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ORIGINAL ARTICLE

# Antibiotic sensitivity pattern of indigenous lactobacilli isolated from curd and human milk samples

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**Abstract** The gut microbiota plays a vital role in host well-being and lactic acid bacteria (LAB) have gained an overwhelming attention as health promoter. This perception has evolved from traditional dairy products to a money-spinning market of probiotics. The safety of probiotics is coupled to their intended use and LAB may act as pool of antimicrobial resistance genes that could be transferred to pathogens, either in food matrix or in gastrointestinal tract, which could be detrimental to host. This study evaluated the antibiotic susceptibility patterns of LAB isolated from curd (20) and human milk (11) samples. Antibiotic susceptibility was determined against 26 common antibiotics, following reference disc diffusion assay. A varied response in terms of susceptibility and resistance towards antibiotics was recorded. Among curd isolates, D7 (*Lactobacillus plantarum*) was the most resistant followed by D4, D8, D10 and D25. Among human milk isolates, HM-1 (*L. casei*) showed the highest resistance profile. All LAB isolates displayed high susceptibility pattern towards imipenem and meropenem. In general, high resistivity was exhibited by human milk isolates. The present study showed that antibiotic resistance is widespread among different lactobacilli, which may pose a food safety concern. Therefore, antibiotic sensitivity should be considered as a vital tool for safety assessment of probiotics.

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**Keywords** Antibiotic resistance · Susceptibility · Lactic acid bacteria · *Lactobacillus* · Probiotics

**Abbreviations**

| | |
|---|---|
| ABR | Antibiotic resistance |
| ABS | Antibiotic susceptibility |
| LAB | Lactic acid bacteria |

## Introduction

The human and animal gut harbor an essential, complex, yet adaptive component that allows host adaptation at different timescales (Quercia et al. 2014). The complex consortium of living microbes, dominated by phyla, firmicutes (60–65%); bacteroidetes (20–25%); proteobacteria (5–10%) and actinobacteria (3%) (Rosenbaum et al. 2015), once established is relatively stable throughout adult life; but a shift is observed as a result of bacterial infections, antibiotic treatment, lifestyle, surgical, dietary shift, host physiology, age, environmental or endogenous stress and other factors (Quercia et al. 2014). Activity of these gut inhabitants immensely affects host health through the nutritional, physiological and protective processes including fermentation of unabsorbed dietary carbohydrate, production of vital vitamins (biotin and vitamin K), immune response mediation, energy metabolism, protection of the host against pathogen invasion, etc. (Kau et al. 2011). A balance between beneficial and pathogenic constituents of the microbiota exists in a healthy host and any variation in this balance can lead to a state of dysbiosis mainly responsible for illness and morbidity. Metchnikoff, the Russian Nobel laureate, was the first to relate lactic acid

bacteria of fermented yogurt with health and longevity of certain Balkan communities. The concept of beneficial microbes that he introduced has led to an extensive consumption of food preparations containing LAB and/or bifidobacteria, with the belief that they will confer health benefits. Moreover, controlled changes in normal diet, e.g., introduction of probiotics, prebiotics, polyphenols, high-fat/protein diet can result in changes in gut microbiome (Maukonen and Saarela 2015). Likewise, consumption of food/dairy products with high LAB count can help to maintain/replenish the beneficial microbiota. Furthermore, time honored Generally Recognized as Safe (GRAS) status by FDA, candidature for Qualified Presumption of Safety status (QPS) by EFSA (Arioli et al. 2013) and safety assurance from number of research studies and reports have resulted in a noteworthy attention towards probiotics as health promoters. The concept of probiotics has grown from traditional dairy products to a profitable market in which probiotic bacteria are incorporated in dairy products such as yogurt, fermented milk drinks, health supplements and functional foods.

Usually, LAB colonizes human digestive tract, urinary tract and genital systems and very rarely causes any infection. Besides lacking pathogenicity, they confer several health benefits (Manzoor et al. 2016). Now-a-days probiotics are globally consumed in food, dietary supplements, or as active components of a registered medication, and are commercially available in various forms. However, there is a need to re-assure their safety, especially in terms of spreading antibiotic resistance (ABR). Although antibiotic resistance is a hot topic of the hour, it is yet not being paid much attention in terms of LABs, the most freely consumed bacterial group. There is a high possibility of horizontal gene transfer among bacteria in nature and further spread of these resistant strains between populations (Sukmarini et al. 2014). The last decade has witnessed an increase in the number of reports documenting antibiotic resistance in LAB strains. Although, LAB are safe, there is concern towards possible mobility of resistance determinants to human and animal pathogenic and opportunistic bacteria. Few researchers acknowledge the presence of antibiotic resistance in LAB and appreciate the possibility of their co-administration with antibiotic therapy, ensuring replenishment of the healthy gut flora, which is otherwise at high risk (Dixit et al. 2013). However, this statement is divisive and a matter of conflict. The presence of resistance coding genes and transfer of the same through plasmids and conjugative transposons have also been reported in *Lactobacillus* species (Jose et al. 2015). Genes conferring resistance to several antimicrobials have been reported to be located on transferable genetic elements in several LABs (Gfeller et al. 2003). For a number of lactobacilli, a very high frequency of spontaneous mutation to kanamycin and streptomycin was reported (Curragh and Collins 1992). Therefore, there is a probability of transferring antibiotic resistance from probiotic strains to other bacteria, either commensal and/or pathogens that are undesirable and detrimental. Fermented milk products such as curd and yoghurt and human milk are among the common source of LAB that assures establishment and replenishment of healthy gut flora after antibiotic treatment to adults and infants, respectively. Human milk is vital for host health and is the primary deciding factor for selection and establishment of gut microflora. It is important to understand the resistance profile of bacterial inhabitants of these two (curd and human milk) primary foods. Resistance towards current antibiotics has been reported in LAB of several commercially available dairy/food products. It would be further interesting to assess the spectra of same in cultivable aerobic microflora of human milk. Humans are exposed to antibiotics in day-to-day life and this treatment may influence the susceptibility/resistance profile of human milk microflora, especially LAB group. Henceforth, the present study was conceived to assess the antibiotic susceptibility pattern of LAB isolates of curd and human milk origin. Also efforts have been laid to compare the same with reference probiotics and food-borne pathogens.

