Antimicrobial activity of probiotic *Lactobacillus* strains towards gram-negative enteropathogens

Moysés Estevão de Souza Freitas Pehrson[1]* - PEHRSON, M. E. S. F.
E-mail: moyses.freitas@gmail.com

Ismael Maciel de Mancilha[1] - MANCILHA, I. M.
E-mail: mancilha@debiq.eel.usp.br

Carlos Alberto Sanches Pereira[2] – PEREIRA, C. A. S.
E-mail: sanches68@gmail.com

[1] Laboratório de Probióticos, Departamento de Biotecnologia, Escola de Engenharia de Lorena, Universidade de São Paulo, Lorena, SP, Brasil. –
Adress: Estrada Municipal do Campinho, s/n Ponte Nova - Lorena – SP. Postal code: 12602-871

[2] Laboratório de Biotecnologia, Fundação Oswaldo Aranha, Centro Universitário de Volta Redonda (UniFOA), Volta Redonda, RJ, Brasil.
Adress: Avenida Paulo Erlei Alves Abrantes, 1325, Três Poços, Volta Redonda – RJ.
Postal code: 27240-560

*** Corresponding Author**

Sponsoring: CAPES (Scholarship)

ABSTRACT

Gram-negative enteropathogens are among the main bacteria in severe livestock and human intestinal illnesses. Here we evaluated the antimicrobial activity of L. acidophilus ATCC 4356L. rhamnosus ATCC 7469, L. fermentum ATCC 9338and L. plantarum ATCC 8014towards six strains of enteropathogens bacteria. Antimicrobial activity assay was undertaken by evaluating pathogen growth in the presence of cell-free supernatants of lactobacilli strains. Subsequently, the supernatants underwent several treatments (catalase, proteases, heating and neutralization) to assess the nature of the substance involved in antimicrobial activity. The supernatants of L. acidophilus, L. casei and L. plantarum significantly inhibited growth of 5 pathogens. On the other hand, the supernatant obtained from L. fermentum inhibited all evaluated enteropathogens. In all cases, antimicrobial activity was associated to the drop in pH due to the production of organic acids. We also demonstrated that antimicrobial effect was not associated with antimicrobial peptides, hydrogen peroxide or thermolabile substances.

Keywords: Lactobacillus; antagonism; antimicrobial activity; enteropathogens; probiotics

1 INTRODUCTION

The intestinal lumen is a nutritionally rich environment, in which microbial concentrations of up to 10^{12} CFU/ml can be found (Whitman et al., 1998). Gut microbial interactions are highly complex and dynamic; therefore, composition of the microbial community fluctuates constantly due to a series of factors. Large or selective shifts in the gut microbiota composition promoted by changes in diet, medications and environment may trigger specific microbial interactions or host-microbiota associated events, which can significantly influence host health. (Sun; Chang, 2014). In this context, it is well known that the predominance of beneficial microorganisms may contribute to immune function as well as to prevent the emergence of pathogenic and/or opportunistic species.

According to Šušković et al. (2010), the ability to inhibit pathogenic species and undesirable microorganisms is the most important property of probiotic and other functional lactic acid bacteria. In addition, probiotic bacteria have been associated to a number of other beneficial effects, which are observed when they are consumed regularly in adequate amounts (Table 1).

According to O'Brien and Wright (2011), the main objective in the production of antimicrobial compounds is establishing an adaptive advantage in highly competitive environments. In such cases, according to Maróti et al.(2011), antagonism constitutes an important mean by which a particular species overthrow competing species, though this ability comes at an energetic cost.

Fermented foods have been used throughout millennia by several different world cultures. Their primordial function was to preserve the nutritional properties of the food while providing a suitable form for long-term storage (Wood, 1998). Fermentation metabolism reduces the availability of carbohydrates, resulting in the formation of inhibitory compounds such as organic acids (Blom; Mørtvedt, 1991), and other compounds which can be produced by specific lactic acid bacteria (Ouehand; Vesterlund, 2004).

Organic acids, especially lactic acid, have been reported as the main metabolites responsible for antagonist activity of probiotic bacteria towards pathogenic species (Ogawa et al., 2001; Tsai et al., 2005; Tsai et al., 2008a; Zhang et al., 2011). However, many other compounds such as diacetyl, reuterin and bacteriocins have been associated with the antimicrobial effect of probiotic bacteria as well (Lanciottiet al., 2003; Martín et al., 2005; Xieet al, 2011; Jiang et al., 2012). Therefore, the present study aimed to evaluate, *in vitro,* the antimicrobial effect of four probiotic *Lactobacillus* strains upon six strains of enteric pathogens. Additionally, presumptive identification of compounds responsible for pathogen inhibition was undertaken.

