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Isolation and Identification of Probiotic Microbes from Finger Millet

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A B S T R A C T

Crop growth and productivity has for the most part been at risk of varied a-biotic and organic phenomenon stresses that are solely set to be combined thanks to world climate change. So developing improved varieties and planning newer approaches for crop improvement against stress tolerance became a priority now-a-days. Millets have varied nutrition qualities, and have justifiably been known as "nutri-cereals". The bulk of the world's millet crop is created by India, Nigeria, Niger, Mali, Burkina Faso, Chad, and China. millet (*Eleusine coracana* (L.) Gaertn), very little millet (*Panicum sumatrense* Philip Roth ex Roem. & Schult.), foxtail (*Setaria italica* (L.) P. Beauvois) and proso millet (*Panicum miliaceum* L.) are most ordinarily found species among varied millet varieties. In India, finger millet occupy the biggest space below cultivation among the tiny millets. This minor millet includes crucial amino acids viz isoleucine, leucine, methionine and phenyl alanine which can be poor in other starchy meals. It is likewise recognized for numerous fitness advantages inclusive of anti-diabetic, anti-tumerogenic, atherosclerogenic effects, antioxidant, which can be specially attributed due to its polyphenol and nutritional fiber contents. Being indigenous minor millet it's far used within side the instruction of diverse ingredients each in herbal and malted forms. Single colonies have been isolated through enriching in MRS broth and subsequent streaking on MRS agar plate. Total ten isolated micro organism have been diagnosed. All isolated strains have been characterised for probiotic houses consisting of acid and salt tolerance, phenol tolerance, sugar fermentation, lactose fermentation. Acid tolerance check changed into achieved at pH 2, 4, 6, 8 in MRS broth. Salt tolerance check changed into achieved at 2%, 4% 6% and 8% NaCl in MRS broth. Phenol tolerance check changed into achieved in MRS broth with 0.1%, 0.2%, 0.3% and 0.4% phenol concentration. Sugars consisting of glucose, fructose, sucrose, xylose and lactose have been used for fermentation tests. Results of fermentation check confirmed that maximum isolates fermented all sugars. This observe indicated that isolated species from finger millet batter have capability probiotic houses. All the isolates fermented glucose and lactose with evolution of gas. 90%, 80% and 70% of isolates fermented xylose, fructose and sucrose respectively. The probiotic bacteria were capable of fermenting different sugars and end product is lactic acid. Selected isolates can survive at low pH as well as in high salt concentrations and low concentrations of phenol. Further study is needed to find specific probiotics with specific benefit from finger millet based fermented batter.

Introduction

Agriculture productivity is adversely have an effect oned with serious impact on production and productivity due to uneven weather conditions raised temperature and fewer availability of irrigation water. World temperature change beside the speedily increasing population is mounting considerable pressure on agriculture sector to provide a lot of food from less land. The anticipated increase in temperature can principally affect the new tropics, in the main inhabited by developing countries as they're probably to suffer most loss in food production (Cline *et al.,* 2007). A key trouble is whether or not we may be capable of feed the projected international populace of 9 billion in 2050 equitably, healthily and sustainably (Beddington *et al.,* 2010). Even if an individual consumes enough calories, it's possible that he might have associate inadequate consumption of significant matters comparable to vitamins, minerals and trace components resulting in micronutrient malnourishment or what are often termed as hidden hunger. Pests and diseases also are likely to be greatly wedged by ever-changing temperatures (Stireman *et al.,* 2005).

Thus development of types with increased neutralceutical worth and improved stress tolerance has been one in every of the priority areas of analysis these days. Trendy crop improvement techniques like genomics-assisted breeding and genetic engineering play vital role in understanding the complexities of stress response and tolerance further as in providing measures for enhanced crop productivity. However, one of the potential solutions to counter these tribulations will be characteristic and rising native crops that are extremely adaptive to native climate, have high nutritive value and might with efficiency face up to organic phenomenon and/or abiotic stresses. (Shukla *et al.*, 2015). Millets are recognized as one of the most significant cereal grain. Millets are consumed by more than 1/3rd of the world residents. It is the 6th cereal crop in terms of world's agricultural making (Kimeera Ambati *et al.,* 2019).