## Materials and methods

### Bacterial strains

Twenty lactic acid bacteria strains of *Lactobacillus* spp. were isolated from curd samples, and 11 from healthy human lactating volunteers (≥3 months) with no recent history of medication from rural and urban areas of Punjab, India (Table 1). Mothers were explained and convinced before sample collection and written consent for the same was taken. Samples were transported to laboratory under refrigerated condition (4–5 °C), enriched, serially diluted and plated over de-Man, Rogosa and Sharpe (MRS) agar followed by an overnight incubation at 37 °C. Colonies were picked, purified and morphology determined by Gram's and negative staining. Gram-positive, catalase-negative rods were shortlisted as *Lactobacillus* spp. Identity of *Lactobacillus* isolates was further ascertained using standard genus-specific PCR as described earlier (Panwar et al. 2014). Species level identification of the confirmed *Lactobacillus* isolates was further carried out through 16S rRNA-based sequencing (Panwar et al. 2016), using DNA sequencing service of Sci Genom Pvt. Ltd. Chennai, India. Nucleotide sequences were BLAST against NCBI database (http://blast.ncbi.nlm.nih.gov) and sequences were deposited in the NCBI Gene Bank and accession numbers for the same were obtained (Table 1).

**Table 1** List of bacterial isolates used in this study. IPhp denotes lab code; D (curd) and HM (human milk) signifies isolate number

| Code | Identification name | NCBI accession numbers |
|---|---|---|
| Curd isolates | | |
| IPhp-D2 | *Lactobacillus plantarum* | KX129818 |
| IPhp-D4 | *L. plantarum* | KX146485 |
| IPhp-D5 | *L. plantarum* | KX943015 |
| IPhp-D6 | *L. delbrueckii* subsp. *indicus* | KX228834 |
| IPhp-D7 | *L. plantarum* | KX228835 |
| IPhp-D8 | *L. plantarum* subsp. *plantarum* | KX228836 |
| IPhp-D9 | *L. delbrueckii* subsp. *indicus* | KX228837 |
| IPhp-D10 | *L. plantarum* | KX228838 |
| IPhp-D11 | *L. fermentum* | KX228839 |
| IPhp-D12 | *L. plantarum* | KX228840 |
| IPhp-D14 | *L. plantarum* | KX943016 |
| IPhp-D17 | *L. plantarum* | KX943017 |
| IPhp-D18 | *L. delbrueckii* subsp. *indicus* | KX228841 |
| IPhp-D19 | *L. delbrueckii* subsp. *delbrueckii* | KX228842 |
| IPhp-D24 | *L. plantarum* | KX943018 |
| IPhp-D25 | *L. plantarum* | KX943019 |
| IPhp-D26 | *L. plantarum* | KX228843 |
| IPhp-D27 | *L. plantarum* | KX943020 |
| IPhp-D28 | *L. fermentum* | KX228844 |
| IPhp-D29 | *L. plantarum* | KX228845 |
| Human milk isolates | | |
| IPhp-HM1 | *L. casei* | KX714820 |
| IPhp-HM2 | *L. plantarum* | KX943021 |
| IPhp-HM3 | *Lactobacillus* sp. | KX301286 |
| IPhp-HM6 | *L. pentosus* | KX301287 |
| IPhp-HM7 | *L. plantarum* | KX301288 |
| IPhp-HM8 | *L. plantarum* | KX301289 |
| IPhp-HM9 | *L. plantarum* | KX714821 |
| IPhp-HM10 | *L. plantarum* | KX301290 |
| IPhp-HM11 | *L. plantarum* | KX714822 |
| IPhp-HM12 | *Lactobacillus* sp. | KX301291 |
| IPhp-HM13 | *L. pentosus* | KX301292 |

Six reference *Lactobacillus* strains, viz. *Lactobacillus fermentum* (NCDC 214), *L. helveticus* (NCDC 194), *L. plantarum* (NCDC 20), *L. bulgaricus* (NCDC 27), *L. delbrueckii* ssp. *lactis* (NCDC 3), *L. rhamnosus* (NCDC 19), and two lactococci, viz. *Lactococcus lactis* (NCDC 91) and *Lactococcus cremoris* (NCDC 81), were procured from the repository of National Collection of Dairy Cultures (NCDC), ICAR-NDRI, Karnal, India. Two standard probiotic strains, i.e., *L. rhamnosus* GG (ATCC 53103) and *L. casei* (ATCC 393) were procured from American Type Culture Collection (ATCC), USA.

Pathogenic strains used in the study, viz. *Bacillus cereus* (MTCC 1272), *Listeria monocytogenes* (MTCC 1143), *Staphylococcus aureus* (MTCC 96), *Salmonella enterica* serovar Typhi (MTCC 733), *Escherichia coli* (MTCC 723) and *Shigella flexneri* (MTCC 1457) were procured from Microbial Type Culture Collection (MTCC), Chandigarh, India. All lactobacilli were maintained and propagated in MRS broth; lactococci in M17 broth and pathogens in Brain Heart Infusion (BHI) broth. All the bacterial cultures were preserved as glycerol stocks at −80 °C. Prior to the susceptibility assays, all the cultures were sub-cultured thrice in respective growth medium, followed by plating over MRS for *Lactobacillus* and Mueller–Hinton Agar (MHA) for pathogens, where the antibiotic susceptibility assay was performed.

### Test antibiotic disks

Twenty-six commonly used antibacterial(s) tested in this study were procured from Hi-media Laboratories Pvt. Ltd. Mumbai, India. The concentration of various drugs, antibiotic group and mode of action has been depicted in Table 2.

