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Antimicrobial Activity of *Lactobacilli* against *Staphylococcus aureus* MRSA Strains Isolated from Mastitis in Dairy Animals

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A B S T R A C T

Methicillin resistant *Staphylococcus aureus* (MRSA) strains being a major pathogen associated with mastitis of dairy animals, the study was proposed for investigating antimicrobial activity of lactobacilli of dairy animals having activity against the MRSA isolates of subclinical mastitis. Twenty two *Lactobacilli* were isolated from milk of healthy animal and screened for inherent antimicrobial activity with agar spot technique against indicator strain *L. acidophilus*, out of which eleven isolates exhibited strong inhibitory activity. The cell free filtrates of these isolates were then tested *in vitro* against MRSA by employing agar well diffusion and agar disc diffusion tests. Zone size indicative antimicrobial activity against MRSA possessing a very strong (15-18 mm) to moderately strong (10-14 mm) efficacy was observed in *L. plantarum* strains belonging to buffaloes (B5 and B8) and goats (G2 and G6), followed by sheep (S3). Moderate inhibitory activity (6-9 mm) was also observed for *L. paraplantarum* strain from cow (C2).

Introduction

Bovine mastitis is an economically most important disease of dairy industry which is thriving on a very marginal profit in India. Especially subclinical form of disease in the dairy animals is very widely prevalent and is responsible for heavy losses due to reduced milk production and also has public health significance (Esslemont *et al.,* 2000). The major pathogen associated with bovine mastitis is *S. aureus*. Non judicious use of the over the counter antimicrobial in treatment of subclinical mastitis (SCM) has led to a risk of emergence of antimicrobial resistant (AMR) phenotypes. Emergence of increasing AMR in coagulase positive Staphylococci (COPS) associated with mastitic cows against several antimicrobial agents has been reported earlier (Asfour and Darwish, 2011; Chandrasekaran *et al.,* 2014) and emerging multiple AMR especially for methicillin and vancomycin group is known worldwide. Lactic acid bacteria (LAB) having property to produce lactic acid from milk sugars, constitute the predominant micro flora of healthy animal milk. LAB are well known for their use as probiotics and prebiotics in human and veterinary medicine and proved to be producing antibacterial substances like bacteriocin that replace them inhibiting the growth of pathogenic bacteria (Eid *et al.,* 2016). CSF of LAB contains highly specific antibacterial proteins presenting a broad range

of antimicrobial activity against pathogens resulting from production of organic acids, hydrogen peroxide, diacetyl, inhibitory enzymes as well as the bacteriocin (Mohankumar and Murugalatha, 2011).

Materials and Methods

Staphylococcus aureus from subclinical mastitis

Total 100 dairy animals comprised of cow (n=40) and buffaloes (n=60) belonging to geographical area of Pune and Satara districts of Western Maharashtra, India, presented for SCM were screened using California mastitis test (CMT). Each of CMT positive (60) milk samples was inoculated in BHI broth and incubated at 37°C for 24 hours. The cultures were streaked on nutrient agar and incubated at 37°C for 24 hours. Smears of identical colonies were stained and only gram positive cocci found in clusters were further processed for selective growth of *S. aureus* strains on mannitol salt agar (MSA) and incubated at 37°C overnight. Characteristic yellowish colonies on MSA were considered as presumptive *S. aureus* and maintained in pure culture. Phenotypic typing of *S. aureus* was performed by staining, cultural characteristics and biochemical tests (Quinn *et al.*, 2002).

Antibiogram profile of *Staphylococcus aureus*

Antibiotic sensitivity profiles of *S. aureus* isolates as well as other organisms were studied against nine different antibiotics. Antibiotics were randomly selected preferably considering their use in field for treatment of mastitis by clinicians in our study area and performed by the disc diffusion technique Bauer *et al.*, (1966) with minor modifications (CLSI, 2014). The commercially available antibiotic discs used were methicillin (10mcg), vancomycin (10mcg), ciprofloxacin (5mcg), enrofloxacin (10mcg), gentamycin (10mcg), chloramphenicol (30mcg), amoxicillin/clavonic acid (20/10mcg), ceftriaxone/tazobactum (30/10mcg) and penicillin G (10unit) were procured from HiMedia Pvt Ltd., India.

