

## Effect of *Lactobacillus* sp. isolates supernatant on *Escherichia coli* O157:H7 enhances the role of organic acids production as a factor for pathogen control<sup>1</sup>

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**ABSTRACT.-** Poppi L.B., Rivaldi J.D., Coutinho T.S., Astolfi-Ferreira C.S., Ferreira A.J.P. & Mancilha I.M. 2015. **Effect of *Lactobacillus* sp. isolates supernatant on *Escherichia coli* O157:H7 enhances the role of organic acids production as a factor for pathogen control.** *Pesquisa Veterinária Brasileira* 35(4):353-359. Departamento de Patologia, Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, Av. Prof. Dr. Orlando Marques de Paiva 87, São Paulo, SP 05508-270, Brazil. E-mail: [ajpferr@usp.br](mailto:ajpferr@usp.br)

Many attempts have been made to establish the control of foodborne pathogens through *Lactobacillus* isolates and their metabolism products with success being obtained in several situations. The aim of this study was to investigate the antagonistic effect of eight *Lactobacillus* isolates, including *L. casei* subsp. *pseudopantarum*, *L. plantarum*, *L. reuteri* and *L. delbrueckii* subsp. *delbrueckii*, on the pathogenic *Escherichia coli* strain O157:H7. The inhibitory effect of pure cultures and two pooled cultures supernatants of *Lactobacillus* on the growth of pathogenic bacteria was evaluated by the spot agar method and by monitoring turbidity. Antimicrobial activity was confirmed for *L. reuteri* and *L. delbrueckii* subsp. *delbrueckii* and for a pool of lactic acid bacteria. The neutralized supernatant of the pool exerted a higher antimicrobial activity than that of the individual strains. Furthermore, D-lactic acid and acetic acid were produced during growth of the *Lactobacillus* isolates studied.

INDEX TERMS: *Lactobacillus*, antagonism, *Escherichia coli* O157:H7, organic acid.

**RESUMO.- [Efeito do sobrenadante de isolados de *Lactobacillus* sp. sobre *Escherichia coli* O157:H7 reforça o papel da produção de ácidos orgânicos como fator de controle patogênico.]** Muitas tentativas têm sido feitas para se estabelecer o controle de patógenos de origem alimentar através do uso de estirpes de *Lactobacillus* e dos seus produtos de metabolismo, com sucesso sendo sucedido em várias situações. O objetivo deste trabalho foi investigar o efeito antagônico do sobrenadante de culturas de oito

isolados de *Lactobacillus*, incluindo *L. casei* subsp. *pseudopantarum*, *L. plantarum*, *L. reuteri* e *L. delbrueckii* subsp. *delbrueckii*, sobre *Escherichia coli* amostra O157:H7. Os efeitos inibidores de culturas puras e de dois "pools" de cultura de *Lactobacillus* sobre o crescimento da bactéria foram avaliados através do método de inibição em ágar e através do monitoramento da turbidez da cultura bacteriana. A atividade antimicrobiana foi confirmada para *Lactobacillus reuteri* e *Lactobacillus delbrueckii* subsp. *delbrueckii* e para o "pool" de bactérias ácido-láctica. O sobrenadante neutralizado do "pool" de *Lactobacillus* exerceu uma atividade antimicrobiana mais elevada do que aquela das estirpes individuais. Além disso, ácido D-láctico e ácido acético foram produzidos durante o crescimento dos *Lactobacillus* estudados.

TERMOS DE INDEXAÇÃO: *Lactobacillus*, antagonismo, *Escherichia coli* O157:H7, ácido orgânico.

### INTRODUCTION

*Escherichia coli* O157:H7 represents one of the most important enteropathogenic bacteria; it is responsible for numerous reports of diarrhea is transmitted through food, water

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and the environment, affecting mainly infants and immunosuppressed adults, all over the world (Monteiro-Neto et al. 1997, Fábrega et al. 2002). The pathogenicity of *E. coli* O157:H7 is attributed to the production of potent enterotoxins, the Shiga-like toxins (Stx1 and Stx2, also called verocytotoxins), that directly affect the activity of cells in the intestinal wall, resulting in hemorrhage and thousands of deaths annually (Kudva et al. 1996, Cantarelli et al. 2000, Garcia et al. 2010). Many different types of food were identified as potential sources of Shiga-toxin-producing *Escherichia coli* (STEC), including raw and undercooked foodstuffs (Garcia et al. 2010). The natural hosts of *Escherichia coli* O157:H7 are wildlife and farm ruminants, mainly cattle and swine. In the state of Rio de Janeiro (Brazil), studies conducted by Cerqueira et al (1999) reported the presence of STEC in 71 % of the fecal samples of healthy cattle from dairy farms, beef farms and slaughterhouses. This was the first report concerning the isolation of STEC from the intestines of dairy and beef cattle in Brazil, although several studies had already reported the presence of other enteropathogenic strains in the food industry (Cerqueira et al. 1999).

