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Antimicrobial activity of lactic acid bacteria isolated from garri on *Escherichia coli* strains isolated from clinical and environmental samples

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Abstract. *The production of bacteriocins by lactic acid bacteria affords them the ability to inhibit the growth of bacteria; they are particularly important in the biocontrol of human and plant pathogens. Lactic acid bacteria have been frequently isolated from fermented foods due to the high acidity these foods contain. In this study, lactic acid bacteria were isolated from garri, a popular Nigerian staple food, which is fermented from cassava, and their antagonistic activity against clinical and environmental isolates of Escherichia coli was determined. The species of Lactobacillus isolated include: Lactobacillus plantarum (50%), Lactobacillus fermentum (20%), Lactobacillus acidophilus (20%), and Lactobacillus salivarius (10%). Growth inhibition of the strains of E.coli was observed in Lactobacillus plantarum that inhibited the growth of both. The clinical and environmental isolates of E. coli were inhibited by Lactobacillus plantarum, while Lactobacillus acidophilus showed activity against only the clinical isolate. The greatest zone of inhibition against the strains of E. coli was recorded by Lactobacillus acidophilus (22.7±1.53 mm). The bacteriocins produced by Lactobacillus species have a good potential in the biocontrol of pathogens, and should be the focus of further studies on antibiotic resistant bacteria.*

Keywords: antibacterial activity, biocontrol, cassava, fermented food, inhibition, lactobacillus species

Introduction

Garri is a traditional cassava flakes; in simple terms it is flour made from cassava (*Mannihot esculenta*) that is popular among the people of West Africa (Akindele et al., 2018). The production involves peeling of the cassava tuber, soaking in water for about 6 days followed by grinding into a pulp. Afterwards, the pulp is placed in a spongy bag under compression in order to strain out the water content before frying in a calabash-like pot (Adeyemo et al., 2018). The resulting flake from frying is known as Garri which can be used in processing various food delicacies as well as taken raw like cornflakes. This ready-to-eat property of Garri makes it a widely acceptable quick-fix meal (Orji et al., 2016). It should be noted that during the process of manufacturing, the pulp gets exposed to microbiological contamination. However, during the fermentation period, lactic acid bacteria involved in the natural fermentation is suggested to have antimicrobial properties that lower counts of the possible pathogenic bacterial contaminant (Adeyemo et al., 2018).

Lactic acid bacteria are a group of gram positive, catalase negative, non-spore forming and non-motile bacteria that produces lactic acid as a major end product of glucose fermentation. They are widely grouped into three: obligate homofermentative (for example *Lactobacillus acidophilus, Lactobacillus salivarus*), facultative heterofermentative (for example *Lactobacillus plantarum, Lactobacillus curvatus*) and obligate heterofermentative (*Lactobacillus fermentum, Lactobacillus reuteri*) based on their sugar fermentation profile (Buddhiman et al., 2008). Consequently, within LAB group, *Lactobacillus* have been identified as one of the most important in traditional fermented food that are mainly used in functional food production and as a starter culture in food fermentation. They are able to utilize the nutrient in the food matrix for fermentation, producing byproducts such as organic acids, aromatic compounds and antimicrobial peptides termed bacteriocin that limits growth of pathogenic organisms (Mamta et al., 2017).

Although there are several pathogenic bacteria which are food contaminants (Frantamico et al., 2007) that can also be found in garri, *Escherichia coli* is of great concern (Fossi and Ndjouenkeu, 2017). This is due to the fact that one of the most important causative agents of diarrhoeal disease has mainly been *Escherichia coli* (Frantamico et al., 2007) and the process of handling garri during production predisposes it to contamination by *E. coli.* Furthermore, it has been a serious public health issue worldwide; irrespective of the source of transmission either environmental or clinical *E. coli* are always of great health concern (Steven and David, 2014). Among the enteric infection caused by *E. coli*, Enteroinvasive *E. coli* (EIEC),

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Enteropathogenic *E. coli* (EPEC) and Enteroaggregative *E. coli* (EAggEC) are transmitted by inter-human contacts while those described as Enterotoxigenic *E. coli* (ETEC) or Shiga toxin-producing *E. coli* (STEC), are primarily transmitted to humans through the consumption of contaminated water or food (Prere et al. 2006 and Lawan et al., 2015).

