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Potential of Lactic Acid Bacteria Isolated from Fermented Foods for Yogurt Production and Antimicrobial Activity.

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ABSTRACT

Objective: The main objective is to determine potential of Lactic acid bacteria isolated from fermented foods for yogurt production and antimicrobial activity.

Methods: A total of 20 samples of 4 different types of fermented foods (*Gundruk, Sinki, Pickles* and *Cheese*) were collected from different district and research was carried out in microbiological laboratory of D.A.V. College. Lactic acid bacteria (LAB) were isolated from fermented foods and identified through gram staining, catalase, motility, O/F, carbohydrate fermentation and salt concentrations test. Yogurt was prepared using isolated LAB. Probiotic property of isolated LAB was determined by observing growth on 3.1 pH 1.0 N HCl MRS broth and 0.3% sodium taurocholate. Bacteriocin was extracted by centrifugation technique and antimicrobial activity of bacteriocin was performed by agar well diffusion assay.

Result: Among 20 different samples, LAB was isolated from Sinki (S_2 and S_3) and *Gundruk* (S_5) sample only i.e., 15% of total sample. And identified as *Lactobacillus* genera. Isolated LAB showed growth on 3.1 pH 1.0 N HCl MRS broth and 0.3% sodium taurocholate i.e., has probiotic potential. Extracted bacteriocin showed inhibitory action against *Bacillus* spp. and *Escherichia coli*. Yogurt was prepared using isolated LAB and on comparing it with yogurt prepared by using standard thermophilic Freeze-dried lactic culture, the physiochemical properties of yogurt like pH, titratable acidity and syneresis were within suitable standard range during 14 days of storage (4ºC).

Conclusion: The overall, result indicated that LAB can be isolated from traditional fermented foods and isolated LAB have potential as commercial starter culture for yogurt production. The bacteriocin extracted showed inhibitory action against different bacteria. As a result, bacteriocin like molecules can potentially increase shelf life by inhibiting growth of spoilage causing microbes.

Keywords: Lactic acid bacteria, bacteriocins, yogurt, Gundruk, antimicrobial activity, probiotic potential.

INTRODUCTION

Lactic acid bacteria (LAB) are gram positive, catalase negative, fermentative (O/F) and shows zone of hydrolysis around the colonies in MRS (De Man, Rogosa and Sharpe agar) media. It uses carbohydrate as main carbon source (George et al., 2018). LAB are the group of bacteria that are able to ferment carbohydrate to produce lactic acid. These group of bacteria are currently widely used in fermentation of food. In the recent time, significance of LAB has grown a noteworthy contribution not only in food industry but also as a probiotic function (Wang et al., 2021).

LAB are able to breakdown complex polysaccharides and transforms undesirable flavor substances. During metabolic processes, LAB can generate variety of products like amines, bacteriocins, vitamins, exopolysaccharides. Due to such metabolic characteristics, LAB have been found to have diverse application in food sector (Zheng et al., 2020).

LAB are used as starter culture in food fermentation. *Lactobacillus bulgaricus* and *Streptococcus thermophilus* are dominant LAB in dairy industry (Rahman et al., 2016). Yogurt called “Dahi” in Nepal holds significant importance as a key dairy product in the country. Yogurt traces its origin back many centuries to the nomadic tribes of eastern Europe and western Asia, where herders store milk in containers made from animal stomach. The milk’s inherent enzyme caused it to coagulate within container, thus successfully producing yogurt. As time passed, yogurt making techniques evolved into familiar yogurt we enjoy today. Yogurt is valuable source of protein and calcium; with fermentation it enhances the absorption of nutrients in our body. Ongoing research has unveiled numerous health benefits of yogurt consumption, including immune system enhancement, reduced yeast infection and decreased colon cancer (Diary council of California, 2015).

