Annals of R.S.C.B., ISSN:1583-6258, Vol. 24, Issue 2, 2020, Pages. 1189 - 1200
Received 24 October 2020; Accepted 15 December 2020.

Evaluation of Probiotic Properties of Lactic Acid Bacteria Isolated from Curd

J. Jayachitra[1], G. Kumaresan[1], and E. Babu[2]
1. Assistant Professors, Department of Agricultural Microbiology, Faculty of Agriculture, Annamalai University, Annamalai Nagar – 608002, Tamil Nadu – India.
2.Assistant professor,Department of Chemical Engineering, FEAT,Annamalai University, (Deputed to Department of Polymer Technology, Tamil Nadu Government Polytechnic College Madurai, Tamil Nadu – 625 011.)
Mail: microjayachitra@gmail.com

ABSTRACT

The aim of this study is to characterize probiotic properties of isolated lactic acid bacteria isolated from curd sample. Curd sample was cultured on MRS Agar media with appropriate dilution for the isolation of potential probiotics strains and pure cultures were obtained by continuous subculturing. Identification of lactic acid bacterial (LAB) was done by Gram's staining and catalase test and further confirmation was based on morphological identification and different biochemical tests. Three lactic acid bacterial isolates viz. *Lactobacillus fermentum, L. casei, L.acidophilus*were identified after different biochemical analysis which also showed reliable probiotic properties. These isolates were also subjected to further tests to find out the probiotic properties including tolerance to different concentrations of phenol,tolerance to bile salt, tolerance to different concentrations of NaCl and antimicrobial activity. Probiotics are supposed to have beneficial effects for the host.The results proved that LAB isolated from curd exhibited promising probiotic properties and seems to be favourable for use in functional fermented foods as bio preservatives.

Keywords: Probiotic; curd,Lactic acid bacteria; Antimicrobial activity

INTRODUCTION

The lactic acid bacteria display the ability to ferment carbohydrates and degrade proteins and lipids which results in the synthesis of a wide range of compounds such as organic acids, peptides, antimicrobial compounds (Mozzi *et al.,* 2010). Thus, this group of bacteria live in various ecological niches with probably probiotic properties as they can be used in the formulation of new probiotic products (Merrified*et al.,* 2014; Sonsa-Ard*et al.,* 2015). According to WHO (2001), probiotics are living microorganisms which,when ingested in adequate amounts, provide a beneficial effect on the health of their host. Currently, considerable interest has been

Annals of R.S.C.B., ISSN:1583-6258, Vol. 24, Issue 2, 2020, Pages. 1189 - 1200
Received 24 October 2020; Accepted 15 December 2020.

developed around the use of lactic cultures for food and pharmaceutical applications (Kabir et al., 2004; Houndonougbo*et al.*, 2011).

Probiotics are used to stimulate the immune system in stock farming and to increase the productivity (Bahoua, 2008). Other studies have also shown that probiotics exert antibacterial activities against several pathogenic bacteria including those responsible both for infections in humans and animals (Sharifuzzaman*et al.*, 2009). Antibiotics and vaccines are the means commonly used to fight against these diseases which affect the health and productivity of local stock farming. But the misuse of these antibiotics during medical treatment and stock farming induced the increase of bacteria resistant. It is therefore essential to find an alternative therapeutic and (or) protective way to control pathogenic microorganisms. The selected lactic acid bacteria, with probiotic properties, are reported to be used by specialized companies to produce lactic ferments and produce bacteriocins (Beal *et al.*, 2008). Lactic acid bacteria have been isolated from different sources such as dairy products, sewage, plants, human and animalwith probiotic properties. Indeed, before considering a lactic acid bacteria as probiotic, it must displays properties such as: i) colonization and survival in gastrointestinal region, ii) high tolerance to acid and bile, iii) ability to adhere to intestinal surfaces, iv) antibiotic resistance profile and antimicrobial activity and v) some technological (acidification, proteolytic, lipolytic, texturizing, coagulation, thickening and aromatization power) skills (Pitino et al., 2010).Lactic acid bacteria (LAB) are commonly associated with fermented dairy products such as cheese, buttermilk, curd, Shrikhand etc. Curd is fermented milk product which is also good source of lactic acid bacteria.The medical world has long been interested in the nutrient properties of curd. The curd bacteria especially LAB have been well accepted as GRAS (generally regarded as safe). The curd as part of milk that coagulates when the milk sours or is treated with enzymes, curd is used to make cheese; or as a coagulated liquid that resembles milk curd. Curd is also cheap and easily available source of lactic acid bacteria. Thus, the current study has been undertaken to investigate the probiotic properties (acid, bile, and sodium chloride tolerance) of three Lactobacillus strains from locally purchased/homemade curd samples, and their antimicrobial activity.

