



Probiotic Properties of *Lactobacillus casei* and *L. plantarum* Isolated From Overripe Avocado Pear

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Probiotics have been researched for their benefit to gut health. Research for sources of probiotic bacteria has moved from milk to plants, as they have been found to be an inexhaustible source of lactic acid bacteria (LAB). The probiotic properties of lactic acid bacteria isolated from Over ripe Avocado pear on selected bacterial species were assessed. Fifty Unripe Avocado pears were purchased from different markets and allowed to ferment. Enumeration of the microorganisms and pure culture isolation were done on MRS agar supplemented with 1.0% CaCO₃ at 37 °C for 48

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hours; using the pour plate and streak plate methods respectively. The isolates were identified based on their microscopic, macroscopic, biochemical and molecular characteristics. They include *Lactobacillus casei* and *L. plantarum*. LAB isolates were tested for their phenol tolerance, bile salt tolerance, acid tolerance and antimicrobial activity. All the isolates were tolerant to simulated gastrointestinal conditions. Growth of the isolates was observed at different temperature between 15 °C to 60 °C. They exhibited tolerance to phenol ranging from 0.085±0.01% to 2.40±0.02%. Tolerance to bile salt ranged from 1.06x10¹± 0.96x10¹ to 3.04x10³±1.15x10². The acid tolerance level of the LAB obtained ranged from 2.98x10²±1.30x10² to 8.78x10²±2.22x10². Agar-well diffusion method was used to determine the antimicrobial susceptibility of the bacterial species. The test showed that the isolates were susceptible to the LAB isolates as zones of inhibition were observed. The inhibition zones diameter obtained ranged from 8.35±1.34mm to 13.77±2.21mm. Avocado pear, besides from their high nutritional content, also have the potential to serve as source of probiotic bacteria.

Keywords: Probiotics; lactic acid bacteria; avocado pear; inhibition; antimicrobial.

1. INTRODUCTION

Probiotics are live non-pathogenic organisms that have a positive impact on the health of humans and animals when consumed in adequate quantities. Certain criteria are to be met by microorganisms, for them to be classified as probiotic strains. The organisms must be alive at the time of consumption, have beneficial effects on the host, and must be able to reproduce to a sufficiently high dose that would provide health promoting effect on the host. Different microorganisms have probiotic properties, but it is particularly seen among bacteria species [1]. Probiotics confer several health benefits on their hosts. They act as anti-allergic agents, modulating the immune system's response to antigens. They improve the barrier function of the mucosa to reduce the passage of antigen. They stimulate the immune system to produce non-inflammatory cytokines and degrade some antigens [2]. They suppress transplantable or chemically induced tumors, by preventing their initiation, progression, and metastasis [3]. They reduce levels of cholesterol in the body by inhibiting the synthesis of cholesterol and reducing the absorption of cholesterol in the intestine [4]. Probiotics can be used to prevent and manage gastrointestinal diseases like inflammatory bowel disease and diarrhea. They increase the number of healthy microorganisms in the gut and the diversity of the gut microflora. They improve on the permeability of the intestine and on the micro-environment of the immune system [2].

The conditions at which probiotics are cultivated directly impacts on their growth, stability, and activities, and on the conditions under which the probiotic bacteria would be dried and stored.

Fermentation has been identified as a suitable method for the proliferation of probiotic microorganisms [5]. The process of fermentation usually requires starter cultures which consist of some beneficial microorganisms to be added to begin the process and establish the community of microorganisms that would be present in the fermented product. Fermentation provides an environment of competition which helps to establish and maintain a dominant population. The fermentation environment aids the ease of metabolic interaction among microorganisms that promote their growth. Probiotics can be consumed from fermented food products, dietary supplements, or drugs. Fermented dairy products, like cheese and yoghurt, are the most common sources of probiotics [6]. Plants have come into the view of research for its structure and functional attributes, its suitability for the growth of microorganisms, its potential to act as a vector of strains of probiotic microorganism, and its suitability to fermentation [7]. Fermented food products can be produced from cereals, fruits, legumes, tubers, and meat. Cereal-based products account for most traditional fermented foods. Fruits have unique structures that favour the growth of probiotic microorganisms. Fruits like pineapple, dragon fruit, and pear contain prebiotics that favour the proliferation of probiotic bacteria [8]. Fruits can act as vehicles to provide probiotics to humans either in the form of fermented or unfermented fruit juices or as minimally processed fruits. Not all probiotic bacteria can be incorporated into fruits. Some are unable to survive and proliferate in fruits. This poses a challenge to the use of fruit as a source of probiotic [9].

