

Avocado juice (*Persea americana*) with an abundance of bioactive substances on coupling with probiotics revealed potential health benefits

Kanchi Ravi Padma^{1*}, Sankari Mohan¹, Lingareddy Nikitha Reddy¹, Dhanasekar Chitra²

¹Department of Biotechnology, Sri Padmavati Mahila Visvavidyalayam (Women's University), Tirupati, India.

²Department of Biotechnology, Sri Sankara Arts and Science College, Enathur, Kanchipuram, India.

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ABSTRACT

Background: The rising trend in consumption of plant-based functional beverages has spurred research into alternative carriers for probiotics other than dairy products. Avocado (*Persea americana*) is rich in bioactive compounds, antioxidants, vitamins, and healthy lipids, making it suitable for use as a substrate in probiotic fermentation. Adding probiotic *Lactobacillus* strains to avocado juice can make it more nutritious and therapeutic.

Objectives: This research assessed the efficacy of *Persea americana* (avocado) juice as a non-dairy medium for probiotic *Lactobacillus* strains.

Materials and methods: The strains (*L. acidophilus* and *L. plantarum*) were added to pasteurized juice and evaluated for their ability to withstand stress conditions, including varying pH levels, bile salt concentrations (0.1-0.6%), and NaCl concentrations (0.5-3%).

Results: The resulting fermented juice exhibited improved functional characteristics when compared to non-fermented juice. The antioxidant activity in the fermented juice was significantly elevated, with lower IC₅₀ values recorded in both ABTS (17.27 µg/mL) and DPPH (24.64 µg/mL) assays, in contrast to pasteurized juice (31.46 µg/mL and 51.95 µg/mL, respectively), and was found to be comparable to gallic acid standards. Biochemical assessments indicated elevated reducing sugar (683.36 mg/mL) and protein levels, suggesting active microbial metabolism during fermentation. Additionally, the fermented juice exhibited moderate antimicrobial activity against selected pathogenic strains, though lower than that of the standard antibiotic control.

Conclusion: These results imply that avocado juice not only supports the viability of probiotics but also enhances bioactive properties through the fermentation process. In conclusion, this study highlights the potential of avocado juice as a plant-based carrier for probiotics, presenting a promising alternative to dairy-based functional beverages and offering additional nutritional and health benefits.

Introduction

At present, individuals regard food as essential not only for their physical well-being but also for their mental health. The primary aim of contemporary meals is to alleviate hunger while also addressing and mitigating chronic diseases, malnutrition, and the deterioration of health that accompanies aging.¹ The rising expenses associated with healthcare, coupled with an increased understanding of the impact of food

* Corresponding contributor.

Author's Address: Department of Biotechnology, Sri Padmavati Mahila Visvavidyalayam (Women's University), Tirupati, India.

E-mail address: thulasipadi@gmail.com

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on long-term health, have led people globally to take an interest in functional foods, which provide health advantages beyond mere nutritional value.

The avocado (*Persea americana* Mill., Lauraceae) is cultivated in tropical and subtropical regions worldwide. Avocados vary by type depending on their growing climate, with the Hass variety being the most prevalent and widely available. During the production of guacamole and essential oils, the avocado processing industry generates a solid waste byproduct consisting of seeds, peels, and residual pulp, which constitutes approximately 21-30% of the fruit. These byproducts are frequently discarded as waste, presenting an environmental challenge; however, they may also contain bioactive compounds. The avocado seed comprises the endocarp and the kernel, and its triterpenoid constituents have been associated with anticancer properties in studies involving oral, breast, and liver cells.²⁻⁴

Every day, humans are exposed to a significant quantity of pathogens, predominantly comprising bacteria. Probiotic bacteria have been incorporated into various dietary regimens for several decades owing to their advantageous effects on human health.⁵ In 2001, the World Health Organization, in conjunction with the United States Department of Agriculture, delineated probiotics as microorganisms that, when administered to a host in adequate concentrations, promote their overall well-being. *Lactobacillus* and *Bifidobacterium* are the two most commonly used genera in probiotics.⁶ These bacteria are generally regarded as benign since they can persist within the human body and, in contrast to conventional pathogens, possess the capacity to both ameliorate and avert disease. Furthermore, these microorganisms have historically played a crucial role in the fermentation of dairy products and in the preservation of food.

Innovations in the alimentary sector are currently focused on the novel functions that food assumes, notably in mitigating lifestyle-related health issues, predominantly through the development of dietary supplements that modulate the microbial composition of the gastrointestinal tract. Furthermore, the food and beverage sector has successfully launched and advanced a diverse array of pioneering products recognized as functional foods, a trend driven by consumers' increasing inclination towards products without artificial additives. Foods designated as functional can exert beneficial effects on specific physiological systems in addition to providing adequate nutritional value, thereby contributing to human health in numerous ways.⁷ The principal classifications of functional foods encompass prebiotics, probiotics, and, more recently, symbiotics.⁸

Probiotic foods are commonly associated with dairy products, including cheese, sour milk, and yogurt. However, recent developments have introduced various food ingredients as potential probiotic substrates.^{9,10}

This shift can be attributed to the adverse characteristics of dairy products that influence consumer perceptions, such as lactose intolerance, allergies, and milk's cholesterol content.^{11,12} Consequently, alternative food substrates, including cereals, vegetables, and fruits, both fermented and unfermented, have been proposed as optimal carriers for probiotics to mitigate these concerns.¹³ Notably, fruit juices are regarded as particularly attractive and suitable substrates for probiotic delivery. They offer several advantages as probiotic "vehicles," including: 1) high nutritional value, 2) beneficial health effects, and 3) broad consumer acceptance and consumption worldwide.^{14,15}

Among the diverse range of fruits, *Persea americana*, commonly known as avocado, offers a distinctive potential as a functional food base. Originating in Central America and widely grown in tropical and subtropical regions, the avocado is appreciated for its creamy texture, rich flavor, and remarkable nutrient profile. It is a source of heart-healthy monounsaturated fats, dietary fiber, potassium, and a broad spectrum of vitamins, including E, C, and several B-complex vitamins. Significantly, different parts of the avocado, such as the pulp, peel, and seed, are abundant in polyphenols, flavonoids, and triterpenes, which have shown antioxidant, antimicrobial, and anticancer effects.^{2,3}

The global probiotic industry is primarily composed of three main product categories: foods, dietary supplements, and pharmaceutical products. The quality of probiotic products can vary significantly by type and geographical origin.¹⁵ Probiotics predominantly include various species such as *Saccharomyces*, *Propionibacterium*, *Bifidobacterium*, and *Lactobacillus*.¹⁶ In recent years, there has been notable progress in the production of milk and other dairy items that contain probiotic bacteria. These products encompass ice cream, frozen dairy desserts, buttermilk, both plain and flavored liquid milk, milk powder, baby food, and a variety of cheeses.^{17,18} The primary advantage of probiotic microorganisms is their ability to coexist and help maintain a balance between harmful infections and beneficial bacteria essential for normal bodily functions.¹⁹ Currently, there is a marked increase in the consumption of probiotic microorganisms, particularly through probiotic dairy products. However, for many individuals who are unable or unwilling to incorporate dairy into their diets, fruit-based beverages may serve as an ideal alternative for delivering probiotics.²⁰

Numerous studies indicate that consuming fruit juice, which is rich in nutrients and supports the growth of probiotics, is the most effective way to obtain them. Recognizing these advantages, several companies have invested in developing and marketing fruit-flavored probiotic beverages, including pineapple, apple, orange, and grape varieties. The avocado tree, scientifically known as *Persea americana* and part of

the Laurel family, is the focus of this research. Avocados are not only flavorful but also possess a rich, creamy consistency.

However, there is currently a scarcity of data regarding the use of *Lactobacillus* probiotics in conjunction with avocado fruit.²¹ By performing this study, we hope to explore if avocado juice can be a new option for growing *Lactobacillus* strains and to assess the beverage's nutritional, antioxidant, and antimicrobial properties for possible use as a probiotic drink.

Materials and methods

Isolation and identification of lactic acid bacteria

Sample collection

The isolation was performed by taking fresh raw milk samples under aseptic conditions and using a local dairy source as the source of lactic acid bacteria. To eliminate contamination, about 50 mL of milk was collected into airtight, sterile containers. The samples were put in an ice box and taken to the laboratory within 2-4 hrs of collection. The samples were kept at 4 °C upon arrival and processed within 24 hrs. Handling procedures were performed using sterile methods to preserve sample integrity and ensure reproducibility.

