

***In vitro* selection of some lactic acid bacteria strains with probiotic potential**

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Abstract

Developing new strategies for the prevention and treatment of bacterial infections became a major international problem, the probiotic products based on lactic acid bacteria (LAB) strains representing an alternative. The aim of this study was to investigate the antimicrobial activity of LAB strains, the virulence factors, the ability to survive the simulated gastro-intestinal (GI) conditions and antibiotic susceptibility, in order to select effective and safe probiotic strains.

A number of 170 LAB strains were subjected to screening tests regarding the safety assessment and probiotic properties. The probiotic potential was investigated by detection of bacteriocin production, antimicrobial and gastro-intestinal survival tests. The safety of probiotic LAB strains was evaluated by antibiotic susceptibility tests and screening for soluble virulence factors.

Over 75% of strains showed antimicrobial activity and were selected for future studies. About 40% of LAB presenting hemolytic activity were removed from the study. From the remaining strains, 44 (42.7%) showed high resistance to GI condition, and were selected for antibiotic susceptibility tests. To prevent the risk of horizontal genes transfer to the intestinal pathogens, 23 strains presenting acquired antibiotic resistance were removed from the study. Finally, we selected 21 safe probiotic LAB strains which can be used in probiotic products.

Keywords: antimicrobial activity, bacteriocins, antibiotic susceptibility, gastro-intestinal conditions, survival, hemolysins, safety.

1. Introduction

The emergence of bacterial resistance to antibiotics is a major public health problem in the entire world. Possibility of developing alternative strategies for prevention and treatment of the infections with resistant and biofilm forming bacteria has become a priority issue. Probiotics products are viable alternatives to antibiotic treatment because in very rare cases have side effects.

Probiotics influence the composition of intestinal microbiota by competition for nutrients and binding sites and through synthesis of antimicrobial substances, like biosurfactants, organic acids, hydrogen peroxide and bacteriocin/bacteriocin-like compounds. Bacteriocins are proteinaceous antimicrobial substances and are used primarily in food industry as biopreservatives.

According to guidelines developed by FAO and WHO (FAO/WHO [1]), the characteristics of a good probiotic are: benefic effects, lack of pathogenicity and toxicity, the ability to survive in the gastrointestinal tract, resistance at low pH, bile salts and enzymes and the ability to persist in the host organism, adherence to intestinal epithelium to resist

peristalsis, and the ability to interact with immune cells in the gut (DE VUYST & VANDAMME [2]).

Therefore, is important to evaluate the safety of each strain intended to be used in probiotic products. An essential safety criterion in probiotic LAB strains selection is the investigation of antibiotic susceptibility, because strains carrying acquired antibiotic resistance genes may provoke transmission of antibiotic resistance to the pathogenic microorganisms from gastro-intestinal (GI) tract. The safety of selected probiotic strains should also be evaluated for potential virulence factors producing ability and hemolysin activity is the most potent virulence factor playing an important role in the severity of human infections (LJUNGH & WADSTRÖM [3], RADULOVIĆ & al. [4], LIU & al. [5], TOME & al. [6]). Also, in order to exert their beneficial effects, probiotic strains must be able to survive in the acidic and proteolytic digestion conditions from the stomach environment, as well as to resist the effects of bile and pancreatic juice from the upper small intestine. Hence, the evaluation of viability and sufficient survival through GI passage is one of the crucial tests needed in selection of potentially probiotic strains (LJUNGH & WADSTRÖM [3], SAHADEVA & al. [7], CORCORAN & al. [8]).

The most common types of microorganisms used as probiotics are lactic acid bacteria (LAB) strains, such as: *Lactococcus*, *Lactobacillus*, *Pediococcus*, *Bifidobacterium*, *Leuconostoc*, *Carnobacterium*, *Streptococcus* and *Enterococcus*. The main argument for lactic bacteria strains use is their presence in the intestinal normal microbiota and their GRAS (Generally Recognized As Safe) status, presenting very low risks to trigger infections (DE VUYST & VANDAMME [2], O'MAHONY & al. [9], TANNOCK [10]).

Health benefits of probiotics include: maintaining the balance of the normal microbiota (WALKER [11]), prevention of infectious diseases (GALDEANO & PERDIGON [12]) and allergies (WANG & al. [13]), reduced serum cholesterol (LIM & al. [14]), anticancer activity (REDDY [15]), stabilizing intestinal mucosa barrier (SALMINEN & al. [16]), alleviating the symptoms of IBD (inflammatory bowel disease) (SCHULTZ & al. [17]), the immunomodulation ability (YOSHINORI & HIROMI [18]) and reduce the lactose intolerance (HE & al. [19]). Live probiotic cultures are available in fermented dairy products and probiotic foods. However, tablets, capsules and powders containing the bacteria in freeze or dried form are also available.

Health benefits of lactic acid bacteria (LAB) and the increasing antibiotic resistance of pathogenic bacteria have led to the selection and use of probiotic strains as effective alternatives to the conventional treatments.

