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ORIGINAL ARTICLE

Association of mutation patterns in *GyrA* and *ParC* genes with quinolone resistance levels in lactic acid bacteria

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The quinolone resistance of 19 lactic acid bacterial strains belonging to the genera *Enterococcus* and *Lactobacillus* isolated from the natural fermented koumiss and yoghurt were investigated. The objective of this study was to determine the quinolone resistance levels and to explore the association of the resistance with the mutation patterns in *gyrA* and *parC* genes, as is currently recommended by the Food and Agriculture Organization/World Health Organization Joint Expert Committee in Guidelines for Evaluation of Probiotics in Food for probiotic lactic acid bacteria drug resistance in 2001. The Oxford Cup method and double-tube dilution method were used to determine the quinolone resistance levels of the isolated strains. Generally, all of the 19 strains showed resistance towards norfloxacin and ciprofloxacin when the Oxford cup method was used, whereas the incidence was lower (to norfloxacin 89.5% and to ciprofloxacin 68.4%) when minimum inhibitory concentration breakpoints (CLSI M100-S23) were tested. Furthermore, gene sequencing was conducted on *gyrA* and *parC* of topoisomerase II of these isolated strains. The genetic basis for quinolone resistance may be closely related to mutations in *gyrA* genes as there were 10 mutation sites in amino-acid sequences encoded by *gyrA* genes in 10 quinolone resistance strains and 14 mutation sites in *Enterococcus durans* HZ28, whereas no typical mutations were detected in *parC* genes.
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INTRODUCTION

Lactic acid bacteria (LAB) are Gram-positive bacteria that can ferment carbohydrates and produce lactic acids. LAB have a long and safe history of use in the production and consumption of fermented foods and beverages.[1–4] They can not only extend the shelf life of foods and make foods more delicious due to their acidity, but also improve the body's health level and longevity when they are used as medicines or health foods. Numerous studies showed that LAB can prevent intestinal disorders, maintain flora balance, lower blood cholesterol, enhance immunity, antibacterial and anti-infection.[5–8] In recent years, more and more consumers are concerned about LAB, which have been widely used in foods, medicines, animal husbandry and many other fields, such as yogurt, Jinshuangqi capsules, as well as bifido bacteria and synbiotics made of Chinese traditional medicine. Generally, LAB are considered to be safe internationally. But as viable exogenous bacteria, there is a growing emphasis on the security of their application, as is suggested by the Food and Agriculture Organization/World Health Organization Joint Expert Committee of probiotic LAB drug resistances in Guidelines for Evaluation of Probiotics in Food in 2001.[9] Accordingly, it is important and necessary to breed strains with better safety.

Quinolones are synthetic antibacterial drugs that can selectively inhibit DNA gyrase in bacteria. Various kinds of quinolone have been widely used in clinical studies, as they have a broad spectrum and have potent antibacterial activity, are widely distributed in the body, and have good oral bioavailability, convenience and lower cost. The main mechanism of quinolone killing microorganisms is to inhibit DNA gyrase and topoisomerase IV, thereby interfering with DNA replication and leading to the death of bacteria.[10–12] DNA gyrase is a tetramer that is composed of 2 A and 2 B subunits, in which the A and B subunits are encoded by *gyrA* and *gyrB* genes, respectively. A subunits are responsible for DNA double-strand breaks and rejoining, whereas B subunits are in charge of catalyzing the hydrolysis of ATP. Therefore, DNA gyrase-catalyzed negative supercoiled DNA requires ATP. In summary, DNA gyrase has an important role in the initial stages of DNA replication and transcription. Just as DNA gyrase, topoisomerase IV is also a tetramer that is composed of 2 C and 2 E subunits. C subunits, encoded by *parC*, like A, are responsible for DNA double-strand breaks and rejoining, whereas E subunits,

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encoded by *parE*, like B, are in charge of catalyzing the hydrolysis of ATP. Thus, topoisomerase IV has a significant role in the separation of sister chromosomes in the late period of DNA replication.[13] Therefore, DNA gyrase and topoisomerase IV are essential for bacterial growth and reproduction.

