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ORIGINAL ARTICLE

Characterization of quinolone resistance mechanisms in lactic acid bacteria isolated from yogurts in China

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Abstract Twenty-five strains of ciprofloxacin-resistant lactic acid bacteria (LAB; 14 *Lactobacillus* spp. and 11 *Streptococcus thermophiles* spp.) isolated from commercial yogurts in China were analyzed in this study. For each of these strains, amino acid changes associated with quinolone resistance-determining regions (QRDRs) were investigated using PCR-based detection methods. The same methodology was used to identify the presence of plasmid-mediated quinolone resistance (PMQR) genes in LAB. Sequencing analyses and an efflux pump inhibition test (using reserpine) were also performed as part of the analysis. Our results showed that typical mutations corresponding to quinolone resistance were found in the QRDRs of LAB strains. Detected mutations included Ser80Leu in *parC*, and Ser83Leu and Glu87Asp in *gyrA*. In addition, a Tyr74Phe substitution in *parC*, which had not previously been reported to be associated with quinolone resistance, was observed in two *Lactobacillus delbrueckii* subsp. *bulgaricus* strains. For each of the tested strains, the presence of the efflux pump inhibitor, reserpine, resulted in a two- to eightfold reduction in the minimum inhibitory concentrations (MICs) of ciprofloxacin. However, PMQR genes were not observed in any of the strains analyzed. Our results suggest that mutations in the QRDRs or efflux pump could be involved in ciprofloxacin resistance, and that a combination of these mechanisms may lead to increased ciprofloxacin resistance in LAB strains.

Keywords Lactic acid bacteria · Quinolone resistance · Yogurt

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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 produce lactic acid (Klaenhammer et al. 2005). LAB are commonly found in foods and are also natural intestinal inhabitants in humans and most animals (Zhou et al. 2012). Among LAB, *Lactobacillus* species and *Streptococcus thermophilus* are used as starter cultures for probiotic yogurt production. These microorganism species can facilitate acid production along with a number of other important biological metabolic activities, including glycolysis. The resultant activities give rise to milk protein transformations, thereby contributing to the flavor and texture of yogurt (Bourdichon et al. 2012).

There is an extended history associated with the safe consumption of traditionally fermented foods, and as a result, most LAB species have been deemed safe (Arioli et al. 2013). Consequently, the characterization of these bacteria, particularly in the area of antimicrobial resistance, has been neglected. However, several studies have recently reported that LAB strains isolated from foods exhibit high resistance to certain antimicrobial agents, such as aminoglycosides and fluoroquinolones (D'Aimmo et al. 2007; Hummel et al. 2007; Rodriquez-Alonso et al. 2009; Zhang et al. 2013; Zhou et al. 2012). Some studies have suggested that resistant LAB strains facilitate the transmission of resistance genes to pathogenic

bacteria through horizontal gene transfer, thereby potentiating the risk to public health (Jacobsen et al. 2007; Nawaz et al. 2011; Toomey et al. 2010).

Quinolones are broad-spectrum antimicrobial agents that have been widely used in the clinical domain. However, a sharp rise in resistance to quinolones has been seen in China in recent years (Wang et al. 2003). To date, three major mechanisms of quinolone resistance have been established: (i) point mutations in the quinolone resistance-determining regions (QRDRs) of the genes encoding gyrase (*gyrA* and *gyrB*) and topoisomerase (*parC* and *parE*); (ii) decreased accumulation inside bacteria due to overexpression of efflux pump systems; and (iii) plasmid-mediated quinolone resistance (PMQR; Hernández et al. 2011). Previous studies have reported that resistance to ciprofloxacin, a common fluoroquinolone, was frequently observed in LAB strains (D'Aimmo et al. 2007; Hummel et al. 2007; Rodriquez-Alonso et al. 2009). To the best of our knowledge, little data has been generated pertaining to the characterization of quinolone resistance mechanisms in LAB strains (Hummel et al. 2007). In contrast, many similar studies have been performed in relation to other bacterial species such as *Escherichia coli* (Jiang et al. 2014a), *Salmonella* (Kim et al. 2011), *Pseudomonas aeruginosa* (Jiang et al. 2014b), and *Staphylococcus aureus* (Ito et al. 1994). In summary, the objective of this study was to investigate the characteristics associated with quinolone resistance mechanisms in yogurt-based LAB strains.