### Antibiotic susceptibility assay

Antibiogram tests were performed to record the sensitivity or resistance of LAB towards conventional antibiotics. The standard disc diffusion assay was employed to analyze the antibiotic susceptibility pattern through modified Kirby–Bauer method (Bauer et al. 1966). In brief, broth culture (100 µl, 0.5 McFarland equivalent to $10^8$cfu/ml) of all the tested strains was mixed with 8 ml of soft agar, over-layered on pre-solidified agar plate, allowed to solidify, and antibiotic discs were aseptically placed equidistant to each other using sterile forceps. Plates were pre-incubated at room temperature (25 °C) for 1 h for ensuring proper diffusion, and then incubated overnight at 37 °C. After overnight incubation, the diameter (mm) of zone of inhibition (ZOI) was measured by zone reader (Hi Antibiotic zone scale, Hi-Media) on the undersurface of the Petri dish and results were depicted as sensitive/susceptible (*S*), intermediate (*I*) and resistant (*R*). According to Clinical and Laboratory Standards Institute CLSI (2015) interpretive category, *S* signifies that the tested strains are inhibited by the usually achievable concentrations of the antimicrobial agent, upon application of recommended dose. The intermediate category (*I*) includes isolates for which the antimicrobial activity is lower than susceptible isolates. The resistant category (*R*) implies that isolates are resistant towards usually achievable concentrations of the antimicrobial agent present in the commercial disks used.

The results were interpreted according to the breakpoints recommended by CLSI (2015) guidelines as follows: the isolates with a zone of inhibition less than or equal to 14 mm were considered as resistant (*R*) and those

**Table 2** List of antibiotics used in the study

| S. no. | Name of drug | Concentration (mcg) | Antibiotic group | Mode of action |
|---|---|---|---|---|
| 1 | Ampicillin | 10 | β-Lactams | Inhibitors of the cell wall synthesis |
| 2 | Imipenem | 10 | | |
| 3 | Meropenem | 10 | | |
| 4 | Methicillin | 5 | | |
| 5 | Oxacillin | 1 | | |
| 6 | Penicillin | 10[a] | | |
| 7 | Cefuroxime | 30 | Second generation | |
| 8 | Cefoxitin | 30 | Cephalosporins | |
| 9 | Ceftazidime | 30 | Third generation | |
| 10 | Cefotaxime | 30 | Cephalosporins | |
| 11 | Teicoplanin | 30 | Glycopeptides | |
| 12 | Vancomycin | 30 | | |
| 13 | Ciprofloxacin | 5 | Quinolones | Inhibiting DNA replication and transcription |
| 14 | Ofloxacin | 5 | | |
| 15 | Gentamicin | 10 | Aminoglycosides | Inhibitors of protein synthesis |
| 16 | Streptomycin | 300 | | |
| 17 | Tobramycin | 10 | | |
| 18 | Chloramphenicol | 30 | Other | |
| 19 | Clindamycin | 2 | Lincosamide | |
| 20 | Erythromycin | 15 | Macrolides | |
| 21 | Fusidic acid | 10 | Fusidane | |
| 22 | Nitrofurantoin | 300 | Other | |
| 23 | Tetracycline | 30 | Tetracyclines | |
| 24 | Tigecycline | 15 | Glycylcycline | |
| 25 | Co-Trimoxazole | 25 | Other | Folic acid synthesis inhibitors or anti-metabolites |
| 26 | Trimethoprim | 5 | | |

[a] Concentration in units

with more than 20 mm diameter as susceptible (*S*) and those having ZOI between 15 and 19 mm as intermediate (*I*). Antibiotic susceptibility testing was carried out over MRS for lactobacilli, M17 for lactococci and Mueller–Hinton for pathogens. MRS and M17 had been used to assure proper growth of all the lactic acid bacteria (LAB) isolates, which were not equally good over Mueller–Hinton. Few earlier studies have compared activity of LAB over both MRS and Mueller–Hinton and documented poor and irregular growth of LABs over Mueller–Hinton, due to special growth requirement(s) for these cells in terms of nutrient supplementation and medium acidity. Mueller–Hinton, the conventional antibiotic susceptibility testing media, is not uniformly suitable for LABs. However, MRS and M17 agar, owing to have slightly lower pH (6.2 ± 0.2) offers slight resistance to the antimicrobial activity of various drugs having activity optima at neutral or alkaline range. Hence, the zone diameter values may slightly differ to other studies where different nutrient media and incubation conditions may have been used (Klare et al. 2005, 2007; Ocana et al. 2006). Size of the inhibition zones is known to be dependent over the diffusion media; hence, observations made herein cannot be directly co-related with Mueller–Hinton agar, but can be used for categorization purpose.

### Statistical evaluation

The disc diffusion method was performed in triplicate and the diameters recorded are presented either as resistant (*R*), sensitive (*S*) or intermediate (zone diameter ± SD).

## Results

Antimicrobial disk susceptibility tests were performed in accordance with the procedures outlined by CLSI (2015). The growths of all tested LAB isolates were homogenous over MRS and M17 and inhibition halos were noticeably defined. The results for the curd, human milk, reference LAB, standard probiotics, lactococci and pathogenic isolates/strains have been documented in tabular form (Tables 3, 4, 5) in terms of resistant (*R*) and susceptible (*S*). Strains showing intermediate susceptibility (ZOI ~15 to 19 mm) have been depicted by numerical values, clearly depicting the marginal values inclined towards either sensitivity or resistance.

LAB isolates of curd origin showed sensitivity towards ampicillin, imipenem, meropenem (β-lactams), chloramphenicol and erythromycin (macrolide), and resistance towards methicillin (β-lactams) and tobramycin (aminoglycoside). A varied response, viz. resistance–intermediate (cefoxitin, ciprofloxacin, ofloxacin, gentamicin and streptomycin), sensitive–intermediate (penicillin, cefuroxime, cefotaxime, nitrofurantoin, tetracycline, tigecycline and trimethoprim), and resistance–intermediate–sensitive (oxacillin, ceftazidime, teicoplanin, vancomycin, clindamycin, fusidic acid and co-trimoxazole) pattern was recorded against different antibiotics. Out of the isolates screened, D7 (*L. plantarum*) was most resistant, with resistance to intermediate pattern documented against 18/26 antibiotics, closely followed by D4, D8, D10 and D25, showing resistance to intermediate pattern against 17/26 antibiotics. However, isolate D27 (*L. plantarum*) was the most sensitive, with clear resistance only against methicillin and tobramycin (Table 3).

