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CHARACTERIZATION OF PROBIOTIC POTENTIAL OF SOME LACTIC ACID BACTERIA STRAINS ISOLATED FROM FERMENTED VEGETABLES

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Abstract
The probiotics strains are available in a variety of food products and supplements. Most of them are lactic acid bacteria, microorganisms considered commensal and harmless for the host organism (lactic acid bacteria are generally recognized as safe- GRAS). Despite the important progress made in the field of probiotics, the selection of probiotic strains that would be capable of performing effectively in the gastrointestinal tract and the understanding of the precise mechanism of action are still a significant challenge.
The purpose of the present work was to characterize the probiotic potential of seven lactic acid bacteria strains isolated from fermented vegetables and from cereals. The study comprised bacterial growth to pH variations, antibiotic susceptibility and growth to different sodium chloride/ bile salt/ digestive enzymes concentration.
Obtained results revealed that newly isolated lactic acid bacteria strains presented high resistance to different concentration of bile salt and sodium chloride and also resistance to digestive enzymes.Regarding resistance to different pH values, our studies showed that a part of tested lactic acid bacteria strains have a low resistance to acid pH values (pH 3.0; 4.0) and high resistance to alkaline pH values such as 10.0 and 12.0; antibiotic susceptibility profile was strain dependent.
Our studies allowed us to select the most resistant strains in conditions similar to those from the gastrointestinal tract and to exclude the ones with potential risk for human health.

Keywords: probiotic effect, *Lactobacillus*, gastro-intestinal tract, antibiotic susceptibility, human and animal health

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Introduction

Probiotics are "viable non-pathogenic microorganisms which, when there are supplied in adequate amounts, confer health benefits to host" and they could represent a possible alternative to conventional treatment of various disorders (Fuller, 1989; Goktepe et al., 2006; Walker, 2008).
The benefits of probiotics have been recognized and explored for over a century. They has been used as a treatment for several medical conditions, such as gastrointestinal disorders, allergic diseases or bacterial vaginosis (Verna et al., 2010), but they also have a significant biotechnological application. The fermented foods are an important part of the food processing industry and are often prepared using selected strains that allows the achieving of desired and standardized products. (McKay et al., 1990)
In the last years, were developed in vivo and in vitro studies in order to obtaining more efficient probiotic products. The scientific dates have demonstrated that the beneficial effects of probiotics include: improved digestion (Shanahan et al., 2009), reduction of serum cholesterol levels (Noh et al., 1997; Park et al., 2008), maintaining the intestinal microbial balance in human and animal hosts (Reller et al., 2009; Lazar et al.,2005; Hove et al., 1999), amelioration of lactose intolerance (Pereira et al., 2002), modulation of the host immune system, production of antimicrobial compounds which are antagonistic to pathogenic growth (Azizpour et al., 2009) and cancer suppression (Singh et al., 1997).
The importance of these microorganisms on human and animal health was underlined by some international organizations, such as FAO (The Food and Agriculture Organization of the United Nations) and WHO (World Health

Organization) by publishing a Guidelines for the evaluation of probiotics for human and animal use. (WHO, 2006; WHO, 2001)
Because lactic acid bacteria (LAB) strains are widely used as starter cultures or as probiotics and they have the potential to transfer the antibiotic resistance genes to other pathogenic bacteria, this is the reason why it is so important to evaluate the antibiotic susceptibility profile before to introduce them on market. The purpose of the present work was to analyze the resistance of LAB strains to various conditions of gastro-intestinal tract (resistance to pH variations, sodium chloride/ bile salt concentrations and digestive enzymes), and to evaluate the potential risk for the antibiotic resistance genes transfer.

