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RESEARCH ARTICLE OPEN ACCESS

Isolation and Characterization of Lactic Acid Bacteria Isolated from Fermented Food of North-West Himalayas

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Abstract

Lactic acid bacteria (LAB) are ubiquitous and are one of the major microbial groups involved in the fermentation of various types of food. They are the most dominant microbes present in milk or milk products and fermented foods where they play vital roles in both the manufacturing and ripening processes. *Kaladhi* is one of the traditional fermented products of the North-West Himalaya region. It is a hard and dry cheese. In our research, a total of 9 isolates was isolated and was evaluated on the basis of preliminary characterization viz. morphological as well as biochemical characterization and was examined for their antagonistic activity against following pathogens. On the basis of their maximum antagonistic potential against food-borne pathogenic bacteria, isolate K1 is characterized by 16S rRNA sequencing. The isolate was identified as *Lactobacillus paracasei* subsp. *Tolerans* strain NBRC 15906 K1|MN814072|.This research was aimed to study the unexplored microflora of *Kaladhi* and to determine its probiotic potential.

Keywords: Lactic acid bacteria, rRNA, Kaladhi, Probiotics, *Lactobacillus*

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INTRODUCTION

Microorganisms are present almost in every habitat because of their ubiquitous property. Among them, some microbes exhibit various beneficial properties viz. in improving human health or in food production. Probiotics are one of those microbes that have an extensive role in maintaining the host's health and controlling some diseases. According to FAO/ WHO, probiotics are: "Live microorganisms which when administered in adequate amounts confer a health benefit on the host". They are also considered as "friendly bacteria" or "good bacteria". Probiotic bacteria are non-pathogenic and non-toxic microbes and show important functions for well being and health of the host[1]. Therefore, it's important to maintain the ecological flora in order to prevent diseases, especially infections of the gastrointestinal tract[2,3].

The fermented foods are one of the major sources of probiotics[4]. The fermented foods are that food that has undergone a lactic fermentation process in which the natural microflora feeds upon the sugar and starch present in the food creating lactic acid. Traditional fermented food products are important in nutrition as sources of carbohydrates, proteins, fibers, minerals and vitamins[4].

In India, about 50-55% of milk is converted into several traditional products utilizing various processes such as fermentation, coagulation and desiccation[5]. *Kaladhi* is one of the culinary preparations of dairy in Jammu and Kashmir state[6].

Kaladhi is a traditional fermented food product of state Jammu and Kashmir (Fig. 1). It is prepared by coagulating the milk and the coagulum was working out into the pat and finally small ball-like product is prepared which are manually spread out to a circular shape and then dried into the sun light to absorb the moisture. Ahmad in his studies defined *kaladhi* as an acid coagulant product that is prepared by directly acidifying the milk with some coagulating agents like organic acids[7]. The product is made either from buffalo's or cow's milk. *Kaladhi* is also known as '*Maush kraer*' in Kashmir division of the state. *Kaladhi* doesn't require the use of rennet or any starter during its preparation. The most popular coagulant used traditionally for the *Kaladhi* preparation is tartaric acid but either citric acid or whey water can also be used to coagulate the raw or boiled milk. It is consumed after frying it with some suitable frying medium along with spices and condiments. This product is believed to possess anti-diarrheal and anti-cold properties besides being a salubrious food, thus have tremendous market potential[8].

Fig. 1. *Kaladhi*- A traditional fermented food of North-West Himalayas region

Nutritional composition of *Kaladhi* includes 412kj Energy, 3.3gms of Carbohydrate, 2.67 gms. Sugar, 11.12 gms of Proteins, equiv. 5% of Vitamin A, 83mg Calcium, 0.07mg Iron, 8mg Magnesium, 159mg Phosphorus, 104mg Potassium, 3.64mg Sodium and 0.40mg Zinc[16]. So *Kaladhi* possess so many nutrients and can be healthy to consume but because of its low pH, its shelf life is limited caused by various microbes especially molds[17]. *Kaladhi* cheese can be a source to isolate novel probiotics but unpasteurized cheese also produces food borne- pathogens like *S. aureus, L. monocytogenes, Salmonella* and *E. coli* strains[18].