As documented in Table 4, all the test LAB isolates of human milk origin had sensitivity to β-lactams, imipenem and meropenem. Ampicillin could also significantly inhibit majority of isolates, except HM-1 (*L. casei*), showing resistant profile. All the human milk isolates showed resistance towards methicillin and oxacillin, other antimicrobials of same family. Mixed response was observed with penicillin. Among cephalosporins, varied response was observed, with resistance towards cefoxitin, and cefotaxime being most potent against 10 of 11 screened isolates. HM-1 showed intermediate susceptibility to cefotaxime. All the human milk isolates showed clear resistance against glycopeptides (teicoplanin, vancomycin) and quinolones (ciprofloxacin, ofloxacin). Interestingly, among aminoglycoside group, only gentamicin could retard the growth of all human milk isolates, that too at moderate level. Majority of isolates were sensitive towards chloramphenicol, erythromycin and tigecycline. Nitrofurantoin was effective against all isolates except HM-1. All the isolates were resistant to co-trimoxazole, and varied response was recorded against clindamycin, fusidic acid, tetracycline and trimethoprim. Overall, HM-1 (*L. casei*) showed highest resistant/intermediate profile towards 15/08 antimicrobials tested.

Interestingly, reference *Lactobacillus* cultures showed variable activity against different antimicrobials, with no clear trend against any particular group. All the reference cultures were sensitive towards only imipenem (β-lactam) and erythromycin (macrolide), and resistant towards co-trimoxazole and trimethoprim. Out of the six *Lactobacillus* species, *L. brevis* emerged as the most resistant species with resistance/intermediate pattern against 20/6 antibiotics, closely followed by *L. delbrueckii* (13/6), *L. rhamnosus* (13/4) and *L. helveticus* (12/8). *L. fermentum* and *L. plantarum* were most sensitive, with resistant/intermediate spectra recorded against 6/5 and 8/8 antimicrobials, respectively. Standard probiotic culture, *L. rhamnosus* GG and *L. casei* depicted mild resistance towards 11 and 8 antibiotics tested (Table 5). Gram-positive lactococci (*L. lactis* and *L. cremoris*) included as non-probiotic control

**Table 3** Antibacterial susceptibility profile of curd *Lactobacillus* isolates to commercial antibiotics

| S. no. | Antibiotics | D2 | D4 | D5 | D6 | D7 | D8 | D9 | D10 | D11 | D12 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | Ampicillin | S | S | S | S | S | S | S | S | S | S |
| 2 | Imipenem | S | S | S | S | S | S | S | S | S | S |
| 3 | Meropenem | S | S | S | S | S | S | S | S | S | S |
| 4 | Methicillin | R | R | R | R | R | R | R | R | R | R |
| 5 | Oxacillin | R | R | R | R | R | R | R | 15.6 ± 0.57 | 16 ± 1 | R |
| 6 | Penicillin | S | 19 ± 0 | S | S | 16.3 ± 0.57 | 16.6 ± 0.57 | S | S | S | S |
| 7 | Cefuroxime | S | S | S | S | 16 ± 1 | 16.6 ± 1.5 | S | 16.3 ± 0.57 | S | S |
| 8 | Cefoxitin | R | R | R | R | R | R | R | R | R | R |
| 9 | Ceftazidime | S | 17.6 ± 0.57 | 18 ± 1 | S | 16.6 ± 1.52 | R | 18.3 ± 0.57 | R | 17.6 ± 0.57 | 19 ± 1 |
| 10 | Cefotaxime | S | S | S | S | S | S | S | S | S | S |
| 11 | Teicoplanin | R | R | R | R | R | R | R | R | R | R |
| 12 | Vancomycin | R | R | R | R | R | R | R | R | R | R |
| 13 | Ciprofloxacin | R | R | R | R | R | R | R | R | 15.3 ± 0.57 | R |
| 14 | Ofloxacin | R | R | R | R | R | R | R | R | R | R |
| 15 | Gentamicin | 17 ± 1 | R | 15.3 ± 0.57 | 17.6 ± 0.5 | R | R | 17 ± 0 | 15.3 ± 0.57 | 16.6 ± 0.57 | 16 ± 1 |
| 16 | Streptomycin | R | R | R | R | R | R | R | R | R | R |
| 17 | Tobramycin | R | R | R | R | R | R | R | R | R | R |
| 18 | Chloramphenicol | S | S | S | S | S | S | S | S | S | S |
| 19 | Clindamycin | 17.3 ± 0.57 | R | R | 17 ± 1 | R | R | R | R | R | R |
| 20 | Erythromycin | S | S | S | S | S | S | S | S | S | S |
| 21 | Fusidic acid | 19 ± 1 | 15.3 ± 0.57 | 16.3 ± 0.57 | 17 ± 0 | R | R | R | R | 17.3 ± 0.57 | 16.6 ± 0.57 |
| 22 | Nitrofurantoin | 19.3 ± 0.57 | R | S | S | 15.3 ± 0.57 | 15.6 ± 0.57 | S | 16.6 ± 0.57 | S | 19 ± 0 |
| 23 | Tetracycline | S | 19.3 ± 0.57 | S | S | 19 ± 0 | 17.3 ± 0.57 | S | S | S | 17.6 ± 0.57 |
| 24 | Tigecycline | S | S | S | S | S | S | S | S | S | S |
| 25 | Co-Trimoxazole | S | 15 ± 1 | S | S | 18.3 ± 0.57 | S | S | 18.6 ± 0.57 | S | S |
| 26 | Trimethoprim | S | 19 ± 0 | S | S | S | S | S | 18.3 ± 0.57 | S | S |

| S. no. | Antibiotics | D14 | D17 | D18 | D19 | D24 | D25 | D26 | D27 | D28 | D29 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | Ampicillin | S | S | S | S | S | S | S | S | S | S |
| 2 | Imipenem | S | S | S | S | S | S | S | S | S | S |
| 3 | Meropenem | S | S | S | S | S | S | S | S | S | S |
| 4 | Methicillin | R | R | R | R | R | R | R | R | R | R |
| 5 | Oxacillin | R | 16.3 ± 0.57 | S | R | 16 ± 1 | R | 16.6 ± 1.5 | S | R | 18.6 ± 0.57 |
| 6 | Penicillin | 18.3 ± 0.57 | S | S | S | S | 17.6 ± 0.57 | S | S | 18.3 ± 0.57 | S |
| 7 | Cefuroxime | 17.3 ± 0.57 | S | S | S | S | 18.6 ± 0.57 | S | S | S | S |
| 8 | Cefoxitin | R | 16 ± 1 | 18.3 ± 0.57 | R | R | R | R | 16.6 ± 0.57 | R | R |

