Probiotic properties and stress response of thermotolerant lactic acid bacteria isolated from cooked meat products

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Abstract

The aim of this study was to evaluate the probiotic properties of six thermotolerant lactic acid bacteria isolated from cooked meat products. The bacteria were typed, by determination of the DNA sequence of their 16S rRNA coding genes, as one *Enterococcus faecium* (UAM1 strain) and five *Pediococcus pentosaceus* (UAM2-UAM6 strains). Under gastric stress conditions the viability of the *Pediococci* decreased more than five-fold, whereas *E. faecium* showed a high resistance (61% survival). Exposure to small intestine stress did not drastically affect the survival of any of the strains (less than one-fold decrease), which were able to grow in the presence of 0.3% bile. A hydrophilic surface profile was observed, with higher affinity for chloroform than for xylene. Strains showed high levels of auto-aggregation as well as co-aggregation with Gram-positive and Gram-negative bacterial pathogens. The adherence of *E faecium* UAM1 to human Caco-2 cells (around 20%) was significantly higher than that obtained with the *P. pentosaceus* strains (2%-5%) and *Lactobacillus acidophilus* LA-5 (6%). The overall results indicate that *E. faecium* UAM1, has probiotic properties that predict its capability to colonize in competition with pathogens in the intestinal tract. This bacterium deserves further investigation for its potential as a component of functional food.

Keywords

Lactic acid bacteria, thermotolerant, probiotic properties, adhesion.

1. Introduction

Probiotics are defined as "live microorganisms which, when administered in adequate amounts, confer a health benefit on the host" (FAO & WHO, 2001). The majority of probiotics are bacteria, with lactic acid bacteria (LAB) being the most representative, and are used for the manufacture of fermented dairy, meat and vegetable-based foods. Probiotic strains include members of the genera *Pediococcus*, *Lactobacillus*, *Bifidobacterium* and *Enterococcus* (Buntin *et al.* 2008).

Enterococcus is a genus used as a probiotic which may improve the microbial balance of the intestine, and is ubiquitous in nature. Pieniz *et al.* (2013) studied the probiotic potential and antioxidant properties of *Enterococcus durans* LAB18s, a strain capable of selenium bioaccumulation, concluding that these strains could be used as dietary selenium supplementation. Also, Rao *et al*. (2013) examined the adhesion of *Enterococcus faecium in vitro* and concluded that this strain had an effective barrier function in the small intestinal mucus layer of pigs. Carasi *et al*. (2014) isolated and identified a strain of *E. durans* from kefir, and their results showed the potential functionality of this bacteria as probiotic. Moreover, they indicated that the presence of *E. durans* in kefir does not represent a threat to consumer health. In addition, Li *et al*. (2014) identified and evaluated the probiotic properties of five *Enteroccus* strains isolated from silage, and one of those (L2) seems to be a promising candidate for future use as a probiotic in humans.

Strains belonging to the genus *Pedioccocus* have been tested and already used as probiotic bacteria. Vidhyasagar and Jeevaratnam (2013) evaluated six strains of *Pediococcus pentosaceus* for probiotic properties *in vitro*. They concluded that the strains exhibited effective growth inhibition of intestinal Gram positive and Gram negative pathogens and could be used in functional foods as a probiotic strains. Furthermore, similar results were found with *P. pentosaceus* strains isolated from fermented vegetables (Savedboworn *et al.* 2014). Also, Dubey *et al*. (2015) reported about *P. pentosaceus* strains with high survival in simulated gastrointestinal fluid, and antioxidative and biohydrogenation properties. In addition, Chen *et al.* (2017) stated that *P. pentosaceus* is a promising probiotic bacteria with potentially superior biological properties, especially improving

Comentario [SE1]: He colocado el párrafo de enterococcus antes del de Pediococcus, para emplamar major al final

Comentario [SE2]: Falta esta referencia

69 growth performance, intestinal microbiota balance, meat quality and microenvironment in chicken,
70 and decreasing ammonia content in the medium.

71 Thus, the state of the art supports the significance of *enterococci* and *pediococci* in the field of
72 probiotics and indicates that new strains belonging to these genera and isolated from food have
73 potential for their usage in generation of functional food.

74 In a previous study we isolated and identified ten LAB strains from Mexican sausages, which were
75 selected for further characterization as potential probiotics (Ramírez-Chavarin *et al.*, 2010). In
76 general, these strains showed a high adherence capacity as well as high tolerance to gastric pH
77 (Ramírez-Chavarin *et al.*, 2013). In the current work we have identified six thermotolerant LAB,
78 one *E. faecium* and five *P. pentosaceus* strains, isolated from meat products, and have evaluated *in*
79 *vitro* their probiotic potential with a future aim of using them as bioactive starters for the
80 development of Mexican cooked meat products.

2. Materials and Methods

2.1 Bacterial strains and culture conditions. The six lactic acidf bacteria (LAB) strains studied in this work were isolated from Vienna sausages. In addition, *Lactobacillus plantarum* 8014 was obtained from the Universidad Nacional Autónoma de México (UNAM) culture collection, Mexico, and *Lactobacillus acidophilus* LA-5 was kindly provided by Chr. Hansen A/S (Hørsholm, Denmark). These latter two strains were used as controls for the probiotic tests. The bacterial pathogens used in this study were *Escherichia coli* DH5α (Invitrogen, USA), *Bacillus cereus* CFQ-B-230, *Listeria innocua* CFQ-B-232, *Pseudomonas aeruginosa* ATCC 27853, S*taphylococcus aureus* ATCC 6538 and *Salmonella typhimurium* ATCC 14028, all obtained from the UNAM culture collection.

