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Isolation and *in vitro* evaluation of probiotic properties of lactic acid bacteria from locally available pineapple fruits

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Abstract
Probiotics are the live microorganisms that when administered in adequate amounts, confers health benefits on the host. The lactic acid bacteria exist in various environments like water, soil, sewage, plants, fruits, vegetables, as well as in humans and animals. An attempt was made to isolate and evaluate the probiotic characteristics of LAB from pineapple fruits. Totally twenty LAB isolates were obtained from twenty pineapple samples. The isolates were rods, cocci, cocco-bacilli and diplococci in shape and the colonies of the isolates were small, round, white-dull white coloured and creamy with smooth edges. The isolates were Gram positive and catalase negative. They were tolerant and viable at different pH and bile salt conditions. They also showed antimicrobial activity against tested pathogens *viz*., *Escherichia coli* and *Staphylococcus aureus*. The isolates were resistant for most of the antibiotics tested. Hence, the isolates PLAB 10 and PLAB 15 showed potential probiotic properties.

Keywords: Probiotics, lactic acid bacteria, pineapple, antimicrobial activity, antibiotics

1. Introduction
Probiotics are defined as "live microbes" that, when ingested in the right quantity, provide beneficial health function for the host (FAO/WHO, 2006) [9]. LAB comprises of large and diverse group of Gram positive, non-spore forming, anaerobic or facultative aerobic cocci or rod shaped catalase negative bacteria which are considered as "generally recognized as safe" (GRAS) and can be safely used as probiotics (Mathew *et al*., 2017) [23]. The main genera which can be used as probiotic microorganisms are *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Pediococcus* and *Streptococcus* (Schnurer and Magnusson, 2005) [31]. LAB exists in various environments like water, soil, sewage, plants, fruits, vegetables, as well as in humans and animals (Leyva *et al*., 2017) [17]. The carbohydrate rich environment is suitable for the growth of LAB. The LAB can be isolated from different sources. They exhibit resistance to low pH and bile concentration. They show inhibitory effect against other organisms by producing some organic acids and bacteriocins (Kailaspathy and Chin, 2000) [15].
Pineapple [*Ananas comosus* (L). Family: Bromeliaceae] is one of the important commercial fruits in the world. It is known as the queen of fruits and it is the third most important tropical fruit after banana and citrus in the world (Parvej *et al*., 2020) [26]. It contains good amount of calcium, potassium, vitamin C, carbohydrates, crude fiber, water and different minerals. It is a highly perishable fruit due to its higher moisture content (75-90%) and soft texture which results in huge post-harvest losses (Hossain *et al*., 2015) [13]. Therefore, the use of probiotic organisms can also help to reduce the increased use of chemical additives during the processing of fruits to increase its shelf life and confers health benefits when consumed (Mahajan *et al*., 2018) [19]. It is suggested that the minimum concentration of at least 10^9 CFU / mL should be required to achieve beneficial effects on host when probiotic product is consumed (Hill *et al*., 2014) [12].
Hitherto, most of the probiotics were isolated from fermented dairy products or the human GI tract. With the increasing demand for novel probiotics, the search for organisms from non-traditional sources such as fermented foods and beverages, vegetables, fruits *etc*., has been intensified. Hence, this study mainly concerned with the isolation and evaluation of the probiotic characteristics of LAB from pineapple fruits.

2. Material and Methods

2.1 Isolation of lactic acid bacteria

Pineapple samples were collected from different locations of Bengaluru for the isolation of probiotic lactic acid bacteria. 10 g of samples were inoculated in 100 mL of MRS (de Man, Rogosa and Sharpe) broth to enrich lactic acid bacteria at 37 ^{o} C for 24 hours (Karami *et al*., 2017) [16]. After 24 hours, 1 mL of enriched broth was plated using spread plate method. *Lactobacillus plantarum* NCIM 2656 procured from National Collection of Industrial Microorganisms (NCIM), Pune was used as reference probiotic strain of lactic acid bacteria

2.2 Characterization of lactic acid bacteria (LAB)

All the LAB isolates were examined for their colony morphological characteristics, gram staining, biochemical characteristics of LAB and probiotic activity.

