International Food Research Journal 20(2): 991-1000 (2013)
Journal homepage: *http://www.ifrj.upm.edu.my*

Probiotic potential of thermotolerant lactic acid bacteria strains isolated from cooked meat products

Ramirez-Chavarin, M.L., Wacher, C., Eslava-Campos, C.A. and Perez-Chabela, M.L.

[1]*Departmento de Biotecnología, Universidad Autónoma Metropolitana Iztapalapa. Av. San Rafael Atlixco #186, Iztapalapa 09340, Distrito Federal, México*
[2]*Departamento de Alimentos y Biotecnología, Facultad de Química, Universidad Nacional Autónoma de México, Ciudad Universitaria, Coyoacán, 04510, D.F., México*
[3]*Departamento de Salud Pública, Facultad de Medicina, Universidad Nacional Autónoma de México, Ap. Postal 70-433, Ciudad Universitaria, Coyoacán, 04510, D.F., México*

Article history

Received: 26 September 2012
Received in revised form: 19 October 2012
Accepted: 24 October 2012

Keywords

Probiotic
adherence
taurocholic acid
thermotolerant lactic acid bacteria

Abstract

Ten thermotolerant lactic acid bacteria - *Pediococcus pentosaceus* (4 strains), *Lactobacillus plantarum* (3 strains), *Enterococcus faecium* (2 strains) and *Aerococcus viridans* (1 strains) – isolated from cooked meat products were evaluated to identify their probiotic properties, including tolerance to low pH, tolerance to taurocholic acid and bile, coaggregation, autoaggregation and adherence to HEp-2 epithelial cells. Fifty percent of the strains were intolerant to low pH and simulated gastric juice. All strains grew in taurocholic acid and bile concentrations greater than 0.3%, indicating they are good probiotic candidates. Less than 20% of the strains coaggregated with an *E. coli* indicator while 30% coaggregated with a *Salmonella* strain. Eight of the strains exhibited good autoaggregation capacity at 24 h and all ten had a high adherence capacity for HEp-2 cells. The studied thermotolerant lactic acid bacteria are promising ingredients in the production of cooked meat products with probiotic potential.

Introduction

Probiotics are living microorganisms which provide beneficial effects for human or animal health by improving the gut microflora balance and are administered alone or incorporated into food or feed systems (Fuller, 1989). They exhibit antagonist action, produce antimicrobial substances and help to modulate immune response (Dunne *et al*., 1999). For a strain to qualify as a probiotic it must fulfill certain physiological characteristics, mainly survival in the gastrointestinal tract, tolerance to low pH, tolerance to bile in the form of glycocholic or taurocholic acid and sodium desoxycholate (Tomasik and Tomasik, 2003; Madigan *et al*., 2006). It must also have the capacity to adhere to the intestinal mucus and epithelial cells. This is important based on the two proposed mechanisms for lactic acid bacteria's beneficial effects in the intestine: a) production of antimicrobial substances such as lactic acid and bacteriocins; and b) adherence to the mucus, coaggregation and autoaggregation to form a barrier which blocks colonization by pathogens (Ehrmann *et al*., 2002). Lactic acid bacteria (LAB) are natural inhabitants of the gastrointestinal (GI) tract and are the most frequently used probiotics. Some *Lactobacillus* and *Bifidobacterium* strains have been used due to their beneficial effects on health (Lee and Salminen, 1995), although probiotic characteristics and resistance to biological barriers vary widely between species and among strains of the same species (Vinderola and Reinheimer, 2003).

The use of native lactic acid bacteria as bioprotective cultures is important for cooked meat products. Contrary to dry meat products where the starter culture become dominant changing the environment to ensure microbiological quality, cooked meat products need a thermal processing to develop texture and destroy vegetative forms in order to ensure the adequate shelf life. The lactic acid strains employed in this research have been previously studied as a biopreservative culture in cooked meat products, inoculated as free cells (Pérez-Chabela *et al*., 2008) or spray-dry encapsulated (Pérez-Chabela *et al*., 2012). These strains improved the texture of low fat sausages excreting exopolysaccharides

*Corresponding author.
Email: *lpch@xanum.uam.mx*

(Pérez-Chabela *et al*., 2013).

In this view, the thermotolerant capacity of these lactic acid strains could allow their inoculation in heat processed foods like cooked sausages, becoming dominant flora during vacuum-packed cold storage, acting like biopreservatives agents. The determination of the probiotic properties, like tolerance to gastric conditions and aggregation/coaggregation, is important since the characterization of these strains as probiotics can be useful to formulate functional cooked meat products.

Materials and Methods

Ten thermotolerant lactic acid bacteria (T-LAB), previously genotypic identified by 16S rRNA analysis (Ramirez-Chavarin *et al*., 2010), were employed. The strains included 4 *Pediococcus pentosaceus* strains; 3 *Lactobacillus plantarum* strains; 2 *Enterococcus faecium* strains; and 1 *Aerococcus viridans* strain.

Low pH tolerance

Strain tolerance to low pH levels was evaluated following the method of Ehrmann *et al*. (2002). Briefly, each T-LAB strain was inoculated in MRS broth (De Man *et al*., 1960), and incubated at 35 ± 2°C for 24 h. They were then inoculated into fresh MRS culture medium and again incubated at 35 ± 2°C for 24 h. After incubation, they were centrifuged at 5000 x g for 10 min at 4°C, the biomass rinsed twice with sterile 1X PBS solution [Na_2HPO_4 (10.9 g / L), NaH_2PO_4 (3.2 g / L), NaCl (90 g / L), pH 7.2], pH adjusted to 7.2 and then resuspended in 1X PBS. Each strain was diluted to 1/10 in 1X PBS and pH adjusted to 0.5, 1, 2, 3, 4 or 5 before incubating for 1, 2 and 4 h. The strains were then inoculated into MRS medium, incubated under anaerobic conditions at 35 ± 2°C for 24 h, and survival quantified by plate counts. According to the reported by Victoria-Leon *et al*. (2006), abundant growth was considered above 300 CFU/mL.

