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Comparative Microbial Assessment of Fermented Lima Bean (*Phaseolus lunatus*) and Locust Bean (*Parkia biglobossa*) in Production of *Daddawa*

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Authors' contributions

This work was carried out in collaboration between all authors. Author HAA designed the study in conjunction with author SHA while author EOF carried out the laboratory work, analyzed the results and wrote the first draft of the manuscript. Author HAA supervised the study while author SHA provided some literature and technical assistance. All authors read and approved the final manuscript.

Original Research Article

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ABSTRACT

Aim: *Daddawa* is a condiment traditionally produced from fermentation of locust bean (*Parkia biglobossa)*.This study is directed at exploitation of lima bean–an underutilized but cultivated legume for production of *daddawa* analogue-a popular West African food condiment. Microbiological characteristics of the two condiments are compared.
Methodology: Lima bean and locust bean seeds were separately heat processed and fermented into *daddawa* analogue and *daddawa* respectively. The microbial load, microbial types and their succession in fermented samples were monitored over a fermentation period of 72h.
Results: Results showed that total viable bacteria count increased from 4.74 to 9.25 and 5.87 to 8.10 (log CFUg-1) within 72h of fermentation in fermenting lima bean and locust bean respectively. The bacteria isolates from the fermenting lima bean and locust bean

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were identified as *Bacillus subtilis, B. licheniformis, B. brevis, B. coagulans, B. pumilus* and *B. megaterium.*
B. licheniformis occurred virtually throughout the fermentation period in the tested legumes while *B. subtilis and B*. *pumilus* were isolated from 24th and 36th *h* till the end of fermentation in the fermented lima bean samples respectively.
Conclusions: The study has established that *daddawa* produced from the two legumes have similar microbiological characteristics. Production of *daddawa* from locust bean has not been commercialized to date as result of unreliable source of raw material. Locust bean is presently obtained from the wild. Lima bean which is cultivated could be a sustainable raw material for this food condiment which is popular in many West African communities.

Keywords: Lima bean; locust bean; fermentation; daddawa analogue; Micro biological characteristics.

1. INTRODUCTION

Daddawa is a condiment traditionally produced from fermentation of locust bean (*Parkia biglobossa)*. *Daddawa* enhances taste in soup and also serves as low cost protein source in diet of low income families in Nigeria and some West African communities [1]. Fermentation is a process whereby food materials are subjected to the actions of microorganisms or their enzymes for biochemical changes to take place and modify the food and produce desirable attributes such as flavor and textural changes in the food [2]. During fermentation some physical characteristics of the food are modified, and nutrient density is enhanced [3]. In addition, some desirable antimicrobial properties are imparted and nutritional value and wholesomeness are increased over the starting material. Furthermore, level of nutrients, their digestibility and bio availability increase [4]. Fermentation has also been documented to be capable of lowering anti-nutrients [2]. Fermentation is a promising food processing method that can be used to diversify the food uses of some under exploited plant foods such as lima bean [5].

Lima bean is leguminous food stuff, which is cultivated primarily for its immature vegetables or mature dry seeds [6]. Lima bean is a cultivated lesser legume that is not well utilized in Nigeria and as such wasted even in the areas where it is being cultivated. In Africa, about 120,000 to 200,000 hectare is devoted to lima bean cultivation in the sub-humid and humid areas indicating the need for its maximum utilization. In Nigeria, lima bean is consumed as cooked whole bean but the long period of cooking time limits its acceptability [7,8]. It could be fermented into condiments that are added to food in form of sauce, powder, spread, seed or similar thing to enhance and improve the flavor and taste of such food [9].

Lima bean contains some anti-nutritional factors including trypsin and chymotrypsin inhibitors, haemagglutinins, cyanogenic glucoseides, tannin, lecithin, polyphenols, phytic acid and various oligosaccharides which cause flatulence [10,11]. Several food processing methods such as soaking, germination, dehulling, cooking and fermentation have been documented to be capable of reducing these anti-nutritional factors in legumes [11-13].

