Lactobacillus paracasei subsp. *paracasei*: an Algerian isolate with antibacterial activity against enteric pathogens and probiotic fitness

FARIDA BENDALI – ALEXANDRA DURAND – MICHEL HÉBRAUD – DJAMILA SADOUN

Summary

The interest in probiotics as remedies for a broad number of gastrointestinal and other infectious diseases has gained wide interest over the last years. The goal of this study is the investigation of the probiotic potential of a human isolate of *Lactobacillus paracasei* subsp. *paracasei*. Its resistance to simulated gastrointestinal conditions and adhesion to Caco-2 cells were examined and its influence on the growth of the enteropathogenic *Escherichia coli* (EPEC) strain E2348/69 and *Salmonella* Typhimurium LT2 in mixed cultures was investigated. The strain was unaffected by pH 3, pancreatin or bile salts, and was able to survive at pH 2 in the presence of pepsin. A strong inhibition of EPEC and *Salmonella* populations was observed within 24 h, indicating a high antagonistic activity of the strain towards the two enteropathogens. In addition, the strain was able to adhere to Caco-2 cells and to inhibit the adhesion of *E. coli* E2348/69 and *Salmonella* Typhimurium LT2 using either pre-incubation or co-incubation procedures. This strain was therefore found, in vitro, to possess desirable probiotic properties. Moreover, the growth of five potential pathogens (*Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Bacillus cereus*, *Staphylococcus aureus* and *Enterococcus faecalis*) was found to be inhibited.

Keywords

Lactobacillus paracasei subsp. *paracasei*; enteropathogenic *Escherichia coli*; *Salmonella* Typhimurium; antagonism; mixed cultures; Caco-2 cells; probiotics

Maintenance of the intestinal microbiota is important in preventing diseases by controlling the overgrowth of potentially pathogenic bacteria. Wide-spread prescription of antibiotics not only has led to an increase in antibiotic-resistant pathogenic strains, but is often associated with the disruption of the protective flora, leading to predisposition to infections. For these reasons, the control of infections through a non-antibiotic approach is urgently needed and bacterial replacement therapy using non-pathogenic bacteria, from the natural microbiota or probiotics, represents a promising alternative. It is accepted that these bacteria might represent an effective tool for controlling the overgrowth of pathogens and maintaining the integrity of the gut mucosal barrier [1]. *Lactobacillus* and *Bifidobacterium*, which have a long history of safe use in the manufacture of dairy products, are traditionally included as probiotics to protect against intestinal disorders [2]. Both are thought to prevent the adherence, establishment, replication and/or virulence of specific enteropathogens [3]. The effectiveness of selected *Lactobacillus* strains used as probiotics to prevent and treat infectious bacterial and viral diarrhea has been demonstrated in well-designed in vitro and in vivo experimental studies and double-blind, placebo-controlled clinical trials [4, 5]. Diarrhoeal disease is a major public health problem throughout the world, with over two million deaths occurring each year, mostly children under 5-years old in developing countries [6]. There is a wide range of recognized enteric pathogens but diarrheagenic *E. coli* (DEC) and mainly enteropathogenic *E. coli* (EPEC) are the most common causes of diarrhoeal diseases in these countries [7, 8]. Nevertheless, diarrhea is frequently associated with gastroenteritis and *Salmonella enterica*, an important facultative intracellular pathogen, is recognized as the major cause of gastroenteritis in humans [9].

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The *Salmonella* genus contains over 2500 serotypes [10], all of which are potentially pathogenic to humans [11], mainly *S. enterica* serovar Typhimurium (*S*. Typhimurium). Inhibition of pathogen adhesion to the intestinal epithelium may prevent colonization and limit the opportunity for systemic infection [12, 13]. Indeed, initial adhesion to the intestine is the critical first step in establishing colonization or infection of the host [14]. Some probiotic bacteria, including *Lactobacillus*, may be effective in preventing adhesion and invasion by enteric pathogens [15, 16]. Although the exact mechanism of action is unknown, a number of hypothetical mechanisms have been proposed: probiotics could reduce intestinal infections by (i) competing with pathogens for binding sites on the intestinal wall, (ii) competing for nutrients within the intestinal lumen, (iii) producing specific inhibitory compounds such as bacteriocins, (iv) decreasing the luminal pH via the production of volatile short-chain fatty acids or lactic acid, or (v) stimulating the host immune system [17, 18]. For a probiotic strain to exert its beneficial effect on the host, it must be able to survive the passage through the digestive tract of the host. Tolerance to the presence of bile is an important attribute to microbial survival in the intestinal tract. So far, research has mainly focused on strains sensitivity towards a low pH, proteolytic enzymes and bile salts [19–22]. The ability of *Lactobacillus* strains to adhere to mucosal surfaces of the intestine and the subsequent long or short-term colonization have long been the most commonly encountered criteria for the selection of probiotic strains. Adhesive probiotic lactobacilli have been reported to possess beneficial health effects, especially related to the inhibition of pathogens adhesion to intestinal cell lines [23, 24]. Despite numerous studies that demonstrate anti-pathogenic properties of probiotics, great variability exists in their reported effectiveness in reducing intestinal infection [25, 26]. This variability may depend on the health status of the host and the probiotic organism used. Therefore, in the current study, we examined the influence of *Lb. paracasei* subsp. *paracasei* on the EPEC and *Salmonella*-epithelial interaction. The aim of the study was to investigate the influence of a strain of *Lb. paracasei* subsp. *paracasei* on the populations of two human pathogens, EPEC and *S.* Typhimurium in mixed cultures. We investigated further the in vitro resistance capacities of the strain to a simulated gastrointestinal environment and its in vitro adherence properties using human intestinal cells as well as its ability to impair adherence of the two pathogens. Furthermore, the antimicrobial activity of metabolic products from this probiotic strain was determined against a wide variety of Gram-positive and Gram-negative human pathogens.

MATERIALS AND METHODS

Bacterial strains and culture conditions

Lb. paracasei subsp. *paracasei* was isolated from breast-fed infant faeces. It was identified phenotypically by classical tests (growth temperature range, pH, fermentation pattern) and genotypically by sequencing the 16S rDNA. The strain was previously demonstrated to be active against *Listeria*, *E. coli, Salmonella* sp. and *Staph. aureus* strains [27]. The target strains used in this study are *Salmonella enterica* Typhimurium LT2 (ATCC700720), *E. coli* O127:H6 strain E2348/69 (EPEC) (INRA of Clermont Ferrand, France), *Pseudomonas aeruginosa* ATCC 27853, *Enterococcus faecalis* ATCC 29212, and *Staph. aureus* ATCC 25923 (Pasteur Institute of Algeria, Algiers, Algeria), *Bacillus cereus, Klebsiella pneumoniae* (Collection of Applied Microbiology Laboratory, Bejaia University, Bejaia, Algeria). *Lb. paracasei* was cultured in de Man, Rogosa and Sharpe (MRS) broth (Fluka Chemie, Buchs, Switzerland) and MRS agar (Carl Roth, Lauterbourg, France), at 37 °C. Brain Heart Infusion (BHI) broth and BHI agar (Merck, Darmstadt, Germany) were used for growth of the target strains at 37 °C in aerobiosis. Crystal violet neutral red bile glucose (VRBG) agar (Merck) was used for *Enterobacteriaceae* strains counts.