The present study assessed the probiotic properties of lactic acid bacteria isolated from over ripe avocado pear.

2. MATERIALS AND METHODS

2.1 Study Area

The study was carried out at the Laboratory Unit of Department of Applied Microbiology and Brewing, Nnamdi Azikiwe University, Awka, Anambra State, South-East geopolitical zone of Nigeria, between September, 2023 – February, 2024.

2.2 Specimen Collection

Fifty unripe Avocado pears were purchased from Amansea, Eke Awka, Owerri, and Ifite markets, all located in southeastern Nigeria. The samples were placed in sterile bags and transported to the laboratory for further analysis [10].

2.3 Isolation of Lactic Acid Bacteria

De Man, Rogosa, and Sharpe (MRS) media was prepared according to manufacturer's manual. Stock solutions of the fruits were prepared by dissolving 1g of crushed avocado pear in 10ml of sterile normal (w/v) saline solution. The flask was vigorously shaken to mix the contents. Ten-fold serial dilution was done to reduce the microbial load of the samples. Using pour plate method, aliquots (100 μ L) of the samples at dilution 10⁻⁴ were introduced into sterile petri dish. The MRS agar plates were incubated under anaerobic conditions at 37°C for 48 hours. Discrete colonies were selected and sub-cultured onto plates of MRS agar using streak plate method to obtain pure cultures. The pure cultures were stored at 4 °C on MRS agar in Bijou bottles [11].

2.4 Identification of Isolates

The isolates were identified using standard methods which include; Colony morphological, Gram staining, Catalase, Motility [12], Urease Test [13], Citrate utilization, Oxidase tests [14], sugar fermentation tests [15] and Nucleic acid sequence analysis [16].

2.5 Growth at Different Salt Concentrations

The isolates were inoculated on MRS agar plates supplemented with varying concentrations of NaCl: 1.5%, 3.5%, 5.5%, 7.5%, 9.5%, and 10.5% (w/v) respectively and incubated anaerobically at 37 °C for 3 days. Growth at

different salt concentrations was determined by measuring the optical density at 590nm (OD₅₉₀) [17].

2.6 Growth at Different Temperatures

The isolates were inoculated into 10 mL of MRS broth and incubated under anaerobic conditions at 15 °C, 30 °C, 45 °C, and 60 °C for 72 hours. Growth was determined by the formation of sediments at the bottom of the test tube [17].

2.7 Phenol Tolerance

The isolates were inoculated into MRS broths; a set of 3 was supplemented with 0.2% (w/v) phenol and another set of 3 with 0.5% (w/v) phenol. Each set of 3 was supplemented with 1.5, 2.5, and 3.5% (w/v) NaCl respectively and MRS broth without phenol or NaCl (to serve as control). Inoculated plates were incubated anaerobically at 37°C for 48 hours. Growth of LAB in the culture broth was determined by measuring the optical density (OD₅₉₀) [18].

2.8 Bile Salt Tolerance

The isolates were inoculated into 10mL of MRS broth and incubated anaerobically at 37°C for 24 hours. The cultures were centrifuged at 2,800 x g for 15 minutes and filtered to obtain the bacteria cells. Cells of isolates were washed twice with phosphate buffer saline(PBS), resuspended in MRS broth supplemented with 0.3% (w/v) bile salt, and incubated anaerobically at 37°C for 3 hours. Aliquots (100 μ l) of the test cultures were taken at different time intervals (t = 0, 1, 2, 3, 4, 5, 6 h) and plated on MRS agar using the spread plate method. The plates were incubated anaerobically at 37°C for 72 hours. Bile Salt Tolerance was determined by total viable count of cells on the MRS agar plates (CFU/mL) [18].