Materials and Methods

The seven LAB strains used in this study: *Lactobacillus* (*L.*) *oryzae* P49, *L. amylolyticus* P40, *L. fermentum* SE25, *Enterococcus* (*E.*) *faecium* VL47, *Weisella* (*W.*) *confusa* P126, *Leuconostoc* (*L.*) *citreum* 126 and *L. plantarum* 16, were isolated from fermented vegetables and from the surface of cereals. The LAB strains have been identified using conventional and molecular methods and preserved in microbial culture collections of Institute of Biology Bucharest - Romanian Academy and Faculty of Biology University of Bucharest.
Microbial strains were cultivated in Man, Rogosa and Sharpe (MRS: 1.0 % peptone, 0.8 % egg extract, 0.4 % yeast extract, 2.0 % glucose, 0.5 % sodium acetate trihydrate, 0.1 % Tween 80, 0.2 % dipotassium hydrogen phosphate, 0.2 % triammonium citrate, 0.02 % magnesium sulfate heptahydrate, 0.005 % manganese sulfate tetrahydrate, pH 6.2) liquid broth for 24 hours, at 37^{o} C.

Antibiotic susceptibility

The LAB strains were cultivated in MRS liquid broth for 12 hours, at 37^{o} C.
Fresh overnight cultures were centrifuged 10 min. at 10,000 rpm and the cellular sediment was used to obtain a suspension of 0.5 McFarland in phosphate buffered saline (PBS). The suspension was dispersed on ISO-Sensitest Agar plates, using a sterile swab.The following antibiotic disks were used: tobramycin (NN 10 µg), fosfomycin (FFL 50 µg), kanamycin (K 30 µg), cefepime (FEP 30 µg), ceftriaxone (CRO 30 µg), amoxicillin (AMX 25 µg), vancomycin (VA 30 µg), erythromycin (E 15 µg), chloramphenicol (C 30 µg), gentamicin (GM 10 µg), neomycin (N 30 µg), trimethoprim-sulfamethoxazole (SXT 1.25 µg + 23.75 µg), clindamycin (CC 2 µg), streptomycin (S 10 µg), linezolid (LZD 30 µg) and tetracycline (TE 30 µg).
The diameter of inhibition zone was measured after 24 hours of incubation at 37^{o} C and the results was analyze according to CLSI 2011 recommendations.

Growth to different pH values

LAB strains ability to grow to different pH values: 3.0; 4.0; 5.0; 8.0; 9.0; 10.0 and 12.0, was assessed on 96 well microplate using 20 % inoculum, then the samples were incubated for 18-24 hours at 37^{o} C. The pH was adjusted using a solution of acetic acid 99% (for pH < 6) and a solution of NaOH 40% (for pH > 6).
The cell growth was estimated by measuring the absorbance at 600 nm using Synergy HTX Multi-Mode Reader (Bio-Tek).

Growth to various concentrations of bile salts and sodium chloride (NaCl)

In order to determinate growth to various concentrations of bile salt (Ox-bile, Fluka), and sodium chloride, LAB strains were cultivated in MRS liquid broth with 0.25%, 0.5%, 1%, 2%, 4% and 8 % bile salt or sodium chloride. 150 µl of broth were distributed in 96 well microplates and inoculated with 20% bacterial strains culture in logarithmic phase of growth.
The plates were incubated for 18-24 hours at 37^{o} C, and the cell growth was determined by measuring the absorbance at 600 nm using Synergy HTX Multi-Mode Reader (Bio-Tek).

Growth to various concentrations of digestive enzymes

The growth of LAB strains in presence of various concentrations of enzymes was determined by measuring the absorbance at 600

nm using Synergy HTX Multi-Mode Reader (Bio-Tek).
For this purpose, LAB strains were cultivated in MRS liquid broth with 20mg/ml, 10 mg/ml, 5 mg/ml, 2.5 mg/ml, 1.25 mg/ml, 0.625 mg/ml and 0.312 mg/ml enzyme using 20% inoculum and incubated for 24 hours at 37^{o} C.

Results and Discussions

Susceptibility to antimicrobial compounds

In order to determinate a probiotic potential of LAB strains isolated from fermented vegetables has been established their antibiotic susceptibility profile and the ability of these strains to survive in fermented foods and in similar conditions to those of the gastrointestinal tract.
The antibiotic susceptibility results have revealed that LAB strains involved in this study have different antibiotic susceptibility profiles (Table 1).

