

ACID AND BILE TOLERANCE, ADHESION TO EPITHELIAL CELLS OF PROBIOTIC MICROORGANISMS

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*Toleranța față de aciditatea gastrică și față de sărurile biliare, precum și adeziunea bacterilor probiotice la celulele epiteliale sunt considerate condiții minime necesare pentru un efect benefic asupra stării de sănătate. Rezistența tulpinilor *Lactobacillus casei* 01 și *Lactobacillus acidophilus* La-5 la pH=2-4 și la o concentrație de săruri biliare 0,3-1,0%, precum și adeziunea lor la celule IEC-6 a fost verificată in vitro. Capacitatea de adeziune a tulpinilor a fost evidențiată cu metoda colorației Gram. Tulpina *L. casei* 01 prezintă o sensibilitate mai ridicată la aciditatea gastrică decât *L. acidophilus* La-5. *L. casei* este foarte sensibilă față de săruri biliare. Ambele tulpini prezintă o capacitate bună de adeziune la celule epiteliale.*

*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. Resistance to pH 2-4 and 0,3-1% bile salt concentration of *Lactobacillus casei* 01, *Lactobacillus acidophilus* La-5, as well as adhesion of strains to IEC-6 cell line were investigated in vitro. The adhesion ability of the tested strains was quantified with Gram-staining method. *L. casei* 01 presents increased sensibility to gastric acidity than *L. acidophilus* La-5. *L. casei* 01 is very sensitive to action of bile salts. Both strains show good adhesion ability to epithelial cells.*

Keywords: probiotics, tolerance, adhesion, epithelial cells

1. Introduction

Probiotics are commonly defined as living microorganisms which, when administered in adequate amounts, confer health benefit to the host. Probiotics are living bacteria exhibiting health-promoting activities. Recent research has

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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 and immune cells of the gut, but the details of these effects are still being unravelled. Therefore, probiotics, through their effects on the host immune system, might ameliorate diseases triggered by disordered immune responses. Caveats remain and, because the beneficial effects of probiotics can vary between strains, the selection of the most suitable ones will be crucial for their use in the prevention or treatment of specific diseases [1].

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 [2].

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 [3].

2. Experimental

2.1. Acid tolerance determination of probiotic bacteria in presence of different concentrations of hydrochloric acid

Bacterial cultures were obtained on MRS agar media (this medium is named by its inventors: de Man, Rogosa and Sharpe, developed in 1960, it was designed to favour the growth of *Lactobacilli* for lab study). After an incubation time of 48 hours at 37°C are suspended in sterile 0.5w/v% sodium chloride solution (10^5 CFU= colony forming units/mL). Gastric juice is prepared by regulation of pH at 2.0, 3.0 and 4.0 values with cc. hydrochloric acid or with sterile solution of 0.1M sodium hydroxide, using a pH-metre.

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

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

2.2. Bile salt tolerance determination of probiotic bacteria in presence of different concentrations of bile salts

Bacterial cultures obtained on MRS agar media after an incubation time of 48 hours at 37°C are suspended in sterile 0.5w/v% sodium chloride solution (10⁵CFU= colony forming units/mL). Intestinal juice is prepared by resolving in sterile sodium chloride solution (0.5w/v%) bile salts in different concentrations (0.3%, 0.5%, 1.0%), pH is regulated at 8.0.

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

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

2.3. *In vitro* adhesion study

IEC-6 cells were grown in Minimal Essential Medium (MEM) Earle's Base, supplemented with 5% (v/v) fetal bovine serum (FBS), 0.1IU/mL insulin and 1% gentamicin. Incubation was at 37°C in the presence of 5%CO₂. 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 10⁵ cells per well in 12-well microtitre plates to obtain confluence. Incubation was at 37°C in the presence of 5%CO₂. Before the adherence assay, IEC-6 cells were washed twice with sterile phosphate-buffered saline (PBS, 6.0mM Na₂HPO₄, 1.5mM KH₂PO₄, 0.14M NaCl, 3.0mM KCl, pH 7.3). Cultures of bacterial strains were harvested (10,000Xg, 10min, 4°C), the cells washed twice with sterile PBS, and diluted in MEM (without FBS and gentamicin) to OD₆₀₀=0.5, i.e. approximately 10⁶CFU/mL. Wells with IEC-6 cells were inoculated with 10⁵ viable cells of each bacterial cell suspension and incubated at 37°C for 2h. Non-adhering bacterial cells were then withdrawn from the wells and the IEC-6 cells washed twice with 1mL sterile PBS, followed by 1mL 0.5% (v/v) Triton X-100. then they were 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.

