Original Research Article

EFFECTS OF NATURAL AND INOCULATION FERMENTATION ON THE ANTINUTRIENT COMPOSITION OF AFRICAN WALNUT ENRICHED WITH CARROT

ABSTRACT

The effects of fermentation on the antinutrient composition of carrot enriched and non-enriched African walnut were carried out in this study. The submerged natural fermentation of non- enriched and carrot enriched (walnut 70% ;carrot 30%) African walnut was carried out and lasted for 5 days during which microorganisms were isolated and identified. The most predominant bacteria (*Bacillus subtillis* strain b17a and *Lactobacillus lactis* strain SFL8) were then used singly and in consortium during inoculation fermentation. During the fermentation processes, the antinutritional analysis were carried out on the fermented samples on daily basis. Result revealed that the bacteria *B. subtilis* strain b17a and *L. lactis* SFL8 were the most predominant. Raw walnut had a higher anti-nutrient composition than carrot except in saponins (2.05 ± 0.050^{b}). Fermentation led to the reduction in antinutrient composition in the fermented sample. Natural fermented African walnut (NFW) and carrot enriched natural fermented African walnut (NFWC) resulted in the highest reduction in antinutrient composition. NFW and NFWC had a reduction in tannin, saponins, Phytate and oxalate from (2.47 ± 0.008^{b}, 1.46 ± 0.010^{a}, 14.41 ± 0.026^{d}, 3.29 ± 0.05^{c}) to (0.98 ± 0.05^{a}, 0.61 ± 0.05^{a}, 4.35 ± 0.03^{a}, 0.27 ± 0.05^{a}) and (0.91±0.005a, 0.45 ± 0.005^{a}, 3.61 ± 0.03^{a}, 0.17 ± 0.005^{a}) respectively. Inoculation fermentation in which a monoculture of *L. lactis* or *B.subtillis* was used as starter culture resulted in the least reduction in the antinutrient content. Carrot enrichment of fermented African walnut had a reducing effect on the antinutrient composition of African walnut as all the fermentation enriched with carrot all had a reduced tannin, Phytate and oxalate composition compared to those that were not enriched. Hence, fermentation and enrichment with carrot can be employed in improving the nutritional quality of African walnut thereby helping in effectively utilising their potential as human food.

Keywords: Fermentation; Antinutrient; Natural; Inoculation; Composition

1. INTRODUCTION

African walnut (*T. conophorum*) is a tropical perennial woody plant of the family Euphorbiaceae that is widely distributed and consumed by the inhabitants of African (20). The African walnut is often found growing wild as a climber in the forest regions of African and India (10). African walnut has a long history as food plant and is grown by peasant farmers. Its plant is mostly cultivated for its nuts which can be cooked or consumed as snacks. In Nigeria, it is called “Asala or Awusa” in Yoruba, “Ukpa” in Igbo and “Okhue or Okwe” in Edo. African walnut, like many plants in African and other parts of the world has been proven to have decorative, nutritive, medicinal, agricultural and industrial values over the years.

African walnuts are rich in protein, carbohydrate, fats and oils, and minerals (20,5). Its seed are rich in fat, nearly eighty per-cent of unpolysaturated fat with proven cholesterol lowering properties. They can be cooked, roasted or sun dried and the roasted seeds could be ground and used as a thickener in soup preparation. Dried walnuts can be ground and turned into flour which can be used as composite flour during baking or in place of milk in tea preparation (11). However, despite its nutritive values, African Walnut is still included in the list of lesser known food stuff in Nigeria. African walnuts have been demonstrated to have high anti-nutrient content (22). Anti-nutrients are protective, synthetic or natural compounds produced by plants against harsh environments or damage by microbes as a defence mechanism against their seeds being eaten (26). Antinutrient, when consumed can upset the digestive process and interfere with the absorption of beneficial essential organic nutrients and inorganic minerals (5). They either bind to nutrients to prevent their absorption or react with nutrient so it can’t be used by the

body (26). Removals of undesirable components are very essentials in improving the nutritional quality thereby helping in effectively utilise their potential as human food. Several food processing methods such as germination, soaking, cooking and fermentation are known to reduce antinutritional factors effectively and upgrade their nutritional quality (15). Among the Several food processing methods which can reduce anti-nutritional factors, fermentation has been demonstrated to be the most effective (17).