Survival in simulated gastric juice

Following the method of Beumer *et al*. (1992), each strain was inoculated into MRS broth and incubated at 35 ± 2°C for 24 h until reaching an optical density (OD) of 1.0 at λ=600 nm (concentration ~ 10^8 cfu / mL). After initial incubation, 10 µL of each bacterial suspension were inoculated into 10 mL simulated gastric juice (2.05 g / L NaCl; 0.60 g / L KH_2PO4; 0.11 g / L $CaCl_2$; 0.37 g / L KCl; 0.05 g / L porcine bile; 0.1 g / L lisozyme; and 0.0133 g / L pepsin) at pH 2.0 and incubated at 35 ± 2°C for 90 min. Samples were taken at 0, 30, 60 and 90 min, diluted to 10^{-1} and 10^{-2}, 100 µL inoculated into dishes containing MRS agar and incubated at 35 ± 2°C for 48 h. Survival was quantified by plate counts on MRS agar. According to the reported by Victoria-Leon *et al*. (2006), abundant growth was considered above 300 CFU/mL.

Tolerance to bile salts

Strain growth in bile was evaluated according to Pedersen *et al*. (2004) and growth in taurocholate according to Ehrmann *et al*. (2002). Two types of bile salts (porcine bile extract and taurocholic acid) were used to analyze growth in both forms (i.e. conjugated as glycholic acid and taurocholic acid, and deconjugated as sodium desoxycholate). Each T-LAB strain was inoculated into MRS broth and incubated at 35 ± 2°C for 24 h. After initial incubation, 100 µL of each suspension were inoculated into 10 mL MRS broth supplemented with 0.1, 0.3, 0.5, 1.0 or 2.0% porcine bile extract and 7 mmol / L sodium taurocholate, and incubated at 35 ± 2°C for 7 h. Growth was monitored hourly and measured at 600 nm absorbance.

Coaggregation

Analysis of coaggregation was done following Yuki *et al*. (2000), with slight modifications. Each T-LAB strain was inoculated into MRS broth, the indicator strains *E. coli* O139:H26 and *Salmonella parera* IV O11:Z4Z23 inoculated into BT (biotryptase) broth and all were incubated at 35 ± 2°C for 24 h. Dilutions were then done of the T-LAB suspensions until approximately 1.0 OD (λ=600 nm) (corresponding to 10^8 cfu / mL), and of the *E. coli* and *Salmonella parera* strains until 0.6 OD (λ=600 nm) (corresponding to 10^6 cfu / mL). The bacterial suspensions were then centrifuged at 7000 g for 10 min at 4°C, the cells washed three times with 0.1 M PBS and resuspended in the same volume of buffer. Optical density (OD; λ=600 nm) was adjusted to 0.5 cells for the T-LAB, *E. coli* and *Salmonella parera* strains. Mixtures were then made of each T-LAB strain with each indicator strain at a 1:1 total volume and incubated at 35 ± 2°C for 4 h. Suspension absorbance (λ=600 nm) was measured for the mixtures, and each of the individual strains (i.e. T-LABs, *E. coli* O139:H26 and *Salmonella parera* IV O11:Z4Z23). Coaggregation percentage was calculated according to Handley *et al*. (1987):

$$\text{Coaggregation} = \frac{\left[\frac{(A_{\text{Pathogen}} + A_{\text{LAB}})}{2}\right] - A_{\text{Mix}}}{\frac{(A_{\text{Pathogen}} + A_{\text{LAB}})}{2}} \text{x} 100$$

Results were analyzed by a one way ANOVA and significantly difference was determined by Duncan's mean test in SAS Software v. 8.0 (SAS Institute, Cary, USA).

Autoaggregation

Analysis of autoaggregation was done following Collado *et al*. (2007), with slight modifications. Each T-LAB strain was inoculated into MRS broth and incubated at 35 ± 2°C for 24 h. Dilutions were then done until approximately 1.0 OD (λ=600 nm) (corresponding to 108 cfu / mL), and these suspensions centrifuged at 3200 g for 20 min at 4°C. The cells were washed three times with 1.0 M PBS, resuspended in the same volume of buffer, OD (λ=600 nm) adjusted to 1.0 and incubated at 35 ± 2°C for 2 or 24 h. Absorbance was measured after incubation and the autoaggregation percentage calculated according to Reniero *et al*. (1992):

$$\text{Autoaggregation} = 1 - \left(A_{time} / A_{initial} \right) \text{x} 100$$

Where:

A_{time} = Absorbance (λ=600 nm) at different incubation times (2 and 24 h).

$A_{initial}$ = Absorbance (λ=600 nm) before incubation period.

Results of incubation time and lactic acid bacteria strain were analyzed by an ANOVA and significantly difference was determined by Duncan's mean test in SAS Software v. 8.0 (SAS Institute, Cary, USA).

HEp-2 adherence assay

Adherence was evaluated following Cravioto *et al*. (1979) using a 75 mL cell culture flask containing 20 mL Eagle minimum essential medium (MEM) and a HEp-2 cell monolayer at 80% confluence.

Day 1. Bacterial suspension

Each T-LAB strain was inoculated into tubes containing 3 mL MRS broth with 1% D-mannose (Sigma) and incubated at 35 ± 2°C for 2 or 18 h.

Day 2. Preparation of 24-well propylene plate

After flaming, a tweezers was used to place plastic beads in each well. The MEM was emptied from the HEp-2 cell culture flask, and 1 mL trypsin added to rinse the flask and then discarded. Again, 1 mL trypsin was added to the flask and left for 5 min to detach the cell monolayer. After adding 29 mL MEM, 1 mL of suspension (2.5 x 10^5 cells / mL) was added to each well in the plate. After filling six wells, the cell suspension was repipetted to maintain homogeneity. The plate was incubated at 37°C in a 5% CO_2 atmosphere and 85% humidity for 24 h to produce a 90% confluence monolayer.

Day 3. Adherence assay

Using a Pasteur pipette under sterile conditions, 1 mL of medium was removed from each well. The cells in the wells were washed with 1 mL 1X PBS, resuspended three times and the supernatant discarded. After washing, 1 mL 1X PBS was added, the bacterial culture centrifuged at 2000 g for 15 min and the supernatant discarded. The bacterial pellet was resuspended in 1 mL 1X PBS, and the tubes placed in an agitator to dissolve the pellet. Of this bacterial suspension, 50 µL (final concentration = 1.0 x 10^8 cfu / mL) was added to a sterile 1 mL microtube, as well as 850 µL MEM (without serum or antibiotic) and 100 µL 10% D-mannose (1% final concentration). The 1X PBS was removed from the plate wells containing the cell monolayer, 1 mL bacterial suspension added to each well and the plate incubated at 37°C in a 5% CO_2 atmosphere for 3 h. After incubation, the wells were washed in triplicate with 1 mL 1X PBS. Concentrated methanol (500 µL) was added for 1 min to fix, 1 mL Giemsa dye added for 20 min and the wells washed in triplicate with 1 mL deionized water to eliminate excess dye. Cells were dehydrated by picking up each bead and running it for 30 s each through a series of acetone, acetone 50 / xylene 50 and xylol 100. The beads were immediately mounted in Canada balsam on a slide, a drop of resin placed over each bead and allowed to dry for 24 h. Once dry the slides were viewed with a microscope and photographed with an integrated HBO 50 camera. Adherence was indicated to be positive if more than ten T-LAB adhered per HEp-2 epithelial cell and degree of adherence was noted. This analysis was done in triplicate.

