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Characterization of lactic acid bacteria isolated from jackfruit and beet root juices for their probiotic properties

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Abstract
Lactic acid bacterial population in jackfruit and beet root juice varied from 1.9 to 2.1 x 10^6 and 1.1 to 1.3 x 10^6 respectively. The colony colour varied from whitish to cream. Cell shape varied from rod to cocci. LAB isolates JCVL-II and BDVL-III were tolerant to pH 1.5 to 8.5 and bile salt concentration upto 2.0%. Most of the LAB isolates were resistant to antibiotics Streptomycin (10 µg/disc), Gentamycin (10 µg/disc), Kanamycin (30 µg/disc) and Ciprofloxacin (5 µg/ disc) and susceptible to Ampicillin (10 µg/disc) and Chloramphenicol (30 µg/disc). Antimicrobial activity with highest zone of inhibition against *Escherichia coli* NCIM 2065 and *Staphylococcus aureus* NCIM 2079 was produced by JCVL-II *i.e.*, 25.33 mm and 18.67 mm (in diameter) respectively. Molecular characterization of JCVL-II and BDVY-III isolates identified it as *Lactiplantibacillus plantarum* and *Levilactobacillus brevis* respectively.

Keywords: Lactic acid bacteria, probiotics, molecular characterization, jackfruit juice, beet root juice

1. Introduction
Surplus quantity of fruits and vegetables are lost every year due to lack of proper harvesting practises and post-harvest technologies. Improper harvesting, handling and storage in unhygienic conditions often leads to infection by various pathogens, insects and mites. In developing countries, it is assessed that 50% of production is lost during storage due to infection caused by soft rot bacteria and fungi (Gunny *et al*., 2021) [6]. India ranks second in production of fruits and vegetables in the world. During the year 2018-19, the annual production of fruits and vegetables was estimated to be 97.97 million tonnes and 183.17 million tonnes respectively (FAO, 2019) [6].

Jackfruit (*Artocarpus heterophyllus* L.) a desirable tropical fruit that is underutilised, having vitamins A and C, thiamin, riboflavin, calcium, potassium, iron, sodium, zinc, niacin and antioxidants. It has dietary fibres which acts as a good bulk laxative. Vitamin C is required in collagen development, oral health and provides firmness and strength to the skin. Its potassium levels helps in lowering blood pressure and reduces effects of sodium. (Ranasinghe, 2019) [12].

Beet root (*Beta vulgaris* L. ssp. *vulgaris*.) is an edible vegetable taproot. Beet roots have more of sucrose and small amounts of glucose and fructose (Bavec *et al.*, 2010) [2]. It has significant amounts of vitamin C and vitamins B1, B2, niacin, B6, B12. The leaves of beet root are an excellent source of vitamin A. The juice of beetroot is consumed as a natural remedy for sexual weakness and to reduce kidney and bladder stones. The therapeutic use of beetroot includes its antitumor, carminative, emmenagogue, hemostatic and renal protective properties and is a potential herb used in cardiovascular conditions (Dambalkar *et al.*, 2015) [3].

Fruits and vegetables that are important part of healthy balanced diet have essential vitamins, minerals, dietary fibres, antioxidants and bioactive compounds. Lactic acid bacteria (LAB) such as *Lactobacillus, Leuconostoc, Enterococcus, Pediococcus, Fructobacillus,* and *Weissella* are found on fruits and vegetables with microbial population ranging between 10^2 to 10^4 CFU/g (Pimentel *et al*., 2021) [10]. According to FAO/WHO (2006) [4], probiotics are defined as "live microbes" that, when ingested in the right quantity, provide beneficial health function for the host. Lactic acid bacteria with potential probiotic activity builds a proper balance of beneficial microbial population in the gastro-intestinal tract and exhibit antimicrobial activity against food borne human pathogens (Pundir *et al*., 2013) [11].

2. Material and Methods

2.1. Isolation of lactic acid bacteria (LAB)

Jackfruits, beet roots and other fruits and vegetables were obtained from different parts of Karnataka and local markets of Bangalore. Lactic acid bacteria (LAB) were isolated from the juices of jackfruit, beet root juices by employing Standard Plate Count Method. The morphological and biochemical characteristics of LAB were analyzed.

2.2. Probiotic properties of lactic acid bacterial isolates pH tolerance test

The pH tolerance of LAB isolates were evaluated by inoculating LAB in MRS broth and adjusting pH to 1.5, 2.5, 3.5, 4.5, 6.5, 7.5 and 8.5 using 1N HCl and 1N NaOH. The test tubes were incubated at 37 °C for 24 hours. The viability were observed by inoculating on solidified MRS medium and the population of LAB isolates (CFU/ml) were recorded (Hawaz, 2014) [9].