LAB can be isolated from traditional fermented foods like *Gundruk* and *Sinki* but extremely few studies have been carried out in Nepal to isolate and identify LAB from natural niches (Sharma et al., 2021). Promoting the growth of native culture for yogurt production, rather than relying on imported cultures, can offer significant role in dairy industry (Kilic et al., 2022).

During metabolic process some LAB produces bacteriocin, an antimicrobial peptide, act on other bacteria with certain zone of inhibition. LAB isolates from *Lactobacillus* genera with bacteriocin producing ability have been applied in food preservation and in control of spoilage causing pathogens (Parada et al., 2007). Bacteriocins are bioactive compounds, these natural substances are being explored as promising substitutes for conventional antibiotics and are also being evaluated for their potential applications as preservatives within the realms of both the food and pharmaceutical sectors (Leslie et al., 2021).

From long history, Nepal has adapted use of fermented foods as dietary source in their daily consumption. It includes Gundruk, Sinki, Pickles, Cheese, Yogurt etc. This study aims to investigate yogurt production potential of isolated LAB from natural niches, probiotics potential and antimicrobial activity. Foodborne illnesses remain one of the global health interests, through investigating the antimicrobial activity of LAB isolated from fermented foods may contribute to the development of safer and healthy foods.

METHODS

Sample collection

Prior to each collection, reviewed the test description appropriately about sample type (Gundruk, Sinki, Vegetable Pickle, Cheese), quantity (each of 25 gram), aseptically collected in sterilized zip lock plastic bag from different districts of Nepal and transported to Microbiology laboratory of D.A.V. campus. Samples were kept in refrigerator (around 5ºC) until laboratory identification of Lactic Acid Bacteria.

Isolation of Lactic acid bacteria

For isolation of LAB, pour plate technique was used. Then, it was serially diluted up to 10^{-6} dilution. The samples from 10^{-2}, 10^{-4}, 10^{-6} dilution were pipetted out in the petriplate. Then, De Man, Rogosa and Sharpe (MRS) media incorporated with 1% calcium carbonate was poured properly and incubated at 35ºC for 48 hrs. The isolated colony was sub-cultured again in MRS agar then the result was observed. The isolated pure cultures were examined for their colony appearance and subjected to Gram staining for characterization. Only Gram positive, non-motile, rod or cocci-shaped bacteria, showing phenotypic characters similar to *Lactobacillus* and *Streptococci* species on MRS agar media with zone of hydrolysis around colonies were selected for further experiments of biochemical test. The Biochemical test used for identification of LAB are catalase, O/F, MR, VP, carbohydrate fermentation, and different salt concentrations test. The cultures were stored and maintained at 4°C on MRS agar slants for further studies (Sharma, et al., 2021).

Extraction of bacteriocin

The selected LAB isolates were inoculated from slants to fresh 250ml MRS broth and incubated at 37ºC for 5 days. The culture broth of each isolate was centrifuged separately at 10,000 ×g for 20 minutes. The supernatant was collected after centrifugation and passed through Whatman's no.1 filter paper. To confirm bacteriocin production, the cell free neutral supernatant broth was collected for antibacterial activity (Kazemipor, et al., 2012)

Antimicrobial activity of bacteriocin

Fresh cultures of test organisms were swabbed on the Muller Hinton agar (MHA). Wells of 6 mm were made on the media to load samples and control. 35µl of bacteriocin solutions and sterile water (control) was loaded on the respective well. The loaded samples were allowed to diffuse. Then the plates were incubated at 37ºC for 24 hours in upright position and then the zone of inhibition were measured (Zhennai, 2000).

Carbohydrate fermentation test

In this test, fresh culture of test organism was inoculated into glucose broth, lactose broth and sucrose broth aseptically. It was incubated at 37ºC for 24 hours and look for the color change of the medium.

Acid tolerance

An acid tolerance test was studied for selected lactic acid bacteria. The pH of the MRS broth was adjusted to 3.1 with 1.0 N HCl and control set (pH 7.0) was used along with the same. Further, the broth was inoculated with isolated lactic acid bacteria ubated at 37 ºC for 24hrs and the growth was observed.

