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In Vitro Screening of Selected Probiotic Properties of *Lactobacillus* Strains Isolated from Traditional Fermented Cabbage and Cucumber

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Abstract Most important during probiotic selection are gastric acid and bile tolerance, the adhesion to the luminal epithelium to colonize the lower gastrointestinal tract of a human and safety for human consumption. The aim of this study was to evaluate the selected probiotic in vitro properties of *Lactobacillus* spp. Strains isolated from traditional fermented food. A total 38 strains were isolated from the pickled samples and 14 were identified as *Lactobacillus* spp. The survival of almost all strains after incubation at pH 2.5 did not change markedly, and remained at above 90 % (10^9 CFU/mL). The strains also exhibited a high survival rate at pH 3.5 (>90 %), whereas pH 1.5 all were died. Just four strains could survive 90 min. at pH 1.5 (<39 %). The incubation with 0.2 % bile salt solution resulted in a survival rates of 81–94 % after 24 h, whereas after incubation in 2 and 4 % bile salt solution it was 59–94 %. All tested strains showed very good and good resistance to 0.4 % phenol addition, however only *Lb. johnsonii* K4 was able to multiply. The hydrophobic nature of the cell surface of the tested strains was moderated recording hydrophobicity of *Lb. johnsonii* K4 and *Lb. rhamnosus* K3 above 60 %. Safety evaluation excluded four of tested strains as candidate probiotics, according to antibiotic resistance patterns and certain metabolic activities. On the basis on the results 10 of the selected *Lactobacillus* strains are safe and can survive under gastrointestinal conditions, which requires them to future in vitro and in vivo probiotic studies.

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Introduction

Lactic acid bacteria (LAB) are characterized by their production of lactic acid and are predominant participants in many industrial and artisanal plant, meat, and dairy fermentations. LAB group include mainly cocci and rods belonging to the genera: *Streptococcus*, *Leuconostoc, Lactococcus, Oenococcus, Carnobacterium, Lactobacillus, Pediococcus, Enterococcus, Tetragenococcus, Vagococcus* i *Weisella.* The *Bifidobacterium* genus some authors include also to LAB group due to the reason that produce lactic acid and present in a similar environment, while *Bifidobacterium* belongs to the Actinomycetes [30]. In general, LAB occur in habitats with a rich nutrition supply. Carbohydrates are used as carbon and energy source for these bacteria by a homofermentative or heterofermentative pathway. LAB are characterized by the production of 0.6–3 % lactic acid as a major catabolic end product from glucose. Some strains can also produce acetate, ethanol, and formate from pyruvate under low substrate concentrations and strictly anaerobic conditions [2].

LAB, particularly *Lactobacillus* genera, have recently received much attention due to their "generally recognized as safe" (GRAS) status and to their potential health-promoting effects as probiotics. According to FAO/WHO Report [19] probiotics are "live microorganisms which when administered in adequate amounts confer a health benefit on the host" and they should meet certain requirements in vitro and in vivo. Two of the currently most widely used in vitro tests are resistance to gastric acidity and bile salts, as based on both survival and growth studies. Approximately 2.5 l of gastric juice [10] and 1 l of bile [5] are secreted into the human digestive tract every day. Thus, it is essential for the bacteria to have protection systems to withstand the low pH in the stomach, digestive enzymes and

bile of the small intestine [28, 65]. Other functional properties used to characterize probiotics are the production of antimicrobial compounds, the ability to modulate immune responses and bacterial adherence to intestinal epithelial cells and/or mucus [9, 33, 37, 54]. The mechanisms of adhesion is not fully understood, however the process appears to be multifactorial as adhesion cannot be attributed to one component [27] and includes electrostatic interactions, hydrophobic interactions and specific bacterial structures such as external appendages [57]. Regarding the safety of probiotic strains, FAO/WHO Working Group recommends some of the following tests: determination of antibiotic resistance patterns and assessment of certain metabolic activities [19]. It was found that probiotic strains may harbor resistance genes which may be transferred to pathogenic bacteria [65], as well as enzyme activities which may have negative effects in the colon [23].

Strains of *Lactobacillus* spp. occur naturally in the human intestine, and for this reason, such strains are also preferentially developed for commercial use as probiotics. However, some authors report that bacteria isolated from traditional fermented food products also possess potential probiotic properties and could be developed as a commercial starter cultures in food industry [1, 6, 13, 31, 34, 35, 40, 50, 63, 65].

Currently, much attention is paid to the obtaining new probiotic strains of bacteria with satisfactory technological properties in the food industry [26, 46, 47, 64]. Researches effort allowed to obtain sufficient numbers of well-characterized probiotic strains available for commercial use around the world [18, 52, 55]. However, the isolation and characterization of new strains are still needed, especially in developing countries, mainly to the design of new probiotic foods, where it is still limited access to probiotic strains, especially among small manufactories, such as dairies [64].

The aim of this study was identification of *Lactobacillus* spp. bacteria isolated from plant material (pickled cucumbers and cabbage) and evaluation of the safety and behavior of bacteria under gastrointestinal track conditions as selected probiotic properties.

Materials and Methods

Isolation of Bacterial Strains

Six samples of cucumber pickles and cabbage pickles were obtained from household of Poland central region. Samples were collected aseptically in pre-sterile poly-bags kept in an icebox and transported to the laboratory for analyses. 10 g of sample were weighed aseptically and homogenized for 2 min in stomacher (Stomacher 80 Biomaster, Seward) containing 90 ml of peptone water. Briefly, the homogenized samples were serially diluted with sterile peptone water and pour-plated on MRS (de Man, Rogosa and Sharpe) agar (Merck) and were incubated at 30 °C for 72 h. The colonies were selected randomly. Purity of the isolates was checked by streaking twice again on fresh MRS agar plates and further test.

