ISSN PRINT 2319 1775 Online 2320 7876

Research paper UGC CARE Listed (Group -I) Journal Volume 11, S Iss 8, D e c 2022

Isolation and Characterization of potential probiotic lactic acid bacteria from fermented fish and its anti-bacterial efficacy.

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Abstract

The traditional Indian food practices offer various health benefits as they food contains probiotics and maintains healthy gut. Lactic acid bacteria (LAB) have an important role in a great variety of fermented foods. Fermented fish an ethnic food of north eastern states of India, is one such product of probiotics which offers various health benefits. India is a country which is more diversified in its traditional food practice. The current study focuses on isolating and characterising lactic acid bacteria from fermented Ngari fish products by morphological, biochemical tests and 16S rRNA gene sequencing. The results showed the isolates from fermented fish products are dominated by *Lactobacillus Sakei* and *Lactobacillus Curvatus*. All the isolates were Catalase negative and evident in beta type haemolytic activity. The probiotic properties of isolated strains were asseses by acid (pH 2 and 3) and bile salt (0.3%) tolerances. The potential LAB strains showed good anti-bacterialal efficacy against various food borne human pathogens. Overall the potential LAB strains were further utilised for the food formulations and for therapeutic application.

Key words: Probiotics, Lactic acid bacteria, Fermented fish, Anti-bacterial

INTRODUCTION:

Historically people being consumed lactic acid bacteria (LAB) from various fermented food products. LAB are currently gaining interest and the subject of extensive international research due to their critical involvement in most fermented foods, as well as their potential to create antimicrobial chemicals that promote probiotic characteristics (Parlindungan *et al.*, 2023). Probiotics are defined as “live micro-organisms which when administered in adequate amounts confer a health benefit on the host” (FAO/ WHO, 2001 & Hill *et al.*, 2014). Because of their tremendous health benefits, it is gaining popularity in the present time. Lactic acid bacteria occupy dominant position in the category of probiotics.

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They are the most ubiquitous and found almost everywhere (Schoster *et al.*, 2013; Duar *et al.*, 2017). Apart from maintaining homeostasis they offer other strain specific nutraceutical health benefits such as enhancing lactose digestion in children (de Vrese *et al.*, 2001), suppress immune disorders (Kwon *et al.*, 2010), prevent inflammatory response (Turcanu and Lack 2006), other medical disorders like obesity, diabetes, cancer etc (Redman *et al.*, 2014). Naturally fermented foods exhibit a rich biodiversity of microorganisms which make them a good source of potential probiotic LAB (Rezac *et al.*, 2018). Despite of the wide range of fermented foods with diverse therapeutic aspects, still there are many traditional fermented foods that have not been exploited for potential probiotic applications (Peres *et al.*, 2014). Indian traditional food is home to such health promoting beneficial bacteria. India is a diverse country, thus multiple food culture (Somashekaraiah *et al.*, 2019). We use fermentation process in wide range of foods viz. cereals, pulses, fruits vegetables, dairy based and meat based food. Fermented fish is one such ethnic food which is consumed largely in North Eastern region of India. The North eastern region is a treasure of indigenous traditional knowledge. One of such knowledge is their fermentation methods (Satish kumar *et al.*, 2012). Though they lives in bio diverse rich environment, away from the sea, high humid atmosphere, frequent heavy rainfall, etc. created a compulsion to preserve the foods like fish for their long term use which offers them huge functional health benefits Keeping all the above facts the aim of the present study was to isolate and to determine the probiotic properties, safety, and functional effects of LAB strains isolated from fermented fish of north east India. Further, the anti-bacterial potential against various human pathogens of LAB isolates were investigated.

