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In vitro evalution of antibiotic resistance of *Lactobacillus bulgaricus* strains isolated from traditional dairy products

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Abstract: The antimicrobial susceptibility of 20 *Lactobacillus bulgaricus* isolates from traditional fermented milk-originated was assessed and then determined the ability to transfer antibiotic resistance genes to other bacteria. The minimum inhibitory concentration of each strain was determined using a standardized dilution method. All the tested strains were found to be susceptible to gentamicin, erythromycin, clindamycin, neomycin, tetracycline, linezolid, chloramphenicol, rifampicin, and quinupristin/dalfopristin, while their susceptibilities to kanamycin, ciprofloxacin, streptomycin, trimethoprim, ampicillin, and vancomycin varied. Polymerase chain reaction (PCR) was used to check whether specific antibiotic resistance genes were present in these *Lb. bulgaricus*. We detected the *rpoB*, *erm(B)*, *aadA*, *bla*, *cat* and *vanX*. Finally, a filter mating assay was applied to investigate the transferability of these resistance markers; and we observe no antibiotic resistance transfer between bacteria. This work demonstrates a low risk of lateral transfer of the antibiotic resistance gene of *Lb. bulgaricus*.

Keywords: antibiotic susceptibility; resistance genes; transferability

Lactobacillus bulgaricus (*Lb. bulgaricus*) is commonly used as a starter culture for manufacturing of fermented dairy products, particularly fermented milk (Silva *et al*. 2005). Apart from being a starter culture for fermented foods, they also play a key role in the human health (Sánchez *et al*. 2017).With such extensive use of antibiotics, there are increasing concerns regarding bacterial resistance and transfer of microbial resistant genes (Rossi *et al*. 2015). However, adequate risk assessment has to be performed to ensure the safety of this practice before it is widely adopted. Particularly, it would be crucial to consider such risk from two aspects: (1) the intrinsic antibiotic resistance of LAB; (2) the transferability of resistance genes from LAB (Additives and Feed 2008). Although several previous studies have reported the genotype of the LAB in regard to the presence or absence of certain antibiotic resistance genes (Nawaz *et al*. 2011) and the transferability of some of these genes between bacteria (Toomey *et al*. 2010), this kind of study remains limited. The overall objective of this work was to assess the risk of antibiotic gene transfer from this species.

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Table 1. *Lb. bulgaricus* isolates used in this study

Region	Source of isolation	Strains
Xinjiang (China)	fermented cow milk	IMAU32096
		IMAU32076
		IMAU32379
		IMAU32370
		IMAU32368
		IMAU32298
	fermented yak milk	IMAU62159
		IMAU62091
		IMAU62161
Khovsgol (Mongolia)	fermented yak milk	IMAU20450
Selenge (Mongolia)	fermented cow milk	IMAU20290
		IMAU20289
		IMAU20291
Zavkhan (Mongolia)	fermented cow milk	IMAU20515
Bulgan (Mongolia)	fermented cow milk	IMAU20366
Orkhon (Mongolia)	fermented cow milk	IMAU20310
Tov (Mongolia)	fermented cow milk	IMAU20743
Moscow (Russia)	fermented cow milk	IMAU95110
Qinghai (China)	fermented yak milk	IMAU40106
Gansu (China)	fermented yak milk	IMAU80827

Table 2. The final concentration for the determine MIC values and the solvent used to dissolve each antibiotic

Antibiotic	Solvent	Range (μg/ml)
Gentamicin	LSM	0.5–256
Kanamycin		2–1024
Streptomycin		0.5–256
Neomycin		0.5–256
Tetracycline		0.125–64
Clindamycin		0.032–16
Ampicillin		0.032–16
Vancomycin		0.25–128
Quinupristin/ dalfopristin		0.016–8
Linezolid		0.032–16
Erythromycin	95% ethanol	0.016–8
Chloramphenicol		0.125–64
Trimethoprim	glacial acetic acid	0.125–64
Ciprofloxacin	0.05M HCl	0.25–128
Rifampicin	methanol	0.125–64

