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Antibiotic Susceptibility of *Lactobacillus* sp. Isolated from Commercial Probiotic Products by E-Test Strip Method

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A B S T R A C T

Minimum Inhibitory Concentration (MIC) of 29 *Lactobacillus* isolates was determined by E-test strip method according to CLSI and EFSA, 2012 standards. All isolates displayed resistance towards aminoglycosides while 63% of isolates were found resistant to amikacin (MIC 16-256 µg/ml). Strains S8a and S8b (*L. rhamnosus* and *L. acidophilus*) were resistant to tetracycline (MIC 256 µg/ml). Isolates displayed resistance to quinolones [nalidixic acid with MIC 256 µg/ml; norfloxacin, ofloxacin, ciprofloxacin and levofloxacin with MIC 6-256 µg/ml; and sparofloxacin (MIC 0.25-4 µg/ml)], azolidiones [nitrofurantoin (MIC 3-256 µg/ml)] and cephalosporins (MIC 256µg/ml). Seventy-three percent of isolates displayed resistance against vancomycin while 66% to teicoplanin. Isolates, S1b (*L. reutri*), S4 (*L. reuteri*); S5 (*L. plantarum*); S8a (*L. rhamnosus*) and S8b (*L. acidophilus*) exhibited resistance towards beta-lactams. Very few strains exhibited susceptibility to class macrolides [roxithromycin (S10b- *L. plantarum*; MIC 0.5 µg/ml); clarithromycin (S6a- *L. plantarum* MIC 0.125 µg/ml); (S12- *L. acidophilus* MIC 0.25 µg/ml) and azithromycin (S12- *L. acidophilus* MIC 0.75 µg/ml)]. The present study found the prevalence of antibiotic resistance among commercial available probiotics, which may pose a safety risk among humans. Hence, antibiotic sensitivity should be considered as essential for the evaluation of safety assessment of probiotics.

Introduction

Lactic acid bacteria (LAB) constitute a major group of bacteria, known for their potential health benefits which they impart to the host and have a long history of safe use or generally regarded as safe (GRAS) status. A subset of LAB is generally known as clinically relevant 'probiotic organisms' having health benefits on host. The health benefits of consuming these organisms range from improved intestinal health and immunity, prevention of antibiotic associated diarrhea and even cancer. Probiotic foods are having global popularity and widespread acceptability as depicted by an estimated growth of more than 10% in last one decade (Wong *et al.*, 2015). Accelerated by increased consumer demand, these health beneficial microorganisms have been comprehensively

included in a large number of food and pharmaceutical products. Probiotic organisms as pharmaceutical formulations have been prescribed as an adjunct therapy to maintain homeostasis in intestinal microflora, disrupted due to an antibiotic therapy. To re-establish the normal microflora of the intestine, antibiotic resistance in these probiotics is prerequisite, provided of course that they do not pose a threat by way of the transfer of antibiotic resistance genes to other bacteria. The resistance towards antibiotics, if intrinsic, is desirable, however their transfer to pathogenic bacteria offers serious clinical threats. Antibiotic resistance in probiotic organisms is a boon and a ban at the same time. Serious concerns have been raised with this trait of probiotic organisms. A reservoir of antibiotic resistance genes has been established over time because of the extensive use of probiotic organisms in conjunction with antibiotics. Resistance of lactic acid bacteria towards the major groups of antibiotics like beta-lactams, macrolide, aminoglycoside, chloramphenicol and tetracycline has been extensively reviewed (Sharma *et al.,* 2014). The transfer of important antibiotic resistance genes across generations have also been demonstrated among lactobacilli and from lactobacilli to pathogens (Tannock, 1994). These reports have established probiotic bacteria as reservoirs of antibiotic resistant genes that can be transferred to pathogenic strains (Mater, 2008). This raises the question of antibiotic resistances among desired food borne bacteria such as starter and probiotic cultures. The close contact with other bacteria in the human intestine is an excellent pre-condition for horizontal gene transfer with the aid of conjugative transposons and plasmids (Teuber *et al.,* 1999). Therefore, it is very important to validate that probiotic and nutritional LAB strains lack acquired antimicrobial resistance properties prior to considering them safe for human and animal consumption. In the present study, antibiotic resistance of *Lactobacillus* strains present in market probiotic products, both in pharmaceutical and dairy products in an attempt to contribute to biosafety surveillance of LAB for human consumption was evaluated. The antibiotic resistance of 29 commercial probiotic strains was evaluated by determination of Minimum Inhibitory Concentration (MIC) value using the E-test strip method.