Isolation of the organism

The sample was inoculated into de Man Rogosa, Sharpe (MRS) medium and cultured for 24 hrs to isolate the microorganisms. A Petri plate containing 100 µL of sample was used. Fifteen mL of MRS agar (HiMedia Laboratories Pvt. Ltd., India) was poured on the petri plate containing the sample and undisturbed for some time to solidify. After solidification, the plates were incubated for 24 hrs. Once bacterial colonies were visible, they were subcultured on MRS media by the streaking method.

The pour plate method under aseptic conditions was used to isolate microorganisms. Sterile physiological saline was used to serially dilute the sample (10^{-1} to 10^{-6}). Aseptically, 1 mL of appropriate dilutions was transferred to sterile Petri plates. Each plate was poured with sterile MRS agar that was allowed to cool at 45-50 °C. The plates were left to solidify at ambient temperature (25-28 °C) for 15-20 minutes, after which they were incubated in an inverted position at 37 °C for 48-24 hrs. All steps were performed in a laminar airflow cabinet with sterile materials. Specific colonies were picked after incubation and subcultured onto new MRS agar plates to obtain pure isolates.

Stock solution preparation

Milk samples were collected, and bacterial isolation was performed on MRS agar at 37 °C for 24 hrs under aseptic conditions. The medium of MRS (gm/L) was as follows: beef extract (10), yeast extract (5), proteose peptone (10), dextrose (20), sodium acetate (5), polysorbate-80 (1), magnesium sulfate (0.1), ammonium citrate (2), dipotassium phosphate (2),

manganese sulfate. They were sterilized and the pH was adjusted to 6.5 ± 0.2 .²² The isolates were characterized at the species level on the basis of colony morphology on MRS agar, Gram staining, catalase test, and typical physiological and biochemical features, such as growth patterns and fermentation of carbohydrates, which were similar to *Lactobacillus acidophilus* and *Lactobacillus plantarum*. The pure cultures were grown in MRS broth as stock cultures for further use.

Fruit juice preparation

Fresh avocado (*Persea americana*) fruit was purchased from the local market in Bangalore. The fruits were thoroughly washed with tap water and distilled water. Then the fruit was cut into small pieces and made into juice; the juice was transferred to autoclaved conical flasks and covered with a cotton plug to prevent contamination, pasteurized at 75 °C for 30 minute, and allowed to cool to room temperature.

Fermentation of fruit Juice

A volume of 100 mL of pasteurized avocado (*Persea americana*) juice was moved to sterile Erlenmeyer flasks. The juice was inoculated under aseptic conditions with 1% (v/v) actively growing cultures of *Lactobacillus acidophilus* and *Lactobacillus plantarum* (one culture at a time). The size inoculated was about 10^7 - 10^8 CFU/mL. The control flasks were kept uninoculated. Incubation at 37 °C was performed at 24-hr intervals, during which 10 mL samples were withdrawn aseptically and analyzed for biochemical parameters, such as pH, titratable acidity, and sugar content. A calibrated pH meter (µ PH systems 361, Systronics, New Delhi, India) was used to measure the pH.

Sensory evaluation

Sensory analysis was conducted to determine the organoleptic characteristics of fermented and non-fermented *Persea americana* juice, including appearance, aroma, taste, texture, and overall acceptability. Semi-trained volunteers (20-35 years old) used to basic sensory evaluation methods were involved in the study (15 in total). Before taking part, all participants signed an informed consent form using a student consent form, and the study procedures were in line with the general ethical standards of sensory evaluation. To avoid bias, freshly made samples (fermented juice (inoculated with *Lactobacillus* and incubated 48 hrs) and non-fermented pasteurized juice) were served at room temperature (22-25 °C) in coded, odorless (30 mL) plastic cups. The samples were rated on a 5-point hedonic scale (1=dislike extremely, 5=like extremely) by panelists. Palate cleansing between samples was done with water, and assessments were done in an odor-free, neutral environment. One-way ANOVA and post-hoc Tukey test were used to statistically compare the results with $p < 0.05$.²³

Biochemical tests

Biochemical characterization of the isolates was carried out using standard microbiological procedures. All media and reagents were obtained from HiMedia Laboratories Pvt. Ltd. and prepared according to the manufacturer's instructions. All tests were performed under aseptic conditions and incubated at 37 °C for 24-48 hrs.

Indole production test

A loopful of culture was inoculated into 5 mL of sterile tryptone broth and incubated for 24 hrs. After incubation, 0.5 mL of Kovac's reagent was added gently along the sides of the tube. Formation of a red-colored ring at the surface indicated a positive result.

Methyl red (MR) test

The isolate was inoculated into MR-VP broth and incubated for 48 hrs. After incubation, 2-3 drops of methyl red indicator were added. Immediate development of a red indicated a positive result.

Voges-Proskauer (VP) test

After 48 hrs of incubation in MR-VP broth, 0.6 mL of Barritt's reagent A (α -naphthol) and 0.2 mL of reagent B (40% KOH) were added. The tube was shaken and allowed to stand for 15-30 minutes. A pink-to-red color change indicated a positive result.

Citrate utilization test

The isolate was streaked onto Simmons citrate agar slants and incubated. A change from green to blue, along with growth, indicated a positive result.

Hydrogen sulfide (H_2S) test

The culture was inoculated onto TSI agar by stabbing the butt and streaking the slant, then incubated. Black precipitate formation indicated H_2S production.

Nitrate reduction test

The isolate was inoculated into nitrate broth and incubated for 24 hrs. After incubation, 2-3 drops each of sulfanilic acid and α -naphthylamine were added. The development of red indicated nitrate reduction. If no color developed, a small amount of zinc dust was added. The absence of red after zinc addition confirmed a positive result.

Triple sugar iron (TSI) agar test

The culture was stabbed into the butt and streaked over the slant of TSI agar and incubated. Acid production was indicated by yellow coloration, alkaline reaction by red coloration, gas production by cracks or bubbles, and H_2S production by blackening.

Glucose fermentation test

The isolate was inoculated into glucose broth

containing a Durham tube and incubated. A change of the medium color indicated acid production, while gas accumulation in the Durham tube indicated gas production.

Casein hydrolysis test

The culture was streaked onto skim milk agar plates and incubated. Formation of a clear zone around the colonies indicated casein hydrolysis.

Cellulose degradation test

The isolate was spot-inoculated onto cellulose agar plates and incubated. After incubation, plates were flooded with a Congo red solution for 15 minutes, then rinsed with 1M NaCl. Clear zones around colonies indicated cellulose degradation.

Urease test

The isolate was streaked onto urea agar slants and incubated. A change from yellow/orange to pink indicated the presence of urease activity.

Catalase test

A small amount of fresh culture was placed on a clean glass slide, and 1-2 drops of 3% hydrogen peroxide were added. Immediate effervescence (bubble formation) indicated a positive result.

Oxidase test

A colony was smeared onto filter paper impregnated with oxidase reagent (tetramethyl-p-phenylenediamine). The development of dark purple within 10-30 sec indicated a positive result.

All observations were recorded and interpreted using standard microbiological identification criteria.

Effect of pH

The effect of pH on the growth of isolates was evaluated by adjusting the pH of pasteurized *Persea americana* juice to 5, 6, 7, 8, 9, and 10 using sterile 1 N NaOH. A volume of 15 mL of juice was dispensed into sterile conical flasks for each pH level. Each flask was inoculated with 2 mL ($\approx 1\%$ v/v) of actively growing cultures of *Lactobacillus acidophilus* (approximately 10^7 - 10^8 CFU/mL), prepared in MRS broth under aseptic conditions. After inoculation, 200 μ L of each sample was aseptically spread onto sterile MRS agar plates (prepared separately and autoclaved). The plates were allowed to solidify, then incubated at 37 °C for 24-48 hrs. Microbial growth at different pH levels was assessed by colony formation and density on agar plates.

