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Study of Probiotic and Antioxidant activity of *Lactobacillus* spp.

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ABSTRACT

Curd is a source of probiotic *Lactobacilli*. In the present study, ten homemade curd samples were collected from different regions of Vellore district, Tamil Nadu, India. Among the samples, four different strains of *Lactobacillus* were isolated and identified based on their colony morphology and biochemical characteristics. It was observed that isolated *Lactobacillus* spp. were resistant to inhibitory substances like NaCl (1-9%), bile-salt (0.05-0.3%), and showed good growth in the acidic condition, while maximum growth was observed at pH around 6. The isolates were examined for their antibacterial activity against four different test pathogens, and found that growth of all pathogens are inhibited to some extent but maximum zone of inhibition was observed against *E.coli* (33mm) and no zone of inhibition against *Pseudomonas aeruginosa* after 24 hour incubation. The isolated *Lactobacillus* spp. showed good survival abilities in acidic (pH 4) and alkaline (pH 8) conditions. Isolated *Lactobacilli* were able to produce organic acid in skim milk which was determined by titrimetric method. In the antibiotic Sensitivity test, LB06, LBS3, LB02 and LBS1 were found to be sensitive against Ciprofloxacin and Erythromycin. LB06 was resistant against Ampicillin and Bacitracin while LB02 was resistant against Ampicillin. In the DPPH scavenging assay, LB06 and LBS3 showed highest inhibition of 56.84% and 55.86% in 1000 µl/ml compared to the positive control (BHT) 69.29%. The IC_{50} value was found to be 750µl/ml.

Keywords: *Lactobacillus* spp., Antimicrobial, Antioxidant, Probiotic

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INTRODUCTION

Lactobacillus are considered as generally recognized as safe (GRAS) organisms and can be safely used as probiotics for medical and veterinary applications [1]. *Lactobacilli* belong to a large and diverse group of Lactic acid bacteria (LAB), gram positive, non-spore forming, facultative anaerobic, catalase negative, rod shaped bacteria, able to produce lactic acid as the main end-product of the fermentation of carbohydrates [2]. Levels and types of organic acids produced during the fermentation process depend on LAB species or strains, culture composition and growth conditions. The production of organic acids is undoubtedly the determining factor on which the shelf life and the safety of the final product depend while the inhibition of pathogenic and spoilage flora is also dependent on a rapid and adequate formation of these organic acids [3].

Dairy and soy foods may serve as the ideal system for delivery of probiotic bacteria to the human gastrointestinal tract due to provision of a favourable environment that promotes the growth and enhances the viability of these microorganisms [4]. Consumers expect more than the specified nutritional value from food. Currently, the largest segment of the functional food market is provided by the foods targeted towards improving the balance and activity of the intestinal microflora. Most probiotic bacteria belong to the group of lactic acid bacteria and among them *Lactobacilli* and *Bifidobacteria* reportedly play a significant role in maintaining the intestinal ecosystem and in stimulating the immune system of the host [5].

LAB display a wide range of antimicrobial activities. Certain strains of LAB are further known to produce bioactive molecules such as ethanol, formic acid, fatty acids, hydrogen peroxide, diacetyl, reuterin, and reutericyclin. Many strains also produce bacteriocins and bacteriocin-like molecules that display antibacterial activity [6]. Probiotics such as *Lactobacillus* spp. are reported to have inhibitory activity against a variety of human gastrointestinal tract pathogens such as *L. Monocytogens* and *H. pylori* as well as against other pathogens of oral cavity and vagina [7]. It is possible that in future, probiotics will be used for different gastrointestinal diseases, vaginosis, or as delivery systems for vaccines, immunoglobulin, and other therapies [8]. Health benefits of probiotic organisms include reduction of blood cholesterol, improvement of immunity, alleviation of symptoms of lactose tolerance, treatment of diarrhoea, anti-carcinogenic and antihypertensive properties and biotransformation of isoflavone phytoestrogen to improve hormonal balance in postmenopausal women [9],[10].