Materials and Methods

Determination of MIC by E-test strip method

E-test experiments were performed according to the instruction of the manufacturer and the recommendation of Standard Operating Procedure (SOP) by Kushiro *et al.,* (2009) and Mayerhofer *et al.,* (2008). A total of 30 isolates were isolated from 19 commercial products (Table 1). All isolates were characterized and were subjected to antibiotic susceptibility test using disc diffusion method against a total of 45 antibiotics. Many of them were found to exhibit multiple resistance against commonly used antibiotics. Out of 30 tested isolates, 29 were found to have resistance against a wide array of antibiotics (Sharma *et al.,* 2015). The MIC of 29 probiotic isolates which showed resistance in disc diffusion assay was further tested to determine their MIC values against 30 antibiotics by E-test strip method. List of antibiotics and their concentrations range used in this study are mentioned in Table 2. In brief, single colony of respective isolate was picked from previous streaked De Man Ragosa Sharpe (MRS) agar plate and inoculated in MRS broth for 18 h at 37°C. After incubation, cells were harvested by centrifugation (12000 rpm at 4°C for 10 min) and resuspended in sterile saline to achieve optical density equivalent to 0.5 McFarland standards (cell density of 10^8 cfu/ml). A

sterile cotton swab was immersed into the saline suspension, excess fluid was removed, and used to evenly swab the entire surface of the agar plate. One E-test strip containing the particular antibiotic was applied onto the agar using sterile forceps. Plate was inverted and incubated at 37°C for 24h in an anaerobic jar containing an anaerobic gas pack (Himedia, Mumbai, India), and zone of inhibition (elliptical) was measured. Plates without an antibiotic strip were taken as control plate. The experiments were replicated three times to verify the methodology reproducibility.

Results and Discussion

According to E-test strip method, MIC of 29 *Lactobacillus* strains from different commercial probiotic products towards selected antibiotics is summarized in Table 3. Resistance was defined using the previously described breakpoints for *Lactobacillus* strains (CLSI, 2012; EFSA, 2012). All the strains were found resistant to antibiotics.

The minimal inhibitory concentration is the lowest antibiotic concentration that inhibits the visible bacterial growth after overnight incubation using defined cut-off values for experimentally determined MICs (Philips *et al.,* 1991). Food grade LAB can be categorized as 'susceptible' or 'resistant' to each antibiotic tested (Andrews, 2001). It is considered that when MICs are ≥ 8-16 µg/ml the bacteria may be considered as "moderately resistant"; when MIC is >32 µg/ml it may be classified as "clinically resistant" to antibiotics.[10] Results were interpreted and the resistant strains were selected after being compared with known standard given by the CLSI. MIC breakpoints vary considerably depending on the medium and the antibiotics used (Franz *et al.,* 2005). Klare *et al.,* (2005) reported a "general" broth medium for determining LAB antibiotic susceptibilities and showed that this medium, consisting of Iso-Sensitest (90%) and MRS (10%) broth, optimally supported the growth of *Lactobacillus, Pediococcus, Lactococcus* and *Bifidobacterium* sp. Although, MRS has been used in most studies, as it is suited for the growth of many LAB and their antibiotic susceptibility determinations. In the present study, MIC of the 30 antimicrobial agents against 29 isolates was determined by the E-test strip method using MRS broth. Overall, our results are in good agreement with data from other studies for a broad range of *Lactobacillus* species and antibiotics (Danielsen and Wind, 2003; Gevers *et al.,* 2003; Perez *et al.,* 2005).

