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Microbiological Quality and Shelf Life of Pickled African Walnut (*Tetracarpidium conophorum*) Preserved with Lactic and Citric Acids

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Authors' contributions

This work was carried out in collaboration between all authors. Authors IA and NNO designed and supervised the study. Author NM performed the statistical analysis, wrote the protocol, and wrote the first draft of the manuscript. Author VCE managed the analyses of the study and the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

The short shelf life of locally processed ready-to-eat African walnut is a challenge limiting its utilisation. Pickling is an ancient traditional practice of preserving certain types of food. Therefore, the addition of organic acids to African walnut pickle is aimed at elongating shelf life of the product. In this study, raw African walnut was prepared, then pickled using brine solution (5 % NaCl) that separately contain 1 %, 3 %, 5 % citric and 1 %, 3 % and 5 % lactic acid. The samples were stored for 6 Weeks at room temperature (28±2ºC). Total bacterial and fungal counts of the product were monitored weekly using standard methods. Microbiological challenge test study of the pickles using *Bacillus cereus* and *Staphylococcus aureus* isolated from fresh African walnut was also performed. Pickle without organic acid was the control sample. Using molecular identification methods, *Bacillus cereus* strain EV-1 (KY689737), *Ochrobactrum ciceri* strain EV-2 (KY689738) and *Bacillus amyloliquefaciens* strain EV-3 (KY689739) were isolated from the pickles. Fungi isolated were *Penicillium* sp. and *Fusarium* sp. Total bacterial and fungal count of the pickles range between 3.52-

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7.34 log_{10}cfu/g and 3.48-5.73 log_{10}cfu/g, respectively was higher than that of the control sample. Importantly, lactic acid demonstrated more antibacterial effect than citric acid in preserving African walnut pickles. The challenge test result revealed that *B. cereus* was more dominant than *S. aureus*. Based on Microbiological Guidelines for Ready-to-Eat Food that *B. cereus* and *S. aureus* must be less than 5 log_{10}cfu/g and 4 log_{10}cfu/g, respectively, the developed pickle is best consumed within 2 weeks.

Keywords: Pickled; lactic acid; African walnut; citric acid; microbiological quality; preserved.

1. INTRODUCTION

Nuts are rich in nutrients and have numerous health benefits [1,2]. African walnut (*Tetracarpidium conophorum*) is an edible nut. It gives a bitter taste when it is eaten raw [3]. African walnut grows in some parts of Nigeria and West Africa. The literal meaning of walnut is 'foreign nut' derived from *wealhhnutu* which is an old English word [4]. African walnut is usually cooked and consumed as a snack [5]. Assessment of microflora of the shells of ready-to-eat African walnut was done by Nwabunnia and Otogbor [6] to ascertain if the product available in the market is microbially safe for human consumption. This is critical because cooked African walnut has a short shelf life of three days [7]. It is underutilised because of a lack of storage facilities [8,9]. According to Sejiny et al. [10] and Akin-Osanaiye and Ahmad [11], stored African walnuts are exposed to microbial contamination which could cause spoilage.

Pickling is a food preservation method commonly used to preserve meat, vegetables, eggs and fruits. It involves covering the food product with a heated brine solution. Organic acids (usually acetic or citric acid), spices, salt and food colouring is usually added to the brine solution. Pickling process prevents growth and survival of spoilage microorganisms. Malaysia Food Act 1983 and Food Regulations 1985 listed characteristics of pickles of good quality [12]. The term 'hurdle technology' refers to multiple processing factors that inhibit the growth of microorganisms in food. This concept is important to ensure pickles are safe for human consumption [13,14].

For several decades, the pickling process involves a traditional process which does not identify possible sources of microbial contamination of pickles. In recent years, a scientific and systematic approach known as hazard analysis critical control points (HACCP) is being put in place to identify hazards associated with food processing, production or preparation, assessment of the risks involved and the establishment of effective control procedures against microbial contamination. Determination of critical control points (CCPs) is fundamental to the successful implementation of HACCP plan [15]. Therefore, implementation of HACCP plan during the pickling process could guarantee microbially safe pickles. Bulla [16] listed some traditional practices pickles stored for a long period is usually subjected to.

There is a dearth of information about the microbiological quality and shelf life of pickled African walnut with added preservatives. Therefore, this study is aimed at using cultural and molecular identification methods to identify and enumerate microorganisms associated with pickled African walnut preserved with organic acids, assessment of the pickle's ability to support the growth of pathogenic bacteria as well as determine the shelf life of the product.

2. MATERIALS AND METHODS

Fresh raw African walnuts were purchased from Eke Akpara market, Abia state using sterile plastic bags. The nuts were quickly taken to Food and Industrial Microbiology Laboratory, University of Port Harcourt for analysis.

2.1 Preparation of African Walnut

The procedure described by [17] with modifications shown in Fig. 1 was adopted in preparing pickled African walnut. It shows critical control points (CCP) involved in the process.

2.2 Preparation of Brine Solution

It involves adding 5 g of NaCl into 100 ml of distilled water contained in seven clean glass jars [17].

