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VERIFICATION OF PROBIOTIC BACTERIAL PROPERTIES: TOLERANCE TO DIGESTIVE JUICES AND ADHESION TO EPITHELIAL CELLS OF *Lactobacillus acidophilus La-5* AND *Lactobacillus casei 01*

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ABSTRACT. Tolerance to gastric acidity, bile salts and adhesion of probiotic bacteria to intestinal epithelial cells is regarded as a prerequisite to exert beneficial health effects, with this object were examined frecvent utilized probiotic strains. Resistance to gastric acidity and intestinal conditions of *Lactobacillus casei 01, Lactobacillus acidophilus La-5* and adhesion of strains to epithelial cells were investigated in vitro. L. acidophilus *La-5* presents increased tolerance to gastric acidity and intestinal bile salt concentration than *L. casei 01.* Both strains show good adhesion ability to epithelial cells in vitro.

Keywords: probiotics, tolerance, epithelial cells, adhesion

INTRODUCTION

Probiotics are defined as viable microorganisms, which, in sufficient numbers, alter the microflora of a host body compartment and thereby exert beneficial health effect (Shida-Nanno, 2008). The use of probiotics to enhance intestinal health has been proposed for many years. As recently revisited by the Joint Food and Agriculture Organization/World Health Organization: probiotic strains are defined as live micro-organisms that, when consumed in an adequate amount as part of food, confer a health benefit on the host. Probiotic strains are considered non-pathogenic and safe (Servin, 2003).

Recent research has demonstrated that probiotics can prevent pathogen colonization of the gut and reduce the incidence or relieve the symptoms of various diseases caused by dysregulated immune responses. Probiotics seem to function by influencing both intestinal epithelial cells and immune cells of the gut, but the details of these effects are still being unraveled. The selection of the most suitable ones is crucial for their use in the prevention or treatment of specific diseases (Shida-Nanno, 2008).

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Lactic acid bacteria are regarded as a major group of probiotic bacteria. Lactic acid bacteria are usually described as Gram-positive microorganisms, devoid of cytochromes and preferring anaerobic conditions but are aerotolerant, fastidious, acid-tolerant, and strictly fermentative, producing lactic acid as a main product. The most important genera are: *Lactobacillus, Lactococcus, Enterococcoccus, Streptococcus, Pediococcus, Leuconostoc,* and *Bifidobacterium* (Vasiljevic-Shah, 2008).

In order to exert their functional properties, probiotics need to be delivered to the desired sites in an active and viable form. The viability and activity of probiotics in the products have been frequently cited as a prerequisite for achieving numerous beneficial health benefits (Aswathy, 2008).

One of the most important criteria for the potential of putative probiotic strain is to overcome the gastric environment, the presence of bile salts and to resist, through adhesion, the flux of the intestinal content (Vasiljevic-Shah, 2008). Strains belonging to species normally inhabiting the human gut have been shown to be better when assayed for their *in vitro* resistance to low pH or to simulated gastric juice (Morelli, 2007).

Our aim is to study the essential probiotic properties of two lactic acid bacterial strains, *Lactobacillus acidophilus* La-5 and *Lactobacillus casei* 01*.* These strains are nowadays frequently utilized in probiotic products and their surviving rate in digestive track is important to colonize the intestine and to exert their beneficial properties on the host. These properties are: tolerance to gastric and intestinal environment and adhesion capacity to epithelial cells *in vitro*.

RESULTS AND DISCUSSIONS

Tolerance to simulated gastric and intestinal juice

On the basis of the obtained results in determination of tolerance to simulated gastric juice, which contains hydrochloric acid and pepsin, it can be observed that in case of both studied probiotic strains at pH=2 after an incubation period of 30 minutes there were no viable cells (Figure 1., Figure 2.). In acidic conditions at pH=3 in presence of hydrochloric acid and pepsin in case of *Lactobacillus acidophilus* La-5, after 30 minutes of incubation increased the number of viable cells, after that the number of cells remained constant. If the period of exposure is more than 60 minutes, the number of viable cells decreased (Figure 1.). In conditions of pH=4 the number of viable *Lactobacillus acidophilus* La-5 cells decreased in time in simulated gastric juice (Figure 1.).