Table 1 – Beneficial properties associated with probiotics intake

Property	Reference
Vitamin production; mineral bioavailability increase	(NARVA et al., 2004;POMPEI et al., 2007)
Reduction of cholesterol and/or triglyceride levels	(LIONG; SHAH, 2005; GUO et al., 2011)
Immunomodulation	(TSAI et al., 2008b; TSAI et al., 2010a; TSAI et al., 2010b)
Increased intestinal motility/alleviation in constipation cases	(GUERRA et al., 2010; RIEZZO et al., 2012)
Adherence to intestinal mucosa and maintenance of its integrity	(UCHIDA; KURAKASU, 2004;LAM et al., 2007)
Production of digestive enzymes	(HONDA et al., 2007)
Pathogen inhibition and modulation of intestinal microbiota through bacteriocin production .	(CURSINO et al.,2006; PINGITORE et al., 2009; TODOROV et al., 2011)
Reduction of obesity by inducing significant reduction in adipocyte size	(CHOI et al., 2007; TAKEMURA et al., 2009; KADOOKA et al., 2010)
Attenuation of intestinal allergic reactions	(YOSHIDA et al., 2011)
Accelerated wound healing (topicaluse)	(PERAL; MARTINEZ; VALDEZ, 2009; NASRABADI et al., 2011; HUSEINI et al., 2012;)
Anticarcinogenic effect	(KUMAR et al., 2012)

2 MATERIAL AND METHODS

2.1 Microorganisms and media

In this study the following probiotic *Lactobacillus* strains were evaluated: *L. acidophilus* ATCC 4356, *L. rhamnosus* ATCC 7469, *L.fermentum* ATCC 9338, and *L. plantarum* ATCC 8014. These strains were stored at -20 ^{o}C in MRS broth (De Man; Rogosa; Sharpe, 1960) supplemented with 20% (v/v) glycerol and were activated by subsequently growing them in MRS broth at 37°C / 24hthree times.

Enteropathogenic *Escherichia coli* 0112, enteropathogenic *E. coli* 0124, *E. coli* enteropathogenic 0127, *Shigellasonnei* ATCC 25931, *Shigelladysenteriae* ATCC 13313 and *Salmonella enteritidis* ATCC 13076 were used as indicator microorganisms. These strains were kept under refrigeration (5ºC) in agar slant test tubes containing stock agar, which consisted of 23g/L bacteriological agar, 5 g/L NaCl, 2 g/L Na_2HPO_4 , sterilized at 121ºC/15min. Prior to testing these strains were activated by subsequently growing them in BHI (Brain Heart Infusion) broth at 37°C/ 24h three times.

2.2 Obtention of cell-free supernatants

Lactobacilli suspensions were obtainedby inoculating 10 µL in tubes containing 7ml of MRS broth followed by incubation for 48 hoursat 37 ºC prior to separating the cells from the supernatants. At that point, cell suspensions were centrifuged at 7000xG for 15 min at 4 ºC and then supernatants were collected. Afterwards, the supernatants were sterilized by filtration in 0,22 µm Millex® membranes(JBR610209) and stored in cryotubesat -20ºC.

2.3 Screening of antimicrobial activity and expression of inhibition effect

The antimicrobial activities were determined by means of Microscale Optical Density Assay (MODA) method, as described by Lash et al. (2002). In the present study we used a high viable population of the pathogenic strains (10^8 CFU total), suspended in 100 µL BHI broth in a microplate well. To this suspension was added 30 µL of the original supernatant, followed by incubating the microplates at 37 ºC / 24 h**,** afterwards, the absorbance (630nm) was measured in a microplate reader.

From here on referred as MRS control, in one well,30 µL of sterile MRS broth (pH 7.0) were added to the pathogenic cell suspension in order to verify the presence of a possible inhibitory compound in the MRS medium (e.g. sodium acetate, citrate). As a Negative control, only 100 µL of the pathogenic cell suspension were transferred to another well, and the microplate was incubated at the same conditions described above. These procedures were undertaken in three repetitions with analytical triplicates.

The inhibition effect of original supernatants was calculated by the following formula:

$$\%\ \text{inhibition} = 100 \times \left\{1 - \left(\frac{\text{DOs}}{\text{DOc}}\right)\right\}$$

DOs – Optical density observed with the addition of the supernatant

DOc – Optical density observed in the Negative control

2.4 Evaluation of pH effect on antimicrobial activity of the supernatants

In order to evaluate the effect of the supernatants pH, those which showed a significant inhibition effect in the previous experiment, were reevaluated after neutralizing the pH with NaOH 0.1M. Additionally, a test was carried out as a positive control, evaluating the growth of the pathogen species in the presence of 30 µL acidified MRS broth, in the same conditions. The pH of MRS broth was adjusted with glacial acetic acid to the same pH values of probiotic supernatants, as follows: *L. acidophilus*: 4,2; *L. casei*: 4.3; *L. fermentum*: 4.1; *L. plantarum*: 4.4.

2.5 Effect ofH_2O_2, proteolytic enzymes and heating on the antimicrobial activity of the supernatants

In order to verify the possible inhibition effect of hydrogen peroxide, the supernatants were treated with 1mg/ml catalase (Sigma) at 37°C for 1,5 h in a water bath, sterilized by filtration, and reevaluated at the same conditions. To study the contribution of antimicrobial peptides such as bacteriocins, the supernatants were individually treated with 1mg/ml Pronase (Sigma), α-Chymotrypsin (Sigma) and Trypsin (Sigma) at 37°C / 1,5 h in a water bath. Afterwards the supernatants were reevaluated at the same conditions. To investigate the presence of thermolabile antimicrobial compounds, the supernatants were heated to 100 ºC or 121 º for 15 min, and then tested at the same conditions. All tests were also carried out in three repetitions, with analytical triplicates.

2.6 Statistical analysis

The results were statistically analyzed by means of ANOVA and Tukey's post-hoc test with 95% confidence.