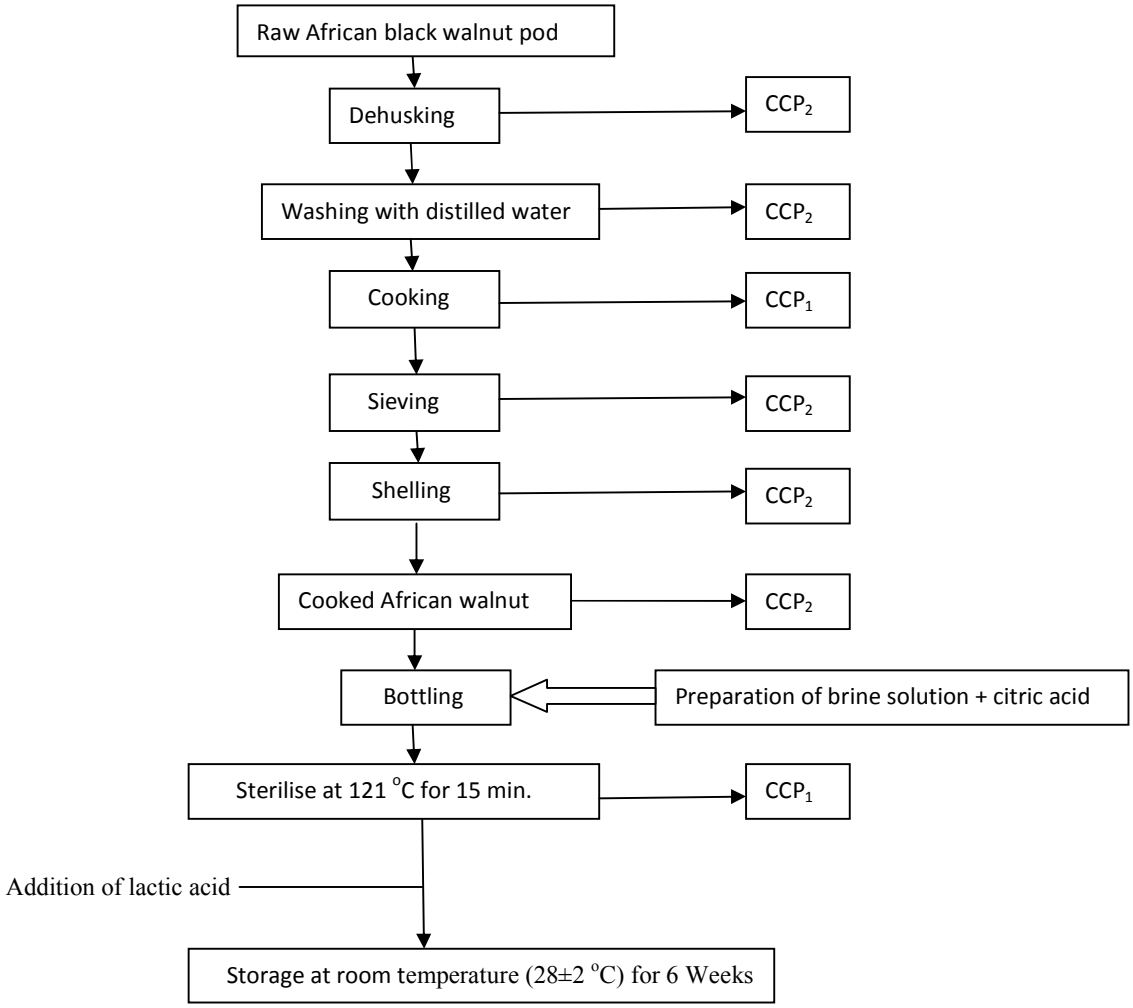

Fig. 1. Flow chart for preparation of pickled African walnut
Key: CCP means critical control points

2.3 Pickle Preparation

The method adopted was described by Sultana et al. [18] and Ibrahim et al. [12] with slight modifications. Pickling of African walnut involved adding 100 g of the sample into already prepared brine solution in seven clean glass jars. Thereafter, 1 % (w/v), 3 % (w/v) and 5 % (w/v) citric acid which serve as preservative were separately added to the brine solution, followed by covering the glass jars with a lid and shaken with two hands before being sterilized at 121ºC for 15 min. Another set of brine solution in 3 clean glass jars containing 100 g African walnut was also sterilised at 121ºC for 15 min. followed by addition of 1 % (v/v), 3 % (v/v), and 5 % (v/v) lactic acid, respectively. Pickled African walnut without preservative was the control sample. The seven glass jars containing pickled African walnut was stored at room temperature (28±2ºC) for a period of 6 Weeks.

2.4 Microbial Analysis

Total aerobic bacteria count and total fungi count of pickled African walnut formulations were monitored weekly for 6 Weeks following the procedure described by Khaskheli et al. [19]. Ten grams (10 g) of each pickled African walnut formulation was pulverised with mortar and pestle sterilised with 70 % ethanol and then homogenised in 90 ml of sterile peptone water to obtain a stock solution. From the stock solution, 1 ml was collected using a sterile pipette and transferred to the first test tube containing 9 ml of sterile peptone water (ten folds serial dilution).

Further dilutions were obtained by transferring 1 ml solution from the first tube to the next test tube until the sixth using separate sterile pipettes for each transfer. These tubes were labelled accordingly as follows (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}). From the 10^{-1}, 10^{-2}, 10^{-3} diluents, 0.1 ml was spread plated on sterilised culture media plates - Nutrient Agar (NA) for isolation and enumeration of the total bacterial count and Sabouraud Dextrose Agar (SDA) for isolation and enumeration of the total fungal count. The NA culture plates were inverted and incubated at 37 ^{0}C for 24 – 48 h. Similarly, SDA culture plates were incubated at room temperature (28±2ºC) for 24-72 h. After the incubation period, culture plates with 30-300 colonies were selected, counted and expressed as colony forming unit per gram (cfu/g) of the sample.

2.4.1 Bacterial identification of isolates

Different morphological and cultural attributes of the colonies such as pigmentation, elevation, texture, shape and size were observed and recorded. Discrete colonies were isolated and purified by repeated sub-culturing and pure isolates were stored at 4ºC on agar slants. The bacterial isolates were subjected to motility test, oxidase test, catalase test, indole test, methyl red test, Voges-Proskauer test, citrate utilisation test, urease test, triple sugar iron test as well as Gram and spore staining using the methods described by Isu and Onyeagba [20]. The dominant isolates were identified to strain level using molecular identification methods as described by Dubbey [21].

2.4.2 Fungal identification of isolates

The cultural and morphological characteristics of the fungal isolates were noted. Fungal microscopy was based on the method described by Isu and Onyeagba [20]. A drop of lactophenol was placed on a clean grease free glass slide. A sterile inoculating needle was used to pick the fungal isolate and then teased on the glass slide containing lactophenol. A coverslip was placed on top of the lactophenol and the slide was viewed under the microscope using 10X and 40X objective lens.

2.5 Molecular Identification of Bacterial Isolates

The dominant bacterial isolates from pickled African walnut formulations were identified to species and strain levels using polymerase chain reaction (PCR) with appropriate primers at the Federal Institute of Industrial Research, Oshodi (FIIRO), Lagos Nigeria.

2.5.1 Isolation of bacterial deoxyribonucleic acid (DNA)

The procedure reported by Dubbey [21] was used with slight modifications. Deoxyribonucleic acid (DNA) isolation was done using universal zymo research (ZR) fungi/ bacteria QIA amp DNA mini kit. Fifty (50) ml (wet weight) of bacterial cells from 12 h Luria Bertani broth (oxoid) that have been re-suspended in 200 ml of water was transferred into a lysis tube. About 750 µl lysis solution was poured into the tube. The lysis tube was centrifuged using microcentrifuge at > 6000xg for 1 min. The flow through the connection tube was discarded and the above step was repeated. Two hundred (200) µl DNA prewashed buffer was added to the spin column in a new collection tube and centrifuged at 6000xg for 1 min. The content of the spin column was transferred to a clean 1.5 ml microcentrifuge and 100 µl DNA elution buffer was directly added to the column matrix. Centrifugation at 6000xg for I min. was done. Ultra-pure DNA was ready for use in the experiments. The high quality of eluted DNA was confirmed using the ratio of 260/280. Samples with 260/280 value greater than 1.8 noted were of high quality.

2.5.2 Polymerase chain reaction (PCR) using 16S primer

PCR analysis was run using 16S universal primer for bacteria. The universal primers used were Eub 27f (AGA GTT TGA TCC TGG CTC AG) forward primer and Eub 1525r (5'-3'.AAG GAG GTG ATC CTC CCG CA) for reverse primers. The PCR mix comprise 1.0 µl of 10x buffer, 1.0 µl of 25 Mm $MgCl_2$, 0.8 µl of 2.5 mMdNTPs, 0.5µl 5 PM forward primer, 0,5 µl of 5 PM reverse primer, 0.1µl of 5 units/µl Taq with 2.0 µl of 10 mg/µl DNA and 3.1 µl of distilled water to make up 10 µl reaction mix. The PCR profile was determined at an initial denaturation temperature of 94ºC for 5 min., followed by 36 cycles of 94ºC for 30 sec, 56ºC for 30 sec, 72 ^{0}C for 45 sec and the final extension temperature of 72ºC for 7 min. and the100ºC hold.

2.5.3 Purification of PCR products

An equal volume of 2 ml sodium acetate at pH 5.2 was added to the PCR products, followed by 20 µl absolute ethanol. The solution was kept at -

20ºC for 1 h after which it was spun at 1000 rpm for 15 min. and then washed with 40 µl of 70 % ethanol before it was air dried. The air-dried sample was re-suspended in 10µl sterile distilled water and kept at 4ºC in readiness for sequencing.

