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Original Article

Characterization and transfer of antimicrobial resistance in lactic acid bacteria from fermented dairy products in China

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Abstract

Introduction: Lactic acid bacteria (LAB) are commonly found in foods and are also natural intestinal inhabitants in humans and most animals. However, information regarding antimicrobial resistance and the transfer of resistance genes of LAB from fermented dairy products in China is limited.

Methodology: In this study, LAB isolates (n = 82) of *Lactobacillus* (n = 43) and *Streptococcus thermophilus* (n = 39) were isolated from 51 commercial fermented food samples in China. All isolates were subjected to pulsed-field gel electrophoresis (PFGE), antimicrobial susceptibility, detecting resistance genes, as well as investigating the transferability of resistance genes.

Results: The 43 *Lactobacillus* isolates yielded 24 PFGE patterns and the 34 isolates of *S. thermophilus* generated 32 different PFGE patterns. Among the 43 *Lactobacillus* strains, the most commonly observed resistance was that to streptomycin (83.7%) and gentamycin (83.7%). Among the 39 *S. thermophilus* strains, the most frequently observed resistance was that to streptomycin (92.3%), gentamycin (87.2%), ciprofloxacin (79.5%), and chloramphenicol (71.8%), whereas the lowest level of resistance was that against erythromycin (7.7%). Antimicrobial resistance genes for erythromycin (*emrB*), gentamycin (*aac(6')-aph(2")*), streptomycin (*ant(6)*), sulfamethoxazole (*sulI* and *sulII*), tetracycline (*tetM* and *tetS*) were detected in the 18 resistance LAB strains. Conjugation experiments showed that *tetM* from *L. delbrueckii* subsp. *bulgaricus* R6 and *tetS* from *L. plantarum* R41 were successfully transferred to *L. monocytogenes* by filter mating.

Conclusions: LAB strains could potentially act as reservoirs of resistance genes and play an active role in the transfer of resistance to humans via the food chain.

Key words: *Lactobacillus* spp.; *Streptococcus thermophilus*; antimicrobial resistance; transfer; fermented dairy products.

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Introduction

Lactic acid bacteria (LAB) are a group of gram-positive bacteria that are non-spore-forming and can ferment hexose sugars and a variety of nutrients to primarily produce lactic acid. Some LAB species constitute the natural intestinal microbiota of humans and are beneficial to various physiological processes, particularly digestion, as well as in preventing gastrointestinal disorders [1]. Thus, LAB have been extensively used in the production of fermented foods and beverages in the food industry for decades and have acquired the "generally recognized as safe" (GRAS) status [2,3]. GRAS is a U.S. Food and Drug Administration (FDA) designation for a substance that, when added to food, is considered safe and exempt from the food additive tolerance requirements.

Due to the extensive use of antimicrobials around the world, the emergence of resistance microorganisms has become a major public health problem. Antimicrobial resistance of foodborne pathogens has been well studied and documented [3]. Researchers have also investigated the antimicrobial resistance of commensal bacteria such as LAB [4,5]. Antimicrobial resistance in bacteria may be intrinsic or acquired [6,7]. Several species of *Lactobacillus* exhibit intrinsic resistance to quinolones, trimethoprim, sulphonamides, and vancomycin [2]. In contrast, acquired resistance is present in some strains within a species usually susceptible to the antimicrobial under consideration, and might be horizontally spread among bacteria [2]. Earlier studies have described the development of antimicrobial resistance in LAB from fermented foods [4]. Several investigators have speculated that LAB could serve as reservoir for antimicrobial resistance genes similar to those found in human pathogens [8,9]. The main threat associated with these bacteria is that these can transfer resistance genes to other microorganisms, including pathogens, through the food

chain. The Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) of the European Food Safety Authority (EFSA) recommends that bacterial strains carrying transferable antimicrobial resistance genes should not be used in fermented and probiotic foods for human [10].

Fermented dairy products are popular products in China and are generally produced by adding *Lactobacillus* species and *Streptococcus thermophilus* as fermenting starter or probiotic strains. Such products are consumed without further heating, thereby providing a potential vehicle for antimicrobial resistant bacteria. However, information regarding antimicrobial resistance and the transfer of resistance genes of LAB from fermented dairy products in China is limited. Therefore, the objectives of the present study were to investigate the occurrence of antimicrobial resistance and the presence and transferability of resistance genes among LAB strains isolated from fermented dairy products in China.

Methodology

Sample collection

A total of 51 commercial fermented food samples, including yogurt (n = 30), old yogurt (n = 13), and fermented dairy drink (n = 8), were purchased from supermarkets between May and October 2014 in Henan Province, located in the central part of China. All samples come from products produced by ten different manufacturers. Old yogurt is a traditional Chinese fermented dairy product, belonging to the set-style yogurt. The difference between old yogurt and normal yogurt is the processing steps. Old yogurt is made by pouring the cultured milk into individual containers and then incubating without any further stirring, while normal yogurt is made by incubating the cultured milk mixture in a large vat and then stirring prior to packaging. Fermented dairy drink has the lower protein content than that of normal yogurt and old yogurt. The samples were transported to the laboratory in an icebox and then immediately used for bacterial isolation.

