Antibiotic Resistance of Probiotic Strains of Lactic Acid Bacteria Isolated from Marketed Foods and Drugs

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Objective To identify the antimicrobial resistance of commercial lactic acid bacteria present in microbial foods and drug additives by analyzing their isolated strains used for fermentation and probiotics. **Methods** Antimicrobial susceptibility of 41 screened isolates was tested with disc diffusion and E-test methods after species-level identification. Resistant strains were selected and examined for the presence of resistance genes by PCR. **Results** Distribution of resistance was found in different species. All isolates were susceptible to chloramphenicol, tetracycline, ampicillin, amoxicillin/clavulanic acid, cephalothin, and imipenem. In addition, isolates resistant to vancomycin, rifampicin, streptomycin, bacitracin, and erythromycin were detected, although the incidence of resistance to these antibiotics was relatively low. In contrast, most strains were resistant to ciprofloxacin, amikacin, trimethoprim/sulphamethoxazole, and gentamycin. The genes msrC, vanX, and dfrA were detected in strains of Enterococcus faecium, Lactobacillus plantarum, Streptococcus thermophilus, and Lactococcus lactis. **Conclusion** Antibiotic resistance is present in different species of probiotic strains, which poses a threat to food safety. Evaluation of the safety of lactic acid bacteria for human consumption should be guided by established criteria, guidelines and regulations.

Key words: Disc diffusion; E-test; MICs; vanX; msrC; dfrA

INTRODUCTION

Lactic acid bacteria (LAB) form a taxonomically diverse group of microorganisms that can convert fermentable carbohydrates into lactic acids[1]. A large number of LAB species are involved in the production and consumption of fermented foods and beverages. Most LAB are omnipresent members of the intestinal flora. Bacteria in the human intestine play an important role in human physiology, most of which are beneficial or neutral for the host.

Antibiotic resistance can occur in two ways in a bacterial population: mutation of an endogenous gene or acquisition of a resistance gene from an exogenous source. Mutations, which may cause genetic changes in multiple regions of the genome, play only a minor role in the development of resistance[2-3]. Horizontal transfer enhances the evolution of antibiotic resistance in microbial communities by moving resistance genes across species and genus borders through conjugative plasmids, transposons, integrons, insertional elements, lytic and temperate bacteriophages[4]. Thus, intestinal bacteria can acquire resistance either by mutation or by horizontal transfer of resistance genes from another intestinal species or any species that passes through the colon.

Although the use of LAB has a long and safe history and has acquired the 'generally regarded as safe' (GRAS) status, the safety of selected strains should be evaluated before use, not only for virulence factors and other potential disease-causing traits, but also for their capability of acquiring and disseminating resistance determinants. The transfer of antibiotic resistance genes from LAB reservoir strains to bacteria in the resident microflora of human gastrointestinal tract and hence to pathogenic bacteria, has not been fully addressed. LAB are not generally targeted by antibiotic treatments as they are considered to be non-pathogenic and non-opportunistic pathogens. Several reports are available on the susceptibility of LAB to antibiotics of diverse origins[5-7]. In contrast, Only a few reports can be found on isolates from food and intestinal samples carrying antibiotic resistance determinants,

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either on chromosome or on plasmids[6, 8-12]. Nonetheless, conjugative transfer of resistance plasmids to LAB from Enterococcal and Staphylococcal species has been achieved[12-14], indicating that marketed strains may have the ability to transmit resistance.

The aim of present studies aims was to evaluate the antibiotic resistance of LAB strains present in Chinese markets and analyze their phenotype and genotype, in an attempt to contribute to the biosafety surveillance of LAB for human consumption.

MATERIALS AND METHODS

Isolation of Bacterial Strains

A total of 41 strains of lactic acid bacteria were evaluated in this study, of which 36 were isolated from commercial dairy and pharmaceutical products and 5 were from probiotic products obtained from the Cobtt Company (Shanghai, China).

Samples were diluted at 1:10 and plated onto a non-selective solid Gifu anaerobic medium (Nissui). The plates were incubated under anaerobic conditions for 48 h at 37 ℃. Distinct colonies per sample were morphologically selected and categorized as rods or cocci under a light microscope. Pure colonies were isolated after they were plated on appropriate agar plates. *Lactobacilli* were selected under anaerobic conditions on MRS agar plates containing MRS broth with Tween 80 and 1.5% agar. *Lactococci and Enterococci* were inoculated on M17 agar (Difco). MRS supplemented with 0.05% (w/v) cysteine hydrochloride was used for *Bifidobacteria*. Cultures were incubated for 48 h at 37 ℃. Pure cultures were obtained after subcultivation in MRS broth (Difco), M17 broth (Difco), or MRS broth supplemented with 0.05% (w/v) cysteine hydrochloride, respectively. Liquid cultures grown for 48 h were stored in 30% glycerol at -80 ℃ and vacuum freeze-drying method was also applied.

Staphylococcus aureus ATCC 25923 and *Escherichia coli* ATCC 25922 were used as reference strains.

Identification Bacteria at Genes Level

All isolated strains were initially identified with the classical microbiological methods of colony appearance, Gram stain, oxidase and catalase reactions.

Genomic DNA used for the PCR template was extracted with a bacteria DNA mini kit (Watson). PCR amplification and subsequent sequencing of 16S rDNA were performed for the genus level identification Universal primers 27F and 1492R[15] were used. The amplified products were then separated by electrophoresis on a 1.5% (w/v) agarose gel and purified using a QIAquick gel extraction kit (QIAGEN) before sequencing. The purified products were sequenced with an ABI DNA sequencer 3730. Alignment with known 16S rDNA sequences in the NCBI database (http://www.ncbi.nlm.nih.gov/BLAST) was done with the basic local alignment search tool, an online software.

