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SELECTION OF LACTIC ACID BACTERIA ISOLATED FROM TRADITIONALLY MANUFACTURED CHEESE VARIETIES

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ABSTRACT. In this report lactic acid bacteria, isolated from traditionally manufactured cheeses were selected by their resistance to gastric acidity and bile salts, adhesion to epithelial cells and inhibition of pathogen growth. 39 bacterial strains were isolated on selective media. Four bacterial strains were selected by their increased capacity to tolerate gastric acid and bile salts, each strains showing good adhesion capacity to IEC-6 intestinal epithelium cells. Some of selected strains inhibit growth of pathogenic bacteria by their metabolites.

Keywords: *probiotics, isolation, tolerance, epithelial cells, adhesion*

INTRODUCTION

Lactic acid bacteria and other probiotic microorganisms have many documented health effects [1]. Lactic acid bacteria (LAB) are present in the intestine of most animals, the beneficial effects of these microorganisms on human and animal health, including the effect on the immune system, has been extensively reported. LAB are present in many functional foods and are frequently used as probiotics to improve their biological effects on the host. The activation of immune response by LAB requires many complex interactions among the different constituents of the intestinal ecosystem (microbiota, epithelial cells and immune cells). They are acid-tolerant, strictly fermentative, gram-positive microorganisms, which produce lactic acid as main product. They are anaerobe and aerotolerant, as well. The most frequently cited definition is Fuller's [2], who defined probiotics as "a live microbial feed supplement, which beneficially affects the host animal by improving its intestinal microbial balance" [3].

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One of the most important criteria for probiotic bacteria is that the strains belonging to bacterial species that are present in the intestinal microbiota could have a better chance of survival [4]. Many mechanisms have been described by which probiotics could enhance intestinal health, such as competition for nutrients, inhibition of the epithelial and mucosal adherence of pathogens, inhibition of epithelial invasion by pathogens, the production of antimicrobial substances (lactic/acetic acid, hydrogen peroxide, bacteriocins) and the stimulation of mucosal immunity [5].

The prevention of pathogen colonization of the gut by probiotic bacteria is demonstrated in many recent researches. Probiotics influences both intestinal epithelial cells and immune cells of the gut, but these effects are still being unraveled. Probiotics, through their effects on the host's immune system, might ameliorate diseases triggered by disordered immune responses [6].

Our aim is to isolate lactic acid bacteria from cheese and to select them basing on their essential probiotic properties. 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 the following: tolerance to gastric acidity and bile salts, adhesion to epithelial cells and inhibiting pathogen bacterial growth.

RESULTS AND DISCUSSIONS

Isolation of lactic acid bacteria

LAB were isolated from traditionally manufactured cheese by appropriate dilutions with saline, plated on MRS (de Man Rogosa Sharpe) [7] agar. Cheese varieties were collected from sheepfolds (Harghita county, Roumania), from traditional manufacturers. Cheese varieties raw material was sheep milk, products were fresh-made (ripened 24 h).

Thirty-nine well-isolated colonies were picked up and transferred to MRS agar in test tubes and reinoculated to obtain pure cultures. Then they were stored in MRS agar at $4^{o}C$.

The isolated strains were tested for their biochemical and morphological properties. The strains were Gram-stained and examined microscopically for cellular morphology and Gram-stain phenotype, tested for catalase and oxidase-activity, lactose digestion, oxygen utilization and colony morphology. All of the examined strains were gram-positive (14 cocci and 25 rods), catalase-negative, oxidase-negative (37 of 39 strains). 30 of 39 strains ferment lactose to acids. Most of them are anaerobes (23 strains), aerobe-facultative anaerobes (5 strains), anaerobe-facultative aerobes (9 strains) and microaerofiles (2 strains). To describe the colony morphology, the following aspects must be taken into account: diameter, form, margin, elevation, color, surface and

density. The diameter of colonies varies between 0.1-6.0 mm, their form is round, and their margin is regulated in 29 cases, dentated at 4 strain-colonies and irregular in the case of 6 strains. The color of colonies are white at 30 strains, cream-colored at 8 strains and cream-colored with yellow center in the case of one strain colony. The surface in both cases was bright, the density of colonies varied from translucent to opaque (17 translucent, 5 opaque and 17 translucent, but opaque at center).

The selected strains were identified genetically by 16S ribosomal DNA sequencing, strain Szj102.1., SRj104.2.: *Lactobacillus brevis,* strain SRj102.2.2.*: Serratia quinivorans*, SGt102.2.2: *Enterococcus faecium.*

Tolerance to simulated gastric and intestinal juice

Basing on the results obtained in the determination of tolerance to simulated gastric juice, which contains hydrochloric acid (pH=2), it can be observed that 26 bacterial strains have high sensitivity to hydrochloric acid (i.e. there are no viable cells after 90 minutes of incubation), at 4 strains high tolerance to acidic conditions can be established, (the viability rate of these strains is higher than 40%), 7 strains show viability between 10-30%, and two of the isolated strains have viability under 10% compared to the initial colony forming unit (CFU) numbers (Figure 1.).

