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ISOLATION AND CHARACTERIZATION OF THE PROBIOTIC POTENTIAL OF LACTOBACILLUS SPECIES FROM *ZINGIBER OFFICINALE*

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Abstract*—Probiotics have been developed with the aim of promoting health and wellness. The probiotic characteristics are likely to be found in the beneficially important medicinal plant, Zingiber officinale (Ginger) for its antimicrobial, anti-oxidant and anti-inflammatory property. The present study is aimed at isolating Lactobacillus species from ginger using 5% glucose solution. The colonies were characterized and confirmed through biochemical screening of the in vitro growth potential among the probiotic species. The best probiotic candidate was selected and further characterization was carried out to isolate the genomic DNA and 16s RNA sequencing was done. Lactic Acid Bacteria (LAB) isolates showed growth at 37°C, pH 6.5 & NaCl free medium. β-Galactosidase activity of LAB isolates was 1.75-3.0 Miller units in an uninduced medium and also the LAB isolates exhibited antimicrobial activity against E. Coli. Nucleotide sequence analysis of LAB amplicons was performed by BLASTN and was found to be closely related to *Lactobacillus iners, Pediococcus acidilactici, Lactobacillus casei, Lactobacillus paracasei* and *Weissella koreensis.

Keywords—**Probiotics, Lactic Acid Bacteria, β-Galactosidase, ginger, Antimicrobial Activity**

I. INTRODUCTION

The demand for production of more health-promoting foods is on the rise throughout the world. Probiotics have been developed for its numerous health benefits such as prevention of colon cancer, lowering BP, reduction of cholesterol and alleviate lactose intolerance (Berner and O'Donnell 1998)(St-Onge, Farnworth, and Jones 2000). Lactose can be hydrolysed into glucose and galactose by the activity of β-Galactosidase (Wang and Sakakibara 1997). Thus, high β-Gal activity is an essential criterion for a probiotic strain. Earlier times, Probiotics were traditionally obtained from fermented fruits, vegetables, and dairy products. At present, the probiotic bacteria are acquired from the plant surface as an epiphyte, within plant organs or tissues as endophytes or in the thin soil zone in the rhizosphere. Probiotic bacteria found below the ground and rhizosphere is higher than the bacteria above the soil (Rosenblueth and Martínez 2006). Lactic acid bacteria are a group of Gram-positive microorganisms that include the genera Carnobacterium, Lactobacillus, Leuconostoc, Pediococcus and Streptococcus (Kandler 1983). They are non-sporous, non-motile, catalase negative rods or cocci and can ferment various carbohydrates to lactic acid and other by-products (Sieladie et al. 2011). Many studies claim the use of ginger for the preparation of fermented dairy products such as soft cheese and herbal ice-cream (Okwute and Olafiaji 2013). Ginger is commonly consumed as a spice and also noted for its anti-inflammatory, cholesterol lowering, and antithrombotic properties (Zaika, Laura and James S 1975; Ahmad et al. 2018; Tan, Benny and Vanitha 2004). Ginger has been given the GRAS (Generally Recognised As Safe) status by The Ailment and Drug administration as a pablum supplement.

Thus, the objective of the study was to isolate the Lactic acid bacteria that naturally colonize in ginger and to study its functional and molecular characteristics. Furthermore, the isolated LAB was investigated for its antibacterial potential and its β-galactosidase activity.

II. MATERIALS AND METHODS

A. Sample Collection

Fresh ginger rhizomes were obtained from a local market. It was washed well with double distilled water to remove mud particles and then air dried and stored at 4°C in an airtight container.

B. Collection of LAB

5% Glucose solution was prepared and maintained at pH 5 since it favours the growth of lactobacilli sp. The dried ginger was taken, the skin was peeled, and immersed in 5% glucose solution at pH 5. The raw ginger with skin was also immersed in a beaker containing 5% glucose solution at pH 5. Both were kept immersed for an hour. The ginger was taken and it was

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swabbed on to the MRS agar labelled as ginger with skin and without skin, respectively. These plates were kept incubated at 37°C in the incubator for 24h.

C. Genomic DNA Extraction from Lab Isolates

After 24h of incubation at 37°C, cultures of suspected LAB isolates (1 ml) in the exponential phase of growth (approximately 1.6 unit of OD 600 nm) were centrifuged for three minutes at 12000 rpm. These bacterial pellets were used for total DNA genomic extraction. One millilitre of distilled water was added to the pellet. After vortexing, the sample was boiled at 100°C for 15 min by placing it in a water bath. The suspension was cooled immediately to −20°C for 20 minutes and centrifuged at 13000 rpm for five minutes and the supernatant was kept frozen until use (Abdulla 2014).