3. Results and discussions

3.1. Acid and bile tolerance

On the basis of the obtained results after 30 minutes at pH 2 in gastric juice, there were no viable cells (Fig. 1, Fig. 2). At pH 3 the number of viable *Lactobacillus acidophilus* La-5 cells decreases in time (Fig. 1), in case of *Lactobacillus casei* 01, the number of viable cells remains constant (Fig. 2.). pH 4 is the optimal pH in case of *Lactobacillus acidophilus* La-5; after a period of adaptation (30min), the number of viable cells is increasing (Fig. 1.). *Lactobacillus casei* 01 is sensible at this pH value until 30 min of incubation when the number of viable cells is decreasing (Fig. 2.).

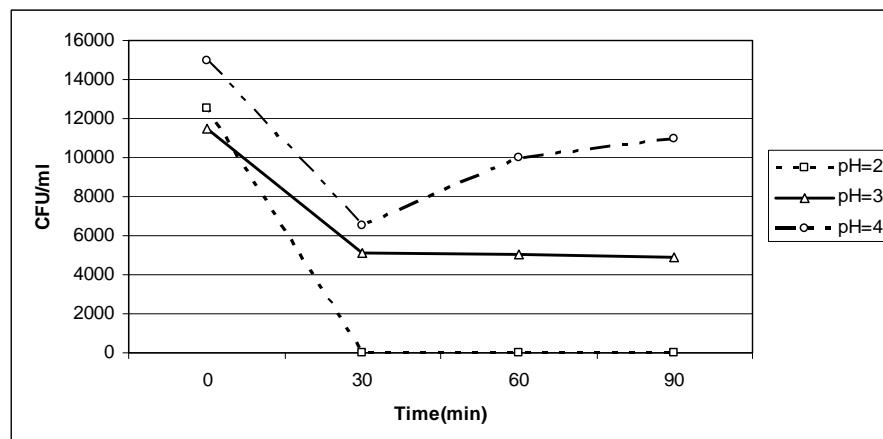


Fig. 1. Variation of CFU/mL at different pH values of *Lactobacillus acidophilus* La-5 incubated in simulated gastric juice

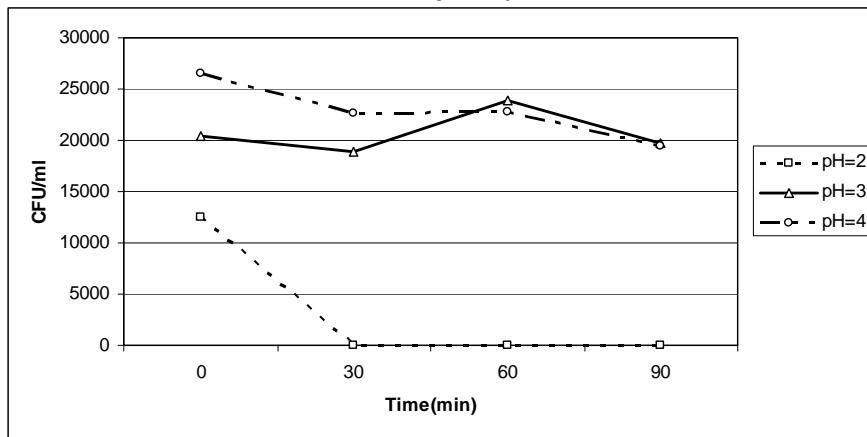


Fig. 2. Variation of CFU/mL at different pH values of *Lactobacillus casei* 01 incubated in simulated gastric juice

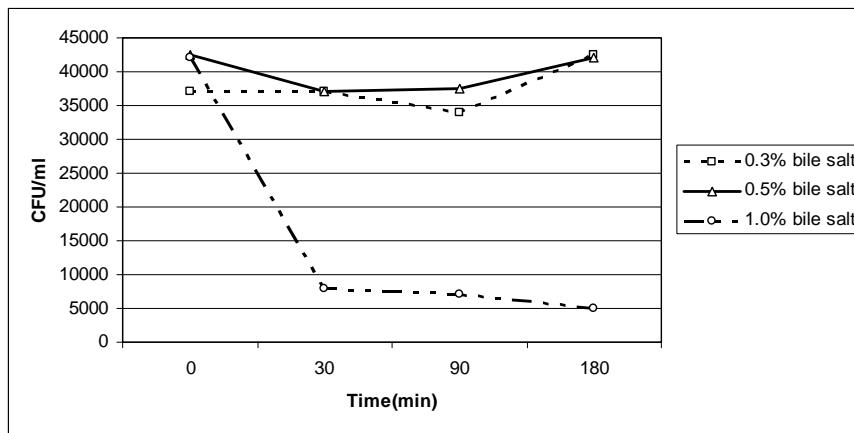


Fig. 3. Variation of CFU/mL at different bile salt concentrations of *Lactobacillus acidophilus* La-5 incubated in simulated intestinal juice

