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Isolation and characterization of dominant lactic acid bacteria from Dahi at Medinipur and evaluation of their antibacterial activity

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Abstract

Lactic acid bacteria (LAB) were isolated and identified from dahi samples and their antibacterial activity was investigated. A total of 92 LAB strains were isolated from 8 dahi samples taken at randomly around different areas of Medinipur town, India. They were identified and divided into 7 groups based on their phenotypic characteristics. The isolates were identified as Streptococcus, Lactobacillus, Enterococcus, Leuconostoc, Pediococcus and Weisella. First two groups comprised of 80% of LAB, whereas, two third (62) were homofermentative in nature.
Isolated LAB strains were tested for their antagonistic activity against pathogenic indicator bacterial strains Staphylococcus aureus (MTCC 3160), Bacillus subtilis (MTCC 1790), Salmonella enterica (MTCC 3223), and Escherichia coli (MTCC 443) using well diffusion method. Staphylococcus aureus was inhibited by all groups of isolated LAB, whereas, Bacillus subtilis was inhibited by least. Lactobacillus plantarum strains identified in this study showed antagonistic activity against all tested pathogens. The inhibition zone diameter by LAB ranged between 0.1 to 3.8 mm. The study showed that dahi samples in Medinipur town region contained wide variety of LAB that had an antimicrobial activity against pathogen and the inhibitory products were extracellular and diffusible.

Key words: dahi, lactic acid bacteria, strain characterization, pathogenic strain, antimicrobial activity

Introduction

Dahi is a traditional fermented milk product and a common menu in Indian subcontinent dishes. Commonly, it is produced from cow, buffalo or goat milk by traditional method using indigenous starter culture containing predominantly lactic acid bacteria (LAB). It has unique colour, texture and flavour. After production, dahi is taken alone or used as co-ingredients in other dishes. Lactic acid bacteria are the dominant microorganisms found in dahi (Rashid et al. 2007). These are a group of gram positive, low GC containing, acid tolerant, generally non-sporulating, catalase negative, coccoid or rod shaped bacteria. They are being used for thousands of years for the production of fermented food because of their ability to produce desirable changes in taste, flavour and texture. Most representatives of LAB do not pose any health risk to human and are designated as 'Generally Regarded as safe' (GRAS).
LAB are also used in making starter cultures for dairy product. Starter culture may consist of single or mixed strains. Lactic starters always include bacteria that convert lactose to lactic acid.
The reason behind selecting dahi as an obvious menu at the end of a meal is the belief that it supports digestion and alleviates the intestinal disorders. This doctrine can be supported by the fact that along with various organics for desirable flavour, LAB produces a number of antimicrobial substances also (Davidson and Hoover 1993). These substances act as natural competitor to inhibit other microorganisms sharing the same niche and thereby maintain the intestinal microbial balance. However, very few reports are available about the variety of LAB in dahi in India and their antibacterial activity.
The objectives of this study were to isolate and characterize the LAB from dahi collected from unorganized sectors of Medinipur town region in West Bengal, India, and to study their antimicrobial activity against various gram positive

and gram negative pathogenic bacteria responsible for serious human diseases.

Material and Methods

Chemicals and Bacterial strain. All chemicals and dyes used in this study were of analytical grade, purchased from Merck, India. The bacteriological media were obtained from HiMedia laboratories Pvt Ltd, India. The various gram positive i.e. Staphylococcus aureus (MTCC 3160), Bacillus subtilis (MTCC 1790) and gram negative bacterial strains i.e. Salmonella enterica (MTCC 3223), Escherichia coli (MTCC 443) were procured from Institute of Microbial Technology, Chandigarh and used for in vitro antimicrobial study.

Isolation and screening of LAB. Eight dahi samples from in and around different market areas of Medinipur town were collected randomly, transported to the laboratory immediately using cool box (4℃) and tested directly. Approximately 10.0 g of each dahi sample was mixed with 90 ml of sterile normal saline solution (8 g NaCИ), homogenized gently, diluted appropriately and spread plated aseptically on de Mann Rogosa Sharpe (MRS) agar media. Duplicate plates were incubated at 37℃ for 48 hours in anaerobic condition. Plates having less than 30 colonies were selected. Colonies differ in morphology, pigmentation, shape, and size were subcultured in MRS broth. Each individual subcultured sample was examined by gram staining and catalase production activity. Only gram positive and catalase negative samples were then purified by streaking and were stored in 0.8% MRS agar overlaid with 20% glycerol at −20°C for further experiment.