Results and Discussion

Low pH tolerance

Tolerance to low pH levels and bile salts is vital for bacteria to survive and grow in the GI tract, making these the main requirements for bacteria to be considered probiotic (Havenaar *et al*., 1992). Among the ten studied T-LAB, all exhibited good growth after 4 h incubation at pH 4 and 5 since optimum pH for LAB is between 4.5 and 6.5 (Prescott *et al*., 1999) (Table 1). At pH values of 1, 2 and 3, *Enterococcus faecium* (10a) exhibited only slight growth after 1 h and then died. *Enterococcus faecium* (18) had slight growth at pH 0.5, 1, 2 and 3 after 1, 2 and 4 h incubation. In contrast, *Lactobacillus plantarum* (17) exhibited good growth values at different pH values for 1 h but did not grow further at 2 and 4 h.

Bacteria are generally sensitive to the stomach's low pH values (Conway *et al*., 1987), however, some LAB can survive and grow at relatively low pH because they have a system that simultaneously transports lactic acid and protons to the cell's exterior.

Table 1. Thermorolerant lactic acid bacteria tolerance to low pH

pH	Time																	
	1 h						2 h						3 h					
Strain	0.5	1	2	3	4	5	0.5	1	2	3	4	5	0.5	1	2	3	4	5
A. viridans (21)	-	-	-	-	+++	+++	-	-	-	++	+++	+++	-	-	-	-	+++	+++
E. faecium (10a)	-	+	+	+	+++	+++	-	-	-	-	+++	+++	-	-	-	-	+++	+++
E. faecium (18)	+	+	+	+	+++	+++	+	+	+	+	+++	+++	-	-	+	+	+++	+++
L. plantarum (15c)	-	-	-	+++	+++	+++	-	-	-	-	+++	+++	-	-	-	+	+++	+++
L. plantarum (17)	++	+++	+++	+++	+++	+++	-	-	-	-	+++	+++	-	-	-	-	+++	+++
P. pentosaceus (11)	-	-	-	+	+++	+++	-	-	-	+	+++	+++	-	-	-	++	+++	+++
P. pentosaceus (12)	-	-	-	-	+++	+++	-	-	-	-	+++	+++	-	-	-	-	+++	+++
P. pentosaceus (15a)	-	-	-	-	+++	+++	-	-	-	-	+++	+++	-	-	-	-	+++	+++
P. pentosaceus (15L)	-	-	-	-	+++	+++	-	-	-	-	+++	+++	-	-	-	-	+++	+++
P. pentosaceus (22)	-	-	-	-	+++	+++	-	-	-	-	+++	+++	-	-	-	-	+++	+++

+++ Abundant growth, ++ Moderated growth, + Poor growth, - No growth

Table 2 Thermorolerant lactic acid bacteria tolerance to different bile concentrations

Strain	Bile concentrations (%)				
	0.1	0.3	0.5	1.0	2.0
A. viridans (21)	++	++	++	+	+
E. faecium (10a)	++	++	++	++	++
E. faecium (18)	++	++	++	+	+
L. plantarum (15c)	++	++	++	+	+
L. plantarum (17)	++	++	++	+	+
P. pentosaceus (11)	++	++	++	+	+
P. pentosaceus (12)	++	++	++	++	++
P. pentosaceus (15a)	++	++	++	++	++
P. pentosaceus (15L)	++	++	++	++	++
P. pentosaceus (22)	++	++	++	++	++

++ Abundant growth, + Poor growth

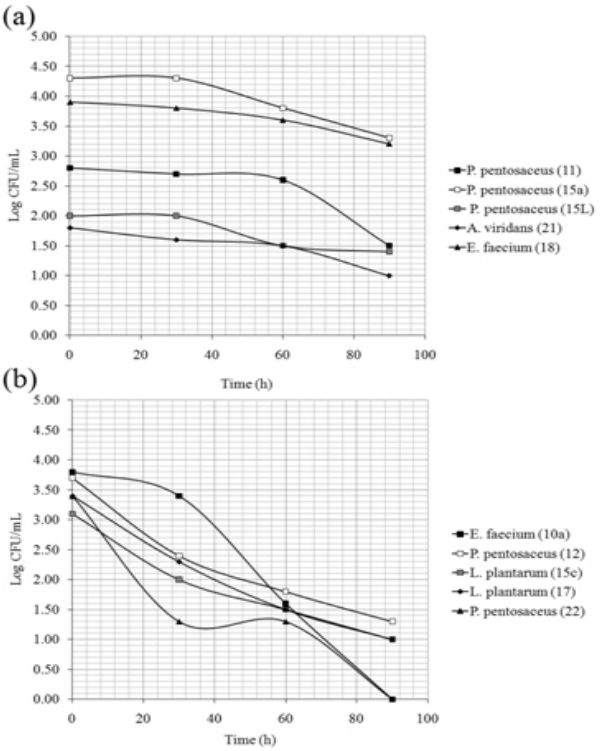

Figure 1. Survival of the employed thermotolerant lactic acid bacteria in simulated gastric juice at pH 2.0; a) *Pedicoccus pentosaceus* (UAM11), *Pedicoccus pentosaceus* (UAM15 a), *Pediococcus pentosaceus* (UAM15L), *Aerococcus viridans* (UAM21), *Enterococcus faecium* (UAM18); b) *Enterococcus faecium* (UAM10a), *Pediococcus pentosaceus* (UAM12), *Pediococcus pentosaceus* (UAM15c), *Lactobacillus plantarum* (UAM17), *Pediococcus pentosaceus* (UAM22).