Effect of temperature

Fifteen mL of pasteurized *Persea americana* juice and 2 mL of microbial broth were added to each of the 6 conical flasks. After that, each conical flask was exposed to a range of temperatures for roughly 24 hrs, including room temperature, the refrigerator, and 30 °C,

35 °C, 40 °C, and 45 °C. After a 24-hr period, 200 µL of juice was pipetted onto sterile petri plates, followed by the addition of MRS agar, and the plates were solidified. Following that, the plates were incubated at 37 °C for 24-48 hrs.

Effect of bile salt

The tolerance of probiotic isolates to bile salt was evaluated using different concentrations of bile salt (0.1-0.6% w/v). The study was done using bile salt (HiMedia Laboratories Pvt. Ltd., Mumbai, India). Six sterile conical flasks with 0.1, 0.2, 0.3, 0.4, 0.5, and 0.6 (w/v) bile salt solutions were prepared, and one control flask was prepared with no bile salt. Aseptic conditions were maintained by filling each flask with 15 mL of pasteurized *Persea americana* juice. *Lactobacillus acidophilus* with an approximate initial microbial load of 10^7 - 10^8 CFU/mL, then in 2 mL (about 1% v/v) were inoculated into each flask to achieve homogeneous initial microbial load.⁴⁴ The flasks were left at 37 °C for 24 hrs. Each sample was aseptically placed on sterile MRS agar plates (HiMedia Laboratories Pvt. Ltd., India) after incubation. They were left to solidify, after which they were incubated at 37 °C for 24-48 hrs. The growth of microbes was evaluated in terms of colony formation and viability across varying bile salt concentrations.

Effect of sodium chloride

The effect of sodium chloride on the growth of probiotic isolates was evaluated using different NaCl concentrations (0.5-3.0% w/v). Six sterile conical flasks containing NaCl at concentrations of 0.5%, 1.0%, 1.5%, 2.0%, 2.5%, and 3.0% (w/v), along with one control flask without NaCl, were prepared. Each flask was filled with 15 mL of pasteurized *Persea americana* juice under aseptic conditions. The flasks were inoculated with 2 mL ($\approx$ 1% v/v) of actively growing cultures of *Lactobacillus acidophilus* or *Lactobacillus plantarum* (approximately 10^7 - 10^8 CFU/mL). The experiments were performed in triplicate to ensure reproducibility and to support statistical analysis. All flasks were incubated at 37 °C for 24 hrs. After incubation, samples were aseptically plated using the pour-plate method onto MRS agar (HiMedia Laboratories Pvt. Ltd.). The plates were allowed to solidify and then incubated at 37 °C for 48-24 hrs. Microbial growth was assessed based on colony formation and viability at different NaCl concentrations. The results obtained from triplicate experiments were used for statistical analysis (ANOVA followed by Tukey's test).²⁴

Biochemical assay

Reducing Sugars by the DNS method were estimated using the following methodology. The amount of sugar present was reduced by the use of dinitrosalicylic acid (DNS) method Miller.²⁹ Preparation of reagents: A glucose stock solution (1 mg/mL) was made by dissolving 10 mg of glucose in 10 mL of

distilled water. A standard solution was then made by adding 1 mL of stock solution to 5 mL of distilled water. DNS reagent was prepared by combining 1 gm of 3, 5-dinitrosalicylic acid, 1 gm NaOH, 200 mg phenol, and 50 mg sodium sulfite in 100 mL of distilled water. In another beaker, 40% (w/v) sodium potassium tartrate solution was prepared. To conduct the assay, 1 mL of DNS reagent was added to 1 mL of glucose standard or sample in a test tube that had been appropriately diluted. This mixture was heated in a boiling water bath (90-100 °C) 10-5 minutes to enable color development. Following the heating procedure, 1 mL of 40% sodium potassium tartrate solution was placed to stabilize the color, and the tubes were allowed to cool down to room temperature. The distilled water (8-10 mL) was used to dilute the reaction mixture, and the absorbance was recorded at 540 nm on a spectrophotometer. A standard curve was drawn, and the glucose and reducing sugar content of the samples was calculated.

Estimation of protein

The protein content was assessed through the Lowry method²⁵

Preparation of reagents

A stock solution of bovine serum albumin (BSA) at a concentration of 1 mg/mL was created by dissolving 10 mg of BSA in 10 mL of distilled water. A working standard was generated by diluting 1 mL of the stock solution to a final volume of 5 mL with distilled water.

Reagent A was formulated by dissolving 2% sodium carbonate (Na_2CO_3) in 0.1N NaOH. Reagent B was made by dissolving 0.5% copper sulfate in 1% potassium sodium tartrate. Reagent C, known as the alkaline copper reagent, was prepared by combining 50 mL of reagent A with 1 mL of reagent B. The Folin-Ciocalteu reagent, commercially available, was diluted to 1N before use.

Procedure

In the assay, 1 mL of the standard or a suitably diluted sample was placed in a test tube, followed by the addition of 5 mL of reagent C. The mixture was vortexed and incubated at room temperature for 10 minutes. Following the incubation period, 0.5 mL of the diluted Folin-Ciocalteu reagent was added swiftly, and the mixture was thoroughly mixed immediately. The tubes were then incubated in the dark at room temperature for 30 min to allow for color development. The absorbance was recorded at 660 nm using a spectrophotometer. A standard curve was established using BSA, and the protein concentration of the samples was calculated accordingly.

Anti-oxidant assay

ABTS

Gallic acid (10 mg) was dissolved in 10 mL of methanol to produce a stock solution. Methanol (9 mL) was added to 1 mL of stock to generate the working

solution. Three sets of test tubes were used; one set was pipetted with 10-50 µL of standard. In addition, the other two sets of test tubes received 10-50 µL samples of regular juice and of pasteurized probiotic juice. After preparing each test tube solution to a final volume of 100 µL with methanol, 3 mL of the ABTS solution was added. Prior to adding ABTS, a concentrated ABTS solution was added to a diluted ABTS solution to set the absorbance to 1. After 30 minutes of dark incubation, the test tubes were removed, and autozero was used to compute the absorbance at 734 nm. To calculate the percentage (%) of inhibition, a formula was used:

$$\text{Percentage (\%) inhibition} = (\text{Abs of control}-\text{Abs of Test}/\text{Abs of control})\times 100$$

DPPH

To prepare the stock, 10 mg of gallic acid and 10 ml of methanol were combined. Methanol (9 mL) was added to 1 mL of stock to create the working solution. They took three sets of test tubes. An empty tube containing 100 µL of methanol was used as the blank, and one set was pipetted with 10-50 µL of the standard; the remaining two sets contained regular juice and pasteurized probiotic juice. Methanol was added to each standard tube to a capacity of 100 µL. Next, 3 mL of DPPH solution was added to each test tube. Prior to adding DPPH, concentrated DPPH was added to diluted DPPH to set the DPPH solution's absorbance to 1. For 15 minutes, all the tubes were incubated in the dark. At 517 nm, the absorbance was measured following incubation.²⁶ To calculate the percentage (%) of inhibition, a formula was used:

$$\text{Percentage (\%) inhibition} = (\text{Abs of control}-\text{Abs of Test}/\text{Abs of control})\times 100$$

Anti-microbial activity

Probiotic avocado juice was prepared by blending 100 gm of ripe avocado with 100 mL of distilled water, then pasteurized at 72 °C for 15 sec. A 10% (v/v) probiotic culture was added and incubated at 37 °C for 24 hrs. Luria-Bertani (LB) agar and Sabouraud Dextrose Agar (SDA) were prepared using commercial media (HiMedia Laboratories Pvt. Ltd., Mumbai, India), autoclaved, and poured into sterile Petri plates. After solidification, pathogenic bacteria were swabbed, and wells were filled with either probiotic or regular avocado juice (50-100 µL per well). Each setup was prepared in triplicate. Plates were incubated for 24 hrs at 37 °C and examined for zones of inhibition.

Data analysis

ANOVA with $p < 0.05$ and Duncan's Multiple Range Test (DMRT) were used to identify significant differences among mean values. Data are reported as the mean and standard deviation (mean±SD) of triplicate measurements. Version 12 of the SPSS program was used for all statistical analyses. Pearson's correlation coefficient (r) was used to obtain correlations in bivariate linear correlations.

