CARINA TERUMI TSURUDA

AVALIAÇÃO DO EFEITO PROBIÓTICO DE *LACTOBACILLUS* SP. NA INVASÃO POR *SALMONELLA ENTERICA* SOROVARIEDADE TYPHIMURIUM

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Dissertação apresentada ao curso de Pós-graduação em Microbiologia da Universidade Estadual de Londrina, para a obtenção do título de Mestre em Microbiologia.
Orientador**:** Prof. Dr. Gerson Nakazato.

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“O sucesso nasce do querer, da determinação e persistência em se chegar a um objetivo. Mesmo não atingindo o alvo, quem busca e vence obstáculos, no mínimo fará coisas admiráveis".

(José de Alencar)

TSURUDA, Carina Terumi. **Avaliação do efeito probiótico de *Lactobacillus* sp. na invasão por *Salmonella enterica* sorovariedade Typhimurium**. 2017. 59f. Dissertação (Mestrado em Microbiologia) – Universidade Estadual de Londrina. Londrina, 2017.

RESUMO

Os probióticos são microrganismos vivos que quando administrados em quantidades adequadas conferem benefícios para a saúde do hospedeiro, impedindo a colonização e invasão de patógenos no intestino. *Lactobacillus* é um dos principais agentes probióticos utilizados na prevenção de infecções e na restauração da microbiota intestinal. *Salmonella enterica* sorovariedade Typhimurium é um importante agente causador de infecção intestinal de origem alimentar. O objetivo deste trabalho foi avaliar a capacidade protetora do *Lactobacillus rhamnosus* produtor de exopolissacarídeos (EPS) contra a invasão *in vitro* e *in vivo* por *S.* Typhimurium. Para isso foi realizada a pesquisa de bacteriocinas, ensaios de adesão e invasão celular *in vitro.* Para o teste de desafio em camundongos BALB/c, realizou-se análise histopatológica e microbiológica do fígado, baço, placas de Peyer e íleo. Os resultados mostraram que os lactobacilos foram capazes de produzir compostos antimicrobianos contra *S.* Typhimurium*.* Estes lactobacilos inibiram a adesão e invasão de *S.* Typhimurium em células HeLa e HEp-2, respectivamente. O ensaio de desafio no modelo murino mostrou uma diminuição da translocação do patógeno para o baço e fígado de camundongos tratados com probiótico, bem como a proteção no tecido intestinal íleo de camundongos (análise histopatológica). Esses resultados mostraram que os ensaios *in vitro* são interessantes para avaliação prévia de candidatos a probióticos.

Palavras-chave: Probiótico. *Lactobacillus. Salmonella* Typhimurium. Efeito protetor.

TSURUDA, Carina Terumi. **Evaluation of the probiotic effect of *Lactobacillus* sp. on the invasion by *Salmonella enterica* serovar Typhimurium**. 2017. 59p. Dissertation (Master Science in Microbiology) – Universidade Estadual de Londrina. Londrina, 2017.

ABSTRACT

Probiotics are live microorganisms that when administered in suitable amounts confer benefits to the host's health, preventing colonization and invasion of pathogens in the intestine. *Lactobacillus* is one of the main probiotic agents used in the prevention of infections and in the restoration of the intestinal microbiota. *Salmonella enterica* serovar Typhimurium is an important agent causing food-borne intestinal infection. The aim of this study was to evaluate the protective capacity of the exopolyssaccharide-producing *Lactobacillus rhamnosus* (EPS) against invasion *in vitro* and *in vivo* by *S.*Typhimurium. The bacteriocins research, in vitro cell adhesion and invasion assays were carried out. For the challenge in BALB/c mice, histopathological and microbiological analysis of the liver, spleen, Peyer's patches and ileus were performed. The results showed that lactobacilli were able to produce antimicrobial compound against *S.* Typhimurium. These lactobacilli inhibited the adhesion and invasion of Salmonella in HeLa and HEp-2 cells, respectively. The challenge assay in the murine model showed a decrease the pathogen translocation in the spleen and liver from mice treated with probiotic as well as protection of ileus intestinal tissue from mice lactobacilli-treated (histopathological analysis). These results showed that in vitro assays are of interest for prior evaluation of probiotic applicants.

Key words: Probiotic*. Lactobacillus. Salmonella* Typhimurium. Protective effect.

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LISTA DE ABREVIATURAS E SIGLAS

AN	Ágar nutriente
CAPES	Coordenação de Aperfeiçoamento de Pessoal de Nível Superior
CFU	Colony Forming Unit
CLSI	Clinical and Laboratory Standards Institute
CO_2	Dióxido de Carbono
DMEM	Dulbecco's Modified Eagle Medium
EPS	exopolissacarídeos
g	Gramas
h	Horas
HE	Hematoxilina e eosina
HEp-2	Células de carcinoma de laringe humana
HeLa	Células de carcinoma de cérvix humana
kDa	Quilodalton
KH_2PO_4	Fosfato de Potássio Monobásico
LB	Luria-Bertani
log	Logaritmo
MC	MacConkey
MH	Mueller-Hinton
MIC	Minimal Inhibitory Concentration
mL	Mililitros
min.	Minutos
mm	Milímetros
MRS	Man Rogosa Sharpe
NaCl	Cloreto de sódio
Na_2HPO_4	Fosfato dissódico
NaOH	Hidróxido de sódio
p	Nível de significância estatística
PBS	Phosphate buffered saline
pH	Potencial hidrogeniônico
PR	Paraná
rpm	Rotações por minuto
sp.	Espécie

SVC	Salmonella-containing vacuoles
USA	United States of America
µL	Microlitro
µm	Micrometro
mM	Milimolar
%	Porcentagem
ºC	Graus Celsius

SUMÁRIO

1. INTRODUÇÃO

O uso de probióticos, microrganismos vivos, exerce efeitos benéficos para a saúde, e ao serem administrados em doses efetivas podem prevenir a invasão e colonização de patógenos intestinais. Os principais gêneros empregados como probióticos são *Bifidobacterium* e *Lactobacillus*, e tem como preferência o íleo terminal e o cólon, para a colonização intestinal.

Os probióticos são capazes de modular a microbiota intestinal, estimulando seletivamente a proliferação de bactéria benéficas. Acredita-se que os probióticos podem competir pelos sítios de adesão, impedindo a colonização de patógenos; estimular o sistema imunológico do hospedeiro; e produzir compostos com atividade antimicrobiana.

Salmonella enterica, é um dos patógenos de origem alimentar de grande importância, pois causam doenças em humanos e outros animais, através do consumo de água e alimentos contaminados. A salmonelose pode variar desde uma gastroenterite a uma infecção sistêmica grave, como a febre tifóide.

Nos últimos anos, nosso grupo de pesquisa tem estudado os efeitos probióticos de alguns lactobacilos contra importantes patógenos como *S. enterica*, com o intuito de prevenir infecções bacterianas sistêmicas.

Nesse contexto, o presente estudo teve como objetivo avaliar a capacidade protetora de uma cepa de *Lactobacillus* produtora de exopolissacarídeos (EPS) contra a invasão *in vitro* e *in vivo* por *Salmonella enterica* sorovariedade Typhimurium.

2. REVISÃO BIBLIOGRÁFICA

2.1 PROBIÓTICOS E PREBIÓTICOS

Uma das prioridades da pesquisa na indústria de alimentos é o desenvolvimento de produtos alimentícios que promovem a saúde e o bem-estar, esta tendência tem favorecido ao consumo de alimentos enriquecidos com componentes fisiologicamente ativos tais como os probióticos (DUARTE et al., 2016).

O termo probiótico é de origem grega e significa “para a vida”, sendo que atualmente, a definição aceita internacionalmente é que são microrganismos vivos que quando ingeridos em quantidades adequadas, conferem um benefício ao hospedeiro (FAO/ WHO, 2002). Os gêneros bacterianos mais comumente utilizados como probióticos são *Lactobacillus*, *Bifidobacterium* e *Enterococcus;* outro probiótico utilizado com frequência é o fungo *Saccharomyces boulardii* (VARAVALLO et al., 2008).

Para que um microrganismo seja considerado como probiótico, alguns critérios são necessários, como: não ser patogênico; ser isolado de humanos, manter sua viabilidade após a secreção de enzimas digestivas; capacidade de aderir a mucosa intestinal; assim como persistir no trato gastrointestinal (MAHAJAN & SINGH, 2014; MAVROUDI, 2012).

Os prebióticos são definidos como fibras hidrossolúveis, ou seja, carboidratos não metabolizados pelos humanos, como por exemplo: os frutooligosacarídeos, a pectina, as ligninas e a inulina (SOUZA et al., 2010). Portanto, devem resistir à acidez gástrica, à hidrólise por enzimas intestinais e não serem absorvidos pelo trato gastrintestinal. Desta forma, são capazes de modular a

microbiota intestinal estimulando seletivamente o crescimento de bactérias que colaboram para o bem-estar e saúde do hospedeiro (FERREIRA & SILVA, 2012).

Uma microbiota intestinal saudável e equilibrada é fundamental para o desempenho normal das funções fisiológicas. A associação de microrganismos probióticos com prebióticos pode potencializar os seus efeitos benéficos na microbiota intestinal, essa associação tem é denominada simbióticos (SAAD, 2006).