Annals of R.S.C.B., ISSN:1583-6258, Vol. 24, Issue 2, 2020, Pages. 1189 - 1200
Received 24 October 2020; Accepted 15 December 2020.

Materials and Methods

Sample collection

Four curd samples were collected from different areas in Chidambaram town of Cuddalore district in Tamil Nadu, India. After collection, the samples were stored aseptically at 4°C in refrigerator to defend from deterioration and contamination.

Sample was collected in autoclaved sterile plasticvials. At each time of collection, provision was taken to prevent (or) avoid cross contamination of samples.

Isolation Lactic Acid Bacteria (LAB)

One gram of curd sample was dissolved in 9 ml of 0.15% buffered peptone water solution and diluted up to ten logarithmic (10^{-10}) fold. About 1 ml of appropriate dilution of the sample was pipette into sterile Petri dishes. MRS agar media were poured and incubate at room temperature for 48 hrs. The LAB was identified based on growth on selective MRS agar (pH 5.2), cell morphology, gram staining, catalase activity and biochemical identification of LAB. Further identification of the species of this LAB was performed according to carbohydrate fermentation patterns and growth on MRS broth (HI Media) as described in Bergey's manual of systematic bacteriology. The isolated LAB wassub cultured and the purified cultures maintained at MRS agar slants. Well isolated bacterial strains were picked up and stored in MRS broth for further studies.

Probiotic Properties Analysis

Resistant to low pH

Resistance to pH 2.0, 3.0 and 4.0 are often used in *invitro* assays to determine the resistance to the pH of stomach. Food usually stays in the stomach for 3-4 hours and this time limit was considered. All the three LAB isolates namely LC1,LC2and LC3 were grown for 16 - 18 hours in MRS broth. The cells were centrifuged at 5,000 rpm for 10-15 min at 4°C and the pellet were collected in a sterile tube. The pellet was washed twice with phosphate saline buffer (PBS at pH 7.0) and inoculated in MRS broth and the pH is adjusted3.0 (by addition of 1 M HCl) and incubated at 37°C. Viable microorganisms were enumerated at the 0, 1, 2, 3 and 4 hours by recording the absorbance at 620 nm in spectrophotometer.

Assay for NaCl Tolerance

For the determination of NaCl tolerance of isolated LAB, test tubes containing MRS broth were adjusted with different concentrations (1-10%) of NaCl. After sterilization, each test

Annals of R.S.C.B., ISSN:1583-6258, Vol. 24, Issue 2, 2020, Pages. 1189 - 1200
Received 24 October 2020; Accepted 15 December 2020.

tube was inoculated with 1% fresh overnight culture of LABs and incubated at 37°C for 24 h. After 24 h of incubation their growth were determined by observing their turbidity (Hoque *et al.*(2010).

Tolerance againstPhenol

For the determination of phenol tolerance, test tubes containing MRS broth were adjusted with different concentration (0.1-0.4%) of phenol. After sterilization, each test tube was inoculated with 1% (v/v) fresh overnight culture of LABs and incubated at 37°C for 24 h.

After 24 h of incubation their growth were determined by 620 nm filter absorbance of cell concentration by spectrophotometer turbidity (Hoque *et al.,* (2010).

Tolerance against Bile Salts

The mean intestinal bile salt concentration is believed to be 0.3% and staying time of food is suggested to be 4 hrs. The experiment was applied at this concentration of bile for 4 h. For this purpose, active cultures were used. Cells were harvested by centrifugation and MRS broth containing 0.3% bile salts were added to pellets. During incubation of 4 hrs, at every hour inoculations were carried out into MRS broths and they were incubated at 37°C for 48 hrs and growth was monitored after incubation at OD620by spectrophotometer.