Results

Isolation, screening, and identification of lactic acid bacteria

Lactic acid bacteria (LAB) were isolated using de Man, Rogosa, and Sharpe (MRS) agar medium under anaerobic incubation conditions. The obtained colonies were screened for morphological and biochemical characteristics, including Gram staining, catalase negativity, and acid production. Selected isolates were further evaluated for probiotic properties, including tolerance to acidic pH, bile salts, and NaCl concentrations. The potent isolates showing maximum survivability and functional activity were identified as *Lactobacillus acidophilus* and *Lactobacillus plantarum*. Identification was confirmed through standard microbiological and biochemical characterization methods.

Isolation, subculturing, and screening of a probiotic strain in avocado juice

This investigation indicates that morphological traits were used to identify the lactic acid bacteria (LAB). In addition, all of the LAB were identified to the generic level by other biochemical tests, which showed negative (data not shown), showing that *Lactobacillus* spp. were whitish, small to large in size, with a circular margin on MRS media (Figure 1), and *Lactococcus* spp. were creamy white to yellowish in color, small to large in size, with a circular margin. To subculture, pure cultures of specific, morphologically distinct bacterial colonies were inoculated onto solidified LB agar plates. To prevent contamination, do this in the laminar air flow chamber. The plates were maintained at 37 °C in an incubator for 24-48 hrs. The juice was screened to ensure sterility. LB agar was prepared and autoclaved before being used for screening. Following autoclaving, 200 µL of pasteurized avocado juice was pipetted onto the Petri plates. Agar was then added to the juice and left to solidify. The plates were incubated at 37 °C for 24 to 48 hrs after solidification.

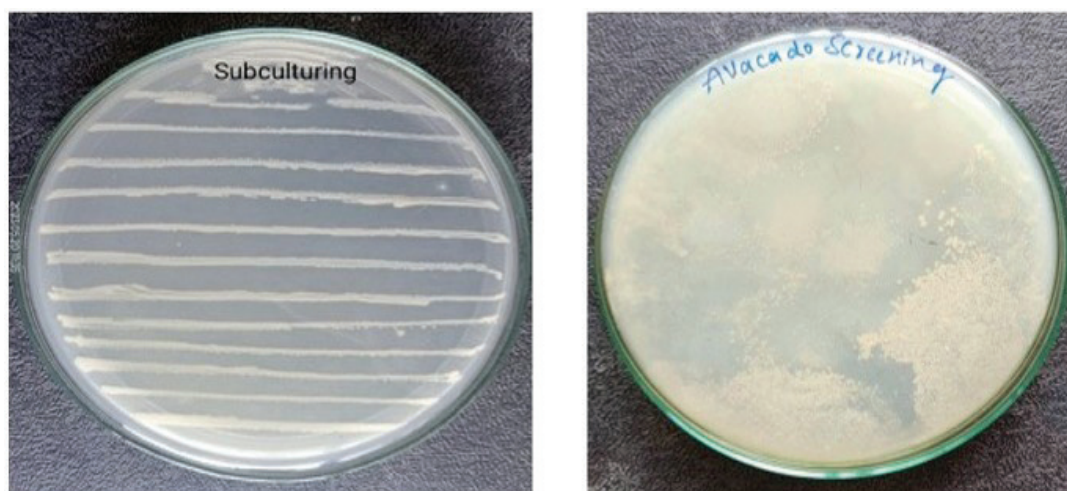


Figure 1. Isolation, subculturing, and screening of probiotic *Lactobacillus* strains in avocado juice. A: subculturing of *Lactobacillus*, B: screening of probiotic strain in avocado juice.

The organisms were isolated from the milk sample on MRS media by the pour-plate method. The isolated bacterial colonies were identified as Gram-positive

rods based on their morphological features. The microscopic view of the Gram-stained organisms is shown in Figure 2.

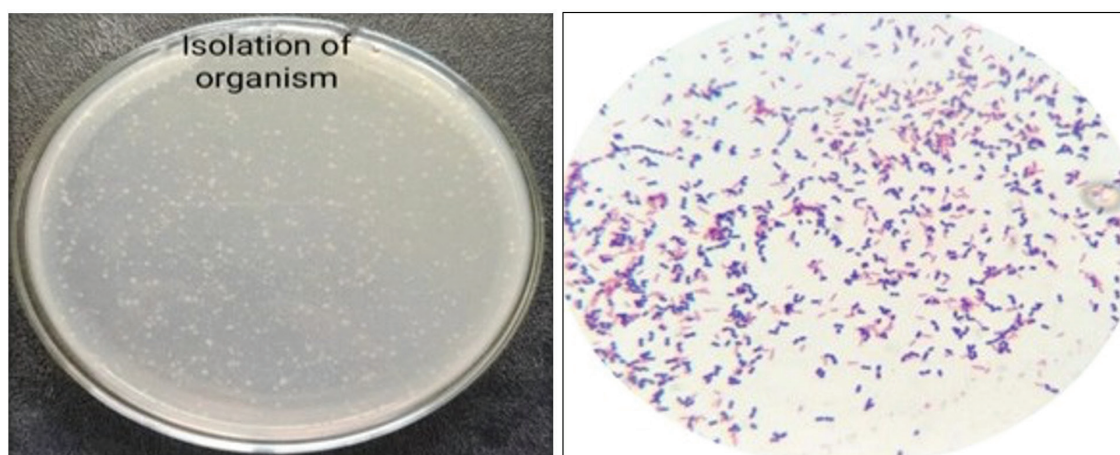


Figure 2. Isolation of LAB on MRS media and bacterial colonies were identified as Gram-positive rods under a 40X magnification microscope.

Biochemical characterization of isolates

The biochemical tests were performed to identify and characterize the bacterial isolates, particularly to confirm their affiliation with lactic acid bacteria (LAB) and to support their classification as *Lactobacillus* species. These tests help differentiate bacteria based on metabolic activities, enzyme production, and substrate utilization patterns.

The observed results-specifically, Gram positive (presumed), catalase-negative, oxidase-negative characteristics, along with positive carbohydrate fermentation patterns-are indicative of *Lactobacillus* species. The lack of indole production, citrate utilization, urease activity, and H_2S production further reinforces this classification. Consequently, these biochemical tests were used to verify the identity and functional traits of the isolates, thereby confirming their suitability as probiotic strains for fermentation research.

Probiotic juice using *Persea americana* (avocado) Extraction and fermentation of fruit juice

The Juice from fruit was extracted and pasteurized at 72 °C for 30 minutes, and the sterility of the pasteurized juice is depicted in Figure 3. It is claimed that pasteurized juice is free of contamination because no colonies are present on the Petri plate. The juice was given a 7-day fermentation period by adding 20 mL of a broth containing microorganisms to 200 mL of pasteurized juice.

Optimization of parameters

The viability of probiotic *Lactobacillus* at a pH range of 5-10, different temperatures from fridge to room temperature, 0.1-0.5% concentration of bile salt, and 0.5-3% NaCl concentration is shown in Figure 4.

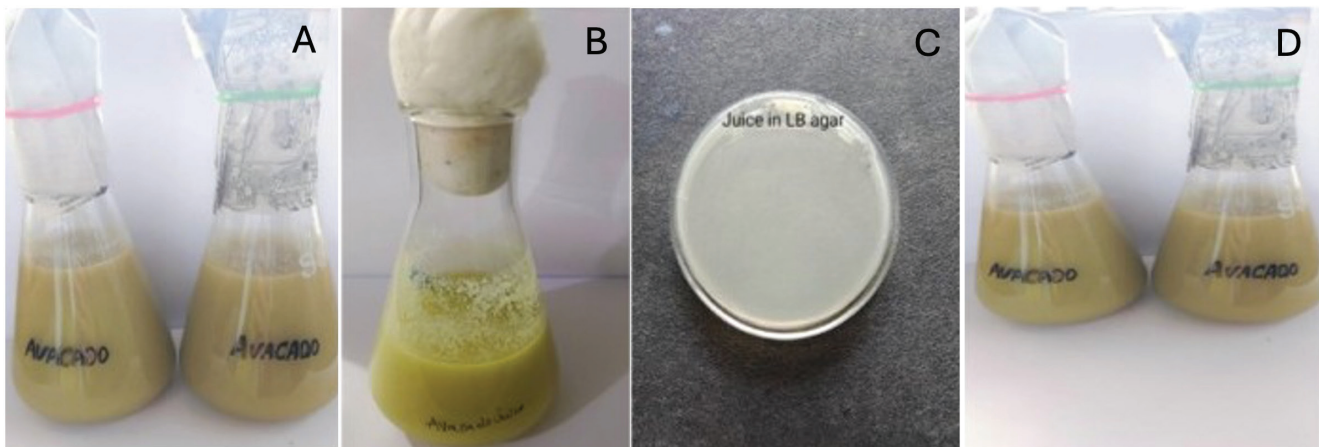


Figure 3. Preparation and fermentation process of avocado juice.