Fermentation is the chemical breakdown of substance by bacteria, yeast or other microorganism into alcohol, carbon dioxide or organic acids (17). Fermentation in food processing serves five main purposes which are to enrich the diet through development of a diversity of flavours, aromas, and textures in food substrates; to preserve substantial amounts of food through lactic acid, alcohol, acetic acid, and alkaline fermentations; to reduce cooking time and the associated use of fuel; to retains food substrates with protein, enzymes, essential amino acids, vitamins and other nutrients that are usually destroyed by food processing since fermentation doesn't involve the use of heat and as a matter of fact to eliminate antinutrient (27). Fruits have been used to improved human diet for centuries, enriching it medicinally, nutritionally and sensorially. Carrot has been demonstrated to have smaller amount of anti-nutrient, zero cholesterol, low to no fat and high levels of vitamins, minerals and fibre (30). Hence, this project will aim at determining the effect of fermentation on the antinutrient composition of African walnut enriched with carrot.

2. MATERIALS AND METHODS

2.1. Sample materials

The African walnuts used for this study were obtained from a farm in Itapa, Ekiti State, Nigeria while the carrots used were bought from Oja Oba Market, Akure, Ondo State, Nigeria. The apparently healthy African walnut and Carrots were transported to the laboratory in separate clean and sterile black opaque polythene bags screwed at the tip and kept at refrigerated temperature (5^oC) in the laboratory

2.2. Preparation of samples

The fruits (raw carrot and raw African walnut with pod) were gently washed with running tap water to remove dust and debris, then successively surface sterilized by washing with sterile distilled water and dipping in sodium hypochlorite. After which, the nuts were removed from the pod with the aid of a knife and the unshelled walnuts were then divided into two parts. The first part was cracked open and blended into powder while the second part was roasted in an oven at 130^oc for one hour and later blended into powder. The unroasted blended walnuts were further subdivided into two parts; one portion was enriched with blended carrot while the other subdivision was not enriched with carrot. While the roasted blended walnut was further subdivide into six portions (three portions were enriched with carrot while the other three portions weren't enriched with carrot).

2.3. Preparation of the starter cultures

The microorganisms used were the most predominant microorganisms isolated from the natural fermentation of African walnut and they include *B. subtilis* b17a and *L. lactis* SFL8. Fresh starter cultures were prepared by reactivating the stock cultured of the pre-isolated microorganisms on Nutrient agar and Man- Rogosa-Sharpe (MRS) agar for *B. subtillis* and *L. lactis* respectively and cultured at 37^oC for 48 hours followed by two successive rounds of sub-culturing in their respective broth with incubation at 37^oC for 24 hours. The organisms were each harvested by centrifugation at 4000 for 20min and pellets were added prior to fermentation in cell concentrations of 10^8 cfu/g (18). Mixed fermentation by both organisms was initiated by using equal proportion of each pure strain (18).

2.4. Submerged Fermentation of Substrates

Natural fermentation of unroasted walnut (NFW) and carrot enriched unroasted walnut (NFWC) was carried out in an enclosed rubber container by soaking 40.0g of blended African walnut inside 200.0ml of water for the non- enriched fermentation while for the enriched sample, 28.0 (70%) gram of African walnut

and 12.0g (30%) of carrot was soaked in 200.0ml of water. The fermentation lasted for 5 days during which microorganisms were isolated and identified using both cultural and molecular methods.

the most predominant microorganisms during the natural fermentation (*B. subtilis* strain b17a and *L. lactis* strain SFL8) were then used singly and in consortium during Inoculation fermentation of non-enriched and carrot enriched African walnut (roasted in an oven at 130^{o}C for 1hr) which was also carried out in an enclosed rubber container by soaking the same grams as used in natural fermentation for the enriched and non- enriched fermentation [roasted African walnut fermented with *L. lactis* (WFL); roasted African walnut fermented with *B. subtillis* (WFB); roasted African walnut fermented with the combination of both *Bacillus subtillis and L. lactis* (WFBL); carrot enriched roasted African walnut fermented with *L. lactis* (WCFL); carrot enriched roasted African walnut fermented with *B. subtillis* (WCFB); carrot enriched roasted African walnut fermented with the combination of both *B. subtillis and L. lactis* (WCFBL)]. In order to prevent contaminations, a container per day was used for different samples. During the five days fermentation, the antinutrient analysis was carried out on daily basis.