The main mechanism of bacterial resistance to quinolone is the occurrence of mutated genes encoding DNA gyrase and topoisomerase IV, which may change the amino-acid sequence, or the codon formed encodes the same amino acid, which may cause the variability of the enzyme subunit. All the above can affect the combination of quinolones and enzymes and reduce bacterial sensitivity to quinolone, finally leading to resistance to quinolone.[14]

Among the LAB, antibiotic resistance of *Enterococci* has been subjected to intensive study,[15–17] particularly because strains of these bacteria cause numerous and serious infections in humans.[18,19] In contrast, fewer physiological and molecular data are available on the antibiotic resistances of LAB present in fermented foods.[1] In the present study, 19 strains of LAB were isolated from the koumiss and yoghurt in Xilinguole pastoral. On the basis of research on the biological characteristics and quinolone resistance, the drug-resistant strains were selected to analyze the association of mutation patterns in *gyrA* and *parC* genes and quinolone resistance levels in LAB. These results can provide not only safety data for the fermentation industry, but also experimental basis for the mechanism of LAB quinolone resistance.

MATERIALS AND METHODS

Bacterial strains and culture conditions

MRS medium contained peptone 10 g, beef extract 10 g, yeast extract 5 g, $CH_3COONa \cdot 3H_2O$ 5 g, K_2HPO_4 2 g, $(NH_4)_2HC_6H_5O_7$ 2 g, Tween 80 1 ml, $MgSO_4 \cdot 7H_2O$ 0.0575 g, $MnSO_4 \cdot 4H_2O$ 0.014 g, glucose 20 g and agar 15 g in 1000 ml distilled water, pH 6.8, and sterilized at 121 °C for 20 min. TPY medium contained trypticase BBL 10 g, phytone BBL 5 g, Difco 2.5 g, Tween 80 1 ml, $MgCl_2 \cdot 6H_2O$ 0.5 g, $ZnSO_4 \cdot 7H_2O$ 0.25 g, cysteine HCl 0.5 g, glucose 15 g, $CaCl_2$ 0.15 g, agar 15 g, K_2HPO_4 2 g in 1000 ml distilled water, pH 6.8, and sterilized at 121 °C for 15 min.

All of the 19 LAB strains belonging to the genera *Enterococcus* and *Lactobacillus* were isolated from Xilinguole Pastoral in Inner Mongolia, China, provided by the National Natural Science Foundation of China, including 14 strains isolated from the koumiss and 5 strains from yogurt (Table 1). In the present study, we also chose *Lactobacillus plantarum* (CICC:6238), *Bifidobacterium infantis* (JCM 01217) and *E. faecalis* (CICC:23658), which served as standard strains, and *Escherichia coli* (ATCC 25922), which was purchased from China Industrial Microbiology Culture Collection and served as quality control strain. All of these strains were routinely cultured aerobically at 37 °C in TPY or MRS broth with pH 6.8, except for *E. coli* pH 7.8.

Preparation of bacterial suspension

The bacterial suspension was prepared by the following process. Firstly, freeze-dried powder was activated in TPY broth at 36 ± 1 °C for 24 h. Then the products were inoculated in MRS (36 ± 1 °C, 24 h) broth to obtain the second and third generations, which had higher activity. Subsequently, the third generation was centrifuged for 10 min at 3000 r.p.m., and the supernatant was discarded. Then sterile saline (NaCl, mass fraction of 0.85%) was added, centrifuged and the supernatant was discarded. The centrifugation and washing were repeated three times. After that, sterile saline was added to the pellet and suspended to reach a cell density of 10^7 CFU ml^{-1}. All of the operations above were carried out under sterile conditions.

Quinolone susceptibility test and minimum inhibitory concentration (MIC) determination

The Oxford Cup method was used to determine norfloxacin (the Food and Drug Verification Research Institute of China, Batch No. 130450-20070) and ciprofloxacin (the Food and Drug Verification Research Institute of China, Batch No. 130451-20030) susceptibility of 19 lactic acid bacterial strains. According to CLSI M100-S23[20] the drug concentrations tested were 1600, 160, 80, 50 (25 μg ml^{-1} for ciprofloxacin) and 0 μg ml^{-1}. Briefly, ~10 ml sterile agar culture was poured into 90 mm diameter sterile plate. After solidification, five Oxford cups were placed equidistantly on the surface of individual plates. Then 20 ml MRS agar culture containing 10^8 CFU ml^{-1} *Lactobacillus* was poured slowly into the plates mentioned above. After solidification, 200 μl of the same drug in different concentrations was added to four cups whereas 200 μl of sterile saline was added to the other cup as comparison. The plates were incubated in an aerobic chamber at 36 ± 1 °C for 24 h before measuring the diameter of the inhibition zone. This was repeated three times for each antibiotic drug per strain. The results of susceptibilities test were interpreted by CLSI M100-S23.[20]

