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Synergistic Assessment of Lactobacillus Plantarum, Aspergillus Fumigatus and Some Yeasts Isolated from "Omidun" Against Diarrheagenic Bacteria

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Abstract:- Supernatant solution of Ogi or Akamu, as known in Yoruba ("omidun") was investigated for microorganisms associated with cereal mash "ogi". The isolates were further tested for antimicrobial activities against two primary diarrheagenic organisms (Salmonella abaetetuba ATCC 35460 and Escherichia coli ATCC 25922). Microorganisms from commercially obtained "omidun" samples and control samples from yellow and white maize varieties were isolated and identified. Types of isolates and their percentage incidences were compared among all the investigated "omidun" samples. Antimicrobial activities of the isolates were further tested using disc diffusion assay. Results showed that the varieties of "omidun" samples tested harbour some microorganisms (bacteria, yeasts and mould), which were occur in varying incidence among the tested samples. Inhibition zones expressed by the isolates in this work were greater than that expressed by the control (commercial antibiotics) with values ranging from 4.00mm-21.00mm in diameter. Synergistic antimicrobial effect was observed when the isolates were combined with zone of inhibition (38.00mm), higher than that expressed when each of these organisms when used independently (32.00mm for Lactobacillus plantarum and 34.00mm for Aspergillus fumigatus). Higher antimicrobial activity (38.00mm) was observed when A. fumigatus and different yeast isolates were combined and highest activity was observed when Lactobacillus plantarum was combined with five different yeast isolates (39.00mm). Hence, these findings clearly demonstrated synergistic activity of "omidun" isolates against diarrheagenic organisms.

Keywords:- *Diarrheagenic; Disc diffusion; Zone of Inhibition; Synergistic effect.*

Short heading:- *Synergy among microorganisms associated with omidun.*

I. INTRODUCTION

Diarrhea disease represents a major public health problem apart from malaria and pneumonia in developing countries and it is also a high risk to travellers who visit these countries [1]. Bacteria are the major cause of about 80% of diarrhea occurrence in travellers, caused principally by primary diarrhea organisms namely; Salmonella spp, Escherichia coli strains, Shigella and Campylobacter [2]. There are over 2,500 different types of Salmonella, a causative agent of one of the commonest forms of food poisoning worldwide but they all produce clinical symptoms similar to other forms of infective gastroenteritis [1].

Medically, bacterial infections are usually controlled by the use of synthetic drugs. But the recent increases in antibiotic resistant strains of clinically important pathogens including those causing diarrhea has led to the emergence of multi-resistant strains of bacteria [3]. Factors such as, non-availability, high cost and limited effective span of new generation antibiotics have resulted in increase in morbidity and mortality [4]. This research provides additional information on an effective antimicrobial agent from plant origin and with the aim of discovering potentially alternative antimicrobial agent that can be used in the synthesis of new antimicrobial drugs [5] [6].

Traditionally, some cereal foods play important role in the diet of the people in cereal producing zones of Africa. One of such cereal based staple is "Ogi" (fermented cereal porridge made from maize) produced using simple processing methods. It is very smooth in texture and has a sour taste reminiscent of that of yoghurt [7].The top water obtained after filtration of "ogi" is called "omi-eko" or "omikan" or "omidun". "Omidun" has been traditionally found to be of medicinal importance in the South-Western part of Nigeria and has been used in herbal extraction [8].

Previous studies have concentrated mainly on the role of lactic acid bacteria and their use as starter culture in "ogi" production [9][10][11]. Several antimicrobial compounds are produced by LAB, among which are bacteriocins (ribosomally synthesized antimicrobial peptides), which are considered to be safe natural biopreservatives. Also because it is assumed that they are degraded by the proteases in the

gastrointestinal tract and may be useful as a primary hurdle for controlling food borne pathogens [12].

Therefore, Lactobacilli are not only important in acidification of foods, but are also important in the preservation of food, prevention of pathogens and improve the palatability of foods [13]. Reports have confirmed the presence and antimicrobial activities of A. fumigatus and lactic acid bacteria associated with "omidun", but the possible synergy between organisms associated with omidun has not been well exposed. Hence, this study evaluates the possible synergy among microorganisms associated with "omidun" on some selected pathogens.

