Curr Microbiol (2011) 62:1081–1089
DOI 10.1007/s00284-010-9856-2

Characterization and Transfer of Antibiotic Resistance in Lactic Acid Bacteria from Fermented Food Products

Muhammad Nawaz · Juan Wang · Aiping Zhou · Chaofeng Ma · Xiaokang Wu · John E. Moore · B. Cherie Millar · Jiru Xu

Received: 1 November 2010 / Accepted: 16 December 2010 / Published online: 7 January 2011

Abstract The study provides phenotypic and molecular analyses of the antibiotic resistance in lactic acid bacteria (LAB) from fermented foods in Xi'an, China. LAB strains ($n = 84$) belonging to 16 species of *Lactobacillus* ($n = 73$), and *Streptococcus thermophilus* ($n = 11$) were isolated and identified by sequencing their 16S rRNA gene. All strains were susceptible to ampicillin, bacitracin, and cefsulodin, and intrinsically resistant to nalidixic acid, kanamycin, and vancomycin (except *L. bulgaricus*, *L. acidophilus*, and *S. thermophilus*, which were susceptible to vancomycin). Some strains had acquired resistance for penicillin ($n = 2$), erythromycin ($n = 9$), clindamycin ($n = 5$), and tetracycline ($n = 14$), while resistance to gentamycin, ciprofloxacin, streptomycin, and chloramphenicol was species dependant. Minimum inhibitory concentrations presented in this study will help to review microbiological breakpoints for some of the species of *Lactobacillus*. The *erm*(B) gene was detected from two strains of each of *L. fermentum* and *L. vaginalis,* and one strain of each of *L. plantarum*, *L. salivarius*, *L. acidophilus*, *L. animalis*, and *S. thermophilus.* The *tet* genes were identified from 12 strains of lactobacilli from traditional foods. This is the first time, the authors identified *tet*(S) gene from *L. brevis* and *L. kefiri*. The *erm*(B) gene from *L. fermentum* NWL24 and *L. salivarius* NWL33, and *tet*(M) gene from *L. plantarum* NWL22 and *L. brevis* NWL59 were successfully transferred to *Enterococcus faecalis* 181 by filter mating. It was concluded that acquired antibiotic resistance is well dispersed in fermented food products in Xi'an, China and its transferability to other genera should be monitored closely.

M. Nawaz · J. Wang · A. Zhou · C. Ma · X. Wu · J. Xu (✉)
Department of Immunology and Pathogenic Biology, Molecular Bacteriology Laboratory, Key Laboratory of Environment and Genes Related to Diseases of Chinese Ministry of Education, School of Medicine, Xi'an Jiaotong University, Xi'an 710061, Shaanxi, People's Republic of China
e-mail: xujiru@mail.xjtu.edu.cn

C. Ma
Center for Disease Control and Prevention of Xi'an, Xi'an 710054, Shaanxi, People's Republic of China

J. E. Moore · B. Cherie Millar
Department of Bacteriology, Northern Ireland Public Health Laboratory, Belfast City Hospital, Lisburn Road, Belfast, Northern Ireland BT9 7AD, UK

Introduction

Lactic acid bacteria (LAB) have a long history of safe use as fermenting natural products and probiotics intended for health benefits and have acquired the "Generally recognized as safe" (GRAS) status [32]. Since the introduction of antibiotics more than 50 years ago, emergence of resistant microorganisms has become a major threat to public health [34]. Antibiotic resistance is well studied and documented for human pathogenic species [32]. However, since the last decade, researchers have also focused on characterizing antibiotic resistance in LAB [4]. LAB can serve as reservoir for antibiotic resistance genes and transfer it to other microorganisms including pathogens [23, 43]. This situation has become more hazardous by the overuse and misuse of antibiotics in feed, agriculture, and veterinary applications [2, 42]. European Food Safety Authority (EFSA) recommends that bacterial strains harboring transferable antibiotic resistance genes should not be used in animal feeds, fermented and probiotic foods for human [14].

Lactobacilli are dominant bacteria in several fermented foods such as meat and dairy products, which interact with

gut microflora on ingestion [2]. Lactobacilli are generally intrinsically resistant for quinolones, trimethoprim, and sulphonamides, and susceptible to all protein synthesis inhibitors except aminoglycosides. Resistance of many species of lactobacilli except *L. delbrueckii subsp. bulgaricus*, *L. acidophilus*, *L. johnsonii*, and *L. crispatus* to glycopeptides is also considered intrinsic [8, 32]. Intrinsic resistance is not horizontally transferable as it is chromosomally encoded and related to the general physiology or anatomy of an organism. Acquired resistance is horizontally transferable, which emerges from genetic changes by mutations or acquisition of genetic elements (plasmids or transposons) most probably by conjugation [2, 4, 32]. Acquired antibiotic resistance in lactobacilli has been reported frequently in recent years from fermented milk products [10, 11, 29, 35, 41], pickles, and meat but there are no reports from China in this regard.

The objective of this study was a safety assessment of bacterial isolates from starter cultures and naturally fermented foods by phenotypic screening, polymerase chain reaction (PCR) and filter mating. This study provides data on the current resistance situation and insinuate for further monitoring and development of regulations for antibiotic resistance in food associated LAB in China.

Materials and Methods

Isolation of LAB and Growth Conditions

Bacterial strains were isolated from commercial and traditionally fermented food samples including Yogurt ($n = 13$), Whey or fermented milk ($n = 10$), Jiang shui ($n = 3$), and fermented vegetables ($n = 15$). Jiang shui is a traditional Chinese drink, made by fermenting a mixture of vegetables. Food samples obtained from local markets were homogenized and serial dilutions were plated on selective media and incubated at 37°C for 48 h. Lactobacilli were selected on MRS agar (Oxoid) plates incubated in anaerobic conditions (Anaerogen, Oxoid), while streptococci were isolated on M17 (Oxoid) agar plates in aerobic environment. After the incubation, distinguished colonies were selected, purified, and stored in MRS broth with 15% (w/v) glycerol at −20°C. *Enterococcus faecalis* ATCC 29212 and *E. faecium* ATCC 35667 were used as control strains in antibiotic sensitivity testing. Prior to all experiment bacterial isolates were subcultured at least twice.