Raw milk and traditional fermented foods act as highly rich environments for a diverse and indigenous microbiota, including LAB, that further contribute to the bio-preservation and development of nutraceutical or functional final products. Among LAB, the *Lactobacillus* strains are predominantly exploited to acquire the probiotic properties as well as utilized in the form of adjunct cultures. Attributing to their historical view of safe use and natural presence in the human gut, they are generally regarded as safe (GRAS). Moreover, several species of *Lactobacillus* have attained the status of qualified presumption of safety (QPS) status.

Such food products act as an alternative source of novel probiotic candidates that can

be utilized for the manufacturing of functional probiotic products that are further protected from gastrointestinal stress. So these foods can be a way to represent novel candidates of probiotics.

As the microflora of this product is not that much explore so in this research an attempt was made to isolate the potential probiotic strains in order to meet the promising results as therapeutic effects.

MATERIALS AND METHODS

Chemicals

All the chemicals utilized in this study were obtained from HI-Media and of analytical grade.

Sample collection

Samples of *Kaladhi* were collected from the North-West Himalayas region mentioned in Fig. 1.

Isolation of LAB

1gm of the sample was diluted with 9ml of sterile water and homogenized for the 60s. The homogenates were followed by serial dilution with sterile distilled water and then plated on the MRS (De Man, Rogosa, and Sharpe media) plates. Incubation was given at 37°C for 24 hrs. After incubated plates, individual similar colonies were selected and further purified by using the streak method on the selected medium[13].

Phenotypic and biochemical characterization

Preliminary identification of LAB strains was done based on their potential to grow in MRS broth, the positive gram staining, absence of endospores. MRS is a media used for the cultivation of *Lactobacillus* species. Biochemical studies of strains were done to evaluate the production of Catalase, oxidase, methyl red voges proskauer (MR-VP test), casein hydrolysis and starch hydrolysis tests[14].

Genotypic characterization

Molecular Identification of the Selected LAB Isolates: The bacterial strains were identified at the genomic level by using the 16s rRNA gene technique.

Antibacterial assay

The antagonistic activity of each isolates against spoilage causing microbes i.e pathogenic test indicators were analyzed by using the agar well diffusion assay. The results were examined in the terms of zone of inhibition around the wells. Test indicators used are *Klebseilla pneumonae, Enterococcus faecalis, St L. paracasei subsp. Tolerans reptococcus dysgalactiae* and *Pseudomonas aeruginosa*[9].

Haemolytic activity

The haemolytic activity of potential probiotic isolates was determined by the method of Marakoudakis[10]. The isolates were grown on respective broth for 37°C for 24h and were streaked on the blood agar plates that were supplemented with 5% (v/v) of whole human blood and the plates were incubated at their respective temperatures then examined on the basis of zones[10].

Inter compatibility testing

Inter compatibility of the bacterial isolates was checked by using the Cross streak method[11].

Acid tolerance assay

Survival of lactobacilli at low pH was checked by inoculating 1% of culture in 20ml of respective broth with the pH of 2.0 and 5.0 and incubation was given at 37°C for 24 hrs. Growth was measured by taking optical density at 620nm[12].

Co-aggregation assay

Co-aggregation ability of the strains to form a barrier against pathogens was assessed according to Del Re[19].

RESULTS AND DISCUSSIONS

Isolation and characterization of LAB

A total of 9 isolates were isolated from the sample *Kaladhi* and were recognized on the basis of morphological characteristics and biochemical analysis. Physiological and Biochemical characterization of the microbial isolates had been done and their characteristics were noted down in Table 1-2. Most of the microbial strains were off white Gram-positive, rod-shaped and some of them gave negative Catalase and oxidase results and hence considered as lactobacilli. All the isolates showed negative results of MR-VP test and were not able to hydrolyse amylase