II. MATERIALS AND METHODS

A. Sample collection

"Omidun" was obtained from sellers of "ogi" in southwest area of Abeokuta, Nigeria. Two varieties of "omidun" samples prepared from white and yellow varieties of "ogi" were collected in sterile bottles with caps. The bottles were labelled for identification and control "omidun" sample was prepared for comparison. Preparation stages include soaking of maize grains in water for 2 days followed by washing of the soaked grains thoroughly with clean water and wet milling to paste. The maize mash was sieved to remove bran, hulls and germ (9, 10).The filtrate is fermented for 2-3days to yield "ogi", which is a sour, starchy sediment with water on top. This top water called "omidun" was transferred into sterile bottles with screw cap and labelled for analysis.

B. Test organisms

The diarrhea organisms used in this study were typed cultures obtained from Lagos State University Teaching Hospital (LUTH) Idi Araba, Lagos. They (S. abaetetuba ATCC 35460 and E. coli ATCC 25922) were collected onto Agar slants and sub-cultured on fresh agar medium for purity.

III. MICROBIOLOGICAL ANALYSIS

A. Determination of Antimicrobial Effect of "omidun"

Antimicrobial effects of the "omidun" samples were determined by agar diffusion method. Few colonies from pure culture of the test isolates was added into 9ml nutrient broth and incubated for 18 hours. A portion of 1mm of 18hr broth culture of E. coli ATCC 25922 was transferred into sterile petri-dish with the aid of sterile syringe, 20ml nutrient agar was added and swirled carefully for the organism to be evenly distributed into the agar and allowed to gel. A sterile cork borer was used to make wells in the agar and 0.1ml "omidun" sample was added into each well. Sterile distilled water as control and the procedure was repeated using S. abaetetuba ATCC 35460 as the test organism. The plates were incubated at 37ºC for 24hrs. The diameter of clear zones around the wells were measured as zones of inhibition and recorded.

B. Isolation of Lb. plantarum

Serial dilutions up to 10^{-5} of "omidun" samples were prepared for enumeration of Lb. plantarum. From each dilution, 1ml was plated on 10-15 ml MRS agar, each set-up was replicated and incubated anaerobically at 37ºC for 48 hours. After 48hrs incubation period, discrete colonies were sub cultured on fresh agar plates of the isolation medium. Further characterization and identification was done on pure isolates stored on Nutrient Agar slants.

C. Identification of Lb. plantarum

Colonies from the MRS were examined for gram strain, catalase reaction, motility and cell morphology. Representative bacteria isolates were identified based on Catalase, indole, motility test, citrate utilization, oxidase test and fermentation of sugars to produce ethanol.

D. Isolation of A. fumigatus

Different dilutions of the serially diluted "omidun" samples were plated onto SDA (containing 60µg/ml of chloramphenicol, to inhibit growth of bacteria). The plates were incubated at 25-30ºC for 48-72 hours after which discrete colonies were sub-cultured on fresh SDA agar plates.

E. Microscopic characteristics

To a drop of sterile distilled water on a clean glass slide was added a small portion of the isolates, mixed and observed microscopically for size, shape and presence or absence of pseudohypha.

F. Identification of A. fumigatus

Fungi isolates were identified by comparing colonial morphological appearance on plates and microscopic examination. For colonial morphology, identification was made according to colony colour, structure, shape, size and pigment. Structural morphology was observed by the Wet mount technique. Growth of each fungus in pure culture was picked using a sterile inoculating needle and placed on a clean glass slide containing a drop of 10% of Potassium hydroxide (KOH) solution. The fungal mycelium was teased out properly. Two drops of lactophenol in cotton blue was added and then covered with cover slip. The slides were then observed under the microscope. Identification was according to structure of mycelium, conditions of branches, presence of conidiophores, sclerotia and shape as compared with literature [14][15][16].