Morphological characteristics like colour, shape, size and position were recorded for all LAB isolates. LAB cultures of 48 hours old were stained with crystal violet and observed for cell shape and their attachment (Becking, 1974) [3]. Gram's staining was performed for all the isolates as per the method followed by Harrigan, 1998 [10] and the cellular morphology was recorded after Gram reaction (Harrigan, 1998) [10]. Different biochemical parameters such as Catalase activity (Balazevic and Ederes, 1975) [2] and acid and gas production (Seeley and Vandemark, 1970) [32] were carried out for all the isolates. LAB isolates were further screened for probiotic activity by subjecting to the pH tolerance test (Mannan *et al*., 2017) [22], bile salt tolerance test (Mandal, 2015) [20], antimicrobial activity (Hussein *et al*., 2013) [14] and antibiotic susceptibility test (Liasi *et al*., 2009) [18].

3. Results and Discussion

The lactic acid bacteria were isolated from pineapple samples and the isolates were screened for their probiotic properties. The results were presented here under.

3.1 Isolation of lactic acid bacteria from pineapple samples

The LAB population of pineapple samples ranged between 3.34×10^{6} CFU / g to 15.58×10^{6} CFU / g. Total of 20 LAB isolates were purified and were further selected based on colony characteristics, microscopic observations and Gram reaction. Similarly, Spurr (1994) [33] also reported that the microbial population of vegetables and fruits fluctuate between 5 and 7 log CFU / g. Whereas, Dicagno *et al*. (2010) [6] inferred that LAB isolated from pineapple was presumptively mesophilic and their population ranged between 5.75±0.91 log CFU / g to 4.32±0.64 log CFU / g.

3.2 Biochemical characterization of lactic acid bacterial isolates

The isolates studied were rods and some were cocci in shape. Fifteen LAB isolates were Gram positive and five isolates were Gram negative. Gram positive isolates were further selected for screening. All the Gram positive isolates were negative for catalase activity and gas production but, they were positive for acid production (Table 1). Amer *et al*. (2017) [1] isolated LAB from dairy products and identified three rod shaped isolates and five cocci shaped isolates. Further, Dallal *et al*. (2017) [5] reported that the isolates from pickled vegetables were Gram positive. Rahman *et al*. (2016) [28] also found that the isolated LAB were Gram positive, catalase negative and possessed bile salt hydrolase activity.

3.4 *In vitro* screening of lactic acid bacterial isolates for their probiotic properties

3.4.1 pH tolerance test of LAB isolates

Among 15 LAB isolates, 12 isolates were tolerant and viable against pH 2 and pH 3 (Table 2). However, seven isolates of these were culturable but non-viable at pH 1 and other five isolates were non-culturable and non-viable at pH 1. The viability of the isolates decreased with decrease in pH. On the other hand, the reference strain was tolerant and viable at all the three pH conditions *viz*., 1, 2 and 3.

According to Kailasapathy and Chin (2000) [15], the tolerance of LAB to acidic condition was due to their cytoplasmic buffering capacity at pH 3.72-7.74 which enabled them to keep constant and higher alkalinity of cytoplasm relative to that of extracellular conditions. Sahadeva *et al*. (2011) [30] also suggested that the threshold point to state acid resistance was set at pH 2 and pH 3 for three hours.

3.4.2 Bile salt tolerance test of LAB isolates

Twelve isolates along with reference strain were tested against different bile salt concentrations *viz*., 0.2%, 0.3% and 0.4% (Table 3). Three isolates (PLAB 2, PLAB 10 and PLAB 15) along with reference strain were viable at 0.2% to 0.4% bile salt. The viability of LAB isolates decreased with increase in bile salt concentration.

According to Begley *et al*. (2006) [4], the tolerance of LAB against bile salt was attributed to the presence of bile salt hydrolase activity. But, the viability of free cells of LAB isolates decreased with increase in bile salt concentration because of disruption of cellular homeostasis which caused the dissociation of lipid bilayer and integral protein of the cell membranes (Mandal *et al*., 2006) [21]. Bile salt hydrolase activity helps in deconjugation and detoxification of bile salt (Messaoudi *et al*., 2012) [24]. Patel *et al*. (2012) [22] concluded that the LAB isolates which were isolated from vegetables were tolerant for 0.3% of bile salt.