3 Results and discussion

Inhibition effects related to the supernatants of the respective lactobacilli strains are represented in Figure 1. As it can be observed, all *Lactobacillus* strains exerted significant ($p<0.05$) growth inhibition effect on *E. coli* 0112, *E. coli* 0124, *E. coli* 0127, *S. enteritidis* ATCC 13076 and S. sonnei ATCC 25931 growth, varying from 24 to 51% of inhibition. However, *S. dysenteriae* ATCC 13313 was not inhibited by

L. plantarum ATCC 8014, *L. rhamnosus* ATCC 7469 and *L. acidophilus* ATCC 4356 supernatants, but it was significantly inhibited (21%) when cultivated in the presence of *L. fermentum* ATCC 9338supernatant. It can also be noted that the inhibition effects of *L. fermentum* ATCC 9338 on the growth of others pathogenic strains were significantly higher ($P<0.05$) and varied between 43 and 57% growth inhibition.

Regarding the presumptive identification steps, the results (Tables 2, 3, 4 and 5) showed no significant effects of enzymatically and heat treated supernatants on the growth of the pathogenic strains. Thus, none of the enzymatic and heat treatments applied to the supernatants were able to neutralize the antimicrobial activity of the supernatants. Therefore, this indicates that the nature of the inhibition effect is not due to the presence of of hydrogen peroxide, antimicrobial peptides and thermolabile compounds in the supernatants.

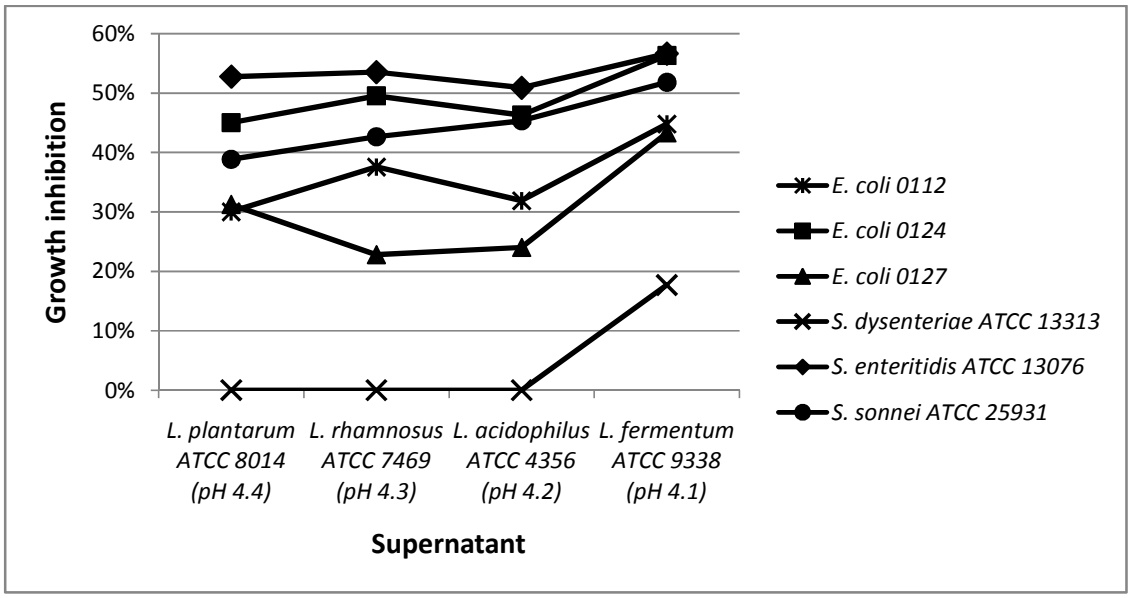

Figure 1 – Inhibition effects observed by culturing indicator pathogenic strains in thepresence of unaltered lactobacilli supernatants.

Table 2 – Effects of different treatments on antimicrobial activity of *L. acidophilus* ATCC 4356 supernatant.

Treatment	Growth inhibition					
	E. coli 0112	*E. coli* 0124	*E. coli* 0127	*S. enteritidis*	*S. dysenteriae*	*S. sonnei*
100 ºC	+	+	+	+	-	+
121 ºC	+	+	+	+	-	+
α-chymotrypsin	+	+	+	+	-	+
Pronase	+	+	+	+	-	+
Trypsin	+	+	+	+	-	+
Catalase	+	+	+	+	-	+
MRS pH 4.2	+	+	+	+	-	+
SN *in natura*	+	+	+	+	-	+
MRS Control	-	-	-	-	-	-
Control	-	-	-	-	-	-
SN pH 7.0	-	-	-	-	-	-

+: Significant inhibition; **-**: No inhibition **SN**: Supernatant.

Table 3 - Effects of different treatments on antimicrobial activity of *L. rhamnosus* ATCC 7469 supernatant.

Treatment	Growth inhibition					
	E. coli 0112	*E. coli* 0124	*E. coli* 0127	*S. enteritidis*	*S. dysenteriae*	*S. sonnei*
100 ºC	+	+	+	+	-	+
121 ºC	+	+	+	+	-	+
α-chymotrypsin	+	+	+	+	-	+
Pronase	+	+	+	+	-	+
Trypsin	+	+	+	+	-	+
Catalase	+	+	+	+	-	+
MRS pH 4.3	+	+	+	+	-	+
SN *in natura*	+	+	+	+	-	+
MRS Control	-	-	-	-	-	-
Control	-	-	-	-	-	-
SN pH 7.0	-	-	-	-	-	-

+: Significant inhibition; **-**: No inhibition **SN**: Supernatant.

Table 4 – Effects of different treatments on antimicrobial activity of *L. fermentum* ATCC 9338 supernatant.

