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Antibiotic Resistance of *LACTOBACILLUS* Strains

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Abstract

The study provides phenotypic and molecular analyses of the antibiotic resistance in 20 *Lactobacillus* strains including 11 strains newly isolated from fermented plant material. According to the results of disc diffusion method, 90% of tested lactobacilli demonstrated sensitivity to clindamycin and 95% of strains were susceptible to tetracycline, erythromycin, and rifampicin. Ampicillin and chloramphenicol were found to inhibit all bacteria used in this study. The vast majority of tested strains revealed phenotypic resistance to vancomycin, ciprofloxacin, and aminoglycosides. Most of *Lactobacillus* strains showed high minimum inhibitory concentrations (MICs) of cefotaxime, ceftriaxone, and cefazolin and therefore were considered resistant to cephalosporins. All the strains exhibited multidrug resistance. The occurrence of resistance genes was associated with phenotypic resistance, with the exception of phenotypically susceptible strains that contained genes for tetracycline (*tet*K, *tet*L) and erythromycin (*erm*B, *mef*A) resistance. The *van*X gene for vancomycin resistance was among the most frequently identified among the lactobacilli (75% of strains), but the occurrence of the *par*C gene for ciprofloxacin resistance was sporadic (20% of strains). Our results mainly evidence the intrinsic nature of the resistance to aminoglycosides in lactobacilli, though genes for enzymatic modification of streptomycin *aad*A and *aad*E were found in 20% of tested strains. The occurrence of extended spectrum beta-lactamases (ESBL) was unknown in *Lactobacillus*, but our results revealed the *bla*TEM gene in 80% of strains, whereas *bla*SHV and *bla*OXA-1 genes were less frequent (20% and 15% of strains, respectively).

Introduction

Lactobacilli are Gram-positive bacteria of high biotechnological and natural significance. They populate nutrient-rich habitats associated with food, feed, plants, animals, and humans [15]. In latter, *Lactobacillus* spp. belong to the resident gut microbiota being responsible for many of its beneficial effects on human health [28]. As a result, lactobacilli are widely used as probiotics to maintain or to replenish the gut microbiota after antibiotic treatment [23].

With regard to antibiotic resistance of the lactobacilli, vancomycin and ciprofloxacin have low inhibitory effect among the majority of *Lactobacillus* species [23, 54]. Lactobacilli are generally susceptible to the inhibitors of protein synthesis, such as chloramphenicol, macrolides, lincosamides, and tetracycline, but their resistance to aminoglycosides is often high [1, 23, 27, 32]. Moreover, lactobacilli are usually sensitive to the cell wall-targeting β-lactams such as penicillin, but are more resistant to cephalosporins [1, 23]. Resistance to other antibiotics varies greatly among lactobacilli.

Antibiotic resistance of probiotic *Lactobacillus* strains is an essential property for their application to reinforce the concomitant antibiotic therapy. On the other hand, intake of bacteria with acquired antibiotic resistance poses the risk of its dissemination in the gastrointestinal microbiota and totally in the environment. Studying of antibiotic susceptibility pattern and resistance genes in *Lactobacillus* spp. is a comparably recent approach [30]. Up to now, the presence of several antibiotic resistance genes, both intrinsic and acquired, has been reported in *Lactobacillus* spp. For example, chloramphenicol-resistance *cat* gene has been found in *L. reuteri* [16] and *L. plantarum* [55]. Different erythromycin-resistance genes (*erm*B, *erm*A, *erm*C, *erm*T) and at least 11 tetracycline resistance genes (*tet*W, *tet*M, *tet*S,

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*tet*O, *tet*Q, *tet*36, *tet*Z, *tet*O/W/32/O/W/O, *tet*W/O, *tet*K, and *tet*L) have been detected to date in lactobacilli [38, 42, 57], among which *tet*M and *erm*B were suggested to be the most widely-distributed among *Lactobacillus* spp.[3, 4, 6, 14]. Some of them were found to be located on plasmids and in transposons and thus were considered acquired [14, 18, 42, 44, 47]. These findings evidence the view on the food and probiotic bacteria as reservoirs of antibiotic resistance genes and facilitate the revision of GRAS (generally recognized as safe) status of lactic acid bacteria (LAB).

However, there is still much to be explored about the problem of antibiotic resistance of lactobacilli. Assessment of antibiotic resistances among lactobacilli is confounded by the lack of standards for susceptibility testing, as well as susceptibility breakpoints for most antibiotics. Determination of antibiotic susceptibility patterns of a representative number of different strains from each species is necessary for working out of reliable criteria for the differentiation between the intrinsic and the acquired antibiotic resistance in probiotic bacteria. Although some effort has been made to this end, work has only been carried out for some antibiotics and particular *Lactobacillus* species, such as *L. casei, L. acidophilus, L. reuteri, L. rhamnosus, L. delbrueckii, L. brevis, L. fermentum* [2, 4, 20, 37, 42]. Besides, antibiotic resistance profile of *Lactobacillus* strains for practical application should be studied by both phenotypic and genotypic methods, because a susceptible phenotype may carry silent genes, which are detected by PCR-based molecular methods [54].

In the present study, antibiotic resistance pattern of 20 *Lactobacillus* strains was investigated through the disc diffusion method and microdilution method as well as molecular methods to check the presence of different antibiotic resistance genes.

Materials and Methods

Isolation of Bacteria and Growth Conditions

Lactobacilli were isolated from probiotics, commercial dairy products and fermented plant material and identified in our previous studies [5, 7]. *L. brevis* DSM 20,054, *L. buchneri* DSM 20,057, *L. brevis* ssp. *gravesensis* LMG 7934, and *L. rhamnosus* B-8238, obtained from Culture Collections, were used as reference strains (Table 1). The cultures preserved as glycerol stocks were activated by growing in sterile de Man, Rogosa, Sharpe (MRS) broth [12] under microaerophilic conditions at 37 °C for 24 h. The cultures thus activated were stored at 5 °C with fortnightly subculture in MRS broth. Working cultures were prepared in sterilized MRS broth using 1% inocula followed by incubation under microaerophilic conditions at 37 °C for 16–18 h.