The aim of this study was to investigate the virulence factors of LAB strains, antibiotic susceptibility, the antimicrobial activity and the ability to survive the simulated gastro-intestinal conditions, in order to select effective probiotic strains with minimal risks of infections, even to immunocompromised hosts.

2. Materials and Methods

Bacterial strains and growth conditions

A set of 170 strains of lactic acid bacteria were evaluated in this study, originated (previously isolated) from newborn feces, fed with breast milk and from fermented dairy products. The pure cultures were growth and preserved in liquid MRS (De man, Rogosa, Sharpe) broth and stored at -70°C in the presence of 20% of glycerol as cryoprotectant. Beside these isolates, other 5 ATCC (American Type Culture Collection) strains of lactic acid bacteria belonging to *Lactobacillus*, *Enterococcus*, *Leuconostoc* and *Bifidobacterium* genera

(microbial culture collection of the Department of Genetics, Faculty of Biology, University of Bucharest) were included as reference strains. Prior to use, LAB strains were subcultivated in MRS broth and incubated overnight at 37°C.

Antimicrobial activity assay of LAB

The antimicrobial activity was tested against five virulent strains, selected after series of virulence tests: *Bacillus cereus* 53(100), *Escherichia coli* 15, *Salmonella arizonae* 18, *Escherichia coli* 159 and *Escherichia coli* FQa2 (strains collected from NIRDMI Cantacuzino Zoonosis Laboratory Collection).

Antimicrobial activity was assessed by measuring the size (diameter) of the inhibition zones, consisting in absence of visible pathogen growth around the lactic colonies.

Two methods were used for evaluation of antimicrobial activity. Overnight cultures of pathogens were grown in liquid *Luria Bertani* (LB) broth for a few hours, until they reached a cellular density around 4×10^8 cells/mL ($OD_{600nm} = 0.4 - 0.6$), then were uniformly dispersed on solid MRS. After drying, 10 µl of each lactic acid bacteria strain was spotted on the plates and incubated at 37 °C for 24h.

Pathogen cultures were grown in liquid LB up to an OD_{600nm} of 0.4 – 0.6, then 1.5 mL of this suspension was mixed with 40mL of semisolid BHI (Brain Heart Infusion) medium and poured (5mL/plate) over the solid MRS plates. After solidification, 10 µL of each lactic acid bacteria strain was spotted on the plates and incubated at 37 °C for 24h.

Screening for bacteriocin or bacteriocin-like production

Antimicrobial activity may be a result of organic acids which determine a reduction in pH, but may also be due to the production of proteinaceous compounds like bacteriocins. Hence, the cell-free supernatants (CFS) from LAB strains showing antimicrobial activity was checked for proteinaceous nature of the antimicrobial compounds after excluding inhibition due to organic acids. Bacteriocin or bacteriocin-like production was tested against the following strains: *Listeria monocytogenes* 333; *Enterobacter faecium* GM6 and *Lactobacillus bulgaricus* 10260. LAB strains for testing were cultured overnight at 37°C in liquid MRS broth, then the cells were removed by two centrifugations at 14000rpm for 15 minutes. The remaining CFS was adjusted to pH 6.5-7 with 40% NaOH in order to eliminate microbial inhibition resulted from the production of organic acids. To determine the possible presence of proteinaceous compounds, supernatants still showing antimicrobial activity after pH neutralization were treated with proteinase K (200 µg/mL) at 37°C for 2h. Supernatants not subjected to proteolytic treatment served as controls. Because of the proteinaceous nature of bacteriocins, the LAB strains whose supernatant loses the antimicrobial activity following treatment with proteinase K are most likely bacteriocin producing strains.

Expression of soluble virulence factors

All strains grown overnight for 18-24h in MRS broth, were tested for hemolysin production, starch hydrolysis, lipase, lecithinase, caseinase, gelatinase, DN-ase activity and siderophore-like synthesis, by cultivating on different specific media for detection of enzymatic activity.

Hemolysin production was revealed by cultivating the strains for 24 hours at 37°C on Mueller Hinton (MH) agar plates supplemented with 5% sheep blood. The haemolytic activity due to hemolysin synthesis was observed after incubation, by the appearance of a clear zone around the colonies (complete or β-haemolysis) or a dark-greenish zone which was correlated with partial lysis of the blood cells (α-haemolysis).

The ability to produce gelatinase was tested by plating the strains on a gelatin containing agar with the following composition: 10 g/L peptone; 1g/L yeast extract; 5 g/L sodium taurocolate; 10 g/L NaCl; 30 g/L gelatin and 15 g/L agar. After 48h of incubation at 37°C, the gelatinase production can be observed by the formation of a clear zone around the gelatinase producing colonies, due to gelatin hydrolysis.

The production of lecithinase enzyme was determined using a nutritive egg-yolk agar medium, containing 2% agar, 0,48% peptone, 4% dextrose, 7,3% sodium chloride, 0,06% calcium chloride and 10% sterile egg yolk. The strains were plated and incubated at 37°C for 48h. The degradation of the lecithin from the egg yolk results in apparition of a precipitate surrounding the colonies that produced lecithinase.