LAB from raw milk samples

Raw unpasteurized milk samples (n=10 each) from apparently healthy CMT negative cow, buffalo, goat and sheep were collected from the animals belonging to study area, aseptically. Methodology for harvesting of LAB was performed as described by Mohankumar and Murugalatha (2011). Milk samples were serially diluted in peptone medium and incubated at 23°C for 30 min before plating and diluted samples were plated onto De Mann Rogosa Sharpe (MRS) medium plates which were incubated at 37°C for 48-72 hours, for isolation of LAB. Characteristic small (2-3 mm) colonies having pure white colour and with entire margin were picked up from each plate and transferred to MRS broth. Purity of the isolates was checked by streaking several times and sub culturing on fresh MRS agar, as well as MRS broth. The isolates were designated as C (cow), B (buffalo), G (goat) and S (Sheep). Identification of LAB was attempted upto species level according to their morphological, cultural, physiological and biochemical characteristics like gram reaction, production of catalase, carbohydrate fermentation, growth at 15°C and 45°C (Bergey's Manual of Systemic Bacteriology, 1986)

MALDI-TOF MS analysis of LAB

The bacterial DNA of LAB was extracted according to the procedure of Behiry *et al.*, (2014). 1µl supernatant was taken in sterile pipette and directly applied onto each spot of ground steel MSP target plate of MALDI-TOF and were air dried. Matrix (1µl) was overlaid

on these spots, air-dried and plates were introduced into the mass spectrometer for analysis. Results were displayed as a series of lines (spectrum) corresponding to different fragment of protein. Measurements were performed on a Microflex LT MALDI-TOF/TOF Mass Spectrometer equipped with a smart beam laser. Spectra were recorded in the linear, positive mode at a frequency of 200 Hz within a mass range from 2,000 to 20,000 Da. Once a spectrum were generated and captured by the software, the whole identification process was performed automatically, without any user intervention. Each peak list generated was matched directly against reference libraries (4,623 species) using the integrated pattern-matching algorithm of the Biotype OC 3.1.66 software. The peak intensity ≥ 1.5 was considered at level of genus and probable species

Detection of antimicrobial activity of LAB by Agar spot test

Pure colonies of LAB were subcultured in 5 ml of MRS broth at 30°C for 16 hours. Aliquots (2µl) of the culture were spotted onto agar plates containing 10ml of MRS medium. After 18 hours at 30°C, the plates were overlaid with the 5ml of the appropriate soft agar (1%) inoculated with the cell suspension of the indicator strain of *L. acidophilus* ATCC 4356 (10^5 CFU/ml). The plates were incubated at 37°C for 24-48 hours, depending on the growth of the indicator strain and the diameters of zones of inhibition were measured. Zone size wider than 2 mm was scored positive.

Antimicrobial efficacy of LAB against MRSA

Each LAB strain showing efficacy in Agar spot assay were selected as potential candidates and their CFS were prepared for further evaluation as per Norroozi and Mirzaii (2004). Isolates were cultivated in MRS broth for 24-48 hours at 37°C. CFS was obtained by centrifuging the culture at 10000 rpm for 10 min followed by filtration of the supernatant through a 0.2 µm pore size filter.

Agar well diffusion test

Efficacy of LAB was investigated by agar well diffusion method as per Mohankumar and Murugalatha (2011). An overnight incubated fresh culture of MRSA was prepared. These bacteria (10 cfu/ml^{-1}) were inoculated by streaking the swab over the entire MHA surface. Around 6mm diameter wells were punched on pre-inoculated agar plates and 100µl of each CFS of LAB was placed into each well after neutralization with NaOH. The plates were incubated for 24 hours at 37°C and inhibition of growth was examined by clear zone surrounding each well.