Detection of inhibitory activity

Lb. paracasei was assessed for its ability to inhibit the target strains using the spot-on-lawn and the well-diffusion methods as described previously [27]. Briefly, active MRS culture of *Lb. paracasei* was spotted on MRS agar. Plates were incubated anaerobically at 37 °C for 18 h, before being overlaid with soft BHI agar inoculated with the target strain (approx. 10^6 CFU·ml^{-1}) and incubated aerobically at 37 °C for further 18 h. For the well-diffusion assay, 20 ml of MRS agar poured into a sterile Petri dish was overlaid with 5 ml of soft BHI agar inoculated with the target strain (10^6 CFU·ml^{-1}). After solidification, the filtered supernatant was placed in triplicate into wells made in the agar. The plates were incubated for 18 h at 37 °C. Absence or presence of any inhibitory zone around the well was recorded. The active supernatant was further characterized by adjusting the pH to 6.5 with 1 mol·l^{-1} NaOH (Merck-eurolab, Briare Le Canal, France) to rule out acid inhibition.

MRS broth either at pH 6.5 or 4.5 was used as a control. In order to determine whether the active supernatant caused a cidal or static effect on the target strains, the method described by LEWUS et al. [28] was used. Briefly, an agar plug was removed from the inhibition zone on the agar plate. The agar plug was dissolved in 1 ml of 50 mmol·l^{-1} phosphate buffer (pH 6.5) and used to inoculate duplicate tubes containing 10 ml of BHI broth. The tubes were incubated at 37 °C for up to 48 h to reveal the presence of viable cells.

Co-culture experiments

The pathogens (EPEC, *S.* Typhimurium) were separately co-cultured with *Lb. paracasei* in two flasks containing 100 ml BHI broth at 37 °C for approx. 24 h. Two distinct experiments were carried out. In the first experiment, pathogenic strains were inoculated with 10^6 CFU·ml^{-1}, while *Lb. paracasei* had final counts of approximately 10^8 CFU·ml^{-1}. In this experiment, Dulbecco's modified Eagle medium (DMEM, GIBCO/BRL Division of Life Technologies, Invitrogen, Cergy Pantoise, France) was also tested as a culture medium for the bacteria. In the second experiment, all counts were 10^3 CFU·ml^{-1}. Pure cultures of the pathogens were included as controls. Counts of the pathogens were enumerated on VRBG agar, incubated aerobically at 37 °C for 24–48 h. The pH values were measured at the end of the incubation period. The experiment was repeated three times.

SURVIVAL UNDER CONDITIONS SIMULATING THE HUMAN GASTRO-INTESTINAL TRACT

Resistance to pH and digestive enzymes

The resistance of *Lb. paracasei* in a low-pH environment was tested as described by CONWAY et al. [19]. Briefly, bacterial cells from overnight (18 h) cultures were harvested (8000 ×*g*, 20 min, 4 °C), washed twice with phosphate buffered saline (PBS) buffer (Sigma-Aldrich, Steinheim, Germany), pH 7.2, and then were resuspended either in PBS or in MRS broth, adjusted to pH 1, pH 2 or pH 3. The initial population was 10^9 CFU·ml^{-1}. The resistance was assessed in terms of viable colony counts and enumerated after incubation at 37 °C for 0, 1 and 3 h, simulating the time spent by food in the stomach. The resistance of *Lb. paracasei* to pepsin and pancreatin was tested as described by CHARTERIS et al. [20]. Briefly, bacterial cells prepared as explained above were resuspended either in PBS solution or MRS broth at pH 2 containing pepsin (3 mg·ml^{-1}; Sigma-Aldrich), or in PBS or MRS broth at pH 8 containing pancreatin (1 mg·ml^{-1}; Sigma-Aldrich). The initial population was 10^9 CFU·ml^{-1}. In parallel, the effect of alkaline pH 8.0 without pancreatin was evaluated by re-suspending cells either in PBS or in MRS broth adjusted to this pH value. The resistance was assessed in terms of viable colony counts and enumerated after incubation at 37 °C for 0, 1 and 3 h with pepsin, and 0 and 4 h with and without pancreatin, simulating the time spent by food in the stomach and small intestine, respectively [29].

Resistance to bile salts

In order to test the survival in the presence of bile, cells were grown during 18 h, serially diluted, and plated on MRS agar containing 0%, 0.5%, 1.0%, 2.0%, 5% and 7% (w/v) Oxgall (Merck). In parallel, fresh cultures (10^9 CFU·ml^{-1}) were inoculated to MRS broth supplemented with 0.3% (w/v) Oxgall. The concentrations of Oxgall (0.3%, 1.0% and 2.0%) were chosen to simulate the concentrations found in the human intestinal tract, which range from 0.2 to 2.0% [30]. High concentrations (5.0% and 7.0%) were also tested. The resistance was assessed in terms of viable colony counts enumerated after incubation at 37 °C for 0 and 4 h, simulating the time spent by food in the small intestine.

ADHESION TO CACO-2 CELLS

Caco-2 cells culture

The cells used in this study were the human-derived Caco-2 cells. All chemicals for the cell culture medium were from the GIBCO/BRL Division of Life Technologies (Invitrogen). Cells were routinely grown at 37 °C in a 95% air/5% CO_2 atmosphere in DMEM containing 4.5 g·l^{-1} D-(+)-glucose, 110 mg·l^{-1} sodium pyruvate, L-glutamate and red phenol. Before use, the medium was supplemented with 10% heat-inactivated (56 °C, 30 min) fetal bovine serum (FBS), 1% (v/v) non-essential amino acids (100×), 1% Pen-Strep solution 100× (100 U·ml^{-1} of penicillin G and 100 μg·ml^{-1} of streptomycin sulfate). Monolayers of Caco-2 cells, which were used in the adherence assays, were prepared by inoculating six-well tissue culture plates with 10^6 cells per well in 4.0 ml of culture medium. Cells were grown in 75 cm^2 flasks until they were confluent before culture in the plates. The concentration of the cells was determined under optical microscopy using a Malassez chamber, the number of cells inoculated in each well was within the range of 1×10^6 to 2×10^6 per well. The tissue culture plates were covered with collagen before use. The culture medium was renewed every two days, and the monolayers were used in the adherence assays after 7 days of incubation. The cells were cultured until they were confluent

and the culture medium was replaced with fresh medium without antibiotics prior to addition of the bacterial cells [31].

Adhesion of *Lb. paracasei* to Caco-2 cells

The study of the probiotic adhesion potential was performed as described by MARAGKOUDAKIS et al. [29] with some modifications. *Lb. paracasei* MRS broth cultures (18 h, 10 ml) were harvested (8000 ×*g*, 10 min, 4 °C) and washed twice with 5 ml PBS buffer, pH 7.2. Cells were re-suspended in 1 ml of PBS and then diluted in DMEM to the final concentration of 10^8 CFU·ml^{-1}. The growth medium in the six-well tissue culture plates of Caco-2 monolayers (7 days old) was aspirated and the cells were washed twice with PBS. Subsequently, 1 ml of bacterial suspension in DMEM was transferred onto the Caco-2 monolayers. The plates were incubated at 37 °C in a 95% air/5% CO_2 atmosphere for 60, 90 and 120 min, then the bacterial suspension was aspirated and the Caco-2 monolayers were washed twice with PBS, before 1 ml of Tween 80 (0.04%, w/v; Sigma-Aldrich) was added to detach the adhered bacterial cells. The bacterial suspension was enumerated as described before. The adhesion of *Lb. paracasei* to Caco-2 cells was expressed as a percentage of viable bacterial cells compared to their initial population in the DMEM suspension. Adhesion experiments were performed in triplicate. The enumeration of the adhered *Lb. paracasei* cells was performed in duplicate.