Identification of Bacteria at Species Level

Enterococci and Lactobacilli were identified at species level using the API 20 strep system and the API 50 CH system (bioMerieux), respectively. API tests were performed following the manufacturer's instructions.

Since the API 50 CH system cannot distinguish *Lactobacillus casei* from *Lactobacillus paracasei*, specific primers W1 and Y2 were used to discriminate these two species as previously described[16].

Species-specific PCR analyses were also used for the identification of *Bifidobacterium longum* and *Bifidobacterium animalis* species[17]. Ten pairs of primers targeting nine species were employed to identify the Bifidobacteria strains[18-19].

PCR of specific genes, the glycogen phosphorylase *glgp* gene and the alpha amylase *amyl* gene, was done to identify *Streptococcus thermophilus* and *Lactococcus lactis* species, respectively. PCR profiles are listed in Table 1.

Screening for Antibiotic Resistant Phenotypes

Disc diffusion method was used to sreen for the antibiotic susceptibility of isolates with 16 discs (BD) containing ampicillin (AM 10 μg), amoxicillin/clavulanic acid (AMC 30 μg), cephalothin (CF 30 μg), cefotaxime (CTX 30 μg), imipenem (IPM 10 μg), erythromycin (E 15 μg), vancomycin (VA 30 μg), chloramphenicol (C 30 μg), rifampin (RA 5 μg), tetracycline (TE 30 μg), amikacin (AK 30 μg), gentamycin (GM 10 μg), streptomycin (S 300 μg), ciprofloxacin (CIP 5 μg), bacitracin (B 10 μg), and trimethoprim/sulphamethoxazole (SXT). Tests were done according to the criteria of the National Committee of Clinical Laboratory Standards (NCCLS) with M17 agar, MRS agar, and MRS agar supplemented with 0.05% (w/v) cysteine hydrochloride for *Lactococci and Streptococci*, *Lactobacilli*, and *Bifidobacteri*, respectively. Inhibition-zone diameters were measured after anaerobic incubation at 37 ℃ for 24 h, as previously described[20] and used as an indication for the

borderline between susceptible and resistant isolates. Resistant strains were selected after compared with known standard. Minimum inhibitory concentration (MIC) of antimicrobial agents in the resistant strains was measured by E-test (AB Biodisk, Solna, Sweden). The culture conditions were identical to those in disc diffusion. Each experiment was performed in triplicate.

Verification of the Presence of Antibiotic Resistant Genes

Genomic DNA of the strains exhibiting phenotypic resistance was extracted for the detection of genes coding for antibiotic resistance. Primers were designed to amplify 57 resistance determinants. Primers and PCR conditions are listed in Table 1. All positive amplicons were purified and sequenced. The obtained sequences were compared with those in GenBank.

RESULTS

Isolation and Identification of Bacterial Strains

Forty-one strains were identified as *Streptococcus thermophilus*, *Lactobacillus acidophilus, Lactobacillus casei*, *Lactobacillus delbrueckii spp. bulgaricus*, *Lactobacillus plantarum*, *Lactobacillus rhamnosus*, *Lactococcus lactis*, *Enterococcus faecalis*, *Enterococcus faecium*, *Bifidobacterium animalis*, and *Bifidobacterium longum* (Table 2). The identified strains were not always indicated on the product label. For example, in *Lb. plantarum* and *E. faecalis* were isolated from two pharmaceutical products, but the product label declared the presence of *Lb. acidophilus* and *Lb. delbrueckii spp. Bulgaricus* instead.

Antibiotic Susceptibility

Antimicrobial disc-diffusion susceptibility of the 41 strains of lactic acid bacteria is summarized in Table 3. A total of 35 strains were resistant to antibiotic agents, some of which were resistant to multiple drugs. The MIC of 9 antimicrobial agents was measured in 35 strains to confirm the results of disc diffusion (Table 4). The breakpoints were calculated as previously described[1, 8, 21-22].

All isolates were susceptible to chloramphenicol, tetracycline, erythromycin, and β-lactams except for one strain of *E. faecium* and one strain of *E. faecalis* were resistant to erythromycin (MIC 24 mg/L) and cefotaxime (MIC 64 mg/L), respectively. Strains of *Lactobacilli* (*n*=7) and *Enterococci* (*n*=1) were resistant to vancomycin. Rifampicin-resistant strains were detected in all *Lactococci* isolates and in one strain of *Enterococci*. Strains with a high resistance level to streptomycin (*n*=2) and bacitracin (*n*=6) were also observed. In contrast, most strains were resistant to ciprofloxacin (*n*=14), amikacin (*n*=19), trimethoprim/ sulphamethoxazole (*n*=24), and gentamycin (*n*=16).

TABLE 1

Oligonucleotide Primers for PCR Identification of Bacteria Strains and Detection of Antibiotic Resistant Genes