2.5. Isolation and conventional identification of microorganisms during fermentation

Fermented broth cultures were cultured on nutrient agar, Man de, Rogosa and Sharpe (MRS) agar, yeast extract agar and potato dextrose agar. Pure isolates were subjected to some biochemical tests and identified according to Cheesbrough (8). The identification of fungi in fermented samples was also carried out using standard morphological characteristics, which include morphology, surface characteristic and microscopic examination (6).

2.6. Molecular identification of bacteria isolate

Extraction of DNA using CTAB method was done according to Akinrotoye (2), PCR analysis was run with a universal primer for fungi called 1TS1 and ITS4 and bacteria which was run with a universal primer called 16S rRNA. The amplicon was further purified before the sequencing using 2M Sodium Acetate wash techniques.

2.7. Antinutrient Determination

Tannin and Phytate was determining according to the method of Wheeler and Ferrel (29). Oxalate was determined using the method of Day and Underwood (9). The spectrophotometric method of Brunner (7) was used for Saponin determination.

2.8. Statistical analysis

Data are presented as mean ± standard error (SE). Significance of difference between different treatment groups was tested using one-way analysis of variance (ANOVA) using SPSS (Statistical Package for Social Science) version 20 software. For all tests, the significance was determined at the level of $P<0.05$

3. RESULTS

(Table 3) show the biochemical and morphological characterisation of the bacterial isolates. All the isolates showed different morphological features and biochemical reactions. The Morphological characteristics of fungi isolated from the fermented broth cultures is shown on table 4

The frequecy distribution of bacterial and fungal isolates in the fermented samples are represented on tables 1 and 2 respectively**.** The result revealed that *B. subtilis* was the most predominant bacteria in the fermented samples, while *Rhizopus oryzae* was the most predominant fungi isolated from the fermented sample.

Molecular identification of the bacterial isolates is shown in Table 5. The lengths of amplified products were 1453, 1133, 1450, 1525 and 1509 base pair for *B. subtilis, L. lactis, L. plantarum, Micrococcus roseus* and *L. fermentum* respectively (Plate 2) and base pair 510 for *Candida albican* (Plate 1). The sequence

obtained was analysed with BLAST in National Centre for Biotechnology Information (NCBI) database. Based on the 16SrRNA sequences, the bacteria *B. subtilis, L. lactis, L. planterium, M. roseus* and *L. fermentum* were confirmed to be *B. subtilis* b17a, *L. lactis* SFL8, *L. paracasei* KLDS 1.0651, *M. luteus* NCTC and *L. casei* JCM 8621 while the yeast candida albican was confirmed to be *C. albicans* h70b. The result also revealed a difference in cultural identification of *L. plantarum, M. roseus* and *L. fermentum*. The Phylogenetic tree of the organisms isolated is shown in figure 1

