

## Identifying the Diversity of Dominant LABs from Fermented Dairy Products *Dahi* and Yoghurt in Eastern Region of Nepal

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*The aim of this study was to isolate and identify lactic acid bacteria diversity from dahi/ yoghurt. Curd, also known as commercially prepared yoghurt or homemade Dahi, is formed during the slow lactic fermentation of lactose from milk by thermophilic lactic acid bacteria (LAB). Thirty-five samples of yoghurt and curd were collected from the Biratnagar and isolation and identification of bacteria were done by various microbiological techniques like MRS Agar inoculation, colony characteristics, microscopic and biochemical examination. A total of sixty-six strains of lactobacilli were isolated from curd and identification of strains was done by biochemical and carbohydrate utilization test. Lactobacillus bulgaricus, L. casei, L. fermentum, L. acidophilus, and Streptococcus thermophilus were identified from curd. pH of samples was between 3.0 to 4.0 for homemade dahi; while 4.34 to 4.5 for commercially available yoghurt. The mean colony count of lactic acid bacteria was  $1.4 \times 10^7$ – $4.9 \times 10^7$  cfu/g. 37.9% of samples contained Streptococcus thermophilus, 30.3% had Lactobacillus bulgaricus. Forty-five isolates from 24 industrial yoghurt samples showed 37.5% of the yoghurt contained both Lactobacillus bulgaricus and Streptococcus thermophilus followed by 25% samples having S. thermophilus and L. acidophilus. Other species like L. fermentum and L. casei were less common. From 11 homemade dahi samples, 54.5% of curd possessed both S. thermophilus and L. bulgaricus; 18.2% curd had both S. thermophilus and L. fermentum. The study concludes that L. bulgaricus and Streptococcus thermophilus are prevalent potent lactic acid bacteria. This study provides an account of the diversity of lactic acid bacteria in dahi/ yoghurt which will provide useful information about the variable nature of curd in this region to future researchers.*

**Keywords:** Antibiotic sensitivity, *Dahi*, Fermented dairy product, Isolation, LAB, Yoghurt

### Introduction

Curd is the most commonly used fermented milk product. The introduction of fermented milk product such as yoghurts into the diet of man is thought to date back to the dawn of civilization (Madhu, 2013). Curd is also known as commercially prepared yoghurt or homemade prepared dahi (Hossain et al., 2012). Curd is a semi-solid lactic acid fermented milk product that results due to semi-solid gel formation from coagulated milk proteins (Dekumpitiya et al., 2016). Lactic acid bacteria (LAB) including *Lactobacillus* spp. are generally recognized as safe (GRAS). LAB can act as cell factories for the production of food additive and aroma compounds. Different *Lactobacillus* spp. are used as probiotics (Mokoena et al., 2016).

Lactic acid bacteria (LAB) are gram-positive rod or cocci shaped, commonly non-spore forming, facultative anaerobes, catalase negative, and can breakdown sugars like glucose, fructose, maltose, lactose, etc. into lactic acid (Thakur et al., 2017). The optimum temperature for the growth of *Lactobacillus* is 37 °C to 45 °C. As the acid accumulates in a solution, the milk thickens and takes a "sour" taste. A variation in pH can make the yoghurt sweeter and thinner (higher pH) or thicker and more sour (lower pH) (Mena-Chalas et al., 2013). Yoghurt comes in a variety of textures (e.g. liquid, set and stirred curd), fat contents (e.g. regular fat, low-fat and fat-free) and flavors (e.g. natural, fruit, cereal, chocolate) can be consumed as a snack or part of a meal as a sweet or savory food (Modhu, 2016). Yoghurt contains thermophilic starter culture such as *Lactobacillus* spp. and

*Streptococcus thermophilus*. Milk is normally heated at high temperatures (e.g. 85 °C for 30 min), which causes the denaturation of whey proteins (Burton et al., 2017). There is increased casein-casein attraction as the pH of milk decreases from ~ 6.6 to ~ 4.6 during yoghurt fermentation. While fermentation, the pH of yoghurt is 4.0 to 4.2 (Aswal et al., 2012). Curds can also have *Lactobacillus acidophilus*, *Lactobacillus casei*, *Lactobacillus fermentum*, *Lactobacillus bulgaricus*, *Streptococcus thermophilus*, etc (Dekumpitiya et al., 2016).

Very limited studies had been performed on microbial diversity and safety on Nepalese *dahi* and yoghurt. This paper was, therefore, aimed at isolating and identifying lactic acid bacteria associated with indigenous *dahi* and industrial yoghurt from eastern Nepal.

