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VERSITA

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ANTIMICROBIAL SUSCEPTIBILITY AND IMMUNOMODULATORY PROPERTIES OF LAMB ISOLATE OF *LACTOBACILLUS MUCOSAE*, NEW PROBIOTIC CANDIDATE

CITLIVOSŤ NA ANTIMIKRÓBNE LÁTKY A IMUNOMODULAČNÉ VLASTNOATI JAHŇACIEHO IZOLÁTU *LACTOBACILLUS MUCOSAE*, NOVÉHO PROBIOTICKÉHO KANDIDÁTA

Original research article

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Abstract In the process of selecting a new probiotic candidate, several bacteria were isolated from the stomach mucosa of a lamb. Among them, three lactobacilli strains were identified and partially characterised. The strain, *Lactobacillus mucosae* D, showed several characteristics appropriate to the probiotics. In this study, we have focused on the further characterisation of *L. mucosae* D and testing of its ability to modulate metabolic and immunomodulatory activities of human mononuclear cells *in vitro*. *L. mucosae* D is resistant to antibiotics, like penicillin G, oxacillin, vancomycin and chemotherapeutics ofloxacin and ciprofloxacin. In *in vitro* conditions, *L. mucosae* D caused a significant increase in phagocytic activity and index (relative activities 1.05 and 1.44, respectively) of human monocytes. It decreased bactericidal activities of monocytes against *Escherichia coli* (relative activity 0.73) and *Staphylococcus aureus* (relative activity 0.36), whereas, candidacidal activity was enhanced (relative activity 1.15). Metabolic activities, lysozyme and peroxidase activity, of mononuclear cells were not changed or increased, respectively. *L. mucosae* D displayed the ability to enhance production of pro-inflammatory cytokine, IL-1β, in monocytes *in vitro* (relative activity 2.60). Therefore, we state that lamb isolate, *L. mucosae* D, has the required attributes for being a potential probiotic candidate.

Slovak abstract S cieľom získať nových probiotických kandidátov, bolo zo žalúdočnej sliznice jahňaťa izolovaných niekoľko baktérií. Tri z nich boli identifikované ako laktobacily a boli čiastočne charakterizované. Kmeň *Lactobacillus mucosae* D preukázal v predošlých experimentoch niekoľko vlastností typických pre probiotiká. V tejto práci sme sa zamerali na ďalšiu charakterizáciu *Lactobacillus mucosae* Da sledovanie jeho schopnosti modulovať metabolické a imunomodulačné vlastnosti ľudských mononukleových buniek v podmienkach *in vitro*. Zistili sme, že *L. mucosae* D je rezistentný voči nasledujúcim antibiotikám: penicilín G, oxacilín, vankomycín a chemoterapeutikám ofloxacín a ciprofloxacin. V podmienkach *in vitro L. mucosae* D spôsobil signifikantné zvýšenie fagocytovej aktivity a indexu (relatívne aktivity 1,05 a 1,44) ľudských monocytov. Baktericídnu aktivitu monocytov voči *Escherichia coli* znížil (relatívna aktivita 0,73) rovnako ako aktivitu voči *Staphylococcus aureus* (relatívna aktivita 0,36), zatiaľ čo kandidacídnu aktivitu zvýšil (relatívna aktivita 1,15). Z metabolických aktivít mononukleových buniek sme u lyzozýmovej aktivity nepozorovali žiadnu zmenu, naopak peroxidázová aktivita bola zvýšená. *L. mucosae* D preukázal schopnosť potencovať produkciu prozápalového cytokínu IL-1β monocytmi v podmienkach *in vitro* (relatívna aktivita 2,60). Na základe získaných výsledkov považujeme jahňací izolát *L. mucosae* D za potenciálne probiotický.

Keywords *Lactobacillus mucosae; probiotics; immunomodulation*

Kľúčové slová: *Lactobacillus mucosae; probiotiká; imunomodulácia*

INTRODUCTION

Strains of the genus, *Lactobacillus*, were involved in the selection of probiotic candidates for human and veterinary use. Probiotics are defined as "live microorganisms which when administered in adequate amounts confer health benefit on the host" (FAO/WHO, 2001). The beneficial influence of a probiotic micro-organism on its host is indisputable. It encompasses numerous factors, like moderation of lactose intolerance; lowering the risk of traveller's diarrhoea; postantibiotic diarrhoea associated with *Clostridium difficile*; modulation of the immune system; lowering the levels of cholesterolemia etc. (Begley et al., 2006; Lin et al., 2009; Sepp et al., 2011). A Probiotic strain should be unerringly identified and characterised before use in clinical practice (FAO/WHO, 2002). This characterisation should involve phenotypic and microbiological tests and determination of biochemical attributes.