Probiotics must be tolerant to acid and bile, which enables selected strains to survive, grow and perform its therapeutic benefits in the small intestinal tract [11],[12]. Increased antibiotic usage is a key factor in the emergence of antibiotic resistant pathogens. Thus there is an urgent need to develop alternatives to antibiotics [13]. Some lactobacilli have been shown possessing antioxidative activity, and are able to decrease the risk of accumulation of ROS during the ingestion of food. Several mechanisms of action have been proposed to explain how probiotic organisms may act within a host to yield beneficial effects. These can be grouped into two categories: suppression of certain gastrointestinal microflora, and immunomodulation of

the host. Possible mechanisms of floral suppression include release of antimicrobial compounds, competition for nutrients, or competition for adhesion sites in the intestines [14].

MATERIALS AND METHODS

Sample collection:

Ten homemade curd samples were collected from different region of Vellore district, Tamil Nadu, India. The samples were stored aseptically in low temperature (-4°C) refrigerator to protect from contamination and deterioration.

Isolation and identification of bacteria:

One gram of each sample was dissolved into 100 ml of MRS broth at pH 6.5. After dissolving into MRS broth they were shaken homogeneously and were incubated at 37°C for 24 hour in aerobic condition. The cultures were subjected to subculture at 37°C under low pH (pH 4.5) and anaerobic condition. After subcultures, the bacterial culture was streak onto MRS agar media at pH 4.8. Finally, the single colony of *Lactobacillus* was isolated and identified by observing their colony morphology by Gram staining, Endospore staining (**Schaeffer-Fulton method)** , Hanging Drop method and some biochemical tests such as Indole test, Methyl Red (MR) and Voges-Proskauer (VP) test, Citrate utilization test, Catalase activity, Oxidase test, Sugar fermentation test, Triple sugar iron test. The cultures were then maintained in MRS broth at pH 5.5 for further use.

Determination of optimal growth at different pH:

To determine the optimal growth of *Lactobacillus* at different pH, 1% (v/v) fresh overnight cultures of *Lactobacillus* were inoculated into MRS broth with varying pH ranging from 4 to 8. The pH were adjusted with concentrated acetic acid (99%) and 5 N NaOH. The inoculated broths were incubated in anaerobic condition 24 hours at 37°C in the presence of 10% CO_2. After 24 hours of incubation, growth of the bacteria were measured using a spectrophotometer, reading the optical density at 600 nm (OD_{600}) against the un-inoculated broth.

NaCl tolerance assay:

To determine NaCl tolerance, all the isolates were grown in MRS broth supplemented with different concentrations of NaCl (1-10%). The broths were inoculated with 100 µl overnight culture of the isolates and incubated in anaerobic condition at 37°C for 24 hours. After 24 hours incubation, growth was determined using a spectrophotometer and reading the optical density at 600 nm.

Bile salt tolerance assay:

Bile salt tolerance of the isolates were investigated by determining their growth in MRS broth containing different levels (0.05, 0.1, 0.15, 0.3 and 0.5) of bile salts (Sodium thioglycolate). Freshly prepared cultures were inoculated (1%) into medium and incubated at 37°C for 24 hours under anaerobic condition. Optical densities were measured using a spectrophotometer at 600 nm after 24 hours incubation.

Quantification of organic acid and determination of pH value:

One percent (v/v) 24 hours active culture of *Lactobacillus* was used to inoculate 10% sterilized skim milk and initial pH (6.6) was determined by a digital electrode pH meter. The inoculated skim milk was incubated at 37°C for 72 hours and samples were collected in every 24 hours, 48 hours and 72 hours and liquids of coagulated milk were separated by filtration. pH of the separated liquid was recorded using a digital electrode pH meter and quantification of organic acid was performed through titration with 0.1 N NaOH using phenolphthalein as pH indicator.

Antimicrobial activity:

Antimicrobial screening of *Lactobacillus* spp. was detected by agar well diffusion method on MH agar against following bacterial cultures include *Staphylococcus aureus, Bacillus cereus, Pseudomonas aeruginosa, Escherichia coli*. The bacterial cultures were inoculated on MH agar plates using sterilized cotton swabs. In each of these plates, wells were made using a sterilized gel borer. The 100 µl of *Lactobacilli* inoculum were loaded into each well. Plates were incubated at 37°C for 24 hours. After incubation, all plates were examined for the presence of zone of inhibition around the Wells.

