# Evaluation of Spontaneously Fermenting Soybean Daddawa Microbiota's Potential for Starter Culture Application

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***Abstract:*** **- The traditional aroma of soybean daddawa has not been perfectly replicated through controlled fermentation which made use of only *Bacillus* species. The present study therefore focused on selection of suitable starter cultures for the controlled fermentation of soybean daddawa with primary focus on using *Bacillus* species, heterofermentative Lactic acid bacteria (LAB) and *Staphylococcus* strains. The effect of mono and mixed starter cultures on the sensorial qualities of the produced soybean daddawa was also evaluated. A total of sixty-six bacteria strains isolated from spontaneously fermented soybean daddawa obtained from four sites were evaluated for their amylolytic, proteolytic, heterofermentation and coagulase activities. Based on the result of the screening exercise *Bacillus subtilis* LB3, *Staphylococcus xylosus* SAU3 and *Leuconostoc mesenteroides* ssp *cremoris* LAB5 were selected for controlled fermentation studies. The result of the antagonistic test between the selected microorganisms showed that the microorganisms were not antagonistic to one another. The enzyme profiles of the selected organisms indicated wide arrays of eleven enzymes. Prominent among these werealkaline and acid phosphatase, leucine arylamidase, trypsin, Naphthol-AS-B1-phosphohydrolase, valine arylamidase, α-chymotrypsin and α-glucosidase and β-galactosidase activities. The overall acceptability of soybean daddawa produced using the three starter bacteria was rated next best to naturally fermented soybean daddawa. This holds a great promise for subsequent industrialization of soybean daddawa production as natural fermentation of soybean daddawa is now nearly replicated in a controlled setting.**

***Keywords*****: *Bacillus subtilis*, condiment, *Leuconostoc mesenteroides* ssp *cremoris*, optimization, *Staphylococcus xylosus*, Sensory attributes and Soybean daddawa,**

## I. INTRODUCTION

The diets of people in many developing countries comprise mainly starchy materials. Unfortunately, animal sources of proteins, which are used to compliment the starchy diets are expensive and out of reach for low-income families (Obatolu et al. 2007). Traditional diets in West Africa often lack variety and consist of large quantities of staple food (such as cassava, yam, maize). Soups eaten with the staples are an essential component of the diet and may contain a variety of seeds, nuts, pulses and leaves (Campbell-Platt, 1980). The staple foods provide the calories but are poor in other nutrients. Soups are the main sources of proteins and minerals and one of the ways to improve the diet have been to improve the nutrient content of soups (Achi, 2005a). Similarly, Adedeji et al., (2017) submitted that the protein requirements of most poor households in rural population across Africa and Asia are often met by traditional fermented foods of legumes and oilseeds origin.

Different types of West African *Bacillus* fermented legume products include *Dawadawa/Daddawa* and *Ogiri* (Adedeji et al., 2017), Soybean daddawa (Popoola and Akueshi, 1985, Omafuvbe et al., 2000; Kolapo et al., 2007 a,b) and *Ugba* (Okorie and Olasupo (2013). In addition to *Bacillus*, *Micrococcus* spp., *Staphylococcus* spp, *Leuconostoc mesenteroides* and *L. dextranicus* have been reported to be involved in this alkaline fermentation (Antai and Ibrahim ,1986; Ogbadu and Okagbue 1988; Dakwa et. al. 2005; Edema and Fawole, 2006 ).

The fermentation process for condiment production is still being carried out by the traditional village-art method. There is need to apply modern biotechnological techniques like the use of starter cultures in improving traditional food processing technologies (Achi, 2005b). It has been suggested that even though hitherto most fermentation processes used in developing countries do not use innocula or extrinsic cultures, these processes could be improved on by using starter cultures, and also by backslopping, which entails application of brine from previous fermentation cycles (Holzapfel, 2002; Niba, 2003).