The anti- nutrient composition of raw walnut, raw carrot and roasted walnut are shown in figure 2. Roasted African walnut had the highest anti-nutrient composition of tannin (3.45 ± 0.053^{b}); Phytate (16.5314 ± 0.025^{c}) and oxalate (3.5317 ± 0.025^{b}) while raw carrot had the highest saponins content (2.05 ± 0.050^{b}). Raw walnut generally had a higher anti-nutrient composition than carrot, except in saponins. Fermentation led to daily reduction in anti-nutrient composition in the fermented sample with the least reduction recorded on the last day of fermentation. Natural fermented African walnut (NFW) and carrot enriched natural fermented African walnut (NFWC) resulted in the highest reduction in anti-nutrient composition. NFW and NFWC had a reduction in tannin, saponins, Phytate and oxalate from (2.47 ± 0.008^{b}, 1.46 ± 0.010^{a}, 14.41 ± 0.026^{d}, 3.29 ± 0.05^{c}) to (0.98 ± 0.05^{a}, 0.61 ± 0.05^{a}, 4.35 ± 0.03^{a}, 0.27 ± 0.05^{a}) and (0.91 ± 0.005a, 0.45 ± 0.005^{a}, 3.61 ± 0.03^{a}, 0.17 ± 0.005^{a}) respectively (Figure 3 to 6). Inoculation fermentation in which a monoculture of *L. lactis* or *B. subtillis* was used as starter culture resulted in the least reduction in the anti-nutrient content. Carrot enrichment of fermented African walnut had significant effect $p<0.05$ in reducing the antinutrient composition of African walnut as all the fermentation enriched with carrot all had a reduced tannin, Phytate and oxalate composition compared to the corresponding natural and inoculation fermentation of African walnut that were not enriched (Figure 3 to 6).

Table 1: Frequency distribution of bacteria in the fermented samples

Isolates	NFW	NFWC	Occurrence (%)
Bacillus subtilis	+	+	65
Lactobacillus plantarum	-	+	5
Lactobacillus lactis	+	+	20
Micococcus roseus	+	+	5
Lactobacillus fermentum	+	+	5

Keys: + = Present, - = Absent**, NFW**: Natural fermentation of African walnut, **NFWC**: Natural fermentation of African walnut enriched with carrot,

Table 2: Frequency distribution of fungi isolate in the fermented samples

Isolates	NFW	NFWC	Occurrence (%)
Rhizopus oryzae	+	+	50
Penicillium notatum	+	+	15
Penicillium chrysogenium	+	+	15
Candida albicans	+	+	10
Alternaria alternate	_	+	5
Rhizopus stolonifer	_	+	5

Keys: + = Present, - = Absent**, NFW**: Natural fermentation of African walnut , **NFWC**: Natural fermentation of African walnut enriched with carrot,

Table 3: Colonial, morphological and biochemical characterization of bacterial isolates from fermented broth cultures

Isolate No	Colony Morphology	Gram's Reaction	Catalase	Coagulas	Motilitv	Mannitol	Glucose	Fructose	Maltose	Lactose	Sucrose	Citrate	Indole	Spore Forming	Methyl	Starch	Urease	Probable Identity
1	Cream, circular, opaque, flat, rough	+	+	**NA**	+	+	AG	AG	AG	AG	AG	+	-	+	-	+	-	*Bacillus subtilis*
2	Circular, opaque, convex, cream, smooth colonies	+	-	-	-		A	AG	AG	A	AG	-	-		+	-	+	*Lactobacillus Plantarum*

3	Cream, circular, smooth, entire	+	+	**NA**	-	-	AG	A	-	-	AG	-	-		-	*Lactobacillus lactis*
4	Cream, circular, raised and smooth	-	+	-	+	-	A	A	-	-	A		-	**NA**	+	*Micococcus roseus*
5	Circular, translucent, convex, creamy, smooth colonies	+	-	-	-		AG	A	AG	AG	AG	-	-		-	*Lactobacillus fermentum*

Keyword: (+) = positive, (AG) = Acid and Gas, (-) = negative, (A) = Acid, (NA) = not applicable

Table 4: Morphological characteristics of fungal isolates from fermented broth cultures

