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POTENTIAL PROBIOTIC PROPERTIES OF LACTOBACILLI ISOLATED FROM GOAT´S MILK

Martin Tomáška, Maroš Drončovský, Lýdia Klapáčová, Anna Slottová, Miroslav Kološta

ABSTRACT

The three strains of lactobacilli isolated from goat's milk – *Lactobacillus (Lbc.) casei* 21L10, *Lbc. johnsonii* KB2-1 and *Lbc. plantarum* 25/1L were selected in previous studies because they showed good processing and protective properties in production of cheeses or lactic acid beverages from heat-treated milk or in model conditions. The aim of this study was to evaluate their potential probiotic properties: resistance to lysozyme, survival in gastrointestinal tract, and hydrophobicity. Testing was carried out *in vitro* methods: comparison of growth and metabolic characteristics of the strains when cultivated in Man, Rogosa and Sharpe medium with and without the addition of lysozyme (400 $\mu g.mL^{-1}$); viability of strains after incubation in simulated environment of gastric juice (pH = 2.0, pepsin) and subsequently in simulated small intestinal juice (pH = 8.0, pancreatin, bile salts) and an ability to adhere to the non-polar solvent in the two-phase system: xylene-water. *Lbc. casei* 21L10 and *Lbc. johnsonii* KB2-1 were resistant to the effect of lysozyme, the strain *Lbc. plantarum* 25/1L was moderately susceptible. To the action of simulated gastric juice was resistant only the *Lbc. johnsonii* KB2-1, that was subsequently inhibited in simulated small intestinal juice. When using xylene as a model agent, all strains were not hydrophobic. From all the tested strains, *Lbc. johnsonii* KB2-1 showed good potential probiotic properties, particularly in relation to resistance to lysozyme and the simulated environment of gastric juice.

Keywords: *Lactobacillus*; lysozyme; simulated gastro-intestinal tract; microbial adhesion to solvent; probiotic

INTRODUCTION

FAO/WHO (2002) defined probiotics as "live microorganisms which when administered in adequate amounts confer a health benefit on the host". It is required that these organisms are clearly described, are particularly safe and have certain features that are presumption for their beneficial effect on the host organism. Additionally, the benefits must be clearly demonstrated in clinical trials **(FAO/WHO, 2002; Verdenelli et al., 2009)**. Selected strains of lactobacilli belong to the typical representatives of lactic acid bacteria **(LAB)**, which may have probiotic properties. Besides human origin, they can be isolated also from the milk processing **(Bao et al., 2010)**. They may be added to the milk products as starter adjuncts, which is one of possible methods (other than the pharmaceutical form) of their consumption (**Vinderola et al., 2009)**.

Lysozyme is an enzyme muramidase, which is commonly found in human saliva, tears, breast milk and mucus. It can hydrolyse 1,4-beta-linkages between N-acetylmuramic acid and N-acetyl-D-glucosamine residues in a peptidoglycan, a common part of cell wall in Gram-positive bacteria **(McKenzie and White, 1991)**. They are therefore more sensitive to the action of lysozyme than Gram-negative bacteria that have a different composition of the cell wall and several protective barriers, protecting of peptidoglycan layer. Lactobacilli are the Gram-positive bacteria. Thus, their resistance to lysozyme is a probiotic criterion **(Kunová et al., 2011)**.

Some microorganisms commonly found in food, either pathogenic or beneficial, are able to survive in the low pH of the stomach, higher pH of digestive juices in the small intestine, activity of enzymes (pepsin, pancreatin), and bile salts. Thus there were created several models of simulated gastro-intestinal environment in which they are tested whether the strains of LAB are able to survive in such conditions. A number of potential probiotic strains of lactobacilli have a high survival rate **(SR)** in simulated gastro intestinal tract **(GIT)**. Milk matrix itself **(Guglielmottia et al., 2007)**, may or may not have protective effect **(Mäkeläinen et al., 2009)**.

