliżewska, 2017). Safety features include accurate taxonomic identification and the non-use of new strains with transferable antibiotic resistance genes, production of harmful metabolites, infectivity and virulence determinants (e.g., gelatinase, hemolysin, or DNAse activities) that should be evaluated by *in vitro* studies prior to *in vivo* testing (FAO/WHO, 2002, Sanders et al., 2010; Pereira et al., 2018).

In addition, new candidates for probiotics may preferably have technological properties that will influence the characteristics of the matrices to which they will be inserted. To do so, the strains must remain viable and stable during the processing of these foods throughout their shelf life so that they can contribute to the sensory attributes of the final product (Santos et al., 2015). Regarding functionality, probiotics are selected based on stress resistance phenotypes that ensure their survival through the GIT (resistance to gastric juice and bile), and the subsequent ability to adhere to the epithelium, colonization and persistence in the intestine. They must have antimicrobial

activity against pathogens and the ability to compete with the resident microbiota with consequent modulation of the immune system and promote a balance in the intestinal microbiota (FAO/WHO, 2002, Pereira et al., 2018).

Each probiotic strain has specific functional properties and its beneficial effects on host health should be provided on the basis of solid evidence and cannot be generalized to other strains (Hill et al., 2014). Significant progress has been observed lately in the field of probiotic studies, particularly in terms of selection and individual characteristics of these cultures, their possible use and their effects on health (Markowiak, & Śliżewska, 2017).

Thus, important positive responses to clinical treatment in physiological and pathological conditions were attributed to probiotics from several studies, such as the control of intestinal infections, anticancer effects, relief of irritated bowel syndrome, reduction of allergies, prevention and treatment of diabetes and obesity (Mushtaq, Gani, & Masoodi, 2019; Kerry et al., 2018), vitamin synthesis (Heenan et al., 2004), antibacterial activities (Li et al., 2015), improvement of inflammatory processes in inflammatory bowel diseases (Rodrigues et al., 2018; Assis et al., 2016), lowering cholesterol and triglyceride levels (Sperry et al., 2018), preventing infections in newborns (Tancred, 2017), modulating the intestinal microbiota (Li et al., 2015), modulating the immune system (Torres-Maravilla et al., 2016; Jiang et al., 2016; Abildgaard et al., 2017; Penha Filho et al., 2015), improved behavior related to psychiatric disorders (Abildgaard et al., 2017), and reduced oxidative stress (Amaretti et al., 2013).

Despite the wide evolution of research conducted through extensive scientific evidence, many products have been developed and named as probiotics without meeting the required criteria. At the same time, probiotic products have drawn the justified attention of regulatory bodies that protect consumers from undue health claims. However, the approach to these issues is not identical throughout the world (Zielinska, & Kolohyn-Krajewska, 2018). These agencies are responsible for ensuring that the announced health benefits have been sufficiently demonstrated with an efficient level of scientific evidence. They should also regulate how these benefits are disclosed in commercial communications and on labels (Gonzalez-Díaz, Gil-Gonzalez, & Alvarez-Dardet, 2018). The European Food Safety Authority (EFSA) which evaluates the safe

use of biological agents in food in the European Union (EU), has so far not considered any microorganism as a probiotic because the health claims submitted for consideration to the evaluation committee were not authorized due to insufficient evidence (EFSA, 2011).

According to Reid (2011), the committee was made up of people with no experience in the field of probiotics, and may have made arbitrary decisions without adequate guidelines. As a result, the EU has banned the use of the term probiotic on product labels so that they are not associated with foods that promote health benefits. Allied to the rigor in this evaluation by EFSA, there is the fear of evaluators facing the difficulty of working with biological agents, wherein the standardization of batches at 100% becomes a challenge and whose mechanism does not occur through the host itself, but through interactions with other microorganisms in the host, which in turn generates the impossibility of controlling or measuring the relationship risk versus benefit of these agents (Reid, 2016).

In the United States, the FDA (Food and Drug Administration) regulatory agency also has not approved any probiotics as a live biotherapeutic product (LBP), which is defined as a biological product other than a vaccine which contains living organisms used to prevent or treat a disease or condition in humans. However, there are foods regulated by the FDA including food supplements containing microorganisms that are legally available, although these products cannot be associated with the cure, treatment or prevention of disease. After a long history of safe use, these microorganisms are considered as GRAS (Generally Recognized as Safe) (FDA, 2018). It is worth noting that some countries, such as Italy and Canada, have more specific legislation and incorporate a list of species considered as probiotics in their regulatory guidelines (Marco et al., 2017).

On the other hand, some established criteria must be complied to prove functional or health properties by the National Health Surveillance Agency (ANVISA) for a certain probiotic food to be released for consumption in Brazil. Specific legislation was adopted (Resolution RDC ANVISA n° 241/2018) in order to establish inspection control and standardize the procedures for safety evaluation, registration and marketing of probiotic food. Among these evaluations, characterization of the microorganism, the antimicrobial resistance profile and information on the genetic basis of antimicrobial

resistance, a determination of hemolytic activity for species with hemolytic potential, studies available in the literature which describe any adverse effects observed regarding the strain in question, and a demonstration of efficacy and viability are included (ANVISA, 2019). However, like the other regulatory agencies mentioned, ANVISA prohibits the use of probiotics for drug or therapeutic purposes, whatever the presentation or administration form.

In view of the above, the adequate scientific validation of health and/or functionality, safety and biocompatibility claims still remains a critical issue for the field of probiotic food/functional beverages, as they are often ignored or assessed as insufficient methodologies by regulatory institutions (Turkmen, Akal, & Özer, 2019). Confirming the mechanisms of action used by the strains to confer their beneficial effects stands out among the difficulties faced by these organs in releasing the use of microorganisms as probiotics, which has become a challenge. The interaction of these agents with the host and it is autochthonous microbiota involves several mechanisms which together act in its final result, thus making it difficult to determine a beneficial effect to only one mechanism in isolation, meaning that the possible mechanisms should be suggested in certainty that they act in a synchronous and not an isolated manner.

Moreover, it is not possible to state with conviction that the presence of certain metabolic pathways or molecular mechanisms will confer a given clinical benefit. A strain may be deficient in overall physiological fitness or may otherwise have characteristics that cancel out the proper expression of coded properties, which prevents detecting an overall health benefit. However, as research continues to evolve, the associations between the presence of specific mechanisms and clinical benefits will continue to be more effectively identified (Sanders et al., 2018). For Walter, Maldonado-Gómez, & Martínez (2018), what is currently lacking in this area is a conceptual understanding of the effect of living microorganisms on the intestinal microbiota and their potential to modulate the host’s indigenous community.

This modulation can be evaluated and identified by means of "-omics" approaches (metagenomics, transcriptomics, metaproteomics and metabolomics), which promise to accelerate progress in understanding the interactions between diet and human intestinal microbiota (Scott et al., 2013). Investigations using modern sequencing technologies, i.e. next generation sequencing, aim to obtain more reliable

results on the composition and evolution of intestinal microbiota before and after the use of probiotics. This approach should be correlated with beneficial results to gain a better understanding of the role of each probiotic and its influence on restoring and maintaining the intestinal microbiota balance (Carmo et al., 2018). It is already known that the intestinal microbiota exerts influence on a series of biochemical reactions of the host, and (when in balance) prevents potentially pathogenic microorganisms from causing diseases. On the other hand, its imbalance may result in the proliferation of pathogens, with consequent bacterial infection (Carding et al., 2015; Leblanc, & Leblanc, 2014).

Thus, the efficacy and action mechanisms of a probiotic depend on interactions with the host microbiome and immunocompetent cells of the intestinal mucosa, because the intestine-associated lymphoid system (GALT) is the largest immunologically competent organ in the body, and its proper maturation and development depend on the microbiome composition (Guarner & Malagelada, 2003). In addition to synergistic interactions with commensal microbiota, probiotics exert anti-pathogenic effects by increasing epithelial barrier function, competitive exclusion and production of antimicrobial compounds (Zhang et al., 2018).

The mucosal barrier in the intestines of humans and animals separates the systemic from the luminal environment and protects the epithelium from direct contact with the luminal contents in order to prevent invasion by pathogenic microorganisms. Local protection of the mucosal surface is mediated by epithelial secretion products such as mucus, antimicrobial peptides (defensins) and antibody secreting lymphocytes, mainly immunoglobulin A (IgA). Mucus consists of glycoproteins and mucins, which are synthesized and secreted by caliciform cells in response to physiological (normal microbiota) or pathological stimuli (enteric pathogens) (Hooper, & Macpherson, 2010), and even during the ingestion of probiotics.

Through competitive exclusion, the adhesion of probiotics in the intestinal epithelium could result in competition between probiotics and pathogens for the same receptor (binding site in the intestinal epithelium) (Oelschlaeger, 2010), and finally, the production of different antimicrobial substances by these agents reduces pathogen proliferation such as hydrogen peroxide, carbon dioxide and antibacterial compounds, including bacteriocins and non-bacteriocins (Marianelli, Cifani, & Pasquali, 2010), as

well as organic acids (lactic acid and acetic acid) which promote a reduction in fecal pH (Wohlgemuth, Loh, & Blaut, 2010).

Probiotics also exert their effect as an immunological adjuvant by interacting with receptors expressed on the surface of intestinal epithelial cells and thus triggering a cascade of immune defense mechanisms or can be transported by cells located in follicles associated with the epithelium that cover the peyer plaques in the subepithelial region. An uptake of antigens and microorganisms can also occur through active trans-epithelial vesicular transport in enterocytes and M cells (Snoeck, Goddeeris, & Cox, 2005). Finally, dendritic cells on the lamina propria can actively extend their dendrites between epithelial junctions and thus come into direct contact with the probiotic in the intestinal lumen (Marco et al., 2006; Rescigno et al., 2001), and antigenic molecules, after their transport through the intestinal barrier, stimulate the innate or adaptive immune system. In addition, probiotics can improve the functions of the innate immune system, including phagocytic activity of neutrophils and cytotoxic activity of natural killer cells (Galdeano et al., 2007).

In adaptive immunity, many probiotic bacteria are able to stimulate the secretion of IgA by B lymphocytes and the activation of T lymphocytes (Th1/Th2) and macrophages, modulating the production of cytokines (TNF-α (tumor necrosis factor), IFN-γ (Interferon) and interleukins: IL-2, IL-8, IL-6, IL-4, IL-5, IL-12), which are the molecules involved in communication between lymphocytes, macrophages and other cells that act on inflammatory reactions and immune responses. Moreover, there is considerable variation in cytokine responses induced by different strains or species of probiotics (Arseneau et al., 2007). As new functional properties are discovered, their physiological mechanisms must be investigated by means of interdisciplinary analyses established to form a solid scientific basis.

3. Cheeses as potential food matrices to carry probiotics

Functional products are foods that contain bioactive compounds with a potential beneficial effect on health when consumed as part of a balanced diet (Kumar et al., 2015). Among them, dairy products are more popular than other foods due to the well-

established image of healthiness, with an inclusion of probiotic strains which has promoted greater consumer demand with guaranteed success in the market (Turkmen, Akal, & Özer, 2019). Considering these aspects, probiotic bacteria have been incorporated as ingredients in dairy products such as yoghurts, fermented milk, dairy beverages, dairy desserts and cheeses (Sperry et al., 2018; Assis et al., 2016; Ranadheera et al., 2010; Granato et al., 2010).

These foods, especially cheeses, play a prominent role in the diets of all population groups, and their daily consumption is recommended by health organizations and world authorities (Lovayová et al., 2015). Allied to this, these matrices provide a favorable environment to maintain the viability of probiotic strains until their consumption (Felicio et al., 2016). Thus, producing cheeses with added probiotics can provide benefits to consumer health in addition to aggregating nutritional and sensory value to products, and has been addressed in several studies with different varieties of cheeses, thus suggesting that these matrices may offer some advantages over other dairy products (Felicio et al., 2016; Rodriguez-Serrano et al., 2014; Oliveira et al., 2014; Santillo et al., 2014).

Among the advantages, cheeses have the highest pH in relation to fermented milk which can contribute to preserving the viability of the probiotic in the maturation processes and during the refrigerated storage of the cheeses. Furthermore, they have good nutrient availability, less water activity, and high fat content, which in combination with protein density makes the matrix more solid; this in turn provides better buffering capacity and low oxygen content which can offer greater protection to microbial cells during passage through the stomach to the intestine (see Fig. 1) (Cichosz, Aljewicz, & Nalepa, 2014; Pitino et al., 2012).

However, the influence of these microorganisms on the nutritional and sensory characteristics of the matrices should be evaluated when developing this type of product. These agents can contribute to biochemical transformations that influence the organoleptic characteristics of dairy products (Moraes et al., 2018). In addition to improving sensory characteristics by helping in the fermentation process of cheeses, they can also act as starter cultures, replacing the use of conventional cultures. Therefore, sensory and physical-chemical evaluations are fundamental for success in producing new products among other viability analyses of the probiotic strain as well as

in vivo tests in animals and clinical trials in humans in order to evaluate the functional potential and provide the necessary answers for a complete evaluation of these matrices.

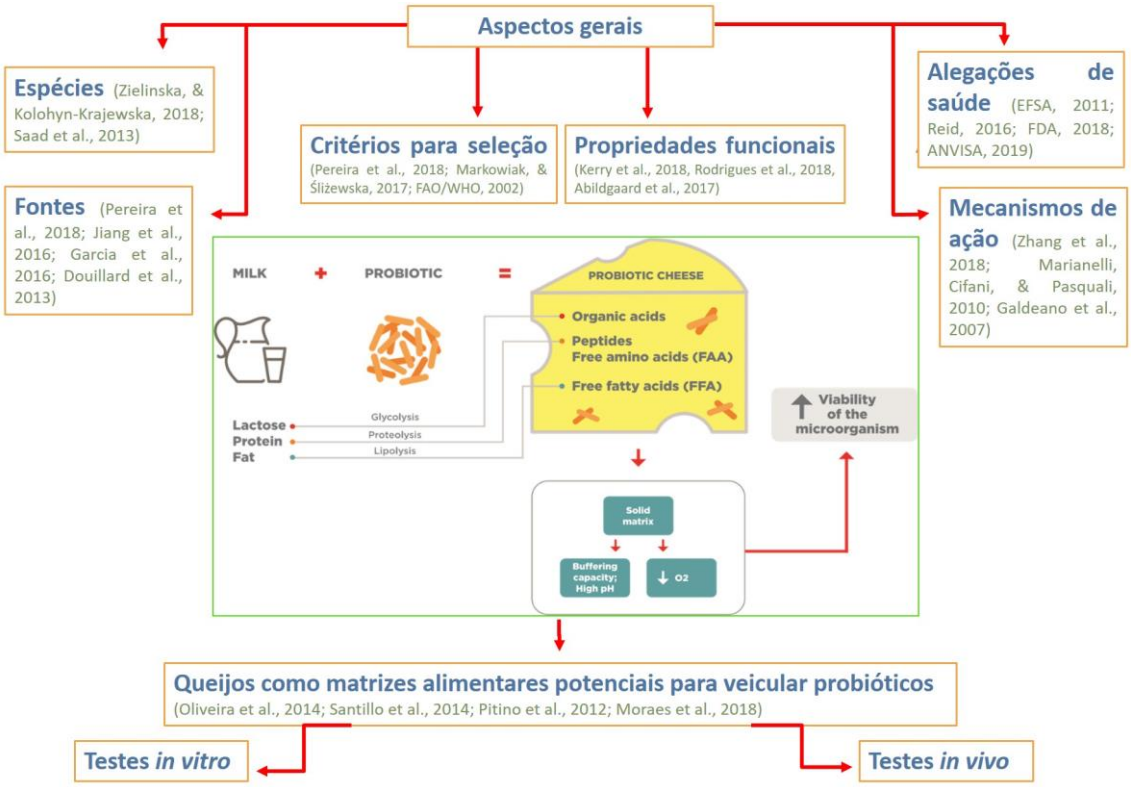

Fig. 1. General aspects of probiotics and cheeses as potential food matrices to carry probiotics, *in vitro* and *in vivo* tests of viability of probiotic strains and their influence on functional cheeses.

4. *In vitro* and *in vivo* tests of viability of probiotic strains and their influence on functional cheeses

4.1. *In vitro* tests

The behavior of each probiotic strain must be adequately tested in the different food matrices during the development of new products. Therefore, extrapolation from similar products or strains instead of adequate verification of the functionality and quality of new products should not be accepted by food manufacturers and regulatory authorities (Ranadheera et al., 2012). Accordingly, after producing a new probiotic product, its properties such as: fat content, concentration and type of proteins and sugars, pH, oxygen level, water activity, presence of inhibiting and competing

microorganisms must be evaluated to ensure the survival of the strains in the food matrix (Coman et al., 2012; Vesterlund; Salminen; Salminen, 2012).

In addition to ensuring the viability of these microorganisms in relation to the food characteristics and constituents, the technological processing and storage conditions of the matrix must also be taken into account. Thus, several parameters are evaluated, such as: type of probiotic culture, addition dose necessary to obtain a physiological response, survival during product processing and stability throughout the food maturation and storage. The possible effects of strains on the sensory and nutritional properties of food matrices must also be assessed as they are fundamental to the success of a new product, such as cheeses (Coman et al., 2012; Reid et al., 2007).

In fact, these strains produce a broad spectrum of enzymes that may be able to modify the biochemical profile of protein, lipid and carbohydrate fractions of cheeses during their maturation and/or storage (see Fig. 1) (Albenzio et al., 2013). Protein metabolism products generate different free amino acids and peptides which can be potentially bioactive depending on the strain used (Cuffia et al., 2017). The metabolism of lipids by probiotic strains generates free fatty acids which include long, medium and short chain (Albenzio et al., 2013). These products generated by the metabolism of probiotics can influence the taste, aroma and texture of cheeses depending on their maturation time and storage (Patrignani et al., 2019; Marco et al., 2017; Cuffia et al., 2017; Dantas et al., 2016). Free amino acids are the main factor responsible for the background flavor of cheeses and contribute as precursors to forming volatile compounds (Patrignani et al., 2019; Cuffia et al., 2017). In addition, free amino acids have been shown to have antioxidant and antimicrobial activity, increasing the shelf life of kalari cheese (Mushtaq et al., 2016). According to Sperry et al. (2018), short chain fatty acids promoted a reduction in cholesterol and bioactive peptides may have contributed to antihypertensive activity in women who consumed minas frescal cheese containing *L. casei* 01 during 28 days.