G. Minimum Inhibitory Concentration (MIC) of the Isolates

Lb. plantarum was serially diluted in Mueller – Hinton broth to decrease concentrations up $10^{-6.}$ One milliliter (1m) 18hr- broth of each test organism was added to each dilution and incubated at 37°C for 18hrs. The turbidity obtained after 18hrs was adjusted to McFarland standard. This was repeated for A. fumigatus against the test organisms.

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H. Preparation and Impregnation of discs

Paper discs were prepared from Whatman filter paper (No.1), autoclaved for 15 minutes at 15 lbs pressure and allowed to cool. The sterile discs were placed in Petri-dishes (approximately 5mm apart). 0.02ml suspension of the antimicrobial agent (Lb. plantarum) was loaded on the discs using a mechanical pipette. This impregnation was repeated for A. fumigatus. The discs were allowed to dry at 35ºC for 2-3 hours and stored in sterile air tight containers for further use.

I. Antibiotics Sensitivity Pattern of the Test Organisms

Disc diffusion method was employed to assay antimicrobial activities [17]. A quantity of 0.5ml of 18hr broth culture of each test organism was transferred into sterile Petri-dishes using sterile syringe. Nutrient agar (20ml) was added into each plate, swirled carefully for even distribution of the organisms within the agar and allowed to gel. Commercial antibiotic was placed on the impregnated plates and incubated at 37ºC for 24hrs. Clear zone around the disc was measured and the diameter was recorded as zones of inhibition.

J. Antimicrobial Effect of each Isolate

A quantity of 18hr broth culture of each of E.coli ATCC25922 and S. abaetetuba ATCC 35460 was transferred into separate sterile Petri dishes respectively using sterile syringe. This was overlaid with 20 ml Nutrient broth, swirled for even distribution and allowed to gel. Impregnated discs of A. fumigatus and Lb. plantarum were distributed on the gel. The discs were gently pressed for uniform contact with the surface of the medium, incubated at 37°C for 24 hours and the zones of inhibition observed was measured and recorded.

K. Antimicrobial Effects of Different Combinations of the Isolates

Antimicrobial effects of different combinations of the isolates impregnated in discs were distributed on mediums containing E. coli ATCC25922 and S. abaetetuba ATCC 35460 respectively. They were incubated at 37°C for 24 hours and the zone diameter was measured.

L. Statistical Analysis

Data obtained were subjected to Analysis of Variance (ANOVA) and Duncan Multiple Range Test to separate the means and it was determined at 5% probability level using SPSS 16.0 for Windows [18].

IV. RESULTS

Microorganisms isolated from “omidun” obtained from commercial sellers of white and yellow “ogi” include bacterium species (Lb. plantarum), yeasts strains (Candida albicans, C. parasilopsis, C. pseudotropicalis, C. tropicalis, S. cerevisiae) and moulds (Aspergillus fumigatus) while bacteria (Lb. plantarum) and yeasts (Candida albicans and S. cerevisiae) were the only groups of microorganisms isolated from control samples. A total of 44 strains including LAB (5), mould (1) and Yeasts (38) were identified from “omidun” from white “ogi” varieties with percentage incidence as Lb. plantarum (11%), A. fumigatus (2%), S. cerevisiae (14%), C. albicans (34%), C. tropicalis (7%), C. pseudotropicalis (14%) and C. parasilopsis (18%). Likewise from “omidun” from yellow “ogi” varieties, 41 strains including LAB (5), mould (2) and Yeasts (34) were identified as Lb. plantarum (12%), A. fumigatus (5%), S. cerevisiae (15%), C. albicans (36%), C. tropicalis (15%), C. pseudotropicalis (5%) and C. parasilopsis (12%).

For the control samples of “omidun” from yellow and white “ogi”, 27 and 28 strains of microorganisms were identified respectively as LAB (26%), S. cerevisiae (30%) and C. albicans (44%) from “omidun” from yellow “ogi” and 21% LAB, 29% S. cerevisiae and 50%C. albicans were identified from “omidun” from white variety of “ogi”. No organisms was isolated from sterile distilled water (Table 1).

