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Characterization of lactic acid bacteria isolated from indigenous dahi samples for potential source of starter culture

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Diversity and density of lactic acid bacteria from indigenous dahi were studied by the determination of morphological, cultural, physiological and biochemical characteristics. A total of 143 isolates were identified phenotypically and divided into three genera: *Lactobacillus, Lactococcus* and *Streptococcus*. The microorganisms isolated were *Lactobacillus bulgaricus* (23.77%), *Streptococcus thermophilus* (26.57%), *Lactococcus lactis* (13.9%), *Lactobacillus acidophilus* (9.79%), *Lactobacillus lactis* (9.79%), *Lactobacillus delbreuckii* (4.89%), *Lactobacillus helveticus* (2.79%), *Lactobacillus casei* (1.39%), *Lactobacillus casei* ssp. *psuedoplantarum* (2.79%), *Streptococcus cremoris* (2.09%) and *Streptococcus lactis* sub. *diacetylactis* (2.09%).

Key words: Lactic acid bacteria, fermented milk, dahi, characterization.

INTRODUCTION

Dahi analogue to yoghurt is a popular fermented milk product of Indo-Pak subcontinent. It's consumption stands next to whole milk especially during summer. Dahi has been reported to contain a mixture of lactic acid bacteria (LAB) in addition to *Lactobacillus bulgaricus* and *Streptococcus thermophilus* mostly used cultures for yoghurt making (Masud et al., 1991).

The developing interest in microorganisms found in food is primarily due to the biotechnological potential of new bacterial species and strains (Leisner et al., 1999). LAB widely distributed in nature and found naturally as indigenous micro flora in raw milk are Gram positive, catalase negative microorganisms that play an important role in many food and feed fermentation. For many centuries, LAB has served to provide an effective form of natural preservation. In addition, they strongly determine the flavor, texture and frequently, the nutritional value of the food and feed products.

LAB is also capable of producing inhibitory substances known as bacteriocins that are antagonistic towards other microorganisms. In several, fermented dairy products, LAB contribute to the characteristic structure of the product by producing exo-polysaccharides (Talarico and Dobrogosz, 1989).

In Pakistan, 70% of the dairy products viz dahi, butter and cheese available in the local market are prepared from buffalo milk in addition to cow, sheep, camel and goat's milk. In these products, the species combination of LAB is more varying and inconsistent as compared to those of the trade products. In biotechnological aspects, the wild strains of the LAB are prospective bacteriocins producers (Padmanabha-Reddy et al., 1994; Park et al., 2003) and probiotics (Rinkinen et al., 2003).

The aim of this work was the isolation and taxonomic determination of a large number of LAB from dahi samples in order to develop an indigenous culture collection

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of LAB strains and to use them as starter for improving the quality of different traditional fermented milk products such as yoghurt, cheese and allied products.

MATERIALS AND METHODS

Isolation of bacterial strains

The LAB was isolated from indigenous dahi samples. The isolation was performed by the routine microbiological procedures and inoculation on a solid medium. Selective media for LAB used were MRS and M17 agar plates. The samples were inoculated on the media and then incubated for 48 h at 37°C. The colonies that showed different morphological characteristics were then identified by using various biochemical tests as described by Collins and Lyne (1980).

Physiological and biochemical tests

All strains were initially tested for Gram reaction and catalase production (Harrigan and McCance, 1976; Sharpe 1979). Cell morphology and colony characteristics on MRS and M17 agar plates were also examined and separation into phenotypic groups was undertaken. Only the Gram positive, catalase negative isolates were further identified. Growth at different temperatures was ob-served in MRS and M17 broth after incubation for five days at 15, 37 and 45°C. Supplemented test was performed and resistance at 63°C for 30 min was done in order to discard enterococcus bacteria. Growth in the presence of 4 and 6.5% NaCl was performed in MRS and M17 broth for five days. Production of CO_2 from glucose was done in MRS broth containing inverted Durham tubes.