For the assays, LAB were grown in Man Rogosa Sharpe (MRS) broth (De Man *et al*., 1960) and incubated at 35ºC, while pathogens were cultured in brain heart infusion broth (BHI) at 37ºC. The stock cultures were stored at -80ºC in medium supplemented with glycerol (20% v/v).

2.2 Isolation and identification of thermotolerant LAB strains. Ten different brands of Vienna type sausages from Mexico City supermarkets were analysed in search of LAB. Thirty-five strains were selected for further studies, after isolation by several cycles of anaerobic growth in MRS solid and liquid media at 37ºC, on the basis of Gram-staining, as well as catalase and oxidase production (Harrigan, 1998). The thermotolerance of these strains was determined by growth in MRS at 37ºC to A_{600nm} of 0.8-1.0 (1×10^8 colony forming unit per mL, cfu/mL), heat shock at 70ºC for 30 min, and recovering in MRS-agar plates at 35ºC for 24-48 h. Six strains were found to have a survival rate higher than 3×10^2 cfu/mL and were considered thermotolerant and selected for subsequent characterization. They were identified as one *Enterococcus faecium* (UAM1 with accession number in GenBank: KY992877) and five *Pediococcus pentosaceus* (UAM2-UAM6 with accession numbers in GenBank: KY992876, KY992875, MF000324, MF000322 and MF000323) by sequencing their 16S rRNA coding genes at Secugen (Madrid, Spain).

2.3 Antibiotic sensitivity and gastrointestinal tolerance. The antibiotic resistance profile against fourteen antibiotics was determined using Clairo Combo Discs for Gram positive bacteria (Accutrack, México).

To test resistance to low pH, LAB were grown in MRS at 37ºC to an A_{600nm} of 0.8-1.0 concentrated ten-fold in phosphate buffer solution (PBS, 10 mM Na_2HPO_4, 1 mM KH_2PO_4, 140 mM NaCl, 3 mM KCl) and evaluated as described by Conway *et al.* (1987).

The ability of the strains to grow in the presence of bile was determined as described by Walker & Gilliland (1993). The bile tolerance was estimated from the differences between growth in presence or absence of bile and calculating the time required for an increase of 0.3 units of absorbance at 620 nm in either condition.

The tolerance of BAL to simulated gastric (pepsin at 3 mg/mL, pH 2.0) and small intestinal (pancreatin at 1 mg/mL, pH 8.0) transits was determined as described by Charteris *et al.*, (1998). Prior to the assay and at the times indicated, the cfu/mL were determined by plating.

2.4 Binding properties. Bacterial adhesion to solvents was adapted from a previously described method (Sánchez & Tromps, 2014). Briefly, exponential growth cultures were sedimented and resuspended to give an ansorbance at 560 nm close to 0.6-0.7, then mixed (v/v) with an organic solvent (xylene or $CHCl_3$) and vortexed for 30 sec. After 1 h incubation at room temperature, the aqueous phase was removed and its absorbance (560 nm) measured. The hydrophobicity of LAB was calculated as: $H = [(A_o - A) / A_o] \times 100$, where A_o and A are the absorbances before and after extraction with organic solvents. Strains were considered strongly hydrophobic when values were > 60%, moderate hydrophobic with values in the range of 40% to 60% and hydrophilic when values were < 40% (Basson *et al*., 2008).

Auto-aggregation abilities of LAB were measured by adapting the method of Collado *et al.* (2008). Bacterial cells were harvested by centrifugation and washed twice with PBS (pH 7.2), then resuspended in the same buffer to an absorbance at 600 nm close to 0.50 ± 0.10 to standardize the

number of bacteria (10^7-10^8 cfu/mL). The bacterial suspensions were incubated at room temperature and monitored at 0 h, and at the times indicated. The percentage of auto-aggregation was expressed as: *A%* = $[(A_o – A_t) / A_o] \times 100$, where A_o represents the absorbance at 0 h and A_t represents the absorbance at the different time intervals.

For co-aggregation assays, bacterial suspensions were prepared as described above. Equal volumes of cells (500 µL) of the different probiotic and pathogen strains were mixed and incubated at room temperature without agitation. The absorabance (600 nm) of the mixtures were monitored at the indicated times and co-aggregation was calculated with the equation of Malik *et al*. (2003): *C%* = $[(A_{pat} + A_{probio}) - (A_{mix})] / [(A_{pat} + A_{probio})] \times 100$, where A_{pat} and A_{probio} represent the absorbance of the independent bacterial suspensions at 0 h and A_{mix} represents the absorbance of the mixed bacterial suspension at the times tested.

2.5 Caco-2 cell culture and adhesion assays. The human enterocyte cell line, obtained from the cell bank at CIB, was seeded in 96-well tissue culture plates (Falcon Microtest^{TM}, USA) at a final concentration of 1.25×10^5 cells/mL and grown as monolayers of differentiated and polarised cells for 21 days as previously described (Nácher-Vázquez *et al*., 2017). Cell concentrations were determined as previously described (Garai-Ibabe *et al*., 2010).

For the adhesion assays, exponential-phase LAB cultures grown in MRS were sedimented by centrifugation (12,000 × *g*, 10 min, 4ºC), resuspended in the appropriate volume of Dulbecco's Modified Eagle medium (DMEM, Invitrogen) to give a final concentration of 1.25×10^6 cfu/ mL. 0.1 mL of bacterial suspension was added per well (ratio 10:1, bacteria: Caco-2 cells) and the plates were incubated for 1 h at 37ºC. The unadhered bacteria were then removed and the cell-associated bacteria processed and quantified by counting, after plating onto MRS plates as previously described (Nácher-Vázquez et al., 2017). All adhesion assays were conducted in triplicate.

