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The role of *Staphylococcus* species in the production of iru during the fermentation of African locust beans (*Parkia biglobosa*)

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Abstract

Staphylococcus spp. are regularly isolated from iru, but the role(s) they play in the fermentation process has not yet been determined; this work thus seeks to determine if *Staphylococcus* spp. isolated from iru play any role in the fermentation of African locust bean. *Bacillus* spp. and *Staphylococcus* spp. isolated from spontaneously fermented African locust bean (iru) were used to ferment African locust beans. The temperature, pH and moisture content were determined as fermentation progress while the total soluble sugar and total free amino acid were determined after fermentation. The microbial load for the three iru products increased gradually until the end of fermentation. The total free amino acids increased in all three iru (1.10, 1.51 and 2.35 mg leucine ml^{-1} for *Staphylococcus* spp. iru, *Bacillus* spp. iru and iru produced with combination of the two species of bacteria) when compared with that of the unfermented bean, while the total soluble sugars reduced after fermentation with *Staphylococcus* spp. iru having 3.84, *Bacillus* spp. iru has 3.60, and the unfermented bean has 5.30 mg glucose ml^{-1} total soluble sugar. The increased free amino acids in the iru fermented with *Staphylococcus* spp. and the ability of the *Staphylococcus* spp. to produce lipase showed that *Staphylococcus* spp. isolated from iru has the ability to ferment African locust bean and carry out the lipolytic activity during the fermentation.

1. Introduction

Iru-a condiment produced from the fermentation of the dried, dehulled boiled seeds of African locust beans tree- *Parkia biglobosa*, which is a perennial leguminous tree of the sub-family *Mimosoideae* family *Leguminosae*. Local production of iru involves the boiling of the dried seeds on fire for about 24 h to soften the testa and cotyledons, the boiled, soft seeds are pressed in the mortar with feet to remove the softened testa; after which the seeds are washed to remove the testa from the cotyledon and then boiled for another 1-2 hours then fermented (Odunfa and Oyewole, 1986).

The popularity and acceptance of Locust bean dawadawa were not in doubt as it is used in soups by communities in several parts of Nigeria (Kolapo *et al.,* 2007). Apart from the improvement of sensory properties, condiments add nutritional values to foods, providing dietary fiber, energy, minerals, and vitamins (Kolapo *et al.,* 2007). Iru is an important source of riboflavin and it contains the highest riboflavin content when compared to some other common plant foods (Pelig-Ba, 2009).

It is clear that the fermentation of vegetable proteins into condiments is usually initially mediated by diverse microbial flora, which at the long-run eventually becomes gram positive flora (Achi, 2005); though the contributions of this microbial flora to the properties of the products are not totally understood (Iwuoha and Eke, 1996).

The co-dominance of *Staphylococcus* and *Bacillus* species was the typical microflora of the fermenting beans (Achi, 1992), but the contribution of the accompanying flora of the fermenting substrate is determined by the composition of the substrate and the hygiene or hygienic measures put in place during production (Achi, 2005).

Bacillus species were reported to be most responsible for alkaline fermentation of vegetable condiments, but several works also have identified some other microorganisms in iru production to include *Micrococcus*, *Leuconostoc,* and *Enterobacteriaceae*, (Omafuvbe *et al.,* 2002; Ogunshe *et al.,* 2008) with *Bacillus* spp. and *Staphylococcus* spp. being the major microflora (Odunfa, 1991).

Lues *et al.* (2011), reported the presence of *Staphylococcus* in a commercially produced traditional

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beer like *circa,* which had 10^4 CFU/ml and *scomfana* which had 10^5 CFU/ml; he discovered that the *Staphylococcus* spp. include *S. aureus, S. epidermidis, S. xylosus, S. hominis, S. capitis* and *S. saprophyticus.* Omafuvbe *et al.* (2000) found out that *Staphylococcus* was only present at the early stage of soybean fermentation to produce soy-iru. Ogunshe *et al.* (2007) isolated non-sporing *Staphylococcus* spp. and some *Bacillus* spp. from the fermenting *Prosopis Africana* cotyledons during the controlled fermentation of afiyo and he concluded that *Staphylococcus* did not appear to play a major role in the production of the condiment. *Bacillus* spp., *Staphylococcus aureus* and *S. saprophyticus* were the characterized bacterial species during the microbial studies on aisa- a fermented food condiment from *Albizia saman*; fermented in the laboratory (Ogunshe *et al.*, 2006). Cultures of *B. subtilis*, *B. licheniformis*, and *S. xylosus* had been isolated and identified as predominant micro-organisms associated with spontaneous fermentation of *Lanhouin* condiment (Anihouvi *et al.,* 2007).

