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Characterization of autochthonous *Lactobacillus paracasei* strains on potential probiotic ability

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Summary

Lactic acid bacteria strains isolated from traditional made cheeses constitute a reservoir of unexplored potential in biotechnology. In this study four autochthonous lactobacilli strains, isolated from traditional white brined cheeses and identified as *Lactobacillus paracasei* (08, 564, 05 and 02), were investigated on potential probiotic ability. The investigation comprised sensitivity to simulated gastrointestinal tract conditions, antimicrobial activity against wide range of pathogens, antibiotic resistance as well as autoaggregation ability. *Lactobacillus rhamnosus* GG was used as referent strain. Three tested strains grew well in simulated gastrointestinal conditions, but their sensitivity was greater on bile acids and pancreatin compared with pepsin low pH 2.5. The examined strains had different sensitivity to antibiotics, but three strains showed very good antimicrobial activity to pathogens. All strains demonstrated very good autoaggregation ability. For three of four examined strains of *Lb. paracasei* probiotic potential was similar with referent strain *Lb. rhamnosus* GG, determined in vitro.

Key words: *Lactobacillus paracasei*, probiotic ability, white brined cheese

Introduction

The variety of autochthonous microflora in white brine cheeses is due to presence of wide range of lactic acid bacteria (LAB) strains, with different metabolic properties (Radulović et al., 2007a)

Considering the fast growing interest for application of probiotic strains in dairy products, it could be presumed that it is possible to isolate some strains, with potential probiotic ability, among the autochthonous strains. Nowadays the criteria for LAB strains selection from traditional products are extend and besides technological and biochemical criteria they also include investigation of their probiotic ability (Ortu et. al., 2007).

Probiotics have been defined as "live microbial food supplements which beneficially affect the host by improving the intestinal microbial balance" (Fuller, 1989).

In recent years, the probiotic activity of lactic acid bacteria (LAB) has been emphasized and an increasing number of food supplements as well as pharmaceutical preparations are being promoted with health claims based on several characteristics of certain strains of lactic acid bacteria (LAB), particularly from the genera *Lactobacillus* and *Bifidobacterium* (McFarland et al., 1997; Kaur et al., 2002).

The health benefits (for customers) attributed to probiotic bacteria in the literature can be categorized as either nutritional benefits or therapeutic benefits (Tamime et al., 2003). Nutritional benefits include their role in enhancing the bioavailability of calcium, zinc, iron, manganese, copper, and phosphorus (Annuk et al., 1999); and an increase of the

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digestibility of protein and synthesis of vitamins in yogurt (Barbara et al., 2000).

The therapeutic benefits of probiotics reported include treatments of conditions including gastrointestinal disorders, hypercholesterolemia, and lactose intolerance; suppression of procarcinogenic enzyme; inhibitory effects on Ehrlich ascites tumour cells; immunomodulation; and treatment of food-related allergies (Begley et al., 2005; Chon and Choi, 2010; Ouwehand et al., 2003; Rodriguez et al., 2010).

The essential characteristics for lactic acid bacteria (LAB) to be used as probiotics during manufacturing, include the following: (i) recognition as safe (GRAS; generally recognized as safe); (ii) viability during processing and storage; (iii) antagonistic effect against pathogens; (iv) capability to survive in the intestinal ecosystem; and (v) adherence to the intestinal epithelium of the host among others (McFarlane et al., 2002; Begley et al., 2005; Vesterlund et al., 2005; Lin et al., 2006).

The aim of this research was to isolate, identify and characterize new strains with high potential for applications as probiotic cultures in foods and improve healthy products from the biodiversity of local high quality traditional products. New probiotic bacterial strains from the genera *Lactobacillus* sp. exhibiting antimicrobial properties were targeted and screened from traditional fermented products and local ecosystems in Serbia.

Materials and methods

Cheese isolates and reference strain

Four autochthonous strains *Lb. paracasei* (08, 564, 05 and 02) isolated from traditionally made white brined cheeses (Radulović, 2007b), were used for examination. These strains belong to the strains collection of the Department for Industrial Microbiology, Faculty of Agriculture, Belgrade. *Lb. rhamnosus* GG is used as reference strain and it was received from the strains collection of the Institute of Food Science and Nutrition, Laboratory of Food Biotechnology, ETH Zürich

Technological characterization of isolates

The examination of selected strains for potential using in food productions was supported by detecting some technological characteristics. Growth ability at various NaCl concentrations (2, 4 and 6.5 %) and growth ability at 15 i 45 °C was determined, by inoculation in appropriate broth media.