Salt tolerance

NaCl tolerance was studied for isolated lactic acid bacteria. The concentration of MRS broth was adjusted to 2%, 4% and 6% NaCl. The broth was inoculated with lactic acid bacteria and incubated at 37 ºC for 24hrs and the growth was observed.

Bile tolerance

Bile tolerance was studied for isolated lactic acid bacteria. The concentration of MRS broth was adjusted to 0.3% sodium taurocholate. The broth was inoculated with lactic acid bacteria and incubated at 37 ºC for 24hrs and growth was observed.

Yogurt preparation

1 liter of commercial pasteurized cow's milk was purchased and dry matter of milk was standardized to 16% with skimmed milk powder. The milk was heated to 95ºC for 5 minutes and cooled down to 42ºC.The starter culture of isolated LAB was added to the initial cell as the count of 10^8 CFU/g by adjusting with 0.5 Mcfarland scale (Kilic, et al., 2022). It was incubated at 35ºC for 8hrs. Physiochemical properties (pH, titratable acidity and syneresis) of yoghurt was measured in the 1, 7 and 14 days.

RESULTS

Lactic acid bacteria were isolated from Gundruk (S_5) and Sinki (S_2 and S_3) sample. A total of 3 LAB was isolated from 20 samples of 4 different traditional fermented food sample. A total of 5 samples of each Gundruk, Sinki, Pickle and Cheese were tested. The highest occurrence of LAB was found in Sinki (S_2 and S_3) i.e. 2, followed by Gundruk (S_5) i.e. 1. Among the total sample, from only 15% sample LAB was isolated.

The isolated LAB on MRS agar incorporated with 1% $CaCo_3$ showed transparent area around the colony. Upon investigating morphology, dimensions and coloring of these LAB isolates through gram staining and spore staining, the gram-positive rod shaped, non-spore former bacteria were further assessed through biochemical test and identified as *Lactobacillus* genera.

The isolated LAB i.e., *Lactobacillus* genera from Sinki (S_2) only showed growth at 3.1 pH 1.0 N HCl MRS broth and 0.3% sodium taurocholate (bile salt). The HCl under pH 3.0 and 0.3% bile salt has been preferred, as survival of bacterial strains in low pH and bile salt condition is more accurate indication of the ability of strains to survive passage through the stomach i.e., probiotic potential whereas other two isolates Gundruk (S_5) and Sinki (S_3) did not show any potential growth.

None of the above isolates showed gas in durham's tude i.e. homofermentative but showed growth in 2%, 4% and 6% Nacl concentrations i.e. it reflects adaptability to osmotic stress.

Sinki (S_2 and S_3), and Gundruk (S_5) was further analyzed in downstream process of this research.

The bacteriocin was extracted from isolated *Lactobacillus* genera of Sinki (S_2) only, which showed antimicrobial activity against *Bacillus* spp and *Escherichia coli*. However, LAB did not show inhibition zone against *Staphylococcus aureus* and *Klebsiella* spp. The yogurt, thus prepared from isolated LAB (Sinki, S_2) as a count of 10^8 CFU/g by adjusting with 0.5 Mcfarland scale showed suitable standard of pH, titratable acidity and syneresis during 14 days of storage at 4ºC whereas other two isolates Gundruk (S_5) and Sinki (S_3) was unable to produce yogurt. The yogurt thus produced from isolated LAB is compared with yogurt produced from standard thermophilic Freeze-dried lactic culture of brand CHR-HANSEN.