Moreover, these microorganisms can also modify the lactose concentration present in food matrices generating different organic acids such as lactic, acetic, and propionic (among others), depending on the probiotic strain. Meira et al. (2015) reported that the reduction in pH caused by organic acids, such as lactic acid generated by probiotics, provided a more acidic taste in ricotta cheese produced in their study.

Aljewicz & Cichosz (2017) found that probiotic bacteria converted Swiss and Dutch cheese lactose into lactic acid and then lactate. This lactate was converted through the peptidolytic activity of *Propionibacterium* sp. during maturation and storage into acetate, propionate, carbon dioxide and water. Cuffia et al. (2017) noted that *Lactobacillus rhamnosus* GG added to cheese converted citric acid to succinic acid and lactose to lactic acid. In addition, organic acids produced by probiotic strains can also promote an inhibition of deteriorating and/or pathogenic bacteria by reducing pH, extending the shelf life of products (Coman et al., 2012).

In order for strains to promote these specific modifications and for cheeses to be classified as functional foods, these microorganisms should be kept in appropriate counts at the end of the shelf life of the product. Probiotics must grow in large quantities during maturation after cheese-making or be incorporated in high concentrations to survive this process, with due care that these strains do not produce a quantity of metabolites that may cause defects such as excessive production of organic acids or interfere with the activity of other essential microorganisms that constitute the native microbiota of these matrices or the added starter cultures (González-Olivares et al., 2014).

It was recommended for some time that the minimum number of viable bacterial cells ingested in the food should be 6 log CFU/g or mL of food, and the daily consumption of products containing probiotics should not be less than 100 g or mL to promote health benefits (Lovayová et al., 2015; Cichosz, Aljewicz, & Nalepa, 2014; Vinderola et al., 2009). However, many studies have shown that the recommended probiotic dose in food will depend on the strain studied, and therefore these products should be evaluated individually in relation to the daily dose, as well as the time period needed for the product to promote its beneficial effects (Rodrigues et al., 2018; Sperry et al., 2018; Martins et al., 2018).

In view of the aspects mentioned above, several studies have evaluated the viability of probiotic strains added to different types of cheeses after processing and during storage and/or product maturation. These studies demonstrated that the cheeses were considered adequate to carry viable bacteria during the studied period, as shown in Table 1, where information is also available regarding the influence of the strains on the sensory and nutritional characteristics of the cheeses.

Table 1. Summary of last 10 years studies about evaluation of probiotics survival in diferent type of cheese after manufacture and during storage and ripening.

Type of cheese	Probiotic strain	Assessed period	Initial Count (CFU g^{-1})	Final count (CFU g^{-1})	Influence of probiotic on the cheese	Research
Argentinian Ovine Cheese	Mix of *L. acidophilus* La-5 and *B. lactis* BB12	Ripening at 12 °C (45 d)	7	La-5: 7.75 BB12: 6.50	Probiotic strains in cheese did not seem to affect the counts of the lactic microflora and did not modify the different parameters evaluated: gross composition, nitrogen fractions, lipolysis, fatty acids profiles of cheese fat including CLA and volatile profile of cheese.	Perotti et al. (2014)
Buscion, Caciotta and Pecorino	SYNBIO® combination of *L. rhamnosus* IMC 501®, *L. paracasei* IMC 502®	Storage: Buscion (21 d) at 4 °C, Caciotta and Pecorino (60 d) at 8 to 10 °C	9	Buscion: 7 Caciotta: 8.8 Pecorino: 7.5	SYNBIO® does not modify the sensory features of the cheeses. The presence of SYNBIO® allows the extension of shelf-life preserving the probiotic-enriched foods longer than the original foods.	Coman et al. (2012)
Buffalo Kalari cheese	*L. casei* 279, *L. brevis* 021 and *L. plantarum* 01	Storage at 4 °C (30 d)	Cheese with: 279: 9.4 021:9.4 01: 9.2	Cheese with: 279: 6.8 021: 6.5 01: 6.2	Probiotics did not affect the water activity, moisture, protein and fat content when compared with control, but pH of probiotiv cheese was lower and acidity was higher than control cheese. Probiotic strains also increased the antioxidant activity of Kalari cheese due to the formation of bioactive peptides; retarded lipid and protein oxidation; inhibited the growth of spoilage microrganisms and probiotics had a positive influence on palatability.	Mushtaq et al. (2016)
Buffalo Minas Frescal cheese	*B. lactis* BB-12	Storage at 5 °C (30 d)	8.15	8.36	Probiotic cheese did not affect yield, physicochemical composition, pH, syneresis and their rheological properties, but showed a greenish white color.	Verruck et al. (2015)
Cheddar cheese	*L. plantarum* K25	After manufacture and Ripening at 4 °C (84 d)	Before manufactu-re: 7.96 and Before ripening: 8.95	After manufac-ture: 9.05 After ripening: 9.31	*L. plantarum* did not influence the chemical composition (weight of the solids (FDM), moisture, protein and fat), levels of salt-in-moisture and pH of cheddar cheese; caused a moderately proteolysis, but did not alter the manufacturing procedure or changed the chemical compositions of cheese; the concentration of most FAAs in probiotic cheeses was higher than the control cheese, except for the aspartic acid.	Zhang et al., 2013

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Table 1 *(continue)*

Type of cheese	Probiotic strain	Assessed period	Initial Count (CFU g^{-1})	Final count (CFU g^{-1})	Influence of probiotic on the cheese	Research
Cottage cheese	*L. casei* 373, *L. rhamnosus* GG and mix YO-MIX^{TM} 205 (*L. bulgaricus, L. acidophilus, Bifidobacterium* spp. and *Streptococcus thermophilus*)	Storage at 8 °C/ 28 d	6	373: 7 YO-MIX^{TM} 205: 7 GG: 6	No differences were observed of cheese with respect to their moisture, fat and total protein. The lactic acid concentration increased slightly during the storage time with decreased of pH. The probiotics released more peptides that could potentially be bioactive because of the proteolytic activity of this probiotics strain.	Abadía-García et al. (2013)
Cream cheese	*L. paracasei* LPC37	Storage at 5 °C/ 21 d	8	8	The acidity was smaller in probiotic cheese; this cheese had a high index of sensory acceptance by the consumers (sensory analysis was performed 7 days after manufacture) and did not present changes in the sensory characteristics compared with the control cheese.	Santini et al. (2012)
Cream mini-cheeses	*B. animalis* subsp. *lactis* 10140 and *L. reuteri* 20016	Storage at 4 °C/ 28 d	Cheese with 10140: 9.78 Cheese with 20016: 9.7	Cheese with 10140: 8.31 Cheese with 20016: 8.26	The probiotic did not negatively affect the cheeses sensory acceptability.	Speranza et al. (2018)
Dutch-type (DE) and Swiss-type (SE) cheese	*L. rhamnosus* HN001	Ripening: DE: 12 °C/ 10 wks; SE: 12-14 °C/ 2 wks, 19-20 °C/ 2 wks and 12-14 °C/ 2 or 6 wks. Storage: at 4 °C/3 mths	8	DE: 9.20 (ripening); 7.60 (storage) SE: 8.51 (ripening); 7.7 (storage)	DE cheese was characterized by a higher protein and fat content than control cheese. No such trends were observed in SE cheese.	Cichosz et al. (2014)

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Table 1 *(continue)*

Type of cheese	Probiotic strain	Assessed period	Initial Count (CFU g^{-1})	Final count (CFU g^{-1})	Influence of probiotic on the cheese	Research
Dutch-type (DE) and Swiss-type (SE) cheese	*L. acidophilus* NCFM, *L. paracasei* LPC37, and *L. rhamnosus* HN001	Ripening: DE: 12 °C/ 6 wks and SE: 12-14 °C/ 2 wks, 19-20 °C/ 2 wks, 12-14 °C/ 2 wks. Storage: at 4 °C/ 3 mths	8	Ripening: NCFM (DE: 7.82 and SE: 7.39); LPC37 (DE: 9.43 and SE: 9.6); HN001 (DE: 9.32 and SE: 9). Storage: NCFM (DE: 7.08 and SE: 5.92); LPC37 (DE: 9.12 and SE: 8.90);HN001 (DE: 8.48 and SE:8.42)	A slight difference in chemical composition was noted between experimental DE cheese and control cheese. The probiotics did not negatively influence on starter cultures used in cheese production. The greatest antimicrobial activity of HN001 and LPC37 against secondary microflora was noted in SE cheese and DE cheese, respectively. The counts of nonstarter lactic acid bacteria and coliforms decreased during storage in both of cheeses. In addition, the counts of yeasts, molds and anaerobic bacteria decreased in DE cheese (with a lower water and higher salt content). The use of probiotic cultures contributed to higher storage stability of experimental cheeses because of de lower of pH due to release of ammonia by slow degradation of casein, and degradation of lactic acid to lactate.	Aljewicz & Cichosz (2017)
Edam cheese	*L. acidophilus* NCFM, *L. rhamnosus* HN001	Ripening at 12 °C/ 10 wks	8	7	Probiotic culture did not lead to differences in the chemical composition (water content, protein, fat and sodium chloride content) of the cheese but the acidity was lower than the control cheese; the probiotic cultures changed the proteolysis pattern by intensifying proteolysis and peptidolysis in the edam cheese.	Aljewicz et al. (2014)
Fior di Latte cheese	*L. rhamnosus* GG	Storage at 4°C/ 15 d	>7	7.55	The addition of the probiotic increased secondary proteolysis, production of diacetyl, acetoin, lactic and acetic acids. Sensory characteristics (smell, astringency, acid taste and residual flavor) were also modified.	Cuffia et al. (2017)

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Table 1 *(continue)*

Type of cheese	Probiotic strain	Assessed period	Initial Count (CFU g^{-1})	Final count (CFU g^{-1})	Influence of probiotic on the cheese	Research
Goat coalho cheese	*L. acidophilus* LA-5, *L. paracasei (L. casei* 01), *B. lactis* BB-12	Storage at 10 °C/ 21 d	LA-5 cheese (C2): 6.5; *L. casei* 01 cheese (C3): 7; BB-12 cheese (C4): 6.5; mix of the 3 strains cheese (C5): 8	C2: 8.5 to 9 C3: 8 to 8.5 C4: 7 to 7.5 C5: 7.5 to 8	Differences in gross composition and physicochemical characteristics (moisture content, dry matter, ashes, total protein, fat, lactose, NaCl, pH, titratable acidity, water activity) were observed in all probiotic cheese during the time of storage. In C5 increased proteolysis, release of peptides (medium and small) and free amino acids; C2 and C4 increased levels of secondary proteolysis. After storage, an increase in hardness was observed for C1, C3 and C5; control cheese presented the lowest values for springiness and cohesiveness; the gumminess increased in C3 and C5 while chewiness increased only in C5 during storage. All probiotic cheeses were better accepted in the sensory evaluation than control cheeses.	Oliveira et al. (2012)
Goat ricota cheese	*L. acidophilus* LA-05 and *B. lactis* BB-12	Storage at 7 °C/ 7 d	LA-05: 8.5 BB-12: 8.0	LA-05: 6.54 BB-12: 6.22	The probiotics did not affect the yield, syneresis rate or physicochemical characteristics of the product, but reduced pH level, increased yellowish color and hardness level.	Meira et al. (2015)
Kwark cheese	*B. longum* KACC 91563	Storage at 4 °C/ 10 d	6.5	7.58	The compositional analysis was not show any differences in probiotic cheese; chemical analysis showed that pH of kwark cheese with commercial starter and *B. longum* was lower than that of control; no differences in lactic acid bacterial counts were detected in the cheeses. The probiotic did not alter the color, flavor, texture, taste, or overall acceptance of the kwark cheese.	Song et al. (2017)
Minas Frescal	*L. acidophilus* LA-5	Storage at 5 °C/ 14 d	Qc (100% NaCl): 7.63 QI (75/25% NaCl/KCl): 7.23; QII (50/ 50%): 7.86; QIII 50/50%, 1% arginine): 9.21	Qc: 8.45 QI: 8.04 QII: 7.4 QIII: 7.76	An increase in lactic and citric acid levels was observed while no significant differences were observed for the acetic acid levels in probiotic cheeses; probiotic bacteria weren't capable of changing the lipolytic profile of cheeses during storage.	Felicio et al. (2016)

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Table 1 *(continue)*

Type of cheese	Probiotic strain	Assessed period	Initial Count (CFU g^{-1})	Final count (CFU g^{-1})	Influence of probiotic on the cheese	Research
Mascar-pone type cheese	*B. lactis* BB-12	Storage at 5 °C/ 30 d	BB-12 cheese: 8.09 BB-12 and inulin cheese: 8.83	BB-12 cheese: 7.57; BB-12 and inulin cheese: 7.94	No differences were noted between yield, moisture, fat, protein and ash contentes in probiotic cheeses; the pH of the cheeses with probiotics was lower than that of control; the probiotic did not affect colour and texture of the cheeses.	Almeida et al. (2018)
Minas Frescal cheese	*L. casei* 01	After manufacture Storage at 5 °C	8	8	*L. casei* in Minas Frescal cheese affected the pH, proteolysis, organic acid levels, fatty acid profile, antioxidant, and ACE inhibitory activities, but did not affect the proximate composition (moisture, protein, and fat).	Sperry et al. (2018)
Minas Frescal cheese	*L. casei* Zhang	Storage at 5 °C/ 20 d	8.1	Zhang cheese: 8.28 Zhang and starter culture cheese: 9.02	*L. casei* Zhang provided lower pH and high proteolysis indexes; higher lactic acid and acetic acid levels during storage. No differences were observed for protein and fat contents between cheese treatments. Probiotic cheeses presented lower values for all sensory attributes probably due content and profile of organic acids or different texture and rheological properties.	Dantas et al. (2016)
Ovine Pecorino Cheese	*L. acidophilus* LA-5 (AML-La, AM-La, AML); Mix: *B. longum* BL-46 and *B. lactis* BB-12 (AMB-Bb, AM-Bb, AMB)	Ripening (120 d)	7	AML-La: 8.2; AM-La: 7.44; AML: 8.2. AMB-Bb: 8.28; AM-Bb: 8.28; AMB: 8.64	Lower values of pH were reach in probiotic cheese; probiotic strain was also able to influence specifically peptidase activities and free amino acid profile in cheese at 120 days of ripening. Cheese lactic microflora seemed to be influenced by the presence of the probiotic strains.	Santillo et al. (2014)
Pecorino ewe milk cheese	*L. acidophilus* LA-5 and Mix of *B. lactis* BB-12 and *B. longum* BB-46	Ripening (30 d)	11	LA-5 cheese: 7; mix of BB-12 and BB-46 cheese: 5	Probiotic cheeses affected the acidification and coagulation phases leading to the lowest pH during ripening. The proteolytic profile was higher in probiotic cheeses probably promoting a lower cheeses hardness; more intense lipolysis was detected in cheese containing probiotics in terms of both FFA and CLA content. The probiotics in cheeses did not adversely affect preference or acceptability.	Albenzio et al. (2010)

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Table 1 *(continue)*

Type of cheese	Probiotic strain	Assessed period	Initial Count (CFU g^{-1})	Final count (CFU g^{-1})	Influence of probiotic on the cheese	Research
Ripening cheese	*L. rhamnosus* GG	Ripening at 4 °C and 14 °C/ 28 d	Ripening at 4 °C: 8 and 14 °C: 7	Ripening at 4 °C: 7 and 14 °C: 8	At 14 °C was observed a higher degradation of proteins into low molecular weight peptides compared with 4 °C, but even in refrigerated conditions, LAB has proteolytic activity, generating low molecular weight peptides during the entire storage period.	González-Olivares et al. (2014)
Semi-hard cheese	*L. acidophilus* LA-3, *L. casei* BGP 93, *L. plantarum* 96	Ripening at 10 °C/ 180 d	8	LA-3: 6.61;BGP 93: 7.71 96: 7.39	There were no differences among experimental cheeses and control for levels of dry matter, salt in dry matter and protein and lactic acid content.	Lovayová et al. (2015)
Scamorza ewe milk cheese	*L. acidophilus* LA-5; mix of *B. longum* BL-46 and *B. lactis* BB-12	Ripening at 8-10 °C/ 15 d	8.5 - 9.0	LA-5: 7.55; Mix:: 9.09	The pH values were lower in cheeses containing the mix of probiotics and the proteolysis were also greater in this cheeses leading to more complex soluble peptide and free amino acids profiles. *L. acidophilus* strain ruled lipolysis and was able to significantly increase vaccenic and oleic acids and CLA content in cheese.	Albenzio et al. (2013)
Sheep feta cheese	*Propionibacterium freudenreichii* subsp. *shermanii* LMG 16424	Ripening at 18 °C/ 10 d and 3 °C/ 50 d	6.8	9	Lower concentrations of lactate were detected in probiotic cheese and the strain produced adequate amounts of propionic acid that it may possess anti-cancer properties. The control cheese scored better appearance and texture than probiotic cheese and there was no difference in the flavour;	Angelopoulou et al. (2016)
Soft goat cheese	*L. plantarum* 564	Storage at 4 °C/ 8 wks	Free 564: 7.68;Spray-dried 564:7.76	Free 564: 6.9;Spray-dried 564:8.82	The chemical composition and pH values of goat cheeses containing live and spray-dried cells were within the typical range for soft cheeses, with excellent sensory quality.	Radulovic et al. (2017)
Squacquerone cheese	*L. crispatus* BC4	Storage at 4 °C/ 13 d	6.8	7	BC4 in the cheese positively affected the starter culture viability and the pH of this cheese was lower than the control; the probiotic improved the sensory properties of the cheese in terms of creaminess, flavour and overall acceptance and affected the proteolytic, lipolytic patterns and the volatile molecule profiles of cheese.	Patrignani et al. (2019)

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Table 1 *(continue)*

Type of cheese	Probiotic strain	Assessed period	Initial Count (CFU g^{-1})	Final count (CFU g^{-1})	Influence of probiotic on the cheese	Research
Whey cheese	*L. casei* LAFTI® L26, *L. acidophilus* LAFTI® L10 ou *B. animalis* LAFTI® B94	Storage at 7 °C/ 21 d)	*L. casei*: 7.10; *L. acidophilus*: 7.86; *B. animalis*: 7.97	*L. casei*: 9.04 *L. acidophilus*: 8.32 *B. animalis*: 8.59	No physicochemical changes occurred in fat, protein and moisture contents, besides a slight decrease in pH (especially in matrices inoculated with *L. casei*) as compared with the control, because of the acidity observed by lactic and acetic acids (*B. animalis* and *L. casei* produced both acids (acetic and lactic), while *L. acidophilus* produced only lactic acid). During the storage, there was a release of new peptides or increase in concentration of already existing ones in cheeses; the most peptidolytic strains were *L. casei* and *B. animalis*. Higher ACE-inhibitory activity also occurred in matrices inoculated with either of these strains.	Madureira et al. (2013)
White brined cheese	*L. paracasei* K5	Ripening at 18-22 °C and Storage at 4 °C/ 70 d)	7	7 to 8	Total nitrogen in DM was higher in cheese with K5 compared to control cheese samples but moisture and fat content did not be affect by probiotic. Lactose, lactic acid concentration and pH were affecting by adjunct culture (lactic acid concentration was higher in immobilized culture than the free culture). Aromatic compounds were was enhanced by the free or immobilized K5.	Terpou et al. (2018)
White cheese	*B. bifidum* DSMZ 20456, *L. acidophilus* DSMZ 20079	Ripening at 4 °C/ 120 d	7 to 8	Cheese in brine with: *B. bifidum*: 6 to 7; *L. acidophilus*: 6 to 7;Cheese in vacuum pack with: *B. bifidum*: 6 to 7; *L. acidophilus*: 6 to 7	The dry matter, fat and protein contents of the cheese samples weren't affected by probiotics. The probiotics, particularly *L. acidophilus* improved proteolytic characteristics and bioactivity of white cheese. The levels of proteolysis and ACE-inhibitory activity were higher in the Cheese in vacuum pack with *L. acidophilus* at 120 days of ripening.	Erkaya & Sengul (2015)

Note: d: days; wks: weeks; mths: months.