O gênero *Lactobacillus* pertence ao grupo das bactérias do ácido láctico (LAB), que é composto por bactérias Gram-positivas que produzem ácido láctico como um dos principais produtos da fermentação do catabolismo de carboidratos (HAYEK & IBRAHIM, 2013). Muitas cepas de LAB são capazes de produzir polímeros de monossacarídeos na maioria das vezes viscosos, denominados exopolissacarídeos (EPS), sendo de grande importância na indústria de laticínios, como na produção de iogurtes, aumentando a consistência e a viscosidade do produto (HASSAN, 2008). O *L. rhamnosus* produz EPS, acredita-se que este composto possa auxiliar a bactéria produtora na ligação ao muco intestinal, assim como proteger as células epiteliais e estimular o sistema imune (KLOPPER et al., 2018).

Os alimentos que contém probióticos e/ou prebióticos são chamados de “Alimentos Funcionais”, pois além do valor nutritivo oferecem benefícios à saúde. O interesse pelo consumo de alimentos funcionais vem crescendo, devido a sociedade estar mais preocupada com a saúde e bem-estar. Os gêneros *Bifidobacterium* e *Lactobacillus* têm sido frequentemente utilizados na produção de leite fermentado e iogurtes (GULDAS & IRKIN, 2010).

O grande benefício da terapia com os probióticos é a ausência de efeitos secundários, como a seleção de bactérias multirresistentes aos antimicrobianos de amplo espectro (FERREIRA & SILVA, 2012; SANDERS, et al. 2013). Assim, os probióticos assumem um papel importante na prevenção de infecções, e eles podem apresentar alguns mecanismos de ação associadas aos seus efeitos protetores.

2.2 MECANISMOS DE AÇÃO DOS PROBIÓTICOS

Os mecanismos exatos de ação dos probióticos ainda não foram inteiramente estabelecidos, mas estudos indicam que os probióticos são capazes de produzir substâncias com atividade antimicrobiana, como os ácidos orgânicos e bacteriocinas, inibindo o crescimento de patógenos (FERREIRA & SILVA, 2012; LEBEER et al., 2008). A modulação da microbiota intestinal pelos microrganismos probióticos ocorre através de um mecanismo denominado “exclusão competitiva”. Esse mecanismo impede a colonização dessa mucosa por microrganismos potencialmente patogênicos, pela competição por sítios de adesão. (LEBEER et al. 2008; SAAD, 2006).

Segundo SAAD (2006), grande parte das evidências de sistemas *in vitro* e de modelos animais e humanos sugere que os probióticos podem estimular a resposta imune sistêmica aumentando o número e a atividade de células fagocíticas do hospedeiro. Acredita-se que esses efeitos sejam mediados por uma ativação dos macrófagos, por um aumento nos níveis de citocinas, por um aumento da atividade das células destruidoras naturais (NK - “natural killer”) e/ou dos níveis de imunoglobulinas (MAHAJAN & SINGH, 2014; OELSCHLAEGER, 2010).

2.3 BACTERIOCINAS DE BACTÉRIAS LÁCTICAS

O interesse da indústria alimentícia em pesquisas com bacteriocinas, especialmente de bactérias ácidas (LAB), é pelo fato de muitas destas bactérias já desempenharem um papel crucial numa variedade de fermentações de alimentos, serem consideradas como bactérias GRAS (Generally Recognized as Safe) e produzir uma variedade de substancias antimicrobianas tais como ácidos orgânicos e bacteriocinas, podendo ser utilizadas na bioconservação de alimentos. (PEREZ et al., 2014; EGAN et al., 2016).

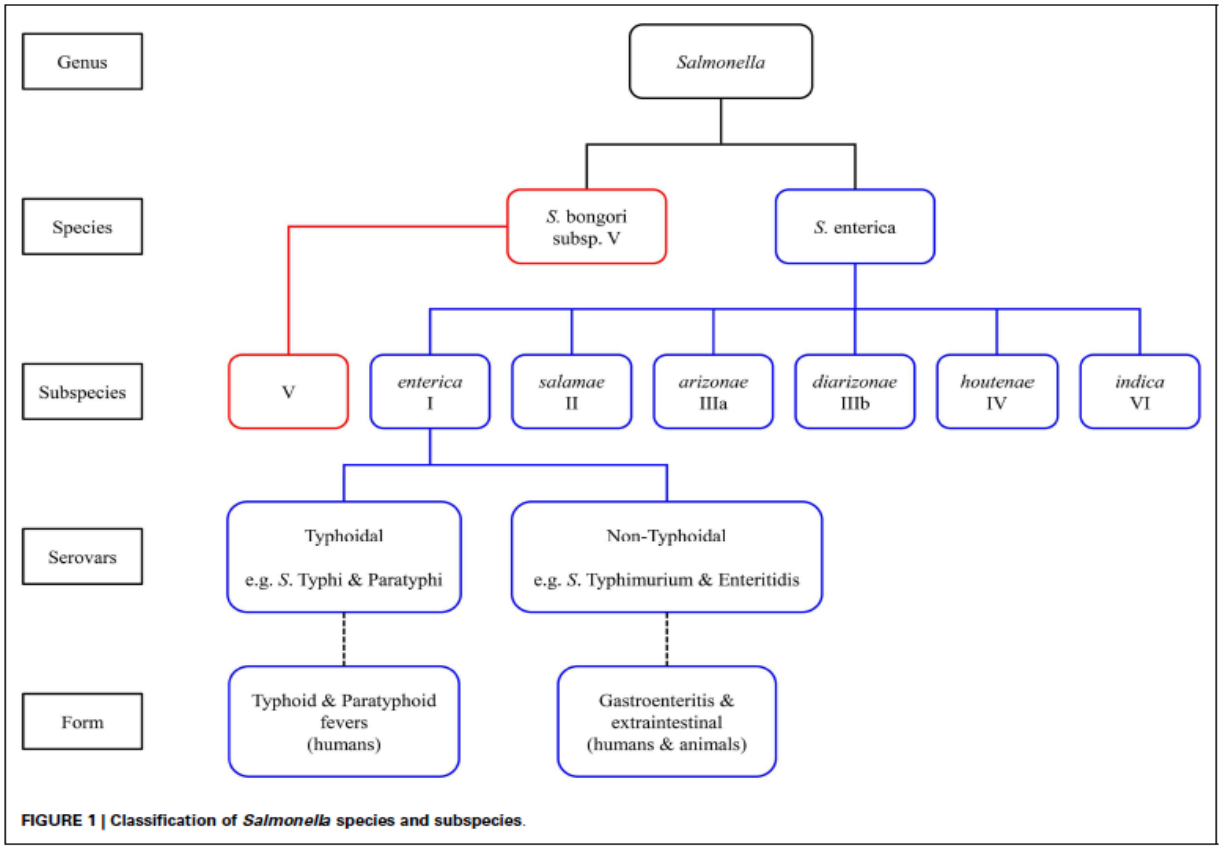
As bacteriocinas são peptídeos antimicrobianos, com ação bactericida ou bacteriostática sobre outros microrganismos. São sintetizadas nos ribossomos, durante a fase inicial de crescimento bacteriano. A bactéria produtora de bacteriocinas possui um mecanismo de proteção, é capaz de produzir proteína de imunidade que se liga aos receptores da membrana citoplasmática impedindo que a bacteriocina se ligue e forme poros que levaria a lise celular (PEREZ et al., 2014; OGAKI et al., 2016).

As bacteriocinas produzidas por LAB são de baixo peso molecular (<10 kDa) e geralmente, atuam na membrana citoplasmática da bactéria alvo em duas etapas distintas. A primeira etapa consiste na adsorção da bacteriocina em receptores presentes na membrana celular da bactéria. A segunda etapa ocorre com a inserção da bacteriocina na membrana ocasionando modificações no seu potencial e no gradiente das concentrações de hidrogênio. Isto faz com que comprometa a viabilidade da célula bacteriana devido a formação de poros na membrana e alterações na produção de ATP (PEREZ et al., 2014; EGAN et al., 2016).

2.4 O GÊNERO *SALMONELLA*

As bactérias do gênero *Salmonella*, são caracterizados como bacilo Gram-negativo, anaeróbio facultativo, fermentador de glicose, geralmente flagelado, e intracelular facultativo. Atualmente o gênero *Salmonella* consiste em apenas duas espécies, *Salmonella bongori* e *Salmonella enterica*, sendo esta subdividida em seis subespécies (*enterica*, *salamae*, *arizonae*, *diarizonae*, *houtenae*, *indica*). Na subespécie *enterica* encontram-se a maioria das sorovariedades patogênicas para o homem, pois são mais frequente para animais de sangue quente, enquanto as outras podem infectar somente animais de sangue frio (GARAI et al., 2012).

Figura 1. Classificação do gênero *Salmonella* em espécies e subespécies



Fonte: Hurley, et al. (2014).

A classificação a nível de gênero é realizada através de testes bioquímicos juntamente com técnicas de biologia molecular, permitindo a uma análise taxonômica mais apurada.

A classificação sorológica de *Salmonella* é obtida através da detecção de antígenos flagelares (H), somáticos (O) e capsulares (Vi). Os antígenos capsulares são característicos de alguns isolados e quando presentes definem uma sorovariedade. De acordo com o sistema de classificação Kauffman-White, atualmente há mais de 2600 sorovariedades de *S. enterica* (HURLEY et al., 2014).

2.5 SALMONELOSE

S. enterica é um dos patógenos de origem alimentar de grande importância, visto que apresenta capacidade de infectar diferentes animais. A infecção ocorre através da ingestão de água e alimentos contaminados, principalmente de origem animal, podendo estar relacionada a praticamente todos os tipos de alimentos, devido a sua capacidade ubíqua podendo crescer em temperaturas que variam de 7 a 45 °C (ALVARÉZ-ORDÓÑEZ et al., 2011; RUBY et al., 2012).