LSM consisting of 90% Iso-Sensitest medium and 10% MRS broth

MATERIAL AND METHODS

Bacterial strains and growth conditions. The strains were identified as *Lb. bulgaricus* by the traditional morphological characters, physiological, biochemical properties and 16S rRNA sequences. The isolates were obtained from the Lactic Acid Bacteria Cell Collection (Key Laboratory of Dairy Biotechnology and Engineering, Ministry of Education, Inner Mongolia Agricultural University, China) (Table 1). *Lactobacillus paracasei* ATCC334 was used as a quality control strain for ensuring the precision and accuracy of the susceptibility testing procedures. All lactobacilli strains were routinely cultured in de Man, Rogosa, Sharpe (MRS) broth or agar (CM0359, CM0361, OXOID) at 37°C.

Determination of minimum inhibitory concentrations (MICs). Fifteen antibiotics were tested. Each antibiotic stock was dissolved in the respective solvent (Table 2). The antibiotic stocks were kept at –80°C until use. The LAB susceptibility test medium (LSM), consisting of 90% Iso-Sensitest medium (OXOID, CM0473) and 10% MRS broth, was used for diluting the antibiotic stocks. The working concentration range of each antibiotic is given in Table 2, which was either 2-fold (for water-soluble antibiotics) or 10-fold (for water-insoluble antibiotics) of the final concentrations in the MIC assays.

The MICs were determined based on the standard method (ISO10932/IDF223 2010). For each strain, a bacterial suspension was prepared by inoculating a single colony randomly picked up from the agar plate. The culture was grown to McFarland standard 1 (~3×10^8 CFU/ml). To determine the MICs of water-soluble and water-insouble antibiotics, bacterial suspensions were diluted 500-fold (~6×10^5 CFU/ ml) and 100-fold (~3×10^6 CFU/ ml) with LSM. The procedures led to a final cell concentration of 3×10^5 CFU/ml in all cases. The water-insoluble antibiotics were diluted more (100-times) in these procedures to minimize the effect of organic solvents on cell growth. All the bacterial suspensions with antibiotics were incubated anaerobically at 37°C for 48 hours. Every assay was repeated three times. Based on the EFSA guidelines (EFSA 2012), resistant bacteria can grow at an antibiotic concentration higher than the cut-off MIC value, while susceptible bacteria are those suppressed with an antibiotic concentration equal to or lower than the cut-off MIC value. However, the European Committee (EUC

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2002) defined the resistant strains as those that can grow at the cut-off MIC level (Table 3).

DNA extraction and detection of antibiotic resistance genes. Genomic DNA was extracted from each isolate using a DNA Extraction Kit (QIAGEN) following the manufacturer's instructions. The quality of extracted DNA was checked by 1% agarose gel electrophoresis and spectrophotometry (260 nm / 280 nm). The presence of antibiotic resistance genes was detected by PCR using methods and gene-specific primers described by Table 4 (Guo *et al*. 2017). All amplified PCR products were analysed on 1% agarose gel to confirm the DNA fragment size.

Transferability of detected resistance. Transferability of the detected resistance genes was assessed by a filter mating technique described by Toomey *et al*. (2010). The donor and recipient bacteria were separately cultured overnight. The cultures were inoculated into fresh culture media (in 2 % density) and cultured for 4 h to reach the mid-exponential phase of growth (OD_{600} 0.2 to 0.5). One millilitre each of the donor and the recipient bacterial solutions were mixed and filtered through a 0.45 µm MF-Millipore membrane filter of 2.5 cm diameter (HAWP02500; Millipore, USA). The cells were retained on the membrane, and the membrane was incubated on MRS agar with the side of cells facing upward at 37°C. After incubation for 20 h, the membrane was washed with PBS buffer and vortex mixed to remove the cells. The washed suspension was cultured on an agar plate containing the respective antibiotics for 48 h at 30°C or 37°C. Only trans-conjugants would be able to grow on the selected agar. All mating experiments were repeated three times in duplicate.