3.4.3 Antimicrobial activity of LAB isolates

Eight isolates which were tolerant to at least one of the bile salt concentrations were further selected for testing the antimicrobial activity. The isolates PLAB 1, PLAB 6, PLAB 10, PLAB 13 and PLAB 15 and reference strain showed inhibitory effect against both the pathogens. The area of zone of inhibition of isolates against two pathogens varied between 245.07 Sq.mm to 834.10 Sq.mm (Table 4).

Further, the findings of Dinoto *et al*. (2020) [7] showed that the LAB from wild fruits inhibited the growth of *E. coli*, *S. aureus* and *Mycobacterium smegmatis*. The antimicrobial activity of LAB was attributed to the production of lactic acid, bacteriocins, antifungal peptides, H_2O_2 and deacetyl which can inhibit the growth of microorganisms (Hassanzadazar *et al*., 2012; Reis *et al*. 2012) [11, 29].

3.4.4Antibiotic susceptibility test of LAB isolates

The isolates which showed inhibitory effect against both the pathogens were further selected for their antibiotic susceptibility test using antibiotic discs (Table 5). The isolates showed varying range of resistance to different antibiotics of different concentrations. Most of the isolates were resistant against some of the antibiotics like Streptomycin, Kanamycin, Gentamycin and Ciprofloxacin, they showed intermediate

resistance against Chloromphenicol and most of them were susceptible for Azithromycin.
Similarly, Naeem *et al*. (2012) [25] reported that LAB isolates from fruits were resistant against kanamycin and oxacillin. While, Erginkaya *et al*. (2018) [8] reported that *Lactobacillus* sp. was resistant to vancomycin, erythromycin, tetracycline, gentamicin and ciprofloxacin.

Table 1: Biochemical characterization of lactic acid bacterial isolates

Sl. No.	Lactic acid bacterial isolates	Gram reaction	Catalase test	Acid production	Gas Production
1	PLAB 1	+	-	+	-
2	PLAB 2	+	-	+	-
3	PLAB 3	+	-	+	-
4	PLAB 4	-	ND	ND	ND
5	PLAB 5	+	-	+	-
6	PLAB 6	+	-	+	-
7	PLAB 7	+	-	+	-
8	PLAB 8	+	-	+	-
9	PLAB 9	-	ND	ND	ND
10	PLAB 10	+	-	+	-
11	PLAB 11	+	-	+	-
12	PLAB 12	+	-	+	-
13	PLAB 13	+	-	+	-
14	PLAB 14	+	-	+	-
15	PLAB 15	+	-	+	-
16	PLAB 16	-	ND	ND	ND
17	PLAB 17	-	ND	ND	ND
18	PLAB 18	+	-	+	-
19	PLAB 19	-	ND	ND	ND
20	PLAB 20	+	-	+	-
21	*Lactobacillus plantarum* NCIM 2656	+	-	+	-

Note: PLAB = Pineapple LAB, - = Negative, ND = Not determined + = Positive

Table 2: Screening of lactic acid bacterial isolates for growth and survivability at different pH levels

Sl. No.	Lactic acid bacterial isolates	Turbidity			Viability of LAB isolates on MRS agar plates after 48 hrs (x 10^6 CFU / mL)		
		pH 1.0	pH 2.0	pH 3.0	pH 1.0	pH 2.0	pH 3.0
1	Control	-	-	-	0	0	0
2	PLAB 1	+	+	+	0	7.98	26.67
3	PLAB 2	+	+	+	0	15.69	20.79
4	PLAB 3	-	+	+	0	6.57	20.54
5	PLAB 5	-	-	+	0	0	0
6	PLAB 6	-	-	+	0	7.50	9.97
7	PLAB 7	+	+	+	0	4.68	7.82
8	PLAB 8	+	+	+	0	3.92	10.95
9	PLAB 10	+	+	+	0	17.26	29.67
10	PLAB 11	-	-	+	0	4.35	26.97
11	PLAB 12	+	+	+	0	3.67	14.67
12	PLAB 13	-	+	+	0	4.43	19.48
13	PLAB 14	-	+	+	0	3.97	15.79
14	PLAB 15	-	+	+	0	13.87	29.12
15	PLAB 18	-	+	+	0	0	0
16	PLAB 20	+	+	+	0	0	0
17	*Lactobacillus plantarum* NCIM 2656	+	+	+	22.36	24.65	30.63