As infecções causadas pela *S. enterica* podem variar desde uma gastroenterite localizada na mucosa intestinal à uma infecção sistêmica grave, podendo levar a morte (GARAI et al., 2012). Todos os anos, milhares de casos de salmonelose são relatados em todo o mundo, estima a ocorrência de 16 a 33 milhões de casos de febre tifóide no mundo, com 500 a 600 mil fatalidades anuais. Entretanto, o número real de infecções pode ser muito diferente e muitas vezes maior do que o esperado, visto que a maioria dos casos de gastroenterites não tão graves, ou seja com sintomas mais brandos, não são diagnosticados ou relatados (WHO 2013).

As sorovariedades Typhi e Paratyphi são responsáveis por causar uma infecção sistêmica grave conhecida como febre tifóide, sendo mais prevalentes em países em desenvolvimento, onde a qualidade da água potável e o tratamento de esgoto são inadequadas (RUBY et al., 2012).

Os casos de salmonelose por sorovares não tifoides (NTS) são muito frequentes, estima-se que milhões de casos em humanos ocorram no mundo a cada ano, resultando em mais de cem mil mortes (ANDREWS-POLYMENIS et al., 2010). As sorovariedades de *S. enterica* Typhimurium e Enteritidis, são de grande importância, visto que são patógenos frequentes de gastroenterites e infecções extraintestinais em humanos. Em modelo murino, essas duas sorovariedades são muito estudadas, pois causam um tipo de infecção muito semelhante à febre tifóide humana.

No Brasil, no período 1999 a 2008, ocorreram 6602 surtos causados por patógenos entéricos, sendo *S. enterica* presente em 43% dos casos no qual o agente etiológico foi identificado (MEDEIROS et al., 2011). No estado do Rio Grande do Sul, Brasil, entre os anos de 2007 a 2012 foram isoladas 163 amostras de *Salmonella* das quais 138 (84,7%) foram identificados como *S.* Enteritidis e 6 (3,7%) como S. Typhimurium, sendo a maionese caseira e carnes processadas as principais fontes de transmissão (CAPALONGA et al., 2014).

2.6 PATOGENICIDADE DA *SALMONELLA*

A infecção por *S. enterica* ocorre com a ingestão de água ou alimentos contaminados, para que ocorra a infecção, a dose infectante média (DI_{50}) está entre 10^5 a 10^{10} bactérias ingeridas, os sintomas são basicamente diarreia, vômito e dores abdominais que podem aparecer dentro de 6-72 horas após a ingestão do alimento contaminado (FÀBREGA & VILA, 2013). Entretanto a

quantidade exata requerida para causar a doença varia de acordo com o estado imunológico do paciente, do patógeno encontrar local adequado, para que possa se estabelecer, replicar e expressar seus fatores de virulência, assim como a sorovariedade envolvida (OCHOA & RODRÍGUEZ, 2005).

O primeiro obstáculo a ser superado dentro do hospedeiro é resistir ao pH ácido do estômago e aos sais biliares intestinais, propriedades essenciais, para que ocorra a infecção. Para se proteger, o patógeno possui um mecanismo ácido-tolerância, que regula a homeostase do pH, que induz manter o pH intracelular em valores superiores aos do ambiente extracelular (FOSTER & HALL, 1991).

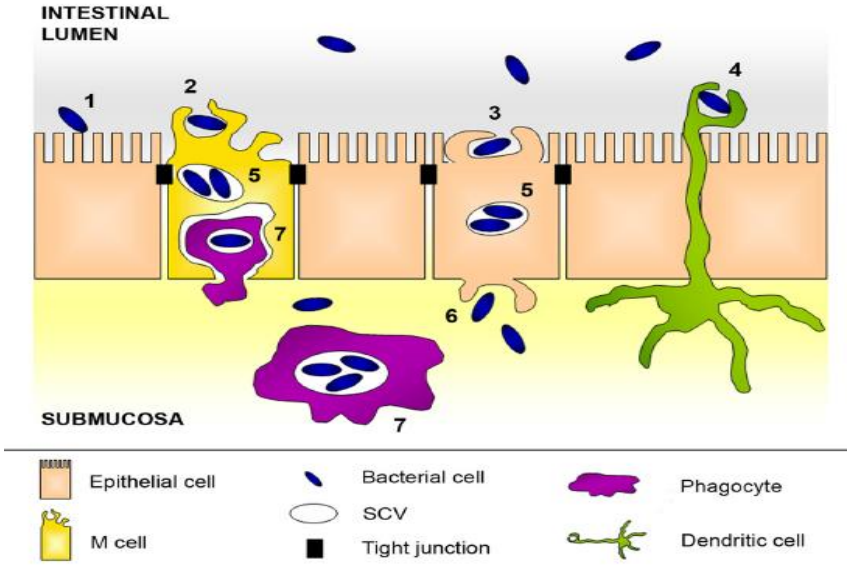
S. enterica após resistir ao pH ácido e aos sais biliares, é no intestino delgado seu local de infecção, onde vai se aderir ao epitélio intestinal por meio de adesinas e invadir os enterócitos, células dendríticas e principalmente as células M, que tem como função, capturar antígenos intestinais por endocitose e transportar para células linfóides presentes nas placas de Peyer. A invasão das células M também permite que as bactérias infectem células epiteliais adjacentes através de sua superfície basolateral (JONES et al., 1994).

Durante a invasão, *S. enterica* altera o citoesqueleto sendo engolfada pela célula do hospedeiro em vesículas chamadas Salmonella-containing vacuoles (SCV), simultaneamente, ocorre uma intensa resposta inflamatória no epitélio intestinal iniciando o recrutamento e transmigração de fagócitos do espaço submucoso para dentro do lúmen intestinal (FÀBREGA & VILA, 2013).

S. enterica atravessa o epitélio intestinal e os macrófagos da submucosa detectam a bactéria e a internalizam para eliminá-la do hospedeiro, no entanto nem todas as bactérias são eliminadas, as que sobrevivem são capazes de

se replicar no interior dos SCV dos fagócitos, mecanismo essencial para a sua sobrevivência (FÀBREGA & VILA, 2013). Após esse processo a bactéria induz a célula sofrer apoptose sendo liberada para invadir novas células. Por fim, estes fagócitos infectados podem disseminar-se através do sistema linfático e da corrente sanguínea (Figura 2).

Figura 2. Modelo de patogenicidade de *S.* Typhimurium



Fonte: Fàbrega & Vila, (2013)

Todo esse mecanismo de invasão da Salmonella, provoca uma intensa resposta inflamatória, que leva a liberação de prostaglandinas que estimulam a adenilato ciclase nas células intestinais a inibir a absorção de sódio e aumentar a secreção de cloro ocasionando diarreia aquosa (FÀBREGA & VILA, 2013).

Sendo assim, devido à importância dos probióticos em prevenir infecções intestinais causadas por patógenos como *S. enterica*, mostra a relevância deste trabalho, onde foi avaliado os efeitos de *L. rhamnosus* contra a invasão por *S.* Typhimurium por meio de testes *in vivo*.

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4. RESULTADOS E DISCUSSÃO

Os resultados e discussão, juntamente com a metodologia da presente dissertação foi apresentado na forma de artigo.

4.2 *Lactobacillus rhamnosus* V5 prevents *Salmonella enterica* serovar Typhimurium invasion in cell culture and mice infection

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ABSTRACT

Lactobacillus is one of the main probiotic agents used in the prevention of infections intestinal. The aim of this study was to evaluate the protective capacity of the exopolysaccharide-producing *Lactobacillus rhamnosus* against invasion *in vitro* and *in vivo* with *S.* Typhimurium. The results showed that lactobacilli were able to produce antimicrobial compounds against *S.* Typhimurium. These lactobacilli inhibited the adhesion and invasion of *S.* Typhimurium in HeLa and HEp-2 cells, respectively. The challenge assay in the murine model demonstrated a decrease in pathogen translocation in the spleen and liver from mice treated with probiotic as well as protection of ileal tissue in lactobacilli-treated mice. The histopathological analysis demonstrated the presence of prominent lymphoid nodules in the ileum from the non-treated lactobacilli mice. Our results suggest that *L. rhamnosus* improved the effectiveness of the intestinal barrier and, thus, could be used as a probiotic in order to control salmonellosis.

Keywords: bacteriocinas, protection, adherence, murine model, histopathological

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INTRODUCTION

Probiotics are live microorganisms that confer a health benefit for the host by means of producing bioactive compounds or equilibrating the gastrointestinal tract (Costa and Miglioranza, 2012) when administered in adequate amounts (FAO/WHO, 2002). Probiotics are usually incorporated into nutritional supplements and pharmaceutical products (Sanders et al., 2013).

The use of probiotics has not been associated with side effects, especially the selection of multi-resistant bacteria for broad-spectrum antimicrobials (Ferreira de Luces Fortes, 2012). Probiotics rarely cause problems in healthy people, but they should be used with caution in patients with serious illnesses or in severely immunocompromised people (Williams, 2010). Probiotics can act as an alternative growth promoter in animal production (Higgins et al., 2010)

Besides these positive effects, probiotics help in the prevention or treatment of gastrointestinal diseases such as irritable bowel syndrome (Clarke et al., 2012), inflammatory bowel disease (Tursi et al., 2010), necrotizing enterocolitis (Deshpande, et al., 2010), food allergy (Fölster-Holst, 2010), and infectious diarrhea (Guandalini, 2011), as well as presenting great effectiveness in the treatment of rotavirus and pouchitis (Williams, 2010).