Starter cultures have been found to reduce fermentation time as well as guarantee good product quality, and for this purpose controlled fermentation of soybeans was achieved by using pure single cultures of *Bacillus subtilis*, *B. licheniformis* or in combinations (Sarkar et al. 1993; Suberu and Akinyanju, 1996). Mixed cultures of *Bacillus subtilis*, and *B. licheniformis* were highly recommended by the same authors and fermentation was achieved in 72h. Omafuvbe et

al.(2002) on the other hand, tested three *Bacillus* species namely *B. subtilis*, *B. licheniformis* and *B. pumilus* singly and in combination for their ability to ferment soybean for the production of daddawa. *B. subtilis* as single or member of a mixed starter produced soybean daddawa, which was considered most suitable as it gave acceptable pure culture condiment supposedly due to its proteolytic enzyme activity as shown by high level of free amino acid in the fermenting substrates. A common denominator to all the previous daddawa optimization attempts is the restrictive use of *Bacillus* species. It is not until very lately that inclusion of lactic acid bacteria is being considered (Afolabi and Abdulkadir, 2016). However, it is important to note that despite the fact that controlled fermentation achieved good quality end product, the traditional aroma of soybean daddawa has not been perfectly replicated through controlled fermentation which made use of only *Bacillus* species.

Achi,( 2005a) opined that the use of a single strain would seem too restrictive for the production of a foodstuff with generous range of organoleptic characteristics. Rather, the use of mixture of microorganisms with complimentary physiological and metabolic properties seems to be the best approach for obtaining a product with the nutritional and sensory properties desired. This becomes more plausible on the understanding that fermented condiments have characteristic organoleptic properties that determine its quality and which probably are the most important factors for consumers. There is therefore a need for further research on the possibility of using mixed cultures of *Bacillus* species, Lactic acid bacteria (LAB) and other members of Staphylococcaceae to effect controlled fermentation of soybean daddawa and subsequently evaluate the effect of such mixed cultures on the sensorial qualities of the ensued soybean daddawa. The present study therefore focused on selection of suitable starter cultures for the controlled fermentation of soybean daddawa with primary focus on using *Bacillus* species, heterofermentative Lactic acid bacteria (LAB) and *Staphylococcus* strains. The effect of mono and mixed starter cultures on the sensorial qualities of the produced soybean daddawa was also evaluated.

## II. MATERIALS AND METHODS

### *A. Laboratory Preparation of Soybean Daddawa and Sample Collection from Field*

Preparation of soybean daddawa by natural fermentation in the laboratory was done following the traditional method described by Popoola and Akueshi (1985). Approximately 800g of soybean seeds were sorted, washed thoroughly and soaked in tap water for 12h. The soaked beans were dehusked, boiled in excess water for 2h, allowed to cool down and transferred to calabash lined with banana (*Musa sapientum*) leaves and covered with more leaves. The whole set up was left on the bench at ambient temperature ($31\pm1^0$C) to ferment naturally for 72h. Samples were also collected aseptically from three sites of traditional production that were using similar procedure. They were transported to the laboratory in an iced bag.

### *B. Isolation and Identification of Spontaneously Fermenting Soybean Daddawa Microbiota*

Replicate portions of ten-fold dilutions of samples in sterile peptone water were made for all the samples collected at 36 and 72h of fermentation which correspond to the peak and end of fermentation process. The preparations were homogenized using a stomacher and 1ml each of appropriate dilution plated using the pour plate method (Harrigan and McCance, 1976) on Nutrient Agar and MRS agar.

Nutrient agar and MRS agar plates were incubated at $35^0$C and $33^0$C respectively for 48h. Colonies were grouped using their cultural and morphological features. Representative colonies from the incubated plates were purified by repeated streaking. In order to determine the genera of the different isolated microorganisms, preliminary characterization of the isolates was carried out by Gram staining , spore staining, catalase test, Voges-Proskauer test, Indole test and Oxygen relationship test as described by Skerman (1967). Also, growth in 7% NaCl, sugar utilization, nitrate reduction, casein and starch hydrolytic potentials of the different strains were investigated.

The actual identity of the various isolates was determined using API fermentation galleries (Bio Merieux, France). Different strains of *Bacillus, Staphylococcus, Lactobacillus* and *Leuconostoc* were identified by assaying isolated cultures in API fermentation galleries. *Bacillus* strains were assayed on API 50 CHB galleries.Strains of *Staphylococcus* were assayed on API Staph galleries. *Lactobacillus and Leuconostoc* strains were assayed on API 50 CHL galleries using the manufacturer's protocol. Different strains of *Bacillus, Staphylococcus, Lactobacillus and Leuconostoc* were maintained on appropriate agar slants and stored in the refrigerator ($6\pm2^0$C). The technological characteristics of the different isolates were evaluated to determine their potentials for use in control fermentation of soybean daddawa.