**Table 3** continued

| S. no. | Antibiotics | D14 | D17 | D18 | D19 | D24 | D25 | D26 | D27 | D28 | D29 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 9 | Ceftazidime | 16.6 ± 0.57 | S | 16.3 ± 0.57 | S | 18 ± 1 | 16 ± 0 | 18.6 ± 0.57 | 18.3 ± 0.57 | 17 ± 1 | 18.6 ± 0.57 |
| 10 | Cefotaxime | S | S | 16.6 ± 1.52 | S | R | S | S | S | S | S |
| 11 | Teicoplanin | R | R | 19.3 ± 0.57 | R | 18.6 ± 0.57 | R | R | S | R | R |
| 12 | Vancomycin | R | R | S | R | R | R | R | 16 ± 1 | R | R |
| 13 | Ciprofloxacin | R | R | R | R | R | R | R | 19 ± 0 | R | S |
| 14 | Ofloxacin | R | R | 17.6 ± 0.57 | R | R | R | R | 19.3 ± 0.57 | R | 15 ± 1 |
| 15 | Gentamicin | R | 15.3 ± 0.57 | 17.3 ± 0.57 | 15 ± 0 | 16 ± 1 | R | R | 19 ± 1 | R | 18.6 ± 0.57 |
| 16 | Streptomycin | R | R | 18.3 ± 0.57 | R | R | R | R | 19 ± 0 | R | R |
| 17 | Tobramycin | R | R | R | R | R | R | R | R | R | R |
| 18 | Chloramphenicol | S | S | S | S | S | S | S | S | S | S |
| 19 | Clindamycin | R | R | S | R | 19 ± 0 | R | R | S | 15.6 ± 1.52 | S |
| 20 | Erythromycin | S | S | S | S | S | S | S | S | S | S |
| 21 | Fusidic acid | S | 16.3 ± 0.57 | R | 15 ± 0 | S | 15 ± 1 | 16.3 ± 0.57 | S | 16 ± 1 | S |
| 22 | Nitrofurantoin | S | R | 16.3 ± 0.57 | S | S | 16 ± 1 | S | 15.3 ± 0.57 | 18 ± 0 | S |
| 23 | Tetracycline | S | S | S | 19 ± 0 | S | 19.3 ± 0.57 | 18.6 ± 0.57 | S | S | S |
| 24 | Tigecycline | S | 19.3 ± 0.57 | 17.6 ± 0.57 | S | S | S | S | S | S | S |
| 25 | Co-Trimoxazole | 17.3 ± 0.57 | S | R | S | S | S | S | S | S | S |
| 26 | Trimethoprim | S | S | 18 ± 1 | S | S | S | S | S | S | 17.6 ± 0.57 |

**Table 4** Antibacterial susceptibility profile of human milk *Lactobacillus* isolates to commercial antibiotics

| S. no. | Antibiotics | HM1 | HM2 | HM3 | HM6 | HM7 | HM8 | HM9 | HM10 | HM11 | HM12 | HM13 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | Ampicillin | R | S | S | S | S | S | S | S | S | S | S |
| 2 | Imipenem | S | S | S | S | S | S | S | S | S | S | S |
| 3 | Meropenem | S | S | S | S | S | S | S | S | S | S | S |
| 4 | Methicillin | R | R | R | R | R | R | R | R | R | R | R |
| 5 | Oxacillin | R | R | R | R | R | R | R | R | R | R | R |
| 6 | Penicillin | R | S | 15.3 ± 0.57 | 19 ± 0 | S | 17.6 ± 0.57 | 19 ± 1 | S | 16.3 ± 0.57 | 16.6 ± 0.57 | S |
| 7 | Cefuroxime | 17.6 ± 0.57 | R | R | R | S | 16.3 ± 0.57 | R | 16 ± 0 | R | R | R |
| 8 | Cefoxitin | R | R | R | R | R | R | R | R | R | R | R |
| 9 | Ceftazidime | 15.3 ± 0.57 | R | R | R | 18.3 ± 0.57 | 17.3 ± 0.57 | 16.3 ± 0.57 | R | 16.3 ± 0.57 | 16 ± 1 | R |
| 10 | Cefotaxime | 15.6 ± 0.57 | S | S | S | S | S | S | S | S | S | S |
| 11 | Teicoplanin | R | R | R | R | R | R | R | R | R | R | R |
| 12 | Vancomycin | R | R | R | R | R | R | R | R | R | R | R |
| 13 | Ciprofloxacin | R | R | R | R | R | R | R | R | R | R | R |
| 14 | Ofloxacin | R | R | R | R | R | R | R | R | R | R | R |
| 15 | Gentamicin | 17.3 ± 0.57 | 16.3 ± 0.57 | 16 ± 0 | 15.3 ± 0.57 | 16.6 ± 0.57 | 17.3 ± 0.57 | 17 ± 1 | 16.3 ± 0.57 | 15.6 ± 0.57 | 16.3 ± 0.57 | R |
| 16 | Streptomycin | R | R | R | R | R | R | R | R | R | R | R |
| 17 | Tobramycin | R | R | R | R | R | R | R | R | R | R | R |
| 18 | Chloramphenicol | 17.3 ± 0.57 | S | S | S | S | S | S | S | S | S | S |
| 19 | Clindamycin | S | R | R | R | R | R | 15.6 ± 0.57 | R | R | 16.3 ± 0.57 | R |
| 20 | Erythromycin | 17.6 ± 0.57 | S | S | S | S | S | S | S | S | S | S |
| 21 | Fusidic acid | R | 16.3 ± 0.57 | 17.3 ± 0.57 | 16.6 ± 0.57 | 17 ± 1 | 16.3 ± 0.57 | R | 17 ± 0 | R | 17.6 ± 0.57 | 15.6 ± 0.57 |
| 22 | Nitrofurantoin | R | S | S | S | S | S | S | S | S | S | S |
| 23 | Tetracycline | 19.3 ± 0.57 | S | 17.6 ± 0.57 | 18.3 ± 0.57 | S | 19 ± 0 | 17.3 ± 0.57 | 18.6 ± 0.57 | 16.3 ± 0.57 | S | 19 ± 0 |
| 24 | Tigecycline | 16.6 ± 0.57 | S | S | S | S | S | S | S | S | S | S |
| 25 | Co-Trimoxazole | R | R | R | R | R | R | R | R | R | R | R |
| 26 | Trimethoprim | R | S | R | R | R | R | R | R | R | 19 ± 0 | 18.3 ± 0.57 |