In a study similar to the present study, *Lactobacillus* GG survived pH 3 for 4 h (Goldin *et al*., 1992), while in another study *L. casei* 212.3 and *Lactobacillus* GG exhibited good growth at pH 2.3 for 3 h (Charteris *et a*l., 1998). In the latter study, the authors classified the strains as intrinsically tolerant, meaning that when ingested under fasting conditions they were intrinsically resistant to transit through the human GI tract, meaning a large amount of microorganisms could therefore arrive at the upper intestine. Under the same conditions, *L. casei* F19 decreased from 10^9 to 10^3 cfu / mL, causing the authors to classify it as intrinsically sensitive, that is, intolerant of GI tract conditions (Charteris *et al*., 1998). In another study, 99 *Lactobacillus casei* strains were shown to resist pH 3 for 3 h, but the strains *Lactobacillus casei* NCDC 17 and *Lactobacillus casei* C1 were able to resist pH 2 for 3 h (Mishra and Prasad, 2005).

Overall, the ten T-LAB strains studied here were comparatively intolerant of low pH conditions. Nonetheless, systems such as microencapsulation con improve probiotic survival when exposed to acid conditions, bile salts and thermal treatments (Ding & Shah, 2007).

Survival in simulated gastric juice

In the simulated gastric juice survival trial, three *Pediococcus pentosaceus* strains (11, 15L and 15a), *Aerococcus viridans* (21) and *Enterococcus faecium* (18) remained viable during the 90 min incubation (Figure 1a). *Pediococcus pentosaceus* (12) and

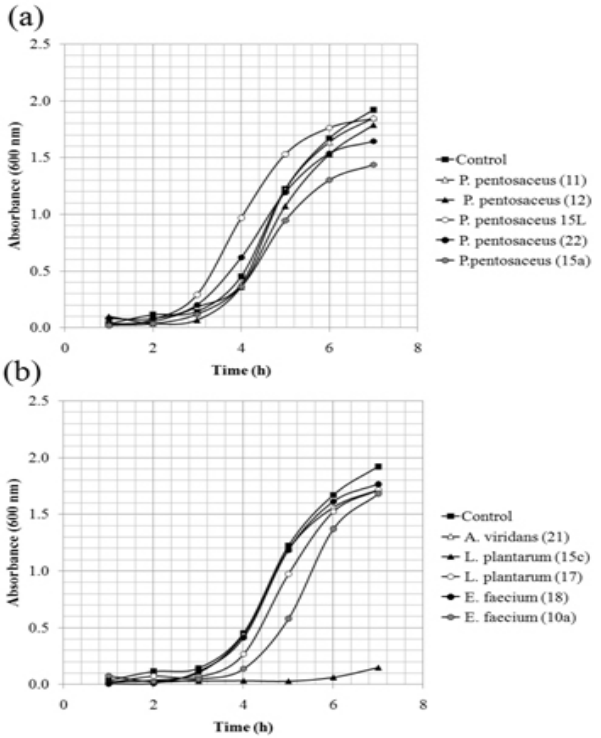

Figure 2 Taurocholic acid tolerance of thermotolerant lactic acid bacteria; a) *Pedicoccus pentosaceus* (UAM11), *Pediococcus pentosaceus* (UAM12), *Pediococcus pentosaceus* (UAM15L), *Pediococcus pentosaceus* (UAM22), *Pedicoccus pentosaceus* (UAM15a); b) *Aerococcus viridans* (UAM21), *Pediococcus pentosaceus* (UAM15c), *Lactobacillus plantarum* (UAM17), *Enterococcus faecium* (UAM18). b) *Enterococcus faecium* (UAM10a).

two *Lactobacillus plantarum* strains (15c and 17) exhibited a reduction of one logarithmic cycle during the first 30 min. At 60 min incubation, *Pediococcus pentosaceus* (22) experienced a reduction of two logarithmic cycles and *Enterococcus faecium* (10a) one of 1.5 logarithmic cycles (Figure 1b).

Bacteria intended to exercise beneficial effects in the intestines must survive transit through the stomach's acid environment. The present results demonstrate that 50% of the studied T-LAB strains could survive these conditions for 90 min, long enough to reach their action site in the intestine (Fernandez *et al*., 2003).

Bile tolerance

Some *Lactobacillus* strains are capable of surviving high bile concentrations and can therefore adapt to GI tract conditions (Gilliland *et al*., 1984). Of the ten studied T-LAB strains, five grew effectively at all five tested bile concentrations (0.1, 0.3, 0.5, 1.0 and 2.0%) (Table 2): *Pediococcus pentosaceus* (12); *Pediococcus pentosaceus* (15L); *Pediococcus pentosaceus* (22); *Enterococcus faecium* (10a); and *Pediococcus pentosaceus* (15a). The remaining five strains exhibited abundant growth at the 0.5% concentration, but lower growth rates at the 1 and 2% concentrations: *Pediococcus pentosaceus* (11), *Aerococcus viridans* (21), *Lactobacillus plantarum* (15c), *Lactobacillus plantarum* (17) and *Enterococcus faecium* (18). These results coincide with an evaluation of *Lactobacillus* strains isolated from the chicken GI tract in which strains resistant to acid pH and bile salts were selected (Rondón *et al*., 2008): twenty strains were identified with probiotic activity, six of which had the best tolerance to low pH and bile salts. This is similar to an earlier study in which twelve *Lactobacillus* strains isolated from the chicken GI tract were reported to survive low pH and a 0.3% bile concentration (Jin *et al*., 1998). This bile salt concentration is critical for detecting resistant bacterial strains (Gilliland *et al*., 1984), meaning all ten T-LAB tested in the present study are candidates for use as probiotics since all grew at a bile concentration greater than 0.3%.

Taurocholic acid tolerance

Bile is a steroid produced by the liver and secreted through the bile duct in the form of bile salts. These salts can occur as conjugates between cholic acid and the amino acids glycine or taurine (forming glycholic or taurocholic acid), or as deconjugates such as sodium desoxycholate (Madigan *et al*., 2006). The five *Pediococcus* sp. tested in the present study grew well in 7 mM taurocholic acid compared to the control (Figure 2a), as did the strains *Aerococcus viridans* (21), *Lactobacillus plantarum* (17), *Enterococcus faecium* (18) and *Enterococcus faecium* (10a) (Figure 2b). The exception was *Lactobacillus plantarum* (15c), which did not survive this hostile medium. This coincides with previous reports of some *Lactobacillus* strains not surviving conjugated bile salts, probably due to an absence of the enzymes needed to hydrolyze them, but achieving growth in the presence of deconjugated bile salts (Suskovic *et al*., 1997). Similar results were reported in an *in vitro* study of *Lactobacillus reuteri* strains with hydrolytic activity for bile salts in which the bile salts had no negative effect on strain growth probably because the deconjugated salts were adsorbed onto the lactobacillus cell surface, thus diminishing their bioavailability (De Boever *et al*., 2000). In another study, most probiotic bacteria were reported to grow in MRS medium supplemented with more than 0.5% conjugated bile salts (Noriega *et al*., 2006).