2.6 Statistics. Results are expressed as the mean and standard deviation of three determinations. Statistical analysis was performed using the SPSS 24.0 software (IBM SPSS, Trial version, USA).

Data were subjected to one-way analysis of variance (ANOVA) and the Duncan test was used for comparison of the means. $P< 0.05$ was considered statistically significant.

3. RESULTS AND DISCUSSION

3.1 Antibiotic susceptibility. The absence of transferable antibiotic resistant genes in the bacterial genome is recommended and, for some scientific committees, even considered a prerequisite for approval of the use of a bacterium as probiotic in foods and feeds (Jansen *et al.*, 2006). Thus, we tested the antibiotic resistance of the six thermotolerant LAB isolated from Vienna sausages, *Enterococcus faecium* UAM1, and five *Pediococcus pentosaceus* (UAM2-UAM6 strains) isolated and typed in this work (Supplementary Table 1S). Resistance against inhibitory protein synthesis antibiotics, such as chloramphenicol, erythromycin and tetracycline is plasmid encoded and varies among LAB strains. Our results revealed that almost all strains were to some extent susceptible to these types of antibiotics including erythromycin, azithromycin, clarithromycin, tetracycline and chloramphenicol. The strains were also susceptible to β-lactam antibiotics (penicillin G, cephalothin, cefuroxime, ceftizoxime and cephalexin), which inhibit cell wall synthesis. *L. plantarum* 8014 (control strain) and *P. pentosaceus* UAM2 showed an intermediate resistance to cefazolin (cephalosporin class), and the strain 8014 also for chloramphenicol and clarithromycin, both inhibitors of protein synthesis. In addition, among the *P. pentosaceus* strains, UAM4 and UAM5 showed intermediate resistance to amoxicillin. *Enterococcus* spp. strains are known to be resistant to cephalosporins, low levels of amino-glycoside and clindamycin (Teuber, 1999), and the *E. faecium* UAM1 strain as well as the *Pediococci* UAM 2, UAM4 and UAM5 showed an intermediate resistance to cephalexin. Also, our results revealed that UAM6 was sensitive to all antibiotics tested with the exception of co-trimoxazole and cephalexin.

3.2 Resistance to low pH conditions. Before reaching the intestinal tract, probiotic bacteria must first survive transit through the stomach. The average pH of the stomach is 3.0-2.0; during digestion a pH gradient (4.0-1.8) is generated and the food has to travel through the digestive tract for a

period of 2 h to 3 h (Maragkoudakis *et al*., 2006). Thus, acidic pH values (4.0-2.0) were selected to examine the acid tolerance of UAM1-UAM6 strains as well as *L. plantarum* 8014 (Table 1). All strains were able to survive after an exposure to pH 4.0 or 3.0, but only 8014 and UAM1 strains showed around 50% viability after 1 h treatment at pH 2.0, and 40% of the *E. faecium* population was recovered after 3 h exposure to pH 2.0.

Osmanagaoglu *et al*. (2010) reported that *P. pentosaceus* OZF, isolated from human breast milk, is able to survive after 3 h of exposure at pH 3.0 and retained a viability of 6.41 log cfu/mL, when the initial populations ranged from 8.2 to 9.0 log cfu/mL. Also, Lee *et al*. (2014) showed that three strains of *P. pentosaceus* isolated from a salted and fermented Korean sea-food tolerated a 2 h exposure to pH 3.0 with survival rates between 7.5% and 32.6%. In addition, Guo *et al*. (2016) described that four strains of *Enterococcus* were tolerant to pH 3.0 and could survive for 2 h under this stress. One of them, *E. durans* KLDS 6.0930, was the most acid-tolerant and its viability remained stable (10^7 cfu/mL) after 2 h of incubation at pH 2.0.

Thus, the high degree of acid resistance detected for the UAM strains was in the same range as that of other potential probiotic LAB belonging to the same species and isolated from food and milk.

3.3. Bile tolerance. Bile plays a fundamental role in specific and non-specific defence mechanisms of the gut, and the magnitude of its inhibitory effect is determined by the bile salt concentrations (Charteris *et al*., 1998). The physiological concentrations of human bile range from 0.3% to 0.5% (Dunne *et al*., 2001), therefore, the effect of 0.3% bile on the growth of the BAL in liquid medium was evaluated and the results are shown in Fig. 1. The growth of each strain in medium without bile was used as control. The data revealed that all strains were able to grow in both media. In the case of the *L. plantarum* 8014 and the *P. pentosaceus* UAM2, the presence of bile did not significantly affect the growth. By contrast, *E. faecium* UAM1 and the other *P. pentosaceus* strains exhibited various increased latent periods in presence of bile and the time required to increase the absorbance by 0.3 units ranged from 1.5 h to 3 h in MRS, and from 3 h to 4 h in MRS supplemented with bile.

207 This type of evaluation (delay to reach an increase of 0.3 units of absorbance in presence of bile
208 salt) has been used previously to test other *lactobacilli* and *pediococci*. Zeng *et al*. (2010) reported
209 that *Lb. buchneri* P2 isolated from pickled juice needed nearly 6 h to reach the absorbance increase
210 when incubated in MRS supplemented with either 0.2% or 0.3% oxgall, 2 h more than in medium
211 lacking oxgall. Vidhyasagar & Jeevaratnam (2013) reported that some *P. pentosaceus* strains
212 isolated from a traditional fermented food of South India had a delay time between 2 h and 8 h in
213 the presence of bile, time values which are within the average transit time of food in the intestine.
214 For *Enterococcus* strains, Guo *et al*. (2016) reported that KLDS 6.0930 exhibited the highest
215 tolerance to bile, since it required less time (4.7 h) to reach the absorbance increase than other
216 strains of *Enterococci* exposed to oxgall, which needed more than 5.4 h.