Agar disc diffusion test

Each of the bacterial isolates were subjected to *in-vitro* antibacterial sensitivity testing by disc diffusion technique with different concentrations like 10µl, 20µ, and 30 µl of supernatant as per the method of Bauer *et al.,* (1996). In this method sterile paper disc (6mm) were placed over MHA agar plates seeded with MRSA, and 30µl of cell free supernatant was added to the sterile paper disc. Plates were incubated at 37°C for 48 hrs. After incubation antimicrobial activity was measured in diameter (mm) around the paper disc as per the method of Tajehmiri *et al.,* (2014).

Results and Discussion

Detection of bovine subclinical mastitis with cultural method

Total 60 CMT positive (> 1 CMT) milk samples were collected aseptically that were

processed for bacterial culture as described by the National Mastitis Council (1999). The results of bacterial isolation, in the milk samples from Pune region 09/20 (45.00%) cow and 14/25 (56.00 %) buffaloes were found positive. While from Satara region 07/20 (35.00%) cow and 27/35 (77.14%) buffaloes yielded positive results. Overall 16/40 (4000%) cow and 41/60 (68.33%) buffaloes tested culturally positive. Thus, out of total 100 animals tested 60 (60.00%) were CMT positive and 57 (57.00%) were culturally found positive for mastitis thus, CMT test remained 95.00 percent in agreement with cultural method. Similar inferences were drawn by Sharma *et al.,* (2007) and Saidi *et al.,* (2013) in their studies on subclinical mastitis in bovines.

Phenotypic characterization of *Staphylococcus aureus* from bovine subclinical mastitis

Amongst all culturally positive samples 35/57 (61.40%) isolates were phenotypically identified as *Staphylococcus* spp., amongst which 29/57 (50.87%) were typed as *S. aureus* on MSA and remaining 06/57 (10.53%) were minor gram positive cocci. Our findings of staphylococci, especially *S.aureus* as major pathogen in SCM were in close accordance with Hamed *et al.,* (2014).

Antibiogram profile of organisms associated with SCM

In addition to 35 staphylococci the other 22/57 isolates included other 09 (15.79%) non coccoid gram positive and 13 (22.81%) gram negative bacteria. All these isolates were processed for their antibiogram profiling and the overall results of antimicrobial sensitivity profile as depicted indicated gentamicin as most effective drug against 25/57 (43.85%) isolates, followed by ciprofloxacin against 22/57 (38.59%), ceftriaxone + tazobactum against 19/57 (33.33%), chloramphenicol against 18/57 (31.57%), enrofloxacin against 16/57 (28.07), methicillin against 11/57 (19.29%), amoxicillin + clavulanicacid against 09/57 (15.78%), vancomycin against 07/57 (12.28%) and penicillin against none (zero %) of these isolates. Profile exhibited by *S. aureus* indicated highest resistance towards methicillin in 26/29 (89.60%) its isolates followed by enrofloxacin in 20/29 (68.96%), vancomycin and gentamicin in 18/29 (62.06%) each, chloramphenicol in 17/29 (58.60%), ceftriaxone + tazobactum and amoxicillin + clavulanic acid in 15/29 each (51.72%) and ciprofloxacin resistance in 08/29 (27.58%) isolates, respectively. Total 26/29 (75.80 %).

In close agreement with Gentilini *et al.,* (2000) and Jian ping *et al.,* (2009), very high percentage of *S. aureus* were identified as MRSA which also exhibited a very high prevalence of multiple drug resistant (MDR) strains. In all 46.00 percent *S. aureus* exhibited multiple antibiotics resistance for two to five different antibiotics used.

Phenotypic characterization of LAB isolated from milk samples

Total 40 raw milk samples were processed for isolation of LAB, 22 had resulted positive. From cow milk samples, 04/10 (40.00%) LAB were isolated while from buffaloes milk 09/10 (90.00%) LAB were grown. The milk samples of sheep could generate 03/10 (30.00%) LAB isolates while goats generated 06/10 (60.00%). This outcome was in concurrence with results published by Nair and Surendaran (2005). All LAB (n=22) were attempted for identification by growth and morphological characteristics and biochemical tests as per Bergey's Manual of Determinative bacteriology (Table 1). Results of biochemical tests indicated these isolates as citrate positive and negative in MR-VP, indol, catalase and citrate.