2.2.1 Bile salt tolerance test

Bile salt tolerance of LAB isolates were evaluated by inoculating LAB in MRS broth at various concentrations levels 0.25, 0.5%, 1.0% and 2.0%. The test tubes were incubated at 37 °C for 24 hours. The viability were observed by inoculating on solidified MRS medium and the population of LAB isolates (CFU/ml) were recorded (Hawaz, 2014) [9].

2.2.2 Antimicrobial activity

The antimicrobial activity of LAB isolates were tested against gram negative bacteria *Escherichia coli* NCIM 2065 and gram positive bacteria *Staphylococcus aureus* NCIM 2079 by using agar well diffusion assay. Petri dishes with nutrient agar that was previously inoculated with 0.1 ml of 24 hours old nutrient broth culture of individual test bacteria were poured. After solidifying, wells of 7 mm diameter were made and filled with 100µl of culture supernatant of LAB. The plates were incubated at 37 °C for 48 h and the diameter of zone of inhibition (in mm) was measured (Bali *et al*., 2011) [1].

2.2.3 Antibiotic sensitivity test

The lactic acid bacterial isolates tolerant to acidic pH and higher bile concentration were assessed for its antibiotic resistance by the disc diffusion method against different antibiotics such as ampicillin (10 µg/disc), gentamycin (10 µg/disc), ciprofloxacin (5 µg/disc), streptomycin (10 µg/disc), chloramphenicol (30 µg/disc), tetracycline (30 µg/disc), kanamycin (25 µg/disc) and azithromycin (15 µg/disc). Actively growing inoculum of LAB (100 µl) were spread on solidified MRS agar medium and the respective antibiotic discs were placed on the medium and were incubated at 37 °C for 48 hrs. The diameter of zone of inhibition (in mm) for each antibiotic was measured and expressed as susceptible (≥21 mm), intermediate (16-20 mm) and resistance (≤15 mm) (Zhang *et al*., 2016) [15].

2.3. Molecular identification of Lactic acid bacteria

Total genomic DNA of the two LAB isolates were extracted by alkaline lysis method. Using two primers (22 bp forward primer 5' GGAGAGTTAGATCTTGGCTCAG 3' and 20 bp reverse primer 5' AAGGAGGGGATCCAGCCGCA 3') already reported for 16S rRNA sequences from the NCBI, Polymerase Chain Reactions (PCR) were carried out. Agarose gel electrophoresis was performed and gel was visualized under UV trans-illuminator and documented using gel documentation unit. The Gene JET™ Gel Extraction Kit (Thermo Scientific) was used for rapid and efficient separation of DNA fragments from agarose gel. The DNA eluted was checked for its concentration using nano drop and got sequenced by Barcode Bio Sciences, Bengaluru, Karnataka. The sequence data received was analyzed for homology.

3. Results and Discussion

Population of lactic acid bacteria (LAB) in jackfruit and beet root juice varied from 1.9 to 2.1 x 10^6 and 1.1 to 1.3 x 10^6 respectively. The colony colour of LAB isolates varied from whitish to cream. The cell shape of LAB varied from rod to cocci when observed under microscope (100 X). Most LAB isolates were Gram positive (+) and few isolates were Gram negative (-). All the isolates were non-motile. All the LAB isolates were catalase negative (-) and did not produce endospores. Most of the LAB isolates produced acid and no gases. The results are presented in table 1. The microbial population on raw fruits and vegetables were estimated to be 10^5 to 10^7 CFU/g. Yeasts are most dominant group having population between 10^2 to 10^6 CFU/g while the population of LAB ranges between 10^2 to 10^4 CFU/g (Pimentel *et al*., 2021) [10].

LAB isolates JCVL – II and BDVL – III along with *Lactobacillus acidophilus* MTCC 10307 and *Lactobacillus casei* Shirota were tolerant to acidic pH (1.5 to 3.5). The growth and viability of LAB strains was more in the pH 5.5 and 6.5. Only LAB isolates JCVL – II and BDVL – III were viable in 8.5. The results are presented in Table 2. Stasiak *et al*., (2021) [14] also reported on acid tolerance of commercial probiotic LAB. *Lactobacillus* GG in the MRS with pH 5.0 after 48 hours of the experiment increased by about 1.34 log order, while at the optimal pH (pH 6.2) by 2.2 log order.

