IDENTIFICATION AND EXAMINATION OF SOME PROBIOTIC PROPERTIES OF THE STRAIN *LACTOBACILLUS* LBRZ12

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Abstract: *In order to be included in the composition of probiotic preparations, a strain should have certain probiotic characteristics, so that when administered as a probiotic it would exercise its beneficial effects on the health of the host. A Lactobacillus strain, Lactobacillus LBRZ12, was identified using contemporary biochemical (API 50 CHL) and molecular genetic (ARDRA) methods. The antimicrobial activity of the strain was determined by the diameter of the hydrolysis zones against 4 saprophytic microorganisms – Bacillus subtilis, Bacillus mesentericus, Aspergillus niger and Saccharomyces cerevisiae. Its antibiotic resistance was determined with the use of 22 of the most commonly used in medical practice antibiotics. The resistance of Lactobacillus LBRZ12 to the conditions in the gastrointestinal tract was tested in vitro with solutions with pH=2 + pepsin, pH+8 + pancreatin, as well as with solutions with different concentrations of bile salts – 0.15%, 0.3%, 0.6%, 1% . The Lactobacillus strain, isolated from fermented cabbage, was identified as Lactobacillus plantarum. Some of the probiotic properties of the selected culture were examined. Lactobacillus LBRZ12 has high antimicrobial activity against saprophytic microorganisms, resistance to most of the antibiotics, currently applied in medical practice, resistance to the conditions in the gastrointestinal tract (pH 2 + pepsin, pH 8 + pancreatin, more than 0.3% bile salts). The probiotic properties of Lactobacillus LBRZ12 define the strain as a potentially probiotic culture suitable for inclusion in the composition of starter cultures for meat and bakery products.*

Keywords: *API 50 CHL, API ZYM, antimicrobial activity, antibiotic resistance, stomach juice, intestinal juice, bile salts*

1. Introduction

Lactobacilli belong to the natural microflora of human and animal organisms. Together with bifidobacteria they maintain the balance between the microbial species in the gastrointestinal tract through the production of short chain acids (lactic, acetic, etc.), bacteriocins [2] and other metabolites that inhibit enteropathogens [4, 6, 7]. In acidic medium lactobacilli stop the putrefactive processes, thus ensuring the normal functioning of the digestive system [9, 10]. The intake of lactic acid bacteria in the composition of fermented foods or probiotics helps to restore the gastrointestinal microflora and to overcome disbacteriosis [8]. Not all lactobacilli can be included in the composition of probiotics and probiotic foods, but only those that have certain properties [9, 10]. Some of these properties are the ability of the strains to survive in the conditions of the gastrointestinal tract,

sustaining high concentration of viable cells, antimicrobial activity against saprophytes and antibiotic resistance.
The aim of this study is to examine some of the probiotic properties of the strain *Lactobacillus* LBRZ12 of plant origin: sustainability in artificial gastric and bile juice, antimicrobial activity and antibiotic resistance.