List of millets

From various types of millets the finger millet and sorghum millets are used for consuming and the other are used as animal feedstuff. In several states of India they are using different varieties of millets. All the millets are higher in their nutrition content as compared to nutritional content of generally used rice and wheat. Wheat and rice deliver with safety of food while millets give many safeties like food, health, nutrition, livelihood, animal feed etc, production of millets as yield of agricultural benefits and they also help in handling health complications such as diabetes mellitus, hyper-lipidemia, etc.

Nutritional value of Millets

Eleusine coracana L. (finger millet), also known as "ragi" belonging from family *poaceae*, and originated in Ethopia. Although finger millet does not enter the international markets as an item of trade, but finger millet is an important crop in the areas of adaptation (Gull *et al.,* 2014), and is contains various vital nutritional components, such as carbohydrates, calcium, methionine, phyto-chemicals, seed storage proteins along with essential amino acids as well as dietary fiber. Finger millet is a strong crop because it can able to grow in semi-dry regions and exhibits a high level of tolerance to drought stress and diseases (Chagam *et al.,* 2017). Because of good nutrition quality of finger millet, its consumption increasing day by day in various forms (Chagam *et al.,* 2017).

Finger Millet (per 100 g edible portion, 12% moisture content)

Processing of millets

The common household practices of processing these foods include grinding, sprouting, cooking, malting and fermentation. Each of these processes qualitatively modifies the nutritive value of the food (Singh and Rita Singh Raghuvanshi, 2012).

Fermentation

Processing of millets, cereals and legume based compounds modifies their composition of nutrients and beneficially affects the growth of lactic acid bacteria (Minelly *et al.,* 2014). The fermented foods have characteristics flavours, taste, colour, nutrients and functional properties, which are generated by the microflora produced from microorganisms i.e., molds, Bacillus spp., lactic acid bacteria and yeast during fermentations (Minelly *et al.,* 2014).

During fermentation, microorganisms contribute to the development of characteristic properties such as taste, aroma, appearance, texture, shelf life and safety. The microbiota of fermented foods is dominated by lactic acid bacteria (LAB), which contribute to their nutritional and health properties (Nout and Motarjemi, 1997). Lactic acid micro organism play an vital function in conventional fermented meals fed on in unique countries.

Microbial fermentation is liable for many favorable food characteristics, for example flavor, shelf-life and texture. Varied categories of fermented vegetable product are offered throughout the globe (Swain *et al.,* 2014).

Probiotics are defined as "live microorganisms which, once administrated in adequate amounts, confer a health profit upon the host" (FAO/WHO, 2002). Probiotics should fulfill sure basic criteria, i.e. tolerance to enteral digestive fluid and pH scale concentration, susceptibleness to antibiotics and inhibition of growth of alternative enteric pathogens. It's well established that probiotics confer various health edges upon humans and animals, together with antihypertensive, antimutagenic, hypocholesterolemic, anticarcinogenic, anti-osteoporosis and immune modulatory effects (Anandharaj *et al.,* 2014; Chiang *et al.,* 2012).

Malolactic fermentation is a process that not only improves the organoleptic and hygienic quality, but also the nutritional quality of food; in particular, it allows good preservation (Ampe *et al.,* 1999). Other microorganisms such as Enterobacteriaceae, yeasts and molds have been isolated from many fermented grain-based foods and can affect their quality.

Studies on the microflora ecology of crabgrass-based sourdough can help to understand microbial dynamics and differences between groups of closely related microbial populations in grain (sourdough) fermentations.

In most natural fermentations, little is known about the starters used., the characterization and identification of the microorganisms involved in the fermentation of cereals with prospective selection as starter cultures is important, hence our research.

Materials and Methods

Procurement of raw materials

Urad dal, chana dal, and finger millet were bought from the local market. Millets should be kept at room temperature in airtight containers until they're ready to use.

Batter preparation

Variant 1

108 grams of chana dal, 72 grams of finger millet, and 60 grams of urad dal were soaked overnight in 200 ml of water, then drained. In a mixer, the soaking mixture was blended. The blended mixture was kept at room temperature for 8 hours to ferment.