Inhibition of pathogen adhesion to Caco-2 cells

The effect of *Lb. paracasei* on the adhesion of two pathogens was investigated using the strains of enteropathogenic *E. coli* E2348/69 and *S.* Typhimurium LT2. Two different procedures were used in order to differentiate exclusion by and competition with *Lb. paracasei*. For exclusion tests, Caco-2 cell monolayers were cultured and washed as previously described before incubation with *Lb. paracasei* (10^8 CFU·ml^{-1}, multiplicity of infection MOI 100) for 60 and 90 min. Afterwards, non-adhering bacteria were removed and EPEC (10^8 CFU·ml^{-1}, MOI 100) or *Salmonella* (10^8 CFU·ml^{-1}, MOI 100 and 10^7 CFU·ml^{-1}, MOI 10) were added and incubation was continued for further 60 and 90 min. For competition tests, *Lb. paracasei* (10^8 CFU·ml^{-1}, MOI 100) and any of the pathogens (10^9 CFU·ml^{-1}, MOI 1 000) were mixed and added to the intestinal cells and then incubated for 90 min. The pathogens (10^9 CFU·ml^{-1}, MOI 1 000) were also incubated with the filter-sterilized culture supernatant of *Lb. paracasei* to investigate if metabolic substances contributed to the interference. The number of bacteria adhering to the intestinal cells was determined as described above, by plating serial dilutions on VRBG agar plates. Each assay was conducted at least twice with two determinations per assay [32].

Statistical analysis

All results were expressed as mean ± standard deviation. Statistical analysis was performed using the one-way ANOVA procedure of Statistica software (StatSoft, Tulsa, Oklahoma, USA). The differences among means were detected by paired Student's test. Values of $P < 0.05$ were considered statistically significant.

RESULTS AND DISCUSSION

Antibacterial activity against enteropathogens

Lb. paracasei inhibited the growth of the pathogenic strains tested, using either the spot-on-lawn (diameter, < 10 mm) or the well-diffusion assay (diameter, < 20 mm). In the well-diffusion assay, the supernatant had an inhibitory effect at pH 6.5 and 4.5 (Fig. 1). Since inhibition was observed when the pathogens were grown in the presence of near-neutral supernatant (pH 6.5), inhibition effects could not be explained by only organic acids production (along with the low pH) and were probably due to bacteriocin-like substances production, as expected previously [27]. In the controls, the growth of the two pathogens was inhibited by MRS adjusted at pH 4.5 but not with MRS at pH 6.5. The antibacterial activity of *Lb. paracasei* was examined using five bacterial pathogens, either Gram-negative or Gram-posi-

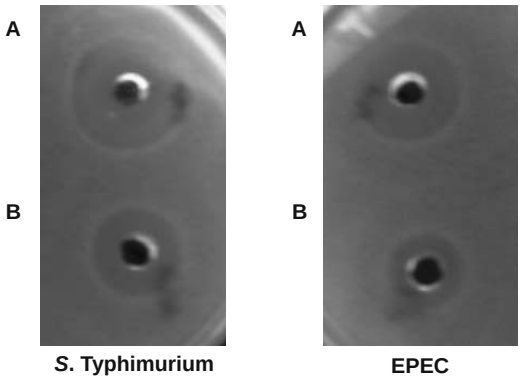

Fig. 1. Well-diffusion assay showing activity of native (pH 4.5) (A) and neutralized (pH 6.5) supernatant (B) of a strain of *Lb. paracasei* subsp. *paracasei* against *S.* Typhimurium and EPEC.

tive: *Ps. aeruginosa*, *K. pneumoniae*, *B. cereus*, *E. faecalis* and *Staph. aureus*. Growth inhibition of these microorganisms was assessed by the spot-on-lawn and the well-diffusion tests using the native and neutralized *Lb. paracasei* culture supernatant. A high inhibitory effect was observed with the spot-on-lawn assay and using the native supernatant (pH 4.5). However, only slight inhibition effect was observed after neutralization of the *Lb. paracasei* culture supernatant. The cells in the inhibition zones remained viable, which indicated that the substance present in the *Lb. paracasei* culture supernatant did not kill the bacteria but rather impaired their division. Many authors reported the production by different species of *Lactobacilli* of bacteriocin-like compounds that exhibit broad activities [33–36]. Moreover, in another study, treatment of *Lb. paracasei* culture supernatant with either proteases or heat (80 °C for 2 h) affected its activity. Purification trials using Sepack cartridges and ion-exchange chromatography procedures indicated a high hydrophobicity and a cationic nature of the active substances (data not shown). Nevertheless, we cannot exclude the implication of organic acids in the antagonistic effect observed. Further experiments are necessary to identify the chemical nature of the antibacterial compounds responsible for the observed effects. We previously showed that this strain was able to kill several pathogens (*Staph. aureus*, *L. monocytogenes*, *E. coli* and *Salmonella* sp.) with implication of proteinaceous substances [27].

Co-culture assays

The growth of EPEC and *S.* Typhimurium in the presence of *Lb. paracasei* was monitored during 24 h incubation in BHI broth and DMEM. Pure cultures of either EPEC or *Salmonella*, obtained in the same conditions, were used as controls. In BHI, EPEC grew at a faster rate in the mono-cultures (2.0×10^8 CFU·ml^{-1}) than in the co-cultures (2.0×10^4 CFU·ml^{-1}; Fig. 2A). A difference of about 4 orders of magnitude was registered in the term of the incubation period (24 h). The average growth rate was 1.18 h^{-1} for EPEC in mono-cultures and 0.16 h^{-1} in co-cultures. A difference of about 1.02 h^{-1} in the growth rate was registered between the two cultures. Significant differences ($P < 0.05$) were noticed between mono- and co-cultures during 8 h incubation. In the case of *Salmonella*, the same growth dynamic was registered (Fig. 2B). The strain grew at a faster rate in mono-cultures (10^8 CFU·ml^{-1}) than in co-cultures (4.0×10^5 CFU·ml^{-1}) with a reduction level of 2.4 log10. The average growth rate was 1.08 h^{-1} in mono-cultures and 0.44 h^{-1} in co-cultures. A difference of about 0.64 h^{-1} in the growth rate was registered between the two cultures. As for EPEC, the *Salmonella* cultures grew in the same manner (Fig. 2B) in the mono- and co-cultures during the first 6 h. After this, significant differences ($P < 0.05$) were noticed. Fig. 3 shows the behaviour of these two pathogens in mono- and co-cultures with *Lb. paracasei* in BHI broth and DMEM during incubation for 1, 2 and 24 h at 37 °C. The two strains grew well in DMEM (Fig. 3A and Fig. 3B). A slight difference in the behaviour of the two pathogens was registered in the co-cultures in DMEM and BHI broth during 2 h incubation. Indeed, a reduction and an increase in the EPEC and *S.* Typhimurium cell numbers were observed after 1-h and 2-h co-cultures in DMEM and BHI broth, respectively. Moreover, pH values of 4.2 and 4.4 were registered after 24-h co-cultures in BHI broth with EPEC and *S.* Typhimurium respectively, whereas pH values of 5.6 and 5.8, res-