D. Molecular Characterization of the Lab Isolates

16S rRNA gene sequencing was used to study bacterial phylogeny and taxonomy. Semi-universal primers L1/L2 based on the variable loop in 16S rRNA sequence of Lactobacillus species 21mer forward primer coupled with 21mer universal sequences (L2) from the flanking terminal of the 16S rRNA gene were applied to determine the genera of the type strains. The lactococcal species was identified using semi-universal primers (PI/P2) that were designed based on the inconstant region of V1 lactococcal 16s rRNA (Klijn, Weerkamp, and De Vos 1995). The corresponding regions were PCR amplified with genomic DNA as the template using the primers and the primer sequences given below:

P1(coccus)F- GCGGCGTGCCTAATACATGC
P2(coccus)R- TTGTTGCCTCCCGTAGGAGT
L1(Bacillus)F- CTCAAAACTAAACAAAGTTC
L2(Bacillus)R- CTTGTACACACCGCCCGTCA
PCR amplification and 16s rRNA sequencing were performed. The cycling conditions were 95°C for 5 min, followed by 36 cycles of 95°C for 1 min, 55°C for 1 min, followed by an extension at 72°C for 1 min, and the final extension at 72°C for 10 min. The PCR products were resolved on 1% agarose along with the 100 bp DNA ladder and visualized under UV-Spectrophotometer.

E. Phylogenetic tree using MEGA X

The DNA sequences were acquired and aligned using BLASTN. A phylogenetic tree was constructed from the aligned sequences using MEGA X software using the neighbour joining method.

F. Morphological and Biochemical Characteristics of LAB isolates

The isolates with highest sequence similarity from the phylogenetic analysis were chosen and confirmed morphologically and through biochemical tests. Those colonies were picked and inoculated in MRS broth. Further purification was done by streaking again on MRS agar. The colonies were tested for its morphology by gram staining and catalase activity.

G. Phenol Red Carbohydrate Fermentation Broth

Trypticase, Sodium chloride, and Phenol red was weighed and dissolved in 100 ml distilled water and transferred into conical flasks. 0.5% to 1% of the desired carbohydrate was added to all flasks. The sugar was transferred into screw-capped tubes or fermentation tubes and labelled. Inverted Durham tubes were inserted into all tubes and the Durham tubes were fully filled with broth and sterilized at 115°C for 15 minutes. Bacterial culture was inoculated in each of the labelled tube (uninoculated tubes was kept as control), and it was incubated for 18–24 hours at 37°C.

H. Temperature, pH, Salt Tolerance Activity of Lab Isolates

The growth of the isolates was assessed in MRS broth at different temperatures 20°C and 45°C and distinctive pH 4.4 and 9.6. Salt tolerances of the isolated strains were tested by incorporating 6.5% and 15% NaCl in MRS broth.

I. Milk Curdling Test

Fresh raw milk was collected and taken in a beaker. A loop full of culture was inoculated in MRS broth and incubated at 37°C. The grown culture was taken and inoculated in the fresh milk taken in a falcon tube. It was incubated at 37°C for 24 hours and the results were observed.

J. Antibacterial Activity of LAB Isolates

E. coli was inoculated in LB medium and grown at 37°C till O.D reached 0.4. Lab isolates were also grown at 37°C till O.D reaches 0.6. E. coli culture of 100µl was mixed with 5–50µl of LAB supernatants and it was incubated at 37°C for 3 hours at 120 rpm and O.D was measured at 630nm and percentage inhibition was calculated (Ashrethalatha et al. 2016). The inhibition was found to be significant with 20µl of LAB supernatants.

K. Statistical Analysis

Statistical analysis was performed for two significant concentration of LAB isolates using one-way ANOVA followed by Tukey's multiple comparison test using GraphPad Prism software (version 5.09. $p \leq 0.05$ was considered significant).

L. β -Galactosidase Activity of LAB Isolates

Bacterial cells were grown until the O.D ranges between 0.75-0.95 at 610nm. These cells were harvested by centrifugation at 6000g at 4°C for 10 min. Z-buffer (composed of 0.06M Na2HPO4; 0.04M NaH2PO4; 0.01M KCl; 0.001M MgSO4. 7H2O) was used to wash the cells twice and were suspended in the same buffer. 10µl of chloroform was added to this suspension, mixed well, and incubated at 37°C with open caps for 30 min. 200µl of β-ONPG was added to each tube and

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vortexed to start the reaction. Stop reaction was initiated by adding 0.5 mL of 1N Na2CO3 after yellow colour had developed. OD was measured at OD420 and OD550. Units of β-Gal were calculated as described by Miller.

III. RESULT

Molecular Characterization of LAB

The genomic DNA was isolated from the LAB isolates using the boiling method and run on 1% agarose gel electrophoresis as shown in Figure 1. The isolates were PCR amplified. Isolate 1 showed amplification with both lactobacillus and lactococcus gene. Isolate 2 and 3 showed amplification with lactobacillus specific genes.