Table.1 Results of sugar fermentation tests performed for identification of lactobacilli isolated from raw milk

Sr no	*Lactobacillus* Spp.	Growth of MRS Agar	Methyl red	VP	Indol	Catalase	Oxidase	Citrate	Arabinose	Cellobiose	Mannitol	Raffinose	Lactose	Sallicin	Melibiose	Sorbitol	Trehalose	Sucrose	Species Identified
1	C1	+	-	-	-	-	-	+	-	+	+	+	-	+	+	-	+	+	*L. plantarum*
2	C2	+	-	-	-	-	-	+	-	+	+	+	-	+	+	-	+	+	*L. plantarum*
3	C8	+	-	-	-	-	-	+	-	+	+	+	-	+	+	-	+	+	*L. plantarum*
4	C10	+	-	-	-	-	-	+	-	+	+	+	-	+	+	-	+	+	*L.plantarum*
5	B1	+	-	-	-	-	-	+	+	+	+	-	+	+	-	-	+	+	*L.plantarum*
6	B2	+	-	-	-	-	-	+	-	+	+	+	-	+	+	-	+	+	*L.plantarum*
7	B3	+	-	-	-	-	-	-	+	-	-	+	d	-	+	d	+	-	*Pediococcus* Spp
8	B4	+	-	-	-	-	-	+	+	+	+	-	+	+	-	-	+	+	*L.plantarum*
9	B5	+	-	-	-	-	-	+	+	-	-	+	d	-	+	d	+	-	*Pediococcus* Spp
10	B6	+	-	-	-	-	-	+	d	+	+	+	-	+	+	-	+	d	Other *Lactobacillus* spp
11	B8	+	-	-	-	-	-	-	+	-	-	+	d	-	+	d	+	-	*Pediococcus* Spp
12	B9	+																	Other *Lactobacillus* spp
13	B10	+	-	-	-	-	-	+	d	+	+	+	-	+	+	-	+	d	Other *Lactobacillus* spp.

14	**G1**	**+**	**-**	**-**	**-**	**-**	**-**	**+**	**-**	**+**	**+**	**+**	**-**	**+**	**+**	**-**	**+**	**+**	***L.plantarum***
15	**G2**	**+**	**-**	**-**	**-**	**-**	**-**	+	**-**	**+**	**+**	**+**	**-**	**+**	**+**	**-**	**+**	**+**	*L.plantarum*
16	**G3**	**+**	**-**	**-**	**-**	**-**	**-**	+	**-**	**+**	**+**	**+**	**-**	**+**	**+**	**-**	**+**	**+**	*L.plantarum*
17	**G5**	**+**	**-**	**-**	**-**	**-**	**-**	+	**-**	**+**	**+**	**+**	**-**	**+**	**+**	**-**	**+**	**+**	*L.plantarum*
18	**G6**	**+**	**-**	**-**	**-**	**-**	**-**	+	**d**	**+**	**+**	**+**	**-**	**+**	**+**	**-**	**+**	**d**	Other *Lactobacillus* spp
19	**S1**	**+**	**-**	**-**	**-**	**-**	**-**	**-**	**+**	**-**	**-**	**+**	**d**	**-**	**+**	**d**	**+**	**-**	*Pediococcus* Spp
20	**S3**	**+**	**-**	**-**	**-**	**-**	**-**	+	**d**	**+**	**+**	**+**	**-**	**+**	**+**	**-**	**+**	**d**	Other *Lactobacillus* spp
21	**S9**	**+**	**-**	**-**	**-**	**-**	**-**	**+**	**-**	**+**	**+**	**+**	**-**	**+**	**+**	**-**	**+**	**+**	*L.plantarum*
22	**S10**	**+**	**-**	**-**	**-**	**-**	**-**	+	**-**	**+**	**+**	**+**	**-**	**+**	**+**	**-**	**+**	**+**	*L.plantarum*
13	**B10**	**+**	**-**	**-**	**-**	**-**	**-**	**+**	**d**	**+**	**+**	**+**	**-**	**+**	**+**	**-**	**+**	**d**	Other *Lactobacillus* spp.
14	**G1**	**+**	**-**	**-**	**-**	**-**	**-**	+	**-**	**+**	**+**	**+**	**-**	**+**	**+**	**-**	**+**	**+**	*L.plantarum*
15	**G2**	**+**	**-**	**-**	**-**	**-**	**-**	+	**-**	**+**	**+**	**+**	**-**	**+**	**+**	**-**	**+**	**+**	*L.plantarum*
16	**G3**	**+**	**-**	**-**	**-**	**-**	**-**	+	**-**	**+**	**+**	**+**	**-**	**+**	**+**	**-**	**+**	**+**	*L.plantarum*
17	**G5**	**+**	**-**	**-**	**-**	**-**	**-**	+	**-**	**+**	**+**	**+**	**-**	**+**	**+**	**-**	**+**	**+**	*L.plantarum*
18	**G6**	**+**	**-**	**-**	**-**	**-**	**-**	+	**d**	**+**	**+**	**+**	**-**	**+**	**+**	**-**	**+**	**d**	Other *Lactobacillus* spp
19	**S1**	**+**	**-**	**-**	**-**	**-**	**-**	**-**	**+**	**-**	**-**	**+**	**d**	**-**	**+**	**d**	**+**	**-**	*Pediococcus* Spp
20	**S3**	**+**	**-**	**-**	**-**	**-**	**-**	+	**d**	**+**	**+**	**+**	**-**	**+**	**+**	**-**	**+**	**d**	Other *Lactobacillus* spp
21	**S9**	**+**	**-**	**-**	**-**	**-**	**-**	+	**-**	**+**	**+**	**+**	**-**	**+**	**+**	**-**	**+**	**+**	*L.plantarum*
22	**S10**	**+**	**-**	**-**	**-**	**-**	**-**	+	**-**	**+**	**+**	**+**	**-**	**+**	**+**	**-**	**+**	**+**	*L.plantarum*