Identification of LAB. Each pure isolate was propagated twice in MRS broth before use and an overnight culture was used in each test. All isolates were initially gram stained and examined for cell size, cell form and cell arrangement. Growths of the isolates were assessed in MRS broth in different temperatures (15 and 45℃) and NaCl concentrations (4, 6.5 and 8%). Fermentative nature was evaluated by growing the isolates in homofermentative-heterofermentative differential (HDD) medium described by McDonald et al (1987). Homofermentative isolates produced blue colonies where heterofermentative produced white. Ammonia production by arginine hydrolysis was performed in MRS broth containing 0.3% arginine and 0.2% Na-citrate replacing ammonium citrate and esculine hydrolysis was performed in bile esculine azide agar medium. Exopolysaccharide (EPS) production by the isolated strain was examined by growing them on MRS agar medium containing 20 g⁄l sucrose (formation of slimy colony indicate positive result). Filter sterilized (0.22 µm nylon membrane) solution of sugar (D-glucose, D-fructose, lactose, sucrose, manitol, D-cellobiose, D-ribose, and D-mannose) was added to basal medium (MRS devoid of glucose and meat extract and contain 0.004% chromophenol red indicator) to a final concentration of 2% (w/v) for the evaluation of acid production from carbohydrate by the isolates.

Detection of antimicrobial activity. For the detection of antimicrobial activity, well diffusion method was used. LAB isolates were separately grown in MRS broth at 30℃ for 48 hours. After incubation, cell free solutions of bacterial cultures were obtained by centrifugation (10 min × 15000g at 4℃) followed by filtration of the supernatant by 0.22 µm cellulose acetate filter.

Overnight broth culture of target strains were inoculated (0.1 ml) on solid Mueller-Hinton agar medium by spreading. After 10 minutes of contact, the plates were dried for 20 minutes. Four wells were made and filled with 100 µl of previously prepared cell free solutions. Target strain inoculated plate with un-inoculated MRS broth served as control. Plates were incubated at 37℃ for 24 hours and diameter of inhibition zones were measured with callipers. The antimicrobial tests were done in duplicate and the mean values were recorded.

Results

Isolation and identification of the isolates. A total of 136 strains were isolated from the dahi samples. All except 44 were Gram positive, catalase negative, non-sporulating, non-motile facultative anaerobic strain. A final number of 92 strains were divided into 7 groups based on morphological, cultural, physiological and biochemical characteristics (Table 1). The isolates belonging to a given group were identified on the basis of species literature description.

More than 38% (35) of the isolates were rod shaped and the rest (57) were either spherical or ovoid shaped bacteria. Rod shaped isolates, strain A1 and A2 appeared singly or in short chains and unable to produce exopolosaccharides (EPS). They were regarded as belonging to the genus Lactobacillus.

All LAB isolates from A1 were homofermentative rod; showed incapability to hydrolyze arginine, to grow at 45℃ and at 8% NaCl. They were related to Lactobacillus plantarum. Their capability to hydrolyze esculine and acid production from all tested carbohydrates showed a high similarity profile with the mentioned species described in the literatures (Wheater 1955; Duk-Mo et al. 1994; Phalakornkule and Tanasupawat 2007).

The second type of Lactobacillus isolates (A2), accounting 65% of the genus and representating 25% of the total isolated starins were heterofermentative in nature. They could grow at 45°C and produced NH3 from arginine. They were also differing from group A1 in the deficiency of producing acid from manitol and cellobiose. They could be identified as Lactobacillus fermentum by comparing with the species description (Sanchez et al. 2000; Sanni et al.

2002; Rashid et al. 2007; Phalakornkule and Tanasupawat 2007).

Two types of heterofermentative cocci were isolated. Group A3 contained only two isolates. They were sphere-shaped, arranged in pair or tetrads, produced EPS, did not grow at 45°C but grew at 6.5% NaCl. They were identified as belonging to the genus Weissella. Based on other biochemical characteristics they could be identified as Weissella confusa or Weissella kimchii. (Savic et al. 2007; Phalakornkule and Tanasupawat 2007; Patil et al. 2010).

All 5 isolates of group A4 grew at 10°C and 4% NaCl; none grew at 45°C or in 6.5% NaCl, and none hydrolyzed arginine were identified as Leuconostoc (Centeno et al. 1996). All isolates produce exopolysaccharides and could be identified as Leuconostoc mesenteroides (Duk-Mo et al. 1994; Savic et al. 2007).