Another *in vitro* test often used to assess the potential of probiotic strains is resistance to simulated gastrointestinal conditions (Reale et al., 2015). For this purpose, digestion models are used to provide rapid screening and evaluation of food products. An optimal *in vitro* digestion method can yield accurate results in a short time (Coles et al., 2005). Testing the efficacy of newly developed foods depends on the use of digestion models which accurately simulate the physical-chemical and physiological complex of events that occur in the human GIT (Hur et al., 2011).

However, there is no international consensus on a standardized protocol to evaluate the resistance to conditions found in the GIT (Burns et al., 2014). *In vitro* digestion characteristics such as digestion time, enzyme content or composition should be adjusted according to the sample characteristics. For example, if the concentration of the target substance (protein, lipid, or carbohydrate) is increased, the concentration of enzymes or the digestion time should also be increased, even while maintaining the other steps of the *in vitro* digestion procedure (Hur et al., 2011). In view of this, the digestion time at each stage (e.g., mouth, stomach, and small bowel) is an important factor in determining an *in vitro* model (Mcclements et al., 2009). Regarding the use of digestive enzymes, the more complete the model including from α-amylase in the mouth to the bile salts present in the intestine (for example), the more reliable the GIT simulation will be when compared to reality (Madureira et al., 2013).

However, the large intestine is generally not taken into account in digestion models because the absorption of compounds mainly occurs in the small intestine (Brandon et al., 2006). Table 2 shows some research on the models used to evaluate the survival of probiotic bacteria in cheeses against simulated gastrointestinal conditions. The viability of probiotics may be favored during their passage in the GIT after consumption due to the physical and chemical characteristics of the cheeses (Felicio et al., 2016; Pitino et al., 2012).

Table 2. Studies on the evaluation of the viability of probiotics in cheeses under simulated gastrointestinal conditions.

Simulated gastrointestinal model					Type of cheese	Probiotic strain	Initial Count (CFU g^{-1})	Final Count (CFU g^{-1})	Research
Model*	**Compart-ment**	**Conditions**	**pH**	**Time (min)**					
1	Mouth	Saliva (α-amilase)	6.9	2	**Whey cheese**	*L. casei* (LAFTI® L26), *L. acidophilus* (LAFTI® L10) ou *B. animalis* (LAFTI® Bo)	LAFTI® L26: 9 to 10; LAFTI® L10: 8 to 9; LAFTI® Bo: 8	LAFTI® L26:7 to 8; LAFTI® L10: 6 to 7; LAFTI® Bo: 8 to 9	Madureira et al. (2011)
	Oesophagus – stomach	Pepsin	5.5-2.0	90	**Goat coalho cheese**	*L. acidophilus* La-5; *L. paracasei* (*L. casei* 01); *B. lactis* BB-12	La-5: 7 to 8; *L. casei* 01: 8 to 9; BB-12: 7	La-5: 6; *L. casei* 01: 5.7; BB-12: 5.5	Oliveira et al. (2014)
	Duodenum	Pancreatin + bile salts	5.0	20	**Goat coalho cheese**	*L. rhamnosus* EM1107	EM1107: 6.53	EM1107: 6.75	Rolim et al. (2015)
					Goat ricota cheese	*L. acidophilus* La-5 e *B. lactis* BB-12	La-05: 6.54; Bb-12: 6.22	La-05: 6.01; Bb-12: 6.27	Meira et al. (2015)
					Buffalo Minas Frescal cheese	*B. lactis* BB-12	8.4	8 to 9	Verruck et al. (2015)
	Ileum	-	6.5	90	**Mascar-pone-type cheese**	*B. lactis* BB-12	BB-12 cheese: 8.07; BB-12 and inulin cheese with: 8.6	BB-12 cheese: 7.65; BB-12 and inulin cheese: 8.42	Almeida et al. (2018)

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Table 2 *(continue)*

Simulated gastrointestinal model					Type of cheese	Probiotic strain	Initial Count (CFU g^{-1})	Final Count (CFU g^{-1})	Research
Model*	**Compart-ment**	**Conditions**	**pH**	**Time (min)**					
2	Oral	-	Sample pH	2	**Single-strain cheese**	*Propionibacterium freudenreichii* strain CIRM-BIA 129	10	9	Rabah et al. (2018)
	Stomach	Pepsin	3	120					
	Duodenum	Pancreatin, trypsin and bile salt	7	120					
3	Stomach	Artificial saliva and Pepsin	2.0	180	**Cottage**	*L. casei* 373, *L. rhamnosus* GG and mix YO-MIX™ 205 (*L. bulgaricus*, *L. acidophilus*, *Bifidobacterium* spp. and *S. thermophilus*)	373: 6 YO-MIX™ 205: 6 GG: 7	373: 3; YO-MIX™ 205: 2 GG: 3	Abadía-García et al. (2013)
	Intestine	Pancreatin + bile salts	8.0	240					
4	Stomach	Pepsin and lipase	5.9-1.8	78	**Pasta filata**	*L. rhamnosus* (D44, F17, H12, H25, N24, R61)	D44: 11.09 F17:11.55 H12: 10.95 H25: 11.54 N24: 11.45 R61: 11.44 GG: 11.42	D44: 10.67 F17: 8.3 H12: 9.42 H25: 9.97 N24: 10.24 R61: 9.44 GG: 10.61	Pitino et al. (2012)
	Intestine	Lecitina hepática, lipase pancreática, colipase, tripsina, quimiotripsina e α-amilase	7	60 e 120					

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Table 2 *(continue)*

Simulated gastrointestinal model					Type of cheese	Probiotic strain	Initial Count (CFU g^{-1})	Final Count (CFU g^{-1})	Research
Model*	**Compart-ment**	**Conditions**	**pH**	**Time (min)**					
5	Stomach	Gastric juice (0.2% NaCl)	2.0	30	**Minas Frescal**	*L. acidophilus* LA-5	Qc (NaCl, 100%): 6.82; QI (NaCl/KCl 75/25%): 6.89; QII (50/50%,): 7.1; QIII (50/50%, 1% arginine): 7.05	Qc: 6,16 QI: 6.66 QII: 7.92 QIII: 7.79	Felicio et al. (2016)
	Intestine	Bile salts	7.0	60					
6	Stomach	Saliva-gastric solution (pepsin)	3.0, 2.7, 2,5	120	**Squac-querone cheese**	*L. crispatus* BC4	7	6	Patrignani et al. (2019)
	Intestine	Pancreatic enzymes and bile extract	7.4	180					
7	Stomach	Saliva-gastric (pepsin)	3.0	90	**Fior di Latte cheese**	*L. rhamnosus* GG	6	5 to 6	Cuffia et al. (2017)
	Intestine	Duodenal digestion (bovine bile)	8	10					
		Intestinal digestion (bovine bile, pancreatin)	8	90					

*Model: 1 Madureira et al. (2011); 2 Minekus et al. (2014); 3 Charteris et al. (1998); 4 Pitino et al. (2010); 5 Fernandes et al. (2013); 6 Vinderola et al. (2011); Vinderola et al. (2011) with some modifications.

Table 3 shows some studies involving inhibition analysis of pathogenic bacteria most commonly found in possible contamination of cheeses by probiotics added to cheeses. The ability of these agents to inhibit colonization of pathogens is well known and may have several applications, such as preventing microbial food spoilage (Zhang et al., 2018) or preventing the development of pathogenic bacteria in food. The consequence of this is the extension of a product's validity without using any synthetic substances (preservatives or antioxidants), which are a measure of concern to consumers because of the possible health hazards that these compounds may cause (Mushtaq et al., 2016). Other *in vitro* evaluations were performed on a smaller scale in research using cheeses as food matrices. For example, Mushtaq, Gani, & Masoodi (2019) evaluated the immunomodulatory, antidiabetic, antiproliferative and antimicrobial potential of probiotic strains in kalari cheese using carcinogenic cell lines; while Liu et al. (2018) assessed the antioxidant activity of an added probiotic in cheddar cheese after GIT simulation.

4.2. *In vivo* tests

In vitro cell culture studies and/or experimental animal testing models, such as laboratory mice, are useful tools for a preliminary assessment of the health impacts of probiotics and aim to select strains to verify their specific properties, as well as to investigate the possible mechanism of action used by the microorganism to promote the health benefit by providing a scientific basis for prevention or treatment of various diseases (Servin & Coconnier, 2003; Castillo et al., 2013). However, these studies do not provide sufficient data to reach a fair and concrete conclusion on the health impacts of these microorganisms on the human body system. Any health claim associated with a probiotic product (solid foods or beverages) must be substantiated by means of human clinical trials, which are conducted using appropriate methodologies (Turkmen, Akal, & Özer, 2019).

Tabela 3. *In vitro* studies on inhibition of pathogenic and deteriorating bacteria, antioxidant activity and functional properties of probiotics in cheeses.

Type of cheese	Probiotic strain	Pathogenic bacteria	Assessed period	Result	Research
Cottage cheese	*L. casei* 373, *L. rhamnosus* GG; YO-MIX^{TM} 205 (*L. bulgaricus*, *L. acidophilus*, *Bifidobacterium* spp. and *S. thermophilus*)	*Listeria monocytogenes*	Storage at 8 °C (28 days)	The lactic acid concentration increased slightly during the storage time with decreased of pH that explain the decreased significantly of the viable counts of *L. monocytogenes* in the all probiotic cheeses compared with control cheese.	Abadía-García et al. (2013)
Goat coalho cheese	*L. acidophilus* La-5; *L. paracasei* (*L. casei* 01); *B. lactis* BB-12	*L. monocytogenes* and *Staphylococcus aureus*	Storage at 7 °C (21 days)	*L. casei* 01 stood out in inhibiting the growth of *S. aureus* and or *L. monocytogenes* in goat coalho cheese over refrigerated storage relative to the inhibitory effects caused by BB-12 and La-5.	Oliveira et al. (2014)
Goat coalho cheese	*L. rhamnosus* EM1107	*L. monocytogenes*, *S. aureus*, *Escherichia coli* and *Salmonella* Enteritidis	Storage at 7 °C (21 days)	The probiotic strain delayed the growth of the pathogenic bacterias in cheese. Therefore, this study demonstrated the potential of this strain of EM1107 to be used as a protective culture, particularly *S. aureus* and *L. monocytogenes*.	Rolim et al. (2015)
Minas Frescal cheese	*L. plantarum* 49, *L. plantarum* 201 and *L. paracasei* 108	*L. monocytogenes*	Storage at 4 °C (14 days)	*L. plantarum* 49 and *L. paracasei* 108 decreased the counts of *L. monocytogenes*, while *L. plantarum* 201 exerted bacteriostatic effects against this pathogen during refrigerated storage.	Costa et al.(2018)
Type of cheese	**Probiotic strain**	**Objective**		**Result**	**Research**
Bufallo Kalari cheese	*L. casei* 279, *L. brevis* 021 and *L. plantarum* 01	To investigate the effect of probiotics cheese on immuno-modulatory, antidiabetic, antiproliferative, and antimicrobial potential *in vitro*		Probiotic cheese exhibited profound cell proliferation inhibition against different cancer lines (MCF-7, HCT 116, IMR 32, and HEK-T), increased of immuno-modulatory, antidiabetic and antimicrobial activity.	Mushtaq Gani, & Masoodi (2019)

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Table 3 *(continue)*

Type of cheese	Probiotic strain	Objective	Result	Research
Cheddar cheese	*L. rhamnosus* 6134	Determination of antioxidant activity of Cheddar cheese during the ripening period in simulated gastrointestinal digestion and changes in polypeptide content after simulated gastrointestinal digestion	The antioxidant capacity increased significantly after simulated gastrointestinal digestion may be due to degradation of casein in cheese by pepsin, trypsin, and protease produced by the probiotics conferring antioxidant peptides.	Liu et al. (2018)
Burrata Cheese	*L. plantarum* LPAL and *L. rhamnosus* LRB	Improving the functional attributes and shelf life of burrata cheese by using protective lactobacilli	The addition of protective lactobacilli improved the flavor of the burrata cheeses. The use of protective lactobacilli strains increased the shelf life of burrata cheese by 3 days; This study provided more in-depth knowledge of the microbiome of burrata cheese	Minervini et al. (2017)

Thus, animal models provide proof of the effects of *in vitro* evaluation and their results must be confirmed by clinical trials in humans. These results may be: a significant improvement in the physiological condition of individuals, i.e. in symptoms, well-being and quality of life; a reduction in the risk of disease; or a faster recovery from the disease. Each should have a proven relationship with the tested probiotic (FAO/WHO, 2002). *In vivo* methods using animals or humans generally provide more accurate results when compared to *in vitro* tests, but are more time-consuming and expensive (Coles et al., 2005).

Thus, data generated by animal models should be obtained from a coherent chain of well-designed experimental and clinical studies before being extrapolated to humans (Servin & Coconnier, 2003). These studies have their importance, especially considering the limitations of directly investigating certain diseases in human beings, which often involves ethical issues and/or risks related to the disease under study (Da Matta, 2010; Fagundes & Taha, 2004).

A particular probiotic strain may have a beneficial effect, show no effect, or result in an adverse effect when tested in different clinical situations. Negative or adverse effects do not attest to the probiotic condition (status) of the strain in certain situations. However, these results should be documented by specifying their possible effects and emphasizing their use free of health risks (FAO/WHO, 2002). The resulting clinical effect of a live bacterium will be dictated by the complex set of genetic expression and technological production factors for probiotic cultures that affect viability and shelf life. Tests must be performed in humans to confirm that a particular mechanism conducts the clinical effects observed with adequate evidence of attributing a clinical benefit to a probiotic taxonomic group (Sanders et al., 2018).

Table 4 presents some studies involving the evaluation of probiotic cheeses in animal and human models to investigate the functional properties of the strains and their mechanisms of action to obtain a beneficial effect.

Table 4. Studies involving *in vivo* evaluation of probiotic potential in cheeses.