2. Experimental

Determination of the biochemical profile
The system API 50 CHL (BioMerieux SA, France) is used for the identification of the species of the genus *Lactobacillus* based on their ability to utilize 49 carbon sources. Fresh 24-hour culture of the studied strain is centrifuged for 15 min at 5000xg. The obtained sludge, containing biomass, is washed twice with PBS-buffer and resuspended in medium API 50 CHL, an integral part of the used kit. The API strips are placed in the incubation boxes, the microtubules are inoculated with the prepared cell suspension and sealed with sterile liquid paraffin. The results are reported on the 24^{th} and the 48^{th} hour of incubation at 37°C ± 1°C. Reporting is done, based on the colour change of each microtubule, compared to the colour of the control microtubule (microtubule 0). Positive results were recorded in the cases of color change from blue to green or bright yellow. The obtained results are processed with apiweb® identification software.
Determination of the profile of antibiotic sensitivity
The profile of antibiotic susceptibility is determined by the disc diffusion method according to Bauer, Kirby et al., 1966 [1]. Fresh 24-hour culture of the tested strain is used to inoculate the plates with MRS-agar medium. Standard discs impregnated with antibiotic are placed in the plates. Then the plates are incubated for 48 hours at optimal temperature. The diameter (in mm) of the sterile zones formed around each antibiotic disc is recorded. The following indications are used: R - resistant (zone diameter < 8 mm), SR - intermediate sensitive (zone diameter - 8-16 mm), S - sensitive (zone diameter > 16 mm).
Determination of the antimicrobial activity
To determine the antimicrobial activity of the studied strain cultural liquid (CL), cultural acellular supernatant without pH adjustment (CASN) and neutralized acellular supernatant (NASN) (pH 6.5), obtained from a 24 hour culture of the strain are used. The antimicrobial activity of the strain is tested against the following test microorganisms: *Bacillus subtilis, Bacillus mesentericus*, *Saccharomyces cerevisiae* and *Aspergillus niger*. Each of the test microorganisms (10^8cfu/cm^3) is inoculated in agar medium and then the medium is poured in the plates. After hardening of the agar, wells are prepared (6 mm). 0,1 cm^3 of CL, CASN and NASN are dropped in the wells and the plates are placed at 4ºC for 1h to allow diffusion in the agar. For each combination of saprophyte + *Lactobacillus* strain two plates are prepared. Then one of the plates is incubated at 30ºC and the other plate at 37ºC for 24 h and the inhibition zones in mm are reported.
Determination of the survival at low pH in the presence of pepsin and at weakly alkaline pH in the presence of pancreatin [3].
Fresh 24-hour culture of the tested strain is centrifuged for 15 min at 5000xg. The sludge, containing biomass, is washed twice with PBS-buffer and resuspended to the initial volume in PBS-buffer. 0.2 cm^3 of the cell suspension is incubated with 5 cm^3 buffer solution with pH = 2, containing 0,5% NaCl and pepsin (at a concentration of 3.2 g/dm^3) (Sigma, 2,500-3,500 U/mg protein) and buffer solution with pH=8 containing 0,5% NaCl and pancreatin (at a concentration 1 g/dm^3)

(Sigma, 2,500-3,500 U/mg protein) at a suitable temperature for the studied strain for 24h. Aliquots are taken for determination of the total number of viable cells (cfu/cm^3) at the 0, 2, 4 and 24th hour.

Determination of the tolerance to bile salts (method modified by Denkova Z., 2005 [5]).

MRS-broth medium, containing bile salts at different concentrations (0%, 0.15%, 0.3%, 0.6% and 1%), is inoculated with 4% inoculum of a 24 hour culture of the tested strain. During the incubation for 24h at the optimum temperature for the tested strain aliquots are taken to determine the total number of viable cells (cfu/cm^3) at the 0, 2nd, 4th, 6th, 8th and 24th hour.

Profile of enzyme activity

The system API ZYM (BioMericux, France) is used for semi-quantitative examination of the enzyme profile of the studied strain. Fresh 24-hour culture of the strain is centrifuged for 15 min at 5000xg, the resulting sludge, containing biomass, is washed twice and resuspended in API suspension medium. The API ZYM strips are placed in the incubation boxes and the wells are inoculated with the prepared cell suspension. The samples are incubated for 4 hours at 37°C. Then one drop of reagent A and reagent B are added to each well. After 5 min staining is recorded as described in the color scheme in the manufacturer's instructions. Enzyme activity is determined by the color scale from 0 (no enzyme activity) to 5 (maximum enzyme activity).

3.Results and discussion

Biochemical characteristics of *Lactobacillus* LBRZ12

The biochemical profile of the strain *Lactobacillus* LBRZ12 is examined using the system for rapid identification API 50 CHL (Biomerieux, France).