Identification of LAB

Preliminary identification of isolates was performed by gram staining and catalase test. DNA was extracted from bacterial isolates using a commercial kit (TianGen, Beijing, China) according to the manufacturer's instructions. *Lactobacillus* genus was identified by PCR using genus specific primers XB5 and LbLMA1-R. For the identification of species, amplification of partial 16S rRNA gene was performed by universal primers (Table 1) in an automated thermocycler (ABI 2720 USA) using the following program: initial denaturation at 95°C for 5 min; followed by 35 cycles of 95°C for 45 s, 55°C for 45 s, 72°C for 1 min, and a final extension at 72°C for 7 min. Amplified products were resolved by 1.5% (w/v) agarose gel electrophoresis and visualized by ethidium bromide staining (0.5 μg/ml) under UV transillumination. PCR products (nearly 1,380 bp) were purified and sequenced directly by Beijing genomics institute (BGI) by using the same universal primers. LAB isolates were identified to the species level by comparing their sequences with those in the NCBI databases by BLAST. All sequences were submitted to GenBank and accession numbers were obtained.

Antibiotic Resistance

Minimum inhibitory concentrations (MICs) of 14 antibiotics were determined by broth microdilution method using LAB susceptibility test medium (LSM) [30], a mixed formulation containing Iso-Sensitest broth (90%) and MRS broth (10%). Penicillin G, ampicillin, erythromycin, clindamycin, tetracycline, cefsulodin, bacitracin, and ciprofloxacin were tested in concentration range of (0.032–64 mg/l) while, gentamicin, kanamycin, fusidic acid, streptomycin, chloramphenicol, nalidixic acid, and vancomycin in concentration range of (0.125–256 mg/l). In brief, 50 μl of antibiotics, doubly diluted in LSM broth were inoculated with 100 μl of test strain inoculum. Inoculum of the test strain was prepared by suspending colonies from LSM agar plates in 5 ml 0.85% NaCl solution to a turbidity of McFarland 1 standard and subsequently diluting them 1:1000 in LSM broth. Plates were incubated under anaerobic conditions at 37°C for 48 h. MICs were read as the lowest concentration of an antimicrobial agent at which visible growth was inhibited. Breakpoints were adopted from EFSA [15]. Breakpoints for antibiotic not covered by EFSA were adopted from Danielsen and Wind [10].

Amplification of Antibiotic Resistance Genes

Antibiotic resistance genes for β-lactam (*bla*), erythromycin [*erm*(A), *erm*(B), and *mef*(A)], tetracycline [*tet*(M), *tet*(O), *tet*(Q), *tet*(S), *tet*(W), *tet*(K), and *tet*(L)], gentamycin [*aac*(6′)-*aph*(2″)], and chloramphenicol (*cat*) were amplified by PCR using primers given in Table 1. The reaction mixtures (50 μl) contained 25 pmol of each primer, 1.5, 2.0, 2.5, or 3.0 mM $MgCl_2$ depending on

Table 1 Nucleotide sequences of primer sets used for PCR in this study

Target gene	Primer sequence (5′ → 3′)	T_a°C	Expected size (bp)	Reference
16S/23S spacer	XB5-F :GCCTTGTACACACCGCCCGT	55	250	[This study, 13]
	LbLMA1-R:CTCAAAACTAAACAAAGTTTC			
16S rRNA	8FLP-F: GGATCCGCGGCCGCTGCAGAGTTTGATCCTGGCTCAG	55	1,380	[37, 44]
	XB4-R: GTGTGTACAAGGCCCGGGAAC			
bla	Bla-forward: CATARTTCCGATAATASMGCC	51	297	[26]
	Bla-reverse:CGTSTTTAACTAAGTATSGY-3			
erm(A)	ermAI: AAGCGGTAAACCCCTCTGA	55	190	[31]
	ermAII: TTCGCAAATCCCTTCTCAAC			
erm(B)	F:GAAAAGRTACTCAACCAAATA	52	642	[40]
	R: AGTAACGGTACTTAAATTGTTTAC			
tet(M)	F:GTTAAATAGTGTTCTTGGAG	55	576	[1]
	R:CTAAGATATGGCTCTAACAA			
tet(K)	F:TTAGGTGAAGGGTTAGGTCC	55	697	[1]
	R:GCAAACTCATTCCAGAAGCA			
tet(L)	F:CATTTGGTCTTATTGGATCG	50	456	[1]
	R:ATTACACTTCCGATTTCGG			
tet(S)	TetS-FWT 1: ATCAAGATATTAAGGAC	56	573	[22]
	TetS-RVT 2:TTCTCTATGTGGTAATC			
tet(O)	teto-F: AACTTAGGCATTCTGGCTCAC	52	515	[22]
	tetO-R: TCCCACTGTTCCATATCGTCA			
tet(Q)	TetQ-FW: AGAATCTGCTGTTTGCCAGTG	56	169	[22]
	TetQ-RV: CGGAGTGTCAATGATATTGCA			
tet(W)	tetW-FW GAGAGCCTGCTATATGCCAGC	64	168	[29]
	tetW-RV GGGCGTATCCACAATGTTAAC			
mef(A)	mefAfw: CTATGACAGCCTCAATGCG	52	1,400	[29]
	mefArv: ACCGATTCTATCAGCAAAG			
cat	F: GGATATGAAATTTATCCCTC	50	486	[1]
	R: CAATCATCTACCCTATGAAT			
aac(6′)-*aph*(2″)	F: CCAAGAGCAATAAGGGCATA	60	220	[38]
	R: CACTATCATAACCACTACCG			

experiment, 50–100 ng bacterial DNA, and 2.5 U of Taq DNA Polymerase. DNA fragments were amplified in a thermal cycler up to 40 cycles by using annealing temperatures given in Table 1. Positive and negative controls form our lab were used for all PCR reactions. PCR products were subjected to agarose gel electrophoresis in TAE buffer. Gels were stained with ethidium bromide (5 μg/ml) and visualized under UV transillumination.