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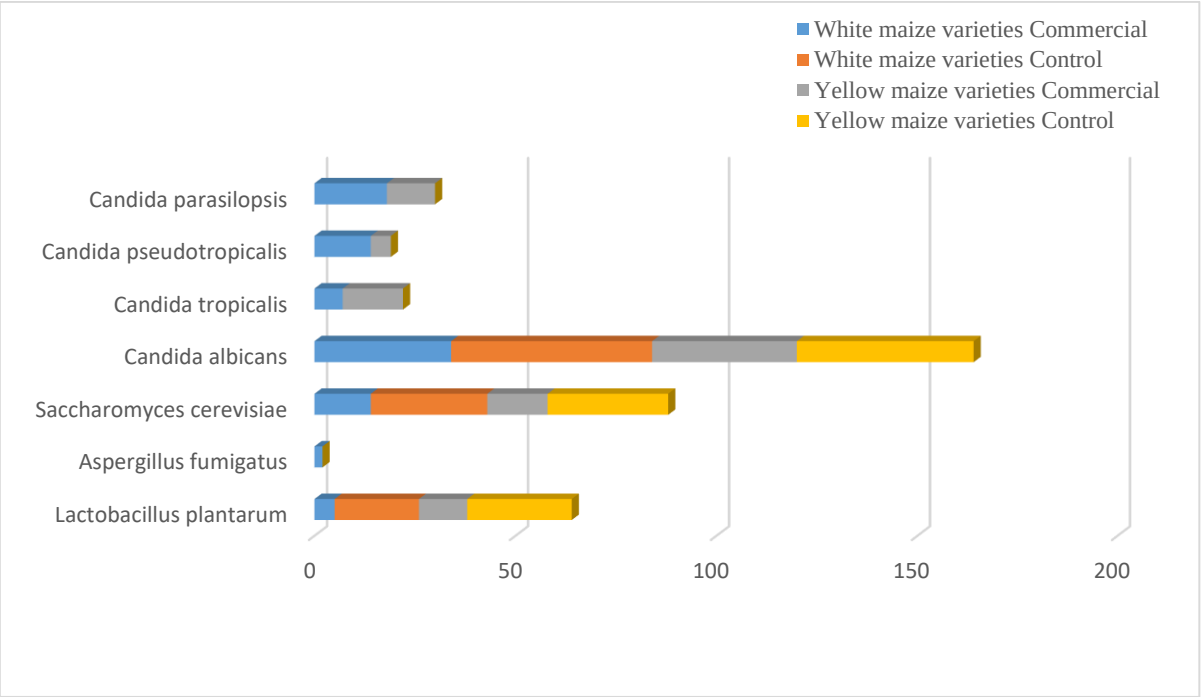

Figure 1:- Percentage Incidence of Isolates from "omidun" from White and Yellow "ogi" Varieties

The Minimum Inhibitory Concentration (MIC) of A. fumigatus was lower (0.07 µg/mL on E. coli and 0.03-0.13 on S. abaetetuba), while that of Lb. plantarum was 0.25 µg/mL on E. coli and 0.13 on S. abaetetuba) but higher MIC value was obtained when A. fumigatus was combined with Lb. plantarum (0.13 µg/mL) and when A. fumigatus was combined with 5 yeasts (0.25 µg/mL) as shown in Table 1.

Diameter of inhibition zone observed on Lb.plantarum against E. coli ATCC 25922 was 32 mm and 28 mm on S. abaetetuba ATCC 35460. A significantly ($p<0.05$) higher inhibition zones (34 mm respectively) was obtained when A fumigatus was used against E. coli ATCC 25922 and S. abaetetuba ATCC 35460. However, combination of Lb. Plantarum and A. fumigatus showed wider zone of inhibition of 35 mm on both E. coli ATCC 25922 and S. abaetetuba ATCC 35460. Widest zone of inhibition was observed when all the yeast isolates were combined with Lb. plantarum (38mm against E. coli ATCC 25922 and 39mm against S. Abaetetuba ATCC 35460) and A. fumigatus (39mm against the two tested organisms) respectively. Ciprofloxacin gave inhibition zone of 21mm and 20mm respectively on E. Coli and S. abaetetuba (Figures 2 and 3)