38 strains of presumptive LAB strains were finally selected from these media. Stock cultures of each strain were transferred frozen at −80 °C in 20 % (w/v) glycerol for further studies.

Phenotypic Characterization

The selected strains were examined for cell morphology, Gram staining and catalase test. Strains were characterized phenotypically by testing their growth in MRS broth at 10, 15, and 45 °C and at pH 3.9 and 9.6. 17 of isolated strains identified as *Lactobacillus* spp. have been tested to carbohydrate fermentation profiles using the API 50CH identification system (BioMérieux).

Genotypic Characterization

Molecular identification was carried out by amplification of 16S rDNA gene of all identify phenotypically *Lactobacillus* strains using polymerase chain reaction (PCR). Bacterial DNA was isolated according to manufacturer's description (Isolate Genomic DNA Kit, Bioline). The primer combination 27F/1492R (5′-AGA GTT TGA TCC TGG CTC AG-3′/5′- GGT TAC CTT GTT ACG ACT T-3′) was used for the amplification of 16S rDNA. 16S rDNA sequencing was performed with the superior strain screened from the study by Illumina technique. The homology search was carried out by BLAST software.

Probiotic Control Strain, Culture Media, and Growth Conditions

The well-studied commercial probiotic strain *Lactobacillus plantarum* 299v was used as a reference strain. All bacterial strains used in this study are listed in Table 1. Strains were cultivated in MRS broth using a 1 % inoculum, incubation was provided at 30 °C for 72 h.

Studies on Resistance to Gastrointestinal Conditions

Resistance to the Low pH Conditions

Cultures of all *Lactobacillus* strains were cultivated for 24 h in MRS medium, centrifuged, washed twice by PBS buffer and re-suspended to final concentration 10^8–10^9 CFU/mL in PBS. Bacteria were incubated in 0.85 % NaCl

Table 1 Strains included in this study

Origin	Strain	Phenotypic characterisation	API CH50	16S rDNA	No. of NCBI accesion
pickled cucumber	O12	*Lactobacillus* spp.	*Lactobacillus paracasei* ssp. *paracasei*	*Lactobacillus casei*	KM186152
	O13	*Lactobacillus* spp.	*Lactobacillus paracasei* ssp. *paracasei*	*Lactobacillus casei*	KM186153
	O14	*Lactobacillus* spp.	*Lactobacillus paracasei* ssp. *paracasei*	*Lactobacillus casei*	KM186154
	O15	*Lactobacillus* spp.	not identified	not identified	not identified
	O16	*Lactobacillus* spp.	*Lactobacillus brevis*	*Lactobacillus casei*	KM186155
	O18	*Lactobacillus* spp.	*Lactobacillus paracasei* ssp. *paracasei*	*Lactobacillus casei*	KM186156
	O19	*Lactobacillus* spp.	*Lactobacillus plantarum*	*Lactobacillus plantarum*	KM186157
	O20	*Lactobacillus* spp.	*Lactobacillus plantarum*	*Lactobacillus plantarum*	KM186158
	O21	*Lactobacillus* spp.	*Lactobacillus plantarum*	*Lactobacillus plantarum*	KM186159
	O22	*Lactobacillus* spp.	*Lactobacillus pentosus*	*Lactobacillus brevis*	KM186160
	O23	*Lactobacillus* spp.	*Lactobacillus plantarum*	*Lactobacillus plantarum*	KM186161
	O24	*Lactobacillus* spp.	*Lactobacillus pentosus*	*Lactobacillus brevis*	KM186162
pickled cabbage	K1	*Lactobacillus* spp.	*Lactobacillus plantarum*	*Lactobacillus plantarum*	KM186163
	K2	*Lactobacillus* spp.	not identified	not identified	not identified
	K3	*Lactobacillus* spp.	*Lactobacillus rhamnosus*	*Lactobacillus rhamnosus*	KM186164
	K4	*Lactobacillus* spp.	*Lactobacillus rhamnosus*	*Lactobacillus johnsonii*	KM186165
	K5	*Lactobacillus* spp.	not identified	not identified	not identified

at pH 1.5, 2.5 and 3.5 for 2 h at 37 °C to study the impact of pH [11]. The bacteria were then spread-plated (100 µl aliquots) onto MRS agar medium and incubate for 72 h at 30 °C. *Lactobacillus* survival (in %) was calculated as follows:

Ni/*Nx* × 100, where *Ni* = log CFU/mL after incubation, *Nx* = log CFU/mL before incubation.

Resistance to High Concentration of Bile Salts

Cultures of all *Lactobacillus* strains were cultivated for 24 h in MRS medium than 1 % of cultures were transferred into fresh MRS broth with 0.2, 2 and 4 % (w/v) bile salt addition (Ox gall powder; Sigma Aldrich). Effect of the bile was estimated after 24 and 48 h treatment [11]. The bacteria were then spread-plated (100 µl aliquots) onto MRS agar medium and incubate for 72 h at 30 °C. *Lactobacillus* survival (in %) was calculated as follows:

Ni/*Nx* × 100, where *Ni* = log CFU/mL after incubation, *Nx* = log CFU/mL before incubation.