Isolation and identification of LAB

LAB isolation was performed using standard procedures described in the National Standards of the People's Republic of China (GB 4789.35-2010). Briefly, 25 g of each sample was aseptically collected, mixed with 225 mL of sterilized physiological saline, and homogenized in a stomacher for 1 minute (Interscience, Saint Nom, France). The mixture was serially diluted with physiological saline and plated on MRS agar (Land Bridge Technology Co. Ltd., Beijing, China) and Modified Chalmers (MC) agar (Land Bridge Technology Co. Ltd., Beijing, China) and incubated at 37°C for 48 hours. Lactobacilli were selected on MRS agar plates incubated in anaerobic conditions, whereas *S. thermophilus* was isolated on MC agar plates in an aerobic environment [3]. After the incubation, colonies were selected and subjected to Gram staining and identified by *Lactobacillus* biochemical identification kit or *S. thermophilus* biochemical identification kit (Land Bridge Technology Co. Ltd., Beijing, China). The biochemical identification for lactobacilli included fermentation tests from esculin, cellobiose, maltose, mannitol, salicin, sorbitol, sucrose and raffinose; for *S. thermophilus*, it included fermentation tests from inulin, lactose, mannitol, salicin, sorbitol, hippuric acid and esculin. Then, the LAB isolates was further confirmed by PCR-based 16S rDNA sequencing using a pair of universal primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3') [11].

Pulsed-field gel electrophoresis (PFGE)

PFGE typing of all LAB isolates was performed as previously described [12,13]. Briefly, after adding 10 μg/μL of lysozyme solution (Sigma-Aldrich, St. Louis, MO, USA), each bacterial suspension was incubated at 37°C for 10 min. The treated bacterial suspension was then mixed with low-melting point agarose (Bio-Rad, Hercules, CA, USA) in buffer and pipetted into plug molds. The plugs were lysed with a cell lysis buffer and washed 4 times by TE buffer. Next, agarose-embedded DNA of *Lactobacillus* species and *S. thermophilus* was digested with 30 U of *Asc*I (Takara Bio Inc., Otsu, Shiga, Japan) for 12 hours at 37 °C and 20 U of *Sma*I (Takara Bio Inc., Otsu, Shiga, Japan) at 30°C overnight, respectively. The choice of enzymes used in our study was performed according to the previous studies [12–15]. The restriction fragments were separated using the CHEF MAPPER apparatus (Bio-Rad, Hercules, CA, USA) at 6 V/cm for 22 hours, with switch time ranging from 1 to 15 seconds for *Lactobacillus* and 5.3 to 34.9 seconds for *S. thermophilus*. The gel was stained in an ethidium bromide solution and imaged using a Bio-Rad Gel Doc. PFGE patterns were compared using Quantity One software (Bio-Rad, Hercules, CA, USA). A dendrogram was deduced from the matrix of similarities by using the unweighted pair-group method with arithmetic means (UPGMA). This specific computer-assisted analysis was performed according to the manufacturer's instructions.

Antimicrobial susceptibility testing

The minimum inhibitory concentrations (MICs) of 9 antimicrobials, including ampicillin (0.032-64 μg/mL), chloramphenicol (0.125-256 μg/mL), ciprofloxacin (0.125-256 μg/mL), erythromycin (0.032-8 μg/mL), gentamycin (1-256 μg/mL), streptomycin (1-256 μg/mL), sulfamethoxazole (8-512 μg/mL), tetracycline (1-64 μg/mL), and vancomycin

Table 1. MIC breakpoints for *Lactobacillus* species and *Streptococcus thermophilus* in this study.