One approach that led to the reduction and, in a number of cases, the elimination of intestinal pathogenic bacteria in humans and animals includes the ingestion of probiotics in the dairy diet (Guarner & Schaafsma 1998, Gopal et al. 2001). Probiotics are live microorganisms that, when administered in adequate amounts, confer beneficial effects on the host by altering indigenous microbiota and preventing infections (FAO/WHO 2001). Lactic acid bacteria (LAB) with probiotic properties, such as *Bifidobacterium* spp. and *Lactobacillus* spp. were used to prevent some intestinal pathogenic infections and to stimulate the host's immune system in both humans and animals (Perdigón et al. 1999, Fang et al. 2000, Nakazato et al. 2011). It is well documented that *Lactobacillus* spp. with probiotic properties prevent the growth and toxin production of bacteria such as *Campylobacter jejuni*, *Listeria monocytogenes*, *Helicobacter pylori*, *Salmonella*, *Shigella* and *Escherichia coli* (Jin et al. 1996, Kalantzopoulos 1997, Servin & Coconnier 2003, Poppi et al. 2008, Scapin et al. 2013).

The antagonist activity of probiotics on pathogenic bacteria could be associated with the competition for nutrients and sites of adhesion in the mucosa of the small intestine and the production of carbon dioxide, hydrogen peroxide and diacetyl (Gopal et al. 2001). Furthermore, the inhibitory effect on the growth of several enteropathogenic bacteria is likely associated with the antimicrobial compounds produced by lactic acid bacteria, such as bacteriocin and lactic, acetic and other short-chain organic acids, which are responsible for a reduction in the intestinal pH (Servin & Coconnier 2003, Cheng et al. 2003, Varalakshmi et al. 2013). Lactic acid represents the main antimicrobial compound present in cultures of lactic acid bacteria (Earnshaw 1992, Navarro et al. 2000, Todorov & Dicks 2005, Rosslund et al. 2005, Moraes et al. 2013). Weak acids possess higher antimicrobial activity than strong acids, which ionize completely in an aqueous solution (Axe & Bailey 1995). The non-dissociated forms of organic acids are able to function as protonophores, inducing the acidification of the cytoplasm and the accumulation

of toxic anions. The decrease in the cell's internal pH affects the influx of protons through the cell membrane, which dissipates the proton-motive force, reducing cellular energy (ATP) and affecting substrate uptake in the cell (Axe & Bailey 1995, Diez-Gonzalez & Russell 1997).

Several *in vitro* and *in vivo* studies demonstrated the antagonism of numerous strains of *Lactobacillus*, including *L. delbrueckii* var *delbrueckii*, *L. plantarum*, *L. acidophilus*, *L. reuteri* and *L. casei*, against a variety of pathogens (Poppi et al. 2008, Servin & Coconnier 2003, Kalantzopoulos 1997, Jin et al. 1996). In spite of many detailed studies concerning the antagonistic effects of these bacteria on pathogens, there is still a need for new bacterial strains with antimicrobial properties for clinical and commercial benefits (Chaucheyras-Durand & Durand 2010). *In vitro* screening methods such as agar spotting and the monitoring of turbidity represent a fast and effective tool for this purpose. Therefore, it is desirable to use these methods to select promising strains of *Lactobacillus* for the development of new probiotic preparations at the industrial scale.

The main objective of this work was to evaluate the *in vitro* performance of eight strains of *Lactobacillus* isolated from poultry litter with respect to their inhibitory effect on the growth of *Escherichia coli* O157:H7.

## MATERIALS AND METHODS

### *Lactobacillus* isolates and *Escherichia coli* strain O157:H7

Eight isolates of *Lactobacillus*, previously isolated from poultry litter (Paço et al. 2003), were selected to study their antagonistic effects on an *Escherichia coli* O157:H7 strain that was kindly provided by Dr. Isabel Scaletsky (Department of Microbiology, Immunology and Parasitology, Federal University of São Paulo, São Paulo, Brazil). The bacterial isolates *L. casei* subsp. *pseudoplantarum* (isolates 30b and 30c), *L. plantarum* (isolates 11fb, 22c and 41b), *L. reuteri* (isolates 18fa and 19fa) and *L. delbrueckii* subsp. *delbrueckii* (isolate 17fb) were obtained from the Laboratory of Avian Diseases at the School of Veterinary Medicine and Animal Science of the University of São Paulo, Brazil. The *Lactobacillus* isolates were maintained at 4°C in MRS (Manosa-Rogosa and Sharpe) agar slant. The pathogenic strain of *Escherichia coli* O157:H7 was grown in BHI broth (Difco, Sparks, MD, USA) under aerobic conditions at 37°C for 18h.

The physiological characteristics and purity of the *Lactobacillus* isolates were analyzed by observation of their morphological characteristics using a light microscope, Gram staining, a catalase test, a motility test and their ability to ferment various substrates. Sugar and sugar-alcohol assimilation tests were performed in test tubes by inoculation of the isolates into sterilized media, as described. The following substrates were used: peptone (10 g.L<sup>-1</sup>), yeast extract (5 g.L<sup>-1</sup>), potassium phosphate (5 g.L<sup>-1</sup>), ammonium citrate (2 g.L<sup>-1</sup>), sodium acetate (5 g.L<sup>-1</sup>), manganese sulfate (0.5 g.L<sup>-1</sup>), magnesium sulfate (0.01 g.L<sup>-1</sup>), Tween 80 (0.05 g.L<sup>-1</sup>), phenol red (0.5 g.L<sup>-1</sup>). All products have been purchased from Merck, Darmstadt, Germany. The carbohydrate sources used: arabinose, cellobiose, galactose, glucose, lactose, maltose, mannitol, mannose, melezitose, melibiose, raffinose, rhamnose, salicin, sucrose, trehalose and xylose (Sigma, St. Louis, MO, USA) at concentration 10g.L<sup>-1</sup> for each sugar type. The stock solutions were prepared from a 20% (w/v) solution previously filtered through a 0.45 µm membrane filter (Millipore Corp., Billerica, MA, USA). The tubes were inoculated and incubated at 37°C for 7 days to observe red phenol reduction by the sugar test (Kandler & Weiss 1986).

