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Assessment of Probiotic Activities of Lactic Acid Bacteria Isolated from Cassava Mill Effluent in Awka Metropolis

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Abstract

Lactic Acid Bacteria (LAB) are renowned for their probiotic benefits, including immune function enhancement, gut microbiota balance, and prevention of gastrointestinal disorders. While traditionally sourced from fermented foods and dairy products, environmental niches have emerged as potential reservoirs for novel LAB strains. This study assessed the probiotic potential of LAB strains isolated from Cassava Mill Effluent (CME) in Awka metropolis. LAB strains were isolated from CME samples using MRS agar supplemented with 1.0% CaCO3 and incubated micro-aerophilically at 37oC for 48 hours. The isolates were identified based on microscopic, macroscopic, biochemical, and molecular characteristics. Probiotic properties, including acid tolerance, bile salt tolerance, phenol tolerance, salt concentration tolerance, temperature tolerance, and antimicrobial activities, were evaluated. The LAB counts ranged from 5.45 x 104 ± 1.11 cfu/ml to 9.93 x 107 ± 1.87 cfu/ml. The isolates were identified as *Lactococcus lactis*, *Lactobacillus acidophilus*, and *Lactobacillus gallinarum*. *L. lactis* and *L. acidophilus* exhibited acid tolerance, remaining viable for 3 h, while *L. gallinarum* was sensitive to acid conditions. All isolates showed bile salt tolerance, phenol tolerance, and temperature tolerance. The LAB strains demonstrated antimicrobial activities, attributed to bacteriocin production, against *E. coli, K. pneumonia, S. aureus, E. aerogenes*, and *E. faecalis*. The findings suggest that CMF can serve as a natural reservoir for LAB strains with probiotic potential, useful for reclamation of microbiota balance in the human gut and intestines, promoting health and preventing diseases.

Keywords: Probiotics; lactic acid bacteria; Cassava mill effluent; antimicrobial activity.

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Introduction

Probiotics are live microorganisms which when consumed in adequate amounts provide health benefits to the host (Anwar *et al*. 2021). They are symbiotic microorganisms that generally reside in the gastrointestinal tract of the hosts which have beneficial effect. Probiotic cultures have been widely used in food, medical treatments, animal feed, etc (Naray and Surender, 2019).

Live organisms used as probiotics are mostly lactic acid producing bacteria. They cause rapid acidification of the raw material through the production of organic acids, mainly lactic acid. Lactic acid bacteria have a long history of application in fermented foods because of their beneficial influence on nutritional, organoleptic, and shelf- life enhancements (Sanlier *et al*. 2019).Their ability to enhance gut health, modulate the immune system, prevent allergies, and exert antimicrobial effect makes them valuable in both clinical and everyday dietary applications. Lactic acid bacteria help maintain a healthy balance of gut microbiota, which is crucial for proper digestive function. They can inhibit the growth of harmful bacteria by producing substances like lactic acid, hydrogen peroxide and bacteriocin (Rijkers *et al*. 2010). They can enhance the body's immune response by simulating the activity of macrophages, increasing the levels of cytokines and improving the intestinal barrier (Lucano *et al*. 2011).

For probiotic bacteria to be effective they must possess a number of specific properties. Such property is the ability to survive in acidic and bile-containing media as they have to undergo these conditions during their passage through the gastrointestinal tract.The beneficial effects of probiotics on human health among other properties include to show antibacterial behaviours towards pathogenic strains, either by producing antimicrobial agents (bacteriocins, organic acids, etc) or by reducing the adhesion of pathogenic bacteria (Gareau *et al*. 2010). Supplementation of probiotic products can improve feed efficiency and growth rate and reduce intestinal infections in various animals (Wang *et al*. 2019). There is a need for improved global healthy microbiota for several reasons which include enhanced immune function, prevention and management of immune disease, enhanced digestion and nutrients absorption and antibiotics resistance prevention.

Cassava (*Manihot esculenta crantz*) is a drought tolerant, staple food crop grown in tropical and subtropical areas where many people are afflicted with under-nutrition making it a potentially valuable food source in developing countries. Cassava is cultivated in the tropical and subtropical regions as an annual crop for its edible starchy root tuber, a major source of carbohydrate, and may be the main valuable root crop in terms of overall output and cultivated area (Ano, 2003).