Isolate No	Cultural and Microscopy description	Probable Identity
1	Yellowish green to dark green hyphae. Conidiophores arising from the mycelium singly or less often in synnemata, branched near the apex, penicillate, ending in a group of phialides	*Penicillium notatum*
2	Colonies are very fast growing with some tendency to collapse, white cottony at first becoming brownish grey to blackish-grey, sporangiophores are non-septate, arising from stolons opposite rhizoids usually in groups of 3 or more. Sporangia are globose with a flattened base, greyish black, powdery in appearance, Columellae and apophysis together are globose. Sporangiospores are angular, subglobose to ellipsoidal, with ridges on the surface.	*Rhizopus oryzae*
3	Greenish mycelium , Conidiophore hyaline, slender with spairing upper part, branched conidia and septate hyphae	*Penicillium chrysogenium*
4	Greenish growth, Conidiophore hyaline, slender with spairing upper part, branched conidia and septate hyphae	*Candida albicans*
5	White greenish growth with Conidia have a globose shape with rough surface wall	*Alternaria alternate*
6	Hyphae broad, not or scarcely septate; rhizoids and stolons present; sporangiophores brown, solitary or in tufts on the stolons, diverging from the point at which the rhizoids form; sporangia rather round; apophysis absent or scarcely apparent; sporangiophores ovoid.	*Rhizopus stolonifer*

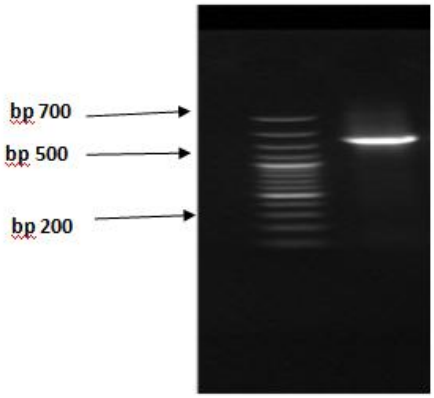

Plate 1: PCR amplification of genomic DNA targeted to amplify the 18SrNA gene of Yeast isolate on 1.0% agarose gel electrophoresis.

Key: **M** = Molecular marker

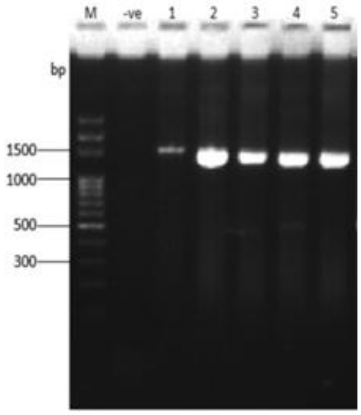

Plate 2: PCR amplification of genomic DNA targeted to amplify the 16SrRNA gene of bacterial isolate on agarose gel electrophoresis.

Key: **M** = Molecular marker
1= *Micrococcus roseus*
2= *Lactobacillus fermentum*
3= *Lactobacillus plantarum*
4= *Bacillus subtilis*
5= *Lactobacillus* lactis

Cultural and biochemical identification	**Gene sequence identification**	**Max Identity**	**Accession number**
Bacillus subtilis	*Bacillus subtilis* b17a	100	JX406823.1
Lactobacillus lactis	*Lactobacillus lactis SFL8*	99	KT757263.1
Lactobacillus Plantarum	*Lactobacillus paracasei* KLDS 1.0651	99	FJ861111.1
Micrococcus roseus	*Micrococcus luteus* NCTC 2665	100	NR075062.2
Lactobacillus fermentum	*Lactobacillus casei* JCM 8621	100	AB690208.1
Candida albican	*Candida albican* h70b	100	KP674768.1

