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The Isolation, Identification and Analyses of *Lactobacillus* Genus Bacteria with Probiotic Potential

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Abstract

103 strains of lactic acid bacteria of *Lactobacillus* genus were isolated from natural sources and identified for genus and species level with API tests and 16S rRNA sequencing. However, only 27 strains from isolated cultures demonstrated a high stability to gastric stress and from that – only 15 strains were highly resistant to intestinal stress. Results indicated that only some isolated cultures of lactobacilli possessed potential probiotical properties and could serve as new probiotics for dairy industry with high resistance to gastro-intestinal stresses.

Keywords

Isolation of lactic acid bacteria • Molecular identification • Probiotic properties • Gastro-intestinal stress

1 Introduction

Scientific interest to lactic acid bacteria (LAB) emerged at the end of nineteenth century. Nowadays, live microorganisms if used in right quantities and benefit to host organism's health were defined as probiotics (Reid et al. 2003). In food industry the fortification of sour milk products with LAB probiotics was widely used (Buss 2004) that explained the interest for the studies of these microorganism survival and activity in the human intestinal tract. Food industry and Academy continued the research aimed at more profound studies of probiotic physiology and mechanisms which allowed them to benefit to human's health and wellbeing from intestinal tract (Vaughan et al. 2002; Reid et al. 2003; Boclé and Thomann 2005).

A wealth of information had been gathered over the past years on probiotics through experimental, animal and human studies, with the aim to understand the mechanism of actions and elucidate their beneficial health effects on human host. Significant amount of evidence existed for their ability to increase the bioavailability of minerals and to stimulate the immune system, although there were less clear evidences so far for their prophylactic or therapeutic role in gastrointestinal infections. Moreover, the effect of the food

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delivery vehicle on the efficacy of probiotics was an area that had been hardly investigated (Charalampopoulos and Rastall 2011). It was demonstrated that food-grade LAB could be used for the interacting with the human gut with benefits to its health (Sanchez et al. 2011).

Plenty of LAB strains were isolated from different sources: human milk (Langa et al. 2012) and dairy products (Bujňáková and Kmeť 2012), Italian and Argentinean cheeses (Zago et al. 2011), human and animal feces (Lee et al. 2011a; Martin et al. 2012; Zeng et al. 2011), fresh and fermented fruit and vegetable products (Kumar et al. 2012; Lee et al. 2011b; Vitali et al. 2012). Many of them had probiotic properties. They had to survive in the gastric juice and bile acids salts in the gastrointestinal tract of humans and animals (Lee et al. 2011a; Zeng et al. 2011; Jensen et al. 2012). In addition, some strains were found to be resistant to lysozyme (Kimoto-Nira et al. 2010). However, these properties were expressed in different degrees (Koll et al. 2010; Zago et al. 2011).

The aim of this study was to isolate the new LAB of *Lactobacillus* genus from different kinds of natural sources, to analyze the probiotic potential of the obtained cultures with testing of the strain resistance level to gastric and intestinal stresses.

2 Materials and Methods

2.1 Sampling

According to physiological features of LAB, four main sources for biological sampling were determined: (1) feces of adult humans and children of different ages; (2) feces of various animals; (3) hand-made dairy products from Moscow region and other areas of Russia; (4) anaerobic sludge of natural ponds and food manufacture waste waters.

2.2 Isolation of Lactobacilli from Natural Sources

MRS medium was used for the isolation of bacteria of genus *Lactobacillus* (Man et al. 1960). Isolation of pure cultures was performed in several stages.

The 1st stage – enrichment cultures. For obtaining of lactobacilli enrichment cultures, 1 g of sample was placed into 10 ml sterile serum vial, 5 ml of sterile liquid anaerobic MRS medium was added, serum vial was closed with sterile rubber stopper and fixed with aluminum crimple cap. Then, inoculated serum vial was placed into the thermostat at the temperature of 37 °C and was incubated for 1–3 days until the signs of growth appeared (dimness, change of medium color, appearance of the smell, gas formation).

The 2nd stage – isolation of pure cultures. To obtain pure culture, 0.1 ml of enrichment culture was plated onto solid MRS medium, according to Koch method, following the spreading with sterile spatula on three Petri dishes. The dishes were cultivated at the temperature of 37 °C under anaerobic conditions in anaerostat. The colonies were described, studied under microscope, their Gram stain, catalase and oxidase activities were determined. Only Gram-positive, oxidase- and catalase-negative colonies were selected; these cultures were inoculated into serum vials with liquid MRS medium. The isolate purity was confirmed by further spreading of cells on solid MRS medium to obtain single uniform colonies. In order of a long-time storage, the cultures were frozen at −70 °C in MRS broth containing 25 % (v/v) of glycerol and lyophilized in Free Zone freeze-dryer (Labconco, USA) at T = −51 °C and P = 49 kPa for 24 h. Dried cultures were kept at +4–6 °C in refrigerator.