Table 1 Strains used in this study

Strain category and species	*Strain number*
Strain sources	
Koumiss	
E. raffinosus	HZ1, HZ17, HZ18, HZ27
E. durans	HZ9, HZ15, HZ28
E. gallinarum	HZ22, HZ29
E. pseudoavium	HZ23
L. sake	HZ16
L. plantarum	HZ24, HZ25
L. casei	HZ26
Yoghurt	
E villorum	NN, NN1
E. dispar	NN2, NN3, NN4
Standard strains for quinolone resistance study	
L. plantarum	CICC 6238
B. infantis	JCM 01217
E. faecalis	CICC 23658
Control strains for quinolone resistance study	
E. coli	ATCC 25922

The MICs of the resistant strains to norfloxacin and ciprofloxacin were measured. Briefly, the double-tube dilution method was used to dilute the norfloxacin and ciprofloxacin. In all, 0.2 ml diluted drugs were mixed with 5 ml MRS broth that contained 10^8 CFU ml^{-1} of tested bacteria. Meanwhile, the control medium (without drugs and bacteria), drugs (drugs and medium, without bacteria) and inoculated bacteria (bacteria and medium, without drugs) were set as blank, positive and negative control, respectively. The tubes were incubated in an aerobic chamber at 36 ± 1 °C for 24 h before observing. The MIC was defined as the lowest concentration of each antibiotic that prevented growth of the tested isolate. Both norfloxacin and ciprofloxacin concentrations were used at 6400, 3200, 1600, 800, 400, 200, 100, 50, 25 and 0 μg ml^{-1} for drug susceptibility tests.

Total DNA preparation

Total genomic DNA of each strain was extracted from the third generation suspension by using bacterial genomic DNA extraction kits (centrifugal columns) (BioTeke Biotechnology Co., Ltd, Beijing, China) according to the manufacturer's instructions. 1% agarose gel electrophoresis (Sigma Corporation) was carried out by using 5 μl DNA. The purified genomic DNA was stored at −20 °C until it was used for polymerase chain reaction (PCR) analysis.

PCR amplification of *gyrA* and *parC* genes

In the present study, in order to figure out whether quinolone resistance was contributed by mutations in quinolone resistance-determining regions

(QRDR) of *gyrA* and *parC* genes, PCR amplification was done in 100 µl volumes that contained 2 µl of each specific primer, 50 µl of 2 × Easy Taq PCR Supermix (TransGen Biotech, Beijing, China) and 8 µl of DNA template.

Primers of *parC* were designed by using Primer 5 according to *parC* genes of *E. faecalis* in GenBank (GenBank/EMBL/DDBJ accession number: NC-004668, NC-019770), then synthesized by Shanghai Life Technologies Biotechnology Co., Ltd, while the primer of *gyrA* was designed according to *gyrA* genes of *E. coli* (GenBank/EMBL/DDBJ accession number: EU512996). The total sequence of *parC* gene with the length of 2463 bp was amplified by dividing into three sections, and the custom-designed primers for each section were as follows: section 1 for 30–646 bp, [forward] 5′-AAC CCT TGA AGA AGT AAT G-3′, [reverse] 5′-AAT CTG GTC CTG GAA TGT-3′. Section 2 for 316–1543 bp, [forward] 5′-GGA AAC AAC GGA AGT ATG G-3′, [reverse] 5′-TAT AGC CTT CGT GCG TCA-3′. Section 3 for 1546–2287 bp, [forward] 5′-AAG CGG AGT AGT ATT CGT-3′, [reverse] 5′-AAT CGG TAC TTT CAG ACA T-3′. Primers designed for *gyrA*: [forward] 5′-GAG GGA TAG CGG TTA GAT GAG-3′, [reverse] 5′-CCG TTC ACC AGC AGG TTA GG-3′.

