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Antibacterial Properties of the Predominant Microorganisms Isolated from Fermenting Cassava Tubers during *fufu* Production against Selected Enteropathogenic Bacteria

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Authors' contributions

The research was the collaborative efforts of the authors. Author KTA designed the research, was involved in microbiological, chemical and statistical analyses and wrote the first draft of the work. Author SF was involved in the microbiological and statistical aspects of the work. Author BOM was involved in the microbiological and statistical analyses. Author BSA was involved in literature finding and microbiological analyses. Authors OSF and DOA were involved in the procurement of raw material and microbiological analyses.

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ABSTRACT

Aim: This research investigated the antibacterial activities of the predominant microorganisms isolated from fermenting cassava mash during *fufu* production against selected enteropathogenic bacteria.
Methodology: Microbiological analysis was carried out on the mash on daily basis during the

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three-day fermentation period. The pH, TTA and temperature of the *fufu* were also evaluated. The antibacterial activities of dominant microorganisms from the mash were assayed against the isolated microorganisms and test isolates using disc and agar diffusion methods.

Results: The bacteria isolated from the fermenting mash include *Bacillus subtilis, Lactobacillus fermentum, L. plantarum, Pediococcus acidilactici*, *Micrococcus luteus* and *Staphylococcus aureus* while the fungi were *Aspergillus flavus, A. niger, A. fumigatus, Geotrichum candidum, Penicillium expansum* and *Rhizospus stolonifer*. The predominant microorganisms were *L. plantarum, L. mesenteroides, A. niger, A. fumigatus* and *G. candidum.* The total bacterial, lactic acid bacterial and fungal counts increased from 2.5X10^5 cfu/ml, 2.0X10^5 cfu/ml and 1.5X10^3 cfu/ml to 7.6X10^6 cfu/ml, 6.7X10^6 cfu/ml and 1.0X10^6 cfu/ml respectively. The temperature of cassava mash increased from 26℃ to 30℃. The pH decreased from 6.80 to 4.22 while the total titratable acidity increased from 0.70% to 0.94%. *Escherichia coli, P. mirabilis, S. typhimurium* and *S. aureus* were inhibited by *L. plantarum* and *L. mesenteroides* while *E. agglomerans* and *K. pneumoniae* were resistant to *L. plantarum* and *L. mesenteroides* respectively. *Aspergillus niger* and *G. candidum* inhibited *S. aureus* but *E. agglomerans, K. pneumoniae, P. mirabilis* and *S. typhimurium* were not affected*. Enterobacter agglomerans, E. coli, P. mirabilis* and *S. aureus* were inhibited by *A. fumigatus* while *K. pneumoniae* and *S. typhimurium* were resistant*.*

Conclusion: These results suggested that consumption of *fufu* and other fermented cassava tubers could enhance less susceptibility to diseases caused by the test bacteria and *fufu* may be recommended for people suffering from infections caused by these microorganisms.

Keywords: Fufu; fermentation; cassava; antibacterial; mash.

1. INTRODUCTION

Cassava (*Manihot esculenta* Crantz) is a perennial, root tuber crop that belongs to the family Euphorbiaceae. Cassava is the third-largest source of carbohydrate after rice and maize for people living in the tropics. It provides a basic diet for over half a billion people in the developing countries of the world [1]. Cassava is cultivated in all the tropical and the subtropical regions of the world. In 2014, the global production of cassava tubers was 268 million tonnes with Nigeria as the world largest producer of nearly 55 million tonnes of the world total while Thailand was the largest exporter of dried cassava. Indonesia and Brazil are the other major producers of cassava [2]. Over the years, cassava has been used as food, animal feed and industrially in the manufacture of adhesives, beverages, biofuel, laundry starch and in traditional medicinal practices [3]. They are very rich in carbohydrate and contain significant amounts of calcium, phosphorus and vitamin C. However, they are poor in protein and other nutrients. In contrast, cassava leaves are a good source of an amino acid called lysine [4].