A gradual increase of *S. xylosus* was noted during the first 60 h of fermentation, followed by a slight decrease in the final microbial load (Anihouvi *et al.,* 2012). *S. xylosus* had an important proteolytic activity, leading to the increase of pH during the spontaneous fermentation of fish (Anihouvi *et al*., 2007). The microbial flora isolated from laboratory prepared anyi-fermented *Samanea saman*; include different species of *Bacillus* and a few *Staphylococci* to include *S. saprophyticus*, which did not appear to play any major role in fermentation (Okonko, 2008).

The total sugar decreased significantly in soy-daddawa fermentation while reducing sugar increased in the first 12 hours and then decreased, which was suggested to be as a result of utilization of soluble sugars by the increasing population of the microorganism, but the addition of 1% salt to the substrate resulted in the increase of free amino acids and the proteolytic activities which are thought to enhance flavor (Omafuvbe, 2006).

Ogunshe *et al.* (2007), reported an increase in the total sugars during the first 24h of fermentation, followed by a subsequent decrease on the second day of fermentation; and the peak came on the 3rd and 6th days of fermentation, but the total amino acids level was found to increase throughout the fermentation period. Uaboi-Egbenni *et al.* (2009) reported that the highest mean value obtained for reducing sugar was 1.25 mg at the fourth day of fermentation,

Ogunjobi *et al*. (2005) reported a slight increase in the moisture contents of brined samples of Irish potatoes, Pelig-Ba (2009) reported an increase in moisture during the fermentation of African locust beans.

The objective of this work is to determine what possible role(s) *Staphylococcus* spp. play in the fermentation of African locust beans to produce iru; since *Staphylococcus* spp. are regularly isolated from iru.

2. Materials and methods

2.1 African locust bean seeds

Dried of African locust bean seeds (*Parkia biglobossa*) were purchased at Bodija market in Ibadan.

2.2 Fermentation of iru in the laboratory

Seeds were cooked in a pressure pot for about 2h, the swollen seeds were rubbed in between palms to remove the testa, the hard seeds were removed during washing. The cotyledons were then boiled for another 30 minutes and drained in a sieve. Fifty (50 g) grams of boiled, dehulled seeds were weighed into small DANA plastics with airtight cover and sterilized at 121^0C for 15 minutes, this was then allowed to cool down. 0.5 ml of inoculums was used to inoculate the cooked beans. The inoculated beans were wrapped in towels and incubated for 48 h.

2.3 Determination of inoculums size

The representative of each bacteria group was grown on agar at 37^0C for 18 h; each of the cultures was suspended in 10 ml sterile 1% NaCl solution (the three species of each bacteria is mixed together and well mixed before dilution), diluted to give an absorbance of 0.03 at 540 nm in a spectrophotometer, 0.5 ml of this dilution was used to inoculate the cooked beans (Omafuvbe *et al*., 2002; Omafuvbe, 2006) aseptically (this gives about 10^4 cells g^{-1} wet wt.); the suspension was mixed equally for two bacteria membered mixed culture. Three *Staphylococcus* spp. (*S. saprophyticus*, *S. xylosus* and *S. epidermidis*) and three *Bacillus* spp. (*B. subtilis, B. pumilus* and *B. licheniformis*) previously isolated from iru (gotten from the food microbiology laboratory of Microbiology department of University of Ibadan) were used.

2.4 Microbiological analysis

The microbial load was determined, by aseptically collecting samples at 24-hour interval (0, 24, and 48 hours) during fermentation, 1 g of the samples (each) was transferred to sterile distilled water and well shaken. Ten-fold serial dilutions in the same diluents were prepared and used. Aerobic mesophilic counts were done after incubation at 37^0C on plate count agar (PCA), using 1 ml of different dilutions for the pour plate method; analyses were carried out in triplicates at specified times.

2.5 pH and temperature measurement

The temperature and pH changes during

fermentation were measured at 0, 24 and 48 h. The pH was determined by mixing 1 g of the fermenting mash with 10 ml of distilled water and the suspension was used for the pH determination using a pH meter (using EXTECH pH100).

2.6 Moisture content

The moisture contents were determined for the products as fermentation progresses at 0, 24 and 48 h by weighing 1 g of each fermenting mashes on petri dish and put in the oven at 80^0C, the weight was checked and recorded at intervals till a constant weight was obtained, the final weight is then subtracted from the initial weight and the answer was divided by the initial weight and multiplied by 100 to get the percentage moisture content.