### *C. Evaluation of Potential of Isolated Cultures for Use as Starter Culture*

To obtain strains of bacteria with great potentials in effecting controlled fermentation of soybean daddawa, the amylolytic and proteolytic activities of the different strains were evaluated. Lactic acid bacteria were assessed for heterofermentation potential. The *Staphylococcus* strains were further assayed for coagulase activity. The possible antagonistic activity between the selected cultures was also carried out.

#### *I. Evaluation of Proteolytic Activity of Bacterial Strains*

Nutrient Agar (NA) containing 2% casein was prepared, autoclaved at $121^0$C for 20 min and distributed in Petri dishes. After solidification, a 8-mm deep well was made in the middle of the agar. For each isolate, 100µl of inoculum was transferred to a well and the Petri dishes were incubated at

$35^0$C. The diameter (mm) of the clear zone was measured at 48 h. The experiment was conducted in triplicates. (Ouoba et.al. 2003)

*II. Evaluation of Amylolytic Activity of Bacterial Strains*

Starch agar containing g/l distilled;10, starch and 23, nutrient agar was prepared, autoclaved at $121^0$C for 20 min and distributed in Petri dishes. After solidification a 8-mm deep well was made in the middle of the agar. For each isolate, 100µl of inoculum was transferred to a well and the Petri dishes were incubated at $35^0$C for 48 h. After incubation the agar plates were flooded with iodine solution for observance of amylolytic activities. The diameter (mm) of the clear zone was measured. The experiment was conducted in triplicates (Ouoba et.al. 2003).

*III. Evaluation of Coagulase Activity of Staphylococcus Strains*

Coagulase positive *Staphylococcus* strains are undesirable in food fermentation because of their pathogenicity, hence the necessity for this assay. Active cultures (24 h) of all the *Staphylococcus* isolates were suspended in fresh human serum in separate test tubes. Formation of precipitate of blood protein within 3-5 min indicated a positive test.

*IV. Evaluation of Heterofermentative Potentials of Lactic Acid Bacteria (LAB) Strains*

Hetero-fermenters are known to produce more of other volatiles in addition to lactic acid when compared with the homofermenters. Their use in production of aroma volatiles in food is highly desrable. MRS broth was prepared, autoclaved at $121^0$C and cooled down to about $30^0$C. LAB strain was inoculated into the cooled tube. Molten agar was then poured along the side of the tube in such a way that the top of the broth was sealed up as the agar gel was formed. The set-up was incubated at $33^0$C for 48 h. Formation of air space which pushed up the agar gel indicates an heterofermenter LAB.

*V. Evaluation of Antagonistic Activity of Bacterial Strains Selected for Optimisation Studies.*

NA plates of *Staphylococcus* strain were prepared. Also, MRS plates of the LAB strains were prepared. After solidification a 8-mm deep well was made in the middle of the agar. The growth of the organisms was challenged by adding 100 µl of the *Bacillus* inoculum to a well and incubated at $37^0$C (NA) and $35^0$C (MRS) plates for 72 h. A clear zone around a well indicates antagonistic activity.

*D. Determination of the Enzyme Profile of Microorganisms Selected for Controlled Fermentation of Soybean Daddawa.*

The enzyme profile of the microorganisms selected for controlled fermentation of soybean daddawa was assayed in API ZYM (Bio Merieux, France) galleries. The galleries test for 19 different enzymes. Respective bacterial strains were grown on appropriate agar medium at $37^o$C for 18 h. Sterile swab was used to harvest microbial growth and was suspended in sterile physiological saline and its turbidity adjusted to 2 Mc farland.

The API strips were placed in the incubation trays into which 5 ml of distilled water had been distributed into the honey comb to maintain moist conditions and the cupules of the strips containing the dehydrated substrate inoculated with 65 µl of microbial suspension using a sterile pipette. The boxes were incubated at $37^o$C for 5 h. After incubation 1 drop of each of ZYM A and ZYM B reagents was added to each cupule. The colour change and its intensity within 8-10 min were compared with the standard stated in the reading table to determine the enzyme profile of the test organisms.

