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Probiotic Potentiality of Lactic Acid Bacteria Isolated from "Theki Dahi" around Various Places of Pokhara Valley

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Abstract
This research is undertaken to study the probiotic potential of lactic acid bacteria (LAB) isolated from *theki dahi* of Pokhara valley. A total of 48 samples were collected, pooled together, and examined. From characterization, three types of Lactobacilli (Lb A, LbB, and LbC), two types of Leuconostoc (Leu A and LeuB), two types of Bifidobacterium (Bif A and Bif B), one *Streptococcus* and three types of Lactococci (Lc A, LcB, and LcC) were identified. Also, the probiotic strain of *Lactobacillus casei* sub sp. Shirota was isolated from the yakult sample. All were then subjected to further examination.In terms of acid resistance, all were found to be resistant to low pH 2 and 4 for 2 h. Similarly, all were resistant to bile salt (0.3% and 0.4% w/v) and able to hydrolyze it. With regards to antibiotic resistance, all were sensitive to penicillin G, ampicillin, and amoxicillin but only Lactobacilli and Bifidobacterium showed resistance to ciprofloxacin. Also, the isolates adhered to hexane. This study implies that *theki dahi* comprises various LAB with probiotic properties. Majorly, Lb A and Bif A demonstrated similar probiotic properties to control organism. Thus, they can be considered as potential probiotic candidates in *theki dahi.*
Keywords: *Theki dahi*, probiotic potentiality, LAB, isolation, identification, characterization
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1. Introduction

Probiotics are usually defined as microbial food supplements with beneficial effects on the consumers (**Parvez *et al.*, 2006).** Species of *Lactobacillus*, *Bifidobacterium* and *Streptococcus*, as well as *Lactococcus lactis*, *Leuconostoc* and some *Enterococcus* species are the most commonly used bacterial probiotics. They are safe for consumption, survive intestinal transit (acid and bile tolerant), adhere to mucosa surface and colonize in the intestine. They also confer many health benefits **(Hawaz, 2014).**

Dahi is one of the most popular indigenous fermented foods of South Asia. Indigenously, most flavorful *dahi* is prepared in a *theki* which is a close-necked wooden vessel carved out of wood like *daar* (*Boehmeria rugulosa*) **(Bhattarai and Das, 2013, Semwal *et al.*, 2009).** The main purpose of using *daar theki* is to give flavorful *dahi* and to serve as natural microflora reservoir **(Bhattarai *et al.*, 2016).**

Lactic acid bacteria (LAB) are Gram-positive, occurring naturally as indigenous microflora in raw milk **(Guessas and Kihal, 2004).** LAB found in *dahi* has been concluded to have the potential to exert probiotic effects in the individual consumer **Balamurugan *et al.*, 2014).** The probiotic potentiality of microorganisms can be evaluated from five *in-vitro* tests namely acid resistance, bile salt resistance, bile salt hydrolysis, cell surface hydrophobicity and antibiotic resistance tests **(Hawaz, 2014).**

Even though, *dahi* is consumed as a part of daily diet in Nepal and believed to carter probiotic properties, enough research and documentation regarding its microbiology and health benefits is still lacking **(Bhattarai and Das, 2013; Khanal and Koirala, 2019).** This has endangered one of the famous and potential probiotic food of Nepal; *theki dahi*. Henceforth, this study is undertaken to evaluate the probiotic potentiality of lactic acid bacteria isolated from *theki dahi* so that the findings will be helpful to promote such an easily accessible probiotic food to Nepalese society. Additionally, this research is also aimed at its scientific documentation.

2. Material and Methodology

2.1 Sample collection

A total of 48 samples of the *Theki dahi* (5 to 7 days old) were collected in a sterile glass container from different places around Pokhara valley during the morning. 10 grams of each sample was collected and pooled separately to yield 500 gm of final sample. They were brought to the laboratory where it was stored at refrigeration temperature within 3 h of collection.

Similarly, probiotic strain of *Lactobacillus casei* was isolated from Yakult manufactured by YAKULT DANONE INDIA PRIVAATE LIMITED which was transported to Nepal by FLIPKART through E-Kart

logistics.

2.2 Microbiological media and chemical reagent collection

Lactobacilli was isolated on De Man, Rogosa and Sharpe (MRS) agar media which was purchased from Hi Media, Mumbai, India and imported to Raxual, Nepal by Flipkart, India and finally transported to Pokhara via truck. In order to improve specificity of Lactobacilli, 0.25% of L-cysteine was supplemented to isolate Bifidobacterium which was also purchased from HiMedia, Mumbai, India. Similarly, Vancomycin (20mg/L) was added to specifically isolate *Leuconostoc* only, which was bought from local pharmacy in Pokhara. Similarly, M_{17} agar was also procured from Hi Media, Mumbai, India and imported to Raxual, Nepal by Flipkart, India and finally transported to Pokhara via truck for isolating *Streptococcus thermophilus*. Finally, in order to isolate Lactocooci, differentiating agar media was composed in the laboratory itself wherein the chemicals and reagents required were obtained from Science House P. Ltd., Pokhara, Nepal and Kiran Scientific House, Pokhara, Nepal.

2.3 Isolation of microbes

10 g of the collected sample was homogenized (using an electric stationary blender consisting of blender jar with rotating metal blades) with 90 mL of 0.1% (w/v) sterile peptone water to obtain 1:10 (10^{-1}) dilution. Successive decimal dilutions were carried out with 0.1 % (w/v) sterile peptone water. Finally, aliquot (0.1 mL) from various dilutions was spread plated on various agar plates to isolate the microbe.