The number of viable cells of *Lactobacillus casei 01* in acidic conditions at pH=3 decreased in time and decreased drastic at pH=4 (Figure 2.).

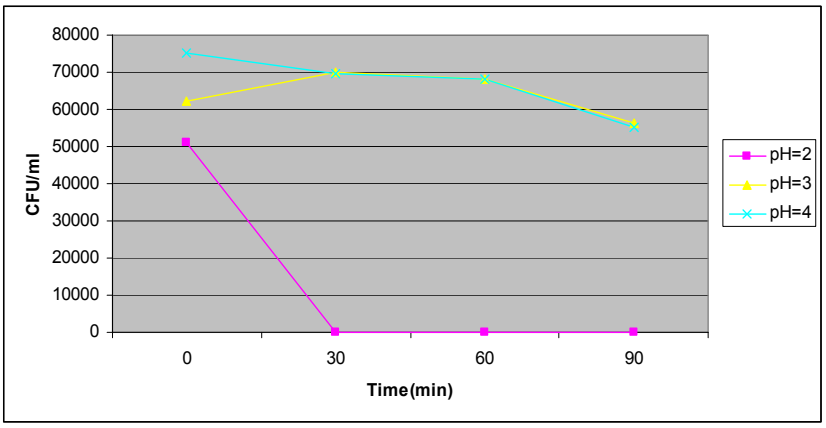

Figure 1. Variation of colony-forming unit (CFU)/ml at different pH values of *Lactobacillus acidophilus* La-5 incubated in simulated gastric juice.

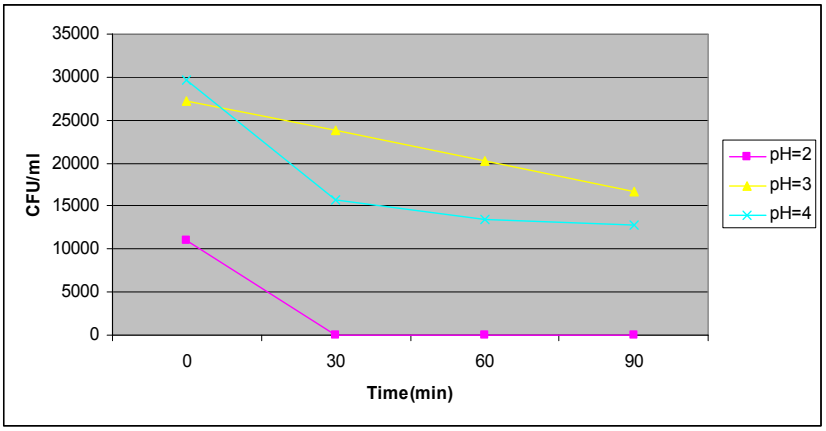

Figure 2. Variation of CFU/ml at different pH values of *Lactobacillus casei* 01 incubated in simulated gastric juice.

Results show that viability of *Lactobacillus casei* 01 strain in simulated intestinal juice - with different concentrations of bile salts (0,3%, 0,5%, 1%) and pancreatin - is very low, after a period of 30 minutes of incubation, there are no colony forming units. *Lactobacillus acidophilus* La-5 is not so sensible, there is a decrease in the number of viable cells after different periods of incubation, but exists the possibility of surviving at low bile salt concentrations (Figure 3.).