The ability of lactobacilli and LAB generally, to adhere to cells of GIT, is important. This feature prevents the peristaltic movements of the digestive system to remove cells immediately so that they can carry out their beneficial activity. The pathogenic microorganisms are capable of binding to GIT, too. Their ability to auto-aggregation correlates with adhesion. These are the basic conditions for colonization and subsequent infection of the GIT. If the probiotic strains are capable to co-aggregate with pathogens or to replace them, then they create a protective barrier **(Collado et al., 2008)**. Besides, the capabilities of potential probiotics occupy binding sites in the GIT and compete for nutrients; their therapeutic effect is also based on immunomodulation **(Collado et al., 2007)**. The rate of bacterial adhesion to human GIT depends on the physico-chemical properties of the bacterial cell surface. One of these properties is hydrophobicity **(Del Re et al.,**

2000). According to this *in vitro* method, it is possible to estimate whether the strains are capable of adhesion to GIT, or not.

In our previous studies the processing characteristics **(Kološta et al., 2014; Slottova et al., 2014; Klapáčová et al., 2014**) and protective properties **(Klapáčová et al., in press)** of three selected strains of lactobacilli (*Lbc. casei* 21L10, *Lbc. johnsonii* KB2-1 and *Lbc. plantarum* 25/1L), isolated from goat's milk, were described. The aim of this work was to study the susceptibility of the strains to lysozyme, model conditions of GIT (simulated gastric and small intestinal juice) and their ability to adhese to a non-polar xylene as an indicator of hydrophobicity. All these properties are important for assessment of the potential probiotic effect of lactobacilli.

MATERIAL AND METHODOLOGY

Lbc. casei 21L10, *Lbc. johnsonii* KB2-1 and *Lbc. plantarum* 25/1L were isolated from raw goat´s milk and were identified with 16S rRNA PCR method **(Slottova et al., 2014)**.

Sensitivity of the lactobacilli to lysozyme was determined by their cultivation (24.5 hours; 37 °C; statically; aerobically) in Man, Rogosa and Sharpe (**MRS)** medium (Merck, Darmstadt, Germany). After one hour of cultivation, the lysozyme was added to the media – Lysozyme from chicken egg white 156733 $U.mg^{-1}$ (Serva Electrophoresis, Heidelberg, Germany) at a concentration of 400 $\mu g.mL^{-1}$. The growth was monitored by measuring optical density **(OD)** at 600 nm by spectrophotometer and their metabolic activity by pH decrease of the cultivation media by pH meter. Control samples did not contain lysozyme. From data measured, the growth lines and graphics dependence of pH on cultivation time were drawn.

The ability of lactobacilli to survive in a simulated GIT was estimated as follows: MRS medium was inoculated with an overnight culture of the strains (1%) and was cultivated for 24 hours at 37 °C, statically, aerobically. After the cultivation, the 5 mL of medium was filled into sterile centrifuge tubes. The supernatant was separated by centrifugation (Relative Centrifugal Force **(RCF)** = 6000; 20 min.; 30 °C) and the biomass was washed with physiological saline. After, the biomass was carefully resuspended by vortexing in 5 mL of simulated gastric juice or in 5 mL of physiological saline (control sample). Simulated gastric juice was prepared as follows: 0.5 g of NaCl (Merck) was dissolved in 100 mL of deionized water and pH was adjusted to 2.0 ±0.1 by 36% HCl (Merck). The solution was sterilized by autoclaving. To the sterile simulated gastric juice 0.3 g of pepsin – Pepsin from porcine gastric mucosa 0.7 $FIP\text{-}U.mg^{-1}$ for biochemistry (Merck) per 100 ml of juice was added and the pH was checked. The strains in the simulated gastric juice were incubated statically at 37 °C for 2 hours. Subsequently, supernatant was separated by centrifugation under the same conditions. Then 5 mL of simulated small intestinal juice was added to the biomass. The simulated small intestinal juice was prepared as follows: 0.5 g NaCl and 0.3 g bile salts (Sigma-Aldrich, St. Louis, USA) were dissolved in 100 mL of 0.1 M potassium phosphate buffer with pH 8.0 ±0.1. The solution was sterilized by autoclaving. To the sterile simulated small intestinal juice 0.1 g of pancreatin – Pancreatin NB from porcine pancreas 5,407 $U.g^{-1}$ (Serva Electrophoresis, Heidelberg, Germany) was added per 100 mL of the juice and the pH was checked. The strains in simulated small intestinal juice were incubated statically at 37 °C for 4 hours. After the incubation in simulated gastric and small intestinal juice, the number of lactobacilli was determined in the samples – by cultivation on MRS agar (Merck) – 72 hours, 37 °C. SR was expressed as percentage share of logarithms of colonies of lactobacilli measured after incubation in different environments in comparison to a control sample.