Firstly, lactobacilli were isolated on MRS agar plate after complete incubation in anaerobic chamber (using gas pack chamber) at 37 ^{o}C for 24-48 h. *Leuconostoc* was isolated on MRS-vancomycin (Vancomycin 20 mg/L) after incubating for 24 h at 30 ℃**(Bhattarai *et al*., 2016).** Similarly, after anaerobically incubating the MRS agar containing 0.25% L-cysteine at 37 ^{o}C for 48 h, Bifidobacterium was isolated**(Zinedine and Faid, 2007).**

In order to isolate *Streptococcus thermophilus*, aliquot of 0.1mL was spread plated on M_{17} agar plate which was then incubated at 42 ℃ for 48 h. In regards to lactococci, *Streptocoocus lactis* differential agar plate (SL) was incubated anaerobically for 48 h to differentiate citrate utilizing and non-utilizing Lactococci. While Differential agar medium (D) plate was used for differentiating *Streptococcus lactis* and *Streptococcus cremoris* based on their ability to hydrolase arginine, which was incubated at 32 ℃ for 48 h **(Kempler adn McKay, 1980; Reddy *et al*., 1969).**

Colonies were randomly selected from the agar plates based on colony morphology but if the plate contained less than 4 colonies, all the colonies were used for sub-culturing. The isolated colonies were streaked and sub-cultured on the respective agar plates followed by visual or microscopic examination to confirm pure culture.

2.4 Identification of isolated colonies

All the preserved isolated colonies were initially characterized on the basis of their cultural or microscopic characteristics. Afterwards, biochemical tests were performed to identify them. Gram staining, catalase, oxidase test and motility test were performed as primary basis of characterization. Moreover, heat resistance at 60 ^{o}C for 30 mins (Sherman test) was also performed **(Bhattarai *et al*., 2016)**. Arginine hydrolysis test was performed by inoculating a loopful of bacterial culture in arginine broth with few drops of Nesseler reagent. A brown coloration of the medium indicated positive result **(Mehmood *et al*., 2009).** regards to citrate utilization test, Simmon's citrate agar slant was used. A small amount of bacterial colony was touched on the center and streaked on the slant followed by incubation aerobically at 35 -37 ℃ for 4 to 5 days. Finally, Prussian blue coloration of the slant was designated as positive response **(Aryal, 2019).** Moreover, color of the bacterial colonies observed on SL and D agars was employed to interpret arginine hydrolysis and citrate utilization as well as distinguish bacteria **(Kempler adn McKay, 1980;Reddy *et al*., 1969).**

2.4.1 Sugar fermentation test

In order to study the sugar fermentation characteristics of isolated microbes, membrane (0.45 µm) filtered 1% (w/v) solutions of different sugars (glucose, fructose, lactose, galactose, sucrose, maltose, and mannitol) were prepared. Nutrient broth (0.8%) with 1mL of phenol red indicator was prepared and transferred equally in various test tubes with Durham tube. The test tubes were then autoclaved at 121 ^{o}C for 30 mins to sterilize them properly. Subsequently, 100 µL of different sugar solution was mixed to the sterilized broth. Finally, freshly cultured colonies were inoculated into the broth enriched with the mentioned sugar solutions and incubated at 37 ^{o}C for 48 h. A color change of broth from red to yellow in the test tubes indicated positive result for sugar fermentation while gas production was noted in the Durham tube **(Mahato and Shahani, 2019).**

2.5 Characterization of identified microbes

Physiological tests were used to characterize the identified bacteria. Growth at different temperature (10 ^{o}C, 30 ^{o}C and 45 ^{o}C) for 5 days, growth at different pH (2, 4 and 7) and growth in the presence of 4 % and 6.5 % (w/v)

NaCl was used to determine whether the isolated microbe can resist high temperature, acidic and halophilic conditions respectively and to detect their optimal growth condition **(Khanal and Koirala, 2019).** Growth was evaluated by sub culturing 1 mL of actively growing bacterial culture to10 mL of the respective broth for testing growth at different temperatures or to the broth whose pH was adjusted to 2 and 4 by using 1 M Hydrochloric acid (HCL) or to the broth supplemented with 4.0% and 6.50% (w/v) NaCl followed by incubation as required. Growth index was interpreted by comparing the change in optical density (OD) of the test sample by using spectrophotometer at 620nm (manufactured by Sky Technology, India) with that of control sample which was prepared for each test using all the optimum conditions **(Menconi *et al*., 2014; Sharama *et al*., 2021).**

$$\text{Growth index} = \frac{\text{OD at 620 nm of test sample}}{\text{OD at 620 nm of control sample}} \times 100\ \%$$

2.6 Test for probiotic potentiality of isolated organisms.

2.6.1 Acid resistance test

Actively growing isolated bacteria (1 mL) was inoculated from their respective medium broth to the medium whose pH has been adjusted to pH of 2.0 and pH of 4.0 with hydrochloric acid (HCl) and later incubated anaerobically for2 hours at 37 ℃. Similarly, the same procedure was also carried out with the pH of 7.0 which was taken as control sample. In the next step, the cells were isolated using centrifugation at 6000 rpm for 10 mins and inoculated in broth cultivation medium (neutral pH). Finally, acid resistance was determined by comparing the change in optical density (OD) of both the samples by using spectrophotometer at 620nm **(Hoque *et al*., 2010).**

$$\text{Acid Resistance \%} = \frac{\text{OD at 620 nm of sample (pH 2.0 and pH 4.0)}}{\text{OD at 620 nm of control sample (pH 7.0)}}$$