Antibiotic sensitivity test:

Pure culture colonies of *Lactobacillus* spp. were inoculated in MRS broth at 37 °C for 24 hours. A sterile cotton wool swab dipped into the bacterial suspension was spread evenly on the surface of the MRS agar plate. The inoculated plate was allowed to dry before placing the diffusion discs containing antibiotics. Susceptibility of the four isolates to four types of antibiotics was performed by the disc diffusion method. Using commercially available antibiotics disc Erythromycin, Ampicillin, Bacitracin and Ciprofloxacin were placed on the surface of the agar plates. Precaution was taken to ensure that there was uniform contact between the antibiotic disc and agar plate. The plates were then incubated at 37 °C for 24 hours. Zone of inhibition diameters were measured inclusive of the diameter of the discs.

DPPH free radical scavenging assay:

The free radical scavenging activity of isolated bacterial strains (LB06, LBS1, LB02 and LBS1) were measured by using the method of Son and Lewis [15]. Percentage inhibition or DPPH scavenging activity was calculated by following expression:

$$\text{Percentage of scavenging} = [(A_0-A_1)/A_0]X100$$

Where, A_0 = Absorbance of control, A_1= Absorbance of sample

The samples were kept in the dark for 30 minutes and the optical density was measured at 517 nm. Ethanol was used as a blank, while DPPH solution in ethanol served as the control.

RESULTS AND DISCUSSION

Various morphological and biochemical characteristics of isolated bacteria from different curd samples are given in Table 1. They were classified as four strains such as LB02, LB06, LBS1 and LBS3. Colony morphology of LB02 was white in colour, big in size, concave and circular in shape. Colony of LB06 strain was smooth, white creamy in colour, small and circular in size and shape respectively. Colony of LBS1 was rough, white in colour, small in size and circular in shape while colony of LBS3 was smooth, white in colour, small and circular in size and shape. Microscopic visualization of the colonies of all strain tell that they are Gram positive, rod shaped, non spore forming, non motile bacteria. Biochemical tests of all the bacterial strains (LB02, LB06, LBS1 and LBS3) show negative result for Indole, MR and VP test, Citrate Utilization test, Catalase activity, oxidase test, sucrose, D- Mannitol. All the four bacterial strain show positive result for Glucose and Dextrose. LBS1 and LBS3 strain show positive result for Lactose while other two strains (LB02 and LB06) show negative results. Only LB02 show positive result for Fructose while other three strain show negative result. LBS1 and LBS3 produce acid in Triple Sugar Iron test and show positive result while other two strains (LB02 and LB06) show negative result. Based on these morphological characteristics four isolates were identified as *Lactobacillus* spp. This result can be compared with the study of Patil et al. [16]

The experimental result represented in Figure 1 shows that the isolated bacterial strains from different curd samples were able to survive in extreme acidic pH (pH 4) to basic pH (pH 8). Maximum growth of isolated strain LB06 (OD= 2.34) was observed at pH 5.5 and maximum growth of isolated strain LBS3 (OD=1.73), LB02 (OD=1.69) and LBS1 (OD=1.55) at pH 6. The reason for choosing this pH range was to determine whether *Lactobacillus* spp. can grow in acidic and alkaline conditions and also to predict the optimum pH value for good growth. Collins *et al*. reported that many in vitro properties, such as adhesion, resistance to pH, etc. were usually investigated to determine if a specific selected strain would be suitable as a probiotic that should survive at human gut pH conditions which supports our result [17].

All the isolates LB06, LBS3, LB02, and LBS1 show the good tolerance over the range of 1-9% w/v concentration of NaCl in the MRS broth (Figure 2). LB06 showed highest growth in the

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salt concentration among other isolated Lactobacillus strains. However significant decrease in the growth with the increase in the salt concentration was observed. This result has similarities with the study of Elezete and Carlos. [18]

