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Antibacterial Potentiality of Commercially Available Probiotic Lactobacilli and Curd Lactobacilli Strains, Alone and in Combination, against Human Pathogenic Bacteria

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Abstract

Background and objective: Lactobacilli are excellent antibacterial agents; however, scientific study on their combined action against human pathogenic bacteria is skimpy. The current study determines the antibacterial activity of commercially available probiotic lactobacilli and the curd lactobacilli, alone and in combination, against clinical bacterial isolates.

Methods and findings: Two curd lactobacilli (*Lactobacillus animalis* and *L. gasseri*) and two commercially available probiotic lactobacilli (*L. acidophilus* and *L. rhamnosus*) strains were tested for their antibacterial activity against the clinical bacterial isolates: *Escherichia coli* and *Klebsiella pneumoniae*, following agar-overlay method. The 'R' values (width of clear zones) due to their action against the indicator bacteria were recorded, and growth inhibitory indices (GIIs) were calculated from their combined action. The commercial lactobacilli strains, for which the 'R' values ranged 8-12 mm, from single action, had synergistic activity when used in combination against *E. coli* (GII: 0.56), while the curd lactobacilli strains having 'R' values 6.5-9.5 mm had synergistic activity against both *E. coli* and *K. pneumoniae* (GIIs: 0.65-0.74). The lactobacilli strains isolated were multidrug resistant.

Conclusion: The current findings suggest the usefulness of natural curd lactobacilli as well as the commercially available lactobacilli strains, alone or in combination, or in combination with some conventionally used antibiotics, as therapeutics against bacterial infection to humans.

Keywords: Lactobacilli; Antibiogram; Antibacterial activity; Synergy; Growth inhibitory indices; 'R' values; Human pathogenic bacteria

Introduction

The three main categories of antibacterial resistances, such as multidrug resistant (MDR: which is the acquisition of non-susceptibility to ≥ 3 antimicrobial agents of different

categories), extensively drug resistant (XDR: means bacterial non-susceptibility to a minimum of one antimicrobial agent from all except ≤ 2 antimicrobial categories) and pandrug resistant (PDR: non-susceptibility to all antimicrobial agents of all categories) create a great threat to the global public health with huge economic burden, treatment failures, and increased morbidity and mortality [1-5]. This rising in the emergence of drug-resistant pathogenic bacteria led to search the non-antibiotic treatment regimens, including the probiotic lactobacilli, as new therapies to combat the bacterial antibiotic resistance and to treat the infection [6-9].

One of the important features of probiotic lactobacilli is to achieve antagonistic activity against bacterial pathogens because of their capacity to produce lactic acid and other organic acids that lower the pH in the human intestine, and to produce H_2O_2 and bacteriocin, thereby establishing a hostile environment for the growth and survival of various human pathogenic bacteria [10,11]. Moreover, antibiotic resistance is a required probiotic property of lactic acid bacilli, for their survival in presence of antibiotics, especially for those strains that are co-administered with antibiotics [12]; however, such resistance among the probiotic strains be essentially innate and non-transferable in nature [13]. The probiotics having innate resistance to kanamycin (K), streptomycin, gentamicin (GEN), vancomycin (VA) and nalidixic acid suggest the preventive and therapeutic administration of such strains in treating bacterial infection in the cases of co-administration with antibiotics [14-16].

Jalilsood et al. [16] evaluated the ability of *Lactobacillus plantarum* to form biofilm, which provided the inhibitory effect against spoilage and pathogenic bacteria. It has been reported that the *L. fermentum* supernatant plus bovine lactoferrin (BLF) had synergistic inhibitory activity against MRSA strains [17]. The BLF in combination with *L. reuteri* cell free culture supernatant demonstrated increased antibacterial activity against pathogenic bacterial strains [18]. Uraipan and Hongpattarakere [19] suggested that the combination of lactic acid bacteria and *Bifidobacterium* probiotic strains from human sources might be promising in the development of probiotic products in order to prevent and control the microbial infection to humans. Anal et al. [20] characterized probiotic properties of *Lactobacillus acidophilus*, *Lactobacillus rhamnosus* and *Bifidobacterium bifidum* from pharmaceutical

