Antibiotic resistance of lactic acid bacteria isolated from Chinese yogurts

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ABSTRACT

The aim of this study was to evaluate the susceptibility of 43 strains of lactic acid bacteria, isolated from Chinese yogurts made in different geographical areas, to 11 antibiotics (ampicillin, penicillin G, roxithromycin, chloramphenicol, tetracycline, chlortetracycline, lincomycin, kanamycin, streptomycin, neomycin, and gentamycin). The 43 isolates (18 *Lactobacillus bulgaricus* and 25 *Streptococcus thermophilus*) were identified at species level and were typed by random amplified polymorphic DNA analysis. Thirty-five genotypically different strains were detected and their antimicrobial resistance to 11 antibiotics was determined using the agar dilution method. Widespread resistance to ampicillin, chloramphenicol, chlortetracycline, tetracyclines, lincomycin, streptomycin, neomycin, and gentamycin was found among the 35 strains tested. All of the *Strep. thermophilus* strains tested were susceptible to penicillin G and roxithromycin, whereas 23.5 and 64.7% of *Lb. bulgaricus* strains, respectively, were resistant. All of the *Strep. thermophilus* and *Lb. bulgaricus* strains were found to be resistant to kanamycin. The presence of the corresponding resistance genes in the resistant isolates was investigated through PCR, with the following genes detected: *tet*(M) in 1 *Lb. bulgaricus* and 2 *Strep. thermophilus* isolates, *ant*(6) in 2 *Lb. bulgaricus* and 2 *Strep. thermophilus* isolates, and *aph*(3')-IIIa in 5 *Lb. bulgaricus* and 2 *Strep. thermophilus* isolates. The main threat associated with these bacteria is that they may transfer resistance genes to pathogenic bacteria, which has been a major cause of concern to human and animal health. To our knowledge, the *aph*(3')-IIIa and *ant*(6) genes were found in *Lb. bulgaricus* and *Strep. thermophilus* for the first time. Further investigations are required to analyze whether the genes identified in *Lb. bulgaricus* and *Strep. thermophilus* isolates might be horizontally transferred to other species.

Key words: lactic acid bacteria, antibiotic resistance, yogurt

INTRODUCTION

Lactic acid bacteria (LAB) are common microorganisms in foods and also constitute the natural intestinal microbiota of humans and most animals. Lactic acid bacteria are involved in various kinds of fermentative processes, including lactic acid fermentation for yogurt production, in which *Lactobacillus bulgaricus* and *Streptococcus thermophilus* are commonly used (Adolfsson et al., 2004). During the fermentation, lactose is converted into lactic acid, which acts on milk protein to give yogurt its texture and its characteristic tang (Lourens-Hattingh and Viljoen, 2001).

The increased use of LAB in food production as probiotics over the last decades has raised safety issues, one of which is the nature of acquiring and distribution of antimicrobial resistance (Teuber et al., 1999; Ammor et al., 2007). Antimicrobial resistance is an increasing problem worldwide, with the effective treatment of bacterial infections being compromised. The extensive use of antibiotics in both human and animal treatment has created a selective pressure for acquisition of resistance phenotypes that can be transmitted via food. The evolution of antibiotic-resistant foodborne pathogens has been widely reported (Zhao et al., 2006; Rahimi et al., 2010; Gousia et al., 2011). Recently, several studies have investigated the role of commensal bacteria (such as LAB) as reservoirs of antibiotic resistance genes similar to those found among human pathogens (Salysers et al., 2004; Toomey et al., 2010). The main threat associated with these bacteria is that they could transfer resistance genes to pathogenic bacteria (Flórez et al., 2005).

