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ISOLATION, CHARACTERIZATION OF LACTIC ACID BACTERIA FROM COW AND BUFFALO MILK AND EVALUATION FOR ANTIBACTERIAL AND ANTIMYCOBACTERIAL ACTIVITY *IN VITRO*

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Abstract – Lactic Acid Bacteria (LAB) are the heterogeneous group of Gram positive bacteria exist in the forms of both cocci and bacilli. Members of the LAB grab - extensive research due to their potential applications in various food industries and Generally Recognized As Safe (GRAS) status. Animal milk is considered as one of the natural habitat of LAB which is known to be responsible for the natural fermentation of milk products. The bacterial count in raw milk accounts around 30% of LAB and it varies depends on animal species, season, production, etc. In our study, we have isolated 50 LAB isolates from fresh cow and buffalo milk using standard protocol with some modification and identified at their genus level as described by *Hemangi et al*. (2012). LAB isolated from cow and buffalo milk samples were belongs to the genus *viz., Aerococcus* (30%), *Pediococcus* (26%), *Lactococcus* (8%), *Streptococcus* (8%), *Enterococcus* (6%), *Tetragenococcus* (12%), *Streptobacterium* (6%) and *Thermobacterium* (4%). Antibacterial and antimycobacterial properties of all the isolates have been evaluated against *S. aureus, B. cereus, E. coli, K. pneumoniae, M. smegmatis, M. fortuitum*. Notably, the inhibitory activity of cell free supernatant (CFS) obtained from 10 LAB isolates out of 50 isolates showed significant antimycobacterial activity against *M. smegmatis and M. fortuitum*. Further extraction and purification of bacteriocin from potential LAB isolates can develop as a good candidate to fight against non-tuberculous mycobacteria.

INTRODUCTION

Lactic Acid Bacteria (LAB) are the heterogenous group of Gram positive bacteria exists in the forms of both cocci and bacilli. LAB have been widely studied and characterized due to their potential use in food industries as biopreservatives and dairy industries as starter culture, etc. Members of the LAB notably the genus *Lactococcus* and *Lactobacillus* grab an extensive research due to their potential application and Generally Recognized As Safe (GRAS) status (Alvarez-Sieiro *et al*., 2016; Perin and Nero, 2014; Gómez-Sala *et al*., 2016). Bacteriocins are ribosomally synthesized antimicrobial peptides possess antibacterial activity against closely related organisms. Few studies reported that the bacteriocins showed antibacterial activity against non closely organisms of bacteriocin producer organisms. Several studies have reported that the LAB have the potential to manage many diseases like Diarrhea, food allergy, Colorectal cancer, etc. (Arqués *et al.,* 2015; Zhong *et al.,* 2014). Animal milk is considered as one of the natural habitat of LAB. It is known to be responsible for the production of different milk products through its natural fermentation. The bacterial count in animal milk accounts around 30% of LAB and it varies depends on animal species, season, production, etc. (Alvarez-Sieiro *et al*., 2016; Delavenne *et al.,* 2012; Wouters *et al.,* 2002; Verdier-Metz *et al.,* 2009). Isolation of LAB from fresh milk rather than its products like ripened cheese gives higher choice of selecting both strains and species since milk possess a wide range of LAB (Franciosi *et al.,* 2009).

Mycobacterium fortuitum is rapidly growing non-tuberculous mycobacteria, known for producing a wide range of clinical diseases. It causes pulmonary, skin, bone/joint infections in immunocompetent and

immunocompromised patients (Wallace *et al.,* 1983; Okamori *et al.,* 2018). In this study, we have isolated and identified LAB from fresh cow and buffalo milk. The antimicrobial activity of cell free supernatant obtained from LAB isolates have also been determined against bacterial pathogens such as *Staphylococcus aureus, Bacillus cereus, Escherichia coli, Klebsiella pneumoniae* and against *Mycobacterium smegmatis, Mycobacterium fortuitum.*

MATERIALS AND METHODS

Chemicals and Strains

MRS broth, Middlebrook 7H9 Broth and catalase were purchased from Himedia (Mumbai). MRS-BCP agar (MRS medium added with 0.004% bromocresol purple) was prepared. Strains such as *Staphylococcus aureus, Bacillus cereus, Escherichia coli, Klebsiella pneumonia* were received from out centre's culture depository. *Mycobacterium fortuitum* (MTCC1902) were obtained from Microbial Type Culture Collection, Chandigarh, India and *Mycobacterium smegmatis* was kindly received from NIRT, Chennai.