E. faecium VL 47 strain exhibited natural resistance to most of the antibiotics used, except amoxicillin, linezolid and tetracycline while the most sensitive LAB tested was *L. citreum* 126 strain. *Enterococcus* strain who shown resistance to vancomycine could not be part of a probiotic products because of the risk for vancomycin genes transfer to other potential pathogenic microbial strains and thus could representa risk for human health.
All strains tested were susceptible to tetracyclin and most of them were also susceptible to cefepime, ceftriaxone, linezolid and cloramphenicol (Table 1).
A considerable number of LAB strains tested were resistant to tobramycin, fosfomycin, kanamycin, gentamicin, neomycin and trimethoprim-sulfamethoxazole.

Table 1. Susceptibility of LAB to different antimicrobial substances

ANTIMICROBIAL COMPOUNDS	*L. oryzae* P 49	*L. amylolyticus* P 40	*L. fermentum* SE 25	*E. faecium* VL 47	*W. confusa* P 126	*L. citreum* 126	*L. plantarum* 16	*L. plantarum* ATCC 8014
Tobramycin	R	R	R	R	S	S	R	R
Fosfomycin	R	R	R	R	R	S	R	R
Kanamycin	R	R	R	R	R	I	R	S
Cefepime	S	S	S	R	S	S	S	S
Ceftriaxone	S	S	S	R	S	S	S	S
Amoxicillin	S	S	S	I	R	R	S	I
Vancomycin	R	S	R	R	R	I	S	S
Erythromycin	S	S	I	R	R	S	I	R
Chloramphenicol	S	S	S	R	I	S	S	R
Gentamicin	R	R	R	R	S	S	R	R
Neomycin	R	R	R	R	S	S	R	R
Trimethoprim-sulfamethoxazole	R	S	R	R	R	S	R	S
Clindamycin	S	S	R	R	R	I	I	S
Streptomycin	S	R	R	R	S	R	R	S
Linezolid	S	S	R	S	S	S	S	S
Tetracycline	S	S	S	S	S	S	S	S

S-sensitive; R-resitant; I- intermediate resistant

Strains growth at different pH values

Growth to various pH values is an essential feature for biotechnological processes and for LAB strains used in probiotic products in order to survive to gastrointestinal condition and to exert their beneficial properties.

They must be able to survive at different pH values that were found in digestive tract starting with the stomach (normally with pH 2.0), continuing with the small intestine where pH value is varying (pH 4.0-7.0 in the proximal part to pH 7.8- 9.0 in distal part) and finally in large intestine where the pH is between 4.0-6.0.

The results of this study (Figure 1), showed that all strains tested had a very low growth at pH 3.0 and 4.0 while an increased resistance to alkaline pH value such as 12.0, was observed only for two strains: *E. faecium* VL 47 and *L. fermentum* SE 25 (OD $_{600nm}$ 0.883 for VL47 and 1.347 for SE 25). Most of the LAB strains analyzed had the optimal pH range between 6.5 and 8.0.

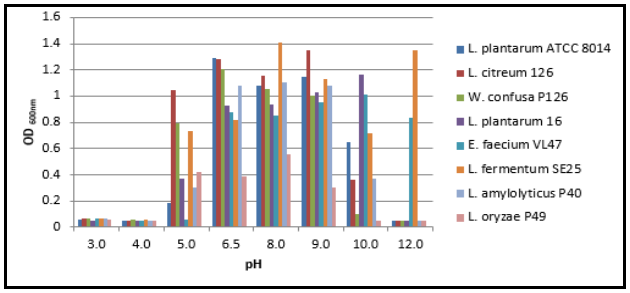

Fig 1. Growth of LAB at different pH values

Growth to various concentrations of bile salts and sodium chloride (NaCl)

The concentration of bile salts is an important factor that may influence the viability of LAB strains in human intestinal tract. Generally, the concentration of bile salts in the gastrointestinal tract is 0.8%, but it can reach up to 4%, before the passage of food from the stomach into the duodenum.