Antimicrobial Activity

The possible probiotic potentials or the antagonistic activity of the isolate against the selected pathogens was investigated by well diffusion method on a solid medium. In the well diffusion assay, isolated colonies of probiotic cultures were inoculated in 5 ml LB broth and grown at 30°C on a shaking incubator at 150 rpm for 72hrs, and cells were removed by centrifugation at 8,000 rpm for 5 min and the culture supernatant were sterilized by passage through 0.45 µm pore size filters (Millipore). Four embattled pathogenic bacteria *Staphylococcus aureus*, *VibrioCholerae*, *Pseudomonas aeruginosa*, *Escherichiacoli*were precultured in LB broth medium, incubated at 30°C for two days and the culture was swabbed over the nutrient agar plate. Wells (4 mm in diameter) were punched into the swabbed nutrient agar plate and 80 µl of culture supernatants from the probiotic isolate were added. The plates were then incubated at 37°C for 24 h. Antibacterial activity was estimated as the diameter (mm) of the clear inhibitory zone formed around the wells.

Annals of R.S.C.B., ISSN:1583-6258, Vol. 24, Issue 2, 2020, Pages. 1189 - 1200
Received 24 October 2020; Accepted 15 December 2020.

Results and Discussion

Table – 1. Morphological and biochemical characteristics of lactic acid bacterial strains

Morphological Characters			
	LC1	**LC2**	**LC3**
Gram reaction	+	+	+
Spores	-	-	-
Shape	Rods	Rods	Short rods
Size	0.5μm X 0.8 μm	0.8 μm X 2 μm	0.7μm X 2μm
Motility	-	-	-
Biochemical characters			
Catalase test	-	-	-
Oxidase test	-	-	-
Gas production from glucose	+	-	-
NH_3 from arginine	+	-	-
Carbohydrates			
Arabinose	D	-	-
Cellobiose	D	+	+
Esculin	-	+	+
Fructose	+	+	+
Galactose	+	+	-
Glucose	+	+	+
Lactose	+	D	+
Maltose	+	+	+
Mannitol	-	+	-
Mannose	+	+	-
Melezitose	-	+	-
Melibiose	+	-	-
Raffinose	+	-	+
Rhamnose	+	-	-
Ribose	+	+	-
Salicin	+	+	-
Sorbitol	-	+	-
Sucrose	+	+	+
Trehalose	D	+	+
Xylose	D	-	-
Tentatively Identified as	*Lactobacillus fermentum*	*Lactobacillus casei*	*Lactobacillus acidophilus*

+ Positive; - negative; D – delayed reaction

Annals of R.S.C.B., ISSN:1583-6258, Vol. 24, Issue 2, 2020, Pages. 1189 - 1200
Received 24 October 2020; Accepted 15 December 2020.

Table – 2. NaCl tolerance test of Lactic Acid Bacterial isolates in MRS broth

Isolates	1%	2%	3%	4%	5%	6%	7%	8%	9%	10%
LC–1	+	+	+	+	+	+	+/-	-	-	-
LC–2	+	+	+	+	+	+	+	-	-	-
LC–3	+	+	+	+	+	+	+/-	-	-	-