217 Thus, our results and current knowledge revealed that the UAM strains could be considered bile-
218 tolerant in the same range as other potential probiotic strains.

219 **3.4 Determination of transit tolerance**

220 **3.4.1 Resistance to gastric stress.** Approximately 2.5 L of gastric juice and 1 L of bile are secreted
221 into the human digestive tract every day. Thus, it is essential for the bacteria to have protection
222 systems to withstand the low pH in the stomach, digestive enzymes and bile in the small intestine
223 (Begley *et al*., 2005). Therefore, the UAM LAB as well as *L. plantarum* 8014 were exposed to
224 gastric stress conditions (pepsin at pH 2.0). All the strains showed a significant decrease of viability
225 upon incubation in the presence of the protease at acidic pH (Fig. 2). *E. faecium* UAM1 exhibited
226 the greatest viability, and after 180 min of treatment a three-fold reduction of viable cells was
227 observed for this strain *versus* approximately a five-fold reduction for the *P. pentosaceus* and the *L.*
228 *plantarum* 8014 strains.

229 Monteagudo-Mera *et al.* (2012) studied the effect of gastric stress on LAB strains isolated from
230 dairy products. Only *L. lactis* ATCC11454, like *E. faecium* UAM1, survived after 180 min gastric
231 stress treatment at pH 2.0, with a 2.5-fold reduction of viability. In addition, under the same

conditions, various *L. lactis*, *L. paracasei*, *L. casei* and *L. rhamnosus* dairy strains lost all viability, a more pronounced sensitivity than that observed for the *P. pentosaceus* UAM2-UAM6 strains.

3.4.2 Resistance to intestinal stress. All strains tolerated the simulated small intestinal juice containing pancreatin (Fig. 3). None of the UAM strains, nor *L. plantarum* 8014, exhibited more than 1.2-fold reduction of viability after treatment for 240 min. Again, *E. faecium* UAM1 showed the highest resistance with a 15% survival rate. Similar behaviour was observed by Jensen *et al*. (2012) when comparing some commercial and potential probiotic LAB. Some *L. plantarum* and *P. pentosaceus* strains retained the same level of viability over 240 min of incubation, (around 6 log cfu/mL). Strains with a decrease in viability of 0.5-1.0 log cfu/mL were *Lactobacillus farciminis*, *Lactobacillus sakei* and the probiotic *Lactobacillus rhamnosus* GG. Monteagudo-Mera *et al.* (2012) also detected no loss of viability of *lactobacilli* and *lactococci* strains after 240 min of incubation with a simulated pancreatin solution. The authors pointed out that these strains appeared to have a natural ability to tolerate this compound and so its presence in the small intestine does not seem to be a barrier for these strains. Thus, the high survival rate detected for the UAM strains indicate that like probiotic strains they can tolerate intestinal stress.

3.5 Adhesion properties

3.5.1 Hydrophobicity (bacterial adhesion to solvents). Hydrophobic/hydrophilic properties and surface charge of bacteria may differ between strains due to variation in the physiological state of cells or the composition of media. In addition, the expression of variable surface-associated proteins between strains might be involved (Schär-Zammaretti *et al*., 2005). Moreover, Pelletier *et al.* (1997) reported that physico-chemical properties of the microbial cell surface, including the presence of (glycol-) proteinaceous material at the cell surface results in higher hydrophobicity, whereas hydrophilic surfaces are associated with the presence of polysaccharides. Thus, xylene and chloroform were used to assess the hydrophobic/hydrophilic and electron donor (basic) characteristics of the bacterial surface (Xu *et al*., 2009). The assay to test the adherence of the LAB to the two solvents showed significant variations (Table 2). Values obtained with chloroform were

higher than those detected with xylene. The UAM strains showed lower hydrophobicity (1.2-2.8%) than strain 8014 (5.89%). Within the UAM strains, the most hydrophobic was UAM4 (2.8%), followed by UAM6 (2.25%) and UAM5 (1.97%), which were not significantly different ($P < 0.05$).

Strong affinity to chloroform was observed only for 8014 (69.38%), indicating that this strain is a strong electron donor. Lower affinities were obtained for UAM1 (9.17%) and UAM3 (9.08%), which did not differ significantly ($P < 0.05$), while the other *P. pentosaceus* strains showed the lowest affinities ranging from 3.44% to 6.33%.

The overall results indicated that the UAM strains had a low hydrophobic surface profile and are weak electron donors. However, this is not a general feature of *P. pentosaceus*, since Lee *et al.* (2014) reported that some strains belonging to this species have hydrophobic surfaces as they showed more affinity to xylene than n-hexadecane, particularly *P. pentosaceus* D56 with an affinity of 33.71% for xylene and 3.67% for n-hexadecane.

3.5.2 Bacterial auto-aggregation and co-aggregation capabilities. Bacterial aggregation between cells of the same strain (auto-aggregation) or between genetically different strains (co-aggregation) is important in several ecological niches, especially in the human gut where such abilities increase the chance of bacterial retention in the gastrointestinal tract (Collado *et al*., 2007). Auto-aggregation determines the ability of the probiotic strain to adhere to the oral cavity as well as the gastrointestinal and urogenital tracts, while co-aggregation ability helps to form a barrier that prevents colonization by pathogens (Abdulla *et al*., 2014).