In order to determine the LAB strains resistance to bile salts we select different concentrations between 0.25% and 8%.

The results showed that all strains tested exhibited resistance to a high level of bile salts concentration, such as 4% and 8%. The cellular growth revealed that *L. citreum* 126 and *W. confusa* P126 strains had the lowest sensitivity to bile salts, while *L. oryzae* P49 strain had the highest sensitivity at the same concentration values (Figure 2).

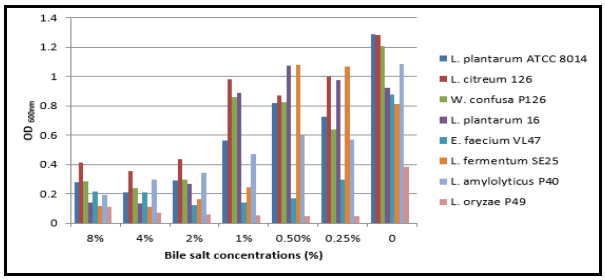

Figure 2. Resistance of LAB strains to different concentrations of bile salts

The tolerance of LAB strains to different concentrations of sodium chloride is an important biotechnological issue especially for fermented vegetables. Considering these the growth of LAB strains was analyzed, in presence of different concentrations of NaCl ranging between 8% and 0.5% (Figure 3).

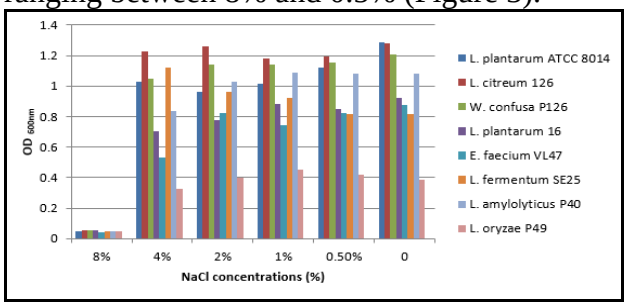

Figure 3. LAB strains growth to various concentrations of sodium chloride

The *L. citreum* 126 and *L. fermentum* SE 25 strains had the highest growth at 4% NaCl concentration (OD $_{600nm}$ 1.225 for 126 strain and 1.224 for SE 25 strain).

Growth to various concentrations of digestive enzymes

Being resistant to different concentrations of digestive enzymes, such as pepsin, pronase E, trypsin, lysozyme, lipase and pancreatin, is essential for LAB strain to survive to gastrointestinal condition. The results of our study (Figure 4) showed that the resistance profile of LAB strains, to digestive enzymes is a strains specific character.

