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Antagonistic effect of probiotic strains against two pathogens: Salmonella Typhimurium and E. coli O157:H7 resistant to antibiotics.

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Efecto antagónico de cepas probióticas contra dos cepas patógenas resistentes a antibióticos: *Salmonella* Thyphimuirum y *E. coli* O157:H7

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RESUMEN. El objetivo de este estudio fue probar la eficiencia de las cepas probióticas *Lactobacillus acidophilus* NCFM (*L. acidophilus), Lactobacillus rhamnosus* HN001 (*L. rhmanosus)* y *Bifidobacterium animalis* BI07 (*B. animalis)* para antagonizar los patógenos resistentes a antibióticos *Salmonella enteritidis* var Thyphimurium (*Salmonella* Thyphimurium) y *Escherichia coli* O157:H7 (*E. coli* O157:H7). Las tres cepas probióticas mostraron poseer un efecto antagónico contra las cepas patógenas *Salmonella* Thyphimurium y *E. coli* O157:H7 resistentes a antibióticos. En la prueba de "Well Diffusion", *L. acidophilus* y *L. rhamnosus* presentaron reducciones estadísticamente semejantes entre ellas (P >0.05) de 37-41 mm para *E. coli* O157:H7 y de 32 – 41 mm para *Salmonella* Thyphimurium**,** mientras que *B. animalis* mostró reducciones menores (P<0.05) para ambas bacterias patógenas de 6 y 5 mm respectivamente. Así mismo, los sobrenadantes de *L. rhamnosus* y *L. acidophilus* tuvieron un efecto significativo (P<0.05) y semejante en la reducción de la población (6-7 LOG UFC para *Salmonella* Thyphimurium y 3-5 LOG UFC para *E. coli* O157:H7), mientras que *B. animalis* solo fue capaz de inhibir el crecimiento de ambas cepas patógenas durante 24 h de incubación. Las diferencias en el efecto antagónico de los sobrenadantes se explica debido a la mayor presencia de ácido láctico de la cepa *L. rhamnosus* (265.69 ± 7.35 mM) seguida de *L. acidophilus* (163.02 ± 17.21 mM), y la menor de *B. animalis* (121.69 ± 5.41 mM), (P <0.05), aunque estas inhibiciones pueden ser explicadas por la posible presencia de bacteriocinas, no determinadas en este estudio. En el ensayo en co-cultivo, *Salmonella* Typhimurium presenta una mayor inhibición que *E. coli* O157:H7 (P <0.05), ya que a las 16 h de incubación, *L. acidophilus* redujo su población al límite de detección. El resto de las combinaciones en esta prueba, no fueron significativas (P >0.05).

PALABRAS CLAVE: Resistencia a antibióticos, Antagonismo bacteriano, ácido láctico.

ABSTRACT. The aim of this study was to prove the effective antagonism of probiotic bacteria *Lactobacillus acidophilus* NCFM (*L. acidophilus), Lactobacillus rhamnosus* HN001 (*L. rhamnosus*) and *Bifidobacterium animalis* BI07 (*B. animalis*) against antibiotic resistant pathogens *Salmonella enteritidis* var Thyphimurium and *Escherichia coli* O157:H7 (*E. coli* O157:H7). The three probiotic strains showed to have an antagonistic effect against pathogen strains *Salmonella* Thyphimurium and *E. coli* O157:H7 resistant to antibiotics. In the test of "Well Diffusion" *L. acidophilus* and *L. rhamnosus* showed similar statistically reductions between them (P > 0.05) of 37 - 41 mm for *E. coli* O157: H7 and a 32 - 41 mm for *Salmonella* Typhimurium, while *B. animalis* showed smaller reductions (P < 0.05) for both pathogen bacteria of 6 and 5 mm respectively. Similarly, the supernatants of *L. rhamnosus* and *L. acidophilus* showed a significant reduction (P <0.05) on the population (6 - 7 LOG CFU for

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Salmonella Typhimurium and 3 - 5 LOG CFU for *E. coli* O157: H7), while *B. animalis* only was able to inhibit the growth of both pathogenic strains during 24 h of incubation. Differences in the antagonistic effect of the supernatants were explained by the presence of lactic acid greater for strain *L. rhamnosus* (265.69 ± 7.35 mM) followed by *L. acidophilus* (163.02 ± 17.21 mM), and less for *B. animalis* (121.69 ± 5.41 mM) ($P <0.05$), although these inhibitions can be explained by the possible presence of bacteriocins not determined in this study. In the co-culture assay, *Salmonella* Typhimurium exhibits greater inhibition than *E. coli* O157: H7 ($P <0.05$) as at 16 h of incubation, *L. acidophilus* reduced its population to the detection limit. The other combinations in this test, were not significant ($P >0.05$).

KEYWORDS: Antibiotic resistance, bacterial antagonism, lactic acid.

Introduction

The foodborne disease is a major cause of morbidity and mortality in the world's population, causing death of about 1.9 million children worldwide each year, even though most of these diarrheal deaths occur in developing countries, although not limited to these countries. It is estimated that in the United States, foodborne diseases are 76 million people sick with 325,000 hospitalizations and 5,000 deaths each year. New forms of transmission of foodborne and increased antibiotic resistance by pathogens, are evading the conventional control measures [1].

During 2009-2010 in the United States, 1,527 foodborne disease outbreaks were reported, of which 7,089 cases were caused by *Salmonella* and 651 cases by *Escherichia coli* O157: H7 [2]. Mexico reported 633 outbreaks with 19,493 cases of diarrhea, of which 107 died during the period 1993 to 2002 [3].

Salmonella enteritidis var Typhimurium is a facultative intracellular bacterial pathogen that infects, replicates and persists in macrophages. This pathogen can cause severe intestinal infections [4]. On the other hand, *Escherichia coli* O157: H7 can cause bloody diarrhea, hemorrhagic colitis, hemolytic uremic syndrome and thrombotic thrombocytopenic purpura. This strain has a unique capacity to survive in an acidity that is lethal to other *Enterobacteriaceae*. [4, 5].