Materials and methods

Strains of LAB

A total of 58 strains of LAB, including *Lactobacillus delbrueckii* subsp. *bulgaricus* ($n=19$), *Lactobacillus plantarum* ($n=5$), *Lactobacillus paracasei* ($n=4$), *Lactobacillus acidophilus* ($n=2$), and *Streptococcus thermophilus* ($n=28$), were isolated from 30 commercial yogurts between May and August, 2014, in the province of Henan. Samples representing seven brands were purchased from four different supermarkets. Isolates were initially identified using standard biochemical methods (including Gram stain, catalase test, and oxidase test). Further characterization was performed using commercial kits (Land Bridge Technology Co. Ltd., Beijing, China). The putative LAB strains identified as a result of this analysis were confirmed by PCR-based 16S rDNA sequencing, using universal primer pair 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3') (Moreno et al. 2002). The PCR mixture consisted of 20 ng of bacterial DNA, 0.6 μM of each primer, 200 μM deoxynucleotide triphosphate (Takara Bio Inc., Otsu, Shiga, Japan), 1× PCR buffer (Takara Bio), and 0.5 U *Taq* DNA polymerase (Takara Bio) in a total volume of 25 μL. The PCR conditions were as follows: initial denaturation at 94 °C for 5 min, followed by 30 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min, extension at 72 °C for 2 min, and a final extension at 72 °C for 7 min. The purified PCR products were sequenced, and DNA sequence data were analyzed using the BLAST program (http://www.ncbi.nlm.nih.gov/BLAST/).

Antimicrobial susceptibility testing

The MICs of ciprofloxacin were determined using the broth microdilution method. This method required LAB susceptibility test medium (LSM; Klare et al. 2005), a mixed formulation containing Iso-Sensitest broth (90 %; Oxoid Ltd., Basingstoke, Hampshire, UK) and MRS (Land Bridge Technology Co. Ltd., Beijing, China) or M17 (Land Bridge) broth medium (10 %). The ciprofloxacin (Sigma-Aldrich, St. Louis, MO, USA) MICs were determined using a concentration range of 0.125 to 64 μg/mL ciprofloxacin. Test strain inocula were prepared by suspending single colonies from LSM agar plates in 5 mL of an 0.85 % NaCl solution until 0.5 McFarland standard turbidity was achieved. The resuspensions were subsequently diluted (1:10) in NaCl. The inoculated plates were incubated in the presence of 5 % CO_2 at 37 °C for 24 h. MIC values for each antimicrobial agent were visually evaluated as the lowest concentration at which no growth was observed. *Enterococcus faecalis* ATCC 29212 and *Staphylococcus aureus* ATCC 29213 were used as control strains (Nawaz et al. 2011). The assay was repeated on three independent occasions. Each assay repeat was performed in duplicate.

PCR amplification and DNA sequencing of the QRDR

Genomic DNA was extracted from LAB strains using a TIANamp Bacteria DNA Kit (Tiangen Biotech, Beijing, China). The QRDRs of the *parC* and *gyrA* genes were amplified by PCR using the primers in Table 1. Both strands of the purified PCR products were sequenced, and the deduced amino acid sequences were aligned using the Geneious (version 8.0.5) Clustal W multiple alignment tool.

Efflux pump inhibition test by using reserpine

To determine the effect of efflux pumps on quinolone resistance, MICs of ciprofloxacin were examined using the broth microdilution method in the presence or absence of the inhibitor reserpine (final concentration, 20 μg/mL; Sigma-Aldrich). Experiments were repeated on three separate occasions. Control cells were grown in the presence of reserpine without ciprofloxacin. This was performed to confirm that reserpine did not have an inhibitory effect on cell growth.