### Growth conditions

The strains of *Lactobacillus* were activated by transferring a full loop from the stock culture to a 125mL Erlenmeyer flask containing 45mL of MRS broth, followed by incubation at 37°C for 18 h. After three successive propagations in the same conditions, strains were grown independently in test tubes containing 5.0mL of MRS broth at 37°C for 16h.

Two different pools of *Lactobacillus* were prepared, which were named pool A (PA) and pool B (PB). PA was prepared by mixing 4.0-mL of each pre-activated culture (32-mL total) in a 500-mL flask containing 268-mL sterile MRS broth and incubating the mixture at 37°C for 18 h. PB consisted of the same mixture as PA, but without the subsequent incubation. Cells of individual and pooled cultures were collected by centrifugation (10,000xg at 4°C for 10 min), washed twice with 0.1 M PBS (phosphate saline buffer, pH 7.4), suspended in 20 % sterile sucrose solution and frozen at -80°C for later use. The supernatants from these cultures were used to study their inhibitory capability against *Escherichia coli* O157:H7. All of the experiments were carried out at least in duplicate. Replicates differ by less than 10%, and typically by less than 5%. The statistical significance was evaluated by the Student's t test.

### Antibacterial activity against *Escherichia coli* O157:H7 - agar spot test

The antimicrobial activity of *Lactobacillus* strains against *Escherichia coli* O157:H7 was analyzed by a spot agar test as described by Schillinger & Lüke (1989). Aliquots of 2µL of concentrated cell suspensions of pure cultures and pool A of *Lactobacillus* were spotted on the surface of MRS agar plates and MRS agar plates buffered with 35 mM sodium bicarbonate. The plates were dried out at room temperature for 30 min and incubated microaerobically at 37°C for 24 h. Afterward, the plates were covered with 10mL of soft BHI (Brain Heart Infusion) agar containing 10<sup>8</sup> CFU/mL of an overnight culture of *Escherichia coli* O157:H7 suspension. Then, the plates were incubated under anaerobic condition at 37°C for an additional 24h. The formation of clear zones of growth inhibition around *Lactobacillus* colonies and their diameters were recorded. Inhibition was considered positive if the diameter of the clear zone that formed around colonies was 5 mm or larger.

### Inhibitory effect of *Lactobacillus* supernatants on *Escherichia coli* O157:H7

The supernatants from each culture and pool of *Lactobacillus* were separated into two groups, a neutralized fraction and an acidic fraction. One fraction was adjusted to pH 7.0 with sodium bicarbonate, while the pH of the other fraction remained unaltered. Both fractions were filtered using a 0.45 µm membrane filter (Millipore Corp., Billerica, MA, USA) and stored at -80°C for further use.

The direct antagonism of compounds contained in *Lactobacillus* cell-free supernatants against *E. coli* O157:H7 was monitored by turbidimetry. Aliquots (300 µL) of the neutralized fraction and the acidic fraction of supernatants from each pure culture and PA and PB were transferred to sterile tubes containing 300µL of an *E. coli* O157:H7 cell suspension (10<sup>8</sup> CFU/mL) and incubated at 37°C for 7h. The blank used for standardization consisted of a mixture of 300µL each of sterile MRS and BHI broth. As a control run, 300µL of sterile MRS broth was added to 300 µL of the pathogenic cell suspension, followed by incubation as indicated above. Samples were taken at intervals of 1 hour, and their OD was determined using a spectrophotometer Biomate 3 (Thermo Fisher Scientific, Waltham, MA, USA) at 600 nm. After each absorbance measurement, cell viability was confirmed using a BHI agar plate incubated in the same conditions.

### Effect of pH on *Escherichia coli* O157:H7

To investigate the survival of *Escherichia coli* O157:H7 in the culture media at different pH values. Afterwards, pH was adjusted to 3.6 and 4.2, respectively, with 1.0 N HCl. The bacterial cultures were inoculated with an aliquot of *Escherichia coli* O157:H7 cell suspension (10<sup>8</sup> CFU/mL) and incubated at 37°C for 7h. The cell growth was monitored following the procedures described above.

### Lactic acid and acetic acid assays

The D-lactic acid and acetic acid concentrations were determined by HPLC Waters 786 (Spectralab Scientific, Ontario, Canada) with a refractive index (IR) detector and Bio-Rad HPX-87-H (300 x 7.8mm) column at 45°C using 5 mM sulfuric acid as the eluent, a flow rate of 0.6ml.min<sup>-1</sup> and a sample volume of 20 µl. All samples were conveniently diluted and filtered using a Sep Pak C18 column (Millipore Corp., Billerica, MA, USA).