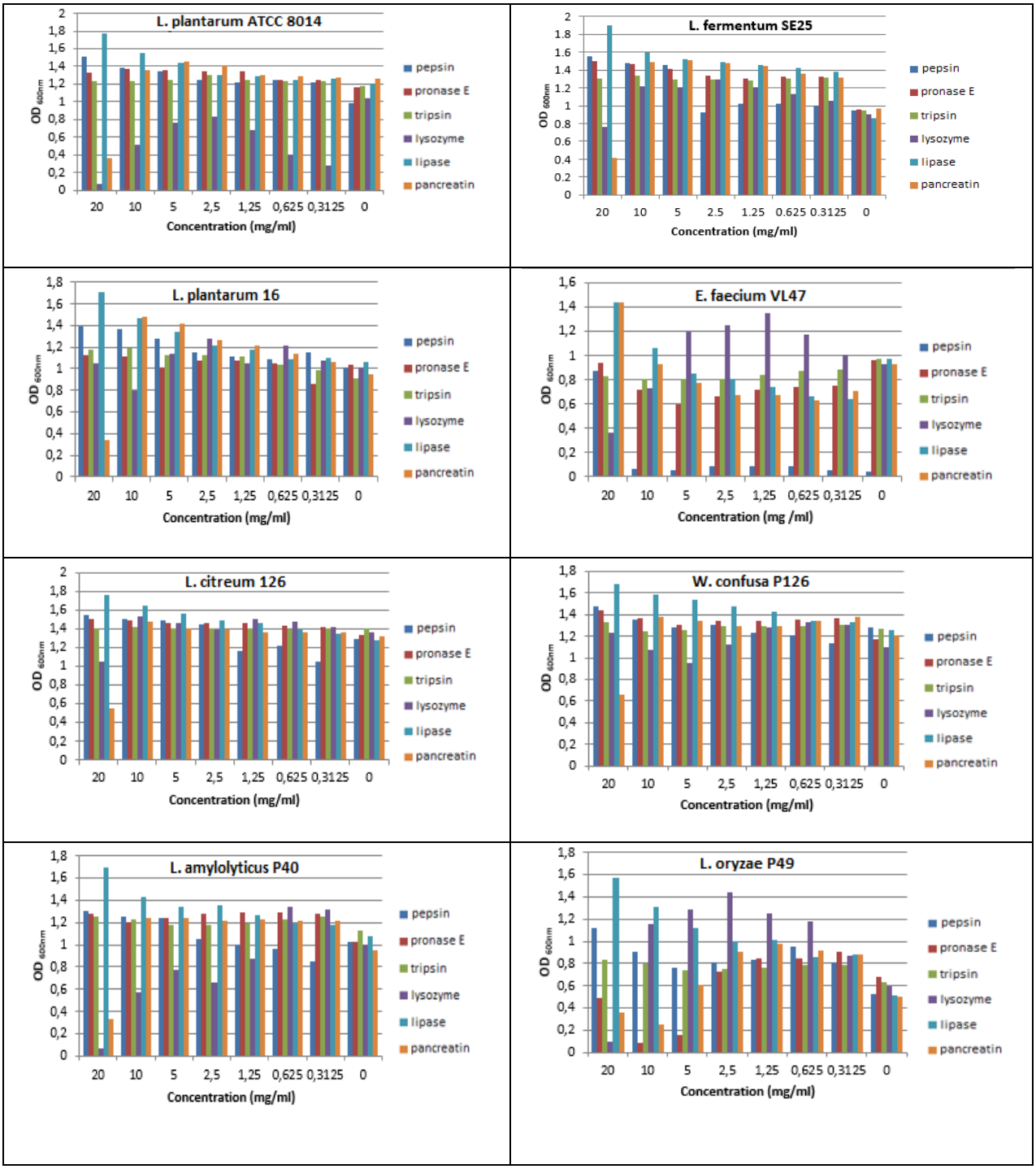

Figure 4. LAB strains growth to different digestive enzymes

The strains who presented high resistance to tested enzymes, even at 20 mg/ml concentration were *L. fermentum* SE 25 and *L. citreum* 126 strains (Figure 4). This shows us their high capacity to survive and to multiply in the human gastrointestinal tract.

Conclusions

Our in vitro studies have demonstrated that the selected LAB strains, isolated from fermented vegetables and from cereals, are exhibiting high resistance to digestive enzymes (lysozyme, pepsin, pronase E, pancreatin, lipase and trypsin), also to different

concentrations of bile salt. These features are very important for the viability of LAB strains in gastrointestinal tract of the human host. A great advantage of our LAB strains is their high pH tolerance and their high resistance to various concentrations of sodium chloride. Most of the tested strains exhibited natural resistance to tobramycine, fosfomycin, gentamicin, trimethoprim-sulfamethoxazole and neomycin, but some of them were sensitive to linezolid, cefepime, ceftriaxone and chloramphenicol. The results of this study have also shown that our selected LAB strains have presented different antibiotic susceptibility profiles and allowed us to exclude a strain with has a potential risk for human health like *E. faecium* VL43.

Acknowledgments

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