2.5.4 Gel electrophoresis of PCR products

Electrophoresis of PCR products was performed on 1.5 % agarose gel and standard markers were run in parallel. The gels were stained with ethidium bromide and UV transilluminator was used to observe the bands.

2.5.5 Software and BLASTN

The 16SrRNA gene sequences obtained were compared with the sequences from the Basic Local Alignment Search Tool (BLAST) of National Center for Biotechnology Information (NCBI) database. The strains that show 80-100 % 16SrRNA gene sequence similarity was considered to be of the same species and strain where necessary.

2.6 Microbiological Challenge Testing of Pickled African Walnut Formulations

It involves testing the ability of pathogenic microorganisms to survive in the pickled African walnut formulations stored at room temperature (28±2ºC). The procedure involves the following steps:

2.6.1 Preparation of the McFarland standards

The procedure described by Moosdeen et al. [22] was adopted. One gram (1 g) of barium chloride was weighed and transferred into 99 ml of sterile distilled water. A sterile pipette was used to add 0.10 ml of 1 % H_2S0_4 to the barium chloride solution which resulted in the formation of barium sulphate precipitate. The turbidity was determined using a spectrophotometer which indicates the bacterial inoculum size used for the challenge test study.

2.6.2 Preparation of bacterial inoculum

The nutrient broth was prepared according to manufacturers' instruction. Based on the identification of bacterial isolates from fresh African walnut using biochemical tests, the pure culture of *Bacillus cereus* and *Staphylococcus aureus* was selected for the challenge test. One loopful of each of the pure cultures was transferred to a tube containing nutrient broth using a sterile inoculating loop and then incubated for 24 h at 37ºC to resuscitate the organisms. The content of the tubes were centrifuged at 600 rev/min and the turbidity was monitored using a spectrophotometer until the turbidity matched with that of barium sulphate precipitate i.e. (McFarland standard). The inoculum size to be cultured corroborate McFarland standard which is 10^8 cfu.

2.6.3 Challenge test study

The method described by Lee and Coates [23] was adopted. A sterile pipette was used to transfer 1 ml each of *Bacillus cereus* and *Staphylococcus aureus* separately into six different formulations of pickled African walnut in a glass jar and then stored at room temperature (28±2ºC) for 4 Weeks. Thereafter, stock solutions of each of the inoculated pickled African walnut formulations were prepared by homogenising 10 g sample in 90 ml of sterile peptone water and then serial dilution up to 10^{-6} was carried out using sterile pipettes. From each dilution, 0.1 ml was spread plated on nutrient agar for *Bacillus cereus* and mannitol salt agar for *Staphylococcus aureus*. The culture plates were incubated at 37ºC for 24-48 h and then examined for colony formation which is an indication that the inoculated microorganism was able to survive in the food product or not. Colonies within 30-300 were counted and recorded. This test was repeated at the 1-week interval for 4 Weeks.

2.7 Statistical Analysis

Average of triplicate results for each sample tested for heterotrophic bacterial and fungal count was calculated and Microsoft Excel 2013 used to plot graphs.

3. RESULTS AND DISCUSSION

3.1 Microorganisms Isolated from fresh African walnut

Colonial, morphological (Gram stain reaction) and biochemical characterisation of the bacterial isolates from freshly cooked African walnut before pickling were presented in Table 1. The isolates were *Staphylococcus aureus* and *Bacillus cereus*. The dominant isolate is *Bacillus cereus* possibly because it is ubiquitous. It is also important to note that *B. cereus* has the ability to survive in harsh environmental conditions. The

presence of *Staphylococcus aureus* in freshly cooked African walnut could have resulted from human handling of African walnut. According to Saranraj et al. [24], the main reservoir of *Staphylococcus* sp. is a nasal cavity and human skin. This bacteria is pathogenic and a causative agent for diseased conditions in humans. Akin-Osanaiye and Ahmad [11] in a related study identified *Bacillus subtilis* and *Staphylococcus aureus* as bacterial species implicated in spoilage of African walnut.

3.2 Microorganisms Isolated from Pickled African Walnut Formulations

Tables 2 and 3 show the colonial, morphological (Gram stain reaction) and biochemical characterisation of the bacterial isolates from pickled African walnut preserved with citric and lactic acids, respectively. Having applied combined treatment (hurdle technology) in the development of pickled African walnut to extend its shelf life, this study has shown that only *Bacillus* spp. and two fungal genera (*Penicillium and Fusarium* spp.) were isolated and identified using cultural techniques while *Ochrabactrum ciceri* was identified using molecular identification methods. The dominance of *Bacillus cereus* in the pickled samples is most likely as a result of *Bacillus* spores is capable of withstanding harsh environmental conditions. Nwabunnia and Ezeimo [25], Oranusi and Braide [26] and Vural and Erkam [27] in their separate studies reported the presence of *Bacillus* sp. in retailed Nigerian walnut (*Tetracarpidium conophorum*). However, there seem not to be any reported study in which *Ochrabactrum ciceri* was isolated and identified from pickled African walnut. To the best of our knowledge, this is the first time *Ochrabactrum ciceri* is associated with pickled African walnut. The use of molecular methods for identification of bacterial isolates might have contributed to its detection in pickled African walnut formulations. Interestingly, *Staphylococcus aureus* isolated from fresh African walnut was eliminated after the pickling process. Oulkheir et al. [28] reported that 0.75 % solution of citric acid was able to drastically reduce the population of *Staphylococcus aureus* on hard-boiled eggs.

Table 4 shows the fungi species isolated weekly throughout the storage period of pickled African walnut formulations while Table 5 shows the colonial morphology and microscopic characteristics of fungal species isolated from both the fresh and pickled African walnut formulations. This study revealed that *Penicillium* sp. was dominant in the pickles. The *Penicillium* sp. isolated from African walnut might produce aflatoxins and ochratoxin A [29]. In a related study, Kazemi et al. [30] isolated *Aspergillus* sp. and *Penicillium* sp. as well as other fungi species from African walnut roasted with salt as well as the untreated sample. Previous studies by Nkwonta et al. [31] revealed that *Penicillium, Aspergillus and Fusarium* spp. is present in the African walnut shell. These fungi are commonly associated with nuts [32,33]. According to Wang and Xing [34], *Penicillium* sp. is present in homemade pickles. The dominance of *Penicillium* sp. in African walnut pickle could be as a result of the fungus being able to tolerate high temperature [35] and also grow where there is relatively low water activity [36]. The presence of *Penicillium* sp. is of public health significance because it is associated with the production of mycotoxin which is detrimental to human health [37]. *Fusarium* sp. was also isolated from pickled African walnut. Saranraj et al. [38] also isolated *Fusarium oxysporium* from pickles. According to Microbiological Guidelines for Ready-to-Eat Food [39], the population of *Bacillus cereus* in ready-to-food must be less than 10^5cfu/g. It is satisfactory if it is less than 10^3cfu/g. The same standard also stipulates that *Staphylococcus aureus* below 20cfu/g is satisfactory but it must not reach 10^4cfu/g.

3.3 Gel Electrophoresis of the Bacterial Isolates

The result of the agarose gel electrophoresis was presented in Plate 1. They all formed the 1500bp. Figs. 2, 3 and 4 show the phylogenetic trees of the bacteria isolated from pickled African walnut which are *Bacillus cereus* strain EV-1 (KY689737), *Ochrobactrum ciceri* strain EV-2 (KY689738) and *Bacillus amyloliquefaciens* strain EV-3 (KY689739), respectively.