Cassava is categorized into two based on their cyanogenic components: the sweet and the bitter cassava. Sweet cassava contains low cyanogenic contents and can be processed by simple boiling while bitter cassava has high cyanogenic which requires processing such as fermentation before consumption [5].

Traditional methods of processing cassava tubers into food vary by region and by ethnic group. Popular methods used in processing cassava tubers before consumption include fermentation, drying, boiling and roasting. These are done to remove the toxic anti-nutritional factors such as cyanogenogic glucosides, oxalates, phytic acids and saponins from the roots [1].

Fermentation of food before consumption has many advantages. They include improved nutritional value, palatability, reduce antinutrient contents and improve safety against enteropathogenic bacteria. These bacteria are diarrhea causing pathogens which employ various mechanisms of action to colonise and cause the inflammation of the intestinal epithelium of infected individuals [6]. They are ingested through food and water, common enteropathogenic bacteria in developing countries include *Escherichia coli, Staphylococcus aureus*, and *Salmonella typhimurium* [7]. Naturally occurring microorganisms during fermentation of cassava tubers such as *Lactobacillus plantarum, L. fermentum*, *Saccharomyces cerevisiae* and *Geotrichum candidum* produce antibacterial substances such as bacteriocin and organic acids that help to inhibit the activities of

enteropathogenic bacteria in fermented foods [8]. Lactic acid bacteria ingested in fermented foods help to improve the health of consumers by modifying their intestinal microflora [9]. *Fufu, garri, lafun* and *pupuru* are the common foods produced from cassava tubers in Nigeria [10,11].

The aim of this research project work was to isolate the microorganisms involved in the fermentation of cassava tubers used for the production *fufu* and to test the antibacterial properties of the predominant microorganisms against selected enteropathogenic bacteria; *Escherichia coli, Enterobacter agglomerans, Klebsiella pneumoneae, Salmonella typhimurium, Staphylococcus aureus* and *Proteus mirabilis.*

2. MATERIALS AND METHODS

Fermenting cassava mash were collected from 5 commercial *fufu* producers on daily basis for 3 days aseptically in sterile conical flasks in Akungba-Akoko, Ondo State, Nigeria. They were transported to the Microbiology Laboratory of Adekunle Ajasin University, Akungba-Akoko, Ondo State, Nigeria for microbiological analysis.

2.1 Collection and Screening of the Test Organisms

The Clinical isolates which were *Escherichia coli, Enterobacter agglomerans, Klebsiella pneumoniae, Salmonella typhimurium, Staphylococcus aureus* and *Proteus mirabilis* were collected from the Microbiology Laboratory of Federal Medical Center (FMC), Owo, Ondo State, Nigeria. All the isolates were of feacal origin. Morphological and biochemical characteristics of the 24-hour old cultures of the microorganisms were carried out to ascertain their identity. They were inoculated into nutrient agar slants and stored at 4ºC in the refrigerator.

2.2 Microbiological Analysis

2.2.1 Isolation procedure for microorganisms

Serial dilution of the cassava mash was carried out by weighing 10 grams of the sample into a sterile beaker containing 100 ml of sterile distilled water. The beaker was shaken properly, and 1 ml of the suspension was dispensed into a test tube containing 9 ml of sterile distilled water and the same procedure continued up to the ninth test tube (10^9). One ml of the appropriate dilution was aseptically pipetted into a sterile Petri dish. Nutrient agar, de Man Rogosa and Sharpe agar and PDA that had been sterilized by autoclaving and cooled to about 45℃ were poured aseptically into the Petri dishes containing the dilutions. The plates were swirled gently on the work bench for even distribution of the inocula in the media and were allowed to set. Bacterial plates were incubated in an inverted position at 37℃ for 24 to 48 hours while fungal plates at room temperatures for 72 to 120 hours. MRS agar plates were incubated under anaerobic conditions.

Enumeration of the microorganisms was carried out on daily basis. Representative colonies were picked from the plates and sub-cultured by repeated streaking on their respective media until pure cultures were isolated. The isolates were characterized by cultural, morphological and biochemical tests. The pure cultures were then transferred to agar slants and stored in the refrigerator for characterization and identification purposes [12].