Lipase activity was analysed by plating the strains onto a nutritive medium supplemented with 1% Tween 80. After 48h of incubation at 37°C, a precipitate appear around the lipase producing colonies, as a result of tween hydrolysis by the lipolytic enzymes.

DN-ase production was studied after 48h of incubation at 37°C in DNA-se Test Agar (Acumedia) with toluidine blue added after autoclaving. In the presence of HCl 1N occurs DNA precipitation, creating an opaque aspect of the medium, revealing the positive reaction consisted on a clear zone around the DN-ase producing colonies,

The caseinase activity was determined using a nutritive agar containing 10% skimmed milk. After 24h of incubation at 37°C, the positive reaction, consisted in precipitation of calcium amino acids from the milk, was observed around the casein producing colonies.

To determine the amylase synthesis, the strains were spotted onto a nutritive agar (meat peptone 10 g/L, meat extract 3 g/L; NaCl 5 g/L) supplemented with 10% of starch and incubated for 24h at 37°C. The positive reaction was revealed after incubation time, adding Lugol above the plates. The interaction between iod and starch triggers a changing in the color of the medium, except the areas around the amylase producing colonies.

Siderophore-like synthesis ability was revealed by cultivating the strains on BEA (Bile Esculine Agar), with the following composition: ox bile 40g/L; meat peptone g/L; ferric citrate 0,5g/L; agar 15g/L. The ferric citrate represents a color indicator of the positive reaction. It reacts with the free esculetin resulted by esculin hidrolising and form an iron complex which turns the agar colour in dark brown to black.

Resistance to gastric and intestinal fluids

The ability of lactic acid bacteria to survive and grow in extreme conditions, such as the gastro-intestinal environment, was tested through analysing strains viability in the presence of simulated gastric and intestinal juices (fluids). The method used to evaluate the effects of simulated gastro-intestinal conditions on probiotic strains, was adapted from Grimoud et al (2010) and Radulovic et al. (2010) (RADULOVIĆ & al. [4], GRIMOUD & al. [20]). Simulated gastric juice (NaCl-125mM; KCl-7mM; NaHCO₃-45 mM; pepsin 3g/L) was adjusted with 1N HCl to a final pH of 2-2.5, and intestinal juice (bile salts 4g/L; pancreatin 2g/L), simulating duodenal conditions, was adjusted to pH 8 using NaOH. Both solutions were made in MRS broth and sterile filtered through a 0.22-µm membrane filter. The assay was performed in a 96 microtiter plate. Simulated fluids were inoculated with overnight cultures of probiotic strains at a final concentration of 1·10⁸ cells/mL. Suspensions were then incubated aerobically at 37 °C for 48 h and the optical density at 590nm was measured for different time intervals (0, 3, 6, 9, 12, and 24h of incubation). Additionally, strains resistance at pH 10 was tested, given the intestinal alkaline environment, which often reaches this pH value. Uninoculated fluids and MRS broth with optimal growth pH (pH 6.5) were used as

controls. Each strain was tested twice and determination values represent the mean of two observations.

Antibiotic susceptibility

The antibiotic susceptibility of the LAB isolates was determined using the Kirby-Bauer disc diffusion method on solid ISO medium previously swabbed with approximately 1.5×10^8 CFU/mL (0.5 McFarland) of each fresh overnight lactic strain. After drying, 12 discs containing the following antibiotics were placed on top of the agar: kanamycin 30 mg (K-30); streptomycin 10 mg (S-10); neomycin 30 mg (N-30); gentamycin 10 mg (CN-10); trimethoprim 30 mg (TMP-30); erythromycin 5 mg (E-5); clindamycin 2 mg (DA-2); ampicillin 10 mg (AM-10); chloramphenicol 30 mg (C-30); tetracycline 30 mg (TE-30); vancomycin 5 mg (VA-5) and linezolid 30 mg (LNZ-30). Inhibition zone diameters around the antibiotic discs were measured after 24h of incubation at 37°C and the result was expressed as sensitive (S), intermediate (I) or resistant (R) strains, according to the international standards CLSI (Clinical and Laboratory Standards Institute).

3. Results and discussions

Several assays were performed in order to select some lactic acid bacteria strains with probiotic potential and without risks to harbour infectious diseases. Antimicrobial activity, bacteriocin production and the ability to grow in gastro-intestinal conditions were investigated to determine the probiotic potential of LAB strains. The safety of probiotic LAB strains was established after screenings for the presence of potential soluble virulence factors and acquired antibiotic resistance.

Determination of LAB antimicrobial activity by agar diffusion method

The antimicrobial activity of lactic acid bacteria changes the intestinal microbiota composition, generating a hostile environment for pathogenic microorganisms. Principal antimicrobial compounds synthesized by lactic acid bacteria are organic acids, hydrogen peroxide and bacteriocins.