A: extraction of fruit juice, B: pasteurization, C: sterility check of the pasteurized milk, D: fermented fruit juice.

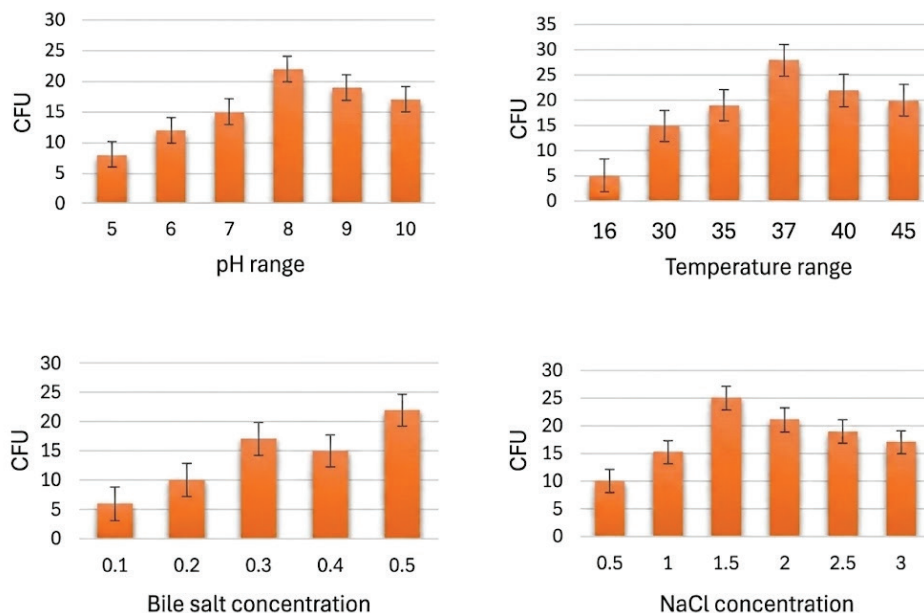


Figure 4. Enhancement of cultural conditions for the viability of *Lactobacillus*.

A: influence of pH (ranging from 5 to 10), B: temperature settings (both refrigerated and ambient temperature), C: concentration of bile salts (0.1 to 0.5% w/v), D: concentration of NaCl (0.5 to 3% w/v) on the survival of probiotic *Lactobacillus*. Viability is quantified in colony-forming units/mL (CFU/mL). The data presented are mean values derived from triplicate experiments.

Sensory evaluation

The sensory evaluation of both fermented and non-fermented *Persea americana* (avocado) juice was conducted using a 5-point hedonic scale to assess consumer preferences for appearance, aroma, taste, texture, and overall acceptability. The fermented juice, inoculated with *Lactobacillus* and incubated for 48 hrs, consistently received higher scores across all sensory characteristics. It attained mean scores of 4.2 ± 0.5 for taste, 4.1 ± 0.6 for aroma, and 4.3 ± 0.5 for overall acceptability, indicating an improved sensory profile

compared with the pasteurized, non-fermented sample, which scored 3.4 ± 0.6 . These enhancements may be linked to the breakdown of sugars and the generation of aromatic compounds during the fermentation process, as similarly noted in earlier research involving probiotic fruit beverages.^{27,28} Statistical evaluation through one-way ANOVA followed by Tukey's post-hoc test validated that the differences were significant at $p < 0.05$. These results highlight the potential of fermented *Persea americana* juice as a more enjoyable and consumer-friendly functional beverage (Figure 5).

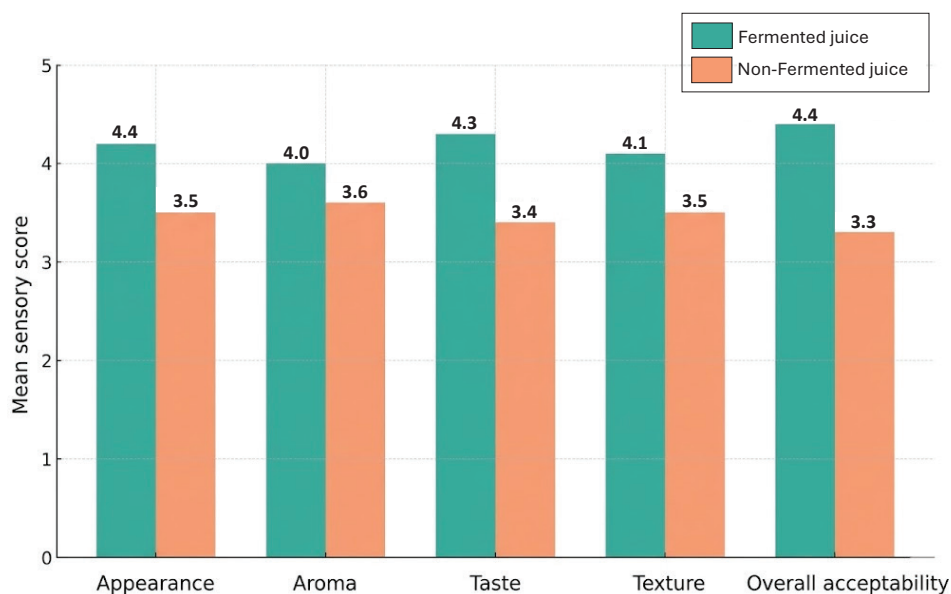


Figure 5. A histogram was employed to illustrate the sensory evaluation scores of both fermented and non-fermented *Persea americana* juice. The assessment utilized a 5-point hedonic scale to evaluate appearance, aroma, taste, texture, and overall acceptability. The green bars denote the fermented juice, while the orange bars signify the non-fermented juice. Error bars represent the standard deviations derived from the responses of 15 panelists.

Biochemical assays

Estimation of reducing sugar by the dinitrosalicylic acid method

The content of reducing sugars (RSC) was determined utilizing the dinitrosalicylic acid (DNS) method as described by Miller and Jain.^{29,30} This technique relies on the reduction of 3,5-dinitrosalicylic acid under alkaline conditions by reducing sugars such as glucose and fructose, resulting in the formation of 3-amino-5-nitrosalicylic acid, which exhibits an orange-red color with peak absorbance at 540 nm. The color intensity is directly proportional to the concentration of

reducing sugars in the sample. A standard calibration curve was established using D-glucose ($Y=0.014x+0.1202$, $R^2=0.9938$), and the reducing sugar content in the samples was calculated and reported in mg/mL. All measurements were conducted in triplicate ($N=3$) to guarantee accuracy and reproducibility. The results are presented as mean $\pm$ SD, and the relative standard deviation (RSD) was computed to assess precision. These results are illustrated in Table 1 and Figure 6.

These RSD values indicate good precision, especially since most are under 5%, which is generally acceptable in biochemical assays.

Table 1. Estimation of reducing sugar by dinitrosalicylic acid.

Sample	O.D. (540 nm)	Concentration (mg/mL)	SD	RSD (%)
Blank	0	0	0	0
1	0.740	40	0.030	4.05
2	1.345	80	0.040	2.98
3	1.820	120	0.040	2.20
4	2.360	160	0.060	2.54
5	2.860	200	0.060	2.10
Normal juice	0.120	83.50	0.005	4.17
Probiotic juice	0.066	37.36	0.002	3.03

Note: RSD values indicate good precision, especially since most are under 5%.