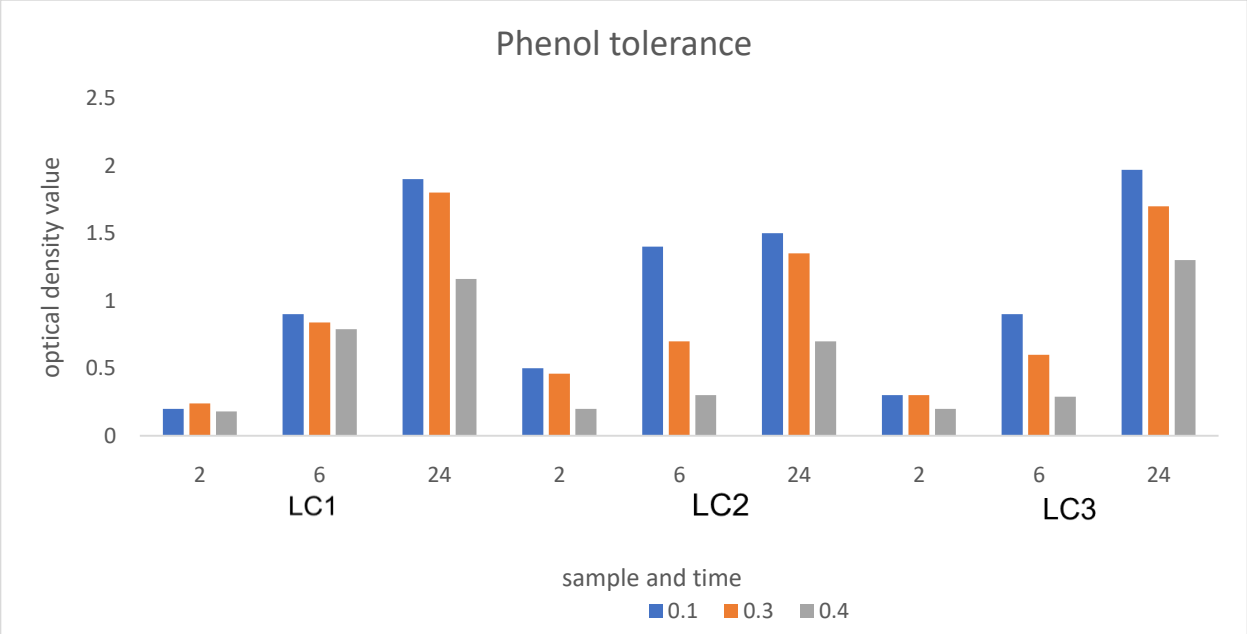
+' indicates normal level growth; '+/-' indicates moderate growth; '-' indicates no growth

In this study, lactic acid bacteria were isolated from curd. Microscopic identification of the isolate could determine the rod-shaped cells, gram positive, catalase negative, non-motile rods and oxidase negative which indicated the typical basic characteristics of Lactobacilli. Based on the carbohydrate utilization pattern of bacterial isolates,LC - 1,LC -2 andLC - 3 were identified *as Lactobacillus fermentum,Lactobacillus casei, Lactobacillus acidophilus*. The results are tabulated in Table - 1. Similar characters for lactic acid bacteria observed earlier by Kandler and Weiss (1986). Jayachitra *et al.*,(2019) in their studies isolated and characterized the lactic acid bacteria from cheese,fish and prawn. (Seema Nair and Surendran (2009). In this study all the isolated lactic acid bacterial isolates were able to grow at 1-6% NaCl concentration, whereas fairly grow at 7% concentration but completely failed at 8-10% NaCl concentration (Table - 2).All probiotic isolates were capable to grow at 1-6% NaCl concentration but failed at 7-10% NaCl concentration Our results are in agreement with *Lactobacillus* strains from buffalo milk cheese (Jeronymo-Ceneviva *et al.,* (2014), which as well found to tolerate 1-7% NaCl.

The isolated probiotic candidates were screened for their ability to withstand phenolic environment. Isolates weredetected in 0.1%, 0.3% and 0.4% phenol solution throughout interval of 2, 6 and 24 hours. The optical density was measured by spectrophotometer, according to the results all the isolatesare defiant to phenol at different concentrations. At 0.1% concentration all isolates showed higher level of tolerance whereas in 0.3% they were moderate but in 0.4% tolerance was much lower (Figure - 1).

Annals of R.S.C.B., ISSN:1583-6258, Vol. 24, Issue 2, 2020, Pages. 1189 - 1200
Received 24 October 2020; Accepted 15 December 2020.