Isolation and identification of LAB from milk samples

Cow and buffalo milk samples were collected from milk vendors in Chennai (13.0827° N; 80.2707° E) and Kanchipuram (12.8342° N; 79.7036° E), Tamil Nadu, India. All the milk samples were collected in sterile vials and immediately transported using cold condition to laboratory and stored at 4 °C until further processing. The collected milk samples were serially diluted then 100µL of aliquot from each dilution were spreaded on MRS-BCP agar. All the plates were incubated at 30 °C under aerobic condition. After 24 hours of incubation, the colonies showing yellow zone around them were chosen for further LAB characterization studies. All isolates were subjected to Gram staining and catalase test by adopting standard procedures (Sharpe, 1979; Harrigan and McCance, 1976). Then isolates were identified at genus level by biochemical methods such as gas production, growth at different pH (4.4, 9.6), temperature (10°C, 45 °C) and NaCl concentrations (6.5%, 18%) according to methods described by Hemangi *et al.,* 2012 and Kalschne *et al., 2015.*

Antimicrobial activity

About 1% of overnight grown LAB isolates were inoculated into MRS broth and incubated at 30ˆC. After 24 hours, cell free supernatant (CFS) was collected from each isolates using centrifugation at 5000rpm for 20mins. The antibacterial activity and anti-NTM activity was performed with obtained CFS as per protocol mentioned. Briefly, the pH of the CFS was adjusted to 7 and treated with catalase (1mg/mL) for 1 hour at 37°C in order to eliminate acidic condition and H_2O_2. The treated CFS was tested against *S. aureus, B. cereus, E. coli*, and *K. pneumoniae* using agar well diffusion assay method as per procedure. The anti-NTM activity of CFS of LAB isolates were performed against *M. smegmatis* and *M. fortuitum*. Briefly, loopful of *M. smegmatis* and *M. fortuitum* cultures from Lowenstein Jensen (LJ) medium was taken and inoculated separately into 300µL of Middlebrook 7H9 broth in sterile Bijou bottle containing glass beads. Then the suspension was vortexed for 30 seconds and adjusted to McFarland unit 1 with Middlebrook 7H9 broth. 200µL of suspension were added into 5 mL of molten agar (1%) and poured onto Middlebrook 7H9 Agar base plate. After solidification, wells (5mm) were cut using sterile cork borer and 100µl of treated CFS was added into each well. The plates were incubated at 37°C and after 48 hours all plates were observed for zone of inhibition and measured in mm.

RESULTS

Totally 50 LAB isolates from the 32 animal milk samples were identified based on Grams staining and catalase test. Of the 50 LAB, 43 and 7 LAB was isolated from 27 cow milk and 5 buffalo milk samples respectively. Forty LAB isolates are cocci in shape, 5 LAB are in bacilli and another 5 LAB were coccobacilli in shapes. (Table 1)

The results of biochemical characterization of LAB revealed that the 50 LAB isolates were belongs to the 8 different genus of LAB. This includes *Aerococcus* (15), *Pediococcus*(13), *Lactococcus*(4), *Streptococcus*(4), *Enterococcus*(3), *Tetragenococcus*(6), *Streptobacterium*(3) and *Thermobacterium*(2) (Table 1; Fig.1). Among the 50 LAB isolate's CFS tested, only one LAB isolate showed inhibition against *S. aureus*, 6 showed inhibition against *B. cereus*, 5 against *E. coli*, whereas *K. pneumonia* was not inhibited by any of the LAB isolates tested. Whereas, the anti_NTM activity results showed 11 LAB was found active against *M. smegmatis* and 17 showed inhibition against *M. fortuitum*. Of them, 7 LAB isolates