Gene	Primer Pair	Sequence (5'-3')	PCR Conditions	PCR Fragments	Reference
16S rDNA	27F	AGAGTTTGATCCTGGCTCAG	95 ℃ for 30 s, 55 ℃ 45 s, 72 ℃ 2 min; 30×	1500 bp	[15]
	1492R	GGTTACCTTGTTACGACTT			
B. adolescentis partial 16S rDNA	BiADO-1	CTCCAGTTGGATGCATGTC	95 ℃ for 20 s, 55 ℃ 20 s, 72 ℃ 30 s; 30×	279 bp	[17]
	BiADO-2	CGAAGGCTTGCTCCCAGT			
B. angulatum partial 16S rDNA	BiANG-1	CAGTCCATCGCATGGTGGT	95 ℃ for 20 s, 55 ℃ 20 s, 72 ℃ 30 s; 30×	275 bp	[17]
	BiANG-2	GAAGGCTTGCTCCCCAAC			
B. bifidum partial 16S rDNA	BiBIF-1	CCACATGATCGCATGTGATTG	95℃ for 20 s, 55℃ 20 s, 72℃ 30 s; 30×	278 bp	[17]
	BiBIF-2	CCGAAGGCTTGCTCCCAAA			
B. breve partial 16S rDNA	BiBRE-1	CCGGATGCTCCATCACAC	95 ℃ for 20 s, 55 ℃ 20 s, 72 ℃ 30 s; 30×	288 bp	[17]
	BiBRE-2	ACAAAGTGCCTTGCTCCCT			
B. catenulatum group partial 16S rDNA	BiCATg-1	CGGATGCTCCGACTCCT	95 ℃ for 20 s, 55 ℃ 20 s, 72 ℃ 30 s; 30×	285 bp	[17]
	BiCATg-2	CGAAGGCTTGCTCCCGAT			
B. longum partial 16S rDNA	BiLON-1	TTCCAGTTGATCGCATGGTC	95 ℃ for 20 s, 55 ℃ 20 s, 72 ℃ 30 s; 30×	831 bp	[17]
	BiLON-2	GGGAAGCCGTATCTCTACGA			

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Gene	Primer Pair	Sequence (5'-3')	PCR Conditions	PCR Fragments	Reference
B. infantis Partial 16S rDNA	BiINF-1	TTCCAGTTGATCGCATGGTC	95 ℃ for 20 s, 55 ℃ 20 s, 72 ℃ 30 s; 30×	828 bp	[17]
	BiINF-2	GGAAACCCCATCTCTGGGAT			
B. dentium Partial 16S rDNA	BiDEN-1	ATCCCGGGGGTTCGCCT	95 ℃ for 20 s, 55 ℃ 20 s, 72 ℃ 30 s; 30×	387 bp	[17]
	BiDEN-2	GAAGGGCTTGCTCCCGA			
B. gallicum Partial 16S rDNA	BiGAL-1	TAATACCGGATGTTCCGCTC	95 ℃ for 20 s, 55 ℃ 20 s, 72 ℃ 30 s; 30×	303 bp	[17]
	BiGAL-2	ACATCCCCGAAAGGACGC			
B. animalis Partial 16S rDNA	BanF2	AACCTGCCCTGTG	95 ℃ for 20 s, 55 ℃ 20 s, 72 ℃ 30 s; 30×	925 bp	[19]
	Pbi R1	GCACCACCTGTGAACCG			
Lb. casei Partial 16S rDNA	W1	TGCACTGAGATTCGACTTAA	95 ℃ for 20 s, 50 ℃ 20 s, 72 ℃ 30 s; 30×	295 bp	[16]
	Y2	CCCACTGCTGCCTCCCGTAGGAGT			
Lc. lactis amyl Gene	LC1	ACACTACACCACAACAA	95 ℃ for 30 s, 47 ℃ 45 s, 72 ℃ 2 min; 30×	1178 bp	This Study
	LC2	TCCTTATCTACCCAAAC			
S.thermophilus glgp Gene	ST1F	GCGAAAAATAAAAACCT	95 ℃ for 30 s, 55 ℃ 45 s, 72 ℃ 2 min; 30×	1606 bp	This Study
	ST1R	AGTGAATGATGTCTTGAGC			
ermA	ermA1	TCTAAAAAGCATGTAAAAGAA	95 ℃ for 30 s, 52 ℃ 45 s, 72 ℃ 2 min; 30×	645 bp	[42]
	ermA2	CTTCGATAGTTTATTAATATTAGT			
ermB	ermB1	GAAAAGGTACTCAACCAAATA	95 ℃ for 30 s, 52 ℃ 45 s, 72 ℃ 2 min; 30×	639 bp	[42]
	ermB2	AGTAACCGTACTTAAATTGTTTAC			
ermC	ermC1	TCAAAACATAATATAGATAAA	95 ℃ for 30 s, 52 ℃ 45 s, 72 ℃ 2 min; 30×	642 bp	[42]
	ermC2	GCTAATATTGTTTAAATCGTCAAT			
ermF	ermF1	CGGGTCAGCACTTTACTATTG	95 ℃ for 30 s, 50 ℃ 45 s, 72 ℃ 2 min; 30×	466 bp	[43]
	ermF2	GGACCTACCTCATAGACAAG			
ermFU	ermFU1	TTTACGGGTCAGCACTTT	95 ℃ for 30 s, 48 ℃ 45 s, 72 ℃ 2 min; 30×	748 bp	This Study
	ermFU2	CAACTTCCAGCATTTCCA			
ermG	ermG1	GAAATAGGTGCAGGGAAAGGTCA	95 ℃ for 30 s, 48 ℃ 45 s, 72 ℃ 2 min; 30×	603 bp	This Study
	ermG2	AAATAGCGATACAAATTGTTCGA			
ermQ	ermQ1	AAGTTATTGGGTTACAGCTA	95 ℃ for 30 s, 50 ℃ 45 s, 72 ℃ 2 min; 30×	624 bp	This Study
	ermQ2	CACCTCCTAATTTAAATCTACTA			
ereA	ereA1	AACACCCTGAACCCAAGGGACG	95 ℃ for 30 s, 50 ℃ 45 s, 72 ℃ 2 min; 30×	420 bp	[42]
	ereA2	CTTCACATCCGGATTCGCTCGA			
ereB	ereB1	AGAAATGGAGGTTCATACTTACCA	95 ℃ for 30 s, 50 ℃ 45 s, 72 ℃ 2 min; 30×	546 bp	[42]
	ereB2	CATATAATCATCACCAATGGCA			
mphA	mphA1	AACTGTACGCACTTGC	95 ℃ for 30 s, 50 ℃ 45 s, 72 ℃ 2 min; 30×	837 bp	[42]
	mphA2	GGTACTCTTCGTTACC			
msrA/B	msrA/B1	GCAAATGGTGTAGGTAAGACAACT	95 ℃ for 30 s, 50 ℃ 45 s, 72 ℃ 2 min; 30×	399 bp	[42]
	msrA/B2	ATCATGTGATGTAAACAAAAT			
msrC	msrC1	TATTGGAACATATCCGCAAACAAG	95 ℃ for 30 s, 52 ℃ 45 s, 72 ℃ 2 min; 30×	316 bp	This Study
	msrC2	GTTGCCATATCAATGAAATTAGTCG			
vga	vga1	TCTAATGGTACAGGAAAGACAACG	95 ℃ for 30 s, 50 ℃ 45 s, 72 ℃ 2 min; 30×	399 bp	[42]
	vga2	ATCGTGAGATACAAAGATTAT			

