Original Research Article

Assessment of Probiotic Properties of Lactic Acid Bacteria Isolated from Overripe Avocado Pear on selected Bacterial species

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 microorganisms that have been found to possess properties that make them suitable to be used as probiotics. Dietary supplements do contain synthetic nutrient which pose serious health effects when taken in excess. 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 using pour plate and streak plate methods respectively, on MRS agar supplemented with1.0% $CaCO_3$ at 37 ^oC for 48 hours. 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 ^oC to 60 ^oC. They exhibited tolerance to phenol ranging from 0.085±0.01% to 2.40±0.02%. Tolerance to bile salt ranged from $1.06x10^1$± $0.96x10^1$ to $3.04x10^3$±$1.15x10^2$. The acid tolerance level of the LAB obtained ranged from $2.98x10^2$±$1.30x10^2$ to $8.78x10^2$±$2.22x10^2$. 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, asides from their high nutritional content, also have the potential to serve as a 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; they 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 prevalent 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 prevent and manage gastrointestinal diseases like inflammatory bowel disease and diarrhoea. 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 because they are unable to survive and proliferate in fruits. This poses a challenge to the use of fruit as a source of probiotic bacteria.

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

2.0 MATERIALS AND METHOD

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 – Febuary, 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 processing and analysis [9].

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 adding 1g of crushed avocado pear into 10ml of sterile normal (w/v) saline solution in a conical flask. The flask was vigorously shaken to mix the contents. Ten-fold serial dilution was done to reduce the concentration of microorganisms in the samples. Using pour plate method, aliquots (100µL) of the samples at dilution 10^{-4} were introduced into sterile petri dish. Already prepared MRS agar supplemented with 1.0% $CaCO_3$ was poured into the plate, swirled and allowed to gel. The

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 [10].

2.4 Identification of isolates

The isolates were identified using standard methods which include; Colony morphological, Gram staining, Catalase, Motility [11], Urease Test [12], Citrate utilization, Oxidase tests [13], sugar fermentation tests [14] and Nucleic acid sequence analysis [15].

2.5 Growth at Different Salt Concentrations

The isolates were inoculated on MRS agar plates supplemented with NaCl concentrations of 1.5%, 3.5%, 5.5%, 7.5%, 9.5%, and 10.5% (w/v) respectively and incubated anaerobically at 37 ºC for 3 days. Growth at different salt concentrations was determined by measuring the optical density at 590nm (OD590) [16].

2.6 Growth at Different Temperatures

The isolates were inoculated into 10 mL of MRS broth and incubated under anaerobic conditions at 15 ºC, 30 ºC, 45 ºC, and 60 ºC for 72 hours. Growth was determined by the formation of sediments at the bottom of the test tube [16].

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 37ºC for 48 hours. Growth of LAB in the culture broth was determined by measuring the optical density (OD590) [17].

2.8 Bile Salt Tolerance

The isolates were inoculated into 10mL of MRS broth and incubated anaerobically at 37ºC 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 37ºC for 3 hours. Aliquots (100µl) 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 37ºC for 72 hours. Bile Salt Tolerance was determined by total viable count of cells on the MRS agar plates (CFU/mL) [17].

2.9 Acid Tolerance

The isolates were inoculated into 10 mL of MRS broth and incubated anaerobically at 37 ºC for 20 hours. The cells were harvested by centrifuging at 2,800 x g for 15 minutes and filtering. 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 37ºC 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 ºC for 72 hours. Acid Tolerance was determined by total viable count of cells on the MRS agar plates (CFU/mL) [17].

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 ^{o}C for 24 hours. The culture was centrifuged at 2,800 x g for 15 minutes, 0.22μm filter was used for filtration to obtain cell free supernatant [16].

2.10.2 Sensitivity testing

The sensitivity test was conducted using agar-well diffusion method. Plates of MRS Agar were aseptically prepared. Twenty four hour old of some 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 wells. The wells were incubated anaerobically at 37 ^{o}C for 48 hours. Antimicrobial activity was determined by measuring the inhibition zone diameter (in mm) [16].

3.0 RESULTS AND DISCUSSION

The growing awareness of the 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 [18], and the absence of cholesterol, lactose, and several other allergens, have placed fruits at the forefront of the search for alternative probiotic sources [19].

This research aims at assessing the probiotic properties of lactic acid bacteria isolated from Avocado pear on some 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 table 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*. [20], 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*.

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	*Lactobacillus casei*

2	Circular	Smooth	Smooth	Convex	Translucent	Positive	Rod	*Lactobacillus plantarum*

Table 3. Biochemical Characteristics of the LAB

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

Key: += Positive reaction; - = Negative reaction

Table 4. Growth of the LAB at Different Temperatures

Isolates	15 ^{o}C	30 ^{o}C	45 ^{o}C	60 ^{o}C
L. casei	+	+	+	-
L. plantarum	+	+	-	-

Key: += growth; -= no growth.