Isolates from omidun	MIC (µg/mL) on E. coli ATCC 25922	Zone diameter(mm) on E. coli ATCC 25922	MIC diameter(µg/mL) on S. abaetetuba ATCC 35460	Zone diameter (mm) on S. abaetetuba ATCC 35460
Lb. plantarum	0.25	32.00±0.1b	0.13	28.00±0.3b
A. fumigatus	0.07	34.00±1.4ab	0.03	34.00±0.7a
Lb. plantarum with A. Fumigatus	0.13	35.00±0.4ab	0.25	35.00±1.3a
Lb. plantarum with 5 yeasts	0.06	39.00±11a	0.13	39.00±0.5 a
A. fumigatus with 5 yeasts	0.13	38.00±0.4a	0.25	39.00±0.2 a

Table 1: Summary of MIC and Zone diameter of Lb. Plantarum and A. Fumigatus individually and in combination

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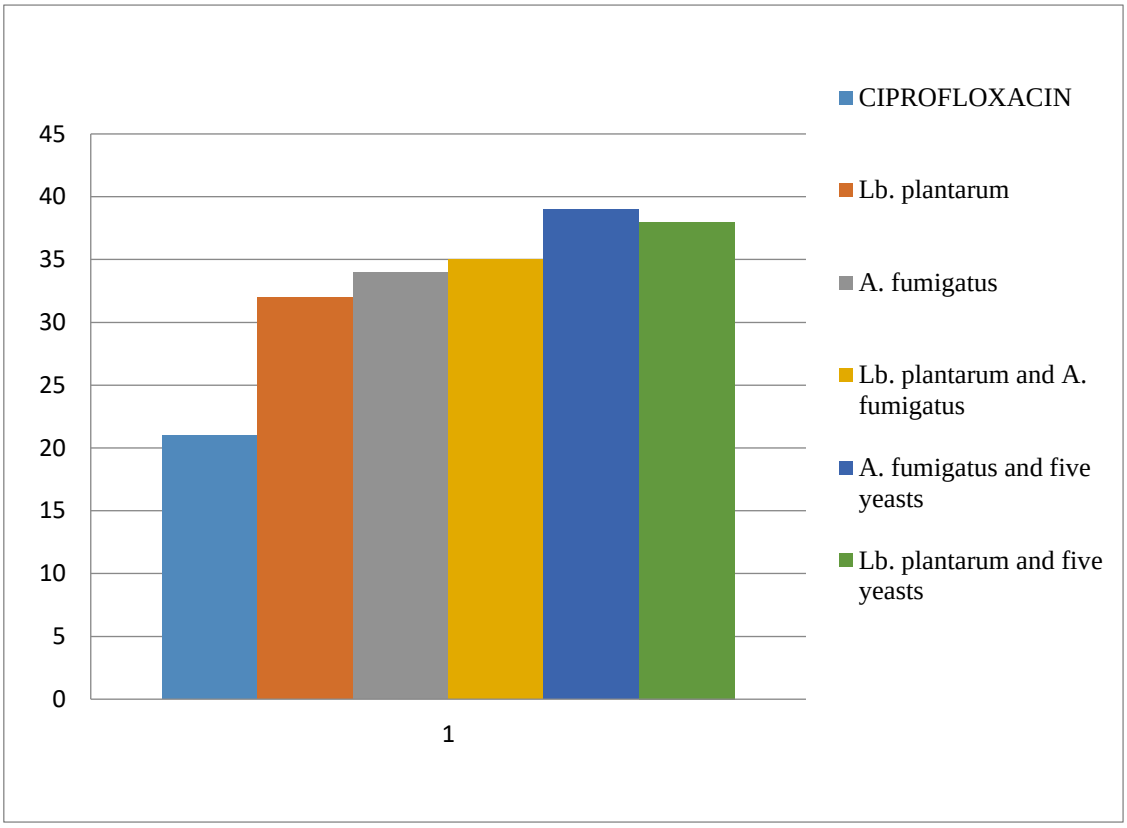