The food chain is considered as one of the main routes of transmission of antibiotic-resistant bacteria between animal and human populations. In particular, fermented dairy products that are not heat treated before consumption provide a vehicle for antibiotic-resistant bacteria, with a direct link between the animal indigenous microflora and the human gastrointestinal tract (Mathur and Singh, 2005). The incidence of antibiotic-resistant *Lactobacillus plantarum*, *Lactobacillus paracasei*, *Lactobacillus reuteri*, and *Lactobacillus acidophilus* in food has been reported (Rojo-Bezares et al.,

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2006; D'Aimmo et al., 2007; Liu et al., 2009). However, only limited information about the occurrence of different resistance genes among *Lb. bulgaricus* and *Strep. thermophilus* from yogurts is available. The aim of this work was to study the occurrence of antimicrobial resistance and presence of selected resistance genes among *Lb. bulgaricus* and *Strep. thermophilus* isolated from yogurts made in different places in China.

MATERIALS AND METHODS

Isolation and Identification of LAB

Forty-three strains of LAB, including 18 strains of *Lb. bulgaricus* and 25 strains of *Strep. thermophilus*, were isolated from yogurts collected from dairy plants located in different places in China. *Lactobacillus bulgaricus* and *Strep. thermophilus* were used as starter cultures. Recovered strains derived from yogurts were isolated on de Man, Rogosa, and Sharpe (MRS) agar (Oxoid Ltd., Basingstoke, UK) supplemented with 0.75% CaCO₃. Isolates with dissolved calcium were initially identified by colonial appearance, Gram staining, cell morphology, catalase reaction, and yogurt coagulation test. The putative LAB identified were further confirmed by PCR-based 16S rDNA sequencing, using a pair of universal primers 27f (5'-AGAGTTTGTATCTGGCTCAG-3') and 1495r (5'-CTACGGCTACCTTGTACGA-3'; Yu et al., 2011).

Strain Typing

Genomic DNA was extracted from different isolates of overnight MRS broth cultures using the CTAB method described by Moore et al. (2004). Eight random primers (Table 1), P2, P15, P17, COC, Lp1, OPL-05, CRA23, and CRA25, were tested for their ability to discriminate between strains of LAB and for their reproducibility. Primer CRA23, which showed higher discriminatory ability, was selected for random ampli-

fied polymorphic DNA (RAPD) analysis. Polymerase chain reaction was conducted in a total volume of 25 µL, containing 10 mmol of Tris-HCl/L (pH 8.3), 50 mmol of KCl/L, 2.0 mmol of MgCl₂/L, 0.2 mmol/L of each deoxyribonucleotide triphosphate (dNTP), 2 µmol of primer/L, 1.25 U of Taq polymerase and 100 ng of extracted DNA. Amplification was carried out in a Peltier thermal cycler (PTC-200; MJ Research Inc., St. Bruno, QC, Canada) under the conditions listed in Table 1. The PCR products were separated by electrophoresis on a 1.5% (wt/vol) agarose gel in 0.5× Tris/borate/EDTA (TBE) buffer. Random amplified polymorphic DNA profiles were obtained directly using a digital camera (Bio-Rad Laboratories Inc., Hercules, CA) and analyzed with the pattern analysis software NTSYS-pc version 2.1e (Exeter Software, Setauket, NY). Calculation of similarity of the PCR fingerprinting profiles was based on the Pearson product-moment correlation coefficient. A dendrogram was deduced from the matrix of similarities by the unweighted pair group method using arithmetic mean (UPGMA). Reproducibility was tested by repeating the PCR amplification at least 3 times under the same amplification conditions.