Thus, beneficial organisms such as *Lactobacillus* with documented safety profile possessing antagonistic potential against pathogens have received increased attention for their possible usage as probiotics in place of antibiotics (Servin, 2004). This study aims to isolate lactic acid bacteria within the garri matrix that has inhibitory effect on *E. coli* isolated from clinical settings.

Material and methods

Samples collection

Ten different varieties (5 yellow and 5 white) of Garri was purchased randomly from different retail traders at Samaru Market, Zaria, Nigeria. The samples were collected in polyethylene aseptic bags at ambient temperature, to prevent contamination with spoilage agents and were labeled before transporting to the laboratory of the Department of Microbiology, Ahmadu Bello University, Zaria for analysis.

Determination of physio-chemical properties of "Garri"

Ten grams of each sample were added to 10 ml of distilled water and allowed to homogenize for 2 min. Afterwards, the temperature and pH value were taken using a thermometer and calibrated pH meter, respectively, followed by the procedure of Olopade et al. (2014). Subsequently, in order to determine the moisture content, 10 g of each sample were weighed and placed in a dry weighed crucible of known weight. Samples were placed in a moisture extraction oven at 105°C for 3h and re-weighed after cooling. This process was repeated until constant weight was achieved; the percentage difference in weight equates the moisture content which was calculated thus:

Moisture Content (%) = $(w_2 – w_3) / (w_2 – w_1) \times 100$,

Where W_1 is Weight of empty petri dish;

W_2 = Weight of petri dish + Sample before;

W_3 = Weight of empty petri dish + Sample after (Orji et al. 2016).

Isolation of lactic acid bacteria

Ten grams of the samples ("Garri") were serially diluted in 90 ml peptone water using a ten-fold dilution under aseptic condition. The suspension was homogenized and filtered through sterile wool (Adejumo and Raji, 2012). Aliquots from 10^{-2} dilutions were inoculated on the already prepared sterile De-Man Rogosa and Sharpe (MRS) Agar (Himedia, U.S.A) and plates were incubated in a candle jar at 37°C for 24h to 48 hours. After incubation, discrete colonies were streaked on MRS Agar in order to obtain pure isolate by incubating anaerobically at 37°C for 24h. The colonies were sub-cultured on MRS Agar slants and stored at 4°C in the refrigerator for further use. The colonial morphology of the isolates cultured on MRS agar were observed and recorded based on colour, shape and size (Fossi and Ndjouenkeu, 2017).

Gram's staining

Smears of the isolates were prepared and heat fixed on clean grease free glass slides. The smears were stained for one minute with crystal violet. This was followed by washing out the stain with a gentle running tap water. The slides were then flooded with dilute Gram's iodine solution; washed off with water and the smears decolorized with 95% alcohol until the blue colour no longer dripped out (about 10 to 20 secs.). The smears were counter stained with safranin solution for 1 min. Finally, the slides were washed with slow running tap water; air dried and observed under oil immersion objectives according to Heil (2009).

Catalase Test

Catalase test was performed on all the isolates in order to know their catalase reactions. 3% hydrogen peroxide solution was added to 1 ml of overnight cultures. The isolates which did not give gas bubbles are catalase negative and selected presumptively as lactic acid bacteria since LAB is known to be catalase negative (Kavitha and Devasena, 2013).

Biochemical characterization of the isolates

After Gram Staining (Cheesbrough, 2006) all the pure cultures of lactic acid bacteria were characterized using the biochemical tests listed below:

Carbohydrate fermentation test: Fermentation patterns of the isolates were determined using modified MRS medium from which meat extract and glucose had been omitted, but containing 0.05% (w/v) bromocresol purple indicator as basal medium. Filter sterilized solution of the sugars was added to the reconstituted MRS broth at a final concentration of 2%. The sugars used were Arabinose, Sucrose, Maltose, Sorbitol, Fructose, Galactose, Mannose and Manitol. The cultures to be tested were first grown on MRS agar plates for 18 hours; the active cultures were then aseptically transferred from plates and inoculated into tubes of the basal medium (10ml) containing the test carbohydrates and incubated at 37°C for 24h. Tubes in which bromocresol purple indicator changed to yellow indicated acid production from carbohydrate (De Man et al., 1960).

Motility test: With the aid of a light microscope using hanging drop method the motility test for each isolate was carried out. A drop of MRS broth containing 18h old LAB suspension was used to make a wet mount by placing the drop on a cover slip. The edges of the cover slip were smeared with jelly and inverted over a depression slide to allow the media drops hang over the depressed point while jelly prevented the suspension from drying out. The wet mount was then viewed with 40X power objectives to observe if there would be true motility, Brownian movement or no movement (Bin Masalam et al., 2018).