Table 1 Bacterial strains used in this study

№	Strain	Source
1	*L. brevis* ssp. *gravesensis* LMG 7934	Belgian collection of microorganisms
2	*L. brevis* DSM 20054	German collection of microorganisms and cell cultures (Leibniz Institute)
3	*L. buchneri* DSM 20057	
4	*L. rhamnosus* B-8238	Russian collection of industrial microorganisms
5	*L. fermentum* BB	Drink yoghurt “Bio Balance”, JUnimilk, Russia
6	*L. fermentum* 1–3	Drink yoghurt “Vamin”, Vamin, Tatarstan, Russia
7	*L. fermentum* Ga	Probiotic “Gastropharm”, Biovet, Bulgary
8	*L. plantarum* Na	Dietary supplement “Narine”, Narex, Armenia
9	*L. plantarum* 8PA3	Probiotic preparation “Lactobacterin dry”, Biomed, Russia
10	*L. plantarum* FCa1L	Sauerkraut collected from a local market (Kazan, Tatarstan, Russia)
11	*L. plantarum* FCa3L	
12	*L. plantarum* S1	Silage collected from the agricultural enterprise “Kulon” (Chistopol district, Tatarstan, Russia)
13	*L. plantarum* S6	
14	*L. plantarum* S7	
15	*L. plantarum* AG1	Silage collected from the agricultural enterprise “Zavolzh’e” (Kaibitsy district, Tatarstan, Russia)
16	*L. plantarum* AG8	
17	*L. plantarum* AG9	
18	*L. plantarum* AG10	
19	*L. plantarum* AG15	
20	*L. fermentum* AG16	

Antibiotic Susceptibility Testing

Antibiotic susceptibility was assessed by the disk diffusion method, as described earlier [5, 7]. In brief, all strains were diluted in 0.85% saline to obtain turbidity equivalent to McFarland scale 0.5 and aliquots were pour-plated on MRS agar plates. Antibiotic discs (Scientific Research Centre of Pharmacotherapy, Russia) were placed on the surface of inoculated plates. After 48 h incubation in anaerobic conditions (Anaerogas GasPak, NIKI MLT, Russia) at 37 °C, inhibition halos were measured in mm (means ± SD of 3 trials) and interpreted as susceptible (S), moderately susceptible (MS), or resistant (R) according to [9, 43], as indicated in Table S1.

The MIC values of cephalosporins were determined by the broth microdilution method in MRS broth in 96-well nontreated cell culture plates (Eppendorf). Cefazolin, ceftriaxone, and cefotaxime (all Sigma-Aldrich) were tested in concentration range of 0.5–1024 µg/ml obtained after a series of two-fold dilutions in MRS broth. Wells were inoculated with 200 µl of the bacterial culture (3×10^7 CFU/ml) and incubated at 37 °C. The MIC was read after 24 h of incubation as the lowest concentration of an antibiotic at which visible growth was inhibited.

Detection of Antibiotic Resistance Genes

Genomic DNA was extracted from lactobacilli cells and purified as described earlier [4]. Antibiotic resistance genes tested by PCR are indicated in Table S2. The PCR reaction was carried out in a total volume of 25 µl containing DNA template, 10 pmol of each primer (Table S2) [10, 16, 17, 21, 25, 27, 31, 40, 48, 50, 52, 59, 60], 1U Taq DNA polymerase, each of four dNTPs at a concentration of 200 µM, and PCR buffer containing Tris–HCl, KCl $(NH_4)_2SO_4$, $MgSO_4$, and Triton X-100. The amplification program was as following: initial denaturation step of 94 °C for 5 min, and then 35 cycles of 94 °C for 30 s, annealing temperature (Table S2) for 30 s, and a final extension step at 72 °C for 7 min. The obtained PCR fragments were analyzed by electrophoreses on a 1% agarose gel, stained with Midori Green DNA Stain (Nippon Genetics Europe, Germany) and visualized with UV light. The positive amplicons obtained in these PCRs were confirmed by sequencing. PCR products were purified with a GenJET Plasmid Miniprep Kit (Thermo Scientific, Lithuania) according to the instructions of the manufacturer. The purified products were sequenced with the forward and reverse primer (Evrogen JSC, Russian). The obtained primary nucleotide sequences were analyzed using the NCBI-BLAST algorithm and GenBank database.

Results

Phenotypic Resistance of Lactobacilli

In this study, 20 *Lactobacillus* strains were analyzed for the antibiotic susceptibility by disc diffusion method and were classified either as resistant (R), moderately susceptible (MS), or sensitive (S) based on zones of growth inhibition. All tested *Lactobacillus* strains were susceptible to ampicillin and chloramphenicol. Sensitivity to rifampicin and to the inhibitors of protein synthesis erythromycin, tetracycline, and clindamycin was also widely distributed among lactobacilli (Fig. 1b). Most of *Lactobacillus* strains were resistant to vancomycin (95% of strains), ciprofloxacin (95%) and aminoglycosides used in this work: amikacin (95%), kanamycin (100%), gentamicin (90%), streptomycin (85%). Noteworthily, that well-known probiotic strain *L. planrarum* 8PA3 possessed an antibiotic resistance profile not typical for *Lactobacillus*. For example, it revealed sensibility to vancomycin, while the vast majority of other tested lactobacilli were resistant to this antibiotic. Besides, unlike most lactobacilli, *L. plantarum* AG15 revealed rifampicin resistance.

The broth microdilution method was used to characterize the resistance of lactobacilli to cephalosporins. Microbiological breakpoints categorizing *Lactobacillus* species as resistant to cefotaxime, ceftriaxone and cefazolin are not determined yet. Therefore, we assumed that strains with high MIC values (64 and 128 µg/ml) were more likely to be resistant while two *L. plantarum* isolates (strains FCa1L and S6) with MIC values 0.5 µg/mL for three tested cephalosporins were considered as sensitive to them.