2.9 Acid Tolerance

The isolates were inoculated into 10 mL of MRS broth and incubated anaerobically at 37 °C for 20 hours. The cells were harvested by centrifuging at 2,800 x g for 15 minutes and filtered. The cells were washed thrice with PBS and then resuspended in 1 mL of phosphate buffer saline (PBS). Five mL (5 mL) of simulated gastric juice and 1.5 mL of 0.5% (w/v) NaCl was added to the resuspension. The resuspension was shaken vigorously for 20 seconds and incubated anaerobically at 37°C for 3 hours.

Aliquots (100µL) of the cultures were taken at time intervals of t = 0, 1, 2, and 3 hours and spread on MRS agar plates. The plates were incubated anaerobically at 37 °C for 72 hours. Acid Tolerance was determined by total viable count of cells on the MRS agar plates (CFU/mL) [18].

2.10 *In vitro* Evaluation of the Antimicrobial Activity of Lactic acid Bacteria

2.10.1 Antibacterial agent preparation

Lactic acid bacteria obtained were inoculated into MRS broth and incubated under anaerobic conditions for 37 °C for 24 hours. The culture was centrifuged at 2,800 x g for 15 minutes and filtered with 0.22µm filter to obtain cell free supernatant [17].

2.10.2 Sensitivity testing

The sensitivity test was performed using agar-well diffusion method. Plates of MRS Agar were aseptically prepared. Twenty four hour old of selected bacterial species each were inoculated into 20 ml of molten soft agar and dispensed into sterile petri dishes. Using 6mm cork-borer, wells were bored through the already gelled media. Hundred µL of the prepared lactic acid bacteria culture extracts were seeded into each well and incubated anaerobically at 37 °C for 48 hours. Antimicrobial activity was determined by measuring the inhibition zone diameter (in mm) as per CLSI guidelines [17].

3. RESULTS AND DISCUSSION

The growing awareness of high levels of fat and cholesterol in milk and the allergenicity of people to lactose, a component of milk, has increased the demand for plant-based sources of probiotics. The nutritional value of fruits; the high amounts of minerals, vitamins, fibre, and bioactive compounds they contain, provide the body with energy [19], and the absence of cholesterol, lactose, and several other allergens, have placed fruits at the forefront of the search for alternative probiotic sources [20].

This research aims at assessing the probiotic properties of lactic acid bacteria isolated from Avocado pear on selected bacterial species. Fifty unripe avocado pears were purchased from three different locations which include: Eke Akwa, Owerri and Ifite markets. Isolation,

characterization and identification of the LAB were carried out as shown in Tables 1, 2 and 3. The LAB obtained are *Lactobacillus casei* and *Lactobacillus plantarum* subsp *plantarum*. This is similar with the work of Filannino et al. [21], who obtained *Lactobacillus plantarum* in their sample of avocado pear, along with *Enterococcus faecalis* which was higher in prevalence as compared to the *L. plantarum*.

During digestion, probiotics have to survive in the presence of phenol, the toxic metabolite produced from the deamination of proteins in the gastrointestinal tract [18]. *L. casei* and *L. plantarum* exhibited unhindered growth at both concentrations of 0.2% and 0.5% phenol. The highest growth for the isolates was seen at 3.5% NaCl (as shown in Table 5). These findings were comparable to those by Parlindungan et al. [18] in which growth of LAB isolates was unhindered at concentrations of 0.2% phenol, but became slightly impaired at exposure to 0.5% phenol. The current findings are also comparable to the result of Tawab et al. [22] in which LAB isolates had good tolerance at 8% concentration of NaCl and also had both variable tolerance to increasing concentrations of phenol and good tolerance to 0.4% phenol.

L. casei and *L. plantarum* were viable in the presence of bile salt for 5 hours as shown in Table 6. This is in accordance with Parlindungan et al. [18] who reported similar bile salt tolerance of *L. plantarum* isolated from fermented meat. The environment of the gastrointestinal tract is acidic to prevent the passage of pathogenic microorganisms; and the bile content exhibits inhibitory action against pathogenic bacteria [23, 24]. *L. casei*, remained viable for 3 hours while *L. plantarum* declined in viability after 2 hours (as shown in Table 7). The result in this study agrees with Hossain et al. [25] who states that the viability of LAB isolates declined after 3 hours of exposure to pH level of 3.0. According to Montville and Matthews [26], mechanism of resistance of microorganisms to acidic conditions is strain and specie dependent.