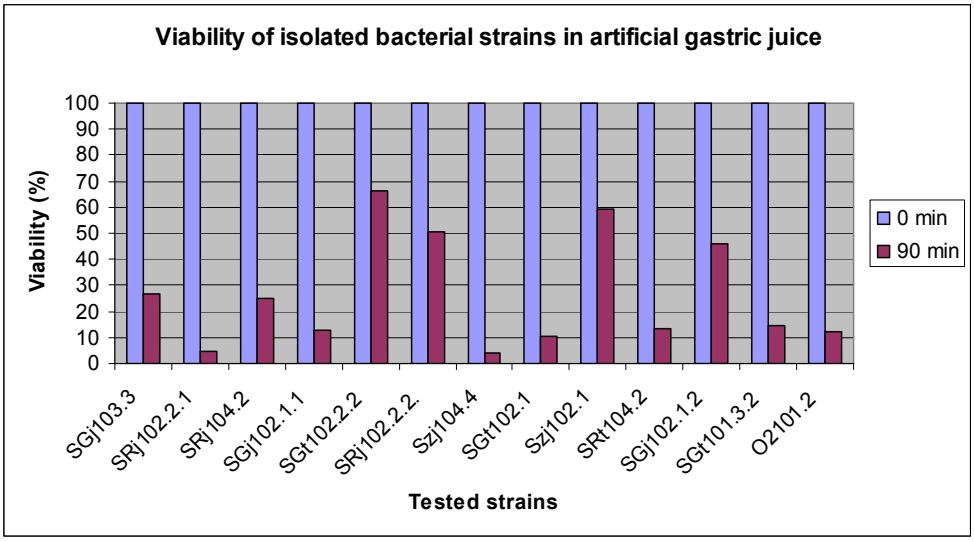

Figure 1. Viability rate of tested bacterial strains incubated in artificial gastric juice.

Simulated intestinal juice has a drastic effect on lactic acid bacteria; in the case of 7 strains there can not be observed viable cells after 180 minutes of incubation. 4 bacterial strains show better tolerance to bile salts, their viability rate is positive (Figure 2).

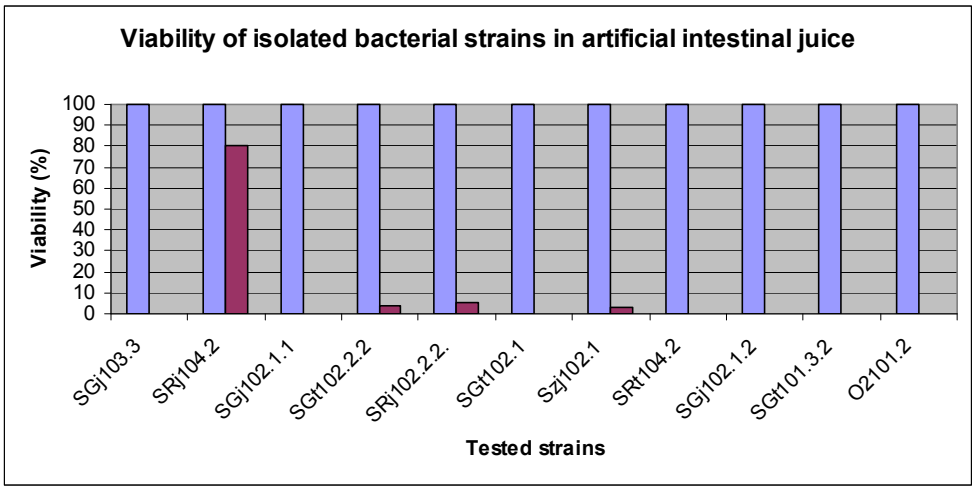

Figure 2. Viability rate of tested bacterial strains incubated in artificial intestinal juice.

In vitro adhesion study

The adhesion ability was determined after gram-staining. The results show that the adherence of the examined probiotic strains is good to IEC-6 epithelial cells in *in vitro* conditions. The adhesion ability results are expressed in the following form: number of adhered bacterial cells/100 epithelial cell.

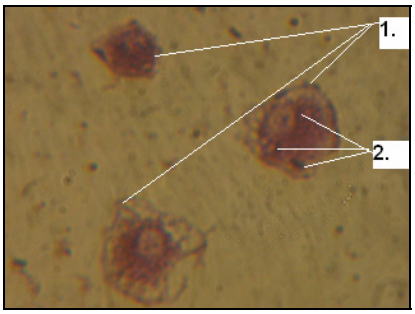

Figure 3. Bacterial cells (2) adhered on the surface of intestinal epithelial cells (1), after gram-staining.

