ISOLATION AND IDENTIFICATION OF DOMINANT LACTIC ACID BACTERIA FROM DAHI: AN INDIGENOUS DAIRY PRODUCT OF NEPAL HIMALAYAS

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ABSTRACT

Morphological, cultural, physiological and biochemical characteristics were employed to identify dominant Lactic acid bacteria (LAB) isolates from 39 dahi (indigenous dairy product) samples collected from different districts of eastern Nepal. The isolates comprised of predominately Lactobacillus fermentum, Lactobacillus delbrueckii subsp. bulgaricus, Streptococcus thermophilus, Lactococcus lactis subspecies cremoris, Lactococcus lactis subsp. lactis biovar diacetylactis and Leuconostoc mesenteroides subsp. mesenteroides. S. thermophilus were found consistently in most of the samples examined. In this study, 59.38% of Lactobacillus, 21% of Streptococci, 8.2 % Lactococci and 11.42 % Leuconostocs were isolated from indigenous dahi. This study revealed diversity of lactic acid bacteria in Nepalese milieus having immense potential in producing qualitative fermented milk.

Keywords: Isolation, Characterization, LAB, fermented dairy product, dahi

INTRODUCTION

Fermentation is one of the ancient food processing and preserving techniques used all around the world. There are many indigenous fermented foods from different regions. Dahi or curd is one of the popular fermented dairy products from South Asia. It is prepared from boiled milk, fermented in a traditional way by natural micro flora (Bhattarai and Das, 2013). Traditionally, dahi is consumed as a dessert or refreshing beverage along with main course in different rituals (Kharel et al., 2010). In Nepal, it has been consumed with boiled rice or “chevra” (rice flakes) (Dewan and Tamang, 2007) or use it to make as lassi (popular, traditional, yogurt-based drink) and dahi-wala (dish consisting of balls made from ground lentils, deep-fried and served in a yogurt sauce) (Bhattarai and Das, 2013). Fermenting milk to dahi itself is a primary step for other indigenous dairy products like nauni (Nepalese traditional butter), ghee (traditional clarified butter), mohi (traditional butter milk) and chhubri (traditional hard cheese).

As much as 45 % of total milk production is fermented into dahi in Eastern Nepal (Bhattarai and Das, 2013). In Nepalese indigenous way, dahi is fermented in theki (a wooden utensil) made from daar (Boerhertia rubogosa) wood. Main purpose of daar theki is believed to give unique flavor in dahi (Bhattarai and Das, 2013) and might serve as natural micro flora reservoir. Flavor chemistry and microbial role are major subjects of interest in dahi. Microbiology and diversity have been studied by some researcher in indigenous dairy products from different parts of world. Knowledge on microbial diversity of various indigenous foods can guide us in to different area. Typical microbial species and strains from indigenous food origin could be used to make starter culture or other industrial product. Among many important species, groups of Lactic Acid Bacteria (LAB) are vital and natural micro flora in different foods due to their unique fermentation mechanism (Gilliland, 1990) relating to their health and nutritional benefits (Francois et al., 2007). LAB isolation from fermented milks have been practiced to screen desirable traits so as to obtain consistent quality, high productivity and safety (Erkus, 2007).

Isolation of microbial strains from ready to eat food products helps in maintaining the food safety standards in food industries (Nawaz and Bhattarai, 2015). Identification and characterization of LAB in various indigenous foods from different parts of the world have been reported, for example: rob of Sudan (Abdelgadir et al., 2001; Abdalla and Hussain, 2010), rayeb of Egypt (Al Rubayyi et al., 2010), amasi of Zimbabwe (Gran et al., 2003), Falali of Burkina Faso (Savadogo et al., 2004), keluny of Kenya (Mathala et al., 2004), laban of Lebanon (Chammas et al., 2006), nyarmie of Ghana (Obodai et al., 2005), fermented milk from the Romania (Zamfir et al., 2006), Tibet (Airdengcailiche et al., 2010) and Mongolia (Oki et al., 2014). Very limited studies have been reported on microbial diversity and safety on Nepalese fermented milk products. This paper is therefore aimed at isolating and identifying lactic acid bacteria associated with indigenous dahi from eastern Nepal.

