Antimicrobial Activity of Lactic Acid Bacteria Isolated from Traditional Fermented Food

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ABSTRACT

Objective: The main objective is to isolate Lactic acid bacteria from traditional fermented food of Kathmandu valley and to study their antimicrobial properties by agar well diffusion method.

Methods: A total of 30 samples of 4 different types of traditional fermented food (*Gundruk* and *sinki, Pickles* and *Dahi*) were obtained from Kathmandu valley and processed in Microbiology Laboratory of Padma Kanya Multiple Campus. For identification of Lactic acid bacteria (LAB), Gram staining, catalase and motile tests were done. In the carbohydrate fermentation test, all isolates were processed for fermentation of glucose, lactose, sucrose and fructose. Bacteriocin was extracted by precipitation method. Antibiotic susceptibility of the isolates was determined by using modified Kirby-Bauer disc diffusion method. The antimicrobial activity of Lactic acid bacterial (LAB) was done by agar well diffusion method.

Results: A total of 21 LAB isolates were identified which were 10 *Lactobacillus* spp (47.6%), 8 *Pediococcus* spp (38.0%) and 3 *Streptococcus* spp (14.3%). The antimicrobial activity of bacteriocin showed inhibitory activity against *Shigella* spp, *Escherichia coli* and *Bacillus* spp but didnot show inhibition to *Staphylococcus aureus*, *Salmonella* spp. and *Klebsiella pneumoniae*. For the quality control, the antimicrobial activity of bacteriocin was done on ATCC (25923) *Staphylococcus aureus.* All isolates were susceptible to ampicillin while resistant to nalidixic acid and Co-trimoxazole.

Conclusion: The present study showed the bacteriocin produced by LAB from traditional fermented food (*Gundruk* and *Sinki,* yogurt and *Pickle*) showed antimicrobial activity against different bacteria which underline its important role in improving food quality and increasing safety.

Keywords: Traditional fermented foods, lactic acid bacteria, bacteriocins, antimicrobial activity, Nepal

INTRODUCTION

Lactic acid bacteria (LAB) play an important role in food as their importance is associated mainly with their safe metabolic activity while growing in foods. They are utilizing available sugar for the production of organic acids and other metabolites which are natural acceptance for human consumption (Bourdichon et al. 2012). Fermented food products like curd and pickles had been an integral part of human diet from ancient time (Nuraida 2018). LAB are groups of Gram-positive bacteria. They are facultative anaerobes, catalase-negative, non-motile, do not produce spores, and create lactic acid as a metabolic end-product during carbohydrate fermentation. Based on their metabolic pathways, LAB is classified either homofermentative or heterofermentative. In homofermentative LAB, they ferment sugars to produce mainly lactic acid under anaerobic conditions. In heterofermentative LAB, sugars are fermented to produce ethanol, CO_2, and less lactic acid (Ayyash et al. 2018).

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Traditionally fermented food like *Pickles, Yogurt* and *Gundruk* and *Sinki* are usually homemade products obtained through spontaneous fermentation (Lee et al. 2004).

LAB are considered as a major group of probiotic bacteria which are known as microbial cell preparations or components of microbial cells that have a beneficial effect on the health and well-being of the host (Salminen et al. 1999). So, LAB has a diverse range of health-promoting effects, including protection against infectious agents, immunomodulatory effects, anti-allergenic effects, anti-obesity effects, anti-oxidant effects, enhancing the bioavailability of vitamins/minerals, anti-anxiety effects (Mathur et al. 2020).

During fermentation, LAB produces compound like Bacteriocin which is ribosomally synthesized antimicrobial peptides that are active against other bacteria, either of the same species (narrow spectrum) or across genera (broad spectrum). It exerts a preservative effect on fermented food as a result of strong antagonistic activity against spoilage organisms as well as pathogenic bacteria like *E. coli, Bacillus* species, *Staphylococcus aureus* etc. (Gorya et al. 2013; Mobolaji & Wuraola 2011).

There are plenty of traditional fermented food in Nepali diet, among which *Pickles, Yogurt,* and *Gundruk* and *Sinki* are most common of Nepali kitchen. So, this study was done for identifying LAB in Nepali traditional fermented food and also analyzing its antimicrobial properties which can be used to increase self-life and quality of food as well as their application as bio preservatives.