2.3 Species Determination of *Lactobacillus*

Pure cultures of isolated LAB were tested for the usage of different carbon sources with API50CH test (BioMerieu Co., France).

Isolated cultures were further identified by partial sequencing of 16S rRNA gene and phylogenetic analysis of nucleotide sequences.

Isolation of DNA from a biomass of bacteria was done according to a technique described by Bulygina et al. (2002). Concentration of the

isolated DNA for the use of this method of was 30–50 mkg/ml.

For polymerase chain reaction and further sequencing of PCR-fragments of 16S rRNA gene, the universal primer system had been used (Lane 1991). The volume of amplification mixture was 50 μl, and it had the following composition: 1× buffer for DNA polymerase BioTaq [17 mM $(NH_4)_2SO_4$, 67 mM Tris-HCl, pH 8.8, 2 mM $MgCl_2$]; 12.5 pmol of each of dNTPs, 50 ng of DNA-matrix; 5 pmol of correspondent primers and 3 units of DNA-polymerase BioTaq (Dialat LTD, Russia). A pair of the following primers was used for amplification: 8-27f – 5-′-AGAGTTTGATCMTGGCTCAG-3′ and 1492r – 5′-TACGGYTACCTTGTTACGACTT-3′.

The temperature-time profile of PCR was as follows: the first cycle – 94 °C × 9 min, 55 °C × 1 min, 72 °C × 2 min; following by 30 cycles – 94 °C × 1 min, 55 °C × 1 min, 72 °C × 2 min; the final cycle – 72 °C × 7 min. The analysis of PCR products was performed with electrophoresis in 2 % agarose gel with electric field strength of 6 V/cm. The isolation and purification of PCR products was executed from low-melt point agarose using reagent kit by Wizard PCR Preps (Promega, USA), according to manufacturer's instructions.

The sequencing of the received PCR gene's fragments encoding 16S rRNA was performed according to Sanger's method (Sanger et al. 1977) using reagents kit of Big Dye Terminator v.3.1 (Applied Biosystems Inc., USA) on automatic sequenator ABI PRIZM 3730 (Applied Biosystems Inc., USA) according to manufacturer's instructions. The following primers were used for the sequencing: 27f 5-′-AGAGTTTGATCMTGGCTCAG, 519r 5-′-GWATTACCGCGGCKGCTG, 530f 5′-GTGCCAGCMGCCGCGG, 1114f 5-′-GCAACGAGCGCAACCC, 1492r 5-′-TACGGYTACCTTGTTACGACTT, 357f 5′-CTCCTACGGGAGGCAGCAG. The reading of amplicons was performed in two directions.

The primary analysis of 16S rRNA gene sequence of the studied strains was performed using BLAST software package (Camacho et al. 2009).

2.4 Resistance Degree Determination of Lactic Acid Bacteria of *Lactobacillus* Genus to Gastric and Intestinal Stresses

Gastric and intestinal stresses had been determined according by Pinto et al. (2006) with some modifications. The studied bacterial strains were cultivated anaerobically in MRS liquid medium, pH 6.5 for 24–48 h at 37 °C and 10 % (v/v) of CO_2.

To imitate gastric stress *in vitro*, 100 μl of the studied culture from a stationary growth phase were added to 1 ml of artificial gastric juice (dilution 1/11). The suspensions were incubated for 10, 30 and 60 min at 37 °C and 10 % CO_2. In the control sample, 1 ml of MRS was added to bacterial suspension instead of gastric juice. The tests were performed three times and the average value was calculated. After bacteria were incubated, the suspensions were diluted up to 10^{-10} in fresh MRS. The dilutions were plated to Petri dishes with MRS agar and incubated for 24–48 h at 37 °C and 10 % CO_2. The quantity of survived cells (with and without stress) was calculated according the colony counts in each dilution.

Artificial gastric juice composition contained: NaCl (Sigma S9625) – 2.2 g/l; L-lactic acid (Sigma L1750) – 9.9 g/l (0.11 M); porcine pepsin (Sigma P7125) – 3.5 g/l (600–1800 units/mg); pH 2.7 ± 0.02 (made up with 35 % NaOH); pH after dilution 1/11 was 3.10 ± 0.10 (pH was controlled for each culture).