Taking into account previous examinations, using known probiotic strains [8] and observing on microscope the adhesion-capacity of 20 randomly selected epithelial cells, we drew the conclusion that their ability proved to be good, from which it follows that the adhesion capacity of the analyzed strains is: SGt 102.2.2.: 240 bacterial cells adhered/100 epithelial cells, Szj102.1.: 180 bacterial cells adhered /100 epithelial cells, SRj104.2.: 133 bacterial cells adhered/100 epithelial cells, SRj 102.2.2.: 233 bacterial cells adhered /100 epithelial cells.

Antibiosis tests

The Petri-dishes containing the nutritive media with pathogen bacteria and the supernatants of lactic acid bacterial strains were incubated for 48 hours. After the incubation the inhibition zone diameter can be analized. In the case of *Salmonella enteritidis* an inhibition of growth can be observed around the following supernatants: SRj102.2.2, Szj102.1 and SGt102.2.2 (see Figure 4.). The diameters of the inhibition zones are listed in Table 1. There is no discernible inhibition on growth in the case of the following bacteria: *Staphylococcus aureus* 5973, *Bacilus cereus* 2, *Escherichia coli* ATCC 13706 and *Listeria monocytogenes.*

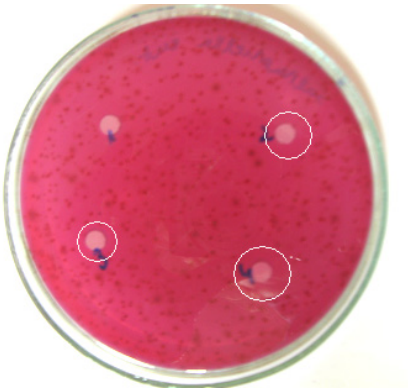

Figure 4. Inhibition of *Salmonella enteritidis* by lactic acid bacterial supernatants (1- SRj104.2., 2- SGt102.2.2., 3- SRj102.2.2., 4- Szj102.1.).

Table 1. Inhibition zones of different lactic acid bacterial strains (n=6).

Tested strain	Diameter (mm) of inhibition zone against *Salmonella enteritidis*
Lactobacillus brevis Szj102.1.	4.333 ± 0.471405
Serratia quinivorans SRj102.2.2.	3.333 ± 0.471405
Enterococcus faecium SGt102.2.2.	4.166 ± 0.235702
Lactobacillus brevis SRj104.2.	0

CONCLUSIONS

39 bacterial isolates were characterized from a biochemical and morphological point of view. The isolates were selected basing on their tolerance to gastric acid an bile salts, as well as on their adhesion to epithelial cells and inhibition of pathogen growth. There are strains with increased gastric acid- and bile salt-tolerance and their adhesion on the epithelial cell- surface is good, three of the strains have bacteriostatic effects on *Salmonella enteritidis.* Basing on the results obtained, after identifying the bacterial isolates and examining their effects on the immune system, we have the chance to find probiotic bacterial strains.

EXPERIMENTAL SECTION

Isolation of lactic acid bacteria

Lactic acid bacteria were isolated from cheeses on selective media (MRS agar), obtaining pure colonies. LAB were isolated from traditionally manufactured cheese by appropriate dilutions with saline, plated on MRS agar and incubated aerobically or anaerobically at 37^{o}C for 2-3 days. Thirty-nine well-isolated colonies were picked up and transferred to MRS agar in test tubes and reinoculated to obtain pure cultures. Then they were stored in MRS agar at 4^{o}C.

The isolated strains were tested for their biochemical and morphological properties. The strains were gram-stained and examined microscopically for cellular morphology and Gram-stain phenotype, tested for catalase and oxidase-activity, lactose digestion, oxygen utilization and colony morphology.

Tolerance to simulated gastric and intestinal juice

The bacterial cultures obtained on MRS agar media after an incubation time of 48 hours at 37^{o}C are suspended in sterile 0.5 w/v% sodium chloride solution (10^5CFU= colony forming units/ml). The tolerance test is carried out as described elsewhere [8].

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 gentamicin, ampicillin and kanamycin. Incubation was at 37^{o}C in the presence of 5% CO_2. The medium 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 microtiter

plates to obtain confluence. The incubation was at 37^{o}C in the presence of 5% CO_2. Before the adherence assay, IEC-6 cells were washed twice with sterile phosphate-buffered saline solution. Adhesion study is described elsewhere [8].

Antibiosis tests

Lactic acid bacterial strains were cultivated in MRS-broth for 10 days at 37^{o}C. Their supernatans were used for this study. Pathogen bacterial strains: *Salmonella enteritidis*, *Staphylococcus aureus* 5973, *Bacilus cereus* 2, *Escherichia coli* ATCC 13706 and *Listeria monocytogenes* were cultivated for 24 hours and suspended in agar media. Then a drop of 0.1 ml of the broth culture of the isolated strains was placed in the wells cut, and incubated at 37°C for 48 hours and observed for inhibition zones.

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