probiotic sachets that on single action showed growth inhibitory property against gram-positive as well as gram-negative bacteria strains, such as *Staphylococcus aureus* (*S. aureus*), *Salmonella typhi* and *Escherichia coli*. Tomioka et al. [21] demonstrated the effect of ofloxacin-*L. casei* combination against *Mycobacterium fortuitum* infection in mice model. Bassyouni et al. [22] studied the effect *L. acidophilus* strains in combination with antibiotics, and found that the strains improved the antibacterial efficacy of the test antibiotics against *E. coli*, *Salmonella typhimurium*, *S. aureus* and *Shigella flexneri*. However, no report has been made on the combined antibacterial activity of two different lactobacilli strains, at least in this part of the globe, against human pathogenic bacteria. Therefore, the current study has been undertaken to procure lactobacilli strains from probiotic sachet available in the local medical shop, to isolate and identify lactobacilli from fermented curd, and to evaluate their antibacterial activity against gram-negative human pathogenic bacteria.

Methods

Isolation and characterization of lactobacilli

The lactic acid bacteria were isolated from two fresh curd samples (commercial curd and homemade curd) following the protocol described earlier [23], and were stored in MRS agar (Hi-Media, India) slants and stabs. The non-motile gram-positive non-spore forming rod-shaped bacteria, from MRS agar plates, presenting negative catalase and oxidase test results were considered as *Lactobacillus*, and were subjected for further phenotypic characterization: biochemical tests and sugar fermentation [23]. The curd lactobacilli thus procured were designated as S3 and DC16. Two lactobacilli strains (named as PBL2B and PBL2C strains) procured from commercially available sachets of probiotics were subjected for their identity confirmation following the methods adopted in identifying the curd lactobacilli, as mentioned above. The control strain used in the study included *Lactobacillus fermentum* MTCC 9748.

Antibacterial activity

Antibacterial activity of the lactobacilli procured from curd samples (S3 and DC16 strains alone and in combination) or probiotic sachet (PBL2B and PBL2C strains alone and in combination) was determined by agar overlay method [24] against the clinical isolates of *E. coli* and *Klebsiella pneumoniae*. Each of the individual *Lactobacillus* strains, of a pair as mentioned above, was spot inoculated alone or in combination onto MRS agar plates, with approximately 10^5 CFU/spot, and were incubated at 37°C for 24 h. The MRS agar plates containing the growth of lactobacilli 'in spot form' (≈ 6 mm diameter) were thereafter overlaid with Muller-Hinton agar (0.8% agar) containing a single indicator stain (*E. coli* or *K. pneumoniae*) on the individual plates and incubated at 37°C for 24 h.

Interpretation of the results

The antibacterial activity of the lactobacilli strains, alone or in combination, after 24 h incubation at 37°C, was recorded by measuring the width of clear zone (R), using the formula suggested by Carasi et al. [25] and Pisano et al. [26]:

$$R = \frac{(d_{\text{Inhib}} - d_{\text{Spot}})}{2}$$

where 'Inhib' denotes the diameter of clear zone around the 'Spot' and 'Spot' denotes the diameter of spot form growth of lactobacilli on MRS agar plate. Inhibition scores were considered as the no inhibition capacity with $R < 2$ mm; low inhibition capacity when $R = 2-5$ mm, and high inhibition capacity with $R > 6$ mm [25,26].

The growth inhibitory indices (GIIs) were calculated using the formula, according to Mandal et al. [27]:

GII =

$$\frac{\text{Clear zone obtained due to the combined action of two test lactobacilli strains}}{\text{Total of clear zones of two test strains in single action}}$$

the synergistic and antagonistic interaction between a pair of test lactobacilli strains were defined with GIIs > 0.5 and < 0.5 , respectively.

Antibiogram of lactobacilli

Susceptibility to 12 antibiotics, such as amikacin (AK), ciprofloxacin (CIP), cefoxitin (CX), cefotaxime (CTX), imipenem (IMP), K, trimethoprim (TR), tetracycline (TE), amoxycylav (AMC), GEN, ampicillin (AMP), vancomycin (VA) (Hi-Media, India), for the lactobacilli strains from curd and probiotic sachets was determined by disc diffusion method [28]. Single colonies of the test lactobacilli strains were inoculated into MRS broth and incubated at 37°C for 24 h. A sterilized cotton-swab with test bacterial MRS broth culture was used to spread bacteria on the surface of MRS agar plate, and the antibiotic discs were placed on the surface of the agar plates. After incubation for 24 h at 37°C, the zone diameter of inhibition (ZDI) obtained around the antibiotic disc were recorded, and isolates were categorized as sensitive (ZDI; ≥ 21 mm), intermediate (ZDI; 16-20 mm), or resistant (ZDI; ≤ 15 mm) [29,30].