Transfer of Antibiotic Resistance

Transfer of antibiotic resistance was analyzed by filter mating experiments as described by Gevers et al [24] with slight modifications. *Enterococcus faecalis* 181 and *E. facium* 258 were used as the recipient, which were sensitive to erythromycin and tetracycline, and resistant to rifampicin and fusidic acid. The *tet*(M), and *erm*(B) positive strains from the study were used as donors. Equal volumes (1 ml each) of donor and recipient strain, at exponential growth were mixed, filtered through a sterile 0.45 μm-pore-size nitrocellulose membrane filter (Millipore, USA), and placed on MRS agar plates. After incubation at 37°C for 24 h, cells were resuspended in phosphate buffer saline and serial dilutions were spread on selective medium. Following incubation for 48 h, transconjugants were selected and analyzed by antimicrobial susceptibility. The transfer of resistant gene *tet*(M) and *erm*(B) was confirmed in transconjugants by the detection of *tet*(M) and *erm*(B) by PCR. The number of transconjugants per recipient cell was given as conjugation frequencies.

The GenBank accession numbers of sequences reported in this article are mainly from HQ293024–HQ293117and HQ111074–HQ111078.

Results

Isolation and Identification of LAB

A total of 22 LAB, including, *L. delbrueckii* subsp. *bulgaricus* ($n = 6$), *L. acidophilus* ($n = 6$), *L. rhamnosus* ($n = 1$), *L. kefiri* ($n = 1$), and *S. thermophilus* (n = 8) were isolated from commercial Yogurts. 62 LAB strains belonging to species *L. delbrueckii* subsp. *bulgaricus* ($n = 7$), *L. acidophilus* (1), *L. casei* (n = 5), *L. rhamnosus* (3), *L. fermentum* (5), *L. plantarum* (7), *L. brevis* (6), *L. salivarius* (5), *L. parabuchneri* (12), *L. alimentarius* (2), *L. anamalis* (2), *L. vaginalis* (2), *L. parabrevis* (1), *L. coryniformis* (1), and *S. thermophilus* ($n = 3$) were isolated from traditional products of whey, yogurt, pickle, and Jiang shui. Species were identified by partially sequencing their 16S rRNA gene and all the sequences were submitted to GenBank. All the commercial products had the probiotic or starter strains of LAB as per label, except one yogurt sample that had *L. kefiri* instead of *L. acidophilus* as indicated on product label.

Phenotypic Profile of Antimicrobial Resistances

Table 2 indicates the number of strains that were subjected to antibiotic susceptibility, 90% MIC, range, and number of resistant strains for each species. All strains were susceptible to ampicillin, bacitracin, and cefsulodin and resistant to nalidixic acid and kanamycin. All the species except, *L. bulgaricus* and *L. acidophilus* and *S. thermophilus* were intrinsically resistant to vancomycin. Two strains, one each of *L. salivarius* and *S. thermophilus* species were resistant to penicillin G. High percentage of strains were resistant to gentamycin (65%) and ciprofloxacin (85%) while, a low percentage was resistant to streptomycin (7%) and chloramphenicol (12%) in species dependant manner. Moderately higher percentage of strains had acquired resistance for erythromycin (11%), clindamycin (6%), and tetracycline (17%). All erythromycin ($n = 9$) and tetracycline ($n = 14$) resistant strains originated from traditional products, except tetracycline resistant *L. kefiri* NWL78 and *S. thermophilus* NWL93, which were from two different commercially produced probiotic yogurts.

Antibiotic Resistance Genes

Antibiotic resistance genes were detected by PCR from resistant strains and results are presented in Table 3. Erythromycin resistance gene *erm*(B) was found from eight lactobacilli strains including *L. fermentum* NWL24 and NWL26, *L. vaginalis* NWL 35 and NWL43, *L. plantarum* NWL22, *L. acidophilus* NWL23, *L. salivarius* NWL33, and *L. animalis* NWL39. One strain of *S. thermophilus* (NWL02), isolated from a traditionally fermented yogurt also had *erm*(B) gene. All the strains with atypical MIC to erythromycin were positive for *erm*(B), while none of the susceptible strains were positive for *erm*(B). Other erythromycin resistant determinants [*erm*(A) and *mef*(A)] were not detected from any strain.

Out of 14 lactobacilli strains resistant to tetracycline, eight strains were positive for *tet*(M) gene only, two strains had *tet*(M) and *tet*(S) together, and two strains harbored only *tet*(S) gene. Two strains, resistant to tetracycline were negative for any of the tested *tet* resistance gene. None of the *tet*(W), *tet*(O), *tet*(Q), *tet*(K), and *tet*(L) genes were present in any strain. All *tet* gene positive strains were isolated from traditional sources except *L. kefiri* NWL78, which was isolated from a probiotic yogurt. One strain of each of *L. plantarum* (NWL16) and *L. salivarius* (NWL33) had *erm*(B) and *tet*(M) together. Penicillin, gentamycin, chloramphenicol, and streptomycin resistant strains were negative for any of the tested genes. Vancomycin resistance was intrinsic, and strains did not harbor acquired resistance gene *van*(B) (data not shown).