Coaggregation

Aggregation between cells of the same strain (autoaggregation) or different species (coaggregation) is an example of bacterial interaction (Kmet *et al*., 1995). Coaggregation may improve pathogen

Table 3 Thermorolerant lactic acid bacteria coaggregation capacity with other strains

Strain	% Coaggregation with *E. coli* O139:H26	% Coaggregation with *Salmonella parera* IV O11:Z4Z23
A. viridans (21)	5.34 [d]	5.53 [e]
E. faecium (10a)	5.06 [d]	16.11 [b]
E. faecium (18)	23.56 [b]	32.71 [a]
L. plantarum (15c)	6.31 [d]	8.81 [d]
L. plantarum (17)	2.49 [e]	0.48 [g]
P. pentosaceus (11)	5.72 [d]	4.87 [f]
P. pentosaceus (12)	26.33 [a]	2.13 [g]
P. pentosaceus (15a)	5.97 [d]	6.71 [c]
P. pentosaceus (15L)	12.55 [c]	17.29 [c]
P. pentosaceus (22)	11.04 [c]	1.80 [g]

[a, b, c] Means with same letter in same column are not significantly different (P<0.05) for lactic acid bacteria strain

Table 4. Thermorolerant lactic acid bacteria autoaggregation capacity (%) after 2 and 24 h incubation time

Strain	Incubation time (h)	
	2	24
A. viridans (21)	20.24 [B, a]	29.77 [A, a]
E. faecium (10a)	7.39 [B, c]	25.10 [A, c]
E. faecium (18)	2.90 [B, d]	9.70 [A, d]
L. plantarum (15c)	1.24 [B, d]	7.40 [A, d]
L. plantarum (17)	15.30 [B, a]	33.53 [A, a]
P. pentosaceus (11)	18.96 [B, a]	28.78 [A, a]
P. pentosaceus (12)	3.59 [B, d]	6.59 [A, d]
P. pentosaceus (15a)	23.25 [B, d]	43.83 [A, d]
P. pentosaceus (15L)	4.26 [B, c]	5.11 [A, c]
P. pentosaceus (22)	17.74 [B, b]	23.80 [A, b]

[A, B] Means with same letter in same row are not significantly different (P<0.05) for incubation time
[a, b, c] Means with same letter in same column are not significantly different (P<0.05) for thermotolerant lactic acid bacteria strain

elimination (via dragging) and/or destruction in the GI tract in that it may optimize the effect of antibacterial substances emitted by probiotic microorganisms against the pathogenic bacteria with which they coaggregate (Charteris *et al*., 1998). All the T-LAB strains tested in the present study coaggregated with the *E. coli* O139:H26 and *Salmonella parera* IV O11:Z4Z23 as indicator strains (Table 3). *E. faecium* (18) obtained the significantly (P<0.05) higher coaggregation percent values for both *E. coli* and *Salmonella parera* strains. Although *P. pentosaceus* (12) obtained the significantly (P<0.05) higher values with *E. coli*, its coaggregation with *S. parera* was significantly (P<0.05) lower than the rest of the T-LAB. Both P. pentosaceus (15L) and (15a) obtained significantly (P<0.05) acceptable coaggregation percent as well.

Coaggregation may be a vital factor in establishing and maintaining microflora with the aim of preventing gastrointestinal and urogenital bacterial infections. The presence of *Lactobacillus* in the urogenital epithelium and their ability to interact with uropathogens may be an important host defense mechanism against infections (Reid *et al*., 1988). In the present study, 20% of the T-LAB stains interacted with *E. coli* and 30% interacted with *Salmonella*, indicating they could function to eliminate pathogenic bacteria from the GI tract.

Autoaggregation

This phenomenon is very important in a number of ecological niches, particularly in the human gut, the most common intended action site for probiotics (Jankovic *et al*., 2003). As expected, all the strains showed a significantly (P<0.05) higher aggregation capacity after 24 h of incubation time (Table 4). The thermotolerant lactic acid strains with significantly (P<0.05) higher autoaggregation percents were *A. viridans* (21), *L. plantarum* (17) and *P. pentosaceus* (11). The significantly (P<0.05) lower autoaggregation was for *E. faecium* (18), *L. plantarum* (12), *L. plantarum* (15c) and *P. pentosaceus* (15a). Their high autoaggregation capacities suggest these strains could be very useful in forming biofilms and/or in GI tract colonization, which are ways of forming a barrier against colonization by pathogenic microorganisms (Schachtsiek *et al*., 2004; Schellenberg *et al*., 2006). This capacity may be the result of their modification of the microenvironment surrounding pathogens and/or the production of inhibitory substances by the T-LAB (Pérez *et al*., 1998; Del Re *et al*., 2000).

In a study of cell surface adherence and aggregative capacity in *Lactobacillus plantarum* and *Enterococcus faecium* strains. Collado *et al*. (2007) reported that autoaggregative capacity is correlated to adherence, which is a prerequisite for GI tract colonization and infection by pathogens. Coaggregation, in contrast, is linked to the ability to interact closely with pathogens. These authors concluded that autoaggregative capacity, together with cell surface hydrophobicity and the ability to coaggregate with pathogenic strains can be used for preliminary selection and identification of probiotic bacteria with potential applications in human and animal systems.

HEp-2 cell adherence

A principal criterion for probiotic strain selection is the capacity to adhere to the intestinal surface since adherence to the intestinal mucus is required for colonization and antagonistic activity against enteropathogens (Granato *et al*., 1999). Upon arriving in the intestine, a probiotic strain must fix itself to the tips of the microvilli and then adhere itself to the mucus to avoid being swept off by peristalsis (Fernández *et al*., 2003). The intestinal mucus is a classic model for testing in vitro adherence since different receptors can be located in the small and large intestine mucus using the specific adherence

