


ISOLATION OF LACTOBACILLUS FROM NON-DAIRY PRODUCTS AND ASSESSMENT OF THEIR PROBIOTIC PROPERTIES

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ABSTRACT

The main objective of the study was to isolate *Lactobacillus sp.* from fruit wastes such as banana peel and grape wastes, its characterisation by physiological, morphological and biochemical characteristics and assessment of probiotic potential of isolated *Lactobacillus sp.* to confirm its usefulness as a probiotic. The use of fruit wastes as a source of probiotics is a light to waste management as well as cheap and an alternative of traditional source. For a good probiotic it should tolerate the low pH of the gut and should tolerate bile salt and phenol each up to 0.3% concentration. All the isolates showed above 85% tolerance of 0.3% bile, while phenol tolerance is about 69-82% is seen among the isolates. It also shows that all the isolates exhibits good tolerance to low pH (above 85%). As the antibiotic resistant strains can be co-administered with antibiotics for treatment of diseases the isolates are being tested for antibiotic susceptibility. The main property of probiotic, that is antagonistic activity against pathogens such as *E. coli* and *Salmonella* was being tested. The experimental results showed that the banana and grape wastes contains *Lactobacilli* which can tolerate inhibitory substances and were able to survive both in acidic and alkaline conditions. They exhibited antibacterial activity against some indicator pathogens. Based on these characteristics the isolates may have potential for probiotic application.

KEYWORDS: *Lactobacillus sp.*, Probiotic, Antagonistic activity, *E. coli*, *Salmonella*.

INTRODUCTION

Lactobacillus is gram positive, facultative anaerobic or microaerophilic, rod-shaped organism. They are normal flora of mouth, intestine, and female genital tract with important role in the control of undesirable microorganisms that can be considered as natural bio-preservatives. Probiotics are considered as non-pathogenic live microorganisms that, when administered in adequate amounts, confer a health benefit on the host such as the improvement of immune system, modification of intestinal microflora, and anti-pathogenic effects (10 Takeda S *et al.*, 2011). The genus *Lactobacillus* belongs to the normal mucosal microbiota of humans and animals (Lahteenen T *et al.*, 2010). This group of bacteria is important for maintaining the stability of the gastrointestinal tract, preventing intestinal infections and generally supporting intestinal health (Gu RX *et al.*, 2008). *Lactobacilli*, primarily facultative or strict anaerobes generally has fastidious growth requirement. They prefer an acidic environment by producing lactic and other acids. In general, *Lactobacilli* have not been associated with disease and have been regarded as non-pathogenic and isolates were able to tolerate the acidic condition of the environment, NaCl

concentration and resistance to bile (Chidre Prabhurajeshwar *et al.*, 2017).

Probiotics, according to the revised definition of Food and Agriculture Organization (FAO) / World Health Organization (WHO), are considered as non-pathogenic live microorganisms that, when administered in adequate amounts, confer a health benefit on the host such as the improvement of immune system, modification of intestinal microflora, and anti-pathogenic effects (10 Takeda S *et al.*, 2011). Probiotics are effective in different clinical conditions including infectious diarrhea, necrotizing enterocolitis, antibiotic associated diarrhea, clostridium difficile colitis, helicobacter pylori infections, inflammatory bowel disease, cancer, female uro-genital infection and surgical infections. Therefore, there is growing interest on both basic and clinical sciences in probiotics (Sanders ME *et al.*, 2013). Some criteria have been identified for a bacterial strain to be a potential probiotic such as resistance to gastric and bile salt are observed in-vitro and are the first host factors that influence strain selection(Tinrat S *et al.*, 2011). The ability to survive and colonization in the gastrointestinal environment, production of anti-bacterial substance, to be of human origin and the possession of at least one

beneficial function and assessment of these criteria is an important part for human use (Verdenelli MC et al., 2009).