A new species of the genus *Lactobacillus*, *Lactobacillus mucosae*, affiliated to the group of *Lactobacillus reuteri – Lactobacillus Fermentum*, was first described in 2000 (Roos et al., 2000). As the probiotic bacteria in the host organism are in contact with commensals, it is important to determine their ability in the potential transmission of antibiotic resistance. Broadly, this is the case when the genes for resistance are localised on

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plasmids. If the possibility or likelihood of such transmission is suspected, then such a strain cannot be accepted in human and/or veterinary use. Understanding the susceptibility of an antibiotic probiotic strain will be useful also in the case of reducing postantibiotic diarrhoea, in choosing the strain resistant to the particular antibiotic/chemotherapeutic used. However, immunocompromised patients may be infected also by the probiotic taken, so the susceptibility levels of the strain against some antimicrobials should be ascertained (Ashraf & Shah, 2011; Clementi & Aquilanti, 2011; Dušková & Karpíšková, 2013).

Lactobacilli are capable of influencing the immune system of a host with the help of several mechanisms, and to also affect innate and adaptive immunity (Hemarajata & Versalovic, 2013). Growth and functions of immune cells are influenced by secreted products and metabolites of these bacteria (Preidis & Versalovic, 2009). Both live and dead probiotic bacteria are able to modulate immune response. Some studies stated that heat-killed bacteria possess pro-inflammatory rather than anti-inflammatory effects on a host organism (induction of cytokines – interleukin 6 (IL-6) or tumour necrosis factor α (TNF-α) – production; Marin et al., 1997). On the other hand, Livingston et al. (2009) observed that heat-killed *L. reuteri* 100-23 induced production of anti-inflammatory cytokine interleukin 10 (IL-10) by dendritic cells. In live bacteria of the genera *Lactobacillus* and *Enterococcus*, induction of B-cells and stimulation of Ig A secretion in the intestine was observed (Vinderola et al., 2005). *In vivo* on *Lactobacillus*-freemice model *L. reuteri* 100-23 recruited regulatory T cells to the gastrointestinal epithelium (Zhang et al., 2008). These abilities are strain-dependent and cannot be generalised.

Therefore, in this study, we have focused on the determination of susceptibility of a potential probiotic bacterial isolate, *L. mucosae* D, originated from the stomach mucosa of lamb, against several antibiotics and chemotherapeutics, and conducted the testing of its immunomodulatory/immunomodulation activity on human mononuclear cells *in vitro*.

MATERIALS AND METHODS

Strains and culture conditions

L. mucosae D was isolated from the stomach mucosa of breast-fed lamb (breeding station Očová, Slovakia), previously identified by Bilková et al., (2008). For comparison, a probiotic strain, *L. reuteri* ATCC 55730 (America Type Culture Collection, Manassas, USA), was included in some experiments. Lactobacilli were cultivated overnight anaerobically at 37°C in MRS broth (Oxoid, Hampshire, Great Britain). In experiments involving metabolic activity, *Escherichia coli* ATCC 11229, *Micrococcus luteus* ATCC 4698, *Enterococcus faecalis* CCM 2122 (Czech Collection of Microorganisms, Brno, Czech Republic) and *Candida albicans* CCM 8180 were used. Bacteria were grown aerobically in nutrient broth and yeast in Sabouraud liquid medium (both Imuna, Šarišské Michaľany, Slovakia) for 24 h at 37°C and 48 h at laboratory temperature, respectively.

Antibiogram of *L. mucosae* D

Susceptibility to selected antimicrobial substances was determined by disc-diffusion method according to Ortez (2005). Discs with antimicrobial substances (Table 1) were purchased from Oxoid (Hampshire, Great Britain).

Table 1. Zones of growth inhibition around discs with antimicrobial substances

Antimicrobial substance	*L. mucosae* D (mm)
PNC	R
AMP	11.3 ± 1.2
OXA	R
COT	20.7 ± 1.2
CTA	19.7 ± 1.5
VAN	R
GEN	10.7 ± 0.6
TET	21.7 ± 0.6
ERY	25.3 ± 1.5
CLI	25.7 ± 0.6
OFL	R
CIP	R

PNC – penicillin G (10 I.U.), AMP – ampicillin (10 μg), OXA – oxacillin (1 μg), COT – cefotaxime (5 μg), CTA – ceftazidime (30 μg), VAN – vancomycin (30 μg), GEN – gentamicin (10 μg), TET – tetracycline (30 μg), ERY – erythromycin (15 μg), CLI – clindamycin (2 μg), OFL – ofloxacin (5 μg), CIP – ciprofloxacin (5 μg), R – resistance. Diameter of disc was 7 mm. Data are arithmetical means of six measurements ± SD.

