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Studies on the Occurrence of Pathogens in Fermented African Oil Bean Seed Ugba

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Abstract
The study was carried out to determine the occurrence of pathogens in fermented African oil bean ugba. Samples of ugba were produced locally using standard procedures. The bacterial population was isolated, characterized and identified. Antibiotic susceptibity tests were conducted on the pathogens. The bacteria isolated were Bacillus licheniformis, Enterobacter spp, Staphylococcus aureus, Shigella spp, Bacillus subtilis, Klebsiella spp. Staphylococcus epidermidis, Salmonella spp. Bacillus cereus, Corynebacterium spp., Escherichia coli, Shigella spp and Bacillus alvei. The coliform count had the highest value of $3.7x10^4$, $7.9 x10^2$, $2.5x10^4$ cfu/g at 72 h for all the samples of ugba produced, except for the ugba bought from the local producer which recorded the highest count ($5.5x10^7$) at 48 h. The highest heterotrophic count of $8.1x10^7$, $1.1x10^5$, $1.3x10^3$, $3.6x10^3$ was obtained at 72 h for all the samples of ugba produced. The Staphylococcus count recorded the highest value of $3.9x10^5$, $4.5x10^3$, $9.5x10^5$ at 48 h for all the samples of ugba produced, except ugba prepared with sterilized water which recorded a maximum of $1.81x10^3$ at 48 h. For Salmonella-Shigella count, the highest value of $5.0x10^6$ and $6.0x10^2$ cfu/g was obtained at 48 h for ugba samples bought from a local producer and prepared with unsterilized water, while the highest values of $3.7x10^4$ and $3.4x10^3$ cfu/g were recorded for samples of ugba prepared with boiled and sterilized water. Some of the bacteria were susceptible to reflacine, ciproflox, gentamycin and streptomycin and all showed multi-drug resistance. It is concluded that ugba contained entero pathogens which are of public health importance.

Keywords: Pathogenic bacteria, Fermentation, African oil bean seeds, ugba, pulic health importance

Introduction
Ugba is rich in protein and is obtained by a solid state fermentation of the seed of African oil bean seed (*Pentaclethra macrophylla* Benth). It is consumed by an estimated 15million people in Eastern Nigeria, majority of whom are igbos(Odunfa and Oyeyiola, 1985). It is an essential food item for various traditional ceremonies where it is mixed with slices of boiled stock fish(*ugba na okpoloko*), garnished with steamed vegetables and consumed by all socio-economic class (Achinewhu, 1982). *Pentaclethra macrophylla* Benth belongs to the family *lenguminosae* and sub family *microsoideae (*Keay, 1989) and is a large wooden plant abundant in the rain forest areas of west and central Africa. Its origin in Nigeria is believed to be around 1937 (Ladipo, 1984), where it was found in the southern Nigeria (Mbajunwa *et al.*, 1998). *Pentaclethra macrophylla* Benth Ugba seeds are irregular and oval; they are flat black-brown and hard pods. It is composed of oil, protein and small amounts of carbohydrates (Obeta, 1983). Their edible seed require tedious but careful processing and fermentation before they can be eaten as food supplement (Enujiugha and Badejo, 2002). Microorganisms in ugba fermentation act upon the seeds to bring about changes in the texture, aroma, colour, taste and nutritional composition. The diversity of the bacteria groups isolated from the fermented oil bean seed product showed that the seeds contain sufficient nutrients to support the growth of these organisms (Enujiugha *et al.*, 2008). The bacteria are able to perform these roles because they can utilize the major constituents of the seed, that is, the proteins, oils and carbohydrates (Enujiugha *et al.*, 2008). During ugba fermentation, the organisms soften the oil bean cotyledons progressively, while at the same time, producing the characteristic aroma and taste of the product (Enujiugha and Badejo, 2002). The natural