Genotypic Resistance of Lactobacilli

The *Lactobacillus* strains were tested for the presence of antibiotic resistance genes by PCR. The results are presented in Table 2 and fig. S1. Four silage *Lactobacillus* isolates were positive for genes encoding erythromycin-resistance *erm*B and *mef*A. The *tet*K gene was detected in *L. buchneri* DSM 20057 and *tet*L was found in *L. plantarum* FCa1L and in two *L. plantarum* silage isolates S1 and AG15 (Fig. S1, b). Detection of *tet*K gene was confirmed by the results of NCBI BLAST search for the homologs of tetracycline-resistance protein of *Staphylococcus aureus* PM1 (YP_006958133.1) in the genome sequence of *L. buchneri* DSM 20057. As a result, we identified nine secondary transporters which belong to the major facilitator superfamily (MFS) proteins and facilitate the transport across bacterial membranes. Among them, the EmrB QacA subfamily drug resistance transporter (KRK67099.1) gave the highest query cover

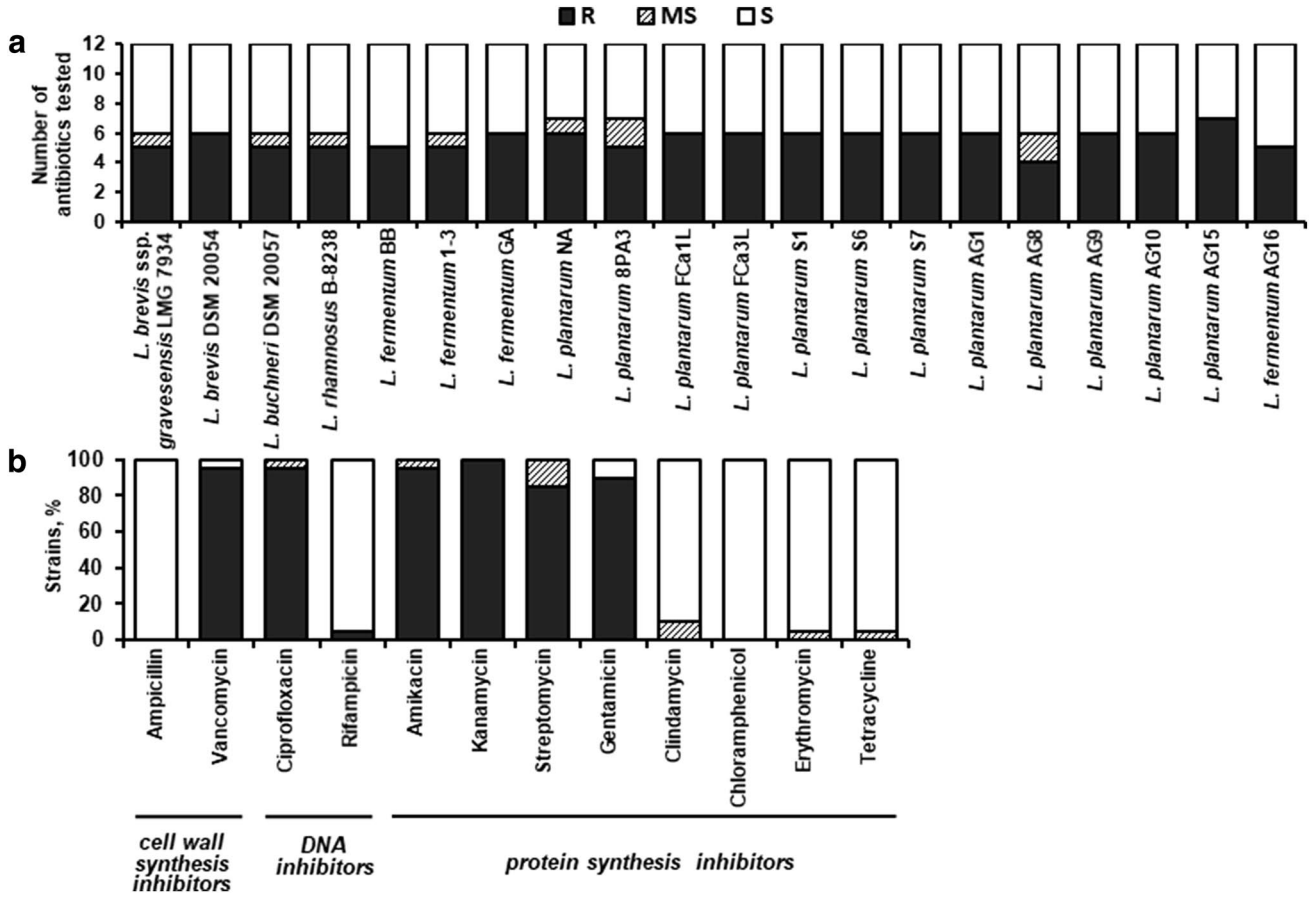

Fig. 1 Antibiotic resistance of lactobacilli. **a** Antibiotic resistance profiles exhibited by *Lactobacillus* strains. **b** Prevalence of the resistance to specific antibiotics among tested *Lactobacillus* strains

(99%) and shared 41% similarity and 24% identity with the typical tetracycline efflux protein TetK.

Other erythromycin and tetracycline-resistance determinants (*erm*A, *mef*E, *tet*M) were not detected in any strain.

The gene *par*C associated with resistance to ciprofloxacin was detected in *L. plantarum* FCa3L, *L. brevis* DSM 20,054, *L. brevis* ssp. *gravesensis* LMG 7934, and L. *buchneri* DSM 20,057. No PCR products were obtained for another ciprofloxacin resistance gene *gyr*A. In addition, the PCR analysis showed that none of tested *Lactobacillus* strains possessed *aac*(6′)-*aph*(2″), *ant*(6), *aph*(3′)-III, and *ant*(2″)-I genes, which encode enzymatic inactivation of aminoglycosides. Yet, streptomycin resistance genes *aad*A and *aad*E were found in *L. rhamnosus* B-8238, *L. plantarum* FCa3L, *L. plantarum* AG1, and *L. plantarum* AG10.

The vancomycin resistance gene *van*X was detected in 15 *Lactobacillus* strains (Table 2, Fig. S1, c), while other genes of this cluster *van*A and *van*E were not revealed in any strain.

Sixteen tested *Lactobacillus* strains gave a 0.5 kbp band, presumably corresponding to *bla*TEM gene of cephalosporins resistance (Fig S1, a). The amplicons were sequenced and a 99% similarity with the *bla*TEM gene of *Acinetobacter baumannii* (GenBank Accession No. MK764360.1) and *Escherichia* sp. (GenBank Accession No. NG050218.1) was revealed by NCBI BLAST algorithm. The genes *bla*OXA-1 and *bla*SHV also related to cephalosporins resistance were less frequent in tested *Lactobacillus* strains and were detected in 3 and 4 strains, correspondingly.

The chloramphenicol resistance gene *cat* as well as the *mec*A gene were not detected in any strain.