The ability to decrease the pH (with lactic acid production), producing antimicrobial compounds and competing with pathogens for adhesion and colonization and for nutrients and other growth factors in the gut helps the probiotics to fight against pathogens (Kim, K.W et al., 2017). Probiotics are widely used in dietary supplements, food, infant formula formulations, and medical devices (Bermúdez-Brito et al., 2012; Pamer E.G et al., 2016). They have demonstrated significant potential as therapeutic options for a variety of diseases, mainly gastrointestinal diseases (including acute infectious diarrhea, antibiotic-associated diarrhea, ulcerative colitis, irritable bowel syndrome, functional gastrointestinal disorders, or necrotizing enterocolitis), but also extra-intestinal disorders, such as hepatic encephalopathy (Wilkins, T et al., 2017). Some strains of probiotic bacteria can also block pathogen entry into the epithelial cells by increasing the mucus barrier, by stimulating the release of mucin granules from Goblet cells; and by maintaining the intestinal permeability, by increasing the intercellular integrity of apical tight junctions (Bermúdez-Brito et al., 2012; Gareau, M.G et al., 2010; Lopetuso, L et al., 2017).

The study focuses on isolation of probiotic *Lactobacillus* from sources other than usual dairy products such as fruit wastes. The use of fruit wastes as a source of probiotics is a light to waste management as well as cheap and an alternative of traditional source. For a good probiotic it should tolerate the low pH of the gut and should tolerate bile salt and phenol. So pH, bile, phenol tolerance of the isolated probiotic strains has been tested. They are also been tested for antibiotic susceptibility and the main property that is antagonistic activity against pathogens such as *E.coli* and *salmonella*.

MATERIALS AND METHOD

ISOLATION OF *Lactobacillus* sp.: On 10/12/19 the sample (fruit waste) was collected from the cool bar 'Mario' in Coimbatore, Tamil Nadu. The sample crushed using a mortar and pestle, serially diluted 6 folds in

distilled water and spread plated in MRS agar media (De Man et al., 1960). Plates were incubated at 37°C for 48 hours.

IDENTIFICATION OF *Lactobacillus* sp.: The isolated bacteria are identified as *Lactobacillus* according to their morphological, cultural, physiological and biochemical characteristics by the procedures as described in Bergey's Manual of Systematic Bacteriology (Holt et al., 1994). The tests carried out were gram reaction, catalase test, motility test, endospore test, IMViC test, sugar fermentation profile, phenol tolerance test and bile tolerance test and pH tolerance test.

PROBIOTIC ASSAY

Antibiotic susceptibility test: Kirby Bauer disc diffusion method (Mathur s and Singh R, 2005) is used, a cell suspension equivalent to 0.5 turbidity McFarland standard is prepared. Lawn culture of *Lactobacillus* made in Mueller-Hinton agar plates and antibiotic disc such as colistin, ampicillin, amoxicillin, penicillin and oxacillin are placed. After 24 hour incubation at 37°C, the inhibition zone was measured (Haghshenas B et al., 2014).

Antimicrobial activity assay: The two pathogenic organism, *E.coli* and *Salmonella*, was collected from sewage and identified by biochemical characterisation. The *Lactobacillus* isolates were grown in nutrient broth at 37°C for 48hrs with constant shaking at 180 rpm in a shaking incubator and is centrifuged at 7000 rpm for 20 min at 4°C. 8 mm diameter well was cut from the Mueller Hinton agar using a sterile cork-borer; subsequently each well was filled with 30µL, 50µL, 80µL of the cell free supernatant of the selected isolates. Sterile water is inoculated at the central well. The plates were incubated at 37°C for 48hrs in upright position, after incubation zone of inhibition is measured.

RESULTS

Isolation and Characterisation of *Lactobacillus*

Four types of isolates was obtained from banana sampling and it is named as B1, B2, B3, B4 and from grapes there were two kinds of isolates and it is been coded as G1and G2.

Table 1: Morphological and physiological characterization of the isolated bacterial strain.