Lactobacillus and *Bifidobacterium* are the most common genus of probiotics (Song et al., 2012). *Lactobacillus* belongs to the group of lactic acid bacteria (LAB) that is composed of Gram-positive, non-sporulating, anaerobic or facultative aerobic cocci or rods, which produce lactic acid as one of the main fermentation end products of the catabolism of simple carbohydrates (Hayek and Ibrahim, 2013). This lactic acid reduces the pH of the intraluminal environment and inhibits multiplication of pathogenic bacteria. In this sense, it is suggested that

organic acids can penetrate the bacterial cell wall and change their normal physiology of species of microorganisms (Ferreira de Luces Fortes, 2012).

LAB can provide immune-modulating and immune-stimulating activities (Pagnini et al., 2010) or non-immune mechanisms (Lebeer et al., 2008). They can exert direct antimicrobial activity against pathogens by increasing phagocytosis (De Moreno De Le Blanc et al., 2010), modifying cytokine production (Castillo et al., 2011), and enhancing interleukin production (Galdeano and Perdigon, 2006). Recent studies have proven that *Lactobacillus* administration prevents intestinal infection with *Salmonella* (Forkus et al., 2017; Hirano et al., 2017).

According to the Centers for Disease Control and Prevention (CDC) of the United States (2014), approximately 1.2 million illnesses are caused by *Salmonella* spp. every year, causing 19,000 hospitalizations and 380 deaths. Children up to four years old are the most likely to contract salmonellosis. The *Salmonella* genus consists of only two species. According to the Kauffman-White classification system, *S. enterica* actually has more than 2,600 serovars (Hurley et al., 2014). The serovar *S.* Typhimurium induces rapid host death, mainly in susceptible hosts (Robinson et al., 2012). It causes a considerable number of human diseases in developed nations (Kingsley et al., 2009) and variants of *S*. *enterica* serovar Typhimurium have been described as causing highly invasive illnesses in Africa (Kingsley et al., 2009; Ley et al., 2014).

S. enteric is one of the most common causal agents of foodborne illnesses associated with the consumption of fresh leafy vegetables (Warriner and Namvar, 2010), tomatoes, alfalfa sprouts, and orange juice (Barton Behravesh et al., 2011; Mody et al., 2011). This pathogen can be ingested in beef, pork, turkey and

principally in chicken, due to the ubiquity of bacteria and its capacity to grow at a wide range of temperatures: from 7 to 45 °C (CDC, 2014).

S. enterica serovar Typhimurium can resist the low pH of gastric secretion, overcome the intestinal barrier, and survive inside macrophages (Gonzalez-Escobedo et al., 2011). Robinson (2012) proposes that this pathogen induces the production of type I interferon, which drives necroptosis of macrophages and allows them to evade the immune response. In this report, we explored the protective ability of a strain of *Lactobacillus rhamnosus* against the invasion *in vitro* and *in vivo* by *S*. *enterica* serovar Typhimurium.

MATERIAL AND METHODS

1. DNA extraction and PCR amplification

The total genomic DNAs of *Lactobacillus rhamnosus* V5 was extracted using the Puregene® Blood core kit B (Qiagen, Hilden, Gemany) according to the manufacturer's instructions. Bacterial ribosomal subunits 16S primers were used in this study (primers sets: 16S Fw: 5'-GAGTTTGATCCTGGCTCAG-3' and 16S Rev: 5'-AGAAAGGAGGTGATCCAGCC-3'). The PCR melting temperature was 59 ºC. A PCR reaction mixture contained 2 µL of extracted template DNA (50 ng), 2 µL of dNTPs (0,2 mM; Invitrogen, USA), 0.2 µL of Taq High Fidelity (5 U/µL; Invitrogen, USA), 5 µL of buffer (10 x; Invitrogen, USA), 3 µL of $MgSO_4$ (50 mM; Invitrogen, USA) and 37 µL of deionized water, totaling a volume of 50 µL. PCR products were analyzed by agarose gel electrophoresis in 1% in TAE (20 mM Tris acetate, pH 8.0; 0.5 mM EDTA) at 80 V and 400 mA for 30 min. After that, the DNA was extracted

from the gel and purified using quick gel extraction Kit PureLink ^{TM} (Invitrogen, USA). The PCR cycles consisted of 94 °C of initial denaturation for 5 min, 35 cycles of 94 °C for 1 min, 59 °C for 1 min and 68 °C for 2 min, followed by 10 min of final extension at 68 °C.

2. Pathogenic bacteria

The mice were infected with an attenuated pathogen, *S.* Typhimurium *χ3985* UK1 *(ΔcyaΔcrp)* strain from the Center for Infectious Diseases and Vaccinology, Biodesign Institute and School of Life Sciences, Arizona State University, Tempe, AZ, United States of America (Curtiss et al., 1989). The use of an attenuated strain allows evaluation of the process of translocation in the mouse, which would not be possible with a virulent strain. *S.* Typhimurium χ3985 UK1 (*ΔcyaΔcrp*) has a deletion in the adenylate cyclase and cyclic AMP receptor protein (Curtiss and Kelly, 1987), however it continues with its immunogenic action, being able to infect and persist in the organs of mice, such as in the Peyer's patches, spleen, and liver. These bacteria were grown in Luria-Bertani (LB) broth (Difco, Franklin Lakes, NJ, USA) at a temperature of 37 ºC for 18 h.

3. Probiotic

The *Lactobacillus rhamnosus* V5 strain was obtained from a mixture of various bacteria from “Viili” given in Department of Food Science and Technology, Agricultural Science Center, State University of Londrina, Londrina, Paraná, Brazil. The *L. rhamnosus* strain was grown in De Man, Rogosa and Sharpe (MRS) broth medium (Difco, Franklin Lakes, NJ, USA), at a temperature of 37 ºC, in an atmosphere of 5% (v/v) CO_2 for 18 h.

4. Adhesion and invasion in cell culture

4.1. Cultivation of HEp-2 and HeLa cells

Cell cultures were grown in a 24-well plate (BD Falcon, Bedford, MA, USA) in Dulbecco's Modified Eagle Medium (DMEM) (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum which was incubated in a 5% CO_2 atmosphere at a temperature of 37 ºC, for 48 h. The cell monolayer was grown for approximately 24 h at 37°C with 5% CO_2 to give at least 80% confluency.

4.2. Inhibition of bacterial adhesion

The assay was performed according to the methodology described by Cravioto and collaborators (1979). The HeLa cells were cultured in a 24-well plate (BD Falcon microplates, Bedford, MA, USA) on sterile round coverslips (13 mm in diameter) that were placed before the cells.

First, we added 10^7 CFU of lactobacilli into a well to 1 mL of DMEM, for 2 h in the CO_2 oven (5%), at a temperature of 37 °C. Next, we added 10^7 CFU of *S.* Typhimurium, leaving the well for 3 h under the same conditions. After the period, the monolayers were washed with sterile 0.05 M phosphate buffered saline (PBS, pH 7.4) and incubated for another 3 h. Then, we washed the coverslips five times with PBS, fixed with absolute methanol (Merck, Darmstadt, Germany) for 10 min, and stained with May-Grunwald (Sigma-Aldrich, St. Louis, MO, USA) and Giemsa (Sigma-Aldrich, St. Louis, MO, USA). The slides were examined under a light microscope using an oil immersion lens. Finally, we quantified added-bacteria for each 100 HeLa cells from different fields of the coverslip.

4.3. Inhibition of bacterial invasion

Invasion testing by *S. enterica* serovar Typhimurium strain and inhibition of invasion by *L. rhamnosus*. were performed according to Sansonetti and collaborators (1986). First the HEp-2 cells were washed twice with Phosphate Buffered Saline - PBS (137 mM NaCl, 2.7 mMKCl, 10 mM Na_2HPO_4, 1.76 mM KH_2PO_4, pH 7.4) after which they were added to 50µL medium and a suspension containing 10^8 CFU/mL of *L. rhamnosus* and incubated for 2 h in a CO_2 incubator (5%) at 37 °C. Next, 100 µL of suspension containing 10^7 CFU / mL of *S*. Typhimurium invading bacteria was added and left to act for 2 h. After this period the plate passed through the washing process as described above. Then 1 mL of gentamicin (Sigma-Aldrich, St. Louis, MO, USA) was added to each well in a concentration of 100 µg /mL and allowed to act for 2 h. The aim of the antibiotic is to kill the bacteria that did not invade the cells.

After the effect of the antibiotic, the plate was passed 3 times through wash steps, after which 500 µL of 1% Triton (Sigma-Aldrich, St. Louis, MO, USA) was added and left to act for 5 min, to lyse the cell function to release the invading bacteria. Next, 100 µL of each well was withdrawn and transferred to a microtube. From this serial dilution (10^{-1}, 10^{-2},10^{-3}) was carried out and plated in triplicate in MacConkey (MC) agar (Difco, Franklin Lakes, NJ, USA). Analysis was performed after incubating for 24 h at a temperature of 37 °C.