Discussion

In the present study, we characterized phenotypic and genotypic antibiotic resistance profiles of 20 *Lactobacillus* strains, including 11 strains newly isolated from fermented plant material. In addition, four reference strains and five probiotic and dairy isolates were included in this study (Table 1). Irrespective of the origin, all the strains exhibited phenotypic resistance to a number of antibiotics revealing multidrug resistance pattern (Fig. 1, a). Moreover, all the

Table 2 Antibiotic resistance of *Lactobacillus* strains and detection of antibiotic resistance genes by polymerase chain reaction (PCR)

№	Strain	Phenotype*												
		Ampicillin	Vancomycin	Ciprofloxacin	Rifampicin	Amikacin	Kanamycin	Streptomycin	Gentamicin	Clindamycin	Chloramphenicol	Erythromycin	Tetracycline	Genotype
1	*L. brevis* ssp. *gravesensis* LMG 7934	S	R	R	S	R	R	MS	R	S	S	S	S	*par*C
2	*L. brevis* DSM 20054	S	R	R	S	R	R	R	R	S	S	S	S	*par*C, vanX
3	*L. buchneri* DSM 20057	S	R	R	S	R	R	R	S	S	S	S	MS	*tet*K, *par*C
4	*L. rhamnosus* B-8238	S	R	MS	S	R	R	R	R	S	S	S	S	*aad*A
5	*L. fermentum* BB	S	R	R	S	R	R	S	R	S	S	S	S	–
6	*L. fermentum* 1–3	S	R	R	S	R	R	MS	R	S	S	S	S	*van*X
7	*L. fermentum* Ga	S	R	R	S	R	R	R	R	S	S	S	S	*van*X
8	*L. plantarum* Na	S	R	R	S	R	R	R	R	MS	S	S	S	*van*X
9	*L. plantarum* 8PA3	S	S	R	S	R	R	R	R	MS	S	MS	S	–
10	*L. plantarum* FCa1L	S	R	R	S	R	R	R	R	S	S	S	S	*van*X, *tet*L
11	*L. plantarum* FCa3L	S	R	R	S	R	R	R	R	S	S	S	S	*par*C, *van*X, *aad*E
12	*L. plantarum* S1	S	R	R	S	R	R	R	R	S	S	S	S	*tet*L, *van*X
13	*L. plantarum* S6	S	R	R	S	R	R	R	R	S	S	S	S	*van*X
14	*L. plantarum* S7	S	R	R	S	R	R	R	R	S	S	S	S	*van*X
15	*L. plantarum* AG1	S	R	R	S	R	R	R	R	S	S	S	S	*van*X, *aad*E, *erm*B
16	*L. plantarum* AG8	S	R	R	S	MS	R	MS	R	S	S	S	S	*van*X
17	*L. plantarum* AG9	S	R	R	S	R	R	R	R	S	S	S	S	*van*X, *erm*B
18	*L. plantarum* AG10	S	R	R	S	R	R	R	R	S	S	S	S	*van*X, *aad*E, *erm*B
19	*L. plantarum* AG15	S	R	R	R	R	R	R	R	S	S	S	S	*van*X, *mef*A, *tet*L

Table 2 (continued)

№	Strain	Phenotype*												Genotype
		Ampicillin	Vancomycin	Ciprofloxacin	Rifampicin	Amikacin	Kanamycin	Streptomycin	Gentamicin	Clindamycin	Chloramphenicol	Erythromycin	Tetracycline	
20	*L. fermentum* AG16	S	R	R	S	R	R	R	S	S	S	S	S	*van*X

*Based on standards mentioned in Materials and methods, lactobacilli were characterized as either *S* susceptible, *MS* moderately susceptible, or *R* resistant to each antibiotic tested in disc diffusion method

strains carried at least one gene for antibiotic resistance (Tables 2, 3).

It is well known that lactobacilli are generally susceptible to antibiotics inhibiting nucleic acid synthesis (except for ciprofloxacin) and protein synthesis (except for aminoglycosides), and to cell wall synthesis inhibitors (except for vancomycin) [1, 11, 27, 34]. Indeed, in our study, 95% of tested *Lactobacillus* strains were susceptible to tetracycline, erythromycin, and rifampicin, and 90% of strains demonstrated sensitivity to clindamycin (Fig. 1b). Ampicillin and chloramphenicol were found to inhibit all bacteria used in this study (Fig. 1b). The latter notion coincided with the genomic program of tested lactobacilli. The chloramphenicol acetyl transferase gene *cat* which is associated with resistance to chloramphenicol was not detected in any of the strains. Gene *mec*A which encodes penicillin-binding protein 2A and confers resistance to penicillin-like antibiotics also was not found in tested *Lactobacillus* strains.

Tetracycline or erythromycin-resistant lactobacilli have been encountered in previous studies [4, 11, 21, 23, 42, 56]. Genes encoding these two resistances are often located on mobile genetic elements such as conjugative plasmids and transposons [3, 14, 18, 44, 47]. Therefore, detection of resistances to tetracycline and erythromycin in bacterial strains for food and agricultural applications always constitutes the risk of spread of antibiotic resistance genes in the environment. The most common determinants for resistance to tetracycline found in lactobacilli are genes *tet* (K, M, O, Q, S, W, 36), sometimes also present in combination [3, 23]. Among erythromycin-resistance genes, the *erm*B gene is the most frequently found among *Lactobacillus* spp.[42, 57].

In the present study, using PCR, we detected tetracycline efflux genes *tet*K (in *L. buchneri* DSM 20,057) and *tet*L (in *L. plantarum* FCa1L, *L. plantarum* S1, and *L. plantarum* AG15). The *erm*B gene, encoding 23S ribosomal rRNA methyltransferase, was found in three silage isolates, and macrolide efflux gene *mef*A was detected in another silage isolate *L. plantarum* AG15. All the strains which contained erythromycin and tetracycline-resistance genes displayed phenotypic susceptibility to these antibiotics. Similarly, in our previous investigation *L. fermentum* 5–1 sensitive to tetracycline was discovered to carry silent genes *tet*K and *tet*M, and *L. fermentum* 3–4 sensitive to erythromycin was positive for *erm*C [4]. These discrepancies between the resistance phenotype and genotype may be due to defective expression of resistance genes and have been described earlier by [41].