Table 1 Primers used to detect the QRDR mutations in this study

Target gene	Primer	Sequence(5' to 3')	Annealing temperature (°C)	Amplicon size (bp)	Reference sequence
L. acidophilus					
gyrA	A1-F	GCGTAGTTCTTTCTTGGA	49	332	NC_006814
	A1-R	TCATTCTTGCTTCGGTAT			
parC	C1-F	CGATGTATCAAGATGGAA	47	412	NC_006814
	C1-R	CCTAAATTATGAGGTGGA			
L. delbrueckii subsp. *bulgaricus*					
gyrA	A2-F	CAAGCCTTACAAGAAGAGTGC	52	152	NC_008054
	A2-R	AACCGAAGTTACCGTGCC			
parC	C2-F	GTGAAATGCCTTTGGAACA	51	241	NC_008054
	C2-R	AGAGCCCCGTAGATAGAGC			
L. paracasei					
gyrA	A3-F	TGCGTAAATCATTCCTTG	50	241	NC_022112
	A3-R	CTGAAGTCCTGTGCCATT			
parC	C3-F	GTTCAGCGTCGCATTTTA	51	336	NC_022112
	C3-R	TTCCTGTGCCGTGTCATC			
L. plantarum					
gyrA	A4-F	ATTCTTTACGGTATGAGTGA	50	312	NC_014554
	A4-R	ATCATCATAGTTTGGTTGC			
parC	C4-F	GTTCCGTAAGTCCGCTAA	50	278	NC_014554
	C4-R	CGTACTCCGTATCATCAAAA			
S. thermophilus					
gyrA	A5-F	TCTTTATGGAATGAATGAG	48	245	NC_006449
	A5-R	TTTTAGACATACGAGCCT			
parC	C5-F	TCGTCCCTTTGTATCTTA	49	339	NC_006449
	C5-R	TAGAACCATTGTTACCGT			

Detection of *qnr* and *aac(6')-Ib-cr* genes

All 58 LAB strains were screened for the presence of *qnrA*, *qnrB*, *qnrC*, *qnrD*, *qnrS*, and *aac(6')-Ib-cr* genes by PCR amplification using previously described primers (Cavaco et al. 2009; Jiang et al. 2008; Wang et al. 2003, 2009).

Results

Antimicrobial susceptibility testing

The MIC distributions of the tested strains are presented in Table 2. As there is no definitive breakpoint of ciprofloxacin for LAB, the definition of ciprofloxacin resistance has varied significantly in previous studies (Table 3). In this study, *L. delbrueckii* subsp. *bulgaricus* and *S. thermophilus* strains with MIC≥8 μg/mL were considered ciprofloxacin-resistant. For the strains of *L. plantarum*, *L. paracasei*, and *L. acidophilus*, the MIC breakpoint (4 μg/mL) was adopted from European Commission (EUC) and other previous studies (Table 3).

According to the MIC breakpoints proposed in our study,25 (43.1 %) LAB strains were considered resistant to ciprofloxacin, including nine strains of *L. delbrueckii* subsp. *bulgaricus*, two strains of *L. plantarum*, two strains of *L. paracasei*, one strain of *L. acidophilus*, and 11 strains of *S. thermophilus*. All of the resistant strains were subjected to further investigation. In addition, 12 strains that were deemed susceptible to ciprofloxacin, including three strains of *L. delbrueckii* subsp. *bulgaricus*, three strains of *L. plantarum*, two strains of *L. paracasei*, one strain of *L. acidophilus*, and three strains of *S. thermophilus*, were included as negative controls.

Identification of mutations in the QRDRs

The QRDRs of *parC* and *gyrA* from ciprofloxacin-resistant strains were aligned with those of sensitive strains (Fig. 1). Mutations in the QRDRs of *parC* were observed in seven ciprofloxacin-resistant strains, including five strains that

Table 2 The distribution of MIC for ciprofloxacin among LAB strains

Species (no. of strains tested)	Number of strains with MIC (μg/mL) as follows									
	≤0.125	0.25	0.5	1	2	4	8	16	32	≥64
L. delbrueckii subsp. *bulgaricus* (19)			1	1	2	6	5	2		2
L. plantarum (5)			1		2		1		1	
L. paracasei (4)			1		1	1	1			
L. acidophilus (2)				1			1			
S. thermophilus (28)			2	1	6	8	6	3	2	

contained a single mutation and two strains that harbored double mutations (Table 4). For each of the four *L. delbrueckii* subsp. *bulgaricus* strains (R9, R11, R12, and R14), serine was substituted with leucine at position 83 (80 in *E. coli* numbering). In addition, the replacement of tyrosine with phenylalanine at position 77 (74 in *E. coli*) was observed in *L. delbrueckii* subsp. *bulgaricus* strains R11 and R12. For *L. acidophilus* R7, serine was substituted with leucine at position 82 (80 in *E. coli*). A similar substitution was observed at position 83 (80 in *E. coli*) in *L. plantarum* strains R3 and R1.

