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Pallavi Shelar
Department of Dairy Microbiology, College of Dairy Science and Food Technology, D.S.V.C.K.V., Raipur, Chhattisgarh, India

Sarang Dilip Pophaly
Department of Dairy Microbiology, College of Dairy Science and Food Technology, D.S.V.C.K.V., Raipur, Chhattisgarh, India

Manorama
Department of Dairy Microbiology, College of Dairy Science and Food Technology, D.S.V.C.K.V., Raipur, Chhattisgarh, India

Awadhesh Kumar Tripathi
Department of Dairy Microbiology, College of Dairy Science and Food Technology, D.S.V.C.K.V., Raipur, Chhattisgarh, India

Shakeel Asgar
Department of Dairy Technology, College of Dairy Science and Food Technology, D.S.V.C.K.V., Raipur, Chhattisgarh, India

Archana Khare
Department of Dairy Chemistry, College of Dairy Science and Food Technology, D.S.V.C.K.V., Raipur, Chhattisgarh, India

Lata Jain
National Institute of Biotic Stress Management (ICAR-NIBSM), Baronda, Raipur, Chhattisgarh, India

Corresponding Author
Pallavi Shelar
Department of Dairy Microbiology, College of Dairy Science and Food Technology, D.S.V.C.K.V., Raipur, Chhattisgarh, India

Isolation and identification of lactococcal cultures exhibiting antibacterial activity against selected pathogens

Pallavi Shelar, Sarang Dilip Pophaly, Manorama, Awadhesh Kumar Tripathi, Shakeel Asgar, Archana Khare and Lata Jain

Abstract
Lactic acid bacteria have been used by mankind for centuries for the production of a variety of dairy based fermented products. *Lactococcus* is one of the most important starter bacteria used in dairy industry. The aim of this study was to determine antibacterial properties of *Lactococcus* strains isolated from dairy and vegetable samples. For this reason 76 samples were collected from different districts of Chhattisgarh State and 76 tentative isolates were obtained by streaking the samples on M-17 Agar. Out of these 76 isolates, as many as 53 isolates were found to be Lactococci based on morphological and biochemical properties (Gram reaction, catalase test, NaCl concentration and carbohydrate fermentation test). These 53 lactococcal isolates were tested for antibacterial properties against four common foodborne pathogens viz. *Escherichia coli*, *Enterococcus faecalis*, *Bacillus cereus* and *Staphylococcus aureus*. *Lactococcus* (KD2 isolate) strain isolated from *dahi*, exhibited highest antibacterial activities against all the selected test pathogens.

Keywords: Lactic acid bacteria, *Lactococccus*, isolation, antibacterial activity, cell free supernatant

1. Introduction
Gram-positive bacteria that ferment carbohydrate into energy and lactic acid are members of the lactic acid bacteria family [1]. Depending on the organism, the metabolic processes are different if glucose is the major source of carbon: homofermentative bacteria, including *Lactococcus* and *Streptococcus* subsp. give two lactates from a single glucose molecule are transformed into lactates with ethanol and carbon dioxide (i.e., *Leuconostoc* and *Weissella* ssp.). The genus *Lactococcus* is part of the lactic acid bacteria, one of the most biotechnologically important groups of lactic acid bacteria [11]. Isolation and screening of lactic acid bacteria from naturally occurring processes have always been the most powerful means for obtaining useful cultures for scientific and commercial purposes and the proper selection and balance of lactic acid bacteria used for starter culture is critical for the manufacture of fermented dairy products [22, 24]. Additionally these species are facultative anaerobic, catalase-negative and non-motile. *Lactococcus lactis* is used as a fermentations starter in dairy or fermented foods and is regarded as "Generally Recognized as safe" microorganisms.
During the last few years health-conscious consumers are looking for natural foods without chemical preservatives that will fit in their healthy lifestyle. Lactic acid bacteria are Gram positive organisms which are safety applied in medical and veterinary functions [13]. Bio-preservation refers to extended shelf life and enhanced safety of foods using microorganisms or their metabolites. Lactic acid bacteria play a key role in food fermentations where they not only contribute to the development of the desired sensory properties in the final product but also to their microbiological safety [7, 23]. The antimicrobial effect of lactic acid bacteria is mainly related to the production of lactic and acetic acids as well as propionic acids, sorbic acids, benzoic acids, hydrogen peroxide, diacetyl, CO_2, acetaldehyde [6], ethanol, phenolic and proteinaceous compounds [8], organic acids and short chain fatty acids is one of the functional properties used to characterize probiotics [9, 10]; Also, some strains are able to synthesize antimicrobial substances- reuterin D-isomers of amino acids and bacteriocins [27].
Therefore, the present study aimed at isolation and identification of lactococcal cultures exhibiting antimicrobial activity against common food borne pathogens.