2.7 Preparation of extracts

For the analysis of total free amino acids and total soluble sugar, approximately 5 g of the samples after drying at 80^0C in the oven for 24 h was mixed with 70% ethanol and ground in a mortar; the suspension was then washed with 5 ml n-hexane to extract the oil, this was then filtered, using Whatman filter paper number 1 and the filtrate was used for analysis.

2.8 Determination of total free amino acids

The total free amino acids of the African locust bean before and after fermentation were determined by the ninhydrin colorimetric method of Rosen (1957) using leucine as the standard. To 1ml of the extract was added 0.5 ml of cyanide-acetate buffer and 0.5 ml of 3% ninhydrin in Methyl Cellosolve (2-Methoxyl Ethanol); the mixture was heated for 15 minutes in 100^0C boiling water, 5 ml of n-Propanol water mixture was added and shaken vigorously. The color was read in a spectrophotometer at 570 nm after cooling and the concentration of amino acids was calculated from a standard curve based on the known concentration of leucine.

2.9 Determination of total soluble sugar

The total soluble sugar of the African locust bean before and after fermentation was determined by anthrone reaction method, with glucose as the standard (Omafuvbe *et al.,* 2004). The extract was suitably diluted and to 1 ml of this was added 4 ml of freshly prepared anthrone reagent and the mixture heated for 10 min and cool rapidly. The color change was read at 630 nm in a spectrophotometer and the concentration of the total soluble sugar was calculated from the standard curve based on the concentration of glucose.

2.10 Enzyme assay

Staphylococcus spp. isolated from iru were cultivated in 20 ml each separately and together nutrient broth and incubated at 37°C in a shaking incubator (150 rpm) for 72 hours. Enzyme production and growth of culture were determined at the end of each cultivation period, the broth was centrifuged at 10,000 rpm at 4°C. The clear supernatant was collected as a source of enzymes. Proteinase (using the modified casein digestion method of Kunitz (1974)), amylase (assayed by the method of Bernfield (1955)) and lipase production (assayed by modifying the method of Yong and Wood (1977)) over the 72-hour cultivation period was determined through assay of enzymes activity.

2.11 Statistical analysis

The data obtained were subjected to analysis of variance (ANOVA) and, Duncan's multiple range tests were used to significantly differentiate the means of the sample treatments ($P \leq 0.05$).

3. Results and Discussion

3.1 Change in microbial count

Table 1 shows the growth pattern of the inocula, change in temperature and pH of the fermenting mash as fermentation progresses. There was an appreciable increase in the microbial count of all the fermenting mash, with that of *Staphylococcus* spp. fermented iru rose from an initial of 4.17 (log CFU/ml) to 7.38 after 24 hours of fermentation and 10.38 after 48 h, *Bacillus* spp. iru had an initial of 4.2 (log CFU/ml) that increased to 7.45 and 10.59 at 24 and 48 h of fermentation respectively; the microbial count for the iru produced with the two bacteria was 4.25 (log CFU/ml) at the start of fermentation, this increased to 7.50 and 10.74 at 24 h and 48 h respectively.

The increased microbial count is in agreement with the result of Odunfa and Komolafe (1989), but the increase in the number of *Staphylococcus* spp. till the end of the fermentation disagrees with that of Ogbadu and Okagbue (1988) who reported the absence of *Staphylococcus* in the 48 h fermentation of dawadawa, and that of Omafuvbe *et al.* (2000) who found out that *Staphylococcus* was only present at the early stage of fermentation, this may be because *Staphylococcus* spp. has the ability to utilize the nutrients found in African locust bean.

3.2 Change in temperature

There was an increase in the temperature of all the fermenting mashes from a start of around 23.6^0C to a final temperature of 28.9^0C for *Bacillus* spp. fermented beans and 29.3^0C for the *Staphylococcus* spp. fermented locust beans while the one fermented with the two species of bacteria has a final temperature of 29.5^0C (Table 1); this is in agreement with the report of Jonathan *et al*. (2011).