Treatment	Growth inhibition					
	E. coli 0112	*E. coli* 0124	*E. coli* 0127	*S. enteritidis*	*S. dysenteriae*	*S. sonnei*
100 ºC	+	+	+	+	+	+
121 ºC	+	+	+	+	+	+
α-chymotrypsin	+	+	+	+	+	+
Pronase	+	+	+	+	+	+
Trypsin	+	+	+	+	+	+
Catalase	+	+	+	+	+	+
MRS pH 4.1	+	+	+	+	+	+
SN *in natura*	+	+	+	+	+	+
MRS Control	-	-	-	-	-	-
Control	-	-	-	-	-	-
SN pH 7.0	-	-	-	-	-	-

+: Significant inhibition; **-**: No inhibition **SN**: Supernatant.

Table 5 - Effects of different treatments on antimicrobial activity of *L. plantarum* ATCC 8014 supernatant.

Treatment	Growth inhibition					
	E. coli 0112	*E. coli* 0124	*E. coli* 0127	*S. enteritidis*	*S. dysenteriae*	*S. sonnei*
100 ºC	+	+	+	+	-	+
121 ºC	+	+	+	+	-	+
α-chymotrypsin	+	+	+	+	-	+
Pronase	+	+	+	+	-	+
Trypsin	+	+	+	+	-	+
Catalase	+	+	+	+	-	+
MRS pH 4.4	+	+	+	+	-	+
SN *in natura*	+	+	+	+	-	+
MRS Control	-	-	-	-	-	-
Control	-	-	-	-	-	-
SN pH 7.0	-	-	-	-	-	-

+: Significant inhibition; **-**: No inhibition **SN**: Supernatant.

Table 6 – Average absorbance values (*) ± standard deviation of pathogen cell suspensions in different conditions

	***E. coli* 0112**			***E. coli* 0124**			***E. coli* 0127**		
	A	**B**	**C**	**A**	**B**	**C**	**A**	**B**	**C**
L. acidophilus	0,70 ±0,05	0,67±0.05	0,47 ±0,06	0,61 ±0,04	0,62±0,03	0,39 ±0,04	0,82 ±0,03	0,83±0,05	0,64 ±0,05
L. casei	0,68 ±0,03	0,67±0,04	0,43 ±0,03	0,61 ±0,05	0,61±0,04	0,36 ±0,02	0,80 ±0,04	0,83±0,02	0,63 ±0,04
L. fermentum	0,69 ±0,05	0,66±0,04	0,37 ±0,07	0,60 ±0,05	0,61±0,04	0,30 ±0,07	0,81 ±0,04	0,82±0,03	0,48 ±0,03
L. plantarum	0,72 ±0,04	0,70±0,06	0,47 ±0,07	0,63 ±0,03	0,62±0,03	0,39 ±0,03	0,82 ±0,04	0,80±0,04	0,60 ±0,03

A: MRScontrol **B**: Neutralized supernatant **C**: Unaltered supernatant **(*)**: 3 readings x 3 repetitions

Tabela 6 - Continued

	***S. dysenteriae* ATCC 13313**			***S. enteritidis* ATCC 13076**			***S. sonnei* ATCC 25931**		
	A	**B**	**C**	**A**	**B**	**C**	**A**	**B**	**C**
L. acidophilus	1,07 ±0,05	1,04±0,05	0,82 ±0,03	0,67 ±0,07	0,66±0,05	0,41 ±0,06	0,51 ±0,04	0,52±0,05	0,31 ±0,04
L. casei	1,12 ±0,03	1,14±0,05	0,81 ±0,03	0,67 ±0,06	0,66±0,05	0,40 ±0,04	0,54 ±0,04	0,51±0,05	0,3 ±0,03
L. fermentum	1,03 ±0,03	1,04±0,05	0,65 ±0,06	0,67 ±0,08	0,71±0,04	0,38 ±0,08	0,54 ±0,05	0,53±0,05	0,30 ±0,03
L. plantarum	1,21 ±0,07	1,19±0,08	0,90 ±0,1	0,68 ±0,06	0,71±0,06	0,41 ±0,05	0,59 ±0,05	0,55±0,05	0,35 ±0,03

A: MRScontrol **B**: Neutralized supernatant **C**: Unaltered supernatant **(*)**: 3 readings x 3 repetitions

The results (Table 6) statistically analyzed by Tukey's test demonstrated that the evaluation of acidified MRS broth (MRS control) showed the same inhibition effects as the original supernatants. Thus, the most probable cause of antimicrobial activity was the drop in supernatant pH caused by the production of organic acids, which did not differ among *L. acidophilus* ATCC 4356, *L. rhamnosus* ATCC 7469 and *L. plantarum* ATCC 8014 (pH 4.2, 4.3, 4.4, respectively). However, the inhibition effect exerted by *L. fermentum* ATCC 9338, which presented the lowest pH (4.1) was significantly higher ($p<0.05$) than the inhibition effects exerted by the other lactobacilli.

In this study, it was demonstrated that among the pathogenic strains tested, *Shigelladysenteriae* ATCC 13313 was the most resistant to inhibition by the probiotic strains evaluated. Conversely, *Shigellasonnei* ATCC 25937 was the most susceptible to the supernatants pH. This indicates that acid tolerance can vary greatly among different species of *Shigella*. Gorden and Small (1993) made the same statement when they found up to 80% differences in acid susceptibility between different strains of *Shigellaflexneri*, and over 95% between different species of *Shigella*.

Similarly to the present study, Zhang et al (2011) found significant inhibitory effect of 4 lactobacilli species on *Shigellasonnei* and *Escherichia coli*, and they also observed that enzymatically treating or boiling (15 min) the supernatants did not eliminate the antimicrobial activity of the supernatants, but it was completely eliminated after pH neutralization.