*E. Preparation of Soybean Daddawa by Controlled and Natural Fermentation*

Preparation of soy daddawa by Natural fermentation was done following the traditional method of Popoola and Akueshi (1985) as earlier described in. In another approach, seven batches of fermented product were produced in a controlled setting replacing the rudimentary equipment used in the traditional method with glassware as described by Omafuvbe *et al*. (2002). Starter cultures selected on the basis of their previously described technological characteristics (*Bacillus. subtilis*, *Staphylococcus xylosus* and *Leuconostoc mesenteroides ssp cremoris*) were introduced into the fermentation medium at the onset of fermentation. The starter cultures were used as a monoculture, double cultures and multiple cultures resulting into seven treatments of controlled fermentation

Preparation and inoculation of cultures was as described by Omafuvbe *et al* (2002). *Bacillussubtilis* and *Staphylococcus xylosus* were grown in Nutrient agar (Oxoids, UK) at $35^0$C for 18h while *Leuconostoc mesenteroides ssp cremoris* was grown on MRS agar at $33^0$C for 24 h. Each of the culture was suspended into 10ml sterile 0.9% NaCl solution and diluted to give an absorbance of 0.03 at 540nm in a spectrophotometer. The suspensions of each culture were mixed equally and 0.5ml of the final mixture was inoculated into 50g sterile cooked beans held in 250ml conical flask. The inoculated beans were incubated at $35^0$C for 65 h. The eighth treatment was produced using natural/spontaneous fermentation as described earlier.

*F. Sensory Evaluation*

All the eight batches of Soybean daddawa samples produced through both natural and controlled fermentation procedures were subjected to organoleptic evaluation. Attributes evaluated include aroma, colour, texture and over all acceptability. The samples were assessed by a

panel of 50 regular consumers of soybean daddawa using a score range of 1 (dislike extremely) to 9 (like extremely). The data obtained were subjected to analysis of variance (ANOVA) and the Duncan's multiple range test was used to separate the means.

## III. RESULTS

### A. *Biochemical and Morphological Characteristics of Different Strains of Bacillus, Staphylococcus and LAB Isolated from Naturally Fermenting Soybean Daddawa.*

A total of sixty six bacterial strains were obtained. Out of these, fifty two were Gram-positive, catalase-positive, spore bearing rods and aerobic. They were assumed to belong to the genus *Bacillus*. Further test showed that majority of these isolates produced acid from D-Glucose, D-Mannitol, D-xylose, hydrolysed casein and starch, reduced nitrate, grew in 7% NaCl, Voges-Proskauer positive and Indole negative. In addition to the above characteristics, their colonies had irregular margins and rough ridged surfaces.

Six isolates presumed to be *Staphylococcus* strains were Gram-positive, catalase-negative cocci. The cocci cells were arranged in clusters. They produced acid from D-Glucose, D-Mannitol, D-xylose, hydrolysed casein and starch, reduced nitrate, grew in 7% NaCl, Voges-Proskauer positive and Indole negative. LAB isolated on MRS agar were non-spore bearing, Gram-positive, catalase-negative rods and coccoid. Some of the isolated LAB were facultatively heterofermentative which were able to produce $CO_2$ from glucose. The true identity of the different presumed strains of *Bacillus, Staphylococcus* and LAB were determined on API fermentation galleries.

In the API galleries, the *Bacillus subtilis* cultures generally fermented glycerol, L-arabinose, ribose, D-xylose, D-glucose, D-fructose, D-mannose, D-galactose, inositol, sorbitol, mannitol, α-methyl-D-glucoside, N-acetyl glucosamine, amygdalin, arbutin, esculin, salicin, cellobiose, maltose, melibiose, saccharose, trehalose, inuline, D-raffinose, amidon, glycogen. In addition to these reactions, *Bacillus licheniformis* fermented methyl-β-D-xylopyraoside, sorbose and reduce potassium gluconate while *Bacillus coagulans* only reduced potassium gluconate in addition to reactions exhibited by *Bacillus subtilis* cultures.