The obtained results show that *Lactobacillus casei* 01 strain is very sensible on different bile salt concentrations (0.3%, 0.5%, 1%), after 30min of incubation of cells with simulated intestinal juice, there are no viable cells. In case of *Lactobacillus acidophilus* La-5 is an important decrease in the number of viable cells, but there is the possibility of surviving at low bile salt concentrations (Fig. 3).

3.2. Adhesion capacity

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

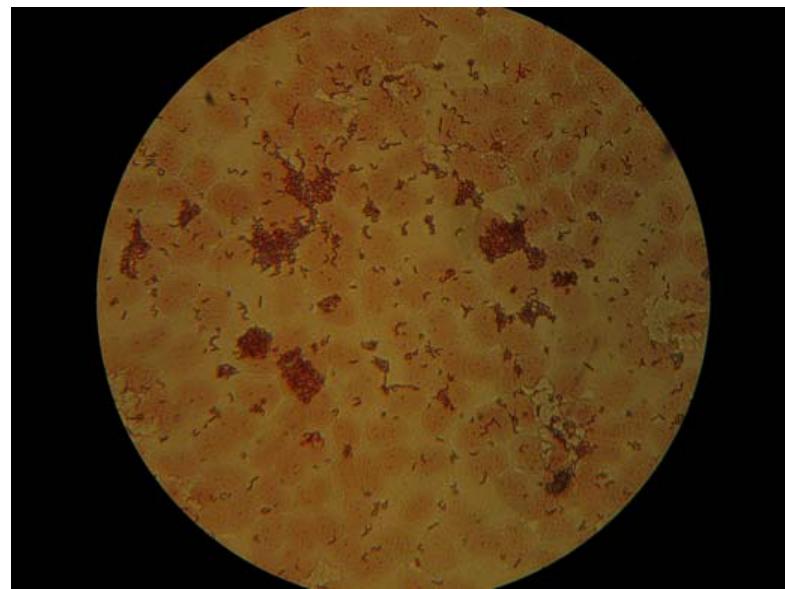


Fig. 4. Adhesion of *Lactobacillus acidophilus* La-5 to IEC-6 cells

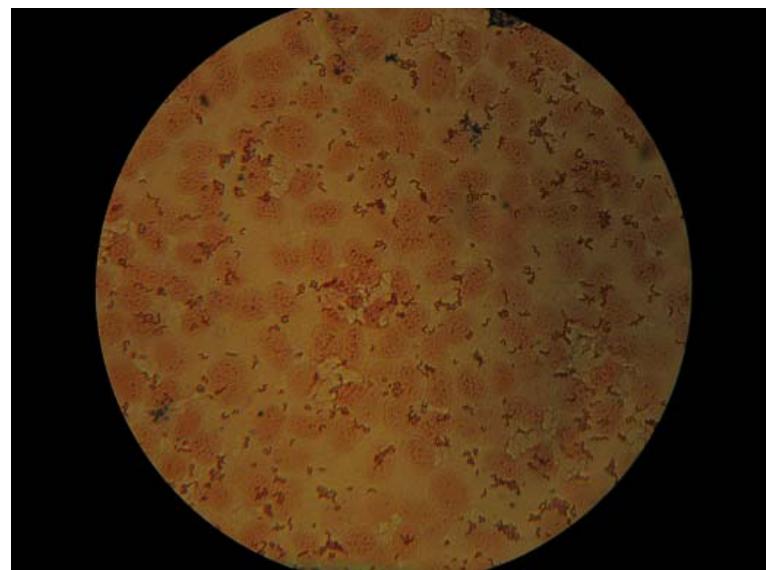


Fig. 5. Adhesion of *Lactobacillus casei* 01 to IEC-6 cells

4. Conclusions

- The acidity of gastric juice, which is determined as hydrochloric acid concentration, influences the viability of probiotic strains, which manifests in different modes, depending on bacterial strains.
- Bacterial strain *Lactobacillus casei* 01 is more sensible than *Lactobacillus acidophilus* La-5 in acidic environment.
- *Lactobacillus casei* 01 is very sensible in the presence of bile salts.
- Bacterial strains are more resistant in gastric juice than in intestinal juice.
- Both bacterial strains show good adhesion ability to epithelial cells in conditions *in vitro*.

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