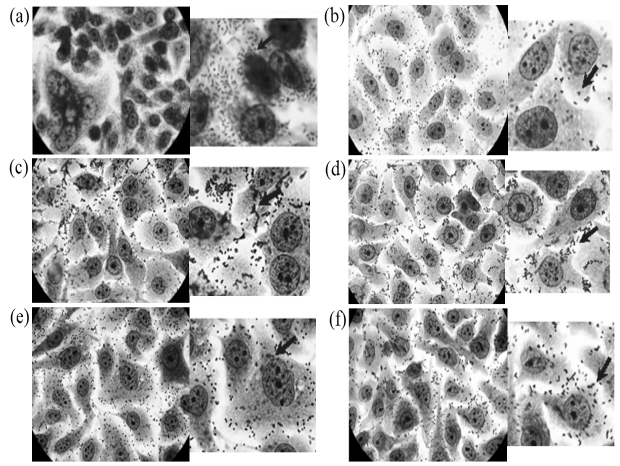

Figure 3. Adherence to HEp-2 epithelial cells at 100 x of: a) Aggregative control of *E. coli* 87125. b) Diffuse adhesion of *Pedicoccus pentosaceus* (UAM11). c) Similar adhesion to the find for *Pedicoccus pentosaceus* (UAM12). d) Diffuse adhesion of *Pediococcus pentosaceus* (UAM15L). e) Diffuse adhesion of *Aerococcus viridans* (UAM21). f) Diffuse adhesion of *Pediococcus pentosaceus* (UAM22).

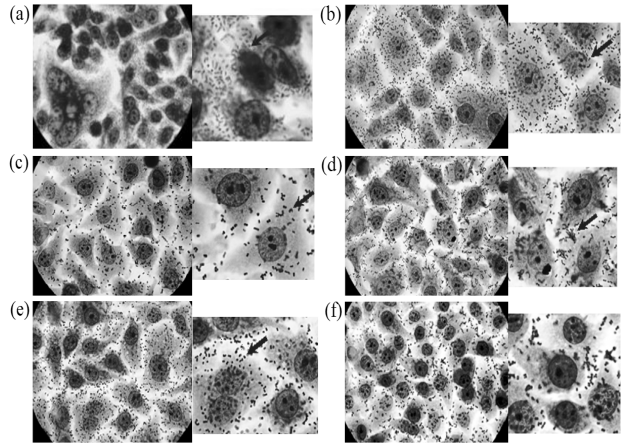

Figure 4. Adherence to HEp-2 epithelial cells at 100 x of: a) Aggregative control of *E. coli* 87125. b) Diffuse adhesion of *Enterococcus faecium* (UAM10a). c) Diffuse adhesion of *Pediococcus pentosaceus* (UAM15a). d) Diffuse adhesion of *Lactobacillus plantarum* (UAM15c). e) Diffuse adhesion of *Lactobacillus plantarum* (UAM17). f) Diffuse adhesion of *Enterococcus faecium* (UAM18).

properties of a number of beneficial and pathogenic bacteria (Li *et al*., 2008).

All ten of the studied T-LAB strains met the minimum adherence criterion of ten bacteria adhered per HEp-2 epithelial cell (derived from human pharynx cancer cells) (Cravioto *et al*., 1979; Ehrmann *et al*., 2002). In addition to this overall positive adherence, over 40% of the cells exhibited levels indicating strong adherence (Figs, 3a-f, Figs. 4a-f). The one exception was the comparatively lower adherence of *Pediococcus pentosaceus* (22) (Figure 3-f), although it still met the criterion for positive adherence (Ehrmann *et al*., 2002).

In contrast to previous adherence studies using *Lactobacillus strains*, which employed Caco-2 and HT-29 intestinal cell lines, the HEp-2 cell line was used in the present study. The results are still comparable to those produced using the intestinal cell lines since the HEp-2 cell line is a model system used to test the adherence of enterotoxic *E. coli* strains which cause diarrheic disorders in humans and animals. Its surface has pili which adhere to intestinal epithelial cells and colonize the small intestine (Smith & Longgood, 1971). This model is therefore valid for evaluating the adherence and colonization of T-LAB in the GI tract.

The adherence observed here for the ten studied T-LAB strains is consistent with previous reports of *Lactobacillus* adhering to human intestinal cells via a mechanism involving different combinations of carbohydrates and proteins in the bacteria cell's surface (Chauviére *et al*., 1992). The present results also contained patterns similar to those reported in other studies in which four adherence patterns are described: aggregative adherence; diffuse adherence; localized adherence; and localized adherence-like (Nataro *et al*., 1987; Vidal *et al*., 2007). Eight of the studied T-LAB strains exhibited a diffuse adherence pattern: *Pedicoccus pentosaceus* (11); *Pediococcus pentosaceus* (15 L); *Aerococcus viridans* (21); *Enterococcus faecium* (10a); *Pediococcus pentosaceus* (15a); *Lactobacillus plantarum* (15c); *Lactobacillus plantarum* (17); and *Enterococcus faecium* (18). *Pediococcus pentosaceus* (12) exhibited a localized adherence-like pattern.

A number of studies have been done on adherence in potential probiotic LAB, using both the HEp-2 and Caco-2 cell lines. Ehrmann *et al*. (2002) evaluated 112 LAB strains from duck using in vitro methods to determine autoaggregation, coaggregation, surface hydrophobicity and adherence to HEp-2 cells, among other characteristics. They observed significant autoaggregation and coaggregation, as well as strong adherence to HEp-2 cells, and concluded that most of the tested *Lactobacilllus* strains were potential probiotics. Using the Caco-2 cell line as an in vitro model, Tuomola and Salminen (1998) compared adherence among twelve *Lactobacilllus* strains, observing the highest adherence in *L. casei* (14.4%) and the lowest in *L. casei* rhamnosus (2.6%). They concluded that these potential probiotic *Lactobacilllus* strains had variable ability to adhere to intestinal cells, possibly attributed to strain source and/or surface properties. In another study of adherence capacity in eleven *Lactobacilllus* strains, Pennacchia *et al*. (2006) used an *in vitro* Caco-2 model to find that all eleven strains had good adherence capacity to the Caco-2 cell monolayer with eight strains from the group *Lactobacillus plantarum* exhibiting the highest number of adhered cells. Delgado *et al*.

(2008) analyzed the probiotic characteristics of 187 *Bifidobacterium* strains isolated from healthy adult mucus and feces, including an *in vitro* adherence assay using the Caco-2 cell line. All the tested strains adhered to the epithelial cells, and they proposed the strains *Bifidobacterium catenulatum*, *Bifidobacterium longum* and *Bifidobacterium pseudocatenulatum* as new probiotic candidates. Based on the present results and compared to the above studies, the ten studied T-LAB strains had good adherence capacity to HEp-2 cells and therefore could adhere to intestinal cells.