Transfer of Antibiotic Resistance Genes

LAB strains positive for *erm*(B) and *tet*(M) were used as donors, while *E. faecalis* 181 and *E. facium*258 were used as recipient strains in filter mating experiments. Results showed that *erm*(B) from *L. fermentum* NWL24 and *L. salivarius* NWL33 and *tet*(M) gene from *L. plantarum* NWL22 and *L. brevis* NWL59 were successfully transferred to *E. faecalis*. All transconjugants were resistant to erythromycin (MIC > 64) or tetracycline (MIC > 128) and positive for *tet*(M) or *erm*(B) genes. Transfer rate in filter mating experiments ranged from 2.9×10^{-6} to 1.39×10^{-5} for different combinations (Table 4).

Discussion

Since 1960s, the emergence of drug resistant pathogens and lack of newly developed antibiotics is a major concern for health professionals and researchers. Magnitude of problem increases significantly by the possibility of horizontal gene transfer, misuse, and overuse of antibiotics in human medicine, veterinary, agriculture, and food products. Food chain is considered as a major route of transmission of antibiotic resistant genes between animal and human populations [32]. LAB, a dominant part of microflora of fermented foods, can exchange genes to enhance their survival in antibiotic-containing environments and may transfer it to other commensal bacteria or pathogens in the intestine, on mucosal surfaces or in food [39, 43]. LAB with acquired resistance should be discouraged from

Table 2 Minimum inhibitory concentration (MIC) values of selected antibiotics against bacterial species

Bacterial species (n^a)	Antibiotic MIC[b] (μg/ml)									
	Penicillin		Ampicillin		Erythromycin		Clindamycin		Tetracycline	
	90%[c]	Range (n^d)	90%	Range (n)	90%	Range (n)	90%	Range (n)	90%	Range (n)
L. bulgaricus (13)	0.25	0.063–0.25 (0)	0.25	0.125–0.25 (0)	0.5	0.063–0.5 (0)	0.5	0.063–0.5 (0)	2	0.125–2 (0)
L. acidophilus (7)	0.5	0.125–0.5 (0)	0.5	0.5 (0)	4	0.063–4 (1)	0.25	0.063–0.25 (0)	2	0.5–2 (0)
L. casei (5)	2	1–2 (0)	1	0.125–1 (0)	0.5	0.063–0.5 (0)	0.5	0.5 (0)	1	0.5–1 (0)
L. rhamnosus (4)	2	1–2 (0)	1	0.5–1 (0)	0.125	0.063–0.5 (0)	0.125	0.063–0.5 (0)	4	1–4 (0)
L. fermentum (5)	0.5	0.125–0.5 (0)	0.5	0.125–0.5 (0)	>64	0.125–>64 (2)	>64	0.125–>64 (2)	4	0.125–4 (0)
L. plantarum (7)	1	0.125–1 (0)	0.5	0.125–0.5 (0)	32	0.063–32 (1)	0.125	0.063–0.125 (1)	>128	1–>128 (3)
L. brevis (6)	2	0.125–2 (0)	0.5	0.125–0.5 (0)	0.125	0.063–0.125 (0)	0.125	0.063–0.5 (0)	8	0.125–8 (2)
L. parabrevis (1)	0.25	0.25 (0)	0.125	0.125 (0)	0.125	0.125 (0)	0.125	0.063–0.5 (0)	0.5	0.5 (0)
L. alimentarius (2)	0.25	0.25 (0)	0.125	0.125 (0)	0.125	0.125 (0)	0.125	0.063–0.5 (0)	2	2 (0)
L. salivarius (5)	64	0.125–>64 (1)	4	0.125–0.25 (0)	>64	0.063–>64 (1)	0.125	0.063–0.5 (1)	>128	>128 (5)
L. animalis (2)	0.5	0.125–5 (0)	0.125	0.125–0.5 (0)	32	0.063–32 (1)	0.125	0.063–0.5 (0)	0.25	0.125–4 (1)
L. parabuchneri (12)	0.25	0.063–0.25 (0)	0.5	0.125–0.5 (0)	0.125	0.063–0.5 (0)	0.125	0.063–0.5 (0)	32	8–32 (1)
L. vaginalis (2)	0.25	0.125–0.25 (0)	0.125	0.125 (0)	32	32(2)	0.125	0.063–0.5 (0)	0.25	0.125–4 (0)
L. kefiri (1)	0.063	0.063 (0)	0.063	0.063 (0)	0.063	0.063 (0)	0.125	0.063–0.5 (0)	64	64 (1)
L. coryniformis (1)	0.125	0.125 (0)	0.125	0.125 (0)	0.063	0.063 (0)	0.125	0.063–0.5 (0)	0.25	0.25(0)
S. thermophilus (11)	0.5	0.125–64 (1)	0.5	0.125–0.25 (0)	0.5	0.063–8 (1)	0.125	0.063–0.5 (1)	0.5	0.125–16 (1)
Total resistant strains	2 (2%)		0 (0%)		9 (11%)		5 (6%)		14 (17%)	
Bacterial species (n^a)	Antibiotic MICb (μg/ml)									
	Gentamycin		Ciprofloxacin		Bacitracin		Cefsulodin		Kanamycin	
	90%[c]	Range (n^d)	90%	Range (n)	90%	Range (n)	90%	Range (n)	90%	Range (n)
L. bulgaricus (13)	64	4–64 (10)	32	2–32 (11)	2	0.05–2 (0)	0.125	0.063–0.125 (0)	>256	>256 (13)
L. acidophilus (7)	64	2– 64 (4)	16	2–16 (6)	2	0.5–2 (0)	1	0.063–1 (0)	>256	>256 (7)
L. casei (5)	128	4–128 (2)	32	8–32 (5)	4	0.5–4 (0)	16	0.125–16 (0)	>256	>256 (5)
L. rhamnosus (4)	128	32–128 (4)	16	4–32 (4)	4	0.5–4 (0)	0.125	0.063–0.5 (0)	>256	>256 (4)
L. fermentum (5)	128	4–128 (3)	8	4–8 (5)	4	2–4 (0)	4	0.125–4 (0)	>256	>256 (5)
L. plantarum (7)	128	4–128 (5)	32	2–32 (5)	8	0.5–8 (0)	4	0.5–4 (0)	>256	>256 (7)
L. brevis (6)	64	4–64 (4)	16	2–16 (4)	4	0.063–0.5 (0)	32	4–32 (0)	>256	>256 (6)
L. parabrevis (1)	128	128 (1)	64	64 (1)	16	16 (0)	16	16 (0)	>256	>256 (1)
L. alimentarius (2)	128	16–128 (1)	64	64 (1)	16	16 (0)	2	2 (0)	>256	>256 (2)
L. salivarius (5)	128	16–128 (3)	32	4–32 (5)	8	2–8 (0)	8	1–8 (0)	>256	>256 (5)
L. animalis (2)	256	128–256 (2)	32	2–32 (1)	4	2–4 (0)	1	1 (0)	>256	>256 (2)