**Table 5** Antibacterial susceptibility pattern of different LAB strains, reference probiotics and pathogens against commercial antibiotics

| S. no. | Antibiotics | *Lactobacillus* sp. | | | | | | Non-probiotic | |
|---|---|---|---|---|---|---|---|---|---|
| | | *Lactobacillus delbrueckii* subsp. *lactis* | *L. rhamnosus* | *L. plantarum* | *L. brevis* | *L. helveticus* | *L. fermentum* | *Lactococcus lactis* | *Lactococcus cremoris* |
| 1 | Ampicillin | S | S | S | 19 ± 0 | 18.3 ± 0.57 | S | S | S |
| 2 | Imipenem | S | S | S | S | S | S | S | S |
| 3 | Meropenem | 18.3 ± 0.57 | R | S | 16.3 ± 0.57 | 16 ± 1 | S | S | S |
| 4 | Methicillin | R | R | 17.3 ± 0.57 | R | R | R | R | R |
| 5 | Oxacillin | R | R | 19.3 ± 0.57 | R | R | 19 ± 0 | R | R |
| 6 | Penicillin | 18.3 ± 0.57 | S | S | S | S | S | S | S |
| 7 | Cefuroxime | 17.3 ± 0.57 | S | S | 19.3 ± 0.57 | S | S | S | S |
| 8 | Cefoxitin | R | R | R | R | R | R | R | R |
| 9 | Ceftazidime | 15.3 ± 0.57 | R | 17.3 ± 0.57 | R | 16.3 ± 0.57 | 18 ± 0 | 17.3 ± 0.57 | 17.6 ± 0.57 |
| 10 | Cefotaxime | 18 ± 1 | S | S | S | 17.3 ± 0.57 | S | S | S |
| 11 | Teicoplanin | R | R | R | R | R | 16.3 ± 0.57 | R | R |
| 12 | Vancomycin | R | R | R | R | R | 15.6 ± 0.57 | R | R |
| 13 | Ciprofloxacin | R | R | 18.3 ± 0.57 | R | R | S | R | R |
| 14 | Ofloxacin | R | R | 16.3 ± 0.57 | R | R | R | R | R |
| 15 | Gentamicin | R | 15.3 ± 0.57 | 16.6 ± 0.57 | R | R | S | 15 ± 1 | 16.6 ± 1.52 |
| 16 | Streptomycin | R | R | 15.3 ± 0.57 | R | R | R | R | R |
| 17 | Tobramycin | R | R | R | R | R | 16.3 ± 0.57 | R | R |
| 18 | Chloramphenicol | S | S | S | S | 19 ± 1 | S | S | S |
| 19 | Clindamycin | S | S | 19 ± 0 | S | S | S | R | R |
| 20 | Erythromycin | S | S | S | S | S | S | S | S |
| 21 | Fusidic acid | 19 ± 0 | 16.6 ± 1.52 | R | 17.3 ± 0.57 | 17.6 ± 0.57 | S | 19 ± 1 | 19.3 ± 0.57 |
| 22 | Nitrofurantoin | R | 18.6 ± 0.57 | S | R | S | S | S | S |
| 23 | Tetracycline | S | 16.3 ± 0.57 | S | 16.6 ± 0.57 | 15.3 ± 0.57 | S | S | S |
| 24 | Tigecycline | S | S | 19.0 ± 0.57 | 18.6 ± 0.57 | 16.3 ± 0.57 | S | S | S |
| 25 | Co-Trimoxazole | R | R | R | R | R | R | S | S |
| 26 | Trimethoprim | R | R | R | R | R | R | S | S |

| S. no. | Antibiotics | Reference probiotic | | Pathogens | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | *L. rhamnosus* GG | *L. casei* | *Bacillus cereus* | *Listeria monocytogenes* | *Staphylococcus aureus* | *Salmonella enterica* serovar Typhi | *Escherichia coli* | *Shigella flexneri* |
| 1 | Ampicillin | S | S | S | S | S | 19.3 ± 0.57 | 19.6 ± 0.57 | S |
| 2 | Imipenem | S | S | S | S | S | S | S | S |
| 3 | Meropenem | S | S | R | S | S | S | S | S |