All isolates in our study were found to be sensitive against tetracycline except strain S8a and S8b (*L. rhamnosus* and *L. acidophilus*), showed MIC 256 µg/ml towards tetracycline. Similarly, Thumu *et al.,* (2012) determined tetracycline resistance in *L. plantarum* and determined that MIC range was 128 µg/ml. The wide range of high MICs obtained was in agreement with a previous study assessing antibiotic susceptibility of 43 *L. reuteri* strains isolated from piglets (Korhonen *et al.,* 2007).

The *Lactobacillus* and *Bifidobacteria* strains showed a wide range of resistance towards streptomycin MICs (2 -256 µg/ml), as reported in some studies (Delgado *et al.,* 2005; Katla *et al.,* 2001). In other studies, *Lactobacillus* sp. are reported to have a high natural resistance to kanamycin, gentamicin and streptomycin (Blandino *et al.,* 2008; Klare *et al.,* 2007; Nawaz *et al.,* 2011; Zhang *et al.,* 2007). In a study by Hummel *et al.,* (2007) more than 70% of the isolates were resistant to gentamicin and streptomycin based on the MIC breakpoint values of Scientific Committee on Animal Nutrition (SCAN) and Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) (European Commission, 2002; FEEDAP, 2005). Variability in *Lactobacillus*

sp. to these antibiotics has also been evidenced by other authors and tested strains to gentamicin isolated from dairy products and determined that MIC values in gentamicin ranging from 0.06 to 8µg/ml while resistance to gentamicin (MIC ≥ 16 mg/L) was present in strains of *L. salivarius*, *L. acidophilus*, and *L. paracasei* (Bujnakova *et al.*, 2014; Hleba *et al.*, 2012; Mayerhofer *et al.*, 2010).

In our study, *Lactobacillus* strains displayed susceptibility towards gentamicin and tobramycin with MIC in the range 0.19-24 µg/ml and (0.19-64 µg/ml) respectively, whereas intermediate resistance to kanamycin (12-256 µg/ml) and streptomycin (3-256 µg/ml). While more than 58% of isolates were resistant to kanamycin and 26% were resistant to streptomycin. Moreover, 63% of the stains were resistant to amikacin and observed to have MIC in the range 16-256 µg/ml and 23% of the strains displayed resistance to tobramycin, though only strain S15a showed susceptibility against these antibiotics. Resistance to aminoglycoside antibiotics was previously reported in LAB and probiotic strains and is considered to be intrinsic in LAB (Charteris *et al.*, 2001; Danielsen and Wind, 2003; Katla *et al.*, 2001). Lower MIC for gentamicin as compared to kanamycin and streptomycin as reported previously and the results was in correlation with our results (Danielsen and Wind, 2003).

In some studies, higher MICs of the aminoglycosides; gentamicin and streptomycin were reported for LAB, which is probably due to the fact that susceptibility testing was performed on MRS agar (E-test) (Charteris *et al.*, 1998). In few studies, streptomycin-resistant *Lactobacillus* sp. was encountered with MICs of streptomycin >256mg/L. The reason for the increased MICs of the aminoglycosides on MRS agar may be due to the medium's low pH (6.2), because the optimum pH of the aminoglycosides is in the alkaline range (pH 7.8) (Amesterdam, 2005).

Some of the isolates in the present study, exhibited complete susceptibility towards macrolides class of antibiotics viz. roxithromycin (S10b- *L. plantarum*- MIC 0.5 µg/ml); clarithromycin (S6a- *L. plantarum*- MIC 0.125 µg/ml; S12- *L. acidophilus*- MIC 0.25 µg/ml) and azithromycin (S12- *L. acidophilus*- MIC 0.75 µg/ml). In the current study, class azolidiones (23.33%) showed resistance towards *Lactobacillus* sp. All the isolates were found to be highly resistant to nitrofurantoin; displayed MIC in the range (3-256 µg/ml).