2.2.2 Identification of bacterial isolates

Bacterial colonies, shape, colour, size, edge, elevation and surface texture were observed after 18-24 hours of incubation. Characterization was based on Gram stain, morphological characteristics and biochemical characteristics of the isolates according to Bergey's Manual of Determinative Bacteriology [13].

2.3 Determination of Total Titratable Acidity (TTA), pH and Temperature

The total titratable acidity was carried out in triplicates. Ten grams of the cassava mash sample was homogenized in 100 ml of distilled water and filtered through Whatman filter paper. Twenty-five milliliters of the filtrate were titrated with 0.1 M NaOH using 2 drops of phenolphthalein as indicator. The percentage titratable acidity (TTA %) was calculated using the formula:

$$TTA = \text{Volume of NaOH used (ml)} \times 0.009 \times 100 / \text{Volume of sample used.}$$

where 0.009 is the lactic acid milliequivalent [14].

The pH and temperature of the cassava mash were determined daily in triplicates by using a standard pH meter and thermometer respectively. Ten grams of the sample was

homogenized in 100 ml of distilled water. The pH was determined by calibrating the electrode that is connected to the meter first in buffer solutions (pH 4 and pH 7) to standardize the meter, before used for homogenized samples. The reading was taken for each day. The temperature of the samples was determined according to Owuamanam et al. [14].

2.4 Standardization of the Test Organisms

All the organisms used were standardized to 0.5 McFarland standards. A 0.2 ml aliquot of 24-hour old broth culture was dispensed in another sterilized Mueller-Hinton broth and incubated for 6 hours. One milliliter from the final broth is equal to 0.5 McFarland standard ($6x10^8$ cfu/ml) [15].

2.5 Inoculation of the Test Organisms

A sterile swab stick was dipped into the standardized broth culture and excess liquid was drained from the swab stick by pressing it gently to the inner side of the test tube containing the broth culture. The surface of the set media (Mueller-Hinton agar, Oxoid, England) was streaked with the swab stick respectively. The plates were incubated at 37℃ for 24 hours and the diameter of zones of inhibition was measured [15].

2.6 Preparation of Cell-free Supernatants

Lactobacillus plantarum and *Leuconostoc mesenteroides* (dominant bacteria) isolated from fermenting cassava were cultured and incubated overnight separately in MRS broths respectively. The broth culture was centrifuged at 14,000 rpm for 15 minutes, the supernatants were filtered through a membrane filter of 0.2 µm pores and the resulting cell free supernatant was tested against the selected enteropathogenic bacteria using agar diffusion assay as described by Yang et al. [16] and Onwuakor et al. [17].

2.7 Preparation of Cell-free Filtrates

Mycelia from 7 days old cultures of *Aspergillus fumigatus, A. niger* and *Geotrichum candidum* (dominant fungi) were inoculated into 100 ml of potato dextrose broth each. They were incubated without agitation for 7 days at 25℃. The broth cultures were filtered through Whatman paper to remove the their mycelia. The crude cell free filtrates from each broth culture were tested against the selected enteropathogenic bacteria for their antimicrobial activity using agar well diffusion assay [18,19].

2.8 Screening of Cell-free Supernatants and Cell Free Filtrates for Antibacterial Activities

Agar well diffusion method was used. Three wells of 5.00 mm in diameter each were made on solidified Mueller-Hinton agar plate seeded with the test bacteria using a sterile cork borer. Equal volume of cell free extracts from the broth cultures was introduced into the wells and sterile distilled water and standard antibiotics (ciprofloxacin 20 mg/ml) were used as the negative and positive controls respectively. Experiment was carried out in triplicates. Zones of inhibition after 24 hours were calculated. Zones of inhibition which were less than 15, between 16 and 22 and from 23 mm and above were recorded as low susceptibility, moderate susceptibility and high susceptibility respectively [18,20].