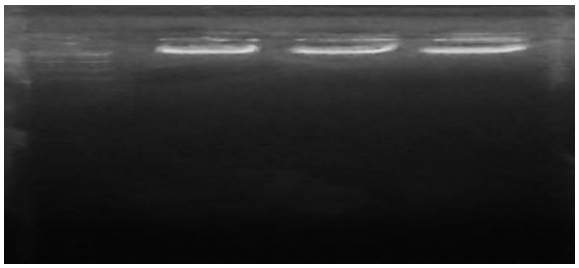

Fig. 1. Genomic DNA of three selected isolates. Lane M: DNA Ladder; Lane 1: Genomic DNA of isolate 1; Lane 2: Genomic DNA of isolate 2; Lane 3: Genomic DNA of isolate 3

Phylogenetic tree using MEGA X

All the tests conducted indicates the probiotic potential of the isolates. The strains identified through 16s rRNA Sequencing and Multiple Sequencing alignments were as follows:

- *Lactobacillus iners*
- *Lactobacillus casei*
- *Lactobacillus paracasei*
- *Weissella koreensis*
- Pediococcus acidilactici

The Lactobacillus isolates showed sequence similarity with *Lactobacillus iners* with 83% identity, *Pediococcus acidilactici* with 93% identity, and *Lactobacillus casei* and *Lactobacillus paracasei* with 97% identity by pairwise alignment using

BLASTN. The Lactococcus isolates showed high sequence similarity with *Weissella koreensis* with 99% identity.

Phylogenetic tree (Shown in Figure 2) constructed using MEGA X software for LAB isolates. 2A: LAB Isolate 1 with the sum of branch length of the tree = 0.07153078 is shown (next to the branches). 2B: LAB Isolate 2 with the sum of branch length of the tree = 0.04038413. 2C-1: LAB Isolate 3(a) with the sum of branch length of the tree = 0.01049509. 2C-2: LAB Isolate 3(b) with the sum of branch length of the tree = 0.00390133. 2D: LAC Isolate 1 with the sum of branch length = 0.04904226.

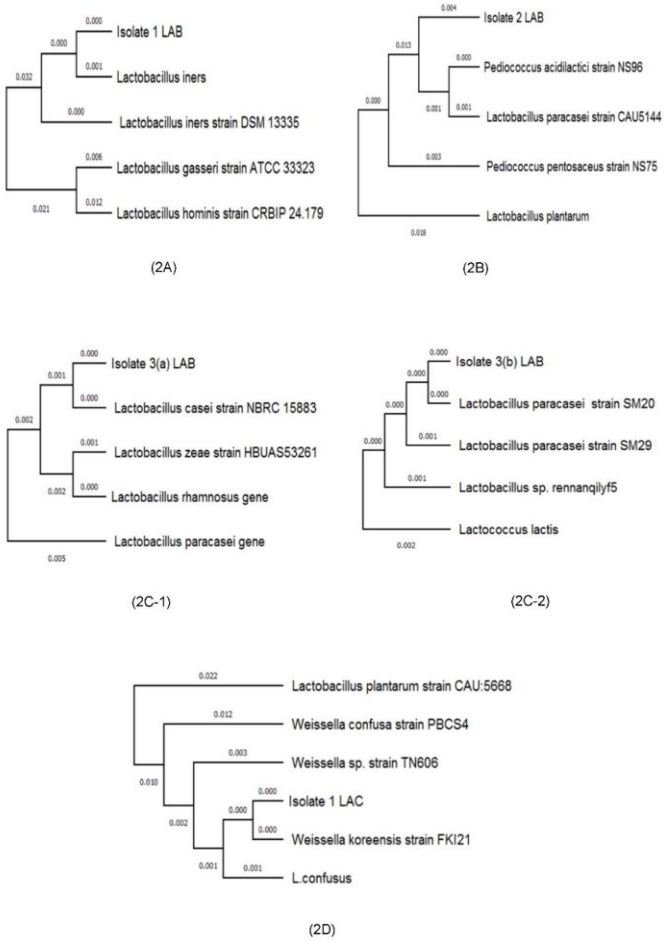

Figure 2 Phylogenetic tree constructed using MEGA X software for LAB isolates

Morphological and Biochemical Characteristics of LAB

A total of three isolates were identified and were classified based on the morphology, biochemical and molecular characteristics as shown in Table 1. All the three isolated LAB species were gram-positive and catalase negative. The isolate 1 contained a mixture of cream coloured short rods and short coccus. The isolate 2 and 3 were cream coloured short rods. All the three isolates fermented glucose. Lactose on fermentation yields acid turning the product into yellow was noticed in one of the isolates and was positive. Similarly, no yellow colour was produced in the other two isolates tested and thus were negative. There was no bubble produced inside the Durham's tube. Only two isolates fermented Sucrose.