*Staphylococcus xylosus* and *S. sciuri* cultures in API galleries fermented D-glucose, D-fructose, D-mannose, D-maltose, trehalose, mannitol, saccharose, N-acetyl glucosamine, β-naphthyl phosphate and reduced potassium nitrate. The additional reactions of *Staphylococcus xylosus* include fermentation of D-raffinose, glucopyranoside and urea while *S. sciuri* only fermented D-lactose in addition . Some strains of isolated LAB which fermented D-glucose, D-fructose, D-mannose, N-acetyl glucosamine, arbutine, esculine, salicine, D-saccharose and D-trehalose were identified as *Lactococcus lactis* ssp *hordiniae.* LAB strains which only fermented D-galactose, D-glucose, N-acetyl glucosamine, D-lactose and D-saccharose were identified as *Leuconostoc mesenteroides* ssp *cremoris*.

### B. *Technological characteristics of Isolated Cultures* .

The agar diffusion test showed that all the isolates produced a clear zone around the wells of casein agar plates. The largest clear zone produced by *Bacillus* culture was 44 mm while the least was 18 mm. The largest clear zone produced by *Staphylococcus* culture was 28 mm while the least was 10 mm. Generally speaking, the clear zones produced by *Bacillus* are larger than those produced by *Staphylococcus* (Table1).

The agar diffusion test showed that all the isolates produced a clear zone around the wells of starch agar plates. The largest clear zone produced by *Bacillus* culture was 32 mm while the least was 18 mm. The largest clear zone produced by *Staphylococcus* culture was 27 mm while the least was 13 mm (Table 2). Among the *Bacillus* and *Staphylococcus* strains, LB3 and SAU3 were respectively found to have demonstrated consistently highest proteolytic and amylolytic activities. They were identified as *Bacillus subtilis* and *Staphylococcus xylosus* strains respectively.

Out of the six isolated *Staphylococcus* starins, only strains SNS4 which was identified as *Staphylococcus sciuri* was coagulase positive while others were coagulase negative. Of all the LAB strains it was only strain LAB5 which was identified as *Leuconostoc mesenteroides* ssp *cremoris* that demonstrated heterofermentation potential. Arising from the screening activities, *Bacillus subtilis* LB3, *Staphylococcus xylosus* SAU3 and *Leuconostoc mesenteroides* ssp *cremoris* LAB5 were selected for controlled fermentation studies. The result of the antagonistic test between the organisms selected for controlled fermentation studies showed that the microorganisms were not antagonistic to one another, as there was no clear zone on all the plates.

Table 1

Zones of inhibition (mm) during the degradation of casein in nutrien agar for 48 h at $35^0$C by strains of *Bacillus* and *Staphylococcus*isolated from fermenting soybean daddawa

| *Bacillus* | | *Staphylococcus* | |
|---|---|---|---|
| Strain | | Strain | |
| LB1 | $20^{e}$ | SS1 | $18^{b}$ |
| LB2 | $20^{e}$ | SAU3 | $28^{a}$ |
| LB3 | $44^{a}$ | SNS4 | $12^{c}$ |
| LB4 | $32^{c}$ | SNS8 | $10^{c}$ |
| LB5 | $24^{de}$ | | |
| LB6 | $39^{b}$ | | |
| LB7 | $18^{f}$ | | |
| LB8 | $27^{d}$ | | |
| SS2 | $26^{d}$ | | |
| SS3 | $21^{e}$ | | |
| SS5 | $21^{e}$ | | |
| SS6 | $30^{c}$ | | |
| SNS5 | $17^{f}$ | | |

Values are means of triplicate determinations. Along column, values with different superscripts are significantly different ($p < .05$)

Table 2

Zones of inhibition (mm) during the degradation of starch in nutrient agar for 48 h at $35^0$C by strains of *Bacillus* and *Staphylococcus*isolated from fermenting soybean daddawa

| | *Bacillus* | | *Staphylococcus* |
|---|---|---|---|
| Strain | | Strain | |
| LB1 | $23^b$ | SS1 | $22^b$ |
| LB2 | $22^b$ | SAU3 | $27^a$ |
| LB3 | $30^a$ | SNS4 | $14^c$ |
| LB4 | $29^a$ | SNS8 | $13^c$ |
| LB5 | $32^a$ | | |
| LB6 | $20^c$ | | |
| LB7 | $19^d$ | | |
| LB8 | $20^c$ | | |
| SS2 | $21^c$ | | |
| SS3 | $27^{ab}$ | | |
| SS5 | $32^a$ | | |
| SS6 | $18^d$ | | |
| SNS5 | $23^b$ | | |

