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Detection of *Lactobacillus* in Pork and Traditional Pork Products of Mizoram, India and Evaluation of its Probiotic Characteristics

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ABSTRACT

Background: Sa-Um, a traditional pork product of Mizoram could be an alternative and easily available source for *Lactobacillus* starter culture with probiotic properties.

Methods: Traditional pork product (Sa-Um) and pig faecal samples from Mizoram were collected and analysed for number of viable *Lactobacillus* bacteria by spread plate method. Samples above 10^6 cfu/ ml were considered for isolation of probiotics. Gram positive and catalase negative presumptive *Lactobacillus* isolates were confirmed by PCR analysis of *16S-rRNA* gene. The *Lactobacillus* strains were studied for viability at different temperatures, species identification by fermentation of sugars and probiotic properties namely tolerance to acid, bile salt and digestive enzymes, antagonistic activity and antimicrobial sensitivity.

Result: Four *Lactobacillus* strains, namely *L. plantarum* SU 2, *L. plantarum* SU 11 and *L. acidophilus* SU 38 from Sa-Um and *L. plantarum* PF 11 from pig faeces were suggested as probiotic bacteria. *Lactobacillus plantarum* SU 2 was the most potential probiotic candidate based on the studied probiotic properties.

Key words: Antimicrobial sensitivity, *Lactobacillus*, Pig faeces, Probiotic, Sa-Um.

INTRODUCTION

Sa-Um is a traditional pork product with its ethnicity of Mizoram in North East India. Fat is collected from the inner abdominal portion and other body parts of pig, cooked, chopped into pieces and are placed in a special container called Sa-Um 'Bur'. The container is then placed over the fireplace and after three days or a bit longer, the Sa-Um is ready for use in the preparation of other foods (Lalthanpuii *et al*., 2015).

Probiotics are defined as live microbes which transit the gastro-intestinal tract and in doing so; they benefit the health of consumers (Bhardwaj *et al*., 2012). *Lactobacillus* is one of the most important probiotic organisms found primarily in the small intestine capable of stimulating immune system and interactions with other microorganisms (Gupta *et al*., 2021). *Lactobacillus* is Gram positive, rod shaped and generally deficient in catalase and use fermentable carbohydrates as energy source (Duar *et al*., 2017; Muhammad *et al*., 2018). The evolution of the genus *Lactobacillus* has counted to nearly 200 species. These bacteria are also involved in the processes of fermentation and preservation of food products and they are generally regarded as safe.

Sa-Um could be an alternative and easily available source for *Lactobacillus* starter inoculums with probiotic potential. Therefore, the present study was aimed to isolate and identify the *Lactobacillus* from pork and Sa-Um and *in vitro* evaluation of its probiotic characteristics.

MATERIALS AND METHODS

The study was conducted on 50 Sa-Um and 50 pig faecal samples collected randomly from Aizawl district of Mizoram, India between April to September, 2021.

Enumeration, isolation and identification of *Lactobacillus*

One gram of each sample was diluted in 9 ml of 0.1% peptone water, mixed and then 10-fold serially diluted. A volume of 0.1 ml of appropriately diluted sample was spread in *Lactobacillus* selection agar (LSA) plate in duplicates and incubated at 37°C for 48 hours and bacterial colonies were enumerated. The bacterial colony forming unit was calculated by multiplying the number of colonies with the dilution factor and was expressed as mean log cfu/ml of the original sample. Morphologically distinct colonies were sub-cultured by using *Lactobacillus* selection broth (LSB) and

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LSA to isolate the pure *Lactobacillus* culture. Presumptive *Lactobacillus* isolates were identified by morphological characteristics, positive Gram's reaction and negative catalase reaction (Mannan *et al*., 2017).

Molecular confirmation of *Lactobacillus*

The presumptive *Lactobacillus* isolates were confirmed by PCR analysis ofgenus specific *16S-rRNA* gene (250bp) using R16-1 F (5′-CTT GTA CAC ACC GCC CGT CA-3′) and LbLMA1-rev R (5′-CTC AAA ACT AAA CAA AGT TTC-3′) primers (Dubernet *et al*., 2002).

Growth of *Lactobacillus* strains in different temperature

The PCR confirmed *Lactobacillus* strains showing bacterial colony count of 10^6 cfu/ml and above in original samples were studied for growth at 20°C and 30°C in LSB for 48 hours.