Antimicrobial activity of the 170 LAB strains (67 isolated from new-born faeces and 103 originated from fermented dairy products) was tested against 5 (previously selected) virulent pathogens: *E. coli* FQa2; *E. coli* 15; *E. coli* 159; *S. arizonae* 18; *B. cereus* 53(100). The positive result may be observed due to the emergence of clear inhibition zones around the colonies (Fig 1).

More than 75% of the strains exerted antimicrobial activity against at least one of the pathogenic strains, especially towards those Gram negative (*E. coli* 15, *E. coli* 159 and *S. arizonae* 18). Generally, LAB had the lowest antimicrobial activity against *E. coli* FQa2 and *B. cereus* 53(100), manifesting none or low inhibitory effects. The highest antimicrobial activity was observed against *E. coli* 159 strain. Figure 2 is showing the antimicrobial activity of LAB strains against pathogens.

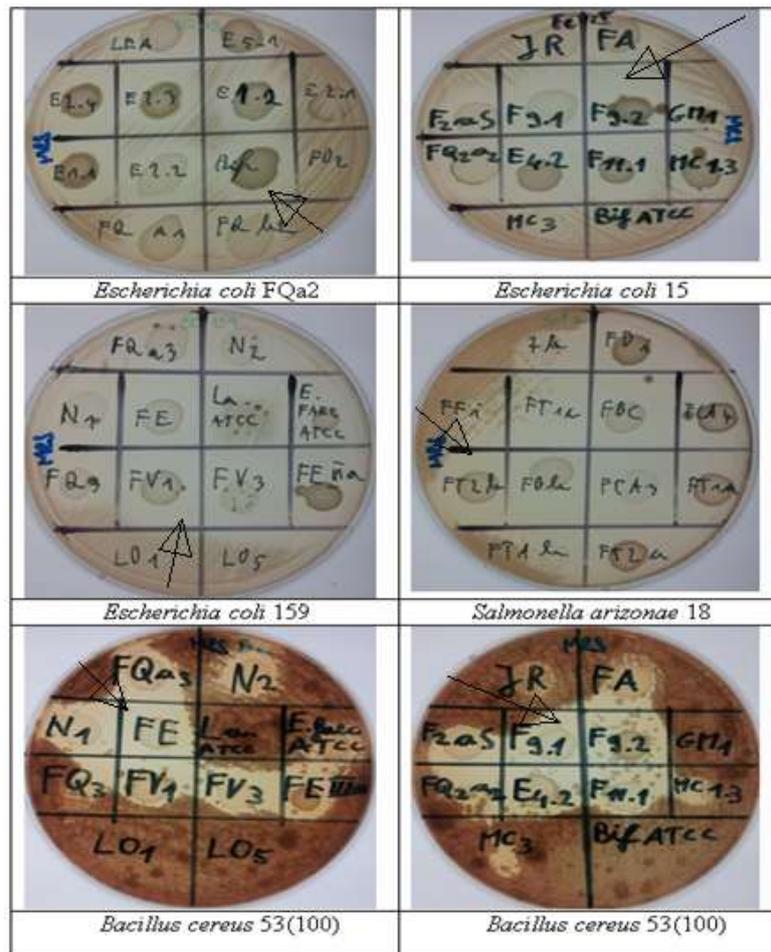


Figure 1. Evaluation of antimicrobial activity of LAB against selected pathogenic strains

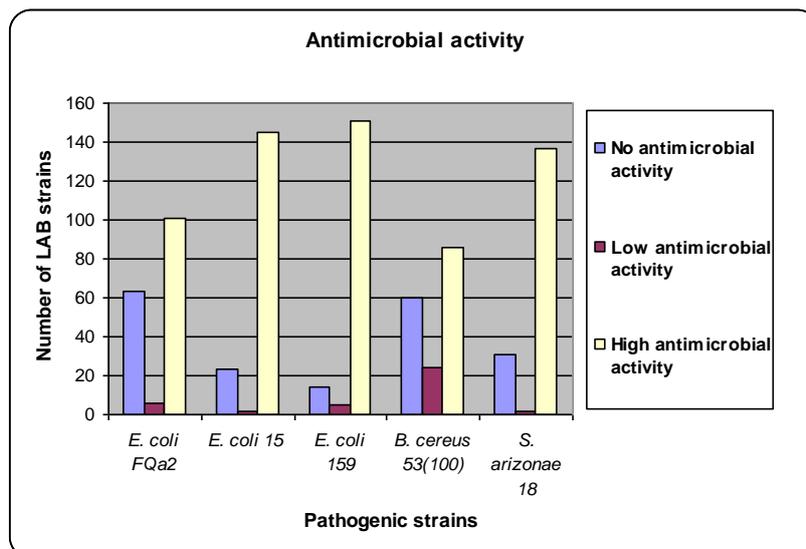


Figure 2. Antimicrobial activity quantification of lactic acid bacteria strains

A total of 143 LAB strains with antimicrobial activity have been selected to determine the chemical nature of the antimicrobial compounds in order to identify the bacteriocin or bacteriocin-like producing strains.