Variant 2

72 grams of chana dal, 108 grams of finger millet, and 60 grams of urad dal were soaked overnight in 200 ml of water, then drained. In a mixer, the soaking mixture was blended. The blended mixture was kept at room temperature for 8 hours to ferment. By dilution with saline, LAB was isolated from the above described fermented batter, namely variant 1 and variant 2, plated on MRS agar, and incubated at 37°C for 24 - 48 hours.

Ten well-isolated colonies were selected and

inoculated to MRS broth. Colonies were also streaked on MRS agar to check the purity of isolates.

On MRS agar plates, one loopful broth culture was streaked and incubated for 48 hours. Individual colonies were isolated and identified using gram staining and biochemical assays.

For subsequent investigation, a single colony was stored in an MRS agar slant.

Gram staining by Hucker's Modification

Gram staining test was performed for all isolated strains according to the standard procedure. A smear of single colony was prepared on a clean glass slide and the smear was allowed to air-dry and then heat fixed. The heat fixed smear was flooded with crystal violet solution and after one minute, extra stain was drainedoff and flooded with mordant Gram's iodine. The smear was decolorized with 95% ethyl alcohol and rinsed with water. Finally safranin was used as counter stains for 7 to 8 minutes and washed with water, and examined under an oil immersion (100X) lens (Mannan *et al.,* 2016).

Catalase test

A drop of 3% hydrogen peroxide was added to a fresh culture on a sterile glass slide and mixed well. Producing bubble or froth indicated catalase-positive and no bubble or froth indicated catalase-negative (Mannan *et al.*, 2016).

pH tolerance test

MRS broth at pH 2, 3, 4, 5, 6, 7 and 8 was prepared by adjusting with 10N HCl and 1N NaOH. Fresh bacterial cultures were inoculated into respective MRS broth in test tubes and incubated at 37o C for 48 h. Only media was used as negative time control. Results were obtained by observing turbidity of the culture media after 24 h and 48 h and no growth was observed in the negative control (Mannan *et al.*, 2016).

NaCl tolerance test

NaCl tolerance of isolated Lactobacillus was determined by using MRS broth with 2%, 4% a, and 8% of NaCl concentration. The fresh culture was inoculated and incubated at 37o C for 48 h. Only media was used as a negative control. Results were determined by observing the turbidity after 24 h and 48 h and no growth was observed in the negative control (Mannan *et al.,* 2016).

Phenol tolerance test

MRS broth containing 0.1%, 0.2%, 0.3% and 0.4% of phenol concentration were prepared for the determination of phenol tolerance. The fresh culture was inoculated and incubated at 37o C for 48 h. Only media was used as a negative control. Results were determined by observing turbidity after 24 h and 48 h and no growth was found in the negative control (Mannan *et al.,* 2016).

Determination of sugar fermentation

A sugar fermentation test was performed using 1% (w/v) sugar in MRS broth. Glucose, fructose, sucrose, xylose, and lactose were used in this test. Phenol red solution was used as an indicator. 10 ml media was dispensed and Durham's tube was inserted invertible in each of the test tubes. The fresh culture was inoculated and incubated at 37°C for 24 h. Only media was used as a negative control. Results were observed by color changing and gas formation (Mannan *et al.,* 2016).

16S rRNA Identification

DNA was isolated from the culture. Its quality was evaluated on 1.0% Agarose Gel, a single band of high – molecular weight DNA has been observed. Fragment of gene was amplified by PCR. A single discrete PCR amplicon band was observed when resolved on Agarose Gel. The PCR amplicon was purified by column purification to remove contaminants. DNA sequencing reaction of PCR amplicon was carried out with 16s using BDT v3.1

Cycle sequencing kit on ABI 3730xl Genetic Analyzer.(Primer Details Given Below). The gene sequence was used to carry out BLAST with the database of NCBI Genbank database. Based on maximum identity score first ten sequences were selected and aligned using multiple alignment software programs.

Results and Discussion

Lactobacillus strains are found in the human gut including the oral cavity. In this study, a survivability study in different pH was performed because pH is an important factor for the growth of bacteria. All strains were tested in acidic conditions at different ranges of pH 2 to 6. From this experiment isolated lactobacillus showed maximum growth at pH 3. Therefore, these bacteria exhibited survival in highly acidic conditions.