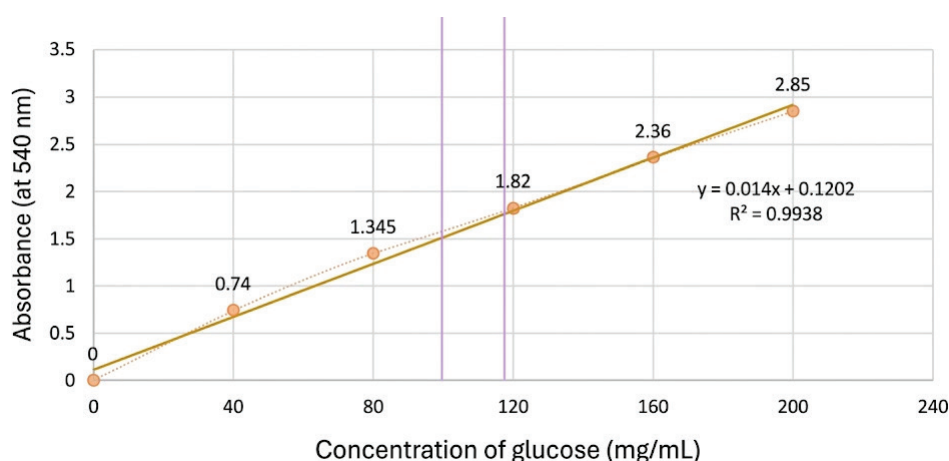


Figure 6. Estimation of reducing sugar by DNS method.

Estimation of protein using Lowry's method

The protein content was assessed using the modified Lowry method, which combines the Biuret reaction (formation of a copper-protein complex under alkaline conditions) with the reduction of the Folin-Ciocalteu reagent by aromatic amino acids. This process yields a stable blue-colored complex that can be measured at wavelengths between 650 and 750 nm.³¹ A standard

calibration curve was established utilizing bovine serum albumin (BSA), and the protein concentration in the samples was calculated accordingly. The findings are illustrated in Table 2 and Figure 7. All measurements were conducted in triplicate (N=3) (technical replicates) to guarantee precision and reproducibility. The results are presented as mean±standard deviation (SD).

Table 2. Estimation of protein by Lowry's method.

Sample	OD (540nm)	Concentration (mg/mL)	SD	RSD %
Blank	0	0	0	0
1	0.057	40	0.0025	4.30
2	0.129	80	0.0055	4.25
3	0.182	120	0.0085	4.66
4	0.22	160	0.0102	4.62
5	0.273	200	0.0135	4.94
Normal juice	0.698	493.3571	0.0165	2.37
Probiotic juice	0.964	683.3571	0.0241	2.51

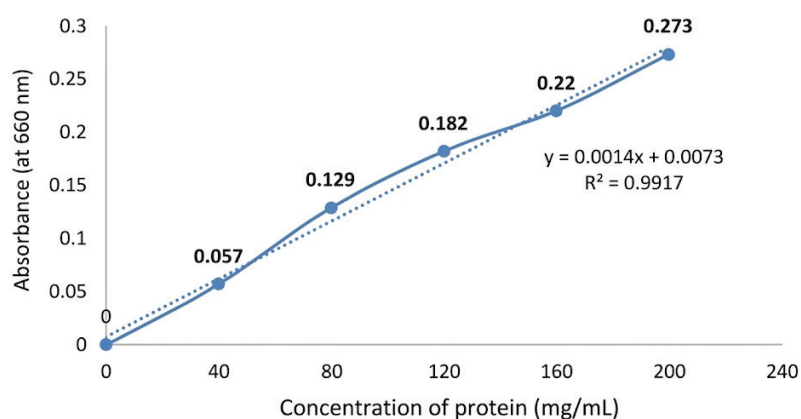


Figure 7. Standard graph for the estimation of proteins.

Antioxidant activity

ABTS assay

The antioxidant activity of the samples was assessed using the ABTS [2,2-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)] radical-scavenging assay, measured at 734 nm.³² This assay relies on the capacity of antioxidants to neutralize the ABTS radical cation, leading to a reduction in absorbance that is proportional to the antioxidant capacity. Various concentrations of the samples (10-50 µg/mL) were evaluated, and the percentage inhibition of ABTS radicals was calculated relative to gallic acid, which served as a standard.

The IC₅₀ values (the concentration needed to inhibit 50% of radicals) were derived from the inhibition curves. The findings indicated that the fermented juice demonstrated superior antioxidant activity (IC₅₀=17.27 µg/mL) when compared to pasteurized avocado juice (IC₅₀=31.46 µg/mL), and it was similar to the standard gallic acid (IC₅₀=18.73 µg/mL).³³ This increased activity may be due to the presence of bioactive compounds such as phenolics and flavonoids. All experiments were conducted in triplicate (N=3), and the results are presented as mean±SD. The data are presented in Table 3 and Figure 8.

Table 3. ABTS assay of fermented avocado and pasteurized avocado juice samples.

Concentration (µg/mL)	Gallic acid (control) Standard	Fermented avocado juice	Pasteurized avocado juice
10	0.786±0.024	0.597±0.018	0.794±0.024
20	0.610±0.018	0.457±0.014	0.611±0.018
30	0.440±0.013	0.359±0.011	0.493±0.015
40	0.268±0.008	0.263±0.008	0.410±0.012
50	0.129±0.004	0.188±0.006	0.282±0.008
IC ₅₀ (µg/mL)	18.73	17.27	31.46

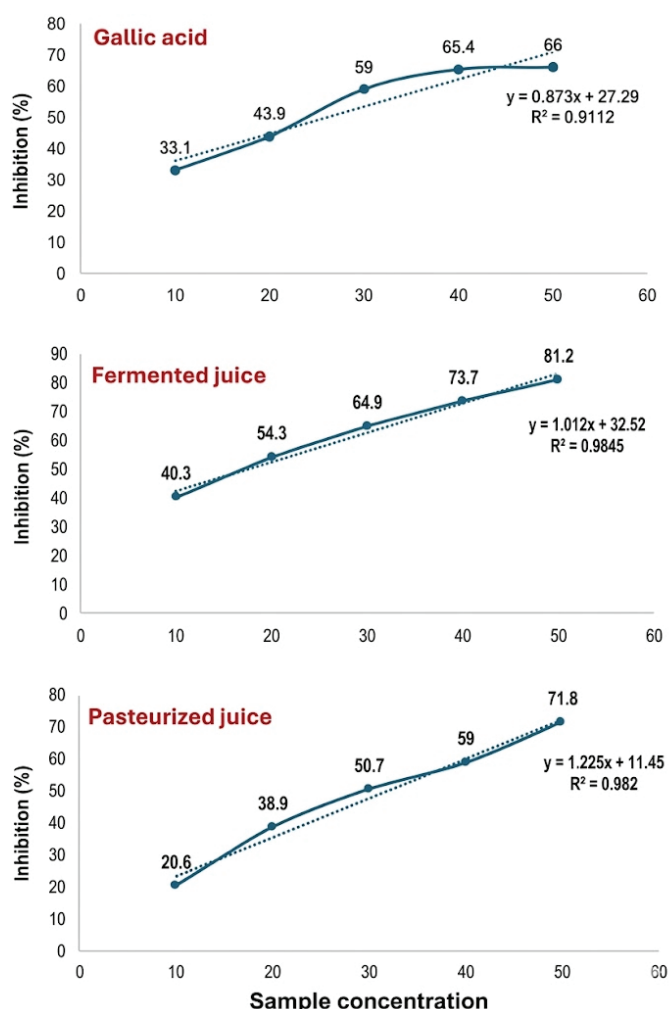


Figure 8. Antioxidant assay (ABTS method). A: gallic acid (control), B: fermented juice, C: pasteurized juice.

DPPH radical scavenging assay

The antioxidant properties of the samples were further assessed using the DPPH (2,2-diphenyl-1-picrylhydrazyl) radical-scavenging assay, measured at 517 nm.^{33,34} This technique relies on the conversion of the purple-hued DPPH radical into a yellow diphenylpicrylhydrazine in the presence of antioxidants, leading to a reduction in absorbance.

Various concentrations of the samples were evaluated, and the percentage of radical-scavenging activity was calculated relative to gallic acid, which served as the standard. The IC₅₀ values (the

concentration needed to inhibit 50% of DPPH radicals) were derived from the inhibition curves.