One of the criteria set by the Food and Agriculture Organization (FAO) and the World Health Organization (WHO) for a bacterium to be classified as a probiotic, is its ability to produce antimicrobial substances that inhibit the growth of or kill pathogenic microorganisms [26,27]. This study examined the action of identified LAB isolates against Gram negative and Gram positive pathogens which include *E. coli*, *K*

pneumonia, *S. enterica*, *E. aerogenes*, *P. aeruginosa*, *S. flexneri*, *E. feacalis* and *S. aureus*. The inhibition zone diameter of the LAB on all the isolates (Table 8) showed that *L. casei* and *L. plantarum* had inhibitory ability against

the selected bacterial species. The study corresponds with Jannah et al. [28] where LAB isolated from the intestinal tract of chickens exhibited antimicrobial action against *Escherichia coli* and *Staphylococcus aureus*.

Table 1. Total Lactic Acid Bacteria (LAB) count

Samples	Colony count	Total count (Cfu/ml)
Eke Awka (1AV)	48	$4.32 \times 10^7 \pm 1.12 \times 10^6$
Owerri (2AV)	31	$3.38 \times 10^7 \pm 1.05 \times 10^6$
Amansea (3AV)	-	-
Ifite (4AV)	-	-

Table 2. Morphological Characteristics of the LAB

Isolate	Form	Surface	Colour	Elevation	Opacity	Gram stain	Cell shape	Suspected organism
1	Circular	Smooth	Smooth	Convex	Moist	Positive	Rod	<i>Lactobacillus casei</i>
2	Circular	Smooth	Smooth	Convex	Translucent	Positive	Rod	<i>Lactobacillus plantarum</i>

Table 3. Biochemical Characteristics of the LAB

Isolates	Catalase test	Oxidase test	Motility test	Urease test	Citrate test	Lactose	Glucose	Sucrose	Fructose
<i>L. casei</i>	-	-	-	-	+	+	+	+	+
<i>L. plantarum</i>	-	-	-	-	+	+	+	+	+

Key: += Positive reaction; - = Negative reaction

Table 4. Growth of the LAB at Different Temperatures

Isolates	15 °C	30 °C	45 °C	60 °C
<i>L. casei</i>	+	+	+	-
<i>L. plantarum</i>	+	+	-	-

Key: += growth; -= no growth

Table 5. Phenol Tolerance of the LAB

Isolate	0.2% phenol			0.5% phenol		
	1.5% NaCl	2.5% NaCl	3.5% NaCl	1.5% NaCl	2.5% NaCl	3.5% NaCl
<i>L. casei</i>	0.112 ± 0.01	0.24 ± 0.01	0.25 ± 0.01	0.270 ± 0.02	1.27 ± 0.73	2.40 ± 0.02
<i>L. plantarum</i>	0.085 ± 0.01	0.20 ± 0.02	0.220 ± 0.01	0.290 ± 0.01	1.32 ± 0.40	1.95 ± 0.07

Table 6. Bile Salt Tolerance of the LAB

Isolate	0hr (CFU/ml)	1hr (CFU/ml)	2hr (CFU/ml)	3hr (CFU/ml)	4hr (CFU/ml)	5hr (CFU/ml)	6hr (CFU/ml)
<i>L. casei</i>	$2.92 \times 10^3 \pm 1.14 \times 10^2$	$2.02 \times 10^3 \pm 1.10 \times 10^2$	$1.72 \times 10^3 \pm 1.08 \times 10^2$	$1.42 \times 10^2 \pm 0.97 \times 10^1$	$1.15 \times 10^2 \pm 1.02 \times 10^1$	$1.06 \times 10^2 \pm 0.96 \times 10^1$	TFTC
<i>L. plantarum</i>	$3.04 \times 10^3 \pm 1.15 \times 10^2$	$2.7 \times 10^3 \pm 1.4 \times 10^2$	$2.04 \times 10^3 \pm 1.02 \times 10^2$	$1.72 \times 10^3 \pm 1.3 \times 10^2$	$1.45 \times 10^2 \pm 0.92 \times 10^1$	$1.12 \times 10^2 \pm 0.25 \times 10^1$	TFTC