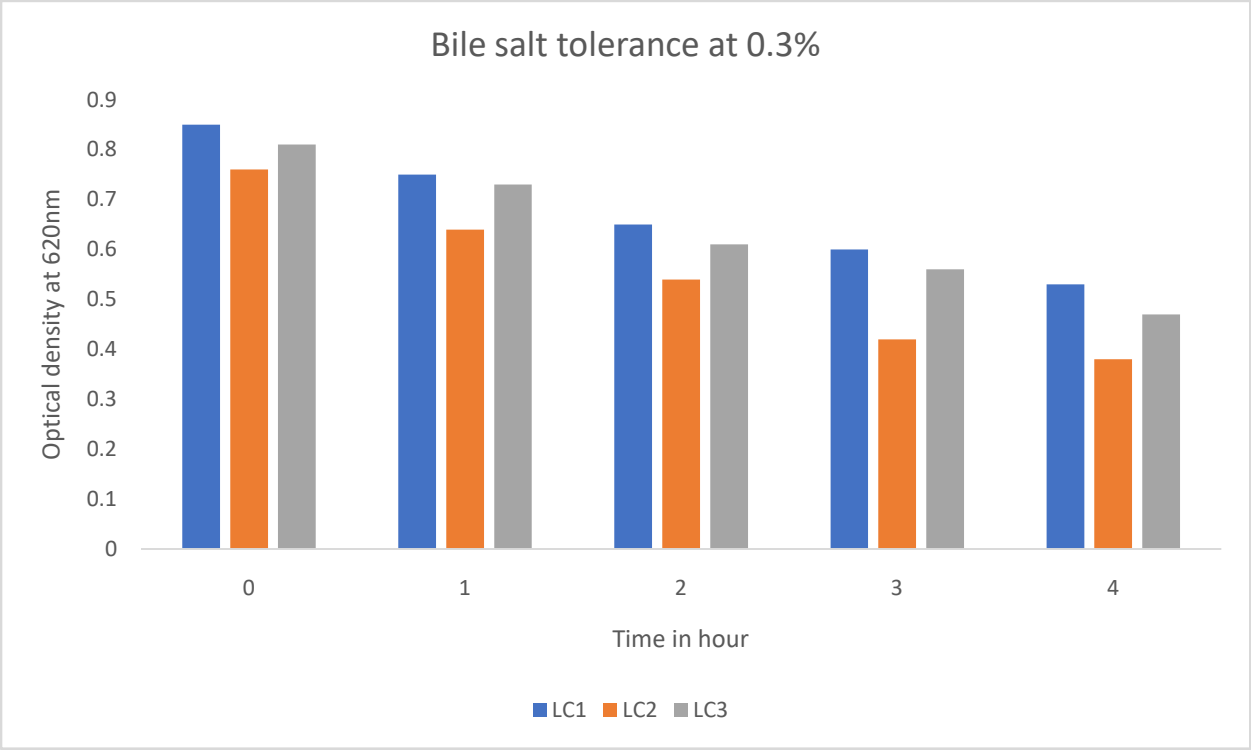
Figure – 1. Tolerance against Phenol (0.1%, 0.3%, 0.4%) at OD 620nm values.



This phenol tolerance is important for isolates to survive in the gastrointestinal conditions, where the gut bacteria could deaminate aromatic amino acids that are derived from dietary proteins and may lead to formation of phenols(Yadav *et al.*, 2016; Divisekera*et al.*, 2019;). There are many instances of phenol tolerance reportedin LAB that were isolated from natural fermented food sources (Ghabbour*et al.*, 2011). The results prove that theisolates evaluated in the present study can survive human gastrointestinal conditions.The isolates were tested for their ability to tolerate bile salt, although the bile concentration of the human gastrointestinal tract varies. Lactic acid bacterial isolates were able to grow at 0.3% of bile salt concentrations. The optical density is measured by spectrophotometer after0,1, 2,3 and4 hrs interval. Their ability to tolerate at 0.3% concentrations of bile salt in different time interval is showed in Figure - 2. Survival rate of LC -1 was higher than LC - 3and LC -2 at 0.3% bile salts concentrations.Considering bile tolerance an important characteristic of probiotic microorganisms, it has been reported that 0.3% oxgall closely appropriates the bile level of the human gastrointestinal tract, and the concentration of 0.3% bile salts has been considered crucial for screening and selection of human probiotics Goldin and Gorbach (1992).

Annals of R.S.C.B., ISSN:1583-6258, Vol. 24, Issue 2, 2020, Pages. 1189 - 1200
Received 24 October 2020; Accepted 15 December 2020.

Figure – 2. Survival of Lactic acid bacterial strains in 0.3%Bile salts at OD620 nm values



Liong and Shah (2005) stated that resistance at pH 3 has been the standards for acid tolerance of probiotic culture. The ability of withstanding low acid level was tested for all the three isolates (pH range: 3.0 and 4.0). The results were recorded for 0, 1, 2, 3, 4 hrs and presented in the Figure - 3 and 4.The isolate LC - 1 recorded the highest growth followed by LC - 3. Lowest was recorded in LC-2 at 4 hours of incubation. (Figure - 1). Same trend was observed at pH 4.0 also. Lowest was observed in LC - 2 at 4 hours of incubation.These results are in lined with findings of Debashis Halder and Shyamapada Mandal (2015) who reported that the curd isolates of *L. fermentum* and *L. casei*, showed tolerance to low-pH values (3.0 and 4.0) and bile salts (0.2 and 0.3%; w/v), and found luxuriant in MRS broth even after 24 hrs under atmospheric carbon dioxide at 37°C.