Table1**.** Biochemical and physiological characteristics of lactic acid bacteria isolated from fermented milk product *Dahi* in Medinipur town, India

Characteristics	**Strain Designation**						
	A1	A2	A3	A4	A5	A6	A7
Gram Stain	+	+	+	+	+	+	+
Catalase test	−	−	−	−	−	−	−
Number of isolates	12	23	2	5	38	5	7
Cell form	Long rod	Long rod	Spherical	Spherical	Spherical	Spherical	Ovoid
Cell arrangement	Single or short chain	Single or short chain	Pair or tetrads	Pair or short chain	Pair or chain	Pair or tetrad	Pair or short chain
Growth at							
Growth at 10°C	+	−	+	+	−	+	+
45°C	−	+	−	−	+	+	+
Growth at 4% NaCl	+	+	+	+	+	+	+
6.5% NaCl	+	−	+	−	−	+	+
8% NaCl	−	−	W±	−	−	+	−
Fermentative Nature	Homo	Hetero	Hetero	Hetero	Homo	Homo	Homo
Arginine hydrolysis	−	+	+	−	W±	+	+
Esculine hydrolysis	+	−	+	+	+	+	+
EPS production	−	−	+	+	−	−	−
Acid production from							
D-glucose	+	+	+	+	+	+	+
D-fructose	+	+	+	+	+	+	+
lactose	+	+	W±	W±	+	+	+
Sucrose	+	+	+	+	+	W±	+
Manitol	+	−	−	W±	+	−	+
D-cellobiose	+	−	+	+	+	+	+
D-ribose	+	W±	W±	W±	−	W±	+
D-mannose	+	W±	+	+	+	+	+
Identified as	*Lactobacillus plantarum*	*Lactobacillus fermentum*	*Weissella sp.*	*Leuconostoc mesenteroides*	*Streptococcus bovis*	*Pediococcus pentosaccus*	*Enterococcus sp.*

Legend: Positive (+), weakly positive (W±), negative reaction (−)

Group A5 contain most isolates (38) in dahi samples. These isolates were homofermentative sphere-shaped, arranged in pairs or short chain of 4 to 10 cells. They were identified as belonging to the genus Streptococcus. They produced orange or white pigmentation in MRS agar media. All the isolates exhibited growth at 45℃ but not at 10℃ and did not grow at 6.5% NaCl. No isolates produced acid from ribose and some strains hydrolyzed arginine. Therefore they could be recognized as Streptococcus bovis (Osawa and Sly 1991; Savic et al. 2007; Rashid et al. 2007).

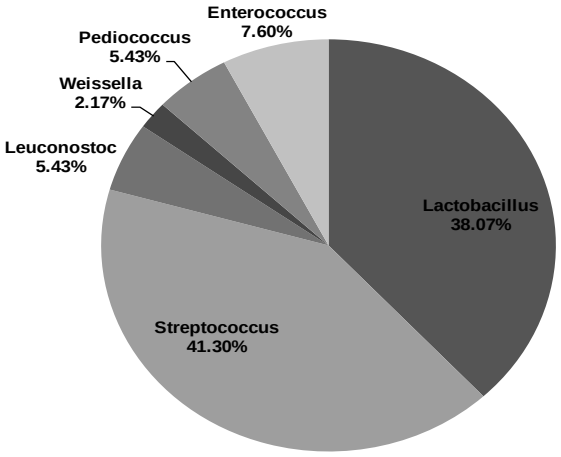

Figure 1. Lactic acid bacteria distribution at the genus level isolated from "Dahi" samples in Medinipur town, India.

Another group of homofermentative coccus (A6) appeared mostly in tetrads, grew both at 10℃ and 45℃ and even at 8% NaCl were identified as Pediococcus sp. They all hydrolyzed arginine and esculine but did not produce EPS. They all produced acid from all tested carbohydrate except manitol. On the basis of these characteristics they could be identified as Pediococcus pentosaccus (Rashid et al. 2007; Phalakornkule and Tanasupawat 2007; Savic et al. 2007).

Last group of the isolates (A7) were also homofermentative in nature. Ovoid cells, elongated in the direction of the chain, were generally appeared in pair or short chain. All isolates grew at both 10℃ and 45℃ and in 6.5% NaCl, hydrolyzed arginine and esculine. Therefore these strains were included in the genus of Enterococcus. Compairing all the characteristics tested in this study it is difficult to conclude about a particular species, however, from the species description they could be identified either as Enterococcus faecium or as Enterococcus faecalis (Schleifer and Kilpper-Balz 1984; Duk-Mo et al. 1994; Savic et al. 2007).

