

Original Research Article

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***In vitro* Assessment of Antibiotic Resistance Pattern among *Lactobacillus* Strains Isolated from Goat Milk**

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A B S T R A C T

This study aimed to assess the antibiotic susceptibility of *Lactobacillus* strains of goat milk origin. Lactobacilli are generally recognized as safe, but can act as reservoir for genes carrying antibiotic resistance traits; which can further spread to commensal, food spoilage or pathogenic organisms. Lactic acid bacteria (LAB) strains coming from animal sources had significant interaction with antibiotics. Goat milk, being second to cow milk in production and due to its close resemblance to human milk composition, is among favorable source for isolating potential probiotic LAB strains. Herein, this study assessed the antibiotic susceptibility pattern of fourteen *Lactobacillus* strains isolated from goat milk. The antibiotic susceptibility was recorded against thirty antibiotics following standard disk-diffusion assay. Imipenem, meropenem and nitrofurantoin turned out to be the most effective antibiotics, displaying high zone of inhibition (ZOI) against all the isolates. In contrast, all lactobacilli strains exhibited resistance pattern towards methicillin, oxacillin, cefoxitin, cefmetazole, teicoplanin, vancomycin, ciprofloxacin, ofloxacin, streptomycin, tobramycin, clindamycin and fusidic acid group antibiotics. Overall, GM4 (*L. plantarum*) displayed highest resistance with clear resistance phenotype against 18/30 antibiotics tested and intermediate resistance against other 5 antibiotics. This study generates new data pertaining to the antibiotic resistance risk of new candidate probiotic strains from goat milk.

Keywords

Lactobacillus, Goat milk, Susceptibility, Resistance, Probiotics

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Introduction

Probiotic, prebiotic and synbiotic research is gaining overwhelming response due to their potential health benefits; noteworthy being emerging as a safe and cost-effective alternative to current day antibiotic therapy. Probiotics are mainly dominated by LAB, especially *Lactobacillus* sp., which constitutes

a diverse clade of Gram-positive, catalase-negative, non-spore forming rods or coccobacilli bacteria (Seddik *et al.*, 2017). Probiotics have long history of safe use, are conferred with GRAS (generally recognized as safe) and QPS (Qualified Presumption of Safety) status; however, these strains may act as a reservoir of antibiotic resistance genes that may be transferable. Several reports

identified genes encoding transferable antibiotic resistance among lactobacilli (Munoz *et al.*, 2014). Therefore, the presence of antibiotic resistance genes in lactobacilli indicates a possible threat to human health. LABs are isolated from several sources including milk, traditional fermented dairy products, faecal matter etc. Strains coming from animal sources *viz.* milk, fecal matter etc., along with food of animal origin had significant interaction with antibiotics. Antimicrobials are administered to dairy animals for treatment, prophylaxis and growth promotion purpose. Such practices are favorable for genetic selection of resistant strains. In comparison to cattle and buffalo, goats are considered as hardy and adaptive animal and are exposed to antimicrobials to a lower extent. Additionally goat milk is being refereed as a treasure trove, with respect to its nutritional and therapeutic components (Silanikove *et al.*, 2010). Goat milk, due to its close resemblance to human milk oligosaccharide composition (Bode, 2006) is among favorable source for isolating potential probiotic LAB strains. In order to assure the safety of candidate probiotic strains, FAO/WHO guidelines suggest testing these strains for antibiotic resistance patterns. Hence, the present study was undertaken to analyze the status of antibiotic resistance among *Lactobacillus* isolates of goat milk origin.

Materials and Methods

Bacterial strains

This study was attempted to isolate *Lactobacillus* spp. from raw goat (Beetal) milk obtained from dairy farm of Guru Angad Dev Veterinary and Animal Sciences University (GADVASU) Ludhiana, Punjab, India. Milk samples were processed and lactobacilli were isolated and identified as discussed previously (Panwar *et al.*, 2016;

Sharma *et al.*, 2017a and 2017b). Nucleotide sequence(s) thus obtained were BLAST for similarity search and submitted to NCBI database for Gene Bank accession number (Table 1). Reference *Lactobacillus* strains *viz.* *Lactobacillus fermentum* (NCDC 214), *L. plantarum* (NCDC 20) and *L. rhamnosus* (NCDC 19) were procured from National Collection of Dairy Cultures (NCDC), ICAR-NDRI, Karnal, India.