2. Material and Methods

2.1 Procedures for Collecting and Handling Samples

A total of 43 milk and milk products (*dahi* & buttermilk) and 10 vegetable samples (chili, cauliflower, ladyfinger, cucumber, tomato, cabbage, and beetroot) were collected from Chhattisgarh. Samples were collected from dairy farms, households, vending shops, supermarket and fresh farms by using a sterile sample bottle. The samples were labeled carefully, stored at 4 ºC, and were used for further isolation of lactococcal strains. Dairy and vegetable samples for the isolation of lactic acid bacteria were collected from rural and urban areas of Chhattisgarh state in order to get a wider diversity of lactic acid bacteria strains.

2.2 Isolation and Identification of *Lactococcus* strains

Bacterial isolation was performed by preparing serial dilutions of the samples with sterilized maximum recovery diluents (pH-7), 0.1 ml of the dilution was spread on M-17 agar + agar powder for *Lactococcus* isolation (Hi-Media), plates were incubated at 30 ºC under aerobic conditions for 24 h. Viable aerobic counts of the samples were determined. The isolation was obtained by morphological characteristics (colony and cell morphology), on the selective media, and biochemical tests used were Gram's reaction, catalase test, NaCl concentration and carbohydrate fermentation. Only Gram positive bacteria with catalase-negative responses were found and representative isolates were purified by streaking them over the same agar substrate several times. Following that, isolates from dairy and vegetable samples were selected for further identification.

Stock cultures were kept in the M-17 broth medium with 1 per cent glycerol at -20 ºC. The microbial culture was regenerated and maintained by regular subculture on M-17 broth. Prior to beginning work, a subculture was made by transferring a loopful of the culture to 10 ml of M-17 broth and incubating it at 30 ºC for 24 h.

At the end of incubation (30 ºC for 24 h), the tentative *Lactococcus* strains were selected based on their morphology in their selective media and biochemical profiles. The carbohydrate fermentation profiles of isolates were determined with the carbohydrate fermentation kit.

2.3 Morphological and Biochemical Characterization

The morphological characteristics (i.e., shape, size and arrangements of cells were determined by negative staining using nigrosine stain) and Gram's reactions of the *Lactococcus* spp.were determined after 24 h of incubation on M-17 medium and cells were examined using microscopy. An array of biochemical tests were performed as per standard methods [17] to identify the morphologically and biochemical profile of the selected isolates. Pure culture were grown in M-17 broth at 30 ºC for 24 h. Cultures activated 24 h before were used as an inoculum for the various biochemical tests employed to identify the isolated cultures.

Catalase activity was determined by slide method [15]. Using an inoculating needle culture from well isolated colony was placed on a clean glass slide. A drop of 3 per cent H_2O_2 solution was added onto culture and closely observed for the effervescence, indicating positive result.

Carbohydrate fermentation test was using Hi-Carbo Kit (Part-A; Hi-Media Laboratories Pvt. L.td., Mumbai, India) which contained different sugar wells. The kit includes twelve immobilized carbohydrates in wells viz. lactose, xylose, maltose, fructose, dextrose, galactose, raffinose, trehalose, melibiose, sucrose, L-arabinose and mannose. The culture was grown in 10 ml M-17 broth at 30 ºC for 24 h cells were harvested by centrifugation in refrigerated centrifuge (10,000 rpm for 10 min at 4 ºC). The supernatant was discarded carefully and pellet was washed using sterilized saline. The washed cell was suspended in 5 ml of saline, a part of which was used for assessing O.D. using spectrophotometer. The prepared inoculums was inoculated @ 50 µl in each well of the kit and incubated at 30 ºC for 24-48 h. after stipulated incubation change in color of immobilized sugar was observed and noted down.

The effect of NaCl concentration on growth of isolates were inoculated in M-17 broth having different NaCl concentration viz., 4 per cent, 6 per cent and 9 per cent, and incubating at 30 ºC for 24-48 h the culture tube was observed for the presence or absence of growth.