Table 1: Morphological and biochemical characteristics of isolates

Characteristics	LB02	LB06	LBS1	LBS3
Colonies morphology	White, concave, big, circular	White-creamy, smooth, small, circular	White, rough, small, circular	White, smooth, small, circular
Gram stain	Gram positive, rod shaped	Gram positive, rod shaped	Gram positive, rod shaped	Gram positive, rod shaped
Spore	Non-spore forming	Non-spore forming	Non-spore forming	Non-spore forming
Motility	Non-motile	Non-motile	Non-motile	Non-motile
Indole	-	-	-	-
Methyl Red	-	-	-	-
VP	-	-	-	-
Citrate Utilization	-	-	-	-
Catalase	-	-	-	-
Oxidase	-	-	-	-
Sucrose	-	-	-	-
Lactose	-	-	+	+
Glucose	+	+	+	+
Fructose	+	-	-	-
D-Mannitol	-	-	-	-
Dextrose	+	+	+	+
Triple Sugar Iron	A -	A -	A +	A+

**** '+'=Positive, '-'= Negative, 'A -' = Acid not produce and 'A+' = Acid produce.**

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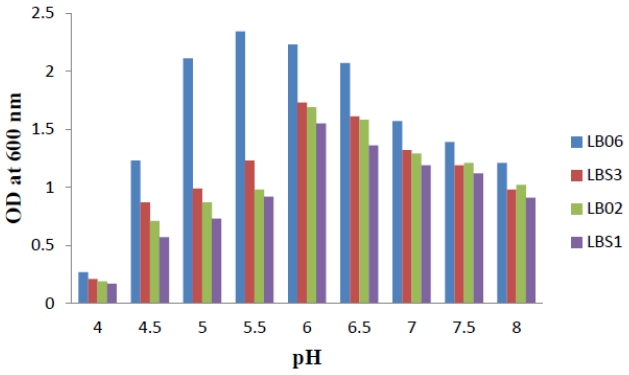

Figure 1: Optimal growth of isolated *Lactobacillus* spp. at different pH

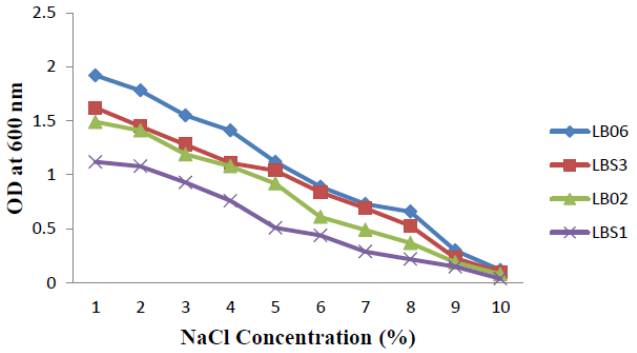

Figure 2: Sodium chloride (NaCl) tolerance of isolated *Lactobacillus* spp.

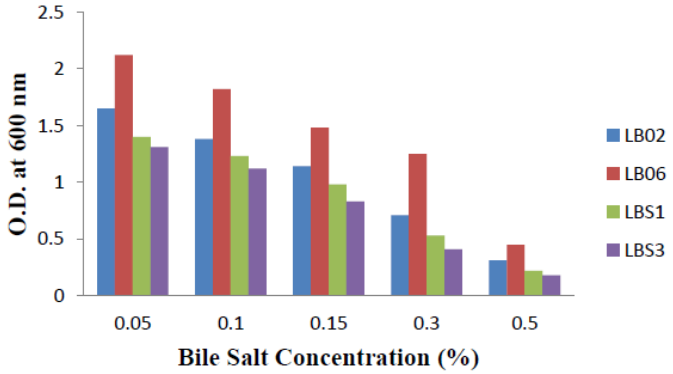

Figure 3: Bile salt tolerance of isolated *Lactobacillus* spp.

All the isolates (LB06, LBS3, LB02, and LBS1) were able to survive over the range of 0.05-0.3% w/v supplementation of bile-salt in MRS broth. The growth of the strains declined with increased bile-salt supplementation. Bacterial isolates were able to maintain good growth and multiplication up to 0.15% w/v supplementation of bile-salt in MRS broth. However, the strain LB06 showed good growth at 0.05, 0.1, 0.15 and 0.3% bile salt concentration, represented in Figure 3. The results indicate that the isolated *Lactobacillus* spp. have potential to be used as

probiotic bacteria. As this result aligns with that found in the human intestinal tract, and the concentration of bile in intestine of healthy men is 0.3%. Therefore, before selection of probiotic bacteria for human consumption make sure that it can endure in 0.3% bile concentration [19].