Table 5: Molecular identification of isolated bacteria and yeast

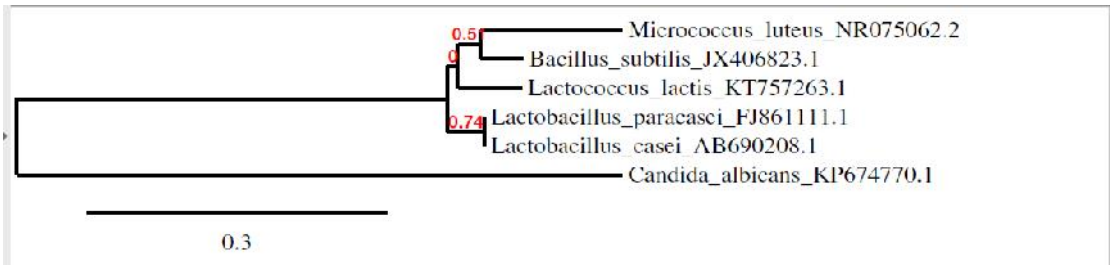

Figure 1: Phylogenetic tree of the microorganism isolated

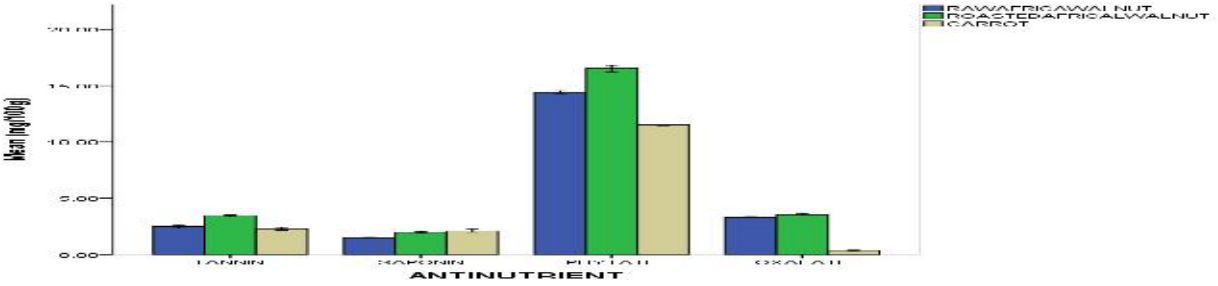

Figure 2: Antinutrient composition of raw walnut, roasted walnut and raw carrot

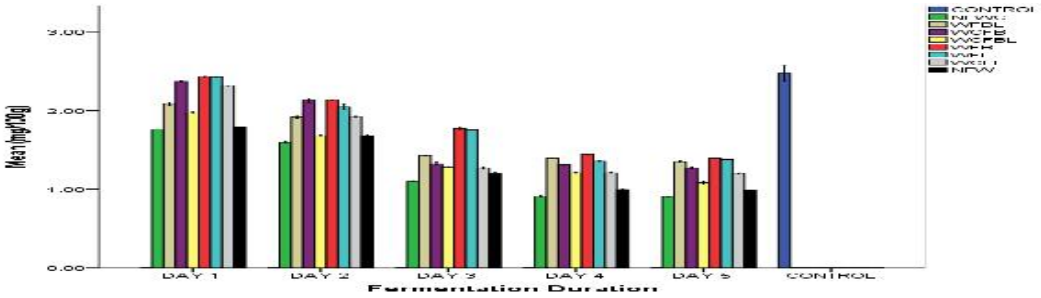

Figure 3: Effects of fermentation on the tannin composition fermented samples

Keys: **NFW**: Natural fermentation of African walnut , **WCFBL**: Carrot enriched African walnut fermented *B. subtillis and L. latics*, **NFWC**: Natural fermentation of African walnut enriched with carrot , **WFB**: African walnut fermented with *B. subtillis* , **WFBL**: African walnut fermented with *B. subtillis and L. latics* , **WFL**: African walnut fermented with *L. latics* , **WCFB**: Carrot enriched African walnut fermented with *B. subtillis* , **WCFL**: Carrot enriched African walnut fermented with *L. latics* Control: Unfermented walnut.