As a consequence of the indiscriminate use of antibiotics to treat human and animal microbial infections, some bacteria have developed new resistances [1, 6, 7]. In order to avoid the use of antibiotics and to control efficiently the proliferation of gastrointestinal disease-causing bacteria, probiotics are successfully employed [8].

Probiotics are live microorganisms that confer a health benefit on the host when administered in appropriate amounts; consequently their use in the formulation of foods is very common and is still increasing [9, 10, 11].

Probiotics genres of *Lactobacillus* and *Bifidobacterium* belong to the gastrointestinal microflora and are utilized in the manufacture of dairy products [11, 12].

Many studies have attempted to identify specific positive health effects of probiotics on human health. It has been revealed that different species or even strains belonging to the same species exert different effects on human health [13]. Several health benefits have been claimed for probiotic bacteria, which include anticarcinogenic properties, lactose digestion, serum cholesterol reduction and immune system stimulation

[11]. Probiotics have also preventive and therapeutic effects on several types of diarrhea of different etiologies [14]. Probiotic bacteria are increasingly used for food and pharmaceutical applications to restore disturbed intestinal microflora and related dysfunction of the human gastrointestinal tract [15]. Probiotic bacteria affect growth of microbial pathogens and favor commensal's microflora proliferation, by synthesizing antibacterial compounds (including bacteriocins, non-bacteriocins and organic acid molecules) and by decreasing pH [14, 16, 17].

Some authors have suggested that the strong antimicrobial activity of *Lactobacillus* and *Bifidobacterium* strains to inhibit intestinal pathogens, included *Salmonella* Typhimurium and *E. coli* O157:H7 is due to the organic acid production, particularly lactic and acetic acids [18-21].

The aim of this study was to prove the effective antagonism of commercial probiotic bacteria *Lactobacillus acidophilus* NCFM (*L. acidophilus), Lactobacillus rhamnosus* HN001 (*L. rhamnosus)* and *Bifidobacterium animalis* BI07 (*B. animalis*) against antibiotic resistant pathogens *Salmonella enteritidis* var Thyphimurium (*Salmonella* Thyphimurium) and *Escherichia coli* O157:H7 (*E. coli* O157:H7).

Materials and Methods

Bacterial strains and culture conditions.

Lactic bacterial strains used on this work belong to the DANISCO Company: *Bifidobacterum animalis* BI07, *Lactobacillus acidophilus* NCFM and *Lactobacillus rhamnosus* HN001. These lyophilized strains were preserved at 4°C and reactivated in Man Rogosa Sharpe medium (MRS, Difco) at 37 °C for 42 h, in anaerobic conditions (BD GasPak^{TM} EZ Anaerobe Container System).

Pathogen bacterial strains *E. coli* O157:H7 and *Salmonella* Typhimurium were donated by the Department of Animal Science, Texas A&M University and by the Secretariat of Health of Jalisco, Mexico, respectively. These strains were preserved at 4°C and reactivated in Trypticase Soy Broth (TSB, Bioxon) at 35°C for 24 h in aerobic conditions.

Antimicrobial Susceptibility Test

The antibiotics susceptibility of *Salmonella* Typhimurium and *E. coli* O157:H7, was determined according to the methodology of the NCCLS M100-S18, published by the Clinical Laboratory Standard Institute [22]. The antibiotics used in the test were: ampicilin, cephalothin, cefoxitin, ceftriaxone, chloramphenicol, ciprofloxacin, imipem, gentamicin, kanamycin, nalidixic acid, streptomycin, tetracycline and trimethoprim-sulfamethoxazole (BD Discs, BBL Sensi-Disc).

Pathogenic strains were reactivated by incubation in Trypticase Soy Broth (TSB, Bioxon) at 35 °C for 24 h. Later, they were cultured in Trypticase Soy Agar (TSA, Bioxon) at 35°C for 6 h; five colonies were placed in 5 ml of TSB and incubated at 35°C. Cultures were stopped when absorbance reached 0.5 MacFarland standards, $(1x10^8$ CFU/ml) [23].

Petri dishes containing TSA were inoculated with *Salmonella* Typhimurium or *E. coli* O157:H7 using sterile swabs over solid medium. Subsequently, impregnated discs with antibiotics were placed on the inoculated surface and incubated at 35 °C for 16-18 h.

The inhibition zones were measured (mm), and the results were interpreted according to the M100-S18 document, indicating the bacterium antibiotic susceptibility: Susceptible (S), Intermediate (I) or Resistant (R) [22, 23].

Inhibition activity assays of pathogenic strains.

Well diffusion assay.

B. animalis, *L. acidophilus* and *L. rhamnosus* were cultivated in duplicate in 10 ml of broth MRS unstirred at 35°C, for 48 h. Probiotic cultures were centrifuged at 6,500 rpm for 15 min at 4°C and the supernatants were distributed in two aliquots, one was non filtered (NFS) and the other one was filtered (FS) with a sterile membrane of 0.22 µm pore size (Millex GS filter, Millipore). These procedures were performed to see the influence of the residual cells of unfiltered samples, in the antagonistic effect.

Wells of 3 mm in diameter were cut into the MRS agar plates and 10 µl of each type of probiotic strain supernatant were placed into each well [16]. On the other hand, *Salmonella* Typhimurium and *E. coli* O157:H7 were cultivated in TSB until to reach 5×10^7 CFU/ml. The MRS plates with the wells were overlaid with 10 ml of Violet Red Bile Agar (VRBA) at 45°C and inoculated with 100 µl of the pathogenic strain culture at concentration of 5×10^7 CFU/ml. They were incubated at 35°C for 24 h, in aerobic or anaerobic conditions. An inhibition of pathogenic strains was considered to be positive if the inhibition zone was equal or higher than 0.5 mm. [16]. Experiments were made in triplicate and results were analyzed by a One Way ANOVA (Statgraphics Centurion XV). Differences were considered significant at *P* value < 0.05.

Inhibitory activity of the culture supernatants of probiotic bacteria in cultures of pathogenic strains.