It is well documented that lactobacilli are usually resistant to aminoglycosides [23, 60]. This resistance is considered intrinsic and originates from the low impermeability of lactobacillar cell surface for aminoglycosides [9, 32, 34]. Yet, resistance genes for enzymatic modification of aminoglycosides such as *aac*(6′)-*aph*(2″), *ant*(6) and *aph*(3′)-III have been reported in several *Lactobacillus* spp.[50]. The

vast majority of tested strains revealed phenotypic resistance to streptomycin, kanamycin, gentamycin, and amikacin (Table 2). The aminoglycoside adenyltransferase genes *aad*A and *aad*E which confer resistance to streptomycin were identified in four strains (Table 2, Fig S1, b), but other aminoglycoside-resistance genes (*aac*(6′)-*aph*(2″), *aph*(3′)-III, *ant*(6), *ant*(2″)-I) were not detected by PCR analysis in any of the tested lactobacilli. Thus, according to our results, among *Lactobacillus* spp. widely distributed resistance to aminoglycosides is likely to be intrinsic, though enzymatic inactivation of streptomycin is possible in some strains.

Lactobacilli have high natural resistance to vancomycin and ciprofloxacin. However, susceptibility to these antibiotics was shown to be species-dependent and varied several folds between species [11]. Hence, some resistant strains may harbor spontaneous mutations or acquired genes. In this work, we studied resistances to vancomycin and ciprofloxacin to find out their genetic determinants and assess their potential transferability.

We demonstrated that all tested *Lactobacillus* strains except for *L. plantarum* 8PA3 were resistant to vancomycin (Table 2). The vancomycin resistance has been reported to be intrinsic, chromosomally encoded and not inducible or transferable in lactobacilli [35, 51]. The most studied mechanism of vancomycin resistance in *Lactobacillus* spp. includes the replacement of the terminal D-alanine residue by D-lactate or D-serine in muramyl-pentapeptide molecule [13, 54]. Vancomycin has low-affinity binding to such altered peptidoglycan termini, and thus lactobacilli are generally resistant to vancomycin. The D-alanyl-D-alanine dipeptidase, a product of *van*X gene, is critical for vancomycin resistance, because it prevents synthesis of the usual D-alanyl-D-alanine termini of the peptidoglycan precursor side chain. Homologs of *van*X were found in genomes of sequenced *Lactobacillus* spp. (e.g. KRK67761.1 in *L. buchneri* DSM 20,057, ERK42887.1 in *L. brevis* DSM 20,054, ARW34669.1 in *L. plantarum* SRCM102022, according to NCBI GenBank database). Besides, in two strains of *L. plantarum* (LP1, LP2), the *van*X gene was detected after sequencing and alignment [40]. In this study, PCR analysis revealed *van*X gene in 15 tested *Lactobacillus* strains (Table 2, fig. S1, c). Guo et al. [24] also showed that *van*X gene was frequent in lactobacilli. Among the genes of the vancomycin resistance cluster, only *van*A gene is considered transferable via conjugation within the plasmid DNA [56, 58] or the conjugative transposon [26, 56]. In our work, no PCR products were amplified with *van*A and *van*E primer sets. Therefore. we conclude that tested *Lactobacillus* strains did not carry these resistance genes.

According to our results, all tested *Lactobacillus* strains showed resistance to ciprofloxacin, except for *L. rhamnosus* B-8238 which was moderately susceptible. Frequently

Table 3 Characterization of cephalosporin resistance of *Lactobacillus* strains

№	Strain	MIC, μg/mL			Genotype *
		Cefazolin	Ceftriaxone	Cefotaxime	
1	*L. brevis* ssp. *gravesensis* LMG 7934	16	0.5	0.5	*bla*OXA-1, *bla*SHV, *bla*TEM
2	*L. brevis* DSM 20054	8	16	4	*bla*TEM
3	*L. buchneri* DSM 20057	8	4	0.5	*bla*OXA-1, *bla*SHV, *bla*TEM
4	*L. rhamnosus* B-8238	16	16	16	*bla*TEM
5	*L. fermentum* BB	32	128	4	*bla*TEM
6	*L. fermentum* 1–3	16	32	0.5	*bla*OXA-1, *bla*TEM
7	*L. fermentum* Ga	1	8	4	*bla*TEM
8	*L. plantarum* Na	16	0.5	1	*bla*TEM
9	*L. plantarum* 8PA3	0.5	16	2	-
10	*L. plantarum* FCa1L	0.5	0.5	0.5	-
11	*L. plantarum* FCa3L	0.5	128	0.5	*bla*TEM
12	*L. plantarum* S1	64	128	2	*bla*TEM
13	*L. plantarum* S6	0.5	0.5	0.5	*bla*TEM
14	*L. plantarum* S7	64	128	0.5	-
15	*L. plantarum* AG1	32	32	8	*bla*TEM *bla*SHV
16	*L. plantarum* AG8	16	64	16	*bla*TEM, *bla*SHV
17	*L. plantarum* AG9	16	128	16	-
18	*L. plantarum* AG10	16	16	4	*bla*TEM
19	*L. plantarum* AG15	64	32	2	*bla*TEM
20	*L. fermentum* AG16	4	16	32	*bla*TEM

*Cephalosporin resistance genes present in *Lactobacillus* strains as detected using PCR

encountered within the genus *Lactobacillus* resistance to ciprofloxacin has been earlier described by [11, 32, 39]. It is considered to arise from intrinsic characteristics, such as cell wall impermeability or efflux mechanism [27]. However, other mechanisms can be involved in the development of resistance to fluoroquinolones in Gram-positive bacteria. Some are the consequence of mutations involving genes encoding DNA gyrase and topoisomerase IV, essential type II topoisomerases necessary for DNA replication, chromosome segregation and DNA compaction in the cell [36, 49]. Here, we tested mutations in the quinolone resistance-determining region (QRDR) of the *par*C (topoisomerase IV) and *gyr*A (DNA gyrase) genes using PCR and consequent DNA sequencing of the obtained amplicons. The PCR fragments for the *par*C gene were obtained in four *Lactobacillus* strains and none of the strains possessed *gyr*A gene. Our data partly corroborate the results of [20, 27], which also demonstrated absence of typical mutations in the QRDR of *gyr*A or *par*C genes for ciprofloxacin resistance in *Lactobacillus* spp.