Molecular Identification of the Isolates

The best-selected LAB isolates on the basis of their antagonistic effect against selected pathogenic bacteria were identified at the genomic level by 16srRNA sequence analysis. The 16S

Table 1. Morphological Characteristic of microbial isolates

No.	Isolate	Source	Color	Surface
1	K1	*Kaladhi*	White	Round
2	K2	*Kaladhi*	Cream	Round
3	K3	*Kaladhi*	Cream	Round
4	K4	*Kaladhi*	Cream	Smooth
5	K5	*Kaladhi*	White	Round
6	K6	*Kaladhi*	White	Round
7	K7	*Kaladhi*	White	Round
8	K8	*Kaladhi*	Cream	Round
9	K9	*Kaladhi*	Cream	Smooth

Table 2. Biochemical characteristics of microbial isolates

No.	Isolate	Grams reaction	Catalase test	Starch hydrolysis test	Casein hydrolysis test
1	K1	+ve	-ve	-ve	-ve
2	K2	+ve	-ve	-ve	+ve
3	K3	+ve	-ve	-ve	+ve
4	K4	+ve	+ve	-ve	+ve
5	K5	+ve	+ve	-ve	-ve
6	K6	-ve	-ve	-ve	-ve
7	K7	-ve	-ve	-ve	-ve
8	K8	+ve	-ve	-ve	-ve
9	K9	+ve	-ve	-ve	-ve

rRNA sequence of isolate K1 showed its close resemblance (98.7%) with other *Lactobacillus* paracasei strains available in the GenBank database (National Centre for Biotechnology Information (NCBI)). Therefore, this isolate was designated as *L. paracasei subsp. Tolerans* K1. The sequence of K1 was deposited in GenBank with accession number MN814072. Phylogenetic relationship of *L. paracasei subsp. Tolerans* K1 based on 16S rRNA sequencing analysis is shown in Fig. 2.

Antimicrobial activity

Many of the potential LAB isolates exhibited antibacterial effect towards food spoilage as well as pathogenic bacteria viz. *Klebseilla pneumonae, Enterococcus faecalis, Streptococcus dysgalactiae* and *Pseudomonas aeruginosa*. Isolates K-1 and K-3 inhibited most of the pathogenic micro-organisms. The data is shown in Table 3.

Hemolytic activity

The hemolytic activity of isolates was recorded by the observation of a clear zone of hydrolysis around the bacterial colonies (β-haemolysis), a partial hydrolysis or greenish zone shows α-haemolysis, or no reaction (γ-haemolysis). No zone is shown by any isolates in our present study which shows that the isolated isolates can be consumed by humans or animals in any form.

Inter compatibility testing

This test was determined by the cross streak method. It was done to check the inter-compatibility of strains in order to formulate the probiotic consortia. Every strain showed 100% compatibility with each other.

Acid tolerance assay

As pH medium of the gastrointestinal tract is acidic, so in order to survive in a raucous environment of the gastrointestinal tract, probiotic strains must show resistance to low pH. So the effect of acidic pH was studied to determine the

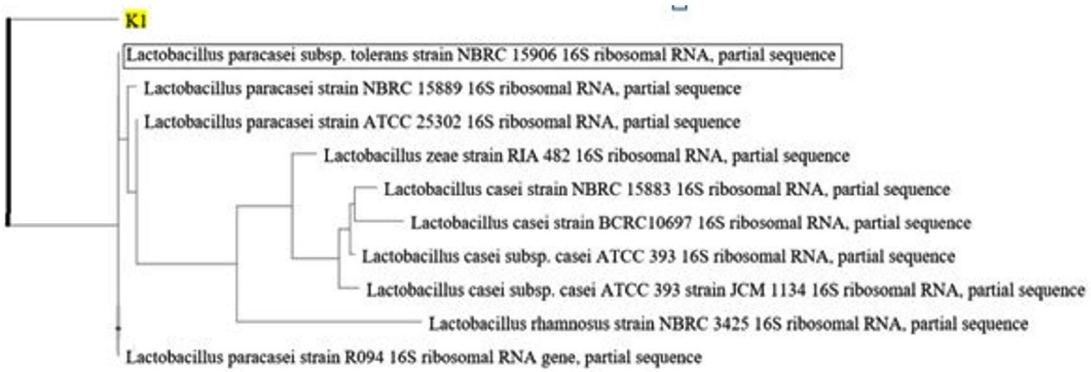

Fig. 2. Phylogenetic relationship of *L. paracasei* subsp. Tolerans K1 based on 16S rRNA sequencing analysis