## RESULTS AND DISCUSSION

The physiological characteristics of *Lactobacillus* strains previously isolated from poultry litter (Paço et al. 2003) were analyzed with regard to their morphology, physiology and biochemical characteristics. All strains were confirmed as *Lactobacillus* using characteristics and properties expected to be on this bacterium, such as a rod shape, positive Gram staining, lack of motility, catalase-negativity, absence of endospores, fermentation of most of the sugars and sugar alcohols tested and resistance to the phenol compound. The strains exhibited a carbohydrate fermentation profile similar to *L. plantarum*, *L. reuteri*, *L. delbruecki* subsp. *delbruecki* and *L. casei* subsp. *pseudoplantarum* (Kandler & Weiss 1986).

Table 1 shows the total viable cells, final pH, lactic and acetic acid concentrations of cell-free supernatants obtained from pure and pooled cultures of *Lactobacillus*. The total number of colony formation units (CFU) of the strains were generally high, ranging from 1.10<sup>7</sup> to 1.10<sup>9</sup> CFU mL<sup>-1</sup>. These results were similar to those obtained by Avonts & De Vuyst (2001) using seven commercial strains of lactobacilli grown in MRS broth under the same conditions, also as shown in Table 2.

**Table 1. Results of colony-forming unit, pH values and organic acid concentrations from *Lactobacillus* isolates after 24 h of cultivation**

<i>Lactobacillus</i> isolates	CFU.mL <sup>-1</sup> pH (x10 <sup>7</sup> )		Organic acid production after cultivation on MRS	
			Lactic acid (g.L <sup>-1</sup> )*	Acetic acid (g.L <sup>-1</sup> )*
<i>L. plantarum</i> isolate 11fb	62	4.10	15.0±0.2	4.4±0.1
<i>L. delbruecki</i> subsp. delbrueckii isolate 17fb	100	3.86	17.6±0.6	4.1±0.2
<i>L. reuteri</i> isolate 18fa	22	4.29	16.1±0.5	4.9±0.1
<i>L. reuteri</i> isolate 19fa	1	3.96	17.8±0.7	4.3±0.4
<i>L. plantarum</i> isolate 22c	90	3.83	18.5±0.3	4.2±0.5
<i>L. casei</i> subsp.	330	3.95	8.6±0.1	2.1±0.2
<i>Pseudoplantarum</i> isolate 30b				
<i>L. casei</i> subsp.	790	3.98	16.7±0.2	2.7±0.1
<i>Pseudoplantarum</i> isolate 30c				
<i>L. plantarum</i> isolate 41b	39	3.88	18.4±0.4	3.7±0.3
Pool A	83	3.93	19.1±0.1	2.8±0.5
Pool B	90	3.90	12.4±0.2	2.7±0.1

\*These values are means of triplicate experiments.

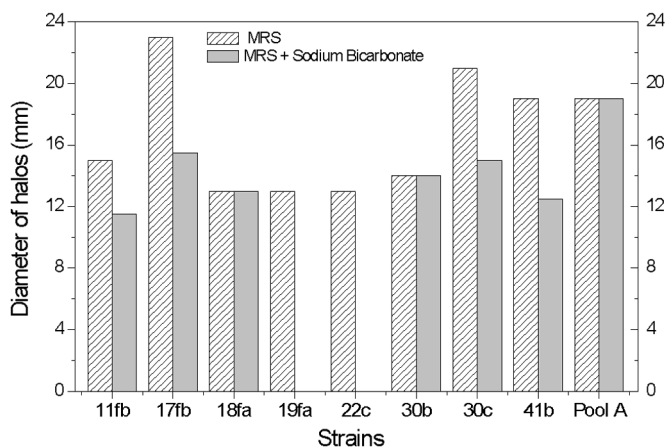
All strains, except *L. casei* subsp. *pseudoplantaum* isolate 30b, were able to produce lactic acid at concentrations higher than 12.4g.L<sup>-1</sup>. The highest concentration was attained with pool A (19.1g.L<sup>-1</sup>), followed by *L. plantarum* isolate 41b (18.4g.L<sup>-1</sup>) and *L. plantarum* isolate 22c (18.5g.L<sup>-1</sup>). In addition, all lactobacilli produced acetic acid at concentrations higher than 2.0g.L<sup>-1</sup>. These short-chain organic acids are known to exhibit high antimicrobial activity against microorganisms due to the easy diffusion of the non-dissociated form through the cell membranes of pathogens (Axe & Bailey 1995). Cheng et al. (2003) studied the influence of organic acids, including acetic acid, propionic acid and lactic acid on the growth and survival of *E. coli* O157:H7. The authors reported that lactic acid exerts greater inhibitory effects on the growth of the pathogenic bacteria than acetic acid and propionic acid; however, the combination of the organic acids exhibited an important synergic effect in the inhibition of this pathogen (Cheng et al. 2003, Varalakshmi et al. 2013). According to Diez-Gonzalez & Russell (1997) small amounts of sodium acetate (1.6g.L<sup>-1</sup>) can inhibit the growth of *E. coli* O157:H7. This level of acetate is similar to the levels observed in the present work.

The antagonistic effects of pure and pooled cultures of *Lactobacillus* strains on *E. coli* O157:H7 were studied using the agar spot method. Based on the data in Figure 1, in non-neutralized media, all of the strains and the pool of *Lactobacillus* strains were antagonistic towards *E. coli* O157:H7

**Table 2. Results of optical density of *Escherichia coli* O157:H7 after 7 h of incubation in acidified BHI and MRS media**

Broth	Optical density* (600 nm)	
	Initial OD	Final OD
MRS, pH 3,6	0.063±0.005	0.076±0.004
MRS, pH 4,2	0.103±0.008	0.117±0.003
MRS, pH 6,3	0.083±0.003	0.212±0.010
BHI, pH 3,6	0.090±0.004	0.113±0.005
BHI, pH 4,2	0.094±0.002	0.120±0.008
BHI, pH 7,4	0.111±0.010	1.015±0.016

MRS =Manosa, Rogosa, Sharpe media, BHI = Brain Heart Infusion, \*Average of three experiments, OD = Optical density.