To imitate intestinal stress *in vitro*, 100 μl of the studied culture in a steady state were added to 1 ml of artificial intestinal juice (dilution 1/11). The suspensions were incubated for 5 h at 37 °C and 10 % CO_2. In the control sample, 1 ml of MRS was added to bacterial suspension instead of intestinal juice. The tests were performed three times and the average value was calculated. After bacteria were incubated, the suspensions were diluted up to 10^{-10} in fresh MRS. The dilutions were plated to Petri dishes with MRS agar and incubated for 24–48 h at 37 °C and 10 %

CO_2. The quantity of survived cells (with and without stress) was calculated according the colony counts in each dilution.

The composition of artificial intestinal juice was as follows: bile salts (porcine bile, Sigma B8631) – 3.3 g/l (final concentration – 0.3 %), carbonate buffer $NaHCO_3$ (Sigma S8875) – 16.5 g/l (final concentration – 1.5 %); pH 6.3 ± 0.10 (pH was controlled for each culture).

The resistance degree to gastric and intestinal stresses – RD (Resistance Degree) was calculated according to formula: $RD = n_1/n_2$, where n_1 – amount of CFU in 1 ml of the control sample; n_2 – amount of CFU in 1 ml of the tested sample.

Cell resistance to gastrointestinal stresses was evaluated in the following ranges: very good – $RD \leq 5$; good – $6 < RD \leq 10$; acceptable – $11 < RD \leq 15$; unacceptable – $RD > 15$.

3 Results

3.1 Isolation and Identification of LAB

More than 1000 samples from different natural sources of biological material were analyzed. The species of 250 pure cultures of *Lactobacillus* were determined with API test. Of them, 103 strains of the isolated lactobacilli were determined as *Lactobacillus* genus bacteria and were further identified up to the species level using method of partial sequencing of 16S rRNA gene.

After identification of 16S rRNA sequences comparison, it was found those 13 strains of *L. brevis*, 9 strains of *L. paracasei*, 21 strains of *L. plantarum*, and 1 strain of each species: *L. delbrueckii*, *L. fermentum*, *L. johnsonii*, were isolated from dairy products and homemade cheeses. Two strains of *L. brevis*, 3 strains of *L. paracasei*, 10 strains of *L. rhamnosus* and 12 strains of *L. plantarum* were isolated from feces of newborns. Three strains of *L. brevis*, 3 strains of *L. paracasei*, 5 strains of *L. reuteri*, 6 strains of *L. rhamnosus*, 6 strains of *L. plantarum* and 1 strain of *L. vaginalis* were isolated from wild and homebred animal feces. Two strains of *L. paracasei* and 4 strains of *L. plantarum* were isolated from anaerobic sludge of natural ponds and food manufacture waste waters.

In total, 43 strains of *L. plantarum*, 18 – of *L. brevis*, 17 – of *L. paracasei*, 16 – of *L. rhamnosus*, 5 – of *L. reuteri* and 1 strain of each: *L. delbrueckii*, *L. fermentum*, *L. johnsonii*, *L. vaginalis* species were isolated from natural sources and homemade dairy products, which apparently reflected their spreading in natural and artificial ecological niches.

The sequences of 16S rRNA genes of 27 strains were deposited to GenBank database, where they were given numbers (Table 1).

3.2 The Determination of Resistance of Lactobacilli to Gastric and Intestinal Stresses

The tolerance of 103 isolated and determined to the species level with 16S rRNA gene sequencing *Lactobacillus* strains to gastric and intestinal stresses was studied. The results of only resistant cultures were presented in the Table 2. From 103 tested strains of lactobacilli, only 27 showed good resistance to gastric acid stress, after which they were further studied for tolerance to intestinal stress. As an example of not tolerant strains, four of them were included in Table 2 (pos. 28–31).

First of all, the isolated lactobacilli cultures were tested for the resistance to gastric juice, because bacteria in the food passed through the stomach first. *Lactobacillus* strains with good and very good resistance to gastric stress were further tested to intestinal stress influence (bile acids with pH 6.3). That corresponded to the task of this research aimed to find lactobacillus strains that could survive the passage through the stomach and duodenal intestine without considerable loss in their vitality.