The amplification for both *gyrA* and *parC* genes was performed for 45 circles at a denaturation temperature of 94 °C for 30 s, annealing temperature of 60 °C for 30 s and extension temperature of 72 °C for 1 min. Finally, the terminal extension was done for 5 min at 72 °C. Five microliters of the PCR products was mixed with 1 µl of 6 × Loading buffer (Takara, Dalian, China). Electrophoresis was carried out on the mixture on 1% agarose gel to detect whether there is a specific band. Electrophoresis conditions were as follows: electrophoresis solution, 1 × TBE buffer (Takara), voltage, 92 V, electric current, 55 mA. Time was determined by the size of gel electrophoresis, usually 40 min.

Sequence of PCR amplification of *gyrA* and *parC*

The PCR products were sequenced bidirectionally at Life Technologies (Invitrogen, Shanghai, China), and the deduced amino-acid sequences were aligned with *E. coli* (for *gyrA*) and *E. faecalis* (for *parC*) retrieved from GenBank database by using BankIt. The DNA sequences of *parC* genes obtained from *E. dispar* NN2, NN3, NN4, *E. raffinosus* HZ27, *E. durans* HZ9, *E. raffinosus* HZ17, HZ18, *E. gallinarum* HZ22, HZ29, *E. pseudoavium* HZ23 and *E. villorum* NN were submitted to GenBank and the accession numbers were KF946547, KF946548, KF946549, KF946550, KF958135, KF958136, KF958137, KF958138, KF958139, KF958140 and KF958141, respectively.

RESULTS

Antibiotic resistance phenotypes

The incidence of resistance to both norfloxacin and ciprofloxacin of the test strains was quite different depending on the methods used in the study. The original quinolone resistance data of the Oxford Cup method and MIC for 22 tested strains are displayed in Table 2. According to CLSI M100-S23[20] all of the isolated strains in Oxford Cup method showed resistance to norfloxacin and ciprofloxacin at much higher concentration than the data provided by CLSI (Table 3a). Only the control strain *E. coli* showed sensitivity to the antibiotic. However, when the MIC test was conducted using the double-tube dilution method, the incidence of resistance to these two antibiotics was lower than in the Oxford Cup method. Interestingly, the resistance to norfloxacin (89.5%) was much higher than ciprofloxacin (68.4%) (Table 3b). In the MIC test, *E. raffinosus* HZ1 and *E. durans* HZ9 showed intermediate sensitivity to both norfloxacin and ciprofloxacin, *E. raffinosus* HZ17, HZ18, HZ27 and *E. gallinarum* HZ22 showed resistance to norfloxacin and intermediate sensitivity to ciprofloxacin, whereas the other test strains showed resistance to the two quinolones. In addition, the MIC breakpoints of 10 isolated strains (HZ15, HZ24, HZ25, HZ28, NN, NN1, NN2, NN3, *L. plantarum*, *Bif. infantis*) were $>237.04\,\mu g\,ml^{-1}$ to both norfloxacin and ciprofloxacin. Moreover, the MIC breakpoints of *L. casei* HZ26 and *E. gallinarum* HZ29 to norfloxacin were $>237.04\,\mu g\,ml^{-1}$ whereas they were $237.04\,\mu g\,ml^{-1}$ and $118.52\,\mu g\,ml^{-1}$ to ciprofloxacin, respectively, which were much higher than that of other strains. For example, $237.04\,\mu g\,ml^{-1}$ is 59.26 times

Table 2 Diameter of inhibition zone (unit: mm) and MIC value ($\mu g\,ml^{-1}$) of quinolone to 22 tested strains