LAB isolates tolerant to acidic pH were evaluated for its bile salt tolerance by growing LAB in MRS broth at various concentrations levels of 0.25%, 0.5%, 1.0% and 2.0%. LAB isolates JCVL – II and BDVL – III along with *Lactobacillus acidophilus* MTCC 10307 and

Lactobacillus casei Shirota were tolerant to bile salt concentration from upto 2.0%. JCVL-II had highest population of 5.00 x 10^6 cfu/ml surviving at 2.0% bile salt concentration. The results are presented in Table 3. Hassanzadazar *et al*., (2012) [8] investigated on *Lactobacillus plantarum*, *Lactobacillus casei* and *Lactobacillus delbrueckii* isolated from Koozeh cheese. *Lactobacillus casei* alone showed survivability at pH 2.0 and bile salt concentration of 0.3% and had antibacterial activity against of *Listeria monocytogenes*.

Most of the LAB isolates were resistant to antibiotics such as streptomycin (10 µg/disc), gentamycin (10 µg/disc), kanamycin (30 µg/disc) and ciprofloxacin (5 µg/disc). Few of the lab isolates showed intermediate resistance to tetracycline (30 µg/disc) and azithromycin (15 µg/disc). Most LAB isolates were susceptible for ampicillin (10 µg/disc) and Chloramphenicol (30 µg/disc) (Fig 1.). Reuben *et al*., (2020) [13] evaluated probiotic lactic acid bacterial strains *viz*., *Lactobacillus casei, Lactobacillus plantarum, Lactobacillus fermentum* and *Lactobacillus paracasei* from indigenous raw milk and previously identified strains. The antibiotic sensitivity of LAB strains was tested against penicillin G (2 units), ceftriaxone (30 µg), ampicillin (25 µg), vancomycin (30 µg), oxacillin (1 µg), streptomycin (10 µg), chloramphenicol (30 µg), gentamicin (10 µg), erythromycin

(10 µg), tetracycline (10 µg), novobiocin (30 µg) and ciprofloxacin (10 µg) using agar disc diffusion method. Strains were resistant to erythromycin, chloramphenicol, oxacillin, vancomycin and streptomycin but were susceptible to ampicillin, erythromycin, novobiocin, and tetracycline.

Highest zone of inhibition against *Escherichia coli* NCIM 2065 and *Staphylococcus aureus* NCIM 2079 was produced by JCVL-II *i.e.*, 25.33 mm and 18.67 mm (in diameter) respectively. Gutierrez *et al.*, (2018) [7] investigated on lactic acid bacterial metabolites inhibiting growth of food spoilage microorganisms. Species of *Lactobacillus* (*L. casei*, *L. brevis*, *L. paracasei* and *L. plantarum*) and *Pediococcus acidilactici* exhibited their antagonistic activity against *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 20 25923 and *Listeria monocytogenes*ATCC7644.

Molecular characterization (Fig 2 and 3) resulted in identification of JCVL-II and BDVY-III isolates as *Lactiplantibacillus plantarum* (Accession number ON860692) and *Levilactobacillus brevis* (Accession number ON860693) respectively.

Table 1: Morphological and biochemical characteristics of lactic acid bacterial isolates

S. No.	LAB Isolates	Colony colour	Cell shape	Gram reaction	Catalase test	Endospore test	Motility test	Acid production	Gas production
1.	JCVL-I	Whitish	Rod	+	_	_	_	+	_
2.	JCVL-II	Whitish	Rod	+	_	_	_	+	_
3.	JCVL-III	Whitish	Rod	+	_	_	+	+	_
4.	JSVL-I	Whitish	Rod	+	_	_	_	+	_
5.	JSVL-II	Whitish	Cocci	+	_	_	_	+	_
6.	JSVL-III	Whitish	Cocci	+	_	_	_	+	_
7.	JBVL-I	Whitish	Rod	+	_	_	_	+	_
8.	JBVL-II	Whitish	Rod	+	_	_	_	+	_
9.	JBVL-III	Whitish	Rod	+	_	_	_	+	_
10.	BDVL-I	Whitish	Rod	+	_	_	+	+	_
11.	BDVL-II	Whitish	Rod	+	_	_	_	+	_
12.	BDVL-III	Creamish	Rod	+	_	_	_	+	_
13.	BCVL-I	Whitish	Rod	+	_	_	_	+	_
14.	BCVL-II	Whitish	Rod	+	_	_	_	+	_
15.	BCVL-III	Whitish	Rod	+	_	_	_	+	_
16.	WML	Creamish	Rod	+	_	_	_	+	_
17.	PAL	Whitish	Rod	+	_	_	_	+	_
18.	MML	Whitish	Rod	+	_	_	_	+	_
19.	*L. acidophilus* MTCC 10307	Whitish	Rod	+	_	_	_	+	_
20.	*L. casei* Shirota (Yakult)	Whitish	Rod	+	_	_	_	+	_