**Fig.1. Inhibitory effects of *Lactobacillus* isolates supernatant on *Escherichia coli* O157:H7 by the spot agar method using MRS agar and MRS after treatment with sodium bicarbonate.**

(Table 2). The strains *L. delbrueckii* isolate 17fb and *L. casei* subsp. *pseudoplantarum* isolate 30c displayed significantly greater ( $p<0.05$ ) inhibitory effects on the pathogen than the other studied strains, with zones of inhibition larger than 20mm (Fig.1). Pool A and *L. plantarum* isolate 41b exhibited a similar pattern (19mm of inhibition halo). Among the strains studied, differences between 10 and 77% of the diameter of clear zone formation were observed. The total colony count in 2 µL of cell suspension obtained by parallel culture on an MRS plate ranged from 1.10<sup>5</sup> to 1.10<sup>8</sup> CFU.mL<sup>-1</sup>, while the inoculum concentration of *E. coli* O157:H7 was 10<sup>8</sup> CFU in 10 mL of liquefied BHI agar. The lower level of inhibition by *L. reuteri* isolate 18fa, *L. reuteri* isolate 19fa and *L. plantarum* isolate 22c in MRS media was likely related to their slower growth capacity (Table 2).

Furthermore, when the same strains and pools of *Lactobacillus* were tested on neutralized MRS medium (with sodium bicarbonate, 3g.L<sup>-1</sup>), the mixed strains (Pool A) exhibited higher inhibitory activity (19mm halo diameter) than the pure cultures (0 to 15mm). Differences in the halo diameter of between 30 and 100% were observed for cultures in MRS and MRS-neutralized agar medium (Fig.1). Similar results have been described in a study that examined the inhibitory effects of different strains of *Bifidobacterium* against *E. coli*; halo diameters in the range of 13 to 30mm were reported for the un-neutralized agar media associated with a considerable reduction in halo size during growth in media containing sodium bicarbonate (Gagnon et al. 2004).

In our study, the *E. coli* O157:H7 inhibition by the *Lactobacillus* isolates in the presence of sodium bicarbonate may be associated with non-acidic substances, such as hydrogen peroxide and bacteriocins. Furthermore, it has been confirmed that the failure of *L. plantarum* isolate 22c and *L. reuteri* isolate 19fa to grow in the media containing sodium bicarbonate was due to an unfavorable pH for the growth of these microorganism.

The combination of strains in pool A displayed greater inhibitory activity toward *E. coli* O157:H7 than the individual cultures in almost all cases, regardless of the presence or absence of sodium bicarbonate (Fig.1). These results demonstrate variation in the inhibitory strength of pure cultures of *Lactobacillus*, as well as in the synergistic effect of co-cultures against *E. coli* O157:H7, emphasizing the importance of the precise selection of the strains for probiotic applications.

Figure 2A shows the optical density of *E. coli* O157:H7 grown in media containing the supernatants from different cultures of *Lactobacillus*. The absorbance values demonstrate that *E. coli* was able to grow in media containing the neutralized fraction of supernatants from pure and pooled cultures of *Lactobacillus*. However, the pathogenic strain exhibited slower growth rate and reached lower final OD values in neutralized media compared to the control (Fig.2A).

The results suggest that pH adjustment results in the loss of the antibacterial properties of the supernatants. However, inhibitory activity was observed in all of the media containing the acidic fractions of the supernatants, as con-