Detection of *Lactobacillus* species

The *Lactobacillus* strains showing growth in both 20°C and 30°C were identified for *Lactobacillus* species using a sugar fermentation panel of 17 sugars, namely arabinose, cellobiose, D- mannose, inositol, lactose, melezitose, melibiose, maltose, mannitol, raffinose, rhamnose, ribose, salicin, starch, sucrose, xylose and glucose (Ahirwar *et al*., 2017).

Evaluation of probiotic characteristics

Overnight cultured bacterial cells were harvested by centrifugation (10,000 × g, 5 minutes, 4°C), washed twice with PBS (pH 7.0) and then the cells were re-suspended applying required conditions for evaluation of following probiotic characteristics.

Acid resistance

The harvested bacterial cells were re-suspended in PBS with pH 2.0 and 3.0 and *Lactobacillus* strains were observed for the acid resistance based on viable colony counts on LSA in triplicates after incubation at 37°C for 0 and 3 hours (Maragkoudakis *et al*., 2006).

Bile salt tolerance

The harvested bacterial cells were re-suspended in PBS (pH 8.0) containing 0.5 and 1.0% bile salts and tolerance to the bile salt was evaluated based on viable colony counts in LSA in triplicates at 0 and 4 hours after incubation at 37°C (Argyri *et al.,* 2013).

Digestive enzyme tolerance

The harvested bacterial cell suspension was prepared in PBS containing the enzymes, trypsin and pepsin, each at 2 and 8 gm/L concentration and incubated at 37°C for 2 hours and then viable colonies were counted in LSA plates in triplicate and incubated at 37°C for 36 hours (Veerapagu and Jeya, 2017).

Antagonistic activity

The antimicrobial activity of the *Lactobacillus* strains was determined against indicator bacterial pathogens, namely *Salmonella* Typhimurium, *Staphylococcus aureus* and *Escherichia coli* by using agar spot test and the average diameters (mm) of zones of inhibition were measured (Sharma *et al*., 2017).

Antimicrobial sensitivity

The *Lactobacillus* strains were subjected to antibiotic sensitivity assay by disk diffusion method against a panel of 12 antimicrobial agents used against Gram positive bacteria namelyAmpicillin (10 µg), Ceftriaxone (30 µg), Ciprofloxacin (5 µg), Chloramphenicol (30 µg), Clindamycin (2 µg), Erythromycin (15 µg), Gentamicin (10 µg), Kanamycin (30 µg), Penicillin(10 µg), Rifampicin (5 µg), Streptomycin (10 µg) and Tetracycline (30 µg) (Angmo *et al*., 2016).

RESULTS AND DISCUSSION

Enumeration, isolation and molecular identification of *Lactobacillus*

A total of 16 Sa-Um and 3 pig faecal samples have shown growth in LSA, out of which, 3 Sa-Um (SU 2, SU 11 and SU 38) and 1 pig faecal (PF 11) samples showed the viable colony count more than 10^6cfu/ ml (Table 1). Nineteen presumptive *Lactobacillus* strains were isolated contributing to 16 from Sa-Um and 3 from pig faeces and subsequently, 4 *Lactobacillus* strains from Sa-Um (SU 2, SU 11 and SU 38) and pig faeces (PF 11) were confirmed by PCR assay of *16S-rRNA* gene (250 bp) (Fig 1). All the4 PCR positive strains have grown at both 20° and 30°C and were suggestive of probiotic *Lactobacillus*. The fermentation of 17 sugars by the 4 *Lactobacillus* strains identified that strains SU 2, SU 11 and PF 11 were *L. plantarum* and SU 38 was *L. acidophilus* (Table 2).

Table 1: Enumeration (log cfu/ml±SE) of *Lactobacillus* isolated from Sa-Um and pig faeces in Aizawl district, Mizoram.