Table 1.
Ability of *Lactobacillus* LBRZ12 to utilize the 49 carbon sources included in API 50 CHL

#	Carbohydrates	Lactobacillus LBRZ12
1	Glycerol	-
2	Erythriol	-
3	D-arabinose	-
4	L-arabinose	+ (90-100%)
5	Ribose	+ (90-100%)
6	D-xylose	-
7	L-xylose	-
8	Adonitol	-
9	β-metil-D-xyloside	-
10	Galactose	+ (90-100%)
11	D-glucose	+ (90-100%)
12	D-fructose	+ (90-100%)
13	D-mannose	+ (90-100%)
14	L-sorbose	-
15	Rhamnose	-
16	Dulcitol	-
17	Inositol	-
18	Manitol	+ (90-100%)
19	Sorbitol	+ (90-100%)
20	α-methyl-D-mannoside	+ (90-100%)
21	α-methyl-D-glucoside	-
22	N-acetyl-glucosamine	+ (90-100%)
23	Amigdalin	+ (90-100%)
24	Arbutin	+ (90-100%)
25	Esculin	+ (90-100%)
26	Salicin	+ (90-100%)
27	Cellobiose	+ (90-100%)
28	Maltose	+ (90-100%)
29	Lactose	+ (90-100%)
30	Melibiose	+ (90-100%)
31	Saccharose	+ (90-100%)
32	Trehalose	+ (90-100%)
33	Inulin	-
34	Melezitose	-
35	D-raffinose	+ (90-100%)
36	Amidon	-
37	Glycogen	-
38	Xylitol	-
39	β-gentiobiose	+ (90-100%)
40	D-turanose	+ (90-100%)
41	D-lyxose	+/- (40-45%)
42	D-tagarose	+/- (40-45%)
43	D-fuccose	+/- (30-35%)
44	L-fuccose	-
45	D-arabitol	+ (65-70%)
46	L-arabitol	-
47	Gluconate	+ (90-100%)
48	2-keto-gluconate	-
49	5-keto-gluconate	-

According to the strain's ability to utilize 49 carbon sources (Table 1) is determined its belonging to the species *Lactobacillus plantarum* 1 with the corresponding percentage of accuracy (99.9%).

In order to confirm the result of the biochemical study the molecular genetic method ARDRA with the restriction enzymes *Eco*RI, *Hap* II and *Hae*III is applied. The restriction profile of the strain

Lactobacillus LBRZ12 with each of the three restriction enzymes coincides with the profile of the type strain *Lactobacillus plantarum* DSM 20174. The results of the molecular genetic analysis confirmed the identity of Lactobacillus LBRZ12 as a representative of the species *Lactobacillus plantarum.*

In a series of experiments some of the strain's probiotic properties have been established.

Antimicrobial activity of *Lactobacillus* LBRZ12

The antimicrobial activity of *Lactobacillus* LBRZ12 is studied using the method of diffusion in agar. The results of the double retries are summarized in Table. 2.

Table 2.
Antimicrobial activity of *Lactobacillus* LBRZ12 against saprophytic microorganisms. The values are in mm. Diameter of the wells - 6 mm.

Saprophytic microorganism Concentration of the suspension	Cultural liquid		Cultural acellular supernatant		Neutralized acellular supernatant	
	30^0C	37^0C	30^0C	37^0C	30^0C	37^0C
Bacillus subtilis $1{,}9x10^7$cfu/cm^3	-	-	-	-	-	-
Bacillus mesentericus $4x10^6$cfu/cm^3	9	11,5	9	11	-	-
Saccharomyces cerevisiae $9{,}2x10^6$cfu/cm^3	-	-	-	-	-	-
Aspergillus niger $1{,}2x10^7$cfu/cm^3	12	12	10	12	-	-

Antimicrobial activity against *Bacillus mesentericus* and *Aspergillus niger*, but not against *Bacillus subtilis* and *Saccharomyces cerevisiae* is established. For *Bacillus mesentericus* the diameters of the sterile zones of the cultural liquid and the cultural acellular supernatant are comparable, which means that the inhibition of the strain *Lactobacillus plantarum* LBRZ12 is due to the production of acids, which leads to a decrease in pH. For *Aspergillus niger* the antimicrobial activity of the cultural liquid is higher than that of the cultural acellular supernatant and the neutralized acellular supernatant, which means that the suppression of the test microorganism is a result of the competition for nutrients and/or attachment sites as well as of the lowering of pH due to the acids synthesized by *Lactobacillus plantarum* LBRZ12.

Antibiotic resistance of *Lactobacillus plantarum* LBRZ12

The initial concentration of viable cells of *Lactobacillus plantarum* LBRZ12 is $1.3x10^{13}$cfu/cm^3.