	Concentration of norfloxacin ($\mu g\,ml^{-1}$)					*Concentration of ciprofloxacin ($\mu g\,ml^{-1}$)*				
Strain number	*1600*	*160*	*80*	*50*	*MIC*	*1600*	*160*	*80*	*25*	*MIC*
HZ1	0	0	0	0	14.81	13.82	12	10.35	9.21	3.7
HZ9	0	0	0	0	14.81	18.1	12.4	10.95	9.87	3.7
HZ15	0	0	0	0	>237.04	10.7	0	0	0	>237.04
HZ16	0	0	0	0	>237.04	12.67	0	0	0	118.52
HZ17	0	0	0	0	29.63	18.71	13.05	11.6	9.56	3.7
HZ18	0	0	0	0	29.63	18.45	13.75	12.95	10.6	3.7
HZ22	0	0	0	0	>237.04	19.84	13.25	12.5	0	3.7
HZ23	0	0	0	0	59.26	0	0	0	0	7.4
HZ24	0	0	0	0	>237.04	0	0	0	0	>237.04
HZ25	0	0	0	0	>237.04	12.03	0	0	0	>237.04
HZ26	0	0	0	0	>237.04	10.23	0	0	0	237.04
HZ27	0	0	0	0	29.63	23	15.06	10.75	0	3.7
HZ28	0	0	0	0	>237.04	14	0	0	0	>237.04
HZ29	0	0	0	0	>237.04	12.05	0	0	0	118.52
NN	0	0	0	0	>237.04	11.65	0	0	0	>237.04
NN1	0	0	0	0	>237.04	13.34	0	0	0	>237.04
NN2	0	0	0	0	>237.04	14.17	0	0	0	>237.04
NN3	0	0	0	0	>237.04	10.7	0	0	0	>237.04
NN4	0	0	0	0	59.26	13.17	0	0	0	14.81
L. plantarum	0	0	0	0	>237.04	0	0	0	0	>237.04
Bif. infantis	0	0	0	0	>237.04	0	0	0	0	>237.04
E. coli	30.85	28.6	26.55	24.32	1.85	34.78	31.56	29.54	28.44	0.93

Abbreviation: MIC, minimum inhibitory concentration.

Table 3 Susceptibility criteria and results of the Oxford cup method and MIC: (a) susceptibility criteria for the Oxford cup method and MIC in CLSI (M100-S23); (b) susceptibility results of 22 tested strains to two quinolones

(a)

		Zone diameter interpretive criteria (mm)			*MIC interpretive criteria ($\mu g\,ml^{-1}$)*		
Antimicrobial agent	*Disk content (μg)*	*R*	*I*	*S*	*R*	*I*	*S*
Ciprofloxacin	5	⩽15	16∼20	⩾21	⩾4	2	⩽1
Norfloxacin	10	⩽12	13∼16	⩾17	⩾16	8	⩽4

(b)

	Norfloxacin		*Ciprofloxacin*	
Strain number	*Oxford Cup method*	*MIC*	*Oxford Cup method*	*MIC*
HZ1	R	I	R	I
HZ9	R	I	R	I
HZ15	R	R	R	R
HZ16	R	R	R	R
HZ17	R	R	R	I
HZ18	R	R	R	I
HZ22	R	R	R	I
HZ23	R	R	R	R
HZ24	R	R	R	R
HZ25	R	R	R	R
HZ26	R	R	R	R
HZ27	R	R	R	I
HZ28	R	R	R	R
HZ29	R	R	R	R
NN	R	R	R	R
NN1	R	R	R	R
NN2	R	R	R	R
NN3	R	R	R	R
NN4	R	R	R	R
L. plantarum	R	R	R	R
Bif. infantis	R	R	R	R
E. coli	S	S	S	S

Abbreviations: I, intermediate sensitive; MIC, minimum inhibitory concentration; R, resistance; S, sensitive.

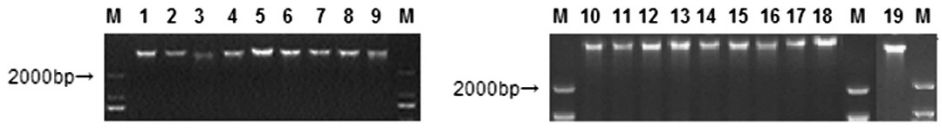

Figure 1 Agarose gel electrophoresis of total DNA. M: DNA marker DL 2000. 1: *L. plantarum*, 2: NN3, 3: NN2, 4: NN1, 5: NN, 6: HZ28,7: HZ25, 8: HZ24, 9: HZ15, 10: *Bif. infantis*, 11: HZ9, 12: HZ17, 13: HZ18, 14: HZ22, 15: HZ23, 16: HZ27, 17: HZ29, 18: NN4, 19: *E. faecalis*.

the resistance standard $4\,\mu g\,ml^{-1}$. This indicated that resistance to quinolone among the lactic acid bacteria was very common and the incidence was generally high. In this study, although a small number of species and antibiotics were investigated, it would be necessary to study more strains and drugs to determine the quinolone resistance of LAB.