Bile Salt Hydrolase (BSH) Activity

BSH activity of the cultures was detected using the plate screening procedure described by Du Toit et al. [15] and Franz et al. [20]. Overnight cultures were spotted onto MRS agar plates containing 0.5 % (w/v) sodium taurodeoxycholate (Sigma Aldrich) and 0.37 g/l $CaCl_2$. Plates were incubated anaerobically, in an anaerobic jar (BioMérieux) at 37 °C. Colonies with precipitation zones were considered as BSH-positive.

Resistance to 0.4 % (v/v) Phenol

The ability of all *Lactobacillus* strains to grow in the presence of phenol was tested by inoculating cultures (1 % of

overnight culture) in MRS broth with and without 0.4 % phenol. Serial dilutions were spread-plated (100 µl aliquots) onto MRS agar at time 0 and after 24 h of incubation at 37 °C to enumerate surviving bacteria [67].

Bacterial Adhesion to Hydrocarbons

The bacterial adhesion to hydrocarbons (BATH) test was performed according to Reniero et al. [53]. Bacterial cells were washed with PBS buffer and resuspended in the same buffer. Absorbance was adjusted to 0.25 ± 0.05 to standardize the number of bacteria (10^8 CFU/mL) at 600 nm. Then, equal proportions of viable bacterial suspension and solvent (xylene) were mixed by vortexing for 5 min. The aqueous phase was removed after 1 h of incubation at room temperature and its absorbance was measured. Results were reported as percentages according to the formula:

BATH % = [(Ao−A)/Ao]*100, where Ao and A are absorbance before and after mixing with xylene, respectively.

Safety Evaluation

The selected strains were investigated for their antibiotic resistance profile using the *E* test (bioMérieux) using MRS agar and anaerobic incubation conditions. The strains were considered resistant when they showed MIC values higher than the MIC breakpoints established by the EFSA [16]. Enzyme activities were examined by API ZYM kit (bioMérieux) according to manufacturer's instruction.

Statistical Analysis

All the experiments were performed in triplicate. Error bars on graphs show the standard deviation. The data were analyzed by analysis of variance (ANOVA) and Student *t* test. Differences were considered significant at $P \leq 0.05$.

Results

Characterization and Identification of Bacterial Strains

A total 38 strains were isolated from the pickled samples and 17 were identified as *Lactobacillus* spp. according to phenotypic characterization. All the strains were Gram-positive, catalase-negative. Most tested strains were able to growth at 15 and 45 °C, just 9 of total 38 have tolerated temperature 10 °C. The growth at pH 9.6 was characteristic of most strains. At pH 3.9, just 7 of total 38 tested strains were able to growth well, and other strains represent moderate or slight growth. According to this tests 17 strains were identified, which were rod shaped and exhibited characteristics typical for *Lactobacillus.* These selected strains were identified to the species level also by sugar fermentation investigations (Table 1).

Almost all strains exhibited the ability to ferment glucose, fructose, mannose, mannitol, maltose, sacharose, and turanose. 12 of the total 17 strain were able to reduce lactose. Three strains were not identified according to API test and did not future investigated.

The nucleotide BLAST analysis showed that all of 14 isolates belong to the phylum Firmicutes and family Bacillaceae. Phylogenetic analyses of the 16S rDNA region identified strains O12, O13, O14, O16, O18 as *Lb. casei,* and K3 as *Lb. rhamnosus,* while strains O19, O20, O21, O23, K1 as *Lb. plantarum*. 2 strains (O22 and O24) were identified as *Lb. brevis* and strain K4 as *Lb. johnsonii*. All strains showed 97–100 % similarity in the nucleotide sequence of the 16S rDNA gene of the identify *Lactobacillus* type strain.

Studies on Resistance to Gastrointestinal Conditions

The ability of survival under simulates gastrointestinal conditions of 14 strains of lactic acid bacteria was examined. This examination included the resistance to low pH of the environment, high concentration of bile salts and 0.4 % phenol addition.

The first step in determining probiotic properties (in vitro tests) of lactic acid bacteria was the study of resistance to low pH of the environment. The effect of the successive incubations in low pH solutions (1.5, 2.5, and 3.5) on the viability of the strains are shown in the Table 2.

Individual strains performed different degrees of sensitivity toward gastric acidity. In the solution at pH 1.5 the highest level of survival (more than 98 %) was observed after 30 min of *Lb. johnsonii* K4 strain incubation. However after 60 min of incubation, other strains selected on the basis of high tolerance to low pH–strains *Lb. casei* O18 and *Lb. plantarum* O23 (levels of survival with 57–68 % cells). After 90 min of incubation only 4 isolates–*Lb. casei* O13 and O18 and *Lb. plantarum* O21 and O23-performed high tolerance to pH 1.5. These strains performed the survival rate of 23–38 %. None of the strains survive at pH 1.5 for 120 min of incubation.

The survival of all tested strains after incubation at pH 2.5 and 3.5 did not change markedly and remained at above 90 % (10^9 CFU/mL) and what is interesting some strains were able to multiply.

If strains survive the synthetic gastric juice, the next major challenge is the survival in the artificial small intestine. The effects of the successive incubations through synthetic bile salts on the viability of the strains are shown in the Fig. 1.

