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ASSESSMENT OF PROBIOTIC PROPERTIES OF LACTIC ACID BACTERIA ISOLATED FROM PASSION FRUIT AND POTATO *IN VITRO*

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ABSTRACT

Probiotics are live microorganisms which when consumed in required number with a food promote the health of the consumer. The aim of this study was to evaluate *in vitro* probiotic properties of lactic acid bacteria (LAB) isolated from indigenous sources such as passion fruit and potato. The two isolates were biochemically characterised. *Weissella cibaria* from passion fruit and *Lactococcus lactis* from potato were deposited in NCBI with accession MK368397 and MK368420, respectively. Both isolates showed positive reaction on qualitative tests for exopolysaccharide production. As a part of assessing the probiotic potential, isolates were exposed to the harsh conditions of acid and bile. *W. cibaria* showed growth even after 3h of exposure to pH 2.0. It also showed remarkable tolerance to 0.6 per cent bile salts. To evaluate the adhesion potential, cell surface hydrophobicity (CSH) and auto aggregation were determined. CSH value and auto aggregation values of *W. cibaria* and *L. lactis* was found to be 89.1% and 79.6 % and 83.8 % and 33.7 % respectively. Both the isolates were non-haemolytic and not capable of liquifying gelatin, which indicates the possible absence of virulence factors.

Keywords: Lactic acid bacteria, probiotics, *W.cibaria, L.lactis*

INTRODUCTION

Lactic acid bacteria (LAB) constitute a group of Gram-positive bacteria united by a constellation of morphological, metabolic and physiological characteristics, including non-sporulating, non-respiring rods or cocci, which produce lactic acid as the major end product during the fermentation of carbohydrates. The term LAB is conventionally reserved for the genera *Lactobacillus, Leuconostoc, Pediococcus, Lactococcus, Streptococcus, Tetragenococcus, Carnobacterium, Weisella, Oenococcus, Vagococcus* and *Enterococcus*. Lactic acid bacteria, generally recognised as safe (GRAS)

inhabits a variety of ecological niches like food matrices: dairy products, meat, vegetables, sour dough, bread, wine and human mucosal surfaces such as oral cavity, gastrointestinal and genital tracts (Stiles and Holzapfel, 1997). Infact, they constitute a major share of probiotics used for the production of fermented foods like yoghurt, cheese, dry sausage, salami and sourdough (Wood, 1997). Probiotics are the most highly acclaimed group of functional foods, defined as "live microorganisms that, when supplied in suitable proportions, impose a health benefit on the host" (ISAPP, 2017). The probiotic organisms must be capable of surviving the stress environment of stomach (acid) and intestine (bile and hydrolytic enzymes) to get established in the intestine. They generally bring about their effect by altering the intestinal microbial balance in a way advantageous to the consumer. Hence, the present study was conducted to evaluate the *in vitro* probiotic properties of lactic acid bacteria isolated from passion fruit and potato.

MATERIALS AND METHODS

Isolation of lactic acid bacteria

The inner contents of passion fruit were scooped taking all aseptic precautions for preparing serial dilutions. Potato was thoroughly washed with hot water, peeled and then smashed well using mortar and pestle as a part of preparation of samples. Appropriate serial dilutions of these samples were pour plated in De Man, Rogosa and Sharpe (MRS) agar and incubated at 37^{o}C for 48h so as to get well isolated discrete colonies.

Physiological and biochemical tests

Preliminary characterisation of the LAB isolates was done using phenotypic (morphological) and biochemical tests that included Grams reaction, catalase test and oxidase test as described by Barrow and Feltham (1993). Arginine dehydrolyase activity, Bile Esculin hydrolysis, osmotic stress (sodium chloride: 4% and 6.5 %) resistance, sugar fermentation ability (Holt *et al.,*1994) and growth of the isolates at different temperatures was also tested.

Colony morphology, microscopic examination (capsule staining) and differential agar plating (Congo red agar) were resorted to, for checking exopolysaccharide (EPS) production potential. The colonies forming long filaments when extended with a loop are generally considered as ropy strains. Colonies showing sliminess but not forming filaments when extended were described as mucoid in nature.

Capsular staining was done as suggested by Anthony (1931). A thin smear of active culture was prepared and air dried

without heat fixing, One percent crystal violet was allowed to act for two minutes and then rinsed with 20 per cent (w/v) copper sulphate solution. After air drying, smears were examined under oil immersion. Crystal violet get dislodged easily from the non-ionic capsule when washed with copper sulphate solution. Light blue to colourless halo around deeply stained bacterial cells indicated the presence of capsules.