Type of cheese	Probiotic strain	Evaluated group/ time	Functional properties	Mechanism of action	Research
Gouda cheese	*L. rhamnosus* HN001 and *L. acidophilus* NCFM	Elderly people (72 to 103 years old)/ 4 weeks	Stimulate the innate immune response	Improvement in the capacity of NK (Natural Killer) cells to act against tumor cells and phagocytosis activity of granulocytes and monocytes	Ibrahim et al. (2010)
Minas Frescal cheese	*L. acidophilus* LA-14 and *B. longum* BL05	Immunosuppressed adult Wistar rats/ 2 weeks	Modulation of the immune system	Increased white blood cells (monocytes, lymphocytes)	Lollo et al. (2012)
Minas Frescal cheese	*L. acidophilus* LA-14 and *B. longum* BL05	Male (7 weeks old, specific pathogen-free) wistar rats/ 15 days	Reduce the development of hypertension measured by the systolic and diastolic blood pressure parameters in spontaneously hypertensive rats. The cheese was able to attenuate as well as it presented a significant decrease in LDL cholesterol and triglycerides levels, and an increase in HDL cholesterol	The reduction of blood pressure may be due to the proteolytic activity of the probiotic in cheese, generating peptides with antihypertensive action that act on renin–angiotensin system and consequently vasoconstriction, reducing blood pressure. The possible mechanisms responsible for cholesterol reduction by probiotics are the incorporation of cholesterol into the cellular membrane during bacterial growth in the small intestine e the active bile salt hydrolase, by the strain increasing their rates of excretion, thereby leaving less cholesterol available for intestinal absorption and to reduced blood cholesterol	Lollo et al. (2015)

(continued on next page)

Table 4 *(continue)*

Type of cheese	Probiotic strain	Evaluated group/ time	Functional properties	Mechanism of action	Research
Minas Frescal cheese	*L. casei* 01	Women (age 32-72) with overweight and diagnosed with arterial hypertension/ 28 days	Probiotic cheese led to an improved lipid (reduction in total cholesterol, LDL cholesterol and triacylglycerides and an increase of HDL cholesterol) and hematological profiles (probiotic was able to attenuate the systolic (SBP) and diastolic (DBP) blood pressure parameters) in hypertensive overweight women. The hemoglobin and hematocrit levels increased after the 4-week consumption of probiotic cheese	The cholesterol-lowering mechanisms of probiotics proposed including deconjugation catalyzed by bile salt hydrolase enzymes, binding of cholesterol to probiotic cellular surface and incorporation into their cell membrane, production of short chain fatty acids from oligosaccharides, co-precipitation of cholesterol with deconjugated bile, and cholesterol conversion to co-prostanol. The reduction of blood pressure may be related to vascular relaxation associated probiotic antioxidant activity and with some peptides derived from milk protein by probiotic fermentation with hypotensive action. The increase in hemoglobin and hematocrit levels could be attributed to increase iron absorption from the diet by probiotic because of reduction of intestine pH	Sperry et al. (2018)
Minas Frescal cheese	Cheese with *L. acidophilus* NCFM and other cheese with *L. rhamnosus* Lr-32	Humans with oral colonization by *Candida* and absence of clinical signs of denture stomatitis or candidiasis undergoing dental treatment/ 8 weeks	The probiotics in cheeses were able to reduce the colonization of oral *Candida* in complete denture wearers, suggesting their potential in reducing the risk of oral candidiasis in these highly susceptible subjects	UNINFORMED	Miyazima et al. (2017)
Cheddar cheese	*L. plantarum* K25	Mice Kunming/ 4 weeks	Reduction of total cholesterol and LDL	UNINFORMED	Zhang et al. (2013)

(continued on next page)

Table 4 *(continue)*

Type of cheese	Probiotic strain	Evaluated group/ time	Functional properties	Mechanism of action	Research
Goat coalho cheese	*L. rhamnosus* EM1107	Female wistar rats/ 14 dias	Reduction of damage caused by acetic acid-induced colitis	Improvement of tissue lesions, inflammatory and immune response with reduced levels of myeloperoxidase, TNF-α, IL-1β, IL-17 and NF-κB in colonic tissue; improvement in oxidative stress parameters (reduced concentration of malondialdehyde, increased total glutathione and suppression of iNOS expression in the colon); improvement of the mucosal barrier (increased expression of ZO-1 and MUC-2 and reduced MMP-2 and MMP-9)	Rodrigues et al. (2018)
Prato Cheese	*L. casei*-01	Male (8 weeks old) Wistar rats/ 25 days	Reduction of the development of implanted renal calculi in experimental model of urolithiasis	It is suggested that probiotic can degrade calcium oxalate in the intestinal lumen, decrease urinary excretion of oxalate and consequently decrease the risk of crystal formation	Martins et al. (2018)

5. Conclusions

The growing number of scientific studies focused on applying probiotics in cheeses and the relationship between these products and the benefits to consumer health allied to sensory attractions has lead to the market for these products becoming more and more competitive. Cheeses act as protective matrices to carry probiotics because some of their ingredients can contribute to maintain the viability of microorganisms in appropriate quantities, such as the availability of nutrients, fat and proteins that form a solid matrix with high buffering capacity, low oxygen content and high pH value compared to fermented milk.

In vitro and *in vivo* tests are compulsory for validating developed probiotic cheese products. *In vitro* trials should evaluate the nutritional and sensory quality of the matrices, the viability of the strains during processing, maturation, shelf life of the cheeses, as well as their passage along the gastrointestinal tract (GIT) until their arrival in the action site, the intestine. Other *in vitro* tests include evaluating the inhibition of pathogenic and deteriorating bacteria by probiotics, which may be effective in minimizing the damage caused by these agents to both food and consumer health. On the other hand, *in vivo* assessments are essential to prove the results of *in vitro* analyses through more precise investigation of the beneficial effects and possible action mechanisms of strains. Finally, clinical evaluations in humans are necessary to verify previous results, thus closing a cycle of analyses aimed at ensuring the safe and effective use of these strains inserted in a product of nutritional and sensory quality which meets consumer expectations.

Thus, much research has shown that certain types of microorganisms are beneficial to human health. However, the market and regulatory institutions will decide which probiotics will be made available to consumers, which limits the variability of available strains. However, it is important that such studies on probiotic products are properly conducted and disseminated so that they provide credibility and are a source of information available to health professionals and consumers.

Conflicts of interest

The authors declare no conflict of interest.

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ARTIGO ORIGINAL

MICROBIOLOGICAL, IMMUNOLOGICAL AND HISTOLOGICAL CHANGES IN THE GUT OF RATS FED GOAT CHEESE WITH *Lactobacillus rhamnosus* EM1107 CHALLENGED WITH *Salmonella* ENTERITIDIS

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Abstract

The present study aimed to evaluate the microbiological, immunological and histological changes in the gut of rats which were fed goat cheese with the probiotic *Lactobacillus rhamnosus* EM1107 and challenged with *Salmonella* Enteritidis. Male albino Wistar rats were randomly distributed into five experimental treatments: negative control (NC), positive control (PtC), control goat cheese (CCh), goat cheese supplemented with *L. rhamnosus* EM1107 (LrCh) and *L. rhamnosus* EM1107 only (EM1107). The strain, either in a cheese matrix or by itself, was able to significantly reduce *Salmonella* colonization in the intestinal lumen; to change the gut microbiota of rats by modulating commensal bacteria populations, such as those belonging to Lachnospiraceae family and genus *Blautia* and *Lactobacillus*; to decrease the production of cytokines (NF-κB, TNF-α, IL-1β and IFN-γ) in different days of infection; and to mitigate damage to intestinal tissue. The findings indicate that the treatment with EM1107 led to better effects when administered alone than probiotic cheese, although the cheese added or not with the probiotic strain could also mitigate damage to the intestinal tissue caised by *Salmonella* Enteritidis.

Keywords: Goat cheese; gut microbiome; sequencing analysis; probiotic; *Salmonella* Enteritidis; *Lactobacillus rhamnosus*

1. Introduction

Salmonella is considered one of the main agents of foodborne infection and its control is very difficult due to its wide distribution and complex epidemiology, high tolerance and adaptability to different environmental conditions and its resistance to multiple antimicrobial agents

(Chen et al., 2013). The indiscriminate use of antibiotics for treatment and prevention of salmonellosis can exert a selective pressure on the gut microbiota and thus contribute to the current emergence of multidrug-resistant strains (Carmo et al., 2018). Therefore, the mitigation of antimicrobial resistance must involve antimicrobial stewardship and the development of alternative products, such as probiotics, active peptides and vaccines. Probiotics have been considered a promising alternative for promoting intestinal health and, consequently, reducing the use of antimicrobials in food chain production systems (Ma et al., 2018).

Probiotics are defined as living microorganisms that provide benefits to the health of the host when administered in adequate concentrations (Hill et al., 2014). Probiotics have been successfully used for the prevention and treatment of gastrointestinal diseases such as salmonellosis, as demonstrated in some *in vivo* studies (Kemgang et al., 2016; Acurcio et al., 2017). Supplementation with probiotics can promote immunomodulatory effects that are characterized by increased innate and adaptive immune functions in response to the modulation of the gut microbiota (Ma et al., 2018). *Lactobacillus*, for example, play an important role in the process of regulating the commensal microbiota by inhibiting the growth of opportunistic pathogens such as *Salmonella* and *Escherichia. coli*, and promoting the growth of beneficial bacteria including *Lactobacillus* and *Bifidobacterium* genus (Kamdar et al., 2016; Zhang et al., 2017).

In this context, it is necessary to understand the microbial shifts triggered by probiotic products in order to elucidate their biological mechanisms on a solid scientific basis (Castillo et al., 2013; Ma et al., 2018). The microbiome plays a key role in many human health conditions. Therefore, we need to better understand the functions of microorganisms in foodstuff, as well as the products derived from their metabolism, which promote effects that go beyond the preservation of the product and its sensory attributes (Marco et al., 2017). In this aspect, fermented foods like cheeses could serve as a suitable matrix for supplying probiotic agents to consumers, enabling

appropriate conditions for bacteria to produce enzymes that promote biochemical processes. These enzymes act in the catabolism of proteins and lipids providing specific texture and flavor (Albenzio et al., 2013; Marco et al., 2017) and reducing protein oxidation by the formation of bioactive peptides (Mushtaq et al., 2016).

We hypothesize that coalho cheese, a typic semi-hard cheese marketed throughout Brazil, can grant protection to probiotic strains during passage through the gastrointestinal tract, as it consists of a solid buffered matrix with high pH and low oxygen concentrations. This rennet cheese has a cooked or pre-cooked mass, with high humidity (36-45%), semi consistency, texture and characteristic taste (Oliveira et al., 2012; Rolim et al., 2015). Furthermore, probiotic-added cheese produced from goat's milk can be an innovative alternative for the goat industry in order to diversify their products on the market (Dantas et al., 2016). The use of goat milk for cheese production could be an interesting alternative because it’s an excellent source of nutrients, which is rich in proteins with high biological value, essential fatty acids, high mineral bioavailability and vitamin contents. Moreover, goat dairy products are considered a delicacy in many countries (Verruck et al., 2019). Goat milk co-products have been reported to promote anti-inflammatory effects, reducing tissue damage (Rodrigues et al., 2018; Verruck et al., 2019).

Lactobacillus rhamnosus EM1107 is a probiotic strain, tested *in vitro* for identification and characterization, assessment of its technological properties, survival tests in food matrices and conditions simulating the gastrointestinal tract (Rolim et al., 2015; Santos et al., 2015). *In vivo* investigation showed that the consumption of cheese with *L. rhamnosus* EM1107 reduced injuries caused by acetic acid-induced colitis in rats. Animals fed *L. rhamnosus* EM107-added cheese had an overall improvement in immunity, reduction of inflammation and oxidative stress (Rodrigues et al., 2018). The promising findings observed *in vitro* and *in vivo* regarding its use as a probiotic starter culture, suitable for cheese, encourage further investigation on the putative protective effects

of this strain in animals infected by enteric pathogens. The present study aimed to evaluate the microbiological, immunological, and histological changes in the gut of rats fed goat cheese added with *L. rhamnosus* EM1107 before challenge with *Salmonella enterica* serovar Enteritidis.

2. Material and methods

2.1. Bacteria and inocula preparations

L. rhamnosus EM1107 was provided to this study by the Brazilian Agricultural Research Corporation (EMBRAPA, Rio de Janeiro). The strain *S.* Enteritidis P125109 was provided by the School of Veterinary Medicine and Science of the University of Nottingham, England. *L. rhamnosus* inocula were prepared according to Rodrigues et al. (2018).

The inoculum of *S.* Enteritidis was prepared on the day before the infection. To briefly describe the methodology applied in this study, the strain was inoculated in Brilliant Green agar (Oxoid), incubated at 37 °C/24 h and then in BHI broth (Sigma-Aldrich) at 37 °C/18 h, under aerobic conditions. After this period, the inoculum was centrifuged (4500G, 15min, 4°C), washed twice and resuspended in sterile saline solution (NaCl at 0.85%). Inoculum count was determined after serial dilutions (10^{-1} to 10^{-9}), followed by inoculation in BHI agar (Sigma-Aldrich) and incubation under aerobic conditions at 37 °C/24 h. The results were expressed as a log ratio of the colony forming units per mL (CFU/mL log) in 10^9 CFU/mL.

For this study, two types of coalho cheeses were produced: probiotic coalho cheese, in which coagulation was performed with addition of the coagulant enzyme (rennet) and *L. rhamnosus* EM1107 as the starter culture, and the coalho cheese without the supplementation of the probiotic bacteria (control cheese). Cheese was produced as previously described (Rodrigues et al., 2018).

2.2. Animals

The experimental protocol was approved by the Animal Ethics Committee of the Federal University of Paraíba (CEUA/UFPB-Protocol n° 021/2016), according to regulations established by the National Council for the Control of Animal Experimentation (CONCEA, Brazil). We used male albino Wistar rats (*Rattus norvegicus*) with 6 weeks of age, kept in cages, at room temperature (± 22°C), with light-dark cycle 12/12 h, *ad libitum* access to food (Presence-Purina®), and water.

2.3. Experimental design

The animals were randomly distributed into five experimental treatments: negative control (NC), positive control (PtC), control goat cheese (CCh), goat cheese added with *L. rhamnosus* EM1107 (LrCh) and *L. rhamnosus* EM1107 only (EM1107). For the NC and PtC groups, 1 mL of saline solution was administered to the animals as placebo; for the CCh and LrCh groups, 1 g of cheese only and cheese suplemented with the probiotic bacteria were administered, respectively. For the EM1107 group, 1 mL of 1 x 10^9 CFU of the probiotic in saline solution was administered. The animals received these treatments during all 24 days of the experiment, by intragastric gavage.

With the exception of the NC group, all other groups were infected with *Salmonella enterica* serovar Enteritidis. Prior to infection, the animals in these groups were pretreated with 20 mg streptomycin (Sigma-Aldrich), resuspended in distilled water and administered intragastrically on the 15th day of the experiment. Distilled water was provided to the animals in the NC group (Barthel et al., 2003). After 24 hours of the streptomycin treatment, the animals were infected with 1 mL of 10^9 CFU of *S.* Enteritidis P125109 and saline was provided to the animals of the NC group by intragastric gavage (Fig. 1A).

Thirty minutes before infection, the groups received 100 µL sterile 10% sodium bicarbonate solution dissolved in saline water by intragastric gavage, and the same amount of saline water was

provided only for NC group (Tennant et al., 2008). Animals were deprived of water and fed 4 hours before and 2 hours after P125109 administration (Moreau et al., 2016). After this time, the animals had access to water and food *ad libitum*. The animals were euthanized on days 1, 4 and 8 post infection (PI). Samples of the organs, faeces and caecal content were collected for further analysis.

2.4. Salmonella Enteritidis *enumeration*

Cecal contents samples were aseptically collected from the euthanized animals on day 1 PI. Liver and spleen fractions were also collected on 3 days of euthanasia for bacterial translocation assessment. Samples were serially diluted in saline water (NaCl at 0.85%) and aliquots transferred to Brilliant Green agar (Oxoid) plates supplemented with nalidixic acid (Sigma-Aldrich) (100 μg/mL), which were incubated under aerobic conditions at 37 °C/24 h for *Salmonella* counting. Counts were logarithmically transformed (log) and results expressed in log of CFU/g. Whenever a negative result was obtained in the counting procedure, a 1 mL aliquot of the first dilution was transferred to Rappaport Vassiliadis R10 broth (Acumedia), incubated at 37 °C/24 h, and then spread onto Brilliant agar plates supplemented with nalidixic acid (Sigma-Aldrich) (100 μg/mL) and incubated at 37 °C/24 h under aerobically conditions. The results were interpreted either as negative or positive.

2.5. 16S rRNA Microbiome sequencing analysis

2.5.*1 Samples and DNA extraction*

Stool samples from animals were collected on day 4 PI (20th experiment day), just before euthanasia. These samples were collected individually, directly transferred to DNAse/RNAse free microtubes and stored at -80 °C. Genomic DNA was extracted using a commercial kit (DNeasy

PowerSoil, Qiagen, Hilden, Germany) according to the manufacturer's recommendations. DNA concentration was determined by micro-volume spectrophotometer (Colibri). The integrity of the DNA was evaluated visually by electrophoresis in 1% agarose gel.

2.5.2 Library preparations and sequencing

DNA libraries were prepared using the Nextera XT Index Kit (Illumina) with multiplex indexing according to the manufacturer's protocol. Subsequently, the DNA fragments were purified with Agencourt AMPure XP reagent (Beckman). Purified PCR products were quantified by fluorometry using Qubit 3.0 (Life Invitrogen, San Diego, CA, USA).

Paired-end sequencing was performed in Illumina Miseq sequencer using V2 kit (Illumina Inc., San Diego, CA, USA) with 2 x 250 bp, according to HMP protocol (Caporaso et al., 2001). Phix (Illumina) kit at 15% run concentration was used as sequencing control. Briefly, the hypervariable regions V3 and V4 of the 16s rRNA gene were used for PCR amplification using primers (16S Amplicon PCR Forward Primer = *5' TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG; 16S Amplicon PCR Reverse Primer = 5' GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATC).*

2.5.3 16S rRNA amplicons pre-processing and ecological studies

Procedures followed raw, demultiplexed, paired-end fastq sequences being uploaded on QIIME 2 v.19.7 (BOLYEN et al., 2018). Thereafter, they were merged, selected through minimum and maximum sizes (200 ~ 500 pb), PHRED Score greater than (Q>20) and finally derreplicated to generate a OTU (Opetarional Taxonomic Unit) table using VSEARCH (ROGNES et al., 2016). Chimeric sequences were filtered out using UCHIME (EDGAR et al., 2011). The clusterization

was set to a default of 99% of simmilarity of the centroid region from the De Novo method. Sequences were aligned using mafft (KATOH et al., 2002) to further construct a phylogenetic tree by fasttree2 (PRICE et al., 2010) that served as input to interpret ecological studies. The number of sequences per sample was normalized to 14900 reads.

Alpha diversity was estimated by Chao1 and Shannon indexes and beta diversity was estimated by classical multidimensional scaling calculated with Unweighted Unifrac distance matrix (LOZUPONE; KNIGHT, 2005), represented in a Principal Coordinate Analysis (PCoA). Relatative abundance was assessed using package phyloseq v.1.8.2 (MCMURDIE; HOLMES, 2013) from R v.3.5.7 software. Taxonomic classification was assigned through Naïve Bayes method right of SILVA v. 132 data base cured for V3-V4 regions with 99% OTUs simmilarities (QUAST et al., 2013).

The Linear discriminant analysis effect size (LEfSe) was performed according to Segata et al. (2011) in order to identify putative OTUs associated with the differences between the treatment groups.