Cassava effluent is the milky colloid pressed out of the fresh tuber paste (the latex and the wash water) generated during the processing of cassava roots into various products such as starch, garri, flour, and other fermented foods (Nwaugo *et al.* 2008,). It is a byproduct that is rich in starch, glucose, carbohydrates, proteins, lipids, and minerals (iron, magnesium), enabling its use as a low-cost culture medium for biotechnological processes. In cassava waste water fermentation, different consortia of organisms are involved. Among them can be the lactic acid producing bacteria. Therefore, this research assessed the probiotic activities of lactic acid bacteria isolated from cassava mill effluent in Awka metropolis.

Materials and Methods

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.

Specimen Collection

Samples of cassava mill effluent were collected from five different garri processing plants within Awka metropolis which include Amansea, Ifite-Awka, Nkwelle, Amaenyi and Amachalla. The samples were collected directly from the outlet pipe of garri processing plant and transported to the Laboratory Unit of Department of Applied Microbiology and Brewing, Nnamdi Azikiwe University, Awka, Anambra State in an air tight sterile container.

Sample Treatment

The cassava waste water samples were filtered to remove debris and solid particles. They were allowed to stand for suspended solids to settle at the bottom and later separated.

Isolation of Lactic Acid Bacteria

Hundred milliliters of the representative samples was dispensed in a conical flask covered with a cotton plug and then incubated for 5 days at 37°C using a laboratory shaker. Stock solution of the cassava effluents were prepared by adding 1ml of each sample into 9ml of sterile water. Ten-fold serial dilution was done to reduce the microbial load of the sample (Obiefuna *et al*., 2025a). Using pour plate method, aliquots (0.1ml) of the sample at dilution 10^{-4} was inoculated on MRS agar plates and incubated micro-aerophilically at 37°C for 48hrs (Savitri *et al.* 2017).The plates were observed for growth colonies and counted. Discrete colonies were selected and sub cultured onto plates of MRS agar using streak plate method to obtain pure cultures (Iheukwumere *et al*., 2025a; Iheukwumere *et al.*, 2025b). The pure cultures were stored at 4 °C on MRS agar in bijou bottles.

Identification of Isolates

The isolates were identified using standard methods which include; Colony morphological (Ekechukwu *et al*., 2025a), Gram staining, other biochemical tests (Ekechukwu *et al.*, 2025b) and Nucleic acid sequence analysis (Ekechukwu *et al*., 2025c).

Determination of the Probiotic Properties of the Isolates

The method of Parlindungan *et al*. (2021) was used to determine the probiotic activities of the isolates.

Acid Tolerance Test

The isolates were inoculated into 10 ml of MRS broth and incubated micro-aerophilically 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 re-suspended in 1ml of phosphate buffer saline (PBS). Five ml (5ml) 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 micro-aerophilically 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 (Obiefuna *et al*., 2025b). Acid tolerance was determined by total viable count of cells on the MRS agar plates (CFU/ml).

Bile Salt Tolerance Test

Isolates were inoculated into 10ml of MRS broth and incubated at 37ºC for 20 hours. The cultures were centrifuged at 2800x g for 15 minutes and filtered to obtain the bacteria cells. The isolate was washed twice with PBS, re-suspended in MRS broth supplemented with 0.3% (w/v) bile salt, and incubated at 37ºC for 3 hours. Aliquots (100µl) of the test culture was taken at regular time intervals (t = 0, 1, 2, 3 h) and spread on MRS agar plates. The plates were incubated at 37ºC for 72 hours to determine total viable cell count (CFU/mL).

Phenol Tolerance Test

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). 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).

Growth at Different Salt Concentrations

The method of Banik *et al*. (2023) was used. 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).

Growth at Different Temperatures

Using the method of Banik *et al*. (2023), the Isolates were inoculated into 10mL of MRS broth and incubated under anaerobic conditions at 15ºC, 30ºC, 45ºC, and 60ºC for 72 hours. Growth was determined by the formation of sediments at the bottom of the test tube (Obiefuna *et al.*, 2025c).

Antimicrobial Activity of the Lactic Acid Bacteria

Preparation of the antibacterial agent

Isolates of lactic acid bacteria obtained were inoculated into MRS broth and incubated under anaerobic conditions at 37ºC for 48 hours. The culture was centrifuged at 2800x g for 15 minutes and filtered with 0.22µm pore-sized filter to obtain cell free supernatant (Banik *et al*. 2023).

Sensitivity testing using the agar-well diffusion method

Plates of MRS Agar were aseptically prepared. Twenty four hours old of bacteria isolates (*E. coli, K. pneumonia, S. aureus, E. aerogenes, E. faecalis*) each were inoculated into 20 ml of molten soft agar and dispensed into sterile petri dishes. Using 6mm cork-borer, wells were bored on the already gelled media. Hundred micro liters (100µl) 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) (Banik *et al*. 2023).