All of isolates were capable of growth with the concentration of 0.2 % bile salts (levels of survival with

Table 2 Resistance of *Lactobacillus* strains to low pH of the environment

Isolate	Initial count of bacteria Log CFU/mL	pH 1.5				pH 2.5		pH 3.5	
		S_{30} Survival [%]	S_{60}	S_{90}	S_{120}	S_{60}	S_{120}	S_{60}	S_{120}
299v	8.82 ± 0.3	50.8*	23.7*	0*	–	98.1	96.5	99.7	98.1
O12	9.45 ± 0.5	46.1*	22.9*	0*	–	99.9	98.3	99.4	99.7
O13	9.42 ± 0.1	49.6*	42.9*	23.1*	0*	98.1*	94.6*	99.0	97.5
O14	9.40 ± 0.1	38.1*	0*	–	–	101.2*	102.9*	100.1	101.3
O16	9.82 ± 0.1	52.7*	0*	–	–	96.9	94.6	102.3	104.2
O18	9.54 ± 0.1	71.6*	68.4*	31.7*	0*	100.3	97.9	99.0	100.3
O19	9.25 ± 0.4	0*	–	–	–	101.1*	103.3*	95.7	95.8
O20	9.46 ± 0.1	43.5*	0*	–	–	99.3	96.3	99.7	99.7
O21	9.47 ± 0.2	60.1*	49.6*	38.8*	0*	98.1	95.1*	98.8	98.6
O22	9.08 ± 0.4	36.9*	0*	–	–	97.8	96.6	100.7	101.8
O23	9.36 ± 0.1	61.2*	57.7*	27.7*	0*	98.3	95.4*	98.1	97.7
O24	9.22 ± 0.2	77.7*	41.4*	0*	–	99.7	101.9	100.1	99.8
K1	8.86 ± 0.1	66.2*	37.1*	0*	–	99.2	96.5	98.8	97.1
K3	9.13 ± 0.1	50.1*	0*	–	–	98.5	96.2	97.9	95.3
K4	9.02 ± 0.1	98.3*	0*	–	–	99.8	99.4	99.7	101.1

S_{30}, S_{60}, S_{90}, S_{120}–survival of population after 30, 60, 90, 120 min of incubation

* $P < 0{,}05$

81–93 % cells after 24 h of incubation and 57–86 % cells after 48 h of incubation). The strains *Lb. brevis* O22, *Lb. plantarum* O23 and K1 tolerated bile salts at concentration of 2 % with levels of survival with more than 85 % of cells after 24 h and 70 % after 48 h of incubation. Strains capable of growth in the presence of up to 4 % of bile salts were *Lb. brevis* O22 and *Lb. plantarum* K1 (more than 84 % cells after 24 and 48 h of incubation).

All *Lactobacillus* strains were tested for their ability to hydrolyse the sodium salt of taurodeoxycholic acid. In almost all cases, we did not observe deconjugation of bile salts. Just one strain *Lb. johnsonii* K4 showed BSH activity, while two strains *Lb brevis* O22 and O24 possessed weak bile salt hydrolase activity (Table 3).

Resistance to phenol was tested as an indicator for survival under intestinal conditions for all 14 *Lactobacillus* spp. strains isolated form pickled vegetables and the reference strain. The results are shown on Fig. 2. All tested strains showed very good and good resistance to this compound. Strains *Lb. johnsonii* K4, *Lb. casei* O16 and O18, *Lb. rhamnosus* K3 and *Lb. plantarum* K1 were distinguished from the other strains, as being the most resistant 0.4 % phenol, what indicate that these strains tolerate stress intestinal conditions. However, just one strain *Lb. johnsonii* K4 was able to growth in the presence of phenol during 24 h incubation at 37 °C.

Cell Surface Hydrophobicity

The hydrophobic nature of the cell surface the tested strains was measured photometrically using BATH assay. The results are listed in Table 3. We have found that two of *Lactobacillus* strains (*Lb. rhamnosus* K3 and *Lb. johnsonii* K4) showed quite high, recording above 60 % hydrophobicity values. However 12 of 14 tested strains showed moderate hydrophobicity character (Table 3).

Safety Evaluation

All tested strains were sensitive to ampicillin and except two strains (*Lb. plantarum* O23 and K1) to tetracycline (Table 4). However, all these strains were considered as resistant to the antibiotics streptomycin, gentamicin (except *Lb. rhamnosus* K3), and vancomycin, when using the MIC breakpoint values as suggested by EFSA [16].

None of the tested isolates produced enzymes such as alkaline phosphatase, esterase lipase (C8), lipase (C14), trypsin, α-chymotrypsin, acid phosphatase, α-galactosidase, α-mannosidase, and α-fucosidase (Table 5). Also the carcinogen enzyme, β-glucuronidase was not produced by any of the isolated lactobacilli. However, esterase (C4), leucine arylamidase, valine arylamidase, cystine arylamidase, naphthol-AS-BI-phosphohydrolase, β-galactosidase, α and β-glucosidase, and *N*-acetyl-β-glucosaminidase were detected.

Discussion

Traditional fermented food can be considered as open ecosystems that contain undefined LAB with high competitive potential. Several of these traditional foods are kept in unrefrigerated conditions and are rich sources for new LAB strains with unprecedented antimicrobial activity