Sensitivity to simulated gastrointestinal conditions

Gastric and bile salts test were performed using a modified version of Doleyres et al. (2004).

Gastric test

0.5 % NaCl - 0.3 % pepsin solution was simulating gastrointestinal conditions. pH of the solution was adjusted to 2.5 by addition of 1 M HCl and solution is filtered sterile. Overnight cell culture (1 mL) was centrifuged at 5000 g at room temperature. The pellet was then washed with 0.1 % peptone water and resuspended in 0.1 % peptone and stored on ice until use. 270 μL of simulated gastric juices and 30 μL of cell suspension were mixed in microtiterplate (corresponding to dilution -1) and stored at 37 °C under anaerobic conditions for 30 min. After that a dilution row was carried out in the PBS buffer pH 7.2 and viable count determined using the spot method. Each strain was tested twice. The plates were incubated anaerobically at 37 °C for 48 h.

Bile salts test

0.4 % bile salts- 0.2 % pancreatin solution was simulating duodenal conditions. Overnight cell culture (1 mL) was centrifuged at 5000 g at room temperature. The pellet was then washed twice with 1X PBS buffer pH 7.2 and resuspended in 1X PBS buffer pH 7.2 and stored on ice until use. 270 μL of simulated duodenal juices and 30 μL of cell suspension were mixed in microtiterplate (corresponding to dilution -1) and stored at 37 °C under anaerobic conditions for 60 min. After that a dilution row was carried out in the PBS buffer pH 7.2 and viable count assessed by the spot method. Each strain was tested twice. The plates were incubated anaerobically at 37 °C for 48 h.

Tolerance to antibiotics

Bacterial antibiotic resistance of four isolated strains was determined on solid MRS medium by the use of 6 different antibiotic discs (Torlak, Serbia). MRS agar plates were overlaid with 6 mL of

TOP MRS agar containing 0.6 % agar and seeded with 0.2 mL of tested cultures. The discs with antibiotic were placed on top of the agar after they became harden. After incubation at 37 °C for 24 h, the diameter (mm) of inhibition zone was measured.

Antimicrobial activity

The antimicrobial activity was achieved by using the spot method described by Bernet et al. (1993).

The selected strains belonging to *Lactobacillus paracasei* and reference strain *Lactobacillus rhamnosus* GG were screened for their ability to inhibit food borne pathogens as followed: *Listeria monocytogenes* IM2000, *Pseudomonas aeruginosa* ATCC5999, *Bacillus subtilis* ATCC6633, *Staphylococcus aureus* ATCC25923, *Candida albicans* ATCC10259, *Escherichia coli* ATCC25922 and *Salmonella enteritidis* ATCC31806.

Autoaggregation assays

Autoaggregation assays were performed according to Del Re et al. (2000) with certain modifications. Bacteria were grown for 18 h at 37 °C with MRS solid or liquid medium. The cells were harvested by centrifugation at 5000 g for 15 min, washed twice and resuspended in their culture supernatant fluid or in phosphate buffered saline (PBS) to give viable counts of approximately 10^8 CFU/mL. Cell suspensions (4 mL) were mixed by vortexing for 10 s and autoaggregation was determined during 5 h of incubation at room temperature. Every hour 0.1 mL of the upper suspension was transferred to another tube with 3.9 mL of PBS and the absorbance (A) was measured at 600 nm.

The autoaggregation percentage is expressed as:

$$\text{Autoaggregation } \% = 1-(A_t/A_0)\ x100$$

where A_t represents the absorbance at time t = 1, 2, 3, 4 or 5 h and A_0 the absorbance at t=0. Each strain was tested in duplicates.

Results

Sensitivity to some technological parameters

Technological characteristics of investigated *Lb. paracasei* strains are shown in Table 1. All strains showed very good ability to grow in the presence of high % of salt and wide range of temperatures, which approve them as good candidate for food application. The strains with these properties could be capable to withstand the technological condition during the food production.