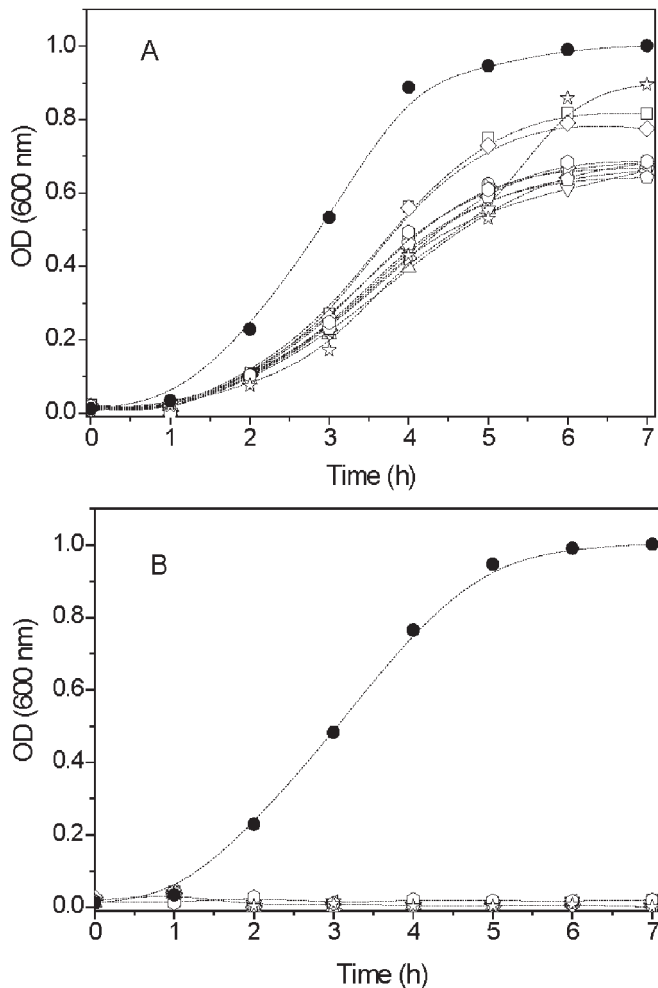


Fig. 2. Growth of *Escherichia coli* O157:H7 in cultural media containing the neutralized fraction of supernatants of *Lactobacillus* cultures (A) and the acidic fraction of supernatants (B) of *L. plantarum*-11fb ( $\square$ ), *L. delbrueckii* subsp. *delbrueckii*-17fb ( $\circ$ ), *L. reuteri* -18fa ( $\triangle$ ), *L. reuteri*-19fa ( $\nabla$ ), *L. plantarum* -22c ( $\triangleleft$ ), *L. casei* subsp. *pseudoplantarum* -30b ( $\triangleright$ ), *L. casei* subsp. *pseudoplantarum*-30c ( $\diamond$ ), *L. plantarum* -41b ( ), pool A ( ) and control ( $\bullet$ ).

firmed by optical density values that were near zero. Indeed, it is well known that most of the antagonistic effects of *Lactobacillus* on *E. coli* growth could be due to the presence of organic acids that are produced during cell growth (Axe & Bailey 1995, Diez-Gonzalez & Russell 1997).

According to the results shown in Figure 2B, substances present in the supernatants exerted bacteriostatic or bactericidal effects on the pathogenic *Escherichia coli*. The supernatants from *L. delbrueckii* subsp. *Delbrueckii* isolate 17fb, *L. reuteri* isolate 19fa and pool B cultures exhibited bactericidal activity on *E. coli*; after 7 h of exposure to these supernatants, no colony formation was observed on specific agar media (data not shown). Some strains, such as *L. reuteri*, can produce reuterin, a non-proteic substance that is soluble in water and possesses antibacterial, antifungal and antiprotozoan properties (Sung et al. 2003).

Lash et al. (2005) studied the inhibitory effects and stability of *L. plantarum* supernatants at different temperatu-

res, pHs and after treatment with proteolytic enzymes. The results revealed that the supernatant from *L. plantarum* lost its antimicrobial activity when the pH was adjusted to values higher than 5.0 and lower than 4.0, suggesting that the compound responsible for the inhibition was active only in this pH range. In the present work, a similar effect was detected with the supernatants from *Lactobacillus* isolates 17fb and 19fa, and pool B, which exhibited antimicrobial activity toward *E. coli* O157:H7 except at pH 7.0, where the pathogenic bacteria grew normally.

Ogawa et al. (2001) reported that *L. casei* strain Shirota and *L. acidophilus* strain YIT 0070 exerted inhibitory effects on *E. coli* growth. Their antimicrobial activity was assayed in co-culture, suggesting that the antimicrobial effect of *Lactobacillus* depends on the pH, due to accumulation of lactic acid in the culture medium. The authors also tested the antagonistic effect of *L. brevis*; however, this organism was unable to affect *E. coli* growth. The lack of antagonistic effects indicates that, as observed in the present work, the antimicrobial activity is dependent on the *Lactobacillus* strain. On the other hand, Aricia et al. (2004) observed a decrease in the antibacterial activity of supernatants of *Lactobacillus* that were adjusted to pH 6.5 and contained catalase, suggesting that hydrogen peroxide and organic acids promoted the inhibition of the growth of pathogens, including *E. coli*.

The pH exerts an important negative effect on bacterial metabolism and growth. In this study, the *E. coli* O157:H7 was subjected to a moderately acidic environment at 37°C for 7 h. Table 2 shows the optical density (OD 600 nm) of the pathogenic *E. coli* inoculated in BHI and MRS broth which had been adjusted to different pH values. The results demonstrate that *E. coli* O157:H7 was unable to grow in both media at pH 3.6 and 4.2; the effect on pathogen growth was bacteriostatic. However, the acidified media did not exert a bactericidal effect on *E. coli*; after 7h of exposure, the pathogenic strains were able to grow on BHI agar plates (data not shown). Previous studies showed that *E. coli* and other pathogenic bacteria are sensitive to pH 3.5 and that exposure to low pH can result in adaptive resistance to acidic media (Koutsoumanis & Sofos 2004; Cheng et al. 2003). *E. coli* and other pathogenic bacteria can survive under conditions of extreme acids stress thanks to an acidic-resistance (AR) systems, which is a group of amino acid decarboxylases and antiporters amino-acid dependent. Cells that have grown in amino-acid deficient medium (minimal medium) can succumb at pH 2.5 given the lack of glutamate or arginine (Foster 2004).