2.6.2 Bile salt resistance test

Bile salt resistance of the isolated bacteria by cultivating the respective medium containing 0.3% and 0.4%(w/v) bile salt with overnight grown culture followed by incubation at 37 ℃ anaerobically for 24 h. The level of resistance was determined by comparing optical absorption of the sample with the control sample (cultivation medium without bile salts) **(Hoque *et al*., 2010).**

$$\text{Bile salt resistance \%} = \frac{\text{OD at 620 nm of sample with 0.3\% bile salt}}{\text{OD at 620 nm of control sample without bile salt}} \times 100\%$$

2.6.3 Bile salt hydrolysis test

For this test, the isolated strains were cultivated in medium containing 0.5 % (w/v) bile salt. The medium was then incubated to allow dissolving for 48 to72 h at 37 ℃ in a CO_2 incubator. Then, plates were examined for presence of white precipitates which was a sign of bile salt hydrolysis **(Hoque *et al*., 2010).**

2.6.4 Antibiotic resistance test

In order to determine antibiotic resistance, disc diffusion method was performed. Firstly, 1 mL of actively growing isolated bacteria was inoculated to sterile MHA agar. Antibiotic disks were then placed at regular intervals on the plate followed by incubation in a CO_2 incubator at 37 ℃. After 24 h, each plate was retrieved and the zone of inhibition was measured by using a metric ruler **(Khanal and Koirala, 2019).**

2.6.5 Cell surface hydrophobicity test

It was evaluated by measuring bacterial cell adhesion to hydrocarbon. Firstly, the overnight grown bacterial cultures in MRS broth were harvested by centrifugation at 8,000 rpm for 10 minutes, washed twice with PBS and resuspended in the PBS buffer solution followed by absorbance (A_o) measurement at 600 nm by using spectrophotometer. A cell suspension of about 3 mL was then mixed with 1 mL of hydrocarbon (hexane) and incubated at 37 ℃ for 1 h for aqueous phase and organic phase separation. 1 mL of aqueous phase was removed carefully and the absorbance (A_1) was measured by spectrophotometer at 600 nm. The percentage hydrophobicity was measured by decrease in the absorbance **(Somashekariah *et al*., 2019).**

$$\text{Cell surface hydrophobicity\%} = \left(1 - \frac{A_1}{A_0} \times 100\%\right)$$

3. Result and Discussion

3.1 Isolation and characterization of Lactic acid bacteria

Out of the numerous organisms isolated from the pooled *theki dahi* sample, 11 were differentiated on the basis of their morphological, biochemical and physiological characteristics. Based on these properties, they were presumed to be 3 varieties of Lactobacilli, 2 varieties of *Leuconostoc*, 2 varieties of Bifidobacterium, 1 variety of *Streptococcus* and 3 varieties of Lactococci (Table 3.1) (Figure 1-8).

Table 3.1 Morphological, biochemical and physiological characterization of isolated microbes from the pooled *theki dahi* sample

Tests	Isolate 1	Isolate 2	Isolate 3	Isolate 4	Isolate 5	Isolate 6	Isolate 7	Isolate 8	Isolate 9	Isolate 10	Isolate 11
Colony morphology	White colored, round shaped, of creamy texture and small sized with entire margin			White colored, round shaped, of creamy texture and slightly bigger in size with entire margin		White colored, round shaped, of creamy texture with entire margin		White colored, round shaped, of creamy texture	Prussian blue colored colonies in SL differentiating agar plates	Yellow colonies surrounded by yellow zones on purple media	White colonies with no surrounding zones
Gram staining	Gram positive and elongated rod shaped			Gram positive and cocci shaped		Gram positive, Y and U shaped with branches		Gram positive, cocci in short chains	Gram positive, cocci in short chains		
Catalase	-	-	-	-	-	-	-	-	-	-	-
Oxidase	-	-	-	-	-	-	-	-	-	-	-
Motility	-	-	-	-	-	-	-	-	-	-	-
Citrate utilization	-	+	-	-	+	-	-	-	+	-	-
Heat resistance at 60 ºC for 30 mins (Sherman test)	+	+	+	+	+	+	+	+	-	-	-
Arginine hydrolysis	-	-	-	-	-	-	-	-	+	-	+
CO_2 production from glucose	-	-	+	+	+	-	-	-	-	-	+
Sugar fermentation											
Glucose	+	+	+	+	+	+	+	+	+	+	+
Lactose	+	+	+	+	+	+	+	+	+	+	+
Galactose	+	+	+	+	+	+	+	-	+	-	+
Fructose	+	+	+	+	+	+	+	+	+	-	+
Sucrose	+	+	+	+	-	+	-	+	+	-	+
Maltose	+	+	+	+	+	+	+	-	-	+	+
Mannitol	+	+	-	-	-	+	-	-	+	-	-
Growth at different temperature											
10 ºC	-	-	+	+	+	+	-	-	++	++	++
30 ºC	+++	+++	+++	++	++	+++	+++	++	+++	+++	+++
45 ºC	+	+	++	-	-	++	++	+++	-	-	-
Growth at different NaCl concentration											
0%	+++	+++	+++	+++	+++	+++	+++	++	+++	+++	+++
4.0%	++	++	++	++	++	++	++	-	++	++	++
6.50%	+	+	++	++	++	++	++	-	-	-	-
Presumed bacteria	Lb A	Lb B	Lb C	Leu A	Leu B	Bif A	Bif B	Streptococci	Lc A	Lc B	Lc C
	Lactobacilli			*Leuconostoc*		Bifidobacterium			Lactococci		