Reference probiotic strains i.e. *L. rhamnosus* GG (ATCC 53103) and *L. casei* (ATCC 393) were procured from American Type Culture Collection (ATCC), USA. All the test strains were maintained and propagated in MRS medium and sub-cultured thrice before antibiotic susceptibility testing.

Test antibiotic disks

A total of thirty Antibiotic discs belonging to different family were procured from HiMedia Laboratories Pvt. Ltd. Mumbai, India. The list of 26 antibiotics, their dosage and family had been summarized in our previous manuscript (Sharma *et al.*, 2017a); except four new antibiotics namely, Azithromycin (Macrolides), Cefmetazole (2nd generation cephalosporins), Cephalothin (1st generation cephalosporins), and Amoxycylav (β -lactams).

Antibiotic susceptibility assay

The susceptibility pattern of different isolates and cultures were evaluated through modified Kirby-Bauer disc-diffusion assay (Sharma *et al.*, 2017a). Isolates with $ZOI \leq 14$ mm were recorded as resistant (R), $ZOI \geq 20$ as susceptible (S), and ZOI in between 15-19mm were considered as intermediate (I). Antibiotic susceptibility assay was performed in triplicate and the diameters of zone of inhibition were recorded either as resistant (R), sensitive (S) or intermediate (zone diameter \pm SD).

Results and Discussion

A total of 14 *Lactobacillus* strains were identified based on genus specific PCR and 16S rRNA sequencing. The identity of isolates along with the NCBI accession numbers has been listed in Table 1. All the isolates were subjected to antibiotic susceptibility testing.

The antibiotic resistance patterns of *Lactobacillus* strains are displayed in Table 2. Resistance and susceptibility are represented as R and S respectively. Intermediate susceptibility has been presented as ZOI±SD reflecting the inclination towards sensitivity or resistance. All the goat milk lactobacilli displayed sensitivity towards β -lactams (imipenem, meropenem) and nitrofurantoin. Macrolide (erythromycin) and glycylicycline (tigecycline) group antibiotics also inhibited majority of *Lactobacillus* sp. isolates, except GM5 and GM10. Ampicillin, cefotaxime and chloramphenicol were also inhibitory towards most of lactobacilli, except GM4, GM5, GM9 and GM10. A high degree of resistance was recorded against selective β -lactam (methicillin and oxacillin), glycopeptide (teicoplanin, vancomycin), quinolone (ciprofloxacin, ofloxacin), aminoglycoside (streptomycin, tobramycin), lincosamide (Clindamycin) and fusidane (Fusidic acid) group antibiotics. Among cephalosporins; cefoxitin and cefmetazole were in-effective, while cefuroxime displayed inhibitory activity against few isolates. Ceftazidime inhibited most of the isolates, except GM5. Majority of isolates showed intermediate susceptibility towards tetracycline. A variable resistance/intermediate/sensitive profile was observed against amoxycylav, azithromycin, penicillin, co-trimoxazole, trimethoprim, gentamycin and cephalothin. Overall, GM4 (*L. plantarum*) showed highest resistance, as 18 out of 30 antibiotics turned out to be in-effective; closely followed by GM8 (*L. plantarum*) and GM13 (*L. plantarum*) (17/30).

Similar to *Lactobacillus* isolates, a varied level of resistance was displayed by reference *Lactobacillus* (*L. plantarum*, *L. rhamnosus* and *L. fermentum*) and probiotic (*L. rhamnosus* GG and *L. casei*) strains. All of the reference and probiotic strains were susceptible towards imipenem, ampicillin, penicillin (β -lactam), erythromycin (macrolide), cefuroxime, cefotaxime (cephalosporin) and chloramphenicol; whereas displayed resistant pattern towards cefoxitin, cefmetazole (cephalosporins), trimethoprim and co-Trimoxazole. Intermediate to resistance profile was displayed against other antibiotics. Among all the antibiotic groups, cell wall synthesis inhibitors (imipenem, meropenem, ampicillin and cefotaxime) displayed better inhibitory activity, followed by protein synthesis inhibitors (erythromycin, chloramphenicol and tigecycline).