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Gene	Primer Pair	Sequence (5'-3')	PCR Conditions	PCR Fragments	Reference
mefA/E	mefA/E1	AGTATCATTAATCACTAGTGC	95 ℃ for 30 s, 50 ℃ 45 s, 72 ℃ 2 min; 30×	348 bp	[42]
	mefA/E2	TTCTTCTGGTACTAAAAGTGG			
mefA	mefA1	CTATGACAGCCTCAATGCG	95 ℃ for 30 s, 52 ℃ 45 s, 72 ℃ 2 min; 30×	1400 bp	This Study
	mefA2	ACCGATTCTATCAGCAAAG			
mefE	mefE1	ATGGAAAAATACAACAATTGGAAACGA	95 ℃ for 30 s, 52 ℃ 45 s, 72 ℃ 2 min; 30×	1191 bp	This Study
	mefE2	TTATTTTAAATCTAATTTTCTAACCTC			
vgb	vgb1	ACTAACCAAGATACAGGACC	95 ℃ for 30 s, 53 ℃ 45 s, 72 ℃ 2 min; 30×	734 bp	[44]
	vgb2	TTATTGCTTGTCAGCCTTCC			
lnuA	lnuA1	GGTGGCTGGGGGGTAGATGTATTAACTGG	95 ℃ for 30 s, 57 ℃ 45 s, 72 ℃ 2 min; 30×	323 bp	[44]
	lnuA2	GCTTCTTTTGAAATACATGGTATTTTTCGA			
lnuB	lnuB1	CCTACCTATTGTTTGTGGAA	95 ℃ for 30 s, 54 ℃ 45 s, 72℃ 2 min; 30×	925 bp	[45]
	lnuB2	ATAACGTTACTCTCCTATTTC			
vatA	vatA1	CAATGACCATGGACCTGATC	95 ℃ for 30 s, 52 ℃ 45 s, 72 ℃ 2 min; 30×	619 bp	[44]
	vatA2	CTTCAGCATTTCGATATCTC			
vatB	vatB1	GGCCCTGATCCAAATAGCAT	95 ℃ for 30 s, 60 ℃ 1 min, 72 ℃ 2 min; 30×	559 bp	[46]
	vatB2	GTGCTGACCAATCCCACCAT			
vatC	vatC1	ATGAATTCGCAAAATCAGGAAGG	95 ℃ for 30 s, 60 ℃ 20 s, 72 ℃ 2 min; 30×	580 bp	[46]
	vatC2	TCGTCTCGAGCTCTAGGTCC			
vatD	vatD1	GCTCAATAGGACCAGGTGTA	95℃ for 30 s, 60℃ 20 s, 72℃ 2 min; 30×	272 bp	[47]
	vatD2	TCCAGCTAACATGTATGGCG			
vatE	vatE1	ACTATACCTGACGCAAATGC	95 ℃ for 30 s, 53 ℃ 20 s, 72 ℃ 2 min; 30×	512 bp	[46]
	vatE2	GGTTCAAATCTTGGTCCG			
vanA	vanA-36F	TTGCTCAGAGGAGCATGACG	95 ℃ for 30 s, 65 ℃ 45 s, 72 ℃ 2 min; 30×	957 bp	[48]
	vanA-992R	TCGGGAAGTGCAATACCTGC			
vanH	vanH-10F	ATCGGCATTACTGTTTATGGA	95 ℃ for 30 s, 60 ℃ 45 s, 72 ℃ 2 min; 30×	943 bp	[48]
	vanH-952R	TCCTTTCAAAATCCAAACAGTTT			
vanR	vanR-4F	AGCGATAAAATACTTATTGTGGA	95 ℃ for 30 s, 65 ℃ 45 s, 72 ℃ 2 min; 30×	645 bp	[48]
	vanR-648R	CGGATTATCAATGGTGTCGTT			
vanS	vanS-28F	AACGACTATTCCAAACTAGAAC	95 ℃ for 30 s, 61 ℃ 45 s, 72 ℃ 2 min; 30×	1094 bp	[48]
	vanS-1121R	GCTGGAAGCTCTACCCTAAA			
vanX	vanX-F	TCGCGGTAGTCCCACCATTCGTT	95 ℃ for 30 s, 55 ℃ 45 s, 72 ℃ 2 min; 30×	454 bp	This Study
	vanX-R	AAATCATCGTTGACCTGCGTTAT			
vanY	vanY-44F	ACTTAGGTTATGACTACGTTAAT	95 ℃ for 30 s, 55 ℃ 45 s, 72℃ 2 min; 30×	866 bp	[48]
	vanY-909R	CCTCCTTGAATTAGTATGTGTT			
vanZ	vanZ-13F	TTATCTAGAGGATTGCTAGC	95 ℃ for 30 s, 64 ℃ 45 s, 72 ℃ 2 min; 30×	454 bp	[48]
	vanZ-466R	AATGGGTACGGTAAACGAGC			
vanB	vanB-23F	TTA TCT TCG GCG GTT GCT CG	95 ℃ for 30 s, 62 ℃ 45 s, 72 ℃ 2 min; 30×	994 bp	[48]
	vanB-1016R	GCC AAT GTA ATC AGG CTG TC			
vanC	vanC-F	CAGTGTCACTAACCTCAGCAGCCG	95 ℃ for 30 s, 56 ℃ 45 s, 72 ℃ 2 min; 30×	934 bp	This Study
	VanC-R	TAGGATAACCCGACTTCCGCCA			