Table 2 continued

Bacterial species (n^a)	Antibiotic MICb (μg/ml)									
	Gentamycin		Ciprofloxacin		Bacitracin		Cefsulodin		Kanamycin	
	90%[c]	Range (n^d)	90%	Range (*n*)	90%	Range (*n*)	90%	Range (*n*)	90%	Range (*n*)
L.parabuchneri (12)	128	1–128 (7)	32	4–32 (12)	2	0.125–2 (0)	16	2–16 (0)	>256	>256 (12)
L. vaginalis (2)	8	4–8 (0)	64	32–64 (2)	4	1–4 (0)	16	16 (0)	>256	>256 (2)
L. kefiri (1)	4	4 (0)	8	8 (1)	0.5	0.5 (0)	2	2 (0)	>256	>256 (1)
L. coryniformis (1)	64	64 (1)	32	32 (1)	8	8 (0)	8	8 (0)	>256	>256 (1)
S. thermophilus (11)	128	16–128 (8)	32	1–32 (7)	1	0.125–1 (0)	1	0.063–1 (0)	0.5	>256 (11)
Total resistant strains	55 (65%)		71 (85%)		0		0		84 (100%)	

Bacterial species (n[a])	Antibiotic MICb (μg/ml)							
	Streptomycin		Chloramphenicol		Nalidixic acid		Vancomycin	
	90%[c]	Range (n^d)	90%	Range (n)	90%	Range (n)	90%	Range (n)
L. bulgaricus (13)	4	1–4 (0)	2	0.25–16 (1)	>256	>256 (13)	0.5	0.125–0.5 (0)
L. acidophilus (7)	8	1–8 (0)	4	0.25–4 (0)	>256	>256 (7)	0.5	0.125–0.5 (0)
L. casei (5)	32	4–32(1)	2	0.25–2 (0)	>256	>256 (5)	>256	>256(5)
L. rhamnosus (4)	>256	4–>256 (1)	2	0.25–2 (0)	>256	>256 (4)	>256	>256 (4)
L. fermentum (5)	>256	8–>256 (2)	8	0.25–8 (0)	>256	>256 (5)	>256	>256 (5)
L. plantarum (7)	>256	32–>256 (2)	2	0.25–2 (0)	>256	>256 (7)	>256	>256 (7)
L. brevis (6)	4	0.5–4 (0)	2	0.25–2 (0)	>256	>256 (6)	>256	>256 (6)
L. parabrevis (1)	4	4 (0)	1	1 (0)	>256	>256 (1)	>256	>256 (1)
L. alimentarius (2)	2	2 (0)	1	0.25–1 (0)	>256	>256 (2)	>256	>256 (2)
L. salivarius (5)	32	16–32 (0)	32	0.25–32 (3)	>256	>256 (5)	>256	>256 (5)
L. animalis (2)	32	32 (0)	32	32 (2)	>256	>256 (2)	>256	>256 (2)
L. parabuchneri (12)	8	2–8 (0)	1	0.25–32 (2)	>256	>256 (12)	>256	>256 (12)
L. vaginalis (2)	16	8–16 (0)	1	0.25–1 (0)	>256	>256 (2)	>256	>256 (2)
L.kefiri (1)	16	16 (0)	2	2 (0)	>256	>256 (1)	>256	>256 (1)
L. coryniformis (1)	16	16 (0)	4	4 (0)	>256	>256 (1)	>256	>256 (1)
S. thermophilus (11)	32	2–32 (0)	2	0.25–64 (2)	0.5	>256 (11)	0.5	0.125–0.5 (0)
Total resistant strains	6 (7%)		10 (12%)		84 (100%)		53 (63%)	

[a] Number of strains of a species tested

[b] The minimal inhibitory concentration (MIC) is the lowest concentration of an antibiotic that inhibits the visible bacterial growth after 24 h incubation

[c] MIC values that inhibit 90% of the strains belonging to the same species

[d] Number of resistant strains

Table 3 Characterization of 21 strains of LAB with acquired antibiotic resistances for erythromycin and tetracycline