Table 1. List of LAB Isolates Obtained

S. No	Reference Name	Shape	Gas Production	Tetrad formation	Growth @10°C	Growth @45°C	Growth @ pH 4.4	Growth @pH 9.6	Growth @6.5% NaCl	Growth @18% NaCl	Genus Identified
1	CMD2-2	Cocci	-	+	-	+	+	-	+	-	*Pediococcus*
2	CMD2-3	Cocci	-	+	-	+	+	-	+	-	*Pediococcus*
3	CMD2-4	Cocci	-	+	+	-	+	+	+	-	*Pediococcus*
4	CMD3-2	Cocci	-	+	+	-	-	+	-	-	*Aerococcus*
5	CMD4-2	Cocci	-	+	-	-	+	-	-	-	*Pediococcus*
6	CMD5-1	Cocci	-	+	-	-	+	-	-	-	*Pediococcus*
7	CMD5-2	Cocci	-	+	-	-	-	-	-	-	*Aerococcus*
8	CMD6-1	Cocci	-	-	+	-	+	+	-	-	*Vagococcus/ Lactococcus*
9	CMD7-1	Coccobacilli	-	-	-	-	+	-	+	+	*Streptococcus*
10	CMD7-2	Cocci	-	+	+	-	-	+	+	±	*Tetragenococcus*
11	CMD7-3	Cocci	-	-	+	-	+	+	-	-	*Vagococcus/ Lactococcus*
12	CMD8-1	Cocci	-	+	+	+	-	-	+	-	*Aerococcus*
13	CMD8-2	Cocci	-	+	-	+	-	-	+	-	*Aerococcus*
14	CMD8-3	Cocci	-	-	-	+	-	-	+	-	*Enterococcus*
15	CMD8-4	Cocci	-	+	-	-	-	+	+	-	*Aerococcus*
16	CMD10-1	Cocci	-	+	-	+	+	+	+	+	*Tetragenococcus*
17	BMD1-1	Cocci	-	+	+	-	-	+	+	+	*Tetragenococcus*
18	BMD1-2	Cocci	-	+	-	-	-	+	+	-	*Aerococcus*
19	BMD2-1	Cocci	-	+	-	-	+	-	+	-	*Pediococcus*
20	BMD2-3	Cocci	-	+	-	+	-	-	-	-	*Aerococcus*
21	BMD3-2	Cocci	-	+	-	-	-	+	-	-	*Aerococcus*
22	BMD5-1	Cocci	-	+	+	+	+	+	+	+	*Tetragenococcus*
23	BMD5-2	Cocci	-	+	+	+	+	+	+	+	*Tetragenococcus*
24	PCMD1	Cocci	-	-	-	-	-	-	-	-	*Lactococcus*
25	PCMD6-2	Cocci	-	+	-	-	+	+	+	-	*Pediococcus*
26	CMP11	Cocci	-	+	+	+	+	+	+	-	*Pediococcus*
27	PCMD9-2	Cocci	-	+	-	+	-	-	-	+	*Tetragenococcus*
28	PCMD11-3	Cocci	-	+	+	-	-	+	-	-	*Aerococcus*
29	PCMD12-2	Coccobacilli	-	-	-	-	±	+	+	-	*Streptococcus*
30	PCMD13-1	Cocci	-	-	-	-	-	-	-	-	*Lactococcus*
31	PCMD13-2	Cocci	-	+	-	-	+	-	-	-	*Pediococcus*
32	PCMD14-1	Cocci	-	+	-	-	+	+	-	-	*Pediococcus*
33	PCMD14-2	Cocci	-	+	±	-	-	+	+	-	*Aerococcus*
34	CMAM5-1	Cocci	-	+	+	+	-	+	-	-	*Aerococcus*
35	CMAM5-2	Cocci	-	+	+	+	-	+	-	-	*Aerococcus*
36	CMP6-1	Bacilli	-	-	-	+	+	+	+	-	*Thermobacterium*
37	CMP6-2	Bacilli	-	-	-	-	+	+	-	-	*Thermobacterium*
38	CMP5-1	Bacilli	-	-	+	+	+	+	+	±	*Streptobacterium*
39	CMP5-2	Bacilli	-	-	+	+	+	+	+	-	*Streptobacterium*
40	CMP5-4	Bacilli	-	-	+	+	+	+	+	-	*Streptobacterium*
41	CMP1-4	Cocci	-	+	-	-	+	+	±	-	*Pediococcus*
42	CMP4-2	Coccobacilli	-	-	-	-	-	-	-	-	*Streptococcus*
43	CMP9-1	Coccobacilli	-	-	+	+	+	+	-	-	*Streptococcus*
44	CMP9-2	Cocci	-	+	+	+	+	+	-	-	*Pediococcus*
45	CMT1-3	Cocci	-	+	-	+	+	-	+	-	*Pediococcus*
46	CMT2-1	Cocci	-	+	-	-	-	+	+	-	*Aerococcus*
47	CMT2-2	Cocci	-	+	-	-	-	+	+	-	*Aerococcus*
48	CMK3-2	Cocci	-	+	+	+	-	-	+	-	*Aerococcus*
49	CMK3-3	Cocci	-	-	-	+	-	-	+	-	*Enterococcus*
50	CMK4-1	Coccobacilli	-	-	+	+	+	+	±	-	*Enterococcus*