Vancomycin is considered one of the last antibiotics in the treatment of multidrug resistant pathogens; therefore, its resistance in commercial lactobacilli is of major concern (Bernardeau *et al.*, 2008). Most of the isolated strains in our study were observed to have resistance against vancomycin (73%) and teicoplanin (66%) except strain S9a, S14a, S14b, S15a, S15b, S16 and S19b for vancomycin and tecioplanin showed susceptibility to these antibiotics leaving S12. Lactic acid bacteria, including *L. paracasei, L. salivarius, L. plantarum, L. delbrueckii* subsp. *bulgaricus, L. casei, L. rhamnosus* were reported to have mechanisms providing resistance to vancomycin (Aslim and Beyatli, 2004; Comunian *et al.*, 2010; Coppola *et al.*, 2005; Devirgiliis *et al.*, 2011). The resistance of these species to vancomycin is intrinsic, due to the presence of D-Ala-D-lactate in their peptidoglycan instead of the normal dipeptide D-Ala-D-Ala (Ammor *et al.*, 2008). D-ala-D-ala dipeptidase encoded by VanX may act only in the presence of D-ala-D-ala precursor. Intrinsic resistance to vancomycin was earlier also confirmed for *L. paracasei*, *L. salivarius* and *L. plantarum* (MIC ≥ 32 µg/L) (Blandino *et al.*, 2008).

Table.1 Commercial probiotic dairy and pharmaceutical products with organisms mentioned on the products and their origin

S. No.	Probiotic capsules and dairy products	Strains isolated	Origin
1	S1	*L. rhamnosus, L. reuteri*	Pharmaceutical product
2	S2	*L. casei*	Dairy product
3	S3	*L. plantarum*	Pharmaceutical product
4	S4	*L. reuteri*	Pharmaceutical product
5	S5	*L. plantarum*	Pharmaceutical product
6	S6	*L. plantarum, L. rhamnosus*	Pharmaceutical product
7	S7	*L. plantarum, L. rhamnosus*	Pharmaceutical product
8	S8	*L. rhamnosus, L. acidophilus*	Pharmaceutical product
9	S9	*L. rhamnosus, L. acidophilus*	Pharmaceutical product
10	S10	*L. fermentum,, L. plantarum*	Dairy product
11	S11	*L. acidophilus, L. plantarum*	Dairy product
12	S12	*L. acidophilus*	Pharmaceutical product
13	S13	*L. acidophilus, L. plantarum*	Dairy product
14	S14	*L. acidophilus, L. plantarum*	Dairy product
15	S15	*L. acidophilus, L. plantarum*	Dairy product
16	S16	*L. rhamnosus*	Pharmaceutical product
17	S17	*L. rhamnosus*	Pharmaceutical product
18	S18	*L. rhamnosus*	Pharmaceutical product
19	S19	*L. rhamnosus, L. acidophilus*	Pharmaceutical product

Table.2 List of antibiotics and their range of concentration used for determination of MIC

List of antibiotics	Concentration range (µg/ml)
Amikacin, Ampicillin, Azithromycin, Cefaclor, Cefotaxime, Cefepime, Ceftriaxone, Cephalothin, Cefoperazone, Ceftazidime, Cefuroxime, Clarithromycin, Cotrimoxazole, Gentamicin, Kanamycin, Nalidixic acid, Norfloxacin, Nitrofurantoin, Oxacillin, Streptomycin, Tetracycline, Roxithromycin, Teicoplanin and Vancomycin	0.016-256
Ofloxacin, Ciprofloxacin, Levofloxacin, Sparofloxacin, Penicillin and Tobramycin	0.002-32

Table.3 Susceptibility of 29 *Lactobacillus* strains to selected antibiotics as determined by the E-test method using MRS medium