For each probiotic strain, three treatments were applied to each pathogen. Probiotic and pathogen strains were cultivated in duplicate in TBS and MRS broth, respectively. All the probiotic bacteria cultures were centrifuged at 6500 rpm for 15 min at 4°C and the supernatants were filtered (FS) using a sterile filter (0.22 µm pore size Millex GS, Millipore) or not filtered (NFS). Moreover, as a control, pH of MRS was adjusted for each probiotic strains with HCl 1 N (pH treatment). Culture medium of *L. rhamnosus* was adjusted at 3.8, *L. acidophilus* at 4.3 and *B. animalis* at 5.6, these pH values were established from the mean values obtained during growth tests performed for each probiotic strain. Cultures of each pathogen strain without probiotic supernatants, were used as a control under the same conditions [17].

Sterilized TSB broth was inoculated with 100 µl of each pathogen strain culture, and mixed with the 1:1 dilution of this broth with the supernatants of each probiotic strains. It was incubated at 35°C and sampled at 0, 3, 6, 9, 12 and 24 h for *B. animalis,* 0, 1, 2, 3, 4, 5, 6, 7 and 8 h for *L. acidophilus* and 0, 1, 2, 3, 4, 5, and 6 h for *L. rhamnosus.* Serial dilutions of culture samples were spread on the surface of Violet Red Bile Agar (VRBA) and incubated at 35°C for 24 h, to quantify the number of living pathogen cells [17]. Experiments were made by triplicate and then reported.

Results were means ± standard deviations. The statistical significance was assessed by a Multifactor ANOVA (Statgraphics Centurion XV). Differences were considered significant at *P* value < 0.05.

Co-cultive of probiotic and pathogen strains.

The probiotic strains were cultivated in 100 ml of MRS broth at 37°C for 48 h in anaerobic condition and the pathogen strains were cultivated in 15 ml of TSB broth (Bioxon) in aerobic conditions at 37°C for 24 h. All the cultures were centrifuged at 4000 rpm for 10 min and biomasses were recovered and washed twice with saline physiological solution. Later, biomasses were resuspended in 15 ml of MRS broth for probiotic strains and 15 ml of TSB for pathogen strains. Cell suspensions of *Salmonella* Typhimurium were diluted at 1:1000 and *E. coli* O157:H7 at 1:10 to obtain a concentration of $5X10^8$ and $1X10^6$ CFU/ml, respectively. Moreover, the cell suspensions of probiotic strains achieved $1x10^{10}$ CFU/ml. In 50 ml of sterilized TBS broth, were added one milliliter of cell suspension of pathogen strain and 10 ml of cell suspension of probiotic strain and then, were incubated under anaerobic conditions at 35°C for 48 h. Samples were taken at 0, 4, 8, 12, 16, 20, 24 and 48 h. Serial dilutions of the culture samples were spread on the surface of VRBA plates for pathogen strains and of MRS agar plates for probiotic strains [24].

Quantification of Organic Acids

The quantification of organic acids was carried out using a HPLC with a Phenomenex Kinetex PFP column, particle size 5 μ, pore size 100Å, length 150 mm, internal diameter 4.60 mm. The mobile phase was water acidified to pH 3 with H_3PO_4. The flow rate was 0.5 ml/min, and the injection volume was 0.5 ml/min. A standard calibration curve for lactic acid (250, 500, 750, 1000, 1250, 1500 and 2000 mg/l) was prepared. There was used a UV diode array detector (DAD-UV) at 210 nm.

Results and discussion

Antibiotic resistance of pathogen strains

The pathogen strains used in this study *Salmonella* Typhimurium and *E. coli* O157: H7, have been involved in foodborne outbreaks [25] and they are considered as cause of zoonotic diseases that often contaminated animal feed, and meat food manufactured in unsanitary conditions, could predispose meat consumers to risks of antibiotic resistant bacteria disease [26, 27]. Antibiotic resistances for both pathogen strains found in this research are showed in Table 1. *Salmonella* Typhimurium showed resistance to ampicillin, cephalothin, cefoxitin, ceftriaxone, chloramphenicol, nalidixic acid, streptomycin and tetracycline, while *E. coli* O157:H7 was resistant only to ampicillin and intermediate resistant to tetracycline (Table 1).

Bacterial antibiotic resistance depends on genus, specie and particularly, on strain. *Salmonella* strains isolated from healthy animals, meat food and hospitalized patients were resistant to cefalothin and susceptible to imipenem; two of them were resistant to cefuroxime and one showed intermediate resistance to cefoxitin. Additional resistances were found against cefalotin, cefoxitin, gentamicin, streptomycin, sulfamethoxazole, tetracycline, nalidixic acid and trimethoprim. *Salmonella* strains obtained from hospitalized patients presented variants in the blaCTX-M gene which suggest that the source of the pathogenic strains could be due to nosocomial infections [28]. Moreover, *Salmonella* strains isolated from animal feed, retail meat and humans, in Yucatan México, showed susceptibility to ampicilin, cefotixin, ceftriaxone, chloramphenicol, gentamicin, kanamycin, nalidixic acid, streptomycin, tetracycline and trimethropim-sulfamethoxazole [27]. Our results exhibit differences in susceptibility of the *Salmonella* Typhimiurium strain to gentamicin, kanamycin and trimethoprim sulfamethoxazole.

Table 1. Antibiotic sensitivity testing of *Salmonella* Typhimurium and *E. coli* O157: H7.

Antibiotic	*E. coli* O157:H7	*Salmonella* Typhimurium
Ampicilin	R	R
Cefalothin	S	R
Cefoxitin	S	R
Ceftriaxone	S	R
Chloramphenicol	S	R
Ciprofloxacin	S	S
Imipem	S	S
Gentamicin	S	S
Kanamycin	S	S
Nalidixic acid	S	R
Streptomycin	S	R
Tetracycline	I	R
Trimethoprim-sulfamethoxazole	S	S

R = resistant, I = intermediate S = susceptible. This test was performed according to Ref. NCCLS M100-
S18 published by Clinical and Laboratory Standards Institute (2008).

In Portugal, *Salmonella* strains obtained from different sources (humans, food and environment) were mostly resistant to nalidixic acid, tetracycline, streptomycin, sulfamethoxazole and ampilicilin. These researchers suggested that food of animal origin might be considered as a reservoir and source of multidrug-resistance pathogens [29].