Cheng et al. (2003) reported that the percentage of *E. coli* O157:H7 cells that survived in MRS broth at pH 3.0 were 4.2%. However, the authors mentioned that the bactericidal effect was not observed after 120 min of exposure to these conditions and that by increasing the pH of the culture broth to 4.0, the percentage of survival grew to 70% of the control growth (pH 7.0). Previous researches showed that many *Lactobacillus* isolates may prevent the binding of pathogens bacteria in the human and animal intestinal cells. After the banning of growth promoters antibiotics (GPA) in worldwide, many alternatives were launched, but

still the *Lactobacillus* strains are the best choice for many situations, like the use as probiotics for chicken, cattle, swine and others production animals, such as meat-type or egg-type. Nevertheless, we must consider the metabolism product of these bacteria for use in animal production feeding. The *Lactobacillus* studied could prove useful as dietary supplements as well as antimicrobial agents in food and packaging applications.

## CONCLUSIONS

Almost all strains of *Lactobacillus* investigated in this study exhibited bactericidal effects on *Escherichia coli* O157:H7.

The highest inhibitory activity corresponded to *Lactobacillus delbrueckii* subsp. *delbrueckii* 17fb and *L. casei* subsp. *pseudopantarum* 30c and, suggesting that antimicrobial compounds production, such as hydrogen peroxide or bacteriocins may be responsible by bacterial inhibition.

All strains tested produced lactic and acetic acids.

The amount of lactic acid produced has no demonstrable influence on the inhibition; in pool A, the supernatants with the highest lactic acid concentrations were unable to exert a more pronounced bactericidal effect on the pathogenic bacteria than the other strains.

Thus, the strains of *Lactobacillus* studied could be used as antimicrobial agents in packaging or as dietary supplements to control pathogenic microorganisms such as *Escherichia coli* O157:H7.

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## REFERENCES

- Aricia M., Bilgin B., Sadgic O. & Ozdemir C. 2004. Some characteristic of *Lactobacillus* isolates from infant faeces. *Food Microbiol.* 21:19-24.
- Avonts L. & De Vuyst L. 2001. Antimicrobial potential of probiotic lactic acid bacteria. *Meded Rijksuniv. Gent., Fak. Landbouwk. Toegep. Biol. Wet.* 66:543-550.
- Axe D.D. & Bailey J.E. 1995. Transport of lactate and acetate through the cytoplasmic membrane of *Escherichia coli*. *Biotechnol. Bioeng.* 47:8-19.
- Cantarelli V., Nagayama K., Takahashi A., Honda T., Cauduro P., Dias G.A.G., Mezzari A. & Brodt T. 2000. Isolation of Shiga toxin-producing *Escherichia coli* (STEC) serotype O91:H21 from a child with diarrhea in Porto Alegre city, RS, Brazil. *Braz. J. Microbiol.* 31:266-270.
- Cerqueira A.M.F., Guth B.E.C., Joaquim R.M. & Andrade J.R.C. 1999. High occurrence of Shiga toxin-producing *Escherichia coli* (STEC) in healthy cattle in Rio de Janeiro State, Brazil. *Vet. Microbiol.* 70:111-121.
- Chaucheyras-Durand F. & Durand H. 2010. Probiotics in animal nutrition and health. *Beneficial Microbes* 1:3-9.
- Cheng H.Y., Yu R.C. & Chou C.C. 2003. Increased acid tolerance of *Escherichia coli* O157:H7 as affected by acid adaptation time and conditions of acid challenge. *Food Res. Int.* 36:49-56.
- Diez-Gonzalez F. & Russell J.B. 1997. The ability of *Escherichia coli* O157:H7 to decrease its intracellular pH and resist the toxicity of acetic acid. *Microbiol.* 143:1175-1180.
- Earnshaw R.G. 1992. The antimicrobial action of lactic acid bacteria: natural food preservation systems, p.211-232. In: Wood B.J.B. (Ed.), *The Lactic Acid Bacteria: the lactic acid bacteria in health and disease*. Chapman and Hall, London, UK.
- Fábrega V.L.A., Ferreira A.J.P., Patrício F.R.S., Brinkley C. & Scaletsky I.C.A. 2002. Cell-detaching *Escherichia coli* (CDEC) strains from children with diarrhea: identification of a protein with toxicogenic activity. *FEMS Microbiol. Lett.* 217:191-197.
- Fang H., Elina T., Heikki A. & Seppo S. 2000. Modulation of humoral immune response through probiotic intake. *FEMS Immunol. Med. Microbiol.* 29:47-52.
- FAO/WHO 2001. Joint FAO/WHO Expert consultation on evaluation of health and nutritional properties of probiotics in food including powder milk with live lactic acid bacteria. Available at <[http://www.who.int/foodsafety/publications/fs\\_management/en/probiotics.pdf](http://www.who.int/foodsafety/publications/fs_management/en/probiotics.pdf)> Accessed Febr. 12, 2014.
- Foster J.W. 2004. *Escherichia coli* acid resistance: tales of an amateur acidophile. *Nature Rev. Microbiol.* 2:898-907.
- Gagnon M., Kheadr E.E., Blay G.L. & Fliss I. 2004. In vitro inhibition of *Escherichia coli* O157:H7 by bifidobacterial strains of human origin. *Int. J. Food Microbiol.* 92:69-78.
- Garcia A., Fox J.G. & Besser T.E. 2010. Zoonotic enterohemorrhagic *Escherichia coli*: a one health perspective. *ILAR J.* 51:221-232.
- Gopal P.K., Prasad J., Smart J. & Gill H.S. 2001. In vitro adherence properties of *Lactobacillus rhamnosus* DR20 and *Bifidobacterium lactis* DR10 strains and their antagonistic activity against enterotoxigenic *Escherichia coli*. *Int. J. Food Microbiol.* 67:207-216.
- Guarner F. & Schaafsma G.J. 1998. Probiotics. *Int. J. Food Microbiol.* 39:237-238.
- Jin L.Z., Ho Y.W., Ali M.A., Abdullah N. & Jalaludin S. 1996. Effect of adherent *Lactobacillus* spp. on in vitro adherence of salmonellae to the intestinal epithelial cells of chicken. *J. Appl. Bacteriol.* 81:201-206.
- Kalantzopoulos G. 1997. Fermented products with probiotic qualities. *Anaerobe* 3:15-19.
- Kandler O. & Weiss N. 1986. Regular, nonsporulating Gram-positive rods, p.1209-1234. In: Sneath P.H.A., Mairm N.S. & Sharpe M.E. (Eds), *Bergey's Manual of Systematic Bacteriology*. Williams and Wilkins, Baltimore, USA.
- Koutsoumanis K.P. & Sofos J.N. 2004. Comparative acid stress response of *Listeria monocytogenes*, *Escherichia coli* O157:H7 and *Salmonella* Typhimurium after habituation at different pH conditions. *Let. Appl. Microbiol.* 38:321-326.
- Kudva T.I., Hatfield P.G. & Hovde C.J. 1996. *Escherichia coli* O157:H7 in microbiota flora of sheep. *J. Clin. Microbiol.* 34:431-433.
- Lash B.W., Mysliwiec T.H., Gourama H. & Mysliwiec T.H. 2005. Detection and partial characterization of a broad-range bacteriocin produced by *Lactobacillus plantarum* (ATCC 8014). *Food Microbiol.* 22:199-204.
- Monteiro-Neto V., Campos L.C., Ferreira A.J.P., Gomes T.A.T. & Trabulsi L.R. 1997. Virulence properties of *Escherichia coli* O111:H12 strains. *FEMS Microbiol. Lett.* 146:123-128.
- Moraes P.M., Perin L.M., Silva Jr A. & Nero L.A. 2013. Comparison of phenotypic and molecular tests to identify lactic acid bacteria. *Braz. J. Microbiol.* 44:109-112.
- Nakazato G., Paganelli F.L., Lago J.C., Aoki F.H., Mabilon C., Brocchi M., Stelling E.G. & Silveira W.D. 2011. *Lactobacillus acidophilus* decreases *Salmonella* Typhimurium invasion *in vivo*. *J. Food Saf.* 31:284-289.
- Navarro L., Zarazaga M., Sáenz J., Ruiz-Larrea F. & Torres C. 2000. Bacteriocin production of by lactic acid bacteria isolated from Rioja red wines. *J. Appl. Microbiol.* 88:44-51.
- 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 bacteria *Lactobacillus* strains due to production of lactic acid. *Int. J. Food Microbiol.* 68:135-140.
- Paço R.S., Leme I.L., Bottino J.A. & Ferreira A.J.P. 2003. Identification of *Lactobacillus* spp. from broiler litter in Brazil. *Braz. J. Microbiol.* 34:236-237.
- Perdigón G., Vintiñi E., Alvarez S., Medina M. & Medici M. 1999. Study of the possible mechanisms involved in the mucosal immune system activation by lactic acid bacteria. *J. Dairy Sci.* 82:1108-1114.