RESULTS AND DISCUSSION

According to the EFSA and EUC guidelines, the antibiotic resistance profiles of the tested and quality control strains were determined (Table 5). All the isolates were susceptible to gentamicin, erythromycin, clindamycin, neomycin, tetracycline, linezolid, chloramphenicol, rifampicin, and quinupristin/dalfopristin. Nawaz *et al*. (2011) found that *Lb. bulgaricus* was susceptible to chloramphenicol, tetracycline,

Table 3. The minimum inhibitory concentration (MIC) breakpoints for *Lb. bulgaricus*

Antibiotics	MIC breakpoints (µg/ml)
Gentamicin	16[a]
Kanamycin	16[a]
Streptomycin	16[a]
Neomycin	32[b]
Tetracycline	4[a]
Erythromycin	1[a]
Clindamycin	1[a]
Chloramphenicol	4[a]
Ampicillin	1[a]
Vancomycin	2[a]
Quinupristin/dalfopristin	4[b]
Linezolid	4[b]
Trimethoprim	32[b]
Ciprofloxacin	4[a]
Rifampicin	32[b]

[a]European Food Safety Authority (EFSA) (2012); [b]European Community (EUC) (2002)

Table 4. Gene specific primers and conditions for polymerase chain reaction (PCR) detection

Antibiotic	Antibiotic resistance gene	Primers (5'–3')	Annealing temperature (°C)	Amplicon size (bp)
Gentamicin	*aac*(6')-*aph*(2'')	CCAAGAGCAATAAGGGCATA CACTATCATAACCACTACCG	60	220
	aac(6')*Ie*-*aph*(2'')*Ia*	CAGAGCCTTGGGAAGATGAAG CCTCGTGTAATTCATGTTCTGGC	58	348
Streptomycin	*aad*A	ATCCTTCGGCGCGATTTTG GCAGCGCAATGACATTCTTG	56	282
	*aad*E	ATGGAATTATTCCCACCTGA TCAAAACCCCTATTAAAGCC	50	565
	ant(6)	ACTGGCTTAATCAATTTGGG GCCTTTCCGCCACCTCACCG	53	597

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Table 4. To be continued

Antibiotic	Antibiotic resistance gene	Primers (5‘–3‘)	Annealing temperature (°C)	Amplicon size (bp)
Kanamycin	*aph*(3”)-*III*	GCCGATGTGGATTGCGAAAA GCTTGATCCCCAGTAAGTCA	52	292
	ant(2”)-*I*	GGGCGCGTCATGGAGGAGTT TATCGCGACCTGAAAGCGGC	67	329
Neomycin	*aph*(3”)-*I*	AACGTCTTGCTCGAGGCCGCG GGCAAGATCCTGGTATCGGTCTGCG	68	670
	aph(3”)-*III*	GCCGATGTGGATTGCGAAAA GCTTGATCCCCAGTAAGTCA	52	292
Tetracycline	*tet*(M)	GGTGAACATCATAGACACGC CTTGTTCGAGTTCCAATGC	55	401
	tet(K)	TCGATAGGAACAGCAGTA CAGCAGATCCTACTCCTT	55	169
	tet(W)	GAGAGCCTGCTATATGCCAGC GGGCGTATCCACAATGTTAAC	64	168
Erythromycin	*erm*(B)	GAAAAGGTACTCAACCAAATA AGTAACGGTACTTAAATTGTTTAC	54	639
	erm(B)-*1*	CATTTAACGACGAAACTGGC GGAACATCTGTGGTATGGCG	54	405
	erm(C)	TCAAAACATAATATAGATAAA GCTAATATTGTTTAAATCGTCAAT	50	642
Clindamycin	*lnu*(A)	GGTGGCTGGGGGGTAGATGTATTAACTGG GCTTCTTTTGAAATACATGGTATTTTTCGATC	55	323
	lnu(B)	CCTACCTATTGTTTGTGGAA ATAACGTTACTCTCCTATTTC	54	925
Chloramphenicol	*cat*A	GGATATGAAATTTATCCCTC CAATCATCTACCCTATGAAT	50	486
	cat	TTAGGTTATTGGGATAAGTTA GCATGRTAACCATCACAWAC	48	300
Ampicillin	*bla*Z	ACTTCAACACCTGCTGCTTTC TAGGTTCAGATTGGCCCTTAG	58	240
	bla	CATARTTCCGATAATASMGCC CGTSTTTAACTAAGTATSGY	51	297
	*mec*A	GGGATCATAGCGTCATTATTC AGTTCTGCAGTACCGGATTTGC	58	1429
Vancomycin	*van*E	TGTGGTATCGGAGCTGCAG GTCGATTCTCGCTAATCC	52	513
	*van*X	TCGCGGTAGTCCCACCATTCGTT AAATCATCGTTGACCTGCGTTAT	55	454
Quinupristin/ dalfopristin	*vat*C	GAAATGGTTGGGAGAAGCATACC CAGCAATCGCGCCCGTTTG	64	392
	*vat*E	CTATACCTGACGCAAATGC GGTTCAAATCTTGGTCCG	52	490