Strain	Source	Phenotype	Genotype
L. plantarum NWL16[a]	Yogurt	ERM, TET	*erm*(B), *tet*(M)
L. plantarum NWL22[a]	Fermented vegetable	TET	*tet*(M)
L. plantarum NWL 61	Jiang Shui	TET	*tet*(S)
L. acidophilus NWL23[a]	Yogurt	ERM	*erm*(B)
L. fermentum NWL24[a]	Yogurt	ERM	*erm*(B)
L. fermentum NWL26[a]	Yogurt	ERM	*erm*(B)
L. salivarius NWL31[a]	Pickle	TET	*tet*(M)
L. salivarius NWL33[a]	Pickle	ERM, TET	*erm*(B), *tet*(M)
L. salivarius NWL34[a]	Dairy	TET	*tet*(M)
L. salivarius NWL38[a]	Dairy	TET	*tet*(M)
L. salivarius NWL42[a]	Fermented vegetable	TET	*tet*(M)
L. animalis NWL39[a]	Fermented vegetable	ERM	*erm*(B)
L. animalis NWL40[a]	Fermented vegetable	TET	*tet*(M)
L. vaginalis NWL43[a]	Fermented vegetable	ERM	*erm*(B)
L. vaginalis NWL3[a]	Dairy	ERM	*erm*(B)
L. brevis NWL51[a]	Yogurt	TET	*tet*(M), *tet*(S)[c]
L. brevis NWL59[a]	Fermented vegetable	TET	*tet*(M), *tet*(S)[c]
L. parabuchneri NWL70	Fermented vegetable	TET	–[b]
L. kefiri NWL78	Yogurt	TET	*tet*(S)[c]
S. thermophilus NWL02[a]	Yogurt	ERM	*erm*(B)
S. thermophilus NWL93	Yogurt	TET	–[b]

ERM erythromycin, *TET* tetracycline

[a] Strain used as donor in filter mating experiment

[b] Negative for all tet genes

[c] First report of gene from that particular species

Table 4 In vitro transfer of *erm*(B) and *tet*(M) from lactobacilli strains to *E. faecalis* 181

Mating organisms	Mean cell density			Transfer rate T/R
	Donor	Recipient	Transconjugants	
E. faecalis 181 × *L. fermentum* NWL24[a]	$5.8 \pm 0.96 \times 10^8$	$2.9 \pm 0.72 \times 10^7$	$7.4 \pm 1.30 \times 10^2$ [a]	$2.62 \pm 0.81 \times 10^{-5}$
E. faecalis 181 × *L. salivarius* NWL33[a]	$4.6 \pm 1.13 \times 10^8$	$3.2 \pm 1.2 \times 10^7$	$7.6 \pm 1.52 \times 10^1$ [a]	$2.9 \pm 0.9 \times 10^{-6}$
E. faecalis 181 × *L. plantarum* NWL22[b]	$5.63 \pm 0.92 \times 10^8$	$3.7 \pm 0.7 \times 10^7$	$5.2 \pm 0.94 \times 10^2$ [b]	$1.39 \pm 0.06 \times 10^{-5}$
E. faecalis 181 × *L. brevis* NWL59[b]	$4.2 \pm 1.1 \times 10^8$	$3.1 \pm 0.29 \times 10^7$	$6.7 \pm 2.67 \times 10^1$ [b]	$2.1 \pm 0.76 \times 10^{-6}$

R recipient; *T* transconjugants

[a] Resistant to erythromycin (MIC > 64) and positive for *erm*(B) gene by PCR

[b] Resistant to tetracycline (MIC > 128) and positive for *tet*(M) gene by PCR

entering into human food chain and a careful monitoring and regulation must be implied to check their antibiotic resistance profiles.

A wide spread susceptibility toward the inhibitors of cell wall synthesis, such as penicillin and ampicillin have been observed in many species of lactobacilli from different sources, such as cheese [4], probiotics or fermented foods [6], and human intestine [7, 11]. All of the lactobacilli from commercial or traditionally fermented foods in this study were sensitive to penicillin and ampicillin, except one strain of *L. salivarius* NWL31 (MIC > 64) from pickles that was resistant to penicillin. Resistance to penicillin has been reported in some species of lactobacilli from European probiotic products [41], from sausages [23] and cheese [19].

Susceptibility of all LAB strains to ampicillin, cefsulodin, and bacitracin, and intrinsic resistance to nalidixic acid, vancomycin, and kanamycin was in accordance with previous studies [10, 31, 32, 45]. Resistance of *Lactobacillus* species to vancomycin is considered as intrinsic [2], except for *L. delbrueckii* subsp. *bulgaricus*, *L. acidophilus*, *L. johnsonii*, and *L. crispatus* [4, 7].

Acquired resistance to erythromycin is generally because of *erm*(B) which has been reported previously in lactobacilli from different sources [2, 4, 5, 11, 18, 21, 25, 26, 31, 32]. Other resistant determinants [*erm*(A), *erm*(C), and *erm*(T)] are rarely detected from LAB, including lactobacilli and *S. themophillus*. Tetracycline resistance is also an acquired resistance in lactobacilli and *S. thermophilus*. Many tetracycline resistant determinants [*tet*(M), *tet*(O),

tet(S), and *tet*(W) or the efflux proteins *tet*(K) and *tet*(L)] have been reported [26, 32]. The authors identified *tet*(M) in *L. plantarum*, *L. salivarius, L. animalis*, and *L. brevis* from different sources. The *tet*(M) has also been reported previously, in *L. plantarum* [17, 23] and *L. salivarius* from meat products [3]. The authors also identified, *tet*(S) gene in *L. kefiri* (NWL78) and *L. brevis* (NWL51 and 59). To the authors' knowledge, the presence of *tet*(S) gene in *L. kefiri* or *L. brevis* has not been described previously; however, it has been detected in members of *L. plantarum* [27]. Other tetracycline resistant genes [*tet*(O), *tet*(Q), and *tet*(W)] have also been identified in lactobacilli such as *tet*(W) from a probiotic *L. reuteri* [29], but we did not find any of these genes in this study.

Although MIC can be considered as a "true" measure, however, it can have low reproducibility between labs. Recently, LAB susceptibility medium (LSM) [30] and microbiological breakpoints have been suggested by different researchers [10, 15, 16, 20, 31, 33]. Here, the authors propose tetracycline breakpoint for *L.parabuchneri*, isolated from fermented vegetables as 16 μg/ml. MIC of tetracycline for all the *L. parabuchneri* isolates ($n = 12$) from different sources, was from 16 to 32. *Lactobacillus parabuchneri* can be isolated frequently from fermented vegetables and pickles [9], from where resistances for different antibiotics have been described in other species of lactobacilli [36, 46]. MICs of bacitracin ranged from 0.063 to 16 in this study but none of the strain was resistant by the breakpoints given by Danielsen and Wind [10].