Otherwise, *E. coli* O157:H7 isolated from chicken and beef in Nigeria, were resistant to one or various antibiotics (91.1% of them were resistant to tetracycline) [30]. In our study, *E. coli* O157:H7 exhibited Intermediate resistance to this antibiotic

Dispersion trough the food chain of *Salmonella* Typhimurium and *E. coli* O157: H7 towards humans, and their multi- resistance against antibiotics, make it mandatory to propose alternatives to prevent and combat gastrointestinal diseases caused by these strains. It has been shown that probiotic bacteria are able to inhibit and prevent gastrointestinal diseases with pathogenic bacteria [31] even when pathogens are antibiotic resistant, like in this case.

Inhibitory activity of probiotic strains on growth and survival of pathogen strains

Well Diffusion Assay of cells free liquid cultures of the probiotic strains on semi-solid cultures of pathogen strains

All the inhibition assays using cell-free liquid cultures of *B. animalis, L. rhamnosus* and *L. acidophilus* on anaerobic semi-solid cultures of pathogen strains, showed a growth inhibition diameter equal or greater than 5 mm (Table 2).

No significant differences (*P* >0.05) were found for the inhibition assays of supernatants obtained from *L. rhamnosus* and *L. acidophilus* on *Salmonella* Typhimurium and *E. coli* O157: H7 cultures. However, the inhibition of pathogen strains by supernatants of *B. animalis* was lower than for the others tested probiotic strains (Table 2). *L. rhamnosus* and *L. acidophilus* synthesized molecules which effectively inhibited the growth of *E. coli* O157:H7 and *Salmonella* Typhimurium; while *B. animalis* was the least effective.

Table 2. Test of growth inhibition on agar plates in anaerobic cultures of *Salmonella* Typhimurium and *E. coli* O156:H7 by well diffusion of the supernatants of liquid cultures of probiotics strains.

	L. acidophilus	*L. rhamnosus*	*B. animalis*
E. coli O157:H7	37 ± 12^a	41 ± 5^a	6 ± 1^b
Salmonella Typhimurium	32 ± 08^a	41 ± 08^a	5 ± 1^b

Data are the means ±standard deviations (*n* = 3) of the diameters of growth inhibition (mm) measured on the surface of dish Petri of pathogen strain cultures. [a] Means with same letter are statistically similar (*P*>0.05).

In one well diffusion assay, in which were used supernatants of *Bifidobacterium* strains isolated from milk against pathogenic strains *Salmonella* DT124, *Salmonella enteritidis* and *Escherichia coli* cultures, researchers found a weak growth inhibition of pathogen strains (3 mm of diameter) only for concentrated supernatants. This inhibition was attributed to lactic and acetic acids. *Bifidobacterium animalis subsp. lactis* was the strain that presented less reduction in well diffusion assay compared with other *Bifidobacterium* strains tested in this research. The authors concluded that it might be due to the concentration or type of individual organic acids derived from their metabolisms [32].

Diameters of inhibition using supernatants of *L. acidophilus* and *L. rhamnosus* were greater than those reported by other authors. *E. coli* has shown inhibitions of 1.90 mm and 3.13 mm in diameter with supernatants of *L. acidophilus* [33]. When supernatants of *L. rhamnosus* have been used, *E. coli* and *Salmonella* Thyphimurium have shown inhibitions of 22.6 mm and 21.1 mm in diameter, respectively [34].

Bifidobacterium spp. supernatants isolated from patients, have presented a high spectrum of inhibition towards Gram positive and Gram negative bacteria and yeasts relevant to foodsafety and human health. Just one of the above mentioned *Bifidobacteria* strain showed antagonistic effect to *E. coli* O157: H7 (10.2 ± 0.2 mm) and remarkably, five of those *Bifidobacterium* strains were able to inhibit *Salmonella* Typhimurium with values of 10.4 ± 0.5, 15.2 ± 0.2, 12.0 ± 0.2, 14.4 ± 0.1, 9.0 ± 0.3 mm respectively [35].

Assay of growth inhibition of pathogenic strains by supernatants of probiotic strains cultures.

Supernatants of *L. acidophilus* and *L. rhamnosus* both types of supernatants (F and NFS) were not statistically different (*P* >0.05) and they affected severely the growth and survival of the pathogenic strains (Figure 1, 2 and 3). This result confirmed that the inhibition effect was caused by the compounds secreted by the probiotic strains and not by the remaining cells in the supernatants. Furthermore, the acidified culture

conditions were not responsible of the growth inhibition, as it can be observed by comparing of pathogenic strains cultures on acidified and control media (Figure 1, 2 and 3).
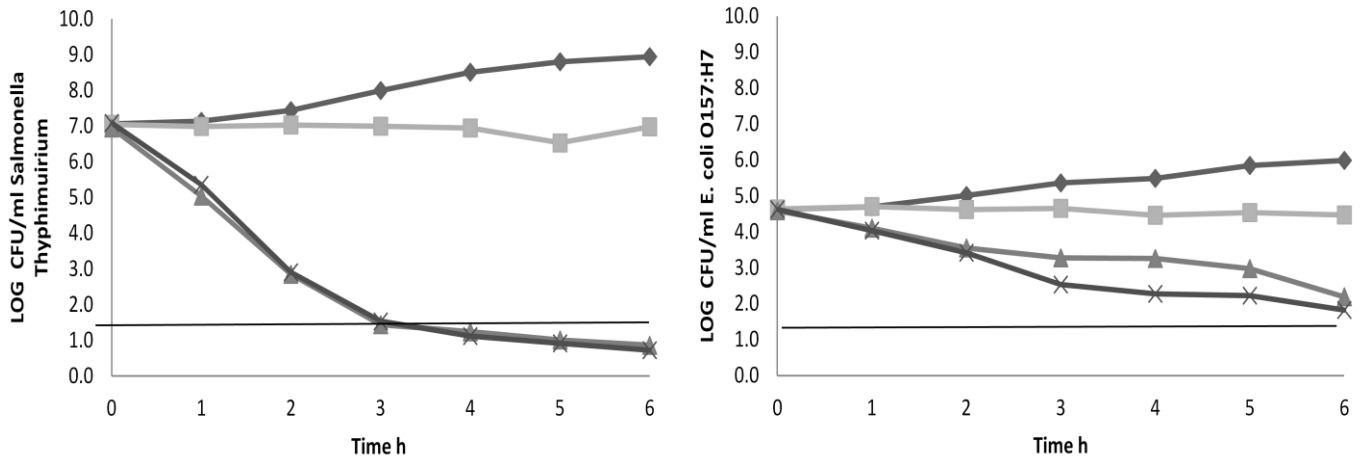