Ragul et al. [40] isolated *Bacillus amyloliquefaciens* from traditional brine pickle which has the potential of being a beneficial probiotic. In a related study, Saranrajet al. [38] isolated *Bacillus cereus* from pickles. *Bacillus cereus* is ubiquitous and capable of causing food poisoning because it is toxigenic. The spores of *Bacillus* sp. are heat resistant [41]. This could be the reason *Bacillus* sp. present in African walnut survived after it was cooked. *Ochrobactrum ciceri* exist in the

Table 1. Colonial, morphology (Gram stain reaction) and biochemical characteristics of bacteria isolated from fresh African walnut before pickling

Isolate code	Shape	Size	Colour	Elevation	Spore stain	Gram morphology	Gram reaction	Catalase test	Coagulase test	Citrate test	Motility test	Gas	Slant	Butts	H_2S production	Indole test	MR test	VP test	Glucose	Lactose test	Sucrose test	Probable organism
A0	Round	0.5 mm	Cream	Raised	-	Cocci	+	+	+	+	+	-	B	A	-	+	+	-	AG	-	AG	*Staphylococcus aureus*
B0	Round	1 mm	Cream	Flat	+	Rod	+	+	-	+	+	-	B	A	-	-	-	+	AG	AG	AG	*Bacillus* sp.
C0	Round	1.5 mm	Cream	Flat	+	Rod	+	+	-	+	+	-	B	B	-	-	-	+	AG	AG	AG	*Bacillus* sp.
D0	Round	1 mm	Cream	Flat	+	Rod	+	-	-	+	+	-	B	B	-	+	-	-	AG	AG	AG	*Bacillus* sp.
E0	Round	0.5 mm	Cream	Flat	-	Cocci	+	+	+	+	+	-	B	A	-	+	+	-	AG	-	AG	*Staphylococcus aureus*

Key: AG=Acid and gas, A = Acid, B = Base.

Table 2. Colonial, morphological (Gram stain reaction) and biochemical characteristics of bacteria isolated from citric acid preserved African walnut pickle throughout the storage period

Isolate code	Shape	Size	Colour	Edge	Elevation	Spore Stain	Gram morphology	Gram Reaction	Catalase test	Citrate test	Motility test	Gas	Slant	Butt	H_2S production	Indole test	MR test	VP test	Glucose	Lactose test	Sucrose test	Probable organism
IA1%W_3	Round	1mm	Cream	Smooth	Flat	+	Rod	+	+	+	+	-	B	A	-	+	+	-	AG	-	AG	*Bacillus sp.*
IB1%W_4	Round	1mm	Cream	Smooth	Flat	+	Rod	+	-	+	+	+	B	A	-	+	+	-	AG	AG	AG	*Bacillus sp.*
IC1%W_5	Round	0.5mm	Cream	Smooth	Flat	+	Rod	+	+	+	+	-	B	A	-	-	+	-	AG	-	AG	*Bacillus sp.*
2A3%W_3	Irregular	1.5mm	Cream	Cerated	Flat	+	Rod	+	+	+	+	-	B	A	-	-	+	-	AG	AG	AG	*Bacillus sp.*
2B3%W_4	Round	0.5mm	Cream	Smooth	Raised	+	Rod	+	-	+	+	+	B	A	-	+	+	-	AG	-	AG	*Bacillus sp.*
2C3%W_6	Irregular	1.5mm	Cream	Cerated	Raised	+	Rod	+	+	+	+	-	B	A	-	+	+	-	AG	-	AG	*Bacillus sp.*
3A5%W_4	Irregular	1.5mm	Cream	Cerated	Flat	+	Rod	+	+	+	+	-	B	A	-	-	+	-	AG	AG	AG	*Bacillus sp.*
3B5%W_5	Round	1mm	Cream	Smooth	Raised	+	Rod	+	-	+	+	+	B	A	-	-	+	-	AG	AG	AG	*Bacillus sp.*
3C5%W_6	Round	1mm	Cream	Smooth	Flat	+	Rod	+	+	+	+	-	B	A	-	+	+	-	AG	AG	AG	*Bacillus sp.*

Key: W_3 = Week 3, W_4 = Week 4, W_5 = Week 5, W_6 =Week 6; 1 % (v/v) citric acid, 3 % (v/v) citric acid, 5 % (v/v) citric acid; A=Acid, AG=Acid and gas, A =Acid B=Base,

Table 3. Colonial, morphology (Gram stain reaction) and biochemical characteristics of bacteria isolated from lactic acid preserved African walnut pickle throughout the storage period

Isolate code	Shape	Size	Colour	Edge	Elevation	Spore stain	Gram morphology	Gram reaction	Catalase test	Citrate test	Motility test	Gas	Slant	Butts	H_2S production	Indole test	MR test	VP test	Glucose	Lactose test	Sucrose test	Probable organism
IA1%W_3	Round	1mm	Cream	Smooth	Flat	+	Rod	+	-	+	+	+	B	A	-	+	+	-	AG	-	AG	*Bacillus* sp.
IB1%W_4	Round	1mm	Cream	Smooth	Flat	+	Rod	+	+	+	+	-	B	A	-	+	+	-	AG	AG	AG	*Bacillus* sp.
IC1%W_5	Round	0.5mm	Cream	Smooth	Flat	+	Rod	+	+	-	+	-	B	A	-	-	+	-	AG	-	AG	*Bacillus* sp.
2A3%W_3	Irregular	1.5mm	Cream	Cerated	Flat	+	Rod	+	+	-	+	-	B	A	-	-	+	-	AG	AG	AG	*Bacillus* sp.
2B3%W_4	Round	0.5mm	Cream	Smooth	Raised	+	Rod	+	-	-	+	+	B	A	-	+	+	-	AG	-	AG	*Bacillus* sp.
2C3%W_6	Irregular	1.5mm	Cream	Cerated	Raised	+	Rod	+	+	+	+	-	B	A	-	+	+	-	AG	-	AG	*Bacillus* sp.
3A5%W_4	Irregular	1.5mm	Cream	Cerated	Flat	+	Rod	+	+	+	+	-	B	A	-	-	+	-	AG	AG	AG	*Bacillus* sp.
3B5%W_5	Round	1mm	Cream	Smooth	Raised	+	Rod	+	-	+	+	+	B	A	-	-	+	-	AG	AG	AG	*Bacillus* sp.
3C5%W_6	Round	1mm	Cream	Smooth	Flat	+	Rod	+	-	+	+	+	B	A	-	+	+	-	AG	AG	AG	*Bacillus* sp.