There are only few reports on conjugative transfer of naturally occurring antibiotic resistance determinants including *erm*(B) and *tet*(M) from lactobacilli to other bacteria in vitro [24, 35] or in gnotobiotic rats [28]. Feld et al. [18] successfully transferred an erythromycin resistant plasmid (pLFE1) in vitro and in vivo, from *L. plantarum* to a broad host-range including *L. rhamnosus*, *L. lactis*, *E. faecalis*, and *L. monocytogenes*. Hummel et al. [26] found *erm*(B) gene involved in erythromycin resistance from *L. salivarius* strain (BFE 7441), which was on genomic DNA. Recently, a transposons Tn916 containing *tet*(M) gene was transferred from *L. paracasei* to *E. faecalis* strain JH2-2 in mating experiments at low conjugation frequency [12]. Erythromycin resistance determinant *Erm*(B) from *L. fermentum* NWL24 and *L. salivarius* NWL33, and *tet*(M) gene from *L. plantarum* NWL22 and *L. brevis* NWL59 were successfully transferred to *E. faecalis* 181. Plasmid free recipient strains were characterized in the lab in another study. The authors also implied broth mating and filter separating techniques but conjugal transfer of *erm*(B) and *tet*(M) was not successful by these methods (data not shown), which further strengthen the notion that a close contact on a solid surface is required for better conjugal transfer in LAB [24].

The authors conclude that the prevalence of antibiotic resistant LAB in Chinese food chain is high and care should be taken whenever introducing any of these strains in functional foods intentionally. Although probiotic products and starter strains rarely had acquired antibiotic resistance, the presence of *tet*(S) gene in *L. kefiri* NWL78, isolated from a probiotic yogurt, draws the attention for a strict monitoring and regulation.

Acknowledgments This study was supported by higher education commission (HEC) of Pakistan.

References

1. Aarestrup FM, Agerso Y, Gerner-Smidt P et al (2000) Comparison of antimicrobial resistance phenotypes and resistance genes in *Enterococcus faecalis* and *Enterococcus faecium* from humans in the community, broilers, and pigs in Denmark. Diagn Microbiol Infect Dis 37:127–137
2. Ammor MS, Florez AB, Mayo B (2007) Antibiotic resistance in non-enterococcal lactic acid bacteria and bifidobacteria. Food Microbiol 24:559–570
3. Aquilanti L, Garofalo C, Osimani A et al (2007) Isolation and molecular characterization of antibiotic-resistant lactic acid bacteria from poultry and swine meat products. J Food Prot 70: 557–565
4. Belletti N, Gatti M, Bottari B et al (2009) Antibiotic resistance of lactobacilli isolated from two italian hard cheeses. J Food Prot 72:2162–2169
5. Cataloluk O, Gogebakan B (2004) Presence of drug resistance in intestinal lactobacilli of dairy and human origin in Turkey. FEMS Microbiol Lett 236:7–12
6. Charteris WP, Kelly PM, Morelli L et al (1998) Antibiotic susceptibility of potentially probiotic *Bifidobacterium* isolates from the human gastrointestinal tract. Lett Appl Microbiol 26:333–337
7. Charteris WP, Kelly PM, Morelli L et al (1998) Antibiotic susceptibility of potentially probiotic *Lactobacillus* species. J Food Prot 61:1636–1643
8. Charteris WP, Kelly PM, Morelli L et al (1998) Development and application of an in vitro methodology to determine the transit tolerance of potentially probiotic *Lactobacillus* and *Bifidobacterium* species in the upper human gastrointestinal tract. J Appl Microbiol 84:759–768
9. Coton M, Berthier F, Coton E (2008) Rapid identification of the three major species of dairy obligate heterofermenters *Lactobacillus brevis*, *Lactobacillus fermentum* and *Lactobacillus parabuchneri* by species-specific duplex PCR. FEMS Microbiol Lett 284:150–157
10. Danielsen M, Wind A (2003) Susceptibility of *Lactobacillus* spp. to antimicrobial agents. Int J Food Microbiol 82:1–11
11. Delgado S, Florez AB, Mayo B (2005) Antibiotic susceptibility of *Lactobacillus* and *Bifidobacterium* species from the human gastrointestinal tract. Curr Microbiol 50:202–207
12. Devirgiliis C, Coppola D, Barile S et al (2009) Characterization of the Tn916 conjugative transposon in a food-borne strain of *Lactobacillus paracasei*. Appl Environ Microbiol 75:3866–3871
13. Dubernet S, Desmasures N, Gueguen M (2002) A PCR-based method for identification of lactobacilli at the genus level. FEMS Microbiol Lett 214:271–275
14. EFSA (2007) Opinion of the scientific committee on a request from EFSA on the introduction of a qualified presumption of