Our study revealed high resistance of goat milk lactobacilli to tested antibiotics. None of the strain was totally susceptible to all targeted antibiotics and displayed multidrug resistance. All the goat milk lactobacilli were inhibited by imipenem and meropenem, both belonging to carbapenems, the most potent class of β -lactams family. Our results are in high accordance with Bousmaha-Marroki and Marroki (2015) who also reported high susceptibility of Algerian goat milk lactobacilli towards imipenem. Among other β -lactams, most of *Lactobacillus* isolates were sensitive towards ampicillin; sensitive to intermediate against amoxycylav, followed by penicillin; and complete resistance towards methicillin and oxacillin. Our results are in partial concordance with Bousmaha-Marroki and Marroki (2015), who also observed the susceptibility pattern of lactobacilli against penicillin, ampicillin and resistance towards oxacillin. Recent study conducted by Sharma and co-workers (2017a) also observed the resistance pattern of curd and human milk LAB towards methicillin and oxacillin.

The genetic basis of such resistance might be due to mutations in the penicillin-binding proteins, or due to the presence of genes coding for β -lactamase (Ashraf and Shah, 2011).

Among cephalosporins, cefotaxime, the 3rd generation cephalosporin was quite effective against majority of goat milk lactobacilli. However, ceftazidime (3rd generation cephalosporin), cefoxitin and cefmetazole (2nd generation cephalosporins) were nearly ineffective. Interestingly, most goat milk lactobacilli displayed intermediate susceptibility towards cephalothin (1st generation cephalosporin). Few other reports also documents high resistance of LAB towards cephalosporins (Gad *et al.*, 2014).

Our results are in agreement to findings of Halder and Shyamapada (2016), who also observed the susceptibility of lactobacilli against cefotaxime. Resistance to cephalosporins can be attributed to the

presence of β -lactamases and/or efflux pumps (Pfeifer *et al.*, 2010), or due to cell wall impermeability (Delgado *et al.*, 2005).

The complete resistance of goat milk *Lactobacillus* isolates towards glycopeptides (teicoplanin, vancomycin) is in line to other reports (Nawaz *et al.*, 2011). Intrinsic resistance towards vancomycin might be due to the presence of D-Ala-D-lactate in the cell wall peptidoglycan, rather than the D-ala-D-ala dipeptide moiety. Vancomycin resistance is chromosomally encoded and non-transmissible (Marroki *et al.*, 2011).

All goat milk lactobacilli exhibited strong resistance pattern towards quinolones (ciprofloxacin and ofloxacin). Resistance towards quinolones may be explained with the nucleotide substitutions (gyrA and/or gyrB) within the quinolone-resistance determining region of DNA gyrase subunits (Baines and Wilcox, 2015), or due to mutation in topoisomerase IV (Hummel *et al.*, 2007).

Table.1 List of goat milk LAB strains used in this study. IPhp denotes lab code and GM (goat milk) signifies isolate number

Lab code	Identity	NCBI Accession numbers
IPhp-GM4	<i>Lactobacillus plantarum</i>	KX943025
IPhp-GM5	<i>L. rhamnosus</i>	KX943026
IPhp-GM6	<i>L. plantarum</i>	KX943027
IPhp-GM7	<i>L. plantarum</i>	KX943028
IPhp-GM8	<i>L. plantarum</i>	KX943029
IPhp-GM9	<i>L. pentosus</i>	KY658473
IPhp-GM10	<i>L. plantarum</i>	KX943030
IPhp-GM11	<i>L. plantarum</i>	KY658474
IPhp-GM12	<i>L. plantarum</i>	KY658475
IPhp-GM13	<i>L. plantarum</i>	KY658476
IPhp-GM14	<i>L. plantarum</i>	KY658477
IPhp-GM15	<i>L. plantarum</i>	KY658478
IPhp-GM17	<i>L. fermentum</i>	KY658479
IPhp-GM18	<i>L. gasseri</i>	KY658480