(+) good growth (++) better growth (+++) excellent growth (-) no growth

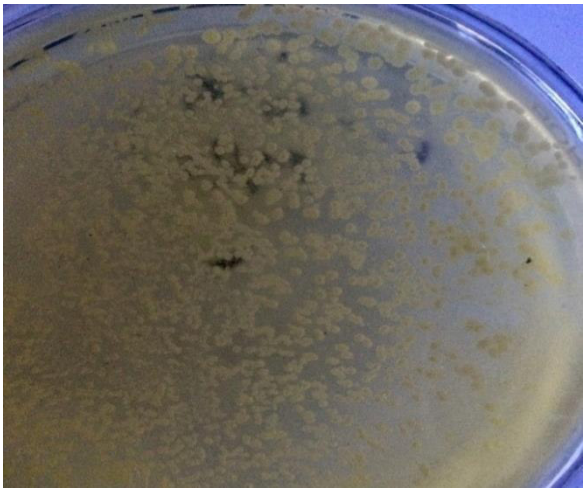

Figure 1: Lactobacilli on MRS plate

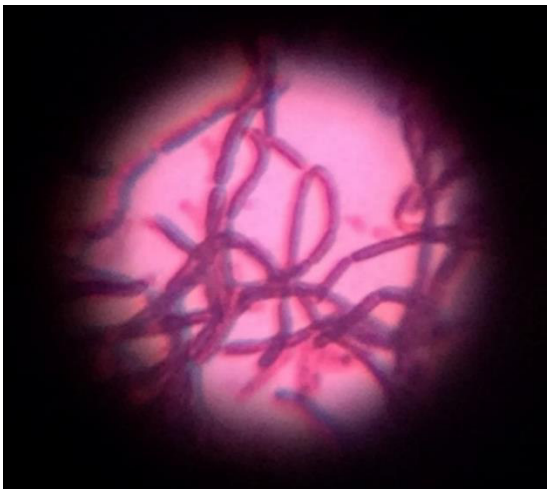

Figure 2: Microscopic observation of Lb A under 100x

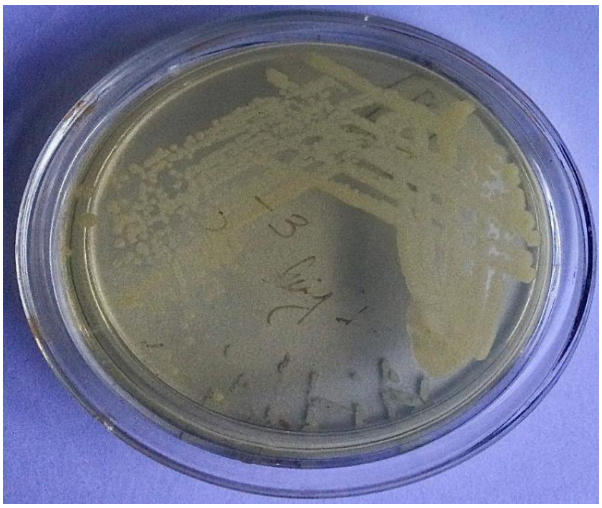

Figure 3:*Leuconostoc* on MRS-vancomycin plate

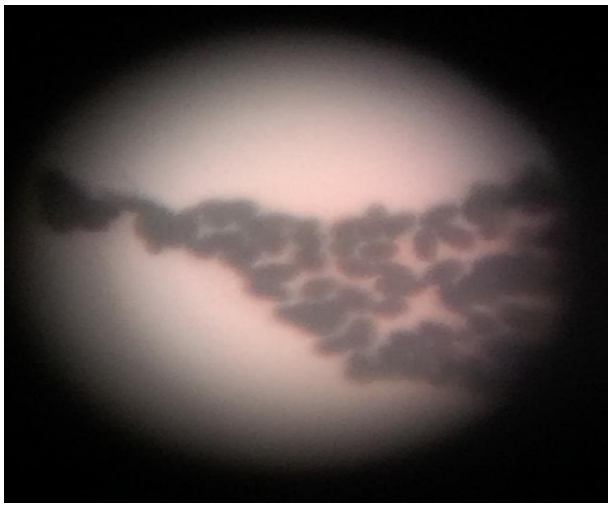

Figure 4: Microscopic observation of Leu A under 100x

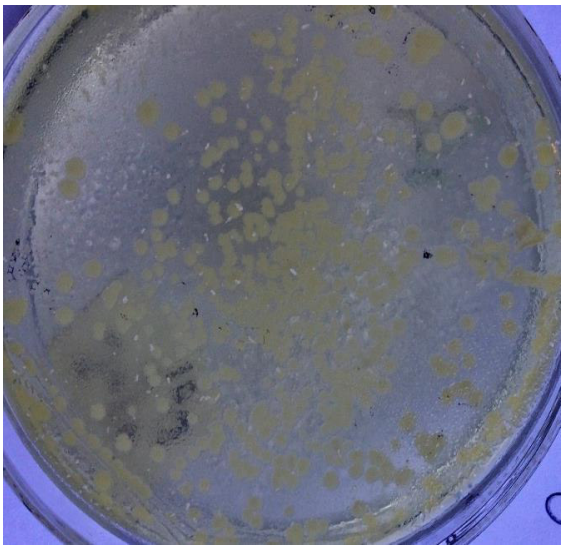

Figure 5: Bifidobacterium on MRS-cysteine

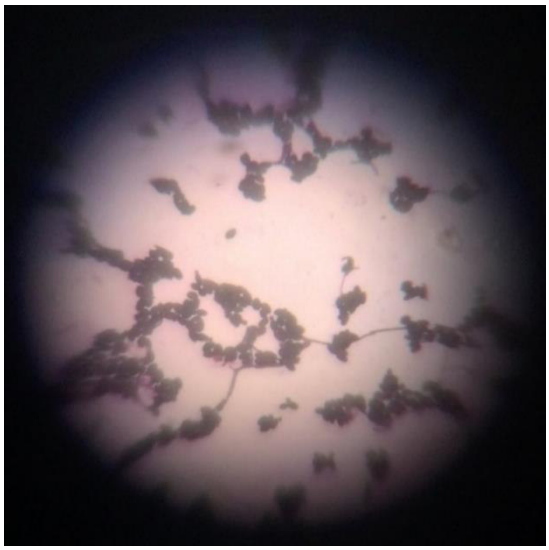

Figure 6: Microcopic obseravtion of Bif A under 100x

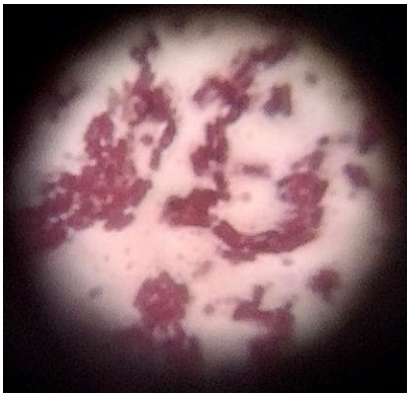

Figure 7: Microscopic observation of *Streptococcus thermophilus* under 100x

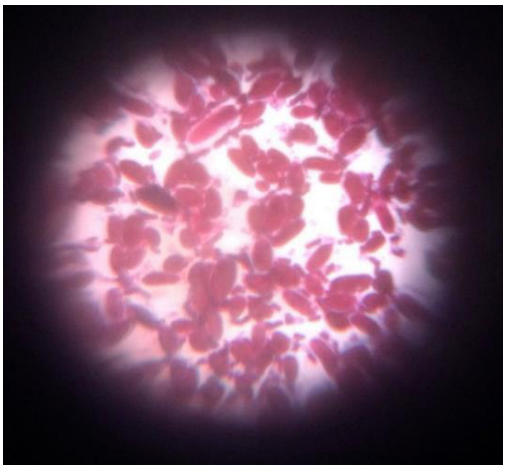

Figure 8: Microscopic observation of Lactococci under 100x

Lactobacilli of similar properties was isolated and identified as the dominant lactic acid bacteria from various traditional fermented dairy products of Nepal **(Bhattarai *et al*., 2016; Dewan and Tamang, 2007; Koirala *et al*., 2014; Mahato and Shahani, 2019).** According to these researches, the most distinctive feature of identification was its bacterial structure observed under microscope. Moreover, various researches also suggested that lactobacilli are resistant of higher temperature but cannot grow at lower temperature (10 ℃) **(Erkus, 2007; Khalil and Anwar, 2016)** Finally, previous finding proposed that lactobacilli were able to survive extreme NaCl conditions **(Koirala *et al*., 2014).**

Leuconostoc was identified as bacteria that produced gas from glucose but could not utilize arginine for NH3 production. They are able to grow well at 10 ℃ but not at 45 ℃b. Previous studies also concluded that *leuconostoc* of similar properties was found in various traditional fermented milk products **(Bhattarai *et al*., 2016; Mathara *et al*., 2004).** It also suggested the lower number of *Leuconostoc* species in the indigenous *dahi* may be due to their complex nutritional requirements and lower adaption capacity in milk**(Bhattarai *et al*., 2016).**