Microbial adhesion to solvent of lactobacilli was assessed in a two-phase system of water-xylene. 50 mL MRS medium was inoculated with 1% of overnight culture of the strains. The medium was cultivated for 24 hours at 37 °C, statically, aerobically. After the cultivation, the medium was centrifuged (RCF = 6000; 20 min.; 30 °C), and the biomass was washed twice with buffer saline. Subsequently, the biomass was resuspended by vortexing into 50 mL of 0.1 M KNO_3 (Lachema, Brno, Czech Republic). OD of the suspension was measured at 600 nm (OD_0). From the suspension 6 mL was removed to a sterile tube and 2 mL of xylene – Xylene extra pure (Merck) was added. The mixture was pre-incubated for 10 minutes at room temperature and then was thoroughly vortexing for 2 minutes. The mixture was kept for 20 minutes at room temperature, to separate the phases. The aqueous phase was removed carefully and their OD was measured at 600 nm (OD_1). The percentage of bacterial adhesion **(BA)** to solvent was calculated as follows: $BA = (1 - OD_1/OD_0) \times 100$.

Each measurement was carried out twice.

RESULTS AND DISCUSSION

From all the tested strains, only *Lbc. plantarum* 25/1L was partially sensitive to lysozyme (400 $\mu g.mL^{-1}$). Figure 1 shows that the addition of lysozyme slightly protracted lag phase and reduced specific growth rate of the strain compared to the control. Also decrease the pH of the culture medium was slightly slower (Figure 2). Growth characteristics of the strains *Lbc. casei* 21L10 and *Lbc. johnsonii* KB2-1 and the production of lactic acid **(Kološta et al., 2014)** were not influenced by addition of lysozyme (data not presented here).

Kunová et al. (2011) tested 13 strains of lactobacilli isolated from dairy products, or human origin for lysozyme (400 $\mu g.mL^{-1}$). The 8 strains were resistant to the effect of lysozyme, in the rest of the strains; authors reported a slight delay in the exponential phase of growth curves. The origin of the strains did not affect their susceptibility to lysozyme. On the contrary, in bifidobacteria, **Kunová et al. (2012)** demonstrated that the strain of *Bifidobacterium bifidum* and *Bifidobacterium longum* isolated from faeces of babies and children were more resistant to the effect of lysozyme (400 $\mu g.mL^{-1}$ and 1000 $\mu g.mL^{-1}$), than the strains of animal origin.

Besides origin of LAB, their sensitivity to lysozyme may also be influenced with the presence of other substances in the environment (e.g. nisin), cultivation temperature, growth phase or mutations of the tested strains (**Neujahr**

et al., 1973; Guglielmotti et al., 2007; Kunová et al., 2012).

Survival of lactobacilli in a simulated environment GIT is summarized in Table 1. The only strain *Lbc. johnsonii* KB2-1 was resistant to the effect of simulated gastric juice (more than the 90% SR), but after subsequent simulation of small intestinal juice was already inhibited (less than 50% SR) – criterion according to **Kejmarová et al.,**