5. Antibacterial activity of the supernatant

5.1. Obtaining supernatant from *Lactobacillus rhamnosus*

L. rhamnosus V5 strain was grown in a tube containing 10mL of De Man Rogosa, Sharpe Medium (MRS) broth (Difco, Franklin Lakes, NJ, USA) at a

temperature of 37°C, for 18 h. After growth, the culture was centrifuged at 7000 rpm for 10 min. The clear supernatants obtained were used in the experimental trials as follows: (I) Clear supernatants were filter sterilized through membrane filtration, 0.22 µm pore size and 25 mm diameter (Millipore, Billerica, MA, USA), and used in the assay. (II) The pH of clear supernatants was adjusted to pH 6.5–7.0 with 0.1 NaOH and used in the assay after filter sterilization. (III) Clear supernatants with no treatment were subjected to heat treatment at 100°C for 10 and 20 min, respectively, and used in the assay after filter sterilization (Aslim et al., 2005).

5.2. Antimicrobial susceptibility testing

After obtaining supernatant (I, II, III), susceptibility testing was performed to determine the Minimal Inhibitory Concentration (MIC) using the microdilution method, as standardized by the National Committee for Clinical Laboratory Standards - CLSI (2011). The test was performed in triplicate in a 96-well plate, with a U-bottom shape.

S. *enterica* serovar Typhimurium was initially cultivated in Nutrient Agar (AN) (Difco, Franklin Lakes, NJ, USA) at 37 °C for 18 h and was then standardized against the 0.5 McFarland standard and diluted 1:100 in saline (0.9% NaCl) until reaching the concentration of 10^6 CFU / mL. In the positive control, Müller-Hinton (MH) broth (Difco, Franklin Lakes, NJ, USA) medium and the bacteria were added, while in the negative control only the MH broth was added. In the first horizontal line of the plate 60 µL of MH broth, and 40 µL of the supernatant were added and in the rest of the board 50 µL of MH broth was added. Microdilutions were made with the supernatant concentrations ranging from 20 to 0.62%. After that, each well was inoculated with 50 µL of the bacterial suspension prepared above and finally the plate was incubated at 37 °C for 24 h and the bacterial growth visually assessed.

5.3. Growth and death curve

After analyzing the MIC of the supernatant (I) the growth and death curve was performed. *S*. Typhimurium assay was grown in NA (Difco, Franklin Lakes, NJ, USA) medium and incubated at 37°C for 18 h after bacterial growth. It was adjusted to a concentration of 1.5 x 10^8 bacteria/mL and 10 µL placed in three microtubes by varying the concentration of the supernatant, 20%, 10%, and the control, containing the bacterium and MH broth.

The microtubes were incubated at 37°C and evaluated at the following moments; 0 h, 2 h, 4 h, 7 h, 10 h, and 24 h. Serial dilutions and triplicate plating in MH agar medium were performed in each period. The CFU count was performed after 24 h of incubation at 37ºC.

5.4 "Spot-on-the-lawn" antagonism method

The antimicrobial activity of lactobacilli against *S.* Typhimurium was determined by the "spot on the lawn" antagonism method, performed according to the methodology described by Lima, Andreatti Filho et al. (2007). The lactobacilli were grown in MRS broth, and incubated at a temperature of 37°C, for 24 h under aerobic conditions. Subsequently, aliquots of this culture were added in point form onto the MRS agar plate. After drying was complete, the plate was incubated under aerobic conditions at a temperature of 37°C, for 8 h.

S. Typhimurium was previously seeded in NA at 37°C for 24 h, and standardized according to the McFarland 0.5 scale (corresponding to 1.5 x 10^8 CFU/ml). Next, 250 µL of the bacterium was transferred to an erlenmeyer flask containing 25 mL of MH semi-solid agar, where it was homogenized to be poured into the *L. rhamnosus* dish. After complete solidification of the upper layer, the plate

was incubated for an additional 24 h at 37°C under aerobic conditions. The presence of inhibition halos indicated the production of substances with antimicrobial activity.

6. *In vivo* assay

6.1. Animals

In total,16 BALB/c female mice were tested (4- to 6-weeks-old), approximately 20 g, purchased from the State University of Londrina (Londrina, PR, Brazil) and maintained in a pathogen-free animal facility. The project was approved by the Ethics Committee for Animal Experimentation of the State University of Londrina (CEUA / UEL), protocol n^{o}104/2013.

6.2. *In vivo* challenge with mice

The *in vivo* assay and microbiological analysis were performed according to the protocol of Coconnier et al. (1998). The mice were divided into two groups, the treated group which received, orally (gavage method), three inoculations of an 18 h grown culture of *L. rhamnosus* containing 10^9 CFU in 0.2 mL, on alternate days, and the control group that received 0.2 mL of PBS.

After treatment, a bacteria suspension with 10^8 UFC of *S. enterica* serovar Typhimurium was used for inoculation. After 10 and 14 days of pathogen inoculation, 4 mice from each group were euthanized by cervical dislocation (treated with lactobacilli and non-treated control) and the spleen, liver, and Peyer's patches removed for microbiological and histopathological analysis.

6.3. Microbiological analysis

Microbiological analysis was performed to evaluate the translocation of *S. enterica* serovar Typhimurium. After collection, a small part of the organs was cut and reserved for histology. The other portion of the organs was weighed, crushed with macerators, homogenized, and individually reserved in Falcon type tubes containing 5 mL of PBS. Serial dilutions (10^{-1}, 10^{-2}, 10^{-3}) were made and 10 µL of these bacterial suspensions were plated in triplicate in MC agar at 37 ºC; after 24 h the CFU was determined by direct counting.

6.4. Histopathological analysis

The collected material was processed and analyzed by the Department of Histology, Center for Biological Sciences, State University of Londrina, PR, Brazil.

The organs were fixed by immersion in Bouin solution for 24h. All collected organs and the ileum were included in paraffin following a conventional protocol; 7-µm- sections were stained with hematoxylin and eosin (HE). The images were captured using photomicroscopy (Zeiss Axiophot) coupled to a high resolution camera (Moticam 2300 3.0 MP). Alterations in the histological structure were investigated.

7. Statistical Analysis

Differences in the *in vitro* and *in vivo* tests were compared using the Student t test. For statistical analysis *in vivo,* data were normalized by total CFU per milliliter (CFU/mL) for the Peyer's patches, spleen, and liver.

For analysis of the growth and death curve data, analysis of variance (ANOVA) was performed, and the Tukey test to compare the means, considering a

factorial design, the factors being the treatments, and the levels the times. The significance level adopted was 10%, and the analyses were performed using software R.

RESULTS

1. Identification of *Lactobacillus rhamnosus* strain V5

The 16S ribosomal RNA gene sequence was deposited in GenBank database under accession number MG209517.

2. Adhesion and invasion in cell culture

The adhesion assays using HeLa cells showed an inhibition of *Salmonella*-adherence in the presence of *L. rhamnosus* (Table 1 and Figure 1). The addition of probiotic together with *Salmonella* presented the highest inhibition when compared with previous treatment (Table 1 and Figure 1).

Table 1. Inhibition of the *Salmonella* adhesion in HeLa cells by *Lactobacillus rhamnosus*

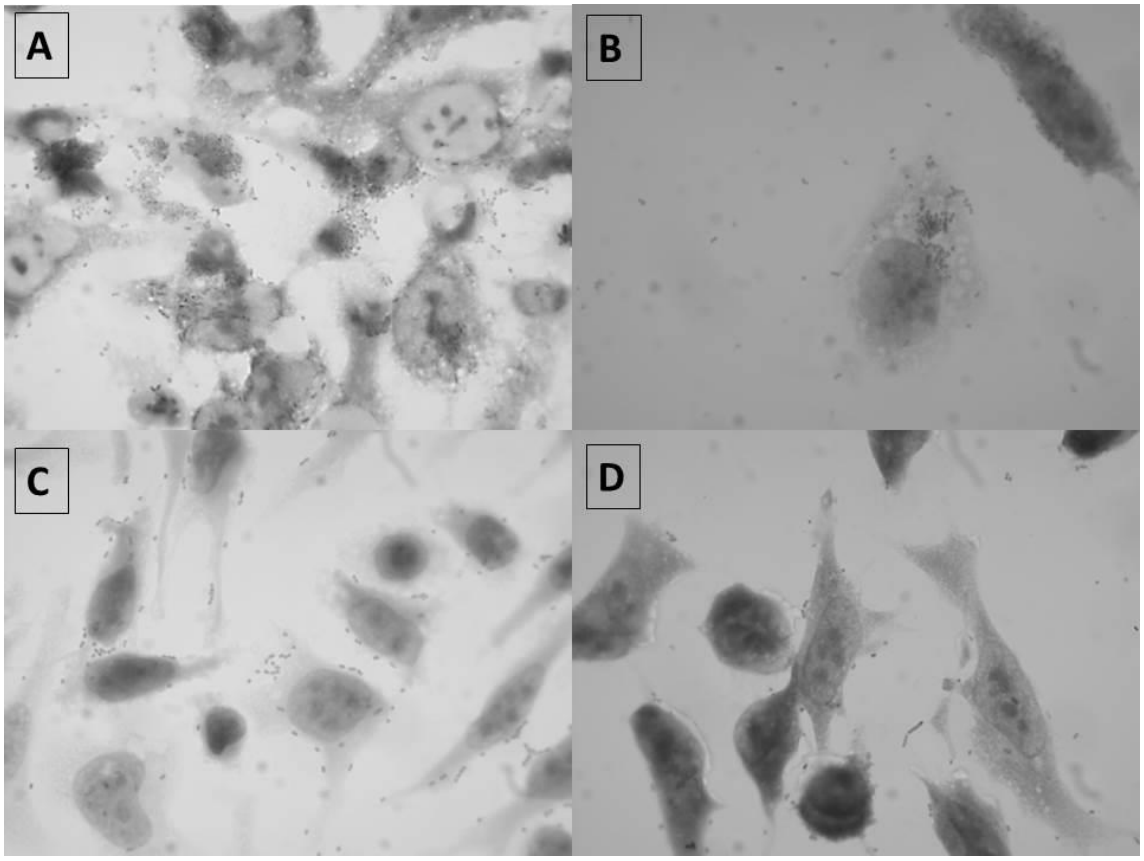
Bacterial treatment	Number of adhered-bacteria/ 100 cells
Lactobacillus rhamnosus	0
***Salmonella* Typhimurium**	782 ± 144,2498
Lactobacillus* + *Salmonella* (0 h)	40 ± 16,32993
Lactobacillus* + *Salmonella* (- 3 h)*	128 ± 45,25483
Lactobacillus* + *Salmonella* (- 5 h)**	370 ± 140,4564