2.9 Statistical Analysis

All analyses were carried out in triplicates. Analysis of variance (ANOVA) plus Duncan's multiple range test was used for comparison of means using SPSS software (version 16.0 for Windows, SPSS Inc., Chicago, USA). Significance was accepted at $P<0.05$.

3. RESULTS

3.1 Microbiological Identification

Eight genera of bacteria were isolated from the fermenting cassava mash during the three days of fermentation period. They include *Bacillus subtilis, Lactobacillus fermentum, L. plantarum, Leuconostoc mesenteroides, Pediococcus acidilactici*, *Micrococcus luteus, Proteus mirabilis, Pseudomonas aeruginosa* and *Staphylococcus aureus.* The genera of fungi isolated were *Aspergillus flavus, A. niger, A. fumigatus, Geotrichum candidum, Penicillium expansum* and *Rhizopus stolonifer* (Table 1).

The predominant microorganisms from the mash were *L. plantarum, L. mesenteroides, A. niger, A. fumigatus* and *G. candidum. Pseudomonas aeruginosa, Pr. mirabilis* and *A. flavus* appeared

only at the zero hour of fermentation and were not observed till the end of the fermentation. *B. subtilis, S. aureus* and *P. expansum* disappeared after 24 hours of fermentation while *R. stolonifer* was isolated at the 48 hour of fermentation and did not appear till the end of the fermentation. *Micrococcus luteus* and *P. acidilactici* appeared only at the 72 hours of fermentation (Table 1).

3.2 Microbial Counts

As the period of fermentation increased, the total bacterial count, lactic acid bacterial and fungal counts increased from 2.5x10^5 cfu/ml, 2.0x10^5 cfu/ml and 1.5x10^3 cfu/mlto 7.6x10^6 cfu/ml, 6.7x10^6 cfu/ml and 1.0x10^6 cfu/ml respectively (Fig. 1).

Table 1. Occurrence of the microorganisms from the fermenting cassava mash

Microorganisms	Hours of fermentation			
	0	24	48	72
Bacteria				
Bacillus subtilis	+	+	-	-
Lactobacillus fermentum	+	+	+	+
Lactobacillus plantarum	+	+	+	+
Leuconostoc mesenteroides	+	+	+	+
Micrococcus luteus	-	-	-	+
Pediococcus acidilactici	-	-	-	+
Proteus mirabilis	+	-	-	+
Pseudomonas aeruginosa	+	-	-	-
Staphylococcus aureus	+	+	-	-
Fungi				
Aspergillus flavus	+	-	-	-
Aspergillus fumigatus	+	+	+	+
Aspergillus niger	+	+	+	+
Geotrichum candidum	+	+	+	+
Penicillium expansum	+	+	-	-
Rhizopus stolonifer	-	-	+	-

Present = +, absent = -

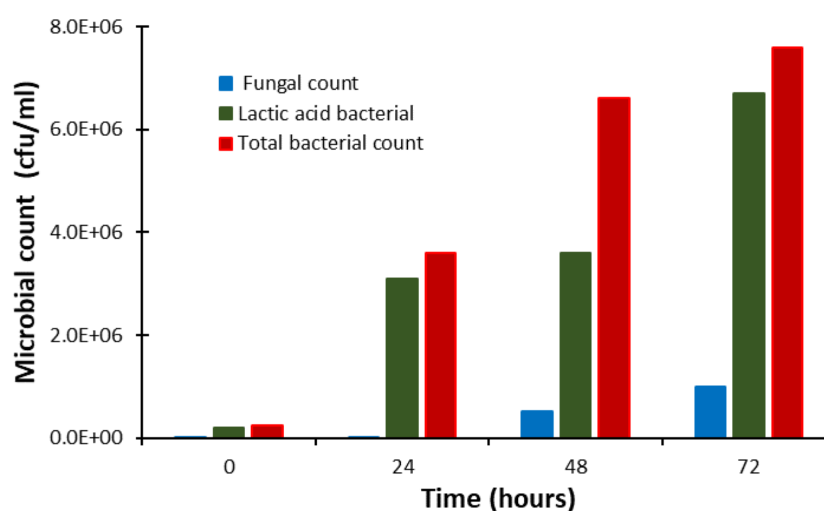

Fig. 1. Microbial counts of the fermenting cassava mash

3.3 The Temperature, pH and Total Titratable Acidities of the Fermenting Mash

The temperature of the cassava mash increased progressively during the period of fermentation from 26°C to 30°C. The pH decreased from 6.80 to 4.22 as the percentage total titratable acidity of the fermenting cassava mash increases from 0.70 to 0.94 (Fig. 2).