Table 1. Changes in the microbial load, temperature and pH during the production of iru with two different bacteria at different sampling hour

	Microbial Load (log CFU/ml)	Temperature (^{0}C)	pH
Control			
0 h	NG	-	-
24 h	NG	-	-
48 h	NG	-	-
Staphylococcal Iru			
0 h	4.17±0.01^a	23.6±0.43^a	6.46±0.03^a
24 h	7.38±0.02^b	26.8±0.36^b	7.59±0.01^b
48 h	10.38±0.05^c	29.3±0.98^c	7.70±0.04^c
Bacillus Iru			
0 h	4.20±0.01^a	23.7±0.43^a	6.45±0.03^a
24 h	7.45±0.02^b	26.8±0.36^b	7.56±0.01^b
48 h	10.59±0.05^c	28.9±0.98^c	7.65±0.04^c
Bacillus + *Staphylococcal* Iru			
0 h	4.25±0.01^a	23.5±0.43^a	6.46±0.03^a
24 h	7.50±0.02^b	26.6±0.36^b	7.55±0.01^b
48 h	10.74±0.05^c	29.5±0.98^c	7.84±0.04^c

NG= No Growth

Mean values followed by the same letter in the column are not significantly different by Duncan's Multiple Range test (P≤0.05)

3.3 Change in pH

The pH of the *Staphylococcus* spp. fermented locust beans increased from 6.46 at 0 h to 7.59 at 12 h and 24 h, then declined to 7.55 at 36 h, after which it rose again to 7.70 at 48 h of fermentation. The pH of the *Bacillus* spp. fermented beans increased from 6.45 at 0 h and 7.61 at 12 h of fermentation then declined to 7.56 and 7.55 at 24 and 36 h of fermentation respectively then increased to 7.65 after 48 h of fermentation. The pH of the locust beans fermented with the combination of the two species of organisms rose from 6.46 at 0 h to 7.62 at 24 h, then declined to 7.55 at 24 h, then rose to 7.57 and 7.84 at 36 h and 48 h of fermentation respectively (Table 1); this agrees with the results of Omafuvbe *et al*. (2002); the increase in pH may be due to the ability of the bacteria to carry out proteolysis, producing ammonia, which increases the pH.

3.4 Free amino acids

Table 2 shows the changes in the free amino acids and total soluble sugars in the fermented mash. The free amino acids of the unfermented mash increased from 0.74 to 1.10 in the *Staphylococcus* spp. fermented iru, 1.51 in the *Bacillus* spp. fermented iru and 2.35 in the iru fermented with the two bacterial species after fermentation. There is a significant difference between the free amino acids of the iru fermented with both species of bacteria and the one fermented with *Staphylococcus* spp. but not between *Staphylococcus* spp. fermented iru and the one fermented with *Bacillus* spp. There was no significant difference between the free amino acids of the *Bacillus* spp. fermented iru and the one fermented with the two species of bacteria.

Table 2. Change in free amino acids and total soluble sugars of iru produced with two different bacteria

Organism	Free Amino Acids (mg leucine ml^{-1})	Total Soluble Sugars (mg glucose ml^{-1})
Staphylococcal iru	1.10±0.26^a	3.84±0.46^{ab}
Bacillus iru	1.51±0.26^{ab}	3.60±0.46^{ab}
Bacillus + *Staphylococcal* iru	2.35±0.26^b	2.83±0.46^a
Unfermented Bean	0.74±0.26^a	5.30±0.46^b

Values are Means Scores ± Standard Error. Mean values followed by the same superscript in the column are not significantly different by Duncan's Multiple Range test (P≤0.05)

The increase in free amino acids in the *Staphylococcus* spp. fermented iru, *Bacillus* spp. fermented iru and the iru fermented with both species is in agreement with that of Odunfa and Adewuyi (1985b) who reported the highest value of 1.6 mg/ml of extract in amino acid content at the 36 h of fermentation; Odunfa and Adewuyi (1985a) who reported an efficient hydrolysis of locust bean protein to amino acids between 1.98 to 2.6 mg/ml and that of Omafuvbe *et al.* (2002), who reported a continuous increase in the free amino acids contents; it also agrees with what Anihouvi *et al.* (2012), reported in the controlled *Lanhouin*.

The relationship shown between the free amino acids of the *Staphylococcal* iru and *Bacillus* iru may be due to the ability of both bacterial species to hydrolyse the African locust bean protein to produce amino acids, but the significant differences shown by the iru produced by the two bacterial species shows that the two bacterial species in combination have the ability to improve the number of free amino acids produced during the production of iru.

3.5 Total soluble sugars

The total soluble sugars of the unfermented beans of 5.30 decreased to 3.84 when fermented with *Staphylococcus* spp. and 3.60 when fermented with *Bacillus* spp. but it was 2.83 when fermented with the two species of bacteria (Table 2). There was no significant difference between the total soluble sugar of the iru fermented with *Bacillus* spp., *Staphylococcus* spp. and the unfermented beans; but there was a significant difference between the iru fermented with both species of bacteria and the unfermented beans. There was no significant difference between the total soluble sugar of the iru fermented with *Bacillus* spp., *Staphylococcus* spp. and the iru fermented with the two species of bacteria. This result agrees with that of Omafuvbe *et al.* (2004), who reported a substantial decrease in the total soluble sugar during the fermentation of melon, also with that of Omafuvbe (2006). The decrease in total soluble sugars may be due to the utilization of the sugars by the bacteria for their growth.