Bifidobacterium was particularly identified by their slightly bifurcated of ˠ-shaped structure **(Mishra *et al*., 2012).** They were isolated and identified from several traditional fermented milk products as anerobic, gram positive, catalase negative, non-gas former with an ability to survive well adverse heat conditions**(Zinedine and Faid, 2007).** In earlier researches also, they were able to grow at high temperature, pH and even high NaCl concentration **(Liu *et al*., 2020).** This may be due to an improved molecular machinery of *Bifidobacterium* to degrade non-digestible sugars allowing them to survive in adverse stress conditions**(Ruiz *et al*., 2011).**

In previous study, *Streptococcus thermophilus* was isolated and identified as one of the prominent LAB of traditional fermented milk of Nepal **(Bhattarai *et al*., 2016).** They were characterized by their distinctive ability to resist high temperature **(Dan *et al*., 2018; Erkus, 2007).**

Lactococci with similar properties was identified from various indigenous fermented milk products**(Bhattarai *et al*., 2016; Guessas and Kihal, 2004; Maqsood *et al*., 2013).** They were distinguished by their ability to hydrolyze arginine **(Guessas and Kihal, 2004).** Similarly, they were reported as mesophile growing well at 10 ºC but not at 45 ºC and not tolerant to high NaCl concentration (**Maqsood *et al*., 2013).**

3.2 Isolation and characterization of Lactobacillus casei sub sp. Shirota

Lactobacillus casei sub sp. Shirota was isolated from the yakult sample. The isolated strain was further subjected to various morphological and biochemical tests for identification purpose (Table 4.5).