Annals of R.S.C.B., ISSN:1583-6258, Vol. 24, Issue 2, 2020, Pages. 1189 - 1200
Received 24 October 2020; Accepted 15 December 2020.

Figure – 3. Survival ofLactic acid bacterial strains in pH 3 at OD620 nm values

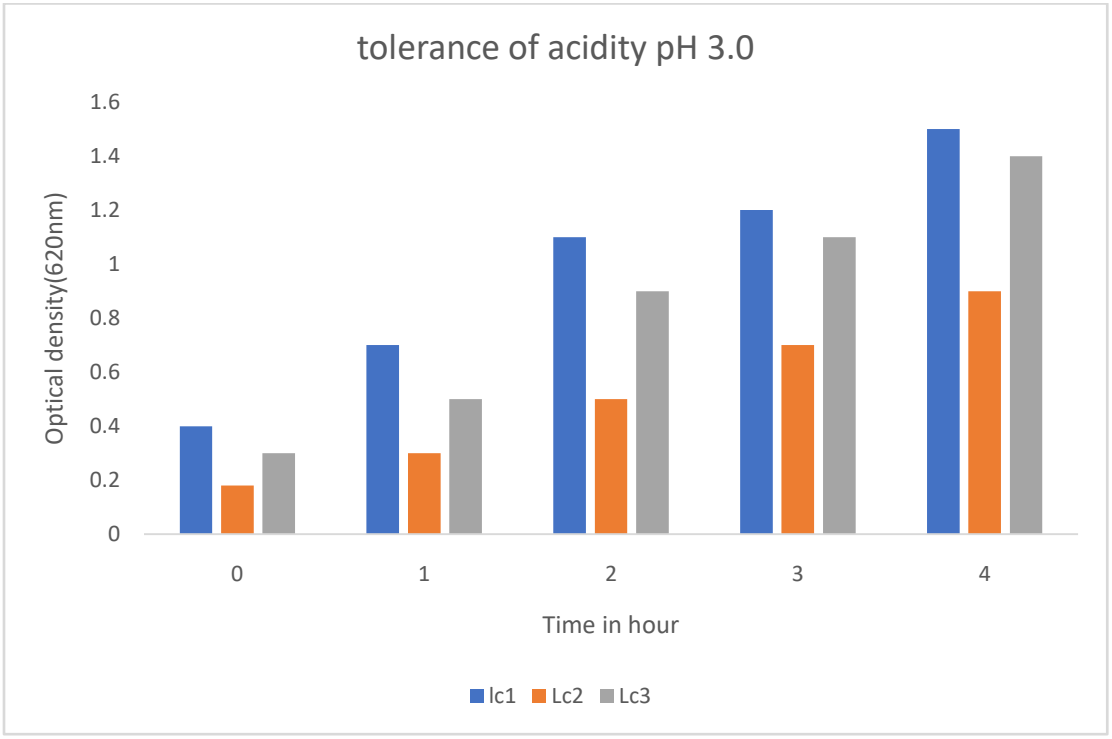
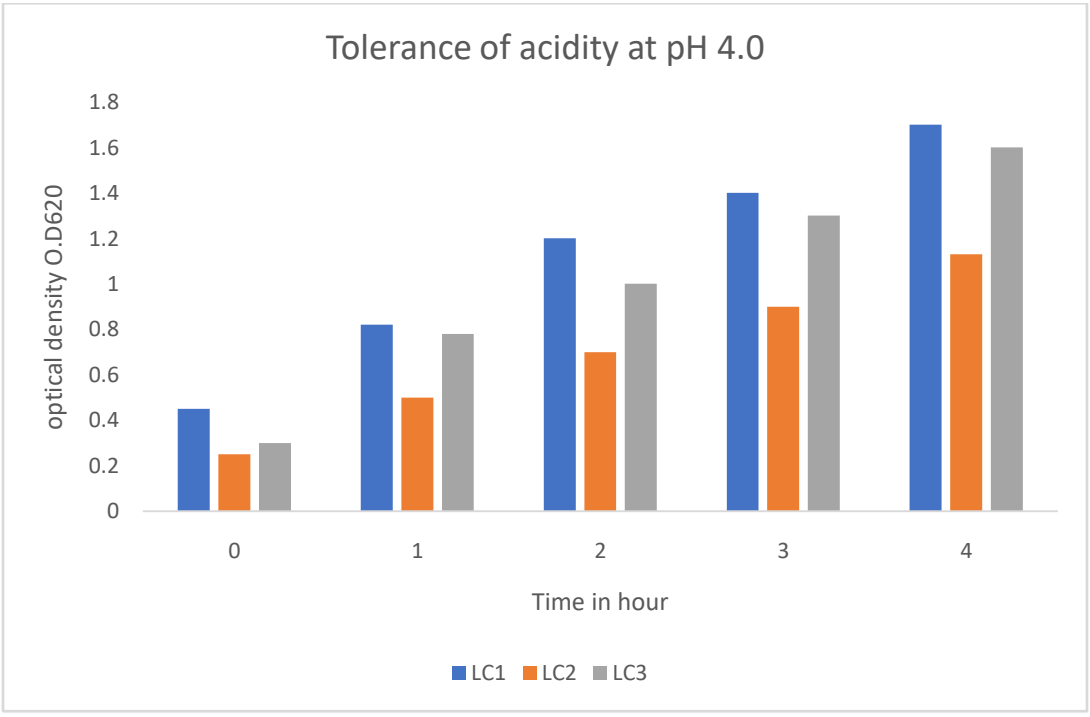