Since the major constituents of the legumes intended for processing into condiments are proteins, fats and carbohydrate, the organisms responsible for fermenting them must be able to utilize the nutrients in these seeds and convert them to products with desired chemical composition and taste. [14] reported some major microorganisms associated with fermented

condiments. They include *Bacillus subtilis, B. pumilus, B. licheniformis* and *B. megatarium*. *Bacillus subtilis* was found as the predominant microorganism common to all the fermented seeds. *Bacillus species* are known to have proteolytic ability and are able to break down oil. *Bacillus subtilis* has been associated with fermentation of locust bean to produce *daddawa* [15,16].

In an earlier study [18] reported on the proximate, mineral and sensory characteristics of *daddawa* analogue from lima bean. The present study evaluates and compares the microorganisms involved in the fermentation of lima bean to produce *daddawa* with the view of understanding the similarities and differences in microbiology of the two products with aim of using the information to develop a starter culture technology for commercial production of *daddawa* analogue from lima bean. This is necessary in the light of knowing that locust bean which is the major raw material for production of *daddawa* is not presently cultivated. Commercial production of locust bean *daddawa* may not be feasible in the future.

2. MATERIALS AND METHODS

2.1 Source of Materials

Lima bean seeds (NSWP 46) were obtained from the Grain legume Improvement Program me of Institute of Agricultural Research and Training, Ibadan, Oyo State, Nigeria. Locust bean seeds and fermenting containers (calabash) were obtained from a local market in Ibadan, Oyo State, Nigeria.

2.2 Lima Bean and Locust Bean Seeds Fermentation

Lima bean fermentation was done by the modification of the method described by [19] for production of soy *daddawa*. Five hundred grams (500g) of lima bean seeds were sorted, roasted in open frying pan for 10min. and dehulled by rubbing the seeds between palms. The dehulled beans were cooked with 1000ml of water for 40min. and excess water was drained. The cooked beans were poured while still hot into clean calabash lined with clean freshly harvested banana leaves. The calabash was covered with another calabash and was incubated at 30±5ºC for 72 hours. Locust bean seeds were fermented using the method described by [15] with slight modification.

Five hundred grams of locust bean seeds were soaked in 2000ml of water overnight (12h). The soaked beans were pressure cooked for 1h at 121ºC at 15 psi. In a pressure cooker (MASTER CHEF MC-PC-4060), seed coats were removed from the cooked beans by rubbing the beans between palms and washing with water. The dehulled beans were again pressure cooked with 1500ml of water for 1h 30mins at 121ºC at 15 psi. Excess water was drained and the cooked locust bean seeds were poured hot into calabash lined with clean freshly harvested banana leaves and fermented as done for lima bean. Samples were taken out at 12h interval for analyses. The samples were prepared in triplicates and replicated three times.

3. MICROBIAL ANALYSES

3.1 Determination of Total Viable Count

Total viable count was determined using the method described by [19]. Pour plate method was employed. Samples (2.0g) from fermented lima bean mashes were aseptically taken and diluted serially in 0.1% peptone water. The aliquots (1.0ml) of appropriate dilution were placed on nutrient agar (Lab M, England), tryptone soy agar (Lab M, England) and potato dextrose agar (Lab M, England) to determine the total viable count and fungi count respectively. Nutrient agar (NA) and tryptone soy agar (TSA) plates were incubated at 35ºC±2ºC aerobically for 24 hours while potato dextrose agar plates were incubated at 28ºC±1ºC for 5 days. Colonies were counted and expressed as colony forming unit per gram (CFUg-1) of the sample. The counts were done in triplicates and the mean taken.

3.2 Isolation and Identification of Microorganisms

Representative colonies from NA and TSA plates counts were purified by repeated streaking to isolate the microorganisms, the isolated microorganisms were characterized and identified by the standard methods [20-23].

3.3 Gram Staining

A heat fixed smear was prepared on a clean slide from an 18h old bacterial culture. The smear was stained with crystal violet solution for 1 minute. The slide was rinsed with tap water, Gram's iodine solution was added. The iodine solution was allowed to stay for 60 seconds. The slide was rinsed with water and 95% ethanol was added, allowed to stay for 30 seconds to decolorize the crystal violet stain. The slide was rinsed with water, counter stained with safranin solution, which was left for 30 seconds. The slide was rinsed with tap water and blot dried. The slide was observed under the microscope with immersion oil.