Antibiotic Susceptibility Testing

The phenotypic antimicrobial resistance of a strain to each antibiotic tested was determined as the MIC, defined as the lowest antibiotic concentration that will inhibit the visible growth of a microorganism after overnight incubation (Andrews, 2001). The MIC for 11 antibiotics (ampicillin, penicillin G, roxithromycin, chloramphenicol, chlortetracycline, tetracycline, lincomycin, kanamycin, streptomycin, neomycin, and gentamycin) was determined by the agar dilution method on MRS agar according to a modification of the Clinical and Laboratory Standards Institute (CLSI) standards (CLSI, 2009). The assay was performed in duplicate and repeated on 3 independent occasions. As no definitive breakpoint list has been established for lactic acid bac-

Table 1. Random primers and PCR conditions used in this study

Random primer	Sequence (5'-3')	Annealing temperature (°C)	Reference ¹
P2	GATCGGACGG	36	Samaržija et al. (2002)
P15	CTGGGCACGA	36	Samaržija et al. (2002)
P17	CAGACAAGCC	35	Samaržija et al. (2002)
Lp1	ACGCGCCCT	33	Johansson et al. (1995)
COC	AGCAGCGTGG	36	Cocconcelli et al. (1995)
OPL-05	ACGCAGGCA	36	Van Reenen and Dicks (1996)
CRA23	GCGATCCCCA	36	Daud Khaled et al. (1997)
CRA25	AACGCGCAAC	36	Daud Khaled et al. (1997)

¹References for random primer sequence only.

Table 2. Microbiological breakpoints for *Lactobacillus bulgaricus* and *Streptococcus thermophilus* used in food or as probiotics

Antibiotic	Species	Proposed breakpoint, MIC ($\mu\text{g/mL}$)		
		This paper	EFSA ¹	Other articles
Ampicillin	<i>Lb. bulgaricus</i>	1	1	2, ² 8, ³ 4 ⁴
	<i>Strep. thermophilus</i>	2	2	8 ³
Penicillin G	<i>Lb. bulgaricus</i>	1		4 ^{3,4}
	<i>Strep. thermophilus</i>	1		4 ³
Roxithromycin	<i>Lb. bulgaricus</i>	1		
	<i>Strep. thermophilus</i>	2		
Chloramphenicol	<i>Lb. bulgaricus</i>	4	4	16 ^{2,3,4}
	<i>Strep. thermophilus</i>	4	4	16 ²
Chlortetracycline	<i>Lb. bulgaricus</i>	1		
	<i>Strep. thermophilus</i>	1		
Tetracycline	<i>Lb. bulgaricus</i>	4	4	16, ² 8, ³ 4 ⁴
	<i>Strep. thermophilus</i>	4	4	8 ²
Lincomycin	<i>Lb. bulgaricus</i>	4		
	<i>Strep. thermophilus</i>	4		
Kanamycin	<i>Lb. bulgaricus</i>	16	16	32, ² >256 ³
	<i>Strep. thermophilus</i>	64	64	
Streptomycin	<i>Lb. bulgaricus</i>	16	16	16, ^{2,3} >256 ⁴
	<i>Strep. thermophilus</i>	64	64	16 ³
Neomycin	<i>Lb. bulgaricus</i>	32		32 ¹
	<i>Strep. thermophilus</i>	32		
Gentamycin	<i>Lb. bulgaricus</i>	16	16	1, ² 16 ³
	<i>Strep. thermophilus</i>	32	32	16 ³

¹Breakpoints defined by the European Food Safety Authority (EFSA, 2008) for *Lb. bulgaricus* and *Strep. thermophilus*.

²Breakpoints defined by the European Commission (EC, 2001) for *Lactobacillus* spp.

³Breakpoints suggested by Katla et al. (2001) for *Lactobacillus* spp. and *Strep. thermophilus*.

⁴Breakpoints suggested by Danielsen and Wind (2003) for *Lactobacillus* spp.

teria, susceptibility to the antibiotics was determined by comparing MIC values to the proposed breakpoints from previous studies. Some breakpoints that were not published anywhere before, such as breakpoints for roxithromycin and chlortetracycline, were defined according to the breakpoints for antibiotics in the same type, as presented in Table 2. Strains with MIC equal to or higher than the breakpoints are considered as resistant (EC, 2001).