In previous study*, Lactobacilluscasei* group were found to be able to survive in adverse stress conditions pH, temperature and osmotic stress conditions **(Das *et al*., 2016; Haddaji *et al*., 2015).**

Table 4.5 Morphological and biochemical characterization of isolated *Lactobacillus casei* sub sp. Shirota from the yakult sample

S No.	Tests	Isolated strain of *Lactobacillus casei* sub sp. Shirota
1	Colony morphology	Creamy blue colored colonies, round shaped with entire margin
2	Gram staining	Gram positive, small rod shaped
3	Catalase	-
4	Oxidase	-
5	Motility	-
6	Citrate utilization	-
7	Heat resistance at 60 ºC for 30 mins (Sherman test)	+
8	Arginine hydrolysis	-
9	CO_2 production from glucose	-
10	Sugar fermentation	
	Glucose	+
	Lactose	-
	Galactose	+
	Fructose	+
	Sucrose	-
	Maltose	+
	Mannitol	-
11	Growth at different temperatures	
	10 ºC	+
	30 ºC	+++
	45 ºC	++
12	Growth at different NaCl concentration	
	0%	+++
	4%	+++
	6.50%	++

3.3 Probiotic potentiality tests of isolated organism

3.3.1 Acid resistance test

Acid resistance at pH 2 and 4 of all 10 isolates obtained from pooled *theki dahi* sample was determined and compared to that of *Lactobacillus casei* sub sp. Shirota. All the tested isolates were able to survive well at both pH 2 and 4 for 2 hours (Figure 9). From the figure, it is clear that Lb A and Bif B gave high acid resistance which is significantly similar to Lb casei Shirota.

Lactobacilli are resistant to acidic condition with the ability to survive well at various pH ranges 2.5 to 8.5**(Khanal and Koirala, 2019).** However, due to low acidification ability of *Leuconostoc*, they are very sensitive to the low pH **(Bhattarai *et al*., 2016).** While in previous study carried out for Bifodobacterium, they showed appreciable acid tolerance **(Awasti *et al*., 2016).** \They have higher ability to survive in adverse stress conditions due to their wide molecular machinery allowing degradation of many non-digestible sugars **(Ruiz *et al*., 2011).** Also, our finding of low acid tolerance of Lactococci was found to collide with earlier researches **(Akbar *et al*., 2019; Bhattarai *et al*., 2016).**

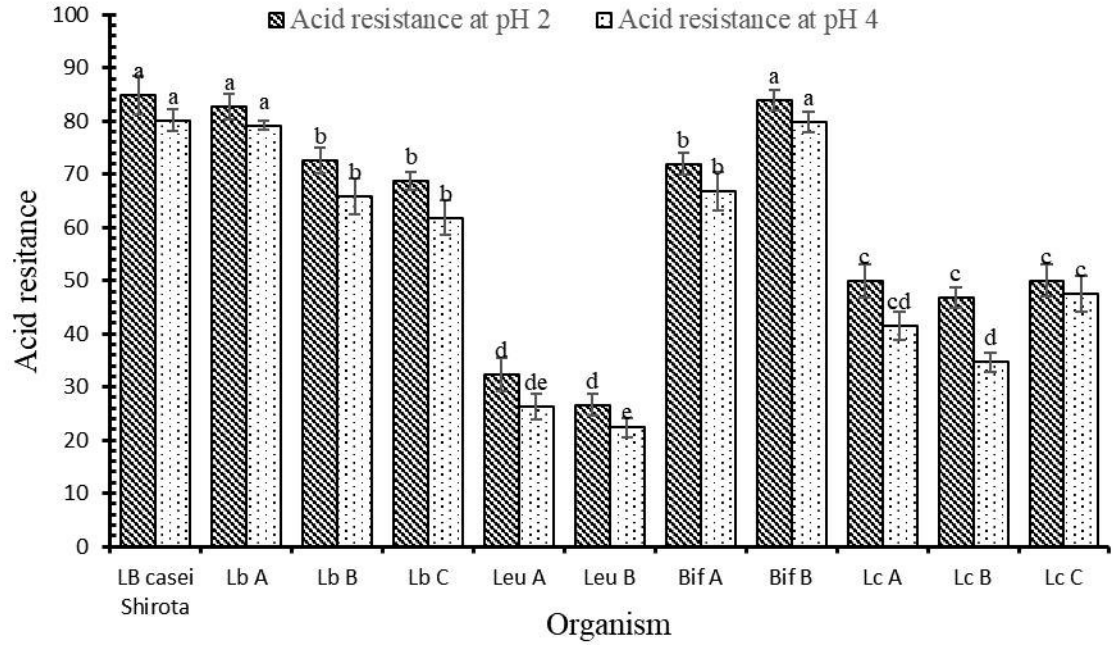

Figure 9 Acid resistance of all isolates obtained from the pooled *theki dahi* sample

Results are expressed as mean ±SD and error bar represents standard deviation. Also, the similar subscript denotes that they are significantly similar while the different subscript represents significant difference between them.

3.3.2 Bile salt resistance test

Bile salt resistance at 0.3% and 0.4% (w/v) bile salt of all 10 isolates obtained from pooled *theki dahi* sample was determined and compared to that of *Lactobacillus casei* sub sp. Shirota. All the tested isolates were able to survive well at bile salt 0.3% and 0.4 % for 4 hours (Figure 10). From the figure, it is clear that Lb A and Bif B resulted high bile salt resistance which is significantly similar to Lb casei Shirota.