fermentation of the seeds which at present is still done at the household level renders the product nutritious, palatable and non-toxic(Ogbulie *et al.*,2014). Several workers have investigated the microorganisms involved in the fermentation and observed that only bacteria were involved (Obeta, 1983; Odunfa and Oyeyiola, 1985; Ejiofor *et al.*, 1987 and Ogueke and Aririatu, 2004). The main fermenting microorganisms have been *Bacillus* species, *Micrococcus* species and *Lactobacillus* species. Other organisms isolated from ugba included *Pseudomonas* species, *Staphylococcus* species, *Enterobacter* species and *Corynebacterium* species (Enujiugha, 2009; Isu and Njoku, 1997; Isu and Ofuya, 2000; Sanni *et al.*, 2000).The traditional processing method of fermenting the African oil bean seed into ugba is riddled with issues of product safety. For instance during the production of ugba pathogens could be introduced through air, water, equipments used during processing such as utensils, leaves used for wrapping, or even through the human handlers (Nwagu *et al.*,2010). The growth and occurrence of these organisms of public health importance in ugba is of great concern. These pathogens if consumed in ugba are capable of causing various diseases. The information on the occurrence and growth of pathogens in African fermented foods is limited. In previous studies, a few pathogens have been detected through the cultural-based method. Most of them except *Escherichia coli* and *Staphylococcus aureus* were identified only to the genus level (Ogueke *et al*. 2010; Eze *et al*. 2014). Anyanwu *et al*. (2016) and Ogbulie *et al*. (2014) have both reported the presence of *E. coli*, *Klebsiella* and *Staphylococcus* species in samples of ugba. The presence of these organisms in the product constitute a source of concern to the consumers who are increasingly becoming safety conscious.

The current study was undertaken to determine the occurrence of pathogens from fermented African oil bean seed ugba and to determine the antibiotic susceptibility profile of them.

Materials and methods

Sample collection

The African oil bean seed (*Pentaclethra macrophylla*) was purchased from Ihiala market, Ihiala town in Eastern Nigeria. Freshly wrapped unfermented slices of African oil bean seeds were bought from a local producer at Obodokoli village, Uli, Anambra state and was promptly taken to the laboratory for analysis.

Traditional preparation of ugba in the laboratory

The method used was described by (Okorie and Olasupo, 2011). The traditional method of preparing ugba was adopted in the laboratory to ferment the seeds for ugba production. One kilogram (1kg) African oil bean seeds were boiled for 6h to soften the hard brown testa. The shells were removed and the cotyledons were cut into long thin slices. The slices were washed and boiled for another 2h. These slices of ugba were cooled and divided into three parts and each was washed using (i) unsterilized water (ii) boiled water and (iii) sterilized water. The washed ugba slices were wrapped accordingly into small packets in banana leaves and were lightly tied and placed in a basket to ferment at room temperature for three days to yield ugba. The freshly wrapped slices of African oil bean seeds bought from a local producer at Obodokoli village Uli were also placed in a basket and allowed to ferment also for three days.

Isolation and characterization of enteropathogens from ugba

Sterile forcep was used to take samples aseptically from the fermenting packets prepared in the laboratory and those bought from the market. Ten grammes of the sample was homogenized with 90ml of sterile saline solution as diluents. Thereafter tenfold serial dilutions of the samples was done and aliquots of 1ml was used to inoculate plates of Nutrient agar, MacConkey agar, Mannitol salt agar, Salmonella-Shigella agar using pour plating techniques. The plates were incubated at 37^0C for 24-48h and colonies that develop were subcultured into freshly prepared agar plates. Pure cultures were stored in agar slants. The bacteria isolates were identified based on cultural, morphological and biochemical characteristics.

Antibiotic sensitivity testing

The disc diffusion technique was adopted for determining the sensitivity of the isolates to different antibiotics. Each isolate (2 loopfuls) was inoculated into sterile nutrient broth and incubated for 24h. Then the broth culture of the isolate was diluted 10^{-4} in sterile normal saline solution and aliquot of 0.1ml was inoculated on nutrient agar plates using spread plate technique. With the aid of sterile forceps the antibiotic impregnated disks were carefully placed on the inoculated plates and firmly pressed with the forcep to ensure contact with the medium. The plates were inverted and incubated for 24h at 35^0C.The zones of inhibitions noticed on the plates were measured and the readings were taken.