MATERIAL AND METHODS

Collection of samples

Thirty nine indigenous dahi samples were collected from sixteen districts of eastern Nepal. Preliminary study was done to confirm the highly dense dahi producing locations in different districts. No specific permissions were required for sample collection in these areas as local people were helpful and were enthusiastic to understand the quality and microbial flora of indigenous dahi. The samples were aseptically collected in sterile screw capped test tubes using sterile latex gloves and kept cool in ice-box until taken to the food microbiology laboratory at Central Campus of Technology, Dharan, Nepal. Samples were kept below 4°C for further use and examinations. No human or animals were used in the experiments as well as the field studies did not involve endangered or protected species.

Isolation of Lactobacilli, Lactococci, Leuconostoc, and Streptococci

Twenty five grams of dahi samples were homogenized with 225 mL Quarter Strength Ringer’s solution to make an initial dilution (10^-3) and decimal dilution techniques were applied according to Public Health England (2014). Aliquots with various dilutions amounting 0.1 mL were spread plated onto duplicate of different media plates (MRS agar, SL agar and D agar). MRS agar (Himedia, Mumbai, India) plates were incubated under anaerobic condition in an Anaerobic Gas-Pack system (Himedia, Mumbai, India) at 30°C for 48–72 h to isolate...
Lactobacilli (Badis et al., 2004a); Streptococcus lactis differential agar plates (SL) (Himedia, Mumbai, India) were incubated at 37°C for 48 h to differentiate citrate utilizing and non-utilizing Lactococci (Kempler, Mckay, 1980); MRS-vancomycin (vancomycin 20 mg L\(^{-1}\)) plates were incubated at 30°C for 24 h to isolate Streptococci and enterococci (Garrido et al., 1994); differential agar mediums (D) were incubated at 32°C for 48 h to differentiate S. lactis and S. cremoris (Reddy et al., 1969) and S. thermophilus (ST) agar plates with added cycloheximide (100 mg L\(^{-1}\)) for inhibition of yeast growth (Beukes et al., 2001) were incubated at 42°C for 24-48 h to isolate Streptococci (Atlas, 2004). Colonies were selected randomly from agar plate. If the plate contained less than 10 colonies, all colonies were isolated. Purity of the isolates were checked by streaking again and sub-culturing on fresh media plates, followed by microscopic examinations.

Isolated strains of Lactobacilli and Streptococci were preserved in MRS and ST broth at -20°C. Purified strains of Lactococci and Leuconostoc were preserved subsequently on the same media through periodic transfer.

Identification of the bacterial strains

Those preserved isolated strains were tested for gram staining, catalase production and spore formation by method from Harrigan and McCance (1976). Hydrolysis of arginine, citrate utilizations, gas formations from glucose in MRS broths containing inverted Durham tubes, dextran productions from sucrose in MRS+ST agar, growths on different temperature (10, 37 and 45°C) for 5 days, resistance to 60°C for 30 min (Sherman test), growths in the presence of 4 and 6.5% (w/v) NaCl and different pH (4.5 and 6.5) and changes in turbidity of MRS broth after 24, 48 and 72 h of incubations were implicated to identify the strains (Mayeux et al., 1962; Sharpe, 1979; Samelis et al., 1994). Arginine MRS medium and Nessler reagent were employed to perform the hydrolysis tests as described by Yavuzdurmaz (2007). Citrate utilization and colored colonies growth were observed in SL and D agars and results were interpreted according to Reddy et al. (1969) and Kempler and Mckay (1980).