LAB are able to survive well even at 0.4%, 0.5% and 0.6% bile salt concentration for 2, 4 and 24 hours **(Menconi *et al*., 2014).** Furthermore, as being popularly known a probiotic organism, Bifidobacterium show appreciable bile salt tolerance even at 1% and 2% concentration **(Awasti *et al*., 2016).** On the other hand, growth of *Leuconostoc* decreased on higher bile salt concentration. It suggested that bile salt affected their growth and limited its viability **(de Paula *et al.*, 2014).** Similarly, in case of Lactococci, they were able to grow well at 0.3% (w/v) bile salt while their growth decreased at higher concentration **(Akbar *et al*., 2019).**

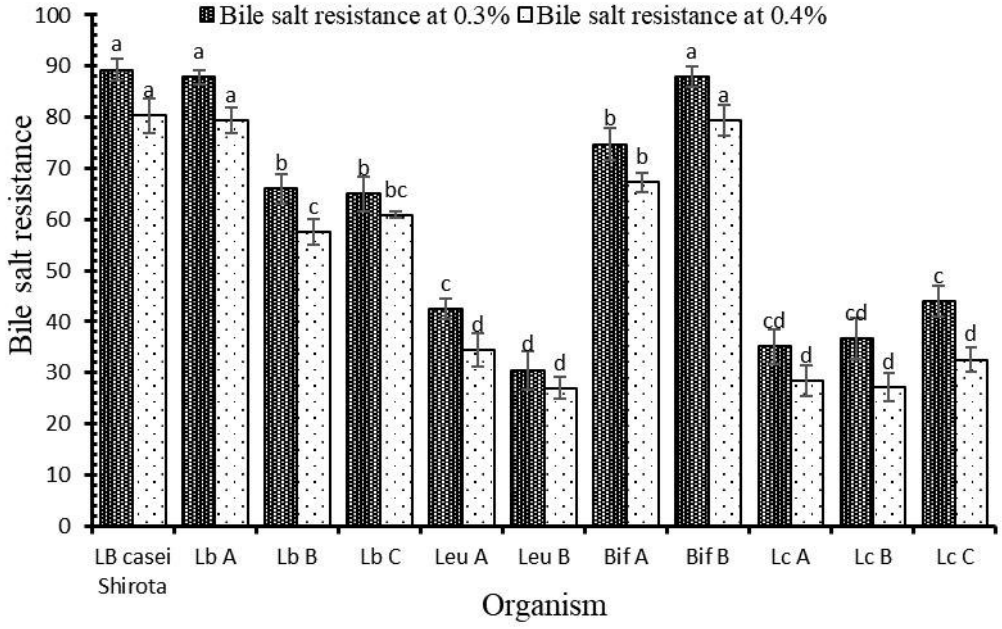

Figure 10: Bile salt resistance of all isolates obtained from the pooled *theki dahi* sample

Results are expressed as mean ±SD and error bar represents standard deviation. Also, the similar subscript

denotes that they are significantly similar while the different subscript represents significant difference between them.

3.3.3 Bile salt hydrolysis test

Bile salt hydrolysis of all the isolates were determined. It was observed that all the isolates including *Lactobacillus casei* sub sp. Shirota was able to hydrolyze bile salt at the concentration of 0.5 % (w/v).

Similar reports were concluded for LAB from camel milk **(Sharma *et al.*, 2021).** for Bifidobacterium of Indian human origin, for *Leuconostoc* from Brazilian mozzarella cheese **(de Paula *et al.*, 2014)** and also for Lactococci isolated from raw milk and kefir grains **(Yerlikaya, 2019).**

3.3.4 Antibiotic resistance test

Antibiotic resistance of all the 10 isolates obtained from pooled *theki dahi* sample was determined and compared to that of *Lactobacillus casei* sub sp. Shirota. The result obtained for all the isolates are shown in the Table 3.2. we can clearly observe that, lactobacilli and Bifidobacterium were similar to Lb casei Shirota while *leuconostoc* were found to be sensitive to all the tested antibiotic and lactococci demonstrated intermediate sensitivity against CIP.

Table 3.2 Antibiotic resistance for all the isolates

Organisms	**Antibiotics**			
	AMX (30 mcg)	**AMP (10 mcg)**	**CIP (10 mcg)**	**PEN- G (10U)**
LB Casei	--	--	+	--
Lb A	--	--	+	--
Lb B	--	--	+	--
Lb C	--	--	+	--
Leu A	--	--	--	--
Leu B	--	--	--	--
Bif A	--	--	+	--
Bif B	--	--	+	--
Lc A	--	--	-	--
Lc B	--	--	-	--
Lc C	--	--	-	--

(+)=Resistant, (-) =Intermediate and (--) =Sensitive

LcS was found to be sensitive to penicillins but quite resistant to ciprofloxacin **(Shao *et al.*, 2015).** Lactobacilli were found to be inhibited by penicillin, ampicillin, amoxicillin, tetracycline, erythromycin nalidixic acid and chloramphenicol, but only resistance to ciprofloxacin **(Khanal and Koirala, 2019).** It also suggested that resistance of lactobacilli to ciprofloxacin might be attributed to their cell wall structural and membrane impermeability.