Sensitivity to simulated gastrointestinal conditions (gastric test and bile salts test)

To develop or select new probiotic organisms, their stability on gastrointestinal conditions should first be assessed. Therefore we tested two effective properties as prerequisites for probiotics: tolerance to low pH and to high bile concentration. Data of survival of isolated strains in gastric conditions are shown in Table 2 and 3. Three isolates have shown very good survival after 30 min in gastric conditions at pH 2.5 (>93.49 %).

Tolerance to bile salts is considered to be a prerequisite for colonization and metabolic activity of bacteria in the small intestine of the host (Havenaar et al., 1992). In this study, bile tolerance of the 4 strains of *L. paracasei* was investigated and 3 strains performed very good survival in these conditions, more than 80 % (Table 3).

Table 1. Technological characteristics of *Lb. paracasei* strains
Tablica 1. Tehnološke karakteristike sojeva *Lb. paracasei*

Strains/Sojevi	NaCl			Temperature Temperatura	
	2 %	4 %	6.5 %	15 °C	45 °C
Lb. paracasei 08	+	+	+	+	+
Lb. paracasei 564	+	+	+	+	+
Lb. paracasei 05	+	+	+	+	+
Lb. paracasei 02	+	+	+	+	+

Table 2. Survival of isolated strains after 30 min incubation in simulated gastric conditions (0.5 % NaCl - 0.3 % pepsine solution, pH 2.5)
Tablica 2. Preživljavanje izoliranih sojeva nakon 30 min. inkubacije u simuliranim gastro uvjetima (0,5 % NaCl - 0,3 % otopina pepsina, pH 2,5)

Strains/Sojevi	Start number Početni broj CFU/mL	Pepsin pH 2.5	
		Survival number Broj preživjelih CFU/mL	Survival rate Postotak preživljavanja %
Lb. paracasei 08	$1.90x10^9$	$5.70x10^8$	94.34
Lb. paracasei 564	$4.10x10^8$	$4.10x10^8$	100.00
Lb. paracasei 05	$2.50x10^8$	$2.30x10^8$	93.49
Lb. paracasei 02	$7.80x10^8$	$4.48x10^8$	57.43
Lb. rhamnosus GG	$1.50x10^9$	$1.50x10^9$	100.00

Table 3. Survival of isolated strains after 60 min incubation in simulated intestinal conditions
Tablica 3. Preživljavanje izoliranih sojeva nakon 60 min. inkubacije u simuliranim intestinalnim uvjetima

Strains/Sojevi	Start number Početni broj CFU/mL	Pancreatin+ bile salts Pankreatin + žučne soli	
		Survival number Broj preživjelih CFU/mL	Survival rate Postotak preživljavanja %
Lb. paracasei 08	$1.90x10^9$	$4.85x10^8$	93.64
Lb. paracasei 564	$4.10x10^8$	$4.05x10^8$	98.78
Lb. paracasei 05	$2.46x10^8$	$2.00x10^8$	81.30
Lb. paracasei 02	$7.80x10^8$	$2.56x10^8$	32.82
Lb. rhamnosus GG	$1.50x10^9$	$6.90x10^8$	96.30

Table 4. Antibiotic susceptibility of the isolated and reference strains
Tablica 4. Osjetljivost izoliranih sojeva i referentnog soja na antibiotike

Antibiotic/Antibiotik	*Lb. paracasei*				*Lb. rhamnosus*
	08	564	05	02	GG
Cloramphenicol, 30 mcg	+	+	+	+	++
Penicillin G, 6 mcg	+	R	R	+	+
Tetracycline, 30 mcg	+	+	+	+	+
Streptomycin, 30 mcg	R	R	R	R	+
Ampicillin, 10 mcg	+	++	+	++	+
Kanamycin, 40 mcg	R	R	R	R	R

++: sensitive - with of halo of inhibition >10 mm; osjetljivo - sa zonom inhibicije >10 mm
+: moderately sensitive - with of halo of inhibition 5 - 10 mm; umjereno osjetljivo - sa zonom inhibicije 5 - 10 mm
R: resistant - with of halo of inhibition <5 mm; otporno - sa zonom inhibicije <5 mm

Table 5. Antimicrobial activity of isolated strains (spot test)
Tablica 5. Antimikrobna aktivnost izoliranih sojeva (spot test)