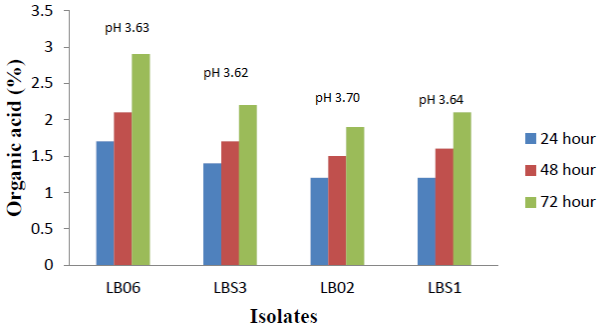

Figure 4: Organic acids produced by isolated *Lactobacillus* spp. at 37°C

Figure 4 represents that the highest acidity (2.9%) and lowest pH (3.63) was observed after 72 h incubation at 37°C for the strain LB06. On the other hand, other isolate LBS3 showed the acid production (2.2%) and lowest pH (3.62), LBS1 also showed the acid production (2.1%), lowest pH (3.64) and LB02 showed acid production (1.98%), lowest pH (3.70) value after 72 h incubation. This experiment indicates that organic acid such as lactic acid production was increased with the incubation time and the pH of the media decreased with the increasing acid production. This investigation indicates that, there is a minor variation in organic acid production by *Lactobacilli* due to their regional variation. This investigation indicates that, there is a minor variation in organic acid production by Lactobacilli due to their regional variation. This finding has the connection with the study of Leroy F and Vuyst LD [20]. According to the studies of Jay M.J., fermentation is a process of breaking down of complex organic compounds into smaller ones, this process is done in the absence of oxygen. Here organic compounds act as final electron acceptors, which will lead to partial oxidation of compounds, only a small amount of energy is released during this process and the product of fermentation consist of some organic acids that are more reduced than others [21].

The antimicrobial activity of the four Lactobacillus isolates (LB06, LBS3, LB02 and LBS1) was screened against four pathogenic bacteria such as *E.coli*, *Staphylococcus aureus*, *Bacillus cereus* and *Peudomonas aeruginosa* were determined by measuring the zone of inhibition (Figure 5 and Table 2). Among four isolates, LB06 was found to be effective against *E. coli* (33 mm), *Staphylococcus aureus* (16 mm) and *Bacillus cereus* (19 mm) in well diffusion method. LBS3 was found to be effective against *E. coli* (29 mm) and *Bacillus cereus* (17 mm). LB02 and LBS1 was found to be effective against only *E. coli* (27 mm and 5 mm respectively). The above isolates did not show any zone of inhibition against *Peudomonas aeruginosa.* This result can be compared with the study of Raja A. et al. where of *Lactobacillus lactis cremoris* isolated from Kefir also shows antimicrobial activity against Food Spoilage Bacteria such as *E.coli*, *Pseudomonas* sp., *S. aureus*, *Bacillus cereus* etc., and its activity is dependent on pH of the

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surrounding media. This antimicrobial activity is observed to be quite predominant at pH 4.5 and 6.5, but no activity is observed at pH 8.5 [22].

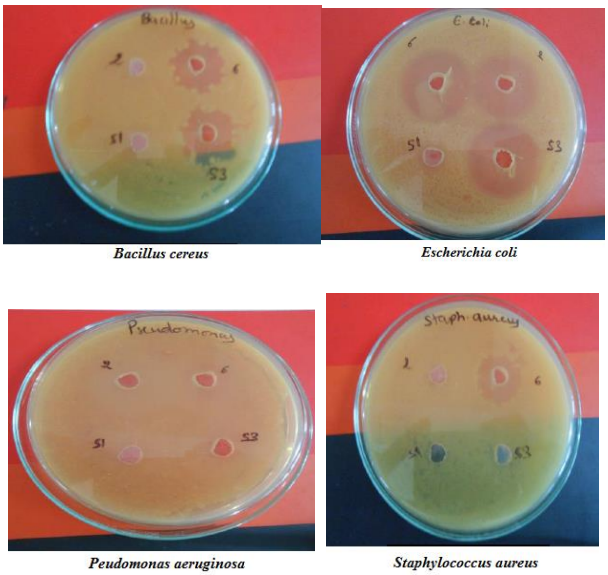

Figure 5: Antimicrobial activity of isolated *Lactobacillus* spp. Clear zone indicates inhibition of bacterial growth