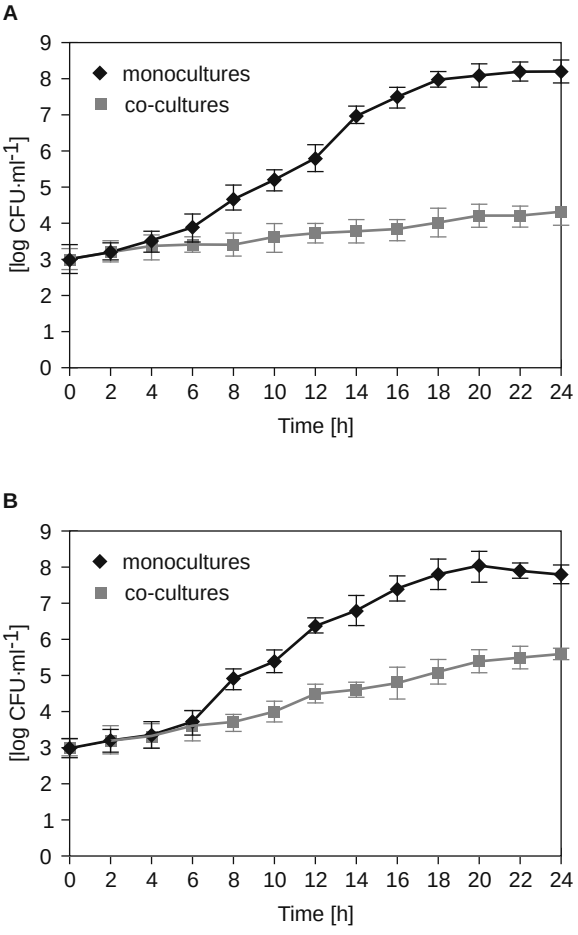

Fig. 2. Kinetics of EPEC (A) and *Salmonella* Typhimurium (B) growth in monocultures and co-cultures with *Lb. paracasei* during 24 h incubation in BHI broth.

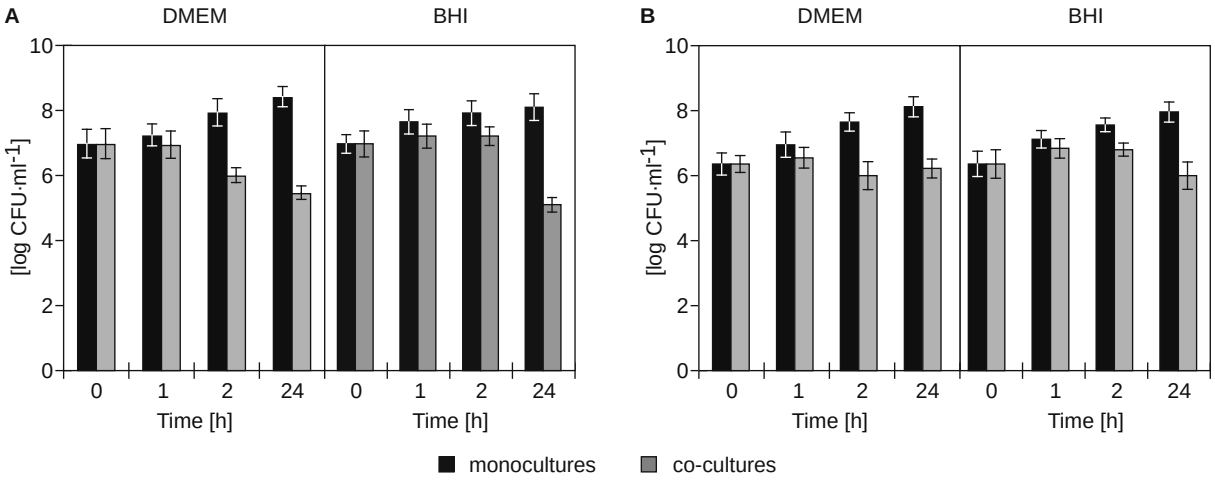

Fig. 3. Behaviour of EPEC (A) and *S*. Typhimurium (B) in mono-cultures and co-cultures with *Lb. paracasei* during incubation in DMEM and BHI broths, respectively.

pectively, were noticed in DMEM. This indicates the implication of specific metabolites other than those responsible for acidic pH in the inhibitory activity of the LAB strain in DMEM. The antagonistic effect of lactic acid bacteria (LAB) on pathogenic microorganisms has a complex character and has not been completely defined yet. In the study of JOHNSON-HENRY et al. [29], there was no difference in growth when *E. coli* O157:H7 was grown alone and when it was incubated with *Lb. rhamnosus* GG or its culture supernatant for 3 h and 18 h. In contrast, FORESTIER et al. [32] have demonstrated that active substances of the culture supernatant of *Lb. casei* subsp. *rhamnosus* after 5 h caused clear inhibition of the growth of *Shigella flexneri*. Similarly in an experiment of FERNANDEZ et al. [36], inactivation of enteric pathogens by lactobacilli occurred as early as after 7 h of co-culture. APELLA et al. [37] in turn observed the elimination of *Shigella sonnei* incubated together with *Lb. casei* and *Lb. acidophilus* for 6 h to 9 h. In our study, inactivation of *Salmonella* and EPEC strains occurred after 8 h of co-culture with *Lb. paracasei*. It was already mentioned that elimination rate of different pathogens was dependent on the antagonistic strain applied [38] and varied in relation to different pathogenic microorganisms [36]. In the present study, EPEC appeared to be more susceptible to the action of *Lb. paracasei* than *S*. Typhimurium LT2.

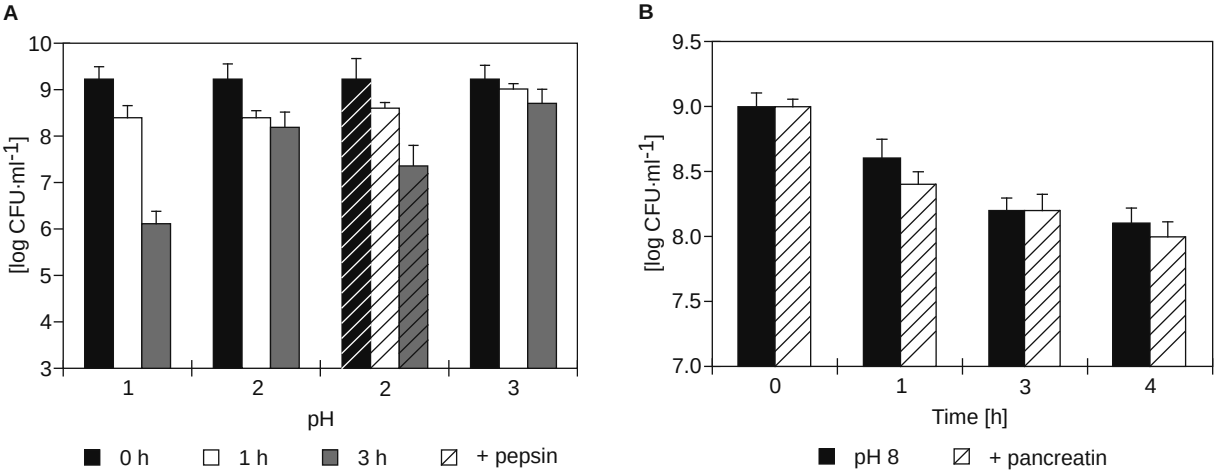

Fig. 4. Survival of the *Lb. paracasei* subsp. *paracasei* strain (A): at different acidic pH and in the presence of pepsin at 0 h, 1 h and 3 h and (B): at pH 8.0 and in the presence of pancreatin, expressed in log CFU·ml^{-1}.