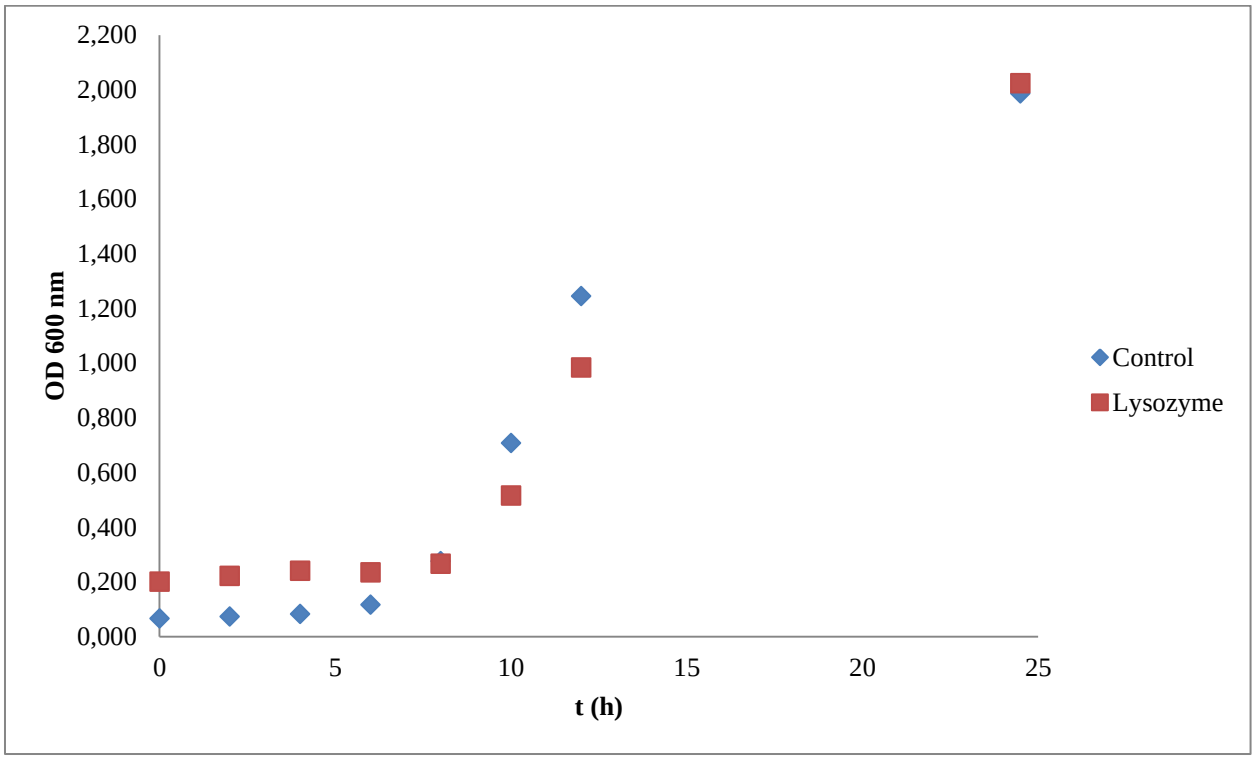

Figure 1 The growth of *Lbc. plantarum* 25/1L (37 °C, statically, aerobically) in MRS medium without and with addition of lysozyme (400 μg.mL^{-1}), detected by measuring the optical density (OD).