Results

The curd isolates of lactobacilli, which were designated as S3 and DC16, were identified, respectively, as *Lactobacillus gasseri* and *Lactobacillus animalis* (Figure 1). The lactobacilli strains, PBL2B and PBL2C, procured from the commercial probiotic sachet were confirmed with phenotypic characterization as *Lactobacillus acidophilus* and *Lactobacillus rhamnosus*, respectively. The sugar fermentation test results of the lactobacilli strains are represented in Table 1.

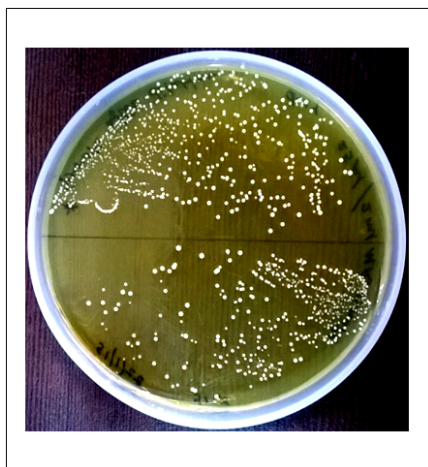


Figure 1 Pure culture of *L. gasseri* S3 (upper portion) and *L. animalis* DC16 (lower portion) strains on MRS agar plate.

The curd lactobacilli as well as the probiotic lactobacilli tested had excellent antibacterial activities against *E. coli* and *K. pneumoniae* clinical isolates (**Figures 2** and **3**). The antibacterial activity, in terms of the 'R' values (i.e. the diameter of clear zones) obtained due to the action of the test lactobacilli strains, alone or in combination, is represented in **Figure 4**. The R values for curd lactobacilli ranged 6.5-9.5 mm against the indicator bacteria, and both the probiotic lactobacilli from sachet had R value of 12 mm for *E. coli*, while the values ranged 8-10.5 mm for *K. pneumoniae*, when tested alone. In combination, the two lactobacilli strains: *L. gasseri* S3 (obtained from commercial curd) and *L. animalis* DC16 (procured from homemade curd prepared with branded milk), had increased activity against *E. coli* and *K. pneumoniae* with R values 10 mm and 10.5 mm, respectively; the PBL2B-PBL2C combined action had increased R value against *E. coli* (13.5 mm), only. For *E. coli* and *K. pneumoniae*, the GIs from the PBL2B-PBL2C combination were 0.56 and 0.48, respectively, and from the S3-DC16 combination, 0.65 and 0.74, respectively.

Table 1 Sugar fermentation test results for curd and sachet isolates of lactobacilli.

Sugar	Curd lactobacilli		Sachet lactobacilli	
	DC16	S3	PBL2B	PBL2C
AD	-	-	-	-
AR	+	-	-	+
CE	+	+	+	+
DE	+	+	+	+
LA	+	+	-	-
MA	+	+	+	+
MB	+	w	-	-
MN	+	+	-	-
MO	+	+	+	+
RF	+	+	-	-
RH	+(gas)	w	W	+
SB	+	+	-	+
SU	+	+	W	+
TE	+	+	+	+
XY	w	w	+	+
Identity	<i>L. animalis</i>	<i>L. gasseri</i>	<i>L. acidophilus</i>	<i>L. rhamnosus</i>

AD: adinitol; AR: arabinose; CE: cellobiose; DE: dextrose; LA: lactose; MA: mannose; MN: manitol; MO: Mannose; MB: melibiose; RF: raffinose; RH: rhamnose; SB: sorbitol; SU: sucrose; TE: trehalose; XY: xylose; +: positive; -: negative; w: weakly positive.