2.4 Determination of Antibacterial Activity

Each isolate was screened for antibacterial activity against four bacterial pathogens, according to Amin *et al*. (2016). All Gram-positive and catalase negative isolates were identified as *Lactococcus* and screened for antibacterial activity (against *Escherichia coli.*, *Enterococcus faecalis*, *Bacillus cereus* and *Staphylococcus aureus*) by agar well diffusion assay [12]. The isolates were propagated at 30 ºC for 48-72 h. The appropriate solid medium was used for each indicator microorganisms [25]. Supernatants were directly used for antagonistic test. The agar plate surface was inoculated by spreading a volume of the microbial inoculums over the entire agar surface. Then, a hole with a diameter of 6 to 8 mm was punched aseptically with a sterile cork-borer, and a volume (20-100 µl) of the cell free culture (supernatant) filtrate of the 24 h, M-17 broth culture of *Lactococcus* was poured into the respective well and incubated at 30 ºC for overnight and resistance was defined as the absence of a growth inhibition zone (mm) around the well was measured. Data of antibacterial activities against bacterial pathogens were recorded according to the following scale: (-) no inhibition, (+) inhibition zone less than 4 mm, (++) inhibition zone 5-7 mm and (+++) inhibition zone 8-10 mm around the punched well.

3. Result and Discussion

3.1 Isolation and Identification of *Lactococcus*

The morphological and biochemical results illustrated that isolates were Gram positive, catalase negative and cocci shaped. According to this study, identification of the *Lactococcus* was performed through morphological characteristics. Besides, other biochemical tests and *Lactococcus* species were found to be creamy white to yellowish color, small to large in size and circular margin on M-17 media (Figure 1). On the basis of morphological and biochemical characterization, the isolates could be identified as *Lactococcus* strains. The isolation of 13 *Lactococcus* strains from *dahi* samples [21]. Vegetable and fruit samples are also known to harbour lactococcal strains. Noruma *et al*. (2006) have been reported the isolation of *Lactococcus* strains from different vegetable samples. The characterization of lactic acid bacterial strains *Lactococcus* from buffalo milk, *Streptococcus thermophilus* from cow milk, *Lactobacillus delbrueckii* subsp. *delbrueckii* from sheep milk were identified capable of producing lactic acid in generous amount.

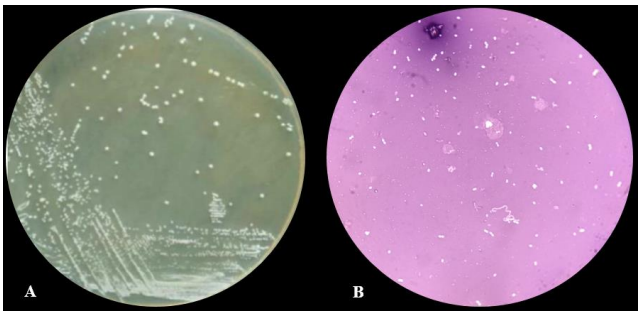

Fig 1: Typical isolated colonies of *Lactococci* on M-17 media (A); and Negative staining of Lactococci (B)

3.2 Morphological Characterization

A total of 76 lactococci isolates were characterized and identified to genus level from 43 dairy samples and 10 vegetable samples collected from the surrounding area of the Chhattisgarh state. Morphological and biochemical characteristics of isolated genus are shown in (Table 1 and 2). All isolates were Gram-positive, catalase negative and non-motile. The cell morphology of all isolate was evaluated through microscopic observation and majority of them, were found to be cocci. Lactic acid bacteria that produce lactic acid as their fermentation product into the culture medium and generally regarded as safe [14, 26].

Catalase is an extracellular enzyme secreted by several microorganisms. The catalase is involved in catalyzing the breakdown of toxic hydrogen peroxide to produce molecular oxygen that generates vigorously while producing effervescence, when a microbial culture is mixed with an equal volume of 3 per cent solution of hydrogen peroxide. Absence of effervescence is taken as negative for catalase enzyme production. In this study, all the isolates were found to be catalase negative (Table 1).