Table 2: Population of LAB isolates at different pH levels

	LAB Isolates	Population of LAB isolates (x 10^6 CFU/ml)							
		pH 1.5	pH 2.5	pH 3.5	pH 4.5	pH 5.5	pH 6.5	pH 7.5	pH 8.5
1.	JCVL-I	0.0	0.0	0.0	4.67	11.67	18.00	0.0	0.0
2.	JCVL-II	12.00	13.67	15.00	18.33	21.67	33.00	12.67	10. 33
3.	JCVL-III	0.0	0.0	0.0	0.0	9.67	13.33	0.0	0.0
4.	JSVL-I	0.0	0.0	0.0	0.0	0.0	9.00	0.0	0.0
5.	JSVL-II	0.0	0.0	0.0	4.00	8.66	11.00	0.0	0.0
6.	JSVL-III	0.0	0.0	0.0	0.0	0.0	6.67	0.0	0.0
7.	JBVL-I	0.0	0.0	0.0	0.0	8.00	14.33	6.33	0.0
8.	JBVL-II	0.0	7.67	9.00	9.67	10.67	16.00	9.00	0.0
9.	JBVL-III	0.0	0.0	0.0	0.0	6.00	11.67	0.0	0.0
10.	BDVL-I	0.0	0.0	2.57	4.00	7.33	12.00	0.0	0.0
11.	BDVL-II	0.0	0.0	6.00	8.67	10.00	14.00	5.00	0.0
12.	BDVL-III	11.00	12.33	13.67	14.00	20.00	27.67	14.00	9.67
13.	BCVL-I	0.0	0.0	0.0	0.0	0.0	7.33	0.0	0.0
14.	BCVL-II	0.0	0.0	0.0	0.0	0.0	8.00	0.0	0.0
15.	BCVL-III	0.0	0.0	0.0	0.0	0.0	11.00	8.00	0.0
16.	WML	0.0	0.0	0.0	0.0	0.0	5.23	0.0	0.0
17.	PAL	0.0	0.0	0.0	0.0	0.0	3.00	0.0	0.0
18.	MML	0.0	0.0	0.0	0.0	0.0	4.67	0.0	0.0
19.	*Lactobacillus acidophilus* MTCC 10307	9.67	10.33	12.67	11.00	17.67	24.00	4.67	0.0
20.	*Lactobacillus casei* Shirota (Yakult)	6.66	8.00	10.33	12.33	13.00	20.33	0.0	0.0

Table 3: Population of LAB isolates at different bile salt concentrations (%)

S. No.	LAB Isolates	Population of LAB isolates (x 10^6 CFU/ml)			
		0.25%	0.5%	1.0%	2.0%
1.	JCVL-I	0.0	0.0	0.0	0.0
2.	JCVL-II	21.67	14.00	12.33	5.00
3.	JCVL-III	0.0	0.0	0.0	0.0
4.	JSVL-I	0.0	0.0	0.0	0.0
5.	JSVL-II	11.67	0.0	0.0	0.0
6.	JSVL-III	0.0	0.0	0.0	0.0
7.	JBVL-I	0.0	0.0	0.0	0.0
8.	JBVL-II	0.0	0.0	0.0	0.0
9.	JBVL-III	0.0	0.0	0.0	0.0
10.	BDVL-I	0.0	0.0	0.0	0.0
11.	BDVL-II	0.0	0.0	0.0	0.0
12.	BDVL-III	18.00	12.67	9.00	3.67
13.	BCVL-I	0.0	0.0	0.0	0.0
14.	BCVL-II	4.67	0.0	0.0	0.0
15.	BCVL-III	0.0	0.0	0.0	0.0
16.	WML	5.67	0.0	0.0	0.0
17.	PAL	0.0	0.0	0.0	0.0
18.	MML	0.0	0.0	0.0	0.0
19.	*Lactobacillus acidophilus* MTCC 10307	20.33	12.00	10.33	4.00
20.	*Lactobacillus casei* Shirota (Yakult)	16.00	10.00	7.67	2.33