Commercial products with bacterial strains										
			(S1a) *L. rhamnosus*	(S1b) *L. reuteri*	(S2) *L. casei*	(S3) *L. plantarum*	(S4) *L. reuteri*	(S5) *L. plantarum*	(S6a) *L. plantarum*	(S6b) *L. rhamnosus*
Different Classes of Antibiotics	Antibiotics	MIC range (µg/ml)	Zone of Inhibition							
Aminoglycosides	Amikacin	0.016-256	64	16	48	256	48	256	32	96
	Tobramycin	0.016-256	-	6	-	-	12	32	-	24
	Gentamicin	0.016-256	6	2	-	12	-	6	6	4
	Kanamycin	0.016-256	64	12	-	256	24	256	32	32
	Streptomycin	0.016-256	24	12	48	12	12	4	64	24
Macrolides	Clarithromycin	0.016-256	-	-	-	-	-	-	.125	-
Azolidiones	Nitrofurantoin	0.016-256	-	32	48	-	48	4	24	-
Glycopeptides	Teicoplanin	0.016-256	256	256	256	256	256	256	256	256
	Vancomycin	0.016-256	256	256	256	256	256	256	256	256
Beta lactams	Ampicillin	0.016-256	1.5	16	-	.75	256	-	-	-
	Penicillin	0.002-32	-	256	-	-	256	256	-	-
	Oxacillin	0.016-256	-	256	-	-	256	-	-	-

Cephalosporins	Cefaclor	0.016-256	48	256	32	-	-	256	-	-
	Cephalothin	0.016-256	-	-	-	-	256	1	-	-
	Ceftazidime	0.002-32	32	6	-	-	-	1	-	-
	Cefepime	0.016-256	256	4	256	-	6	.64	256	-
	Ceftriaxone	0.016-256	64	2	-	-	-	.094	-	-
	Cefuroxime	0.016-256	-	-	-	-	-	.75	-	-
	Cefoperazone	0.016-256	-	-	-	-	-	32	-	-
Quinolones	Norfloxacin	0.016-256	-	256	-	256	256	256	-	256
	Ofloxacin	0.002-32	-	-	-	256	-	256	-	-
	Ciprofloxacin	0.002-32	-	256	-	-	256	256	-	256
	Nalidixic Acid	0.016-256	256	256	256	256	256	256	256	256
	Levofloxacin	0.002-32	-	-	-	-	-	6	-	-
	Sparofloxacin	0.002-32	-	-	-	-	-	4	-	-
Sulfonamides	Co-Trimoxazole	0.016-256	256	256	256	-	256	-	256	256

Commercial products with bacterial strains									
			(S7a) *L. rhamnosus*	(S7b) *L. plantarum*	(S8a) *L. rhamnosus*	(S8b) *L. acidophilus*	(S9) *L. rhamnosus*	(S10a) *L. fermentum*	(S10b) *L. plantarum*
Different Classes of Antibiotics	Antibiotics	MIC range (µg/ml)	Zone of Inhibition (µg/ml)						
Tetracycline	Tetracycline	0.016-256	-	-	256	256	-	-	-
Aminoglycosides	Tobramycin	0.016-256	32	64	32	16	-	-	12
	Gentamicin	0.016-256	4	8	24	16	-	-	-
	Kanamycin	0.016-256	256	64	256	256	-	32	16
	Amikacin	0.016-256	96	48	256	256	-	16	16
	Streptomycin	0.016-256	256	48	48	24	4	-	24
Macrolides	Roxithromycin	0.016-256	-	-	-	-	-	-	.5
Azolidiones	Nitrofurantoin	0.016-256	256	-	256	256	-	-	-
Glycopeptides	Vancomycin	0.016-256	256	256	256	256	-	256	256
	Teicoplanin	0.016-256	256	256	256	256	-	256	256
Beta lactams	Ampicillin	0.016-256	-	2	24	24	.75	-	-
	Penicillin	0.002-32	-	-	16	256	-	-	-