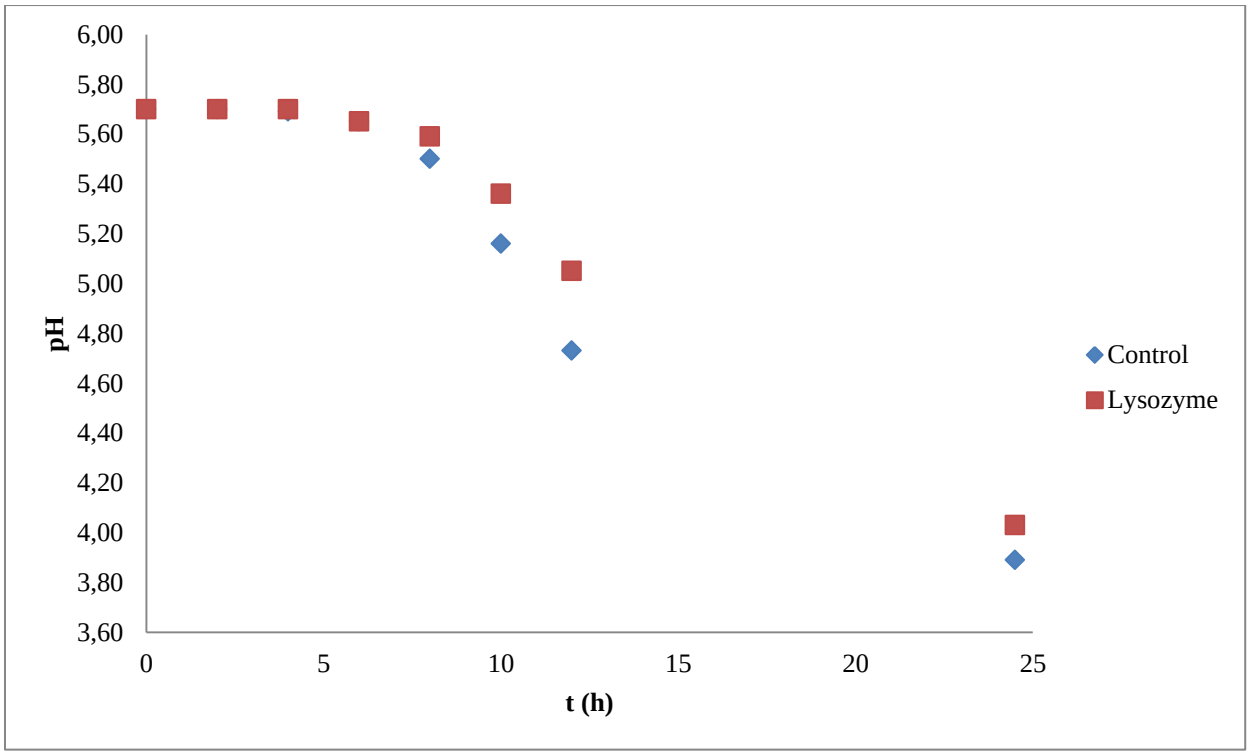

Figure 2 The decrease in pH during cultivation (37 ° C, statically, aerobically) *Lbc. plantarum* 25/1L in MRS medium without and with addition of lysozyme (400 μg.mL^{-1}).

Table 1 Survival of lactobacilli in simulated environment of gastrointestinal tract at 37 °C.

Strain**	CS***		GJ****		GJ + SIJ*****	
	$\log_{10}$(CFU.mL^{-1})	SR* (%)	$\log_{10}$(CFU.mL^{-1})	SR (%)	$\log_{10}$(CFU.mL^{-1})	SR (%)
21L10	9.00 – 9.08	100.0	2.91	32.2	<1.00	<11.1
KB2-1	6.00 – 6.20	100.0	5.49 – 5.52	90.2	2.30 – 2.32	37.9
25/1L	8.66 – 8.74	100.0	<2.00	<23.0	<1.00	<11.5

* Survival rate
** *Lbc. casei* 21L10, *Lbc. johnsonii* KB2-1, *Lbc. plantarum* 25/1L
*** Control sample
**** Simulated gastric juice (2hours)
***** Simulated gastric juice (2hours) and subsequently small intestine juice (4hours)

(2011). Strains *Lbc. casei* 21L10 and *Lbc. plantarum* 25/1L were inhibited after the effect of simulated gastric juice (SR from 32.2% to less than 23%).

Strains of lactobacilli resistant to simulated GIT have been isolated from various matrices, e.g.: from fermented olives (*Lbc. pentosus*, *Lbc. plantarum*, *Lbc. paracasei* subs. *paracasei*) **(Argyri et al., 2013)**, cheese (*Lbc. rhamnosus*, *Lbc. paracasei*, *Lbc. casei*, *Lbc. harbinensis*, *Lbc. fermentum*) **(Solieri et al., 2014)**, fermented meat products (*Lbc. sakei*, *Lbc. curvatus*, *Lbc. plantarum*) **(Papamanoli et al., 2003)**. Methodology of survival varied in studies – they tested various pH of simulated gastric juice (from about 1 to 3), different additions of enzymes or bile salts, the environment, where the influence was observed (MRS medium, various buffers, etc.), and the exposure time.