MATERIALS AND METHODS:

Fermented Ngari fish samples were obtained from cities of north east region of India. Sample was extracted with physiological saline and subjected the serial dilution. Samples of each dilution were spread directly onto deMann Rogosa Sharpe (MRS, Hi Media) plates and incubated at 37^0C for 24 to 48 hours in an anaerobic condition. Isolated colonies were further checked for purity following few more isolations and gram staining. Colonies that represented putative lactobacilli according to Bergey‟s manual of systematic bacteriology (2009) were chosen for the different tests. Six potential isolates were obtained from fermented Ngari fish which could be maintained on repeated sub-culturing on MRS media.

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PRELIMINARY ANALYSIS

Potential LAB isolates were subjected to catalase test using H_2O_2, survavility test at different temperature was tested using culturing in MRS broth. The isolates were also further categorized for their type of fermentation pathway using Gibson‟s semisolid media (Collins et al., 2004; Harrigan, 2013). Furthertypes of haemolytic activity of the isolates were evaluated using blood agar method.

TOLERENCE TO ACIDIC pH

Overnight grown probiotic cultures were inoculated (10^7 cfu/ml) into MRS broth of pH 2.0 & 3.0 (adjusted using 1N HCl) to simulate acidic conditions of gut. The lactobacilli viable counts, present initially and after exposure upto 3 hours with an interval one hour were carried out. Serial dilutions (10-fold) were done using saline and the cultures were C°plated onto MRS agar. The plates were incubated at 37 ^{0}C for 24-48 hours and numbers of live organisms expressed as cfu/ml.

RESISTANCE TO BILE

The potential isolates showing good tolerance to acidic conditions were tested for their bile tolerance. Overnight grown cultures were inoculated (10^7 cfu/ml) into MRS broth containing 0.3% of Oxgall (HiMedia). The viable counts of the cultures were determined after two hours of exposure, as in the earlier method and numbers of live organisms expressed in cfu/ml.

MOLECULAR CHARACTERIZATION DENTIFICATION BY 16S rRNA SEQUENCING

The molecular characterization of LAB strains was evaluated 16S rRNA amplification, sequencing and analysis, using the 16S rRNA universal forward and reverse primers 27F: AGAGTTTGATCMTGGCTCAG and 1492R: TACGGCTACCTTGTTACGACTT with 1500 bp product as previously described by Shokryazdan *et al.*, 2014. with slight modification. PCR reactions were conducted using a total volume of 25 µl, containing 15 µl of NZYTaq 2× Green Master Mix, 1 µl each of forward and reverse primers, 6 µl of DNase free water and 2 µl of DNA template. After amplification, 5 µl of PCR products were analyzed for electrophoresis and then visualizaed by transillumination under UV light. The

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PCR products (1.5 kB) sequenced and data obtained were further compared with the

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database in the NCBI Genbank using the basic local alignment search tool (BLAST) for the final identification of the potential LAB strains.

ANTIBACTERIAL ACTIVITY

Antibacterial activity of LAB isolates were assessed using culture supernatants against human pathogens by agar well diffusion method as previously described by Schoster et al., 2013. Selected strains were incubated in MRS agar medium at 37 ℃ for 48 h. Culture supernatants were obtained through centrifugation (8,000×g for 20 min at 4℃) and used as an antagonistic substance. Wells (8 mm) impregnated with 60 µL of culture supernatant were placed on reinforced nutrient agar plates seeded various human pathogens (10^7 CFU/mL) and subsequently incubated under anaerobic conditions at 37℃ for 24 hours. The diameter of each zone of inhibition was measured in millimeters to assess the antagonistic effect of LAB strains.

RESULTS:

All the six isolates were found to be gram positive, catalase negative rod shaped bacteria. Their survivalance at different temperature, ability to hydrolyse bile salt, pattern of fermentation and type of haemolytic activity is represented in table 1. The acedic pH tolerance response of all the potential isolates depicted in table 2 and the graphical representation in figure 1 and 2. The molecular identification using 16S rRNA universal primers which yields the PCR product of 1.5 kB of all LAB isolates are separated using 1.2% agarose gel electrophoresis was shown in Fig 2. The DNA sequence alignment data in NCBI portal confirming the presence of *Lactobacillus Sakei* and *Lactobacillus Curvatus* species predominantly, summary of the data were shown in table 3. The antagonistic effect of LAB isolated evaluated using five food borne human pathogen, all isolates showing anti-bacterial effect among that isolated NFSG09 showed highest potential table 4.