Results

Table 1 shows the bacteria isolated from ugba using different types of water. The bacteria isolated from ugba bought from local producer were *Bacillus licheniformis, Enterobacter* spp, *Staphylococcus aureus* and *Shigella* species while those isolated from ugba produced using unsterilized water were *Bacillus licheniformis, Bacillus subtilis, Klebsiella* spp. *Staphylococcus epidermidis* and *Salmonella* spp. For ugba produced using boiled water, the bacteria isolated were *Bacillus cereus, Bacillus licheniformis, Corynebacterium spp., Escherichia coli, Staphylococcus aureus* and *Shigella* spp, while for ugba produced using sterilized water the bacteria isolated were *Esherichia coli, Bacillus alvei, Bacillus licheniformis, Staphylococcus aureus* and *Shigella* spp.

Table 2 shows the microbial count for ugba sample bought from a local producer. The total coliform count increased to the highest at 48 h (5.5 x10^7cfu/g), while the total heterotrophic count increased maximally at 72 h (8.1 x 10^7 cfu/g). The total staphylococcus count increased to maximum at 48 h (3.9 x10^5cfu/g), while the total Salmonella-Shigella count increased to the highest level at 48 h (5.0 x 10^6cfu/g).

Table 3 show the microbial count of ugba sample produced with unsterilized water. The total coliform count increased to the maximum at 72 h (3.7 x 10^4cfu/g), while the total heterotrophic count increased to the maximum at 48 h (1.5 x 10^4cfu/g). The total staphylococcus count increased to highest at 48 h (4.5 x 10^3cfu/g), while the total Salmonella-Shigella count increased to the highest level at 48 h (6.0 x 10^2cfu/g).

Table 4 show the microbial count for ugba sample produced with boiled water. The total coliform count

increased maximally at 72 h (7.9 x 10^2 cfu/g), while the total heterotrophic count increased to the highest at 72 h (1.3 x 10^3 cfu/g). The total staphylococcus count increased to the maximum at 48 h (9.5 x 10^3 cfu/g), while the total Salmonella-Shigella count increased maximally at 72 h (3.7 x 10^4 cfu/g).

Table 5 shows the microbial count for ugba sample produced with sterilized water. The highest total coliform count was observed at 72 h (2.5 x 10^4cfu/g), while the total heterotrophic count was maximum at 72 h (3.6 x 10^3cfu/g). The maximum total staphylococcal count was recorded at 48 h (1.8 x 10^3cfu/g), while the total Salmonella-Shigella increased to the highest at 72 h (3.4 x 10^3cfu/g).

Table 6 shows the antibiotic susceptibility testing of enteropathogens isolated from ugba. *Klebsiella* sp. was resistant to all the antibiotic while 100% of *Enterobacter* and *Salmonella* species were susceptible to ciproflox. *Shigella* spp (33.33%) were sensitive to reflacine and gentamycin, while 33.33% of *Escherichia coli* were sensitive to ciproflox and 66.66% were sensitive to gentamycin. *Bacillus cereus* and *Staphylococcus epidermidis* were resistant to all the antibiotics except rifampicin which was effective against *Staphylococcus epidermidis.* Ciproflox, gentamycin and rifampicin were effective against *Staphylococcus aureus.*

Table 1: Bacteria Isolated From ugba prepared using different types of water

Types of water used for production of ugba	Bacteria Isolated
Ugba bought from the local producer (control)	*Bacillus licheniformis, Enterobacter* species, *Staphylococcus aureus, Shigella* species.
Production of ugba with unsterilized water	*Bacillus licheniformis, Bacillus subtilis, Klebsiella* species, *Staphylococcus epidermidis, salmonella* species
Production of ugba with boiled water	*Bacillus cereus, Bacillus licheniformis, Corynebacterium* species, *Escherichia coli, Staphylococcus aureus, Shigella* species.
Production of ugba with sterilized water	*Escherichia coli, Bacillus alvei, Bacillus licheniformis, Staphylococcus aureus, shigella* species