Figure 1. Assays of growth inhibition of *Salmonella* Typhimurium and *E. coli* O157: H7 by adding supernatants of *L. acidophilus* NCFMS. Cultures of both pathogen strains were performed using different treatments (probiotic supernatants): Media (MRS broth) with acidified pH at 4.3 (■); Media with filtered supernatants (▲); Media with non- filtered supernatants (x); and Media without treatment (♦). Solid lines indicate detection limit (*n* = 3).
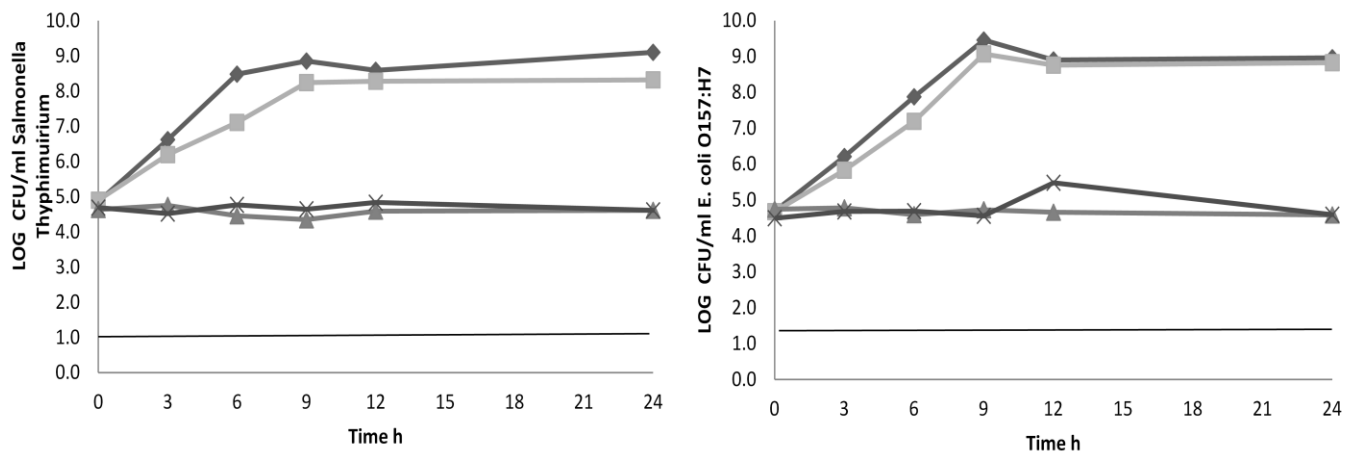

Figure 2. Assays of growth inhibition of *Salmonella* Typhimurium and *E. coli* O157: H7 by adding supernatants of *B. animalis* BI07 cultures. Cultures of both pathogen strains were performed using different treatments (probiotic supernatants): Media (MRS broth) with

acidified pH at 5.6 (■); Media with filtered supernatants (▲); Media with non- filtered supernatants (x); and Media without treatment (♦). Solid lines indicate detection limit (*n* = 3).
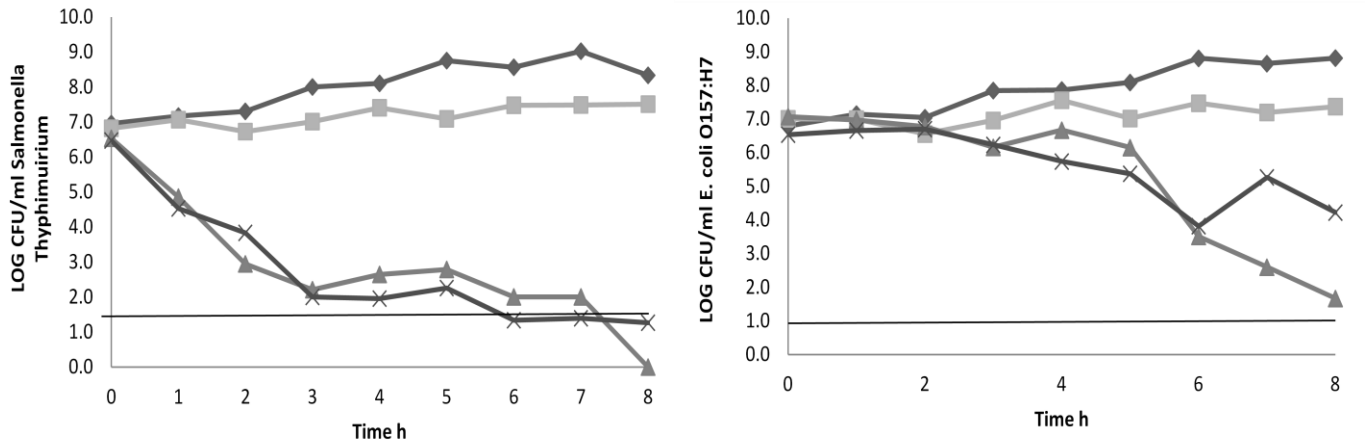

Figure 3. Assays of growth inhibition of *Salmonella* Typhimurium and *E. coli* O157: H7 by adding supernatants of *L. rhamnosus* HN001. Cultures of both pathogen strains were performed using different treatments (probiotic supernatants): Media (MRS broth) with acidified pH at 3.8 control, respectability (■); Media with filtered supernatants (▲); Media with non- filtered supernatants (x); and Media without treatment (♦). Solid lines indicate detection limit (*n* = 3).