Total DNA extraction test

The electrophoresis in 1% agarose gel was carried on the total genomic DNA of each strain extracted by using bacterial genomic DNA extraction kits (centrifugal columns). Figure 1 indicates that the sizes of electrophoretic bands were in line with the expected size.

PCR amplification of *gyrA* gene

Quinolone resistance is known to be associated with mutations in the QRDR of the *gyrA* or *parC* genes in various Gram-positive or Gram-negative bacteria, which lead to amino-acid substitutions and result in the quinolone resistance phenotype.[1,21,22] After PCR amplification of *gyrA* on 11 drug resistance strains, 3 intermediate susceptible strains

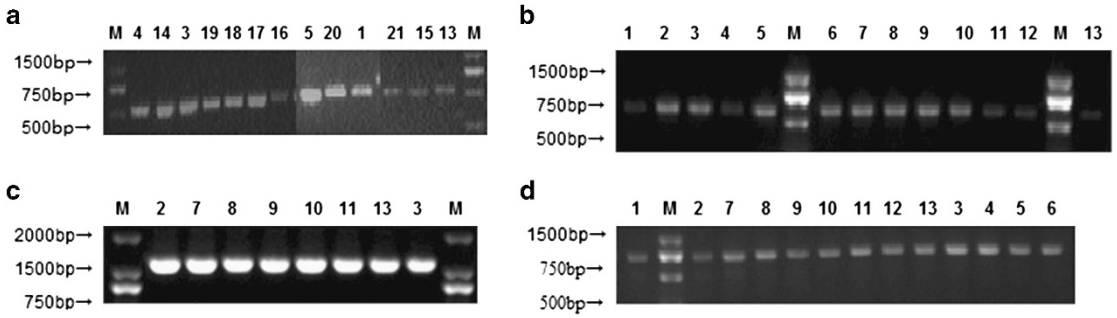

Figure 2 (**a**) represent the agarose gel electrophoresis of *gyrA* in QRDR of 13 strains. Figures **b**–**d** represent the 1,2,3 fragment of *parC* gene, respectively. M: DNA Marker DL 2000. 1: *Bif. infantis*, 2: *E. faecalis*, 3: NN, 4: NN2, 5: NN3, 6: NN4 7, HZ9, 8: HZ17, 9: HZ18, 10: HZ22, 11: HZ23, 12: HZ27, 13: HZ29, 14: NN1, 15: HZ1, 16:HZ15,17: HZ24, 18:HZ25, 19: HZ28, 20: *L. plantarum*, 21: *E. coli*.

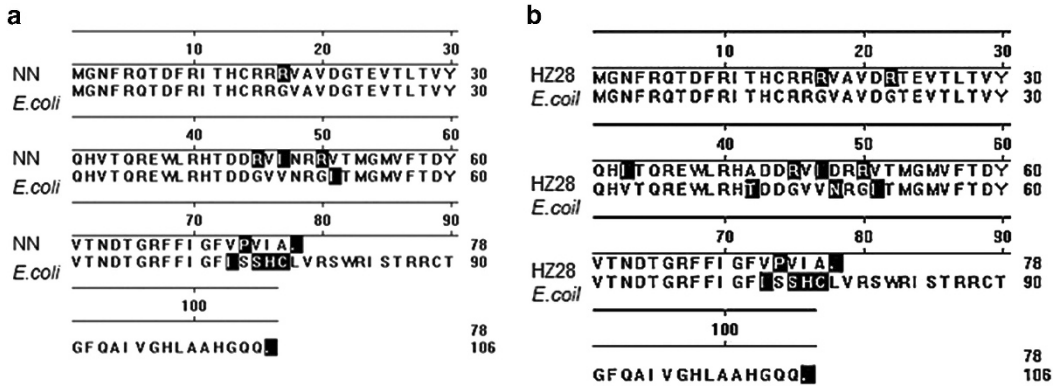

Figure 3 (**a**) Amino-acid sequence comparing results of *gyrA* genes between NN and *E. coli*. (**b**) Amino-acid sequence comparing results of *gyrA* genes between HZ28 and *E. coli*. A: Ala, C: Cys, D: Asp, E: Glu, F: Phe, G: Gly, H: His, I: Ile, K: Lys, L: Leu, M: Met, N: Asn, P: Pro, Q: Gln, R: Arg, S: Ser, T: Thr, V: Val, W: Trp, Y: Tyr. The shaded areas indicate that the corresponding nucleotides are different.

and 1 susceptible strain (*E. coli*), the electrophoresis was carried out in 1% agarose gel. As shown in Figure 2a, the amplified fragments of the selected strains were ~800 bp, which is consistent with the size of the reported *gyrA* gene.