Sample no.	Sa-Um	Pig faeces
SU1	1.99 ±0.08	-
SU2	7.02±0.12	-
SU5	3.11±0.09	-
SU7	1.17±0.16	-
SU10	2.03±0.01	-
SU11	7.05±0.13	-
SU15	4.07±0.02	-
SU19	3.02±0.07	-
SU21	5.01±0.08	-
SU24	4.05±0.07	-
SU27	3.11±0.08	-
SU30	3.09±0.07	-
SU33	4.28±0.11	-
SU34	2.32±0.21	-
SU38	6.16±0.12	-
SU46	1.12±0.08	-
PF11	-	6.09±0.04
PF24	-	1.41±0.13
PF38	-	1.37±0.03

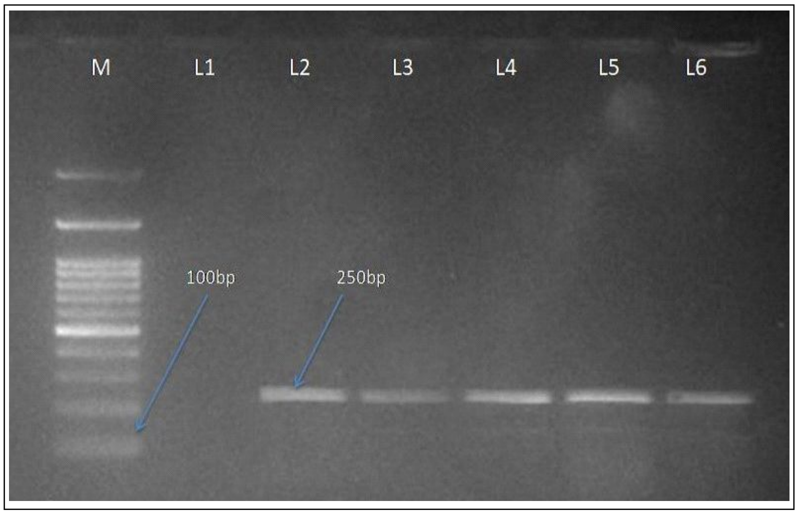

Fig 1: Agarose gel electrophoresis plate showing PCR amplicons of *16S-rRNA* gene (250 bp) of *Lactobacillus*
M: 100 bp ladder; L1: Negative control; L2: Positive control; L3 to L6: Positive samples.

Table 2: Species detection of *Lactobacillus* strains isolatedfrom Sa-Um and pig faeces by sugar fermentation test.

Isolate no.	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	Species detected
SU2	-ve	+ve	+ve	+ve	+ve	+ve	+ve	-ve	+ve	+ve	-ve	+ve	+ve	+ve	+ve	+ve	-ve	*L.plantarum*
SU11	-ve	+ve	+ve	+ve	+ve	+ve	+ve	-ve	+ve	+ve	-ve	+ve	+ve	+ve	+ve	+ve	-ve	*L.plantarum*
SU38	-ve	+ve	+ve	+ve	-ve	+ve	+ve	-ve	+ve	+ve	-ve	-ve	+ve	+ve	-ve	+ve	-ve	*L.acidophilus*
PF11	-ve	+ve	+ve	+ve	+ve	+ve	+ve	-ve	+ve	+ve	-ve	+ve	+ve	+ve	+ve	+ve	-ve	*L.plantarum*

A-Arabinose; B-Mannitol; C-Lactose; D-Maltose; E-Rhamnose; F-Sucrose; G-Glucose; H-Xylose; I-Cellobiose; J-D-Mannose; K-Inositol; L-Melezitose; M-Melibios; N-Raffinose; O-Ribose; P-Salicin; Q-Starch.

Acid resistance

The viable colony counts of the 4 *Lactobacillus* strains ranged between 3.41±0.06 to 6.28±0.36 log cfu/ml after 3 hours of exposure to pH 3.0 and 2.0 (Table 3) indicating that all the strains were acid tolerant at pH 3.0 and 2.0. The *L. plantarum* SU2 strain showed the highest growth (5.17 ±0.29) and the growth was significantly ($P \leq 0.01$) higher than the *L. acidophilus* SU 38 strain (3.41±0.06)at lower pH 2 after 3 hours. The ability to transit through the low pH of stomach (1.5-2) before reaching the intestine is a crucial characteristic of any probiotic bacteria (Gupta *et al*., 2021). The high survival rate of *L. plantarum and L. acidophilus* strains at gastric pH of 2.0 and 3.0 has been reported by Moreno *et al.* (2018); Betancur *et al*. (2020); Nath *et al.* (2020); Barzegar *et al.* (2021).