In other study, Zhang et al (2012), selected 14 lactobacilli strains, which presented inhibitory activity on *Shigellasonnei* ATCC 25931, also tested in the present work. They showed that neither treating the supernatants with proteinase K nor boiling them for 15 minutes affected the antimicrobial activity of the supernatants. However, neutralizing the supernatants pH also eliminated the inhibition effect exerted upon *Shigellasonnei* ATCC 25931. The authors attributed the inhibition effects to the production of organic acids. Additionally in the present study, the supernatants were also treated with pronase, α-chymotrypsin, trypsin, catalase or boiling and autoclaving, in order do find out the possible nature of the inhibition effect. However, only the neutralization of the supernatants pH eliminated the inhibition effect, which indicates its association with the production of organic acids.

Regarding the inhibition effect caused by bacteriocins, it is well established that gram-negative bacteria are intrinsically resistant to bacteriocins produced by lactic acid bacteria. This occurs due to the presence of the external membrane, which constitutes a physical barrier to the passage and binding of bacteriocins. However, it has been reported that the destabilization of the outer membrane can make gram-negative bacteria susceptible to such bacteriocins (Kalchayanandet al., 1992; Kalchayanandet al., 1998; Belfioreet al., 2007).

According to Alakomi et al. (2000), lactic acid exerts a strong effect on the outer membrane of gram-negative bacteria, and in many cases, may cause its rupture. Corroborating this observation, it has been reported cases in which bacteriocins produced by lactic acid bacteria are effective against gram-negative bacteria only at low pH (Messiet al., 2001; Lash et al., 2005). Lash et al. (2005) observed that low pH (<5.0) was essential to the inhibition of gram-negative pathogens mediated by a peptidic substance synthesized by *Lactobacillus plantarum* ATCC 8014. However, in the present work, it was not observed any inhibitory effect originating from a peptidic substance even though the supernatants pH was below 5.0.

As pointed out by Makras and De Vuyst (2006), organic acids, in particular lactic and acetic acids, exert a strong inhibitory effect on gram-negative bacteria. Fooks and Gibson (2001) observed that probiotic-mediated inhibition effect of *Escherichia coli* and *Salmonella enteritidis* increased proportionally to the concentration of organic acid in the medium. They also stated that low pH may not be the sole cause of the observed inhibition effects. This however may be an important condition for the passage of organic acids

through the membrane to the intracellular environment, where they will accumulate and exert inhibitory activity.

According to Kashket (1987), organic acids-mediated antimicrobial activity is due to the lowering of the intracellular pH caused by the cyclic dissociation of organic acids present in the medium. By this mechanism, organic acids act as transporters of H^+ to the intracellular environment, where they dissociate. After that, they are excreted to the extracellular medium in their anionic form, which then acquires another H^+, returns to the intracellular environment, dissociates and so forth. Consequently, the increased rate of H^+ accumulation surpasses the cell's ability to excrete H^+, therefore dissipating the membrane's electrochemical gradient. Also, there is an intrinsic increase in ATP consumption due to the continuous exporting of H^+ through active transporters, which generates an energetic deficit.

It is important to emphasize that the absence of an antimicrobial effect caused by peptidic substances does not imply that the tested strains are unable to produce bacteriocins. As previously stated by Cotter Hill and Ross (2005), these compounds present a variable antimicrobial spectrum and may not be effective against an entire group of bacteria. Our results support only that the evaluated lactobacilli did not produce antimicrobial peptides which are active against the tested pathogenic species. However, they still may produce antimicrobial peptides that are active against other bacteria.

The premise of modulating the intestinal microbiota through probiotic administration is considered promising. In this context, according to Claesson et al (2009), the intestinal microbiotain composed of about 1000 species of microorganisms and that diversity is found at the species level, with relatively few different genera. Kim et al (2013) demonstrated that probiotic administration virtually does not alter the taxonomic structure of the intestinal microbiota. However, the authors reported that probiotic ingestion can significantly reduce up to tenfold the concentration of 88 species in a community of 1,175 species. Therefore, they emphasize that probiotic therapy may be successfully used in the control, but not in the total elimination of potentially pathogenic bacteria in the gut.

Corroborating this observation, the results of the present work showed that there was not a complete inhibition of pathogenic strains, but a significant inhibition rate up to 57% was observed based on a population of 10^8 viable cells. However, the related literature reports much lower infective population for gram-negative enteropathogenic species. Populations may vary from $5x10^2$ UFC for *Shigellasonnei*53G (Munoz et al., 1995), 10^2–10^4 UFC for *Shigelladysenteriae* M 131 (Levine et al., 1973), approximately 10^6 UFC for several strains of *Echerichia coli* (FDA, 2012) and 10^2-10^8 for *Salmonella*spp (Humphrey, 2004).

4 CONCLUSIONS

We found out that some of the evaluated lactobacilli can significantly reduce a microbial load of10^8 viablecells of most of the gram-negativebacteria evaluated in this work through the production of organic acids. Therefore, considering the low infective dosages that can be present in foods reported on the literature, these lactobacilli strains may be able to protect the host from infections caused by a number of gram-negative foodborne pathogens.

5 ACKNOWLEDGEMENTS

The authors would like to thank CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior) for the financial support.The authors declare that there are no conflicts of interest which could influence interpretation of the results reported in this study.