Table 3.
Antibiotic resistance of *Lactobacillus* LBRZ12

Mechanism of action	#	Antibiotic		Concentration	Lactobacillus LBRZ12
Inhibitors of the synthesis of the cell walls	1	Penicillin	P	10 E/disc	R
	2	Azlocillin	Az	75 µg/disc	R
	3	Piperacillin	Pi	100 µg/disc	R
	4	Ampicillin	A	10 µg/disc	S
	5	Oxacillin	O	1 µg/disc	R
	6	Amoxicillin	Ax	25 µg/disc	R
	7	Vancomycin	V	30 µg/disc	R
	8	Cefamandole	Cm	30 µg/disc	SR
Inhibitors of the protein synthesis	9	Tetracycline	T	30 µg/disc	R
	10	Doxycycline	D	30 µg/disc	S
	11	Gentamycin	G	10 µg/disc	R
	12	Streptomycin	S	30 mg/disc	R
	13	Clindomycin	Cl	2 µg/disc	S
	14	Kanamycin	K	30 µg/disc	R
	15	Tobramycin	Tb	10 µg/disc	SR
	16	Amikacin	Am	30 µg/disc	R
	17	Rifampin	R	5 µg/disc	SR
	18	Lincomycin	L	15 µg/disc	S
	19	Chloramphenicol	C	30 µg/disc	SR
	20	Erythromycin	E	15 µg/disc	S
Inhibitors of the synthesis of DNA and/or of the binary fission	21	Nalidixic acid	Nx	30 µg/disc	R
	22	Ciprofloxacin	Cp	5 µg/disc	R

Legend: R-resistant, SR - intermediate (zone 7-16 mm), S - sensitive (zone> 16 mm)

In order to study the spectrum of antibiotic resistance of the strain 22 antibiotics with different mechanisms of action belonging to the main groups used in medical practice were selected. The results of the conducted diffusion method according to Bauer, Kirby et al., 1966 [1] for 24 h are summarized in Table. 3. The strain *Lactobacillus plantarum* LBRZ12 is resistant to penicillin, cefamandole, nalidixic acid, ampicillin, amoxicillin, tetracycline, oxacillin, gentamycin,

kanamycin, amikacin, vancomycin, rifampin, streptomycin and chloramphenicol and ciprofloxacin but it is sensitive to clindomycin, doxycycline, lincomycin, tobramycin, azlocillin, piperacillin and erythromycin.

In vitro determining the ability of *Lactobacillus plantarum* LBRZ12 to survive in the conditions simulating the departments of the gastrointestinal tract

In order to be included in the composition of probiotic preparations, the probiotic strains must possess the ability to survive in the conditions of the gastrointestinal tract. The stability of the cells of *Lactobacillus plantarum* LBRZ12 in artificial conditions, simulating the conditions of the gastrointestinal tract - pH = 2 + pepsin and pH = 8 + pancreatin, is examined. In a parallel experiment the tolerance of the strain to different concentrations of bile salts is tested. All three experiments - survival in artificial gastric and pancreatic juice and tolerance to high concentrations of bile salts - were held for 24 h. The results of the experiments are presented on Fig. 1 and Fig.2.

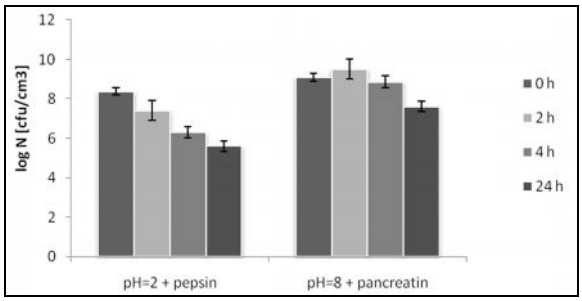

Figure 1. Survival of the cells of *Lactobacillus plantarum* LBRZ12 at low pH (pH = 2) + pepsin and weakly alkaline pH (pH=8) + pancreatin.