	Amoxycillin	0.016-256	-	-	-	3	-	-	-
Cephalosporins	Cefaclor	0.016-256	256	48	256	256	-	64	32
	Cefotaxime	0.016-256	-	-	256	256	-	-	-
	Cephalothin	0.016-256	-	-	-	256	-	-	-
	Cefoperazone	0.016-256	-	-	256	256	-	-	-
	Ceftazidime	0.016-256	-	16	256	256	256	8	6
	Cefepime	0.016-256	256	256	256	256	256	-	-
	Ceftriaxone	0.016-256	256	48	256	256	-	-	8
	Cefuroxime	0.016-256	4	2	-	256	-	-	-
Quinolones	Norfloxacin	0.016-256	-	-	8	4	-	24	24
	Ofloxacin	0.002-32	-	-	-	-	-	-	12
	Nalidixic Acid	0.016-256	256	256	256	256	256	256	256
Sulfonamides	Cotrimoxazole	0.016-256	256	256	256	256	.38	256	1

Commercial products with bacterial strains										
			(S11a) *L. acidophilus*	(S11b) *L. plantarum*	(S12) *L. acidophilus*	(S13a) *L. acidophilus*	(S13b) *L. plantarum*	(S14a) *L. plantarum*	(S14b) *L. acidophilus*	(S15a) *L. plantarum*
Different Classes of Antibiotics	Antibiotics	MIC range (µg/ml)	Zone of Inhibition (µg/ml)							
Amino-glycosides	Gentamicin	0.016-256	2	3	6	-	-	8	-	.19
	Amikacin	0.016-256	24	16	256	24	32	32	64	.5
	Tobramycin	0.002-32	-	12	-	32	-	64	24	.19
	Kanamycin	0.016-256	32	32	256	48	24	-	-	-
	Streptomycin	0.016-256	12	16	256	32	24	16	3	-
Macrolides	Clarithromycin	0.016-256	-	-	.25	-	-	-	-	-
	Azithromycin	0.016-256	-	-	.75	-	-	-	-	-
Azolidiones	Nitrofurantoin	0.016-256	-	-	-	-	4	-	256	4
Glycopeptides	Vancomycin	0.016-256	256	256	256	256	256	-	-	-
	Teicoplanin	0.016-256	256	256	-	256	.75	-	-	-
Beta lactams	Ampicillin	0.016-256	-	-	-	-	-	.19	.75	-
Cephalosporins	Cefepime	0.016-256	-	-	-	-	-	-	-	256

Quinolones	Norfloxacin	0.016-256	-	12	256	256	32	24	-	2
	Ofloxacin	0.016-256	-	6	256	-	-	-	-	-
	Ciprofloxacin	0.002-32	-	3	-	-	-	-	256	.25
	Nalidixic Acid	0.016-256	256	256	256	256	256	256	256	256
	Levofloxacin	0.002-32	-	-	-	-	-	-	256	-
	Sparofloxacin	0.002-32	-	-	-	-	-	3	.25	-
Sulfonamides	Cotrimoxazole	0.016-256	256	256	-	-	256	256	2	.19

Commercial products with bacterial strains								
			(S15b) *L. acidophilus*	(S16) *L. rhamnosus*	(S17) *L. rhamnosus*	(S18) *L. rhamnosus*	(S19a) *L. rhamnosus*	(S19b) *L. acidophilus*
Different Classes of Antibiotics	Antibiotics	Minimum Inhibitory Concentration (MIC) µg/ml	Zone of Inhibition (µg/ml)					
Aminoglycosides	Gentamicin	0.016-256	12	-	12	4	-	-
	Amikacin	0.016-256	64	-	256	32	256	-
	Tobramycin	0.016-256	32	-	24	8	-	-
	Kanamycin	0.016-256	256	-	-	32	24	-
	Streptomycin	0.016-256	12	-	-	16	32	-
Azolidiones	Nitrofurantoin	0.016-256	16	4	12	3	-	-
Glycopeptides	Vancomycin	0.016-256	-	-	256	256	256	-
	Teicoplanin	0.016-256	-	-	256	256	256	-
Cephalosporins	Ceftazidime	0.002-32	-	-	32	16	-	-
	Cefaclor	0.016-256	16	-	-	32	-	-
	Cefepime	0.016-256	2	2	-	256	-	.75
	Ceftriaxone	0.016-256	-	-	-	256	-	-
Quinolones	Norfloxacin	0.016-256	32	-	4	4	256	-
	Ofloxacin	0.002-32	-	-	-	-	256	-
	Ciprofloxacin	0.016-256	256	-	-	-	256	-
	Nalidixic Acid	0.016-256	256	-	-	-	256	256
Sulfonamides	Cotrimox-azole	0.016-256	8	-	256	256	-	-