METHODS

Sample collection

A convenience sampling technique was used for samples collection. A total of 30 samples (10 homemade *Yoghurt*, 10 *Pickles, 5 Sinki and 5 Gundruk* each 25 gms) were aseptically collected in sterilized zip lock plastic bags and transported to microbiology laboratory of Padma Kanya Multiple Campus. Samples were kept in a refrigerator (around 4^0C) until laboratory identification of Lactic acid bacteria.

Isolation of LAB

For isolation of LAB, 1gm of each sample (*Gundruk* and *Sinki*, *Yogurt* and *Pickle*) was added to 9ml of phosphate buffered saline to make 1/10 dilution and mixed the sample properly. The 1/10 dilution was then 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 35^0C for 48 hours. The isolated colony was sub-cultured again in MRS agar then the result was observed (Kazemipoor et al 2012).

Identifying LAB from sample

LAB was identified by Gram staining and biochemical tests. Further, carbohydrates fermentation for *Lactobacillus* species, *Streptococcus thermophillus* and *Pediococcus* species were done by observing the colour change and gas formation. In this study, all isolates were incubated at different temperature like 10˚C, 35˚C, 37˚C and 45˚C (Cheesbrough 2000).

Extraction of bacteriocin

The isolated LAB having inhibition property was propagated in 500ml MRS broth (pH 7.0) for 48 hours at 35^0c.The cultures were centrifuged at 10,000 rpm for 20 minutes at 4^0C to obtain a cell free suspension (CFS). Then pH of the CFS was adjusted to 7.0 by means of 1M NaOH. After adjusting pH, CFS was precipitated with ammonium sulphate (40% saturation). The bacteriocin was filtered through the Whatman's no.1 filter paper and eluted in potassium phosphate buffer (Yang et al 1992).

Determination of antimicrobial activity of bacteriocin

Fresh cultures of all test organisms were swabbed on the Muller Hinton agar. Wells were made on the media to load samples and control. Bacteriocin solutions and sterile water (control) were loaded on the respective well. The loaded samples were allowed to diffuse. Then the plates were incubated at 37^0C for 24 hours then the zone of inhibition were measured (Zhennai 2000).

Antibiotic susceptibility tests

A modified method of Kirby-Bauer disc diffusion method according to the CLSI (2006) was used for the study. The culture densities of isolated lactic acid bacteria were adjusted to 0.5McFarland. A suspension of the isolated LAB was spread over the MHA agar plates, antibiotic discs of ampicillin (10 µg), nalidixic acid (30 µg), azithromycin (15 µg), co-trimoxazole (30 µg) and cephalexin (30 µg) were placed on the surface of the agar plates. In an inverted position, the plates were incubated at 37^0C for 24 hours. The diameters of the inhibition zones around the discs were measured. The result was expressed as susceptible, intermediate, or resistant according to the standard chart.

RESULTS

In the present study, 21 LAB were isolated from 30 samples with 4 traditional fermented food samples. A total of 10 samples of *Gundruk and Sinki*, 10 samples of *Pickle* and 10

samples of *Yogurt* were included. The highest occurrence of LAB was found in *Yogurt* samples 11(36.6%) and lowest number of LAB was found in *Pickle* samples 4 (13.4%). The higher LAB isolates were *Lactobacillus* species (47.6%) and lower were *Streptococcus* species (14.3%).

Morphological characteristics of the LAB isolates were identified as Gram positive, catalase negative and non-motile. The cell morphology of all isolates was evaluated through microscopic observation. In the carbohydrate fermentation test, all isolates were able to ferment Sucrose and fructose. Among the isolates, only *Pediococcus* species were not able to ferment glucose and lactose. In this study, only one isolate (*Streptococcus thermophillus*) was able to grow at 45^{0}C. All the isolates of *Lactobacillus* species and *Pediococcus* species were able to grow both at 35^{0}C and 37^{0}C. In addition, the isolates of both *Pediococcus* species and *Lactobacillus* species show slow growth at higher temperature.

Isolated LAB (cell free filtrate) showed antimicrobial activity by zone of inhibition against *S. aureus*, *Shigella* species, *Salmonella* species, *E. coli*, *Klebsiella* species and *Bacillus* species. However, LAB did not show inhibition zone against *Staphylococcus aureus*, *Salmonella* species and *Klebsiella pnumoniae*.