The antibiogram test results for the lactobacilli are shown in **Figure 5**; the *L. animalis* DC16 strain showed resistance to CX and VA; moderate susceptibility to AMC, CIP and K, and sensitivity to AK, AMP, CTX, GEN, TE, TR and IMP. The *L. gasseri* S3 strain was resistant to CX, CIP, K, VA and TR, moderately susceptible to GEN and AK, and sensitive to AMP, AMC, CTX, TE

and IMP. The *L. acidophilus* PBL2B strain was resistant to AK, CX, CIP, GEN, K, VA and TR, and sensitive to AMP, AMC, CTX, TE and IMP, while the *L. rhamnosus* PBL2C strain had resistivity to AK, CX, CIP, GEN, K, VA and TR, moderately susceptibility to AMC and TE, and sensitivity to AMP, CTX and IMP.

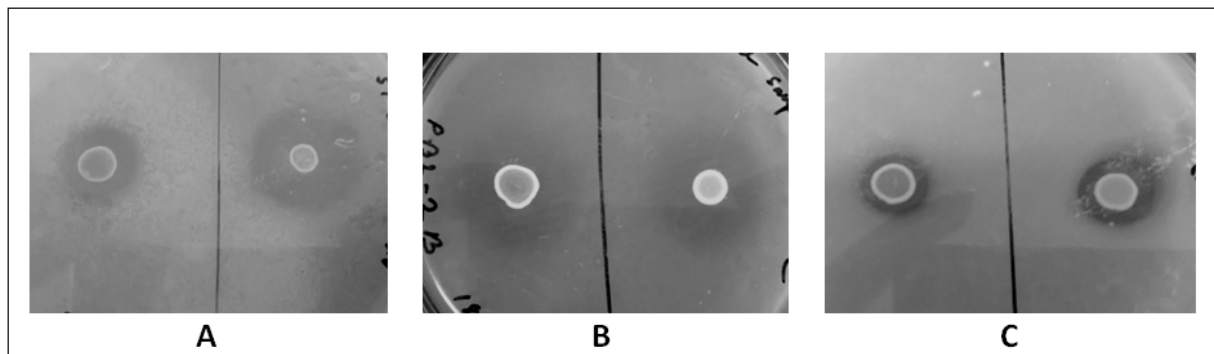


Figure 2 Antibacterial activity of lactobacilli: (A) PBL2B strain (spot on the right side) PBL2C strain (spot on the left side) against *K. pneumoniae*; (B) PBL2B strain (spot on the left side) PBL2C strain (spot on the right side) against *E. coli*; (C) S3 strain (spot on the left side) DC16 strain (spot on the right side) against *K. pneumoniae*.

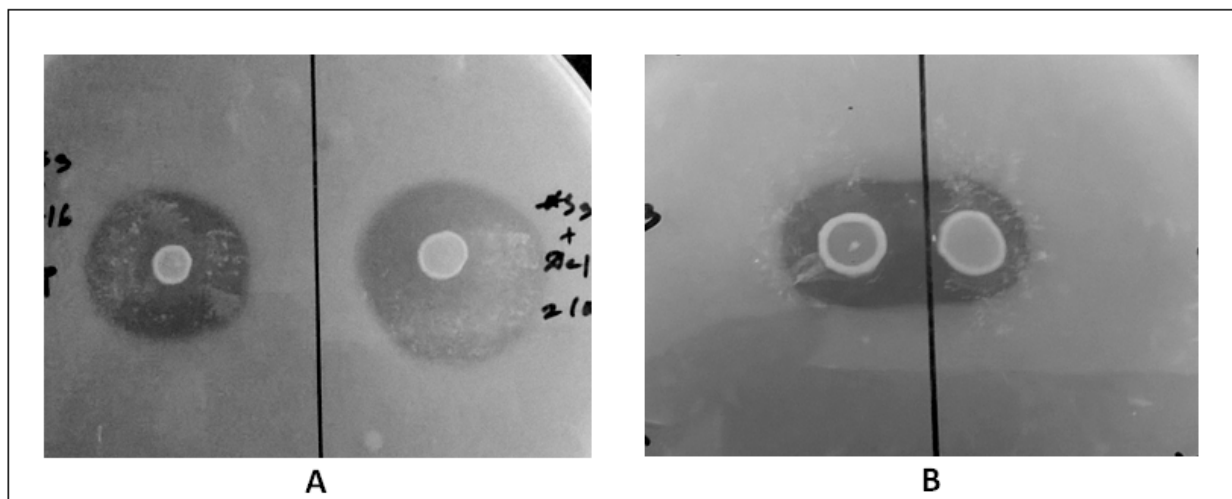


Figure 3 Combined antibacterial activity of curd lactobacilli S3 and DC16 strains against *K. pneumoniae*: (A) combined spot of the two strains S3 and DC16, one loop-full of culture (10^5 CFU) on the left spot and two loop-full of culture (2×10^5 CFU) on the right spot; the increased size of zone of inhibitions against the indicator strain is the indicative of synergism between two test lactobacilli (B) Double spot approximation of the two strains, S3 (left side) and DC16 (right side), the characteristic fused zone of inhibition is indicative of synergistic action between the two lactobacilli strains.