Table 1: Cell morphology and biochemical characteristics of lactococci strains isolated from dairy and vegetable samples

Isolates	Gram's Reaction	Shape and Arrangement	Catalase
MD1, RD1, LT1, KD1, KD2,BD4, RD3, TD1, BD6, IT1	+ve	Cocci in short chain	-ve
AD1, AB1, SD3, ZD1, PD2, BB1, TD1, TD2 MD4, JD1, ST1, MF1, PD3, SD5, LC2	+ve	Cocci in pairs	-ve
BD2, SD2, DD2, RD2, SB1, MD2, MB1, LD1, SD4, BD7 UD1, CD1, MC1	+ve	Cocci in medium chain	-ve
BD1, SD1, LL1, GD2, LC1	+ve	Cocci in pair and short chain	-ve
BB1, IC2, AC1	+ve	Cocci in pair and medium chain	-ve
GD1, PD1, MD3	+ve	Cocci in pair and single	-ve
DD1, BD3, KD3, MB2	+ve	Cocci in medium and short chain	-ve

The sugar utilization patterns of the isolates suspected to be the *Lactococci* after their growth on M-17 medium. The carbohydrate fermentation Hi-Carbo Kit tests were performed using (Part- A; Hi-Media Laboratories Pvt. Ltd., Mumbai, India) with different sugars. Out of 76 pure isolates, 53 cultures were confirmed Lactococci on the basis of polyphasic morphological and biochemical identification (Table 2). Lactococci isolates showed similar sugar utilization patterns, where some wells containing isolates and sugar turned yellow whereas the other remained brown colored indicating the positive and negative tests, respectively for carbohydrate utilization tests. *Lactococcus* strains were capable of metabolising a wide range of carbohydrates in that they fermented D-mannitol, amygdalin, potassium gluconate, L-arabinose, D-xylose, sucrose and gentibiose (Alemayehu *et al*., 2014).

Table 2: Sugar profiling characteristics of lactococci strains isolated from dairy and vegetable samples

Isolates	Carbohydrates											
	Lac	Xyl	Mal	Fru	Dex	Gal	Raf	Tre	Mel	Suc	L-ar	Man
MD1, RD1, LT1, KD1, BD4, RD3, TD1, BD6, IT1	+	-	-	+	+	+/-	-	+	-	+	-	+
AD1, AB1, SD3, ZD1, PD2, BB1, TD1, KD2, TD2, MD4, JD1, ST1, MF1, PD3,SD5, LC2	+	-	+	+	+	+	-	+	+	+	-	+
BD2, SD2, DD2, RD2, SB1, MD2, MB1, LD1, SD4, BD7 UD1, CD1, MC1	+	-	-	+	+	+	-	+	-	+	+	+
BD1, SD1, LL1, GD2, LC1	-	-	-	+	+	+	-	+	+	+	+	+
BB1, IC2, AC1	-	+	+	+	+	+	+	-	-	+	+	+
GD1, PD1, MD3	-	+	+	+	+	+	-	+	-	+	+	+
DD1, BD3, KD3, MB2	+	-	+	+	+	+	-	+	-	+	+	+

The effect of NaCl concentration on the isolates was studied. Lactococci cultures were incubated in M-17 broth at 30 ºC for 24 h to assess the effect of varying NaCl concentration viz., 4 per cent, 6 per cent and 9 per cent and were results evaluated in terms of turbidity as an indication of microbial growth. All the 54 isolates were tolerant to 2 per cent and 4 percent NaCl concentration and only 10 of them were able to tolerate a NaCl concentration of 6.5 per cent. None of the 53 isolates were able to grow at 9 per cent salt concentration. Some are able to grow in a broad range of temperature, tolerate low pH, high salt concentrations and atmospheric pressure. Tetragenococci have extreme salt tolerance (>18 per cent NaCl) and generally require 5 per cent NaCl for growth (Axelsson, 2004; Liu *et al.*, 2011).

3.3 Antibacterial Activity

The cell free supernatant of *Lactococcus* strains were tested for antibacterial activity against selected pathogenic strains (*Escherichia coli*., *Enterococcus faecalis*, *Bacillus cereus* and *Staphylococcus aureus*) by using agar well diffusion assayas presented in Table 3. The diameters of inhibition zones were measured and compared. Results showed that the majority of *Lactococcus* isolates were able to inhibit the growth of all selected bacterial pathogens. A few of the *Lactococcus* isolates TD1, MD4, LC2 and MC1 strains exhibited inhibitory effects against the *Escherichia coli*., *Enterococcus faecalis*, *Bacillus cereus* and *Staphylococcus aureus*. Of these the *Lactococcus* isolate (isolate KD2) showed maximum inhibition zone (zone diameter was 8-10 mm) against the