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Table 4. To be continued

Antibiotic	Antibiotic resistance gene	Primers (5'–3')	Annealing temperature (°C)	Amplicon size (bp)
Linezolid	*cfr*	TGAAGTATAAAGCAGGTTGGGAGTCA ACCATATAATTGACCACAAGCAGC	55	746
Trimethoprim	*dfr*A	CTTTTCTACGCACTAAATGTAAG CATTATCAATAATTGTCGCTCAC	50	474
	*dfr*D	GGAAGGGCTTTACCTGACAGAAG CGACATAAGGCAAGAACATAACATA	50	175
Rifampicin	*rpo*B	TAACCGTGGTGCTTGGCTDGAATWYGAAAC ATCAAACCAATGTTAGGNCCTTCWGGDGTTTC	59	1100
Ciprofloxacin	*gyr*A	GAYTATGCWATGTCAGTTATTGT GGAATRTTRGAYGTCATACCAAC	45	286
	*par*C	TATTCYAAATAYATCATTCARGA GCYTCNGTATAACGCATMGCCG	50	286

and linezolid, which was in agreement with the present results. Lactobacilli have been reported to be susceptible to chloramphenicol, erythromycin, clindamycin, and tetracycline mainly via the inhibition of protein synthesis (Coppola *et al*. 2005; Zhou *et al*. 2005). However, they showed different degree of resistance to other antibiotics. The only strain that was resistant to ampicillin was IMAU62161. Two strains, IMAU62161 and IMAU62091, were resistant to vancomycin with an MIC value of up to 128 µg/ ml. Five strains were resistant to kanamycin with MIC values higher than 32 µg/ml, while the other strains were susceptible with MIC values ranging from 2 to 4 µg/ml. Zhou *et al*. (2012) showed that all the tested *Lb*. *bulgaricus* strains were resistant to kanamycin, contrasting to our observation. The susceptibilities of the tested strains to trimethoprim, streptomycin, and ciprofloxacin widely varied. Five percent of currently tested *Lb. bulgaricus* strains were resistant to ampicillin. Penicillin and ampicillin are cell wall synthesis inhibitors of LAB, which may explain the high rate of *Lb. bulgaricus* susceptible to these antibiotics (Danielsen & Wind 2003).

Antibiotic resistance genes were detected by PCR (Table 6). None of the target antibiotic resistance genes was detected in 10 of the tested strains. The *rpoB* gene was detected in 7 of the tested strains. The vancomycin resistance gene, *vanX*, was detected in 2 strains, IMAU32368 and IMAU62091. The *ermB* gene was detected in 4 tested strains, IMAU20450, IMAU20290, IMAU20289 and IMAU95110. The streptomycin resistance gene, *aadA*, was detected in IMAU62091 and IMAU62161. Four antibiotic resistance genes were detected in the strain IMAU62091. Moreover, the *cat* and *bla* genes were uniquely present in IMAU62091, but *cat* gene was not detected in lactobacilli isolated from fermented sausages (Hummel *et al*. 2007). Four of the currently tested strains were found to carry the erythromycin resistance gene *ermB*, although these strains were not erythromycin resistant. The discrepancy between the bacterial genotype and phenotype is indicative of the presence of other unidentified resistant genes or mechanisms e.g. multi-drug efflux pump or gene mutation at the target gene (Lubelski *et al*. 2007). Such discrepancy is not unique to lactobacilli, the species *Lactococcus lactis* was resistant to 6 antibiotics (ampicillin, chloramphenicol, erythromycin, streptomycin, tetracycline, and vancomycin) (Toomey *et al*. 2010). Although the bacterium was resistant to streptomycin, the authors failed to detect any of the known streptomycin resistance genes (*strA*, *strB*, *aadA*, and *aadE*) by PCR.