Genotypic Detection of Antimicrobial Resistance Genes

Determination of the genetic background to phenotypic resistance was achieved by applying PCR with specific primers for known antibiotic resistance genes. Genes responsible for resistance to tetracycline [*tet*(K), *tet*(L), *tet*(M), *tet*(O), *tet*(S), and *tet*(W)], streptomycin [*str*(A), *str*(B), and *ant*(6)], chloramphenicol [*cat*(A)], gentamycin [*aac*(6')-*aph*(2'')], and kanamycin [*aph*(3')-IIIa] were amplified by PCR using primers listed in Table 3, according to amplification conditions described by the references. Polymerase chain reaction products (7 μL) were separated by 1.5% (wt/vol) agarose gel

electrophoresis in 0.5 $\times$ TBE buffer and visualized by ethidium bromide (10 mg/mL) staining.

RESULTS

Biodiversity of LAB Isolates

A notable genotypic heterogeneity among the 43 LAB isolates was observed by RAPD fingerprinting (Figure 1). The majority of fingerprinting profiles represented unique genotypes with rather low correlation values (49%), resulting in a high degree of biodiversity. The profiles of some isolates, however, were found to cluster together in the same branch of the dendrogram. Overall, a total of 35 genotypically different strains were detected at the level of reproducibility (92% similarity; data not shown), and were then subjected to further analysis.

Antibiotic Susceptibility Testing

The agar dilution method was used to assess the susceptibility of the 35 LAB strains isolated from yogurts to 11 antibiotics, ampicillin, penicillin G, roxithromy-

Table 3. Primers for selected antibiotic resistance genes and PCR conditions used in this study

Target gene	Primer ¹	Sequence (5'-3')	Annealing temperature (°C)	Size (bp)	Reference ²
<i>tet</i> (M)	tetM-F	GTGGACAAAGGTACAACGAG	53	406	Ng et al. (2001)
	tetM-R	CGGTAAAGTTTCGTACACAC			
<i>tet</i> (S)	tetS-F	ATCAAGATATTAAGGAC	55	573	Gevers et al. (2003)
	tetS-R	TTCTCTATGTGGTAATC			
<i>tet</i> (W)	tetW-F	GAGAGCCTGCTATATGCCAGC	64	168	Aminov et al. (2001)
	tetW-R	GGGCGTATCCACAATGTTAAC			
<i>tet</i> (K)	tetK-F	TTAGGTGAAGGGTTAGGTCC	55	697	Aarestrup et al. (2000)
	tetK-R	GCAAACCTCATTCCAGAAGCA			
<i>tet</i> (L)	tetL-F	CATTTGGTCTTATTGGATCG	50	456	Aarestrup et al. (2000)
	tetL-R	ATTACACTCCGATTTCGG			
<i>tet</i> (O)	tetO-F	AACTTAGGCATTCTGGCTCAC	55	515	Ng et al. (2001)
	tetO-R	TCCCACGTGTTCCATATCGTCA			
<i>str</i> (A)	strA-F	CTTGGTGATAACGGCAATTC	55	548	Ouoba et al. (2008)
	strA-R	CCAATCGCAGATAGAAGGC			
<i>str</i> (B)	strB-F	ATCGTCAAGGGATTGAAACC	56	509	Ouoba et al. (2008)
	strB-R	GGATCGTAGAACATATTGGC			
<i>ant</i> (6)	ant6-F	ACTGGCTTAATCAATTTGGG	53	597	Rojo-Bezares et al. (2006)
	ant6-R	GCCTTTCCGCCACCTCACCG			
<i>cat</i> (A)	catA-F	GGATATGAAATTTATCCCTC	50	486	Aarestrup et al. (2000)
	catA-R	GGATATGAAATTTATCCCTC			
<i>aac</i> (6')-aph(2'')	aac-F	CCAAGAGCAATAAGGGCATACC	58	675	Kobayashi et al. (2001)
	aac-R	ACCCTCAAAAACGTGTTGTGC			
<i>aph</i> (3')-IIIa	aph3'-F	GCCGATGTGGATTGCGAAAA	55	292	Rojo-Bezares et al. (2006)
	aph3'-R	GCTTGATCCCCAGTAAGTCA			