Growth at different temperatures

Growth at 15 and 45°C are most frequently used criteria for the classification of bacilli (Hammes and Vogel, 1995). *Lactobacilli* cannot grow at 15°C, however can grow at 45°C. For the classification of cocci isolates, the growth temperatures of 10 and 45°C were used. *S. thermophilus* cannot grow at 10°C, but can grow well at 45°C. In order to determine the growth at given temperatures, the modified MRS and M17 media given in the Appendix B were used. Basically, all ingredients were the same, except for Bromocresol purple. Bromocresol purple was used to determine the color change in acidity with from purple to yellow, indicating lactic acid production and cell growth. Fifty microliters of overnight activated cultures were inoculated into 5 ml test media and the incubation period at the given temperatures was observed for seven days to determine the growth and color change. For performing the biochemical tests, all strains were tested for fermentation of the following seven sugars: Lactose, Glucose, Sucrose, Maltose, Mannitol, Galactose, and Fructose. To ensure anaerobic conditions; two drops of sterile liquid paraffin were placed in each tube after inoculation. For further identification of the LAB, API 50 CH tests (bioMerieux) were also used.

Growth at different NaCl concentration

Unlike other bacilli and cocci, *Lb. delbrueckii* ssp*. bulgaricus Lb. acidophilus* and *S. thermophilus* are highly sensitive to NaCl. *S. thermophilus* does not grow even at 2% NaCl concentration, but there is no data available for *Lb*. delbrueckii ssp. bulgaricus. The most frequently used NaCl concentration for the identification of bacilli are 4 and 6.5% salt concentrations. Hence growth at 2 and 4% NaCl concentrations were used for cocci isolates and the growth at 4 and 6.5% NaCl concentrations were used for bacilli isolates in NaCl test medium (Appendix B). Fifty microliters of overnight activated cultures were inoculated into 5 ml NaCl test media and the incubation at 42°C was observed for 7 days to determine the growth with the indication of color change from purple to yellow.

Unlike other bacilli and cocci *Lb. bulgaricus* and *S. thermophilus* are highly sensitive to NaCl. *S. thermophillus* does not grow even at 2% NaCl concentration, but there is no data available for *Lb. bulgaricus*. The most frequently used NaCl concentration used for identification of bacteria is 4 and 6.5% salt concentration. Hence growth at 2 and 4% NaCl concontration were used for cocci isolates and growth at 4 and 6.5% NaCl concentration for bacilli isolates in Nacl test medium. (50 µl of overnight activated culture was inoculated at 5 ml NaCl test medium and incubation at 42°C was observed for seven days so as to determine growth with indication of color change from purple to yellow).

RESULTS AND DISCUSSION

The lactic acid bacteria were isolated from indigenous dahi samples. The isolation was performed by the routine microbiological procedure and inoculation on a solid medium. Selective media were used for LAB were M17 and MRS plates. In most of the cases more than one colony was observed on the surface of M17 and MRS plates. In most of the cases more than one colony was observed. The cultural and morphological characteristics were examined within the help of microscope. Different types of microorganisms were observed, majority of them belonged to Gram positive rods and cocci shaped bacteria.

The Gram positive rods and cocci shaped bacteria were specifically transferred to the plates of selective media MRS and M17 respectively to purify the isolated. Subculturing of the isolate was done until pure isolates were obtained. Once pure colonies were obtained they were cultured in MRS and M17 and stored at 4°C in refrigerator until it was used.

From fifty samples, a total of 143 isolates were Gram positive and catalase negative bacteria were recorded. The organisms identified in this study are listed in Table 1. The Majority of the isolates identified belongs to genus *Lactobacillus* and the rest were referred to genus *Lactococcus* respectively. Results reveal that LAB dominated the microbial flora of dahi. It might be due to the reason that two specific media MRS and M17 agar were used to study the morphological characteristics of rods and cocci isolates respectively. This selective media allows only specific type of microorganisms to grow therefore the ability of bacterial species to grow on specific media is regarded as an important characteristic in identification. MRS and M17 media are the best suitable media for the isolation of LAB as reported earlier by Ghoddusi (2002).

All the isolates lactobacilli grew at 37 and 45°C but non at 15°C and also produced no gas from glucose and no growth at 4 and 6.5% concentration except *Lb helvitus*. None of the *Lactobacilli* isolates grew at 15°C and all characteristics of LAB, attention will be given to the

Table 1. Incidence of Lactic acid bacteria isolated from indigenous dahi samples.

Isolated specie	Number of strains/(percentage)
Lb. delbrueckii ssp. *bulgaricus*	34 (23.77)
Lb. delbrueckii ssp. *lactis*	14 (9.79)
Lb. acidophilus	14 (9.79)
Lb. helveticus	4 (2.79)
Lb. delbrueckii	7 (4.89)
Lb. casei	2 (1.39)
Lb. casei ssp. *psuedoplantarum*	4 (2.79)
L. lactis	20 (13.9)
S. thermophilus	38 (26.57)
S. cremoris	3 (2.09)
S. lactis ssp. diacetylactis	3 (2.09)
Total number of strains	143

distinguish sugars for the purpose of indicating which were more relevant to the identification. All the dahi isolates fermented Lactose and glucose except four isolates of *Lb. acidophilus* which were unable to ferment glucose, whereas all the isolates of *Lb. acidophilus* ferment sucrose, maltose, galactose and fructose (Table 2).