3.4 Catalase Test

A loopful of bacterial culture was emulsified with hydrogen peroxide on a clean slide. The slide was observed for effervescence caused by libration of free oxygen as gas bubbles and result taken.

3.5 Oxidase Test

Few drops of 1% aqueous of tetramethyl-p-phenylenediamine hydrochloride were added to a loopful of bacterial culture on a clean slide. The slide was observed for development of a purple color within 5 seconds and result taken.

3.6 Growth at 50ºC, 60ºC and 65ºC

Bacterial culture was streaked on fresh nutrient agar plates in triplicates and each set of the plates was incubated at different temperatures of 50ºC, 60ºC and 65ºC respectively. The plates were observed after 24 hours for growth and result taken.

3.7 Production of Ammonia from Peptone

Tubes of Tryptone water were inoculated with bacterial culture. The tubes were incubated with a control tube at 35^{o}C for 7 days. After incubation, 1ml of culture was added to 1ml of Nessler's reagent in a test tube. The tubes were observed for development of an orange or brown color.

3.8 Nitrate Reduction

Nitrate peptone water with the addition of 0.2% potassium nitrate was dispensed into tubes, each with an inverted Durham tubes. The tubes were sterilized by autoclaving for 25 mins at 121ºC. The sterile medium was inoculated with bacterial culture and incubated with a sterile control tubes at 35ºC for 7 days. After incubation, Iml of Griess-Ilosvay's reagent was added to the culture and the control tube. The tubes were observed for development of red color within few minutes.

3.9 Starch Hydrolysis

Solidified starch nutrient agar plates were inoculated with bacterial culture by streaking once across the surface of the agar. The plates were incubated at 35ºC for 7 days. After incubation, the plates were flooded with Gram's iodine solution and starch hydrolysis was observed as clear zone on the plates.

3.10 Gelatin Hydrolysis

Nutrient broth with the addition of 10% gelatin was dispensed into tubes, sterilized in an autoclave at 115ºC for 25mins. Each sterile gelatin medium in tubes was stabbed-inoculated with bacterial culture and incubated with a control tube at 35ºC for 7 days. After incubation the tubes were put in the refrigerator for 5mins. After which the tubes were observed for solidification of the gelatin medium.

3.11 Production of Indole from Tryptophan

Sterile peptone water in tubes was inoculated with bacterial culture from an agar slant. The inoculated tubes and the control were incubated at 35ºC for 7 days. After incubation, Kovac's reagent (0.5ml) was added and the tubes were shaken gently and allowed to stand for about 30mins. The tubes were observed for development of red color which separated out in the alcohol layer.

3.12 Oxidation and Fermentation of Carbohydrates

Carbohydrate (D-Glucose) was added to basal medium (Hugh and Leifson's medium). This was dispensed into tubes, and sterilized by autoclaving at 115ºC for 25mins. The sterile medium was stabbed- inoculated with bacterial culture. The inoculated tubes were prepared in duplicates. One set was covered with liquid paraffin while the other tubes were left open. The tubes were incubated at 35ºC for 7 days. After incubation, the tubes were observed for change in color from blue to yellow which indicate acid production. Open tubes were observed for color change, an indication of oxidative bacteria, while open and closed tubes were observed for color change, an indication of fermentative bacteria.

3.13 Citrate Utilization

Koser's citrate medium was dispensed into clean test tubes and sterilized in an autoclave at 115ºC for 20mins. The sterile medium was inoculated with peptone water culture using a wire needle. The inoculated medium in tubes was incubated at 35ºC for 7 days. After incubation, the tubes were observed for turbidity indicating growth and utilization of citrate.

3.14 Methyl Red Test

Glucose phosphate broth was prepared, dispensed into tubes and sterilized in an autoclave at 115ºC for 20mins. The sterile medium was inoculated with bacterial culture and incubated at 35ºC for 7 days. After incubation, five drops of methyl red indicator was added to 5ml of broth culture in tubes. The tubes were observed for development of red color.

3.15 Voges-proskauer Test

Sterile Glucose phosphate broth was inoculated with bacterial culture and incubated at 35ºC for 7 days. After incubation, a pinch of creatine was added to the culture followed by 5ml of 40% sodium hydroxide. The tubes were shaken very well and allowed to stand for 30 minutes. The tubes were observed for development of red color within 30mins which is an indication of production of acetyl methyl carbinol from glucose.