Regarding beta-lactams, lactobacilli are generally susceptible to penicillins, but more resistant or variable to cephalosporins [1, 53]. With few exceptions, *Lactobacillus* strains showed high MIC values of cefotaxime, ceftriaxone, and cefazolin (Table 3), as previously reported by [1, 22, 29]. Notably, among tested *Lactobacillus* strain sensitivity to cefotaxime was more frequent rather than to two other cephalosporins (Table 3). Indeed, cefotaxime has been shown to be the most active type I β-lactamase inhibitor, in comparison to the other β-lactam antibiotics [19]. The understanding of the mechanisms underlying resistance to cephalosporins is still very limited in lactobacilli. Although multiple beta-lactamases can be identified in the available genomic sequences of *Lactobacillus* spp., the presence of extended spectrum beta-lactamase (ESBL) in Gram-positive lactic acid bacteria remains obscure [33]. The major ESBL enzymes are TEM, SHV, CTX-M KPC, VIM, IMP, NDM-1, and OXA [8, 46]. Using PCR amplification and subsequent sequencing we identified the *bla*TEM gene in 80% of tested *Lactobacillus* trains, whereas *bla*SHV and *bla*OXA-1 genes were less frequent. To our knowledge, this is the first data on the detection of ESBL in lactobacilli. Although it is believed, that SHV enzymes confer much higher resistance than do TEM enzymes [45], according to our results TEM enzyme was the most frequent lactamase responsible for resistance to cephalosporins in lactobacilli.

Conclusions

Phenotypic multidrug resistance was revealed in all tested *Lactobacillus* strains. Studying of corresponding resistance genes showed that *bla*TEM (80% of strains) and *van*X (75%) were the most frequently identified, and the occurrence of *erm*B (15%), *mef*A (5%), *tet*K (5%), *tet*L (15%), *par*C (20%), *aad*A (5%), *aad*E (15%), *bla*SHV (20%), and *bla*OXA-1 (15%) was less frequent. To our knowledge, genes for ESBLs were found in lactobacilli for the first time. Consideration should be given to the potentially transferable resistance determinants such as *erm*B, *tet*K, and *tet*L which were found in this work, fueling the debate about the safe use of lactobacilli in food and their potential to spread resistance in the environment. Future studies should be focused on horizontal transfer of detected resistance genes to other species.

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Author Contributions EA designed the experiments, carried out the study, interpreted the data and drafted the manuscript. DY designed the study, supervised all experiments and reviewed the manuscript. All authors read and approved the final manuscript.

Data Availability The data used to support the findings of this study are included within the article.

Compliance with Ethical Standards

Conflict of interests The authors declare that there is no conflict of interests regarding the publication of this paper.

Ethical Approval Ethical approval was not required.

References

1. Ammor MS, Flórez AB, Mayo B (2007) Antibiotic resistance in non-enterococcal lactic acid bacteria and bifidobacterial. Food Microbiol 24:559–570. https://doi.org/10.1016/j.fm.2006.11.001
2. Ammor MS, Flórez AB, Van Hoek AHAM, de los Reyes-Gavilán CG, Aarts HJM, Margolles A, et al (2008) Molecular characterization of intrinsic and acquired antibiotic resistance in lactic acid bacteria and bifidobacteria. J Mol Microbiol Biotechnol 14:6–15. https://doi.org/10.1159/000106077
3. Ammor MS, Gueimonde M, Danielsen M, Zagorec M, van Hoek AH, de Los Reyes-Gavilán CG, Mayo B, Margolles A (2008) Two different tetracycline resistance mechanisms, plasmid-carried *tet*(L) and chromosomally located transposon-associated *tet*(M), coexist in *Lactobacillus sakei* Rits 9. Appl Environ Microbiol 74(5):1394–1401. https://doi.org/10.1128/AEM.01463-07
4. Anisimova E, Yarullina D (2018) Characterization of erythromycin and tetracycline resistance in *Lactobacillus fermentum* strains. Int J Microbiol. https://doi.org/10.1155/2018/3912326
5. Anisimova EA, Yarullina DR, Ilinskaya ON (2017) Antagonistic activity of lactobacilli isolated from natural ecotopes. Microbiology 86(6):708–713. https://doi.org/10.1134/S0026261717060054
6. Bernardeau M, Vernoux JP, Henri-Dubernet S, Guéguen M (2008) Safety assessment of dairy microorganisms: the *Lactobacillus* genus. Int J Food Microbiol 126:278–285. https://doi.org/10.1016/j.ijfoodmicro.2007.08.015
7. Bruslik NL, Akhatova DR, Toimentseva AA, Abdulkhakov SR, Yarullina DR (2015) Estimation of probiotic lactobacilli drug resistance. J Antibiotics and Chemotherapy 60(3–4):6–13