In this study, another test was conducted for NaCl tolerance evaluation of Lactobacillus species. NaCl is an inhibitor of some of the bacteria's growth. The NaCl tolerance test was conducted at 2%, 4%, 6%, and 8% NaCl levels. All isolates were able to grow at 2%, 4%, and 6% some were able to grow at NaCl levels of 8%.

Phenol is also an inhibitory compound that is produced within bowels during amino acid deamination reactions. Probiotic bacterial strains survive at low concentrations of phenol. In the present study, phenol tolerance test was performed in 0.1%, 0.2%, 0.3% and 0.4% of phenol concentrations. All the isolates survived in 0.1% phenol concentration.

Lactobacillus is a world-famous food fermenter. The various monosaccharides and disaccharides were fermented by the lactobacillus bacteria. The sugar fermentation results have been shown in the table in this study. Glucose and lactose with the evolution of gas fermented by all isolates. 90%, 80%, and 70% of isolates fermented fructose, xylose, and sucrose respectively.

Microbial Identification using 16s rRNA

Sample which was labeled as L1 showed high similarity with *Priestia aryabhattai* based on nucleotide homology and phylogenetic analysis.

The evolutionary history was inferred using the Neighbor-Joining method (Saitou *et al.*, 1987). The optimal tree with the sum of branch Length = 0.04530117 is shown. The confidence probability (multiplied by 100) that the interior branch length is greater than 0, as estimated using the bootstrap test (500 replicates is shown next to the branches (Dopazo, 1994; Rzhetsky *et al.*, 1992).

The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura *et al.*, 2004) and are in the units of the number of base substitutions per site. The analysis involved 11 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding.

Table.1 List of millets (Source: Veena *et al.*, 2003)

Type of millets	Common name
Sorghum	*Jowar*
Little millet	*Sama*
Finger millet	*Ragi*
Foxtail millet	*Kora*
Proso millet	*variga*

Table.2 Nutritional value of Millets (Source: Singh *et al.*, 2012)

Nutrient	Amount (%)
Carbohydrates	65%
Proteins	09%
Fat	03%
Crude Fibre	2-7%

Table.3 Finger Millet (per 100 g edible portion, 12% moisture content)*Source: USDA Nutrient database (Amir Gull *et al.*, 2014)

S.No	Particulars	Amount
1	Carbohydrates (g)	72.6
2	Protein (g)	7.7
3	Fat (g)	1.5
4	Crude fibre (g)	3.6
5	Ash (g)	2.7
6	Calcium (mg)	344
7	Phosphorus (mg)	250
8	Iron (mg)	6.3
9	Manganese (mg)	3.5
10	Magnesium (mg)	130

Table.4 Batter preparation

Variant	1	2
Chana daal	108 grams	72 grams
Finger millet	72 grams	108 grams
Urad daal	60 grams	60 grams

Table.5 Colony characteristics of LAB on MRS agar plate

Variant – 1	2 hours	4 hours	6 hours	8 hours
Colony characteristics				
Size	Large	Pinpoint	Medium	Large
Shape	Round	Round	Round	Round
Margin	Irregular	Entire	Entire	Irregular
Elevation	Flat	Raised	Flat	Flat
Texture	Smooth	Smooth	Smooth	Smooth
Appearance	Mucoid	Mucoid	Mucoid	Mucoid
Pigment	No	No	No	No
Opacity	Opaque	Opaque	Opaque	Opaque
Gram's staining	Gram's positive coccobacilli	Gram's positive coccobacilli	Gram's positive coccobacilli	Gram's positive coccobacilli
No. of c.f.u	$44*10^{-6}$	$60*10^{-6}$	$80*10^{-6}$	$84*10^{-6}$