Conclusions

Most of the studied T-LAB strains presented an adequate resistant to low pH and bile salt concentrations. These characteristics are important requisites for a probiotic strain, and also exhibited high adherence capacity to HEp-2 epithelial cells. The inherent thermotolerant capacity (probably resulting from their isolation from cooked emulsified meat products) plus the demonstrated probiotic properties made these strains a viable bioprotective culture that can be inoculated in cooked meat products before thermal processing, in order to ensure their prevalence as dominant probiotic flora during and before shelf-life.

References

Arihara, K., Ota, H., Itoh, M., Kondo, Y., Sameshima, T., Yahanaka, H., Akimoto, M., Kanai, S. and Miki, T. 1998. *Lactobacillus acidophilus* group lactic acid bacteria applied to meat fermentation. Journal of Food Science 63: 544-547.

Beumer, R.R., de Vries, J. and Rombouts, F.M. 1992. *Campylobacter jejuni* non-culturable coccoid cells. International Journal of Food Microbiology 15: 153-163.

Charteris, W.P., Kelly, P.M., Morelli, L. and Collins, J.K. 1998. Development and application of an *in vitro* methodology to determine the transit tolerance of potentially probiotic *Lactobacillus* and *Bifidobacterium* species in the upper human gastrointestinal tract. Journal of Applied Microbiology 84: 759-768.

Chauviére, G., Coconnier, M.H., Kerneis, S., Fourniat, S. and Servin, A.L. 1992. Adhesion of human *Lactobacillus acidophilus* strain LB to human enterocyte-like Caco-2 cells. Journal of General Microbiology 138: 1689-1696.

Collado, M.C., Surono, I., Meriluoto, J and Salminen, S. 2007. Indigenous dadih lactic acid bacteria: cells-surface properties and interactions with pathogens. Journal of Food Science 72: 89-93.

Conway, P.L., Gorbach, S.L. and Goldin, B.R. 1987. Survival of lactic acid bacteria in the human stomach and adhesion to intestinal cells. Journal of Dairy Science 70: 1-12.

Cravioto, A., Gross, R.J., Scotland, S.M. and Rowe, B. 1979. An adhesive factor found in strains of *Escherichia coli* belonging to the traditional infantile enteropathogenic serotypes. Current Microbiology 3: 95-99.

De Boever, P., Wouters, R., Verschaeve, L., Berckmans, P., Schoeters, G., & Verstraete, W. (2000). Protective effect of the bile salt hydrolase–active *Lactobacillus reuteri* against bile salt cytotoxity. Applied and Microbiology Biotechnology, 53, 709-714.

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

Del Re, B., Sgorbati, B. Miglioli, M., & Palenzona, D. (2000). Adhesión, autoaggregation and hydrophobicity of 13 strains of *Bifidobacterium longum*. Letters in Applied Microbiology, 31, 438-442.

Dunne, C., Murphy, L., Flynn, S., O'Mahony, L., O'Halloran, S., Feeney, M., Morrissey, D., Thornton, G., Fitzgerald, G., Daly, C., Kiely, B., Quigley, E.M.M., O'Sullivan, G.C., Shanahan, F., & Kevin, J. (1999). Probiotics: from myth to reality. Demonstration of functionality in animal models of disease and in human clinical trials. Antonie Van Leeuwenhoek, 76, 279-292.

Ehrmann, M.A., Kurzak, P., Bauer, J., & Vogel, R.F. (2002). Characterization of *lactobacilli* towards their use as probiotic adjuncts in poultry. Journal of Applied Microbiology, 92, 966-975.

Fernandez, M., F. Boris, S., & Barbés, C. (2003). Probiotic properties of human *lactobacilli* strains to be used in the gastrointestinal tract. Journal of Applied Microbiology, 94, 449-455.

Fuller, R. (1989). A review. Probiotics in man and animals. Journal of Applied Bacteriology, 66, 365-378.

Gilliland, S.E., Staley, T.E., & Bush, L.J. (1984). Importance in bile tolerans of *Lactobacillus acidophilus* used as a diatery adjunt. Journal of Dairy Science, 67, 3045-3051.

Goldin, B.R., Gorbach, S.L., Saxelin, M., Barakat, S. Gaulthieri, L., & Salminen, S., (1992). Survival of *Lactobacillus* species (strain GG) in human gastrointestinal tract. Digestive Diseases and Sciences, 37, 121-128.

Granato, F., Perotti, I., Masserey, M., Rpouvet, M., Golliard, & Servin, A. (1999). Cell surface-associated lipoteichoic acid acts as an adhesion factor for attachment of *Lactobacillus johnsonii* La1 to human enterocyte-like Caco-2 cells. Applied and Environmental Microbiology, 65, 1071-1077.

Handley, P.S., Harty, D.W., Wyatt, J.E., Brown, C.R., Doran, J.P., & Gibbs, A.C. (1987). A comparison of the adhesion, coaggregation and cell-surface hydrophobicity properties of fibrillar and fimbriate strains of *Streptococcus salivarius*. Journal of General Microbiology, 133, 3207-3217.

Havenaar, R., Ten Brink, B., & Huis in y Veld, J. (1992). Selection of strains for probiotic use. In R. Fuller (Ed.), Probiotics: The Scientific Basis (pp. 209-224).

Chapman & Hall, London.

Huggett, A.C., & Verschuren, P.M. (1996). The safety assurance of functional foods. Nutrition Reviews, 54, S132–S140.

Jankovic, I., Ventura, M., Meylan, V., Rouvet, M., Elli, M., & Zink, R. (2003). Contribution of aggregation-promoting factor to maintenance of cell shape in *Lactobacillus gasseri* 4B2. Journal of Bacteriology, 185, 3288-3296.

Jin, L., Ho, Y., Abdullah, N., & Jalaludin, S. (1998). Acid and bile tolerance of *Lactobacillus* isolated from chicken intestine. Letters in Applied Microbiology, 27, 183-185.

Kmet, V., Callegari, M.L., Bottazzi, V., & Morelli, L. (1995). Aggregation-promoting factor in pig intestinal Lactobacillus strains. Letters in Applied Microbiology, 21, 351-353.

Li, X.J., Yue, L.Y., Guan, X.F., & Qiao, S.Y. (2008). The adhesion of putative probiotic *lactobacillus* to cultured epithelial cells and porcine intestinal. Journal of Applied Microbiology, 104, 1082-1091.

Madigan, M., Martinko, J., & Parker, J. (2006). Control del crecimiento microbiano. In Brock, Biología de los microorganismos, 10a ed. (pp. 725-727). Prentice Hall Iberia, Madrid.