Note: d- Doubtful

Table.2 Detection of LAB isolated from raw milk samples by MALDI-TOF MS

Sr No.	Score Value	Detected species	No. of species	Percentage
1	2.301-2.129	*L. plantarum*	10	45.45
2	2.195-2.032	*Pediococcus acidilactici*	05	22.72
3	2.227-2.024	*L. plantarum ssp plantarum*	02	09.09
4	2.123-1.848	*L. paraplantarum*	02	09.09
5	2.207-1.91	*L. plantarum ssp argentoratensis*	02	09.09
6	0.00-1.142	Unidentified	01	04.54
Total			**22**	**100**

Table.3 Selective potent *Lactobacillus* isolates having highest activity in Agar spot test

Sr no.	Source	Total no of positive isolates	Isolate with potency	Potent Isolate (code)	Zone of inhibition (mm)
1	Cow	04	01	*L. paraplantarum* (C2)	16
2	Buffalo	09	05	*L. plantarum ssp argentoratensis* (B1)	13-14
				L. plantarum (B5)	15
				L. plantarum ssp argentoratensis (B6)	14
				L. plantarum (B8)	**18**
				L. paraplantarum (B9)	**17**
3	Goat	05	03	*L.plantarum* (G1)	14-15
				L.plantarum (G2)	15
				L. plantarum (G6)	**18**
4	Sheep	04	02	*L. plantarum* (S3)	09
				L. plantarum (S10)	11
	TOTAL	22	11		

Table.4 Zones of Inhibition of individual CFS of LAB against COPS MRSA by well diffusion method