Figure 2: Summary of Zones of Inhibition (mm) of Ciprofloxacin, Lb. Plantarum and A. fumigatus independently and in combination with five yeasts on E. coli

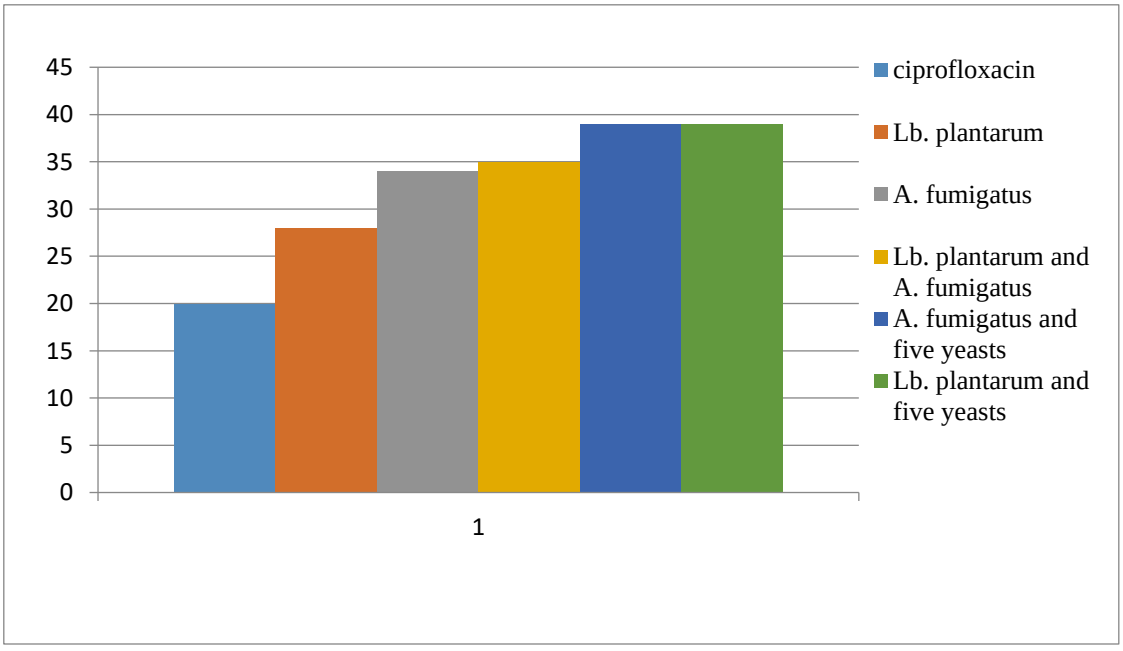

Figure 3:- Summary of Zones of Inhibition (mm) of Ciprofloxacin, Lb. Plantarum and A. fumigatus independently and in combination with five yeasts on S. abaetetuba.

V. DISCUSSION

In Nigeria, "omidun" has been used to soak some plants for medicinal purposes. In this study, "omidun" was found to harbour some microorganisms (bacteria, yeasts and mould) identified as Lactobacillus plantarum, Candida albicans, C. tropicalis, C. pseudotropicalis, C. Parasilopsis, Sacchcaromyces cerevisiae and Aspergillus fumigates as shown in Figure 1. Presence of Lactobacilli has also been reported in the fermentation of "ogi" [19] while Omemu et al. [20] reported the involvement of yeast strains in the fermentation of "ogi". Aspergillus fumigatus was the only mould isolated from commercial "omidun" but not isolated in the control "omidun" sample. This might be probably from the contact the maize kernels had with the soil during the process of drying and milling or due to poor quality of maize kernel used in "ogi" production. Presence of A. fumigatus in this work conforms with the findings of Omemu and Omeike [21], who stated that there is likelihood of pathogenic contamination in improperly cooked Ogi which cannot be ruled out in "omidun" (the supernatant of ogi). Cases of microbial pathogens have also been reported in association with several fermented foods, such as cheese, sausages, fermented fish and fermented cereals [22].