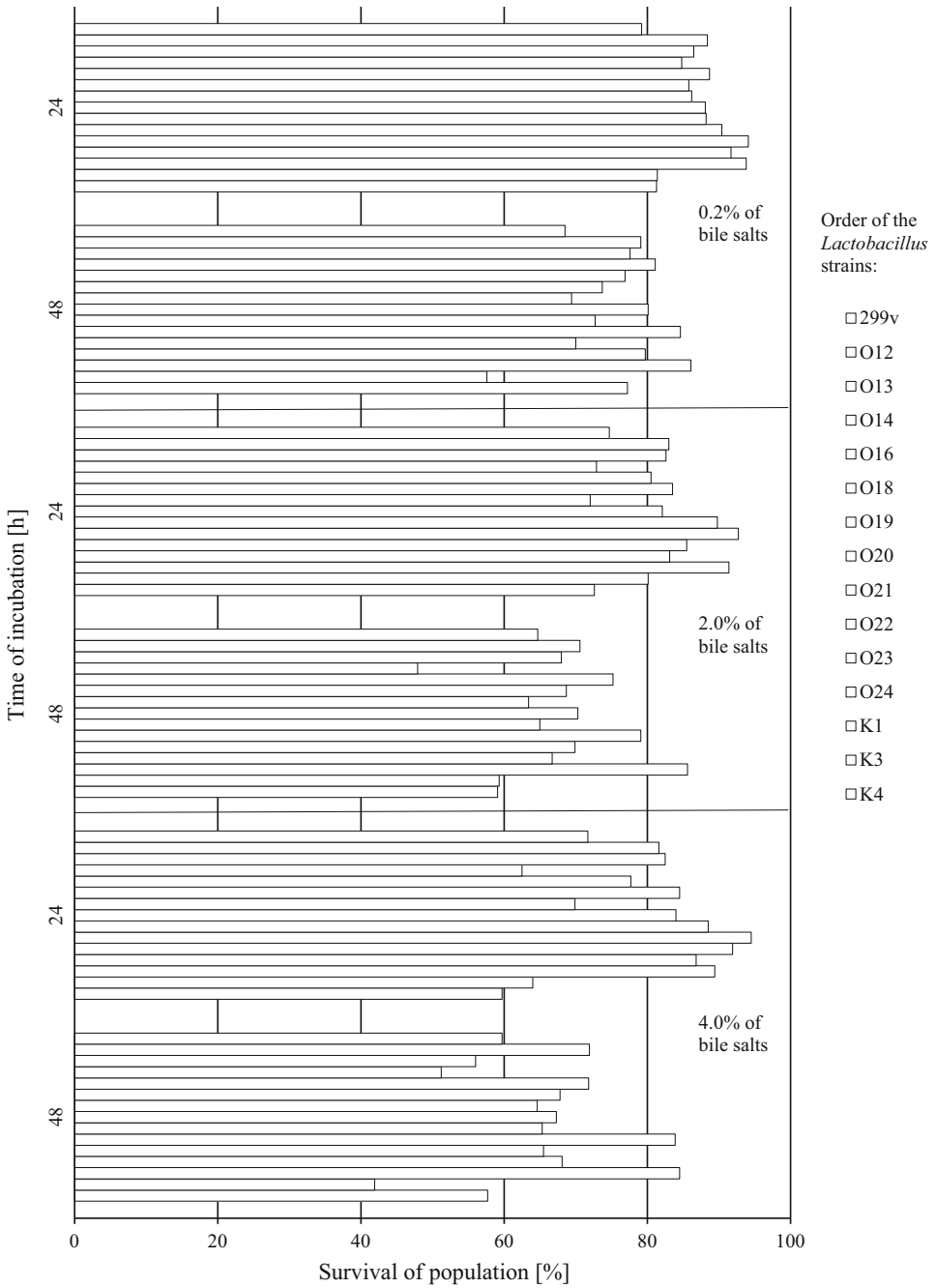

Fig. 1 Resistance of *Lactobacillus* strains to high percentage concentrations of bile salts Initial count of bacteria was approx. 9.5 log CFU/ml. All differences of survival the all tested strains at 0.2, 2.0 and 4.0 % of bile salts were significant ($P < 0.05$)

as well as probiotic characteristics [51]. According to Holzapfel [25], there is an increasing need to select new strains of bacteria with functional properties for commercial production and for improvement of quality of existing traditional fermented food.

The lactic acid bacteria isolated and identified in our pickled cucumber and cabbage samples have been also reported in other investigation of fermented foods. *Lactobacillus plantarum* has been predominantly isolated from natural fermented cabbage and cucumber in earlier studies. Other authors isolate also such species as *Lactobacillus brevis, Lactobacillus buchneri, Lactococcus lactis* subsp. *lactis*, and *Lactobacillus acidophilus* [4, 17, 21, 36, 48, 49, 60, 61, 66, 69]. The dominance of *Lb. plantarum* in pickled vegetables has been attributed to its high acid tolerance [3, 43].

Ability to overcome physical and chemical barriers such as gastric acidity and bile toxicity in the gastrointestinal tract and adherence to mucus or human epithelial cell lines are among the in vitro tests that are frequently suggested for the evaluation of the probiotic potential of the bacterial strain [7, 38, 56].

Table 3 Adhesion to hydrocarbons measured using the BATH test and BSH activity of *Lactobacillus* isolates

Isolate	BSH activity[a]	BATH [%]
299v	+	25.4 ± 4.2
O12	−	32.3 ± 6.2
O13	−	32.7 ± 8.1
O14	−	41.4 ± 5.8
O16	−	39.4 ± 4.3
O18	−	40.1 ± 9.2
O19	−	41.2 ± 2.2
O20	−	39.5 ± 5.9
O21	−	27.2 ± 6.8
O22	∓	51.7 ± 2.9
O23	−	24.7 ± 3.5
O24	∓	57.9 ± 8.7
K1	−	41.6 ± 5.2
K3	−	64.7 ± 6.4
K4	+	73.4 ± 2.3

[a] Capacity to express bile hydrolase activity as detected when precipitation zones of deoxycholic acid appeared around bacterial colonies growing on MRS agar plates supplemented with 0.5 % sodium salt of taurodeoxycholic acid

Stresses to microorganisms begin in the stomach, with pH between 1.5 and 3.0 and in the upper intestine which contains bile [58]. Stomach pH can be as low as 1.0 [68]. However, survival of ingested bacteria in the stomach obviously is influenced by the buffering capacity of food matrix, that is why in most in vitro assays pH 3.0 has been preferred [56]. Del Piano et al. [14] tested seven of *Lactobacillus plantarum* strains for their resistance to both simulated gastric juice and human gastric juice withdrawn on an empty stomach from healthy individuals. It was noted that less than 20 % of the bacteria survived after an hour of exposure to simulated gastric juice, while human gastric juice allowed a survival rate between 15 and 45 %.