¹F = forward; R = reverse.²References for primer sequence and size only.

cin, chloramphenicol, chlortetracycline, tetracycline, lincomycin, kanamycin, streptomycin, neomycin, and gentamycin. The MIC distributions of the tested strains are presented in Table 4. The strains were characterized as either resistant or sensitive to a specific antibiotic according to the proposed breakpoints listed in Table 2. Irrespective of the medium of isolation, all of the *Strep. thermophilus* isolates were found to be susceptible to penicillin G and roxithromycin, whereas 23.5 and 64.7% of *Lb. bulgaricus* isolates, respectively, were resistant. Moderate levels of resistance to ampicillin and chlortetracycline were observed among *Lb. bulgaricus* (29.4 and 47.1%, respectively) and *Strep. thermophilus* (44.4 and 27.8%, respectively). Resistance to tetracycline varied significantly between the 2 species, as 88.2% for *Lb. bulgaricus* and 44.4% for *Strep. thermophilus*.

High-level resistance to chloramphenicol and lincomycin was observed for *Lb. bulgaricus* (88.2 and 94.1%, respectively) and for *Strep. thermophilus* (88.9 and 88.9%, respectively). Both *Lb. bulgaricus* and *Strep. thermophilus* strains tend to be strongly resistant to aminoglycosides (kanamycin, streptomycin, neomycin, and gentamycin). In fact, only 5.9% of *Lb. bulgaricus* isolates showed susceptibility to streptomycin, neomycin, or gentamycin and 33.3 and 11.1% of *Strep. thermophilus* isolates were susceptible to streptomycin and gentamycin, respectively.

Resistance Phenotypes Analysis

The isolates tested were found to exhibit combined resistance among the 11 antibiotics (ampicillin, penicillin G, roxithromycin, chloramphenicol, chlortetracycline, tetracycline, lincomycin, kanamycin, streptomycin, neomycin, and gentamycin). Most isolates (91.4%) showed resistance to 5 to 10 of the antibiotics, whereas certain isolates (51.4%) were resistant to 8 or more antibiotics.

Detection of Antibiotic Resistance Genes

All of the antibiotic-resistant strains were tested for the presence of antibiotic resistance genes most frequently occurring in LAB by PCR amplification. Table 5 presents the tested strains in which antibiotic resistance genes have been detected, as well as the MIC of the antibiotics affected by the products of these resistance genes.

The *tet*(M) gene was detected in 1 (6.67%) of the 15 tetracycline-resistant *Lb. bulgaricus* isolates and in 2 (25%) of the 8 tetracycline-resistant *Strep. thermophilus* isolates. The incidence of *ant*(6) was 12.5% among the streptomycin-resistant *Lb. bulgaricus* isolates, whereas the incidence was 16.7% among the streptomycin-resistant *Strep. thermophilus* isolates. Five (29.4%) of the 17 kanamycin-resistant *Lb. bulgaricus* isolates and

Figure 1. Unweighted pair group method using arithmetic mean (UPGMA)-based dendrogram from the random amplified polymorphic DNA (RAPD) profiles generated with primer CRA23 of 18 *Lactobacillus bulgaricus* (a) and 25 *Streptococcus thermophilus* (b) isolates collected from Chinese yogurts.

2 (11.1%) of the 18 kanamycin-resistant *Strep. thermophilus* isolates were found to contain the *aph(3')-IIIa* gene.