Key: W_3 = Week 3, W_4 = Week 4, W_5 = Week 5, W_6 =Week 6; 1 % (v/v) citric acid, 3 % (v/v) citric acid, 5 % (v/v) citric acid; A=Acid, AG=Acid and gas, B = Base

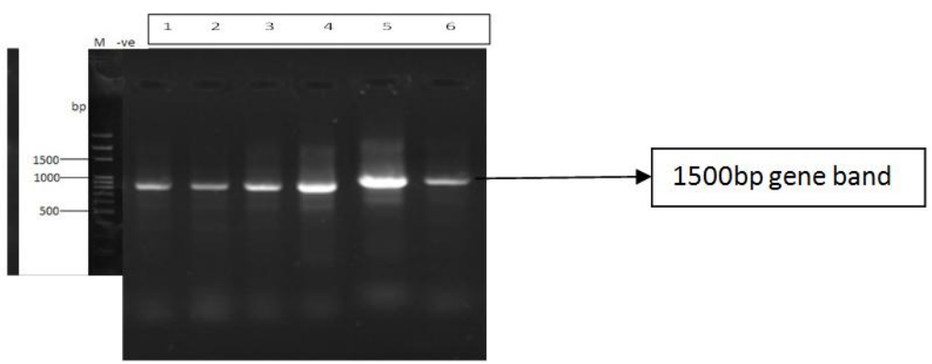

Plate 1. Agarose gel electrophoresis plate showing the 16S gene band (1500bp) of bacterial isolates.
Lane 1-6 represents the isolates while lane M represents Quick- load 1kb molecular ladder

Table 4. Fungi species isolated from pickled African walnut formulations throughout the storage period

S/N	Sample code	Week	Fungi identified
1	PAWFA	1	No Growth observed
		2	No Growth observed
		3	No growth observed
		4	*Penicillium* sp*, Fusarium* sp.
		5	*Penicillium,* sp *Fusarium* sp.
		6	*Penicillium* sp, *Fusarium* sp.
2	PAWFB	1	No Growth observed
		2	No Growth observed
		3	No growth observed
		4	*Penicillium* sp*, Fusarium* sp.
		5	*Penicillium,* sp *Fusarium* sp.
		6	*Penicillium* sp, *Fusarium* sp.
3	PAWFC	1	No Growth observed
		2	No Growth observed
		3	No Growth observed
		4	*Penicillium* sp*, Fusarium* sp.
		5	*Penicillium* sp*, Fusarium* sp.
		6	*Penicillium* sp*, Fusarium* sp.
4	PAWFD	1	No Growth observed
		2	No Growth observed
		3	No Growth observed
		4	*Penicillium* sp*, Fusarium* sp.
		5	*Penicillium* sp*, Fusarium* sp.
		6	*Penicillium* sp*, Fusarium* sp.
5	PAWFE	1	No Growth observed
		2	No Growth observed
		3	No Growth observed
		4	No Growth observed
		5	*Penicillium* sp.
		6	*Penicillium* sp.
6	PAWFF	1	No Growth observed
		2	No Growth observed
		3	No Growth observed
		4	No Growth observed
		5	*Penicillium* sp.
		6	*Penicillium* sp.

PAWFA represent 100 g of African walnut + 5 % NaCl + 1 % Citric acid; PAWFB represent 100 g of African walnut + 5 % NaCl + 3 % Citric acid; PAWFC represent 100 g of African walnut + 5 % NaCl + 5 % Citric acid; PAWFD represent 100 g of African walnut + 5 % NaCl + 1 % Lactic acid; PAWFE represent 100 g of African walnut + 5 % NaCl + 3 % Lactic acid; PAWFF represent 100 g of African walnut + 5 % NaCl + 5 % Lactic acid.

Table 5. Colonial morphology and microscopic characteristics of fungal species isolated from both the fresh and pickled African walnut formulations

Isolate code	Colonial morphology	Microscopic appearance	Fungi identified
1A	Green colonies that are dry in front. Back is yellow in colour and is cracked	Simple branched septate hyphae	*Penicillium* sp.
1B	Whitish fluffy mold, cotton wool-like and back is not cracked	Septate hyphae	*Fusarium* sp.

environment. Imran et al. [42] isolated *Ochrobactrum ciceri* from nodules of chickpea. *Ochrobactrum* sp. GDOS strain isolated from contaminated sites has the potential to degrade

glyphosate in pesticide-contaminated water systems and soils [43]. Table 6 shows the percentage similarity, accession number and bacterial strains isolated from pickled African walnut formulations using molecular identification methods.

3.4 Total Heterotrophic Bacterial Count of Pickled African Walnut Formulations during Storage

The total heterotrophic bacterial count in seven formulations of pickled African walnut stored for a period of six weeks at room temperature (28±2 ^{o}C) are presented in Fig. 5. Throughout the storage period, total bacterial count was highest in African walnut pickle preserved with 1 % citric acid and lowest in that which was preserved with 5 % lactic acid. However, the control sample had the highest heterotrophic bacterial count when compared with that of pickled African walnut formulations preserved with the organic acids throughout the storage period. This study showed that no culturable bacteria were present in developed African walnut pickle preserved with citric and lactic acid in Week 1 and 2, except the control sample. At Week 3, bacterial growth was observed in pickled African walnut formulations including the control sample which had no preservative (citric or lactic acid) added. The allowable limit in terms of microbial count for specific spoilage microorganisms in ready-to-eat food is between 10^5 to 10^8 cfu/ml. Based on PHLSG (2008) manual, Fylde Borough Council stated that maximum level of total aerobic colony permissible in ready-to-eat foods must be less