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Table 1: Morphological, physiological and biochemical characterization of potential Probiotic LAB isolates

Probiotic LAB isolates from Ngari Fermented Fish (NFSG)						
	NFSG02	NFSG06	NFSG08	NFSG09	NFSG14	NFSG18
Morphology	Rod	Rod	Rod	Rod	Rod	Rod
Grams Staining	+	+	+	+	+	+
Catalase Test	-	-	-	-	-	-
Survival at 10 ^{0}C 37 ^{0}C 45 ^{0}C	 + + +	 - + -	 + + -	 - + -	 + + +	 + + +
Bile salt (0.3% Oxgal) hydrolysis	+	+	+	+	+	+
Ho/He	Ho	Ho	Ho	Ho	Ho	Ho
Haemolytic activity	β-type	β-type	β-type	β-type	β-type	β-type

Note: +: Positive, -: Negative, Ho: Homofermentative

Table 2: Acid pH tolerance test of potential LAB isolates

Log CFU ml^{-1}								
Isolates	pH 2				pH 3			
	0 hr	1 hr	2 hr	3 hr	0 hr	1 hr	2 hr	3 hr
NFSG02	8.36±1.28	7.38±2.42	5.62±1.68	3.45±3.12	8.48±1.38	7.56±1.42	6.21±1.34	4.54±2.32
NFSG06	7.84±1.82	6.68±1.42	4.42±2.43	2.22±1.21	7.74±1.82	6.56±2.12	5.18±2.82	3.84±2.54
NFSG08	7.28±1.21	6.45±1.86	5.23±2.18	2.88±1.61	7.32±1.56	6.78±2.86	5.58±1.81	4.13±1.61
NFSG09	8.96±2.14	7.84±1.22	5.86±1.28	4.28±2.18	8.82±1.64	7.84±1.78	6.48±1.21	4.88±1.42
NFSG14	8.48±2.18	7.12±2.16	5.18±1.28	2.12±1.28	8.42±2.48	6.67±1.56	5.48±1.28	4.02±2.28
NFSG18	8.58±1.48	7.28±2.21	5.45±2.82	3.72±2.42	8.48±1.26	7.42±1.82	6.12±2.12	4.36±1.42

(n=3 ^{a}Mean±SD of log Value of viable cell number)

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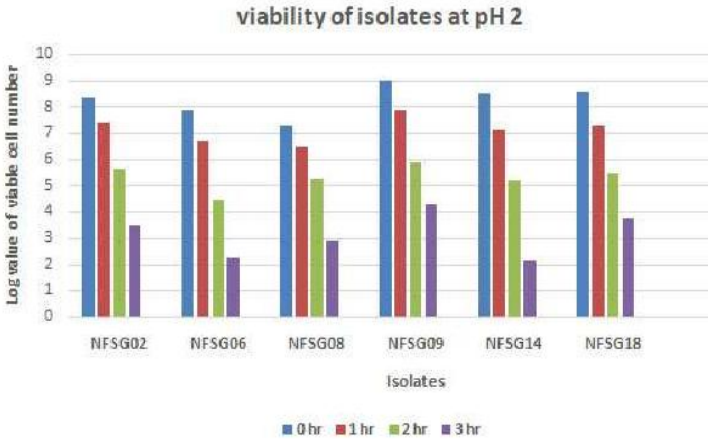

Figure 1. Graphical representation of log value of viability of LAB isolates at pH 2.