The intrinsic resistance of lactobacilli to some antibiotics may be considered as an advantage when they are in adjunct use with antibiotics for treating gastrointestinal tract conditions (Charteris *et al*. 2001). The important concern is the risk of transfer of LAB-originated antibiotic resistance genes to other bacteria, especially at the gut environment where a complex microbial community resides. In this study, the 8 antibiotic resistant *Lb. bulgaricus* were conjugally mated with recipient recipients by filter mating (Table 7). No colony was found on the selective agar plate, suggesting there was no transconjugant after the mating. At least one study has observed

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Table 5. Minimum inhibitory concentration (MIC) and antibiotic resistance profiles for the tested strains

Strain number	GEN	KAN	STR	NEO	TET	CLI	AMP	VAN	QVI	LINE	CIP	ERY	CHL	TRI	RIF
IMAU32096	< 0.5S	16S	4S	2S	1S	< 0.032S	0.125S	0.5S	0.5S	1S	> 128R	0.064S	4S	16S	< 0.125S
IMAU32076	< 0.5S	< 2S	2S	< 0.5S	0.25S	< 0.032S	< 0.032S	< 0.25S	0.25S	0.25S	4S	< 0.016S	< 0.125S	< 0.125S	< 0.125S
IMAU32379	< 0.5S	4S	2S	< 0.5S	0.25S	< 0.032S	< 0.032S	< 0.25S	0.25S	0.25S	8R	0.032S	2S	16S	0.125S
IMAU32370	< 0.5S	< 2S	2S	< 0.5S	< 0.125S	< 0.032S	< 0.032S	< 0.25S	0.5S	0.25S	< 0.25S	< 0.016S	< 0.125S	16S	< 0.125S
IMAU32368	< 0.5S	4S	32R	1S	< 0.125S	< 0.032S	< 0.032S	< 0.25S	0.064S	0.125S	0.5S	< 0.016S	1S	16S	< 0.125S
IMAU32298	< 0.5S	< 2S	64R	< 0.5S	< 0.125S	< 0.032S	< 0.032S	< 0.25S	< 0.016S	< 0.032S	> 128R	0.032S	< 0.125S	32R	< 0.125S
IMAU20450	< 0.5S	< 2S	64R	< 0.5S	0.5S	< 0.032S	< 0.032S	< 0.25S	< 0.016S	0.25S	< 0.25S	< 0.016S	< 0.125S	16S	< 0.125S
IMAU20290	< 0.5S	4S	16S	1S	0.25S	< 0.032S	0.125S	0.5S	0.25S	0.25S	> 128R	0.032S	1S	16S	0.25S
IMAU20289	1S	32R	16S	2S	0.5S	< 0.032S	0.25S	0.5S	0.25S	0.5S	> 128R	0.064S	2S	32R	0.5S
IMAU20515	< 0.5S	32R	8S	1S	0.25S	< 0.032S	0.064S	< 0.25S	0.25S	0.125S	> 128R	0.032S	0.5S	16S	< 0.125S
IMAU20366	< 0.5S	8S	4S	2S	0.25S	< 0.032S	0.125S	< 0.25S	0.25S	0.25S	128R	0.032S	1S	16S	< 0.125S
IMAU20291	1S	32R	8S	4S	0.5S	< 0.032S	0.125S	< 0.25S	0.25S	0.25S	> 128R	0.032S	2S	16S	< 0.125S
IMAU20310	< 0.5S	< 2S	32R	< 0.5S	< 0.125S	< 0.032S	< 0.032S	< 0.25S	0.032S	< 0.032S	32R	0.032S	< 0.125S	16S	< 0.125S
IMAU20743	< 0.5S	< 2S	< 0.5S	< 0.5S	< 0.125S	< 0.032S	< 0.032S	0.5S	0.5S	0.25S	2S	< 0.016S	< 0.125S	< 0.125S	< 0.125S
IMAU62159	1S	32R	8S	4S	< 0.125S	< 0.032S	< 0.032S	0.5S	0.25S	0.5S	> 128R	< 0.016S	1S	8S	< 0.125S
IMAU62091	< 0.5S	16S	16S	1S	4S	0.25S	0.5S	> 128R	1S	2S	> 128R	0.5S	4S	64R	0.5S
IMAU62161	< 0.5S	32R	16S	2S	0.5S	< 0.032S	2R	> 128R	2S	1S	32R	0.25S	4S	64R	0.25S
IMAU95110	< 0.5S	< 2S	< 0.5S	< 0.5S	< 0.125S	< 0.032S	< 0.032S	< 0.25S	< 0.016S	< 0.032S	< 0.25S	< 0.016S	< 0.125S	16S	< 0.125S
IMAU40106	< 0.5S	< 2S	< 0.5S	< 0.5S	< 0.125S	< 0.032S	< 0.032S	< 0.25S	0.032S	< 0.032S	< 0.25S	< 0.016S	< 0.125S	16S	< 0.125S
IMAU80827	< 0.5S	< 2S	64R	< 0.5S	< 0.125S	< 0.032S	0.064S	< 0.25S	0.25S	0.5S	64R	< 0.016S	2S	16S	< 0.125S