The auto-aggregation rate of LAB was measured at different time intervals (Table 3). The auto-aggregation percentages during the shortest incubation times (2-6 h) were similar in all cases, but after 20 h of incubation percentages ranged from 46.13% to 68.02%, and after 24 h these percentages significantly increased, ranging from 62.61% to 87.70%. These results showed that all the strains possessed strong auto-aggregation phenotypes. After 20 h of incubation, the most auto-aggregative strain was UAM3 (68.05%), followed by UAM6 (59.53%) and UAM1 (55.22%). The lowest percentages were observed with strains 8014 (49.70%), UAM5 (51.0%), UAM4 (47.65%)

284 and UAM2 (46.14%). This profile changed after 24 h of incubation, when strains UAM1 and 8014
285 exhibited the lowest auto-aggregation abilities, 62.61% and 64.29% respectively, while the rest of
286 the strains exhibited more than 70% levels.

287 Xu *et al.* (2009) evaluated the auto-aggregation abilities of some probiotic LAB, of which
288 *Bifidobacterium longum* B6 showed the greatest rate (51.8%) after 2 h incubation time.
289 Furthermore, Bao *et al.* (2010) studied the auto-aggregation abilities of eleven strains of
290 *Lactobacillus fermentum*, selected because they showed the greatest tolerance to low pH. Between
291 them the highest auto-aggregation percentage (20 h incubation) was reached by strains IMAU60151
292 (51.5%), IMAU60145 (28.1%) and F6 (27.0%). Thus, UAM strains seem to be in the same range as
293 probiotics and other potential probiotic strains.

294 To test the ability of the UAM strains to co-aggregate with pathogenic bacteria, they were cultured
295 with Gram-positive (*Bacillus cereus*, *Listeria innocua* and *Staphylococcus aureus*) and Gram-
296 negative (*Escherichia coli*, *Pseudomonas aeruginosa* and *Salmonella typhimurium*) bacteria. The
297 results showed that all LAB tested were able to co-aggregate with all the pathogenic bacteria (Table
298 2S). Moreover, they revealed that this property is strain-specific and the degree of interaction
299 gradually increased with time, matching the observations described by Collado *et al*. (2007) and
300 Bao *et al*. (2010). When we compared the initial (2 h) and final (24 h) determinations of co-
301 aggregation, three patterns were observed (Fig. 4). After 2 h, high co-aggregation values were
302 detected for all BAL and pathogens tested, far superior to the levels of BAL auto-aggregation
303 (control in Fig. 4). After 24 h of incubation, *L. plantarum* 8014 and *E. faecium* UAM1 showed a
304 very similar pattern of co-aggregation for all pathogens tested and with similar levels. Almost all
305 LAB strains presented a higher co-aggregation ability (> 80%) when mixed with pathogens than
306 when each one was incubated alone (auto-aggregation, control), with the exception of the UAM5
307 strain that showed similar values in both trials. UAM3 was the only *P. pentosaceus* for which auto-
308 aggregation values were lower than those of co-aggregation (Table 2S). Moreover, all strains co-
309 aggregated with *P. aeruginosa*, followed by *S. typhimurium*, whereas less co-aggregative abilities

were observed with *E. coli.* Among Gram positive pathogens, the major co-aggregation abilities were observed with *S. aureus* and *L. innocua.*

Todorov *et al.* (2008) described very strong co-aggregation of several bacteriocin producing LAB, isolated from Boza, with the pathogen *L. innocua* (80.67-95.68%). Xu *et al*. (2009) showed that *Pediococcus acidilactici* had the highest co-aggregation with *S. typhimurium* (55.4%), whereas *Lactobacillus casei* demonstrated the lowest co-aggregation ability with *S. aureus* (28.7%). In another study, Vidhyasagar and Jeevaratnam (2013) reported a strain of *P. pentosaceus* VJ13 which exhibited high rates of co-aggregation with *L. monocytogenes* and *E. coli* as high as 90% and 81%, respectively. These reports confirm that auto-aggregation and co-aggregation abilities seem to be strain-specific, a property shown by the LAB strains analyzed in this study.

3.5.3 Adherence to Caco-2 cells *in vitro.* An important criterion in the selection of probiotic strains is their ability to adhere to the intestinal epithelium, as it has been established that this determines their interactions with the host and the gut microbiota (Alander *et al*., 1999). In the current study, the ability of the LAB to adhere to epithelial intestinal cells was tested *in vitro* by performing binding assays of the bacteria to Caco-2 cell lines (Fig. 5). The results showed that UAM1 was able to adhere to the enterocytes with a level significantly higher to that of the probiotic strain *L. acidophilus* LA-5 (19.62 $\pm$ 2.24% *versus* 5.97 $\pm$ 0.31%) and to the dairy *E. durans* 655 (2%) previously studied by us (Fernández de Palencia *et al*., 2011). Rao *et al*. (2013) reported similar results using a strain of *Enterococcus faecium*. The Caco-2 cell adhesion activity of *E. faecium* was significantly higher than *L. johnsonii* JCM 8791 ($p < 0:01$), and the authors concluded that *E. faecium* exhibited adhesion to Caco-2 cells to a certain extent.

The above results indicates that *E. faecium* UAM1 possesses high adhesion capacity, which might be advantageous for colonization in the human gastrointestinal tract. Additionally, this strain has significant resistance to low pH and bile, with auto-aggregation and co-aggregation capacities that may qualify it as a probiotic strain.

335 The five *P. pentosaceus* strains presented lower levels of adhesion, ranging from 2.4 ± 0.28% to
336 4.03 ± 0.83% (Fig. 5). The adhesion ability of probiotic microorganisms is closely associated with
337 their surface properties, as these influence the interactions within the gut ecosystem (Deepika &
338 Charalampopoulus, 2010). We have previously shown that the β-glucan exopolysaccharide
339 synthesized by *Pediococcus parvulus* strains isolated from cider increases the adhesion levels of the
340 producing strains (Fernández de Palencia et al., 2009; Garai-Ibabe *et al.*, 2010). Our unpublished
341 results indicate that the *Pediococcus* UAM strains do not produce high levels of β-glucan, although
342 the adhesion level of the meat strains are higher to that previously detected for the cider *P. parvulus*
343 strains (1.2%-0.25%) in the absence of their exopolysaccharide and close to the levels of the low
344 producers (3.5%) (Fernández de Palencia et al., 2009; Garai-Ibabe *et al.*, 2010).