DISCUSSION

Dissemination of antibiotic resistance through the food chain is a serious public health issue, and resistance in fermented dairy products is a component of this paradigm. *Lactobacillus bulgaricus* as well as *Strep. thermophilus*, generally regarded as safe (GRAS), are the main starter cultures for the production of yogurts. Although their probiotic functions have rendered them common food additives (Irkin and Eren, 2008), recent reports have pointed out a possible pathogenic relevance of several LAB species, including *Lb. bulgaricus* and *Strep. thermophilus* (D'Aimmo et al., 2007; Klare et al., 2007; Liu et al., 2009). Up to the present, only a few studies have focused on the identification of antibiotic resistance determinants in these species (Liu et al., 2009), although their widespread presence in fermented foods, as well as the strong selection of antibiotic resistance due to extensive use of antibiotics (Teuber, 2001; Wegener, 2003), make them potential vehicles for the spread of antibiotic resistance genes to human pathogens (Teuber and Perreten, 2000).

To study intraspecies diversity of *Lb. bulgaricus* and *Strep. Thermophilus* isolated from yogurts, all 43 strains were subjected to RAPD analysis with primer CRA23. As a result, a total of 35 genotypically different strains were selected for further analysis. The reproducibility of the RAPD assay and running conditions estimated by analysis of repeated DNA extracts of several type strains was 92% (data not shown). Random amplified polymorphic DNA fingerprinting is one of the most commonly used typing techniques, and is widely used for typing dairy LAB (Andrighetto et al., 2002; de Candia et al., 2007). It has been widely reported as a rapid, sensitive, and inexpensive method for genetic typing of different strains of LAB, although the reproducibility and discriminatory power of the RAPD fingerprints is lower than that of pulsed-field gel electrophoresis (PFGE) and can be influenced by several factors, such as annealing temperature, DNA template purity and concentration, and primer combinations (Amor et al., 2007). However, high reproducibility and discriminatory power can be obtained with optimized protocols.

At present, widespread susceptibility toward the inhibitors of cell wall synthesis (such as penicillin and ampicillin) has been observed in many species of LAB from different sources, including cheese (Belletti et al., 2009), probiotics or fermented foods (Charteris et al., 1998), and human intestine (Delgado et al., 2005). Most of the *Lb. bulgaricus* isolates tested in the current study

Table 4. Minimum inhibitory concentration distributions for 11 antibiotics of *Lactobacillus bulgaricus* and *Streptococcus thermophilus* isolates from yogurts in the Chinese market

Antibiotic ¹	Species (no. of strains tested)	Number of strains with MIC (μg/mL) as follows:													Resistance (%)
		≤0.125	0.25	0.5	1	2	4	8	16	32	64	128	256	≥512	
AMP	<i>Lb. bulgaricus</i> (17)	3	2	7	3	2									29.4
	<i>Strep. thermophilus</i> (18)	4		3	3	7		1							44.4
PEN	<i>Lb. bulgaricus</i> (17)	5	4	4	2	2									23.5
	<i>Strep. thermophilus</i> (18)	2	6	10											0
ROX	<i>Lb. bulgaricus</i> (17)		2	4	11										64.7
	<i>Strep. thermophilus</i> (18)		1	1	16										0
CHL	<i>Lb. bulgaricus</i> (17)					2	10	4	1						88.2
	<i>Strep. thermophilus</i> (18)					2	9	4	1	2					88.9
CHT	<i>Lb. bulgaricus</i> (17)	4	1	4	6	1	1								47.1
	<i>Strep. thermophilus</i> (18)	5	8		5										27.8
TET	<i>Lb. bulgaricus</i> (17)					2	6	8	1						88.2
	<i>Strep. thermophilus</i> (18)				2	8	2	2	4						44.4
LIN	<i>Lb. bulgaricus</i> (17)	1					6	5	5						94.1
	<i>Strep. thermophilus</i> (18)				1	1	7	6	2					1	88.9
KAN	<i>Lb. bulgaricus</i> (17)											3	2	12	100
	<i>Strep. thermophilus</i> (18)											3	4	11	100
STR	<i>Lb. bulgaricus</i> (17)							1		1	5	1	3	6	94.1
	<i>Strep. thermophilus</i> (18)									6	5	4	2	1	66.7
NEO	<i>Lb. bulgaricus</i> (17)							1		2	1	10	3		94.1
	<i>Strep. thermophilus</i> (18)									1	3	5	9		100
GEN	<i>Lb. bulgaricus</i> (17)						1		3	2	5	6			94.1
	<i>Strep. thermophilus</i> (18)								2	7	4	3	2		88.9