The presence of such bacteria has been reported in earlier studies (Samelis et al., 1994; Masud et al., 1993; Naeem and Rizvi, 1983). Moreover, it was observed that all the isolated bacteria from indigenous dahi were thermophilic and mesophilic in nature (Kosikowski, 1982). This diversity of species is relative and dependent primarily on the nature of the material isolated and different criteria used for each study as reported by Fitzsimmons et al. (1999) and Bissonnette et al. (2000).

Out of 143 isolates, 38 (26.57%) were of *S. thermophilus* followed by 34 (23.77%) *Lb. bulgaricus,* 20 (13.9%) *L. lactis,* 14 (9.79%) *Lb. acidophilus,* 14 (9.79%) *Lb. delbreuckii* sub *lactis*, 7 (4.89%) *Lb. delbreuckii,* 4 (2.79%) *Lb. helveticus,* 4 (2.79%) *Lb. casei* ssp. *psuedoplantarum,* 3 (2.09%) *S. cremoris*, 3 (2.09%) *S. lactis* ssp. *diacetylactis* and 2 (1.39%) *Lb. casei*.

It was observed that *S. thermophilus* and *Lb. bulgaricus* along with *L. lactis* constituted the dominant micro flora of dahi. Thus these species play an important role for the preparation of fermented milk products, as also noted by Warsey (1983).

S. thermophilus is mostly used in the manufacture of yoghurt, Mozzarella cheese and in some other cheeses such as cheddar cheese etc. It is further reported by Hitchener et al. (1982) that it provide protection against phages when used in combination with lactococci and produce acid rapidly during scalding.

The presence of large number of *Lb. bulgaricus* as recorded in the present investigation could be attributed to the presence of old inoculums containing large number of *Lb. bulgaricus.* Similar views are expressed by Mohanan et al. (1983). They are mainly used for acid and flavor production in yoghurt making (Kosikowski, 1982). For the thermophilic lactobacilli, the production of flavor is largely due to acetaldehyde, which is regarded as being the most characteristic flavoring compound in dairy products. Indeed, *Lb. bulgaricus* plays an active part in the production of flavor resulting from threonine (Zourari et al., 1991). The data about the phenotypic characterization, identification and biochemical characteristics of isolates is presented in Table 2.

The presence of *Lactococcus lactis* and *Streptococcus cremoris* in our study is of great importance. Several studies reported that *L. lactis* was more frequently isolated from raw milk samples (Weerkamp et al., 1996; Badis et al., 2004), raw milk cheese (Centeno et al., 1996), Pecornio Sardo cheese (Mannu et al., 2000), Moroccan traditional fermented milk Raib (Hamama, 1992), Dahi and buttermilk samples from India (Padmanabha-Reddy et al., 1994) and Amazi, fermented milk in Zimbabwe (Mutukumira, 1996), similar findings have been reported by Guessas and Kihal (2004), who on the basis of morphological, cultural, physiological and biochemical characteristics found that Algerian raw goat milk carried different LAB with majority of *Lactococcus lactis*. These results are also in agreement with the findings of El Shafei et al. (2002).

Kosikowski (1982) reported that *L. lactis* and *S. cremoris* are used for the preparation of different types of cheese. However, they may be used for the preparation of yoghurt where multi-strains starter culture is used to produce the desired acidity. The role of these strains have not been yet characterized, however, they may play a role in the preparation of this product in winter season. Different studies elsewhere reported that these strains have ability to produce slime characteristics, which may be used to improve the quality of the final product.

The low incidence of *S. cremoris* strains in this study might be due to the reason that the incubation temperature was 37°C during isolation and identification of the bacterial strains and *S. cremoris* strains are mesophilic in nature. In addition, the study demonstrates the problem in attempting to classify the LAB found in dahi samples using the classical methods, which were developed largely

Table 2. Phenotypical characteristics of lactic acid bacteria isolated from indigenous Dahi.