Antimicrobial	Species	Proposed breakpoint, MIC (μg/mL)			
		This paper	EFSA[a]	FEEDAP[b]	Other articles
Ampicillin	*L. delbrueckii* subsp. *bulgaricus*	1	1	1	2[c], 4[d], 8[e]
	L. plantarum	2	2	2	2[c], 4[d], 8[e]
	L. paracasei	2	2	4	2[c], 4[d], 8[e]
	L. acidophilus	1	1	1	2[c], 4[d], 8[e]
	S. thermophilus	2	2	2	8[e]
Chloramphenicol	*L. delbrueckii* subsp. *bulgaricus*	4	4	4	16[c,d,e]
	L. plantarum	8	8	8	16[c,d,e]
	L. paracasei	4	4	4	16[c,d,e]
	L. acidophilus	4	4	4	16[c,d,e]
	S. thermophilus	4	4	4	16[e]
Ciprofloxacin	*L. delbrueckii* subsp. *bulgaricus*	4			4[c,e,f], > 32[d]
	L. plantarum	4			4[c,e,f], > 32[d]
	L. paracasei	4			4[c,e,f], > 32[d]
	L. acidophilus	4			4[c,e,f], > 32[d]
	S. thermophilus	4			4[c,e]
Erythromycin	*L. delbrueckii* subsp. *bulgaricus*	1	1	1	4[c], 1[e]
	L. plantarum	1	1	1	4[c, d], 1[e]
	L. paracasei	1	1	1	4[c], 2[d], 1[e]
	L. acidophilus	1	1	1	4[c], 1[d], 1[e]
	S. thermophilus	2	2	2	1[e]
Gentamycin	*L. delbrueckii* subsp. *bulgaricus*	16	16	16	1[c]
	L. plantarum	16	16	16	1[c], 128[d], 16[e]
	L. paracasei	32	32	32	1[c], 128[d], 16[e]
	L. acidophilus	16	16	16	1[c], 256[d], 16[e]
	S. thermophilus	32	32	32	16[e]
Streptomycin	*L. delbrueckii* subsp. *bulgaricus*	16	16	16	16[c], > 256[d]
	L. plantarum	16			16[c], > 256[d]
	L. paracasei	16		64	16[c], > 256[d]
	L. acidophilus	16	16	16	16[c], > 256[d]
	S. thermophilus	64	64	64	16[e]
Sulfamethoxazole	*L. delbrueckii* subsp. *bulgaricus*	512			512[e]
	L. plantarum	512			512[e]
	L. paracasei	512			512[e]
	L. acidophilus	512			512[e]
	S. thermophilus	512			512[e]
Tetracycline	*L. delbrueckii* subsp. *bulgaricus*	4	4	4	16[c], 8[e]
	L. plantarum	32	32	32	16[c], 64[d], 8[e]
	L. paracasei	4	4	4	16[c], 4[d], 8[e]
	L. acidophilus	4	4	4	16[c], 4[d], 8[e]
	S. thermophilus	4	4	4	8[e]
Vancomycin	*L. delbrueckii* subsp. *bulgaricus*	2	2	2	4[c], > 1[e]
	L. plantarum	4			4[c], > 1[e]
	L. paracasei	4			4[c], > 1[e]
	L. acidophilus	2	2	2	4[c,d], > 1[e]
	S. thermophilus	4	4	4	> 1[e]

[a] Breakpoints defined by the European Food Safety Authority [36] for *Lactobacillus* spp. and *S. thermophilus*; [b] Breakpoints defined by the EFSA panel on additives and products or substances used in animal feed (FEEDAP) [37] for *Lactobacillus* spp. and *S. thermophilus*; [c] Breakpoints defined by the European Commission [38] for *Lactobacillus* spp.; [d] Breakpoints suggested by Danielsen and Wind [39] for *Lactobacillus* spp.; [e] Breakpoints suggested by Katla *et al*. [40] for *Lactobacillus* spp. and *Streptococcus* spp.; [f] Breakpoints suggested by Zarazaga *et al*. [41] for *Lactobacillus* spp.

(0.032-256 µg/mL), were determined by using the broth microdilution method. For *Lactobacillus*, LAB susceptibility test medium (LSM), a mixed formulation containing Iso-Sensitest broth (Oxoid Ltd., Basingstoke, Hampshire, England) (90%) and MRS (10%) was used [16]. For *S. thermophilus*, Iso-Sensitest broth (90%) supplemented with M17 broth medium (10%) and lactose (0.5%) was used [17,18]. The inoculum of each test strain was prepared by suspending single colonies from the LSM agar plates in 5 mL of 0.85% NaCl solution to a turbidity of McFarland 0.5 standard and subsequently diluting these to a ratio of 1:10 in NaCl solution. Then the inoculated plates were incubated in the presence of 5% CO_2 at 37°C for 24 hours. The MIC values of each antimicrobial were visually evaluated as the lowest concentrations at which no growth was observed. *Enterococcus faecalis* ATCC 29212 and *Staphylococcus aureus* ATCC 29213 were used as control strains. The assay was repeated on 3 independent occasions and in duplicate each time. Because there is no definitive breakpoint list for LAB, the interpretation for susceptibility status was determined by comparing the MIC values to the proposed breakpoints of previous studies (Table 1). Strains with MIC equal to or higher than the reported breakpoints were considered resistant.

Amplification of antimicrobial resistance genes

DNA was extracted from LAB strains using a commercial kit (Tiangen Biotech, Beijing, China) according to the manufacturer's instructions. Antimicrobial resistance genes for *β*-lactam (*bla*), chloramphenicol (*cat*), ciprofloxacin (*qnrA*, *qnrB*, and

Table 2. Primers used for PCR in this study.