This study showed, all the isolates were susceptible to ampicillin, but resistant to nalidixic acid and co-trimoxazole.

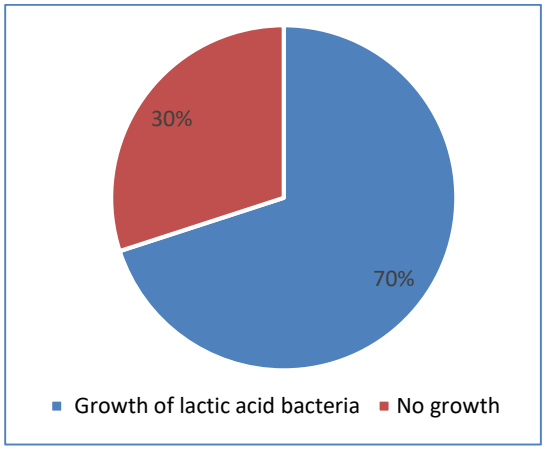

Figure 1: Growth profile of LAB

Table 1: Types of LAB isolated from fermented food samples

Fermented food samples	LAB isolates			Total N (%)
	Lactobacillus Species N (%)	*Pediococcus* *Species* N (%)	*Streptococcus thermophillus* N (%)	
Gundruk	0	6	0	6 (20.0)
Pickle	2	2	0	4 (13.4)
Yogurt	8	0	3	11(36.6)
Total	**10 (47.6)**	**8 (38.0)**	**3 (14.3)**	**21(70.0)**

Table 2: Morphology, physiological and biochemical characteristics of isolated genera of LAB

Characteristics	*Lactobacillus* species	*Pediococcus* species	*Streptococcus thermophillus*
Cell morphology	Rods	Cocci	Cocci/chain
Gram test	+	+	+
Motility test	-	-	-
Catalase	-	-	-

Fermentation of carbohydrates			
Glucose	+	-	+
Lactose	+	-	+
Sucrose	+	+	+
Fructose	+	+	+
Growth at different temperature			
10^0C	-	-	-
35^0C	+	+	+
37^0C	+	+	+
45^0C	-	-	+

+ = Positive, - = Negative

Table 3: Antimicrobial activity of test organism by LAB

Test organisms	**Zone of inhibition (mm)**		
	***Pediococcus* species**	***Lactobacillus* species**	***S. thermophillus***
Staphylococcus aureus ATCC (25923)	30	26	25
Staphylococcus aureus	-	-	-
Shigella species	26	27	29
Salmonella Typhi	-	-	-
Salmonella Paratyphi	-	-	-
Escherichia coli	25	26	30
Klebsiella pneumonia	-	-	-
Bacillus species	15	24	17

- = No zone of inhibition.

Table 4: Antibiotic susceptibility tests

LAB	**Inhibition zone (mm)**				
	NA	**COT**	**AMP**	**AZI**	**CFX**
Pediococcus species	R	R	S	S	R
Lactobacillus species	R	R	S	S	S
Streptococcus thermophillus	R	R	S	R	S

AMP=Ampicillin (10 µg), NA=Nalidixic acid (30 µg), AZI=Azithromycin (15 µg), COT=Co-trimoxazole (30 µg) and CFX= Cephalexin (30 µg)

DISCUSSION

In the present study, altogether 30 samples with 3 different types of fermented foods were included in which 21 LAB (70%) were isolated from traditional fermented food samples. By comparing the result of morphological, physiological and biochemical tests, the isolates were identified as 8 *Pediococcus* species (38.0%), 10 *Lactobacillus* species (47.6%) and 3 *Streptococcus thermophillus* (14.3%). Among total isolates, only one isolate (*Streptococcus thermophillus*) was able to grow at 45^{0}C whereas *Lactobacillus* and *Pediococcus* species were able to grow at 35^{0}C and 37^{0}C but not at 45^{0}C. The result of the present study is in accordance with the report of (Galvez et al. 2007) who revealed that members of LAB could be detected in a variety of habitats including fermented foods. LAB are able to ferment different carbohydrates like glucose, fructose, lactose etc. as they use the nutrients in the substrate for their own metabolism and cell growth and multiplies in food (from one million per millilitre to one billion per millilitre) (Robinson 1991). Lindquist (1998) reported that a medium that would support their growth must contain a fermentable carbohydrate and many growth factors.

