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Bile tolerance and its effect on antibiotic susceptibility of probiotic *Lactobacillus* candidates

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Abstract Before use in practice, it is necessary to precisely identify and characterize a new probiotic candidate. Eight animal lactobacilli and collection strain *Lactobacillus reuteri* CCM 3625 were studied from the point of saccharide fermentation profiles, bile salt resistance, antibiogram profiles, and influence of bile on sensitivity to antibiotics. Studied lactobacilli differed in their sugar fermentation ability determined by API 50CHL and their identification based on these profiles did not correspond with molecular-biological one in most cases. Survival of strains *Lactobacillus murinus* C and *L. reuteri* KO4b was not affected by presence of bile. The resistance of genus *Lactobacillus* to vancomycin and quinolones (ofloxacin, ciprofloxacin) was confirmed in all strains tested. This study provides the new information about oxgall (0.5 and 1 %) effect on the lactobacilli antibiotic susceptibility. Antibiotic profiles were not noticeably affected, and both bile concentrations tested had comparable impact on the lactobacilli antibiotic sensitivity. Interesting change was noticed in *L. murinus* C, where the resistance to cephalosporins was reverted to susceptibility. Similarly, susceptibility of *L. reuteri* E to ceftazidime arose after incubation in both concentration of bile. After influence of 1 % bile, *Lactobacillus mucosae* D lost its resistance to gentamicin. On the base of gained outcomes, the best probiotic properties manifested *L. reuteri* KO4b, *Lactobacillus plantarum* KG4, and *L. reuteri* E due to their survival in the presence of bile.

Introduction

Changeable physiological conditions along human gastrointestinal tract (GIT) determine its microbiota composition. Each microbial inhabitant has to adapt to specific environmental niche and develops appropriate mechanisms for withstanding of diverse stress types (Lebeer et al. 2008). *Lactobacillus* spp. that play a pivotal role in human gut and represents the most commonly used probiotic genus has to survive GIT passage in vital form. Except acid in the stomach (average pH 2) (Siciliano and Mazzeo 2012) and bile in the intestine and colon (concentration varies between 40 and 1 mmol/L) (Fontana et al. 2013), digestion enzymes also affect microbial composition of GIT. Surrounding commensal and opportune pathogenic microflora represents also a rival competing for nutrients and host cell receptors with probiotic cells (Horošová et al. 2006).

All GIT stress factors influence the ability of probiotic bacteria to maintain their health benefits to host macroorganism. Lactobacilli stress coping is evolved in several mechanisms. Acid stress tolerance is provided by three main manners: (i) maintaining the intracellular pH homeostasis; (ii) reparation of DNA and proteins damaged due to low pH; (iii) modification of cell envelope architecture (Lebeer et al. 2008). The cross-adaptation between acid and bile resistance is present, considering some of the common adaptation mechanisms (Lorca and de Valdez 2009). Bile salt hydrolases (coding by *bsh* genes) are responsible for specific adaptation mechanism of GIT lactobacilli to bile (Begley et al. 2005a; Patel et al. 2010). The host may also profit from this adaptation mechanism. Cholesterol level decrease, as one of probiotic mechanisms of action, can be partially ascribed to bile salt hydrolase activity (Liong and Shah 2005; Ebringer et al. 2008; Turková et al. 2013).

Probiotic bacteria are potential reservoir of antibiotic resistance genes that can be spread to surrounding microbiota, even pathogenic. In comparison with intrinsic resistance (genes located on bacterial chromosome), the acquired resistance (genes located on plasmids and transposons) represents the real risk in practical use due to possible resistance transfer

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(Ashraf and Shah 2011; Fukao and Yajima 2012). Postantibiotic diarrhea is one of the health disorders that probiotic bacteria are able to moderate (Sepp et al. 2011). In the case of postantibiotic diarrhea recovery, use of probiotics is very common. Certain strain to be applied should be resistant to antibiotic used in therapy (Clementi and Aquilanti 2011; Dušková and Karpíšková 2013). For these reasons, it is essential to know the antibiogram profile of probiotic bacterium.

The present study is focused on antibiotic susceptibility determination, ability to survive in presence of bile, bile effect on antibiotic susceptibility, and comparison of API 50CHL and molecular-biological identification (previous works: Bilková et al. 2008; Kiňová Sepová and Bilková 2013) of potential probiotic lactobacilli for human or veterinary use.