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Gene	Primer Pair	Sequence (5'-3')	PCR Conditions	PCR Fragments	Reference
vanE	vanE-F	TGTGGTATCGGAGCTGCAG	95 ℃ for 30 s, 52 ℃ 45 s, 72 ℃ 2 min; 30×	513 bp	[10]
	vanE-R	GTCGATTCTCGCTAATCC			
qnrA	qnrA1	AGCAAGAGGATTTCTCACGC	95 ℃ for 30 s, 55 ℃ 45 s, 72 ℃ 2 min; 30×	623 bp	This Study
	qnrA2	CAGCACTATTACTCCCAAGG			
qnrB1	qnrB1-F	AGGTACAAATATGGCTCTG	95 ℃ for 30 s, 51 ℃ 45 s, 72 ℃ 2 min; 30×	619 bp	This Study
	qnrB1-R	CAACGATGCCTGGTAGT			
qnrB2	qnrB2-F	CTCTGGCACTCGTTGGC	95 ℃ for 30 s, 53 ℃ 45 s, 72 ℃ 2 min; 30×	586 bp	This Study
	qnrB2-R	TCCAACTTAACGCCTTGTAAAT			
qnrS	qnrS-F	GGAAACCTACAATCATACATATCGG	95 ℃ for 30 s, 54 ℃ 45 s, 72 ℃ 2 min; 30×	648 bp	This Study
	qnrS-R	GGATAAACAACAATACCCAGTGCTT			
mfpA	mfpA-F	GGCGATGTTCAGCGAATGCG	95 ℃ for 30 s, 61 ℃ 45 s, 72 ℃ 2 min; 30×	463 bp	This Study
	mfpA-R	CAAGCACAGCCCGTGCGCC			
norA	norA-F	TATCGGTTTAGTAATACCAGTCT	95 ℃ for 30 s, 48 ℃ 45 s, 72 ℃ 2 min; 30×	1103 bp	This Study
	norA-R	GTTCTTTCAATTTTGCTCTATGT			
ant(6)	ant(6)F	ACTGGCTTAATCAATTTGGG	95 ℃ for 30 s, 58 ℃ 45 s, 72 ℃ 2 min; 30×	597 bp	[6]
	ant(6)R	GCCTTTCCGCCACCTCACCG			
ant(4')-Ia	ant(4')-IaF	TAAGGCTATTGGTGTTTATGGCTCT	95 ℃ for 30 s, 54 ℃ 45 s, 72 ℃ 2 min; 30×	635 bp	This Study
	ant(4')-IaR	ATCCGTGTCGTTCTGTCCACTCCTG			
aac(6')-Ie	aac(6')-IeF	GATGATGATTTTCCTTTGATGTT	95 ℃ for 30 s, 47 ℃ 45 s, 72 ℃ 2 min; 30×	1046 bp	This Study
	aac(6')-IeR	ACTGTTGTTGCATTTAGTCTTTC			
aac(6')-Im	aac(6')-ImF	AATGGCTGACAGATGACCGTGTT	95 ℃ for 30 s, 53 ℃ 45 s, 72 ℃ 2 min; 30×	430 bp	This Study
	aac(6')-ImR	TCGTGTAGCTCATGTTCGGGAAG			
aac (6')-aph(2')	aac(6')-aph(2')F	CCAAGAGCAATAAGGGCATA	95 ℃ for 30 s, 60 ℃ 45 s, 72 ℃ 2 min; 30×	220 bp	[6]
	aac(6')-aph(2')R	CACTATCATAACCACTACCG			
aph(2')-Ia	aph(2')-IaF	TAAGACAAATGCACGGTTTAGAT	95 ℃ for 30 s, 47 ℃ 45 s, 72 ℃ 2 min; 30×	489 bp	This Study
	aph(2')-IaR	TACCATTTTCGATAAATTCCTGT			
aph(2')-Ib	aph(2')-IbF	ATGAACTCCGTTATTTATCGTCC	95 ℃ for 30 s, 50 ℃ 45 s, 72 ℃ 2 min; 30×	799 bp	This Study
	aph(2')-IbR	CCCTTAATCAACATTTCCCTATC			
aph(2')-Ic	aph(2')-IcF	GTCGCTTGGTGAGGGCTTTAGGA	95 ℃ for 30 s, 55 ℃ 45 s, 72 ℃ 2 min; 30×	654 bp	This Study
	aph(2')-IcR	GTAAACAGCTCGCCGCAATCTTC			
aph(2')-Id	aph(2')-IdF	ATGCCATCAGAAACGTACCAAAT	95 ℃ for 30 s, 50 ℃ 45 s, 72 ℃ 2 min; 30×	631 bp	This Study
	aph(2')-IdR	TTAATCCCTCTTCATACCAATCC			
aph(3')-IIIa	aph(3')-IIIaF	GCCGATGTGGATTGCGAAAA	95 ℃ for 30 s, 60 ℃ 45 s, 72 ℃ 2 min; 30×	292 bp	[6]
	aph(3')-IIIaR	GCTTGATCCCCAGTAAGTCA			
aph(3')-Iva	aph(3')-IvaF	CTTCTTGAGCTTCTCGGGCAGAC	95 ℃ for 30 s, 59 ℃ 45 s, 72 ℃ 2 min; 30×	740 bp	This Study
	aph(3')-IvaR	AGCCGGATGTAATACCGGACCTT			
aadA	aadA-F	CAACTATCAGAGGTGCTAAGCGTCAT	95 ℃ for 30 s, 57 ℃ 45 s, 72℃ 2 min; 30×	735 bp	This Study
	aadA-R	CTCGCCTTTCACAAAGCGAATAA			
aadE	aadE-F	AAAGCCGGAGGATATGGA	95 ℃ for 30 s, 50 ℃ 45 s, 72 ℃ 2 min; 30×	565 bp	This Study
	aadE-R	ATGAAGCCTTTCCGCCAC			