Table 1: Morphological characteristics of isolated LAB

S.N.	Sample	Spore stain	Gram stain	Cell morphology	Cellular arrangement
1.	Gundruk (S_5)	-	+	Rod	Paired rod
2.	Sinki (S_2)	-	+	Rod	Paired rod
3.	Sinki (S_3)	-	+	Rod	Paired rod

+ = Positive, - = Negative

Table 2: Biochemical and physiological characteristic of LAB isolated

S.N.	Sample	Catalase	O/F	MR	VP	Glucose	Lactose	Sucrose	LAB genus detected
1.	Gundruk (S_5)	-	Fermentative	-	-	+	+	+	Lactobacillus
2.	Sinki (S_2)	-	Fermentative	-	-	+	+	+	Lactobacillus
3.	Sinki (S_3)	-	Fermentative	-	-	+	+	+	Lactobacillus

+ = Positive, - = Negative

Table 3: Antimicrobial activity of Bacteriocin against certain bacteria

S. N	Test organisms	Agent used	Method	Zone of inhibition
1.	*Bacillus* spp.	Bacteriocin	Agar well diffusion assay	16mm
2.	*E. coli*	Bacteriocin	Agar well diffusion assay	12mm
3.	*S. aureus*	Bacteriocin	Agar well diffusion assay	0 mm
4.	*Klebsiella* spp.	Bacteriocin	Agar well diffusion assay	0 mm

Table 4: Physiochemical properties of yogurt during storage at 4ºC

S. N	Time	pH	Titrable acidity	Syneresis
1.	1 day	4.52	0.72%	44.21%
2.	7 days	4.45	0.75%	45.89%
3.	14 days	4.36	0.79%	47.23%

% = Percentage

DISCUSSION

In the present study, a total of 20 samples of 4 different fermented foods included in which only 3 LAB (15%) was isolated from traditional fermented food samples. After comparing the result of biochemical, morphological and physiological, the isolated organism was identified as *Lactobacillus* spp. The result of this study is in accordance with the research conducted by (Galvez et al., 2007) which revealed that LAB could be detected in variety of habitats including fermented foods. In a similar way, study conducted in Argentina, where selected 31 fermented dairy and vegetable foods as sources of LAB for production of pickles, 64 isolates presumptively identified as *Lactobacillus* strains (Saez, et al., 2018). The result of this study was similar to the study conducted in kathmandu where among 30 samples of 4 different types of traditional fermented foods, 21 isolates were identified which also possessed bacteriocins like activity (Sharma, et al., 2021). In this study, *Lactobacillus* genera was identified from locally fermented mustard green, Gundruk (S_5) and Sinki (S_2 and S_5), which is similar with result obtained in the study conducted in Turkey (Kilic, et al., 2022). And also similar with study conducted in Banglore (Bhardwaj, et al., 2012) with identification and isolation of similar *Lactobacillus* spp.

In this study, LAB was isolated from Gundruk (S_5) and Sinki (S_2 and S_3) only, which is 15% of total sample used. Isolated number of LAB was comparatively less in compare with previous studies. There can be various reason behind this. The cheese used for isolation of LAB may not have been ripened or there could be use of preservatives like salt. Similarly, in Pickle sample the use of turmeric and mustard oil could have inhibited LAB. In most of the Gundruk and sinki sample, the isolated colonies on microbiological analysis, identified as yeast. The growth of yeast on these fermented sample could have inhibited LAB proliferation. These might be reason that could have hindered in the isolation of LAB.