After PCR amplification and DNA sequencing of *gyrA* genes, the amino-acid sequences of the selected strains were translated by using EditSeq in DNASTAR. Then MegAlign were used to compare the amino-acid sequences of quinolone resistance strains or intermediate sensitive strains and control strain *E. coli*. Specific results are shown in Figure 3. Typically Ser83-to-Arg and Glu87-to-Gly or Lys substitutions within the *gyrA* subunit of DNA gyrase in the QRDR were not observed in the resistance strains mentioned above. However, there are 10 sites that were changed in the translated amino-acid sequences of 10 quinolone resistance strains (eight of the isolated strains NN, NN1, NN2, NN3, HZ1, HZ15, HZ24, HZ25 and two standard strains *L. plantarum*, *Bif. infantis*) (Figure 3a). They are Arg-(17,45,50)-to-Gly, Val-(51,73)-to-Ile, Ile-47-to-Val, Pro-74-to-Ser, Val-75-to-Ser, Ile-76-to-His and Ala-77-to-Cys. In the case of *E. durans* HZ28, in addition to the same 10 sites, there were another 4 sites mutated in the translated amino-acid sequences: Arg-22-to-Gly, Ile-33-to-Val, Ala-42-to-Thr and Asp-48-to-Asn (Figure 3b). Generally, the incidence of mutant strains is extremely high (78.6%). Among the 14 tested strains there were 11 strains whose amino-acid sequences were changed. Of the 11 strains represented by NN, 10 had 10 sites mutated in the translated amino-acid sequences while the other strain HZ28 had 14 sites mutated.

PCR amplification of *parC* gene

Just like *gyrA*, mutations in *parC* gene are also known to be associated with quinolone resistance in various Gram-positive or Gram-negative bacteria.[1,21,22] Several studies suggested that topoisomerase IV is the primary target of ciprofloxacin in *Enterococci* and *Streptococci*.[23–25] The total sequence of *parC* gene with the length of 2463 bp of 13 tested strains was amplified by dividing into three sections and the results are shown in Figures 2b–d. As can be seen from the figure, the size of all of the three amplified sections was consistent with the designed size. Software MegAlign in DNAStar was used to compare the *parC* sequences of the isolated strains and the standard strains *Bif. infantis* or *E. faecalis*, after stitching the three segments per plant using SeqMan in DNASTAR.

However, section 1 was designed for 30–646 bp in the total length 2463 bp of *parC* gene, while the actual sequencing results were 41–628 bp. Section 2 was designed for 316–1543 bp in the total length 2463 bp of *parC* gene, while the actual sequencing results were 371–1481 bp. Section 3 was designed for 1546–2287 bp in the total length 2463 bp of *parC* gene, while the actual sequencing results were 1588–2275 bp. Interestingly, for each section the isolated strains and the standard strains showed identical nucleotide sequences when compared.

DISCUSSION

Knowledge on the antibiotic resistance of lactic acid bacteria is still limited, which may be because the large numbers of genera and species were related in this group.[1] What is becoming obvious, also

consistent with the results of the present study, is that the bacterial resistance to norfloxacin and ciprofloxacin is on the rise.[26,27] In the present study, all of the 19 isolated strains were determined as resistance to norfloxacin and ciprofloxacin by using the Oxford Cup method, while the incidence of resistance of the test strains by the MIC determination method was 89.5% for norfloxacin and 68.4% for ciprofloxacin. Similarly, Hummel *et al.*[1] also described that >70% of isolated strains from the lactic acid bacteria starter or probiotic strains belonging to the genera *Lactobacillus*, *Pediococcus*, *Leuconostoc* and *Streptococcus* were resistant to ciprofloxacin based on the MIC breakpoint values of SCAN and FEEDAP.[28,29] In addition, ciprofloxacin resistance was reported for >60% of the *Lactobacillus* examined by Zarazaga *et al.*[30]