The findings revealed that the fermented juice demonstrated superior antioxidant activity (IC₅₀=24.64 µg/mL) when compared to pasteurized avocado juice (IC₅₀=51.95 µg/mL) and exhibited activity comparable to that of gallic acid (IC₅₀=28.23 µg/mL). The increased antioxidant activity may be due to the presence of bioactive compounds such as phenolics and flavonoids. All experiments were conducted in triplicate (N=3), and the results are presented as the mean±SD. The data are in Table 4 and Figure 9.

Table 4. DPPH assay of pasteurised juice and fermented *P. americana* juice samples.

Concentration (µg/mL)	Gallic acid (control)	Fermented juice	Pasteurized juice
10	0.728±0.022	0.694±0.021	0.899±0.027
20	0.629±0.019	0.542±0.016	0.772±0.023
30	0.465±0.014	0.421±0.013	0.683±0.020
40	0.332±0.010	0.309±0.009	0.572±0.017
50	0.232±0.007	0.215±0.006	0.553±0.017
IC ₅₀ (µg/mL)	28.23	24.64	51.95

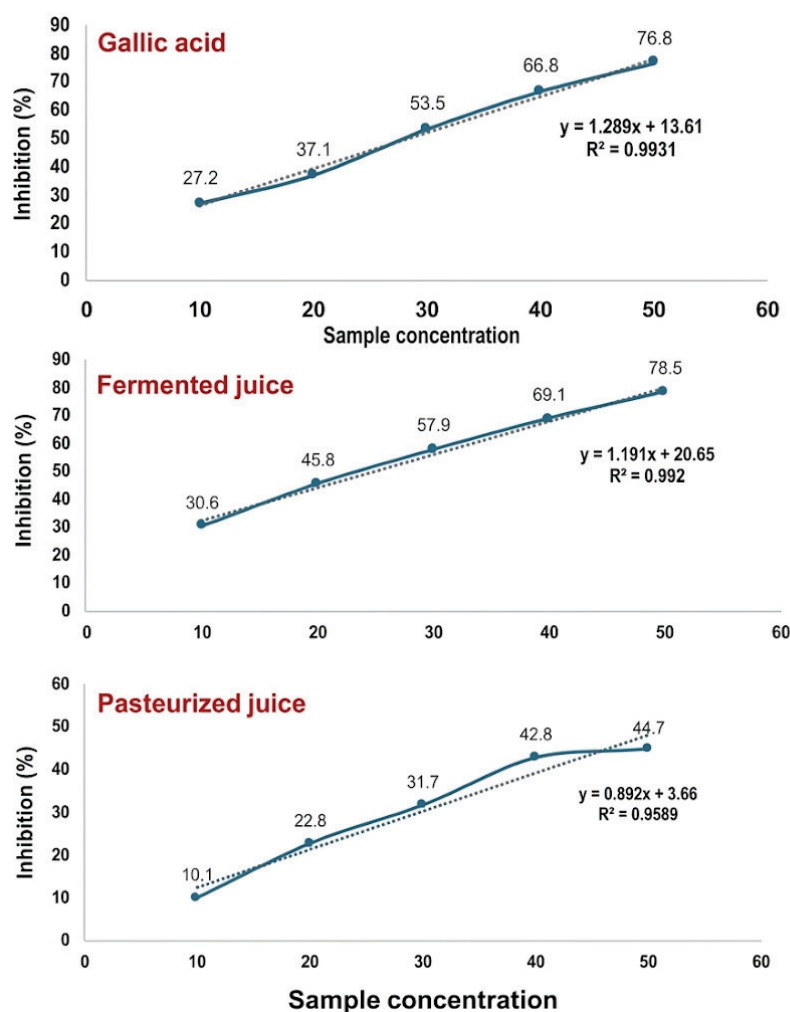


Figure 9. DPPH assay. A: gallic acid standard, B: fermented juice, C: pasteurized juice.

Antimicrobial assay

The antibacterial efficacy of fermented and pasteurized *Persea americana* juice was assessed utilizing the agar well diffusion technique.³⁴ Six pathogenic indicator strains: *E.coli*, *B.cereus*, *S.aureus*, *S.typhimurium*, *S.mutans*, and *P.aeruginosa*, were sourced from the stock cultures of the Dairy Microbiological Laboratory at the National Research Center. Each bacterial strain was activated in tryptone soy broth and incubated at 37 °C for 24 hrs. Approximately 1 mL of the activated culture ($\approx 10^4$ CFU/mL) was combined with 20 mL of molten Mueller-Hinton agar (Becton Dickinson) and poured into sterile Petri plates. Following solidification, wells measuring 5 mm in diameter were aseptically created using a

sterile cork borer. A volume of 50 μ L from each juice sample was introduced into the wells. Penicillin (potassium penicillin, 20%) at concentrations ranging from 50 to 200 mg/mL was used as a positive control, while sterile phosphate buffer served as a negative control. The plates were incubated at 37 °C for 24 hrs. All experiments were conducted in triplicate (N=3) to guarantee reproducibility. The antimicrobial activity was assessed by measuring the zone of inhibition (ZOI, mm) surrounding each well. Results were reported as mean $\pm$ SD. A larger zone of inhibition signified a more potent antibacterial activity. The data are presented in Table 5 and Figure 10, and statistical analysis was conducted using ANOVA.

Table 5. Antimicrobial activity of pasteurised and fermented *P. americana* juice

Strains	Control (mm)	Pasteurized juice (mm)	Fermented juice (mm)
<i>Salmonella typhi</i>	2.3 $\pm$ 0.07	1.2 $\pm$ 0.04	1.5 $\pm$ 0.05
<i>Pseudomonas aeruginosa</i>	2.8 $\pm$ 0.08	1.3 $\pm$ 0.04	1.1 $\pm$ 0.03
<i>Streptococcus mutans</i>	3.0 $\pm$ 0.09	0.0 $\pm$ 0.00	0.0 $\pm$ 0.00
<i>Staphylococcus aureus</i>	1.8 $\pm$ 0.05	2.9 $\pm$ 0.09	1.3 $\pm$ 0.04
<i>Bacillus cereus</i>	2.5 $\pm$ 0.08	2.9 $\pm$ 0.09	0.0 $\pm$ 0.00
<i>Escherichia coli</i>	4.3 $\pm$ 0.13	0.0 $\pm$ 0.00	1.3 $\pm$ 0.04

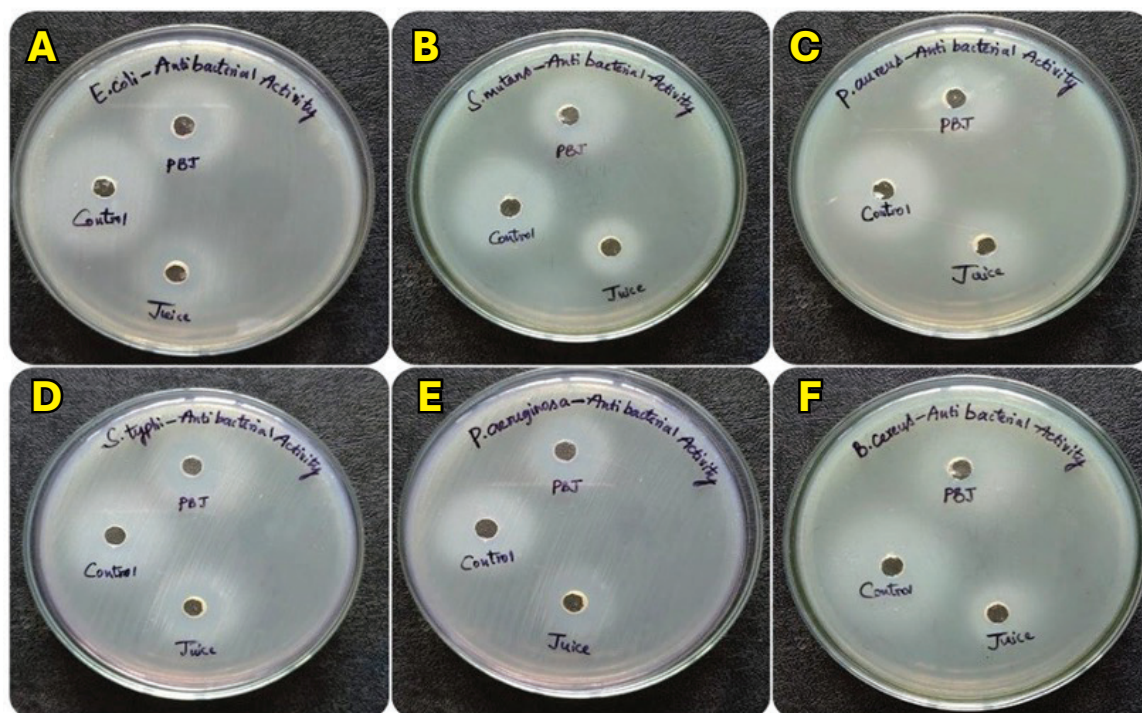


Figure 10. Antimicrobial property.