Strains/Sojevi	Diameter of inhibition halo* / Promjer zone inhibicije* mm						
	Listeria monocytogenes	*Pseudomonas aeruginosa*	*Bacillus subtilis*	*Staphylococcus aureus*	*Candida albicans*	*Escherichia coli*	*Salmonella enteritidis*
Lb. paracasei 08	21	15	17	24	17	25	26
Lb. paracasei 564	23	16	16	24	16	25	26
Lb. paracasei 05	21	16	17	22	16	24	25
Lb. paracasei 02	11	0	0	7	13	0	0
Lb. rhamnosus GG	23	NI	NI	25	12	26	27

*diameter of inhibition halo include 5 mm diameter of culture spot; NI - not investigated
*dijametar zone inhibicije uključuje 5 mm dijametra nanijete kulture; NI - nije istraženo

Table 6. Autoaggregation ability of isolated and reference strains
Tablica 6. Autoagregacijska sposobnost izoliranih sojeva i referentnog soja

Strains/Sojevi	% of autoaggregation at indicated time points (h) / % autoagregacije u određenom vremenskom intervalu (sati)		
	1	3	5
Lb. paracasei 08	95.88	94.91	93.90
Lb. paracasei 564	98.55	95.12	94.15
Lb. paracasei 05	95.15	94.45	94.26
Lb. paracasei 02	69.54	69.50	67.55
Lb. rhamnosus GG	76.42	75.77	70.60

Tolerance to antibiotics

Table 4 shows the results obtained for antibiotic susceptibility of the 4 isolated strains and referent strain. All strains were susceptible to cloramphenicol, ampicillin, tetracycline, and all isolates were resistant on streptomycin and kanamycin.

Antimicrobial activity

Three strains 08, 564, 05 have performed good antimicrobial activity against a wide-range of target strains and a quite similar profile of inhibition to reference strain *Lb. rhamnosus* GG which is proved to have beneficial, probiotic effects. Table 5 is showing antimicrobial activity of isolated and reference strain obtained with full cell culture (spot test).

Autoaggregation assays

The sedimentation rate of isolated and reference strains were measured over a period of 5 h. According to the results shown in Table 6 all tested strains performed a strong autoaggregation phenotype.

Discussion

There are lots of problems in reliable characterization of probiotic strains using *in vitro* methods, but the initial screening of strains in this manner remains a useful first step in the detection of probiotic candidates. Different probiotic characteristics were determined in this study including tolerance to low pH and high bile concentration. Acid and bile tolerance of isolated strains is important in order to predict the strain performance during gastric transit. Presence of pepsin and low pH turns out to be very restrictive conditions for growth of isolated lactobacilli. Three strains (08, 564 and 05) have performed survival over 93.49 % during gastric test.

These findings support the importance of investing the sensitivity of microorganisms to gastric juices as a selection step for potential probiotic.

Resistance to bile salts is also an important property in selection for potential probiotic strains. However, there is still no consensus about the precise concentration to which selected strains should be tolerant. In the present study, 0.4 % bile salts - 0.2 % pancreatin was used for simulation of duodenal juice (Doleyres et al., 2004). Three tested strains showed very good survival in these conditions (more then 81.30 %). These values are very high and similar to those for *Lb. rhamnosus* GG, in this paper as well as in previous investigation (Martin et al., 2005; Perea Velez et al., 2007).

Klaenhammer et al. (2004) reported that probiotic bacteria vary considerably in their level of bile tolerance. Also, they explained that the mechanism of tolerance is not understood and the minimum acceptable level of bile tolerance for a probiotic candidate remains unknown.

Bile resistance of some strains is related to specific enzyme activity-bile salt hydrolase (BSH) which helps hydrolyze conjugated bile, thus reducing its toxic effect (Du Toit et al., 1998). According to Tanaka et al. (1999), BSH activity has most often been found in lactobacilli isolated from the intestines or faeces of animals because these strains come from an intestinal environment in which they are exposed to bile salts. This is not in accordance with our results, since the strains tested here were isolated from traditional cheese and have performed excellent bile tolerance.

The investigation of antibiotic susceptibility is very important criteria for probiotic characteristic of lactobacilli. Antibiotic-resistant strains could meliorate the microbial balance in patients with intestinal disorder provoked by antibiotic therapy (Salminen et al., 1998), but on the other hand these probiotic strains could contribute in transmission of antibiotic-resistant genes on pathogen bacteria in GI tract (Morelli and Wright, 1997; Saarelo et al., 2000).