CFS *Lactobacillus* spp. / MRSA	Mean zone of Inhibition (mm diameter)										
	Lparaplantarum	*L.plantarum spp argentoratesis*	*L.plantarum*	*L.plantarum spp argentoratesis*	*L.plantarum*	*L.paraplantarum*	*L.plantarum*	*L.plantarum*	*L.plantarum*	*L.plantarum*	*L.plantarum*
S1	04	02	10	07	10	02	04	**18**	13	02	12
S3	09	07	02	11	**17**	04	02	11	07	06	10
S4	02	09	14	09	14	08	06	15	**18**	09	04
S5	10	11	11	10	02	04	02	08	10	02	04
S6	08	08	10	04	19	09	08	18	13	09	02
S9	02	05	08	07	11	02	04	12	02	13	07
S10	07	02	14	05	08	04	07	02	10	11	06
S11	10	04	11	02	15	07	09	12	11	06	04
S13	05	09	05	09	04	02	02	17	10	11	14
S14	09	07	10	11	12	02	10	09	17	02	06
S16	02	06	16	04	12	07	11	18	06	04	04
S17	10	12	11	02	17	04	02	12	14	07	06
S18	04	02	02	08	15	02	06	09	12	11	04
S21	02	12	18	07	10	02	14	17	06	08	11
S23	12	02	11	09	16	04	02	04	15	09	12
S26	07	10	16	11	02	02	07	15	06	07	08
S27	09	07	10	02	17	07	05	12	10	04	06
S28	02	05	14	05	11	06	10	08	02	08	07
S29	02	04	15	10	17	12	04	15	14	11	02
S30	05	02	09	05	02	04	12	02	17	11	04
S33	07	10	12	02	16	08	02	11	04	02	11
S35	02	11	10	05	14	02	09	08	12	09	10
Mean± SE	**5.91 ±0.72^{bc}**	**6.68± 0.74^{bc}**	**10.86 ±0.88^{a}**	**6.59± 0.67^{bc}**	**11.6 ±1.13^{a}**	**4.73 ±0.61^{c}**	**6.27 ±0.79^{bc}**	**11.5 ±1.05^{a}**	**10.4 ±1.0^{a}**	**7.363 ±0.73^{b}**	**7±0.747^{bc}**
Level of significance	**	**	*	**	*	**	**	*	*	**	**

Note: a, b, c Means with different superscripts in a column differ significantly *P < 0.05; ** P < 0.01

Table.5 Mean Inhibition zone of lactobacilli against *S. aureus* by agar disc diffusion method

CSF of *Lactobacillus* species / MRSA	Inhibition zone diameter (mm)										
	Lparaplantarum	*L.plantarum spp argentoratesis*	*L.plantarum*	*L.plantarum spp argentoratesis*	*L.plantarum*	*L.paraplantarum*	*L.plantarum*	*L.plantarum*	*L.plantarum*	*L.plantarum*	*L.plantarum*
S1	12	4	11	14	14	02	02	02	11	02	10
S3	07	09	04	09	17	02	04	07	06	16	11
S4	12	07	11	08	12	07	04	02	08	09	02
S5	09	07	15	05	04	02	02	08	09	12	02
S6	07	5	19	12	17	09	07	06	11	09	02
S9	11	07	07	05	19	02	04	09	02	11	07
S10	07	02	12	15	07	02	05	02	09	09	07
S11	07	02	19	12	12	05	07	11	09	17	02
S13	15	07	13	09	12	02	04	07	09	09	11
S14	16	05	17	09	11	02	07	07	05	12	05
S16	02	05	09	14	14	05	09	07	07	02	02
S17	16	09	11	12	02	05	02	04	11	17	05
S18	02	04	12	08	15	02	05	07	11	09	02
S21	12	09	15	12	19	04	11	07	03	17	07
S23	09	02	09	07	15	02	02	04	04	17	07
S26	15	09	12	09	02	02	05	02	07	07	05
S27	12	05	18	12	12	07	05	02	09	12	05
S28	02	03	15	12	19	05	09	07	02	12	07
S29	03	02	14	07	17	11	04	02	02	11	02
S30	12	02	07	12	12	02	11	02	05	09	02
S33	15	09	12	02	15	09	02	09	02	12	09
S35	02	02	02	02	17	02	07	07	11	09	09
Mean±SE	**9.32 ±1.04^{b}**	**5.27± 0.57^{bc}**	**12± 0. 96^{a}**	**9.40 ±0.78^{b}**	**12± 0.96^{a}**	**4.13 ±0.60^{c}**	**5.36 ±0.60^{c}**	**5.5 ±0.61^{bc}**	**6.95±0. 71^{bc}**	**10.9 ±0.91^{a}**	**5.5 ±0.68^{bc}**
Level of significance	**	**	*	**	*	**	*	**	**	*	**