Survival of bacterial strains in low pH conditions is a more accurate indication of the ability of strains to survive passage through the stomach. For this reason, in vitro survival tests in three different low pH (1.5, 2.5, 3.5) values were conducted. Analysis of results in this study suggest that all tested strains are able to survive in great number of cells under the conditions simulated gastric juice at pH 2.5 and 3.5. The applied in the present study experimental conditions are the simulation of the human gastric juice environment, not quite adequately, but are usually used by many researchers to quickly screening of potential probiotic properties of the bacteria [3, 38, 56, 65]. The other researchers have obtained similar results, noting that the survival rate of bacteria of the genus *Lactobacillus* in a low pH ranges from 30–100 %. In the studies conducted by Casey et al. [7] LAB isolates were examined for ability to survive with simulated gastric juice at pH 1.85. Only one of the strains (*Lactobacillus johnsonii* 4) was able to survive after 30 min of incubation. Also in similar studies Kumar et al. [31] observed that only four strains of *Lactobacillus* (out of 34) could survive up to 2 h at pH 1.85. Schillinger et al. [56] suggested that *Lb. casei* group were more susceptible to gastric acidity than strains of the *Lb. acidophilus* group. On the other hand Mathara et al. [38] observed that

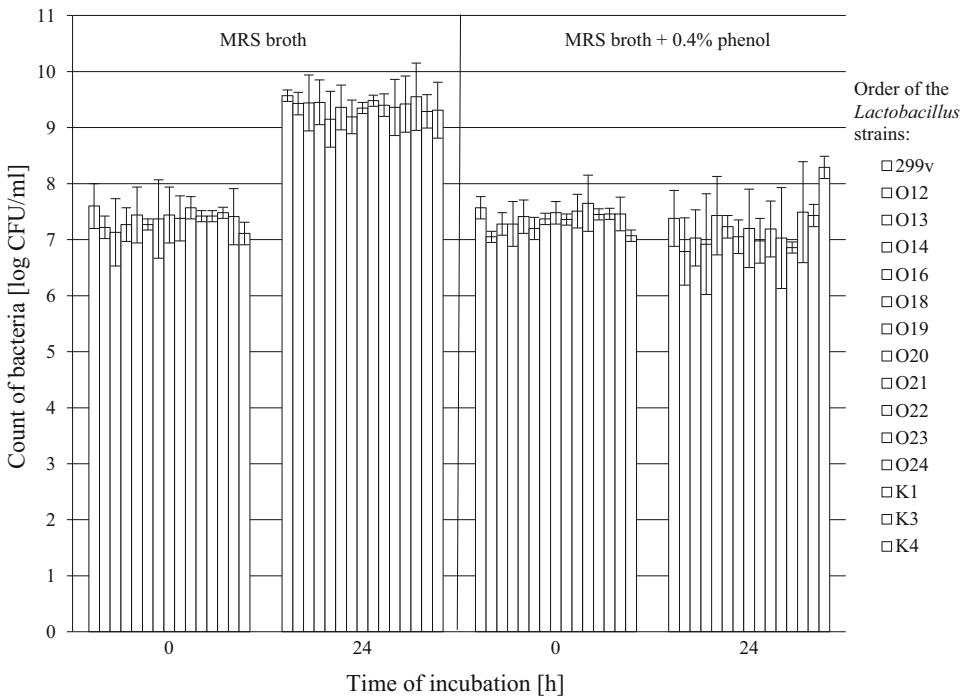

Fig. 2 Tolerance of *Lactobacillus* strains to 0.4 % phenol

Table 4 Minimal inhibitory concentration (MIC) in [μg/ml] of selected antibiotics in *Lactobacillus* strains

Isolate	MIC [μg/mL]				
	SM	VA	TC	AM	GM
O12	384 (R)	>256 (R)	0.125 (S)	0.064 (S)	>256 (R)
O13	>1,024 (R)	>256 (R)	0.125 (S)	0.25 (S)	>256 (R)
O14	512 (R)	>256 (R)	0.5 (S)	0.38 (S)	>256 (R)
O16	>1,024 (R)	>256 (R)	0.5 (S)	0.38 (S)	>256 (R)
O18	>1,024 (R)	>256 (R)	8 (S)	0.38 (S)	>256 (R)
O19	>1,024 (R)	>256 (R)	0.75 (S)	0.094 (S)	>256 (R)
O20	>1,024 (R)	>256 (R)	2 (S)	0.094 (S)	64 (R)
O21	>1,024 (R)	>256 (R)	6 (S)	0.032 (S)	256 (R)
O22	64 (R)	>256 (R)	2 (S)	0.25 (S)	256 (R)
O23	>1,024 (R)	>256 (R)	>256 (R)	0.64 (S)	>256 (R)
O24	>1,024 (R)	>256 (R)	2 (S)	0.25 (S)	>256 (R)
K1	>1,024 (R)	>256 (R)	>256 (R)	0.016 (S)	24 (R)
K3	128 (R)	>256 (R)	1.5 (S)	0.94 (S)	12 (S)
K4	32 (R)	24 (R)	0.19 (S)	0.125 (S)	96 (R)

SM streptomycin, *VA* vancomycin, *TC* tetracycline, *AM* ampicillin, *GM* gentamicin

(S) and (R): Sensitive and Resistant strains according to the break-points established by EFSA (2008)

Lb. fermentum strains showed better survival when exposed to pH 2.0 and 2.5 solution than *Lb. acidophilus* strains, while *Lb. plantarum* represent poor viability [40].