In the present study, norfloxacin and ciprofloxacin were selected to study the quinolone susceptibility phenotype of lactic acid bacteria. Norfloxacin has been widely used for the treatment of intestinal infections and urinary tract infections due to its activity against aerobic Gram-negative bacilli. Ciprofloxacin, a second-generation quinolone, is a quinolone that is widely used clinically. Several studies have confirmed that both norfloxacin and ciprofloxacin were acting on the A subunit of *gyrA* gene in DNA gyrase, thus leading to the death of the bacteria by inhibition of DNA synthesis and replication.[11,12]

In this study, the incidence of mutant strains in *gyrA* was high (78.6%) and among the 14 test strains there were 11 strains whose amino-acid sequences were changed. Of the 11 strains represented by NN, 10 have 10 sites mutated in the translated amino-acid sequences, whereas the other strain, *E. durans* HZ28, has 14 sites mutated. However, the typically Ser83-to-Arg and Glu87-to-Gly or Lys substitutions within the *gyrA* subunit of DNA gyrase in the QRDR were not observed for the resistance strains mentioned above. As for the *parC* gene, surprisingly, the test strains and the reference control strains shared the same nucleotide sequences of the tested fragments. These results indicated that the genetic basis for norfloxacin and ciprofloxacin resistance in lactic acid fermenting bacteria may be closely related to mutations in the *gyrA* gene of DNA gyrase in the QRDR whereas it may be unrelated to the *parC* gene as no typical mutations were detected.

Numerous studies have shown that *gyrA* and *parC* genes in almost all bacteria were in the quinolone resistance-determining region.[31] In Gram-negative bacteria DNA gyrase is the initial target of quinolone. The typical mutations in *E. coli* were Ser83-to-Arg and Glu87-to-Gly or Lys substitutions within the *gyrA* subunit of DNA gyrase in the QRDR. In Gram-positive bacteria, mutations in *gyrA* gene also contribute to the resistance of bacteria to quinolone although it is not the main target. It is reported that the mutation sites of *S. aureus* in *gyrA* of DNA gyrase are located between 68 and 108 amino-acid residues, which is similar to the *E. coli*.[32] The main target of quinolone in Gram-positive bacteria is topoisomerase IV, especially the *parC* gene, and the most commonly occurring mutations are serine 80 and glutamate 84, similar to the serine 83 and glutamate 87 in *gyrA*. In addition, the other mutation site in *parC* gene is glycine 78.[33]

DNA gyrase and topoisomerase IV are necessary for bacterial growth and reproduction. If either of them is not properly involved in DNA replication, it may inhibit the growth of bacteria, thereby resulting in its death. In 1997, Cabral *et al.*[34] found that mutations often occurred in *gyrA* and *parC* in QRDR—the binding region of DNA and the enzyme, in which amino-acid mutations can affect the binding of quinolone and bacteria. In 2003, Gillespie *et al.*[35] reported that the resistance to sparfloxacin and clinafloxacin of *S. pneumoniae* was caused by mutations in *gyrA*. Generally, several mutations in QRDR can result in obvious resistance in clinical strains of enteric pathogens. For example, in the case of *Campylobacter jejuni*,[35] mutations of Ala-70-Thr, Thr-86-Phe, Asp-90-Asn were detected in DNA *gyrA*, which is inconsistent with the present study.

In the present study, all of the 19 lactic acid bacterial strains were separated from the natural fermented koumiss and yoghurt in Xilinguole Pastoral Area, Inner Mongolia, China, in which the fresh milk and fresh mare's milk were produced by the ancient and traditional processing methods—fermented naturally without any processing, with no added preservatives. Why did the lactic acid fermenting bacteria isolated from the particular products show resistance to quinolone? Are they the results of some nucleotide mismatch in its evolution,[14] or are they the result of selection by antibiotic pressure in the environment, or acquired by the transfer of resistance gene[36,37] between bacteria, such as plasmids, transposons and integrons? More importantly, are these mutations transferable? All of these questions need further investigation. It will be of great significance to explore these issues in the future. Meanwhile, it will provide valuable experimental data to explore the mechanism of drug resistance for the application of beneficial lactic acid bacteria in the industry.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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