Screening for LAB bacteriocin or bacteriocin-like production

Some probiotic LAB strains are known to produce small proteic antimicrobial molecules, named *bacteriocins*, which have antagonistic effects mainly against close related species, but also against pathogenic bacteria from the gut, preventing their colonization.

In this study, the nature of antimicrobial compounds was determined by testing LAB strains supernatants for antimicrobial effects against *Listeria monocytogenes* 333; *Enterobacter faecium* GM6 and *Lactobacillus bulgaricus* 10260.

The results obtained showed that supernatants of 49 out of 143 strains (34%) had antimicrobial effects, mainly against *L. monocytogenes* 333, followed by *E. faecium* and *Lb. bulgaricus* 10260 (Fig 3).

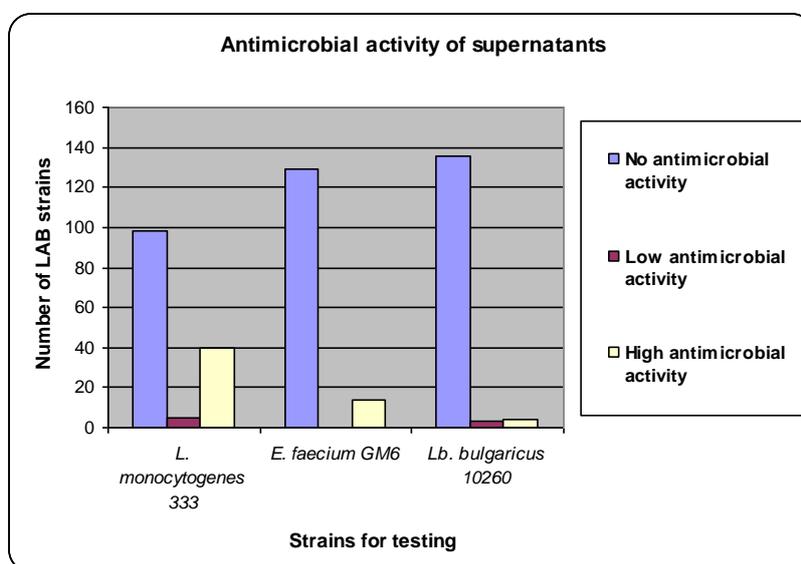


Figure 3. Antibacterial effects of LAB supernatants towards *L. monocytogenes* 333; *E. faecium* and *Lb. bulgaricus* 10260

After pH neutralization, only 7 strains still preserved their antimicrobial activity, which means that rest of the strains had inhibitory effects as a result of lowering pH by organic acids, hydrogen peroxide synthesis or other compounds instead of bacteriocin production. Excepting *LAB F9.1* and *LAB FEM*, the remaining 5 strains supernatants have lost their antimicrobial properties after proteinase K treatment, revealing the proteinaceous nature of the antimicrobial compounds (Table 1).

The fifth most likely bacteriocin or bacteriocin-like producing LAB strains (*LAB 41.2*, *LAB 19.3*, *LAB F2aS*, *LAB F11.1* and *LAB E5.1*) may find applications in pharmaceutical and food industries, as natural biopreservatives, so further studies including: taxonomical identification, bacteriocin purification, heat stability tests as well as bacteriocin encoding genes determination are needed.

Table 1. LAB strains that kept their inhibitory effects after supernatant neutralization; * - supernatants from *LAB F9.1* and *LAB FEM* did not lose antimicrobial effects after proteinase K treatment, which means that inhibition observed was not caused by proteinaceous compounds like bacteriocins.

LAB strains	<i>Listeria monocytogenes</i> 333	<i>Enterobacter faecium</i> GM6	<i>Lactobacillus bulgaricus</i> 10260
<i>LAB 41.2</i>	+	-	-
<i>LAB 19.3</i>	+	+	+
<i>LAB F2aS</i>	+	+	-
* <i>LAB F9.1</i>	+	+	-
<i>LAB F11.1</i>	+	+	-
<i>LAB E5.1</i>	+	+	-
* <i>LAB FEM</i>	+	-	-

Soluble enzymatic virulence factors production

In case of expressing different soluble virulent factors, lactic acid bacteria used in probiotic products could be a risk factor for human health, especially for those with weak immunity.

The safety of LAB strains as probiotics was assessed by testing their ability to produce the following soluble compounds that confer them metabolic advantages: haemolysins, gelatinases, lecithinases, lipases, DN-ases, caseinases, amylases and siderophore-like compounds (Fig 4).