345

346 **4. Conclusions**. *E. faecium* UAM1 and *P. pentosaceus* strains (UAM2-UAM6) showed, *in vitro*,
347 desirable probiotic properties, although the *Pediococci* do not have a very high resistance to acid.
348 Therefore, *E. faecium* UAM1 seems to be the best candidate for further investigation, since it also
349 exhibited a substantial adherence to Caco-2 cells, higher than the commercial probiotic *L.*
350 *acidophilus* LA-5, and good resistance to low pH and gastrointestinal tract conditions. These trials
351 are promising for its application as a novel probiotic strain in the food industry, since their can be
352 employed as bioprotective culture due to their thermotolerant capacity in functional foods.

353

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488 **Figure captions**

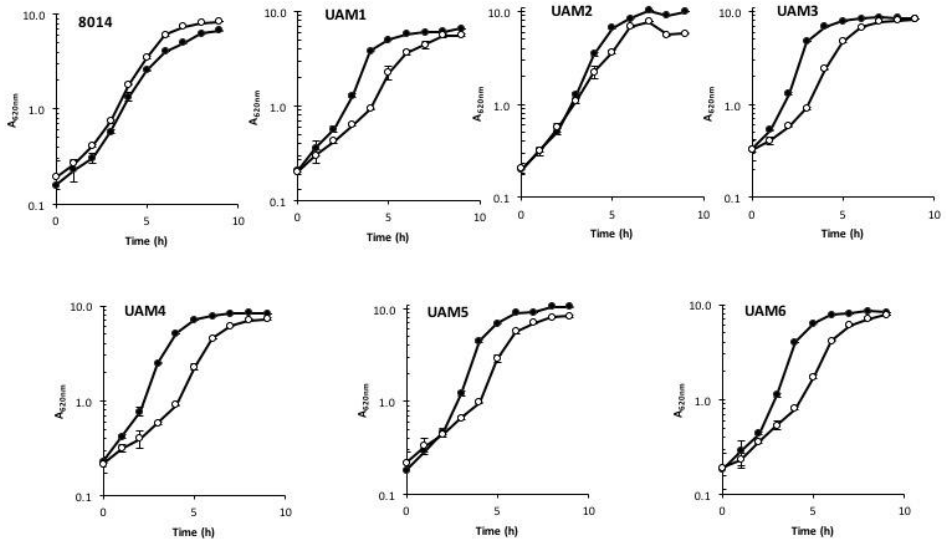
489 **Figure 1. Detection of the influence of bile salt treatment on LAB growth.** The indicated *L.*
490 *plantarum* (8014), *E. faecium* (UAM1) and *P. pentosaceus* (UAM2-AUM6) strains were grown in
491 MRS (o) or MRS supplemented with 0.3% (w/v) of porcine bile (●). The growth rate was
492 determined by measuring the absorbance of the cultures. The determinations were performed in
493 duplicate and the values depicted are the mean with the standard deviations of two independent
494 experiments performed with two different cultures of each bacterium.

495 **Figure 2**. **Analysis of cell survival after simulated gastrointestinal stress**. The indicated LAB
496 strains were challenged with pepsin (3 mg/mL) at pH 2.0 for 3 h at 37ºC. Bacterial viability was
497 analysed by plate count and results are expressed as cfu/mL. The determinations were performed in
498 duplicate and the values depicted are the mean with the standard deviations of two independent
499 experiments performed with two different cultures of each bacterium.

500 **Figure 3**. **Analysis of cell survival after simulated intestinal stress**. The indicated LAB strains
501 were challenged with pancreatin (1 mg/mL) at pH 8.0 for 4 h at 37ºC. Bacterial viability was
502 analyzed by plate count and results are expressed as cfu/mL. The determinations were performed in
503 duplicate and the values depicted are the mean with the standard deviations of two independent
504 experiments performed with two different cultures of each bacterium.

505 **Figure 4. Analysis of the co-aggregation of LAB with pathogenic bacteria.** The results are
506 shown in Supplementary Table 1S. As an example, the results obtained with the indicated strains
507 after 2 h and 24 h of treatment are depicted in the figure. The co-aggregation capacity of each LAB
508 is expressed in percentages and was determined at the indicated times by changes in absorbance
509 A_{600nm} for each LAB and pathogen cultured together and individually. The determinations were
510 performed in duplicate and the values depicted are the mean of two independent experiments
511 performed with two different cultures of each bacterium.

Figure 5. Adhesion of LAB to Caco-2 cells. The enterocytes (1:10) were exposed independently to the indicated UAM strains or to *L. acidophilus* La-5 (La-5). Adhesion levels are expressed as the percentage of the total number of bacteria (adhered plus unadhered) detected after exposure for 1 h to Caco-2 cells. Each adhesion assay was conducted in triplicate. The values are the mean of three independent experiments performed with three different cultures of each bacterium and each experiment with different Caco-2 culture. ANOVA one-way test analysis was carried out, and differences were considered statistically significant at $P < 0.05$.