Material and methods

Bacterial strains and growth conditions Several bacterial strains were isolated from stomach mucosas of breast-fed lamb and goatling. Lamb isolates were identified as *Lactobacillus murinus* C, *Lactobacillus mucosae* D, and *Lactobacillus reuteri* E (Bilková et al. 2008); goatling as *L. reuteri* KO4b, *L. reuteri* KO4m, *L. reuteri* KO5, *Lactobacillus plantarum* KG1z, and *L. plantarum* KG4 (Kiňová Sepová and Bilková 2013). *L. reuteri* CCM 3625 was purchased from Czech Collection of Microorganisms (Brno, Czech Republic). Lactobacilli were cultivated in MRS broth (Oxoid, Great Britain) at 37 °C in anaerobic conditions for 18 h.

API 50CHL Carbohydrate fermentation profiles were found out using API 50CHL kit (bioMérieux, France) according to the manufacturer's recommendations.

Antibiotic susceptibility Susceptibility to selected antimicrobial substances was determined by disc diffusion method according to Coyle (2005). Discs with antimicrobial substances (Tables 2 and 3) were purchased from Oxoid (Great Britain). Testing of antimicrobial susceptibility of lactobacilli after influence of 0.5 and 1.0 % bile salts was performed according to Elkins and Mullis (2004). Preincubation with bile salts lasted 12 h at 37 °C.

Bile resistance Experiment was performed by modified method according to De Boever and Verstraete (1999). Briefly, bacterial cultures after 18 h anaerobical incubation were settled by centrifugation, washed twice in physiological saline, and adjusted to ca 4.8×10^7 CFU/mL. Cultures were further diluted in MRS broth (Oxoid, Great Britain) supplemented with 0 (control sample), 0.5 or 1 % oxgall (Biomark™ Laboratories, India) in ratio 1:9. After 12 h of incubation, the number of survived bacteria was determined by serial dilutions and plating on MRS agar. The results were expressed by the percentage of growth in the presence of bile salts compared to the control.

Statistical analysis Experiments were repeated in three or six parallels. The statistical comparison between control and tested samples was performed by Student's *t*-test. Statistical significant differences between control and sample are expressed as ns non-significant difference; $*p<0.05$; $**p<0.01$; and $***p<0.001$.

Results and discussion

Antibiograms and survival in bile and its effect on antibiotic susceptibility of eight lactobacilli probiotic candidates were studied. As the exact identification of probiotic microorganism is essential (FAO/WHO 2001), molecular-biological identification (partial 16S rDNA sequencing (Bilková et al. 2008; Kiňová Sepová and Bilková 2013)) was compared with biochemical one (API 50CHL; Table 1). The cohesive results were gained only in *L. plantarum* isolates. In other isolates, some discrepancies using API 50CHL were observed. Number of *Lactobacillus* species is still increasing (to date 201 species, 29 subspecies) (Euzéby 2014), and therefore not all of them are covered in database. Actually, API 50CHL database includes 16 species and 3 subspecies of lactobacilli and for example *L. reuteri,* which was firstly described in 1980 (Kandler et al. 1980), is still missing in version V5.1. Heterogeneity of genus *Lactobacillus* (Collins et al. 1991; Claesson et al. 2007) gives also the evidence of various results.

To overcome the stress conditions in GIT, gut-associated lactobacilli are able to metabolize conjugated bile salts by hydrolysis (De Boever and Verstraete 1999). Furthermore, GIT strains adapt more progressively to the bile salts presence (Ruiz et al. 2013) in comparison with bacteria originated from other niches. The probiotic candidates isolated from Slovak bryndza cheese did not show such high rate of survival in the presence of 0.5 and 1 % bile (Belicová et al. 2013), than our strains originating from animals GIT. Survival of *L. reuteri* E in the presence of 1 % bile was significantly better than in 0.5 % (Fig. 1). Survival of strains *L. murinus* C and *L. reuteri* KO4b was not significantly impacted by the presence of bile in both concentrations used. The rest strains were more sensitive to higher bile concentrations.