All the probiotic strains supernatants were effective to inhibit the pathogenic strains growth; however, the *L. acidophilus* and *L. rhamnosus* supernatants were also effective to reduce the populations of both pathogenic bacteria, presenting a greater effect on the inhibition of *Salmonella* Typhimurium. The treatment consisted of adjusting the pH of the medium, did not show significant reductions compared with the treatments F and NFS, as mentioned above, indicating that reductions of pathogens cannot be explained by the decrease in pH.

L. acidophilus and *L. rhamnosus* supernatants showed a greater reduction of *Salmonella* Typhimurium population (about 6 - 7 LOG CFU reduction) compared to *E. coli* O157: H7 (3 - 5 LOG CFU reduction). In fact, *Salmonella* Typhimurium population became undetectable after 4 and 8 h of culture with *L. rhamnosus* and *L. acidophilus* supernatants, respectively.

In this study, *E. coli* O157: H7 population was reduced 5 and 3 LOG by *L. acidophilus* and *L. rhamnosus* respectively. In the case of cultures in supernatants of *B. animalis*, no reductions of *E. coli* O157: H7 and *Salmonella* Typhimurium are shown, but it is remarkable the absence of growth, whereas in controls and in broth adjusted to pH with HCl, growth is observed in both pathogens, were it increase almost 5 LOG.

Death of both pathogens by probiotic strains supernatants was observed as a function of the culture time. In fact, *L. rhamnosus* supernatant reduced 7 and 5 LOG the populations of *Salmonella* Typhimurium and *E. coli* O157:H7, respectively in 6 h, while *L. acidophilus* supernatant reduced both pathogens populations at the same levels in 8 h (Figure 3). Other studies showed that *L. rhamnosus* GG supernatant reduced *Salmonella* Thyphimurium population 2 and 8 LOG after 1 and 3 h of culture, respectively. On the other hand, *B. animalis* inhibited the growth of both pathogens during 24 h of incubation [21].

The killing effect against *E. coli* C1845 reported by other authors, resulting in a 2.67 ± 0.30 LOG CFU/ml decrease of the viability of the pathogenic indicator after 18 h of contact. This inhibition was attributed to the low pH of the culture medium and the organic acids production. No others antibacterial substances were found in the probiotic supernatants [19]. In our results, this killing effect reported by the authors could be duplicated by MRS medium (pH 4.5).

The inhibition of *Salmonella* Thyphimurium SL 1344 cells with CFCS (pH 4.5) or all tested *Lactobacillus* strains resulted in a significant decrease (P<0.001) in *Salmonella* viability after 4 h of contact. The pH control sample gave only a 2 ± 1 % reduction in the viability of the pathogen, indicating that the anti-*Salmonella* activity of the lactobacilli tested was not simply due to a decrease in pH [20].

E. coli O157: H7 has the ability to regulate the cytoplasm pH. Glutamic acid and arginine decarboxylases are considered as essential enzymes involved in the pathogen survival at low pH and anaerobic conditions [5]. However, a low inhibition in *E. coli* wt 555 and *Salmonella* Typhimurium cultures by *Bifidobacterium animalis* supernatants has been found by other authors [36].

Organic acids, in particular acetic acid and lactic acid, have a strong inhibitory effect against Gram-negative bacteria [18]. Other authors have found that the acidified pH of culture media has an antimicrobial effect [19, 20]. It is well known that the probiotic antagonism is due to a combination of factors, such as the production of organic acids and bacteriocins [17]. Even more, lactic acid acts as a premeabilizer of the outer membrane of gram-negative pathogens, thus increasing their susceptibility to antimicrobial molecules by allowing these molecules to penetrate the bacteria [37].

Although in other research was reported that *Lactobacillus rhamnosus* GG supernatants inhibit *Salmonella* Thyphimurium by the produced lactic acid, in this study bacteriocins were not found [21]. Moreover, *E. coli* O157:H7 is considered to be one of the greatest microbial contaminants in the food industry and a severe public health problem, since it provokes sharp human infections at very low dose and survives in acidified foods [39].

The mechanism by which *Lactobacillus* and *Bifidobacterium* supernatants inhibited the pathogen bacteria, appears to be multi factorial, and includes production of lactic acid and antibacterial compounds, including bacteriocins, non-bacteriocins and other organic acids [17, 45]. *L. jhonsonii* La1*, L. rhamonosus* GG*, L. rhamnosus* GR1*, L. casei* Shirota YT9029, *L. casei* DN 114 001, and *L. sake* CWBI 030202, dramatically decreased the viability of *Salmonella* Typhimurium by non-lactic acid molecules; it was observed a decrease of 3 LOG in viable serovar Typhiumurium SL1344 after 8 h of contact with a substance that neutralized the organic acid present in de supernatants [17]. We did not analyze the presence of bacteriocins, however, as mentioned by other authors [17, 45], their possible influence on the inhibition of both pathogens, must be considered.

Organic acids in the probiotic supernatants were assayed from probiotic strains after 48 h of culture in MRS broth (Table 3). Lactic acid production of *B. animalis* was the lowest (*P*<0.05) which could explain its low antimicrobial effect against tested pathogen strains, stopping their growth but not affecting their survival.

Table 3. Lactic acids produced by probiotics strains after 48 h of culture in MRS broth.

	Lactic acid mM
L. acidophilus	163.02 ± 17.21^{b}
L. rhamnosus	265.69 ± 7.35^{a}
B. animalis	121.69 ± 5.41^{c}

[a] Means with same letter are statistically similar for each type of acid (*P*<0.05), n=3.

The concentration of lactic acid present in the cell-free culture supernatants of different *Bifidobacterium* strains reported in other research, was between 36.4 to 20.9 mM [19]. These results, compared with those observed in Table 3, are lower. This means that the strain *B. animalis* used in this study is capable of producing more lactic acid in the MRS broth.

Other researchers found that the lactic acid concentration increased throughout the incubation period, obtaining approximately 70 mM at 7.5 h, 136 mM at 12 h, and 215 mM at 24, 34 and 48 h [21]. In our study, detection of lactic acid was only at 48 h of incubation, and it was less than the concentration found by these authors at the same time of incubation.

Lactic acid production of 15 to 60 mM by *Lactobacillus* strains was reported in other study. *Salmonella* strain decreased of 3.5 LOG with 60 mM of lactic acid [17], less than the results obtained in this work.