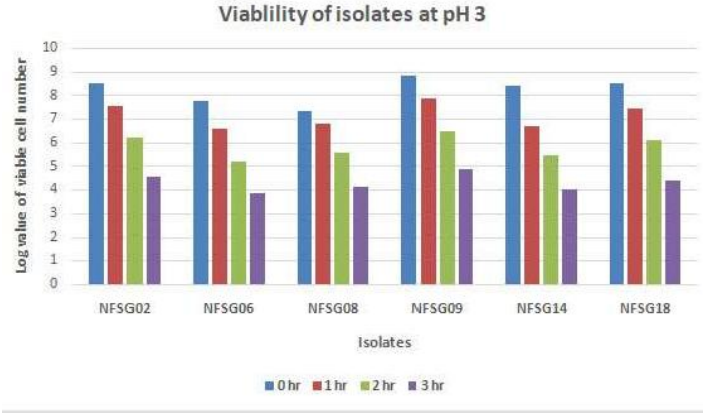

Figure 2. Graphical representation of log value of viability of LAB isolates at pH 3.

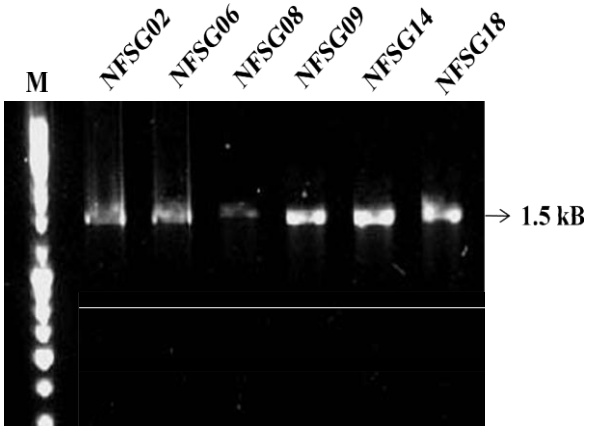

Fig 3: PCR amplified products (1.5 kB) of LAB isolates using 16S rRNA universal primers were resolved on 1.2% agarose gel electrophoresis.

Table 3: Potential probiotic LAB isolates designation, their respective strain and NCBI Gene Bank Accession numbers

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Sl. No	Isolates Designation	Strains	NCBI Accession No.	Identity (%) with Gene Bank Database
1	NFSG02	*Lactobacillus Sakei*	CP048116.1 LT960788.1 LT960784.1	100.00% 99.82% 99.82%
2	NFSG06	*Lactobacillus Sakei*	CP048116.1 CP046037.1 CP032652.1	100.00% 100.00% 100.00%
3	NFSG08	*Lactobacillus Sakei*	CP048116.1 CP046037.1 CP032652.1	99.33% 99.33% 99.33%
4	NFSG09	*Lactobacillus Sakei*	CP048116.1 CP032652.1 CP032633.1	100.00% 100.00% 100.00%
5	NFSG14	*Lactobacillus Curvatus*	CP022474.1 CP015490.1 CP017124.1	100.00% 98.61% 98.23%
6	NFSG18	*Lactobacillus Curvatus*	CP031003.1 CP026116.1 CP029966.1	98.21% 98.21% 98.21%

Table 4: Antibacterial activity of the potential LAB isolates against human pathogens

Indicator Strains	Antibacterial activity (CFS at pH 7.0)[a]				
	SA	EC	BS	PA	LM
NFSG02	15±2.14	13±2.14	13±1.41	12±2.28	14±1.21
NFSG06	12±2.82	11±1.41	10±2.82	10±2.82	12±2.82
NFSG08	14±2.41	12±2.41	12±2.82	12±2.12	14±2.41
NFSG09	16±1.21	14±1.21	15±1.21	14±1.21	16±1.41
NFSG14	10±2.12	09±2.12	12±2.12	09±1.21	07±1.21
NFSG18	14±1.41	14±1.41	12±1.21	13±2.68	14±2.68

([a]Mean±SD of diameter of inhibition zone in mm); SA, *Staphylococcus aureus* MTCC 737; EC, *Escherichia coli* MTCC 1687; BS, *Bacillus subtilis* MTCC 736; *Pseudomanas aeruginosa* MTCC 1688; LM, *Listeria monocytogenes* MTCC 1143.