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Gene	Primer Pair	Sequence (5'-3')	PCR Conditions	PCR Fragments	Reference
aadK	aadK-F	GTACAAACAGAAATATCCCTCCT	95 ℃ for 30 s, 49 ℃ 45 s, 72 ℃ 2 min; 30×	766 bp	This Study
	aadK-R	CACTTTACTGAGCAATAAATACC			
blaZ	blaZ-F	TACTTCAACACCTGCTGCTTTCG	95 ℃ for 30 s, 50 ℃ 45 s, 72 ℃ 2 min; 30×	325 bp	This Study
	blaZ-R	CATTACACTCTTGGCGGTTTCAC			
dfrA	dfrA1	CTTTTCTACGCACTAAATGTAAG	95 ℃ for 30 s, 50 ℃ 45 s, 72 ℃ 2 min; 30×	474 bp	This Study
	dfrA2	CATTATCAATAATTGTCGCTCAC			
dfrD	dfrD1	GGAAGGGCTTTACCTGACAGAAG	95 ℃ for 30 s, 50 ℃ 45 s, 72 ℃ 2 min; 30×	175 bp	This Study
	dfrD2	CGACATAAGGCAAGAACATAACATA			

TABLE 2

Lactic Acid Bacterial Strains Used in This Study

Origins	Bacterial Strains
Fermented Milk	
Lactococcus lactis subsp. lactis (*n*=3)	*Lc. lactis* LC1 ~ *Lc. lactis* LC3
Lactobacillus plantarum (*n*=2)	*Lb. planterum* LP1, *Lb. planterum* LP2
Lactobacillus acidophilus (*n*=5)	*Lb. acidophilus* LA2, *Lb. acidophilus* LA3, *Lb. acidophilus* LA5 ~ *Lb. acidophilus* LA7
Lactobacillus delbrrueckii spp. bulgaricus (*n*=7)	*Lb. bulgaricus* LDB1~ *Lb. bulgaricus* LDB7
Streptococcus thermophilus (*n*=12)	*S.thermophilus* ST1 ~ *S.thermophilus* ST9, *S.thermophilus* ST11 ~ *S.thermophilus* ST13
Beverage	
Lactobacillus casei (*n*=3)	*Lb. casei* LCA1 ~ *Lb. casei* LCA3
Streptococcus thermophilus (*n*=1)	*S.thermophilus* ST10
Drugs	
Enterococcus faecalis (*n*=2)	*E. faecalis* FA1, *E. faecalis* FA2
Enterococcus faecium (*n*=1)	*E. faecium* FM1
Lactobacillus acidophilus (*n*=2)	*Lb. acidophilus* LA1, *Lb. acidophilus* LA4
Lactobacillus rhamnosus (*n*=1)	*Lb. rhamnosus* LR1
Bifidobacterium longum (*n*=1)	*B. longum* BL1
Bifidobacterium animalis (*n*=1)	*B. animalis* BA1
Reference Strains	
Staphylococcus aureus ATCC 25923	
Escherichia coli ATCC 25922	

TABLE 3

Diameters of the Inhibition Zones for 41 Lactic Acid Bacterial Strains in Disc Diffusion Testing of 16 Antimicrobial Agents

Species (Number of Strains)	Inhibiton Zone Diameter Range (mm)															
	AM	AMC	CF	CTX	IPM	E	VA	C	RA	TE	AK	GM	S	CIP	B	SXT
Lb. acidophilus (7)	36~56	41~58	35~45	34~46	38~52	36~48	25~38	30~40	28~38	40~48	10~19	11~23	38~46	6~25*	28~36	6
Lb. casei (3)	28~37	29~37	20~25	26~40	25~31	32~40	6	26~34	29~37	34~40	13~18	12~18	28~32	15~20	22~29	6
Lb. bulgaricus (7)	35~55	34~53	37~53	36~53	30~50	17~44	21~38	28~44	25~43	37~46	6~36	6~26	15~39	6~19	12~40	6
Lb. planterum (2)	25~46	35~43	20~35	36~52	42~55	28~32	6	23~34	15~24	20~27	9~15	9~17	19~28	6	11~13	6
Lb. rhamnosus (1)	29	40	26	30	34	38	6	26	33	34	16	16	30	19	23	6
Lc. Lactis (3)	27~42	20~37	25~32	28~31	32~39	25~30	19~23	23~36	9~12	31~36	10~15	11~14	14~27	14~21	22~29	6
S. thermophilus (13)	40~54	42~56	43~56	40~54	44~58	34~46	26~35	30~40	31~48	26~48	6~21	9~21	24~34	20~30	34~48	6~22
E. faecalis (2)	22~40	35~39	20~32	6~32	33~51	17~24	6~20	22~30	12~26	21~35	9~11	6~13	14~22	6~20	6~11	6~15
E. faecium (1)	26	33	27	29	33	10	19	25	13	30	11	10	16	21	19	6
B. longum (1)	56	57	59	41	45	58	47	56	44	57	6	6	31	6	39	6
B. animalis (1)	58	53	36	58	46	44	38	42	32	37	6	6	29	16	44	47

Note. *Diameter of the disc is 6 mm.