Susceptibility of tested lactobacilli to 11 commonly used antibiotics was determined by disc diffusion assay and interpreted according to Charteris et al. (1998). Since the official breakpoints for lactobacilli are not given, diameters of inhibitory zones are also introduced (Table 2). MRS agar

Table 1 Carbohydrate fermentation and identification of isolates according to API 50CHL

Strain	Number of fermentable sugars	API 50CHL identification		
		Species	Percent	*T* index
Lactobacillus murinus C	26	*L. plantarum*	99.9	0.97
Lactobacillus mucosae D	28	*L. plantarum*	94.1	0.69
Lactobacillus reuteri E	20	*L. brevis*	89.2	0.48
Lactobacillus reuteri KO4b	25	*L. plantarum*	99.9	1.00
Lactobacillus reuteri KO4m	11	*L. fermentum*	89.7	0.97
Lactobacillus reuteri KO5	12	*L. fermentum*	78.2	0.31
Lactobacillus plantarum KG1z	26	*L. plantarum*	99.9	0.95
Lactobacillus plantarum KG4	26	*L. plantarum*	98.9	0.91
Lactobacillus reuteri CCM 3625	26	*L. pentosus*	72.4	0.45

The accuracy of identification is evaluated as following: excellent identification percent id≥99.9 and $T \geq 0.75$; very good identification percent id≥99.0 and $T \geq 0.50$; good identification percent id≥90.0 and $T \geq 0.25$; acceptable identification percent id≥80.0 and $T \geq 0$

was used due to the poor growth of strains on Müller-Hinton agar, although, components of MRS medium can inactivate some antibiotics, e.g., imipenem (Ammor et al. 2007). The selection of appropriate cultivation medium for lactic acid bacteria was proclaimed also by the other authors (Klare et al. 2005; Dušková and Karpíšková 2013). All tested strains were resistant to vancomycin, ofloxacin, and ciprofloxacin (Table 2) in accordance with the literature findings. Vancomycin resistance was described as intrinsic in this genus (Bernardeau et al. 2008). Resistance to quinolones (ofloxacin and ciprofloxacin) corresponds with findings of other authors (Hummel et al. 2007), and it is also proposed to be intrinsic in lactobacilli. Detected susceptibility in lactobacilli to penicillin, ampicillin, and erythromycin was also described before (Danielsen and Wind 2003; Klare et al. 2005)

Influence of bile on antibiotics susceptibility was tested after preincubation of lactobacilli in the presence of 0.5 and 1 % bile. In comparison to previous experiment, antibiotic susceptibility profiles were not markedly modified and both bile concentrations had comparable effect (Table 3). Interesting result was noticed in *L. murinus* C which lost its resistance to ceftazidime and cefotaxime. After preincubation with 1 % bile, *L. mucosae* D lost its gentamicin resistance. Similarly, susceptibility of *L. reuteri* E to ceftazidime arose after exposition to bile. Increased diameter of inhibition zones of *L. mucosae* D to quinolones, penicillin, and ampicillin was detected.

Bile itself has surfactant properties and is able to emulsify and solubilize lipids. These detergent properties result also in an antimicrobial effect due to its membrane activity (Begley et al. 2005b). Modification of bacterial antibiotic susceptibility