The cell wall impermeability seems to have mechanism of resistance to beta-lactam antibiotics (Condon, 1983). Furthermore, the cooperation of non-specific mechanisms, such as multi-drug transporters (Putman *et al.*, 2001) and defective cell wall autolytic systems (Kim *et al.*, 1982) may also account for differences between strains within the same species. The strains tested in this study showed the resistance towards cell wall synthesis inhibitors (beta-lactams) while strains named S1b and S4 (*L. reuteri*), S5 (*L. plantarum*), S8a (*L. rhamnosus*) and S8b (*L. acidophilus*) displayed resistance to ampicillin, penicillin and oxacillin with MIC in the range (MIC 16-256 µg/ml) while the rest of the strains were found to be sensitive against these antibiotics. Strains S1b and S4 (*L. reuteri*), S5 (*L. plantarum*), S8a (*L. rhamnosus*) and S8b (*L. acidophilus*) were resistant to penicillin (MIC 16-256 µg/ml). While for oxacillin two strains were resistant (S1b and S4) and displayed MIC 256 µg/ml, although low resistance towards this antibiotic has also been observed among other strains of *Lactobacillus* sp. and for amoxicillin strain S8b was found to be susceptible and exhibited MIC 3 µg/ml. On the other hand, a widespread resistance to penicillins, especially penicillin G, has already been observed in lactobacilli used as probiotics or starter cultures in *L. rhamnosus, L. reuteri* and *L. plantarum* isolated from cheese (Flórez *et al.*, 2005; Čanžek Majhenič *et al.*, 2007; Belleti *et al.*, 2009), in *L. delbrueckii* subsp. *bulgaricus* from Chinese yogurts (Zhou *et al.*, 2012), in *L. casei* from fermented milk "Dahi" (Soomro and Masud, 2012), in *L. casei, L. helveticus* and *L. plantarum* from fermented milk and vegetables (Lapsiri *et al.*, 2011; Yüksekdağ and Beyatli, 2008), in *L. salivarius, L. curvatus* and *L. sakei* from different fermented foods and beverages (Gevers *et al.*, 2003; Nawaz *et al.*, 2011). While other studies showed that certain ampicillin-resistant lactobacilli were also isolated from Nigerian fermented foods and beverages (Olukoya *et al.*, 1993) as well as fermented milk in India (Lavanya *et al.*, 2011; Soomro and Masud, 2012). Resistance to oxacillin was also shown to occur in lactobacilli (especially *L. rhamnosus*) isolated from dairy products such as different types of cheeses (Coppola *et al.*, 2005; Herreros *et al.*, 2005; Hummel *et al.*, 2007).

Lactobacillus strains exhibited MIC in the range (16-256 µg/ml) against cefaclor, followed by cephalothin and ceftazidime (1-256 µg/ml), cefepime (4-256 µg/ml), ceftriaxone (0.094-256 µg/ml), and for cefuroxime (0.75-256 µg/ml). Generally, *Lactobacillus* species were found more resistant to cephalosporins; cephalothin, cefuroxime, ceftriaxone and cefoxitin (Abriouel *et al.*, 2015). The resistance mechanism is not fully elaborated, but cell wall impermeability and non-specific multidrug transporters may be involved (Ammor *et al.*, 2007). Resistance to these compounds was also shown in *L. paracasei* from yogurts (Honi *et al.*, 2013), in *L. plantarum* and *L. pentosus* from fermented olives (Casado *et al.*, 2014) and in *L. curvatus* and *L. fermentum* from fermented sausages (Zdolec *et al.*, 2011).