TABLE 4

The Applied Breakpoints for Resistance and MIC Values for the Resistant Strains Screened by Disc Diffusion Method (MIC Value mg/L)

Bacterial Strains	Antimicrobial Agents (Breakpoints for Resistance)									
	AK (16)P[a]	GM (8)P[bP]	VA (4)	S	B (n.d.)[c]	CIP (4)	CTX	E	RA	STX(8)
Lb. acidophilus LA4						>32				>32
Lb. acidophilus LA5	96					>32				>32
Lb. acidophilus LA6	96	4				>32				>32
Lb. acidophilus LA7	96	8				>32				>32
Lb. casei LCA1			>256							>32
Lb. casei LCA2			>256			2				>32
Lb. casei LCA3		16	>256							>32
Lb. bulgaricus LDB1	96	24	>256		>256					>32
Lb. bulgaricus LDB2						1.5				>32
Lb. bulgaricus LDB3						12				>32
Lb. bulgaricus LDB4						>32				>32
Lb. bulgaricus LDB5	64	16				>32				>32
Lb. bulgaricus LDB6						>32				>32
Lb. planterum LP1			>256		>256	>32				>32
Lb. planterum LP2	>256	24	>256		>256	>32				>32
Lb. rhamnosus LR1			>256							>32
	AK (8)	GM (8)	VA (4)	S	B (n.d.)[c]	CIP (n.d.)	CTX	E	RA (4)	STX(n.d.)
Lc. lactis LC1	96	12				1.5			16	>32
Lc. lactis LC2		4							12	
Lc. lactis LC3		8							24	
	AK (8)	GM (8)	VA (4)	S	B (n.d.)[c]	CIP (n.d.)	CTX	E	RA (4)	STX(n.d.)
S.thermophilus ST1	192									
S.thermophilus ST2		8								
S.thermophilus ST3	32	4								
S.thermophilus ST4	24									
S.thermophilus ST5	32	12								>32
S.thermophilus ST6	>256	>256								
S.thermophilus ST7	>256	24								
S.thermophilus ST8	>256	12								1.5
	AK (1024)[d]	GM (512)[d]	VA (8)	S (1024)[d]	B (n.d.)[c]	CIP (2)	CTX (64)	E (4)	RA (4)	STX(8)
E. faecalis FA1	>256	96		>256	>256		64		4	>32
E. faecalis FA2	>256	48	>256			>32				2
	AK (8)	GM (4)	VA	S	B	CIP (n.d.)	CTX	E	RA	STX(8)
E. faecium FM1	96	16		>256				24	3	>32
B. longum BL1	>256	>256				>32				>32

Note. a: 64 mg/L was used for *Lb planterum* species; b: 64 mg/L was used for *Lb planterum* species; c: n.d. not determined. d: Maximum concentration of E-test strip is 256 mg/L.

Antibiotic Resistant Genes

Since the strains were resistant to aminoglycosides, β-lactams, fluoroquinolones, glycopeptides, macrolides, and trimethoprim, primer pairs were designed to amplify 57 different resistant determinants. Antibiotic resistant genes were detected in five strains (Fig. 1).

The *msrC* gene, encoding an erythromycin efflux pump, was detected in the *E. faecium* strain with an erythromycin MIC of 24 mg/L. No other erythromycin resistant genes were detected. In two strains of *Lb. plantarum*, a 454 bp amplicon was detected and identified as the *vanX* gene after sequencing and alignment. This gene encodes a D-ala-D-ala dipeptidase that is required for high vancomycin resistance. In addition, a strain of *Lc. Lactis* and a strain of *S. thermophilus* carried a gene

homologous to *dfrA* of *S. aureus*, encoding a drug-resistant dihydrofolate reductase (DHFR) enzyme associated with trimethoprim resistance. No resistant genes included in this study was detected in other resistant strains.

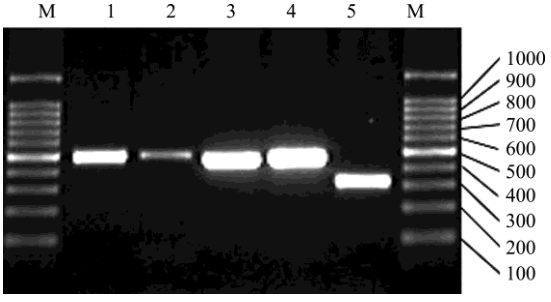

FIG. 1. Results of PCR of resistance genes. M: 100 bp DNA Ladder, lane 1: *dfrA* gene of *S. thermophilus* ST1, lane 2: *dfrA* gene of *Lc.lactis* LC1, lane 3: *vanX* gene of *Lb. plantarum* LP1, lane 4: *vanX* gene of *Lb. plantarum* LP2, lane 5: *msrC* gene of *E. faecium* FM1.