Bile acids are synthesized in the liver from cholesterol and are secreted from the gall bladder into the duodenum in the conjugated form. The physiological concentration of bile acids in the small intestine is between 5.000 and 20.000 lM [24]. The relevant physiological concentration of human bile ranges from 0.3 to 0.5 %.

Many authors accented that there is still no consensus about concentration of bile salts which are used in simulating small intestine [14, 65]. Studies in bile salt must be used with at least 0.15 % concentration [56].

In our selection of *Lactobacillus* strains, we have used the criteria (pH and bile resistance) similar to those had published by Kurman [32]. According to the author the use of 4 % bile salt concentration is a good indicator of the characteristics of probiotics. It has been also suggested that LAB strains vegetable origins are generally less bile-resistant [42]. Mathara et al. [38] have found that strains of *Lb. acidophilus* and *Lb. rhamnosus* group showed highest bile salt tolerance, compared to other lactobacilli.

Table 5 Enzymatic profile of *Lactobacillus* strains

Enzyme	Isolate													
	O12	O13	O14	O16	O18	O19	O20	O21	O22	O23	O24	K1	K3	K4
Control	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Alkaline phosphatase	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Esterase (C4)	0	0	0	*5*	0	0	0	0	0	0	0	0	0	0
Esterase lipase (C8)	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Lipase (C14)	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Leucine arylamidase	*4*	*4*	*4*	*4*	2	*4*	2	*4*	0	3	*4*	*4*	3	*4*
Valine arylamidase	*4*	*4*	*4*	*4*	*4*	3	*4*	3	3	0	3	*4*	*4*	3
Cystine arylamidase	1	*4*	*4*	*4*	2	1	*4*	2	2	0	*4*	*4*	2	*4*
Trypsin	0	0	0	0	0	0	0	0	0	0	0	0	0	0
α-Chymotrypsin	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Acid phosphatase	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Naphthol-AS-BI-phosphohydrolase	0	0	0	0	0	0	0	0	0	0	0	*4*	0	0
α-Galactosidase	0	0	0	0	0	0	0	0	0	0	0	0	0	0
β-Galactosidase	*5*	*5*	*5*	*4*	*5*	*4*	0	0	*5*	3	*5*	*5*	*5*	*5*
β-Glucuronidase	0	0	0	0	0	0	0	0	0	0	0	0	0	0
α-Glucosidase	*5*	*4*	*5*	*4*	*4*	*4*	*4*	3	*4*	*4*	*4*	*5*	3	3
β-Glucosidase	2	2	*4*	2	2	2	2	*4*	2	2	0	*4*	2	0
N-acetyl-β-glucosaminidase	2	2	*4*	2	2	2	2	*4*	2	2	2	*4*	0	2
α-Mannosidase	0	0	3	0	0	0	3	3	3	3	3	3	0	3
α-Fucosidase	0	0	0	0	0	0	3	0	0	0	3	3	0	3

0–3 no enzymatic activity

>3 enzymatic activity

A recent study reported that the strains isolated from pickled vegetables represent good survival under bile salt stress conditions. All numbers of cells of tested strains decrease after 24 and 48 h of incubation. Most resistant to these conditions were *Lb. brevis* O22, *Lb. plantarum* K1, and O23. Suskovic et al. [59] reported that *Lb. plantarum* strains isolated from fermented olives have been tolerance to low pH and high bile salts concentration. Also *Lb. plantarum* strains isolated from fermented milk products were resistant to 0.1–0.5 % concentration of bile salts [39].

It is considered that BSH enzyme might be a detergent shock protein that enables bacteria to survive the intestinal bile stress [12]. While Moser & Savage [41] have proposed that BSH activity facilitates the survival of lactobacilli in the gastrointestinal tract. It is likely that bile salt hydrolase activity may contribute to resistance of lactic acid bacteria to the toxicity of conjugated salts in the small intensity. On the other hand, some data indicate that BSH activity and resistance to bile salts are unrelated in lactobacilli [41], what was also showed in our study. Moreover, most BSH activity relates to strains that have been isolated from the intestines or feces of mammals-rich environment, and non-conjugated bile acids. Strains of other environments, such as milk or vegetables-environment in which bile salts are absent, usually do not possess bile salt hydrolase activity [62] as we have also observed in this study.

Phenols may be formed in the intestine as a result of the bacterial deamination of some aromatic amino acids derived from dietary and endogenous proteins and are believed to act as co-carcinogens [8, 59]. Some LAB create the anti-carcinogenic mechanisms allowing for tolerance to phenols. Vizoso Pinto et al. [65] reported that *Lb. plantarum* strains isolated from children's faeces tolerate phenol at 0.4 % for 24 h as their numbers did not decrease from an initial inoculum, but *Lb. johnsonii* strains were in a great extent inhibited. On the other hand in our present study *Lb. johnsonii* K4 strain showed the best resistant to the phenol from the tested strains. Also Kumar et al. [31] have found that 4 strains isolated form vegetables pickles showed different degrees of sensitivity toward this compound. On the other hand Xanthopoulos et al. [67] reported, that most of the tested strains *Lb. paracasei* subsp. *paracasei, Lb. rhamnosus, Lb. acidophilus, Lb. gasseri*, and *Lb. reuteri* isolates from new-born infants grew, although at lower levels, in the presence of phenol at 0.4 %, during incubation for 24 h. In the present study, the investigated lactobacilli strains survival and growth rates were slightly affected by phenol during 24 h incubation, so it could be supposed that bacteria are able to grow and live in the presence of this compound in colon during the transit time.