Out of 92 lactic acid bacterial strains, 38 belonged to the genus Streptococcus (41.3%), 35 to Lactobacillus (38.1%), 7 to Enterococcus (7.6%), 5 to both Leuconostoc and Pediococcus (5.4%) and 2 to Weissella (2.2%). Figure 1 illustrates the distribution of all 92 isolates from dahi samples in genus level.

Antimicrobial activity. All 92 isolated LAB strains were screened for their antagonistic activity against both gram positive (S. aureus, B. subtilis) and gram negative (S. enterica, E.coli) bacterial strains. Staphylococcus aureus was consistently inhibited by the isolated LAB strains (Table 2). Except Weissella sp. and Enterococcus sp. identified in this study, all other group were active against more than one tested strains. Only Lactobacillus plantarum strains showed wide inhibitory spectrum against all the tested strains, whereas Bacillus subtilis was inhibited by a few (four) Lactobacillus plantarum strains and by a single Weissella strain. A few Streptococcus isolates showed inhibitory activity against tested strains.

Table 2. Antibacterial spectrum of the cell free supernatant of isolated lactic acid bacteria. Number of positive strains are indicated in the parenthesis.

Bacterial Group/ strain identified	**Strain Inhibited**
Lactobacillus plantarum (12)	*Staphylococcus aureus* (8), *Bacillus subtilis* (4), *Salmonella enterica* (1), *Escherichia coli* (2)
Lactobacillus fermentum (23)	*Staphylococcus aureus* (19) , *Salmonella enterica* (4), *Escherichia coli* (11)
Weissella sp.(2)	*Bacillus subtilis* (1)
Leuconostoc mesenteroides (5)	*Staphylococcus aureus* (5), *Salmonella enterica* (2), *Escherichia coli* (1)
Streptococcus bovis (38)	*Staphylococcus aureus* (4), *Salmonella enterica* (7), *Escherichia coli* (2)
Pediococcus pentosaccus (5)	*Staphylococcus aureus* (5), *Salmonella enterica* (2), *Escherichia coli* (1)
Enterococcus sp. (7)	*Staphylococcus aureus* (5)

No LAB isolates showed consistent inhibitory activity against all tested strains (Table 3). Lactobacillus plantarum, however, showed maximum consistency against three tested strains Staphylococcus aureus, Bacillus subtilis and Salmonella enterica. Mean difference in inhibition zone diameter between test and control was ranged between 1.7 to 3.2 mm. Leuconostoc mesenteroides showed most inhibitory activity against Staphylococcus aureus and Escherichia coli. Mean difference in inhibition zone diameters were 3.8 and 3.1 mm respectively. Bacillus subtilis was mostly inhibited by Pediococcus pentosaccus, and, Salmonella enterica by Lactobacillus plantarum. Mean difference in inhibition zone diameters were 2.1 and 3.2 mm respectively.

Discussion

Our experimental results indicate clearly that streptococci and lactobacilli are the dominant LAB in dahi samples characterized here. Near about 80% representative of the LAB are from these two genuses. Streptococcus, Lactobacillus, Lactococcus and Leuconostoc are common lactic acid bacteria in traditional fermented milk products (Beukes et al. 2001; Rashid et al. 2007; El-Baradei et al. 2008) There are a few reports available about the diversity of lactic acid bacteria in curd in Indian subcontinent. However, our result is in agreement with previous study by Rashid et al (2007), where they reported the presence of 77% of these two among the LAB in various curd samples from Bangladesh. Apart from this, they found the presence of Entecoccus, Leuconostoc, Lactococcus and Pediococcus in their samples. In a study, Patil et al (2010) showed the presence of Pediococcus, Weissella and Lactobacillus in curd and cucumber samples from India. However, we did not find the presence of Lactococcus in our samples. Two types of lactobacilli, Lactobacillus plantarum and Lactobacillus fermentum were identified in our study. Lactobacillus plantarum may present in various fermented milk products (Centeno et al. 1996; Beukes et al. 2001; Kongo et al. 2007; Patil et al. 2010), Lactobacillus fermentum is more common in curd (Rashid et al. 2007; Patil et al. 2010). More than 30% of the isolates in our study were heterofermentative LAB that could have an important role in flavour and texture formation in curd

Table 3. Diameter of the zone of inhibition (mm) by different isolated strains against tested bacteria. The values are mean with standard deviation wherever applicable. Control diameter is indicated in the parenthesis.