DISCUSSION:

The recent research on probiotics was attracted the global market for probiotics products with wide diversity in LAB species which offers various health benefits (Meticulous Market Research). Researchers focusing and exploring the various sources for the potential strains especially for LAB from traditional fermented food products (Sieladie *et al.,* 2011; Sirilun *et al.,* 2010). As a probiotics which has to exhibit important characteristic properties to withstand the adverse condition such as acidic pH and bile tolerance. All the six potential strains exhibit the tolerance to acedic pH 2 and pH, also shown bile tolerance. The All the six

ISSN PRINT 2319 1775 Online 2320 7876

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isolates were homofermentative, and exhibited β type haemolytic activity. All isolates grown well at 37 °C. But no growth seen in *Lactobacillus sakei* NFSG08 at 45°C. Also *Lactobacillus sakei*NFSG06 and *Lactobacillus sakei*NFSG09 could not able to survive at both lower (10°C) and higher (45°C) temperatures. All the six isolates show greater tolerance to pH 2 and pH 3 which indicate the ability to survive in acidic condition of gastro intestinal tract. Because stomach pH ranges from 3-7 (Paulo *et al.*, 2014, Sunil and Vora 2015). Naturally fermented foods exhibit a rich biodiversity of microorganisms which make them a good source of potential probiotic LAB. In our study the predominant stains found were *Lactobacillus sakei* and *Lactobacillus Curvatus*. In evident with that study reported that *Lactobacillus sakei, Latilactobacillus curvatus* and *Lactiplantibacillus plantarum* are among the most prevalent LAB species associated with fermented meat products (Van Ba *et al.*, 2018, *Geeraerts et al.*, 2019). These LAB are known to play an important role in food safety and protection through the production of antimicrobial compounds, including organic acids and bacteriocins (Tarman *et al.*, 2017). *Lactobacillus sakei* and *Lactobacillus curvatus* both exhibited good antimicrobial activity. But *Lactobacillus sakei* NFSG09 strain exhibited highest zone of inhibition against all the test pathogen compared to *Lactobacillus curvatus* and other strains. Considering with all the parameters the isolated LAB strains found to be potential probiotics which can be exploited for food preservation especially for the processed meat products and further explored for mechanistic based therapeutic applications.

CONCLUSION

Lactic acid bacteria (LAB) are heterogenous group of bacteria which plays a significant role in a variety of fermentation processes. They ferment food prebiotics and produce lactic acid as the main product of fermentation. Among genera of LAB, both *Lactobacillus* (hereto- and homo-lactic) is the most dominant genus in fermented foods. The status of LAB in foods is termed as generally recognized as safe. Many species of LAB, such as probiotics and antimicrobial, can also exert bio-preservers and have functional properties. Therefore, obtaining potential strains for use in fermentation, to increase food flavour especially in meat products, nutraceuticals or pharmaceuticals, would involve an extensive screening of these sources. The traditional fermented fish products seems to be a great source which fulfils the all the potential characteristic properties when tested for key factors such as resistance to acid, bile environments and antagonistic efficacy against human pathogenic bacteria's.

ISSN PRINT 2319 1775 Online 2320 7876

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ACKNOWLEDGEMENTS

The authors sincerely acknowledge DOS in Microbiology, University of Mysore, Mysuru, India and DOS in Food Technology, Davangere University, Davangere, India for providing facility to carry out this research work.

CONFLICTS OF INTEREST: The authors declare no conflict of interest

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ISSN PRINT 2319 1775 Online 2320 7876

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