Bacteriocin producing LAB genera was isolated from traditional fermented foods. 20 Bacteriocin producing *Lactobacillus* spp. were isolated from traditional Korean fermented food such as *Kimchi* (Haneul, et al., 2014). 12 strains of lactic acid bacteria (LAB) that produce bacteriocin were separated from fermented food originating in Senegal, as documented by (Diop, et al., 2007). Several studies have shown that organism such as *E. coli* and *Bacillus* spp. are adversely affected when present in traditional fermented food (Kawahara et al., 2010). This study is similar to the study conducted by (Sharma, et al., 2021) where extracted bacteriocin showed action against *E.coli* and *Bacillus* spp. with zone of inhibition of 26mm and 24mm respectively. In this study, though the zone of inhibition by Bacteriocin is comparatively lower as compared to other previous studies. This may due the varability in strains of *Lactobacillus* spp used to extract bacteriocins. Few comparative studies were conducted among different species of *Lactobacillus* (Ramos et al., 2015). The results also showed that *Lactobacillus* spp exhibited significant activity against *E. coli* and *Staphylococcus aureus.* Isolated Lactic acid bacteria exhibited antimicrobial activity with varying diameter of inhibition zone on selected bacteria like *Bacillus* spp. and *Escherichia coli* with 16mm and 12 mm respectively. The present study result showed a good zone of inhibition against *Bacillus* spp when compared to the study conducted in Gondor town (Lelise, et al., 2014). The finding of this study also reflects a good inhibition against *Escherichia coli* when compared to study conducted in cameron (Mbawala, et al., 2013)

The central issue of fermenting milk is to extend its self-life and to preserve the nutritious component of milk. The intrinsic characteristics of fermented food items heavily rely on the existence of lactic acid bacteria engaged in fermentation (Savadaraogo, Ouattara, & Savadogo, 2004). Isolation and identification of Lactic acid bacteria from naturally occurring habitat are an important consideration for scientific and commercial purposes.

In this study, it is more likely directed to isolate as many strains of Lactic acid bacteria as possible from fermented foods, yogurt preparation potential of isolated bacteria and their ability to synthesize antibacterial molecules. Lactic acid bacteria isolated from Sinki (S_2), fermented Radish, which showed probiotic potential, was only investigated for yogurt production potential, and their antimicrobial activity against certain bacteria through agar well diffusion assay.

The isolated Lactic acid bacteria exhibited the yogurt production potential with pH, syneresis and titratable acidity ranging from 4.52 – 4.36, 0.72% - 0.79% and 44.21% - 47.23% respectively.

According to the IDFA act of 2023, yogurt is considered to be of high quality if its pH falls between 4 and 4.5, and its titratable acidity ranges from 0.7% to 1.2% within a storage period of 14 days. The titratable acidity of the yogurt samples, measured on the 14th day of storage, was found to be 0.78% in terms of lactic acid. The outcomes observed in terms of pH and titratable acidity changes during storage align with findings from a study conducted by Adhikari and colleagues in 2002. In the realm of the dairy industry, syneresis is a crucial indicator of texture quality. Multiple studies have shown a decrease in the amount of syneresis in yogurt as it undergoes storage. The extent of syneresis is influenced by various factors related to composition and processing, including solid content, fat content, protein content, heat treatment, homogenization, incubation temperature, and pH/acidity.

The results of this research emphasize the significance of LAB in the creation of yogurt and highlight their wider implications in safeguarding food and human well-being. The notable antimicrobial effectiveness of bacteriocin against diverse bacterial types proposes the potential use of these LAB as a substitute for antibiotics, especially amid the escalating challenges of antimicrobial resistance. The evident probiotic qualities of the isolated LAB indicate their potential beneficial influence on intestinal health. This harmonizes with the growing fascination with fermented foods, given their capacity to foster the development of a thriving gut microbiota.

CONCLUSION

The result indicated that isolated *Lactobacillus* genera have potential as commercial starter to improve the quality of yogurt. This study is a good example of the presence of the Lactic acid bacteria strains in different environment including edible fermented mustard green, Gundruk. Developing indigenous cultures as the starter instead of imported culture used in yogurt preparation will be an important gain in yogurt industry.

The isolated Lactic acid bacteria and obtaining cell free supernatant (CFS) from these lactic acid bacteria is an important phenomenon. CFS shows the great inhibitory properties against certain pathogenic organism. Due to which contamination of food by these pathogens like *Bacillus* spp, *Escherichia coli* can be inhibited by the antimicrobial activity of CFS produ*ce*d by the lactic acid bacteria.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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