In previous research, *Leuconostoc* were found to be sensitive to the tested antibiotic. Moreover, it suggested that antibiotic resistance could be strain-dependent and related to the environment in which the strain was isolated **(de Paula *et al.*, 2014).**

In case of Bifidobacterium, none of the isolated strains were found to be resistant to penicillin-G, amoxicillin and ampicillin **(Moubareck *et al.*, 2005).** However, they displayed smaller inhibition zone for ciprofloxacin and thus they were concluded as resistant to this agent **(Masco, 2006).**

In earlier observations, Lactococci were found to be susceptible to most of the tested antibiotic including penicillin-G, ampicillin amoxicillin and ciprofloxacin **(Yerlikaya, 2019).**

3.3.5 Cell surface hydrophobicity test

Cell surface hydrophobicity of all 10 isolates obtained from pooled *theki dahi* sample was determined and compared to that of *Lactobacilli casei* sub sp. Shirota. All the tested isolates were able to adhere to the tested hydrocarbon (hexane) (Figure 11). From the figure, it is clear that Lb A and Bif B demonstrated high cell surface hydrophobicity which is significantly similar to Lb casei Shirota.

In earlier observations, similar to our findings Lactobacilli showed high surface hydrophobicity while Lactococci showed low hydrophobicity **(Sharma *et al.*, 2021).** In case of Bifidobacterium, they showed high hydrophobicity to different hydrocarbons **(Awasti *et al.*, 2016).** However, our results were higher than that reported in the literature. As, cell surface hydrophobicity is strain-specific and it is also highly influenced by the presence of different nutrients or carrier food matrices and also different compounds used to evaluate the hydrophobicity. In earlier researches, *Leuconostoc* demonstrated similar hydrophobicity **(de Paula *et al.*, 2014).**

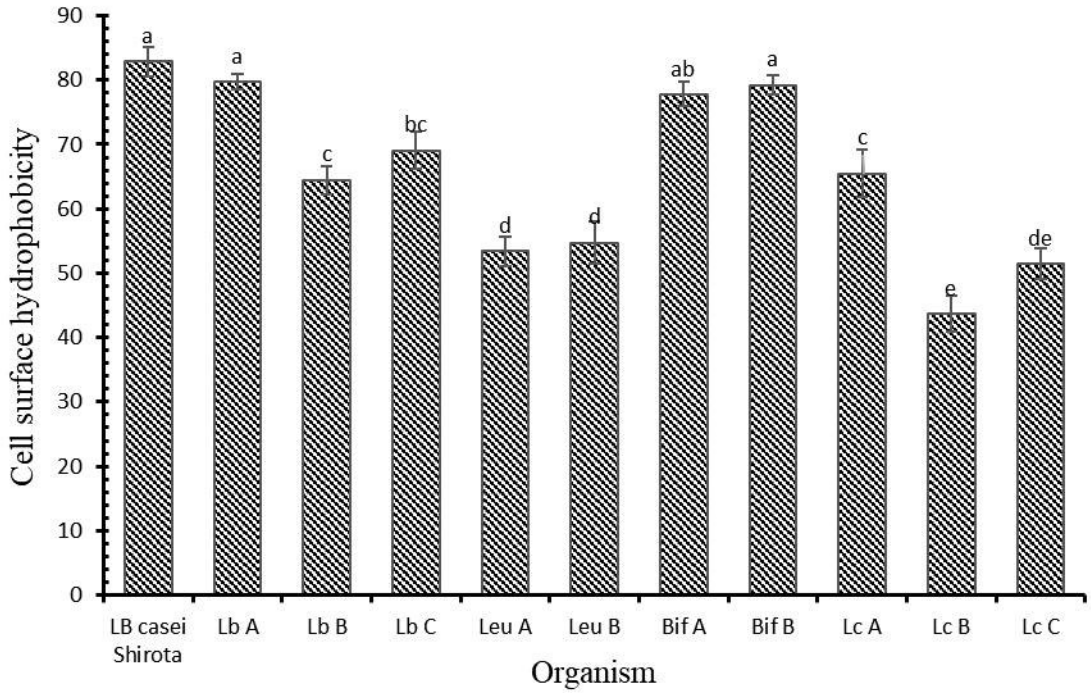

Figure 11: Cell surface hydrophobicity of all isolates obtained from the pooled *theki dahi* sample

Results are expressed as mean ±SD and error bar represents standard deviation. Also, the similar subscript denotes that they are significantly similar while the different subscript represents significant difference between them.

4. Conclusion

Varieties of LAB were isolated and identified from *theki dahi* collected from different locations around Pokhara valley. Based on morphological, biochemical and physiological characterization, three types of Lactobacilli, two types of *Leuconostoc*, two types of Bifidobacterium, one *Streptococcus* and three types of Lactococci were identified. All the isolates were able to resist acid of pH 2.0 and 4.0 for 2 h. Also, they were able to resist bile salt 0.3% and 0.4% (w/v) and hydrolyze bile salt 0.5% (w/v). They were found to be sensitive to penicillin G, ampicillin and amoxicillin. However, they could resist ciprofloxacin except for *Leuconostoc* and Lactococci. Finally, they were able to adhere to tested hydrocarbon (n-hexane).

This research concludes that "*theki dahi*" comprises of varieties of LAB with potential probiotic properties. Majorly, through our study Lb B and Bif A can be considered as potential candidate for probiotic organism in *theki dahi* demonstrating similar characteristics with *Lactobacillus casei* sub sp. Shirota.

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