The antimicrobial activity of *L. acidophilus* IBB 801 and *L. rhamnosus* GG has been shown and it was solely due to the production of lactic, succinic and phenillacetic acid (75 - 250 mM) in MRS broth [20]. Production interval is the same as found in our study. The researchers did not find acetic acid in the products, the main metabolic end product present in the supernatants of all lactobacilli tested was lactic acid in amounts ranging from 150 to 190 mM.

The toxicity of fermentation acids at low pH was traditionally explained by an uncoupling mechanism. Undissociated fermentation acids can pass across the cell membrane and dissociate in the more alkaline interior, but there is little evidence that they can act in a cyclic manner to alkaline interior producing an accumulation of the anionic species, and this accumulation is dependent on the pH gradient (ΔpH) across the membrane [40].

Although both forms can inhibit bacterial growth, the undissociated form of organic acids was reported to be inhibitorier per mole than its corresponding dissociated form [41]. This researcher has found that inhibition of the growth rate was proportional to the concentration of undissociated lactic acid. Complete inhibition of growth occurred consistently at approximately 10 mM undissociated lactic acid for total lactic acid concentration at 25 to 100 mM. This implies that the most significant inhibitory factor under the

conditions tested was the undissociated acid. Moreover, it has been reported [42] that both the dissociated and the undissociated forms of organic acids have inhibitory effects on bacterial growth but that the undissociated form is inhibitorier, per mole, that the dissociated form.

In other study, were probe neutralized and non-neutralized filtered supernatants form cultures of *Lactobacillus acidophilus, Lactobacillus rhamnosus* and *Lactobacillus sake* to antagonist *E. coli, L. monocytogenes* and *S. aureus.* The *L. rhamnosus* and *L. sake* supernatants presented no significant differences on development of pathogens during the observation period (24 h). *L. acidophilus* supernatant presented a better performance than the other two in both cases, with neutralized as well as non-neutralized supernatants [43].

Using non-neutralized and neutralized supernatants it should be possible to discriminate the effects of lactic acid and bacteriocins on growth of pathogenic microorganisms, but in some research there was found no difference between neutralized and non-neutralized in the inhibition of pathogens [19, 43].

Co-culture of pathogen and probiotic strains in MRS broth

Each pathogen strain was co-cultured with a probiotic strain in order to study the antagonism. In general, *Salmonella* Typhimurium was significantly more inhibited than *E. coli* O157:H7 ($P <0.05$). Also, the inhibition of both pathogens was significantly different for each probiotic strain co-cultured, having *L. acidophilus* the highest antagonism effect over *Salmonella* Typhimurim (Figure 4, 5 and 6), more pronounced as incubation time increased, becoming undetectable at 16 h of incubation. The rest of the combinations of the co-cultures were not significant ($P <0.05$), this was observed when compared the growth of each pathogen to the control and to the co-culture, where they were similar.

It is important to mention that the number of the three probiotic bacteria in all experiments remains constant over time at a concentration of 10^8-10^9 LOG CFU/ml, even when they were in the co-culture with pathogenic bacteria.

Other study demonstrated the antagonism of 15 *Bifidobacterium* strains (*B. animalis, B. globosum* and *B. breve*) against six *Salmonella* strains (*Salmonella enteritidis* and *Salmonella* Typhimurium). They found that all strains of *Bifidobacterium* were effectively antagonistic against *Salmonella* strains, which were fully inhibited before of the probiotic strains exponential phase end, then their viability was severely affected at the beginning of the stationary phase. The *Salmonella* strains CFU ranged from zero to 5.13 LOG/ml. Growth of *Bifidobacterium* strains in co-cultures was similar to that observed in mono-cultures [44].
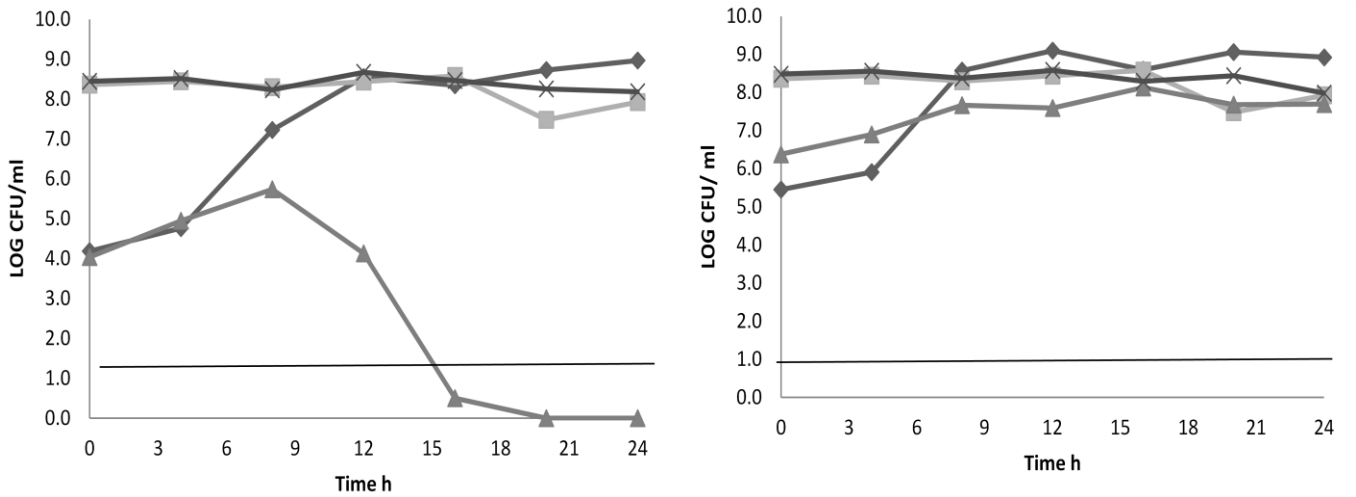
Figure 4. Pathogen strains and *L. acidophilus* NCFM in LMRS broth in co-cultures and mono-cultures, at 35°C in anaerobic conditions. Left graphic: co-culture of *Salmonella* Thyphimurium; right graphic: co-culture of *E. coli* O157:H7. Culture of pathogen strain ▲; Culture of probiotic ■; Pathogen strain in co-culture ♦; Probiotic strain in co-culture ●. Solid lines indicate detection limit (*n*=3).
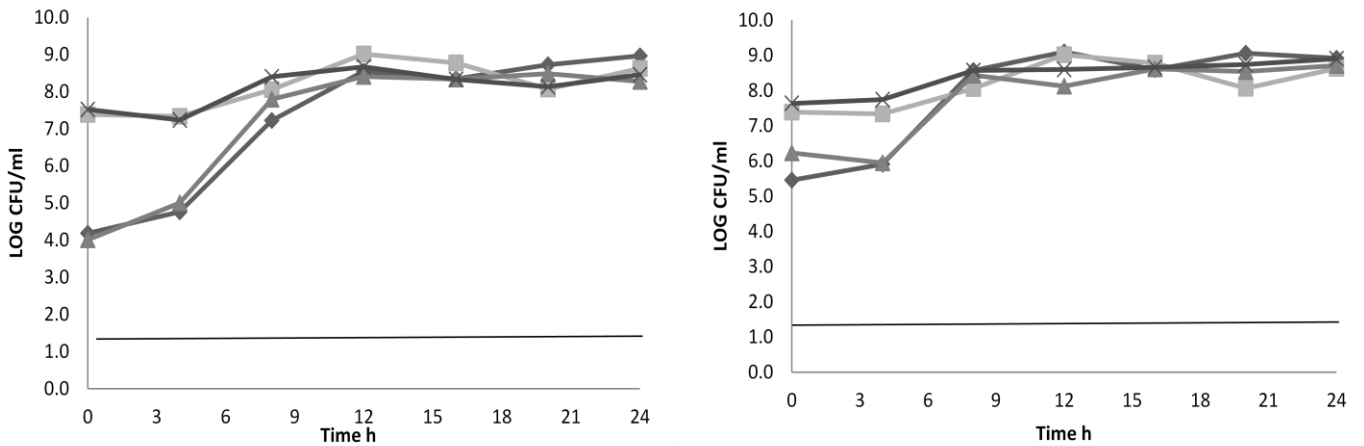