EPS production potential was evaluated based on colony characteristics when streaked on Congo red agar (Freeman *et al*., 1989). Congo red agar was prepared by adding 0.1 per cent Congo red solution at a level of nine per cent to Brain Heart Infusion agar containing five per cent sucrose. The presumptive colonies were streaked on Congo red agar and incubated at 37^{0}C. Formation of slimy and shining black colonies within 24 h of incubation were suggestive of EPS production.

Molecular level confirmation of the isolates was done by 16S rRNA sequencing. The primers used were 16S-RS-F Forward 5'CAGGCCTAACACATGCAAGTC3' and 16S-RS-R Reverse 5'GGGCGGWGTGTACAAGGC3' (Jacob *et al.,* 2017).

Probiotic properties of isolates

Indigenous isolates obtained were evaluated for their probiotic potential in terms of acid tolerance, bile tolerance and adhesion potential.

Acid and bile tolerance

To assess the acid and bile tolerance, the isolates were exposed to pH 2.0 and 3.0 and 0.3 and 0.6 percent bile respectively. Incubation was done at 37 ^{0} C. Tolerance was qualitatively assessed by streaking on MRS agar plates at hourly intervals for four hours (Pundir *et al.,* 2013).

Adhesion Potential

The isolates were further evaluated for their adhesion potential in terms of cell surface hydrophobicity (CSH value) and auto aggregation (%).

Auto-aggregation

Cell surface properties influence auto-aggregation and adhesion of bacteria to different surfaces. To find auto-aggregation, MRS broth was inoculated with freshly activated MRS broth culture at a level of one percent. After incubation at 37^{o}C for 18h, the cells were harvested by refrigerated centrifugation at 5000 g for 15 minutes. The cell pellet was washed twice with phosphate buffered saline (PBS) then resuspended in the same buffer to give a final optical density (OD) of 0.60±0.02 at 600 nm. Four millilitres of this cell suspension were mixed thoroughly by vortexing. From the suspension 0.1 millilitre was transferred

to another tube with 3.9 ml of PBS and the absorbance (A1) was measured at 600 nm. The sample was kept undisturbed 37°C and OD of samples (A2) were determined again exactly at 1h and 6 h (Kos *et al.,* 2003). The auto-aggregation percentage was expressed as:

Auto-aggregation (percentage) =
[(A1 - A2)/ (A1) x 100]

where A1: initial optical density, A2: optical density after incubation.

Bacterial Adhesion to Hydrocarbons (BATH)

BATH assay was done to determine the CSH as described by Collado *et. al*. (2008) with some modifications. The isolates were grown in MRS broth for 16-18 h at 37°C. Bacterial cells in the stationary phase were harvested as pellets by refrigerated centrifugation at 4°C for 12000rpm for 10 minutes. After washing the cell pellet with PBS thrice, pellet was resuspended in the same buffer to have an optical density of 0.25±05 at 600 nm. To 5 ml of this suspension, equal volume of xylene was added and the two-phase system was mixed thoroughly by vortexing for five minutes. Immediately after vortexing, OD at 600 nm was recorded. The vortexed samples were then kept at 37°C for 1h to allow phase separation. The cell suspension (aqueous phase) was pipetted out and OD at 600nm was again found out. Percentage of CSH was calculated using the formula:

[(Initial OD- Final OD) /Initial OD] ×100

Safety assessment

Safety evaluation of the LAB isolates done by investigating their hemolytic potential (Adetoye *et al.,* 2018), gelatin liquefaction and antibiogram by the standard disc diffusion assay as described by Bauer *et al.* (1966).

RESULTS AND DISCUSSION

Isolation of lactic acid bacteria

Potato and passion fruit were screened for the isolation of LAB by pour plating in MRS agar. Well defined gram positive, catalase negative and oxidase negative typical mucoid colonies from passion fruit (isolate 1) and potato (isolate 2) were streaked to purity on MRS agar. The isolates so purified were stored both in glycerol and MRS slants at 4°C for further study.

Physiological and biochemical tests

The ability of the isolates to utilise different carbon sources were assessed by the carbohydrate fermentation test in Phenol red broth. A change in the colour of media from red to yellow was taken as positive. The results (Table **1**) agree with Axelsson

(1998). Both isolates showed maximum growth at 37^0 C and considerable growth at 45^0 C. Isolates were found to be tolerant to high salt concentrations. Osmo tolerance is regarded as an important criterion for the identification and selection of strains for technological applications.

The isolates that showed mucoid colonies with a slimy nature and glistening appearance were selected for microscopic examination. Both showed the presence of capsule as a lightly stained /colourless halo after differential staining.