2.6 Gene expression by Real-time PCR (RT-PCR)

The expression of nuclear factor kappa B (NF-κB) from the animal cecum tissue was obtained by means of real-time PCR analysis according to the methodology described by Rodrigues et al. (2018). The primer used was nuclear factor kappa B p65 (NF-κB p65) (Fw: GAAGAAGCGAGACCTGGAGCAA, – number H9919C11); Rv: GTTGATGGTGCTGAGGGATGCT – number H9919C12) initiators (Forward- Reverse/Fw-Rv), The gene used as a normalizer of the experiments was GAPDH (Fw: AACTTGGCATCGTGGAAGG – number: Fw: I089C12; Rv: GTGGATGCAGGGATGATGATGTTC – number I0892D01). The software used for primers

design was Primers express, Thermofischer® Software 3.0.1. Serial number: 15428. The primers for GADPH and NF-κB were obtained from Invitrogen Life Technologies (Thermo Fisher Scientific Inc., Waltham, MA, USA).

2.7 Biochemical analyses

Cecum samples were also collected for biochemical analyses. Samples were stored at -80 °C until the analyses were performed. For the determination of tumor necrosis factor α (TNF-α), interleukin-1β (IL-1β), interleukin 10 (IL-10) and interferon-γ (IFN-γ), the tissue samples were chopped on an ice-cooled plate, homogenized, kept in tubes with 10 mmol/L phosphate buffer pH 7.4 (1:5 w/V) and centrifuged at 9000 G for 10 min at 4 °C. The concentration of TNF-α, IL-1β, IL-10 and IFN-γ was measured by enzymatic immunoadsorption assay (ELISA) using commercial kits (R & D Systems, Minneapolis, MN, USA), according to the manufacturer's instructions. The results were expressed in ng/g of tissue.

The determination of myeloperoxidase (MPO) was performed by the method described by Krawisz et al. (1984). The results were expressed as U/g tissue, and each MPO (U) unit was defined according to its capacity to degrade 1 nmol/min of hydrogen peroxide at 25 ºC. The absorbance was interpolated in a standard curve performed with human leukocyte myeloperoxidase (M6908, Sigma-Alddrich, São Paulo, Brazil LTDA). The determination of the malondialdehyde content (MDA) was performed by the method described by Esterbauer and Cheeseman (1990), and the results were calculated by interpolation in a standard curve performed with Malonaldehyde-bis (dihetil acetal) (T9889, Sigma-Alddrich, São Paulo, Brazil LTDA) and expressed in nmol/g of tissue. The absorbance was measured in a microplate reader (Polaris, Celer Biotechnology S. A, city) at 450 nm for cytokines and MPO and at 586 nm for MDA.

2.8 Histopathological assessment

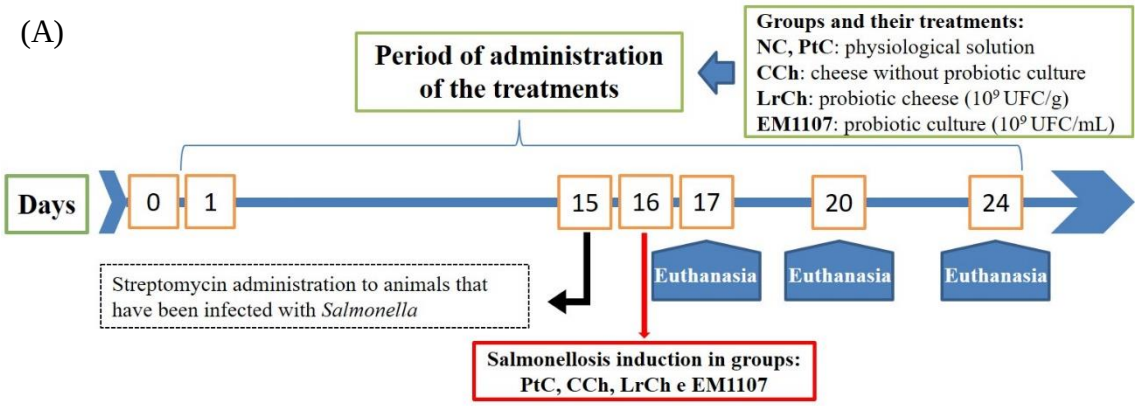
Fragments of the cecum were harvested from the same animals used for biochemical studies. The samples were fixed for 24 h in 10% formalin and then processed according to the routine histological technique. Paraffin-embedded material was sectioned at 5 µm thickness by means of a microtome and stained using Harris hematoxylin and eosin. Slide mounting was performed using synthetic resin (Entellan-Merck).

2.9 Statistical analyses

Conventional microbiological analyses of cecal contents, biochemical determinations and gene expression by RT-PCR were expressed as treatment mean ± standard error of means. Differences among treatment means were tested by analysis of variance (ANOVA), followed by Tukey test at 5% significance level. Statistical analyses were performed using the statistical program Graph Pad Prism 5.0 (GraphPad Software Inc., San Diego, CA, USA).

Alpha diversity index differences were evaluated by non-parametric Kruskall-Wallis pairwise test ($p < 00.5$). Beta diversity dissimilarities between treatments were evaluated by Permutational Multivariate Analysis (PERMANOVA) test (Anderson, 2001).

(A)



(B)

Groups	Log CFU/g
Positive control (PtC)	4,93±0,39[a]
Control cheese (CCh)	4,75±0,28[a]
Probiotic cheese (LrCh)	4,22±0,31[b]
L. rhamnosus (EM1107)	4,32±0,23[b]

Fig. 1. (A) Experimental design showing the treatments performed through chronological order and the main procedures used in the study and (B) *Salmonella* count of animal cecal content 24 hours post infection. Results were expressed as log of colony forming units per ml (log CFU/ g) and values represented as mean ± SEM. Caption: negative control (NC); positive control (PtC); control cheese (CCh); probiotic cheese (LrCh); *L. rhamnosus* EM1107 (EM1107).

3. Results

3.1 Intestinal colonization and systemic Salmonella infection

S. Enteritidis P125109 counts in the cecal contents of euthanized animals 24 hours PI were observed in all treatment groups that were infected, except in the negative control group. There was a difference ($p < 0.05$) in *S.* Enteritidis P125109 counts in cecal contents of rats between positive control (PtC) and control cheese (CCh) groups compared to the treatment groups *L. rhamnosus* (EM1107) and *L. rhamnosus*-added cheese (LrCh) (Fig. 1B). Translocation of *S. Enteritidis* P125109 to the liver and spleen of infected animals was verified by means of the microbial culture of euthanized animals on days 1, 4 and 8 PI (data not shown). The presence of *Salmonella* in the

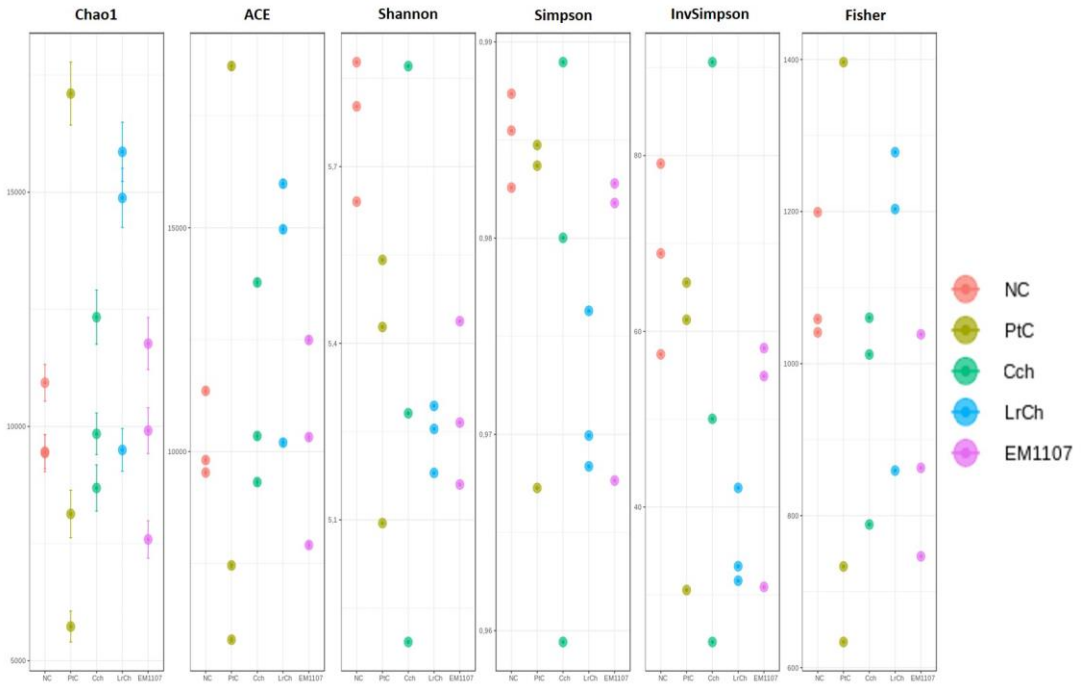
liver and spleen was detected since the beginning of the infection, indicating early penetration through the intestinal barrier and bacterial translocation. However, no statistical difference ($p < 0.05$) between the treatment groups was observed.

3.2 Analysis of the microbial community diversity

A total of 36,600 different OTUs (Operational Taxonomic Unit) were generated and compiled into a table using QIIME 2 platform. The average sequence size was 447.13 base pairs (bp), ranging from 200 bp to 490 bp. It was possible to identify a total of 34,590 different taxa. OTUs classified as Unassigned, Chloroplast, Mitochondria and D_0__Bacteria were removed to improve microbial composition interpretation.

According to alpha diversity indexes (Simpson and Shannon's diversity), samples belonging to NC treatment had the highest values and also presented a significant difference ($p < 0.05$) between LrCh, implying the use of the probiotic cheese might decrease microbial evenness (Figure 1A). On the other hand, the indexes related to microbial richness (Chao1 and ACE) showed an opposite behavior as the LrCh treatment presented the highest values probably due to the increase of LAB specimen in mice gut (Figure 2A). Beta diversity assessed by PCoA showed that the greatest segregation occurred between NC and LrCh clusters and the lowest one between NC and PtC, as the probiotic cheese was the most evident treatment to alter mice gut microbiota (Figure 3A), although no statistical significance was found between pairwise treatments, probably because of the low number of repeats.

(A)



(B)

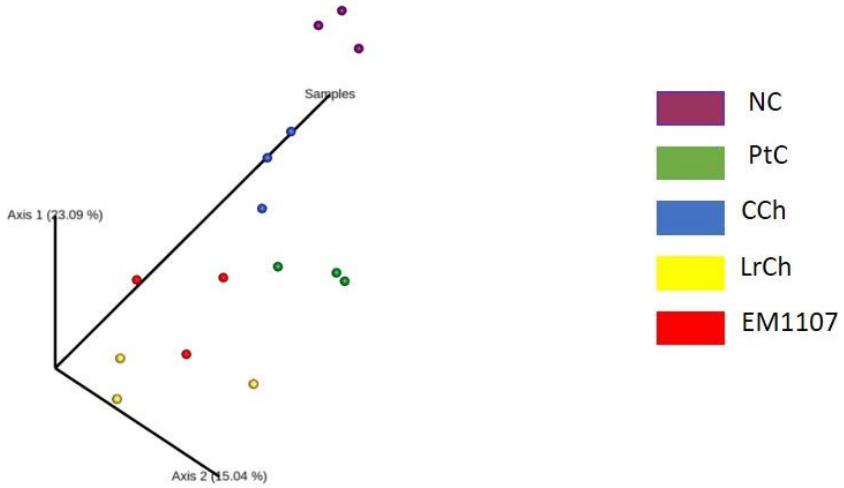


Fig. 2. Effect of administration of treatments on community diversities. (A) Plots showing alpha diversity analysis using Chao1, ACE, Shannon, Simpson, InvSimpson and Fisher indexes and (B) principal coordinate analysis (PCoA) of the community membership using Bray-Curtis distance, for each group. Caption: NC= negative control, PtC= positive control, Cch= control cheese, LrCh= probiotic cheese, EM1107= probiotic alone.

3.3 Metataxonomic composition

Regarding bacterial microbial composition by means of the relative abundances, at the phylum level, it was possible to identify a ratio variance regarding *Firmicutes* in NC (49.43%), CCh (53.14%), EM1107 (58.69%), PtC (67.92%), LrCh (69.82%) and *Bacteroidetes* in PtC (21.58%), LrCh (22.54%), EM1107 (32.15%), NC (39.82%) and CCh (42.88%) (Figure 3). At class level, these same proportion patterns repeated over *Bacteroidia* and *Clostridia* ratio (Figure 14 in Supplementary data), as well as with *Clostridiales* and *Bacteroidales* at the order level (Figure 15 in Supplementary data).

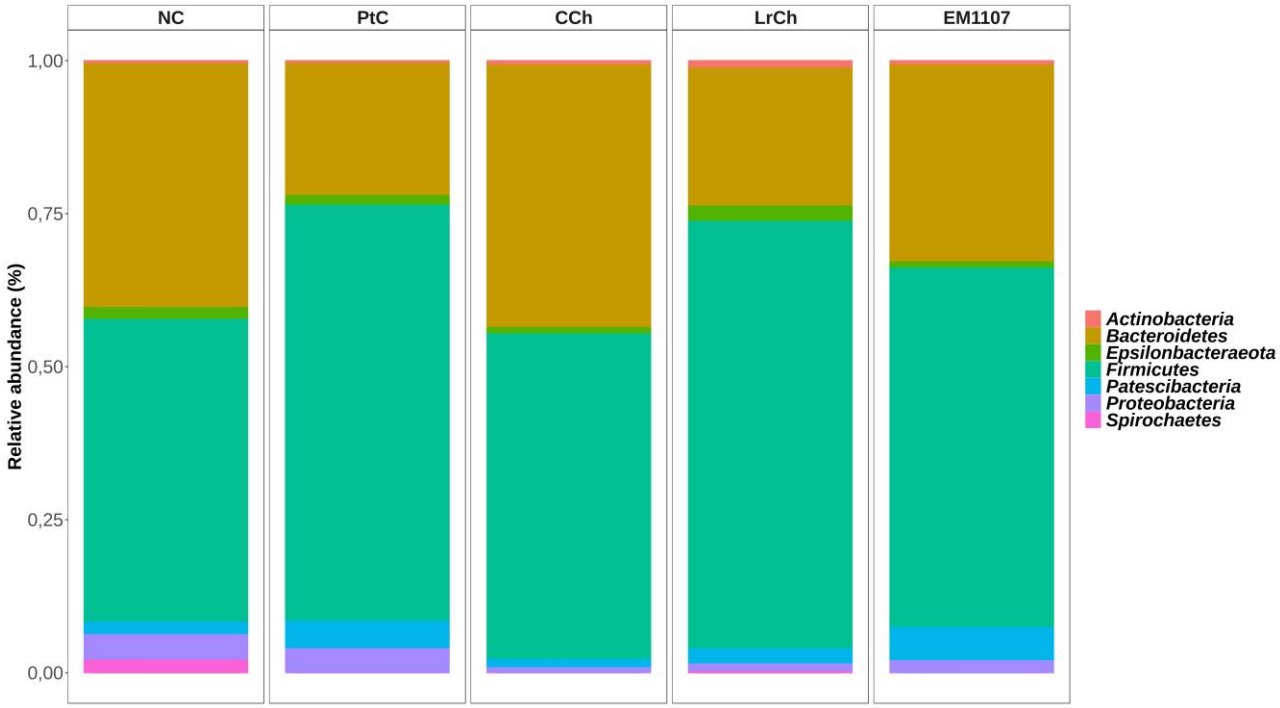


Fig 3. Effect of administration of treatments on bacteria phyla distributions (n = Top 5 abudant taxa) for each group. Caption: NC= negative control, PtC= positive control, Cch= control cheese, LrCh= probiotic cheese, EM1107= probiotic alone.

At the family level, several taxa diverged such as *Lachnospiraceae* in LrCh (40.36%), CCh (37.66%), EMM1107 (36%), PtC (30.49%) and NC (27.46%), *Prevotellaceae* in CCh (37.66%), NC (36.89%), EM1107 (26.58%), LrCh (18.48%) and PtC (17.21%), *Ruminococcaceae* in NC (23.51%), PtC (19.38%), EM1107 (16.98%), LrCh (16.09%) and CCh (14.92%) and *Erysipelotrichaceae* in PtC (8.41%), LrCh (7.29%), EM1107 (3.55%), CCh (3.4%) and NC (1.55%) (Fig. 4). Alongside the genus level, specially *Blautia* in LrCh (28.43%), EM1107 (23.47%), CCh (13.5%), PtC (11.26%) and NC (6.75%), *Prevotella* 9 in CCh (32.69%), NC (31.43%), EM1107 (17.89%), PtC (14.21%) and LrCh (10.74%); finally, *Ruminococcaceae* UCG-014 in PtC (11.27%), CCh (8.05%), NC (6.75%), EM1107 (5.52%) and LrCh (4.04%) (Fig. 5).

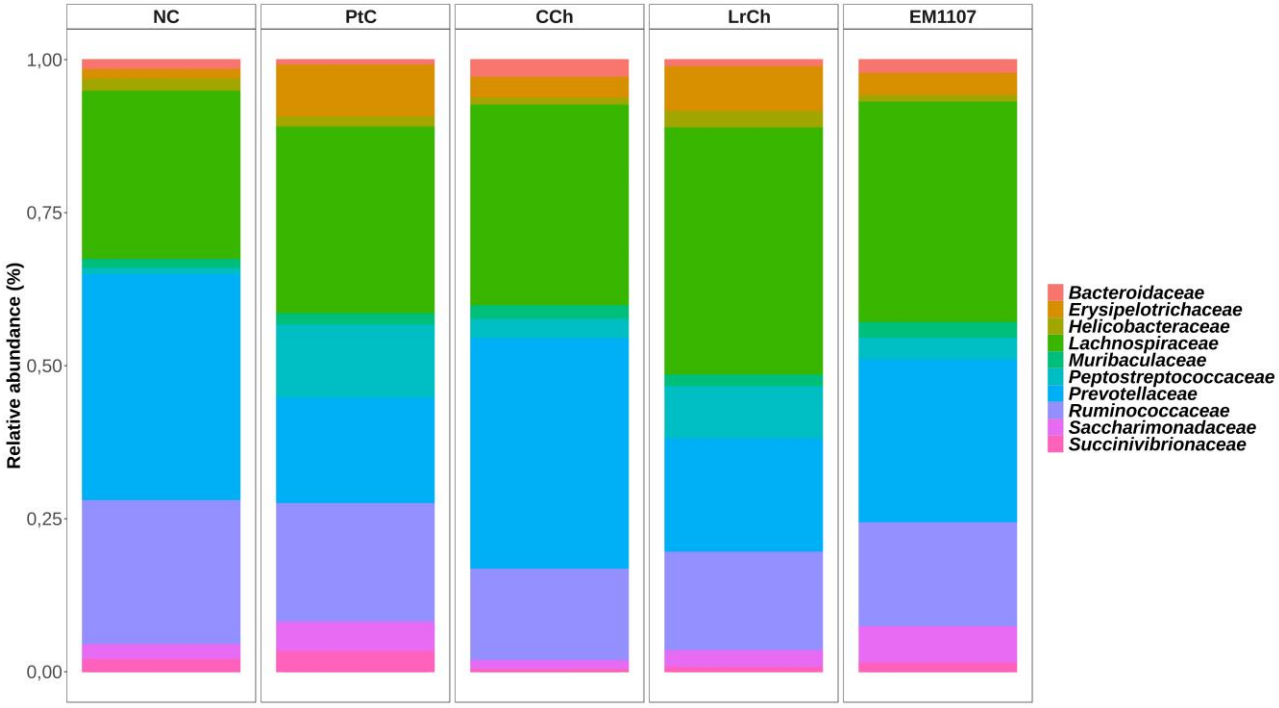


Fig. 4. Bacteria family distributions (n = Top 15 abudant taxa), for each group. Caption: NC= negative control, PtC= positive control, Cch= control cheese, LrCh= probiotic cheese, EM1107= probiotic alone.