Citrate utilization test: This test detects the ability of

an organism to utilize citrate as the sole source of carbon and energy. Lactic acid bacteria isolated were inoculated on a medium containing sodium citrate and a pH indicator bromothymol blue. The medium also contains inorganic ammonium salts, which are utilized as sole source of Nitrogen. The bacterial colonies were picked by a straight wire and inoculated into slant of Simmon's citrate agar (Oxoid, U.K) and incubated overnight at 37°C. The organism that has the ability to utilize citrate changes the medium colour from green to blue.

Confirmation of identity of Escherichia coli

Escherichia coli isolated from clinical settings were collected on nutrient agar (Oxoid, U.K) slants from the university medical center, Ahmadu Bello University, Zaria; and the Department of Microbiology, A.B.U. Zaria. The latter was isolated from hospital sewage and confirmed as an extended spectrum beta lactamase (ESBL) producer. Both cultures were sub-cultured on Eosin Methylene Blue (EMB) agar (Oxoid, U.K) using the streak method and were incubated at 37°C for 24 hours. Gram staining was carried out and the following biochemical tests were conducted; indole, methyl red, voges-proskauer and citrate utilization; in order to confirm their identity (Cheesbrough, 2006).

Antibacterial activity of lactic acid bacteria

Following the methods of Ida et al. (2017), the LAB pure isolates were cultured in MRS broth overnight at 37°C. 200 µL of the *E. coli* isolates were inoculated into nutrient agar by pour plate method and allowed for solidification. Afterwards, wells were bored on the Nutrient agar using cork-borer and the assay performed in triplicate by adding 100 µl of Cell Free Supernatant (CFS) into the wells. The inoculated media were left inside the refrigerator for 30 min for the CFS to diffuse into the media and then incubated at 37°C for 24h. The antimicrobial activity of the lactobacilli was determined based on the development of zones of inhibition around the wells.

Preparation of Mc Farland standard and standardization of the inoculum: A 1% solution of barium chloride was prepared by dissolving 0.5 g of dehydrate $BaCl_2$ and 1% sulphuric acid was prepared by adding 1ml of H_2SO_4 to 99 ml of distilled water. 0.05 ml of the barium chloride was mixed with 9.95 ml of the sulphuric acid to give 0.5 Mc Farland standards which is equivalent to $1.5x10^8$ CFU/ml. Colonies of pure culture of *E. coli* were aseptically emulsified in 5 ml of normal saline water and the turbidity of the suspension was compared to that of 0.5 McFarland standard.

Detection of antimicrobial activity by Agar Well Diffusion Method: The isolates were seeded by spreading the cell suspension aseptically over the surface of Mueller Hinton agar (Oxoid, U.K) using an L-shape glass rod. The plates were allowed to dry and a sterile cork borer was used to bore uniform wells in the agar. Each well was filled with 100 µL broth culture of the LAB isolates and left to diffuse before incubation at 37°C for 24 hours. The plates were observed for zone of inhibition (ZOI) around the wells and the result was recorded. Ciprofloxacin disc (30 µg) was used as control by paper disc assay method (Ahmad et al., 2014).

Results

Characterization of Lactic acid bacteria

Garri samples collected have an average pH of 4.95 at 24.2°C (Table 1) with varying moisture content per sample, between 1.13±0.06% (S1Y) and 12.47±0.06% (S2W) (Figure 1), while the colonial morphology of all the isolates varies in appearance; small, round, smooth, raised, moist, translucent and creamy white in colour and ranges from coccobacilli (short rods) to bacilli when cultured on De Man Rogosa and Sharpe (MRS) agar. All the isolates were Gram positive (Table 2) and catalase negative; as it is the presumptive selection criteria for lactic acid bacteria (Harrigan, 1998) they were selected for further study.