Target gene	Primer	Sequence (5'-3')	Annealing temperature (°C)	Amplicon size (bp)	Reference
strA	strA-F	CTTGGTGATAACGGCAATTC	55	548	[20]
	strA-R	CCAATCGCAGATAGAAGGC			
strB	strB-F	ATCGTCAAGGGATTGAAACC	56	509	[20]
	strB-R	GGATCGTAGAACATATTGGC			
ant(6)	ant6-F	ACTGGCTTAATCAATTTGGG	53	597	[20]
	ant6-R	GCCTTTCCGCCACCTCACCG			
qnrA	qnrA-F	TCAGCAAGAGGATTTCTCA	48	627	[42]
	qnrA-R	GGCAGCACTATTACTCCCA			
qnrB	qnrB-F	ATGACGCCATTACTGTATAA	53	562	[42]
	qnrB-R	GATCGCAATGTGTGAAGTTT			
qnrS	qnrS-F	ACCTTCACCGCTTGCACATT	57	571	[42]
	qnrS-R	CCAGTGCTTCGAGAATCAGT			
aac(6')-aph(2'')	aac-F	CCAAGAGCAATAAGGGCATACC	58	675	[42]
	aac-R	ACCCTCAAAAACTGTTGTTGC			
*tet*K	tetK-F	TCGATAGGAACAGCAGTA	55	169	[1]
	tetK-R	CAG CAG ATC CTA CTC CTT			
*tet*L	tetL-F	TCGTTAGCGTGCTGTCATTC	55	267	[23]
	tetL-R	GTATCCCACCAATGTAGCCG			
tetM	tetM-F	GTGGACAAAGGTACAACGAG	55	406	[23]
	tetM-R	CGGTAAAGTTCGTCACACAC			
tetO	tetO-F	AACTTAGGCATTCTGGCTCAC	55	515	[23]
	tetO-R	TCCCACTGTTCCATATCGTCA			
tetS	tetS-F	CATAGACAAGCCGTTGACC	55	667	[23]
	tetS-R	ATGTTTTTGGAACGCCAGAG			
tetW	tetW-F	GAGAGCCTGCTATATGCCAGC	55	168	[23]
	tetW-R	GGGCGTATCCACAATGTTAAC			
vanA	vanA-F	GCAAGTCAGGTGAAGATGG	58	394	[1]
	vanA-R	ACCTCGCCAACAACTAACGC			
vanB	vanB-F	ACCCTGTCTTTGTGAAGCCGGCAC	58	390	[1]
	vanB-R	CAAAAAAAGATCAACACGAGCAAGCCC			
sulI	sulI-F	TCACCGAGGACTCCTTCTTC	54	331	[21]
	sulI-R	CAGTCCGCCTCAGCAATATC			
sulII	sulII-F	CCTGTTTCGTCCGACACAGA	54	435	[21]
	sulII-R	GAAGCGCAGCCGCAATTCAT			

qnrS), erythromycin (*emrA*, *emrB*, and *emrC*), gentamycin (*aac(6')-aph(2")*), streptomycin (*strA*, *strB*, and *ant(6)*), sulfamethoxazole (*sulI* and *sulII*), tetracycline (*tetK*, *tetL*, *tetM*, *tetO*, *tetS*, and *tetW*), and vancomycin (*vanA* and *vanB*) were PCR amplified by using the primers listed in Table 2. The PCR mixture consisted of 20 ng of bacterial DNA, 0.6 μM of each primer, 200 μM of deoxynucleoside triphosphate (Takara Bio Inc., Otsu, Shiga, Japan), 1× PCR buffer (Takara Bio Inc., Otsu, Shiga, Japan), and 0.5 U *Taq* DNA polymerase (Takara Bio Inc., Otsu, Shiga, Japan) in a total volume of 25 μL. The PCR conditions were as follows: initial denaturation at 94°C for 5 minutes followed by 30 cycles of denaturation at 94°C for 1 minute , annealing at different temperatures depending on the primer set for 1 minute , extension at 72°C for 1 minute, and a final extension at 72°C for 7 minutes. The purified PCR products were sequenced, and the DNA sequence data were analyzed using BLAST (http://www.ncbi.nlm.nih.gov/BLAST/).

Transfer of antimicrobial resistance

Transfer of antimicrobial resistance was analyzed by filter mating experiments as described by Feld *et al*. [19]. *Listeria monocytogenes* strain L82, isolated from quick frozen food made of flour in Hebei province, was used as the recipient. Antimicrobial susceptibility tests showed that L82 was sensitive to erythromycin, gentamycin, streptomycin, sulfamethoxazole, and tetracycline, and resistance to rifampin. Eighteen LAB strains harboring resistance genes were used as donors (Table 3). Briefly, equal volumes of donor and recipient strain were mixed, filtered through a sterile 0.45-μm pore size filter and placed on brain heart infusion (BHI; Land Bridge) agar plates. After incubation at 37°C for 24 h, the cells were suspended in phosphate buffer saline and spread on selective plates containing rifampin (8 μg/mL) and erythromycin (1 μg/mL) or gentamycin (8 μg/mL) or sulfamethoxazole (256 μg/mL) or tetracycline (2 μg/mL). Presumptive *L. monocytogenes* transconjugants were confirmed to be *L. monocytogenes* by using a *Listeria monocytogenes* biochemical identification kit (Land Bridge). PCR was used to confirm that the transconjugants carried the same resistance gene as their donors. Conjugation frequency was calculated as the ratio of the number of transconjugants to the number of recipient cells.

Results

Isolation and identification of LAB

A total of 58 strains of LAB, including *L. delbrueckii* subsp. *bulgaricus* (n=19), *L. plantarum* (n = 5), *L. paracasei* (n = 4), *L. acidophilus* (n = 2), and *S. thermophilus* (n = 28) were isolated from the yogurt samples. Nineteen of the LAB isolates belonging to species *L. delbrueckii* subsp. *bulgaricus* (n = 9) and *S. thermophilus* (n = 10) were isolated from the old yogurt samples. Five LAB isolates belonging to species *L. delbrueckii* subsp. *bulgaricus* (n = 3), *L. plantarum* (n = 1), and *S. thermophilus* (n = 1) were isolated from fermented dairy drink samples.