**Table 5** continued

| S. no. | Antibiotics | Reference probiotic | | Pathogens | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | *L. rhamnosus* GG | *L. casei* | *Bacillus cereus* | *Listeria monocytogenes* | *Staphylococcus aureus* | *Salmonella enterica* serovar Typhi | *Escherichia coli* | *Shigella flexneri* |
| 4 | Methicillin | R | R | R | S | S | R | R | R |
| 5 | Oxacillin | R | R | 19.3 ± 0.57 | S | S | R | R | R |
| 6 | Penicillin | S | S | S | S | S | 17.6 ± 0.57 | R | 17.3 ± 0.57 |
| 7 | Cefuroxime | S | 19 ± 1 | R | S | S | S | 17.6 ± 0.57 | S |
| 8 | Cefoxitin | R | R | S | S | R | S | 15.3 ± 0.57 | 17.6 ± 0.57 |
| 9 | Ceftazidime | R | S | R | S | R | 15.3 ± 0.57 | 18.6 ± 0.57 | S |
| 10 | Cefotaxime | S | S | R | S | S | S | S | S |
| 11 | Teicoplanin | R | 16 ± 1 | 19 ± 0 | 16.3 ± 0.57 | 16.6 ± 0.57 | R | R | R |
| 12 | Vancomycin | R | R | 17.3 ± 0.57 | 18.6 ± 0.57 | 17.3 ± 0.57 | R | R | R |
| 13 | Ciprofloxacin | 16.3 ± 0.57 | R | S | S | S | S | S | S |
| 14 | Ofloxacin | R | 16.6 ± 0.57 | S | S | S | S | S | S |
| 15 | Gentamicin | S | S | S | S | 19.3 ± 0.57 | 18 ± 1 | S | S |
| 16 | Streptomycin | S | 15.6 ± 0.57 | S | 16.3 ± 0.57 | S | S | 19 ± 0 | S |
| 17 | Tobramycin | R | R | S | S | 19 ± 0 | S | 18.6 ± 0.57 | S |
| 18 | Chloramphenicol | 19 ± 0 | 16.3 ± 0.57 | S | S | S | S | S | S |
| 19 | Clindamycin | S | 15.6 ± 0.57 | 16.6 ± 0.57 | S | S | R | 17.3 ± 0.57 | R |
| 20 | Erythromycin | S | S | S | S | 17.3 ± 0.57 | 18.6 ± 0.57 | 18.3 ± 0.57 | S |
| 21 | Fusidic acid | R | R | S | S | S | R | R | R |
| 22 | Nitrofurantoin | S | 19 ± 0 | 17.3 ± 0.57 | S | R | S | R | S |
| 23 | Tetracycline | S | 16.3 ± 0.57 | S | S | S | S | S | S |
| 24 | Tigecycline | S | 15.6 ± 0.57 | S | S | S | S | S | S |
| 25 | Co-Trimoxazole | R | 17.6 ± 0.57 | S | S | S | S | S | S |
| 26 | Trimethoprim | R | R | S | S | R | S | S | S |

were also examined for their antibiotic susceptibility pattern. Both the lactococci showed similar trend, with resistance against 10; sensitive towards 13; and intermediate against 3 antibiotics (Table 5).

All the pathogens tested were observed to be sensitive towards quinolones (ciprofloxacin, ofloxacin), tetracycline, tigecycline and co-trimoxazole. Gram-positive pathogens, viz. *L. monocytogenes*, *S. aureus* and *B. cereus* were recorded to be resistant/intermediate towards only 0/3, 4/5 and 5/5 antibiotics, respectively. Gram-negative pathogens, viz. *S. flexneri*, *S. enteric* serovar Typhi and *E. coli* showed resistance/intermediate profile towards 6/2, 6/5 and 7/8 antibiotics, out of 26 antibiotics, respectively. Overall, a lower resistance pattern was presented by tested pathogens. Gram-positive pathogens gave intermediate pattern towards glycopeptides (teicoplanin, vancomycin), while Gram-negative pathogens were resistant towards the same (Table 5).

## Discussion

The study evaluated the antibiotic susceptibility profile of lactobacilli isolated from curd and human milk samples from the different regions of Punjab, India, against 26 different commercial antibiotic discs belonging to different groups. A general susceptibility of curd and human milk isolates towards β-lactams, except against methicillin and oxacillin, was recorded. Oxacillin was mildly effective against few curd isolates and Gram-positive pathogens; however, the scenario was reverse for all the isolates of human milk origin and Gram-negative pathogens. Overall, our findings are in good agreement with data from earlier studies for a broad range of LAB species. Earlier, Klare et al. (2007), Udhayashree et al. (2012) and Sharma et al. (2015) reported susceptibility of different LAB species from different isolation source towards penicillin and ampicillin. Susceptibility of human milk lactobacilli towards both ampicillin and penicillin has also been documented by few research studies (Martin et al. 2005; Malek et al. 2010). Recently, Balamurugan et al. (2014) recorded sensitivity of curd isolates to imipenem. However, contrary to our results, they observed resistance pattern towards ampicillin. Sharma and Goyal (2015) recorded high sensitivity of *L. rhamnosus* and *L. acidophilus* to meropenem. A general resistance towards oxacillin displayed by all the tested LAB isolates is in line with the pattern observed by Erdourul and Erbulur (2006) in cheese *Lactobacillus* isolates. Another recent study conducted in northern India by Sharma et al. (2015), displayed methicillin-resistant LAB strains in food chain. Earlier, Beyan et al. (2011) too observed methicillin resistance in more than half of the *Lactobacillus* spp. isolated from traditional Ethiopian fermented milk in Ethiopia. Resistance to β-lactam(s) can be attributed to the presence of genes coding for β-lactamases, which has been reported to transfer conjugally within different groups. High frequency of conjugation has been reported in different *Lactobacillus* species (Aquilanti et al. 2007).

High resistance towards glycopeptides (teicoplanin, vancomycin) and quinolones (ciprofloxacin, ofloxacin) exhibited by lactobacilli in our study can be substantiated with the reports supporting the presence of intrinsic resistance mechanism towards both the antibiotic families (Nawaz et al. 2011). Intrinsic resistance refers to the non-sensitivity of bacterial strains towards the approved drug doses, regulated by permeability barriers and active efflux. Intrinsic resistance is usually non-transferable and possesses no risk in LABs. Naturally high resistance to a number of antibiotics, especially vancomycin, is a characteristic feature of lactobacilli. Resistance towards vancomycin is due to the presence of peptidoglycal precursors terminating in D-alanyl-D-lactate, preventing binding of vancomycin (Gueimonde et al. 2013) and to quinolones, it can be attributed to mutation in topoisomerase IV, which is the primary target for ciprofloxacin (Hummel et al. 2007). Most of our LAB isolates were resistant to quinolones. However, both Gram-positive and Gram-negative pathogens presented good sensitivity. Our results are in concurrence with the results of Sharma et al. (2015), who also reported quinolone resistance of maximum numbers of LAB isolates. Several other reports have documented high ciprofloxacin resistance in LAB isolates of food origin (Hummel et al. 2007; Nawaz et al. 2011; Hawaz 2014). In a recent study published by Jiang et al. (2016), resistant pattern of human milk lactobacilli against ciprofloxacin was documented.