During further comparison of the cultures listed in the Table 2, accordingly to the resistance to bile acids (the imitation of duodenum and upper small intestine of human gastrointestinal

Table 1 Accession numbers of the 16S rRNA sequences of isolated strains in GenBank

№	Strain name	Accession No. in GenBank
1	*Lactobacillus plantarum* CM MSU № 503	KJ160508
2	*Lactobacillus plantarum* CM MSU № 504	KJ459015
3	*Lactobacillus plantarum* CM MSU № 505	KJ459016
4	*Lactobacillus plantarum* CM MSU № 506	KJ459017
5	*Lactobacillus plantarum* CM MSU № 507	KJ459018
6	*Lactobacillus plantarum* CM MSU № 508	KJ459019
7	*Lactobacillus plantarum* CM MSU № 509	KJ459020
8	*Lactobacillus plantarum* CM MSU № 510	KJ459021
9	*Lactobacillus plantarum* CM MSU № 511	KJ459022
10	*Lactobacillus plantarum* CM MSU № 512	KJ459023
11	*Lactobacillus plantarum* CM MSU № 513	KJ459024
12	*Lactobacillus plantarum* CM MSU № 514	KJ459025
13	*Lactobacillus plantarum* CM MSU № 515	KJ459026
14	*Lactobacillus plantarum* CM MSU № 516	KJ459027
15	*Lactobacillus plantarum* CM MSU № 517	KJ459028
16	*Lactobacillus plantarum* CM MSU № 518	KJ459029
17	*Lactobacillus plantarum* CM MSU № 519	KJ459030
18	*Lactobacillus plantarum* CM MSU № 520	KJ459031
19	*Lactobacillus brevis* CM MSU № 521	KJ459032
20	*Lactobacillus brevis* CM MSU № 522	KJ459033
21	*Lactobacillus brevis* CM MSU № 523	KJ459034
22	*Lactobacillus brevis* CM MSU № 524	KJ459035
23	*Lactobacillus brevis* CM MSU № 525	KJ459036
24	*Lactobacillus brevis* CM MSU № 526	KJ459037
25	*Lactobacillus parocasei* CM MSU № 527	KJ459038
26	*Lactobacillus rhamnosus* CM MSU № 528	KJ459040
27	*Lactobacillus rhamnosus* CM MSU № 529	KJ459039

tract stress), it was noticed that among 27 cultures, showing high resistance to gastric stress, only 15 cultures had "very good" resistance level to intestinal stress, that reflected the rare occurrence of high resistance value to gastric and intestinal stresses combination.

It also should be noted that 8 of 15 strains, that demonstrated a high resistance to both kinds of stresses applied to them, belonged to *L. plantarum,* and 4 of 15 – to *L. brevis* species, this most likely reflected their confoundedness to natural niches often suffered from hostilities, such as plant surfaces, human and animal gastrointestinal tracts. It was found that *L. plantarum* cells were able to occupy all the ecological niches from where the test samples were taken: sour milk products, human and animal gastrointestinal tract (GIT) and waste water sludge, this was most likely the result of theses bacteria high resistance level to different stresses.

4 Discussion

The results of our study demonstrated that new LAB of *Lactobacillus* genus could be isolated from various kinds of natural and anthropogenic sources. This would open new horizons for the search and isolation of new LAB from many environmental niches, therefore the search for the new probiotic strains was endless. But the isolated strains were different in their survival rates toward gastric and intestinal stresses. Of 103 tested lactobacilli, only 27 strains (ca. 25 %) had shown a high resistance to stresses applied and relative good level of survival, which would make them potential probiotics. These properties would allow the further use of these cultures in functional food products, because of live cells transit through gastric and duodenum parts of GIT would not lead to considerable loss of viability of probiotic cells.

Table 2 Tolerance of the isolated *Lactobacillus* strains to gastric and intestinal stresses

№	Strains	Tolerance to gastric stress	Tolerance to intestinal stress
Strains isolated from human and animal feces			
1.	*L. plantarum* CM MSU № 503	RD = 1	RD = 9
		Very good	Good
2.	*L. plantarum* CM MSU № 504	RD = 1	RD = 4
		Very good	Very good
3.	*L. plantarum* CM MSU № 505	RD = 1	RD = 7
		Very good	Good
4.	*L. plantarum* CM MSU № 518	RD = 1	RD = 1
		Very good	Very good
5.	*L. plantarum* CM MSU № 519	RD = 1	RD = 1
		Very good	Very good
6.	*L. plantarum* CM MSU № 520	RD = 1	RD = 1
		Very good	Very good
7.	*L. rhamnosus* CM MSU № 528	RD = 1.0	RD = 1
		Very good	Very good
8.	*L. rhamnosus* CM MSU № 529	RD = 2	RD = 4
		Very good	Very good
Strains isolated from national dairy products, included in functional nutrition rations in the residence regions of persons with active longevity			
9.	*L. plantarum* CM MSU № 506	RD = 1	RD = 11
		Very good	Acceptable
10.	*L. plantarum* CM MSU № 507	RD = 2	RD = 5
		Very good	Very good
11.	*L. plantarum* CM MSU № 508	RD = 1	RD = 13
		Very good	Acceptable
12.	*L. plantarum* CM MSU № 509	RD = 1	RD = 10
		Very good	Good
13.	*L. plantarum* CM MSU № 510	RD = 1	RD = 6
		Very good	Good
14.	*L. plantarum* CM MSU № 511	RD = 1	RD = 2
		Very good	Very good
15.	*L. plantarum* CM MSU № 512	RD = 1	RD = 2
		Very good	Very good
16.	*L. plantarum* CM MSU № 513	RD = 1	RD = 12
		Very good	Acceptable
17.	*L. plantarum* CM MSU № 514	RD = 2	RD = 14
		Very good	Acceptable
18.	*L. plantarum* CM MSU № 515	RD = 1	RD = 8
		Very good	Good
19.	*L. plantarum* CM MSU № 516	RD = 1	RD = 1
		Very good	Very good
20.	*L. plantarum* CM MSU № 517	RD = 1	RD = 7
		Very good	Good
21.	*L. brevis* CM MSU № 521	RD = 2	RD = 3
		Very good	Very good
22.	*L. brevis* CM MSU № 522	RD = 1	RD = 10
		Very good	Good
23.	*L. brevis* CM MSU № 523	RD = 1	RD =2
		Very good	Very good