### Materials and methods

#### Study area and sampling

Thirty-five samples were collected from commercially prepared yoghurt and homemade curd samples were collected from the Biratnagar city-the eastern region of Nepal. Preliminary study was done to identify highly dense *dahi* producing areas. For yoghurt, available brands in market were selected. The samples were aseptically collected in sterile screw capped test tubes using sterile gloves and preserved in ice box for the transportation to laboratory at Mahendra Morang Adarsh Multiple Campus, Biratnagar, Nepal. Samples were processed, then and there, or kept in refrigerator at 4 °C for further use. The samples were

processed within 24 hours.

### Sample processing and dilution preparation

Twenty-five grams of *dahi* or yoghurt samples were homogenized with 225 mL dipotassium hydrogen phosphate solution to make an initial suspension of dilution ( $10^{-1}$ ). Further dilutions were prepared with peptone saline diluent employing decimal dilution techniques as per Public Health England (2014).

### Spread plate technique on MRS agar plate

0.1 milliliter of each well mixed *dahi* sample was pipetted out in MRS (Mann Rogosa Sharpe; Himedia, Mumbai, India) agar and spread with spreaders throughout the media as per spread plate technique and incubated at 37 °C for 24 hours.

### Colony selection and pure culture isolation

After performing spread plate techniques, the colony count of different curd sample was performed. Multiple colonies having different characters were selected for pure culture of lactobacilli. The selected colonies were sub-cultured in MRS agar plate and incubated again at 37 °C for 48 hours.

### Morphological examination

All pure cultures were subjected to gram staining. Only gram-positive round or cocci shaped bacteria were taken, whereas gram negative bacteria were discarded. White large, mucoid, round and small colonies were selected. As for morphological examination beside gram-staining; capsule staining and spore-staining were also performed on sub-cultured pure colonies.

### Biochemical tests

Motility test was also performed using Sulfide Indole Motility (SIM) medium. Biochemical tests like catalase, oxidase, MR-VP, indole, citrate utilization, urease, triple sugar iron agar, carbohydrate utilization tests were performed (Cheesbrough, 2009). Immediate bubbling shown by bacteria in 3% hydrogen peroxide confirmed catalase positive test. Oxidase paper was used for oxidase test which turned to purple color

when isolate was streaked onto it. Indole positive test was confirmed when SIM media gave red or red-violet color in the surface after addition of Kovac's reagent. The hazy and irregular growth around the stabbed region of SIM media confirmed the motility of bacteria.

### Sugar fermentation test

Different sugars were used to determine the fermentation profile and further characterization of lactobacilli isolates.

For this, lactic acid bacteria were subjected to sugar fermentation reactions using nutrient broth medium. Different sugars such as glucose, fructose, sucrose, lactose, maltose, and mannitol were used to differentiate the different lactic acid bacteria by producing acid and gas in the medium (Cheesbrough, 2009).

Membrane (0.45 µm) filtered 1% (w/v) solutions of different sugars (glucose, fructose, lactose, sucrose, maltose, and mannitol) were used to study fermentation characteristics of the isolates. Nutrient broth (0.8%) with 1 mL phenol red was prepared. Five ml of broth was kept in each test tube with a Durham tube and autoclaved at 121 °C for 15 minutes. Sterilized test tubes with broth were mixed with 100 µL of sugar solutions. The freshly cultured 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 a color change from red to yellow in the test tubes while gas production was noted in Durham tube as mentioned by Mehmood *et al.* (2009).

On glucose fermentation, if acid and gas were formed then it was identified as *Lactobacillus fermentum*; whereas if acid was produced on glucose fermentation, then it was identified as *Lactobacillus casei* and *Lactobacillus acidophilus*. *L. casei* and *L. acidophilus* could be differentiated on mannitol, where only acid production indicated *L. casei*, while only gas evolution pointed to *L. acidophilus*. However, both acid and gas production from mannitol highlighted *L. bulgaricus* (Table 1).