Mutations in *gyrA* were observed in three ciprofloxacin-resistant strains, including two strains that contained a single mutation and one that harbored a double mutation (Table 4). For the two *L. delbrueckii* subsp. *bulgaricus* strains (R11 and R12), serine was substituted with leucine at position 87 (83 in *E. coli*). *L. plantarum* R18 had double mutations at positions 86 (Ser86Leu) and 90 (Glu90Asp), corresponding to positions 83 and 87 in *E. coli*, respectively.

Three strains contained mutations in both *parC* and *gyrA* genes, while mutations in *parC* only were observed for four additional strains. However, mutations in the QRDRs were not identified in any of the *L. paracasei*- or *S. thermophilus*-resistant strains that were analyzed.

Table 3 MIC breakpoints for ciprofloxacin of *Lactobacillus* species and *Streptococcus thermophilus* used in food or as probiotics in this study

Species	Proposed breakpoint, MIC (μg/mL)		
	This article	EUC[a]	Other articles
L. delbrueckii subsp. *bulgaricus*	8	4	4[b,c], >32[d]
L. plantarum	4	4	4[b,c], >32[d]
L. paracasei	4	4	4[b,c], >32[d]
L. acidophilus	4	4	4[b,c], >32[d]
S. thermophilus	8	4	4[b]

[a] Breakpoint for ciprofloxacin defined by the European Commission 2001 for *Lactobacillus* spp.

[b] Breakpoint for ciprofloxacin suggested by Katla et al. 2001 for *Lactobacillus* spp. and *Streptococcus* spp.

[c] Breakpoint for ciprofloxacin suggested by Zarazaga et al. 1999 for *Lactobacillus* spp.

[d] Breakpoint for ciprofloxacin suggested by Danielsen and Wind 2003 for *Lactobacillus* spp.

ParC

Strain		70 … 80 … 90
E. coli str. K-12 substr. MG1655	S	VLGKYHPHGDSACYEAMVLMAQP
L. delbrueckii subsp. *bulgaricus* R4	S	IMGNYHPHGDSSIYGALV
L. delbrueckii subsp. *bulgaricus* R5	S	IMGNYHPHGDSSIYGALV
L. delbrueckii subsp. *bulgaricus* R24	S	IMGNYHPHGDSSIYGALV
L. delbrueckii subsp. *bulgaricus* R9	R	IMGNYHPHGDLSIYGALV
L. delbrueckii subsp. *bulgaricus* R11	R	IMGNFHPHGDLSIYGALV
L. delbrueckii subsp. *bulgaricus* R12	R	IMGNFHPHGDLSIYGALV
L. delbrueckii subsp. *bulgaricus* R14	R	IMGNYHPHGDLSIYGALV
L. plantarum R15	S	VMGNFHPHGDSSIYEAMVRLSQD
L. plantarum R16	S	VMGNFHPHGDSSIYEAMVRLSQD
L. plantarum R25	S	VMGNFHPHGDSSIYEAMVRLSQD
L. plantarum R3	R	VMGNFHPHGDLSIYEAMVRLSQD
L. plantarum R18	R	VMGNFHPHGDLSIYEAMVRLSQD
L. acidophilus R17	S	IMGNYHPHGDSSIYGALVHLSQD
L. acidophilus R7	R	IMGNYHPHGDLSIYGALVHLSQD