Sugar fermentation tests

Membrane (0.45 μm) filtered 1% (w/v) solutions of different sugars (glucose, fructose, lactose, galactose, maltose and mannitol) were deployed to study fermentation characteristics of the isolates. Nutrient broth (0.8%) with 1 mL, phenol red was autoclaved at 121±1°C for 15 minutes then cooled to room temperature. Five ml of broth and 100 μL of sugars were taken into sterilized test tubes. These tubes were checked for contamination by placing at room temperature for 24 hours. After 24 hours, the purified colonies were inoculated into test tubes with specific sugar containing broth and incubated at 37°C for 48 hours. The positive test for sugar fermentation was indicated by color change from red to yellow in the test tubes as mentioned by Mehmood et al. (2009).

RESULTS AND DISCUSSION

Isolation and characterization of thermophilic LAB

Isolation and characterization of thermophilic LAB is highly desirable for dairy manufacturing as the selective species are used as starter cultures. Lactobacilli and Streptococci isolates characterized and counted are presented in Figure 1. Mean counts of all Lactobacilli were 133×10⁴ cfu g\(^{-1}\) representing 59.38% and Streptococci were 47×10⁴ cfu g\(^{-1}\) representing 21% of total isolates from dahi samples. The isolated Lactobacilli were further characterized into different species based on their physiological and biochemical properties (Table 1). In most of our indigenous collected dahi samples, Lactobacilli were dominant bacilli and S. thermophilus were dominant cocci. These findings are according to previous one on Mongolian fermented dairy product where Lactobacilli are dominant (Oki et al., 2014). S. thermophilus plays major roles in the coagulation of milk and is responsible for the production of dahi and its quality. Predominant role of Lactobacilli and Streptococci in the indigenous sample from eastern Nepal were somewhat different from some previous results for fermented milks from different origin (Baldorj et al., 2003; Mathara et al., 2004; Xiao et al., 2004, Harun-ur-Rashid et al., 2007, Watanabe et al., 2008, Yu et al., 2011). Not always Lactobacillus and Streptococcus are dominant in all traditional fermented dairy products such as Leuconostoc are dominant over Lactobacillus South African fermented milk (Beukes et al., 2001). On the other hand, S. thermophilus are important in Greek feta cheese (Manolopoulou et al., 2003), in traditional Ugandan beverage busehra (Muyanja et al., 2003) and commercial Nigerian bottled yogurt (Omuofwe et al., 2011). While, dahi from Bangladesh could be considered different in terms of dominant S. bovis instead of S. thermophilus (Harun-ur-Rashid et al., 2007) than dahi from eastern Nepal.

![Isolated strains](image-url)

**Figure 1** Mean counts (cfu g\(^{-1}\)) of isolated strains of thermophilic lab found in dahi samples obtained from different districts of eastern Nepal

LAB are omnipresent in dairy products. Physiological and biochemical characteristics of isolated LAB strains from dahi of eastern Nepal were done (Table 1). Presence of different Lactobacilli species in dahi samples from Eastern Nepal can be explained by presence of these bacteria as natural micro flora in raw milk sources. To produce different dairy product, it is important to use specific fermentation methodology along with natural micro flora and desired fastidious species such as L. delbrueckii subsp. bulgaricus, L. helveticus etc. L. helveticus has been major contributor in starter cultures of some cheese like Gruyere, Gorgonzola and Mozzarella (Tserovska et al., 2002). This can be explained as the different strains are indigenous micro biota to milk from different animal sources. Raw milk from four races of Algerian goat was abundant of L. helveticus and L. delbrueckii subsp. bulgaricus (Badis et al., 2004b). Other Lactobacilli species like L. plantarum, L. brevis and L. delbrueckii subsp. bulgaricus are isolated from busehra (Muyanja et al., 2003) and L. delbrueckii subsp. bulgaricus from South African traditional fermented milk (Beukes et al., 2001).