***Lactobacillus* strain added together *Salmonella* strain.

****Lactobacillus* strain added 3 h before the addition of *Salmonella* strain.

*****Lactobacillus* strain added 5 h before the addition of *Salmonella* strain.

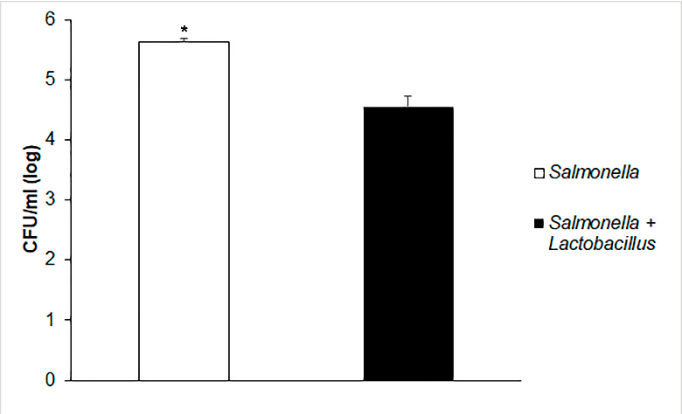
Figure 1. Adhesion of *Salmonella* Typhimurium in HeLa cells in the presence of *Lactobacillus rhamnosus*



A and **B**: Only *Salmonella* Typhimurium. **C**: *Salmonella* plus probiotic (previous treatment – 3 h). **D**: *Salmonella* plus probiotic (previous treatment – 5 h). Magnification of 1,000 x.

The inhibition of Salmonella invasion in HEp-2 cells was also observed in the presence of probiotic (Figure 2), showing a significant reduction of invasive cells in the presence of L. rhamnosus compared to control.

Figure 2. Invasion of *Salmonella* Typhimurium in HEp-2 cells in the presence of *L. rhamnosus*



3. Antibacterial activity of the supernatant

3.1. MIC

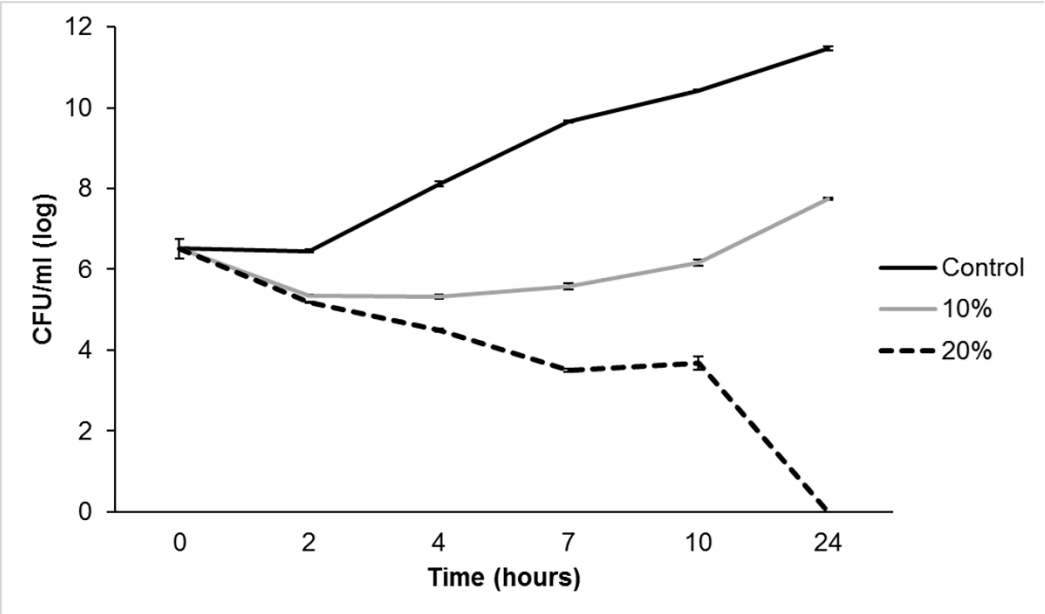
In the present study, the supernatant (I) demonstrated antimicrobial activity. The minimal inhibitory concentration of the supernatant against *S.* Typhimurium was 10%.

The supernatant (II) was sensitive to neutralization with 1N NaOH solution, totally losing its inhibitory capacity, demonstrating that the antimicrobial activity verified in the experiment may have been due to the presence of acids, leading to a drop in the pH of the medium. During the growth of lactic acid bacteria, a fall in pH occurs, making the environment quite acid, mainly due to the production of acids such as lactic acid. The supernatant (III) was resistant to thermal treatments. The minimal inhibitory concentration of the supernatant against *S.* Typhimurium was 10%, demonstrating that the antimicrobial activity verified in the experiment may have been due to the presence of acids. Bacteriocin produced by BAL has low molecular weight and are easily denatured by thermal treatments.

3.2. Growth and death curve

After determining the minimum inhibitory concentration of the supernatant not neutralized against *S*. Typhimurium, the time-kill curve assay was performed. The results showed statistically significant differences considering ($p < 0.10$). The 10% supernatant was able to inhibit the growth of the bacterium, but after the period of 10 h the bacteria began to multiply and at the end of 24 h had a 1 log increase in relation to the initial inoculum, demonstrating a bacteriostatic effect. However, in the time of 24 h showed a difference of 4 logs (Figure 3). The supernatant at 20% presented bactericidal action, gradually decreasing the number of viable cells, eliminating 100% of the bacterial population in 24 h (Figure 3).

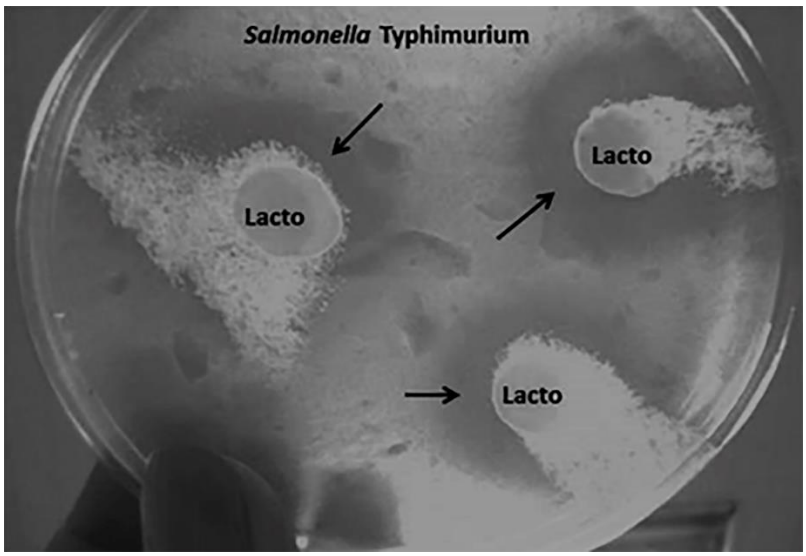
Figure 3. Growth and death curve of *Salmonella* Typhimurium in the presence of supernatant not neutralized from *L. rhamnosus*



3.3. Spot-on-the-lawn antagonism method

The "Spot-on-the-lawn" antagonism method showed the antimicrobial activity of lactobacilli against *S.* Typhimurium, forming zones of inhibition of 21 mm in diameter (Figure 4).

Figure 4. Antagonism assay by "Spot-on-the-lawn" between Salmonella Typhimurium and L. rhamnosus. The arrows indicate halos of inhibition around L. rhamnosus (Lacto) inoculum.



The arrows indicate halos of inhibition around *Lactobacillus* inoculum.

4. *In vivo* assay

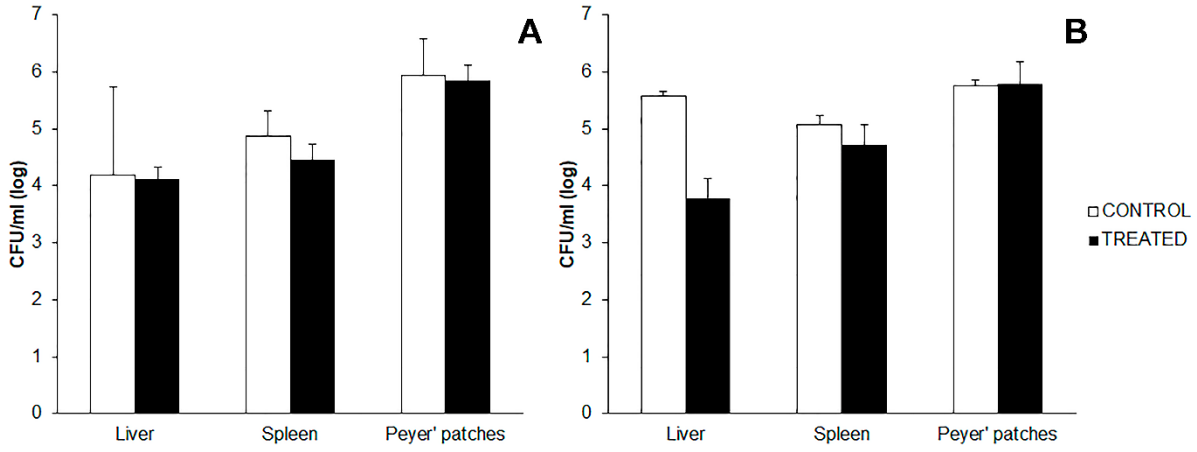
4.1. Microbiological analysis

Ten days post-infection with *S.* Typhimurium, we observed a slight decrease in the number of *Salmonella* colonies in the spleen when treated with probiotic (Figure 5A). Other organs (liver and Peyer's patches) did not present significant differences between the treated and control animals (non-treated).