8. Canton R, Coque TM (2006) The CTX-M beta-lactamase pandemic. Curr Opin Microbiol 9:466–475. https://doi.org/10.1016/j.mib.2006.08.011
9. Charteris WP, Kelly PM, Morelli L, Collins JK (1998) Antibiotic susceptibility of potentially probiotic *Lactobacillus* species. J Food Protection 61(12):1636–1643
10. Colom K, Perez J, Alonson R, Fernandez-Aranguiz A, Larino E, Cisterna R (2003) Simple and reliable multiplex PCR assay for detection of blaTEM, bla SHV and bla OXA-1 genes in *Enterobacteriaceae*. FEMS Microbiol Lett 223:147–151. https://doi.org/10.1016/S0378-1097(03)00306-9
11. Danielsen M, Wind A (2003) Susceptibility of *Lactobacillus* spp. to antimicrobial agents. Int J Food Microbiol 82:1–11
12. De Man JC, Rogosa M, Sharpe MT (1960) A medium for the cultivation of lactobacilli. J Appl Bacteriol 23:130–135
13. Delcour J, Ferain T, Deghorain M, Palumbo E, Hols P (1999) The biosynthesis and functionality of the cell-wall of lactic acid bacteria. Antonie Van Leeuwenhoek 76:159–184
14. Devirgiliis C, Coppola D, Barile S, Colonna B, Perozzi G (2009) Characterization of the *Tn*916 conjugative transposon in a food-borne strain of *Lactobacillus paracasei.* Appl Environ Microbiol 75(12):3866–71. https://doi.org/doi:10.1128/AEM.00589-09
15. Duar RM, Lin XB, Zheng JZ, Martino ME, Grenier T, Pérez-Muñoz ME, Leulier F, Gänzle M, Walter J (2017) Lifestyles in transition: evolution and natural history of the genus *Lactobacillus*. FEMS Microbiol Rev 41:S27–S48. https://doi.org/10.1093/femsre/fux030
16. Egervärn M, Roos S, Lindmark H (2009) Identification and characterization of antibiotic resistance genes in *Lactobacillus reuteri* and *Lactobacillus plantarum*. J Appl Microbiol 107(5):1658–1668. https://doi.org/10.1111/j.1365-2672.2009.04352.x
17. Faridi A, Kareshk AT, Fatahi-Bafghi M, Ziasistani M, Ghahraman MRK, Seyyed-Yousefi SZ, Shakeri N, Kalantar-Neyestanaki D (2018) Detection of methicillin-resistant *Staphylococcus aureus* (MRSA) in clinical samples of patients with external ocular infection. Iranian J Microbiol 10(4):215–219
18. Feld L, Schjorring S, Hammer K, Licht TR, Danielsen M, Krogfelt K, Wilcks A (2008) Selective pressure affects transfer and establishment of a *Lactobacillus plantarum* resistance plasmid in the gastrointestinal environment. J Antimicrob Chemother 61(4):845–852. https://doi.org/10.1093/jac/dkn033
19. Fu KP, Neu HC (1978) Beta-lactamase stability of HR756, a novel cephalosporin compared to that of cefuroxime and cefoxitin. Antimicrob Agents Chemother 14(3):322–326. https://doi.org/10.1128/aac.14.3.322
20. Fukao M, Tomita H, Yakabe T, Nomura T, Ike Y, Yajima N (2009) Assessment of antibiotic resistance in probiotic strain *Lactobacillus brevis* KB290. J Food Prot 72(9):1923–1929
21. Gevers D, Danielsen M, Huys G, Swings J (2003) Molecular characterization of *tet*(M) genes in Lactobacillus isolates from different types of fermented dry sausage. Appl Environ Microbiol 69(2):1270–1275. https://doi.org/10.1128/aem.69.2.1270-1275.2003
22. Gharajalar SN, Firouzamandi M (2017) Molecular detection of antibiotic resistance determinants in *Lactobacillus* bacteria isolated from human dental plaques. J Med Microbiol Infect Dis 5(3–4):51–55
23. Gueimonde M, Sánchez B, de Los Reyes-Gavilán CG, Margolles A (2013) Antibiotic resistance in probiotic bacteria. Front Microbiol 4:202. https://doi.org/10.3389/fmicb.2013.00202
24. Guo H, Pan L, Li L, Lu J, Kwok L, Menghe B, Zhang H, Zhang W (2017) Characterization of antibiotic resistance genes from *Lactobacillus* isolated from traditional dairy products. J Food Sci 82(3):724–730. https://doi.org/10.1111/1750-3841.13645
25. Han JH, Li XF, Gao WH, Walczak P, Zhang BL, Jia YM (2013) Susceptibility of *Lactobacillus pentosus* strains isolated from fermented products to streptomycin and kanamycin. International Food Research Journal 20(4):1927–1931
26. Handwerger S, Skoble J (1995) Identification of chromosomal mobile element conferring high-level vancomycin resistance in *Enterococcus faecium*. Antimicrob Agents Chemother 39(2446):2453
27. Hummel AS, Hertel C, Holzapfel WH, Franz CM (2007) Antibiotic resistances of starter and probiotic strains of lactic acid bacteria. Appl Environ Microbiol 73(3):730–739
28. Ilinskaya ON, Ulyanova VV, Yarullina DR, Gataullin IG (2017) Secretome of intestinal bacilli: a natural guard against pathologies. Front Microbiol 8:1666. https://doi.org/10.3389/fmicb.2017.01666
29. James L, Beena AK, Anupa A, Sreeshma N (2016) Antibiogram of lactobacilli isolated from four different niches. J Microbiol Microb Technol 1(1):4
30. Jorgensen JH, Hindler JF, Reller LB, Weinstein MP (2007) New consensus guidelines from the Clinical and Laboratory Standards Institute for antimicrobial susceptibility testing of infrequently isolated or fastidious bacteria. Clin Infect Dis 44:280–286. https://doi.org/10.1086/510431
31. Kastner S, Perreten V, Bleuler H, Hugenschmidt G, Lacroix C, Meile L (2006) Antibiotic susceptibility patterns and resistance genes of starter cultures and probiotic bacteria used in food. J Syst Appl Microbiol 29(2):145–155. https://doi.org/10.1016/j.syapm.2005.07.009
32. Katla AK, Kruse H, Johnsen G, Herikstad H (2001) Antimicrobial susceptibility of starter culture bacteria used in Norwegian dairy products. Int J Food Microbiol 67(1–2):147–152
33. Khan U, Afsana S, Kibtia M, Hossain M, Choudhury N, Ahsan CR (2019) Presence of blaCTX-M antibiotic resistance gene in Lactobacillus spp. isolated from Hirschsprung diseased infants with stoma. J Infect Dev Ctries 13(5):426–433. https://doi.org/10.3855/jidc.10968
34. Kirtzalidou E, Pramateftaki P, Kotsou M, Kyriacou A (2011) Screening for lactobacilli with probiotic properties in the infant gut microbiota. Anaerobe 17(6):440–443. https://doi.org/10.1016/j.anaerobe.2011.05.007
35. Klein G, Hallman C, Casas IA, Abad J, Lowers J, Reuter G (2000) Exclusion of *vanA, vanB* and *vanC* type glycopeptide resistance in strains of *Lactobacillus reuteri* and *Lactobacillus rhamnosus* used as probiotics by polymerase chain reaction and hybridization methods. J Appl Microbiol 89(5):815–824
36. Klein G, Pack A, Reuter G (1998) Antibiotic resistance patterns of enterococci and occurrence of vancomycin-resistant enterococci in raw minced beef and pork in Germany. Appl Environ Microbiol 64(5):1825–1830
37. Korhonen JM, Danielsen M, Mayo B, Egervarn H, Axelsson L, Huys G et al (2008) Antimicrobial susceptibility and proposed microbiological cut-off values of lactobacilli by phenotypic determination. Int J Probiotics Prebiotics 3:257–268
38. Lahtinen SJ, Boyle RJ, Margolles A, Frías R, Gueimonde M (2009) Safety assessment of probiotics. In: Charalampopoulos D, Rastall RA (eds) Prebiotics and Probiotics Science and Technology. Springer-Verlag, Berlin, pp 1193–1225
39. Li S, Li Z, Wei W, Ma C, Song X, He W, Tian J, Huo X (2015) Association of mutation patterns in GyrA and ParC genes with quinolone resistance levels in lactic acid bacteria. J Antibiot (Tokyo) 68(2):81–87. https://doi.org/10.1038/ja.2014.113
40. Liu C, Zhang ZY, Dong K, Yuan JP, Guo XK (2009) Antibiotic resistance of probiotic strains of lactic acid bacteria isolated from marketed foods and drugs. Biomed Environ Sci 22(5):401–412. https://doi.org/10.1016/S0895-3988(10)60018-9
41. Martel A, Meulenaere V, Devriese LA, Decostere A, Haesebrouck F (2003) Macrolide and lincosamide resistance in the gram-positive nasal and tonsillar flora of pigs. Microb Drug