**Table 1**

Carbohydrate fermentation test of several LABs

Carbohydrates fermentation		<i>Streptococcus thermophilus</i>	<i>Lactobacillus bulgaricus</i>	<i>Lactobacillus acidophilus</i>	<i>L. casei</i>	<i>Lactobacillus fermentum</i>
Glucose	Acid	+	+	+	-	+
	Gas	-	-	-	+	+
Fructose	Acid	+	-	-	-	+
	Gas	-	+	+	-	-
Sucrose	Acid	+	-	+	+	+
	Gas	-	+	+	-	-
Maltose	Acid	-	+	+	-	+
	Gas	-	-	+	+	-
Mannitol	Acid	-	+	-	+	-
	Gas	-	+	+	-	-
Lactose	Acid	-	-	+	+	-
	Gas	-	+	+	-	+

**Table 2**

Morphological characteristics of isolated bacterial culture

SN	Sample code	Colony Characteristics	Shape	Size (mm)
1	L <sub>1</sub> , L <sub>3</sub> , L <sub>5</sub> , L <sub>7</sub> , L <sub>9</sub> , L <sub>11</sub> , L <sub>14</sub> , L <sub>15</sub> , L <sub>18</sub> , L <sub>19</sub> , L <sub>22</sub> , L <sub>27</sub> , L <sub>31</sub> , L <sub>33</sub> , L <sub>36</sub> , L <sub>42</sub> , L <sub>44</sub> , L <sub>46</sub> , L <sub>48</sub> , L <sub>52</sub> , L <sub>55</sub> , L <sub>59</sub> , L <sub>63</sub> , L <sub>65</sub>	Circular, Creamy White	Cocci	2-5
2	L <sub>2</sub> , L <sub>8</sub> , L <sub>13</sub> , L <sub>16</sub> , L <sub>17</sub> , L <sub>26</sub> , L <sub>29</sub> , L <sub>32</sub> , L <sub>34</sub> , L <sub>37</sub> , L <sub>39</sub> , L <sub>41</sub> , L <sub>43</sub> , L <sub>45</sub> , L <sub>47</sub> , L <sub>51</sub> , L <sub>53</sub> , L <sub>56</sub> , L <sub>60</sub> , L <sub>62</sub>	Circular, Creamy Grey	Rod	1-3
3	L <sub>4</sub> , L <sub>12</sub> , L <sub>20</sub> , L <sub>24</sub> , L <sub>28</sub> , L <sub>30</sub> , L <sub>35</sub> , L <sub>57</sub> , L <sub>61</sub> , L <sub>64</sub>	Round, Mucoid	Rod	2-4
4	L <sub>10</sub> , L <sub>23</sub> , L <sub>50</sub> , L <sub>58</sub>	Smooth, Convex, Round	Rod	1-3
5	L <sub>6</sub> , L <sub>21</sub> , L <sub>25</sub> , L <sub>40</sub> , L <sub>49</sub> , L <sub>54</sub> , L <sub>66</sub>	Circular, White mucoid	Rod	1-2

**Curdling of Milk**

A 50 ml milk sample was treated with identified isolated colonies of lactic acid bacteria for further confirmation of *Lactobacillus* spp. The curdling of milk confirmed the isolates as *Lactobacillus* spp.

**Results and Discussion****pH and mean colony count of LABs**

The samples collected were immediately measured for pH after transportation to laboratory which showed a range of pH 3.0 to 4.0 for homemade dahi while pH 4.34 to 4.5 for commercially available yoghurt. The mean colony count of lactic acid bacteria was found to be in a range of  $1.4 \times 10^7$  –  $4.9 \times 10^7$  cfu/g. Sixty-six bacterial isolates were selected from

a total of 35 dahi samples on different colony characteristics (Table 2).

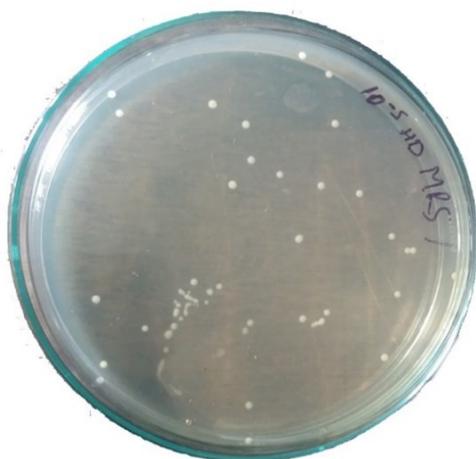
The pH of homemade dahi was 3.0 to 4.0 which was lower than the Bogra dahi (pH 4.25 – 5.01) of Bangladesh (Chanda *et al.*, 2013). pH 4.34 - 4.5 of yoghurt in this study nearly aligned (pH 4.18-4.43) with Tavakoli *et al.* (2019) and (pH 4.25 – 4.5) Ranasinghe and Perera (2016). The mean colony count of LABs  $1.4 \times 10^7$  –  $4.9 \times 10^7$  cfu/g was greater than Lagos  $2.8 \times 10^2$  –  $1.6 \times 10^5$  cfu/gm (Afolabi *et al.*, 2017). Bhattarai *et al.* (2016) reported mean counts of all Lactobacilli as  $133 \times 10^6$  cfu/ g and Streptococci as  $47 \times 10^6$  cfu/g from dahi samples.