6 REFERENCES

Alakomi, H. L., Skyttä, E., Saarela, M., Mattila-Sandholm, T., Latva-Kala, K., Helander, I. M. (2000).Lactic acid permeabilizes Gram-negative bacteria bydisrupting the outer membrane. Applied and Environmental Microbiology,66, 2001-2005.

Belfiore, C.,Castellano, P.,Vignolo, G. (2007). Reduction of *Escherichia coli* population following treatment with bacteriocins from lactic acid bacteria and chelators.Food Microbiology,24, 223-229.

Blom, H.,Mørtvedt, C. (1991). Anti-microbial substances produced by food associated micro-organisms. Biochemical Society Transactions,19, 694-698.

Choi, I.,Kim, Y.,Park, Y.,Seog, H.,Choi, H. (2007).Anti-obesity activities of fermented soygermisoflavones by *Bifidobacteriumbreve*.Biofactors,29, 105-112.

Claesson, M. J.,O'sullivan, O.,Wang, Q.,Nikkilä, J.,Marchesi, J. R.,Smidt, H.,Vos, W. M.,Ross, R. P.,O'toolep. W. (2009).Comparative analysis of pyrosequencing and a phylogenetic microarray for exploring microbial community structures in the human distal intestine. Public Library of Science One, 4, doi: 10.1371/journal.pone.0006669.

Cotter, P. D.,Hill, C.,Ross, P. (2005).Bacteriocins: developing innate immunity for food. Nature Reviews Microbiology, 3, 777-788,

Cursino, L.,Smajs, D.,Smarda, J.,Nardi, R. M.,Nicoli, J.R.,Chartone-Souza, E.,Nascimento, A.M. (2006). Exoproducts of the *Escherichia coli* strain H22 inhibiting some enteric pathogens both in vitro and in vivo. Journal of Applied Microbiology,100, 821-829.

De Man, J. C.,Rogosa, M.,Sharpe, M. E. (1960).A medium for the cultivation of lactobacilli.Journal of Applied Bacteriology,23, 130-135.

Food and Drug Administration.(2012).Pathogenic *Escherichia coli* Group.In:bad bug book,foodborne pathogenic microorganisms and natural toxins handbook, 2ed. Center for Food Safety and Applied Nutrition.

Fooks, L. J.,Gibson, G. R. (2002).*In vitro* investigations of the effect of probiotics and prebiotics on selected human intestinal pathogens. FEMS Microbiology Ecology,39, 67-75.

Gorden, J.,Small, P. L. (1993).Acid resistance in enteric bacteria.Infection and Immunity,61, 364-367.

Guerra, P. V.,Lima, L. N.,Souza, T. C.,Mazochi, V.,Penna, F. J.,Silva, A. M.,Nicoli, J. R.,Guimarães, E, V. (2010). Pediatric functional constipation treatment with Bifidobacterium-containing yogurt: a crossover, double-blind, controlled trial. World Journal of Gastroenterology,17, 3916-3921.

Guo, Z.,Liu, X. M.,Zhang, Q. X.,Shen, Z.,Tian, F. W.,Zhang, H.,Zun, Z. H., Zhang. P., Chen, W. (2011).Influence of consumption of probiotics on the plasma lipid profile: A meta-analysis of randomized controlled trials. Nutrition, Metabolism and Cardiovascular Diseases, 21, 844-850.

Honda,H.,Kataoka, F.,Nagaoka, S.,Kawai, Y.,Kitazawa, H.,Itoh, H.,Kimura, K.,Taketomo, N.,Yamazaki, Y.,Tateno, Y.,Saito, T. (2007).β-Galactosidase, phospho-β-galactosidase and phospho-β-glucosidase activities in lactobacilli strains isolated from human faeces. Letters in Applied Microbiology,45, 461-466.

Humphrey, T. (2004) .*Salmonella*, stress responses and food safety.Nature Reviews Microbiology,2, 504-509.

Huseini, H. F.,Rahimzadeh, G.,Fazeli, M. R.,Mehrazma, M.,Salehi, M. (2012).Evaluation of wound healing activities of kefir products.Burns, 38, 719-723.

Jiang, J.,Shi, B.,Zhu, D.,Cai, Q.,Chen, Y.,Li, J.,Qi, K.,Zhang, M. (2012).Characterization of a novel bacteriocin produced by *Lactobacillus sakei* LSJ618 isolated from traditional Chinese fermented radish. Food Control,23, 338-344.

Kadooka, Y.,Sato, M.,Imaizumi, K.,Ogawa, A.,Ikuyama, K.,Akai, Y.,Okano, M.,Kagoshima, M.,Tsuchida, T. (2010).Regulation of abdominal adiposity by probiotics (*Lactobacillus gasseri* SBT2055) in adults with obese tendencies in a randomized controlled trial.European Journal of Clinical Nutrition, 64, 636-643.

Kalchayanand, N.,Hanlin, M. B.,Ray, B. (1992).Sublethal injury makes Gram-negative and resistant Gram-positive bacteria sensitive to the bacteriocins, pediocinAcH and nisin. Letters in Applied Microbiology,15, 239-243.

Kalchayanand, N.,Sikes, A.,Dunne, C. P.,Ray, B. (1998).Interaction of hydrostatic pressure, time and temperature of pressurization and pediocinAcH on inactivation of foodborne bacteria.Journal of Food Protection, 61, p.425-431.

Kashket, E. R. (1987).Bioenergetics of lactic acid bacteria: cytoplasmic pH and osmotolerance. FEMS Microbiology Letters, 43, 233-244.