Among the cephalosporins, cefotaxime was most effective against the LAB isolates and test strains, followed by cefuroxime. Recently, Halder and Mandel (2015) also observed the susceptibility of curd lactobacilli against cefotaxime. Study carried out by Martin et al. (2005) documented that human milk isolates, i.e., *L. rhamnosus* GG and *L. fermentum* CECT 5716 were resistant to cefoxitin. Several other studies reported high resistance to cephalosporins (Danielsen and Wind 2003; Ammor et al. 2007). Resistance to cephalosporins, a structural sub-type of β-lactam antibiotic can be explained with the presence of variants of broad spectrum β-lactamases and/or efflux pumps (Pfeifer et al. 2010) associated with cell wall impermeability (Delgado et al. 2005).

Various reports corroborate our findings regarding resistance of curd (Hawaz 2014; Halder and Mandal 2015) and human milk (Martin et al. 2005; Malek et al. 2010) lactobacilli towards aminoglycosides (Ammor et al. 2007; Klare et al. 2007; Nawaz et al. 2011). We documented strong resistance of both curd and human milk isolates

against streptomycin and tobramycin. In marked contrast to streptomycin and tobramycin, gentamicin was mildly effective against all the human milk isolates and few isolates of curd origin. Recently, Jiang et al. (2016) also reported the intermediate susceptibility of human milk lactobacilli against gentamicin. Few studies reported presence of aminoglycoside resistance genes in lactobacilli (Zhou et al. 2012). Resistance phenotype to aminoglycosides can be further attributed to the absence of cytochrome-mediated electron transport, enabling antibiotic uptake (Charteris et al. 2001).

Tested LAB isolates showed intermediate susceptibility to fusidic acid, which is in line with the earlier report of Klare et al. (2007). All curd isolates exhibited strong susceptibility against chloramphenicol and erythromycin. The same was true for human milk isolates, except HM1. Susceptibility of lactobacilli to both erythromycin and chloramphenicol has also been reported by earlier studies (Klare et al. 2007; Beyan et al. 2011; Jiang et al. 2016). In the current study, all the *Lactobacillus* isolates, except HM1 were either susceptible or intermediately susceptible to nitrofurantoin, tigecycline and tetracycline. Our results are in accordance with Jiang et al. (2016), who showed the intermediate pattern of human milk lactobacilli against tetracycline and susceptibility to nitrofurantoin. A high resistance to clindamycin, exhibited by majority of LAB isolates is in agreement to previous studies documenting the resistant profile (Klare et al. 2007). Similar to our results, Hoque et al. (2010) showed sensitivity of few *Lactobacillus* spp. towards clindamycin. Earlier, Martin et al. (2005) also observed the resistant pattern of human milk *L. gasseri* isolates towards clindamycin. Belleti et al. (2009) reported that most of the tested *L. helveticus* and *L. delbrueckii* subsp. *lactis* strains were susceptible to erythromycin, tetracycline and clindamycin. Karapetkov et al. (2011) observed that *Lactobacillus* strains were susceptible toward chloramphenicol, erythromycin, tetracycline, and clindamycin.

Interestingly, majority of curd *Lactobacillus* isolates in our study were sensitive to co-trimoxazole and trimethoprim. On the contrary, majority of human milk lactobacilli showed high resistance towards both the drugs. Resistance to inhibitors of nucleic acid synthesis such as trimethoprim and sulphonamides (co-trimoxazole) has been reported to be an intrinsic feature of lactobacilli (Danielsen and Wind 2003). Earlier, Martin et al. (2005) also observed trimethoprim resistance in lactobacilli of human milk origin. A combination of trimethoprim and co-trimoxazole is being extensively employed against different clinical scenarios in humans since late 1960s. Owing to low cost, low toxicity, availability through both oral and intra-venous route and high bactericidal activity, it offers an attractive option, especially for developing world (Goldberg and Bishara 2012). This marked difference between curd and human milk isolates' susceptibility towards trimethoprim and co-trimoxazole may be substantiated with the difference in application and usage of mentioned drug in humans. Probably high exposure to the drugs in humans may have resulted in high resistant phenotype. Of all the tested antibiotic discs, imipenem (*N*-formimidoyl-thienamycin), an antibiotic of β-lactam family was found to be the most effective drug with clear activity against all the tested strains. Although a wide spectrum of antibiotic resistance has been recorded between different study groups; on comparative assessment, human milk isolates have been shown to have high resistance profile over curd lactobacilli. The resistance pattern of human milk and curd LAB isolates cannot be directly correlated, owing to their different ecological niche, but high resistance in human milk isolates over curd isolates may be clarified with the high usage and exposure level.

In our study, standard probiotics *Lactobacillus* strains covering common *Lactobacillus* species along with reference probiotic strains displayed resistance pattern towards different antibiotic functional groups. This along with the findings from lactobacilli from different ecological niches stresses towards developing resistance in microbial group enjoying GRAS status, and having wide application in food and pharma industry. Interestingly, Gram-positive and Gram-negative bacterial pathogens included in the study presented a weak resistance pattern over LAB strains. This marked difference may be due to the environmental niche of the selected strains, which belonging to culture collection center (MTCC, India) lack any direct exposure to the antibiotics for over decades. Hence, these strains probably by-passed the factors responsible for developing drug resistance. It is interesting to note that exposure to antibiotics is a crucial factor for resistance development; rational usage and maximum squeezing of ongoing drugs of choice may retard the developing antibiotic resistance.

Further, the isolates showing high resistance pattern, after screening for their probiotic potential may be a suitable candidate for administration during antibiotic therapy. Judicious application of such strains may expedite recovery and prevent washout of gut microflora. Few researchers support this hypothesis and advise incorporation of resistance probiotic strains in combination to antibiotic therapy achieving best antimicrobial effect (Ketema et al. 2010; Sharma and Goyal 2015). However, it is also important that the resistance should be inherent and non-transferrable. Natural antimicrobial properties of LABs may further pose a synergistic effect to antibiotic therapy in eliminating pathogenic strains (Sharma et al. 2017). Further, synergistic application of different antibiotics groups may display strong activity against resistant strains and may

prevent build-up of strong resistance in strains currently showing intermediate susceptibility. The current study may offer a basis to design/update guidelines for abrogation of rapidly escalating antibiotic resistance in dairy/food strains and also their rational application in food chain.

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**Compliance with ethical standards**

**Conflict of interest** No conflict of interest declared.

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