Figure 5. Pathogen strains and *B.animalis* BI07 in MRS broth in co-cultures and mono-cultures, at 35°C in anaerobic conditions. Left graphic: co-culture of *Salmonella* Thyphimurium; right graphic: co-culture of *E. coli* O157:H7. Culture of pathogen strain ▲; Culture of probiotic ■; Pathogen strain in co-culture ♦; Probiotic strain in co-culture ●. Solid lines indicate detection limit (*n*=3).
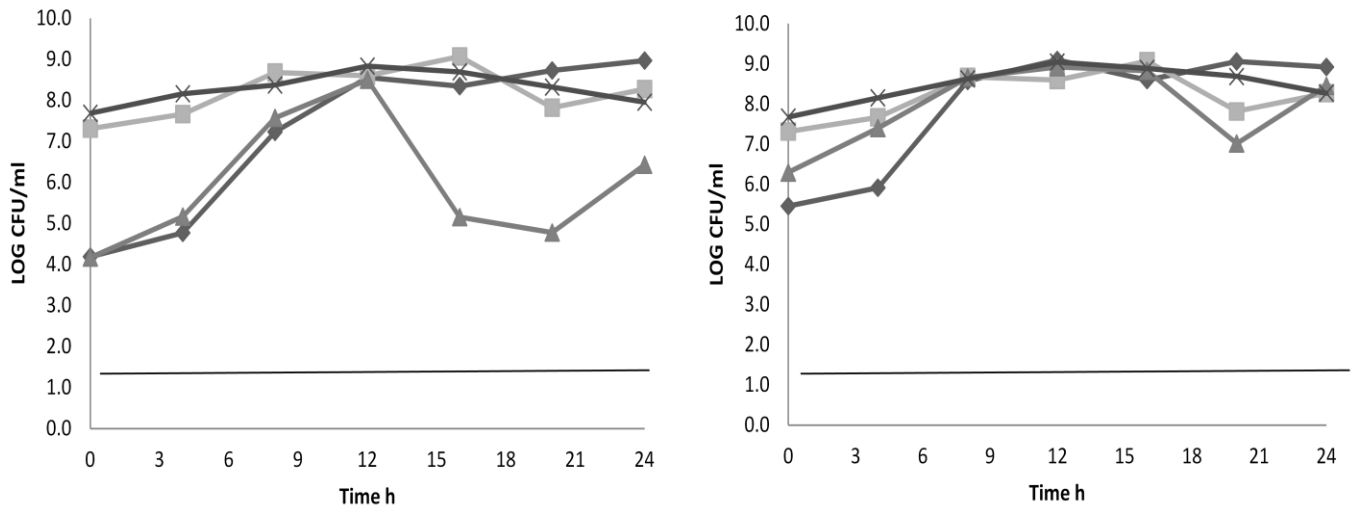
Figure 6. Pathogen strains and *L. rhamnosus* HN001 in LMRS broth in co-cultures and mono-cultures, at 35°C in anaerobic conditions. Left graphic: co-culture of *Salmonella* Thyphimurium; right graphic: co-culture of *E. coli* O157:H7. Culture of pathogen strain ▲; Culture of probiotic ■; Pathogen strain in co-culture ♦; Probiotic strain in co-culture ●. Solid lines indicate detection limit (*n*=3).

Conclusion

L. acidophilus NCFM, *L. rhamnosus* HN001 *and B. animalis* BI07 were shown to be effective antagonists against two antibiotic multiresistant bacteria: *Salmonella* Typhimirium and *E. coli* O157: H7. *Lactobacillus* strains showed a reduction of both pathogen strains, while *B. animalis* had a bacteriostatic effect in both pathogens. Probiotic bacteria synthesized antimicrobials substances having antagonistic effects, including lactic acid. The antagonistic effect against *E. coli* O157:H7, which can resist acidic pH, may be explained by the presence of bacteriocins. Further studies *in vivo* with these probiotic strains are needed to prove a therapeutic or preventive effect against antibiotic-resistant pathogens.

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