Table 3. Characteristics of LAB strains with antimicrobial resistance genes.

Strain	Source	Resistance phenotype[a]	Resistance gene
L. delbrueckii subsp. *bulgaricus* R2	Yogurt	SUL, TET	*sulI*, *tetM*
L. plantarum R3	Yogurt	GEN	*aac(6')-aph(2")*
L. delbrueckii subsp. *bulgaricus* R5	Yogurt	SUL	*sulI*
L. delbrueckii subsp. *bulgaricus* R6	Yogurt	SUL, TET	*sulI*, *tetM*
L. delbrueckii subsp. *bulgaricus* R8	Yogurt	GEN	*aac(6')-aph(2")*
L. delbrueckii subsp. *bulgaricus* R12	Yogurt	SUL	*sulI*
L. plantarum R18	Yogurt	GEN, SUL	*aac(6')-aph(2")*, *sulI*
L. delbrueckii subsp. *bulgaricus* R21	Yogurt	SUL	*sulI*
L. delbrueckii subsp. *bulgaricus* R26	Yogurt	SUL	*sulI*
L. delbrueckii subsp. *bulgaricus* R30	Yogurt	STR	*ant(6)*
L. delbrueckii subsp. *bulgaricus* R36	Old yogurt	TET	*tetM*
L. plantarum R41	Fermented dairy drink	TET	*tetS*
S. thermophilus S7	Yogurt	ERM	*ermB*
S. thermophilus S10	Yogurt	SUL	*sulI*
S. thermophilus S12	Yogurt	ERM	*ermB*
S. thermophilus S13	Yogurt	ERM	*ermB*
S. thermophilus S15	Yogurt	SUL	*sulI*
S. thermophilus S17	Yogurt	SUL	*sulI*, *sulII*

[a] ERM, erythromycin; GEN, gentamycin; STR, streptomycin; SUL, sulfamethoxazole; TET, tetracycline.

Genetic diversity of LAB by using PFGE typing

The genetic fingerprint of the 43 *Lactobacillus* isolates was determined using PFGE, and 24 distinct PFGE profiles were identified (Figure 1). The results of the analysis showed that 10 PFGE types occurred at least 2 times, accounting for 67.4% of the isolates characterized. A total of 14 PFGE types occurred only once and accounted for 32.6% of the strains. PFGE type LP24 predominated and included 6 isolates of *L. delbrueckii* subsp. *bulgaricus*.

PFGE was also conducted to determine the genetic relatedness of the 39 *S. thermophilus* isolates. However, 5 isolates failed to generate distinct PFGE pattern despite repeated attempts. The rest of the 34 isolates could be categorized into 32 different PFGE patterns (Figure 2). Only 2 PFGE types occurred 2 times, accounting for 11.8% of the strains characterized. A total of 30 PFGE types occurred once and accounted for 88.2% of the isolates.

Antimicrobial resistance phenotypes

The results of the sensitivity tests of the strains to 9 different antimicrobials are shown in Table 4. Among the 31 strains of *L. delbrueckii* subsp. *bulgaricus*, the most commonly observed resistance was that to streptomycin (96.8%), followed by gentamycin (93.5%), tetracycline (90.3%), and ciprofloxacin (87.1%). None of the *L. delbrueckii* subsp. *bulgaricus* strains exhibited resistance to erythromycin.

All of the 6 strains of *L. plantarum* exhibited resistance to streptomycin and vancomycin. Resistance to gentamycin, ciprofloxacin, sulfamethoxazole, chloramphenicol, and tetracycline was also observed. None of the strains exhibited resistance to ampicillin and erythromycin.

Four strains of *L. paracasei* exhibited sensitivity to all tested antimicrobials except for vancomycin, ciprofloxacin, and sulfamethoxazole.

For the 2 strains of *L. acidophilus*, resistance to gentamycin, ciprofloxacin, and tetracycline was observed. Both strains were susceptible to the rest of the antimicrobials.

Among the 39 strains of *S. thermophilus*, the most commonly observed resistance was that to streptomycin (92.3%), followed by gentamycin (87.2%), ciprofloxacin (79.5%), and chloramphenicol (71.8%). A small percentage of the strains were resistant to erythromycin.

Detection of antimicrobial resistance genes

The presence of antimicrobial resistance genes in all the LAB strains was determined by PCR analysis (Table 3). None of the resistance genes was detected in corresponding susceptible strains. For example, *sul* genes were found only in sulfamethoxazole-resistant strains, not in sulfamethoxazole-sensitive strains.

Figure 1. Dendrogram of PFGE patterns based on *Asc*I digestion of 43 *Lactobacillus* strains.