A: *E.coli*, B: *S.mutans*, C: *S.aureus*, D: *S.typhi*, E: *P.aeruginosa*, F: *B.cereus*.

Discussion

This research investigated the feasibility of *Lactobacillus* spp. in pasteurized *Persea americana* (avocado) juice and evaluated its potential as a probiotic medium. The results confirmed that *Lactobacillus* can thrive and proliferate in avocado juice without the need for any specialized growth additives. This aligns with the findings of Anil Panghal *et al.*, who highlighted that fruit juices, rich in dietary fiber, vitamins, minerals, and antioxidants, can serve as effective alternative substrates for the cultivation of probiotics.³⁵ The successful proliferation of *Lactobacillus* within the juice matrix illustrates the promise of avocado juice as a non-dairy vehicle for probiotics.

Morphological identification via Gram staining validated the existence of Gram-positive rods, which are indicative of *Lactobacillus* species. MRS medium, recognized as a standard growth medium for lactic acid bacteria, facilitated successful cultivation.³⁶ A variety of environmental factors, including pH, NaCl, and bile salt concentrations, were assessed to identify the conditions most conducive to probiotic survival. The organism exhibited optimal growth at bile salt concentrations of 0.5% and 1.5% NaCl, indicating that the bacteria possess considerable bile and osmotic tolerance, which are crucial for survival within the gastrointestinal tract. Additionally, the analysis of pH ranges showed that pH 8 provided the most favorable conditions for bacterial growth. These findings are consistent with other research indicating that slightly alkaline conditions may be beneficial for certain *Lactobacillus* strains in fruit matrices.

The incubation period significantly influenced bacterial viability. Although 24 hrs of incubation proved inadequate, 48 hrs offered sufficient time for the bacteria to establish and proliferate. The prolonged incubation likely facilitated the accumulation of vital metabolites and adaptation to the juice environment, thereby enhancing colony formation. This observation aligns with findings from other probiotic studies that recommend extended fermentation times to achieve stable growth and functional benefits.

The primary emphasis of the research was on the antioxidant potential of fermented avocado juice. The antioxidant activity was evaluated using DPPH and ABTS spectrophotometric assays, with IC_{50} values indicating that the fermented juice exhibited greater antioxidant capacity than its pasteurized counterpart. This observation supports the findings of Khan *et al.*, who indicated that fermentation frequently enhances the antioxidant potential of foods by generating bioactive compounds such as glutathione, butyrate, and folate.³⁷ Likewise, Martin *et al.* showed that the fermentation of blueberry juice using a novel bacterium significantly enhanced its antioxidant properties.³⁸ Additionally, Bujna *et al.* observed increases in antioxidant activity in apricot juice fermented with *Bifidobacterium lactis* and *Lactobacillus acidophilus*.²⁸ Collectively, these

studies underscore the role of probiotic fermentation in enhancing the health-promoting attributes of fruit-based beverages.

Regarding antimicrobial activity, the fermented and pasteurized juice samples did not show any zones of inhibition (ZI) against mutant *Streptococcus* strains in the well diffusion test, whereas the antibiotic control showed significant ZI. This difference is likely due to the greater potency and concentration of synthetic antibiotics compared to the natural metabolites present in fruit juice. Although probiotic bacteria can produce antimicrobial substances such as bacteriocins and organic acids, they may not generate these compounds at sufficient concentrations under the tested conditions to effectively inhibit resistant or mutant bacterial strains.⁴⁰ Furthermore, juice matrices may lack the synergistic compounds or cofactors that could enhance antimicrobial efficacy. This finding contrasts with studies where probiotic strains have shown inhibitory effects against pathogens, suggesting that the antimicrobial effect is highly dependent on the specific strain and the conditions under which it is tested.

It is widely recognized that distinct strains of probiotic bacteria possess specific antioxidant characteristics that also contribute to their antibacterial properties. These probiotics are known to generate various metabolites with antioxidant capabilities, including glutathione, butyrate, and folate.^{30,39} Martin *et al.* demonstrated that a novel bacterium isolated from the microflora of *Serratia raccinii* fruit significantly enhanced the antioxidant activity of blueberry juice during fermentation.³⁸ In a similar vein, Bujna *et al.* noted a moderate enhancement in the antioxidant levels of apricot juice fermented with *Bifidobacterium lactis* Bb12 and *Lactobacillus acidophilus* La5.²⁸ These results emphasize the essential function of *Lactobacillus* and other probiotic strains in the fermentation of fruit juices and highlight their extensive functional applications.⁴¹ Moreover, recent research has indicated that dairy products enriched with probiotics can inhibit the enzymatic functions of amylase and glucosidase, implying a potential hypoglycemic effect in vitro.⁴²⁻⁴⁵

Another potential explanation for the reduced or nonexistent ZI in the fermented and pasteurized juices may relate to the characteristics of the target organism. The mutant strain of *Streptococcus* utilized could have an intrinsic resistance to mild antimicrobial agents, which accounts for the larger inhibition zones observed with control antibiotics. Furthermore, as fermentation modifies the juice's pH and metabolite composition, the overall antimicrobial effectiveness may be influenced by fermentation dynamics that were not completely captured within the 48-hr duration of this experiment.

In summary, this research demonstrates that *Lactobacillus* spp. can survive and flourish in pasteurized avocado juice without additional supplementation. The fermentation process significantly enhanced the juice's

antioxidant capacity, although the antimicrobial effects were somewhat limited under the tested conditions. These results indicate that avocado juice is a promising medium for developing probiotic beverages, offering considerable functional advantages. Nonetheless, further refinement of fermentation parameters and selection of strains may improve antimicrobial characteristics and broaden the functional potential of these products. This study adds to the growing body of evidence supporting the use of non-dairy matrices for the delivery of probiotics and the development of functional foods.

Limitations

The study was conducted under laboratory conditions, which may not completely reflect industrial-scale production or real market storage conditions. Only two probiotic strains, *Lactobacillus acidophilus* and *Lactobacillus plantarum*, were evaluated. Therefore, the findings cannot be generalized to all probiotic microorganisms. In sensory evaluation, such as taste, aroma, texture, and consumer acceptability of fermented avocado juice, these aspects were not extensively investigated. The antimicrobial activity observed in the fermented juice was moderate and lower than that of standard antibiotic controls. *In vivo* studies and clinical trials were not conducted; therefore, the direct health benefits of fermented avocado juice in humans require further confirmation.

Conclusion

This research emphasizes the potential of *Persea americana* (avocado) juice as a viable medium for creating a functional probiotic beverage. The effective growth and survival of *Lactobacillus* in pasteurized avocado juice without additional nutrients underscores the juice's suitability as a natural carrier for probiotic cultures. Fermentation significantly improved the juice's antioxidant capacity, as evidenced by DPPH and ABTS assays, and also demonstrated enzyme-inhibitory effects, indicating possible hypoglycemic benefits. Although no zones of inhibition were observed against *Streptococcus* in antimicrobial evaluations, this may be due to the limited concentrations of bioactive compounds under the tested conditions. The strain exhibited optimal growth at pH 8, 0.5% bile salt, 1.5% NaCl, and after 48 hrs of incubation, confirming its robustness against gastrointestinal-like stress. These results endorse the development of *Lactobacillus*-fermented *P. americana* juice as an innovative, plant-based functional drink with improved nutraceutical properties, presenting promising implications for the food and health sectors.

Ethical approval

Not applicable

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This research received no external funding.

Conflicts of Interest

The authors declare no conflict of interest.

Availability of data and materials

The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

CRediT authorship contribution statement

Kanchi Ravi Padma: manuscript drafted, supervise; **Lingareddy Nikitha Reddy:** research work; **Sankari Mohan:** supervise.

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