Results and Discussion

Five samples of Cassava mill effluent collected from various mill in Awka metropolis were assessed for the presence of lactic acid bacteria (LAB). LAB growths were observed on three samples from Amansea, Ifite-Awka and Nkwelle while samples from Amenyi and Amachalla showed no growth (Table 1). LAB counts obtained from the effluent samples ranged from 5.45 x 10^4 ± 1.11 to 9.93 x 10^4 ± 1.87 cfu/ml (Table 1). The counts found out in this research agreed with the findings of Atta *et al.* (2020) who reported similar counts from cassava mill effluent from the production of garri in their work Antimicrobial activity of lactic acid bacteria isolated from garri on Escherichia coli strains isolated from clinical and environmental samples. Inability of cassava effluents from Amenyi and Amachalla to grow LAB may be due to treatments given to the tubers there before processing, such as washing tubers with detergents and some antiseptics. Morphological and biochemical characterization of the three isolates showed probable organisms to be *Lactococcus lactis*, *Lactobacillus acidophilus* and *Lactobacillus gallinarum* (Tables 2, 3 and 4*)*. This result agrees with previous reports by Atta *et al.* (2020) who reported that 20% of Lactic acid bacteria (LAB) isolated from cassava mill effluent from the production of garri was identified as *Lactobacillus acidophilus*. Similarly Hutajulu *et al*. (2021) in their study isolated *Lactococcus lactis with* proven probiotic activity from fermented cassava obtained from Sumba, East Nusa Tenggara, Indonesia.

For probiotic bacteria to survive, grow, and perform their beneficial action efficiently, they should be able to withstand certain conditions including acidic, bile salt and phenolic environment, as these are the conditions they would encounter during their passage through the gastrointestinal tract (GIT). *Lactococcus lactis* and *Lactobacillus acidophilus* exhibited significant acid tolerance, maintaining high viability throughout the 3hrs assay period. This gradual decline indicates a high resilience to acidic conditions, suggesting that both strains are capable of surviving the acidic environment of the gastrointestinal tract and the stomach, which is essential for effective probiotic function. In contrast, *Lactobacillus gallinarum* showed poor acid tolerance. Its viability declined rapidly within the first hour and showing no growth thereafter. This indicates that *Lactobacillus gallinarum* is highly sensitive to acidic conditions as shown in Table 5. Previous studies have also reported that LAB strains exhibit varying degrees of acid tolerance as recorded by Shehata *et al. (*2021).

The LAB isolates were observed for their tolerance to bile salt as shown in Table 6. The results revealed distinct tolerance patterns for each strain. *Lactococcus lactis* and *Lactobacillus acidophilus* exhibited moderate levels of tolerance while *Lactobacillus gallinarum* displayed the highest tolerance to bile salts among the three strains. Remarkably, it exhibited a slight increase in viability. These results are consistent with related studies by Koll *et al.* (2008), who reported that test strains of *Lactobacillus* were tolerant to bile concentrations of 0.3%. Similarly, Abriomiel *et al*. (2012) reported that all LAB isolated from fermented olives were able to grow and survive at a 0.3% bile salt concentration, as opposed to a 0.5% concentration. Additionally, it has been reported that *Lactobacillus* strains can thrive in a 0.3% bile salt environment, in contrast to a 0.5% bile condition, where poor tolerance was recorded (Jose *et al*. 2015).

The LAB exhibited a decline in survival as phenol concentration increased, although with varying degrees of tolerance. Notably, *Lactococcus lactis* demonstrated exceptional resistance to higher phenol concentrations, suggesting its potential adaptability to environments rich in this compound as shown in Table 7. The current findings are comparable to the result of Tawab *et al*., (2023) 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.

In the present study, all the LAB strains demonstrated tolerance to salt with varying concentrations. *Lactobacillus gallinarum* demonstrated the highest tolerance as shown in Table 8, while *Lactobacillus lactis* exhibited the lowest tolerance to salt as the concentration reached 10.5%. This is consistent with Gonzales *et* al., (2017) who stated that *Lactobacillus lactis* has less tolerance for salt except under exposure to stress such as mild heat, hydrogen peroxide and ethanol.