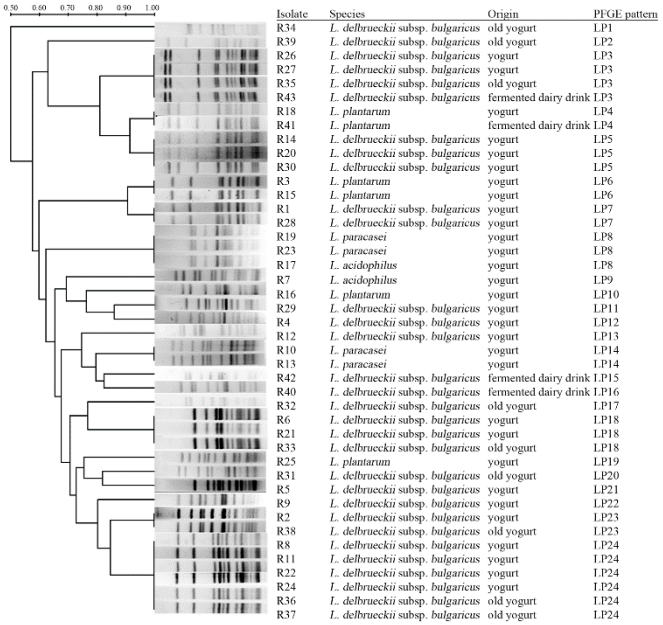

Figure 2. Dendrogram of PFGE patterns based on *Sma*I digestion of 34 *S. thermophilus* strains.

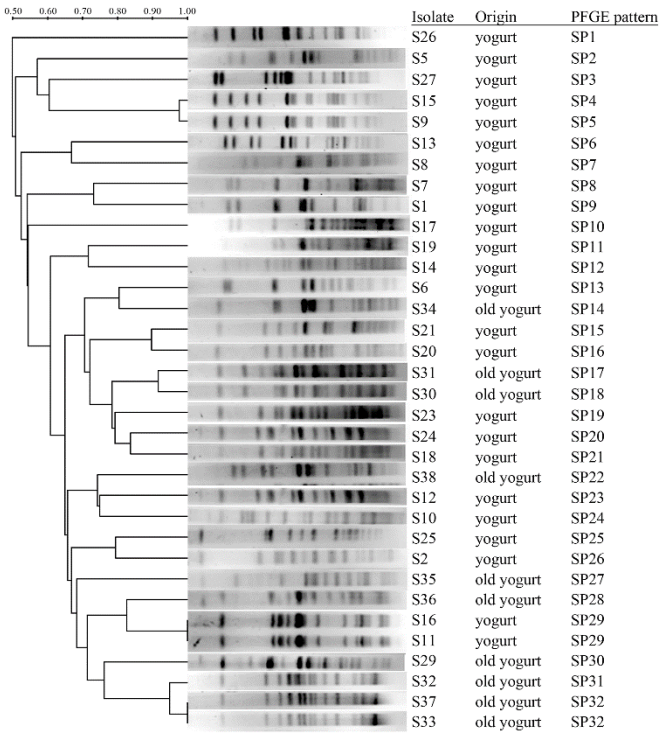

Table 4. Minimum inhibitory concentration (MIC) values of selected antimicrobials against LAB strains.

Antimicrobial	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	
Ampicillin	*L. delbrueckii* subsp. *bulgaricus* (31)	6	4	14	5	2									7 (22.6)
	L. plantarum (6)	4	1	1											0
	L. paracasei (4)	3	1												0
	L. acidophilus (2)		2												0
	S. thermophilus (39)	6	3	8	6	15	1								16 (41.0)
	Total (82)														23 (28.0)
Chloramphenicol	*L. delbrueckii* subsp. *bulgaricus* (31)				3	4	14	8	2						24 (77.4)
	L. plantarum (6)					1	4	1							1 (16.7)
	L. paracasei (4)				1	3									0
	L. acidophilus (2)				1	1									0
	S. thermophilus (39)			2	5	4	15	6	3	4					28 (71.8)
	Total (82)														53 (64.6)
Ciprofloxacin	*L. delbrueckii* subsp. *bulgaricus* (31)			1	1	2	8	5	7	3	4				27 (87.1)
	L. plantarum (6)			1		2		2		1					3 (50.0)
	L. paracasei (4)			1		1	1	1							2 (50.0)
	L. acidophilus (2)				1			1							1 (50.0)
	S. thermophilus (39)			2	1	5	8	7	7	5	4				31 (79.5)
	Total (82)														64 (78.0)
Erythromycin	*L. delbrueckii* subsp. *bulgaricus* (31)	17	5	9											0
	L. plantarum (6)	5		1											0
	L. paracasei (4)		4												0
	L. acidophilus (2)	1	1												0
	S. thermophilus (39)	17	11	6	2	2		1							3 (7.7)
	Total (82)														3 (3.7)
Gentamycin	*L. delbrueckii* subsp. *bulgaricus* (31)						2		5	4	9	11			29 (93.5)
	L. plantarum (6)							1	3	1	1				5 (83.3)
	L. paracasei (4)						1	2	1						0
	L. acidophilus (2)								2						2 (100)
	S. thermophilus (39)								5	15	9	10			34 (87.2)
	Total (82)														70 (85.4)
Streptomycin	*L. delbrueckii* subsp. *bulgaricus* (31)							1	1	2	10	17			30 (96.8)
	L. plantarum (6)									4	1	1			6 (100)
	L. paracasei (4)						2	2							0
	L. acidophilus (2)							2							0
	S. thermophilus (39)								3	12	10	14			36 (92.3)
	Total (82)														72 (87.8)

Table 4 *(continued)*. Minimum inhibitory concentration (MIC) values of selected antimicrobials against LAB strains.