Discussion

In developing countries like India, *E. coli* isolates causing community based infection had elevated resistance to multiple antibiotics of several kinds, and during 2008-2013, antibiotic resistance of *E. coli* to fluoroquinolones and extended spectrum β -lactams increased from 70%-85%, while the situation for carbapenem resistance of *K. pneumoniae* was with an increment from 2% (in the year 2002) to 52% (in the year 2009), as has been documented by Laxminarayan and Chaudhury [31]; the circumstances exert an impact on the outlay and the health penalty [4]. Moreover, the life-threatening infection due to multiple antibiotic resistant bacteria is hard to treat because of the resistance to commonly as well as conventionally used antibiotics; therefore, it is essential to formulate alternative therapeutic protocol with non-antibiotic agents against the bacterial

infection. Combined therapy with a mixture of classic antibiotics [32,33], or classic antibiotics and non-antibiotic antibacterial agents [27,34], has been recommended as an alternative modality for treating pathogenic infections. Zou et al. [35] reported the use of probiotics in conjunction with antibiotic in the treatment of infection with *Helicobacter pylori*.

Herein, we have evaluated the anti-bacterial potential of the combination of lactobacilli strains from varied sources, or from the same source against *E. coli* and *K. pneumoniae*. Davoodabadi et al. [9] reported the inhibitory activity of *Lactobacillus* strains of human origin and suggested their usefulness as probiotics in controlling intestinal infection with *E. coli*. The *Lactobacillus* species are usually chosen as the probiotic microorganisms because of their tolerance to low pH and bile [23], inhibitory effect against pathogenic microbes (antagonistic activity), and innate resistance to antibiotics.

Hence, lactobacilli are one such microorganisms of choice to use as the potential remedial agents, alone or in combination with one another, procured from different sources, or in combination with antibiotics; because of the therapeutic potentiality of probiotic lactobacilli against MDR bacterial infection [36], and treatment with probiotic-antibiotic combination against bacterial infection [37,38].

The antimicrobial combinations (*L. casei* and *L. rhamnosus* with AK and GEN) demonstrated synergistic actions against *Pseudomonas aeruginosa*, as has been reported by Aminnezhad et al. [38]. The antibiotic susceptibility test demonstrated the resistance to ampicillin, CIP and ceftriaxone for *L. plantarum* strain, the cell-free extract of which potentially augmented the efficacy of the test antibiotics against *Salmonella infection* [8]. The antimicrobial combination has been in utilization as an efficient therapeutic approach by using varied mechanisms of action; the probiotic microorganisms produce inhibitory substances, such as bacteriocin, H₂O₂ and organic acids, like lactic and acetic acid [11,39], in the human intestine on consumption, on the body surface on application, or in the culture medium, where they live/colonize, and inhibit the growth of pathogenic bacteria.

In this communication, which as per the knowledge and belief of the authors stands first to undertake the study of its kind at least in our part of the globe, the probiotic lactobacilli were excellent to inhibit the growth of both *E. coli* and *K. pneumoniae* (R value: 8-12 mm), and in combination such strains had synergistic effect against *E. coli* (GII: 0.56); for *K. pneumoniae* the GII was recorded as 0.48. The curd lactobacilli alone had R value of 6.5-9.5 mm, and in combination the strains showed synergism against both the indicator bacterial pathogens (GII: 0.65-0.74); moreover, S3-DC16 combination effectively had synergistic effect by modified disc approximation test (Figure 3). Such differences in the antibacterial activity of the lactobacilli are due to the strain and their source variation, difference in the target bacterial strains, and the variation in the production of antimicrobials by them, as well, which needs further study. Thus, although the probiotics, which are in current usage, are generally regarded as safe, prudence in the selection of probiotic strain(s) for patients has been considered necessary as well as urgent, and full concern for 'risk-benefit' ratio before prescribing is suggested, in compliance with the 'fit for human consumption' protocols [40].