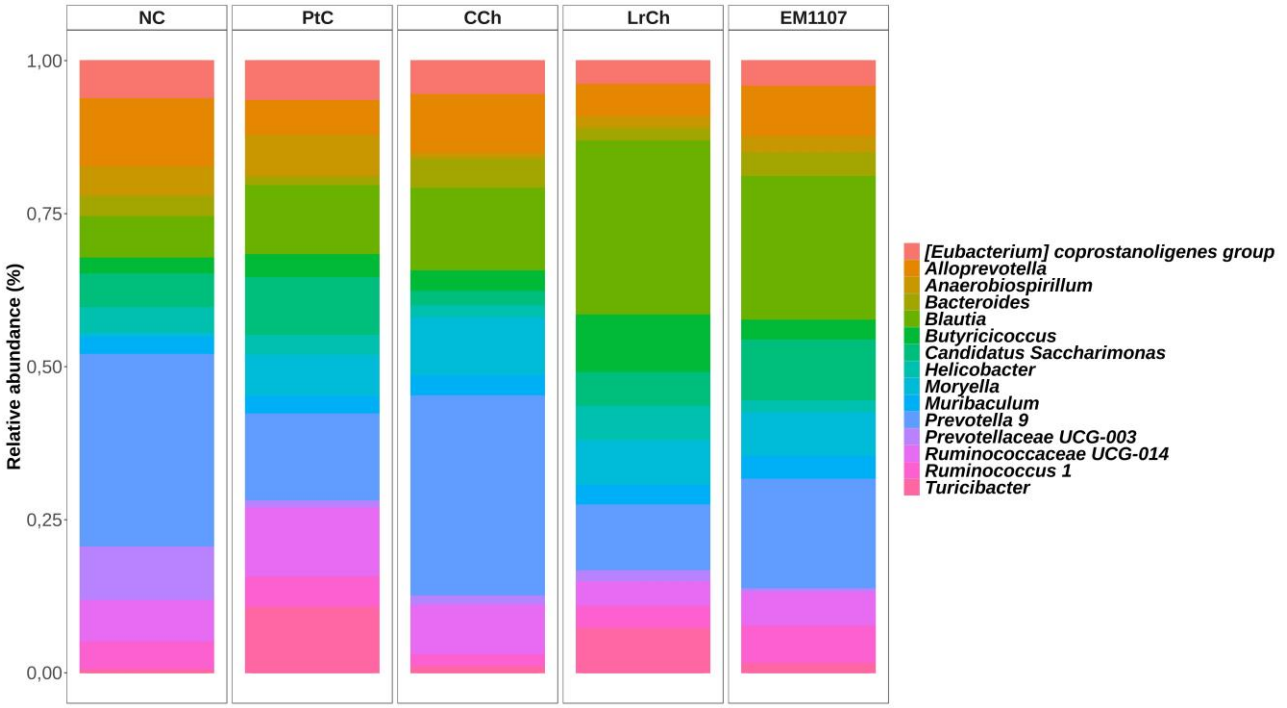


Fig. 5. Effect of administration of treatments on bacteria genera distributions (n = Top 15 abudant taxa) each group. Caption: NC = negative control, PtC = positive control, Cch = control cheese, LrCh = probiotic cheese, EM1107 = probiotic alone.

Although *Lactobacillus* was not among the top genera in any treatment, it was still present, therefore, this genus was selected and its differential abundance chart plotted as shown in Fig. 6, where the LrCh presented the highest values, notwithstanding, it possessed significant differences from PtC and NC. LEfSe results demonstrated several dominant features associated gut microbial uncultured microrganisms related to NC in comparison with other treatments, which presented with a low number of important features (Fig. 7).

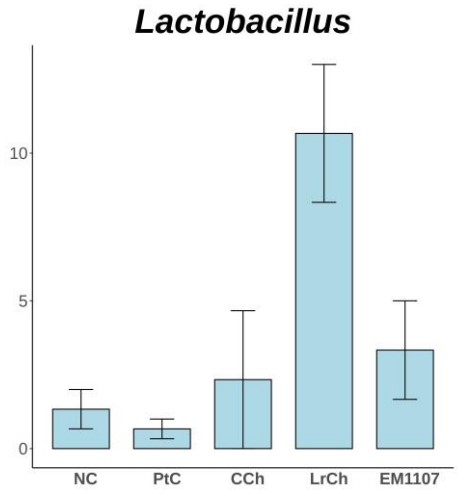


Fig. 6. Effect of administration of treatments on the raw count of the OTUs classified as *Lactobacillus* distributed between each group. Caption: NC = negative control, PtC = positive control, Cch = control cheese, LrCh = probiotic cheese, EM1107 = probiotic alone.

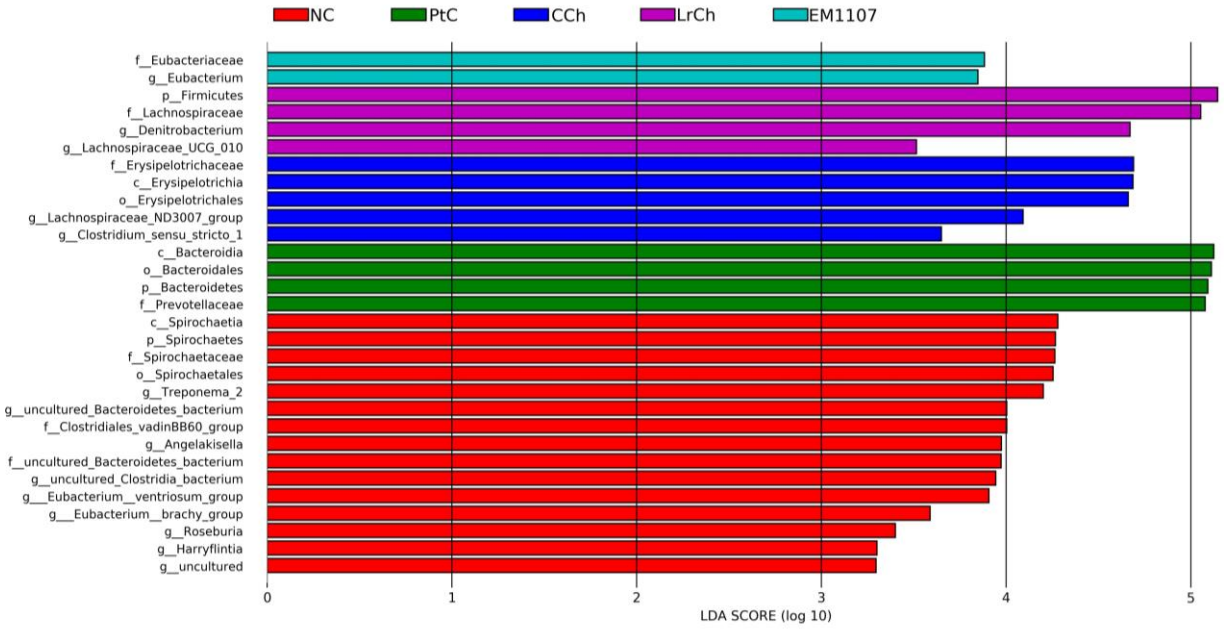


Fig. 7. Effect of administration of treatments on LEfSe results between each group. Caption: NC = negative control, PtC = positive control, Cch = control cheese, LrCh = probiotic cheese, EM1107 = probiotic alone.

3.4 NF-κB determinations

At the beginning of the infection (1st day), it was verified that the expression of NF κB in PtC was higher ($p < 0.05$) compared to the other treatment groups. This difference was observed until Day 4 PI throughout the experimental period in relation to CCh and EM1107 groups. We observed lower ($p < 0.05$) NF-κB determinations in rats fed LrCh compared to PtC on the last day of evaluation (Day 8) (Fig. 8A).

3.5 Cytokine level

At the beginning of the infection (1st day), EM1107 treatment group showed a reduction ($p < 0.05$) in IL-1β and IFN-γ levels compared to PtC. At the 4th day PI, a reduction ($p < 0.05$) of IFN-γ was observed in LrCh and EM1107 groups compared to PtC. On the last day of infection (8° Day), TNF-α was lower in CCh and EM107 treatment groups, while an IFN-γ decreased in both experimental groups in which rats were fed on cheese (CCh and LrCh), in relation to PtC. No statistical difference it was observed on the IL-10 level between *Salmonella*-infected groups (Fig. 8).

3.6 Myeloperoxidase (MPO) and Malondialdehyde (MDA) determinations

There was a reduction ($p < 0.05$) in MPO activity in EM1107 group compared to PtC on the 4th day PI. Higher levels ($p < 0.05$) of MPO were observed in rats that received cheese (LrCh and CCh treatment groups) compared to the PtC group during the course of infection. For MDA levels, no difference ($p \geq 0.05$) was observed among the treatment groups, except for a reduction ($p < 0.05$) in the EM1107 treatment group in the 4th and 8th days PI compared to the PtC (Fig. 9).

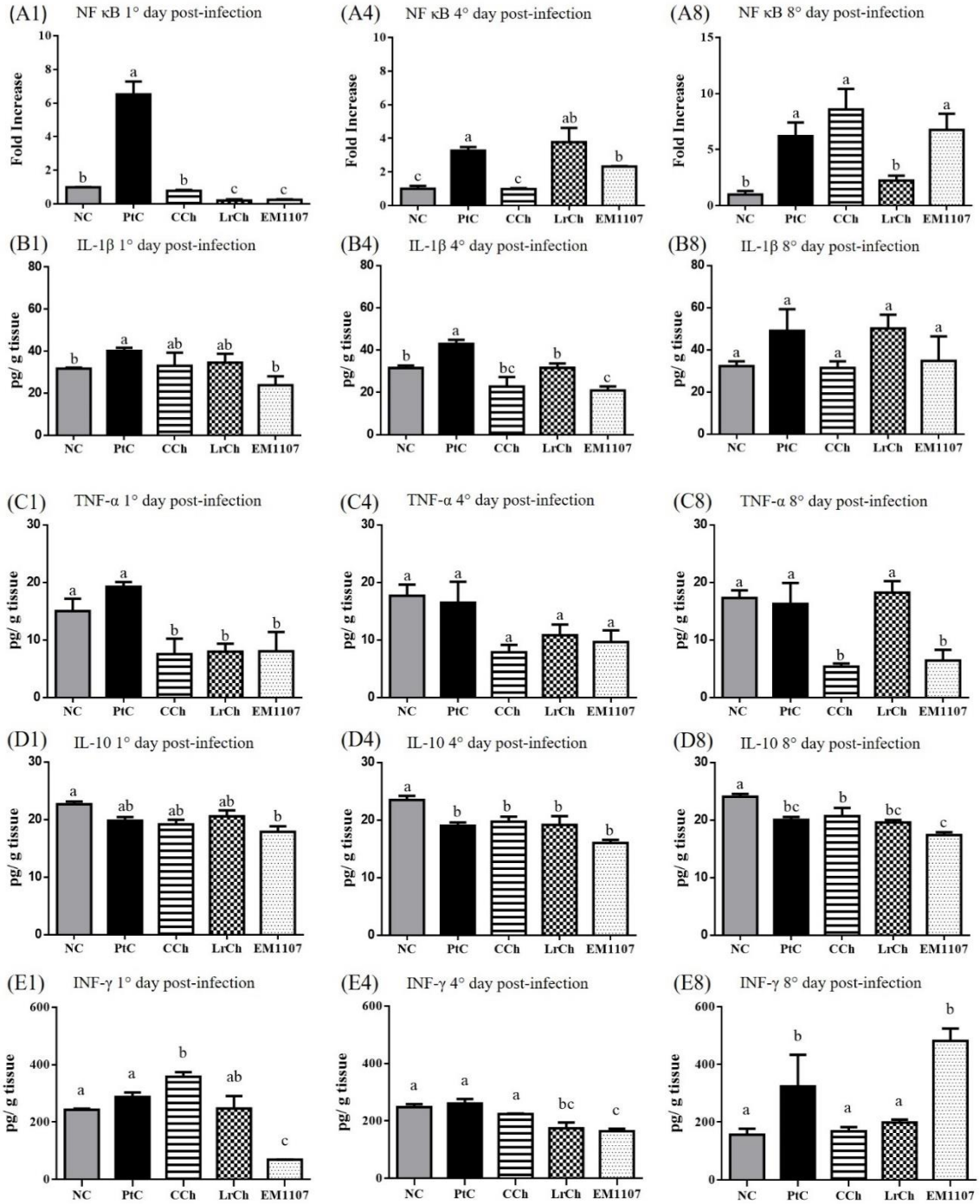


Fig. 8. Effect of administration of treatments on levels of (A) NF kB, (B) IL-1β, (C) TNF-α, (D) IL-10 and (E) interferon gamma (INF-γ) of cecal tissues in euthanized animals on 1° (1), 4° (4) and 8° (8) days after salmonellosis infection. Data are expressed as mean ± SEM. Caption: negative control (NC); positive control (PtC); control cheese (CCh); probiotic cheese (LrCh); *L. rhamnosus* EM1107 (EM1107). Different letters indicate significant differences ($p < 0.05$).

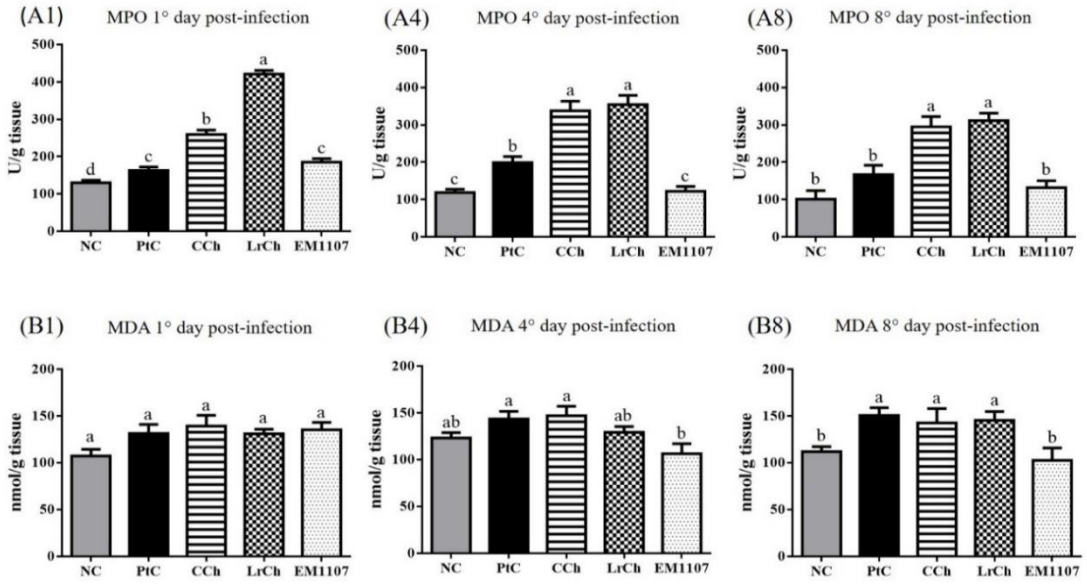


Fig. 9. Effect of administration of treatments on (A) myeloperoxidase (MPO) activity and (B) malondialdehyde (MDA) content in cecal tissues of euthanized animals on 1° (1), 4° (4) and 8° (8) days after salmonellosis infection. Data are expressed as mean ± SEM. Caption: negative control (NC); positive control (PtC); control cheese (CCh); probiotic cheese (LrCh); *L. rhamnosus* EM1107 (EM1107). Different letters indicate significant differences ($p < 0.05$).

3.7 Histopathological assessment

At the beginning of the infection (1st day), there was a reduction of neutrophilic inflammation in the EM1107 group with similar findings in healthy animals (NC). In the LrCh group, discrete lymphohistiocytic inflammation was observed in the cecal mucosa accompanied by discrete edema of the submucosa of the animals. The PtC group presented diffuse lymphohistiocytic inflammation in the cecal mucosa accompanied by severe hemorrhage and submucosal edema in the animals (Fig. 10). With the advance of time (4th day PI), a slight multifocal lymphohistiocytic inflammation in the mucosa of the animals was found in EM1107, while the PtC group presented diffuse lymphohistiocytic inflammation in the cecal mucosa accompanied by edema of submucosa. Like EM1107, the LrCh group presented a reduction of tissue inflammation, with discrete lymphohistiocytic inflammation in the cecal mucosa accompanied by discrete submucosal edema, compared with PtC (Fig. 11). Finally (8th day PI), there was an apparent improvement in

lymphohistium inflammation in the EM1107 group, while in the LrCh group there was a slight improvement at the submucosa level (Fig. 12).