Table 1: Biochemical characterisation of the isolates

	Isolate 1	Isolate 2
Temperature		
15^0 C	+	+
37^0 C	+	+
45^0 C	+	+
NaCl		
4%	+	+
6.5%	+	+
Esculin hydrolysis	+	-
Arginine dehydrolase activity	-	+
Sugar fermentation		
Arabinose	-	+
Cellobiose	+	+
Fructose	+	+
Galactose	+	+
Lactose	+	+
Maltose	+	+
Mannitol	+	+
Mannose	+	+
Melibiose	-	+
Raffinose	-	+
Salicin	+	+
Sucrose	+	+
Trehalose	+	+
Xylose	+	+

(+) positive reaction (-) negative reaction

In the presence of high concentration of sucrose, the isolates synthesised extracellular EPS, which was displayed as black mucoid colonies on Congo red agar (Fig.1). The dye reacts with beta glucans and EPS producing strains formed black colonies while non-EPS producing strains produced colourless colonies (Tsveteslava *et al*., 2017).

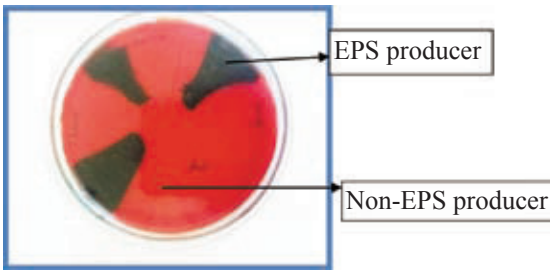

Fig 1: Isolated colonies on congo red agar

The isolates from potato and passion fruit identified *by* 16sRNA as *Weissella cibaria* and *Lactococcus lactis.* The isolates from passion fruit and potato were deposited in NCBI with accession no MK368397 and MK368420, respectively.

3.3. Probiotic Properties of Isolates

3.3.1. Acid tolerance and bile tolerance

In this study, isolates were exposed to pH 2.0 and pH 3.0 for 3 h reflecting the acidity and time spent by the food in the stomach. According to Smith (1995) transit time of food through small intestine

Table 2: Acid tolerance of the isolates.

Isolate	Time							
	pH 3.0				pH 2.0			
	0h	1h	2h	3h	0h	1h	2h	3h
L. lactis	++++	+++	++	+	++++	+	-	-
W. cibaria	++++	+++	++	++	++++	-	-	+

- no growth + -weak growth ++ -less growth, +++ - moderate growth, ++++ - heavy growth
(El-Sayed *et al.,* 2021)

is 1 to 4h and bile concentration is 0.3 per cent. Observations in this study indicate that *W.cibaria* from passion fruit is capable of surviving pH 3.0 for 3h (Table 2). It was also observed that, pH 2.0 adversely affected the growth of isolates. Interestingly, none of the isolates were significantly affected by 0.6 per cent bile (Table 3, Fig 2). The observations on bile tolerance is in consonance with Shukla and Goyal (2014). EPS seen as the outer capsule gives protection for the cells in the harsh environment. Stack *et al*. (2010) opined that beta glucan EPS afforded 5.5-fold increase in survival under bile stress conditions.

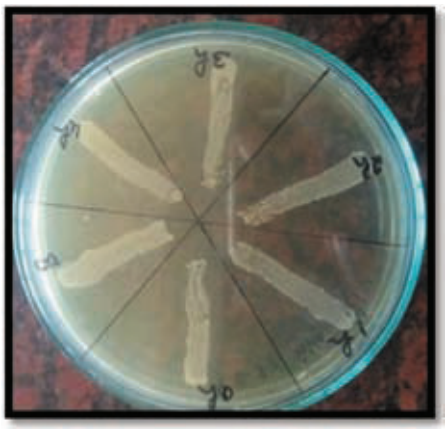

Fig 3: Growth pattern of *W.cibaria* in 0.6% bile

Adhesion Potential

Adherence to intestinal epithelial cells and mucosa are integral for bacterial strains to be used as probiotics. Cell surface properties are of technological importance in determining the interaction of bacterial cells with the gastro intestinal mucosa, thereby influencing their functionality in the gut. The auto-aggregation ability of the isolate was measured based on the principle of sedimentation. Auto-aggregation potential of the bacterial cells can be positively correlated with their adhesion characteristics. According to the results, auto aggregation increased as the time progressed. CSH value and auto -aggregation values of *W. cibaria* and *L. lactis* were found to be 89.1% and 79.6 % and 83.8% and 33.7 % (Figure 3 and 4**)** respectively. According to Wang *et al.* (2010), strains with auto-aggregation values more than 40 per cent is superior and those below cannot be considered. Hydrophobic nature of cell surface plays a key role in auto-aggregation and adhesion of bacteria to various types of surfaces. High CSH

Table 3: Effect of bile salt concentration on the viability of isolate

Isolate	Time							
	0.3%				0.6%			
	0h	1h	2h	3h	0h	1h	2h	3h
L. lactis	++++	++++	+++	+	++++	++	+	+
W. cibaria	++++	++++	++++	++++	++++	++++	++++	++++

- No growth + -Very less growth ++ -less growth, +++ - moderate growth, ++++ - heavy growth

value is linked to glycoprotein and low CSH value is associated with polysaccharide on the bacterial surface. Hydrophobic potential varies between organisms, between strains and is influenced by age of the cells and surface chemistry of cells with growth media components (Garcia-Cayvela *et al*., 2014). A CSH value of more than 40 per cent is suggestive of its hydrophobic nature of cells surface. Todorov *et al*. (2008) opined that, a hydrophobic cell surface may assist but is not at all a prerequisite for colonization.