SURVIVAL UNDER CONDITIONS SIMULATING THE HUMAN GASTROINTESTINAL TRACT

Resistance to pH and digestive enzymes

Before reaching the intestinal tract, probiotic bacteria must first survive transit through the stomach. In this compartment, the secretion of gastric acid constitutes a primary defense mechanism against most of the ingested microorganisms. Therefore, preliminary experiments were completed to determine the degree of acid resistance exhibited by *Lb. paracasei* strain isolated from the human infant faeces. The strain retained its viability even after 3 h of exposure to pH 3.0 (Fig. 4A). After 1 h at pH 1.0, it maintained high viability (log *c* [CFU·ml^{-1}] = 8.2). On the contrary, after 3 h exposure to pH 1.0, only weak viability was recorded (10^6 CFU·ml^{-1}). A high survival was observed when the strain was subjected to pH 2.0 in either the absence or presence of pepsin. After 1 h, only a reduction by less than an order of magnitude was observed in the two cases. After 3 h of exposure to pepsin, the strain of *Lb. paracasei* maintained a weak viability (Fig. 4B). In comparison to the sole effect of pH 2.0, it seems that pepsin effect was greater than that of acidic pH. Indeed, a survival rate of 10% was observed even after 3 h exposure to pH 2.0 in absence of pepsin. In contrast to pepsin, the strain examined in this study could survive well in a pancreatin solution at pH 8.0 simulating the neutral small intestine environment, even after 4 h exposure. No significant differences ($P > 0.05$) could be noticed between the sole effect of pH 8.0 and its combined effect with pancreatin (Fig. 4B).

Previous studies have reported that *Lactobacillus* strains were able to retain their viability when exposed to pH values of 2.5–4.0, but displayed loss of viability at lower pH values [19, 21, 22, 39]. Our results on the viability of the *Lb. paracasei* strain in the presence of pepsin at pH 2 are in agreement with previous data [20, 36]. The combined effect of pepsin and acidic pH aimed at mimicking those of the gastric juice. So it is clear that the decrease in viability conferred by the pepsin solution at pH 2 was due to the action of the enzyme in synergy with low acidity.

Resistance to bile salts

The strain of *Lb. paracasei* retained its entire viability in MRS broth in the presence of 0.3% (w/v) bile salts at pH 8.0 simulating the neutral small intestine environment. Similarly, on the MRS agar plates, this strain could survive well in the presence of 0.5–2% bile salts (Fig. 5). Furthermore, this strain was found to be able to resist high concentrations of bile salts (5% and 7%). A prolonged incubation of 24 h was used to assess the survival capacity of this strain in the case if it colonized the gastro-intestinal tract. From the results obtained (Fig. 5), it is clear that this strain presents a good resistance to bile salts since it could survive well even after 24 h incubation in the presence of high concentrations of bile salts (3, 5 and 7%). This is in agreement with the data reported by Dunne et al. [39], who reported that some *Lactobacillus* and *Bifidobacterium* strains could grow in physiologically relevant concentrations of human bile and exhibited resistance to bovine bile (0.3–7.5%).

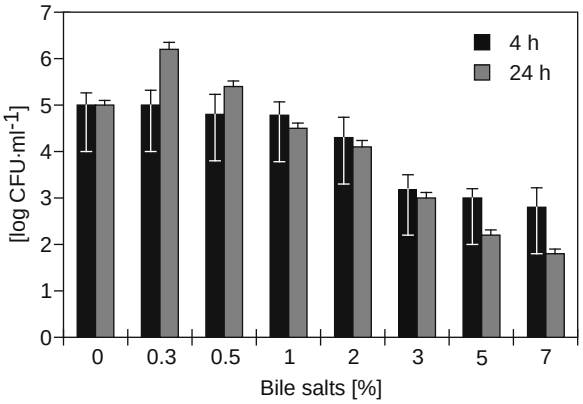

Fig. 5. Survival of the *Lb. paracasei* subsp. *paracasei* strain during 4 h and 24 h incubations in the presence of bile salts, expressed in log CFU·ml^{-1}.

ADHESION PROPERTIES

Adhesion of *Lb. paracasei* to Caco-2 cells

Since bacterial adhesion to intestinal cells is considered as one of the crucial selection criteria for probiotic strains [40], we determined the adherence capacity of the *Lb. paracasei* strain using Caco-2 cells, a cell line used as an in vitro model for intestinal epithelium [41]. The probiotic strain was able to adhere to the cell surface monolayer (Tab. 1). High numbers of adherent bacteria were observed on the intestinal cell line, with an average of 6.0×10^6 CFU·ml^{-1} after 1 h incubation. No major differences were observed between the levels of adhesion obtained after 1 h and 2 h incubation with different MOI, suggesting that adhesion occurred rapidly after the initial contact between cells and bacteria. Adherence of *Lb. paracasei* to Caco-2 cells was measured using a MOI of 100 and 1 000 bacteria/Caco-2 cell (Tab. 1). The number of adhering bacteria reached a maximum of 4.40×10^7 CFU·ml^{-1} with a MOI of 1 000 (2×10^6 cells per well and 2.57×10^9 CFU·ml^{-1}; Tab. 1). Adhesion of *Lb. paracasei* to intestinal

Tab. 1. Adherence of *Lb. paracasei* subsp. *paracasei* to Caco-2 cells.

Incubation time [h]	Initial cells number [CFU·ml^{-1}]	MOI	Adherent cells number [CFU·ml^{-1}]	% of adherence
1	4.50×10^8	100	6.00×10^6	1.33
2	2.57×10^9	1 000	4.40×10^7	1.71

epithelial cells would allow colonization of the intestinal mucosa and therefore could limit the overgrowth of pathogens. Previous studies indicated that *Lactobacillus* species were able to adhere to the surface of intestinal epithelial cells in tissue culture [42].

Adhesion of pathogenic bacteria to Caco-2 cells

Adherence of pathogenic bacteria to Caco-2 cells was measured using different MOI – 100 and 1 000 bacteria/Caco-2 cells. The number of adhering bacteria was dependent on the number of bacteria added and on the contact time, the number reached a maximum of 4.9×10^7 CFU·ml^{-1} and 2.4×10^7 CFU·ml^{-1} after 1.5 h of contact with MOI of 1 000 and 1.5×10^7 CFU·ml^{-1} and 1.2×10^7 CFU·ml^{-1} after 2 h contact with MOI of 100, respectively, with EPEC and *S.* Typhimurium (Fig. 6).