S – susceptible; R – resistant; GEN – gentamicin; KAN – kanamycin; QVI/DAL – quinupristin/dalfopristin; LINE – linezolid; STR – streptomycin; NEO – neomycin; TET – tetracycline; CLI – clindamycin; AMP – ampicillin; VAN – vancomycin; ERY – erythromycin; CHL – chloramphenicol; CIP – ciprofloxacin; TRI – trimethoprim; RIF – rifampicin

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Table 6. Antibitotic resistance genes detected in *Lb. bulgaricus* isolates

Strains number	Resistance genes
IMAU32096	*rpoB*
IMAU32076	–
IMAU32379	–
IMAU32370	*rpoB*
IMAU32368	*vanX, rpoB*
IMAU32298	–
IMAU20450	*erm(B), rpoB*
IMAU20290	*erm(B), rpoB*
IMAU20289	*erm(B), rpoB*
IMAU20515	–
IMAU20366	–
IMAU20291	–
IMAU20310	–
IMAU20743	*rpoB*
IMAU62159	–
IMAU62091	*aadA, bla, vanX, cat*
IMAU62161	*aadA*
IMAU95110	*erm(B)*
IMAU40106	–
IMAU80827	–

Table 7. MIC of donor and recipient used in the filter mating

Donor	MIC (μg/ml)	Recipient
IMAU62161	kan^R (32)	IMAU80827
IMAU62159	kan^R (64)	IMAU80827
IMAU20310	str^R (32)	IMAU20291
IMAU32298	str^R (64)	IMAU20291
IMAU20291	cip^R (> 128)	IMAU32368
IMAU62091	tri^R (64)	IMAU62159
IMAU20289	tri^R (32)	IMAU62159
IMAU80827	cip^R (64)	IMAU32368

the transfer of antibiotic resistance gene from *Lactobacillus* spp. to other bacteria (Devirgiliis *et al.* 2013). Furthermore, Toomey *et al.* (2009) compared the transfer of *tetM* and *ermB* between LAB and other bacteria using *in vitro* (filter mating) and *in vivo* (rumen and alfalfa sprout models) techniques and observed a higher *in vitro* transfer rate. Our results support that there is a limited risk of antibiotic gene transfer between *Lb. bulgaricus* and other bacteria, and thus they are safe for food use.

CONCLUSIONS

We analysed 20 traditional fermented milk-originated *Lb. bulgaricus*. The tested strains show variable antibiotic resistance phenotype and genotype. By using a filter mating assay, we confirmed that the detected antibiotic resistance genes would not be transferred to the recipient bacteria under our assay condition. Our results suggest that the risk of antibiotic gene transfer between *Lb. bulgaricus* and other bacteria is low, and thus they are safe for food use from the perspective of spreading antibiotic resistance.

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