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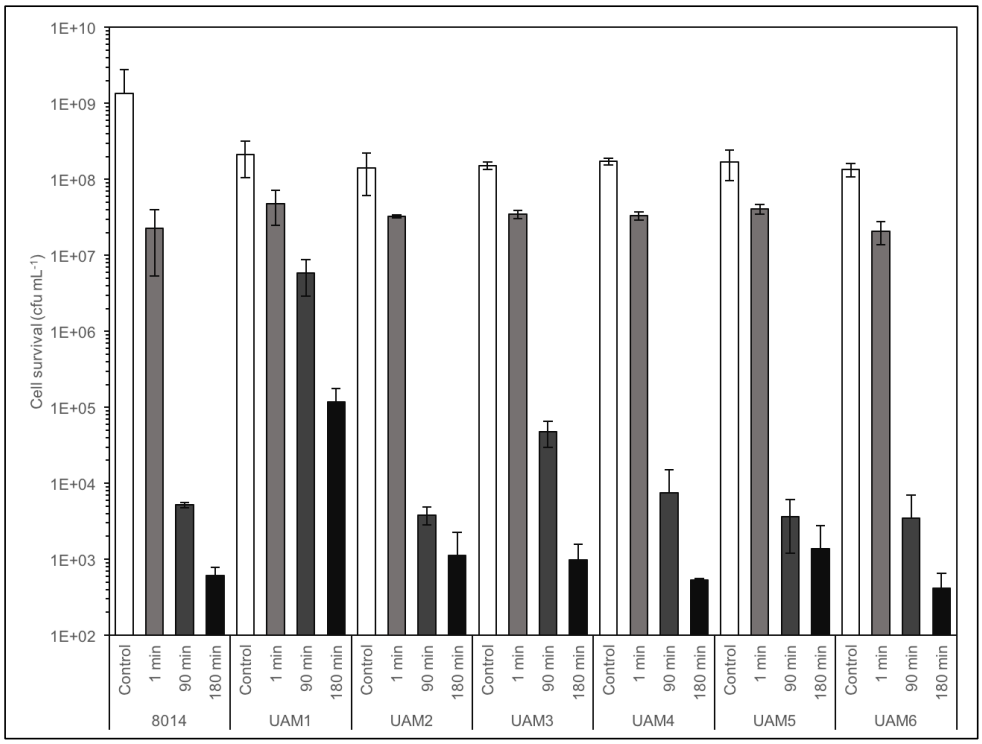


Fig. 2. **Analysis of cell survival after gastrointestinal stress**. The indicated *Lb. plantarum* (8014), *E. faecium* (UAM1) and *P. pentosaceus* (UAM2-AUM6) strains were exposed to pH 2.0 and pepsin at 3 mg/mL for 3 h. Bacterial viability was analyzed by plate count and results are expressed as cfu/mL. The determinations were performed in duplicate and the values depicted are the mean with the standard deviations of two independent experiments performed with two different cultures of each bacterium.

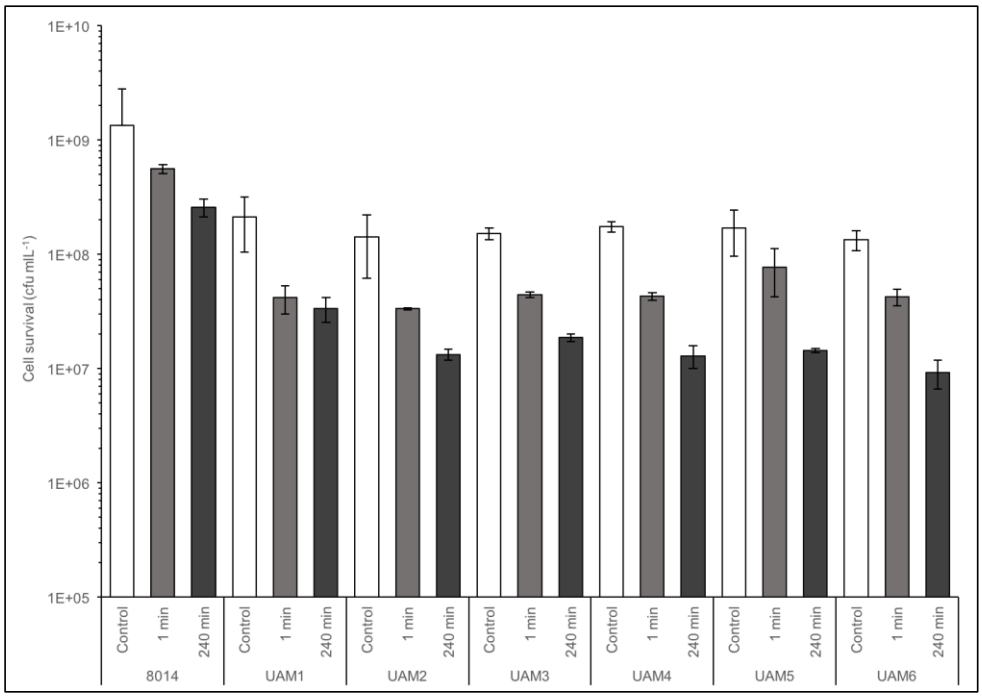


Fig. 3. **Analysis of cell survival after intestinal stress**. The indicated LAB strains were challenged with pancreatin (1 mg/mL) at pH 8.0 for 4 h. Bacterial viability was analyzed by plate count and results are expressed as cfu/mL. The determinations were performed in duplicate and the values depicted are the mean with the standard deviations of two independent experiments performed with two different cultures of each bacterium.