The current research underlines the necessity of antibiotic susceptibility testing for the newly isolated lactobacilli projected for probiotic usage. The isolated curd lactobacilli (*L. animalis* DC16 and *L. gasseri* S3) strains had resistance to one or more of the antibiotics VA, CIP and K, while the commercially available lactobacilli (*L. acidophilus* PBL2B and *L. rhamnosus* PBL2C) strains showed resistance to AK, CIP, GEN, K, VA. As has been reported by Bassyouni et al. [22], the *L. acidophilus*-CIP combined therapy against infective gastroenteritis was effective in terms of decreasing the CIP dose and hence the CIP adverse effect on the balance of microbial flora in human intestine. Thus, the current communication suggests the usefulness of natural curd lactobacilli as well as the commercially available probiotic lactobacilli strains, alone or in combination, or in combination with some conventionally used antibiotics, as therapeutics against bacterial infection to humans. In addition, regular consumption of curd, both homemade and commercial, is required to get probiotic effect among the Indian population, since curd is an excellent source of probiotic lactobacilli;

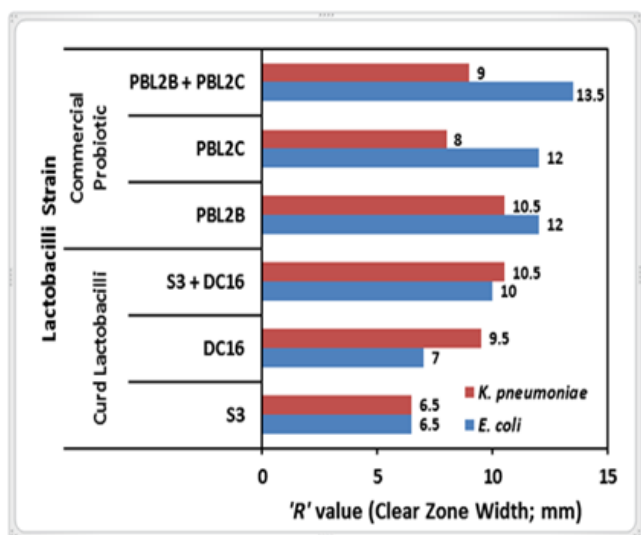


Figure 4 Antibacterial activity of curd lactobacilli (*L. gasseri* S3 and *L. animalis* DC16) and commercially available probiotic lactobacilli (*L. acidophilus* PBL2B and *L. rhamnosus* PBL2C) strains against human pathogenic bacteria.

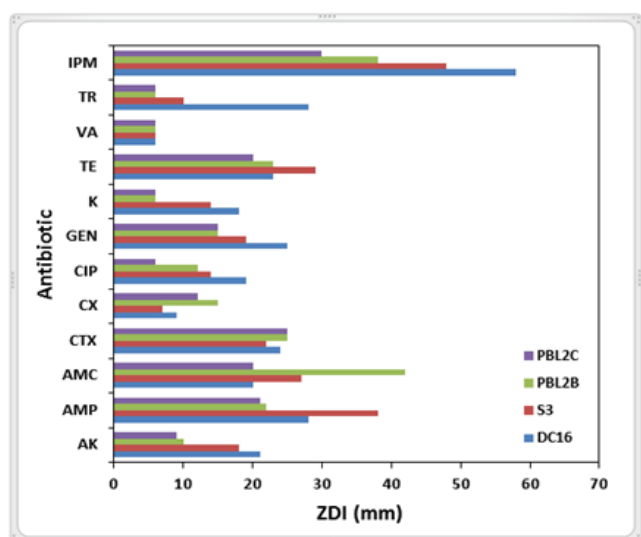


Figure 5 Antibiotic susceptibility test results for lactobacilli strains; PBL2B: *L. acidophilus*, PBL2C: *L. rhamnosus*, S3: *L. gasseri*, DC16: *L. animalis*. AK: amikacin, CIP: ciprofloxacin, CX: cefoxitin, CTX: cefotaxime, IPM: imipenem, K: kanamycin, TR: trimethoprim, TE: tetracycline, AMC: amoxycylav, GEN: gentamycin, AMP: ampicillin, VA: vancomycin.

however, further studies are warranted with local curd samples regarding the current issues.

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Conflict of interest

None

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