Figure – 4.Survival of Lactic acid bacterial strains in pH 4 at OD620 nm values



Annals of R.S.C.B., ISSN:1583-6258, Vol. 24, Issue 2, 2020, Pages. 1189 - 1200
Received 24 October 2020; Accepted 15 December 2020.

Table–3. Inhibitory spectrum of probiotic lactobacillus strains against certain food borne pathogens

	Inhibition zone(in mm)			
Isolate	***Staphylococcus aureus***	***Vibrio cholerae***	***Pseudomonas aeruginosa***	***Escherichia coli***
LC - 1	20.30	14.10	18.60	11.30
LC - 2	16.50	12.50	14.20	9.70
LC - 3	18.40	13.30	16.30	10.20

Isolated lactic acid bacterial strains also showed antibacterial activity against certain food borne bacterial pathogen such as *Staphylococcus aureus, Pseudomonas aeruginosa,Vibrio cholerae*, *E. coli*, by producing zones of inhibition ranged from 9.70 mm to 20.30 mm diameter. This showed that the isolated probiotic strains can produce antimicrobial product which can restrain the growth of pathogenic bacteria.Antimicrobial activity of each lactic acid bacterial isolate affected differently to different species of pathogenic bacteria may be caused by antimicrobial components produced by each isolate were also different antimicrobial activity of lactic acid bacteria caused mainly by organic acids produced because of glucose metabolism.Irma Isnafia*et al.*,(2015) isolated probiotic lactic acid bacterial isolates from Indonesian local beef such as *Lactobacillus plantarum,Lactobacillus acidophilus,Pediococcuspentosaceus* and *Enterococcus faecium* and they were showed antibacterial against *Salmonella typhimurium,E.coli*, *Staphylococcus aureus*. Jayachitra *et al* .,(2019) isolated *Lactobacillus fermentum* from cheese and it showed antimicrobial activity against certain food borne bacterial pathogens such as *Staphylococcus aureus, Pseudomonas aeruginosa,Bacillus subtilis,Escherichia coli*

CONCLUSION

This study allowed us to set up a collection of indigenous strains of lactic bacteria with probable food applications. In this study, resistance of lactic acid bacteria to the acidic media supplemented with bile salts was observed, and which makes possible their living passage in the digestive tract. After various tests conducted, we confirm that the selected strains have probiotic properties and can be used for food applications.

Annals of R.S.C.B., ISSN:1583-6258, Vol. 24, Issue 2, 2020, Pages. 1189 - 1200
Received 24 October 2020; Accepted 15 December 2020.