<table>
<thead>
<tr>
<th>Characteristics of the Strains</th>
<th>L. fermentum</th>
<th>L. casei subsp. casei</th>
<th>L. delbrueckii subsp. bulgaricus</th>
<th>L. helveticus</th>
<th>L. brevis</th>
<th>S. thermophilus</th>
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<td>Gram Strain Reaction</td>
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<td>Glucose Fermentation</td>
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<td>Growth in a Medium With</td>
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<td>Growth at pH</td>
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</table>
Production Of CO₂ from Glucose
Dextran Production
Citrate Utilization
Heat Resistance at 60 °C for 30 min
Sugar fermentation tests
Fructose
Galactose
Glucose
Lactose
Maltose
Sucrose
Mannitol

Growth in a Medium With NaCl, %
NH₃
Glucose Fermentation
Catalase Activity
Dextran Production
Production Of CO₂
Heat Resistance at 60 °C for 30 min
Sugar fermentation tests

Legend: V=Variable.

In Tibetan traditional fermented milk 71.3% bacilli and 28.7% cocci have been screened and two species L. fermentum and L. casei are predominant along with variation between different regions (Airidengcaicike et al., 2010). This could be due to the fact that, micro flora in dairy products is affected by the climatic and other external factors like altitude. In cold climatic regions mesophilic organisms such as Lactococcus and Leuconostoc have been found to be dominant in fermented milk products while thermophile bacteria such as Lactobacillus and Streptococcus in warm regions as explained by Kurman (1984). Differences in the profile of LAB flora in our study as compared to previous findings could be attributed to variations in the specific environmental conditions found in Himalayan region specifically in eastern Nepal. The altitude is considered as one of the important factors that influenced the climate and then temperature variations in the different parts of Nepal. Eastern Nepal is divided into three zones, namely, Mechi, Koshi and Sagarmatha ranging from plain terai to Mt. Everest. Moreover, cold climatic zone in upper Himalayan area and hilly area and hot climatic terai have a significant role in the natural micro flora statistics. The environmental factors, such as dry climate, low temperature, scant oxygen, low atmospheric pressure, strong sunlight and long sunlight radiation in Himalayas may also contribute to these variations. This present findings are according to previous findings on different fermented dairy product from Mongolia (Okè et al., 2014), Kenya (Mathala et al., 2004), Nigeria (Olasupo et al., 2001) and Bangladesh in terms of L. fermentum. It has also been doubted on the negative role of L. fermentum as weak coagulant, gas producer giving bad texture and taste in dairy products (Harun-ur-Rashid et al., 2007). In addition, this bacterium has been connected to many potential probiotic properties, such as acid resistance, bile salt tolerance and indigestible carbohydrate degradation (Airidengcaicike et al., 2010). Therefore, these strains required to be evaluated for probiotic applications and commercialization of indigenous dahi.

Isolation and characterization of Lactococci

Lactococci species contribute to more subtle aromas and flavor that distinguish the fermented dairy products. Gram positive, catalase negative and non-sporo forming isolates were isolated from 39 different samples using SL and D agar. These were further characterized as mesophilic homo-fermentative cocci. The counts of these isolates were as presented in Figure 2. Total 7.282×10⁵±45.19 cfu.g⁻¹ of L. lactis subsp. lactis biovar. diacetylactis, 2.221×10⁸±13.84 cfu.g⁻¹ of L. lactis subsp. lactis and 9.05×10⁵±5.61 cfu.g⁻¹ L. lactis subsp. cremoris were isolated from dahi samples of eastern Nepal. These groups ADH ² (arginine dihydrolase - negative arginine hydrolysis), citrate ³ (negative citrate utilization) isolates from dahi sample were further identified as L. lactis subsp. cremoris and L. lactis subsp. lactis biovar. diacetylactis by physiological and biochemical characteristics (Table 2). Isolated LAB were spherical or ovoid in shape, non-motile and occurred in pairs.