Table 2: Microbial count of ugba sample bought from local producer

Period (h)	Total coliform count (cfu/g)	Total heterotrophic count (cfu/g)	Total staphylococcus count (cfu/g)	Total Salmonella Shigella count(cfu/g)
0	ND	ND	ND	ND
24	$3.0x10^6$	$6.2x10^4$	$3.4x10^5$	$2.9x10^4$
48	$5.5x10^7$	$7.6x10^5$	$3.9x10^5$	$5.0x10^6$
72	$3.6x10^6$	$8.1x10^7$	$1.5x10^3$	$3.2x10^4$

Key: ND = Not determined

Table 3: Microbial count of ugba sample produced with unsterilized water

Period (h)	Total coliform count (cfu/g)	Total heterotrophic count (cfu/g)	Total staphylococcus count (cfu/g)	Total salmonella shigella count (cfu/g)
0	ND	ND	ND	ND
24	$1.8\ 10^2$	$0.6x10^2$	$1.1x10^2$	$2.7x10^2$
48	$1.1x10^3$	$1.5x10^4$	$4.5x10^3$	$6.0x10^2$
72	$3.7x10^4$	$1.1x10^5$	$2.2x10^3$	$4.6x10^2$

Key: ND = Not determined

Table 4: Microbial count for ugba sample produced with boiled water

Period (h)	Total coliform count (cfu/g)	Total heterotrophic count (cfu/g)	Total staphylococcus count (cfu/g)	Total salmonella shigella count(cfu/g)
0	ND	ND	ND	ND
24	2.0×10^1	3.8×10^2	$3.4x10^2$	5.2×10^2
48	$1.4x10^2$	$7.2x10^2$	$9.5x10^3$	$6.8x10^3$
72	$7.9x10^2$	$1.3x10^3$	$7.4x10^2$	$3.7x10^4$

Key: ND = Not determined

Table 5: Microbial count for ugba sample produced with sterilized water

Period (h)	Total coliform count (cfu/g)	Total heterotrophic count (cfu/g)	Total taphylococcal count (cfu/g)	Total salmonella shigella count(cfu/g)
0	ND	ND	ND	ND
24	$0.1 x10^1$	0.4×10^2	$0.4 x10^2$	4.2×10^2
48	$0.8x10^2$	$1.8x10^2$	$1.8x10^3$	$6.8x10^2$
72	$2.5x10^4$	$3.6x10^3$	$4.3x10^2$	$3.4x10^3$

Key: ND = Not determined

Table 6: Susceptibility testing of some enteropathogens isolated from ugba

Antibiotics (µg)	***Klebsiella* sp n=1**	***Enterobacter* sp n=1**	***Shigella* sp n=3**	***Salmonella* sp n=1**	***Escherichia coli* n=3**	***Staphylococcus aureus* n=3**	***Staphylococcus epidermidis* n=1**	***Bacillus cereus* c=1**
Tarivid (10)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	ND	ND	ND
Reflacine (10)	0(0.0)	0(0.0)	1(33.3)	0(0.0)	0(0.0)	ND	ND	ND
Ciproflox (10)	0(0.0)	1(100)	0(0.0)	1(100)	1(33.3)	2(66.7)	0(0.0)	0(0.0)
Augmentin (30)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	ND	ND	ND
Gentamycin (10)	0(0.0)	0(0.0)	1(33.3)	0(0.0)	2(66.7)	1(33.3)	0(0.0)	0(0.0)
Streptomycin (30)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)
Ceporex (10)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	ND	ND	ND
Nalidixic acid (30)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	ND	ND	ND
Septrin (30)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	ND	ND	ND
Ampicillin (30)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	ND	ND	ND

ND = Not determined

Discussion

The occurrence of pathogenic microorganisms has always been attributed to several factors, which include contamination through water and soil, food processing equipments, food contact surfaces and most importantly food handlers(Shamsuddeen and Ameh,2008; Aboloma,2008). The lack of good production practice for fermented African oil bean seeds is a major setback in food safety standards of the product. The lapses in our vigilance for food safety have been responsible for outbreaks of serious food poisoning in different regions particularly in the continent of Africa (Campbell-Platt 1997; Olasupo *et al*.