bacterial pathogens among all the tested isolates. Bassyouni *et al*. (2012) showed that lactic acid bacteria strains tested against *Salmonella typhimurium*, *Escherichia coli.* and *Staphylococcus* species had antibacterial effect against the pathogenic bacteria. The supernatant of *Lactococcus lactis* subsp. *cremoris*, *Lactobacillus plantarum* and *Lactobacillus delbrueckii* subsp. *delbrueckii* inhibit growth of *Escherichia coli*, *Staphylococcus aureus* and *Listeria innocua* respectively [5]. Several *Lactococcus* strains exhibit the two different phenotypic products of exopolysaccharides (EPS), mucoid and ropy. EPS also showed a strong inhibitory activity against *Staphylococcus aureus*, *Pseudomomas aeruginosa*, *Escherichia coli.*, *Listeria monocytogenes*, *Bacillus cereus*, *Proteus mirabilis*, *Acinetobacter baumannii*, *Enterobacter cloacae* and *Candida albicans* [18].

Table 3: Antibacterial activity of *Lactococcus* isolates supernatant

S. No.	Isolates	Diameter of Inhibition Zone (mm)			
		Escherichia coli	***Enterococcus faecalis***	***Bacillus cereus***	***Staphylococcus aureus***
01.	MD1	-	++	++	++
02.	RD1	-	-	-	++
03.	LT1	+++	++	++	-
04.	KD1	++	+++	++	+++
05.	BD4	++	-	-	++
06.	RD3	-	-	-	-
07.	TD1	++	+++	+++	+++
08.	BD6	++	++	++	++
09.	IT1	+++	++	++	+
10.	AD1	++	++	-	++
11.	AB1	++	-	++	-
12.	SD3	-	-	-	-
13.	ZD1	+++	-	-	-
14.	PD2	-	-	-	-
15.	BB1	++	++	-	++
16.	TD1	-	-	-	-
17.	KD2	+++	+++	+++	+++
18.	TD2	-	++	-	-
19.	MD4	+++	+++	-	+++
20.	JD1	++	+++	++	+++
21.	ST1	++	-	-	-
22.	MF1	+++	+++	+	+++
23.	PD3	++	++	++	-
24.	SD5	++	-	+++	+++
25.	LC2	+++	+++	-	+++
26.	BD2	-	++	-	-
27.	SD2	++	+	-	++
28.	DD2	-	++	-	++
29.	RD2	++	+	++	-
30.	SB1	-	++	+++	+++
31.	MD2	-	++	-	-
32.	MB1	+++	-	++	+++
33.	LD1	-	-	+++	-
34.	SD4	-	-	-	++
35.	BD7	+++	-	-	++
36.	UD1	++	-	-	-
37.	CD1	++	-	++	+++
38.	MC1	+++	+++	+++	-
39.	BD1	++	++	-	++
40.	SD1	-	-	++	-
41.	LL1	+	-	+++	+++
42.	GD2	+	+++	++	+++
43.	LC1	++	-	++	++
44.	BB1	-	++	-	++
45.	IC2	-	-	-	-
46.	AC1	+++	+++	++	-
47.	GD1	-	++	-	++
48.	PD1	++	-	-	+++
49.	MD3	-	-	++	++
50.	DD1	++	-	+++	-
51.	BD3	++	+	+++	++
52.	KD3	-	-	-	+++
53.	MB2	+++	++	-	++

The following scale was used: (-) no inhibition zone, (+) inhibition zone =<4 mm, (++) inhibition zone = 5-7 mm, (+++) inhibition zone = 8-10 mm.

Conclusion

Lactic acid bacteria (LAB) are one of the most industrially important groups of bacteria. *Lactococcus* in particular, is a primary constituent of many starter cultures used for the manufacture of cheese, fermented milk and sour cream. Some genera of lactic acid bacteria such as *Lactococcus*, *Lactobacillus*, *Enterococcus* and *Pediococcus* have received attention as beneficial microorganisms. Lactic acid bacteria create a low pH environment, by fermenting several nutrients and produce antimicrobial factors, which can prevent contamination by pathogenic bacteria. During this study, 53 strains of lactococci were isolated from 76 samples of *dahi*, butter milk and fresh vegetables. In our study, the supernatants of *Lactococcus* strains (53 isolates) showed inhibitory effects against pathogenic bacteria. In conclusion, the present study showed that all the tested cell free supernatant isolates showed *in vitro* inhibitory zone against the tested pathogenic bacteria. Among the *Lactococcus* strains, (KD2 isolate) showed strongest inhibition zone.

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