3.4 Antimicrobial Activity of the Predominant Isolates

Escherichia coli, Pr. mirabilis, Salmonella typhimurium and *S. aureus* were inhibited by the antibacterial activities of *L. plantarum* and *L. mesenteroides*. The highest zones of inhibition of *L. plantarum* and *L. mesenteroides* were found against *E. coli* while *Enterobacter agglomerans* and *Klebsiella pneumoniae* were resistant to *L. plantarum* and *L. mesenteroides* respectively (Table 2). The antibacterial activities of *A. niger* and *G. candidum* inhibited *S. aureus*. The highest inhibitions by *A. niger, A. fumigatus* and *G. candidum* were observed against *S. aureus. En. agglomerans, K. pneumoniae* respectively. *Proteus mirabilis* and *S. typhimurium* were resistant to *A. niger* and *G. candidum* respectively. *Enterobacter agglomerans, E. coli, P. mirabilis* and *S. aureus* were inhibited by *A. fumigatus* but did not inhibit *K. pneumoniae* and *S. typhimurium.* All the clinical isolates were inhibited by the positive control (20 mg/ml of ciprofloxacin) and resistant to negative control (distilled water) (Table 3).

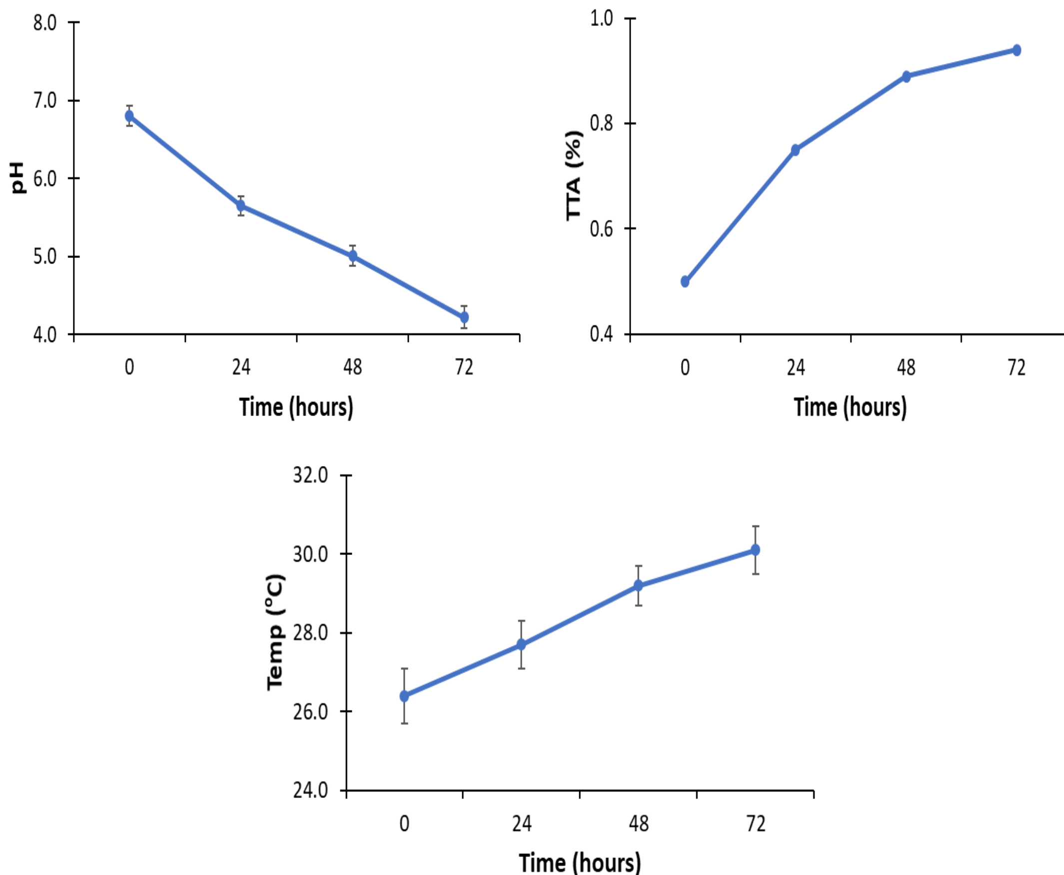

Fig. 2. Description of the physico-chemical properties (pH, TTA and Temperature) of the fermenting cassava mash
TTA = Total Titratable Acidity, Temp = Temperature

Table 2. Antibacterial activities of the predominant bacteria

Test organism	Zones of Inhibition (mm)					
	Lactobacillus plantarum			*Leuconostoc mesenteroides*		
	CFS	CPX (20 mg/ml)	DW	CFS	CPX (20 mg/ml)	DW
Enterobacter agglomerans	0±0	21.00± 0.13	0±0	16.00± 0.04	20.00±0.02	0±0
Escherichia coli	23.00± 0.36	27.00± 0.23	0±0	18.00± 0.03	26.00±0.11	0±0
Klebsiella pneumoniae	15.00± 0.43	26.00± 0.06	0±0	0±0	26.00±0.33	0±0
Proteus mirabilis	14.00± 0.12	25.00± 0.05	0±0	9.00± 0.21	27.00±0.02	0±0
Salmonella typhimurium	19.00± 0.14	23.00± 0.16	0±0	17.00± 0.12	25.00±0.23	0±0
Staphylococcus aureus	21.00± 0.41	28.00± 0.27	0±0	16.00± 0.04	30.00±0.20	0±0

Values are means of three replicates ± standard error.