3.4. Safety assessment

Antibiogram (Table 4) was interpreted according to the guidelines of Clinical and Laboratory Standards Institute

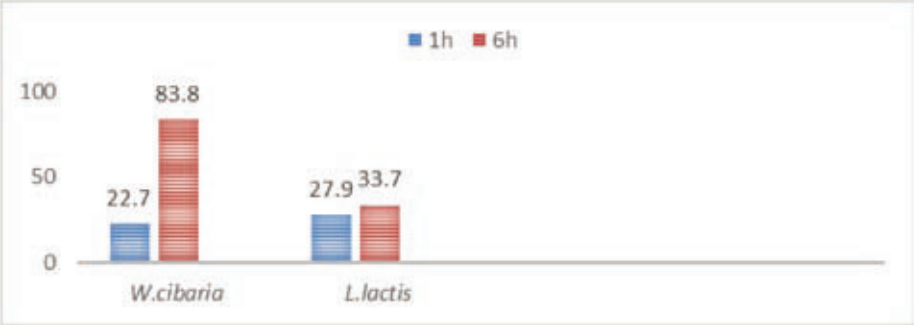

Fig 3. Auto-aggregation of isolates (Each value is the average of triplicate observations)

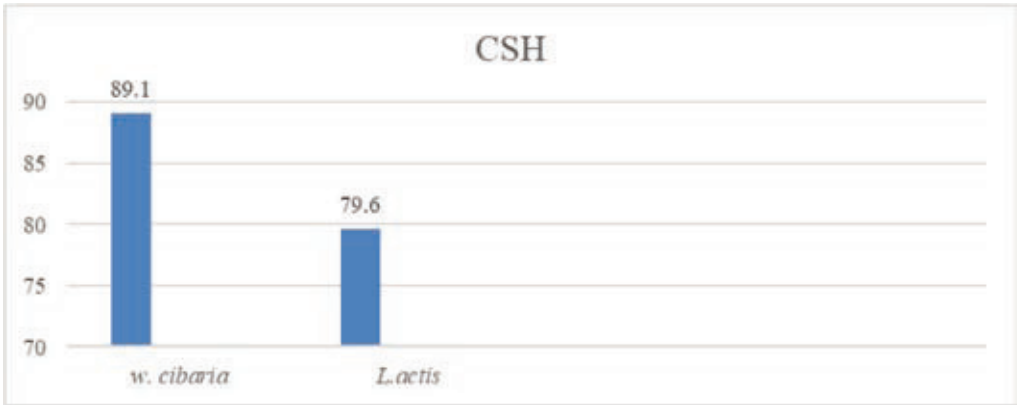

Fig 4: Cell surface hydrophobicity of isolates (Each value is the average of triplicate observations)

(2020) as follows: the isolates with a zone of inhibition less than or equal to 14 mm were considered as resistant (*R*) and those with more than 20 mm diameter as susceptible (*S*) and those having zone diameter between 15 and 19 mm as intermediate (*I*). Both the isolates showed high resistance towards Vancomycin in our study can be substantiated with the reports supporting the presence of intrinsic resistance mechanism towards vancomycin (Gueimonde *et al*., 2013*)*. The intrinsic resistance of the LAB species indicates that the non-sensitivity towards the approved drug doses, regulated by permeability barriers and active efflux (Nawaz *et al.,* 2011).

Table 4: Antibiogram of isolates

Antibiotics	*W.cibaria*	*L.lactis*
Gentamicin	31mm	26mm
Vancomycin	11mm	10mm
Penicillin	20mm	27mm
Erythromycin	26mm	21mm
Tetracycline	19mm	24mm
Streptomycin	27mm	17mm
Chloramphenicol	26mm	28mm

Gelatin liquefaction and hemolysis are linked to pathogenicity. Bacterial pathogenicity is dependent on cell penetration and cell invasion protein degradation ability. Haemolysin is a toxin that damages the cell membrane. Lack of gelatine liquefaction and beta hemolysis are suggestive of the isolate to be non-pathogenic in nature (Kang *et al.,* 2019).

CONCLUSION

The LAB isolates from non -dairy sources demonstrated probiotic attributes with good functional activities *in vitro*, revealing their potential to be used as a functional ingredient in food and feed formulations.

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