It was observed also that, the control "omidun" sample from yellow 'ogi" harboured the highest incidence of Lb. plantarum , followed by the control "omidun" sample from white "ogi" while commercial "omidun" samples from the two "ogi" varieties had the lowest Lb. plantarum incidence. The general incidence of Lb. plantarum observed in this work depicts the sour taste of "omidun". Lactobacilli have been reported to cause acidification, preservation and prevention of pathogens from fermented foods as well as improve the palatability of foods [13]. The aroma of "omidun" can be attributed to the high incidence of some yeast strains discovered in this work. This is in line with the report of Hamad et al. [23] who proclaimed the impact of yeasts in flavor and aroma of fermented foods.

It has been reported that microorganisms may improve or depreciate the safety of food stuffs [24], however, this work revealed antimicrobial properties of microorganisms isolated from "omidun" fermented for 48hours. Varying antimicrobial activities were observed in this work and that was indicated by the different MIC and consequently different zones of inhibitions obtained (Tables 1, Figures 2 and 3). The MIC of the isolates on the tested organisms was 0.03µg/mL, 0.07µg/mL, 0.1µg/mL and 0.25µg/mL. MICs observed in this work were lower than 100µg/mL and this implies high antimicrobial activity of "omidun". Hence, "omidun" is also of great significance in the healthcare delivery system, since it could be used as an alternative to orthodox medicine in the treatment of diarrheagenic infections, especially as microorganisms frequently develop resistance to known antibiotics [25].

Ciprofloxacin antibiotics tested against E. coli ATCC 25922 and S. abaetetuba ATCC 35460 for antimicrobial activity revealed zones of 21mm and 20mm respectively. These zones were greater than 14 mm and the test organisms can be regarded as susceptible to ciprofloxacin antibiotics. When the Lb. plantarum isolated in this work was used alone (Figure 2 and 3), it showed diameter of zone of inhibition greater than that expressed by the commercial antibiotics and this makes Lb. plantarum potentially useful in the control of diarrheagenic bacteria. Similar antimicrobial activity has been reported [26] [27] [28].

A. fumigatus is a commonly known spoilage organism, however, it has been reported to produce fumigacin which shows antimicrobial activity [20] and is used as a health care product. In this work, A. fumigatus showed higher antimicrobial activity than Lb. plantarum against the tested diarhoegenic bacteria (Table 2). Furtado et al. [29] has also reported the production of some antimicrobial metabolites from A. fumigatus. Recently, Kusari et al., [30] reported the production of an anticancaer drug, deoxypodophyllotoxin, from A. fumigatus. The drug has also shown antimicrobial efficacy against some pathogenic microorganisms including E. coli. Interestingly, synergistic antimcicrobial effect was observed when A. fumigatus and Lb. plantarum were combined (Table 2), the antimicrobial effect was higher when the isolates were combined than when each of these organisms were used independently as seen in Table 3. An increase in the antimicrobial activity of A. fumigatus in the presence of bacteria has also been reported by Furtado et al. [31].

Microorganisms isolated from "omidun" have shown zones of inhibition to diarrheagenic bacteria tested in this work. The highest antimicrobial activity in this work was observed when A. fumigatus and Lb. plantarum were combined with five yeasts respectively and this is higher than in any of the commercially produced antibiotics as seen in table 3, this finding may probably be due to synergistic effects exhibited by "omidun" isolates. "Omidun" is often discarded as waste and may become a very cheap source of control of diarrhea.

VI. CONCLUSION

Data from this study has revealed the synergistic antimicrobial properties of microorganisms isolated from "omidun". Although, A. fumigatus have been known to be pathogenic, its presence in this work showed high antimicrobial activity against the tested organisms indicating that it produced antimicrobial compounds. The findings of this study further revealed that apart from the traditional use of "omidun" to soak herb, it has also been confirmed to harbour

some microorganisms with proven synergistic antidiarrheal properties. Hence, consumption of "omidun" obtained from ogi prepared with clean water and under hygienic condition is highly encouraged in the control of diarrhea.

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