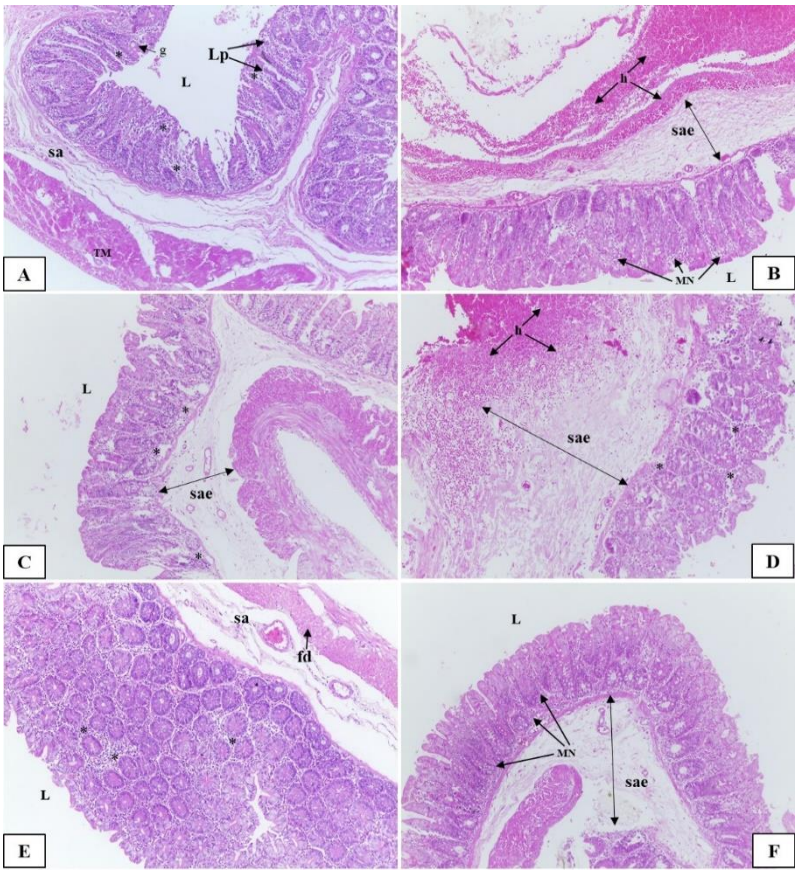


Fig. 10. Histopathological analysis of Wistar rat caeca on the first day after challenge with Salmonella Enteritidis P125109 with different degrees of tiflite. (A) Mild neutrophilic inflammation of the caecum, in addition to submucosal edema in animals of the NC group. (B) Diffuse lymphohistiocytic inflammation in the caecal mucosa accompanied by severe hemorrhage and submucosal edema in PtC group animals. (C) and (D) Focal and discrete neutrophilic and lymphohistiocytic inflammation in the cecal mucosa with marked hemorrhage and edema in the submucosa of the ChC group animals. (E) Discrete multifocal neutrophilic inflammation in the caecum mucosa with slight degeneration of smooth muscle fibers from the EM1107 group. (F) Mild lymphohistiocytic inflammation in the caecal mucosa accompanied by mild submucosal edema of the animals of the LrCh group. Caption: negative control (NC); positive control (PtC); control cheese (CCh); probiotic cheese (LrCh); *L. rhamnosus* EM1107 (EM1107); L, cecal lumen; Lp, lamina propria; g, goblet cells; sa submucosa; TM, muscular tunic; h, hemorrhage; leaves, submucosa edema; NM, lymphohistiocytic and / or lymphohistioplasmicocyte infiltrate; fd, degeneration of smooth muscle fibers; *, neutrophilic infiltrate. H&E coloring. Magnification: 100X.

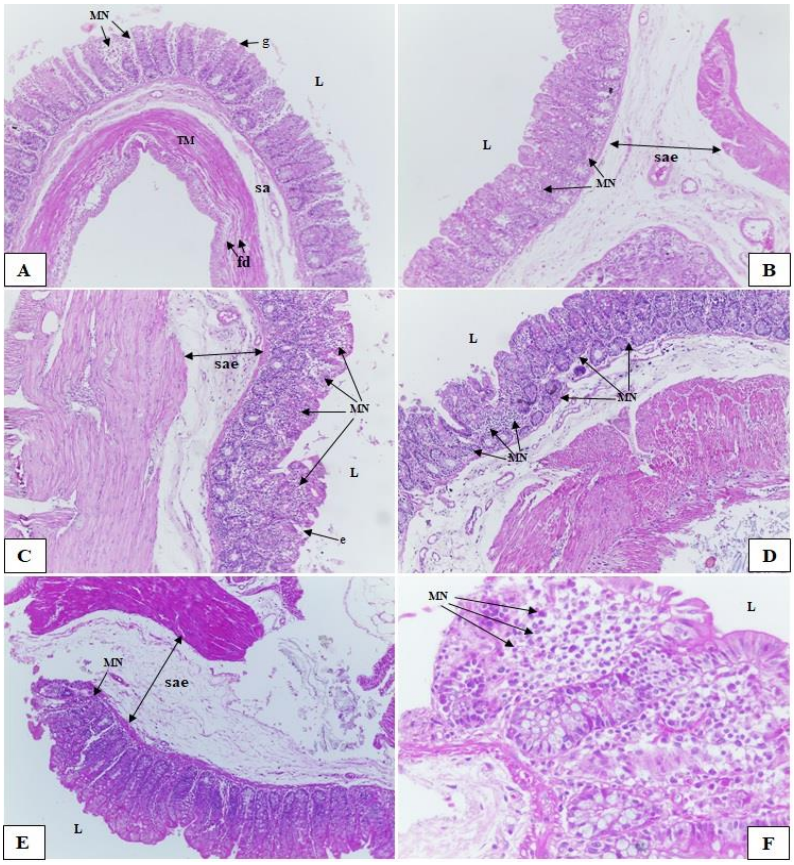


Fig. 11. Histopathological analysis of Wistar rat caeca on the fourth day after challenge with Salmonella Enteritidis P125109 with different degrees of tiflite. (A) Focal lymphohistiocytic inflammation in the caecum mucosa of animals of the NC group. Magnification: 100X. (B) Diffuse lymphohistiocytic inflammation in the caecal mucosa accompanied by submucosal edema in PtC group animals. Magnification: 100X. (C) Lymphohistiocytic inflammation and slight erosion of the epithelium in the cecal mucosa with slight edema in the submucosa of the animals of the ChC group. Magnification: 100X. (D) Discrete multifocal lymphohistiocytic inflammation in the cecum mucosa of animals of group EM1107. Magnification: 100X. (E) Discrete lymphohistiocytic inflammation in the caecal mucosa accompanied by slight edema of the submucosa of the animals of the LrCh group; and (F) Inflammatory lymphohistiocytic infiltrate in the lamina propria of the animals of the LrCh group evidenced at greater magnification (400X). Caption: negative control (NC); positive control (PtC); control cheese (CCh); probiotic cheese (LrCh); L. rhamnosus EM1107 (EM1107); L, cecal lumen; g, goblet cells; sa submucosa; TM, muscular tunic; h, hemorrhage; leaves, submucosa edema; NM, lymphohistiocytic and / or lymphohistioplasmicocyte infiltrate; and, erosion of the lining epithelium; fd, degeneration of smooth muscle fibers. H&E coloring

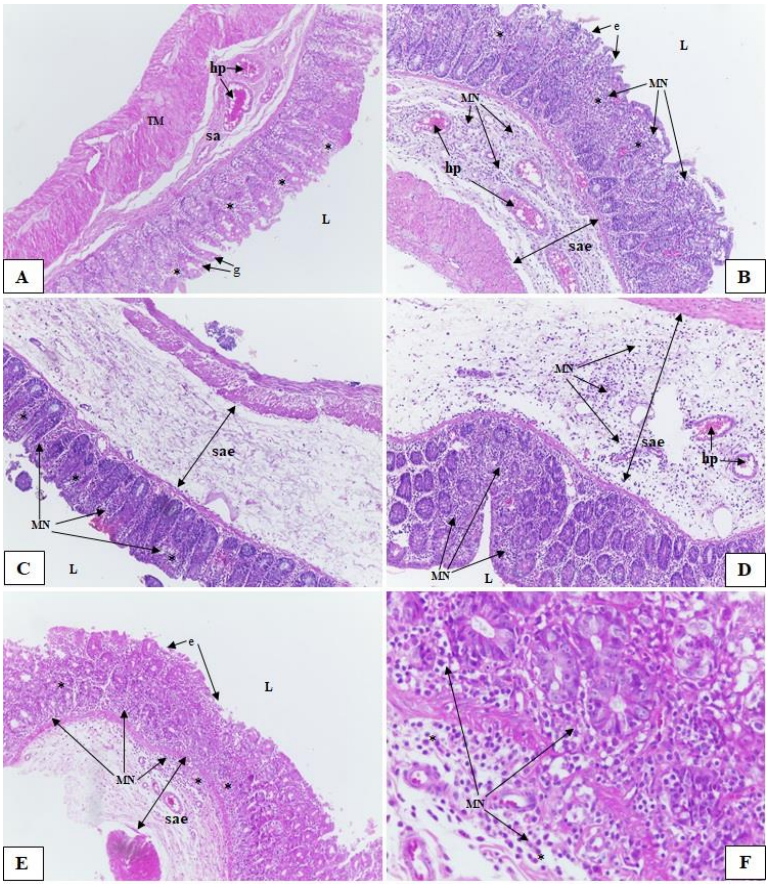


Fig. 12. Histopathological analysis of Wistar rat caeca on the eighth day after challenge with Salmonella Enteritidis P125109 with different degrees of tiflite. (A) Discrete multifocal neutrophilic inflammation in the cecal mucosa and hyperemia in the submucosa of animals in the NC group. Magnification: 100X. (B) Moderate diffuse neutrophilic and lymphohistiocytic inflammation in the cecal mucosa, as well as multifocal erosion of the epithelium and lymphohistioplasmic cell inflammation with diffuse edema in the submucosa of PtC group animals. Magnification: 100X. (C) Multifocal neutrophilic and lymphohistiocytic inflammation in the caecum mucosa with diffuse edema in the submucosa of the ChC group animals. Magnification: 100X. (D) Discrete multifocal lymphohistiocytic inflammation in the cecum mucosa and lymphohistiocytic inflammation, in addition to diffuse edema with focal hyperemia in the submucosa of animals of the EM1107 group. Magnification: 100X. (E) Diffuse neutrophilic and lymphohistiocytic inflammation, as well as erosion of the cecal mucosal epithelium accompanied by mild lymphohistioplasmicocyte infiltrate and edema in the submucosa of the animals of the LrCh group; LrCh group evidenced at higher magnification (400X). Caption: negative control (NC); positive control (PtC); control cheese (CCh); probiotic cheese (LrCh); L. rhamnosus EM1107 (EM1107); L, cecal lumen; g, goblet cells; sa submucosa; TM, muscular tunic; hp, hyperemia; leaves, submucosa edema; NM, lymphohistiocytic and / or lymphohistioplasmicocyte infiltrate; and, erosion of the lining epithelium; *, neutrophilic infiltrate. H&E coloring.

4. Discussion

In the present study, it was found that *L. rhamnosus* EM1107 led to anti-inflammatory effects associated with the ameliorating the parameters of oxidative stress, modulation of the

immune system and intestinal microbiota in rats challenged with *S.* Enteritidis. The administration of *L. rhamnosus* EM1107, either in a cheese matrix or alone, was able to reduce *Salmonella* colonization. A previous *in vitro* study showed that a probiotic *Lactobacillus* sp. strain (KSBT56) led to an inhibitory effect over *S.* Enteritidis P125109 by means of decreasing its multiplication, adhesion and invasion in colon epithelial cells, probably due to the production of lactic acid (Das et al., 2013). Bacteria of the genus *Lactobacillus* metabolize carbohydrates to produce lactic acid that decreases fecal pH. We believe that the lower pH contributed to the reduced *Salmonella* counts in fecal contents of rats receiving the probiotic strain (EM1107 and LrCh). Hydrogen peroxide, carbon dioxide and antibacterial compounds, including bacteriocins and non-bacteriocins and non-lactic acid molecules can also be produced by acid lactic bacteria (Marianelli et al., 2010; Zhang et al., 2018).

In addition, the presence of *Salmonella* in the liver and spleen in the beginning of the infection in the animals of all groups challenged with the pathogen confirms the invasiveness of the *S.* Enteritidis strain used in the present study. According to Finlay and Brumell (2000), *S.* Enteritidis can cross the lining of intestinal epithelial cells and translocate to extra-intestinal organs such as the spleen and liver. *Salmonella*'s ability to spread to other organs and survive in host defense cells, such as macrophages and dendritic cells, is attributed to the Salmonella Pathogenicity Island 2 (SPI-2), which encodes for genes that contribute to cell proliferation in extra-intestinal tissue and therefore causing systemic infections (Silva et al., 2012; Foley et al., 2013). According to our model, the treatment with *L. rhamnosus* EM1107 had no protective effect on the invasiveness of *Salmonella* promoted by SPI-2.

The 16S rRNA metataxonomic analysis revealed that the introduction of *L. rhamnosus* EM1107 led to significant changes in the gut microbiota of rats associated with smaller diversity indexes related to evenness and greater ones accounting for richness if compared with the sham

treatment (NC), probably because of the antimicrobial effect of the probiotic strain acting as a rare specimen colonizing rat's gut. It has been stated that the use of feed supplementation enriched with LAB can cause shifts in gut microbiome in several species, such as shrimps (Sha et al., 2016) and chickens (Wang et al., 2017). The dissimilarities between NC and the other treatments, mostly the LrCh group, indicate that the insertion of an alien bacterium strain into the gut, as determined by beta diversity analysis, can cause a switchover into the microbial composition to the point of decreasing the relatedness in each community. It was clear NC presented plentiful different microrganisms as the most important features as opposed to the narrow scope in the remaining treatments,

L. rhamnosus strain EM1107 supplementation favored the growth the of some commensal bacteria, such as those belonging to the family Lachnospiraceae and the genus *Blautia*. Members of Lachnospiraceae are abundant gut commensal organisms and are consistently depleted in people with intestinal diseases suggesting that these organisms are important in maintaining intestinal homeostasis (Suchodolski, 2013). Reeves, Koenigsknecht et al. (2012) demonstrated that Lachnospiraceae strains were able to partially restore eubiosis in response to *Clostridium difficile* post-infection and improve clinical outcome in germfree mice. According to Jenq et al. (2015), *Blautia* spp. are organisms are associated with reduced lethal-graft-versus-host disease besides they are also negatively correlated with poor health conditions, e.g. malnutrition (Million et al., 2016), Irritable Bowel Syndrom (Weinbergga et al., 2018) and early-stage breast cancer patients, as it's speculated these bacteria can metabolize to estrogen-like substances in hosts organism (Luu et al., 2017).

Milk may pose as a good source of *Prevotella* organisms to consumers, as already seen in neonates of several species (Brink et al., 2019; Petrullo et al., 2019). The fresh cheese probably served as a *Prevotella* source for mice gut colonization. Strains assigned as *Lactobacillus* had a

greater survival/abundance when administered with cheese, i.e. our findings suggest that the supplemented cheese can serve as the ideal delivery product to culture matrix for the probiotic strain *L. rhamnosus* EM1107. Cheese is one of the best food favoring LAB, because its protective effects in comparison to other dairy products, due to its chemical and physical properties such as less acidic environment, high buffering capacity and low oxygen content due to the solid matrix composed both of high fat and protein contents (Cichosz et al., 2014; Haman and Ahmed 2019).

Previous studies showed that the consumption of probiotic-added dairy products are associated with beneficial microbial effects in hosts under several clinical disorders. Non-fermented dairy products added with *Lactobacillus* spp. may halt dysbiosis associated with penicillin post-administration, for example the overgrowth of *Clostridium* spp. (Korpela et al., 2016). Even so, dairy products supplemented with probiotics may promote the growth of butyrate producing strains, which may lead to amelioration of auto-imune illnesses such as atopic dermatitis (Jong-Hwa et al., 2019).

Invasive pathogens, such as *Salmonella*, stimulate the induction of the innate host immune response, so the intestine is in a constant state of moderate inflammation. The intestinal endothelium detects the presence of the invader through receptors and begins activation of mitogen-activated protein kinase (MAPK) that induces gene expression of transcription factors such as activating protein 1 (AP-1) and nuclear factor kappa B (NF-kB). This will result in the production of pro-inflammatory cytokines, IL-18 (Interleukin-18) and IL-12, which stimulate the activation of Ta-1 cells (type 1 helper cells) which in turn synthesize IFN-γ (Interferon-γ), a substance that will increase phagocyte activity and the destruction of internalized bacteria. IL-1β, TNF-α and IL-23 recruit neutrophils that are responsible for the defense of extracellular bacteria (Santos et al., 2009; Thiennimitr et al., 2011). In contrast, the use of probiotics can modulate different pro-inflammatory cytokine profiles such as TNF-α, IFN-γ and IL-1β and anti-inflammatory cytokines such as IL -10

and IL-4, which may promote improved host immune response against invasion by pathogens such as *Salmonella* (Castillo et al., 2012; Alvim et al., 2016).

From the reduction in the expression of NF-κB, at the beginning and throughout the salmonellosis (1st and 4th days PI) in the animals of the EM1107 group, it can be inferred that there was a consequent decrease in TNF-α, IL-1β and IFN-γ; in LrCh, the reduction of the levels of NF-κB, which occurred on 1st and 8th days PI, brought in cytokines on different days: 1° for TNF-α, 4° for IL-1β and 4° and 8° for IFN-γ. This unevenness in the results may have been due to the effect of the lactic matrix, which influenced the postponement of the effects of cytokine suppression. It is important to note that the CCh group was also able to inhibit the expression of NF-κB along salmonellosis (1st and 4th days PI), with consequent reduction in TNF-α levels on the 1st and 8th days PI, IL-1β during infection (4th day PI) and IFN-γ on the last day evaluated (8th day PI).