¹AMP = ampicillin; PEN = penicillin; ROX = roxithromycin; CHL = chloramphenicol; CHT = chlortetracycline; TET = tetracycline; LIN = lincomycin; KAN = kanamycin; STR = streptomycin; NEO = neomycin; GEN = gentamicin.

were found to be sensitive to penicillin and ampicillin. Resistance to roxithromycin and chlortetracycline, although seldom reported, was also found in the current study. Nawaz et al. (2011) found a high portion of the *Lb. bulgaricus* strains susceptible to chloramphenicol, tetracycline, and lincomycin. This is in contrast to our observations that the *Lb. bulgaricus* strains had MIC quite a bit higher than the breakpoint values, and this difference may due to different media used in the MIC testing and also may because isolates tested are from different origins.

A high level of resistance to aminoglycosides was found in almost all of the investigated strains (Table 4). A tendency toward lower MIC was observed for gentamicin, in comparison with kanamycin, streptomycin, and neomycin. Unlike gentamicin, which showed a unimodal MIC distribution at the low-end concentration range, the other aminoglycoside antibiotics (streptomycin, kanamycin, and neomycin) displayed broad MIC distributions at the high-end concentration range. Generally, the reduced susceptibility of lactobacilli to aminoglycosides is thought to occur because of mem-

Table 5. Lactic acid bacteria strains in which antibiotic resistance genes and MIC of specific antibiotics have been detected

Species	Strain	Antibiotic (MIC in μg/mL)	Resistance gene detected
<i>Lactobacillus bulgaricus</i>	B-4	Tetracycline (16)	<i>tet(M)</i>
		Streptomycin (64)	<i>ant(6)</i>
	B-9	Streptomycin (512)	<i>ant(6)</i>
	B-3	Kanamycin (512)	<i>aph(3')-IIIa</i>
	B-10	Kanamycin (≥512)	<i>aph(3')-IIIa</i>
	B-11	Kanamycin (512)	<i>aph(3')-IIIa</i>
	B-15	Kanamycin (512)	<i>aph(3')-IIIa</i>
<i>Streptococcus thermophilus</i>	B-13	Kanamycin (256)	<i>aph(3')-IIIa</i>
	S-1	Tetracycline (16)	<i>tet(M)</i>
	S-3	Tetracycline (8)	<i>tet(M)</i>
	S-4	Streptomycin (256)	<i>ant(6)</i>
	S-10	Streptomycin (256)	<i>ant(6)</i>
	S-7	Kanamycin (512)	<i>aph(3')-IIIa</i>
	S-18	Kanamycin (512)	<i>aph(3')-IIIa</i>

brane impermeability (Elkins and Mullis, 2004). Due to the better ability of gentamicin to cross the membrane, a tendency toward lower MIC for gentamicin is known (Danielsen and Wind, 2003; Egervärn et al., 2007). Additionally, interference by low test medium pH (MRS agar, 6.2 ± 0.2) with aminoglycosides (the optimum pH 7.8) might be attributed to reducing their antibacterial activity (Amsterdam, 2005).

An association between the occurrence of resistance to the different antimicrobial agents tested was found. Among *Lb. bulgaricus* isolates, the combined resistance to chloramphenicol, tetracycline, lincomycin, kanamycin, streptomycin, neomycin, and gentamycin was commonly found. However, *Strep. thermophilus* isolates were much more frequently observed with combined resistance to chloramphenicol, lincomycin, kanamycin, neomycin, and gentamycin, without tetracycline and streptomycin. The significant multidrug resistance reinforces the antimicrobial susceptibility testing in the safety assessment procedure of strains intended for probiotic or nutritional use.