Isolation of human mononuclear cells and their treatment with *L. mucosae* D

Human mononuclear cells (MC) from six healthy random volunteers (OHT-Derer's Hospital, Bratislava, Slovakia) were isolated and purified by HistoPaque-1077 (Sigma, St. Louis, USA) according to Böyum et al., (1968). MC were suspended in RPMI medium (diluted in physiological saline 1:4; Lonza, Basel, Switzerland) supplemented with 10% fetal bovine serum (Sigma, St. Louis, USA) to 2×10^6 cells/ml. In the next step, cells were cultivated over 18 h (at 37°C in 5% CO_2 atmosphere) exposed to 2.5×10^5 CFU/ml of *L. mucosae* D. As a control sample, untreated MC were used.

Phagocytic activity and phagocytic index

For phagocytic activity and index, 2×10^6 of lactobacilli-treated and untreated MC were incubated 1 h at 37°C with 2.5×10^7 CFU of heat-inactivated *E. faecalis* in a total volume 150 μl. Wright's staining was performed according to the conventional method (Wright, 1902). Phagocytic activity and index were determined microscopically. Phagocytic activity

was calculated as a percentage of the phagocyting MC from 100 cells. Phagocytic index was determined as an average number of *E. faecalis* cells ingested per one monocyte.

Microbicidal activity

MC, exposed to L. *mucosae* D *in vitro*, were ultrasonically disintegrated (18 kHz, 10 s; Soniprep 150, MSE, Crawley, Great Britain). The microbicidal activity of crude homogenate was tested on *Staphylococcus aureus*, *E. coli* and *C. albicans*. Cultures of micro-organisms were sedimented by centrifugation and washed twice in physiological saline. Aliquots (100 µl) of MC crude homogenate, and the microbial suspension (2.5×10^8 CFU/ml of bacteria, or 6.6×10^6 CFU/ml of yeast) were incubated for 1 h at 37°C. The number of surviving micro-organisms was estimated after cultivation on appropriate media (*S. aureus* and *E. coli* – 24 h at 37°C on blood agar and Endo agar, respectively, yeast 48 h at 25°C on Sabouraud agar (all Imuna, Šarišské Michaľany, Slovakia). In the control samples, non-treated MC were used. Microbicidal activity was expressed as a difference in number of CFU/ml between control and test samples.

Metabolic activities

The ultrasonically disintegrated MC (18 kHz, 10 s; Soniprep 150, MSE, Crawley, Great Britain) were centrifuged ($2500 \times g$, 10 min, 4°C). For lysozyme activity, 150 µl of supernatant was mixed with 50 µl of *M. luteus* suspension ($OD_{410} = 0.8$) in phosphate buffer (pH 6.2, 70 mmol/l KH_2PO_4 and 70 mmol/l $Na_2HPO_4 \cdot 12H_2O$). Turbidity changes were registered in time 0 and 20 minute at 410 nm (MR 5000, Dynatech, Alexandria, Virginia, USA). For the determination of peroxidase activity 50 µl of the peroxidase substrate (1,2-phenylenediamine, 0.5 mg/ml), freshly diluted H_2O_2 (10 µl/ml) and sodium citrate tribasic dihydrate (100 mmol/l in distilled water, pH 5.0) were added to 150 µl of MC supernatant. After incubation at 22°C for 20 min, the reaction was stopped with H_2SO_4 (50 µl, 4 mol/l), and changes in the absorbance were determined photometrically at 490 nm (Dynatech MR 5000).

IL-1β production

MC (2×10^6 cells/ml) were cultivated with *L. mucosae* D (5×10^5 CFU/ml) in total volume 50 µl in RPMI medium supplemented with 10% fetal bovine serum (Sigma, St. Louis, USA) for 18 h at 37°C. Untreated MC were used as the control. The amount of IL-1β produced by phagocytes was determined photometrically (A450; MR 5000, Dynatech, Alexandria, Virginia, USA) by enzyme immunoassay method according to the manufacturer's recommendations (Immunotech, Marseille, France).

Statistical analysis

All experiments were repeated in six parallels. For calculations and statistical analysis, OriginPro 7.5 was used. Data were expressed as mean values ± SD, or as relative activities (the control sample was considered as 1.00). The statistical comparison between control and tested samples was performed by a Student's t-test. For statistical analysis of adherence ability, a Student's t-test for evaluation of difference of two relative values was used. The value $p \leq 0.05$ was regarded as statistically significant.