The ability of LAB isolates to survive within a temperature range of 25-40°C, similar to the human body temperature, as well as other environment is a critical criterion for probiotic viability. All LAB strains exhibited unhindered growth at 15°C and 30°C. *Lactobacillus acidophilus* and *Lactobacillus gallinarum* remained viable at 45°C, showing moderate heat tolerance. This study is in agreement with previous studies by Makzum *et al.*, (2023) who reported that LAB strains were able to tolerate a temperature of 45°C. In addition, Pundir *et* *al.*, (2013) reported that LAB isolated from food samples could withstand temperatures up to 40°C.

The ability to inhibit pathogenic bacteria is a key characteristic of probiotics. This antimicrobial or antagonistic activity involves several mechanisms, including the production of antimicrobial compounds such as bacteriocins, competitive exclusion of pathogens, enhancement of the intestinal barrier function, and stimulation of the host's immune system to effectively combat pathogens (Fijan (2023).

In this study, crude bacteriocin produced by the identified LAB isolates were tested against some selected pathogenic bacteria species which include *Escherichia coli, Klebsiella pneumonia*, *Staphylococcus aureus*, *Enterobcteraerogenes and Enterococcus feacalis.* The inhibition zone diameter of the bacteriocin produced by the LAB isolates reveals that *Lactococcus lactis*, *Lactobacillus acidophilus* and *Lactobacillus gallinarum* had inhibitory ability against the selected bacterial species. The result as shown in Table 10 showed varying degrees of efficacy of the LAB strains against the various pathogens. The study corresponds with the studies of Atta *et al.* (2020) who reported *Lactobacillus acidophilus* isolated from garri had a significant inhibitory activity against *Escherichia coli*. Rubak *et al.* (2023) also reported moderate inhibitory activity of *Lactobacillus lactis* isolated from Sui wu against *Staphylococcus typhimurium*, *Escherichia coli* and *Staphylococcus aureus*.

Table 1: Total Lactic acid bacteria count

Samples	**Total lactic acid bacteria count (Cfuml^{-1})**
Amansea 1	NG
Amansea 2	9.93 x 10^7 ± 1.87
Ifite-Awka	5.45 x 10^7 ± 1.11
Nkwelle 1	NG
Nkwelle 2	7.00 x 10^7 ± 1.05
Amaenyi	NG
Amachalla	NG

Key: NG= no growth

Table 2: Morphological Characteristics of the Various Lactic Acid Bacteria Isolates.

Isolate	Form	Surface	Colour	Margin	Elevation	Opacity	Gram reaction	Cell shape	Suspected organism
1	Circular	Smooth	Greyish/ colourless	Entire	Convex	Translucent	+	Cocci	*Lactococcus lactis*
2	Circular	Shiny	White	Lobate	Convex	Moist	+	Rod	*Lactobacillus acidophilus*
3	Circular	Irregular	Greyish	Lobate	Low convex		+	Rod	*Lactobacillus gallinarum*

Table 3: Biochemical Characteristics of the Various Lactic Acid Bacteria Isolates.

Isolates	Catalase	Motility	Indole	MR	VP	Citrate	Oxidase	Urease
Lactococcus lactis	-	-	-	+	-	-	-	-
Lactobacillus acidophilus	-	-	-	-	+	+	+	-
Lactobacillus gallinarum	-	+	-	+	-	+	-	-

Key: Gram: MR: Methyl-red, **VP**: Voges-Proskauer,

Table 4: Sugar Fermentation Test of the Various Lactic Acid Bacteria Isolates

Isolates	Lactose	Glucose	Sucrose	Fructose	Maltose
Lactococcus lactis	+	+	-	+	+
Lactobacillus acidophilus	+	+	+	+	-
Lactobacillus gallinarum	+	+	+	+	+

Table 5: Acid Tolerance of the isolates determined as total viable cells on MRS agar for 3 hours

Isolates	0 hr (CFU/ml)	1 hr (CFU/ml)	2 hr (CFU/ml)	3 hr (CFU/ml)
Lactococcus lactis	4.5 x 10^2 ± 0.50	4.2 x 10^2 ± 0.33	3.0 x 10^2 ± 0.11	2.98 x 10^2 ± 0.92
Lactobacillus acidophilus	6.5 x 10^2 ± 0.35	5.0 x 10^2 ± 1.20	4.0 x 10^2 ± 0.20	3.8 x 10^2 ± 0.24
Lactobacillus gallinarum	3.2 x 10^2 ± 0.53	TFTC	NG	NG
P value	0.030	0.10	0.30	0.47