Key: TFTC - too few to count, NG - no growth

Table 7. Acid Tolerance of the LAB

Isolates	0hr	1hr	2hr	3hr
<i>L. casei</i>	$8.78 \times 10^2 \pm 1.57 \times 10^2$	$7.2 \times 10^2 \pm 1.37 \times 10^2$	$5.22 \times 10^2 \pm 1.32 \times 10^2$	$4.2 \times 10^2 \pm 1.07 \times 10^2$
<i>L. plantarum</i>	$6.7 \times 10^2 \pm 2.22 \times 10^2$	$4.98 \times 10^2 \pm 1.22 \times 10^2$	$2.98 \times 10^2 \pm 1.30 \times 10^2$	TFTC

Key: TFTC - too few to count; NG - no growth

Table 8. Antimicrobial Activity of the LAB against different Bacteria

Bacteria	<i>E. coli</i>	<i>K. pneumoniae</i>	<i>S. enterica</i>	<i>E. aerogenes</i>	<i>P. aeruginosa</i>	<i>S. flexneri</i>	<i>E. faecalis</i>	<i>S. aureus</i>
<i>L. casei</i>	10.25 ± 1.27	11.20 ± 2.10	9.88 ± 1.12	13.77 ± 2.21	11.78 ± 3.22	NR	8.89 ± 1.98	10.70 ± 1.35
<i>L. plantarum</i>	13.18 ± 2.33	10.38 ± 1.14	8.35 ± 1.34	9.64 ± 1.24	9.98 ± 1.42	10.32 ± 1.32	12.03 ± 2.05	13.35 ± 1.44

Lactiplantibacillus plantarum subsp. plantarum strain W9-1 16S ribosomal RNA gene, partial sequence

Sequence ID: [OR362767.1](#) Length: 1453 Number of Matches: 1

Range 1: 68 to 1094 [GenBank](#) [Graphics](#)

[▼ Next Match](#) [▲ Previous Match](#)

Score	Expect	Identities	Gaps	Strand
1897 bits(1027)	0.0	1027/1027(100%)	0/1027(0%)	Plus/Plus
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Sbjct 68	TGAGTGAGTGGCGAACTGGTGAGTAACACGTGGGAAACCTGCCAGAAAGCGGGGATAAC	127		
Query 61	ACCTGGAAACAGATGCTAATACCGCATAACAACCTGGACCGCATGGTCCGAGTTTGAAAG	128		
Sbjct 128	ACCTGGAAACAGATGCTAATACCGCATAACAACCTGGACCGCATGGTCCGAGTTTGAAAG	187		
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Sbjct 188	ATGGCTTCGGCTATCACTTTTGGATGGTCCCGCGGCGTATTAGCTAGATGGTGGGTAAC	247		
Query 181	GGCTCACCATGGCAATGATACGTAGCCGACCTGAGAGGGTAATCGGCCACATTGGGACTG	248		
Sbjct 248	GGCTCACCATGGCAATGATACGTAGCCGACCTGAGAGGGTAATCGGCCACATTGGGACTG	307		
Query 241	AGACACGGCCCAAACCTCTACGGGAGGCAGCAGTAGGGAATCTTCCACAATGGACGAAAG	308		
Sbjct 308	AGACACGGCCCAAACCTCTACGGGAGGCAGCAGTAGGGAATCTTCCACAATGGACGAAAG	367		
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Sbjct 368	TCTGATGGAGCAACGCCGCGTGAGTGAAGAAGGGTTTCGGCTCGTAAACTCTGTTGTTA	427		
Query 361	AAGAAGAACATATCTGAGAGTAACTGTTCAAGTATTGACGGTATTTAACAGAAAGCCAC	428		
Sbjct 428	AAGAAGAACATATCTGAGAGTAACTGTTCAAGTATTGACGGTATTTAACAGAAAGCCAC	487		
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Sbjct 488	GGCTAACTACGTGCCAGCAGCCGCGTAATACGTAGGTGGCAAGCGTTGTCCGGATTTAT	547		
Query 481	TGGGCGTAAAGCGAGCCAGCGGTTTTTAAAGTCTGATGTGAAAGCCTTCGGCTCAACC	548		
Sbjct 548	TGGGCGTAAAGCGAGCCAGCGGTTTTTAAAGTCTGATGTGAAAGCCTTCGGCTCAACC	607		
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Sbjct 608	GAAGAAGTGCATCGGAAACTGGGAACTTGAGTGCAGAAGAGGACAGTGGAACTCCATGT	667		
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Sbjct 728	TGTAAGTGCATCGGAAACTGGGAACTTGAGTGCAGAAGAGGACAGTGGAACTCCATGT	787		
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Sbjct 788	CCATACCGTAAACGATGAATGCTAAGTGTGGAGGGTTCCGCCCTTCAGTCTGCAGCT	847		
Query 781	AACGCATTAAGCATTCCGCCTGGGAGTACGGCGCAAGGCTGAAACTCAAAGGAATTGA	848		
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Query 961	ACAGGTGGTGCATGGTGTGCTCAGCTCGTGTGAGATGTTGGGTTAAGTCCCGCAAC	1028		
Sbjct 1028	ACAGGTGGTGCATGGTGTGCTCAGCTCGTGTGAGATGTTGGGTTAAGTCCCGCAAC	1087		
Query 1021	GAGCGCA	1027		
Sbjct 1088	GAGCGCA	1094		