Characteristics	*Lactobacilli*							*Cocci*			
	Lb. delbrueckii ssp bulgaricus	*Lb. delbrueckii ssp lactis*	*Lb. acidophilus*	*Lb. helveticus*	*Lb. delbrueckii*	*Lb. casei*	*Lb. casei ssp. psuedoplantarum*	*S. thermophilus*	*L. lactis*	*S. cremoris*	*S. lactis ssp diacetylactis*
	Number of isolates										
	34	14	14	4	7	2	4	38	20	3	3
Gram stain reaction	+	+	+	+	+	+	+	+	+	+	+
Catalase reaction	-	-	-	-	-	-	-	-	-	-	-
CO_2 from glucose	-	-	-	-	-	-	-	-	-	-	-
Growth at											
10°C	-	-	-	-	-	-	-	-	+	+	+
37°C	+	+	+	+	+	v	+	+	+	-	+
45°C	+	+	+	+	+	v	-	+	-	-	-
Growth in medium											
4% NaCl	-	-	-	+	-	+	-	-	+	-	+
6.5%NaCl	-	-	-	+	-	-	-	-	-	-	-
Sugar fermentation											
Lactose	+	+	+	+	+	+	+	+	+	+	+
Glucose	+	+	10	+	+	+	+	+	+	+	+
Mannitol	-	-	-	-	-	+	+	-	-	-	+
Sucrose	-	+	+	-	+	+	+	+	+	-	+
Maltose	-	+	+	+	d	d	+	+	+	+	-
Galactose	+	+	+	+	w	+	+	-	+	-	+
Fructose	+	+	+	-	+	+	+	-	+	-	+

Positive Reaction (+), Negative Reaction (-), Weak reaction (w), delayed Reaction (d), varying reaction (v) the number designate the amount of positive strains.

for bacteria of dairy origin. In this regard, genotypic tests should confirm the phenotypic results. Identification of *S. cremoris* is becoming scarce in industrial countries but some cases have been described in milk from Morocco or Eastern Europe (Salama, 1995).

Fourteen (14) strains of *Lactobacillus acidophilus* are recorded in the present study. These strains are considered to produce higher titrable acidity and result in the production of low pH that may be considered objectionable (Naeem and Rizvi, 1983). However, the results of these studies report that these strains have the ability to produce bacteriocins and widely used as a probiotic. Its importance is well documented by Isani et al. (1986). Due to the fact that *Lb. acidophilus* produces D-lactate, there have been concerns about its use in infant nutrition. Therefore, we must have to look its role in dahi making with special reference to public health.

Four strains of *Lb. helveticus* and three strains of *S. lactis* ssp. *diacetylactis* are reported in this study. *Lb. helveticus* strains are capable of producing high acidity of 2% or more of lactic acid. They can be used for the production of yoghurt in combination with other strains as reported by Badis et al. (2004). These strains could be used as starters, in the manufacture of dairy products with organoleptic qualities liked by tasters. *S. lactis* ssp. *diacetylactis* are mesophilic strains, which might be the reason for their low incidence in dahi samples. They have low acidifying activity, high diacetyl content and mostly

involved in flavor and aroma production. They are mostly used for cheese and buttermilk production (Badis et al., 2004).

Only two strains of *Lb. casei* have been reported in the present investigation. *Lb. casei* is used as a probiotic although it is found in some starter cultures and is commonly one of the numbers of non-starter lactic acid bacteria found in cheddar cheese (Gomes and Malcata, 1998).

The difference in the microbial composition observed among the tested samples may be contributed to the starter culture used in their preparation, processing techniques and also to the duration of fermentation. The different microbial composition may also be due to the competitive difference between the different microorganisms. The result of this study also reveals that selected LAB can be implicated in the fermentation of dahi. Therefore, there is a need in the selection of most suitable strains for controlled fermentation of dahi. The potential use of our strains as starter culture will depend on further studies on selected isolate and assessment on the effect of the quality of yoghurt.

The identified isolates will undergo tests for lactic acid production and selected for further tests (production of bacteriocins, organic acids and volatile compounds) to asses their potential as starter culture in yoghurt making. The lactic acid bacterial starter culture can greatly contribute in solving the problem of inconsistent quality and short shelf life of dairy product in Pakistan.

On the basis of these results, it can be concluded that indigenous dahi contains a mixture of lactic acid bacteria, and the quality of dahi varies with the type of species predominant in the starter culture. Therefore, efforts should be made to select suitable indigenous strains of LAB in order to produce high quality fermented dahi and its allied products.

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