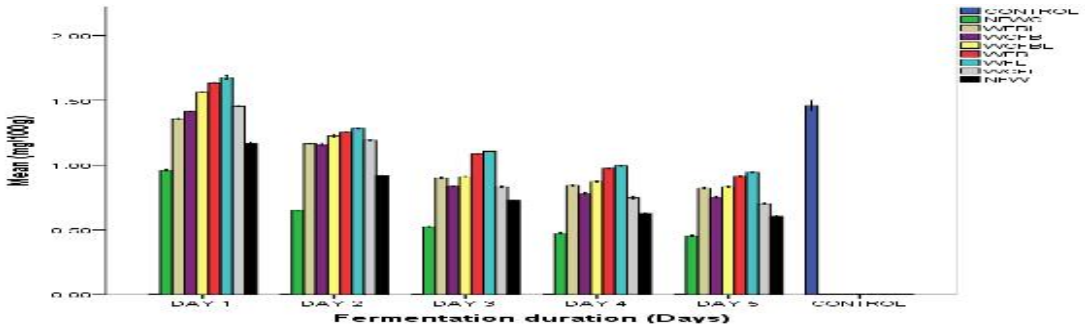

Figure 4: Effects of fermentation on the saponins content of fermented samples

Keys: **NFW**: Natural fermentation of African walnut , **WCFBL**: Carrot enriched African walnut fermented *B. subtillis and L. latics*, **NFWC**: Natural fermentation of African walnut enriched with carrot , **WFB**: African walnut fermented with *B. subtillis* , **WFBL**: African walnut fermented with *B. subtillis and L. latics* , **WFL**: African walnut fermented with *L. latics* , **WCFB**: Carrot enriched African walnut fermented with *B. subtillis* , **WCFL**: Carrot enriched African walnut fermented with *L. latics;* Control : Unfermented walnut

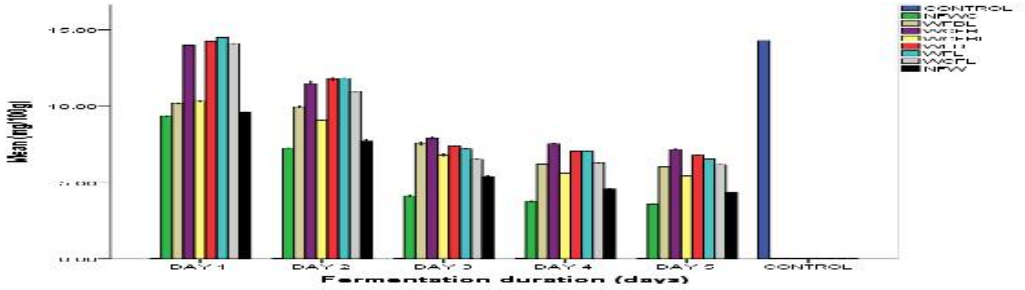

Figure 5: Effects of fermentation on the Phytate composition of fermented samples

Keys: NFW: Natural fermentation of African walnut , **WCFBL**: Carrot enriched African walnut fermented *B. subtillis and L. latics*, **NFWC**: Natural fermentation of African walnut enriched with carrot , **WFB**: African walnut fermented with *B. subtillis* , **WFBL**: African walnut fermented with *B. subtillis and L. latics* , **WFL**: African walnut fermented with *L. latics* , **WCFB**: Carrot enriched African walnut fermented with *B. subtillis* , **WCFL**: Carrot enriched African walnut fermented with *L. latics*, Control: Unfermenteed walnut.

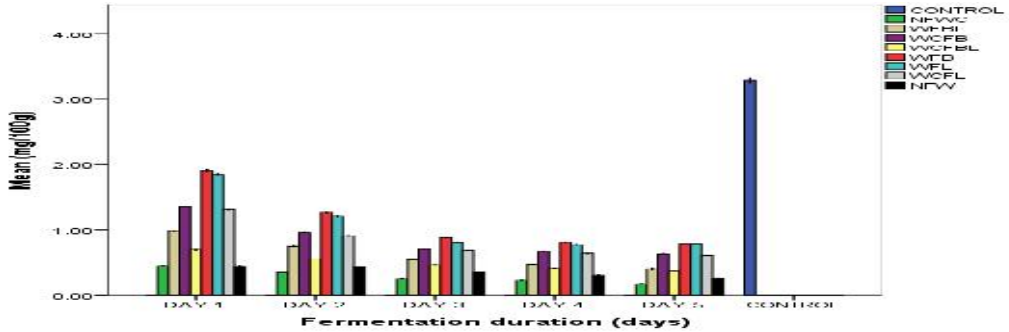

Figure 6: Effects of fermentation on the oxalate composition of carrot enriched and non-enriched African walnut

Keys: NFW: Natural fermentation of African walnut , **WCFBL**: Carrot enriched African walnut fermented *B. subtillis and L. latics*, **NFWC**: Natural fermentation of African walnut enriched with carrot , **WFB**: African walnut fermented with *B. subtillis* , **WFBL**: African walnut fermented with *B. subtillis and L. latics* , **WFL**: African walnut fermented with *L. latics* , **WCFB**: Carrot enriched African walnut fermented with *B. subtillis* , **WCFL**: Carrot enriched African walnut fermented with *L. latic,* Control: Unfermented walnut.