Table 3. Screening of probiotic isolates on the basis of their antagonistic pattern against pathogens by disc diffusion method

No.	Isolate	Source	*K.pneumonae* (mm)	*E.faecalis* (mm)	*S.dysgalactiae* (mm)	*P.aeruginosa* (mm)	Mean (mm)	Percent Inhibition (%)
1	K1	*Kaladhi*	16	13	12	-	10.25	75%
2	K2	*Kaladhi*	-	11	-	-	2.75	25%
3	K3	*Kaladhi*	-	-	-	-	0	0%
4	K4	*Kaladhi*	12	-	-	-	3	25%
5	K5	*Kaladhi*	16	-	-	-	4	25%
6	K6	*Kaladhi*	-	-	-	-	0	0%
7	K7	*Kaladhi*	-	10	-	-	2.5	25%
8	K8	*Kaladhi*	15	11	-	13	9.75	75%
9	K9	*Kaladhi*	13	-	-	-	3.25	25%

Table 4. Effect of different pH on strains at OD 620 nm

No.	Isolate	pH2		pH5	
		O.D after 24 hour	O.D after 48 hour	O.D after 24 hour	O.D after 48 hour
1	K1	0.489	1.160	0.710	0.561
2	K2	0.211	0.177	0.450	0.532
3	K3	0.283	0.191	0.301	0.481
4	K4	0.366	0.113	0.410	1.830
5	K5	0.2770	0.201	0.361	0.374
6	K6	0.210	0.197	0.413	1.910
7	K7	0.401	0.298	1.471	0.620
8	K8	0.129	0.075	0.521	0.601
9	K9	0.393	0.163	0.951	1.590

Table 5. Evaluation of Co-aggregation ability of isolates with test indicators

Isolates	*E. faecalis*	*S. dysgalactiae*	*P. aeruginosa*	Mean
K1	35.23	29.21	46.82	37.08
K2	23.21	41.24	13.00	25.81
K3	14.5	32.18	13.23	19.97
K4	31.23	16.25	4.01	17.61
K5	18.2	15.32	21.44	18.32
K6	9.2	26.21	21.32	18.91
K7	34.2	18.03	12.1	21.44
K8	42.52	16.34	21.74	26.86
K9	28.42	15.32	21.3	21.68

potential of strains at low pH of the stomach. So in this experiment, at pH 2 k1 strain showed excellent results after the incubation of 48 hours. Table 4 shows the result of the acid tolerance assay of all strains.

Co-aggregation

Co-aggregation ability is the process by which 2 strains attached to one another via specific molecules. Probiotic strains shows Co-aggregation activity against pathogens by forming a barrier and thus prevents colonization. Co-aggregation activity of probiotic strains was evaluated against test indicators and shown in Table 5.

CONCLUSIONS

Our present study was an attempt to isolate the potential isolates from the *Kaladhi*, a traditional fermented food of northwest Himalayas. Among all of the isolates, *L. paracasei subsp. Tolerans* showed outstanding results by exhibiting antimicrobial substances and have the ability to survive at the acidic medium of the stomach. These isolates could be considered as a potential probiotics due to their antagonistic effect and can be used in nutraceutical or functional foods due to their therapeutic effect.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHORS' CONTRIBUTION

Both the authors have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

FUNDING

None.

DATA AVAILABILITY

All datasets generated or analyzed during this study are included in the manuscript.

ETHICS STATEMENT

Biosafety assay of the isolates was performed by using blood agar. For its preparation, self-blood (2.5 mL) was used with the proper care in the presence of a medical practitioner.

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