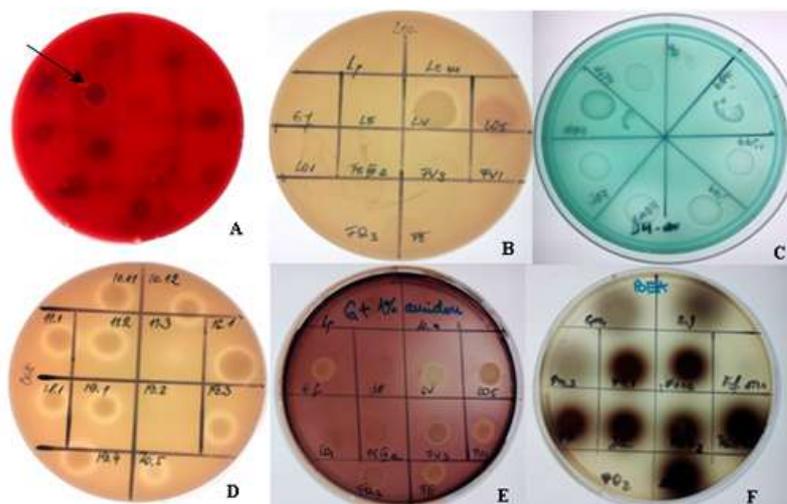


Figure 4. Soluble virulence factors released by LAB strains: A - Hemolysins; B - Lecithinases; C - DN-ases; D - Caseinases; E – Amylases; F - siderophore-like compounds

Most of the strains had the ability to produce caseinases and siderophore-like compounds, while 39.4% presented mild hemolytic activity. Also, the results showed a very low number of amylase, DN-ase and lecithinase producing strains and no strain produced gelatinase (Fig 5).

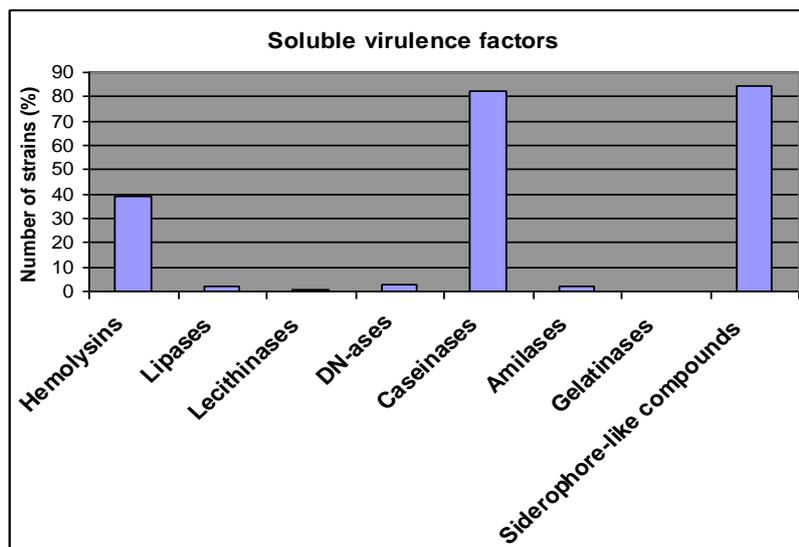


Figure 5. Quantitative assessment of soluble enzymatic virulence factors pattern observed in tested LAB strains

Only hemolysin synthesis represents a significant virulence factor, because hemolysins are pore-forming toxins, which produce lesions in the cell membrane. Even the strains with low hemolytic activity can not be used in probiotic products, because of the risk to determine infectious processes.

The rest of the tested soluble compounds do not affect host health, because they are very weak virulence factors, involved only in nutrients providing and in competition for chemicals or available sources of energy.

Iron requirement is high for the most of microbial pathogens, so harmless siderophore producing strains of LAB can be used as probiotics to compete with pathogens for iron and suppressing their growth, especially in low-iron environments.

The ability of above 82% of the tested LAB strains to hydrolyze milk casein was expected, considering that most of the strains were isolated from fermented dairy products.

Resistance to simulated gastric and intestinal fluids

Survival in harsh gastrointestinal conditions is an essential property of lactic acid bacteria used as probiotics, to maintain their health-promoting effects. Thus, probiotic microorganisms should tolerate the extreme pH conditions from the stomach and small intestine and should resist to pepsin, pancreatin and bile salts. Therefore, 103 nonhemolytic LAB strains with antimicrobial activity, previously selected, were tested for resistance to simulated gastric and intestinal juices and to different pH values (pH 2; pH 8; pH 10), specific to gastric and intestinal conditions.

The results after 24h of incubation in mentioned conditions indicated 16 strains sensitive to all tested conditions; 43 strains with moderate resistance to at least one of the following conditions: simulated intestinal juice, MRS pH8 and MRS pH10, and 44 strains highly resistant, able to develop in the same conditions without any kind of restrictions.

None of the tested strains showed cell multiplication at pH 2 or in the presence of simulated gastric juice (Fig 6).