Due to the complexity of a food matrix in terms of its composition, further studies are necessary to verify which compounds contributed to the result of the animals that received the milk matrix. In a recent study using the same probiotic strain added in cheese and administered alone, a reduction in the expression of NF-κβ, TNF-α and IL-1β, three days after the induction of the disease was observed in a model of acetic acid-induced colitis (Rodrigues et al., 2018).

As a consequence of TNF-α induction by *Salmonella*, neutrophil infiltration into the epithelium generates the release of myeloperoxidase (MPO), an enzyme responsible for the overproduction of reactive oxygen species (ROS) that causes a decrease in intestinal antioxidant defenses, such as glutathione (De Moreno de LeBlanc and Perdigón, 2010) and also, there is an increase in the production of malondialdehyde (MDA), a secondary product of lipid peroxidation. In the present study, there was a reduction in MPO activity during infection (4th day PI) in the EM1107 treatment compared to PtC. Therefore, EM1107 has been shown to reduce the effects of

inflammatory stress caused by the infectious bacteria. In addition, MPO activity was higher in the cheese treatment groups.

Despite the improvement of inflammation observed in the histopathological findings for the cheese-treatment groups, with or without the addition of EM1107, compared to the PtC group, no reduction of MPO activity at the beginning and end of evaluated periods (1st and 8th days) were observed in the EM1107 group. Similar result was found by De Moreno de LeBlanc and Perdigón (2010), who found that the treatment with a probiotic strain reduced the inflammatory response but was not able to reduce the MPO activity. In addition, the improvement of tissue inflammation in cheese groups can be explained by the presence of glutathione peroxidase in goat milk, which is part of a defense system against pathogenic microorganisms (Slačanac et al., 2010).

MDA is a parameter for assessing oxidative stress caused by tissue inflammation, as increased free radicals cause a higher concentration of MDA, a secondary product of lipid peroxidation, which can cause DNA damage, resulting in lysis and cell death (Barreiros and David, 2006). In the present study, there was a reduction in MDA levels throughout the course of infection (4th and 8th days) in the EM1107 treatment compared to PtC group, demonstrating that the probiotic strain alone was able to reduce intestinal oxidative stress. Our results support previous findings showing MDA reduction in rats receiving EM1107 and submitted to the acetic acid-induced colitis (Rodrigues et al., 2018).

The inflammatory response observed in the cecum of PtC group animals are typical *S.* Enteritidis lesions described in previous studies, characterized by enterocolitis with infiltration of polymorphonuclear leukocytes (PMN), and presence of edema, hemorrhages and epithelial erosion (Haraga et al., 2008; Santos et al., 2009). Throughout infection, a general reduction in inflammation was observed in rats from EM1107, LrCh and CCh groups compared to PtC group. Moreover, EM1107 and LrCh groups had similar histological findings than NC group on the 1st and 4th days

PI and 4th day PI, respectively. De Morenod de LeBlanc et al. (2010) reported no inflammation but only increased lymphohistiocytic infiltration in rats receiving *L. casei* 431 for 7 days after they had been challenged with *S.* Typhimurium. Similarly, Kemgang et al. (2016) observed a significant improvement in villi structure of animals fed *L. rhamnosus* S1K3, 5 to 20 days after infection, compared to the control group. The protective action of probiotics in minimizing tissue damage caused by *Salmonella* has also been demonstrated by other authors (Asahara et al., 2011; Castillo et al., 2013; Noto Llana et al., 2013).

Importantly, according to a very similar study using a chemically induced colitis model (Rodrigues et al., 2018), EM1107 treatment increased the expression of MUC-2 indicating that mucus protects the gut epithelium of the animal and it is suggested that this protection reduces the invasiveness of *Salmonella*. Although goat cheese contributed to an improvement against tissue damage caused by *Salmonella*, no synergic effect was observed by adding EM1107 in the form of a probiotic cheese.

5. Conclusions

L. rhamnosus EM1107 administration, alone or added to cheese, was able to reduce Salmonella infection at early stages in the intestinal lumen of rats. It minimized *Salmonella*-induced tissue damage and reduced the production of pro-inflammatory cytokines, while promoted significant changes in the gut microbial composition mainly related to the genus *Blautia* and *Lactobacillus*. The administration of *L. rhamnosus* EM1107 alone stood out in terms of beneficial health effects associated with antioxidant and immunomodulatory properties than the probiotic cheese, although cheese added or not with the probiotic starter culture could also mitigated intestinal tissue damage.

Conflicts of interest

The authors declare no conflict of interest.

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Ethics statements

All procedures were approved by the Animal Ethics Committee of the Federal University of Paraíba (CEUA/UFPB- protocol n°. 021/2016) following the National Institutes of Health (United States) guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978).

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Supplementary data

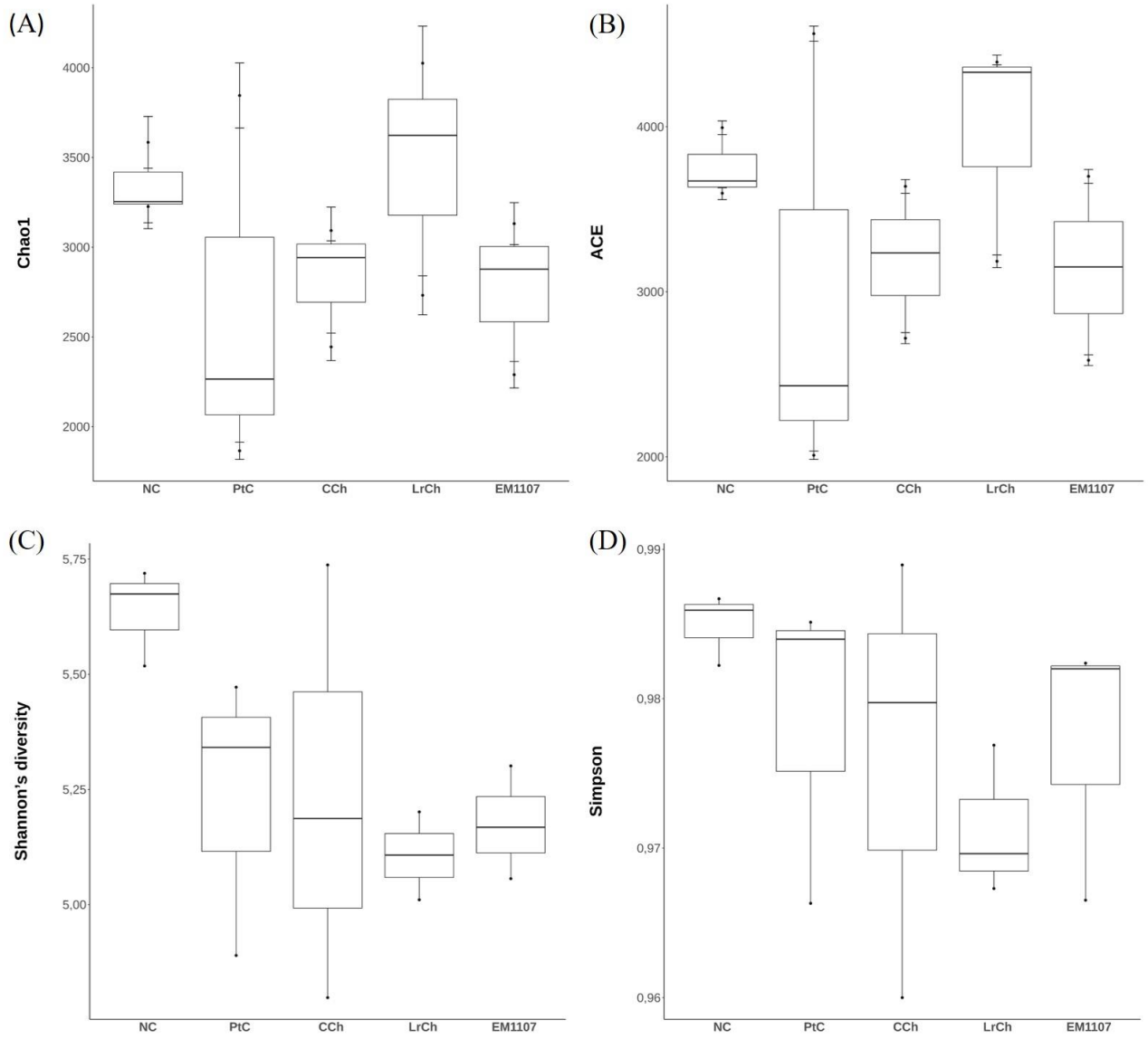

Fig. 13. Statistical effect of administration of treatments on alpha diversity using (A) Chao1, (B) ACE, (C) Shannon and (D) Simpson indexes, for each group. Data are expressed as mean ± SEM; ($p < 0.05$). Caption: NC= negative control, PtC= positive control, Cch= control cheese, LrCh= probiotic cheese, EM1107= probiotic alone.

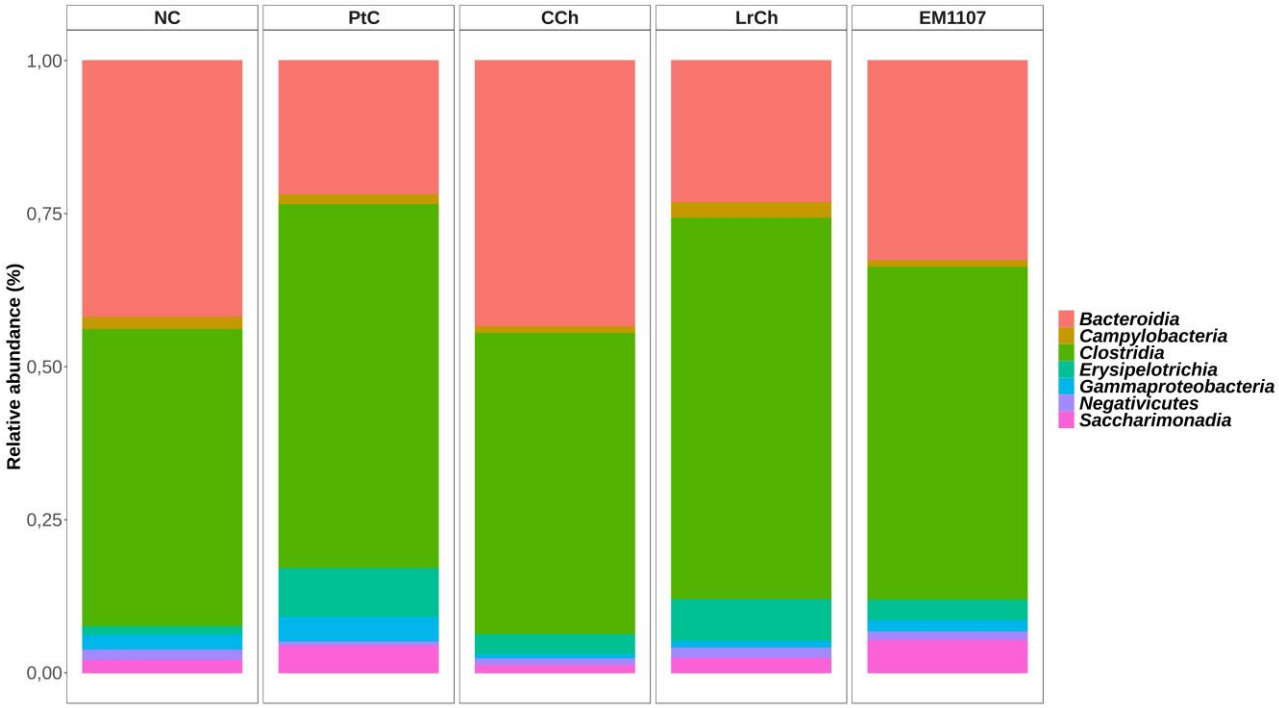

Fig. 14. Bacteria class distributions (n = Top 10 abudant taxa) for each group. Caption: NC= negative control, PtC= positive control, Cch= control cheese, LrCh= probiotic cheese, EM1107= probiotic alone.

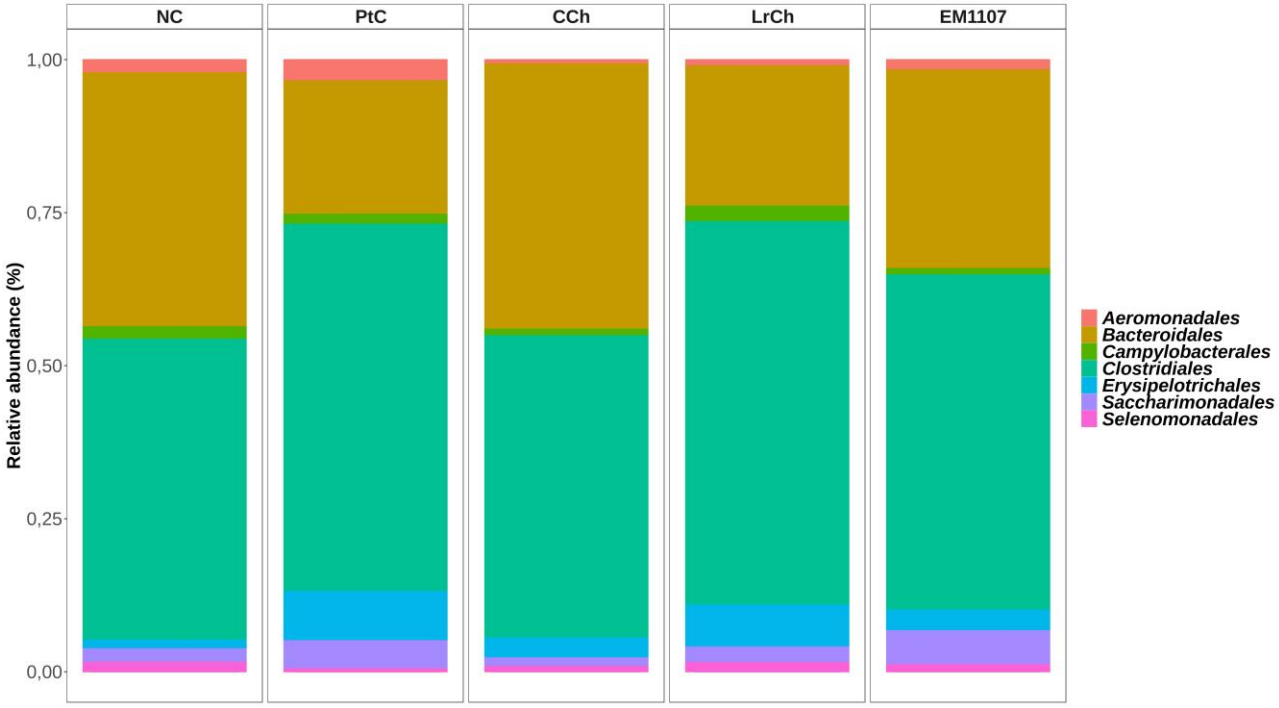

Fig. 15. Bacteria order distributions (n = Top 10 abudant taxa) for each group. Caption: NC= negative control, PtC= positive control, Cch= control cheese, LrCh= probiotic cheese, EM1107= probiotic alone.

5 CONSIDERAÇÕES FINAIS

Alimentos adicionados de estirpes probióticas são uma forma de ingestão desses microrganismos que pode facilitar o seu consumo ao se inserí-los em matrizes consumidas frequentemente no cotidiano, como os queijos. Esses, têm sido demonstrados como veículos adequados para probióticos pois os mantém viáveis ao longo da vida de prateleira dos produtos. Além disso, as estirpes podem influenciar as características organolépticas dos alimentos promovendo textura e sabor diferenciados. Por meio de estudos *in vitro* e *in vivo* avalia-se o desempenho probiótico dos produtos finais, no entanto, é importante que tais estudos sejam conduzidos adequadamente para que eles proporcionem credibilidade e sejam uma fonte de informação disponível aos profissionais de saúde e consumidores.

Os ensaios *in vivo* desenvolvidos nesta pesquisa demonstraram em *L. rhamnosus* EM1107, sozinho ou adicionado ao queijo, foi capaz de reduzir a infecção inicial por *Salmonella*, no lúmen intestinal, minimizando os danos teciduais e promovendo a modulação da microbiota intestinal dos ratos, além de demonstrar propriedades imunomoduladoras. *L. rhamnosus* EM1107, quando administrado isoladamente, destacou-se em relação aos efeitos benéficos à saúde associados às propriedades antioxidantes e imunomoduladoras do que o queijo probiótico, embora o queijo adicionado ou não de EM1107 pudesse mitigar danos aos tecidos intestinais.

Desse modo, sugere-se que novas pesquisas sejam realizadas utilizando-se a estirpe EM1107 em outras matrizes alimentares e/ou avaliação probiótica em diferentes patalogias para se avaliar outras possíveis propriedades benéficas que possam ser atribuídas à estirpe.

ANEXO

CERTIFICADO DE APROVAÇÃO DA PESQUISA PELA COMISSÃO DE ÉTICA NO USO DE ANIMAIS DA UNIVERSIDADE FEDERAL DA PARAÍBA (CEUA/ UFPB - PROTOCOLO N° 021/2016)

UNIVERSIDADE FEDERAL DA PARAÍBA
COMISSÃO DE ÉTICA NO USO DE ANIMAIS (CEUA)

CERTIFICADO

Certificamos que o projeto intitulado **"Avaliação do potencial probiótico de queijo de coalho caprino adicionado de *Lactobacillus rhamnosus* EM1107 em modelo experimental de salmonelose induzida",** protocolo **nº 021/2016** sob a responsabilidade da pesquisadora **Prof. Dr. Rita de Cássia Ramos do Egypto Queiroga** – que envolve a produção, manutenção e/ou a utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto o homem), para fins de pesquisa científica (ou ensino) – encontra-se de acordo com os preceitos da Lei nº 11.794, de 08 de outubro de 2008, do Decreto nº 6.899, de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de controle da Experimentação Animal (CONCEA), e foi aprovado pela Comissão de Ética no Uso de Animais da Universidade Federal da Paraíba (CEUA-UFPB).

Vigência do Projeto	2015 - 2018
Espécie/linhagem	*Rattus Novergicus*
Número de animais	140
Idade/peso	5 semanas – 180-220 g
Sexo	Machos
Origem	Biotério Thomas George - UFPB

Prof. Dr. Ricardo Romão Guerra
Vice-Coordenador CEUA-UFPB