Table 2: Antimicrobial activity of isolated *Lactobacillus* spp. by well-diffusion method

Test organisms	Zone of inhibition (mm)			
	LB06	LBS3	LB02	LBS1
Escherichia coli	33	29	27	5
Staphylococcus aureus	16	-	-	-
Bacillus cereus	19	17	-	-
Peudomonas aeruginosa	-	-	-	-

Lactobacillus isolates tested against four different types of antimicrobial agents (Ampicillin, Bacitracin, Ciprofloxacin, Erythromycin) are shown in Table 3. All the isolated *Lactobacillus* spp. LB06, LBS3, LB02 and LBS1 were found to be sensitive against Ciprofloxacin and Erythromycin. The *Lactobacillus sp*. LB06 was resistant against Ampicillin and Bacitracin. LB02 was resistant against Ampicillin.

Table 3: Antibiotic sensitivity test

Antibiotics	Zone of inhibition (mm)			
	LB06	LBS3	LB02	LBS1
Ampicillin	-	7	-	6
Bacitracin	-	5	16	14
Ciprofloxacin	25	28	29	19
Erythromycin	15	25	24	15

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The four *Lactobacillus* spp. LB06, LBS3, LB02 and LBS1 showed good inhibitory activity in all tested concentrations such as 250 µl/ml, 500 µl/ml, 750 µl/ml, 1000 µl/ml (Figure 6). At higher concentration, 1000 µl/ml, LB06 showed highest 56.84% (±0.14) and LBS3 showed highest 55.86% (±0.12) inhibitory activity in compare to positive control 69.29% (±0.34). Inhibitory activity of the *Lactobacillus* spp. decreased in the order of LB06 56.84% (±0.14) > LBS3 55.86% (±0.12) > LBS1 41.78% (±0.16) > LB02 35.10% (±0.18). The inhibitory activity of the *Lactobacillus* spp. showed good results and the inhibition had increased with the increased concentration. The IC50 values of LB06 and LBS3 was found to be 750 and 850 in µl/ml respectively. Scavenging capacity increase with the increase concentration. This result can be compared with the study of A. Osuntoki and I. Korie. There are variation in the percentage inhibition with the study due to regional variation of strains [23].

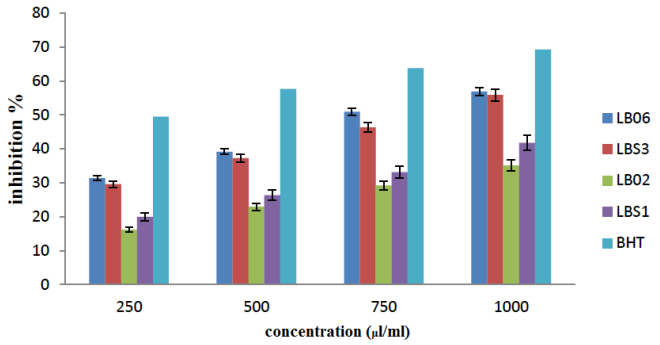

Figure 6: Inhibition percentage of supernatant of *Lactobacillus* spp. and scavenging of DPPH

CONCLUSION

The present study showed that isolated *Lactobacillus* spp. are able to tolerate inhibitory substances such as 0.05-0.3% bile acid, 1-8% NaCl as well as in alkaline (pH 8) and acidic (pH4) condition. The isolated *lactobacilli* were able to produce organic acid in milk which increases texture. The isolated *Lactobacillus* spp. showed antimicrobial activity against the tested pathogens. Therefore it is able to prevent the growth of other pathogenic microorganisms in gut system. The isolated *Lactobacillus* spp. had showed good inhibitory result in antioxidant assay. This shows the presence of antioxidative ability, and prevents the activity of ROS thereby it prevents premature ageing and damage to cells. From the obtained results we can conclude that the isolated *Lactobacillus* spp. have probiotic activities and can be used in dietary supplements.

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