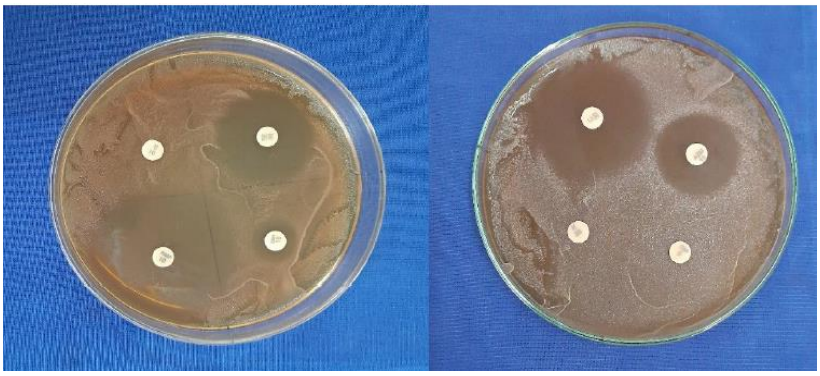

Fig 1: Antibiotic susceptibility of LAB isolates

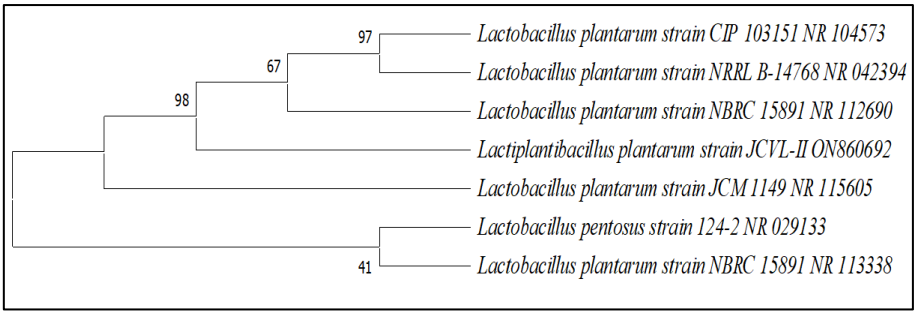

Fig 2: Phylogenetic tree of LAB isolate JCVL-II by Maximum Likelihood method

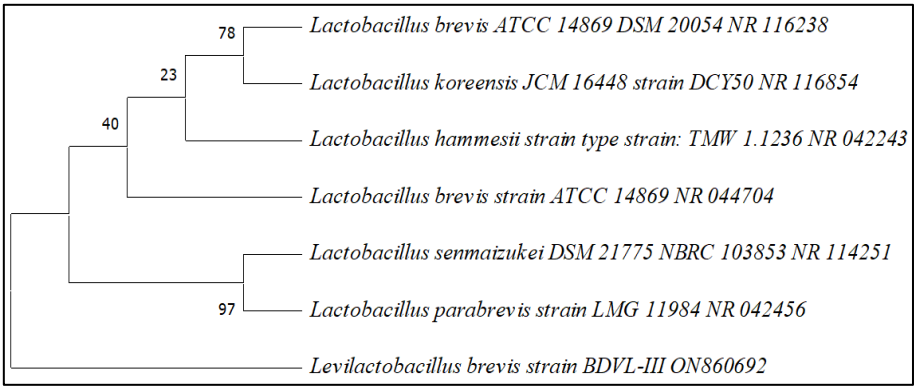

Fig 3: Phylogenetic tree of LAB isolate BDVL-III by Maximum Likelihood method

4. Conclusion

Population of lactic acid bacteria (LAB) varied from 1.9 to 2.1 x 10^6 and 1.1 to 1.3 x 10^6 in jackfruit and beet root juice respectively. The colony colour of LAB isolates varied from whitish to cream. The cell shape of LAB varied from rod to cocci when observed under microscope (100 X). LAB isolates were Gram positive (+) and few isolates were Gram negative (-). All the isolates were non-motile, catalase negative (-) and did not produce endospores. Most of the LAB isolates produced acid and no gases. LAB isolates isolates JCVL – II and BDVL – III along with *Lactobacillus acidophilus* MTCC 10307 and *Lactobacillus casei* Shirota were viable in pH 1.5 to 8.5 and were tolerant to bile salt concentration from upto 2.0%. Antibiotic susceptibility of LAB isolates was tested. Most of the LAB isolates were resistant to antibiotics Streptomycin (10 µg/disc), Gentamycin (10 µg/disc), Kanamycin (30 µg/disc) and Ciprofloxacin (5 µg/disc) and susceptible to Ampicillin (10 µg/disc) and Chloramphenicol (30 µg/disc). LAB isolates exhibited antimicrobial activity also where, highest zone of inhibition against *Escherichia coli* NCIM 2065 and *Staphylococcus aureus* NCIM 2079 was produced by JCVL-II *i.e.,* 25.33 mm and 18.67 mm in diameter respectively. Molecular characterization resulted in identification of JCVL-II and BDVY-III isolates as *Lactiplantibacillus plantarum* and *Levilactobacillus brevis* respectively.

5. Acknowledgement

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