3.16 Acid and Gas Production from Fermentable Sugars

Peptone water and 10% substrate (different sugars: glucose, lactose. maltose, arabinose, xylose and manitol) was taken in a conical flask, 0.1g of bromocresol purple was added. The sugar solution was dispensed into test tubes in which inverted Durham tubes had been placed and sterilized in an autoclave at 115ºC for 25 mins. After sterilization the medium was allowed to cool. Each sterile medium tube was inoculated with bacterial culture and afterwards incubated at 35ºC for 7 days. After incubation, the tubes were observed for acid production by change in color from purple color to pink or any color different from that of the control. The tubes were also observed for gas production in the inverted Durham tubes.

4. RESULT

4.1 Changes in Total Viable Count

The total viable bacteria count increased from 4.74 at 0h to a peak of 10.61 (log CFUg-1) at 36h of fermentation and reduced to 9.25 (log CFUg-1) at 72h in fermenting lima bean. A similar trend was obtained in fermenting locust bean except for the fact that a rise to a peak from 5.75 to 10.00 (log CFUg-1) from 0 to 12h of fermentation was observed. Subsequently, the count reduced to 8.10 (log CFUg-1) at 72h of fermentation in locust bean Table 1.

The logarithmic phase of growth of microorganisms in the fermented lima bean *daddawa* was between 0h and 36h (4.74 to10.61 CFUg-1) during which there was an exponential increase after which the total viable count decreased slightly till the end of fermentation at 7h. The logarithmic phase of growth of microorganisms in the fermented locust bean *daddawa* was between 0h and 12h (5.87 to 10.00 (CFUg-1) after which it decreased gradually till the end of fermentation period.

Table 1. Changes in total viable count during the fermentation of lima bean and locust bean to *daddawa* (log CFU g^{-1})

Sample	Fermentation time (H)						
	0	12	24	36	48	60	72
Lima bean *daddawa*	4.74 ±0.51	9.62 ±0.60	9.98 ±0.18	10.61 ±0.10	9.89 ±0.20	8.34 ±0.30	9.25 ±0.30
Locust bean *daddawa*	5.87 ±0.90	10.00 ±0.25	9.97 ±0.25	8.81 ±0.70	9.57 ±0.65	9.81 ±0.32	8.10 ±0.50

4.2 Isolation and Identification of Microorganisms

Twelve bacteria isolates were obtained from the fermenting lima bean and fermenting locust bean Table 2. The isolates from fermented lima bean were identified as *Bacillus subtilis, B. licheniformis, B. firmus, B. coagulans, B. pumilus, B. megaterium,* and *Corynebacterium species*. The microbial isolates from fermented locust bean were identified as *Bacillus subtilis, B. licheniformis, B. brevis, B. coagulans, B. pumilus, Bacillus species,* and *Staphylococcus aureus.* Species of *Bacillus* were found to be the predominant microorganisms in the fermented lima bean and locust bean.

Staphylococcus aureus was absent throughout the fermentation period of lima bean while it was present at the initial stage of fermentation of locust bean till 36h after which it disappeared Table 3a. *Corynebacterium* sp was isolated during the early stage of the fermentation of lima beans. *Corynebacterium species* were present between the 12 and 48h of fermentation of lima bean Table 3a.

4.3 Succession of Isolates During Fermentation

Succession of the isolated dominating species of bacteria in the fermented lima bean and fermented locust bean is shown in Tables 3a and 3b respectively. The result showed that *Bacillus licheniformis* occurred throughout the fermentation period in lima bean *daddawa* and locust bean *daddawa*. *Bacillus pumilus* and *B. subtilis* were present from 24h and 36h till the end of fermentation period respectively in lima bean *daddawa* Table 3a. *Bacillus brevis* was present during the fermentation of locust bean 24h and 72h while *B. coagulans* was isolated from locust bean at later stages of fermentation Table 3b. *Staphylococuss aureus* was absent throughout the fermentation period of lima bean Table 3a. *S. aureus* was present at the initial stage of fermentation of locust bean till 36h after which it disappeared. *Corynebacterium sp* was isolated during the first 48 hours of fermentation of lima bean while it was not isolated from fermenting locust bean.