Table 3: Screening of lactic acid bacterial isolates for growth and survivability at different bile salt concentrations

Sl. No.	Lactic acid bacterial isolates	Turbidity			Viability of LAB isolates on MRS agar plates after 48 hrs (x 10^6 CFU / mL)		
		0.2%	0.3%	0.4%	0.2%	0.3%	0.4%
1	Control	-	-	-	0	0	0
2	PLAB 1	+	+	-	26.78	0	0
3	PLAB 2	+	+	+	24.97	23.76	10.97
4	PLAB 3	+	+	-	25.54	0	0
5	PLAB 6	+	+	+	0	26.79	0
6	PLAB 7	+	-	-	0	0	0
7	PLAB 8	+	+	+	24.00	25.90	0
8	PLAB 10	+	+	+	28.90	32.59	29.13
9	PLAB 11	+	-	-	0	0	0
10	PLAB 12	+	-	-	0	0	0
11	PLAB 13	+	+	-	20.62	30.65	0
12	PLAB 14	+	-	-	0	0	0
13	PLAB 15	+	+	+	27.98	30.26	24.23
14	*Lactobacillus plantarum* NCIM 2656	+	+	+	32.34	27.83	16.79

Table 4: Screening of lactic acid bacterial isolates for antimicrobial activity

Sl. No.	Lactic acid bacterial isolates	Area of Zone of inhibition (Sq. mm)	
		Escherichia coli	*Staphylococcus aureus*
1	Control	318.88	245.07
2	PLAB 1	275.70	0
3	PLAB 2	0	275.70
4	PLAB 3	286.15	478.70
5	PLAB 6	364.84	0
6	PLAB 8	519.80	834.10
7	PLAB 10	275.70	534.13
8	PLAB 13	364.84	452.44
9	PLAB 15	591.93	799.37
10	*Lactobacillus plantarum* NCIM 2656	534.13	834.10

Table 5: Screening of lactic acid bacterial isolates for antibiotic susceptibility

SL. No.	Lactic acid bacterial isolates	Zone of inhibition (mm)					
		Strepto (10 µg/disc)	Kan (30 µg/disc)	Cip (5 µg/disc)	Gen (10 µg/disc)	Azitro (15 µg/disc)	Chloro (30 µg/disc)
1	Control	0	0	0	0	0	0
2	PLAB 1	9	8	14	10	18	18
3	PLAB 6	10	8	15	13	38	9
4	PLAB 10	7	0	13	12	9	11
5	PLAB 13	8	7	4	10	20	10
6	PLAB 15	5	0	9	8	12	13
7	*Lactobacillus plantarum* NCIM 2656	0	0	0	3	2	0

Note:
Zone diameter (mm): <15mm= Resistant (R); 15-21mm= Intermediate (I); >21mm = Susceptible, (S) Strepto = Streptomycin, Cip = Ciprofloxacin, Azitro = Azithromycin, Kan = Kanamycin, Gen = Gentamycin, Chlor = Chloromphenicol

Conclusion

This study showed that the probiotic lactic acid bacteria (LAB) can be isolated from pineapple samples. Total of 20 LAB isolates were isolated. The isolates were tested for several probiotic properties and were tolerant for different pH and bile salt conditions. They showed potential antimicrobial properties and were resistant against several antibiotics. Thus, concluded that they showed potential probiotic characteristics. Further molecular characterisation and the *in-vivo* studies of the isolates are required for their beneficial use in food industries and there is a need for search of novel probiotics from different sources to use as potential probiotic organisms.

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