Sr. No	Configuration	B1	B2	B3	B4	G1	G2
1	Elevation	flat	convex	convex	flat	flat	Flat
2	Colony color	Creamy white	Creamy white	Off white, shiny	white	Creamy white	Creamy white
3	Opacity	opaque	opaque	translucent	opaque	opaque	Opaque
4	Gram's reaction	+	+	+	+	+	+
5	Spores	-	-	-	-	-	-
6	Motility	-	-	-	-	-	-

It is clear that the bacteria are gram positive, rod shaped occurring single, clusters or in chains. The gram staining results indicated that the isolated bacteria could be

identified as *Lactobacillus* sp. Hanging drop wet method showed that all the isolated bacteria were non motile. The non-motile behaviour is a characteristic of

Lactobacillus. Therefore the sample bacterium resembles characters similar to *Lactobacillus sp*. The catalase test is one of the most useful diagnostic tests for the recognition of bacteria due to their simplicity. In performing catalase test, no bubble was observed indicating that the isolated bacterium is catalase negative and could not mediate the

decomposition of H₂O₂ to produce O₂. It is well known that *Lactobacillus* is catalase negative. Thus, the results obtained coincided with *Lactobacillus* strain characteristics. Their distinguishing features are shown in (Table 1). Their biochemical characters are also shown in the (Table 2).

Table 2: Biochemical characterization of the isolated bacterial strain.

Sr. No.	Tests	B1	B2	B3	B4	G1	G2
1	Catalase test	-	-	-	-	-	-
2	Indole test	-	-	-	-	-	-
3	Methyl red test	-	+	-	+	-	+
4	VP test	-	-	-	-	-	-
5	Citrate utilization test	-	-	-	-	-	-

Table 3: Sugar fermentation profile of the isolated *Lactobacillus* (++sign indicates complete fermentation & +-sign indicates partial fermentation.

Sr. No	Sugar	B1	B2	B3	B4	G1	G2
1	Maltose	++	++	++	+-	+-	++
2	Mannose	++	++	++	++	+-	++
3	Glucose	++	++	++	++	++	++
4	Lactose	+-	++	++	+-	++	+-
5	Fructose	++	++	++	++	++	++

BILE, pH AND PHENOL TOLERANCE OF ISOLATED *Lactobacillus* STRAINS

In order to act as a good probiotic the bacteria should have the ability to survive in gastro-intestinal tract environment so that it should be able to survive under low pH, up to 0.3% bile and phenol concentration. The

tolerance capacity of the isolate against pH, bile and phenol concentrations are shown in (Table.4, 5 & 6). All the isolates showed above 85% tolerance of 0.3% bile, while phenol tolerance is about 69-82% is seen among the isolates. It also shows that all the isolates exhibits good tolerance to low pH (above 85%).

Table 4: Bile tolerance.

Sr. No	Isolates	Control	Untreated culture	0.1% bile concentration	0.2% bile concentration	0.3% bile concentration
1	B1	0.814	1.830	1.689	1.616	1.600
2	B2	0.814	1.616	1.549	1.578	1.546
3	B3	0.814	1.723	1.614	1.573	1.573
4	B4	0.814	1.594	1.502	1.500	1.494
5	G1	0.814	1.784	1.683	1.674	1.600
6	G2	0.814	1.716	1.564	1.543	1.529

Table 5: Phenol tolerance.

Sr. No.	Isolates	Control	Untreated culture	0.1% phenol concentration	0.2% phenol concentration	0.3% phenol concentration
1	B1	0.720	1.830	1.520	1.373	1.274
2	B2	0.720	1.616	1.406	1.406	1.246
3	B3	0.720	1.723	1.290	1.264	1.216
4	B4	0.720	1.594	1.214	1.212	1.200
5	G1	0.720	1.784	1.516	1.454	1.232
6	G2	0.720	1.716	1.494	1.482	1.410

Table 6: pH tolerance.