Inhibition of pathogens adhesion to Caco-2 cells

Lb. paracasei inhibited pathogens adherence (EPEC and *S.* Typhimurium). This inhibition is dependent on MOI, the time of contact, the inoculum size and also the mode of contact (pre-incubation, co-incubation or on the contact of the sole culture supernatant of the probiotic strain). The inhibition level varied from 64.5% to 99.7% for EPEC and from 79.2% to 99.6% for *S*. Typhimurium in the pre-incubation experiments. In the co-incubation experiments, inhibition levels ranging from 92.4% to 94.7% and 70.0% to 90.8% were registered for EPEC and *S*. Typhimurium, respectively. In the pre-incubation experiments, *Lb. paracasei* was maintained in contact to the Caco-2 cells for 1 h and 2 h. After elimination of the non-adherent bacteria, pathogenic species were added and the plates were further incubated for 1 h and 2 h respectively. At the end of the incubation period, adhesion levels of 0.5% and 0.9% were recorded for EPEC and *Salmonella* respectively after 1 h and of 0.01% for both pathogens after 2 h (Fig. 6A). In the co-incubation experiments, two initial inocula for the pathogenic species (approx. 10^8 and approx. 10^9 CFU·ml^{-1}, respectively) were tested with the same inoculum of *Lb. paracasei* subsp. *paracasei* (approx. 10^8 CFU·ml^{-1}). In the case where the three bacteria had the same initial level (approx. 10^8 CFU·ml^{-1}), adhesion levels of 1.3×10^6 and 3.0×10^6 were obtained in the presence of *Lb. paracasei*, with inhibition percentages of 92.4% and 70.0% for EPEC and *Salmonella*, respectively (Fig. 6B). Surprisingly, when the pathogens level was 10-fold greater, higher inhibition levels were recorded. This indicates that the probiotic strain adhered more easily and quickly to the Caco-2 cells than the pathogens.

Lb. paracasei culture supernatant was also examined for its ability to impair the adherence of the two pathogens to Caco-2 cells. Using a MOI of 100, the levels of adhesion of these pathogens (in the absence of the active supernatant) were 4.9×10^7 CFU·ml^{-1} and 2.4×10^7 CFU·ml^{-1}, respectively. In the presence of *Lb. paracasei* culture supernatant, the adhesion of the pathogens was reduced. As shown in Fig. 6C, culture with the *Lb. paracasei* culture supernatant for 1.5 h resulted in reduction levels of 51.1% and 45.8% in the adhesion of EPEC and *S*. Typhimurium to Caco-2 cells, respectively. It was reported that, if *Lb. rhamnosus* blocks the internalization, but not the adherence, of *E. coli* O157:H7 in Caco-2 cells, other LAB (including *Lb. gasseri*, *Lb. casei* or *Lb. plantarum*) had no effect on either binding or internalization of *E. coli* O157:H7 in Caco-2 cell monolayers [43]. This study shows that *Lb. paracasei* adheres to Caco-2 cells and reduces the binding of both *E. coli* E2348/69 and *S.* Typhimurium. The adherence of the two pathogens was decreased by addition of *Lb. paracasei*, regardless of whether the *L. paracasei* was added before or during the incubation with the pathogen. In contrast to the results of Sherman et al. [44], which studied the same EPEC strain, we demonstrated that in the presence of *Lb. paracasei*, the adhesion of EPEC was reduced, regardless to the protocol used: co-incubation or pre-incubation with *Lb. paracasei*. Indeed, in their study, a reduced adherence of the strain E2348/69 was observed only when host epithelial cells were pre-treated with lactobacilli prior to infection. Similarly to the findings of Mack et al. [45], the pre-incubation procedure was more effective than co-incubation at reducing binding of the two strains. Ingrassia et al. [16] observed that adhesion of EPEC was reduced by 73% when

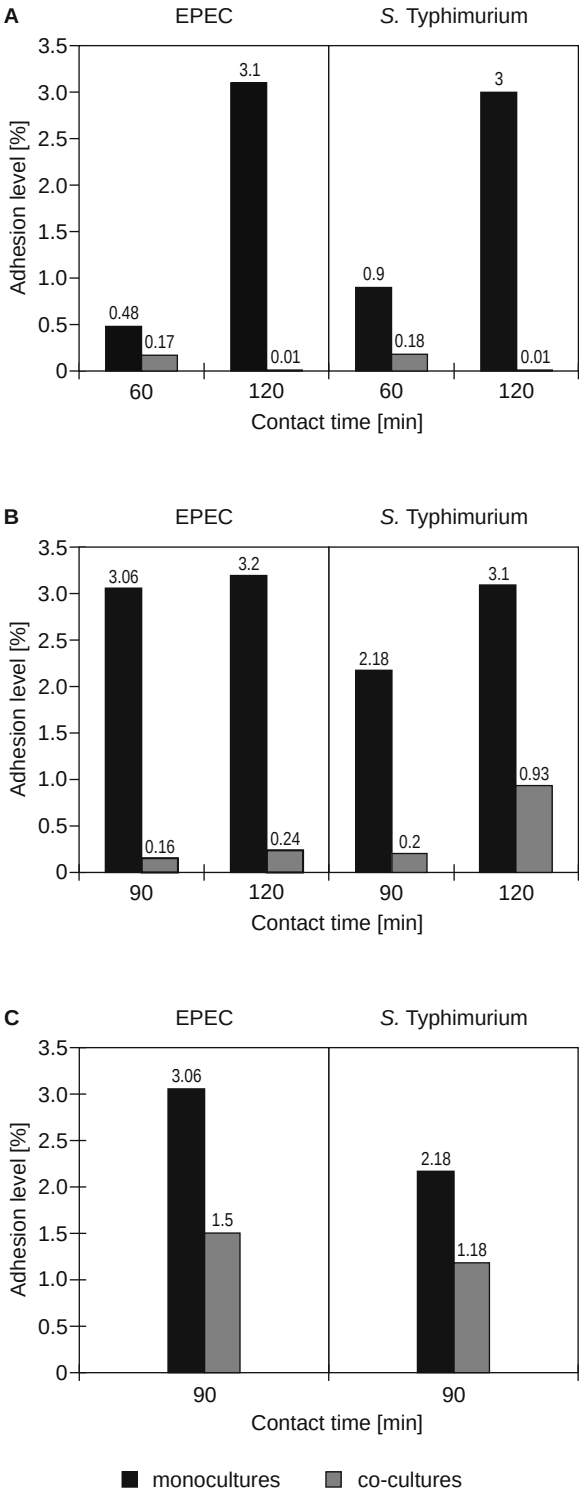

Fig. 6. Adhesion level to Caco-2 cells of the two pathogenic strains (EPEC and *S.* Typhimurium) in the absence and in the presence of *Lb. paracasei* subsp. *paracasei.*

A – pre-incubation (contact 60 min and 120 min, MOI 100); **B** – co-incubation (contact 90 min and 120 min; MOI 1 000 and 100, respectively); **C** – in the presence of *Lb. paracasei* subsp. *paracasei* culture supernatant (contact 90 min, MOI 100).

epithelial cells were exposed to *Lb. casei* prior to infection. In our study, the inhibition levels varied from 64.54% to 99.67% depending on the contact time (1 h or 2 h). The presence of *Lb. paracasei*, as stipulated by FORESTIER et al. [32], may impede the access of pathogens to tissue receptors by steric hindrance and that may explain the decrease of adhesion of the pathogens. It is also possible that *Lactobacillus*-specific products inhibit the adhesion of *Enterobacteriaceae*. It has been previously shown that production of biosurfactants by some strains of *Lactobacillus* can prevent adhesion of pathogens and co-aggregation between lactobacilli and pathogenic microorganisms contributes to creation of a barrier that prevents their adhesion to the epithelia and subsequent access to the tissues, constituting an important host defense mechanism against infections in the urogenital and gastrointestinal tracts [46, 47].