Isolated *Lb. paracasei* strains were susceptible to the some of antibiotics tested. This is not in accordance with various reports indicating that lactic acid bacteria are normally resistant to penicillin G, vancomycin, cloramphenicol, ampicillin (Halami et al., 2000; Coppola et al., 2005). Determination of antibiotic resistant genes location could contribute in safely application of antibiotic-resistant strains as probiotics.

This is very important because one of the required properties of probiotic strains is that they are safe for human consumption.

Lactobacilli reveal different antimicrobial mechanisms shown *in vitro* assays. The antimicrobial activity in liquid media is favoured by rapidly diffusing antimicrobial compounds (Turi et al., 1997; Annuk et al., 1999; 2001). High antagonistic activity of lactobacilli is associated with production of organic acids which leads to pH decrease (Ouwehand and Vesterlund, 2004). The lactobacilli often represent a primary microbial barrier against the infection of intestinal and urogenital tract. Their inhibitory action is due to production of lactic acid, bacteriocins or H_2O_2. There are many strains among lactobacilli with documented probiotic ability, thus they have a huge application in prevention of infection (Bengmark et al., 1998).

In this study, three examined strains have performed very good antimicrobial activity against wide range of food born pathogens, comparable with antagonistic activity of *Lb. rhamnosus* GG. According to this data, isolated *Lb. paracasei* strains might have other antimicrobial mechanisms beside organic acid production. This can be topic for further research.

Another very important property of many bacterial strains used as probiotics is their ability to adhere to epithelial cells and mucosal surfaces. Several researches have investigated the composition, structure and forces of interaction related to bacterial adhesion (Green and Klaenhammer, 1994; Pelletier et al., 1997; Del Re et al., 2000). According to Vandevoorde et al. (1992) and Boris et al. (1997) aggregation ability is related to cell adherence properties. Our isolates have shown good autoaggregation phenotype.

Conclusions

The tested autochthonous strains of *Lb. paracasei* are demonstrated good ability to grow in wide range of temperature and high salt concentrations which is important for their survival during technological production of different cheeses. Three tested strains (08, 564 and 05) showed potential probiotic

ability, which is comparable with referent strain *Lb. rhamnosus* GG *in vitro*. These strains demonstrated very good ability to survive in simulated GI conditions, with 93.49 % of survival in the presence of pepsin, pH 2.5 and 81.30 % in the presence of pancreatin and bile salts, respectively. Further they demonstrated very good inhibition against pathogen microorganisms, they showed sensitivity to majority of antibiotics, and all isolates have shown high autoaggregation properties (93.90-94.26 %). Further investigations are necessary for providing more information about probiotic potential of these species. These probiotic criteria could open a new aspect for strains selection as the starter cultures in cheese production.

Karakterizacija autohtonih sojeva Lactobacillus paracasei na potencijalne probiotičke sposobnosti

Sažetak

Bakterije mliječne kiseline izolirane iz tradicionalnih sireva predstavljaju neiscrpan potencijal u biotehnologiji. U ovom radu 4 soja laktobacila, izolirana iz autohtonih bijelih sireva u salamuri i identificirana kao *Lactobacillus paracasei* (08, 564, 05 and 02), ispitivana su na potencijalne probiotičke sposobnosti. Ispitivanje je obuhvatilo osjetljivost na simulirane gastrointestinalne uvjete, antimikrobnu aktivnost u odnosu na patogene, antibiotsku rezistentnost i sposobnost autoagregacije. *Lactobacillus rhamnosus* GG upotrijebljen je kao referentni soj. Tri testirana soja dobro su rasla u simuliranim gastrointestinalnim uvjetima, ali je njihova osjetljivost bila veća u prisustvu žučne kiseline i pankreatina u usporedbi s pepsinom niskog pH 2,5. Ispitivani sojevi pokazali su različitu osjetljivost na antibiotike, a 3 soja su pokazala dobru antimikrobnu aktivnost u odnosu na patogene. Svi su sojevi pokazali visoku sposobnost autoagregacije. Od 4 ispitana soja *Lb. paracsei*, tri su pokazala dobar probiotički potencijal u usporedbi s referentnim sojem *Lb. rhamnosus* GG, in vitro uvjetima.

Ključne riječi: laktobacili, probiotička sposobnost, tradicionalni bijeli sirevi u salamuri

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