Sr. No.	Isolates	Control	Untreated culture	pH2	pH3	pH4
1	B1	0.416	1.830	1.129	1.499	1.607
2	B2	0.416	1.616	1.101	1.382	1.424
3	B3	0.416	1.723	1.274	1.500	1.663
4	B4	0.416	1.594	1.002	1.172	1.320
5	G1	0.416	1.784	1.116	1.318	1.519
6	G2	0.416	1.716	1.034	1.289	1.477

Antibiotic Susceptibility Test

The isolates are also tested for antibiotic susceptibility as it is very important selection property for probiotic, as the antimicrobial resistant strains can be co-administered with antimicrobial compound for treatment of diseases. The antibiotic susceptibility pattern of the isolates are shown in (Table 7). The isolates are also tested for antibiotic susceptibility as it is very important selection property for probiotic, as the antimicrobial resistant strains can be co-administered with antimicrobial compound for treatment of diseases. The antibiotic

susceptibility pattern of the isolates are shown in (Table 7). Isolate B1 is sensitive to colistin, and ampicillin while resistant to amoxicillin, penicillin and oxacillin. B2 is sensitive to colistin, amoxicillin and penicillin, while resistant to ampicillin and oxacillin. B3 is sensitive to colistin but resistant to ampicillin, amoxicillin, penicillin and oxacillin. B4 is sensitive to colistin and ampicillin but resistant to amoxicillin, penicillin, oxacillin. G1 and G2 are sensitive to colistin and resistant to ampicillin, amoxicillin, penicillin and oxacillin.

Table 7: Antibiotic susceptibility test result (measurement of zone of inhibition).

Sr. No.	Antibiotic disc	B1 (in cm)	B2 (in cm)	B3 (in cm)	B4 (in cm)	G1 (in cm)	G2 (in cm)
1	colistin	2.6	3.4	2.4	2.8	2.5	2.3
2	Ampicillin	1.8	1.5	1.3	1.8	1.2	0.9
3	Amoxicillin	1.5	1.9	1.2	1.2	1	0.9
4	Penicillin	1.2	1.8	1.2	1	1	0.9
5	Oxacillin	0.9	1.6	1	-	0.9	-

Antimicrobial Activity Assay

Antibacterial activities exhibited by *Lactobacillus* species which indicates that the cell free solution of isolated *Lactobacillus* species were able to inhibit the growth of all the test microorganisms. This experiment clearly indicates that the inhibitory metabolites produced by isolated *Lactobacillus* species were extracellular and diffusible. These results are shown in the (Table 8 a) &

b) The experimental results showed that the banana and grape wastes contains *Lactobacilli* which can tolerate inhibitory substances and were able to survive both in acidic and alkaline conditions. They exhibited antibacterial activity against some indicator pathogens. Based on these characteristics the isolates may have potential for probiotic application.

Table 8: Antibacterial activity of the isolates against *E.coli* and *Salmonella*.**a) Result of antibacterial activity of isolates against *E.coli***

Sr. No.	Isolates	Zone of inhibition in 30 µl well (cm)	Zone of inhibition in 50 µl well (cm)	Zone of inhibition in 80 µl well (cm)
1	B1	-	1.1	1.2
2	B2	-	1	1.2
3	B3	-	1.4	1.6
4	B4	-	1	1.5
5	G1	-	0.9	1.3
6	G2	-	1.4	1.5

b) Result of antibacterial activity of isolates against *Salmonella*

Sr. No.	Isolates	Zone of inhibition in 30 µl well (cm)	Zone of inhibition in 50 µl well (cm)	Zone of inhibition in 80 µl well (cm)
1	B1	-	-	1.2
2	B2	-	0.8	1.5
3	B3	-	1.2	1.5
4	B4	-	1.3	1.3
5	G1	-	-	1
6	G2	-	1.3	1.5

DISCUSSION

Probiotic bacteria are used in many health related areas such as the control of inflammation, management of allergic diseases, antibiotic-related diarrhea, gastroenteritis, constipation, lactose intolerance, a preventive role in onset of tumor (Uccello et al., 2012). Up to now, many efforts have been made to produce probiotics from different sources.