Having survived the acid and bile challenges of the gastrointestinal tract, it may be advantageous for LAB to adhere to the luminal epithelium and colonize the lower gastrointestinal tract of a human. High cell surface hydrophobicity may favor the colonization of mucosal surfaces and play a role in the adhesion of bacteria to epithelial cells [22]. On the other hand Mathara et al. [39] did not find any correlations between hydrophobicity and bacterial adhesion. Thus, hydrophobicity may be helpful for adhesion, but it is obviously not a prerequisite for a strong adherence capacity [56]. All isolates examined in this study demonstrated high degrees of adherence to hydrocarbons, but we found the considerable differences between the strains. Strains of *Lb. johnsonii* K4 and *Lb. rhamnosus* K3 and also *Lb. brevis* O22 and O24 exhibit higher hydrophobicity values as compared to *Lb. plantarum* and *Lb. casei* strains. The other strains represent moderate and low character of hydrophobicity (Table 3). The high cell surface hydrophobicity of the *Lb. rhamnosus* K3 and *Lb. johnsonii* K4 strains could play a significant role in interaction with other organic mucin layer of the gut. It needs to be investigated.

The other authors have observed that *Lb acidophilus* and *Lb. fermentum* groups obtained better hydrophobicity values than *Lb. plantarum* and *Lb. rhamnosus* [38, 55]. Studies on the cell surface characteristic of the *Lb. casei* group suggest a relatively hydrophilic nature of these strains [45].

The methods used in this study do not give comprehensive information about the survival bacteria under gastrointestinal conditions. The in vitro assays are quite different from the in vivo conditions, because the human gut food matrix pays the protective role for the bacteria [5]. However, the in vitro tests provide important information about species and strain differences and are very helpful and powerful tools especially for quick screening the bacteria for probiotic activity.

One of the safety considerations in probiotic studies is the verification that a potential probiotic strain does not harbor acquired and transferable antibiotic resistance genes. Studies on the antibiotic resistance of lactobacilli indicated that they were usually resistant to major classes of antibiotics such as β-lactams, aminoglycosides, cephalosporins, and quinolones [29]. In the present study, all strains were resistant to streptomycin, vancomycin, and except *Lb. rhamnosus* K3 to gentamicin. However, according to previous studies [31, 40, 65] such antibiotic resistance observed for *Lactobacillus* strains in this work, were considered to be intrinsic or natural resistances and, therefore, non-transmissible. The resistance of *Lactobacillus* strains to tetracycline has also been previously observed and often considered as potential transferable acquired resistances [44]. In this study only 2 strains (*Lb. plantarum* O23 and K1) were resistant to tetracycline and for this reason, at this stage, they were excluded as candidate

probiotics. Future research should focus on the location and potential transferability of antibiotic resistance genes.

Safety assessment must also include the lack of harmful activities, such as β-glucuronidase activity. β-glucuronidases liberate toxins and mutagens that have been glucuronated in the liver and excreted into the gut with the bile. This can lead to high local concentrations of carcinogenic compounds within the gut, thus increasing the risk of carcinogenesis. Also α-chymotrypsin, β-glucosidase, and *N*-acetyl-β-glucosaminidase activities may have negative effects in the colon [23]. In present study, all tested strains have not possess β-glucuronidase and α-chymotrypsin activity, however 3 of *Lactobacillus* strains (*Lb. casei* O14, *Lb. plantarum* O21 and K1) produce β-glucosidase, and *N*-acetyl-β-glucosaminidase, which excludes them from further probiotic investigations. Among the enzymes perhaps the most significant is β-galactosidase which helps in lactose digestion and ameliorates the disorders associated with lactose intolerance. Fewer (11 of 14 tested strains) possess β-galactosidase activity which suggests the possibility of using the strains in the production of fermented milk products.

Conclusions

In this study, the in vitro tests were used to screening the most important probiotic activity of bacteria isolated from pickled cucumber and cabbage. The findings revealed that strains of *Lactobacillus* spp. investigated at this study were characterized by the high survival under simulates gastrointestinal conditions. Strains *Lb. johnsonii* K4, *Lb. brevis* O22 and *Lb. plantarum* K1 tolerate gastric and small intestinal conditions very well, and also strain *Lb. johnsonii* K4 has a high adhesion capacity. Furthermore, strains group *Lactobacillus plantarum* and *Lactobacillus brevis* tolerate gastric-intestinal conditions better than *Lactobacillus casei* group.

All tested strains probably could be able to enter the intestinal tract alive, which requires them to future studies on in vitro and in vivo probiotic properties. However, four of tested strains (*Lb. casei* O14, *Lb. plantarum* O21, O23, K1) have been excluded as probiotic candidate according to the antibiotic resistant and enzymatic profile study.

We are currently characterizing the antagonistic activity to the pathogenic bacteria and bacteriocins produced by these cultures. On the basis of the results 10 of the *Lactobacillus* spp. strains isolated from pickled cucumber and cabbage have the potential for use in developing probiotic food as a starter culture.

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