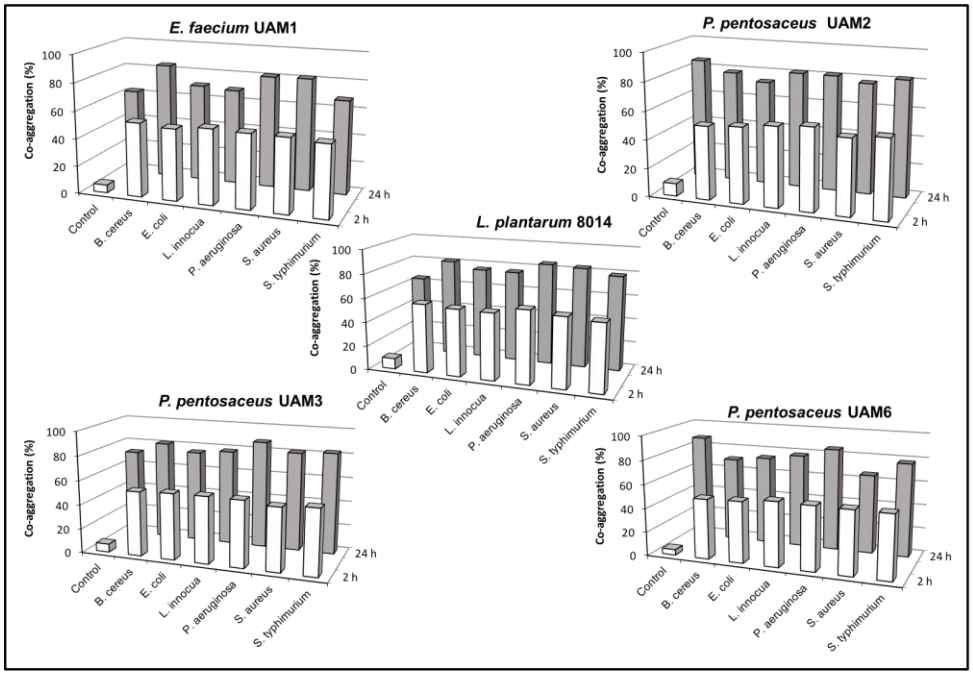
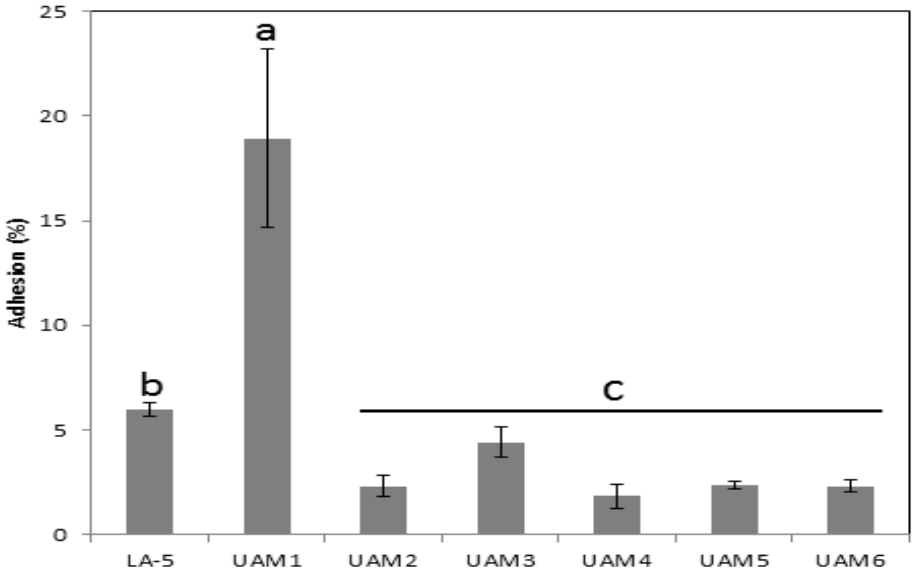


Fig. 4. Analysis of the co-aggregation profile of LAB with pathogenic bacteria. The results are shown in Supplementary Table 1. As an example, the results obtained with the indicated strains after 2 h and 24 h of treatment are depicted in the figure. The co-aggregation capacity of each LAB is expressed in percentages and was determine at the indicated times by changes in absorbance A_{600nm} for each BAL and pathogen cultured together and individually. The determinations were performed in duplicate and the values depicted are the mean of two independent experiments performed with two different cultures of each bacterium.



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562 different Caco-2 culture. ANOVA one-way test analysis was carried out, and differences
563 were considered statistically significant at p <0.05.
564

Table 1. Physiological and biochemical characterization of LAB.

Characteristics	UAM1	UAM2	UAM3	UAM4	UAM5	UAM6
Morphology	Coccobacilli	Coccid	Coccid	Coccid	Coccid	Coccid
Oxidase	-	-	-	-	-	-
Catalase	-	-	-	-	-	-
Growth at 22 °C and 45 °C	+	+	+	+	+	+
Growth in presence of 6.5% NaCl	+	+	+	+	+	+
Hydrolysis of Arginine	+	+	+	+	+	+
Hydrolysis of Esculin	+	+	+	+	+	+
Hydrolysis of Casein	-	-	-	-	-	-
Production of CO_2 from glucose	-	-	-	-	-	-
Nitrate reductase	-	-	-	-	-	-
Production of lactate from						
D-Glucose, D-Mannitol	+	+	+	+	+	+
Inositol, D-Sorbitol	+	+	+	+	+	+
L-rhamnose, D-Melibiose	+	+	+	+	+	+
L-Arabinose	+	+	+	+	+	+
D-Sucrose	-	+	+	+	+	+
Usage of Litmus Milk at 22 °C:						
Acidification	+	+	+	+	+	+
Coagulation	-	-	-	-	-	-
Reduction	+	+	+	+	+	+
Peptonization	-	-	-	-	-	-
Usage of Litmus Milk at 45° C:						
Acidification	+	+	+	+	+	+
Coagulation	+	-	-	-	-	-
Reduction	+	+	+	+	+	+
Peptonization	+	-	-	-	-	-

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