3.6 Moisture content

The moisture content of the products at different hours of fermentation is shown in Table 3, no significant difference was observed in the percentage moisture content of the iru fermented with *Bacillus* spp., *Staphylococcus* spp. and the iru fermented with both *Staphylococcus* spp. and *Bacillus* spp. in combination; but there was a significant difference in their percentage moisture content of iru for each different product at different hours of fermentation as the moisture content of the iru fermented with both *Staphylococcus* spp. increased from an initial of 64.73 to 65.38 at 24 h and 67.43 at 48 h of fermentation, while the moisture content of the iru fermented with *Bacillus* spp. increased from 64.71 at 0 h to 65.76 at 24 h and 67.75 at48 h. The moisture content of the iru fermented with both *Staphylococcus* spp. and *Bacillus* spp. in combination rose from an initial of 64.71 to 65.86 and 67.98 at 24 h and 48 h of fermentation respectively.

The increase observed in the moisture content of African locust beans during fermentation is in line with the report of Ogunjobi *et al*. (2005) and Jonathan *et al.* (2011), who reported an increase in the moisture content of substrates fermentation.

The ability of the *Staphylococcus* spp. isolated from iru to carry out amylolytic, lipolytic and proteolytic activities (Figure 1) was in agreements with the report of Mossel (1982), that *Staphylococcus* spp. isolated from foods produced extracellular enzymes of which lipase is a major; this is however in contrast with the report of Aderibigbe and Odunfa (1990), who reported that no extracellular lipolytic activity was detected for some *Bacillus* spp. isolated from iru, when grown in nutrient broth. *Staphylococcus epidermidis* and the mixed culture of the three *Staphylococcus* spp. showed high lipolytic and proteolytic activities, this is in agreement with the work of Anihouvi *et al.* (2012), who reported that *S. xylosus* could play a significant role in aroma development by the production of ammonia.

Table 3. Change in the moisture content of iru produced by different inocula as fermentation progresses

Product	Hour of fermentation	Moisture Content (%)
Staphylococcus Iru	0h	64.73[a]
	24h	65.38[b]
	48h	67.43[c]
Bacillus Iru	0h	64.71[a]
	24h	65.76[b]
	48h	67.75[c]
Bacillus + *Staphylococcus Iru*	0h	64.71[a]
	24h	65.86[b]
	48h	67.98[c]

Mean values followed by the same superscript are not significantly different by Duncan's Multiple Range test ($P \leq 0.05$)

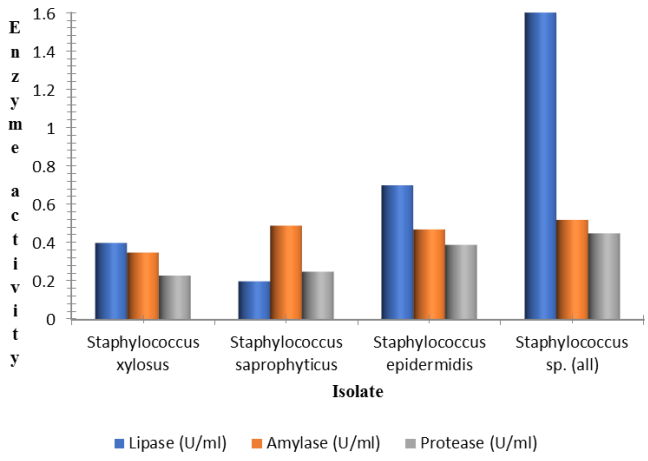

Figure 1. Enzyme assay for three *Staphylococcus* spp. Isolated from iru.

Conclusion

The increase in pH during the fermentation of iru produced with *Staphylococcus* spp. and the increase in free amino acids proved that *Staphylococcus* spp. isolated from iru has the ability to ferment African locust

bean, and thus play a role in the breaking down of proteins in African locust bean to amino acids; but the significant differences in the free amino acids and total soluble sugars observed in the iru produced with single bacterial species compared with that of the iru produced with the two bacterial species shows that they do better together than in singleton. Howbeit, the ability of the three *Staphylococcus* spp. isolated from iru to produce extracellular enzymes (Lipase, Protease, and Amylase) in a relatively high quantity provides a reason for the better products when *Staphylococcus* spp. are involved in the fermentation than when only *Bacillus* spp. is used.

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