GyrA

Strain		73 … 80 … 90
E. coli str. K-12 substr. MG1655	S	VIGKYHPHGDSAVYDTIVRMAQP
L. delbrueckii subsp. *bulgaricus* R4	S	VMGKYHPHGDSSIYLAMAHMAQD
L. delbrueckii subsp. *bulgaricus* R5	S	VMGKYHPHGDSSIYLAMAHMAQD
L. delbrueckii subsp. *bulgaricus* R24	S	VMGKYHPHGDSSIYLAMAHMAQD
L. delbrueckii subsp. *bulgaricus* R11	R	VMGKYHPHGDLSIYLAMAHMAQD
L. delbrueckii subsp. *bulgaricus* R12	R	VMGKYHPHGDLSIYLAMAHMAQD
L. plantarum R15	S	VMGKYHPHGDSAIYESMVRMAQD
L. plantarum R16	S	VMGKYHPHGDSAIYESMVRMAQD
L. plantarum R25	S	VMGKYHPHGDSAIYESMVRMAQD
L. plantarum R18	R	VMGKYHPHGDLAIYDSMVRMAQD

Fig. 1 Comparison of the QRDR of ciprofloxacin-resistant (R) and ciprofloxacin-sensitive (S) strains. Numbering of sequences according to *E. coli*. Substituted amino acids are marked with shading

Efflux pump inhibition with reserpine

The MICs of ciprofloxacin were reduced in all resistant strains following exposure to reserpine. Of those analyzed, four, 15, and four resistant strains demonstrated eight-, four-, and two-fold reductions in the MIC of ciprofloxacin, respectively. Pronounced decreases in the MIC value of ciprofloxacin were not detected for strains R11 or R12. Following the alteration in the ciprofloxacin MIC values observed in the presence of reserpine, the resistance phenotype of 19 strains changed from resistant to susceptible, and six strains remained resistant.

Detection of *qnr* and *aac(6')-Ib-cr* genes

The *qnr* and *aac(6')-Ib-cr* genes were not detected in any of the tested LAB strains.

Discussion

Until recently, the only reported quinolone-resistance mechanisms utilized by bacteria have involved mutations in target genes associated with quinolone activity. Indeed, mutations in gyrase and topoisomerase remain the most prevalent mechanism by which bacterial populations acquire resistance to

Table 4 Characteristics of quinolone resistance in LAB strains

Strain	Organism	QRDR mutations[a]		MIC (μg/mL)[b]	
		ParC	GyrA	CIP	CIP + RES
CIP^R					
R1	*L. delbrueckii* subsp. *bulgaricus*	WT	WT	8	1
R2	*L. delbrueckii* subsp. *bulgaricus*	WT	WT	8	2
R8	*L. delbrueckii* subsp. *bulgaricus*	WT	WT	8	2
R9	*L. delbrueckii* subsp. *bulgaricus*	Ser83Leu	WT	32	8
R11	*L. delbrueckii* subsp. *bulgaricus*	Tyr77Phe, Ser83Leu	Ser87Leu	>32	32
R12	*L. delbrueckii* subsp. *bulgaricus*	Tyr77Phe, Ser83Leu	Ser87Leu	>32	32
R14	*L. delbrueckii* subsp. *bulgaricus*	Ser83Leu	WT	16	8
R20	*L. delbrueckii* subsp. *bulgaricus*	WT	WT	8	2
R30	*L. delbrueckii* subsp. *bulgaricus*	WT	WT	8	2
R3	*L. plantarum*	Ser83Leu	WT	8	2
R18	*L. plantarum*	Ser83Leu	Ser86Leu, Glu90Asp	32	8
R10	*L. paracasei*	WT	WT	4	1
R23	*L. paracasei*	WT	WT	8	1
R7	*L. acidophilus*	Ser82Leu	WT	8	4
S3	*S. thermophilus*	WT	WT	32	4
S6	*S. thermophilus*	WT	WT	32	4
S7	*S. thermophilus*	WT	WT	16	4
S8	*S. thermophilus*	WT	WT	8	2
S9	*S. thermophilus*	WT	WT	8	2
S10	*S. thermophilus*	WT	WT	8	4
S12	*S. thermophilus*	WT	WT	8	4
S14	*S. thermophilus*	WT	WT	8	2
S17	*S. thermophilus*	WT	WT	16	4
S23	*S. thermophilus*	WT	WT	16	4
S24	*S. thermophilus*	WT	WT	8	2
CIP^S					
R4	*L. delbrueckii* subsp. *bulgaricus*	WT	WT	2	ND
R5	*L. delbrueckii* subsp. *bulgaricus*	WT	WT	4	ND
R24	*L. delbrueckii* subsp. *bulgaricus*	WT	WT	0.5	ND
R15	*L. plantarum*	WT	WT	0.5	ND
R16	*L. plantarum*	WT	WT	2	ND
R25	*L. plantarum*	WT	WT	2	ND
R13	*L. paracasei*	WT	WT	0.5	ND
R19	*L. paracasei*	WT	WT	2	ND
R17	*L. acidophilus*	WT	WT	1	ND
S5	*S. thermophilus*	WT	WT	2	ND
S13	*S. thermophilus*	WT	WT	0.5	ND
S21	*S. thermophilus*	WT	WT	0.5	ND