Table 2. Morphological and Biochemical Characteristics of Bacterial isolates

Microscopic observation/ Biochemical Tests	Isolates							
	B1	**B2**	**B3**	**B4**	**B5**	**B6**	**B7**	**B8**
Colony Morphology	Cream, spreading colonies	Cream mucoid jagged edge	Cream mucoid amoeboid, jagged	Cream, rhizoid, jagged edge	Cream, spreading	Transparent, amoeboid, jagged	Cream, lobed	Cream, mucoid, entire
Cell Morphology	Short rods, central spores	Rods, central spores	Long rods central spores	Long rods, central spores	Rods, central spores	Rods, central Spores	Cocci arranged in clusters	Big rods with granules
Gram's Reaction	+	+	+	+	+	+	+	+
Catalase Test	+	+	+	+	+	+	+	+
Starch hydrolysis	+	+	+	+	+	+	-	+
Growth at 50°C	+	+	-	+	+	+	-	-
Growth at 60°C	-	-	-	-	+	+	-	-
Growth at 65°C	-	-	-	-	-	-	-	-
Growth in Hugh-Leifson medium	F/A	A	A	F/A	F/A	A	F/A	A
Nitrate Reduction	+	+	-	+	-	++	-	-
Gelatine liquefaction	+	+	-	+	-	+	-	+
Citrate Utilization	+	+	+	+	-	+	+	ND
Methyl –Red Test	-	-	+	+	+	-	-	+
Acid from Mannitol	-	+	-	+	-	+	+	+
Acid from Xylose	+	+	-	+	-	-	+	+
Acid from Lactose	+	+	-	+	-	-	+	-
Acid from Glucose	+	-	-	+	-	-	+	+
Acid from Trehalose	-	+	-	+	+	-	+	+
Acid from Maltose	-	+	-	+	+	-	+	+
Acid from Arabinose	-	+		+	-	-	+	+
Coagulase Test	ND	ND	ND-	ND	ND	ND	+	ND
Identity of Organism	*Bacillus subtilis*	*Bacillus pumilus*	*Bacillus megaterium*	*Bacillus licheniformis*	*Bacillus coagulans*	*Bacillus brevis*	*Staphylococcus aureus*	*Corynebacterium sp*

Key:+ =positive, -=negative, F/A=facultative anaerobic, A=aerobic, ND=not done

Table 3a. Succession of the isolated bacteria during the fermentation process of lima bean to *daddawa*

Microorganisms	Fermentation time (H)						
	0	12	24	36	48	60	72
B. licheniformis	+	+	+	+	+	+	+
B. coagulans	-	-	-	-	-	-	-
B. subtilis	-	-	+	+	+	+	+
B. pumilus	-	-	-	+	+	+	+
B. megaterium	-	-	-	-	-	+	+
Corynebacterium	-	+	+	+	+	-	-
S. aureus	-	-	-	-	-	-	-

+=*Isolated - =Not Isolated*

Table 3b. Succession of the isolated bacteria in the fermentation process of locust bean to *daddawa*

Microorganisms	Fermentation time (H)						
	0	**12**	**24**	**36**	**48**	**60**	**72**
B. licheniformis	-	+	+	+	+	+	+
B. brevis	-	-	+	+	+	+	
B. coagulans	-	-	-	-	-	+	+
B. subtilis	-	-	+	+	+	+	+
B. pumilus	-	-	+	+	+	+	+
B. megaterium	-	-	-	+	+	+	
Bacillus species	-	-	+	+	+	+	+
Corynebacterium	-	-	-	-	-	-	-
S. aureus	+	+	+	+	-	-	-

+=*Isolated - =Not Isolated*

5. DISCUSSION

5.1 Changes in Total Viable Count

Increase in the total viable count with increasing fermentation time could have been caused by break down of protein, lipids, starch and other nutrients to their simpler forms which were used by these microorganisms as sources of carbon and nitrogen and subsequently to increase their microbial biomass [24]. The total viable counts of microbes have been shown to increase with increasing fermentation time during production of condiments from different vegetable proteins by several authors. [19] Reported increase in total viable count of microorganisms from 3.38 at 0h to 10.29 (log CFUg-1) at 7h of fermentation of soybean to produce soy *daddawa* [24]. Also reported progressive increase in total viable count of microorganisms from $2.8X10^7$ at 24h to$1.8X10^{10}$ CFU g-1 at 7h of fermentation during the production of *anyi* from *Samanena samon* seeds. Also documented was the finding of [25] on the increase in total viable count of microorganisms from $3.2X10^5$ at 0h to $9.2X10^7$ CFU g-1 at 96h of fermentation during the production of *bambara* groundnut to produce *daddawa*.