In this study, only three bacteriocin producing 3 LAB genera were isolated from traditional Neplease fermented foods. Many papers have been published on isolation, characterization of LAB from traditional fermented foods. Fifty bacteriocin producing *Lactobacillus* species were isolated from traditional Nigerian fermented foods such as *Fufu, Garri, Nono* and *Ogi* (Adenike et al. 2007). Twelve bacteriocin producing LAB strains were isolated from Senegal fermented foods (Diop et al. 2007).

Antimicrobial substance (bacteriocin)of *Pediococcus* spp, *Lactobacillus* spp and *Streptococcus thermophillus* isolated from fermented food samples showed antimicrobial activity of against *S. aureus*, *Shigella* species, *Salmonella* species, *E. coli*, *Klebsiella* species and *Bacillus* species. Antimicrobial substance shown inhibited to *S. aureus* ATCC (25923) in 30 mm, 26 mm and 25 mm with respect to diameter of inhibition zone. Similarly, all of these isolates showed zone of inhibition of *Shigella* species in 26 mm, 27 mm and 29 mm while *E. coli* was inhibited by zone of inhibition of 25 mm, 26 mm and 30 mm respectively. Zone of inhibition also shown by *Bacillus* species in the range of 15 mm, 24 mm and 17 mm respectively. However, zone of inhibition did not show against *Staphylococcus aureus*, *Salmonella* species and *Klebsiella pnumoniae*.

Several studies have shown that pathogens such as enterotoxigenic *E. coli*, *Shigella flexneri, S. typhimurium* and *B. cereus* are adversely affected when present in traditional fermented foods (Kingamkono et al. 1995, Kunene et al. 2000, Obadina et al. 2006).

Some of the antimicrobial properties exhibited by these fermented foods may be as a result of the low pH of the food as well as metabolites produced by microorganisms such as LAB involved in the fermentation. Gilliland and Speck (1977) had earlier reported that Lactobacilli showed stronger antibacterial properties against Gram-positive bacteria (*S. aureus* and *Clostridium perfringens*) than Gram-negative bacteria (*E. coli*, *Salmonella* Typhi*, and Salmonella* Paratyphi).

Microbial food safety is an increasing public health concern worldwide and many Gram negative bacteria such as *E. coli, Klebsiella* spp together with Gram positive bacteria such as *S. aureus* have been implicated in food borne diseases (Mead et al. 1999). The LAB isolated from *Yoghurt, Fufu* and *Kunuzaki* in their study probably produced different antimicrobial compounds, the quantity of which might vary with time. Collins et al. (1983) also noted the inhibition of *Psuedomonas fragi* and *S. aureus* against other microorganisms by some LAB strains which contribute to their inhibitory activity.

Similarly, different studies were done for antimicrobial activity of LAB isolated by different fermented food. Kawahara et al. (2010) characterized the antibacterial LAB strain *L. curvatus* isolated from Nozawana-zuke pickles. The strain showed antibacterial activity against some putrefactive bacteria, such as *S. marcescens*, *L. monocytogenes*, and *S. aureus* subsp. *aureus*, without affecting the growth of all the LAB strains tested except for *L. curvatus* strain. In a study of Ozkalp et al. (2009), the lactic acid bacteria were isolated from caper pickle. Thus, bacteriocins produced by LAB are likely to have a much greater potential as preservative (Bromberg et al. 2004). Liasi et al. (2009) emphasized that research on antimicrobial substances produced by LAB, had led to their potential use as natural preservatives, which may be used to combat the growth of pathogenic microorganisms in the food industry. In this study, Antibiotic susceptibility disc test was also done for all LAB isolates which showed susceptible to Ampicillin, but resistant to Nalidixic acid and Co-trimoxazole.

CONCLUSION

The present study showed that the LAB were found to be high in traditional fermented yogurt samples while it was slightly less in two samples i.e. *Gundruk* and *Pickle*. The present study showed the bacteriocin produced by LAB showed antimicrobial activity against different bacteria which underline its important role in improving food quality and increasing safety.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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