Note: a, b, c Means with different superscripts in a column differ significantly *P < 0.05;

Plate.1 Zone of inhibition of *Lactobacillus* spp. in Agar spot assay

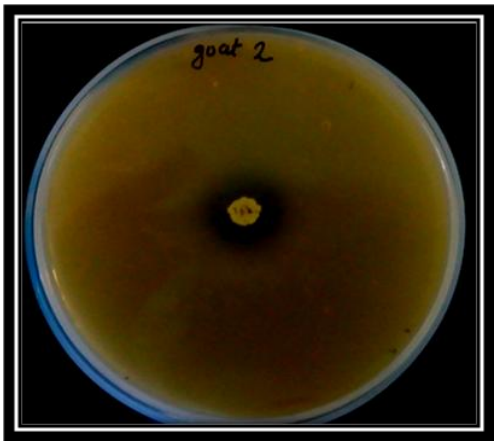


Plate.2 Zones of inhibition of LAB in Agar well diffusion test

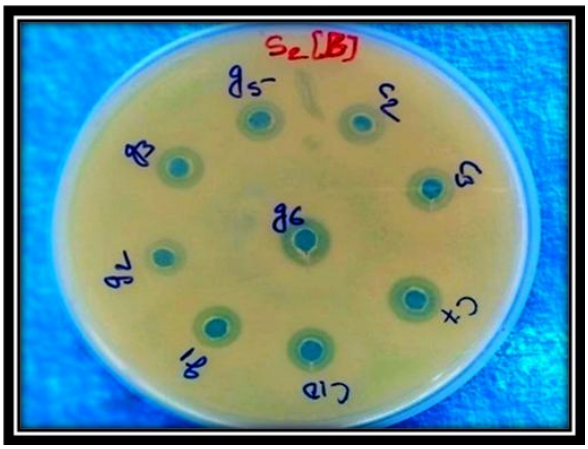
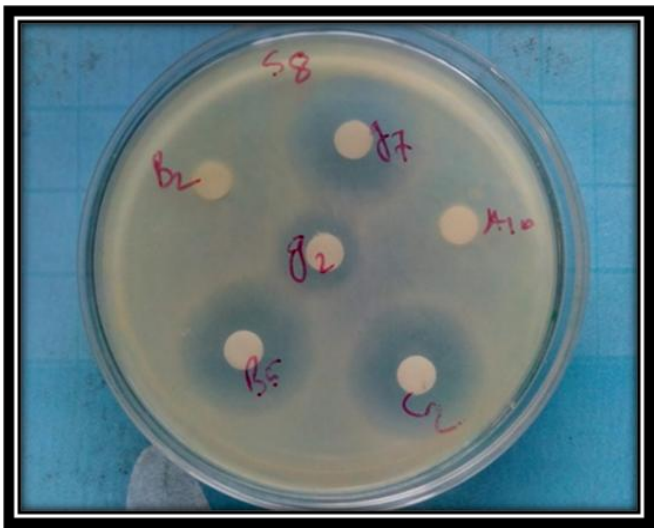


Plate.3 Zones of inhibition of LAB in Agar well diffusion test



In sugar fermentation test 12 out of 22 LAB fermented Cellobiose, Mannitol, Melebiose, Raffinose, Ribose, Salicine, Trehalose and Sucrose, but did not ferment Arabinose, Lactose and Sorbitol, therefore were identified *as L. plantarum*. Six isolates fermented Cellobiose, Mannitol, Melebiose, Raffinose, Ribose, Salicine, but not Lactose and Sorbitol and results of Arabinose and Trehalose remained doubtful, hence, were grouped as other non typable *Lactobacillus* spp. Remaining four isolates fermented Melabiose, Raffinose, Ribose, Arabinose, Trehalose but not Cellobiose and Mannitol, and results of Lactose and Sorbitol were doubtful hence, they were grouped as *Pediococcus* spp. Our findings were consistency with the Mohankumar and Murugalatha (2011) and Sarangdhar *et al.*, (2015).