The exponential growth increase of the microorganisms between 0h and 36h during lima bean fermentation and the increase between 0h and 12h during the locust bean fermentation were probably due to the availability of nutrients at the initial stage of fermentation. The slight

decrease towards the end of fermentation could probably be due to nutrient depletion and accumulation of toxic metabolites as a result of the activities of the microorganisms.

5.2 Isolation and Identification of Microorganisms

The preponderance of *Bacillus species* have been reported in other fermenting legumes [17,26,27]. They are predominant because they are able to utilize carbohydrate, protein and fat which are the major constituents of legumes and oil seed used in production of condiments [28-30] similarly reported that *Bacillus subtilis*, *Bacillus licheniformis*, *Leuconostoc mesenteroides* and *Staphyloccocus sp* were associated with the fermentation of *Cathormion altissimum* seeds to produce *oso* condiment. *Bacillus species* isolated from various plant protein sources have also been reported to be proteolytic and are able to break down oils [29,31]. Hence production of lima bean *daddawa* can be said to be a solid substrate fermentation of protein rich material by *Bacillus* [15].

Staphylococcus species have been found to be associated with fermenting foods of plant origin particularly vegetable proteins [19,32]. Presence of *Staphylococcus* could have been caused by handling of the seeds after boiling. *Corynebacterium species* which was present between the 12 and 48h of fermentation of lima bean is known to be associated with fermentation of cassava to *gari*. It converts starch to lactic acid and formic acid thus lowering the pH [33]. Its role could be that of opportunistic contaminant. Further study in which it should be used as a starter may reveal its real contribution to fermentation of lima bean. High content of carbohydrate (63%) could have encouraged its initial presence in fermenting lima beans. *Corynebacterium* was absent during the fermentation of locust bean to produce *daddawa*. This might be due to the low carbohydrate content of locust bean. [19] Reported low carbohydrate content in locust bean seeds (17%) when proximate composition and sensory value of fermented and unfermented soybean and locust bean were compared.

There were no fungi isolated in any of the fermented lima bean and locust bean samples. This might probably be as a result of the heat process of the beans prior to fermentation and the rapid rise in the pH, both of which may not favor their growth and multiplication. The absence of fungi in the fermented lima bean and locust bean samples makes the samples safe from the risk of mycotoxins [15].

5.3 Succession of Isolates During Fermentation

Since *B. licheniformis* was isolated from lima bean throughout the fermentation period while it was present from 24h of fermentation in locust bean, it is evidently important in the fermentation process. In the fermented samples of lima bean and locust bean, *B. subtilis* was present from 24h till the end of fermentation. *B. licheniformis* has been reported by [34] as having high proteolytic activity when screened on skim milk agar [19]. Also reported the role of *B. subtilis* and *B. licheniformis* in fermentation soy bean to soy-daddawa. It was concluded that the microbes were able to carry out the fermentation because of their high proteolytic activity since biochemical study demonstrated this.

S. aureus and *Corynebacterium* sp were not consistently isolated from the fermenting legume samples probably because they were important in the fermentation process. Some studies have reported on the presence of these microbes in some fermenting food stuffs. Their role should be investigated further with sterile samples.

6. CONCLUSION

The predominant microorganisms in the fermentation process of both samples were found to be *Bacillus species* of which *Bacillus subtilis, B. pumilus and B. licheniformis* were most involved. The study has shown that a *daddawa* analogue, comparable to well accepted locust bean *daddawa* in terms of microbiological characteristics, could be produced from lima bean-an under-utilized legume.

COMPETING INTERESTS

Authors have declared that no competing interests exist

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