Detection of antibiotic resistance genes showed that the *tet(M)* gene was detected in 1 *Lb. bulgaricus* strain and 2 *Strep. thermophilus* strains. The 3 *tet(M)*-possessing strains presented higher MIC values of tetracycline than other LAB strains tested in this study. The *tet(M)* gene encodes a soluble protein to protect the ribosome from the effects of tetracycline, thus conferring tetracycline resistance. These 3 *tet(M)*-owning strains represent examples of acquired resistance in LAB.

The *aph(3')-IIIa* gene, exhibiting high-level resistance to kanamycin, was detected in 5 *Lb. bulgaricus* strains and 2 *Strep. thermophilus* strains in this study. The kanamycin MIC values for the 7 *aph(3')-IIIa*-containing strains were 256 $\mu\text{g/mL}$ or $\geq 512 \mu\text{g/mL}$, similar to the values for most of the other strains tested in this study. The *ant(6)* gene, associated with streptomycin resistance in enterococci (del Campo et al., 2000), was demonstrated in 2 *Lb. bulgaricus* strains and 2 *Strep. thermophilus* strains, which showed streptomycin MIC in the range of 64 to 512 $\mu\text{g/mL}$. The presence of *aph(3')-IIIa* or *ant(6)* genes seems not to be correlated with higher kanamycin or streptomycin MIC values. To our knowledge, the *aph(3')-IIIa* and *ant(6)* genes were detected in strains of *Lb. bulgaricus* and *Strep. thermophilus* for the first time.

Other resistance genes [*tet(K)*, *tet(L)*, *tet(O)*, *tet(S)*, *tet(W)*, *str(A)*, *str(B)*, *cat(A)*, and *aac(6')-aph(2'')*] were not found in any of the resistant strains. This is probably due to, first, these strains possibly being intrinsically resistant to the antibiotics tested. Some resistant strains could inactivate the antibiotic by destroying or modifying the drug itself so that it is no longer toxic. Some others may pump the drug out of

the bacterial cell so that the concentration of the drug is too low to be effective. Second, the emergence of resistance in these organisms may have arrived through evolutionary events, such as mutations. Some resistant species may have an altered form of the target site of the drug (the place on the cell where the drug binds), so the antibiotic cannot find its target. Third, these resistance genes were located on the chromosome but were not incomplete to be detected. Other mechanisms of antibiotic resistance should also be considered; the resistance to some antibiotics may be mediated by transposon or plasmid-carried genes.

Lactic acid bacteria strains have acquired the generally regarded as safe status and are used as fermenting lactic starter cultures in the food industry. When they are used as probiotics and enter into our intestines in large numbers, interaction with the natural intestinal microbiota and gene transfer may occur (Rojo-Bezares et al., 2006). Because LAB can be prone to gene exchange to enhance survival in antibiotic-containing environments (Toomey et al., 2009a,b, 2010), further studies are required to analyze in detail whether the genes identified in our study might be horizontally transferred to other species. In the meantime, a series of measures inspired to a principle of precaution should be undertaken, the most important of which is to systematically test strains for the presence of transferable antibiotic resistance genes before they are used as commercial starters or probiotic cultures in food products. Also, this measure should be complemented by a more prudent use of antibiotics in all fields, including agriculture, veterinary, and human medicine.

CONCLUSIONS

A variety of antibiotic resistant LAB strains was recovered from Chinese yogurt isolates. Potentially transferable resistance determinants such as *tet(M)*, *ant(6)*, and *aph(3')-IIIa*, were found in these isolates, fuelling the debate about the use of these bacteria in food and their potential to spread resistance through the food chain, especially in cases where the transfer of resistance has been demonstrated. Future studies should be focused on horizontal gene transfer of detected resistance genes.

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