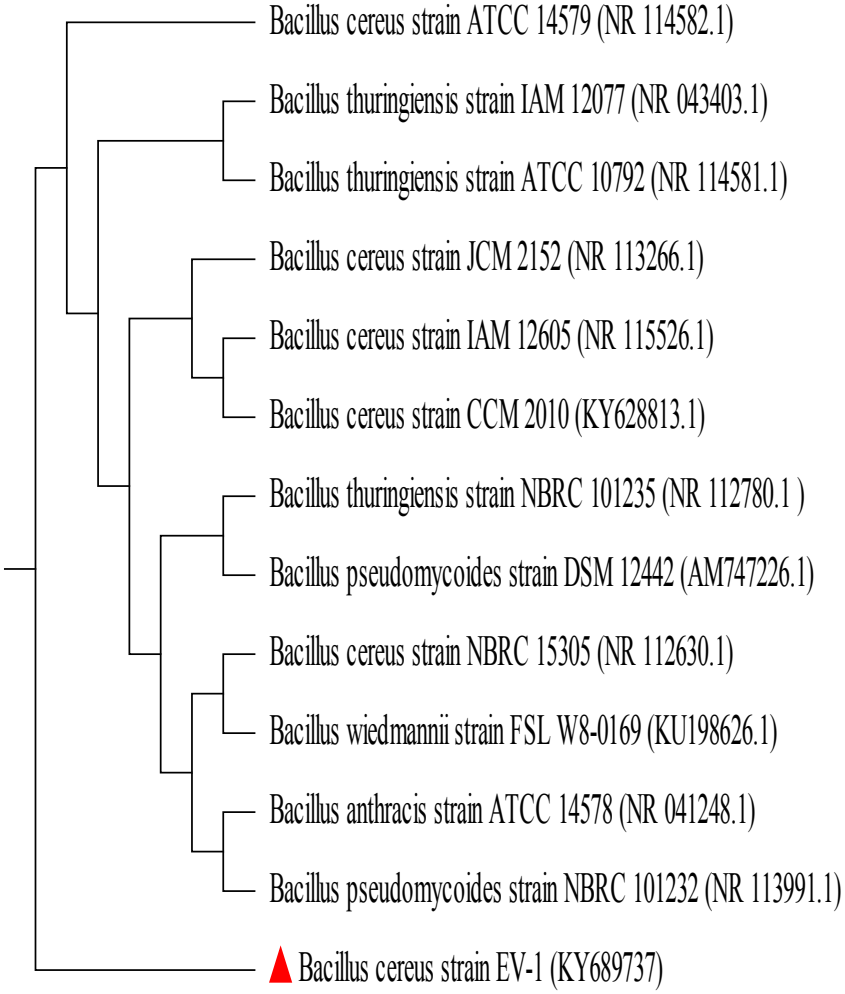

Fig. 2. Phylogenetic tree of *Bacillus cereus*

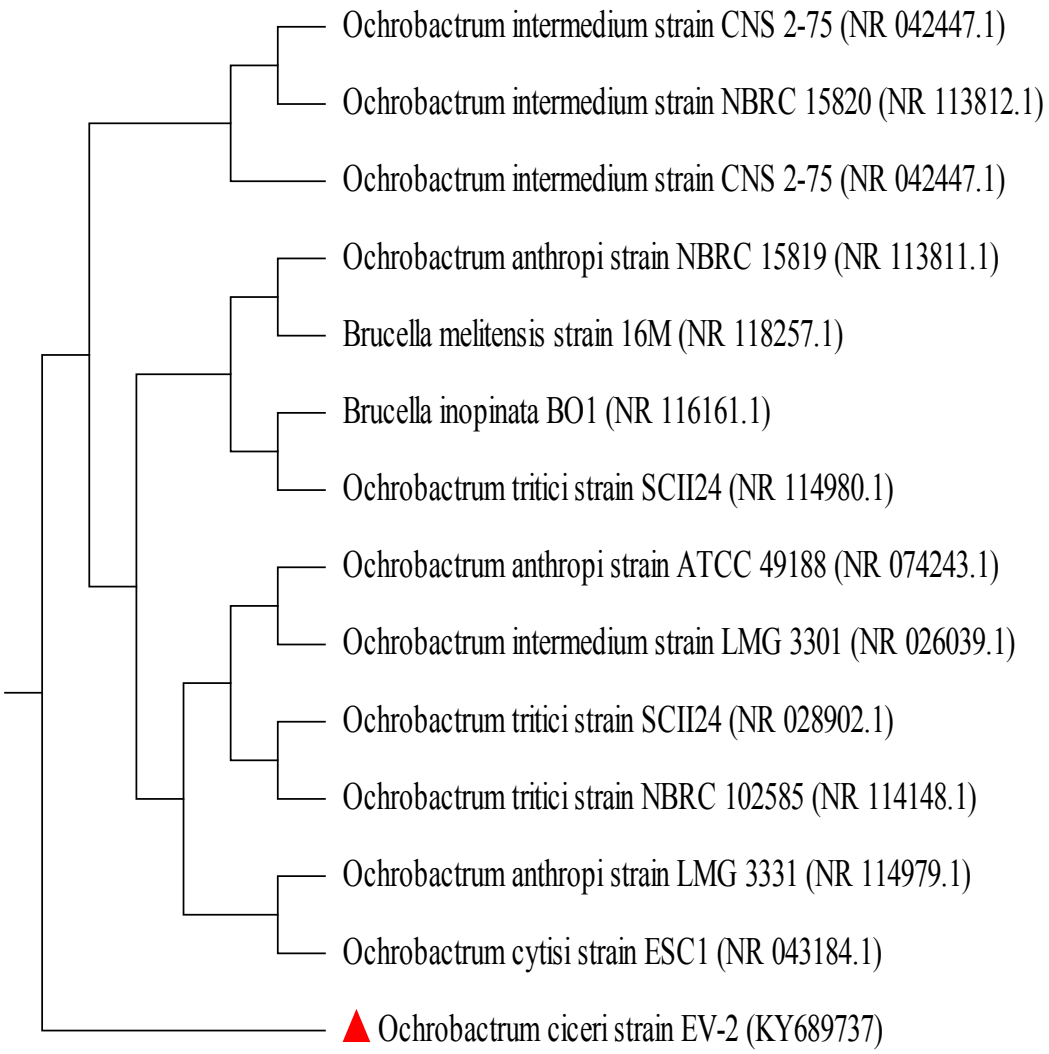

Fig. 3. Phylogenetic tree of *Ochrobactrum ciceri*

than 10^6cfu/g [11]. According to Ogwu et al. [44], a microbial load of fresh and cooked African walnut is 7.4 x 10^2 and 5.3 x 10^1 cfu/g, respectively. The reduction in microbial load could be as a result of heat applied on African walnut during cooking which lasted for 10 min. The combined effect of longer cooking time of African walnut which was 1 h, pickling process as well as the addition of citric or lactic acid as a preservative could have contributed in the absence of culturable bacteria in developed African walnut formulations within two weeks of storage. However, there was microbial growth in the control sample throughout storage. This could be as a result of the absence of preservatives in the heated brine solution.

Table 6. Bacterial isolates from pickled African walnut formulations identified using molecular methods

Isolate code	% Similarity	Accession number	Bacteria identified
1A	99 %	KY689737	*Bacillus cereus*
1B	93 %	KY689738	*Ochrobactrum ciceri*
1C	97 %	KY689739	*Bacillus amyloliquefaciens*