Kim, S. W.,Suda, W.,Kim, S.,Oshima, K.,Fukuda, S.,Ohno, H.,Morita, H.,Hattori, M. (2013). Robustness of gut microbiota of healthy adults in response to probiotic intervention revealed by high-throughput pyrosequencing. DNA Research,20, 241-253.

Kumar, M.,Verma, V.,Nagpal, R.,Kumar, A.,Behare, P. V.,Singh, B.,Aggarwal, P. K. (2012).Anticarcinogenic effect of probiotic fermented milk and chlorophyllin on aflatoxin-B1-induced liver carcinogenesis in rats. British Journal of Nutrition,107, 1006-1016.

Lam, E. K.,Yu, L.,Wong, H. P.,Wu, W. K.,Shin, V. Y.,Tai, E. K.,So, W. H.,Woo, P. C.,Cho, C. H. (2007).Probiotic *Lactobacillus rhamnosus* GG enhances gastric ulcer healing in rats. European Journal of Pharmacology, 565, 171-179.

Lanciotti, R.,Patrignani, F.,Bagnolini, F.,Guerzoni, M. E.,Gardini, F. (2003). Evaluation of diacetyl antimicrobial activity against *Escherichia coli*, *Listeria monocytogenes* and *Staphylococcus aureus.* Food Microbiology, 20, 537-543.

Lash, B. W.,Gourama, H.,Mysliwiec, T. H. (2002).Microscale assay for screening of inhibitory activity of *Lactobacillus.*Biotechniques, 33, 1224-1226.

Lash, B. W.,Mysliwiec, T. H.,(2005).Gourama, H. Detection and partial characterization of a broad-range bacteriocin produced by *Lactobacillus plantarum* (ATCC 8014). Food Microbiology, 22, 199-204.

Levine, M. M.,Dupont, H. L.,Formal, S. B.,Hornick, R. B.,Takeuchi, A.,Gangarosa, E. J.,Snyder, M. J. Libonati, J. P. (1973).Pathogenesis of *Shigelladysenteriae*1 (Shiga) dysentery. The Journal of Infectious Diseases, 127, 261-270.

Liong, M. T.,Shah, N. P. (2002).Bile salt deconjugation ability, bile salt hydrolase activity and cholesterol co-precipitation ability of lactobacilli strains. International Dairy Journal, 15, 391-398.

Makras, L., DE Vuyst, L. (2006). The in vitro inhibition of Gram-negative pathogenic bacteria by bifidobacteria is caused by the production of organic acids. International Dairy Journal, 16, 1049-1057.

Maróti, G.,Kereszt, A.,Kondorosi, E.,Mergaert, P. (2011). Natural roles of antimicrobial peptides in microbes, plants and animals. Research in Microbiology, 162, 363-374.

Martín, R.,Olivares, M.,Marín, M. L.,Xaus, J.,Fernández, L.,Rodríguez, J. M. 2005. Characterization of a reuterin-producing *Lactobacillus coryniformis* strain isolated from a goat's milk cheese. International Journal of Food Microbiology, 104, 267-277.

Messi, P.,Bondi, M.,Sabia, C.,Battini, R.,Manicardi, G. (2001). Detection and preliminary characterization of a bacteriocin (plantaricin 35d) produced by a *Lactobacillus plantarum* strain. International Journal of Food Microbiology, 64, 193-198.

Munoz, C.,Bagar, S.,Verg, L.,Thupari, J.,Goldblum, S.,Olson, J. G.,Taylor, D. N.,Heresi, G. P.,Murphy, J. R. (1995).Characteristics of *Shigellasonnei* infection of volunteers: signs, symptoms, immune responses, changes in selected cytokines and acute-phase substances. American Journal of Tropical Medicine and Hygiene, 53, 47-54.

Nasrabadi, H. M.,Ebrahmini, M. T.,Banadaki, D. S.,Kajousangi, T. M.,Zahedi, F. (2011). Study of cutaneous wound healing in rats treated with *Lactobacillus plantarum* on days 1, 3, 7, 14 and 21. African Journal of Pharmacy and Pharmacology, 5, 2395-2401.

Narva, M.,Collin, M.,Lamberg-Allardt, C.,Kärkkäinen, M.,Poussa, T.,Vapaatalo, H.,Korpela, R.(2004).Effects of long-term intervention with *Lactobacillus helveticus*-fermented milk on bone mineral density and bone mineral content in growing rats. Annals of Nutrition and Metabolism, 48, 228-234.

O'brien, J.,Wright, G. D. (2011).An ecological perspective of microbial secondary metabolism. Current Opinion in Biotechnology, 22, 552-558.

Ogawa, M.,Shimizu, K.,Nomoto, K.,Tanaka, R.,Hamabata, T.,Yamasaki S.,Takeda, T.,Takeda, Y. (2001). Inhibition of in vitro growth of Shiga toxin-producing *Escherichia coli* O157:H7 by probiotic *Lactobacillus* strains due to production of lactic acid. International Journal of Food Microbiology, 68, 135-140.

Ouwehand, A. C.,Vesterlund, S. (2004).Antimicrobial components from lactic acid bacteria (pp. 375-396) In: SALMINEN, S.; WRIGHT, A.; OUWEHAND, A. (Eds) Lactic acid bacteria: microbiological and functional aspects.3.ed New York: Marcel Dekker.

Peral, M. C.,Martinez, M. A. H.,Valdez, J. C. (2009). Bacteriotherapy with *Lactobacillus plantarum*in burns. International Wound Journal, 6, 73-81.