Molecular identification of LAB by MALDI-TOF MS

Employing the MALDI-TOF MS technique LAB species were identified as per table 2; out of which highest number of isolates 10/22 were identified as *L. plantarum* (72.72%) while other six were subspecies of *L. plantarum* identified as two *L. plantarum ssp plantarum* (00.09%), two *L. plantarum ssp argentoratensis* (09.00%) and two *L. paraplantarum* (09.00%) strains.

Another 05/22 (22.72%) isolates identified as *Pediococcus* spp. in cultural method were confirmed with MALDI TOF MS technique as *Pediococcus acidilactici*. While one isolate out of 22 remained unidentified as peak score observed was not optimum. The results of ten bacterial isolate identified as *L. plantarum* with cultural method and genomic PCR remained in cent per cent agreement with MALDI-TOF MS which was in close accordance with Dogan and Ozpinar (2017) and Dissanayake (2017).

Antimicrobial activity

Agar spot assay

As per the results in the table 3 of Agar Spot assay, out of four positive isolates of LAB sourced from cow only one (18.18%) i.e. *L. paraplantarum* (C2) was found highly effective, five out of nine isolates belonging to buffaloes (36.36%) i.e. two *L. plantarum* (B5 and B8), one *L. paraplantarum* (B9) and two *L. plantarum* spp *argentoratensis* (B1 and B6), three out of five isolates belonging goat (27.27%) i.e. *L. plantarum* (G1, G2 and G6) and two out of four isolates belonging to sheep raw milk (13.60 %) i.e. *L. plantarum* (S3 and S10) were having the highest activity. Hence, these isolates were selected for further assessment of their in vitro antibacterial efficacy against COPS MRSA. Our findings closely corroborated with earlier researchers Mohankumar and Murugalatha (2011) and Jothi *et al.*, (2012).

Antibacterial efficacy of LAB against COPS MRSA with Agar Well Diffusion Test

The findings of antibacterial activity of CFS of LAB against COPS MRSA by well diffusion method were as depicted in table 3 (Plate 2). The widest zone of inhibitions (15-18mm) was recorded with strains *L. plantarum* (B5 and B8) isolated from buffalo followed by *L. plantarum* (G2 and G6) from goats (10-14mm). This trend was also seen in sheep *L. plantarum* (S3). Moderate size zones (06-09 mm) were recorded with *L. paraplantarum* (C2) isolated from cow. Overall, out of eleven *L. plantarum*, four strains (B5, B8, G2 and G6) presented very strong to strong antibacterial efficacy and three strains; *L. paraplantarum, L. plantarum* spp *argentoratesis* and *L. plantarum* showed moderate zone of inhibition (C2, B6 and S3). Remaining *Lactobacillus* species recorded

small zones of inhibition of around 05mm. Thus present findings remained in close concurrence with Soleimani *et al.,* (2010) and Eid *et al.,* (2016).

Statistically, the results of treatments with *Lactobacillus* species against COPS MRSA were found significantly different at 1% level of significance at the P-value less than 0.01.

Antibacterial efficacy of LAB against COPS MRSA with Agar Disc Diffusion Test

The antibacterial activity CFS of LAB against COPS MRSA was also examined by disc diffusion method to support the results of agar well diffusion test and the results were as depicted in table 4 (Plate. 3) which indicated that *L. plantarum* (B5, B8, G2 and G6) obtained from buffaloes and goats exhibited highest anti MRSA activity (15-18 mm zone of inhibition), followed by moderate efficacy (10-12 mm zone of inhibition) by *L. plantarum* (S3) and *L. paraplantarum* (C2) from sheep and cow, respectively (Table 5).

Our results were in accordance with the earlier report on antagonistic effect of LAB on gram positive and gram negative bacteria of Tajehmiri and co-workers (2014).

The statistical analyses of results of the treatments were found significant at 1% level of significance when the P-value were less than 0.01.

L. plantarum isolated from buffaloe and goats and *L. paraplantarum* from cows recorded the strongest antimicrobial efficacy against the virulent MRSA, and even *L. plantarum* from sheep also exhibited some efficacy, hence, the *Lactobacillus* strains in our study were found to be potential candidates for further exploiting their antibacterial efficacy against virulent MRSA *in vivo*.

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