Table.2 Antibacterial susceptibility profile of goat milk lactobacilli, standard *Lactobacillus* and reference probiotic strains towards commercially used antibiotics

Antibacterial drugs	Disc code with concentration (mcg)	Goat milk (GM) isolates														Standard <i>Lactobacillus</i> sp.			Probiotic strains		
		GM 4	GM5	GM6	GM7	G M8	GM9	GM 10	GM 11	G M 12	GM 13	GM 14	G M 15	G M 17	G M 18	<i>L. fermentum</i>	<i>L. plantarum</i>	<i>L. rhamnosus</i>	<i>L. rhamnosus</i> GG	<i>L. casei</i>	
Ampicillin	AMP (10)	18±0.57	17±0	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	
Imipenem	IMP (10)	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	
Meropenem	MRP (10)	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	R	S	S	
Methicillin	MET (5)	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	17±0.57	R	R	R
Oxacillin	OX (1)	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	19±0.57	19±0.5	R	R	R
Penicillin	P (10)*	R	S	16±1	18±0	16±0.57	17±0.57	18±1	S	S	S	S	17±0.57	S	S	S	S	S	S	S	
Cefuroxime	AMC (30)	R	S	R	16±0.57	R	R	R	R	R	R	R	R	R	R	R	S	S	S	S	19±0
Cefoxitin	CEP (30)	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
Ceftazidime	CXM (30)	R	S	R	R	R	R	R	R	R	R	R	R	R	R	R	18±0.57	17±0	R	R	S
Cefotaxime	CX (30)	18±0.57	S	S	S	S	S	16±0.57	S	S	S	S	S	S	S	S	S	S	S	S	S
Teicoplanin	CMZ (30)	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	16±0.57	R	R	R	16±0
Vancomycin	CAZ (30)	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	15±0.57	R	R	R	R
Ciprofloxacin	CTX (30)	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	S	18±0.57	R	16±0	R
Ofloxacin	TEI (30)	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	16±1	R	R	16±0
Gentamicin	VA (30)	R	15±	16±1	15±	R	R	15±	15±0	R	R	R	R	R	R	R	S	16±	15	S	S

			0.57		0.57			0.57									0.57			
Streptomycin	CIP (5)	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	15±0.57	R	S	15±0
Tobramycin	OF (5)	R	R	R	R	R	R	R	R	R	R	R	R	R	R	16±0.57	R	R	R	R
Chloramphenicol	GEN (10)	S	R	S	S	S	19±0.57	S	S	S	S	S	S	S	S	S	S	S	19±0	16±1
Clindamycin	S (300)	R	R	R	R	R	R	R	R	R	R	R	R	R	R	S	19	S	S	15±0.57
Erythromycin	TOB (10)	S	R	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
Fusidic acid	CD (2)	R	R	R	R	R	R	R	R	R	R	R	R	R	R	S	R	16±0	R	R
Nitrofurantoin	E (15)	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	18±0	S	19±0.57
Tetracycline	AZM (15)	17±0.57	S	18±1	15±0.57	15±0.57	18±0	16±1	18±0.57	17±0.57	15±0	17±1	16±0.57	19±0	18±0.57	S	S	16±0.57	S	16±0
Tigecycline	FC (10)	S	S	S	S	S	S	19±0.57	S	S	S	S	S	S	S	S	19±0.57	S	S	15±1
Co-Trimoxazole	TGC (15)	R	R	R	R	R	15±0.57	R	17±0.57	19±0	16±0.57	R	15±1	17±0	15±0.57	R	R	R	R	17±0.57
Trimethoprim	COT (25)	R	R	R	R	R	16±0.57	R	16±0	18±0.57	R	15±0.57	16±1	15±0.57	R	R	R	R	R	R
Azithromycin	TR (5)	18±0.57	S	18±0	19±0.57	19±0	18±0	17±1	18±0.57	S	18±0.57	19±0.57	S	19±0	S	S	19	17±0.57	18±0	S
Cefmetazole	C (30)	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
Cephalothin	NIT (300)	17±0.57	19±0.57	16±1	16±0.57	15±0	R	16±0.57	17±0.57	18±0	R	16I	19±0.57	R	19±0	18±0	16±0.57	15±1	16±0.57	19±0
Amoxyclav	TE (30)	S	S	S	18±0.57	19±0	S	16±0.57	S	16±1	17±0.57	18±0.57	S	S	S	S	S	18±0.57	19±0	S