Antimicrobial	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	
Sulfamethoxazole	*L. delbrueckii* subsp. *bulgaricus* (31)											2	17	12	12 (38.7)
	L. plantarum (6)												4	2	2 (33.3)
	L. paracasei (4)											2	1	1	1 (25.0)
	L. acidophilus (2)											2			0
	S. thermophilus (39)										4	11	19	5	5 (12.8)
	Total (82)														20 (24.4)
Tetracycline	*L. delbrueckii* subsp. *bulgaricus* (31)					3	11	16	1						28 (90.3)
	L. plantarum (6)				1	1	2		1	1					1 (16.7)
	L. paracasei (4)				2	2									0
	L. acidophilus (2)					1	1								1 (50.0)
	S. thermophilus (39)				5	17	5	4	8						17 (43.6)
	Total (82)														47 (57.3)
Vancomycin	*L. delbrueckii* subsp. *bulgaricus* (31)	7	3	8	2	4	4	2	1						11 (35.5)
	L. plantarum (6)							2	2	1	1				6 (100)
	L. paracasei (4)							1	3						4 (100)
	L. acidophilus (2)	1		1											0
	S. thermophilus (39)	4	8	8	3	4	4	7	1						12 (30.8)
	Total (82)														33 (40.2)

Seven resistance genes (*ermB*, *aac(6')-aph(2")*, *ant(6)*, *sulI*, *sulII*, *tetM* and *tetS*) conferring resistance to 5 antimicrobials (erythromycin, gentamycin, streptomycin, sulfamethoxazole and tetracycline) were detected in 18 resistant LAB strains. Among these strains, 14 strains carried 1 resistance gene and 4 strains harbored 2 different resistance genes.

Transfer of antimicrobial resistance genes

Eighteen LAB strains that tested positive for resistance genes were used as donors, whereas *L. monocytogenes* L82 was used as the recipient strain in the filter mating experiments. The results showed that the *tetM* gene from *L. delbrueckii* subsp. *bulgaricus* R6 (resistance to tetracycline at 8 μg/mL) and the *tetS* gene from *L. plantarum* R41 (resistance to tetracycline at 32 μg/mL) were successfully transferred to the recipient. All transconjugants were resistant to tetracycline and positive for *tetM* or *tetS* genes. The transfer frequency in the filter mating experiments was 7.3×10^{-7} for *L. delbrueckii* subsp. *bulgaricus* R6 and 2.9×10^{-6} for *L. plantarum* R41.

Discussion

PFGE, which is considered as the gold standard for bacterial molecular typing due to its strong discriminatory power, was employed to investigate genetic diversity of LAB strains from fermented food samples. In the present study, 43 *Lactobacillus* strains yielded 24 PFGE patterns, whereas 55.8% of the strains displayed a different PFGE profile. A similar genetic diversity was also observed among *Lactobacillus* strains from fermented dairy products in China, representing 51.5% of the distinguishable PFGE patterns [12]. PFGE type LP8 consisted of 3 strains of *Lactobacillus* (*L. paracasei* R19 and R23, and *L. acidophilus* R17). Strains belonging to different species exhibited the same PFGE pattern, which has also been reported in other study [12]. The restriction enzyme used in our study could give good results for the majority of *Lactobacillus* strains, except for three strains of LP8. Maybe, the strains of LP8 could be differentiated by PFGE using another enzyme or by other molecular typing methods, such as random amplified polymorphic DNA (RAPD). Here, 32 PFGE patterns were observed, thereby revealing a high genetic diversity among the 34 strains of *S. thermophilus*. Erkus *et al*. [15] recently reported that 61 *S. thermophilus* strains from artisanal Yuruk yogurts yielded 22 distinct PFGE types. However, 5 strains of *S. thermophilus* failed to produce discrete PFGE patterns. There is the possibility that the restriction enzyme used in the present study (*Sma*I) was not optimal for those strains. Further studies are needed to distinguish the LAB strains by employing other restriction enzymes for PFGE or other molecular typing methods.

Resistance to streptomycin, gentamycin, and ciprofloxacin was observed most frequently in the 82 LAB strains (Table 4), which was consistent with the findings of previous studies [9,20,21]. Our results also supported the view that lactobacilli are generally intrinsically resistant to quinolones and aminoglycosides [3,9]. The resistance of lactobacilli to sulphonamides is also considered as intrinsic [22]. However, a low level of resistance to sulfamethoxazole (34.9%, 15/43 strains) was observed in the strains of lactobacilli investigated in the present study. This difference may due to the different media used in the MIC determination as well as differences in the origin of the strains. More than half of the LAB strains exhibited resistance to tetracycline and chloramphenicol, which was in agreement with the findings of Zhou *et al*. [20]. Generally, LAB are susceptible to antimicrobials such as erythromycin, which inhibits protein synthesis [23]. In this study, the majority of the LAB strains were sensitive to erythromycin. A widespread susceptibility toward the inhibitors of cell wall synthesis (such as ampicillin and penicillin) has been observed in various species of lactobacilli that were from different sources, including cheese [24], probiotics or fermented foods [20], and human intestine [25]. Similarly most of the LAB strains tested in the present study were found to be sensitive to ampicillin.