Bile salt tolerance

All the 4 *Lactobacillus* strains tolerated 0.5% and 1%bile salt up to 4 hours of incubation although their growth rate decreased at higher bile salt concentration. The highest growth was visible in *L. plantarum* SU 2 strain (3.25±0.24) after an exposure of 4 hours in 1% bile salt. However, *L. plantarum* SU 11 showed significantly ($P \leq 0.05$) higher growth (4.90±0.30) after an exposure to 4 hours at 0.5% bile salt than *L. plantarum* PF 11 strain (3.46±0.59) (Table 4). It is necessary to evaluate the bile salt resistance of probiotic bacteria for the ability to resist the unfavourable conditions of the intestinal tract for their colonization and metabolic activity. Tolerance to the bile salt among the probiotic *Lactobacillus* strains were also reported by Huang *et al.* (2021).

Digestive enzymes tolerance

The 4 *Lactobacillus* strains were found to be tolerant to the digestive enzymes, trypsin and pepsin at 2 and 8 g concentration up to 2 hours although the viability decreased at higher concentration (8 g) of both the enzymes. Among the strains, *L. plantarum* SU 2 strain showed the highest growth at 8g concentration of trypsin (4.30±0.08) and pepsin (4.26± 0.03) (Table 5). Similarly, Nath *et al. (*2020); Barzegar *et al*. (2021); Huang *et al.* (2021) had reported that *Lactobacillus* could survive different concentrations of trypsin and pepsin.

Antagonistic activity

The antibacterial activity of *Lactobacillus* strains showed weak to moderate inhibition against the three indicator organisms (Table 6). *Lactobacillus acidophilus* SU 38 and *L. plantarum* PF 11 showed weak inhibition of *E. coli* and *L. plantarum* SU 2 and SU 11 showed moderate inhibition against the organism. However, *L. plantarum* SU 2, *L. acidophilus* SU 38 and *L. plantarum* PF 11 showed weak inhibition

Table 3: Acid tolerance of the *Lactobacillus* strains isolated from Sa- Um and pig faeces.

Isolate name and number	Growth of *Lactobacillus* strains (log cfu/ ml) (mean±SE)			
	pH 3		pH 2	
	0hour	3 hours	0hour	3 hours
SU 2 (*L. plantarum*)	6.40±0.28	6.10±0.32	6.23±0.32	5.17 ±0.29^{b}
SU 11 (*L. plantarum*)	6.38±0.35	6.00 ±0.33	5.81±0.37	4.91±0.34^{b}
S U 38 (*L. acidophilus*)	6.37±0.36	6.28±0.36	5.22±0.36	3.41±0.06^{a}
PF11 (*L. plantarum*)	6.04±0.35	5.97±0.35	5.58±0.03	4.19±0.35^{ab}
F-value	0.25^{NS}	0.16^{NS}	1.87^{NS}	7.50**

Values bearing different superscripts in the column differed significantly.

Double (**) asterisk denotes significantly different at $P \leq 0.01$.

NS= Not significant

Table 4: Bile salt tolerance of *Lactobacillus* strains isolated from Sa- Um and pig faeces.

Isolate name and number	Growth of *Lactobacillus* strains (log cfu/ ml) (mean±SE)			
	0.5% bile		1% bile	
	0 hour	4 hours	0 hours	4 hours
SU 2 (*L. plantarum*)	5.25±0.32	4.19±0.34^{ab}	3.64± 0.56	3.25±0.24
SU 11 (*L. plantarum*)	5.60±0.56	4.90±0.30^{b}	4.85±0.35	2.73±0.88
SU 38 (*L. acidophilus*)	4.65±0.33	4.43±0.34^{ab}	4.72±0.31	3.09±0.33
PF 11 (*L. plantarum*)	4.81±0.34	3.46±0.59^{a}	3.77±0.30	3.23±0.35
F-value	1.15^{NS}	2.46*	2.39^{NS}	0.51^{NS}

Values bearing different superscripts in the column differed significantly.

Single asterisk (*) denotes significantly different at ≤0.05.

NS= Not significant.

Table 5: Digestive enzymes tolerance of *Lactobacillus* strains isolated from Sa- Um and pig faeces.