Table 2. Antibacterial Activity of LAB isolates

S. No	Reference Name	Zone of Inhibition					
		Bacterial Pathogens				Mycobacteria	
		S. aureus	*B. cereus*	*E. coli*	*K. pneumoniae*	*M. smegmatis*	*M. fortuitum*
1	CMD2-2	-	-	-	-	±	±
2	CMD2-3	-	-	-	-	±	±
3	CMD2-4	-	-	-	-	-	±
4	CMD3-2	-	-	-	-	±	-
5	CMD4-2	-	-	-	-	-	±
6	CMD5-1	-	-	-	-	±	++
7	CMD5-2	-	-	-	-	+++	-
8	CMD6-1	-	-	-	-	±	-
9	CMD7-1	±	±	±	-	-	±
10	CMD7-2	-	-	+	-	-	-
11	CMD7-3	-	-	+	-	-	±
12	CMD8-1	++	±	-	-	-	-
13	CMD8-2	-	±	-	±	-	±
14	CMD8-3	-	-	-	-	-	-
15	CMD8-4	-	-	±	-	-	-
16	CMD10-1	-	-	-	-	±	-
17	BMD1-1	-	-	-	-	-	-
18	BMD1-2	-	-	-	-	±	-
19	BMD2-1	-	-	±	-	-	-
20	BMD2-3	-	-	-	-	-	-
21	BMD3-2	-	-	-	-	-	-
22	BMD5-1	-	-	±	-	++	++
23	BMD5-2	-	-	±	-	±	+
24	PCMD1	-	-	-	-	-	-
25	PCMD6-2	-	-	-	-	-	-
26	CMP11	-	-	-	-	±	-
27	PCMD9-2	-	-	±	-	-	-
28	PCMD11-3	-	-	-	-	-	-
29	PCMD12-2	-	-	-	-	-	-
30	PCMD13-1	-	-	-	-	-	-
31	PCMD13-2	-	-	-	-	-	-
32	PCMD14-1	-	-	-	-	-	±
33	PCMD14-2	-	-	-	-	±	±
34	CMAM5-1	-	-	-	-	-	+
35	CMAM5-2	-	-	-	-	-	+
36	CMP6-1	-	+	-	-	+	++
37	CMP6-2	-	++	-	-	-	+++
38	CMP5-1	-	+	-	-	+	++
39	CMP5-2	-	-	-	-	+	++
40	CMP5-4	-	++	-	-	+	+
41	CMP1-4	-	-	-	-	+++	++
42	CMP4-2	-	-	-	-	++	++
43	CMP9-1	-	++	+	-	+++	++
44	CMP9-2	-	+	±	-	+++	++
45	CMT1-3	-	-	-	-	-	-
46	CMT2-1	-	-	++	-	-	++
47	CMT2-2	-	-	+	-	-	+
48	CMK3-2	-	-	-	-	±	-
49	CMK3-3	-	-	-	-	±	±
50	CMK4-1	-	-	-	-	++	++