The *ermB* gene was detected in 3 erythromycin-resistant strains. This finding was consistent with the results of previous reports that the *ermB* gene was more frequently detected in LAB strains than other resistance determinants (*ermA* and *ermC*) [1,23]. The *aac(6')-aph(2")* gene encodes the bifunctional enzyme 6'-acetyltransferase-2"-phosphotransferase, which confers resistance to all aminoglycosides, except for streptomycin. This gene is commonly detected in high-level gentamycin-resistant *Enterococcus faecalis* isolates [26]. The *ant(6)* gene, which is associated with streptomycin resistance in enterococci [27], was detected in one *L. delbrueckii* subsp. *bulgaricus* strain. The sulfamethoxazole resistance genes *sulI* and *sulII* have been reported in various bacterial species [28]. However, information on the presence of these genes in LAB strains is limited. It is worth noting that the *sul* gene was detected in lactobacilli and *S. thermophilus* strains in our study. Several tetracycline-resistant

determinants (*tetM*, *tetO*, *tetS*, and *tetW*) or the efflux pump proteins (*tetK* and *tetL*) have also been reported [29]. In the current study, the *tetM* gene was detected in *L. delbrueckii* subsp. *bulgaricus* isolated from yogurt and old yogurt. This gene has also been previously reported in *L. plantarum* that was isolated from cheese [30], fermented vegetables [3], and meat products [1]. In addition, 1 strain of *L. plantarum* from fermented dairy drink harbored the *tetS* gene. Other tetracycline resistance genes have also been identified in lactobacilli and *S. thermophilus* [7,31]; however, these were not detected in any of these strains in the present study. The corresponding resistance genes were absent in various resistant strains, thereby suggesting that other resistance mechanisms may be responsible for their resistance to antimicrobial drugs.

The transfer of conjugative plasmids is known to be the most common mechanism for genetic exchange between bacteria, as plasmid conjugation can occur at high frequency and is able to transfer resistance genes [32]. To date, several *in vitro* studies on conjugative transfer of antimicrobial resistance determinants from lactobacilli to other bacteria have been reported [33,34]. An erythromycin-resistant plasmid (pLFE1) could be transferred from *L. plantarum* to *L. rhamnosus*, *Lactococcus lactis*, and *E. faecalis* [19]. Toomey *et al*. [1] reported that the *tetM* gene of *L. plantarum* was transferred to *L. lactis* BU-2-60 and *E. faecalis* JH2-2 in mating experiments at a low conjugation frequency. In a recent study, the *ermB* gene from *L. fermentum* and *L. salivarius*, and the *tetM* gene from *L. plantarum* and *L. brevis* were successfully transferred to *E. faecalis* 181 [3]. In several previous studies, *E. faecalis* was often used as recipient [3,19]. In the present study, *L. monocytogenes*, an important foodborne pathogen, was selected as recipient. Only the *tetM* gene from *L. delbrueckii* subsp. *bulgaricus* and the *tetS* gene from *L. plantarum* were successfully transferred to the recipient *L. monocytogenes* L82, indicating that the *tet* genes could be located on conjugative plasmids in the two strains. Furthermore, the transfer of resistant genes from commensal bacteria to pathogens in the human intestine has been reported, potentially resulting in food poisoning that is more difficult to treat with conventional antimicrobial agents [35]. Thus, it is possible that the *tet* genes of LAB strains also could be transferred to *L. monocytogenes* in human intestine. Further studies should be performed to provide more evidence for supporting this speculation. Besides, localizing the *tet* genes and investigating the conjugative plasmids in the LAB strains are also our main future work. The rest of the 16 strains failed to produce transconjugants, possibly because the resistance genes of some strains are located on chromosomes. Hummel *et al*. [9] earlier showed that the *ermB* gene, which is involved in erythromycin resistance in a *L. salivarius* strain, occurred in chromosomal DNA. There is also the possibility that in some strains, the resistance genes may be located on non-conjugative plasmids that are not competent to transfer via conjugation.

Conclusion

LAB strains tested in our study exhibited resistance to several antimicrobials that are commonly used in the clinics and veterinary hospitals. However, the data on antimicrobial resistance should be interpreted with caution because our study just evaluated a limited number of LAB strains from three types of samples, indicating that it could not be representative of the LAB from fermented dairy products in China. The distribution and transfer of antimicrobial resistance genes among LAB strains suggests that these strains may potentially act as reservoirs of resistance genes and play an active role in the transfer of resistance to humans through the food chain. Therefore, continuous surveillance of antimicrobial resistance in LAB strains from fermented dairy products is imperative and more attention should be paid to evaluating the safety of LAB strains that are used as starters or probiotics, particularly determining the presence of transferable resistance genes in these strains.

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