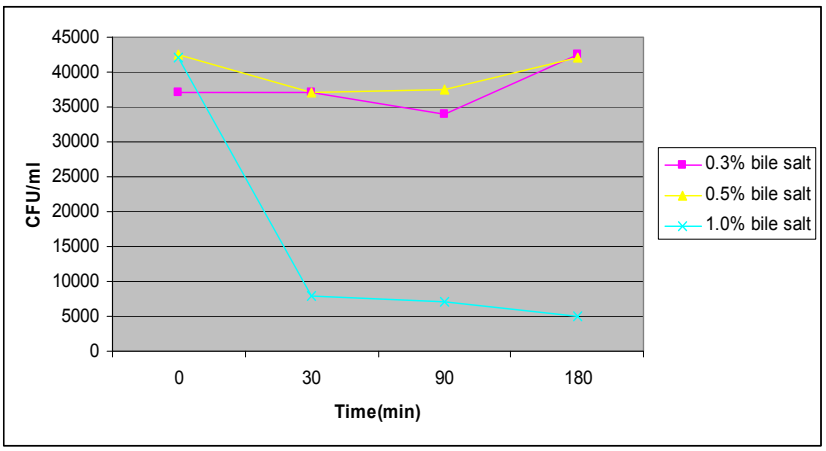

Figure 3. Variation of CFU/ml at different bile salt concentrations of *Lactobacillus acidophilus* La-5 incubated in simulated intestinal juice.

Adhesion capacity

Adhesion ability was determined in 20 random microscopic fields after Gram-staining, results show that adherence of examined probiotic strains is good to IEC-6 epithelial cells in *in vitro* conditions. Adhesion ability results are expressed in next form: number of adhered bacterial cells/100 epithelial cell. In case of *L. acidophilus* La-5 this number is: 265 bacterial cells/100 epithelial cells, adhesion capacity of *L. casei* 01 is 250 bacterial cells/100 epithelial cells. Results confirm that strain *Lactobacillus acidophilus* La-5 has better adhesion capacity to the epithelial cells compared with *Lactobacillus casei* 01, but both probiotic strains show good adhesion capacity compared with results obtained in another studies. Figure 4. and 5. show the microscopical view of *L. acidophilus* La-5 and *L. casei* 01 adhesion to epithelial cells after Gram staining.

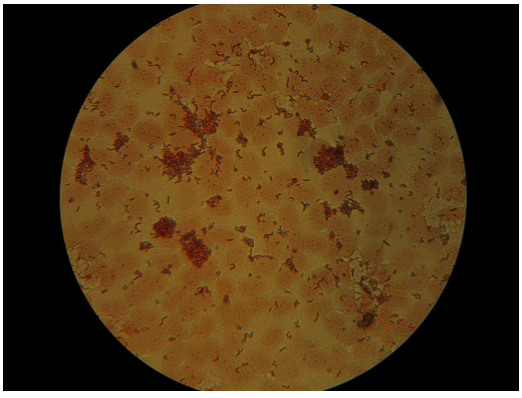

Figure 4. Adhesion of *Lactobacillus acidophilus* La-5 to IEC-6 cells.

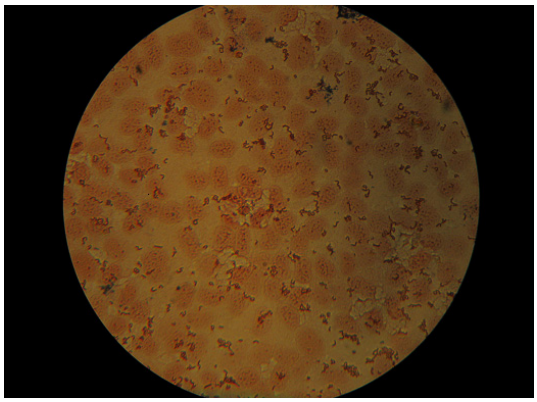

Figure 5. Adhesion of *Lactobacillus casei* 01 to IEC-6 cells.

CONCLUSIONS

The acidity of gastric juice influences the viability of probiotic strains, which manifests in different modes in case of different bacterial stains.

Bacterial strain *Lactobacillus casei* 0.1 is more sensible than *Lactobacillus acidophilus* La-5 at acidic environment.

Lactobacillus casei 01 is very sensitive at bile salts presence.