Legend: CFS = Cell free supernatants, DW = Distilled water, CPX = Ciprofloxacin

4. DISCUSSION

The results of this study clearly showed that a wide array of bacteria and fungi are involved in the fermentation of cassava. The isolation of *B. subtilis* and *S. aureus* in *fufu* from the samples was supported by Umeh and Idibo [11] who reported the presence of *B. subtilis* and *S. aureus* while retting cassava tubers used to produce *fufu*. The occurrence of *S. aureus* might be because of environmental and human contaminations during the processing [20,21]. Ayoade et al. [22] isolated *L. brevis, L. plantarum, A. fumigatus* and *Saccharomyce* spp. As the predominant microorganisms while spontaneously fermenting cassava tubers for producing gari ang fufu. The presence of *A. niger, P. expansum* and *R. stolonifer* had also been reported by Obadina et al. [10] during the microbial assessment of *fufu*. The presence of *Pr. mirabilis, P. aeruginosa* and *Penicillium expansum* during the early stage of fermentation was probably due to soil contaminations [21]. The disappearance of *P. mirabilis* and *P. aeruginosa* during the 24 hours of fermentation as well as the disappearance of *S. aureus* after 24 hours of fermentation was probably due to the antibacterial substances produced by the predominant microorganisms. Charles et al. [9] observed the antifungal ability of lactic acid bacteria. This might be responsible for the disappearance of *A. flavus* and *P. expansum* after 24 and 48 hours of fermentation respectively. A report by Dalie et al. [23] suggested that lactic acid bacteria activities involved in the fermentation of cassava could detoxify possible nephrotoxic mycotoxins produced by molds *A. flavus, A. niger, A. fumigatus* and *P. expansum* during fermentation which may pose a threat to the health of the consumer the fermented product (*fufu*). The aforementioned microorganisms have also been known to be causal agents of post-harvest rots of the cassava tubers during storage [21].