In the present study, 6 gram positive, catalase negative, non-motile, non-sporulated bacilli occurred in single, cluster or in chain with creamy white colonies in MRS agar isolated from 2 different sources such as banana waste and grape waste. MRS agar is used as a selective medium for lactic acid bacteria (De man J.C et al., 1960), besides *Lactobacillus* other lactic acid bacteria such as *Bifidobacterium*, *Streptococcus*, *Pediococcus* can also be grown in MRS medium at the same time MRS pH 5.4 agar incubated at 37 °C for 72 h under anaerobiosis was selective for *Lactobacillus spp.*

The ability to tolerate bile salt at a concentration of 0.3% has a physiological significance because it is a level normally encountered in human intestine (S.E.Gilliland et al., 1984). In many studies conducted, the standard level of 0.3% bile was considered for investigation of bile tolerance of potential probiotic *Lactobacillus* strains. Thus in this study 0.1%, 0.2% and 0.3% of bile salt concentration was used and it is found that all the isolates were able to tolerate bile salt, as all the isolates have grown well when compared with an untreated culture of the same isolate.

Phenol tolerance is important for probiotics to survive the gastrointestinal conditions, where the gut bacteria have the ability to deaminate aromatic amino acids that are derived from dietary proteins and may lead to form phenol (Yadav R et al., 2016) so it is necessary for the probiotics to be able tolerate phenol. In this study the isolates are examined for 0.1%, 0.2% and 0.3% of phenol tolerance and all the isolates exhibits good phenol tolerance capacity

During the passage through stomach, the probiotic microbes have to survive at low pH as of 3.0 before reaching in lower tract and must remain viable for 4h or more. pH 3 was used as a standard pH for investigation of acid tolerance of probiotic strains in many studies (M. T. Liong and N.P. Shah., 2005; R.P.K. Sahadeva et al., 2011). In this study pH 2, pH 3 and pH 4 are used to detect the acid tolerance of the isolates. All the 6 isolates exhibits good acid tolerance capacity it shows growth which is near to growth of untreated culture of the respective isolates.

Resistance to antibiotics is a very important criteria for selection of probiotics, as the antimicrobial resistant strains can be co-administered with antimicrobial compound for treatment of diseases (Petsuriyawong B et al., 2011). All isolates were susceptible to colistin, while

only B1 and B4 are susceptible to ampicillin. All isolates except B2 is resistant to amoxicillin and penicillin and all isolates are resistant to oxacillin. The above discussion clearly indicates that *Lactobacillus* exhibits highly variable sensitivity towards different antimicrobials, so that clears that every probiotic has specific choice of antimicrobial to make a combination.

All the 6 isolates of *Lactobacillus* showed antimicrobial activity at a concentration of 80µl against pathogens such as *E. coli* and *Salmonella*. Antagonistic activity of isolated strains might be due to production of antimicrobial substances like organic acids such as lactic and acetic acid, hydrogen peroxide, bacteriocin, antimicrobial peptides (Venkatesan S et al., 2012).

The study focuses on isolation of probiotic *Lactobacillus* from sources other than usual dairy products such as fruit wastes. The use of fruit wastes as a source of probiotics is a light to waste management as well as cheap and an alternative of traditional dairy source. Through this study it is clear that *Lactobacillus* can also be isolated from fruit wastes thus wastes are no more wastes but is a source of health useful probiotics. As the people are becoming more and more conscious about health the demand for probiotics is also in an increasing state. Thus an alternative source for probiotic organisms is necessary as long as the source is fruit wastes the raw material very cheap and easily available and also with study it states that *Lactobacillus* from fruit wastes have the quality to be used as a probiotic.

CONCLUSION

The current study deals with the potential of *Lactobacillus sp.* isolated from fruit wastes such as banana peel and grape wastes to be used as a probiotic. The bacterium is isolated in selective media and characterization is done by noticing the physiological, morphological and biochemical characteristics. For a good probiotic it is necessary to survive under gastrointestinal tract environment, which, means it should be capable of tolerating low acid pH as well as bile salt and phenol. All the six isolated strains exhibits good tolerance to the three facts and hence it is clear that it can survive in gastro intestinal tract environment. As long as probiotic cannot be used alone as a medicine it is necessary to resist the antimicrobial substance to some extent to co-accommodate with the medications. The antibiotic susceptibility pattern of each organism varies with the strains so that it clear that every probiotic has specific choice of antimicrobial to make a combination. All the isolated strains exhibits antimicrobial activity at 80µl thus it shows that the *Lactobacillus* strains isolated from fruit wastes can also be used as probiotics.

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