RESULTS AND DISCUSSION

Bacterial strain isolated from the stomach mucosa of breast-fed lamb was identified as *L. mucosae* D (JN809247; Bilková et al., 2008) and partially characterised previously by Bilková et al., (2011). *L. mucosae* D was tested for its sensitivity to 10 antibiotics and two chemotherapeutics by disc-diffusion method. For comparison, the probiotic strain, *L. reuteri* ATCC 55730, was used. Up-to-date, standardised breakpoints for testing of lactobacilli susceptibility to antimicrobials using this method have not been published. Some researchers have developed modifications of the semiquantitative disc assay (Delgado et al., 2005; Klare et al., 2005; Ocaña et al., 2006). Different base media and lactobacilli strains have been employed, but reference data are still not available. In our study, only cases with bacterial growth directly around the antimicrobial disc were evaluated as resistant (Table 1). *L. mucosae* D showed resistance to the antibiotics penicillin G, oxacillin and vancomycin, and chemotherapeutics ofloxacin and ciprofloxacin. The evaluation of *L. reuteri* ATCC 55730 resistance/neutrality/susceptibility against several antimicrobials is published on the website (http://www.biogaia.sk/health_tips_antibiotics.php). Our findings for resistance of this bacterium to penicillin G, ampicillin, oxacillin, vancomycin, tetracycline and ciprofloxacin are in good agreement with data published.

Lactobacillus species differ in their resistance to antibiotics. Many of them are resistant to erythromycin, tetracycline and vancomycin (Ashraf & Shah, 2011). Resistance to vancomycin is of major concern, because this antibiotic is one of the last antibiotics broadly efficacious against clinical infections caused by multidrug-resistant pathogens (Woodford et al., 1995). Vancomycin resistance of lactobacilli is species-dependent (Zhou et al., 2005; Delgado et al., 2005; Ocaña et al., 2006), and usually, intrinsic genes of resistance are located on chromosomal DNA (Ashraf & Shah, 2011). Therefore, a risk of their translocation on surrounding microbiota is low (Klein et al., 1998). The findings of Klein et al., (2000) established the safety of the *Lactobacillus* strains exhibiting vancomycin resistance for use as probiotics.

In experimental models, different strains of lactobacilli show diverse ways of physiological action and immune response modulation (Damaskos & Kolios, 2008). Immunomodulation effects of probiotic bacteria depend on the immunologic state of the host organism and may differ according to the probiotic strain used.

Phagocytosis is responsible for early activation of the immune system before antibody production. During various

inflammatory reactions, phagocytes release toxic agents, for example, reactive oxygen intermediates and lytic enzymes. Phagocytic activity results in the further recruitment of immunocompetent cells and the generation of inflammatory response (Isolauri et al., 2001). Several strains of lactobacilli induce the production of macrophages and activate phagocytosis in mice and humans after oral administration (Schiffrin et al., 1995; Perdigón et al., 1998). However, differences were observed in the modulation of phagocytosis in healthy persons, where an immunostimulatory effect was observed, whereas in allergic ones, downregulation of the inflammatory response was detected (Pelto et al., 1998). It was also observed that several strains of live lactic acid bacteria enhance non-specific host resistance to microbial pathogens and induce *in vitro* pro-inflammatory cytokines production, namely TNF-α and IL-6, reflecting stimulation of non-specific immunity (Miettinen et al., 1996; Marin et al., 1997). By contrast, probiotic bacteria can also mediate suppression of lymphocyte proliferation and T-cells cytokine production (Isolauri et al., 2001). Studies have revealed possible collaboration between commensal microbiota and the host immune system in elimination of pathogens (Mowat, 2003). Probiotic bacteria exhibit on their surface molecules, micro-organism-associated molecular patterns (MAMPs). These structures are recognised by pattern recognition receptors on host cells (PRRs; Lebeer et al., 2010) and, finally, by downregulation of NF-κB activity, suppress inflammatory reaction activities (Caballero-Franco et al., 2007).

The results described above indicate that specific strains of probiotic bacteria can possess several immunomodulatory effects. Therefore, evaluation of immunomodulatory potential of *L. mucosae* D on human MC *in vitro* was performed.