Fig. 1. 16S rRNA Gene Sequence of *L. Plantarum*

Lactocaseibacillus casei strain LC130 chromosome, complete genome

Sequence ID: [CP143264.1](#) Length: 2940906 Number of Matches: 10

Range: 1: 223050 to 224220 [CentBank](#) [Graphics](#) [▼ Next Match](#) [▲ Previous Match](#)

Score	Expect	Identical	Gaps	Strand
2154 bits(1166)	0.0	1169/1171(99%)	0/1171(0%)	Plus/Plus
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Sbjct 223110	GTGGGGGATAACATTTGGAAACAGATGCTAATACCGGATAAATCCAAAGAACDGCATGGCTT			223169
Query 338	CTTGGCTGAAAGATGGCGAAGCTATCGCTTTTGGATGCAACCGCGGGCTATTAGCTAGT			389
Sbjct 223170	CTTGGCTGAAAGATGGCGAAGCTATCGCTTTTGGATGCAACCGCGGGCTATTAGCTAGT			223229
Query 398	TGGTGGCTAACGGCTCACCAAGGCGATGATAAGCTAGCGCAACTGACAGGTGATGGGCC			449
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Sbjct 223290	ACATTGGGACTGAGACACGGGCCAAMCTCCTACCGGAGGDCAGENCTAGGGAATETTCAC			223349
Query 518	AATGGACGGAAGTCTGATGGAGCAAGGCCGGCTGACTGAAGAAAGGCTTTGGGGTGGTAA			569
Sbjct 223350	AATGGACGGAAGTCTGATGGAGCAAGGCCGGCTGACTGAAGAAAGGCTTTGGGGTGGTAA			223409
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Sbjct 223410	ACTCTGTGTGAGAGAAATGGTCGGGACAGCTAACTGTTGTGGGGCTGACDGTATCCAA			223469
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Sbjct 224130	TAAAGACTGCGGGTGACAAACCGGAGGAAAGTGGGGATGACGTEAATATCATCATGCCDCTT			224189
Query 1358	ATGACCTGGGCTACACAGGCTGCTACAATGCA		1380	
Sbjct 224190	ATGACCTGGGCTACACAGGCTGCTACAATGCA		224220	

Fig. 2. 16S rRNA Gene Sequence of *L. casei*

4. CONCLUSION AND RECOMMENDATION

This research highlights the potential of avocado pear to serve as sources of lactic acid bacteria, specifically *Lactobacillus casei* and *Lactobacillus plantarum*. The results suggest that incorporating fermented fruits as a natural source of probiotics could be a beneficial alternative to traditional dairy-based probiotics, contributing to improved intestinal health and reduced incidence of gastrointestinal diseases. It is particularly significant for individuals with lactose intolerance or dairy allergies. This study lays the groundwork for future research and development of plant-based probiotics, contributing to the advancement of functional foods and therapeutic interventions.

DISCLAIMER (ARTIFICIAL INTELLIGENCE)

Author(s) hereby declare that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc.) and text-to-image generators have been used during the writing or editing of this manuscript.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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