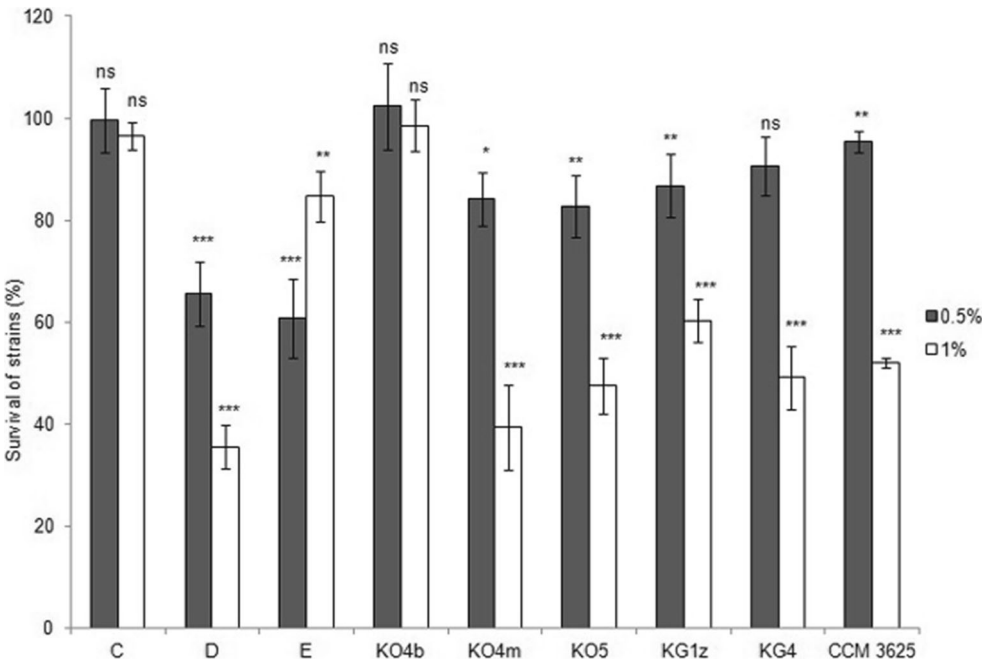

Fig. 1 Survival of tested lactobacilli in the presence of 0.5 and 1 % bile *C Lactobacillus murinus* C, *D Lactobacillus mucosae* D, *E Lactobacillus reuteri* E, *KO4b Lactobacillus reuteri* KO4b, *KO4m Lactobacillus reuteri* KO4m, *KO5 Lactobacillus reuteri* KO5, *KG1z Lactobacillus plantarum* KG1z, *KG4 Lactobacillus plantarum* KG4, *CCM 3625 Lactobacillus reuteri* CCM 3625. Control sample corresponds to 100 %. Values are calculated from six independent experiments as arithmetical means±SD. Statistical significant differences between control and sample are expressed as *ns* non-significant difference; **p*<0.05; ***p*<0.01; ****p*<0.001

Table 2 Susceptibility of tested lactobacilli to selected antibiotics

1 2	C	D	E	KO4b	KO4m	KO5	KG1z	KG4	CCM 3625
P	25 MS	33 S	35 S	30 S	36 S	36 S	33 S	40 S	20 MS
AMP	15 MS	35 S	27 S	30 S	28 S	38 S	30 S	40 S	25 S
CTX	6 R	24 S	24 S	40 S	42 S	32 S	30 S	40 S	25 S
CAZ	11 R	18 MS	6 R	18 MS	30 S	28 S	25 S	22 S	21 S
VA	6 R	6 R	6 R	6 R	6 R	6 R	6 R	6 R	6R
CN	10 R	11 R	14 S	16 S	18 S	15 S	13 S	15 S	15 S
TET	22 S	30 S	27 S	30 S	30 S	30 S	22 S	28 S	21 S
ERY	23 S	33 S	23 S	34 S	36 S	34 S	24 S	32 S	24 S
CLI	22 S	38 S	28 S	34 S	36 S	29 S	22 S	34 S	20 S
OXF	13 R	6 R	6 R	6 R	6 R	6 R	8 R	6 R	6 R
CIP	13 R	6 R	6 R	10 R	6 R	6 R	9 R	6 R	6 R

Susceptibility expressed *R* as resistant, *MS* as moderately susceptible, and *S* as susceptible (Charteris et al. 1998). Diameters of inhibition zones are expressed in millimeter; diameter of disc was 6 mm. Data are arithmetical means of three measurements

1 strain, *2* antimicrobial substance, *P* penicillin G (10 I.U.), *AMP* ampicillin (10 μg), *CTX* cefotaxime (30 μg), *CAZ* ceftazidime (30 μg), *VA* vancomycin (30 μg), *CN* gentamicin (10 μg), *TET* tetracycline (30 μg), *ERY* erythromycin (15 μg), *CLI* clindamycin (2 μg), *OXF* ofloxacin (5 μg), *CIP* ciprofloxacin (5 μg). *C Lactobacillus murinus* C, *D Lactobacillus mucosae* D, *E Lactobacillus reuteri* E, *KO4b Lactobacillus reuteri* KO4b, *KO4m Lactobacillus reuteri* KO4m, *KO5 Lactobacillus reuteri* KO5, *KG1z Lactobacillus plantarum* KG1z, *KG4 Lactobacillus plantarum* KG4, *CCM 3625 Lactobacillus reuteri* CCM 3625

after their exposition to bile could be due the loss of semipermeability, which eases molecules passage. Our observation suggests that this effect could be synergic with activity of beta-lactams. In some cases, the diameters of inhibition zones were larger after action of bile (Elkins and Mullis 2004), and in some cases, the resistance was reverted to susceptibility (ceftazidime and cefotaxime in *L. murinus* C and ceftazidime in *L. reuteri* E).