Tested strains did not show any adhesion to xylene – thus, they are hydrophilic. Calculated BA values were less than 1%.

Kos et al. (2003) using the same methodology, found out that the tested strain *Lbc. acidophilus* M92 was hydrophobic (71% adhesion). Whereas other probiotic strains *Lbc. plantarum* L4 and *Enterococcus faecium* L3 were hydrophilic (7% respectively 0% adhesions). The authors of this study confirmed the link between adhesion and auto aggregation (as model was used porcine ileal epithelial cells), mediated of protein components of cell membrane. **Kejmarova et al. (2011)** tested 15 strains of bifidobacteria (*Bifidobacterium animalis* subsp. *lactis*, *Bifidobacterium longum*, *Bifidobacterium species*, *Bifidobacterium dentium*) for hydrophobicity, which varied from 49% to 68%. **Bhardwaj et al. (2010)** used for testing of the hydrophobicity other agents, besides the xylene also *n*-hexadecane and *n*-octane, while changing their additional amount. *Enterococcus faecium* KH 24 showed low hydrophobicity – from 2% to 17% in case of *n*-hexadecane, but higher using xylene and *n*-octane – from 49% to 86%. It has been therefore proved that the results of such *in vitro* bacterial adhesion assay also depend on of the type and the quantity of used reagents.

It can be summarized that strains *Lbc. casei* 21L10 and *Lbc. johnsonii* KB2-1 were resistant to the effect of lysozyme (400 μg.mL^{-1}) and strain *Lbc. plantarum* 25/1L was affected only marginally by the tested concentration.

Strains *Lbc. casei* 21L10 and *Lbc. plantarum* 25/1L did not survive well in simulated gastric juice environment. On the contrary, strain *Lbc. johnsonii* KB2-1, this environment tolerated well. Surviving of all tested strains was inhibited by the simulated environment of the small intestine digestive juices. Excluding the effects of higher pH, pancreatin and bile salts, also autolysis of cells in the environment of usage of 0.1 M potassium phosphate buffer could occur **(El-Kholy et al., 1998)**.

All the strains were not hydrophobic. Based on the results of the model (xylene), there is not an expectation of adhesion ability to the cells of the GIT. However, it is possible when another reagent is used, the adhesion will achieve different percentage. It is also recommended to use more sophisticated models as e. g. Caco-2 cells **(Candela et al., 2008; Argyri et al., 2013)**.

In previous study it was shown, that the strains did not produce biogenic amines in excessive concentrations **(Klapáčová et al., in press)**. The further safety criteria (resistance to antibiotics, β-haemolysis, etc.) will be evaluated too.

CONCLUSION

From all the tested strains, *Lbc. johnsonii* KB2-1 showed the best potential probiotic properties; however it was not resisted to simulated small intestine juice and was not hydrophobic – under the selected test conditions.

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Contact address:
Martin Tomáška, Dairy Research Institute, JV, Dlhá 95, 010 01 Žilina, Slovakia, E-mail: tomaska@vumza.sk.
Maroš Drončovský, Dairy Research Institute, JV, Dlhá 95, 010 01 Žilina, Slovakia, E-mail: chemia@vumza.sk.
Lýdia Klapáčová, University of Veterinary Medicine and Pharmacy in Košice, Department of Hygiene and Food Industry Technology, Komenského 73, 041 81 Košice, Slovakia, E-mail: klapacol@gmail.com.
Anna Slottová, Dairy Research Institute, JV, Dlhá 95, 010 01 Žilina, Slovakia, E-mail: slottova@vumza.sk.
Miroslav Kološta, Dairy Research Institute, JV, Dlhá 95, 010 01 Žilina, Slovakia, E-mail: kolosta@vumza.sk.