![Figure 2](image)

These findings are accordance with some previous findings reported on isolation of L. lactis subsp. lactis, L. lactis subsp. cremoris from dahi of Himalayas (Devan, Tamang, 2007) and L. lactis subsp. cremoris from Kazerun’s traditional fermented yoghurt (Azadnia et al., 2011). Similarly, L. lactis are abundant in South African fermented milks (Beukes et al., 2001) and Algerian goat milk (Badis et al., 2004b). On the other hand, lactic acid cocci were found to be low in number as compared to lactic acid bacilli in the mixed culture. Low numbered lacto cocci than lacto bacilli might be due to inability to cope with bacilli in dahi fermentation as explained by Azadnia et al. (2011). The present study was executed to design the production of LAB starter culture. Activities of Lactococcus spp. are found in such a way that it might be possible to produce dahi consistently in quality from each batch, with its unique typical indigenous texture and flavor. In future, it might lead through the genetic characterization and selection of the most desirable strains giving highly pure commercialized strain to further elaborate the industrialization of local product.
Isolates were divided into two subgroups and a biovar, on the basis of growth pattern in SL and D agar. In SL agar, two different colored colonies were obtained. One colony was white and other Prussian blue. Totally white colonies in 48 h were identified as *S. lactis* and *S. cremoris*. Prussian blue colonies were identified as *S. lactis* subsp. *lactis* biovar. *diacetylactis*. In D agar, three different colored colonies were obtained. First types were white colonies, second yellow with yellow zones and last ones were Prussian blue. White colonies were identified as *S. lactis*, yellow colonies as *S. cremoris* and Prussian blue as *S. lactis* biovar. *diacetylactis*. The former produced small yellow colonies with yellow zones and the latter produced larger white colonies with no surrounding zones on the finalized medium. It has been justified by evident coloration in extended incubation time as explained in one of the previous studies by Reddy et al. (1969). Occasional variability in the shade or amount of blue color on colonies was recorded with some *S. diacetylactis* strains. This might be due to natural variability on citrate transportation. However, all citrate-positive colonies were blue or large blue centered. Rapid citrate accumulation could be the reason for blue coloration of colonies and this citrate might be utilized by *S. diacetylactis*.

### Isolation and characterization of *Leuconostoc*

The presence of *leuconostoc* species in fermented milk is highly desirable as it would relate to the flavorful fermented products. All grams positive, catalase negative and non-spor forming isolates were further characterized as mesophilic hetero-fermentative cocci using MRS-vancomycin media. The count of these isolates is shown in figure 3. A total of 5.789×10⁵± 14.12 cfu.g⁻¹ of *Leuconostoc mesenteroides* subsp. *cremoris*, and 21.99×10⁵±25.70 cfu.g⁻¹ of *L. mesenteroides* subsp. *mesenteroides* were isolated from indigenous *dahi*. These bacteria represented a reduced fermentative profile, unable to hydrolyze arginine, producing gas from glucose with citrate +/- and dextran negative reactions. Physiological and biochemical characteristic of isolated strains from *dahi* samples were tested and results are presented in table 3. Mean counts (cfu.g⁻¹) of isolated strains found in *dahi* samples obtained from different districts of eastern Nepal.

### Table 3 Physiological and biochemical characteristic of isolated *Leuconostoc* strains from different districts of Eastern Nepal. V=Variable.

<table>
<thead>
<tr>
<th>Characteristic Of The Strains</th>
<th>Results for <em>L. mesenteroides</em> subsp. <em>cremoris</em></th>
<th>Results for <em>L. mesenteroides</em> subsp. <em>mesenteroides</em></th>
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<tr>
<td>Gram Strain Reaction</td>
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<td>Catalase Activity</td>
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<td>Glucose Fermentation</td>
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<td>Growth At Temperature °C</td>
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<td>Growth in a Medium With NaCl, %</td>
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<td>Growth at pH</td>
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Figure 3 Mean counts (cfu g⁻¹) of isolated strains of *leuconostoc* found in *dahi* samples obtained from different districts of eastern Nepal.