± = <10mm; + = 10mm; ++ = >10mm&<=12mm; +++= >12mm

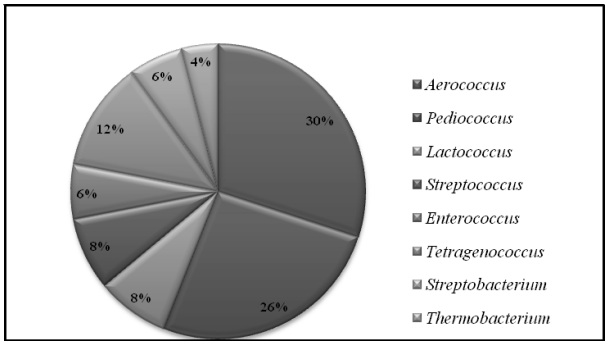

Fig. 1. Different genus of LAB obtained in milk samples

showed significant inhibitory activity against both *M. smegmatis* and *M.fortuitum* (Table 2).

DISCUSSION

Lactic acid bacteria have been widely isolated from milk samples and the diversity of LAB is extensively studied. Various animal milk samples have been used to isolate and identify LAB in which the majority of cow milk samples were analyzed followed by buffalo milk. In the present study, we have obtained 50 bacterial isolates belongs to 8 different LAB genera from cow and buffalo milk samples. The majority of LAB isolates are characterized as gram positive cocci which is considered as predominant in raw milk microbiota (Perin and Nero, 2014). Among 8 different genera, *Aerococcus* sp. found as predominant group followed by *Pediococcus sp.* Our resultsare moderately differs from various studies. Most of the reports found that milk samples contains *Lactobacillus sp*. as predominant group followed by *Streptococcus sp.* (Mohammed and Ijah, 2013; Amir and Shyamapada, 2018; Wassie and Wassie, 2016). Alnakip *et al*., (2016) have reported 27% of *Aerococcus sp.* from cow milk whereas in our study, we have reported 30% *Aerococcus sp.* found in cow milk. The distribution of microbiota in animal milk varies qualitatively and quantitatively due to various factors including bedding material, milking machine, fed habit, etc. (Quigley *et al.*, 2013). Many reports have documented the potential of LAB to inhibit various pathogenic microorganisms due to its bacteriocinogenic potential. LAB has been widely utilized in food industries for fermentation and biopreservation due to their antibacterial activity against various food spoilage microorganisms (Mokoena, 2017). In our study, among 50 LAB isolates, only 10 LAB isolates have showed potential antimicrobial activity. In a study by Perin and Nero (2014), among 423 LAB isolates obtained from goat milk, only 57 isolates showed bacteriocinogenic activity against *Listeria monocytogenes*, Rodriguez *et al*., (2000) have reported that out of 1340 LAB isolates, 321 LAB showed antagonistic activity against *Staphylococcus aureus*. In a study by Martinis *et al*. (2016), they have isolated 815 LAB isolates, none of them showed bacteriocinogenic activity against *Staphylococci*. In our study, among 50 LAB isolates, none of them showed inhibition against *S. aureus*. Few studies focused on the efficacy of LAB against *M. fortuitum* (Saito *et al.*, 1987 and Tomioka *et al.*, 1990). In our study, *Thermobacterium sp. Streptobacterium* sp. and *Aerococcus sp.* showed significant inhibitory activity against *M. fortuitum.*

CONCLUSION

Eight different genus of LAB have been identified from both fresh cow and buffalo milk samples. We found that *Aerococcus sp.* and *Pediococcus sp.* are predominantly present in milk samples. Among 50 LAB, we identified 10 LAB isolates showed significant activity against *M. fortuitum.* This anti-NTM activity reveals that the LAB isolates obtained in this study may produce bacteriocins. Hence, bacteriocin extraction, purification from the potential isolates and *in vitro* testing against NTM particularly *M. fortuitum* may be noteworthy for development of lead molecule for NTM infections in future.

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