The present study completed previous characterization of potential probiotic lactobacilli strains. Due to declared intrinsic resistance to vancomycin (Bernardeau et al. 2008) and quinolones (Hummel et al. 2007) in genus *Lactobacillus*,

Table 3 Effect of bile on susceptibility of tested lactobacilli to selected antibiotics

Strain	C		D		E		KO4b		KO4m		KO5		KG1z		KG4		CCM 3625	
1 2	0.5 %	1 %	0.5 %	1 %	0.5 %	1 %	0.5 %	1 %	0.5 %	1 %	0.5 %	1 %	0.5 %	1 %	0.5 %	1 %	0.5 %	1 %
P	33 S	33 S	36 S	52 S	35 S	35 S	29 S	31 S	32 S	34 S	32 S	33 S	31 S	32 S	30 S	30 S	31 S	32 S
AMP	30 S	28 S	37 S	52 S	33 S	33 S	30 S	29 S	32 S	31 S	29 S	31 S	30 S	31 S	26 S	26 S	30 S	30 S
CTX	22 MS	23 S	52 S	50 S	24 S	24 S	25 S	25 S	24 S	24 S	25 S	25 S	24 S	25 S	24 S	24 S	20 MS	20 MS
CAZ	19 S	19 S	43 S	45 S	28 S	28 S	21 S	22 S	26 S	28 S	28 S	27 S	21 S	22 S	20 S	21 S	22 S	19 S
VA	6 R	6 R	6 R	6 R	6 R	6 R	6 R	6 R	6 R	6 R	6 R	6 R	6 R	6 R	6 R	6 R	6 R	6 R
CN	6 R	6 R	11 R	15 S	14 S	14 S	15 S	15 S	18 S	17 S	15 S	15 S	15 S	16 S	15 S	14 S	15 S	15 S
TET	24 S	22 S	19 S	20 S	24 S	24 S	21 S	19 S	25 S	22 S	22 S	24 S	19 S	20 S	17 MS	20 S	28 S	25 S
ERY	24 S	26 S	35 S	32 S	28 S	28 S	25 S	25 S	23 S	26 S	23 S	25 S	23 S	24 S	25 S	26 S	28 S	25 S
CLI	23 S	23 S	22S	24 S	28 S	26 S	25 S	25 S	27 S	25 S	26 S	27 S	25 S	26 S	25 S	24 S	29 S	30 S
OXF	8 R	8 R	10 R	11 R	7 R	6 R	6 R	6 R	8 R	9 R	8 R	8 R	7 R	6 R	6 R	6 R	8 R	7 R
CIP	6 R	6 R	10 R	9 R	6 R	6 R	6 R	6 R	6 R	6 R	6 R	6 R	6 R	6 R	6 R	6 R	6 R	6 R

Susceptibility expressed *R* as resistant, *MS* as moderately susceptible, and *S* as susceptible (Charteris et al. 1998). Diameters of inhibition zones are expressed in millimeter; diameter of disc was 6 mm. Data are arithmetical means of three measurements

1 concentration of oxgall, *2* antimicrobial substance, *P* penicillin G (10 I.U.), *AMP* ampicillin (10 μg), *CTX* cefotaxime (30 μg), *CAZ* ceftazidime (30 μg), *VA* vancomycin (30 μg), *CN* gentamicin (10 μg), *TET* tetracycline (30 μg), *ERY* erythromycin (15 μg), *CLI* clindamycin (2 μg), *OXF* ofloxacin (5 μg), *CIP* ciprofloxacin (5 μg). *C Lactobacillus murinus* C, *D Lactobacillus mucosae* D, *E Lactobacillus reuteri* E, *KO4b Lactobacillus reuteri* KO4b, *KO4m Lactobacillus reuteri* KO4m, *KO5 Lactobacillus reuteri* KO5, *KG1z Lactobacillus plantarum* KG1z, *KG4 Lactobacillus plantarum* KG4, *CCM 3625 Lactobacillus reuteri* CCM 3625

tested strains do not represent a risk of this resistance genes transfer between bacterial species. There is an assumption that antibiotic resistance of majority of tested strains will be not changed excessively after action of bile in host gut. As possible probiotics, the most promising strains seem to be *L. reuteri* KO4b, *L. plantarum* KG4, and *L. reuteri* E for their good survival in bile comparable to control. However, after complex in vitro studies (e.g., determination of transmissible antibiotic resistance), the in vivo evaluation of beneficial attributes on human and/or animal model is necessary before use in practice.

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Conflict of interest None.

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