[a] WT, wild type

[b] CIP, ciprofloxacin; RES, reserpine; ND, not determined

quinolones (Jacoby 2005). Some researchers have suggested that mutations in the QRDRs of *gyrA* and/or *parC* genes play an important role in ciprofloxacin resistance in various Gram-positive and Gram-negative bacteria (Hernández et al. 2011). In Gram-positive bacteria, different fluoroquinolones exhibit different levels of inhibitory activity against the two enzymes, gyrase and topoisomerase. Topoisomerase IV is usually the primary target of ciprofloxacin in staphylococci, streptococci, and enterococci (Betanzos-Cabrera et al. 2009; Muñoz and de la Campa 1996; Onodera et al. 2002). As part of this study,

ciprofloxacin resistance was observed in *L. delbrueckii* subsp. *bulgaricus*, *L. plantarum*, and *L. acidophilus* strains that harbored substitution mutations (Ser 80 is typically substituted with Leu) in the ParC subunit of topoisomerase IV. Interestingly, a Tyr74-to-Phe substitution was observed in two *L. delbrueckii* subsp. *bulgaricus* strains. Indeed, the same substitution was observed in an *L. acidophilus* strain in a previous study (Hummel et al. 2007). However, to date, there is no evidence to suggest that this substitution is associated with quinolone resistance. Further studies are needed to investigate the relationship between the Tyr74-to-Phe mutation and quinolone resistance in these strains. Mutations in Ser83 and the Glu87 of GyrA have been reported to reduce susceptibility to ciprofloxacin in *Enterococcus faecium* (Petersen and Jensen 2004). In our study, a Ser83-to-Leu substitution of the GyrA subunit of gyrase was observed in *L. delbrueckii* subsp. *bulgaricus* and *L. plantarum* strains. In addition, a single *L. plantarum* strain contained the substitution at position 87. Interestingly, mutations in the *gyrA* gene were only observed in the strains that co-harbored *parC* mutations. These results are consistent with previous findings that mutations initially occurred in *parC* in Gram-positive bacteria (Chen et al. 1996; Hernández et al. 2011; Muñoz and de la Campa 1996).