DISCUSSION

The profiles of antimicrobial susceptibility of LAB have been documented in many countries[1, 10, 23-24]. It was reported that *S. thermophilus* is moderately-highly resistant to aminoglycosides, trimethoprim, and sulphadiazine, and few strains are atypically resistant to tetracycline[23, 25-26]. In our study, aminoglycoside-resistant strains were identified, but only 3 of the 13 strains were resistant to trimethoprim/sulphamethoxazole, and no strain was resistant to tetracycline. It has been shown that *Lactobacillus spp.* is generally susceptible to chloramphenicol, erythromycin and tetracycline[6, 23, 27]. *Lactobacillus* has been reported to be intrinsically resistant to aminoglycosides, fluoroquinolones, and glycopeptides. Nevertheless, 30% of *Lactobacillus* isolates in this study were resistant to amikacin and gentamycin. However, none of them was resistant to high streptomycin levels, and no more than 50% of the isolates were resistant to ciprofloxacin, not supporting the intrinsic resistance of *Lactobacilli* to these antibiotics. All *Lb. plantarum* and *Lb. casei* strains were resistant to vancomycin, supporting the native resistance of *Lb. plantarum* and *Lb. casei* species to vancomycin. In addition, four strains were resistant to bacitracin, a rarely documented finding. *Lc. lactis* strains were all resistant to rifampicin, which is consistent with the reported finding[1]. The mechanism underlying this finding is not yet clear. Most *Bifidobacterium* species are resistant to aminoglycosides and some strains are resistant to vancomycin, erythromycin, tetracycline and cefoxitin, while the resistance of such strains to trimethoprim and sulphadiazine is variable[7, 24, 28]. Such resistance was not confirmed in our study, although one of the strains was resistant to trimethoprim/sulphamethoxazole. Since all *Enterococcal* isolates were resistant to aminoglycosides, trimethoprim/sulphamethoxazole, vancomycin, ciprofloxacin, bacitracin, rifampicin, erythromycin and cefotaxime, it was difficult to compare these findings in our study with those in previous studies[29-30]. *Enterococci* are naturally resistant to all cephalosporins, but susceptible to vancomycin and erythromycin in the clinical environment. However, vancomycin-resistant *Enterococcus faecalis* from a pharmaceutical product was found in our study. Vancomycin-resistant *Enterococci* are commonly associated with nosocomial infections in hospitals. Furthermore, it was reported that the resistance of *Enterococci* to vancomycin-resistant is transferable *in vitro*[31-32], indicating that *Enterococcus* is a controversial species that should not be used for probiotic applications, because of its potential pathogenicity and its notable resistance to some of the widely used antibiotics.

Of the resistant strains in our study, only five carried resistance genes, which may explain why we did not observe more strains with resistant determinant genes. Firstly, these strains may have been intrinsically resistant to the antibiotics tested. Secondly, the emergence of resistance in these organisms may have arrived through evolutionary events, such as mutations. Thirdly, these strains may have acquired resistant genes that could not be detected with the methods we used.

The *vanX* gene, found in two *Lb. plantarum* isolates, encodes a D-ala-D-ala dipeptidase (VanX) that is highly specific for hydrolyzing D-ala-D-ala dipeptides, essential precursors of the cell wall. Normally, this type of resistance is encoded by an entire cluster of genes encoded on a large conjugative plasmid (*vanA*, *vanH*, *vanR*, *vanS*, *vanX*, *vanY*, and *vanZ*)[33]. The *vanA* gene in this cluster usually plays a major role while the other genes play a secondary role in conferring resistance. However, no other gene of the cluster was found in this study. The mechanism by which *vanX* confers resistance in *Lb. plantarum* species is not yet clear, since this particular species is thought to be intrinsically resistant to vancomycin due to its peptidoglycan precursors composed of D-lactate rather than D-alanine at the C-terminus. Since the wild strain can produce D-ala-D-ala precursors[34], vancomycin may be able to inhibit cell wall synthesis even if only a small number of precursors ending in D-alanine are produced. The two *Lb. plantarum* strains in our study may have chosen to strictly use the alternative D-lactate pathway. If so,

this mechanism of acquiring resistance is not as threatening as the inducible, transferable mechanism encoded by the *vanA* plasmid. D-ala-D-ala dipeptidase encoded by *vanX* may act only in the presence of D-ala-D-ala precursor. Further study is required to determine the potential for the *vanX* gene transfer.

The *msrC* gene, found in one strain of *E. faecium*, has 62% identity at DNA level and 72% similarity at amino acid level with *msr(A)*, a plasmid-encoded gene that encodes an ATP-binding cassette (ABC) transporter confering macrolide-lincosamide-streptogramin B (MLS_B) resistance in Staphylococci. The *msrC* gene, an endogenous gene present in the chromosome or on an epidemic plasmid present in all *E. faecium* strains, plays a role in macrolide resistance[35]. Recent studies, however, have suggested that *msrC* is not equally distributed in all *E. faecium* isolates and its inactivation in *E. faecium* leads to a 2-8 fold decrease in the MLS_B MIC. Moreover, *msrC* expression can protect *S. aureus* against erythromycin and other MS_B antibiotics, indicating that the *msrC* gene is not intrinsic to all *E. faecium* isolates[36]. No other resistant gene was found in *E. faecium* strain in our study, revealing that the low resistance of *E. faecium* to erythromycin is induced by the *msrC* gene. Although *msrC* may not be a natural gene in *E. faecium*, transfer is nearly impossible, indicating that the strain seems relatively safe. However, since *msrC* confers a high resistance in *S. aureus*, rather than in its *Enterococcal* hosts, *E. faecium* species should not be used in marketed foods or drugs. While the *msrC* gene shares a significant sequence identity with *msr(A)*, the mechanism of resistance conferred by *msr(A)* remains unclear[37-38]. Additionally, the potential pathogenicity of *E. faecium* species poses a risk factor for its use as a food and drug additive.

The *dfrA* gene, encoding a TMP-resistant DHFR located in the transposon *Tn*4003 in *S. aureus*, induces a high trimethoprim resistance[39-40]. It was reported that such a transposon can be horizontally transferred in nature[41]. Both strains carrying the *dfrA* gene in this study were highly resistant to trimethoprim/sulphamethoxazole.

In conclusion, multiple drug resistance is present in a variety of species. MIC breakpoints of LAB require standardization. Antibiotic resistant genes are detectable in strains with resistant phenotypes. The potential transferability of these resistant genes poses a threat to food safety. Evaluation of the safety of lactic acid bacteria for human consumption must be guided by established criteria, guidelines and regulations, and standardized methods for premarket biosafety testing and post market surveillance should be established.

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