Mishra, V., & Prasad, M.N. (2005). Application of in vitro for selection of *Lactobacillus casei* strains as potencial probiotics. International Journal of Food Microbioology, 103, 109-115.

Nataro, J.P., Koper, B.J., Robins, B.R., Prado, V., Vial, P., & Levine, M.L. (1987). Patterns of adherente of diarrheagenic *Escherichia coli* to HE-p-2 cells. Pediatric Infectious Diseases Journal, 6, 829-831.

Noriega, L., Cuevas, I., Margolles, A., & de los Reyes, C. (2006). Deconjugation and bile salts hydrolase activity by Bifidibacterium strain with acquired resistance to bile. International Dairy Journal, 16, 850-855.

Pedersen, C., Jonsson, H., Lindberg, E., & Roos, S. (2004). Microbiological characterization of wet wheat distillers grain, with focus on isolation of *Lactobacilli* with potential as probiotics. Applied and Environmental Microbiology, 70, 1522-1527.

Pennacchia, C., Ercolini, D., Blaiotta, G., Pepe, O., Mauriello, G., & Villani, F. (2004). Selection of *Lactobacillus* strains from fermented sausage for their potential use as probiotics. Meat Science, 67, 309-317.

Pennacchia, C., Vaughan, E.E.. & Villani, F. (2006). Potential probiotic *Lactobacillus* strains from fermented sausage: Further investigations on their probiotic properties. Meat Science, 73, 90-101.

Pérez, P.F., Minnaard, Y., Disalvo, E.A., & De Antoni, G.L. (1998). Surface properties of *bifidobacterial* strains of human origin. Applied and Environmental Microbiology, 64, 21-26.

Pérez-Chabela, M.L., Díaz-Vela, J., Reyes-Menéndez, C.V., & Totosaus, A. (2013). Improvement of moisture stability and textural properties of fat and salt reduced cooked sausages by inoculation of thermotolerant lactic acid bacteria. International Journal of Food Properties, In press.

Pérez-Chabela, M.L., Lara-Labastida, R., Rodríguez-Huezo, M.E., & Totosaus, A. (2012). Effect of spray drying encapsulation of thermotolerant lactic acid bacteria on meat batters properties. Food Bioprocess and Technology, DOI: 10.1007/s11947-012-0865-y.

Pérez-Chabela, M.L., Totosaus, A., & Guerrero, I. (2008). Evaluation of thermotolerant capacity of lactic acid bacteria isolated from commercial sausages and the effects of their inoculation on the quality of cooked sausages. Ciência e Tecnologia de Alimentos, Campiñas, 28, 132-138.

Prescott, L.M., Harley, J., P. & Klein, D., A. (1999). Crecimiento microbiano. In L.M. Prescott, J.P. Harley, & D.A. Klein (Eds.), Microbiología, 4a edición (pp. 115-136). Editorial McGraw Hill Interamericana, Madrid.

Ramirez-Chavarin, N.L., Wacher-Rodarte, C., & Perez-Chabela, M.L. (2010). Characterization and identification of thermotolerant lactic acid bacteria isolated from cooked sausages as bioprotective cultures. Journal of Muscle Foods, 21, 585-596.

Reid, G.J., McCroarty, A., Amgotti, R., & Cook, R.L. (1988). *Lactobacillus* inhibitor production against E. coli and coaggregation ability with uropathogens. Canadian Journal of Microbiology, 34, 344-351.

Reniero, R., Cocconcelli, P., Bottazzi, V., & Morelli, L. (1992). High frequency of conjugation in *Lactobacillus* mediated by an aggregation-promoting factor. Journal of General Microbiology, 138, 763:768.

Rondón, A.J., Samaniego, L.M., Bocourt, R., Rodríguez, S., Milián, G., Ranilla, M.J., Laurencio, M., & Pérez, M. (2008). Aislamiento, identificación y caracterización parcial de las propiedades probióticas de cepas de *Lactobacillus* sp. procedentes del tracto gastrointestinal de pollo de ceba. CyTA Journal of Food, 61, 56-63.

Schachtsiek, M., Hammes, W.P., & Hertel, C. (2004). Characterization of *Lactobacillus coryniformis* DSM 20001T surface protein Cpf mediating coaggregation with and aggregation among pathogens. Applied and Environmental Microbiology, 70, 7078-85.

Schellenberg, J., Smoragiewicz, W., & Karska-Wysocki, B. (2006). A rapid method combining immunofluorescence and cytometry for improved understanding of competitive interactions between lactic acid bacteria (LAB) and methicillin-resistant S. aureus (MRSA) in mixed culture. Journal of Microbiological Methods, 65, 1-9.

Smith, H.W., & Linggood, M.A. (1971). Observations on the pathogenic properties of the K88, Hly and Ent plasmids of *Escherichia coli* with particular reference to porcine diarrhea. Journal of Medical Microbiology, 4, 467-485.

Suskovic, J., Brkic, B., Matisic, S., & Maric, V. (1997). *Lactobacillus acidophilus* M92 as potential probiotic strain. Milchwissenschaft, 52, 430-435.

Tomasik, P.J., & Tomasik, P. (2003). Probiotics and prebiotics. Cereal Chemistry, 80, 113-117.

Tuomola, E.M., & Salminen, S.J. (1998). Adhesion of

some probiotic and dairy *Lactobacillus* strains to Caco-2 cell cultures. International Journal of Food Microbiology, 41, 45-51.

Vidal, E.J., Canizález, R.A., Gutiérrez, J.J., & Navarro, G.F. (2007). Patogénesis molecular, epidemiología y diagnóstico de *Escherichia coli* enteropatógena. Salud Pública de México, 49, 376-386.

Victoria-León, T., Totosaus, A., Guerrero, I., Pérez-Chabela, M.L. (2006). Efecto de bacterias ácido lácticas termoresistentes en salchichas cocidas. CYTA Journal of Food, 5, 135-141.

Yuki, N., Shimazaki, T., Kushiro, A., Watanabe, K., Uchida, K., Yuyama, T., & Morotomi, M. (2000). Colonization of the stratified squamous epithelium of the nonsecreting area of horse stomach by *Lactobacilli*. Applied and Environmental Microbiology, 66, 5030-5034.

Ziemer, C.J., & Gibson, G.R. (1998). An overview of probiotics, prebiotics and synbiotics in the functional food concept: perspectives and future strategies. International Dairy Journal, 8, 473-479.