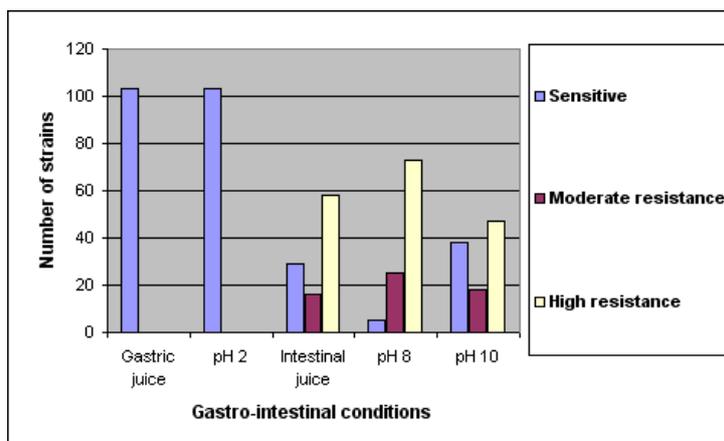


Figure 6. Comparative evaluation of LAB strains resistance to extreme pH and simulated gastro-intestinal conditions

Both acidic pH and pepsin from simulated gastric juice completely inhibited the growth of LAB. Strains developed best in MRS at pH 8 due to lactic acid production that decreased the pH of the medium around neutral value. Although the simulated intestinal fluid had also pH 8, the pancreatin and bile salts were found to have inhibitory effects on LAB strains development (Fig 7). MRS with pH 10 was sufficiently basic to suppress the development of most strains. However, about 45% of the tested lactic acid bacteria strains showed increased resistance to pH 10.

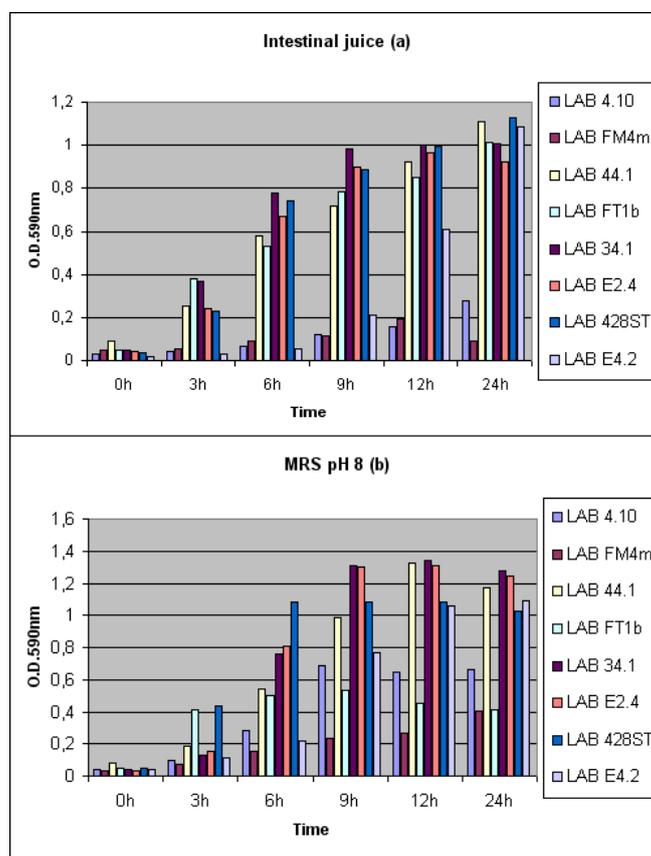


Figure 7. Comparative bacterial growth in: a) simulated intestinal juice and b) MRS at pH 8, within 24h

A total of 44 lactic acid bacteria strains, highly resistant to pH 8 and pH 10 and simulated intestinal juice were selected for further investigations regarding the antibiotic susceptibility and the risk of horizontal transfer of antibiotic resistance genes.

The results obtained *in vitro*, may not truly reflect the *in vivo* rate of bacterial survival, as many other physiological factors, that couldn't be reproduced *in vitro*, might affect the viability of the lactic acid bacteria strains during gastrointestinal passage. Nevertheless, the ability of these strains to develop in the similar conditions mentioned above might reflect the possible temporary human gastro-intestinal colonization capacity, which contributes to their probiotic effects (DANIEL & al. [21]).

Antibiotic susceptibility of the selected LAB strains

From the safety point of view, lactic acid bacteria must not possess acquired resistance to antibiotics, because of the chance of being transmitted to the pathogens from the gut.

Antibiotic resistant, intermediate and sensitive strains were identified by measuring the diameter of inhibition zones around the antibiotic impregnated discs and comparing them with current standards regarding antibiotic resistance (Fig 8).



Figure 8: Determination of antibiotic susceptibility by evaluation of the inhibition zones formed around the antibiotic impregnated discs

Results obtained for antibiotic susceptibility of the 44 lactic acid bacteria strains tested, is presented in Figure 9. The strains showing acquired antibiotic resistance were eliminated from further studies.

According to the literature, lactic acid bacteria may present acquired resistance to chloramphenicol, gentamicin, ampicillin, erythromycin and tetracycline, but may also be natural (intrinsically) resistant to different types of antibiotics.

Enterococci have intrinsic resistance to cephalosporins, aminoglycoside and clindamycin. Lactobacilli, pediococci and *Leuconostoc* spp. have a high natural resistance to vancomycin, and some lactobacilli have also been reported to have high intrinsic resistance to bacitracin, norfloxacin, ciprofloxacin, teicoplanin, fusidic acid, ceftiofur, kanamycin, streptomycin, gentamicin, metronidazole, sulphadiazine, nitrofurantoin and trimethoprim.