*Concentration in units

In our study different degree of sensitivity to resistance pattern has been observed against protein synthesis inhibitors. Previously, it has been documented that lactobacilli are generally resistant to aminoglycosides and susceptible to other protein synthesis inhibitors such as, chloramphenicol, macrolides and tetracycline (Karapetkov *et al.*, 2011). In agreement to above published reports, we have also noted down the high resistance pattern against aminoglycosides (streptomycin, tobramycin), with an exception of gentamicin, against which both resistance and intermediate susceptibility was recorded. Intermediate susceptibility to resistance pattern of lactobacilli against gentamicin has also been reported earlier (Jiang *et al.*, 2016). Among LAB, resistance to aminoglycosides is mainly due to enzymatic inactivation by aminoglycoside modifying enzymes (AMEs). Three main AMEs i.e. N-acetyl-transferases (AACs), O-phospho-transferases (APHs) and O-nucleotidyl-transferases (ANTs) confers resistance towards aminoglycoside. In this study, majority of *Lactobacillus* isolates displayed susceptibility towards erythromycin and intermediate to susceptible pattern towards azithromycin. These findings can be substantiated with earlier reports, wherein LAB strains showed susceptibility towards erythromycin and azithromycin (Bhuiyan *et al.*, 2017).

Among other protein synthesis inhibitors explored in this study, lactobacilli displayed remarkable sensitivity towards nitrofurantoin (a synthetic antimicrobial derived from furan by the addition of a nitro group and side chain containing hydantoin). On the other hand, a high resistance to clindamycin and fusidic acid was observed. Our results are in concurrence with other reports, where lactobacilli displayed susceptibility to nitrofurantoin (Bouridane *et al.*, 2016). Similar to this study, Goldstein *et al.*, (2015) also reported sensitivity of lactobacilli towards

tigecycline and chloramphenicol. In contrary to our results, Bhuiyan *et al.*, (2017) documented resistance pattern of goat milk lactobacilli towards tetracycline. In compliance to our findings, previous studies also revealed that lactobacilli were resistant to clindamycin (Ashraf and Shah, 2011) as well as to fusidic acid (Jose *et al.*, 2015).

Further, most of the goat milk lactobacilli displayed intermediate to resistance phenotype towards folic acid synthesis inhibitors (co-trimoxazole and trimethoprim). These findings are supported by Anas *et al.*, (2014) who also observed that goat milk *L. plantarum* displayed intermediate resistance to co-trimoxazole. Bousmaha-Marroki and Marroki (2015) reported complete resistance of Algerian goat's milk lactobacilli against trimethoprim. Presence of single nucleotide polymorphisms could be responsible for resistance towards trimethoprim (van Hoek *et al.*, 2011).

Overall, goat milk lactobacilli displayed a diverse level of sensitivity/resistance pattern. Therefore, *Lactobacillus* strains depicting antibiotic resistance pattern could be explored in restoring the gut microbiota during and after antibiotic treatment. This assumption is supported by many researchers (Ketema *et al.*, 2010; Sharma and Goyal, 2015). However, such resistance should be intrinsic and non-transferable; as intrinsic resistance and resistance due to mutation of chromosomal genes present a low risk of horizontal dissemination. Further, antimicrobial potential of LAB further enhances the efficacy of antimicrobial drug therapy, as it also helps in eradicating the pathogenic microorganism (Sharma *et al.*, 2017b). In the current study, we have observed a wide range of resistance among goat *Lactobacillus* isolates; therefore further studies are needed to understand the resistance mechanisms on molecular basis.

Also there is a need to put a check and ensure prudent use of antibiotics falling under intermediate susceptibility.

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