During digestion, probiotics have to survive in the presence of phenol, the toxic metabolite produced from the deamination of proteins in the gastrointestinal tract [17]. *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*. [17] 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*. [21] 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.

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
L. casei	0.112 ± 0.01	0.24 ± 0.01	0.25 ± 0.01	0.270 ± 0.02	1.27 ± 0.73	2.40 ± 0.02
L. plantarum	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)
L. casei	2.92 x 10^{3}±	2.02 x 10^{3}±	1.72 x 10^{3}±	1.42 x 10^{2}±	1.15 x 10^{2}±	1.06 x 10^{2}±	TFTC

	1.14 x 10^2	1.10 x 10^2	1.08 x 10^2	0.97 x 10^1	1.02 x 10^1	0.96 x 10^1	
L.	3.04 x 10^3±	2.7 x 10^3±	2.04 x 10^3±	1.72 x 10^3±	1.45 x 10^2±	1.12 x 10^2±	TFTC
plantarum	1.15 x 10^2	1.4 x 10^2	1.02 x 10^2	1.3 x 10^2	0.92 x 10^1	0.25 x 10^1	

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

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*. [17] 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 [22; 23]. *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*. [24] 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 [25], mechanism of resistance of microorganisms to acidic conditions is strain and specie dependent.

Table 7. Acid Tolerance of the LAB

Isolates	**0hr**	**1hr**	**2hr**	**3hr**
L. casei	8.78 x 10^2± 1.57 x 10^2	7.2 x 10^2± 1.37 x 10^2	5.22 x 10^2± 1.32 x 10^2	4.2 x 10^2± 1.07 x 10^2
L. plantarum	6.7 x 10^2± 2.22 x 10^2	4.98 x 10^2± 1.22 x 10^2	2.98 x 10^2± 1.30 x 10^2	TFTC

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

One of the criteria set by the Food and Agriculture Organization (FAO) and the World Health Organization (WHO) for a bacteria to be classified as a probiotic, is its ability to produce antimicrobial substances that inhibit the growth of or kill pathogenic microorganisms [26]. 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 (tables 8) showed that *L. casei* and *L. plantarum* had inhibitory ability against the selected bacterial species. The study corresponds with Jannah *et al*. [27] where LAB isolated from the intestinal tract of chickens exhibited antimicrobial action against *Escherichia coli* and *Staphylococcus aureus*.

Table 8. Antimicrobial Activity of the LAB against Several Bacteria

Bacteria	***E. coli***	***K. pneumoniae***	***S. enterica***	***E. aerogenes***	***P. aeruginosa***	***S. flexneri***	***E. faecalis***	***S. aureus***
L. casei	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
L. plantarum	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

4.0 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.

REFERENCES

1) Zielinska D, Kolozyn-Krajewska D. Food-Origin Lactic Acid Bacteria May Exhibit Probiotic Properties: Review. BioMed Res Intl 2018(5063185). *https://doi.org/10.1155/2018/5063185*
2) Latif A, Shehzad A, Niazi S, Zahid A, Ashraf W, Iqbal MW, *et al*. Probiotics: mechanism of action, health benefits and their application in food industries. *Front Microbiol*. 2023;14(1216674).
3) Samanta S. Potential impacts of prebiotics and probiotics on cancer prevention. Anti-Cancer Agents Med Chem. 2022;22(4): 605–28.
4) Thakkar PN, Modi HA, Prajapati J. Therapeutic impacts of probiotics - as magic bullet. Am J Biomed Sci. 2016;8:97–13.
5) Ankutse P, Kumadoh D, Adase E, AAmeho S. Insights into probiotic fermentation: a promising future for mankind. SSRN Elect J. 2022;10
6) Kaur S, Kaur R, Rani N, Sharma S, Joshi M. Sources and selection criteria of probiotics. *In* G. Goel and A. Kumar (Edn), *Advances in Probiotics as Sustainable Food and Medicine*. Singapore: Springer Nature Singapore Pte Ltd. 2020.
7) Rasika DMD, Vidanarachchi JK, Luiz SF, Azeredo DRP, Cruz AG, Ranadheera CS. Probiotic delivery through non-dairy plant-based food matrices. Agriculture. 2021; 11(599). *https://doi.org/10.3390/agriculture11070599*
8) Min M, Bunt CR, Mason SL, Malik A, Hussain MA. Non-dairy probiotic food products: An emerging group of functional foods. Crit Rev Food Sci Nutrition, 2018;10(2): 3-10.
9) Dhafin AA, Putri EM, Muslikh PA, Prasetyawan F. Total plate count test for bacteria in home industry baby porridge in the Malang City Area. Contemp J Appl Sci. 2023;1(1):25-2.
10) Savitri M, Kumar V, Kumari A, Angmo K, Bhalla TC. Isolation and characterization of lactic acid bacteria from traditional pickles of Himachal Pradesh, India. J Food Sci Technol. 2017;54(7): 1945-52.
11) Ismail YS, Yulvizar C, Mazhitov B. Characterization of lactic acid bacteria from local cow´s milk kefir. IOP Conference Series: Earth and Environmental Science, 2018;130(1): 1315-55.
12) Dahlén G, Hassan H, Blomqvist S, Carlen A. Rapid urease test (RUT) for evaluation of urease activity in oral bacteria in vitro and in supragingival dental plaque ex vivo. *BMC Oral Health*, 2018;10(18): 541-43.