Table 1. Temperature (T), pH and moisture content (MC) values of Garri samples used in isolation of Lactic acid bacteria

S/No.	Sample n = 10	T, °C	pH	MC, % Mean ± SD
1.	S1Y	26	4.8	1.13 ±0.06
2.	S1W	24	5.3	6.20 ±0.15
3.	S2Y	24	4.7	5.17 ±0.06
4.	S2W	25	5.4	12.47±0.06
5.	S3Y	22	4.8	6.20 ±0.15
6.	S3W	25	5.4	5.33 ±0.06
7.	S4Y	22	4.7	4.27 ±0.15
8.	S4W	25	5.0	5.27 ±0.06
9.	S5Y	26	4.9	4.27 ±0.06
10.	S5W	23	4.5	9.53 ±0.06
Average		24.2±0.47	4.95±0.10	

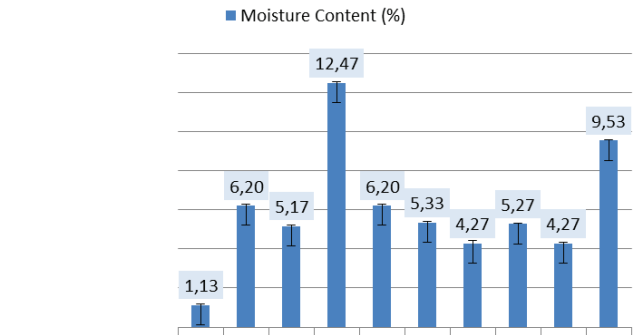


Figure 1. Moisture content of the Garri samples

Biochemical characterization of Lactic acid bacteria

In this study, aside from being Gram's positive and catalase negative all the isolates were non-motile and lacked the ability to utilize citrate. Their ability to utilize Arabinose, Sucrose, Maltose, Sorbitol, Fructose, Galactose, Mannose and Manitol varied according to the strain. Although all isolates were able to utilize glucose and lactose to produce lactic acid (Table 2); based on their ability to produce CO_2 from glucose metabolism

they were classified as homofermenters (30%) - those that did not produce gas from glucose and heterofermenters (70%) – the group that produced gas from glucose. Their sugar fermentation profile serves as the basis for their classification and probable identification when compared to Bergey's Manual of Systematic Bacteriology.

Table 2. Biochemical characterization of Lactic acid bacteria (*Lactobacillus*) isolated from Garri samples

*Isolates	CO_2 Production	Catalase	Motility	Citrate	Glucose	Lactose	Arabinose	Sucrose	Maltose	Sorbitol	Fructose	Galactose	Mannose	Mannitol	Tentative Identity
S1Y	HT	-	-	-	+	+	+	+	+	+	+	+	+	+	*L. plantarum*
S1W	HT	-	-	-	+	+	+	+	+	+	+	+	+	+	*L. plantarum*
S2Y	HM	-	-	-	+	+	-	+	+	-	+	+	+	-	*L. acidophilus*
S2W	HM	-	-	-	+	+	-	+	+	-	+	+	+	-	*L. acidophilus*
S3Y	HM	-	-	-	+	+	-	+	+	+	+	+	-	-	*L. salivarius*
S3W	HT	-	-	-	+	+	+	+	+	+	+	+	+	+	*L. plantarum*
S4Y	HT	-	-	-	+	+	+	+	+	+	+	+	+	+	*L. plantarum*
S4W	HT	-	-	-	+	+	-	+	+	-	+	+	-	-	*L. fermentum*
S5Y	HT	-	-	-	+	+	-	+	+	-	+	+	-	-	*L. fermentum*
S5W	HT	-	-	-	+	+	+	+	+	+	+	+	+	+	*L. plantarum*

*All the isolates are Gram positive rods
HM- Homofermentative, HT- Heterofermentative

The Lactic acid bacteria (LAB) isolated from Garri sample (Table 2) belongs to the genus *Lactobacillus* from our findings. The number present and percentage of occurrence of *Lactobacillus* isolates in Garri sample is as presented in Figure 2 with *Lactobacillus plantarum* having the highest occurrence of 50% followed by *Lactobacillus fermentum* (20%) and *Lactobacillus acidophilus* (20%) with *Lactobacillus salivarus* (10%) having the lowest number of occurrence.