References

1. Bahoua GL (2008). Effect of palm wins yeasts and yogurt probiotics on the growth performance of broilers. LivestResearRur Dev 20: 1-8.
2. Béal C, Marin M, Fontaine E, Fonseca F, Obert JP (2008). Production et conservation desfermentslactiques et probiotiques. In: Bactérieslactiques, de la génétique aux ferments (Corrieu G. &Luquet F.M.). Tec & Doc, Lavoisier. Paris: 661-765.
3. Debashis Halder and Shyamapada Mandal.2015. Curd Lactobacilli with Probiotic Potentiality. Translational Biomedicine Vol. 6 No. 2:8
4. Divisekera, D. M. W. D., Samarasekera, J. K. R. R., Hettiarachchi, C., Gooneratne, J., Choudhary, M. I., Gopalakrishnan, S., et al. (2019). Lactic acid bacteria isolated from fermented flour of finger millet, its probiotic attributes and bioactive properties. Ann. Microbiol. 69, 79–92.
5. Ghabbour, B. N., Lamzira, Z., Thonart, P., Cidalia, P., Markaoui, M., and Asehraou, A. (2011). Selection of oleuropein-degrading lactic acid bacteria strains isolated from fermenting Moroccan green olives. GrasasAceites 62, 84–89.
6. Goldin BR, Gorbach SL (1992) Probiotics for humans. In: Fuller R (ed.), Probiotics. The scientific basis, Chapman and Hall, London, 355-376.
7. Houndonougbo MF, Chrysostome CAAM, Amoussa ZLAO (2011). Tchoukoutou residue and yogurt as feed additives in broilers feed. Res Opin Anim Vet Sci 1: 597-600.
8. Hoque M, et al. (2010) Isolation, identification and analysis of probiotic properties of Lactobacillus spp. from selective regional yoghurts. World Journal of Dairy & Food Sciences 5: 39-46. 1
9. Jayachitra J, E Babu and S Dinakar.2019.Isolation and characterization of bacteriocin produced by *Lactobacillus fermentum* LBC1,Journal of Pharmacognosy and Phytochemistry 2019; SP2: 606-610
10. Jeronymo-Ceneviva AB, et al. (2014) Probiotic properties of lactic acid bacteria isolated from waterbuffalo mozzarella cheese. Probiotics antimicrob proteins 6: 141-156.
11. Kabir SML, Rahman MM, Rahman ME, Ahmed SU (2004). The dynamic of probiotic on growth performance and immune response in broilers. International Journal of Poult Science 3: 361-364.

12. Liong MT, Shah NP (2005) Acid and bile tolerance and cholesterol removal ability of lactobacilli strain. J Dairy Sci 88: 55-66.
13. Merrifield DL, Balcázar JL, Daniels C, Zhou Z, Carnevali O, (2014). Indigenous Lactic Acid Bacteria in Fish and Crustaceans Aquaculture Nutrition: Gut Health, Probiotics and Prebiotics. Published by John Wiley & Sons, Ltd. 6: 128-168.
14. Mozzi F, Raya RR, Vignolo GM (2010). Biotechnology of lactic acid bacteria: Novel applications. Blackwell. Publishing: 3-73
15. Pitino I, Randazzo CL, Mandalari G, Lo Curto A, Faulks RM, (2010). Survival of *Lactobacillusrhamnosus*strains in the upper gastrointestinal tract. Food Microbiology 27: 1121-1127. R
16. Seema Nair P, Puthuvallil Kumaran Surendran. Biochemical characterization of lactic acid bacteria isolated from fish and prawn. J Culture Collections. 2005; 4:48-52
17. Sharifuzzaman SM, Austin B (2009). Influence of probiotic feeding duration on disease resistance and immune parameters in rainbow trout Fish and Shellfish immulogy 27: 440-445.
18. Sonsa-Ard N, Rodtong S, Chikindas ML, Yongsawatdigu J (2015). Characterization of bacteriocin produced by Enterococcus faecium CN-isolated from traditionally Thai fermented fish roe. Food Control 54: 308-316. T
19. Yadav, R., Puniya, A. K., and Shukla, P. (2016). Probiotic properties of *Lactobacillus plantarum* RYPR1 from an indigenous fermented beverage Raabadi. Front. Microbiol. 7:1683. doi: 10.3389/fmicb.2016.01683