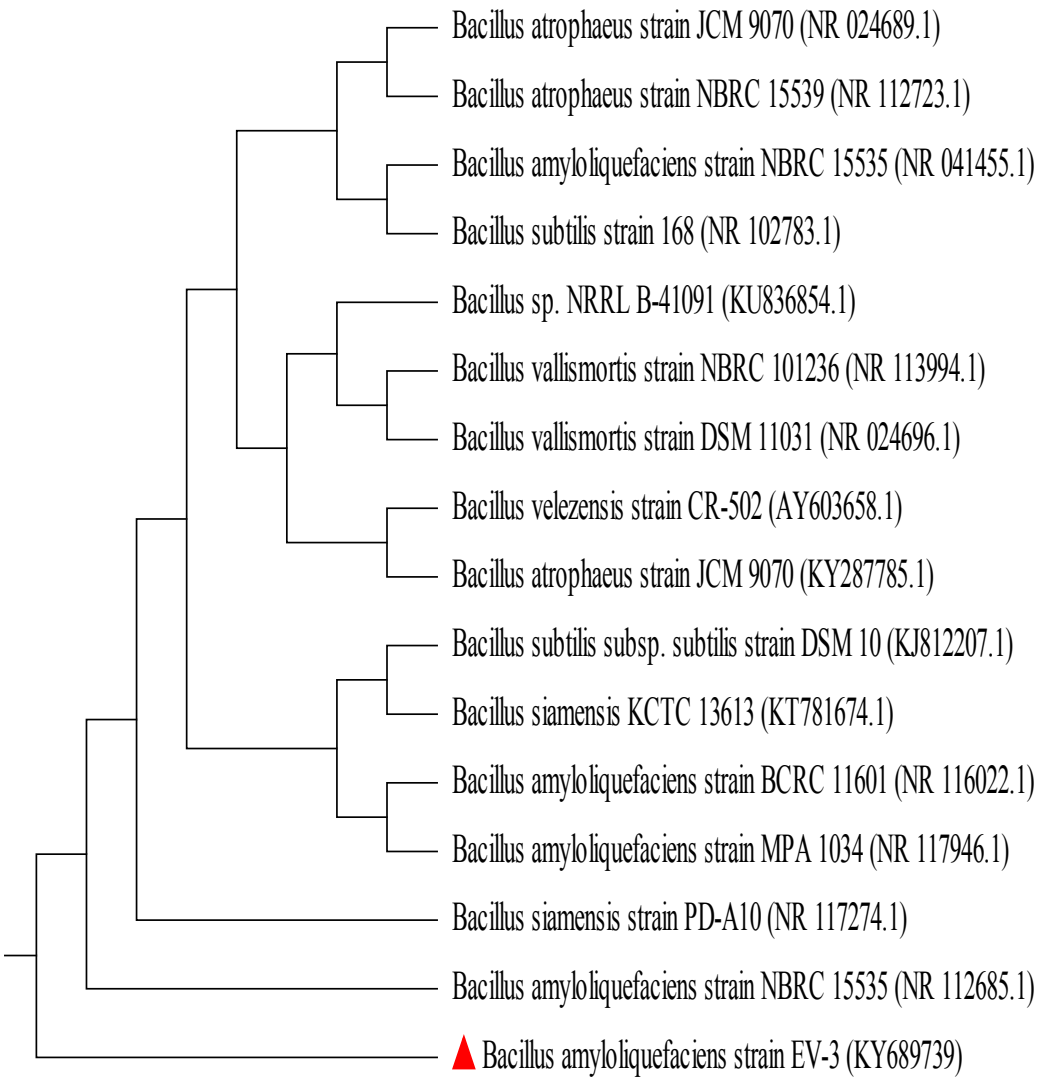

Fig. 4. Phylogenetic tree of *Bacillus amyloliquefaciens*

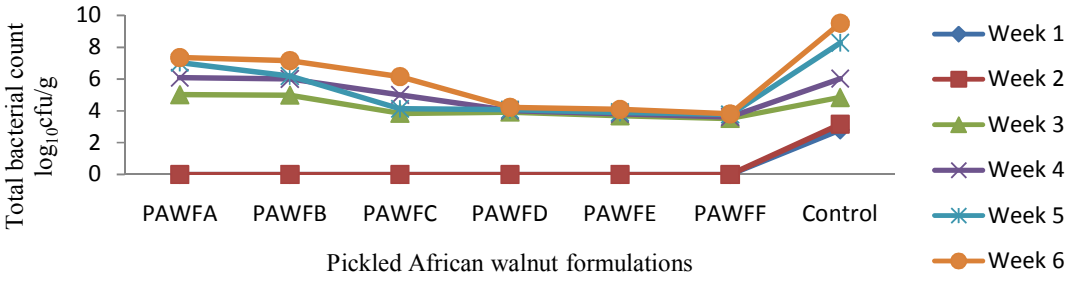

Fig. 5. Total heterotrophic bacterial count of pickled African walnut formulations throughout storage period

Key: PAWF- Pickled African walnut formulation; PAWFA -- 5 % NaCl + 1 % Citric acid; PAWFB –5 % NaCl + 3 % Citric acid; PAWFC – 5 % NaCl + 5 % Citric acid; PAWFD --- 5 % NaCl + 1 % Lactic acid; PAWFE---5 % NaCl + 3 % Lactic acid; PAWFF -- 5 % NaCl + 5 % Lactic acid; Control -- 5 % NaCl.

3.5 Total Heterotrophic Fungal Count of Pickled African Walnut Formulations during Storage

The total heterotrophic fungal count of seven pickled African walnut formulations monitored for six weeks is presented in Figure 6. Among all the African walnut pickle formulations, sample PAWFF had the least frequency of occurrence based on the total heterotrophic fungal count. It only occurred at Week 6 during the period of storage. No fungal growth was observed in all the African walnut pickle formulations between Week 1 and 3, except the control sample. The control sample had the highest heterotrophic fungal count which occurred throughout storage. This could be as a result of citric or lactic acids which function as a preservative was not added to the brine solution used in pickling African walnut. Related studies by Ogwu et al. [44] reported that fungal count of fresh and cooked African walnut which lasted for 10 minutes is 1.1 x 10^2 and 1 x 10^1 cfu/g, respectively. The total heterotrophic fungal count of pickled African walnut formulations reported in this study is lower than that of total heterotrophic bacterial count. This result trend is in agreement with findings by Akin-Osanaiye and Ahmad [11] from a related study.

3.6 Use of lactic and citric acid as preservatives in pickled African walnut formulations

Total viable bacterial counts observed in African walnut pickle preserved with citric acid (PAWFA, PAWFB and PAWFC) is higher when compared with that of lactic acid preserved samples (PAWFD, PAWFE and PAWFF) throughout the six weeks shelf life study. A similar result trend was observed in terms of total viable fungal count of African walnut pickle formulations with few exceptions. This result shows that lactic acid demonstrated more effective antimicrobial effect than citric acid against a microbial load in pickled African walnut formulations stored at room temperature (28±2ºC) for 6 Weeks. This result is in agreement with a related study carried out by Pundir and Jain [45] which involved testing antibacterial activity of lactic and acetic acid against *Bacillus* sp. and *Staphylococcus aureus* isolated from pickles. On the contrary, Oulkheir et al. [28] reported that citric acid had a more antibacterial effect than lactic acid based on the fact that citric acid dissociation constant (pKa 4.8) is higher than that of lactic acid (pKa 3.86) when the two organic acids were tested against *Escherichia coli*. The effectiveness of organic acid depends on the history of the strain and its ability to adapt or not in an acidic environment prior to the contamination of the product [28].

The absence of culturable bacteria observed in the developed pickled African walnut formulations between Week 1 and 2; up to Week 3 regarding culturable fungal count, except the control sample could be attributed to the combination of treatments (salt, acidification and sterilisation) which stressed the cell of the microorganisms. This probably led to sublethal injury on the microorganisms which resulted in an extended lag phase during microbial growth. The significant increase in growth rate of bacterial and fungal population from Week 3 to 6, respectively in the developed pickled African walnut formulations suggest that these microorganisms were able to repair and recover

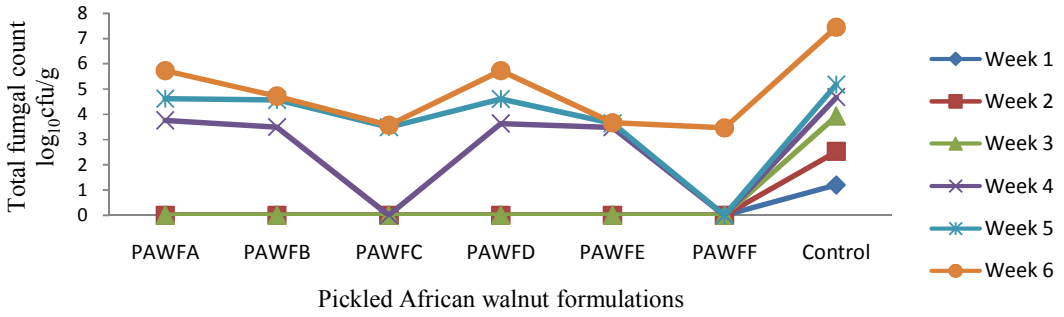

Fig. 6. Total heterotrophic fungal count of pickled African walnut formulations throughout storage period

Key: PAWF- Pickled African walnut formulation; PAWFA -- 5 % NaCl + 1 % Citric acid; PAWFB –5 % NaCl + 3 % Citric acid; PAWFC – 5 % NaCl + 5 % Citric acid; PAWFD --- 5 % NaCl +1 % Lactic acid; PAWFE---5 % NaCl + 3 % Lactic acid; PAWFF -- 5 % NaCl + 5 % Lactic acid; Control -- 5 % NaCl.

from the injury or damage posed by the combination of treatments given to them. This is in agreement with the research findings of Liewen and Martha [46], Everis [47], Alissa et al. [48] and Zhao [49]. They reported that when microorganisms are exposed to lethal and sublethal stresses, they incur structural injuries on their cells. As a result of this, they experience a prolonged recovery period after which they undergo repair from the damages caused by stresses such as cold, acid, heat and osmotic stresses and subsequently cause food spoilage. The presence of injured cells in a food product is of public health risk because many bacterial pathogens can become resistant to cooking and other microbial elimination processes as a result of the sublethal injury.