Lactobacilli seem to be intrinsically resistant to quinolones, e.g. ciprofloxacin and nalidixic acid, by unknown resistance mechanism. The intrinsic resistance to ciprofloxacin was not found to be associated with mutations in the QRDR of gyrA and parC as occurred in gram-positive bacteria, thus the intrinsic resistance could have resulted from intrinsic characteristics such as cell wall structure, permeability, or an efflux mechanism (Hummel *et al.*, 2007). In our study, all the strains were found to be completely resistant towards nalidixic acid (nearly 86%) and found to have MIC 256 µg/ml. Resistance towards norfloxacin has been observed in

almost all strains displayed (MIC 4-256 µg/ml), followed by ofloxacin (S3, S5, S10b, S11b, S12 and S19a; MIC 6-256 µg/ml), ciprofloxacin (S1b, S5, S4, S6b, S11b, S15b and S19a; MIC 0.25-256 µg/ml), levofloxacin (S14b and S15b; MIC 6 and 256 µg/ml) and sparofloxacin (S14a and S14b; MIC 0.25-4 µg/ml) and strain S5 exhibited susceptibility to levofloxacin and sparofloxacin. While ciprofloxacin resistance was reported for more than 60% of the LAB strains examined. A total of 60-77.8% of starter and probiotic strains of LAB was resistant to ciprofloxacin (Hummel *et al.,* 2007; Zarazaga *et al.,* 1999).

In the current study, class sulfonamides (56.66%) showed resistance towards *Lactobacillus* sp. All the isolates were found to be highly resistant towards cotrimoxazole (MIC 0.38-256 µg/ml). Resistance to other inhibitors of nucleic acid synthesis such as trimethoprim and sulfonamides has also been reported as an intrinsic feature (Katla *et al.*, 2001). In some cases, sulfamethoxazole/ trimethoprim phenotypic determination of susceptibility of lactobacilli in some culture media may not be coherent, because certain antagonistic medium components such as p-aminobenzoic acid (PABA) and thymidine may interfere with the antibiotic activity (Turnidge and Bell, 2005). In other cases, the mechanisms of resistance in lactobacilli isolated from fermented foods include cell wall impermeability, alternative metabolic pathways, a dihydrofolate reductase (DHFR), that is insensitive to trimethoprim, overproduction of DHFR and trimethoprim-insensitive transferable DHFRs (Huovinen, 1987).

A number of researchers examined antibiotic resistance of bacteria isolated from different food samples have argued that the results of antibiotic resistance vary from study to study (Lira *et al.*, 2004; Picozzi *et al.*, 2005; Caro *et al.,* 2007). It was concluded that this phenomenon may be due to high spontaneous frequency of mutation to antibiotic resistance which is not uncommon in lactobacilli (Curragh and Collins, 1992). Additionally, the loss of antibacterial activity of unstable antimicrobial agents during incubation may result in sub inhibitory concentrations that could promote the emergence of resistant strains during prolonged exposure (Herra *et al.*, 1995).

Nevertheless, broth micro dilution provides a simple method to determine MICs for a large number of strains and antibiotics, whereas the E-test could be more suitable for testing single strains. However, resistant and susceptible strains were generally more clearly separated by E-test in the present investigation due to the wider and more precise antibiotic concentration range of the E-test. These findings reinforce the antimicrobial susceptibility testing in the safety assessment procedure of strains intended for probiotic or nutritional use especially in humans. All the isolates were having MIC higher than the value prescribed by CLSI against a number of antibiotics. The results have indicated the presence of multiple drug resistance is in most of the isolates in different species of probiotic strains, which is detrimental to food safety.

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