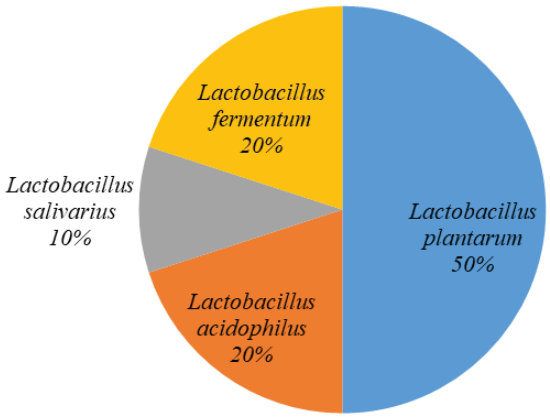


Figure 2. Distribution of Lactic acid bacteria isolated from Garri samples

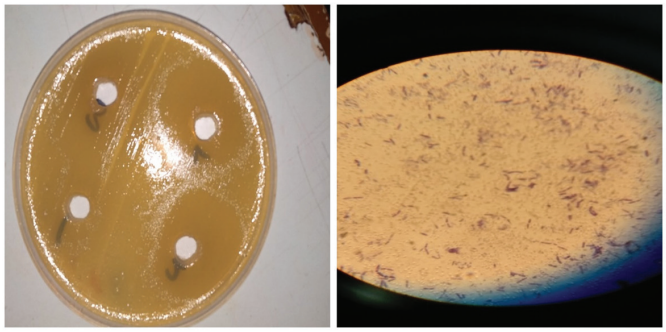
Determination of antimicrobial activity of Lactic acid bacteria

The *Escherichia coli* isolates were cultured on Eosin Methylene Blue Agar that doubles as a confirmatory test for the isolates and maintained on nutrient agar slant. The *E. coli* isolates were gram negative; Indole positive, Methyl Red positive, Voges-Proskauer negative and Citrate negative. From the lactic acid bacteria isolates studied; *Lactobacillus plantarum* (code S1Y) was effective against the environmental *E. coli* with inhibition zone of 20.7±0.58 mm while *Lactobacillus acidophilus* (code S2Y) and *Lactobacillus plantarum* (code S1W) (Figure 3a,b) were effective against the clinical isolate of *E. coli* with zone of inhibition 22.7±1.53 mm and 19.3±2.08 mm, respectively (Table 3). However, *Lactobacillus plantarum* (code S5W) had no significant inhibitory effect on any of the *E. coli* samples and none of the LAB isolate has an inhibitory activity on the growth of the *E. coli* isolates.

Table 3. Antibacterial activity of Lactic acid bacteria isolated from Garri against isolates of *E. coli*

S/No.	Sample code	Isolate	*Zone of inhibition (mm)	
			Clinical isolate	Environmental isolate
1.	S1Y	*Lactobacillus plantarum*	0	20.7 ±0.58
2.	S2Y	*Lactobacillus acidophilus*	22.7 ±1.53	0
3.	S1W	*Lactobacillus plantarum*	19.3 ±2.08	0
4.	Ciprofloxacin (30µg)		45.3 ±0.58	36.3 ±0.58

*Mean ± SD



a) b)

Figure 3. (a) Antimicrobial activity of *Lactobacillus plantarum* against a clinical isolate of *E. coli*; (b) Micrograph of *Lactobacillus plantarum* isolated from Garri sample (X1000 magnification)

Discussion

"Garri" which is a traditional fermented food has no form of preservative added during the production. The samples used in this study were of two varieties, white and yellow; with the exception of moisture content, the variety played no obvious role in their physicochemical properties. The temperature of the samples used in this study was in the range, 22-26°C; with an average of 24.4°C (Table 1). The relatively low temperature is to be expected given the fact that garri is stored in large containers which are left open to prevent spoilage as a result of humidity. All the samples had consistently acidic pH in the range, 4.5-5.4, which is expected due to the fermentation process during the production of garri. The average pH observed for all the samples was found to be 4.95 which provides a favourable environment for lactic acid bacteria to thrive (Table 1). The moisture content of the samples ranged widely from 1.13% - 12.47% (Figure 1). The large disparity in the moisture content of the samples could be due to variations in their mode of preparation and storage conditions. The white garri samples consistently had higher moisture content values than the yellow samples except for sample S3; it is possible that the addition of palm oil during the preparation of the yellow garri could have accounted for the lower moisture content since the presence of oil will naturally prevent absorption of moisture. The moisture content will also affect the organoleptic and shelf life of the garri samples.

The biochemical characterisation (Table 2) of the isolates using conventional tests revealed that fermentation of sugars is a very important metabolic function for distinguishing lactic acid bacteria. With the exception of the fermentation of sorbitol, mannitol, mannose and arabinose, the isolates showed similar fermentation profiles for the other sugars. Four species belonging to the genus *Lactobacillus* were isolated and the most abundant was *Lactobacillus plantarum* (50%), while the others are *Lactobacillus fermentum* (20%), *Lactobacillus acidophilus* (20%), and *Lactobacillus salivarius* (10%) (Figure 2). Aside from samples S1 and S2 which had the same species of *Lactobacillus* in both types of garri, all the other samples showed different species of *Lactobacillus* in both the yellow and white garri varieties.