Table.6 Colony characteristics of LAB on MRS agar plate

Variant – 2	2 hours	4 hours	6 hours	8 hours
Colony characteristics				
Size	Moderate	Moderate	Moderate	Moderate
Shape	Round	Round	Round	Round
Margin	Entire	Entire	Entire	Entire
Elevation	Raised	Raised	Raised	Raised
Texture	Smooth	Smooth	Smooth	Smooth
Appearance	Mucoid	Mucoid	Mucoid	Mucoid
Pigment	No	No	No	No
Opacity	Translucent	Translucent	Translucent	Translucent
Gram's staining	Gram's positive coccobacilli	Gram's positive coccobacilli	Gram's positive coccobacilli	Gram's positive coccobacilli
No. of c.f.u	$32*10^{-6}$	$38*10^{-6}$	$68*10^{-6}$	$80*10^{-6}$

Table.7 Results of pH tolerance tests of selected isolates

	pH 2		pH 3		pH 4		pH 5	
Time (hours)	24	48	24	48	24	48	24	48
L1	+	++	+	+++	+++	+++	+++	+++
L2	+	++	+	++	+++	+++	+++	+++
L3	+	++	+	++	++	+++	+++	+++
L4	+	++	+	++	+++	+++	+++	+++
L5	+	++	+	++	+++	+++	+++	+++
L6	+	++	+	++	++	+++	+++	+++
L7	+	++	+	++	+++	+++	+++	+++
L8	+	++	+	++	+++	+++	+++	+++
L9	+	++	+	++	+++	+++	+++	+++
L10	+	++	++	++	+++	+++	+++	+++

= No growth (negative); += slight growth (positive); ++= moderate growth (positive); +++= dense growth (positive)

Table.8 Results of NaCl (Salt) tolerance tests of selected isolates

Salt conc. (%)	2		4		6		8	
Time (hours)	24	48	24	48	24	48	24	48
L1	+++	+++	+++	+++	+++	+++	+++	+++
L2	+++	+++	+++	+++	+++	+++	+++	+++
L3	+++	+++	+++	+++	+++	+++	+++	+++
L4	++	+++	++	+++	++	+++	+	++
L5	+++	+++	+++	+++	+++	+++	+	++
L6	+++	+++	+++	+++	+++	+++	+++	+++
L7	+++	+++	+++	+++	+++	+++	+	++
L8	+++	+++	+++	+++	++	+++	++	+++
L9	+++	+++	+++	+++	++	+++	+	++
L10	+++	+++	+++	+++	+	+++	+	++

= No growth (negative); += slight growth (positive); ++= moderate growth (positive); +++= dense growth (positive)

Table.9 Results of phenol tolerance tests of selected isolates

Phenol conc (%)	**0.1**		**0.2**		**0.3**		**0.4**	
Time (hours)	24	48	24	48	24	48	24	48
L1	++	+++	-	-	-	-	-	-
L2	++	+++	-	-	-	-	-	-
L3	+	++	-	-	-	-	-	-
L4	++	++	-	-	-	-	-	-
L5	++	++	-	-	-	-	-	-
L6	+	++	-	-	-	-	-	-
L7	+	++	-	-	-	-	-	-
L8	++	++	-	-	-	-	-	-
L9	+	++	-	-	-	-	-	-
L10	+	++	-	-	-	-	-	-

= No growth (negative); += slight growth (positive); ++= moderate growth (positive); +++= dense growth (positive)

Table.10 Result of sugar fermentation test of Dominant bacterial spp.

Isolate	**Sugar**	**Xylose**	**Glucose**	**Fructose**	**Lactose**	**Sucrose**
L1		A^+G^+	A^+G^+	A^+G^+	A^+G^+	A^+G^+
L2		A^+G^-	A^+G^+	A^+G^+	A^+G^+	A^+G^+
L3		A^+G^-	A^+G^+	A^+G^+	A^+G^+	A^+G^+
L4		A^+G^+	A^+G^+	A^+G^+	A^+G^+	A^-G^-
L5		A^+G^+	A^+G^+	A^+G^-	A^+G^+	A^+G^-
L6		A^-G^-	A^+G^+	A^+G^+	A^+G^+	A^-G^-
L7		A^-G^+	A^+G^+	A^-G^-	A^+G^+	A^-G^-
L8		A^+G^+	A^+G^+	A^-G^-	A^+G^+	A^+G^-
L9		A^+G^+	A^+G^+	A^+G^+	A^+G^+	A^+G^+
L10		A^+G^+	A^+G^+	A^+G^+	A^+G^+	A^+G^-