The knowledge of intrinsic resistance of LAB to commonly used antibiotics is needed to recognize acquired resistance traits (MATHUR & SINGH [22]). Antibiotic resistance may be acquired by the mutation of pre-existing genes, or via horizontal transfer of the antibiotic resistance genes. Generally, the natural resistance and acquired resistance by mutations have no risks of dissemination, but horizontal transfer of antibiotic resistance genes, mainly those carried on mobile genetic elements, is more likely to occur (AMMOR & al. [23]).

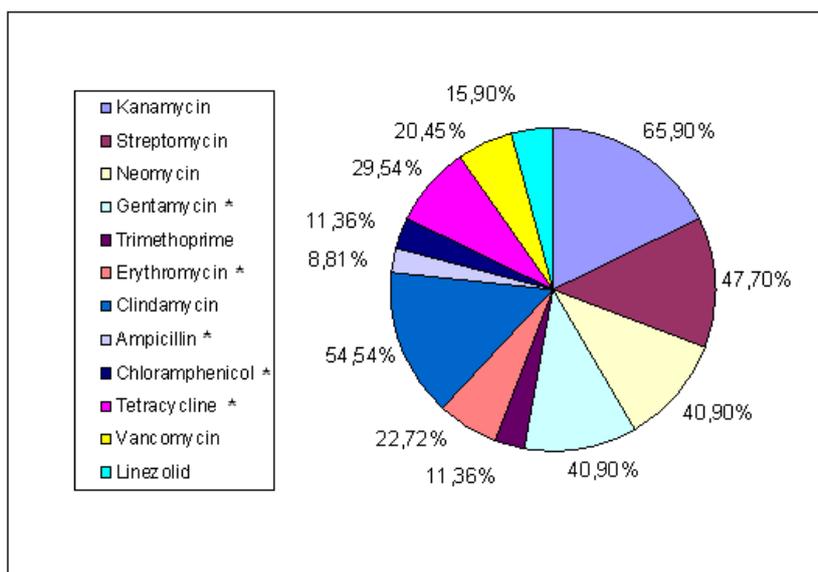


Figure 9. Distribution of LAB antibiotic resistance: * - antibiotics against which lactic acid bacteria have no intrinsic resistance, according to scientific data

In this study, kanamycin resistance was the most prevalent (66%), followed by streptomycin (47%) and neomycin (41%), while less than 16% of the strains have managed to develop under the influence of ampicillin, chloramphenicol, linezolid and trimethoprim. Linezolid and trimethoprim susceptibility observed in our study is an interesting and contradictory finding, considering that lactic acid bacteria are reported to be intrinsically resistant to those antibiotics. Two strains (*LAB 409* and *LAB 413*) were susceptible to all antibiotics tested. About 20% of the lactic bacteria strains showed resistance to a single antibiotic, while multidrug (antibiotic) resistance was detected in 70% of the tested strains mainly combining aminoglycosides, erythromycin and tetracycline resistance. This result may be due to similar ways of antibiotic neutralization, especially for those belonging to the same class.

A total of 23 strains (52.2%) presented acquired resistance to at least one of the following antibiotics, known that lactic acid bacteria have no natural resistant for (gentamycin, erythromycin, ampicillin, chloramphenicol and tetracycline), so were eliminated from the study, because of the antibiotic resistance risk of dissemination by horizontal transfer.

Methodologies used for *in vitro* selection of safe probiotic LAB strains are not standardized, so the results may differ from those obtained in other similar studies (MORELLI [24]).

4. Conclusions

As a safety criterion, phenotypic analysis of LAB virulence factors revealed a predominance of hemolysins, caseinases and siderophore-like compounds synthesis, but only hemolysin producing strains were eliminated from the study, the rest of them being harmless for human health, only providing some nutritional and competitive advantages.

Antimicrobial activity test revealed about 77% of the lactic acid bacteria strains exhibiting high levels of antimicrobial activity against at least one of the pathogenic strains.

Assessment of lactic acid bacteria viability in similar conditions to those from gastro-intestinal tract revealed a complete loss of growth at low pH (2-2.5) and in the presence of pepsin from the gastric juice. Pancreatin and bile salts decreased the viable counts to 56%, compared to the negative control that allowed the growth of 71% of the tested strains. Only 45% of the LAB strains developed in MRS with pH 10. Gastro-intestinal viability assay lead to the selection of 44 resistant strains.

Antibiogram analysis of the 44 selected strains has contributed to elimination of 23 lactic strains with acquired antibiotic resistance and consequently high risk of resistance genes dissemination through horizontal genetic transfer.

The screening studies determined the selection of 21 safe lactic acid bacteria strains with antimicrobial proprieties, resistant to harsh gastrointestinal conditions and lacking acquired antibiotic resistance genes, which will be taxonomically identified and may be used in probiotics production. Moreover, the molecular studies may provide a better understanding of the lactic acid bacteria behavior and mechanisms involved in their probiotic effects.

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