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Characteristics		Isolate		
		1	2	3
Gram Staining		+	+	+
Catalase Test		--	--	--
Phenol Red Broth	Glucose	+	+	+
	Sucrose	+	+	--
	Lactose	+	--	--
Milk Curdling		+	--	+
pH	4.4	+	+	-
	9.6	+	++	++
Temperature (°C)	20	+	++	-
	45	--	--	--
Nacl (%)	65	+	+	+
	15	--	--	-
β-Galactosidase Activity (Miller Units)		1.75	1.83	3.36

Table 1 Characterization of LAB isolates from *Zingiber officinale*

Effect of temperature, pH and NaCl tolerance

Table 1 shows that the LAB isolates can survive at pH 9.6 than at pH4.4. The LAB isolates grew in the presence of 6.5% NaCl and exhibited poor growth with 15% NaCl. LAB isolates grew better at 20°C than at 45 °C.

Antibacterial activity of LAB Isolates

Significant inhibition was noticed with 20 µl concentration (Figure 3), The growth of E. coli was inhibited effectively by *Lactobacillus iners* and *Weissella koreensis*, *Lactobacillus casei* and *Lactobacillus paracasei* at 20 µl.

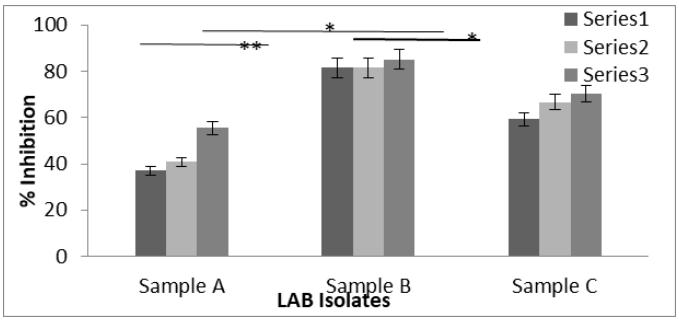

Figure 3 Antibacterial activity of LAB isolates against E. coli at 20 µl from three experiments

β-Galactosidase activity of LAB Isolates

Bacterial Cells of exponential phase were recovered and the activity of β-Galactosidase was evaluated with the addition of β-ONPG. All the three isolates exhibited β-Galactosidase activity with isolate 3 the highest.

In summary, a consortium of the probiotic organism is present in ginger and hence provides the health benefits.

IV. DISCUSSION

Many plant-based foods are utilized as a source of Lactobacillus species. Though these bacteria show positive results for the biochemical and molecular characteristics testing, it is uncertain if those bacteria could sustain in gut microbiota. In the current study, Lactic acid bacteria were isolated from Ginger. The colonies isolated were Gram-positive and catalase negative and concur with previous findings (Hezekiah Adeniran, Henrietta Abiose, and Terkula Ukeyima 2010).

The isolates were tested for its sugar fermentation capacity. Glucose, sucrose and lactose are the primary substrates fermented by LAB. All the isolates were homofermentative fermenting glucose, lactose, and sucrose. The ideal condition for growth of isolates was at 37°C and pH 6.5, which is in accordance with the study carried out with different LAB isolates (Breheny et al. 1975; Nannen and Hutkins 1991; Hutkins and Nannen 1993; Ashrethalatha et al. 2016). Though all the isolates could survive at different salt concentration, it could not show consistent growth in the presence of salt.

BLAST analysis of 16srRNA sequencing revealed Lactobacillus sp. has high sequence similarity to *Lactobacillus iners*, *Pediococcus acidilactici* and *Lactobacillus casei*. Lactococcus sp. is highly related to *Weissella confusa* and *Pediococcus acidilactici*. Likewise, BLAST analysis was performed for the sequenced isolates from Pirandai (Ashrethalatha et al. 2016). The antibacterial activity of Ginger rhizome was tested against gram-negative, E. coli. All the LAB isolates showed effective inhibition against E. coli. Similar findings were observed in a study that showed the antibacterial activity of ginger against various pathogens. The β-Gal activity of three isolates was assessed and was low in *Lactobacillus iners* and *Weissella koreensis.* This could be attributed to the changes in cell wall structure during the stationary phase that could lead to low production of the enzyme (Geciova, Bury, and Jelen 2002).

V. CONCLUSION

In summary, we have identified different strains of probiotic bacteria. These LAB isolates exhibit antimicrobial property and also have β-Gal activity. Thus, the ginger rhizome is a potent source of a consortium of probiotic bacteria and thus beneficial for gut health.

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