Lactobacillus species in the human intestinal system act as a barrier to infection and contribute to the control of the enteric microbiota by competing with other microorganisms for adherence to epithelial cells, displacing pathogen biofilm and/or inhibiting the growth of potential pathogens. Hence, the use of probiotic strains of lactobacilli is potentially interesting both as preventive and curative agents.

CONCLUSIONS

In the light of our experiments, it seems that the probiotic strain of *Lb. paracasei* would be a good candidate as a protective agent against bacterial enteric infections since it was able to adhere to intestinal cells and to antagonize the growth of enteric-associated pathogens. This strain was isolated from a human intestinal microbiota, belongs to the main species of lactobacilli considered to be predominantly linked to the intestinal microbiota and it has been shown to survive within the human gastrointestinal tract. In vivo studies to evaluate its feasibility as such are in progress.

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REFERENCES

1. Bengmark, S.: Ecological control of the gastrointestinal tract. The role of probiotic flora. Gut, *42*, 1998, pp. 2–7.

2. Vaughan, E. E. – Mollet, B. – deVos, W. M.: Functionality of probiotics and intestinal lactobacilli: light in the intestinal tract tunnel. Current Opinion in Biotechnology, *10,* 1999, pp. 605–610.
3. Saavedra, J. M.: Use of probiotics in pediatrics: rationale, mechanisms of action, and practical aspects. Nutrition and Clinical Practices, *22*, 2007, pp. 351–365.
4. Reid, G. – Jass, J. – Sebulsky, M. T. – McCormick, J. K.: Potential uses of probiotics in clinical practice. Clinical Microbiology Reviews, *16*, 2003, pp. 658–672.
5. Servin, A. L.: Antagonistic activities of lactobacilli and bifidobacteria against microbial pathogens. FEMS Microbiology Reviews, *28*, 2004, pp. 405–440.
6. One day (24–28 h) standardized laboratory protocol for molecular subtyping of *Escherichia coli* O157:H7, non-typhoidal *Salmonella* serotypes, and *Shigella sonnei* by pulsed-field gel electrophoresis (PFGE). In: CDC Centers for Disease Control and Prevention : PulseNet [online]. Atlanta, Georgia: CDC Centers for Disease Control and Prevention, June 2004 [cited 4 February 2011]. <http://www.cdc.gov/pulsenet/protocols/ecoli_salmonella_shigella_protocols.pdf>
7. Davidson, G. – Barnes, G. – Bass, D. – Cohen, M. – Fasano, A. – Fontaine, O. – Guandalini S.: Infectious diarrhea in children: working group report of the First World Congress of Pediatric Gastroenterology, Hepatology, and Nutrition. Journal of Pediatric Gastroenterology and Nutrition, *35*, 2002, pp. 143–150.
8. Prere, M. F. – Bacrie, S. C. – Baron, O. – Fayet, O.: Bacterial etiology of diarrhoea in young children: high prevalence of enteropathogenic *Escherichia coli* (EPEC) not belonging to the classical EPEC serogroups. Pathological Biology, *54*, 2006, pp. 600–602.
9. Finlay, B. B. – Falkow, S.: *Salmonella* as an intracellular parasite. Molecular Microbiology, *3*, 1989, pp. 1833–1841.
10. Swaminathan, B. – Barrett, T. J. – Fields, P.: Surveillance for human *Salmonella* infections in the United States. Journal of AOAC International, *89*, 2006, pp. 553–559.
11. Tauxe, R. V. – Pavia, A. T.: Salmonellosis: nontyphoidal. In: Evans, A. –Brachman, P. (Ed.): Bacterial infections of humans: epidemiology and control. New York: Plenum, 1998, pp. 613–628.
12. Humphrey, T.: Science and society – *Salmonella*, stress responses and food safety. Natural Review of Microbiology, *2*, 2004, pp. 504–509.
13. Burkholder, K. M. – Bhunia, A. K.: *Salmonella enterica* serovar Typhimurium adhesion and cytotoxicity during epithelial cell stress is reduced by *Lactobacillus rhamnosus* GG. Gut Pathogensis, *3*, 2009. DOI: 10.1186/1757-4749-1-14.
14. Finlay, B. B. – Falkow, S.: Common themes in microbial pathogenicity revisited. Microbiology and Molecular Biology Review, *61*, 1997, pp. 136–169.
15. Tuomola, E. M. – Ouwehand, A. C. – Salminen, S. J.: The effect of probiotic bacteria on the adhesion of pathogens to human intestinal mucus, FEMS Immunology and Medical Microbiology, *26*, 1999, pp. 137–142.
16. Ingrassia, I. – Leplingard, A. – Darfeuille-Michaud, A.: *Lactobacillus casei* DN-114 001 inhibits the ability of adherent-invasive *Escherichia coli* isolated from Crohn's disease patients to adhere to and to invade intestinal epithelial cells. Applied and Environmental Microbiology, *71*, 2005, pp. 2880–2887.
17. Fuller, R.: Probiotics in man and animals. Journal of Applied Bacteriology, *66*, 1989, pp. 365–378.
18. Coconnier-Polter, M. H. – Moal, V. L. L. – Servin, A. L.: A *Lactobacillus acidophilus* strain of human gastrointestinal microbiota origin elicits killing of enterovirulent *Salmonella enterica* sérovar Typhimurium by triggering lethal bacterial membrane damage. Applied Environmental Microbiology, *71*, 2005, pp. 6115–6120.
19. Conway, P. L. – Gorbach, S. L. – Goldin, B. R.: Survival of lactic acid bacteria in the human stomach and adhesion to intestinal cells. Journal of Dairy Science, *70*, 1987, pp. 1–12.
20. Charteris, W. P. – Kelly, P. M. – Morelli, L. – Collins, J. K.: Development 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*, 1998, pp. 759–768.
21. Du Toit, M. – Franz, C. M. A. P. – Dicks, L. M. T. – Shillinger, U. – Haberer, P. – Warlies, B. – Ahrens, F. – Holzapfel, W. H.: Characterization and selection of probiotic lactobacilli for a preliminary mini pig feeding trial and their effect on serum cholesterol levels, feces pH and feces moisture content. International Journal of Food Microbiology, *40*, 1998, pp. 93–104.
22. Jacobsen, C. N. – Roesnfeldt Nielsen, A. E. – Moller, P. L. – Michaelsen, K. F. – Paerregaard, A. – Sandstrom, B. – Tvede, M. – Jacobsen, M.: Screening of probiotic activities of forty-seven strains of *Lactobacillus* sp. by *in vitro* techniques and evaluation of the colonization ability of five selected strains in humans. Applied and Environmental Microbiology, *65*, 1999, pp. 4949–4956.
23. Hudault, S. – Lievin, V. – Bernet-Camard, M. F. – Servin, A. L.: Antagonistic activity exerted *in vitro* and *in vivo* by *Lactobacillus casei* (Strain GG) against *Salmonella* typhimurium C5 infection. Applied Environmental Microbiology, *63*, 1997, pp. 513–518.
24. Lievin-Le Moal, V. – Amsellem, R. – Servin, A. L. – Coconnier, M. H.: *Lactobacillus acidophilus* (strain LB) from resident human adult gastrointestinal microflora exerts activity against brush border damage promoted by a diarrhoeagenic *Escherichia coli* in human enterocyte-like cells. Gut, *50*, 2002, pp. 803–811.
25. Patterson, J. A. – Burkholder, K. M.: Application of prebiotics and probiotics in poultry production. Poultry Science, *82*, 2003, pp. 627–631.
26. Eutamene, H. – Bueno, L.: Role of probiotics in correcting abnormalities of colonic flora induced by stress. Gut, *56*, 2007, pp. 1495–1497.
27. Bendali, F. – Gaillard-Martinie, B. – Hebraud, M. –