However, after 14 days the number of *Salmonella* colonies was lower in all organs from mice treated with lactobacilli, mainly the liver (Figure 5B).

Some non-treated (control) mice died (data not shown) and their organs were not collected for microbiological analysis.

Figure 5. Counting of colony forming unit from liver, spleen and Peyer' patches after 10 and 14 days post-infection in murine model.



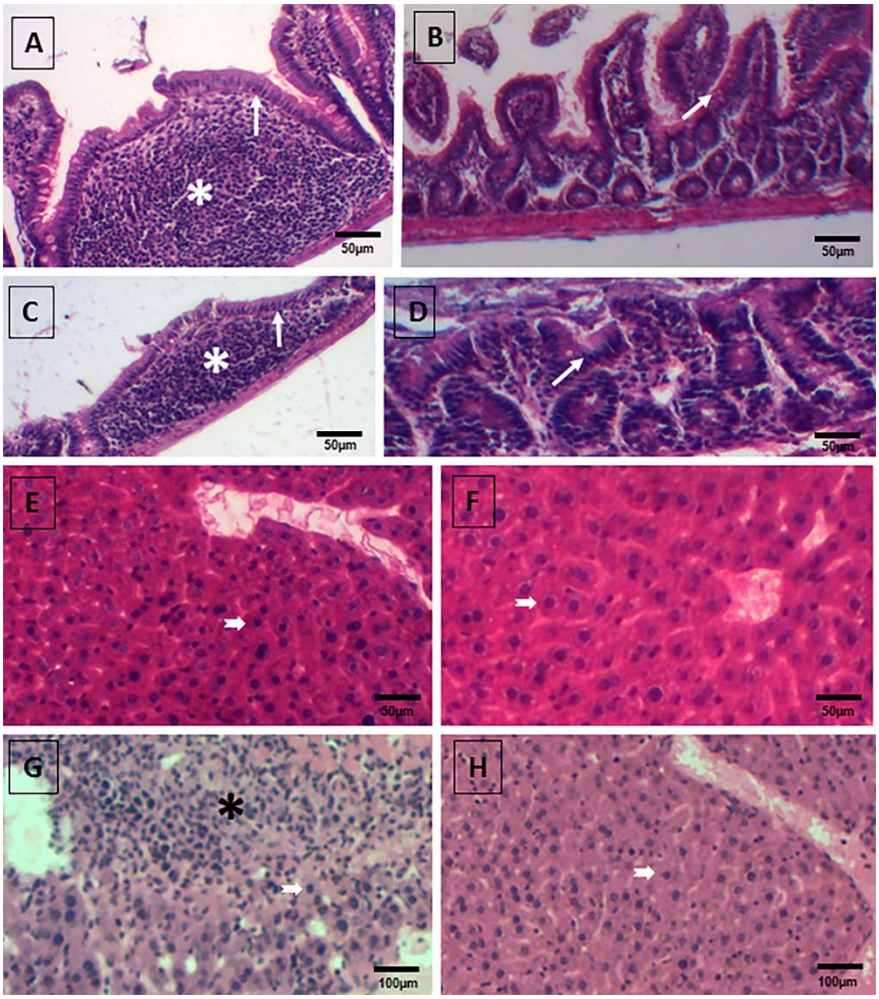
4.2. Histopathological analysis

The ileum samples collected from the control group demonstrated the presence of prominent lymphoid nodules both at 10 days (Figure 6A) and 14 days (Figure 6C) post-infection. Mice treated with *L. rhamnosus* did not present alterations in the histological characteristics of the ileum at either moment (Figures 6B and 6D).

The liver samples of non-treated mice demonstrated the presence of inflammatory foci both at 10 days (Figure 6E) and at 14 days (Figure 6G) post-infection. Mice treated with *L. rhamnosus* did not present inflammatory foci at either moment (Figure 6F and 6H).

Intestinal epithelial cells, and the spleen and liver of all groups did not show detectable histopathological alterations under light microscopy.

Figure 6. Photomicrograph of ileus intestinal tissues and liver of mice treated and non-treated with probiotics at 10 and 14 days post-infection with *S.* Typhimurium.



Photomicrograph of intestine (ileum) and liver of mice infected with *S.* Typhimurium. **A:** Ileum of non-treated mice at 10 days post-infection with *Salmonella*. Note prominent lymphoid nodule (*). **B:** Group treated with *L. rhamnosus* at 10 days post-infection. **C:** Non-treated mice at 14 days post-infection with *Salmonella*. Note prominent lymphoid nodule (*). **D:** Group treated with *L. rhamnosus* at 14 days post-infection. Intestinal epithelium (long arrows). **E:** Liver of non-treated mice at 10 days post-infection with *Salmonella*. **F:** Group treated with *L. rhamnosus* at 10 days of infection. **G:** Non-treated mice at 10 days post-infection with *Salmonella*. Note inflammatory foci (*). **H:** Group treated with *L. rhamnosus* at 14 days of infection. Hepatocyte (short arrows). Stained with Hematolin-Eosin (HE).

DISCUSSION

Several studies have reported different probiotics to prevent infections against foodborne pathogens (Tellez et al., 2013; Hirano et al., 2017; Forkus et al., 2017). In this report, we showed that *L. rhamnosus* V5 promoted protection *in vitro* and *in vivo* against *S. enterica* serovar Typhimurium UK1, attenuated Δ*cya*Δ*crp* (χ3985 UK1 [Δ*cya*Δ*crp*]) (strain from Center for Infectious Diseases and Vaccinology, The Biodesign Institute and School of Life Sciences, Arizona State University, Tempe, AZ (Curtiss et al., 1989). This infection model was used to evaluate the translocation process in mice, although *S*. Typhimurium mutant decreased its virulence by deletion in the receptor protein of the adenylate cyclase and cyclic AMP, this strain keeps invading host cells (Curtiss and Kelly 1987). Due to the characteristic of this pathogen strain, this *in vivo* model evaluated the probiotic effect.

Several mechanisms have been proposed to explain the beneficial effects of probiotics. For example, bacteriocins produced by probiotics can inhibit pathogenic bacteria, preventing infection in humans or other animals (Gómez et al., 2016). However, our results support that the inhibitory activity observed in *in vitro* assays was due to the production of organic acids, which reduced the pH of the medium. This effect was similar to the studies of Ogawa and collaborators (2001) and Pereira and Gómez (2007).

Probiotics can also inhibit pathogen adherence in host cells by competition in linkage to host receptors (Chen et al., 2012). In this sense, we observed that the presence of *L. rhamnosus* decreased the number of *Salmonella*-adhered in HeLa cells, mainly when the probiotic was added together with the pathogen (Table 1). Interestingly, the non-adherence of *L. rhamnosus* in HEp-2 cells suggests that the bacteriocins or presence of acids from probiotics can prevent bacterial adhesion or

colonization. Cell invasion ability is an important virulence characteristic of *Salmonella*. *L. rhamnosus* V5 to reduce the number of invasive bacteria in HEp-2 cells, as also demonstrated in previous studies (Nakazato et al., 2011; Chen et al., 2012). Thus, the results of adhesion in HeLa and invasion assays in HEp-2 cells showed that cell culture is an interesting *in vitro* tool to select an applicant for probiotic.

As *L. rhamnosus* V5 demonstrated a protective effect *in vitro* against infection, we evaluated if this positive effect would also be observed in mice infected with *S. enterica* serovar Typhimurium UK1. In this way, it was verified that the number of salmonella bacteria in the organs of the three treated mice was significantly lower than the untreated mice after 14 days of infection. Thus, the microbiological evaluation after 14 days of infection in this model showed effective partial protection.

Acursio et al. (2017) tested the protective effect of *Lactobacillus plantarum* and *L. rhamnosus* inoculating a single dose of fermented milk containing of 8.0 log10 CFU/mL. Afterwards, live S. Typhimurium was inoculated five days after mono-association with *Lactobacillus* strains. On day 20 post-challenge with *S*. Typhimurium, translocation was found in the liver of mice treated with *L. plantarum* but not in those treated with *L. rhamnosus*. This result is very important to highlight that not all species of the *Lactobacillus* genus are able to present a protective effect.

Our *in vivo* results confirmed the decrease in the invasion *in vitro*, showing that cell culture assays have been used previously to assess the effective probiotic potential. Thus, other tests using alternative models would be interesting in an initial screening assessment of the effectiveness of probiotics (Kim and Mylonakis, 2012; Lee et al., 2011; Trapecar et al., 2014).