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

Table 6: Bile Tolerance of the isolated LAB at different bile concentrations

Isolates	0hr (CFU/ml)	1hr (CFU/ml)	2hr (CFU/ml)	3hr (CFU/ml)	4hr (CFU/ml)	5hr (CFU/ml)	6hr (CFU/ml)
Lactococcuslactis	1.42 x 10^3 ± 0.33	1.05 x 10^3 ± 0.34	8.25 x 10^2 ± 1.21	5.02 x 10^2 ± 0.70	4.0 x 10^2 ± 1.21	NG	NG
Lactobacillus acidophilus	1.70 x 10^3 ± 0.66	1.45 x 10^3 ± 0.44	1.42 x 10^3 ± 0.78	1.23 x 10^3 ± 0.75	NG	NG	NG
Lactobacillus gallinarum	2.03 x 10^3 ± 0.72	2.04 x 10^3 ± 0.76	1.90 x 10^3 ± 0.99	1.52 x 10^3 ± 0.65	6.24 x 10^2 ± 1.25	5.23 x 10^2 ± 0.83	NG

Key: NG= no growth

Table 7: Phenol Tolerance Assay of the isolated LAB

Isolates	0.2% phenol			0.5% phenol		
	1.5% NaCl	2.5% NaCl	3.5% NaCl	1.5% NaCl	2.5% NaCl	3.5% NaCl
Lactococcus lactis	0.11 ± 0.01	0.20 ± 0.01	0.23 ± 0.11	1.1 ± 0.28	1.93 ± 0.13	2.42 ± 0.32
Lactobacillus acidophilus	0.25 ± 0.10	0.195 ± 0.12	0.75 ± 0.12	0.08 ± 0.01	0.16 ± 0.01	0.20 ± 0.01
Lactobacillus gallinarum	0.07 ± 0.01	0.072 ± 0.01	0.20 ± 0.15	0.12 ± 0.05	1.32 ± 0.52	1.60 ± 0.34

Table 8: Growth of the LAB isolates at varying Salt Concentration measured at Optical Density 590nm

Isolates	1.5% NaCl	3.5% NaCl	5.5% NaCl	7.5% NaCl	9.5% NaCl	10.5% NaCl
Lactococcus lactis	1.802 ± 0.22	1.689 ± 0.45	1.340 ± 0.33	0.775 ± 0.24	0.522 ± 0.47	0.211 ± 0.34
Lactobacillus acidophilus	1.942 ± 0.55	1.922 ± 0.47	1.721 ± 0.78	1.571 ± 0.23	0.722 ± 0.32	0.672 ± 0.42
Lactobacillus gallinarum	2.057 ± 0.72	1.887 ± 0.25	1.922 ± 0.25	1.321 ± 0.23	0.770 ± 0.34	0.700 ± 0.50
P value	0.402	0.440	0.512	0.871	0.232	0.224

Table 9: Temperature Tolerance of the isolated LAB grown

Isolates	15 ^{O}C	30 ^{O}C	45 ^{O}C	60 ^{O}C
Lactococcus lactis	G	G	NG	NG
Lactobacillus acidophilus	G	G	G	NG
Lactobacillus gallinarum	G	G	G	NG

key:+: growth**, -:** no growth

Table 10: Antimicrobial Activity Screening of the crude bacteriocin on various pathogenic Gram positive and Gram negative isolates

Bacteriocin	*E. coli*	*K. pneumonia*	*S. aureus*	*E. aerogenes*	*E. faecalis*
Lactococcus lactis	NR	11.05 ± 1.27	12.77 ± 1.87	NR	10.52 ± 0.52
Lactobacillus acidophilus	11.80 ± 2.15	12.38 ± 2.35	NR	13.05 ± 1.19	9.83 ± 1.04
Lactobacillus gallinarum	14.15 ± 1.17	10.38 ± 1.18	11.98 ± 2.07	12.82 ± 1.77	13.23 ± 2.01
P value	0.382	0.311	0.110	0.125	0.042

Conclusion

This research demonstrates the probiotic potential of LAB strains isolated from cassava mill effluent specifically *Lactococcus lactis, Lactobacillus acidophilus* and *Lactobacillus gallinarum*. The study reveals that cassava mill effluent can be a natural reservoir for the isolation of LAB with probiotic potentials for reclamation of microbiota balance in the human gut and intestines necessary for preventing certain diseases and maintaining good health. Further research on these probiotics is therefore highly recommended especially for their potency in solving current emerging lifestyle diseases, their purification and formulation

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