- Poppi L.B., Mancilha I.M., Ferreira A.J.P. & Leal D.D.M. 2008. Evaluation of the antagonistic effect of *Lactobacillus* on *Listeria monocytogenes* in vitro. *Braz. J. Food Technol.* 11:113-119.
- Rosslund E., Langsrud T., Granum P.E. & Sorhaug T. 2005. Production of antimicrobial metabolites by strains of *Lactobacillus* or *Lactococcus* co-cultured with *Bacillus cereus* in milk. *Int. J. Food Microbiol.* 98:193-200.
- Scapin D., Grando W.F., Rossi E.M., Perez K.J., Malheiros P.S. & Tondo E.C. 2013. Antagonistic activity of *Lactobacillus acidophilus* LA10 against *Salmonella enterica* serovar Enteritidis SE86 in mice. *Braz. J. Microbiol.* 44:57-61.
- Servin A.L. & Coconier M.H. 2003. Adhesion of probiotic strains to the intestinal mucosa and interaction with pathogens. *Best Pract. Res.* 17:741-754.
- Schillinger U. & Lücke F.K. 1989. Antibacterial activity of *Lactobacillus sake* isolated from meat. *Appl. Environ. Microbiol.* 55:1901-1906.
- Sung H.W., Chen C.N., Liang H.F. & Hong M.H. 2003. A natural compound (reuterin) produced by *Lactobacillus reuteri* for biological-tissue fixation. *Biomaterials* 24:1335-1347.
- Todorov S.D. & Dicks L.M.T. 2005. *Lactobacillus plantarum* isolated from molasses produces bacteriocins active against Gram-negative bacteria. *Enzyme Microb. Technol.* 36:318-326.
- Varalakshmi S., Balasubramanyam B.V., Surendranath B., Bagath M. & Rajendran D. 2013. Use of novel lactic acid bacterial strains with antagonistic activity for the preparation of safe indigenous fermented dairy foods (Dahi and Raita). *J. Food Safty* 34:26-33.