(continued)

Table 2 (continued)

№	Strains	Tolerance to gastric stress	Tolerance to intestinal stress
24.	*L. brevis* CM MSU № 524	RD = 1	RD = 1
		Very good	Very good
25.	*L. brevis* CM MSU № 525	RD = 1	RD = 6
		Very good	Good
26.	*L. brevis* CM MSU № 526	RD = 1	RD = 3
		Very good	Very good
27.	*L. paracasei* CM MSU № 527	RD = 1	RD = 1
		Very good	Very good
Strains isolated from anaerobic sludge			
28.	*L. paracasei* CM MSU № 531	RD = 390	RD = 80
		Unacceptable	Unacceptable
29.	*L. paracasei* CM MSU № 532	RD = 15	RD = 58
		Acceptable	Unacceptable
30.	*L. plantarum* CM MSU № 533	RD = 39	RD = 75
		Unacceptable	Unacceptable
31.	*L. plantarum* CM MSU № 534	RD = 694	RD = 38
		Unacceptable	Unacceptable

It worth to be mentioned, that among 15 strains that demonstrated high resistance to gastric and intestinal stresses, 8 strains were determined as *L. plantarum* and 4 as *L. brevis* (Table 2), that reflected their affiliation to natural environmental niches, often suffering from natural stresses – leaf surfaces, GIT of animals and humans. Strains of *L. plantarum* could inhabit all the ecological niches, we studied and sampled for the environmental probes: sour milk home-made products, animal and human GITs, waste water mud, etc. This property could put *L. plantarum* strains into the group of most resistant LAB and could be considered as valuable and most potent probiotic strains.

Taking all the aforesaid into consideration, we could prove that lactobacilli isolated in this study had a great potential for serving as probiotics, providing that they were highly resistant to gastric and intestinal stresses (Table 2). It proved that lactobacilli, isolated from natural sources, had a great potential and could be used as probiotic cultures for the treatment and preventive measures against human and animal gastrointestinal distresses.

All LAB isolated in this study from anaerobic sludge of natural ponds and food manufacture waste waters did not demonstrate resistance to gastro-intestinal stresses. As the example one can see the properties of four cultures isolated from anaerobic sludge. Four cultures isolated from sludge are not only not resistant to gastric stress, they are also very sensitive to bile acids of simulated intestine stress (Table 2). It should be also mentioned, that among these four strains two were belonging to *L. plantarum* species, which could reflect that representatives of this species could have and might not have high resistance to GIT stresses depending on the environmental probe they were isolated from and the niche they occupy in the nature.

Most strains (19 of 27) with high resistance to gastric and intestinal stresses were isolated from national diary products included into functional nutrition rations in the residence regions of persons with active longevity (Table 2). That also might reflect the applying the selective methods, which were used for thousands of years to the LAB in home-made sour milk products.

Eight strains isolated from human and animal feces show very good resistance to GIT stresses, among them 6 strains of *L. plantarum* and two – of *L. rhamnosus* species, which again put *L. plantarum* bacteria to the group of most resistant strains to gastric and intestinal environments.

It could be summarized, that potential probiotic LAB with high potential to survive GIT stresses could be isolated not only from human sources and fermented dairy products, but from natural environments with no or little anthropogenic influence. It might open a whole new large area for searching and isolation of new beneficial microbes for human and animal wellbeing and prophylaxis.

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