These micro-aerophilic organisms were also characterized by the fermentation metabolism of fructose, galactose, glucose, lactose, maltose, sucrose and mannitol. *L. mesenteroides* subsp. *cremoris* was fructose, maltose and mannitol negative whereas *L. mesenteroides* subsp. *mesenteroides* were negative to mannitol only. The former utilized citrate while the latter did not (Table 3). *L. cremoris* and *L. mesenteroides* are Arginine hydrolyser and these might be present in *dahi*. Arginine hydrolysis and citrate utilization methods were tested to differentiate *L. mesenteroides* and *L. cremoris*. Arabinose fermentation was also implemented to confirm species. *L. mesenteroides* subsp. *cremoris* and *L. mesenteroides* subsp. *mesenteroides* were isolated from 39 samples but low in counts. Low numbers of these lactic acid cocci might be due to their inability to compete over lactic acid bacilli in mixed cultures. The low percentage of *Leuconostoc* strains isolated from indigenous *dahi* samples could partly be explained by their complex nutritional requirements and lower adaptation (Azadnia et al., 2011) to dairy products. *Leuconostoc* plays important role in flavor development in dairy products (Mataragas et al., 2004) and unique flavor in *dahi* might be attributed to this micro flora in some instances but need to do further detail study on flavor development aspects of this indigenous dairy products from Himalayas.
Presence of *L. mesenteroides* in dahi samples in such numbers is somehow in accordance with previous finding in indigenous fermented dairy products from Kenya (Mathara et al., 2004), Tibet of China (Airidengcaicike et al., 2010), South Africa (Beukes et al., 2001), Bangladesh (Harun-ur-Rashid et al., 2007), Uganda (Muyanja et al., 2003), Sudan (Ali, 2011). In this context, we can say that the *L. mesenteroides* are present in most indigenous milk derived products, either from Himalayas or other part of the world.

**Total LAB count in indigenous dahi of eastern Nepal**

The total LAB counts in indigenous *dahi* of eastern Nepal were found in the range from 221×10⁶ to 225×10⁶ cfu.g⁻¹. Mean LAB counts were 224×10⁶ cfu.g⁻¹. One of the previous studies reported LAB counts range between 132×10⁶ and 246×10⁶ cfu.mL⁻¹ (Gandhi and Natrajan, 2010) in dahi of Indian origin. In this study, 59.38% of Lactobacilli, 21% of Streptococci, 8.2 % Lactococci and 11.42 % Leuconostocs were isolated from indigenous dahi of eastern Nepal (Figure 4). The population and microbial flora of LAB might vary according to climatic condition of different regions. It is obvious that, there is high climatic and temperature variation between Himalayan area of eastern Nepal and southern hot climatic Indian terrain. In this scenario, our finding justifies the differences in microbiology of *dahi* from southern India and eastern Himalayan Nepal. Ultimately flavor dynamics, as flavor is important functions of microbes.

**Figure 4 Distribution of LAB in indigenous dahi of eastern Nepal**

**CONCLUSION**

This work showed a clear picture of microbial diversity and density in indigenous *dahi* of Nepal that might largely contribute to its typical texture and flavor. Dominant LAB comprising of *Lactobacilli*, *Streptococci*, *Lactococci* and *Leuconostocs* were successfully isolated from indigenous *dahi*. The wide diversity in microbial community could be attributed to variations in the specific environmental conditions found in Nepal as well as the manufacturing processes for indigenous *dahi*. Selection, propagation and preservation of the best performing strain to make starter culture could be done in future through genetic characterization. Further study on the volatile flavor compounds-matrix interactions, flavor release mechanisms, synergistic effect of flavor compounds and correlating these compounds to sensory attributes of indigenous *dahi* could be done. The profiles of volatile flavor compounds and unique role of indigenous fermentation strategy practiced could be elaborated in future.

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