The presence of prominent inflammatory foci in the intestinal mucosa was observed only in mice non-treated with *L. rhamnosus* It is known that these inflammatory foci are common in the ileal mucosa, but they tend to increase in quantity and size when the intestinal barrier is ruptured. As no change was observed in the larynx of mice treated with probiotics, it is suggested that *L. rhamnosus*. V5 was more effective for the intestinal barrier.

The literature already describes that *Lactobacillus* protects the integrity of the intestinal epithelial barrier from *Salmonella* infection (Yu et al., 2015), which corroborates our results. Researches showed the efficiency of *Lactobacillus fructosus* and *L. rhamnosus* in maintaining the integrity of Caco-2 culture cells (Kemgang et al., 2016; Yu et al., 2015). Reduction in inflammatory foci leads to a progressive decrease in intestinal inflammation of the Peyer's patches, spleen, and peritoneum of mice treated with *L. casei* (de Moreno de LeBlanc et al., 2010). The absence of changes in the spleen, liver, and Peyer's patches at 10 days post-infection suggests that bacterial translocation was under control.

It is known that salmonella bacteria attack enterocytes, promoting rupture of occlusive junctions (Shen et al., 2006, Yu et al., 2015) and M cells, provoking intense inflammatory response (Jones et al., 1994). Untreated mice infected with *Salmonella* bacteria presented more inflammatory nodules in the ileum and the presence of bacteria in the liver. On the other hand, mice treated with *L. rhamnosus* V5 and infected with *Salmonella* bacteria presented reduced inflammatory nodules in the ileum and no histological alterations in the liver. Considering this, it is reasonable to consider that *L. rhamnosus* V5 used as a probiotic was able to improve the intestinal barrier. Therefore, the use of *L. rhamnosus* V5 as a probiotic could be a viable alternative for controlling salmonellosis. Further studies using transmission

electron microscopy could provide detailed information about the ultrastructure of the intestinal wall of these mice and contribute to understanding the mechanisms involved in the beneficial action of *L. rhamnosus* V5 as a probiotic.

Therefore, we conclude that *L. rhamnosus* V5 was able to control *S. Typhimurium,* inhibiting the adhesion and invasion of *Salmonella* bacteria *in vitro* and in mice *in vivo*. Because of this, *L. rhamnosus* V5 was able to control pathogen translocation in the spleen and liver. Thus, *L. rhamnosus* V5 could be used as a probiotic to control salmonellosis.

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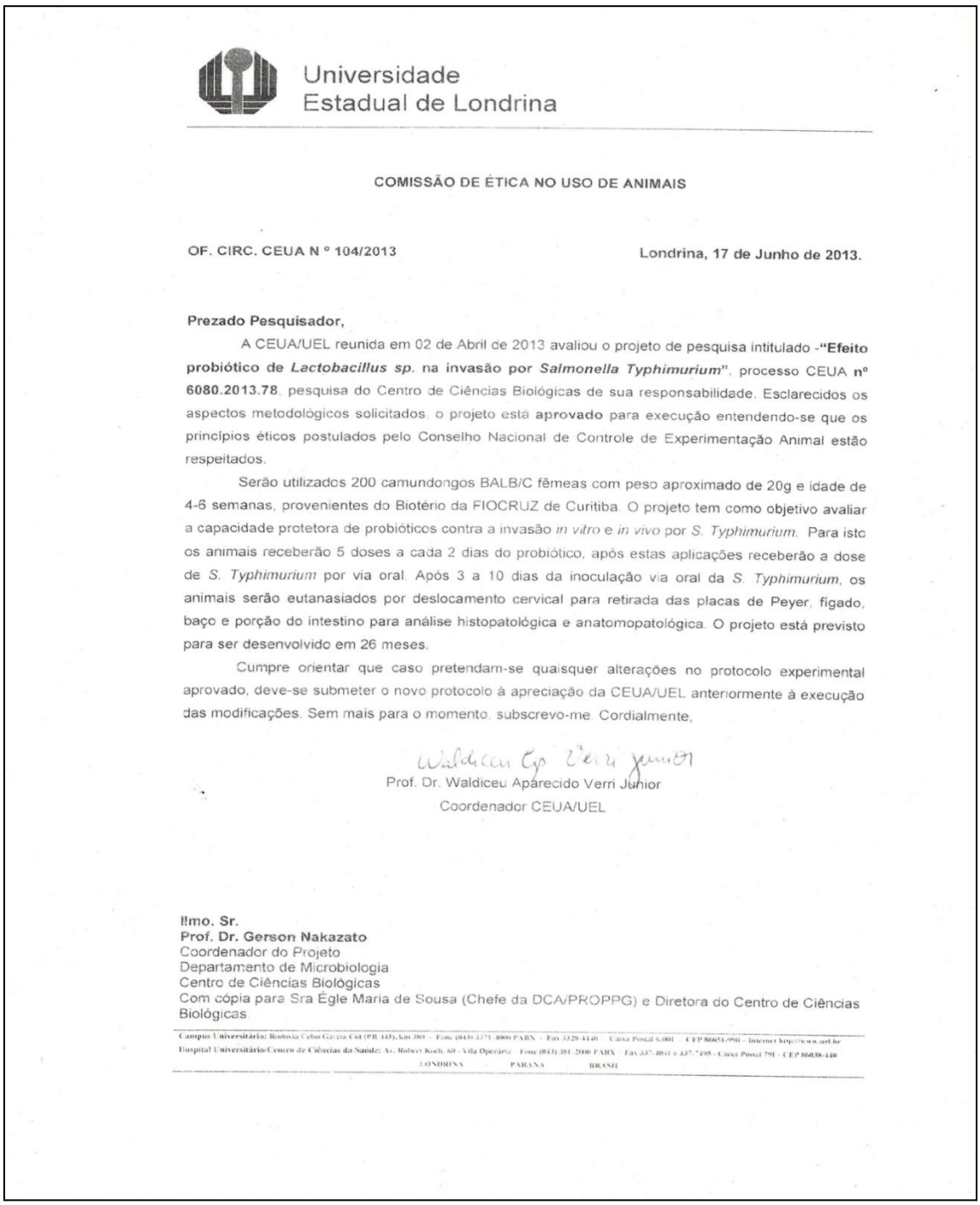
ANEXO

Authorization of the Ethics Committee on Animal Use the State University of Londrina (CEUA / UEL).



Universidade
Estadual de Londrina

COMISSÃO DE ÉTICA NO USO DE ANIMAIS

OF. CIRC. CEUA N º 104/2013 Londrina, 17 de Junho de 2013.

Prezado Pesquisador,

A CEUA/UEL reunida em 02 de Abril de 2013 avaliou o projeto de pesquisa intitulado -**"Efeito probiótico de *Lactobacillus sp.* na invasão por *Salmonella Typhimurium*"**, processo CEUA **nº 6080.2013.78**, pesquisa do Centro de Ciências Biológicas de sua responsabilidade. Esclarecidos os aspectos metodológicos solicitados, o projeto está **aprovado** para execução entendendo-se que os princípios éticos postulados pelo Conselho Nacional de Controle de Experimentação Animal estão respeitados.

Serão utilizados 200 camundongos BALB/C fêmeas com peso aproximado de 20g e idade de 4-6 semanas, provenientes do Biotério da FIOCRUZ de Curitiba. O projeto tem como objetivo avaliar a capacidade protetora de probióticos contra a invasão *in vitro* e *in vivo* por *S. Typhimurium*. Para isto os animais receberão 5 doses a cada 2 dias do probiótico, após estas aplicações receberão a dose de *S. Typhimurium* por via oral. Após 3 a 10 dias da inoculação via oral da *S. Typhimurium*, os animais serão eutanasiados por deslocamento cervical para retirada das placas de Peyer, fígado, baço e porção do intestino para análise histopatológica e anatomopatológica. O projeto está previsto para ser desenvolvido em 26 meses.

Cumpre orientar que caso pretendam-se quaisquer alterações no protocolo experimental aprovado, deve-se submeter o novo protocolo à apreciação da CEUA/UEL anteriormente à execução das modificações. Sem mais para o momento, subscrevo-me. Cordialmente,

Waldiceu Cp Verri Junior

Prof. Dr. Waldiceu Aparecido Verri Junior
Coordenador CEUA/UEL

Ilmo. Sr.
Prof. Dr. Gerson Nakazato
Coordenador do Projeto
Departamento de Microbiologia
Centro de Ciências Biológicas
Com cópia para Sra Égle Maria de Sousa (Chefe da DCA/PROPPG) e Diretora do Centro de Ciências Biológicas

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Comprovante de envio do manuscrito

Dear Ms Tsuruda,

Submission no: FM_2017_959
Submission title: Lactobacillus rhamnosus V5 prevents Salmonella enterica serovar Typhimurium invasion in cell culture and mice infection
Corresponding author: Dr Gerson Nakazato
Listed co-author(s): Dr Sandra Garcia, Dr Ana Angelita Baptista, Professor Ricardo S. Almeida, Dr Luciano Panagio, Professor Renata Kobayashi, Ms Andréia Pupim, Professor Eduardo Jose de Almeida Araujo, Dr Juan Josue Puño-Sarmiento, Dr Daniela Pinheiro, Ms Erick Nishio, Ms Carina Tsuruda, Ms Patrícia De Souza

Dr Nakazato has submitted a manuscript to Food Microbiology and listed you as a co-author. This email is to let you know we will be in contact with updates at each decision stage of the submission process.

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Food Microbiology

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