safety (QPS) approach for assessment of selected microorganisms referred to EFSA. The EFSA Journal 187:1–16
15. EFSA (2008) Technical guidance prepared by the panel on additives and products or substances used in animal feed (FEEDAP) on the update of the criteria used in the assessment of bacterial resistance to antibiotics of human or veterinary importance. The EFSA Journal 732:1–15
16. Egervarn M, Danielsen M, Roos S et al (2007) Antibiotic susceptibility profiles of *Lactobacillus reuteri* and *Lactobacillus fermentum*. J Food Prot 70:412–418
17. Egervarn M, Roos S, Lindmark H (2009) Identification and characterization of antibiotic resistance genes in *Lactobacillus reuteri* and *Lactobacillus plantarum*. J Appl Microbiol 107: 1658–1668
18. Feld L, Bielak E, Hammer K et al (2009) Characterization of a small erythromycin resistance plasmid pLFE1 from the food-isolate *Lactobacillus plantarum* M345. Plasmid 61:159–170
19. Florez AB, Delgado S, Mayo B (2005) Antimicrobial susceptibility of lactic acid bacteria isolated from a cheese environment. Can J Microbiol 51:51–58
20. Florez AB, Egervarn M, Danielsen M et al (2006) Susceptibility of *Lactobacillus plantarum* strains to six antibiotics and definition of new susceptibility-resistance cutoff values. Microb Drug Resist 12:252–256
21. Fons M, Hege T, Ladire M et al (1997) Isolation and characterization of a plasmid from *Lactobacillus fermentum* conferring erythromycin resistance. Plasmid 37:199–203
22. Ge B, Jiang P, Han F et al (2007) Identification and antimicrobial susceptibility of lactic acid bacteria from retail fermented foods. J Food Prot 70:2606–2612
23. Gevers D, Danielsen M, Huys G et al (2003) Molecular characterization of tet(M) genes in *Lactobacillus* isolates from different types of fermented dry sausage. Appl Environ Microbiol 69: 1270–1275
24. Gevers D, Huys G, Swings J (2003) In vitro conjugal transfer of tetracycline resistance from *Lactobacillus* isolates to other Gram-positive bacteria. FEMS Microbiol Lett 225:125–130
25. Gfeller KY, Roth M, Meile L et al (2003) Sequence and genetic organization of the 19.3-kb erythromycin- and dalfopristin-resistance plasmid pLME300 from *Lactobacillus fermentum* ROT1. Plasmid 50:190–201
26. Hummel AS, Hertel C, Holzapfel WH et al (2007) Antibiotic resistances of starter and probiotic strains of lactic acid bacteria. Appl Environ Microbiol 73:730–739
27. Huys G, D'Haene K, Swings J (2006) Genetic basis of tetracycline and minocycline resistance in potentially probiotic *Lactobacillus plantarum* strain CCUG 43738. Antimicrob Agents Chemother 50:1550–1551
28. Jacobsen L, Wilcks A, Hammer K et al (2007) Horizontal transfer of tet(M) and erm(B) resistance plasmids from food strains of Lactobacillus plantarum to Enterococcus faecalis JH2–2 in the gastrointestinal tract of gnotobiotic rats. FEMS Microbiol Ecol 59:158–166
29. Kastner S, Perreten V, Bleuler H et al (2006) Antibiotic susceptibility patterns and resistance genes of starter cultures and probiotic bacteria used in food. Syst Appl Microbiol 29:145–155
30. Klare I, Konstabel C, Muller-Bertling S et al (2005) Evaluation of new broth media for microdilution antibiotic susceptibility testing of Lactobacilli, Pediococci, Lactococci, and Bifidobacteria. Appl Environ Microbiol 71:8982–8986
31. Klare I, Konstabel C, Werner G et al (2007) Antimicrobial susceptibilities of *Lactobacillus*, *Pediococcus* and *Lactococcus* human isolates and cultures intended for probiotic or nutritional use. J Antimicrob Chemother 59:900–912
32. Mathur S, Singh R (2005) Antibiotic resistance in food lactic acid bacteria—a review. Int J Food Microbiol 105:281–295
33. Mayrhofer S, van Hoek AH, Mair C et al (2010) Antibiotic susceptibility of members of the *Lactobacillus acidophilus* group using broth microdilution and molecular identification of their resistance determinants. Int J Food Microbiol 144(1):81–87
34. Mazel D, Davies J (1999) Antibiotic resistance in microbes. Cell Mol Life Sci 56:742–754
35. Ouoba LI, Lei V, Jensen LB (2008) Resistance of potential probiotic lactic acid bacteria and bifidobacteria of African and European origin to antimicrobials: determination and transferability of the resistance genes to other bacteria. Int J Food Microbiol 121:217–224
36. Perez Pulido R, Omar NB, Lucas R et al (2005) Resistance to antimicrobial agents in lactobacilli isolated from caper fermentations. Antonie Van Leeuwenhoek 88:277–281
37. Relman DA, Loutit JS, Schmidt TM et al (1990) The agent of bacillary angiomatosis. An approach to the identification of uncultured pathogens. N Engl J Med 323:1573–1580
38. Rojo-Bezares B, Saenz Y, Poeta P et al (2006) Assessment of antibiotic susceptibility within lactic acid bacteria strains isolated from wine. Int J Food Microbiol 111:234–240
39. Salyers AA, Gupta A, Wang Y (2004) Human intestinal bacteria as reservoirs for antibiotic resistance genes. Trends Microbiol 12:412–416
40. Sutcliffe J, Grebe T, Tait-Kamradt A et al (1996) Detection of erythromycin-resistant determinants by PCR. Antimicrob Agents Chemother 40:2562–2566
41. Temmerman R, Pot B, Huys G et al (2003) Identification and antibiotic susceptibility of bacterial isolates from probiotic products. Int J Food Microbiol 81:1–10
42. Teuber M (2001) Veterinary use and antibiotic resistance. Curr Opin Microbiol 4:493–499
43. Teuber M, Meile L, Schwarz F (1999) Acquired antibiotic resistance in lactic acid bacteria from food. Antonie Van Leeuwenhoek 76:115–137
44. Xu J, Smyth CL, Buchanan JA et al (2004) Employment of 16 S rDNA gene sequencing techniques to identify culturable environmental eubacteria in a tertiary referral hospital. J Hosp Infect 57:52–58
45. Zhou JS, Pillidge CJ, Gopal PK et al (2005) Antibiotic susceptibility profiles of new probiotic *Lactobacillus* and *Bifidobacterium* strains. Int J Food Microbiol 98:211–217
46. Zonenschain D, Rebecchi A, Morelli L (2009) Erythromycin- and tetracycline-resistant lactobacilli in Italian fermented dry sausages. J Appl Microbiol 107:1559–1568