Higher sensitivity to low pH (pH=2) + pepsin than to pH=8 + pancreatin is observed (Fig. 1). For the residence time of food in the stomach - 1.5 to 3 hours - in the acidic environment the number of viable cells of *Lactobacillus plantarum* LBRZ12 is reduced by about 2 log units by the fourth hour and by about 3 log units by the 24th hour, while at pH=8 and pancreatin the number of viable cells decreases by about 1.5 log units by the 24th hour.

Another important factor that influences the survival of probiotic strains in the intestinal tract is the concentration of bile salts. It is known that about three hours after ingestion of food the concentration of bile salts in the small intestine reaches about 0.3%. This requires a study of the influence of different concentrations of bile salts on the development of the strain. This study is conducted in a liquid medium (MRS) with different concentrations of bile salts - 0%, 0.15%, 0.3%, 0.6% and 1% for 24-hour incubation (Fig. 2).

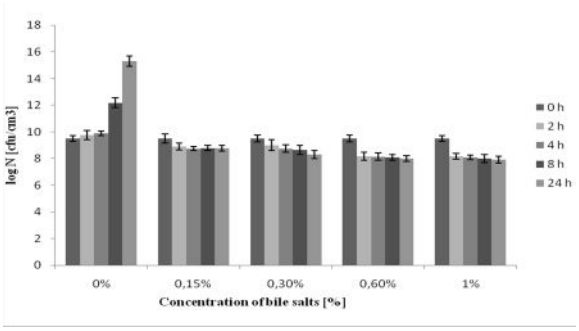

Figure 2. Survival of the cells of *Lactobacillus plantarum* LBRZ12 at different concentrations of bile salts.

The cells of the strain *Lactobacillus plantarum* LBRZ12 are resistant to the presence of bile salts in the medium (Fig. 2). The number of active cells decreases more rapidly in the first two hours at all concentrations of bile salts. Then the reduction of the number of viable cells continues up to the 24th hour, when the concentration of colony forming units is over 10^8cfu/cm^3 at all concentrations of bile salts (Fig. 2).

In the gastrointestinal tract of humans, however, such extreme conditions rarely occur.

Examination of the enzyme profile

The enzyme profile is important for determining the technological characteristics of lactobacilli and their applicability as starter cultures. Therefore, characterization of the strain *Lactobacillus plantarum* LBRZ12 included determining the presence of a set of 19 enzyme activities using the kit API ZYM (BioMerieux, France). The results of this study are shown in Table.4.

Table 4.
Enzymatic profile of *Lactobacillus plantarum* LBRZ12

	Enzyme	Activity*
1	Control	-
2	Alkaline phosphatase	0.5
3	Esterase	0.5
4	Esterase-lipase	1
5	Lipase	1.5
6	Leucine-aminopeptidase	5
7	Valine-aminopeptidase	5
8	Cysteine-aminopeptidase	4.5
9	Trypsin	-
10	Chymotrypsin	-
11	Acid phosphatase	3.5
12	Phosphohydrolase	2.5
13	α-galactosidase	-
14	β-galactosidase	5
15	β-glucoronidase	-
16	α-glucosidase	4
17	β-glucosidase	2.5
18	α-glucoseaminidase	5
19	α-manosidase	-
20	α-fucosidase	-

* enzyme activity is determined by the color scale from 0 (no enzyme activity) to 5 (maximum enzyme activity)

The strain *Lactobacillus plantarum* LBRZ12 has the following enzyme activities: alkaline phosphatase, esterase, esterase-lipase, lipase, leucine-aminopeptidase, valine-aminopeptidase, cysteine-aminopeptidase, acid phosphatase, phosphohydrolase, β-galactosidase, α-glucosidase, β-glucosidase, α-glucoseaminidase (Table 4). The results of the conducted experiments determine the strain *Lactobacillus plantarum* LBRZ12 as potentially probiotic.

4.Conclusion

The strain *Lactobacillus* LBRZ12 of plant origin has been identified as *Lactobacillus plantarum* through the application of physiological, biochemical and molecular genetic methods. The results of the strain for the possession of some probiotic properties - antimicrobial activity, antibiotic resistance and survival in the conditions of artificial gastric and intestinal juice – show the potential of the strain for inclusion in the composition of probiotic preparations.

5. References

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