Sadoun, D.: Kinetic of production and mode of action of the *Lactobacillus paracasei* subsp. *paracasei* antilisterial bacteriocin, an Algerian isolate. LWT Food Science and Technology, *41*, 2008, pp. 1784–1792.
28. Lewus, C. – Sun, S. – Montville, T. J.: Production of an amylase-sensitive bacteriocin by an atypical *Leuconostoc paramesenteroides* strain. Applied and Environmental Microbiology, *58*, 1992, pp. 143–149.
29. Maragkoudakis, P. A. – Zoumpopoulou, G. – Miaris, Ch. – Kalantzopoulos, G. – Pot, B. – Tsakalidou, E.: Probiotic potential of *Lactobacillus* strains isolated from dairy products. International Dairy Journal, *16*, 2006, pp. 189–199.
30. Gunn, J. S.: Mechanisms of bacterial resistance and response to bile. Microbes Infection, *2*, 2000, pp. 907–913.
31. Johnson-Henry, K. C. – Donato, K. A. – Shen-Tu, G. – Gordanpour, M. – Sherman, P. M.: *Lactobacillus rhamnosus* strain GG prevents enterohemorrhagic *Escherichia coli* O157:H7-induced changes in epithelial barrier function. Infection and Immunity, *76*, 2008, pp. 1340–1348.
32. Forestier, Ch. – De Champs, Ch. – Vatoux, C. – Joly, B.: Probiotic activities of *Lactobacillus casei rhamnosus*: *in vitro* adherence to intestinal cells and antimicrobial properties. Research in Microbiology, *152*, 2001, pp. 167–173.
33. Coconnier, M. H. – Liévin, V. – Bernet-Camard, M. F. – Hudault, S. – Servin, A. L.: Antibacterial effect of the adhering human *Lactobacillus acidophilus* strain LB. Antimicrobial Agents and Chemotherapy, *41*, 1997, pp. 1046–1052.
34. Silva, M. – Jacobus, N. V. – Deneke, C. – Gorbach, S. L.: Antimicrobial substance from a human *Lactobacillus* strain. Antimicrobial Agents and Chemotherapy, *31*, 1987, pp. 1231–1233.
35. Ocana, V. S. – Holgado, A. A. P. D. R. – Nader-Macias, M. E.: Characterization of a bacteriocin-like substance produced by a vaginal *Lactobacillus salivarius* strain. Applied and Environmental Microbiology, *65*, 1999, pp. 5631–5635.
36. Schillinger, U. – Geisen, R. – Holzapfell, W.: Potential of antagonistic micro-organisms and bacteriocins for the biological preservation of foods. Trends in Food Science and Technology, *7*, 1996, pp. 158–164.
37. Fernández, M. F. – Boris, S. – Barbés, C.: Probiotic properties of human lactobacilli strains to be used in gastrointestinal tract. Journal of Applied Microbiology, *94*, 2003, pp. 449–455.
38. Apella, M. C. – Gonzalez, S. N. – Nader de Macias, M. E. – Romero, N. – Oliver, G.: *In vitro* studies on the inhibition of the growth of *Shigella sonnei* by *Lactobacillus casei* and *Lact. acidophilus*. Journal of Applied Bacteriology, *73*, 1992, pp. 480– 483.
39. Dunne, C. – O'Mahony, L. – Murphy, L. – Thornton, G. – Morrissey, D. – O'Halloran, S. – Feeney, M. – Flynn, S. – Fitzgerald, G. – Daly, C. – Kiely, B. – O'Sullivan, G. C. – Shanahan, F. – Collins, J. K.: *In vitro* selection criteria for probiotic bacteria of human origin: correlation *in vivo* findings. American Journal of Clinical Nutrition, *73*, 2001, pp. 386–392.
40. Bernet, M. F. – Brassart, D. – Neeser, J. R. – Servin, A. L.: *Lactobacillus acidophilus* LA 1 binds to cultured human intestinal cell lines and inhibits cell-attachment and cell invasion by enterovirulent bacteria. Gut, *35*, 1994, pp. 483–489.
41. Greene, J. D. – Klaenhammer, T. R.: Factors involved in adherence of lactobacilli to human Caco-2 cells. Applied and Environmental Microbiology, *60*, 1994, pp. 4487–4494.
42. Granato, D. – Perotti, F. – Masserey, I. – Rouvet, M. – Golliard, M. – Servin, A. – Brassard, D : 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*, 1999, pp. 1071–1077.
43. Hirano, J. – Yoshida, T. – Sugiyama, T. – Koide, N. – Mori, I. – Yokochi, T.: The effect of *Lactobacillus rhamnosus* on enterohemorrhagic *Escherichia coli* infection of human intestinal cells *in vitro*. Microbiology and Immunology, *47*, 2003, pp. 405–409.
44. Sherman, P. M. – Johnson-Henry, K. C. – Yeung, H. – Ngo, P. – Goulet, J. – Tompkins, T. A.: Probiotics reduce enterohemorrhagic *Escherichia coli* O157:H7 and enteropathogenic *E. coli* O127:H6-induced changes in polarized T84 epithelial cell monolayers by reducing bacterial adhesion and cytoskeletal rearrangements. Infection and Immunity, *73*, 2005, pp. 5183–5188.
45. Mack, D. R. – Michail, S. – Wei, S. – McDougall, L. – Hollingsworth, M. A.: Probiotics inhibit enteropathogenic *E. coli* adherence *in vitro* by inducing intestinal mucin gene expression. American Journal of Physiology, *276*, 1999, pp. 941–950.
46. Gudina, E. J. – Rocha, V. – Teixeira, J. A. – Rodriguez, L. R.: Antimicrobial and antiadhesive properties of a biosurfactant isolated from *Lactobacillus paracasei* ssp. *paracasei* A20. Letters in Applied Microbiology, *50*, 2010, pp. 419–424.
47. Gudina, E. J. – Teixeira, J. A. – Rodriguez, L. R.: Isolation and functional characterization of a biosurfactant produced by *Lactobacillus paracasei*. Colloids and Surfaces B: Biointerfaces, *76*, 2010, pp. 298–304.

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