Pingitore, E. V.,Hébert, E. M.,Nader-Macías, M. E.,Sesma, F. (2009). Characterization of salivaricin CRL 1328, a two-peptide bacteriocin produced by *Lactobacillus salivarius* CRL 1328 isolated from the human vagina. Research in Microbiology, 160, 401-408.

Pompei, A.,Cordisco, L.,Amaretti, A.,Zanoni, S.,Matteuzzi, D.,Rossi, M. (2007). Folate production by bifidobacteria as a potential probiotic property. Applied and Environmental Microbiology, 72, 179-185.

Riezzo, G.,Orlando, A.,D'attoma, B.,Guerra, V.,Valerio, F.,Lavermicocca, P.,De Candia, S.,Russo, F. (2012). Randomized clinical trial: efficacy of *Lactobacillus paracasei* enriched artichokes in the treatment of patients with functional constipation – a double-blind, controlled, crossover study. Alimentary Pharmacology and Therapeutics, 35, 441-450.

Sun, J.,Chang, E. B. (2014).Exploring gut microbes in human health and disease: Pushing the envelope. Genes and Diseases, 1, 132-139.

Šušković, J.,Kos, B., Beganović, J.,Pavund, A. L.,Habjanič, K.,Matošić, S.(2010).Antimicrobial activity – The most important property of probiotic and starter lactic acid bacteria. Food Technology and Biotechnology, 48, 296-307.

Takemura, N.,Okubo, T.,Sonoyama, K. (2010). *Lactobacillus plantarum* strain No. 14 reduces adipocyte size in mice fed high-fat diet. Experimental Biology and Medicine, 235, 849-856.

Todorov, S. D.,Prévost, H.,Lebois, M.,Dousset, X.,Leblanc, J. G.,Franco, B. D. G. M. (2011).Bacteriocinogenic*Lactobacillus plantarum* ST16Pa isolated from papaya (*Carica papaya*) — From isolation to application: Characterization of a bacteriocin. Food Research International, 44, 1351-1363.

Tsai, C.,Hsih, H.,Chiu, H.,Lai, Y.,Liu, J.,Yu, B.,Tsen, H. (2005).Antagonistic activity against *Salmonella*infection in vitro and in vivo for two Lactobacillus strains from swine and poultry. International Journal of Food Microbiology, 102, 185-194.

Tsai, C.,Lin, P.,Hsieh, Y. (2008a).Three *Lactobacillus* strains from healthy infant stool inhibit enterotoxigenic*Escherichia coli* grown *in vitro*. Anaerobe, 14, 61-67.

Tsai, Y. T.,Cheng, P. C.,Fan, C. K.,Pan, T. M. (2008b).Time-dependent persistence of enhanced immune response by a potential probiotic strain*Lactobacillus paracasei* subsp. paracasei NTU 101. International Journal of Food Microbiology, 128, 219-225.

Tsai, Y. T.,Cheng, P. C.,Liao, J. W.,Pan, T. M. (2010a).Effect of the administration of *Lactobacillus paracasei* subsp. paracasei NTU 101 on Peyer's patch-mediated mucosal immunity. International Immunopharmacology, 10, 791-798.

Tsai, Y. T.,Cheng, P. C.,Pan, T. M. (2010b).Immunomodulating activity of *Lactobacillus paracasei* subsp. paracasei NTU 101 in enterohemorrhagic*Escherichia coli* O157:H7-infected mice. Journal of Agricultural and Food Chemistry, 58, 11265–11272.

Uchida, M.,Kurakazu, K. (2004).Yogurt containing *Lactobacillus gasseri* OLL2716 exerts gastroprotective action against acute gastric lesion and antral ulcer in rats. Journal of Pharmacological Sciences, 96, 84-90.

Whitman, W. B.,Coleman, D. C.,Wiebe, W. J. (1998).Prokaryotes: The majority unseen. Proceedings of the National Academy of Sciences of the United States of America, 95, 6578-6583.

Wood, B. J. B. Microbiology of fermented foods. 2nd.ed. London: Thomson Science. 1998. 383 p.

Xie, Y.,An, H.,Hao, Y.,Qin, Q.,Huang, Y.,Luo, Y.,Zhang, L. (2011).Characterization of an anti-*Listeria*bacteriocin produced by *Lactobacillus plantarum* LB-B1 isolated from koumiss, a traditionally fermented dairy product from China. Food Control, 22, 1027-1031.

Yoshida, A.,Aoki, R.,Kimoto-Nira, H.,Kobayashi, M.,Kawasumi, T.,Mizumachi, K.,Suzuki, C. (2011).Oral administration of live *Lactococcuslactis* C59 suppresses IgE antibody production in ovalbumin-sensitised mice via the regulation of interleukin-4 production. FEMS Immunology and Medical Microbiology, 61, 315-322.

Zhang, Y.,Zhang, L.,Du, M.,Yi, H.,Guo, C.,Tuo, Y.,Han, X.,Li, J.,Zhang, L.,Yang, L. (2011).Antimicrobial activity against *Shigellasonnei* and probiotic properties of wild lactobacilli from fermented food. Microbiological Research, 167, 27-31.

Zhang, Y.,Zhang, L.,Ma, W.,Yi, H.,Yang, X.,Du, M.,Shan, Y.,Han, X.,Zhang, L. (2012).Screening of probiotic lactobacilli for inhibition of *Shigellasonnei* and the macromolecules involved in inhibition. Anaerobe, 18, .498-503.