The characterization of efflux pumps in bacteria has facilitated research into the mechanisms involved in quinolone resistance (Li and Nikaido 2009). Quinolones are among the most common substrates associated with efflux pumps (Hooper 1999). To date, efflux pumps such as Lde in *Listeria monocytogenes*, NorA in *Staphylococcus aureus*, and MexAB-OprM in *Pseudomonas aeruginosa* have been shown to be responsible for the extrusion of quinolones in both Gram-positive and Gram-negative bacteria (Godreuil et al. 2003; Masuda et al. 2000; Neyfakh et al. 1993). Chromosomally encoded efflux pumps are usually expressed at low levels, thereby contributing to the intrinsic resistance to quinolones (Grkovic et al. 2002). However, overexpression of these pumps in some mutants can result in an increase in the MICs of quinolones (Jiang et al. 2012; Noquchi et al. 2004; Xia et al. 2011). In order to assess the effect of efflux pumps in relation to ciprofloxacin activity, we compared the MICs of LAB strains in the presence and absence of reserpine. Reserpine is a potent inhibitor that can inhibit efflux pump members of the resistance–nodulation–division family, major facilitator family, and the ATP binding cassette (Stavri et al. 2007). Reserpine has been frequently used to indirectly determine the role of efflux pumps in *L. monocytogenes*, *S. aureus*, and *Streptococcus pneumoniae* (Frempong-Manso et al. 2009; Garvey and Piddock 2008; Godreuil et al. 2003). Following the analysis of resistant strains in the presence of this inhibitor, reserpine reduced the MIC of ciprofloxacin by twofold or greater. This indicates that the activity of efflux pumps was inhibited by reserpine and suggests that efflux pumps might contribute to the high MIC values of ciprofloxacin among these strains. Following exposure to reserpine, all of the 18 ciprofloxacin-resistant strains that did not harbor QRDR mutations were rendered susceptible, suggesting a strong correlation between the activity of efflux pumps and ciprofloxacin resistance in these strains. The ciprofloxacin resistance phenotype was unaltered in the presence of reserpine in six of the seven LAB strains that contained QRDR mutations, which indicates that mutations had an important influence on ciprofloxacin resistance even when the efflux pump was inhibited. Interestingly, although mutations in QRDRs were detected in only a few of the resistant strains, the associated MICs of ciprofloxacin were higher than those observed in strains that lacked mutations. Our data demonstrate that efflux pumps play a role in ciprofloxacin resistance in resistant strains. Moreover, in some strains, mutations in target genes and efflux through the pump system appeared to occur simultaneously, leading to high-level resistance to ciprofloxacin. This finding was in agreement with previous studies demonstrating that quinolone resistance could be caused by more than one mechanism (Jiang et al. 2014a; Kim et al. 2011; Tanaka et al. 2000).

The emergence of PMQR has been reported since 1998, indicating that quinolone resistance can be also acquired through horizontal gene transfer (Martínez-Martínez et al. 1998). To date, several PMQR mechanisms have been identified. These mechanisms utilize a number of effector proteins including the Qnr proteins, the aminoglycoside acetyltransferase AAC(6')-Ib-cr, and the efflux pumps QepA and OqxAB (Poirel et al. 2012). The Qnr proteins protect target enzymes from quinolone inhibition. The AAC(6')-Ib-cr determinant acetylates several fluoroquinolones, including norfloxacin and ciprofloxacin. Finally, the QepA and OqxAB efflux pumps can extrude fluoroquinolones from the bacterial cell. Numerous studies have described the presence of PMQR genes in Gram-negative bacteria, including Enterobacteriaceae, *Aeromonas* spp., *Acinetobacter baumannii*, and *Pseudomonas aeruginosa* (Cattoir et al. 2008; Jiang et al. 2014a, b). To the best of our knowledge, PMQR determinants have not been detected in Gram-positive bacteria. In the current study, LAB strains were screened for *qnr* and *aac(6')-Ib-cr* genes. None of these genes were observed in the 58 strains that were analyzed, demonstrating that PMQR did not participate in ciprofloxacin resistance in the tested strains.

Bacterial resistance mechanisms are quite complicated, and resistance to antimicrobial agents in bacteria is often mediated by more than one mechanism (Jiang et al. 2014a; Kim et al. 2011; Tanaka et al. 2000). Apart from the mechanisms characterized in this study, it is possible that additional unidentified mechanisms exist in the LAB strains examined. Previous studies have reported that quorum sensing and biofilm formation could contribute to antimicrobial resistance in many organisms (Davies 2003; Martins et al. 2011; Zhang et al. 2013). However, it is still unclear whether the latter mechanisms have resulted in quinolone resistance in LAB strains.

In the present study, quinolone resistance mechanisms in LAB were analyzed in some detail. Our results show that efflux pump-mediated quinolone resistance was common in the analyzed strains. It is highly likely that efflux pumps are responsible for ciprofloxacin resistance in the strains that lack mutations. As for the strains that harbored mutations, the mutations in the QRDRs of *parC* and/or *gyrA* could play a leading role in ciprofloxacin resistance. Additionally, these strains could achieve high levels of resistance by using a combination of different mechanisms. Further studies are needed to confirm this hypothesis. In the meantime, the safe utilization of LAB strains in food production should be practiced in conjunction with the establishment of continuous surveillance methodologies for antimicrobial resistance associated with these strains.

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