**Microscopic examination and Biochemical test**

On microscopical examination, isolates were found to be were gram-positive rod and cocci shaped, non-spore former, non-capsulated, and non-motile (Fig 1-2). *Lactobacillus* spp were catalase and oxidase negative, indole negative, methyl red positive, Voges-Proskauer negative, citrate negative, urease negative, TSI negative, and H<sub>2</sub>S negative. Upon biochemical identification of 66 isolates, 25(37.9%) were found to be *Streptococcus thermophilus*, 20 (30.3%) were *Lactobacillus bulgaricus*, 10 (15.2%) *Lactobacillus acidophilus*, 7 (10.6%) *Lactobacillus fermentum*, and 4 (6.1%) were *Lactobacillus casei* (Table 3).

In yoghurt, the microbial share of *S. thermophilus*, *L. bulgaricus*, *L. acidophilus*, *L. fermentum*, and *L. casei* was 35.5%, 31.1%, 20%, 11.1% and 2.2% respectively. In dahi, the microbial share of *S. thermophilus*, *L. bulgaricus*, *L. acidophilus*, *L. casei*, and *L. fermentum* was 42.9%, 28.6%, 14.3%, 9.5% and 4.8% respectively.

From 24 industrial yoghurt samples, 45 isolates were identified. 37.5% of the yoghurt contained both *Lactobacillus bulgaricus* and *Streptococcus thermophilus*; while 25% yoghurt had both *S. thermophilus* and *L. acidophilus*. Other species like *L. bulgaricus* and *L. fermentum* were found in 8.3% yoghurt. *L. bulgaricus* and *L. acidophilus* were also found in 8.3% samples. Similarly, 4.2% of samples had *L. fermentum* and *S. thermophilus*; while 4.2% of samples contained *L. fermentum* and *L. casei*. Only one genus like *L. fermentum*, *L. bulgaricus* and *L. acidophilus* was isolated

**Fig 1.** Spread Plate Technique on MRS Agar**Fig 2.** Sub-culture on MRS agar of lactic acid bacteria

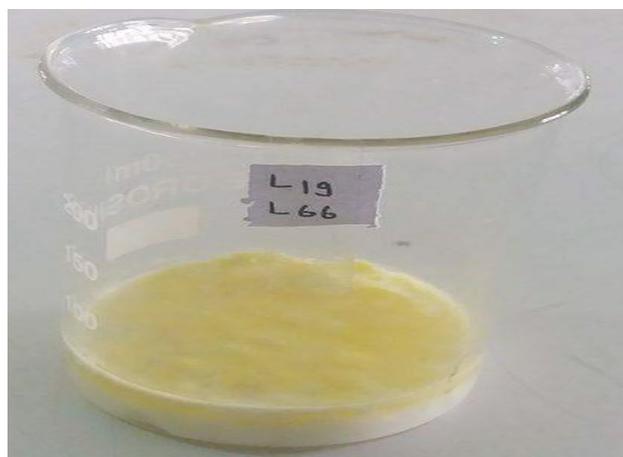
from one sample each. From 11 homemade dahi samples, 21 isolates were identified. Out of 11 samples, 9 samples contained *Streptococcus thermophilus* along with another genus. 54.5% of dahi possessed both *S. thermophilus* and *L. bulgaricus*; 18.2% dahi had both *S. thermophilus* and *L. fermentum*; while 9.1% contained *S. thermophilus* and *L. casei*. While one dahi sample (9.1%) contained *L. casei* and *L. acidophilus*; another sample (9.1%) had only one genus named *L. casei*.