2002).The occurrence of pathogens in the ugba studied is corroborated by the report of Olasupo et al. (2002) which revealed that microorganisms of public health concern have been associated with Nigerian fermented foods which included *Staphylococcus aureus, Klebsiella* sp., *E. coli*, *Salmonella* sp. and *Enterococcus faecalis*. Okorie *et al.* (2017) reported the occurrence of *Staphylococcus aureus*, *Escherichia coli,Klebsiella* species, *Enterobacter* species, *Proteus* species and *Pseudomonas* species in samples of ugba. Other pathogens have been reported in other African fermented foods which included *Bacillus cereus, Salmonella* sp. *Vibrio cholera*, *Aeromonas* sp., *Campylobacter* and *Shigella* spp (Gadaga *et al.* 2004). Anyanwu *et al*. (2016); Eze *et al*. (2014); Isu and Njoku (1997); Ogbulie *et al*. (2014) observed *Escherichia coli* and species of *Staphylococcus*, *Klebsiella*, and *Proteus* in fermented African oil bean seeds. In contrast, *various* microbiological studies conducted on ugba (Obeta, 1983; Odunfa and Oyeyiola, 1985; Ogueke and Aririatu, 2004) showed that pathogens such as *Clostridium perfringes, C. botulinum, Salmonella sp., Shigella sp. and Vibrio sp.* have not been isolated from ugba. However, bacteria such as *E. coli and Staphylococcus aureus* have been isolated. *E coli, Klebsiella sp, Salmonella sp, Shigella sp* and *Proteus sp* are enteric bacteria, whose natural habitat is the intestinal tract of humans and animals (Jawetz et al., 1989). Enteric bacteria are also regarded as indicators of feacal contamination of water and could be introduced in food through water or during the fermented food production.

The occurrence of *Salmonella* in food might be attributed to the water used in preparation of ugba as *Salmonella typhi* has been reported to be transmitted through water (Uzeh and Agboniahor, 2001). *Shigella*, although classically known as a water borne pathogen, is also a significant cause of food borne disease (June *et al.* 1993). The main contributing factors which led to outbreaks of food borne shigellosis or bacillary dysentery, are poor personal hygiene of food handlers and improper holding temperature of contaminated food (Smith, 1987). Consequently, the foods implicated in outbreaks are those that require hand processing and/or are prepared from raw or previously cooked products without re heating. Ugba is an under cooked food that requires hand processing, thus the presence of *Shigella* is expected. *E.coli* if consumed is liable to causing peritonitis, septicemia, gastrointestinal infections and mastitis. *Staphylococcus aureus* is a well-known causative agent of gastro intestinal infection, diarrhea and could be fatal, while *Salmonella typhi* is the causative agent of typhoid fever, an enteric fever that can also be fatal (Nester *et al*., 2004). Whether the pathogens identified by this study were introduced through post-production contamination or they survived the fermentation process, their presence in ugba pose health risks to the consuming public (Okorie *et al*., 2017). This is especially so as ugba purchased in the market place is sometimes eaten without pre-heating or cooking (Okorie *et al*., 2017).

African oil bean are largely composed of oil, protein and relatively small amount of carbohydrate (Enujiugha and Badejo, 2002), the ability to break down these components of the seed is an important characteristic of the organisms able to ferment and persist till the seed rottens. This explains why *Bacillus spp.* which are known to break down oil were prominent throughout the experiment.

Bacillus spp. have been implicated in the fermentation of most vegetable oil protein seeds like African locust bean seeds, soy bean seeds, African oil bean seed etc (Antai and Ibrahim, 1986; Odunfa and Oyewole, 1986).

Conclusion

It is concluded that ugba do contain enteropathogens**.** An important consideration is the fact that most food handlers

do not practice good personal hygiene and good production practices because of lack of continuous awareness and education by relevant authorities concerned with food safety regulations. Post-production contamination of ugba can lead to possible introduction of pathogenic organisms. To ensure food safety, it is critically important that quality control standards including accurate methods for identifying pathogens be adopted and followed, in order to understand the extent of danger posed by the consumption of such food.

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