$^+$ = Positive Acid, A^- = Negative Acid, G^+ = Positive Gas, G^- = Negative Gas

Table.11 Alignment with most coordinate sequence

Alignment with most coordinated sequence:

Priestia aryabhattai B8W22 16S ribosomal RNA, partial sequence Sequence

ID: NR_115953.1 Length: 1533 Number of Matches: 1

Alignment statistics for match #1				
Score	**Expect**	**Identities**	**Gaps**	**Strand**
1938bits(1049)	0.0	1049/1049(100%)	0/1049(0%)	Plus/Plus

Table.12 Alignment with most coordinated sequence

Alignment with most coordinated sequence:

Pediococcus pentosaceus strain DSM 20336 16S ribosomal RNA, partial sequ

Sequence ID: NR_042058.1 Length: 1569 Number of Matches: 1

Alignment statistics for match #1				
Score	**Expect**	**Identities**	**Gaps**	**Strand**
1676bits(907)	0.0	909/910(99%)	0/910(0%)	Plus/Minuus

Chart.1 No of CFU on MRS agar plate in Variant 1 and Variant 2

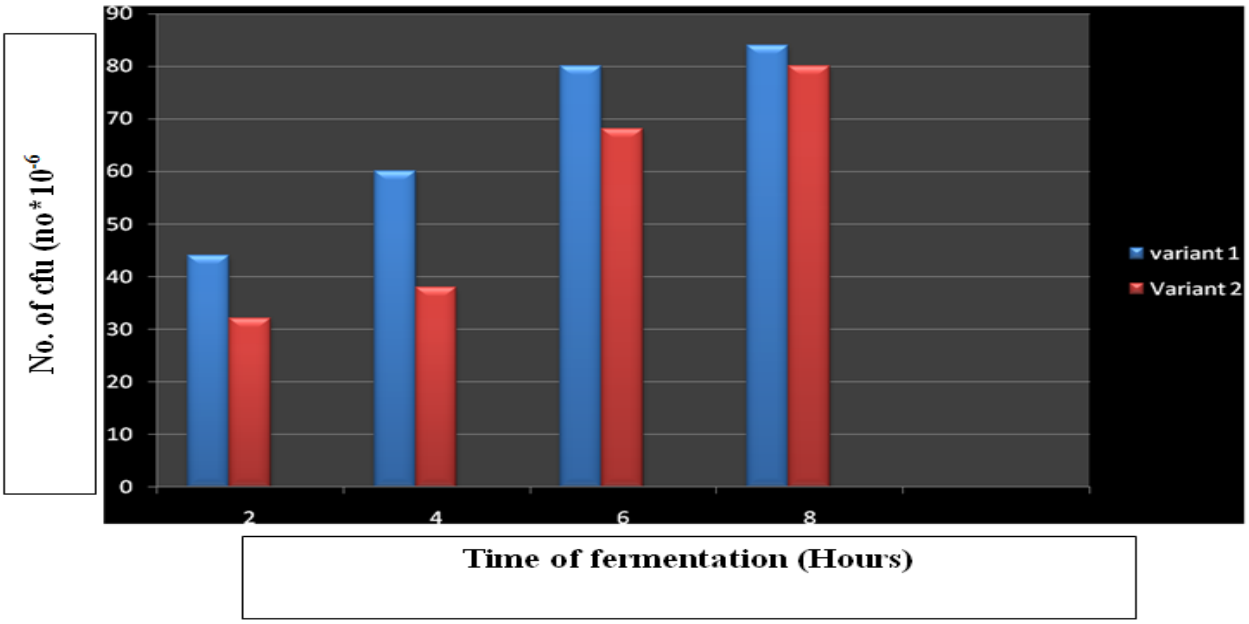


Fig.1 Evolutionary relationships of taxa

Phylogenic Tree:

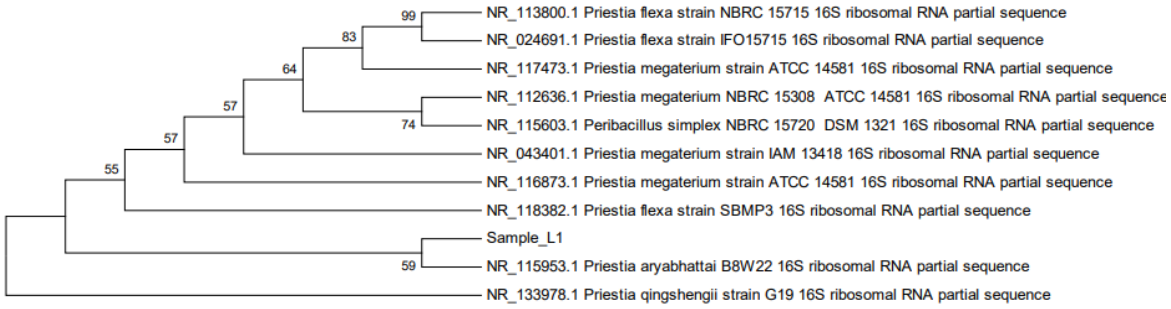
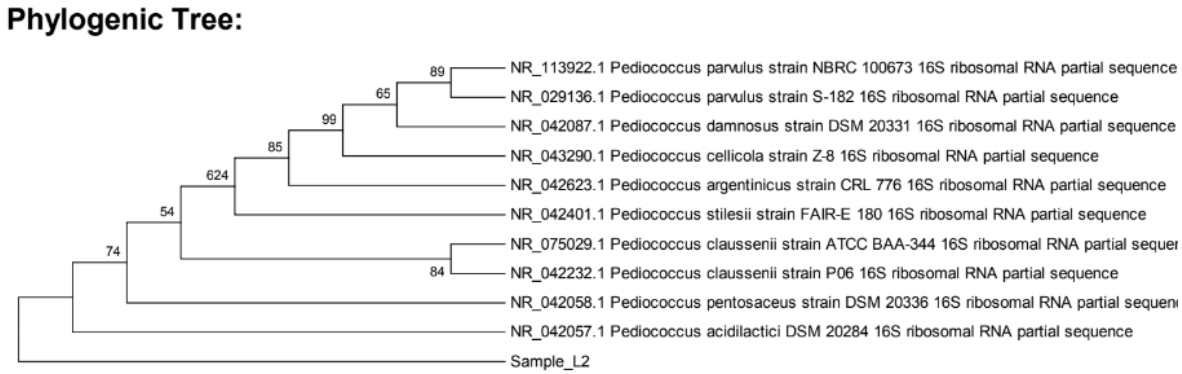


Fig.2 Evolutionary relationships of taxa



All positions containing gaps and missing data were eliminated. There were a total of 1045 positions in the final dataset. Evolutionary analyses were conducted in MEGA6.

Sample which was labeled as L2 showed high similarity with *Pediococcus pentosaceus* based on nucleotide homology and phylogenetic analysis.

The evolutionary history was inferred using the Neighbor-Joining method (Saitou *et al.*, 1987). The optimal tree with the sum of branch. Length = 1.21129054 is shown. The confidence probability (multiplied by 100) that the interior branch length is greater than 0, as estimated using the bootstrap test (500 replicates is shown next to the branches (Dopazo, 1994; Rzhetsky *et al.*, 1992). The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura *et al.*, 2004) and are in the units of the number of base substitutions per site. The analysis involved 11 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 884 positions in the final dataset. Evolutionary analyses were conducted in MEGA6.

Lactobacillus species were isolated and identified from selected fermented batters of finger millet in this study and can survive in extremely acidic conditions, high salt namely low phenolic concentrations. These results showed that the isolated strains of the lactobacillus are suitable in the human gastrointestinal tract environment for survival. The strains have, however, also probiotic sugar fermentation potential. By performing 16S rRNA Sequence analysis the isolates L1 identified as *Priestia aryabhattai* and L2 identified as *Pediococcus pentosaceus.* Both the isolates have potential capabilities to use as probiotic for reducing the risk of many types of the diseases into animals as well as humans. Additional studies are needed to find a specific probiotic with specific benefits from finger millet-based fermented batter.

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