The presence of LAB affects its organoleptic property, shelf life and the inhibition of diarrhoea-causing microorganisms in "garri" as observed in this study. Many of the species of Lactic acid bacteria (LAB) are "generally regarded as safe" (GRAS), and belong to the genus *Lactobacillus*. They are widely studied and applied as probiotics in various aspects of human health (Abubakr and Al-Adiwish, 2017). Lactic acid bacteria used as probiotics have been reported to be effective against common human and food pathogens, and have been harnessed for therapeutics and also food bio-preservatives.

Moreover, it is of great importance that LAB can easily be found in nature and are closely associated with fermented foods such as garri (Okpara et al., 2014) thus, making it easy to isolate and study them for various applications. From this study the ability of some of the LAB strains to inhibit strains of *E.coli* shows their bio-preservative potential and possible usage in food industry which can be tied to any of the antagonistic substances such as organic acids – lactic acid, acetic acid and volatile compounds, hydrogen peroxide, bacteriocins and other antimicrobial peptides produced by them (Agaliya and Kadirvelu, 2013). It is possible that organic acids, bacteriocin or bacteriocin-like substances are the agents (Bendali et al., 2011) that resulted in the inhibition of the growth of *E.coli* as against the control which had no inhibitory effect on the pathogen.

The isolates S1Y, S1W and S2Y that were identified as *Lactobacillus plantarum, L. plantarum and L. acidophilus,* respectively (Table 3), used one of the mechanisms mentioned above in the inhibition of *E. coli* in line with an earlier study by Ahmed (2019) that documented the antagonistic potential of *L. plantarum* isolated from fermented milk (nono) samples against *E. coli*. A strain isolated from raw milk has also shown antimicrobial activity against some bacterial pathogens (Deshmukh and Thorat, 2013); it also showed activity against phytopathogens of tomato plant. *L. plantarum* has been known to produce a variety of bacteriocins, which makes it very proficient in inhibiting the growth of pathogenic bacteria (Beshkova and Frengova, 2012; Zacharof and Lovitt, 2012). *L. plantarum* is frequently isolated from fermented foods and is a common lactic acid producing bacterium, compared to the other species of the same genus. Antimicrobial activity of *L. acidophilus* isolated from food has also been reported in some studies (Karska-Wysocki et al., 2010; Bassyouni et al*.,* 2015; Georgieva et al*.,* 2015; Dinev et al*.,* 2017), further supporting the fact that these lactic acid producing strains could be subjected to further studies involving extraction and purification of the active bacteriocins. *L. acidophilus* has been shown to inhibit the growth of Gram positive (Aween et al*.,* 2012) and Gram negative pathogens (Jamalifar et al*.,* 2011). Interestingly, two out of the three strains showing zones of inhibition against the isolates of *E. coli*, were isolated from the yellow garri, and they also had relatively wider zones of inhibition compared to the isolate from white garri sample. This might imply that the mode of preparation of the yellow variety of garri had a role in enhancing the antimicrobial activities of the lactic acid bacteria present in the sample. These strains could provide a possible alternative to antibiotics and perhaps overcome the challenge of multidrug resistant pathogens and also for the utilization of

antimicrobial peptides in food packaging (López-Cuellar et al., 2016). It is particularly interesting to note that the strain of *E. coli* isolated from hospital waste water in this study is an extended spectrum betalactamase (ESBL) producer thus, it is resistant to third generation cephalosporins which makes it a difficult strain to treat. It is of great interest that one of the strains of *Lactobacillus plantarum* isolated in this study showed relatively strong activity against this resistant bacterium (Figure 3a).

Conclusion

Lactic acid bacteria belonging to the genus, *Lactobacillus*, were successfully isolated from Garri and their CFS tested against clinical and environmental isolates of *Escherichia coli*. *Lactobacillus plantarum* and *Lactobacillus acidophilus* isolated in this study, inhibited the growth of clinical isolates of *E. coli*, as well as an extended spectrum betalactamase (ESBL)-producing strain of *E.coli* isolated from the environment. However, *Lactobacillus acidophilus* showed the widest zone of inhibition against the pathogen.

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Conflict of Interest

No potential conflict of interest to be declared.

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