**Table 3**  
List of identified bacteria of several coded samples

SN	Sample code	Bacteria
1	L <sub>1</sub> , L <sub>3</sub> , L <sub>5</sub> , L <sub>7</sub> , L <sub>9</sub> , L <sub>11</sub> , L <sub>14</sub> , L <sub>15</sub> , L <sub>18</sub> , L <sub>19</sub> , L <sub>22</sub> , L <sub>27</sub> , L <sub>31</sub> , L <sub>33</sub> , L <sub>36</sub> , L <sub>42</sub> , L <sub>44</sub> , L <sub>46</sub> , L <sub>48</sub> , L <sub>52</sub> , L <sub>55</sub> , L <sub>59</sub> , L <sub>63</sub> , L <sub>65</sub>	<i>Streptococcus thermophilus</i>
2	L <sub>2</sub> , L <sub>8</sub> , L <sub>13</sub> , L <sub>16</sub> , L <sub>17</sub> , L <sub>26</sub> , L <sub>29</sub> , L <sub>32</sub> , L <sub>34</sub> , L <sub>37</sub> , L <sub>39</sub> , L <sub>41</sub> , L <sub>43</sub> , L <sub>45</sub> , L <sub>47</sub> , L <sub>51</sub> , L <sub>53</sub> , L <sub>56</sub> , L <sub>60</sub> , L <sub>62</sub>	<i>Lactobacillus bulgaricus</i>
3	L <sub>4</sub> , L <sub>12</sub> , L <sub>20</sub> , L <sub>24</sub> , L <sub>28</sub> , L <sub>30</sub> , L <sub>35</sub> , L <sub>57</sub> , L <sub>61</sub> , L <sub>64</sub>	<i>Lactobacillus acidophilus</i>
4	L <sub>10</sub> , L <sub>23</sub> , L <sub>50</sub> , L <sub>58</sub>	<i>Lactobacillus casei</i>
5	L <sub>6</sub> , L <sub>21</sub> , L <sub>25</sub> , L <sub>40</sub> , L <sub>49</sub> , L <sub>54</sub> , L <sub>66</sub>	<i>Lactobacillus fermentum</i>

**Curdling test**

After 24 hours of incubation of milk sample with identified lactic acid bacteria, milk got curdled and, hence, curd was formed (Fig 3-4). In dahi, *S. thermophilus* and Lactobacilli were 42.9% and 57.1% respectively which was different from Bhattarai et al (2016) who reported 59.38% Lactobacilli and 21% of Streptococci of total isolates from dahi samples. As per Dekumpitiya et al (2016), 69% were identified as *Lactobacillus* spp and 21% as *Streptococcus* spp in buffalo milk curd of Sri Lanka. In yoghurt, 35.5% samples contained *Streptococcus thermophilus* that was much lower (69%) than Dekumpitiya et al (2016).



**Fig. 3.** Curdling of milk with identified lactic acid bacteria *Streptococcus thermophilus* (L19) and *Lactobacillus fermentum* (L66)

Our most indigenous dahi samples had dominant *Lactobacilli* and *S. thermophilus*. These findings aligned with previous study of Mongolian fermented dairy product where *Lactobacilli* were 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 Streptococci and *Lactobacilli* in the indigenous sample from eastern Nepal were somewhat different from several previous studies on fermented milks from different origination (Mathara et al., 2004; Harun-ur-Rashid et al., 2007, Yu et al., 2011).

In dahi, the microbial share of *S. thermophilus*, *L. bulgaricus*, *L. acidophilus*, *L. casei*, and *L. fermentum* was 42.9%, 28.6%, 14.3%, 9.5% and 4.8% respectively. Dahi from Bangladesh are different to dahi from eastern Nepal in terms of dominant *S. bovis* instead of *S. thermophilus* (Harun-ur-Rashid et al., 2007).

Tibetan traditional fermented milk has two predominant species *L. fermentum* and *L. casei* along with a variation between different regions (Airidengcaicike et al., 2010). This could be because microflora in dairy products is affected by external factors like climate and altitude. In region with cold climate mesophilic organisms such as *Lactococcus* and *Leuconostoc* have been found to be dominant in fermented milk products while thermophilic bacteria such as *Lactobacillus* and *Streptococcus* in warm regions as reported by Kurmann (1984). The presence of different *Lactobacillus* species in dahi samples from Eastern Nepal could be endorsed by the presence of these bacteria as natural microflora in raw milk sources.

*L. fermentum* has been suspected as a weak coagulant, gas producer giving bad texture and taste in dairy products (Harun-ur-Rashid et al., 2007), despite having potential probiotic properties, such as acid resistance, indigestible carbohydrate degradation, and bile salt tolerance (Airidengcaicike et al., 2010). Consequently, these strains are needed to be assessed for probiotic applications and commercialization of indigenous dahi.



**Fig. 4.** Curdling of milk with identified lactic acid bacteria *Streptococcus thermophilus* (L19) and *Lactobacillus bulgaricus* (L32).

## Conclusions

The microbial diversity and density in indigenous dahi of eastern Nepal are similar to commercial yoghurt, which indicates a mixing of indigenous dahi culture with industrial culture. However, the typical texture and flavor of indigenous dahi is different from industrial yoghurt. The diversity in microbial community is attributed to variations in the specific environmental conditions found in Nepal as well as the manufacturing processes for indigenous dahi.

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