Bacterial strains from this study are more resistant in gastric juice than in intestinal juice.

Under in vitro conditions, both bacterial strains show good adhesion ability to epithelial cells.

EXPERIMENTAL SECTION

Tolerance determination of probiotic bacteria to simulated gastric juice

Bacterial cultures obtained on MRS agar media (de Man, 1960) after an incubation time of 48 hours at 37^{o}C are suspended in sterile 0.5 w/v% sodium chloride solution (10^{5}CFU= colony forming units/ml). Gastric juice is prepared by suspending pepsin (3 g/l) and regulating the pH at 2.0, 3.0 and 4.0 values with cc. hydrochloric acid or with sterile solution of 0.1 M sodium hydroxide, using a pH-metre.

One aliquot (0.2 ml) from each suspension of bacterial cells is transferred in a 2 ml sterile Eppendorf tube and mixed with 0.3 ml 0.5 w/v% sterile sodium chloride solution and 1.0 ml simulated gastric juice (pH 2.0, pH 3.0 and pH 4.0). This mixture is vortexed for maxim 10 s and incubated at 37^{o}C.

Viability of strains is analysed by determination of CFU/ml after different periods of incubation (0 min, 30 min, 60 min and 90 min in the simulated gastric juice) by inoculation on MRS agar solid media, after an incubation time of 48 hours at 37°C.

Tolerance determination of probiotic bacteria to simulated intestinal juice

Bacterial cultures obtained on MRS agar media after an incubation time of 48 hours at 37°C are suspended in sterile 0.5 w/v% sodium chloride solution (10^5CFU= colony forming units/ml). Intestinal juice is prepared by resolving in sterile sodium chloride solution (0.5 w/v%) bile salts in different concentrations (0.3%, 0.5%, 1.0%) and pancreatin (1 g/l), pH is regulated to 8.0.

One aliquot (0.2 ml) from each suspension of bacterial cells is transferred in a 2 ml sterile Eppendorf tube and mixed with 0.3 ml 0.5 w/v% sterile sodium chloride solution and 1.0 ml simulated intestinal juice (0.3%, 0.5%, 1.0% bile salt). This mixture is vortexed for maxim 10 s and incubated at 37°C.

Viability of strains is analysed by determination of CFU/ml after different periods of incubation (0 min, 30 min, 90 min and 180 min in the simulated intestinal juice) by inoculation on MRS agar solid media, after an incubation time of 48 hours at 37°C.

***In vitro** adhesion study*

IEC-6 cells were grown in Minimal Essential Medium (MEM) Earle's Base, supplemented with 5% (v/v) foetal bovine serum (FBS), 0.1 IU/ml insulin and 1% gentamicin. Incubation was at 37°C in the presence of 5% CO_2. The media was changed every second day. Adhesion assays were performed with cells at late post-confluence (15 days in culture). IEC-6 cells were seeded at 10^5 cells per well in 12-well microtitre plates to obtain confluence. Incubation was at 37°C in the presence of 5%CO_2. Before the adherence assay, IEC-6 cells were washed twice with sterile phosphate-buffered saline (PBS, 6.0 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 0.14 M NaCl, 3.0 mM KCl, pH=7.3). Cultures of bacterial strains were harvested (10,000Xg, 10 min, 4°C), the cells washed twice with sterile PBS, and diluted in MEM (without FBS and gentamicin) to OD_{600}=0.5, i.e. approximately 10^6 CFU/ml. Wells with IEC-6 cells were inoculated with 10^5 viable cells of each bacterial cell suspension and incubated at 37°C for 2 h. Non-adhering bacterial cells were then withdrawn from the wells and the IEC-6 cells washed twice with 1 ml sterile PBS, followed by 1 ml 0.5% (v/v) Triton X-100. Fixed with 90% methanol and Gram stained. The bacterial adhesion was examined microscopically. The number of bacteria adhered to IEC-6 cells was counted in 20 random microscopic fields.

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