Isolate name and number	Growth of *Lactobacillus* strains (log cfu/ ml) (mean±SE)			
	Trypsin		Pepsin	
	2 g	8 g	2 g	8 g
SU 2 (*L. plantarum*)	5.64±0.05	4.30±0.08	5.67± 0.04^{b}	4.25±0.03
SU 11 (*L. plantarum*)	5.54±0.06	4.22±0.06	5.61±0.06^{b}	4.24±0.05
SU 38 (*L. acidophilus*)	5.19±0.45	4.16±0.10	5.50±0.09^{ab}	4.23±0.07
PF 11 (*L. plantarum*)	5.45±0.04	4.18±0.05	5.35±0.03^{a}	4.26±0.04
F-Value	0.68	0.61	5.04*	0.05

Values bearing different superscripts in the column differed significantly.

Single asterisk (*) denotes significantly different at ≤0.05.

NS= Not significant.

against *S. aureus* and *L. plantarum* SU 11 showed moderate inhibition against it. All the 4 strains showed weak inhibition against *S. typhimurium* Antibacterial activity is a functional requirement for probiotic bacteria as they could be used as bio-preservatives. The antibacterial effect exerted by *Lactobacillus* is due to the production of antibacterial metabolites like different organic acids, hydrogen peroxide, bacteriocins and other antibacterial substances (Kabir, 2009). The variable antibacterial activity against common pathogens was also observed by Wang *et al*. (2020); Erdogmus *et al.* (2021).

Antimicrobial sensitivity

All the 4 *Lactobacillus* strains were sensitive to most of the antibiotics namely, Chloramphenicol, Clindamycin, Erythromycin, Penicillin, Ceftriaxone and Amipicillin followed by 3 strains to Tetracycline, Rifampicin and Gentamicin and 2 strainsto Ciprofloxacin and Streptomycin indicating considerable food safety characteristic. However, all the strains were resistant to Kanamycin. *Lactobacilli* possess intrinsic resistance to Kanamycin, Gentamicin and Streptomycin (Fraqueza, 2015). The *L. plantarum* SU2 strain was sensitive to 11 antibiotics except Kanamycin. Variable

Table 6: Antagonistic activity of *Lactobacillus* strains from Sa- Um and pig faeces.

Isolate name and number	Indicator microorganisms		
	Diameter of inhibition zones (mm±SE)		
	E. coli	*S. aureus*	*S. typhimurium*
SU 2 (*L. plantarum*)	16.43 ± 1.10^{bc}	14.66 ± 1.45	12.33 ± 0.88^{ab}
SU 11 (*L. plantarum*)	19.33 ± 1.45^{c}	17.73 ± 1.79	14.83 ± 1.09^{b}
SU 38 (*L. acidophilus*)	14.93 ± 0.61^{ab}	12.00 ± 2.70	10.63 ± 1.16^{a}
PF 11 (*L. plantarum*)	11.86 ± 1.04^{a}	12.66 ± 0.67	12.96 ± 0.53^{ab}
F-Value	8.06^{**}	2.02^{NS}	3.31^{*}

Zone of inhibition: 10-15 mm(Weak inhibition); 16-20 mm (Moderate inhibition).

resistance pattern of *Lactobacilli* to different antibiotics were also reported by Handa and Sharma (2016); Bindu and Devi (2020).

Thus, *in vitro* evaluation of probiotic characteristics of 4 *Lactobacillus* strains has suggested their probiotic potential and indicated that *L. plantarum* SU 2 was the most potential probiotic candidate. Similarly, Moreno *et al.* (2018) and Betancur *et al.* (2020) reported *L. plantarum* with probiotic properties from meat and meat products.

CONCLUSION

The present study primarily confirmed4 *Lactobacillus* with promising probiotic properties from the ethnic pork product (Sa-Um) and pork faeces of Mizoram. Based on the studied probiotic properties, 3Sa-Um Lactobacilli (*L. plantarum* SU2, *L. plantarum* SU 11 and *L. acidophilus* SU 38) and 1 faecal *Lactobacillus* (*L. plantarum* PF 11) were suggested as probiotic strains. The susceptibility of the strains to most of the studied antibiotics is promising from food safety point of view. *Lactobacillus plantarum* SU 2 was the most promising probiotic candidate and Sa-Um may be explored as a potential traditional food source of probiotics. However, to characterize as ideal probiotic microbes, in depth *in vitro* and *in vivo* studies for safe application of these Lactobacilli in food are necessary.

Conflict of interest: None.

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