Resist 9(3):293–297. https://doi.org/10.1089/107662903322286508
42. Mayrhofer S, van Hoeck AHAM, Mair C, Huys G, Arts HJM, Kneifel W 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. https://doi.org/10.1016/j.ijfoodmicro.2010.08.024
43. Melo TA, Dos Santos TF, Pereira LR, Passos HM, Rezende RP, Romano CC (2017) Functional profile evaluation of *Lactobacillus fermentum* TCUESC01: A New Potential Probiotic Strain Isolated during Cocoa Fermentation. Biomed Res Int. https://doi.org/10.1155/2017/5165916
44. Mendonça AA, de Lucena BT., de Morais MM., de Morais MA Jr (2016) First identification of Tn916-like element in industrial strains of *Lactobacillus vini* that spread the *tet*-M resistance gene. FEMS Microbiol Lett https://doi.org/10.1093/femsle/fnv240
45. Moosdeen F (1997) The evoluation of resistance to cephalosporins. Clin Infect Dis 24(3):487–493. https://doi.org/10.1093/clinids/24.3.487
46. Naas T, Cuzon G, Bogaerts P, Glupczynski Y, Nordmann P (2011) Evaluation of a DNA microarray (Check-MDR CT102) for the rapid detection of TEM, SHV and CTX-M extended-spectrum ß-lactamases (ESBLs), and of KPC, OXA-48, VIM, IMP, and NDM-1 carbapenemases. J Clin Microbiol 49(4):1608–1613. https://doi.org/doi:10.1128/JCM.02607-10
47. Nicoloff H, Bringel F (2003) ISLpl1 is a functional IS30-related insertion element in *Lactobacillus plantarum* that is also found in other lactic acid bacteria. Appl Environ Microbiol 69(10):6032–6040. https://doi.org/10.1128/AEM.69.10.6032-6040.2003
48. 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(2):217–224. https://doi.org/10.1016/j.ijfoodmicro.2007.11.018
49. Petersen A, Jensen LB (2004) Analysis of *gyrA* and *parC* mutations in enterococci from environmental samples with reduced susceptibility to ciprofloxacin. FEMS Microbiol Lett 231(1):73–76. https://doi.org/10.1016/S0378-1097(03)00929-7
50. Rojo-Bezares B, Saenz Y, Poeta P, Zarazaga M, Ruiz-Larrea F, Torres C (2006) Assessment of antibiotic susceptibility within lactic acid bacteria strains isolated from wine. Int J Food Microbiol 111(3):234–240. https://doi.org/10.1016/j.ijfoodmicro.2006.06.007
51. Saarela M, Mättö J, Mattila-Sandholm T (2002) Safety aspects of *Lactobacillus* and *Bifidobacterium* species originating from human oro-gastrointestinal tract or from probiotic products. Microbial Ecology in Health and Disease 14(4):233–240. https://doi.org/10.1080/08910600310002127
52. Sabouni F, Movahedi Z, Mahmoudi S, Pourakbari B, Valian SK, Mamishi S (2016) High frequency of vancomycin resistant *Enterococcus faecalis* in children: an alarming concern. Journal of preventive medicine and hygiene 57(4):E201–E204
53. Salminen MK, Rautelin H, Tynkkynen S, Poussa T, Saxelin M, Valtonen V, Järvinen A (2006) Lactobacillus bacteremia, species identification, and antimicrobial susceptibility of 85 blood isolates. A Clin Infect Dis 42(5):e35–44. https://doi.org/10.1086/500214
54. Sharma P, Tomar SK, Goswami P, Sangwan V, Singh R (2014) Antibiotic resistance among commercially available probiotics. Food Res Int 57:176–195. https://doi.org/10.1016/j.foodres.2014.01.025
55. Sukmarini L, Mustopa AZ, Normawati M, Muzdalifah I (2014) Identification of antibiotic-resistance genes from lactic acid bacteria in indonesian fermented foods. HAYATI Journal of Biosciences 21:3:144–150 EISSN: 2086–4094. https://doi.org/10.4308/hjb.21.3.144
56. Thal L, Donabedian S, Robinson-Dunn B, Chow JW, Dembry L, Clewell DB, Alshab D, Zervos MJ (1998) Molecular analysis of glycopeptide-resistant Enterococcus faecium isolates collected from Michigan hospitals over a 6-year period. J Clinical Microbiol 36(11):3303–3308
57. van Hoek AHAM, Margolles A, Damig KJ, Korhonen JM, Zycka-Krzesinka J, Bardowsky JK et al (2008) Molecular assessment of erythromycin and tetracycline resistance genes in lactic acid bacteria and bifidobacteria and their relation to the phenotypic resistance. International Journal of Probiotics and Prebiotics 3(4):271–280
58. Werner G, Klare I, Witte W (1999) Large conjugative vanA plasmids in vancomycin-resistant *Enterococcus faecium*. J Clinical Microbiol 37(7):2383–2384
59. Werner G, Willems RJL, Hildebrandt B, Klare IW (2003) Witte Influence of transferable genetic determinants on the outcome of typing methods commonly used for *Enterococcus faecium*. J Clinical Microbiol 41(4):1499–1506. https://doi.org/10.1128/JCM.41.4.1499-1506.2003
60. Zhou JS, Pillidge CJ, Gopal PK, Gill HS (2005) Antibiotic susceptibility profiles of new probiotic *Lactobacillus* and *Bifidobacterium* strains. Int J Food Microbiology 98(2):211–217. https://doi.org/10.1016/j.ijfoodmicro.2004.05.011