	Lactobacillus plantarum	***Lactobacillus fermentum***	***Weissella***	***Leuconostoc***	***Streptococcus***	***Pediococcus***	***Enterococcus***
Staphylococcus aureus	13.2 ± 0.31 (10.3)	13.3 ± 0.25 (11.0)	13.2 ± 0.32 (10.3)	14.2 ± 0.21 (10.4)	11.2 ± 0.3 (10.6)	14.1 ± 0.17 (10.9)	13.3 ± 0.34 (10.6)
Bacillus subtilis	12.2 ± 0.23 (10.5)	10.9 ± 0.15 (10.7)	10.8 ± 0.21 (10.8)	10.8 ± 0.05 (10.7)	10.6 ± 0.14 (10.5)	13.1 ± 0.33 (11.0)	10.3 ± 0.18 (10.2)
Salmonella enterica	14.1 ± 0.26 (10.9)	13.7 ± 0.11 (10.9)	10.8 ± 0.13 (10.8)	11.5 ± 0.14 (10.7)	13.7 ± 0.2 (10.6)	11.8 ± 0.12 (10.9)	10.4 ± 0.12 (10.3)
Escherichia coli	11.4 ± 0.13 (10.7)	12.9 ± 0.21 (10.9)	10.4 ± 0.16 (10.3)	13.9± 0.22 (10.8)	11.2 ± 0.17 (10.7)	11.1 ± 0.24 (10.5)	10.3 ± 0.05 (10.3)

Lactic acid bacteria are reported to produce some antimicrobial substances that are inhibitory for spoilage and pathogenic bacterial strains. Low molecular mass substances like lactic acid (also lower the medium pH), H2O2, CO2, ethanol, diacetyl (also a flavouring agent) and high molecular mass compounds like bacteriocins are reported to be produced by LAB present in milk or fermented milk products (Alvarado et al. 2006; Ammor et al. 2006; Rattanachaikunsopon and Phumkhachorn 2010). The cell free solution of the isolated LAB were tested to know if the antimicrobial metabolites were extracellular and released into the growth medium. In the test all the isolated LAB genera showed the inhibition of all the tested pathogenic strains indicates that the inhibitory metabolites produced by the isolates were extracellular and diffusible. All the isolates of different genera showed inhibition against tested strains to varying degrees (Table 2). For example, we have isolated and characterized 12 strains as Lactobacillus plantarum. Eight of them showed inhibition against Staphylococcus aureus ,4 against Bacillus subtilis, two against Escherichia coli and only one against Salmonella enterica. Similar results were reported by Alvarado et al (2006) who showed only 25 out of 94 isolated LAB strains from traditional Mexican foods were able to show inhibition against at least one pathogenic indicator microorganism. Ammor et al (2006) reported only 36 strains to do that out of 87 LAB isolated from traditional dry sausage. The inhibition zone diameter by cell free

extracts of LAB including Lactobacillus, Pediococcus, Streptococcus and Leuconostoc against Staphylococus aureus, Salmonella sp and Escherichia coli O157:H7 ranged from less than 3 to 4 mm (Tadesse et al. 2005), where as, extracted bacteriocin from common LAB of fermented milk showed 8 to 12 mm inhibition zone (Savadogo et al. 2004). In our experiment, the additional zone of inhibition (of cell free solutions) over control ranged from 0.5 to 3.8 mm (excluding the incidences where additional zone were up to 0.2 mm). Staphylococus aureus was common in inhibition pattern as it was inhibited by the isolates of all genera (Table 3). Inhibition of this pathogen by common LAB from fermented food was shown in several reports (Savadogo et al. 2004; Tadesse et al. 2005; Alvarado et al. 2006; Ammor et al. 2006; Abdelbasset and Djamila 2008). Bacillus subtilis was least inhibited bacteria in our study, only inhibited by Pediococcus sp. and Lactobacillus plantarum. L. plantarum is reported to show its antagonistic activity of over B. subtilis by producing bacteriocin (Abdelbasset and Djamila 2008). There are also several reports of growth inhibition of Salmonella by Streptococcus, and E.coli by L. mesenteroides and L. fermentum (Savadogo et al. 2004; Tadesse et al. 2005; Abdelbasset and Djamila 2008). Similar results were also observed here. Antimicrobial property shown may be due to various reasons that should be studied later.

In conclusion, curd samples from unorganized sector in Midnapore, West Bengal, India contain a wide diversity of LAB, where Lactobacillus and Streptococcus are dominant and widespread genera found. They have antimicrobial activity against some indicator pathogens. The inhibitory products are extracellular and diffusible. These strains have potential for natural preservative for traditional milk product dahi. Such a collection could be used as starter culture to improve the technological properties for the preparation of dahi from different origin of milk as organised small scale dairy industry in West Bengal, India.

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