4. Discussion

In this study, the effects of fermentation on the anti-nutritional composition of African walnut enriched with carrot were evaluated. In addition, the microorganisms associated with the fermentation of African walnut were also isolated and identified. Since the major constituents of African walnut are proteins, fats and carbohydrates, the organisms responsible for its fermentation must be capable of utilizing these three constituents. Most of the organisms isolated from the fermented seeds are known to possess such characteristics. The organism isolated from the fermented samples were identified using biochemical test to be; *B. cereus, M. roseus, L. lactis, L. plantarum* and *L. fermentum* and the fungi isolated include *R. oryzae, C. albicans, Penicillium chrysogenium, R. stolonifer, Alternaria alternate*, and *Penicillium notatum*. These microorgansisms were found during fermentation due to their ability to utilise the high protein content in African walnut as a source of nutrient for growth. *B. subtillis* was the predominant microorganism present; *B. subtillis* have been known to have proteolytic ability and also possess the

capacity to break down oils (12). *B. subtillis* has been associated with fermenting locust bean for iru production (4) and for fermenting soy bean for natto production (19) and this also agrees with the observation of Isu and Njoku, (21) that *Bacillus* species constitute over 95% of the total microbial population density in ugba fermentation.

Molecular techniques have the advantage: they are rapid, less laborious, and more sensitive, specific and efficient compared to the conventional method (24, 23). This study revealed a difference in cultural identification of *L. plantarum, M. roseus* and *L. fermentum*. A similar observation was also reported by Frickmann *et al.* (16), who reported differences in conventional method and molecular method of bacteria identification. However, the results of this study demonstrate clearly the interest and feasibility to introduce the 16S rRNA gene sequencing method in identification of bacteria, combination of conventional techniques and molecular approach will improve bacteriological investigation and authentication, allowing specific and efficient identification of microorganisms as against cultural method that is probable.

Anti-nutrients are generally known to reduce nutrient utilization and or food intake (14). Roasting generally led to increase in the level of these anti-nutritional components; this pattern of results was similar to what was reported by Enujiugba (13). Ekwe and Ihemeje (11) have also reported that toasting caused an increase in the phytate and tannin content of African walnut. Fermentation significantly led to the reduction in anti-nutrient composition in the fermented sample. This could be as a result of the fact that some anti-nutrient like tannins are water soluble and also the ability of some microorganism to produce enzyme which can break down some of these anti-nutrient e.g. Phytases which has the ability of breakdown phytate content (1). A similar observation was reported by Esekheigbe and Onimawo, (14) where fermentation resulted in the reduction of anti-nutrient. Natural fermentation resulted in the highest reduction in anti-nutrient which maybe as a result of the various array of microorganism present in the natural fermentation as compared to the inoculation fermentation. This was further corroborated as the inoculation fermentation with consortium of two organisms resulting in a better reduction in anti-nutritional composition than when a monoculture was used. Carrot enrichment of fermented African walnut significantly help in reducing the anti-nutrient composition of African walnut as all the fermentation enriched with carrot all had a reduced tannin, Phytate and oxalate composition compared to the corresponding natural and inoculation fermentation of African walnut that were not enriched which maybe as a result of the fact that raw carrot had a very low Phytate, oxalate and tannin content when compared to raw African walnut. By and large, carrot enrichment of African walnut and Natural fermentation brought about maximal anti-nutrient reduction followed by inoculation fermentation with consortium of two organisms. Roasting is definitely not a good choice as the anti-nutrients composition were generally increased.

3. Conclusion

In this study, it is concluded that fermentation and enrichment with carrot helps in significantly reducing the antinutrient composition of African walnut and hence, both method can be employed in improving the nutritional quality of the food thereby helping in effectively utilising their potential as human food.

7. References

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