Effects of *L. mucosae* D on the phagocytic potential of MC showed that similar trends in both parameters, phagocytic activity and index, were significantly elevated (relative values for phagocytic activity and index were 1.05 and 1.44, respectively) (Table 2). Similar results were obtained by Vincenti (2010), who showed that *Lactobacillus* rhamnosus GG supernatants modulate macrophage activity by enhancing the phagocytic activity and index. The number of recovered *E. coli* per macrophage decreased from 6.4 to 0.5 after 60 min incubation with *Lactobacillus* supernatant. Also, the strain, *L. reuteri* E, which has the same origin as *L. mucosae* D (stomach mucosa of lamb), enhanced phagocytic activity and index under similar experimental conditions (Kiňová Sepová et al., 2011).

Human epithelia are permanently exposed to bacteria and fungi, including commensal and pathogenic microbiota. Molecules of innate immunity cells, defensins, protect the host from pathogens and shape the microbial composition of mucosal surfaces. Some probiotic micro-organisms may alter the composition of intestinal microbiota by modification of the immune cells through the production of cytokines and defensins, by competitive exclusion, or due to the stimulation of non-specific immune pathways (Aureli et al., 2011). It was observed that human β-defensin 1 after reduction of disulphide-bridges shields the healthy epithelium against commensal bacteria (*Lactobacillus* and *Bifidobacterium*) and opportunistic pathogenic fungi (e.g. *C. albicans*) *In vivo* (Schroeder et al., 2011).

In this study, we show the difference in microbicidal activity of phagocyting cells affected by *L. mucosae* D against *E. coli*, *S. aureus* and *C. albicans*. Microbicidal activity of pretreated human MC against bacteria was inhibited, whereas against *C. albicans* it was stimulated (Table 2). When the human MC cells were pretreated with *L. reuteri* E, their microbicidal activities against the same indicator micro-organisms were decreased (Kiňová Sepová et al., 2011). In the case of metabolic activities, the peroxidase activity was positively stimulated using both lamb isolates, *L. mucosae* D (this study) and *L. reuteri* E (Kiňová

Table 2. Ability of L. mucosae D to affect metabolic activities of mononuclear cells (MC) in vitro

	Control sample, mean ± SD	Treated sample, mean ± SD	Relative activity
Number of survived MC after coincubation with *Lactobacillus mucosae* D	$1.63 \pm 0.36 \times 10^6$/ml	$1.68 \pm 0.57 \times 10^6$/ml^{NS}	1.03
Phagocytic activity	80 ± 0.1%	84 ± 0.1 %***	1.05
Phagocytic index	5.9 ± 0.6	8.5 ± 0.4***	1.44
Number of killed *Staphylococcus aureus*	$44 \pm 2.0 \times 10^6$/ml	$16 \pm 6.0 \times 10^6$/ml**	0.36
Number of killed *Escherichia coli*	$146 \pm 16.4 \times 10^6$/ml	$106 \pm 10.0 \times 10^6$/ml*	0.73
Number of killed *Candida albicans*	$2.5 \pm 0.1 \times 10^6$/ml	$2.9 \pm 0.0 \times 10^6$/ml**	1.15
Lysozyme activity	0.010 ± 0.004	0.009 ± 0.004^{NS}	0.90
Peroxidase activity	0.163 ± 0.004	0.230 ± 0.007***	1.41
IL-1β	282.6 ± 49.1 pg/ml	734.6 ± 78.4 pg/ml***	2.60

The values are arithmetical means of 6 parallels ± SD. NS – statistically non-significant difference between control and treated samples, p – statistically significant difference between control and treated samples. * – $p \leq 0.05$; ** – $p \leq 0.01$; *** – $p \leq 0.001$.

Sepová et al., 2011). Unlike *L. reuteri* E, *L. mucosae* D decreased lysozyme activity of human MC *in vitro* (Table 2).

To estimate pro-inflammatory properties of *L. mucosae* D, bacterial cells were tested for their ability to affect phagocytes IL-1β production *in vitro*. Pro-inflammatory cytokines, including IL-1β, play a pivotal, yet ambiguous, role in inflammation and in response to pathogens. Increased production of IL-1 has significant impact on stimulation of the phagocytic activity, on activation of T-lymphocytes and duration of B-cells proliferation. Some strains of lactobacilli, bifidobacteria and also *E. coli* (Nissle, 1917), can affect pro- and anti-inflammatory cytokines production in peripheral blood MC (Helwig et al., 2006). *L. mucosae* D significantly stimulated IL-1β production by MC (relative activity 2.6) (Table 2).

CONCLUSION

Based on all our results we estimate that lamb isolate *L. mucosae* D shows several features to be classified as a promising probiotic candidate for human and/or veterinary practice. However, supplementation of *in vivo* tests on animal models is required before application in both human and animals. The risk of transfer of resistant genes to other, even pathogenic bacteria, also need to be evaluated.

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