13) Ngene AC, Onwuakor CE, Aguiyi JC, Ifeanyi VO, Ohaegbu CG, Okwuchukwu CP, Egbere JO. Screening of Some Lactic Acid Bacteria Isolated from Selected Nigerian Fermented Foods for Vitamin Production. Advances Microbiol. 2019;9(11): 943-955.
14) Mamta T, Deshpande HW, Bhate MA. Isolation and identification of lactic acid bacteria and their exploration in non-dairy probiotic drink. Intl J Curr Microbiol Appl Sci. 2017;6(4):1023-1030.
15) Mariyam D, Preetha JS. The evolution of DNA extraction methods. American J Biomed Sci Res. 2020;8(1): 17-47.
16) Banik A, Anjum H, Habib H, Ahmed Z. Characterization of lactic acid bacteria from street pickles of Dhaka, Bangladesh. Heliyon, 2023;9(7): e17508.
17) Parlindungan E, Lugli GA, Ventura M, Sinderen D, Mahony J. Lactic Acid Bacteria Diversity and Characterization of Probiotic Candidates in Fermented Meats. Foods. 2021;10, 1519. https://doi.org/10.3390/foods10071519
18) Amao I. Health benefits of fruits and vegetables: Review from sub-Saharan Africa. In *Vegetables* - Importance of Quality Vegetables to Human Health. *IntechOpen*.2018.
19) Santhosh K, Devi OA, Anto RA, Yadav A. Probiotic foods: Future prospects. *Frontiers in Food Science & Nutrition*. Academic Publishers and Distributors.2023;49-61.
20) Filannino P, Tlaisb AZA, Morozovab K, Cavoskic I, Scampicchio M, Gobbetti M, Di Cagno R. Lactic acid fermentation enriches the profile of biogenic fatty acid derivatives of avocado fruit (*Persea americana* Mill.). Food Chem. 2020;317(126384).
21) Tawab FIA, Abd Elkadr MH, Sultan AM, Hamed EO, El-Zayat AS, Ahmed MN. Probiotic potentials of lactic acid bacteria isolated from Egyptian fermented food. Scientific Reports. 2023;13(16601), *https://doi.org/10.1038/s41598-023-43752-0*
22) Barbieri F, Montanari C, Gardini F, Tabanelli G. Biogenic amine production by lactic acid bacteria: a review. Foods. 2019;8:17.
23) Sun R, Xu C, Feng B, Gao X, Liu Z. Critial roles of bile acids in regulating intestinal mucosal immune responses. Therap Adv Gastroenterol. 2021;14, 17562848211018098. https://doi.org/10.1177/17562848211018098
24) Hossain MI, Kim K, Mizan FR, Toushik SH, Ashrafudoulla M, Roy PK, *et al*. Comprehensive molecular, probiotic, and quorum-sensing characterization of anti-listerial lactic acid bacteria, and application as bioprotective in a food (milk) model. J Dairy Sci. 2021;104: 6516-34.
25) Montville TJ, Matthews KR. Physiology, growth, and inhibition of microbes in foods (pp. 3–18). In Food Microbiology. Am Soc Microbiol. 2013. https://doi.org/10.1128/9781555818463.ch1
26) Binda S, Hill C, Johansen E, Obis D, Pot B., Sanders, M. E, et al. Criteria to qualify microorganisms as "probiotic" in foods and dietary supplements. Front Microbiol. 2020;11(1662).
27) Jannah SN, Saraswati TR, Handayani D, Pujiyanto S. Antibacterial activity of lactic acid bacteria isolated from gastrointesstinal tract of "Ayam Kampung" chicken against food pathogens. *J Phys: Conference Series*, 2018;1025(1):1742-96 .