Lactic acid bacteria such as *L. mesenteroides, L. plantarum* and *L. fermentum* as well as yeast such as *G. candidum* isolated from the fermenting cassava mash had been reported to be responsible for the improvement of the organoleptic property (taste and odour) and increase in the shelf-life of the fermented cassava food [14,21,24].

The antibacterial activities of the predominant lactic acid bacteria against the enteropathogenic test bacteria could be due to their ability to produce antibacterial substances such as bacteriocin, lactic acid, hydrogen peroxide and carbon dioxide [25]. *Lactobacillus plantarum* and *L. mesenteroides* produce bacteriocins called plantaricin and mesenteriocin which might possibly responsible for their antibacterial properties against *E. coli, Pr. mirabilis, S. typhimurium* and *S. aureus* [26]. The antibacterial activities of *A. fumigatus* against *En. agglomerans, E. coli, Pr. mirabilis* and *S. aureus* might be due to its ability to produce a secondary metabolite called gliotoxin. Gliotoxin is currently utilized by pharmaceutical industries as an antibiotic and antiviral agent [27]. The antibacterial activity of *G. candidum* against *S. aureus* might also be because of its ability to produce indole acetic acid and phenyl lactic acids [23]. In addition, Abdulwahid et al. [18] reported that *A. Niger* produces nigerazine and tensyuic acid which are antibacterial; this may be responsible for its inhibition against *S. aureus.*

Table 3. Antibacterial activities of the predominant fungi

Test organism	Zones of Inhibition (mm)								
	Aspergillus niger			***Aspergillus fumigatus***			***Geotrichum candidum***		
	CFS	**CPX (20 mg/ml)**	**DW**	**CFS**	**CPX (20 mg/ml)**	**DW**	**CFS**	**CPX (20 mg/ml)**	**DW**
Enterobacter agglomerans	0±0	23.00±0.05	0±0	13.00±0.03	22.00±0.02	0±0	0±0	21.00±0.23	0±0
Escherichia coli	0±0	25.00±0.03	0±0	12.00±0.02	28.00±0.04	0±0	10.00±0.06	28.00±0.43	0±0
Klebsiella pneumoniae	0±0	26.00±0.15	0±0	0±0	24.00±0.11	0±0	0±0	22.00±0.45	0±0
Proteus mirabilis	0±0	27.00±0.22	0±0	9.00±0.04	27.00±0.33	0±0	0±0	27.00±0.14	0±0
Salmonella typhimurium	0±0	23.00±0.12	0±0	0±0	21.00±0.05	0±0	0±0	23.00±0.36	0±0
Staphylococcus aureus	13.00±0.01	27.00±0.03	0±0	18.00±0.04	25.00±0.04	0±0	11.00±0.05	28.00±0.02	0±0

Values are means of three replicates ± standard error.
Legend CFS = Cell free supernatants, DW = Distilled water, CPX = Ciprofloxacin

5. CONCLUSION AND RECOMMENDATION

The results showed that the predominant bacteria isolated from the fermenting cassava mash are lactic acid bacteria, and the fungi were *G. candidum*, *A. fumigatus* and *A. niger.* The results of *in vitro* antibacterial activities against enteropathogenic bacteria showed that all the predominant microorganisms produced certain antibacterial substances which have inhibitory activities against enteropathogenic bacteria. The consumption of foods with the isolated lactic acid bacteria can serve as a source of probiotics, thereby competing with potential pathogenic bacteria for nutrients and space in the gastrointestinal tract. In addition, with the antibacterial activities observed, there is an indication that the consumption of *fufu* with these predominant microorganisms may have a positive impact on the reduction of the incidence of gastrointestinal illnesses. We have identified the limitation of this study as pertaining to its *in vitro* nature. Therefore, one of the gaps that would be filled in the future studies is the *in vivo* investigation of the survival of the microorganisms present in *fufu* and their interactions with those of the gastrointestinal tracts of animal models. We project that this will establish the link, if any, between the consumption of *fufu* and the reduction in incidences of gastrointestinal illnesses.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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