3.7 Total *Bacillus* and *Staphylococcus* spp. Count (Challenge Test Study) for Pickled African Walnut Formulations

The total *Bacillus* count (challenge test study) of different pickled African walnut formulations which lasted for four weeks is presented in Fig. 7. Regarding frequency of occurrence, sample PAWFF had the lowest value compared with other pickled African walnut formulations. There was no growth of *Bacillus* sp. in all the samples at Week 0. This could be as a result of the bacteria being in the spore stage survived heat treatment during cooking of African walnut before the pickling process. This condition lasted till Week 1 except in sample PAWFA and PAWFD. Growth of culturable *Bacillus* sp. in sample PAWFA and PAWFD at Week 1 could be as a result of lowest concentration 1 % (w/v) of citric and 1 % (v/v) lactic acids added as preservatives to the brine solution compared with higher concentrations -- 3 % and 5 % added to other pickled African walnut formulations.

Fig. 8 shows that within Week 0 and 1, there was no culturable *Staphylococcus* sp. (challenge test study) present in all the pickled African walnut formulations. However, at Week 2 only sample PAWFC and PAWFF had no growth of culturable *Staphylococcus* sp. This could be attributed to the highest concentration 5 % of organic acids added to the pickled African walnut formulations as preservatives. Regarding *Staphylococcus aureus* count (challenge test study), sample PAWFC and PAWFF had a greater ability than other pickled African walnut formulations not to easily support the growth of pathogenic *Staphylococcus aureus*.

In the challenge test study that involved testing the developed pickled African walnut formulations with (*Bacillus cereus* and *Staphylococcus aureus*), the total *Bacillus* counts were higher than that of *Staphylococcus* count. This shows that *Bacillus cereus* proliferated and had greater ability to survive in pickled African walnut formulations than *Staphylococcus aureus*. The result of the challenge test study corroborates the result presented in Table 2 and 3 which shows that *Bacillus* sp. is the dominant heterotrophic bacteria isolated from pickled African walnut formulations. This could largely be attributed to the fact that *Bacillus sp.* has the ability to produce endospores that can survive harsh environmental conditions [50,51].

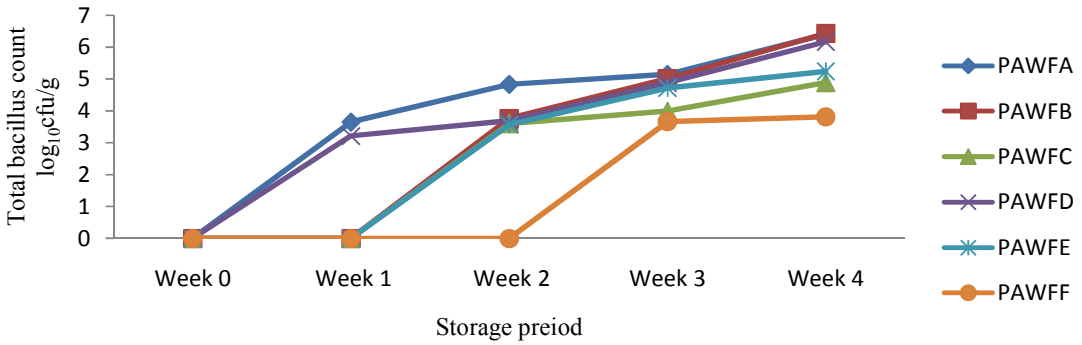

Fig. 7. Total *Bacillus* count (challenge test study) for different pickled African walnut formulations

Key: PAWF- Pickled African walnut formulation; PAWFA -- 5 % NaCl + 1 % Citric acid; PAWFB –5 % NaCl + 3 % Citric acid; PAWFC – 5 % NaCl + 5 % Citric acid; PAWFD --- 5 % NaCl +1 % Lactic acid; PAWFE---5 % NaCl + 3 % Lactic acid; PAWFF -- 5 % NaCl + 5 % Lactic acid.

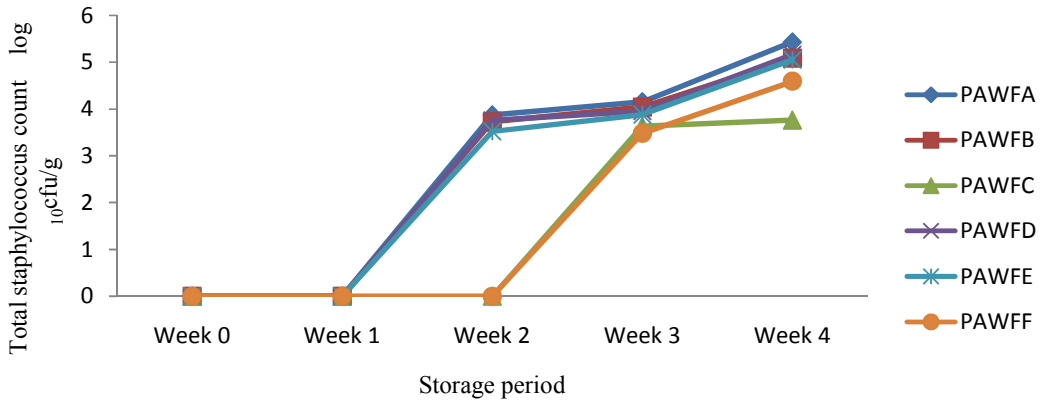

Fig. 8. Total *Staphylococcus* count (challenge test study) for different pickled African walnut formulations

Key: PAWF- Pickled African walnut formulation; PAWFA -- 5 % NaCl + 1 % Citric acid; PAWFB –5 % NaCl + 3 % Citric acid; PAWFC – 5 % NaCl + 5 % Citric acid; PAWFD --- 5 % NaCl + 1 % Lactic acid; PAWFE---5 % NaCl + 3 % Lactic acid; PAWFF -- 5 % NaCl + 5 % Lactic acid.

The intrinsic resistant properties of spores may enhance its survival during food processing. Heating is the commonest treatment applied during food processing to reduce microbial load. Surviving spores may germinate upon exposure to certain environmental triggers and subsequently resume vegetative growth thereby leading to food spoilage [52]. This invariably means that if the storage condition of African walnut pickle formulations is compromised, it could favour the growth of *Bacillus cereus* and result in food spoilage. Consequently, this will lead to foodborne illness since this organism produces enterotoxin when consumed.

4. CONCLUSION

This study was a successful effort to develop pickles using African walnut and give the product extended shelf life using citric and lactic acid as preservatives. Lactic acid proved to be a more effective antibacterial agent as compared with citric acid. It was revealed that pickled African walnut preserved with 5 % lactic acid had the lowest microbial load compared with other pickle formulations. Also, it is the most unfavourable African walnut pickle formulation to support the growth of *Bacillus cereus* and *Staphylococcus aureus* during storage at room temperature compared with other pickle formulations. The pickled African walnut formulations subjected to microbiological challenge test study demonstrated that *Bacillus cereus* had a greater ability than *Staphylococcus aureus* to survive in the developed products during storage at room temperature. Based on Microbiological Guidelines for Ready-to-Eat Foods, pickled African walnut preserved with either lactic or citric acid is best consumed within 2 weeks.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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