Values are means of triplicate determinations. Along column, values in the same column with different superscripts are significantly different ($p < .05$)

The enzyme profile of the three selected organisms is shown in Table 3. *Leuconostoc mesenteroides* ssp *cremoris* LAB5 had the highest alkaline phosphatase and leucine arylamidase activities. Esterase and esterase lipase activities were least in all the three cultures. Only *Bacillus subtilis* LB3 and *Staphylococcus xylosus* SAU3 had trypsin activity with the latter having a higher activity. Among the three organisms, acid phosphatase activity of *Bacillus subtilis* LB3 was the highest. The Naphthol-AS-B1-phosphohydrolase activities of *Bacillus subtilis* LB3 and *Leuconostoc mesenteroides* ssp *cremoris* LAB5 were moderately higher than that exhibited by *Staphylococcus xylosus* SAU3. It was only *Bacillus subtilis* LB3 that had valine arylamidase, α-chymotrypsin and α-glucosidase activities while only *Staphylococcus xylosus* SAU3 exhibited β-galactosidase activities. The wide arrays of enzyme spectrum of the three selected organisms will be an added advantage in the generation of wide arrays of metabolic end-products.

Table 3

Enzyme profiles* of strains of microorganisms used for optimization studies of soybean daddawa production

| | Strain | | |
|---|---|---|---|
| Enzymes Assayed For | LB3 | SAU3 | LAB 5 |
| Alkaline phosphatase | 4 | 2 | 5 |
| Esterase(C4) | 1 | 1 | 1 |
| Esterase lipase(C8) | 1 | 1 | 1 |
| Lipase(C14) | 0 | 0 | 0 |
| Leucine arylamidase | 4 | 3 | 5 |
| Valine arylamidase | 1 | 0 | 0 |
| Cystine arylamidase | 0 | 0 | 0 |
| Trypsin, | 2 | 4 | 0 |
| α-chymotrypsin | 2 | 0 | 0 |
| Acid phosphatase | 4 | 3 | 3 |
| Naphthol-AS-BI-phosphohydrolase | 3 | 1 | 3 |
| α-galactosidase | 0 | 0 | 0 |
| β-galactosidase | 0 | 2 | 0 |
| β-glucuronidase | 0 | 0 | 0 |
| α-glucosidase | 2 | 0 | 0 |
| β-glucosidase | 0 | 0 | 0 |
| N-acetyl- β –glucosaminidase | 0 | 0 | 0 |
| α-mannosidase | 0 | 0 | 0 |
| α-fucosidase | 0 | 0 | 0 |

*0=Negative reaction; 1= Least Activity; 5=Maximum activity

LB3= *Bacillus subtilis;* SAU3= *Staphylococcus xylosus* LAB5 = *Leuconostoc mesenteroides* ssp *cremoris*

### *C. Sensory evaluation of Soybean Daddawa Produced by Starter Cultures and Natural Fermentation.*

The sensory evaluation of soybean daddawa produced by mono and mixed starter cultures as well as natural fermentation is presented in Table 4. There was significant difference ($p<0.05$) in all the attributes (except stickiness) scored for soybean daddawa produced with either mono, mixed or natural fermentation. *Bacillus subtilis* fermented soybean daddawa was rated best in term of colour of the soybean daddawa. The colour of all soybean daddawa samples produced with the use of starter cultures (both mono and mixed fermentation) were more acceptable to the respondents than the one obtained through natural fermentation. In monoculture fermentation, the flavour of *Bacillus subtilis* fermented soybean daddawa was rated best while *Staphylococcus xylosus* fermented soybean daddawa the least. However, Duncan grouping placed the products of the three monoculture fermentation and mixed culture fermentation of *Staphylococcus xylosus* and *Leuconostoc mesenteroides ssp cremoris* in the same group. The flavour of other three products from mixed fermentation was rated to be more acceptable than those from monoculture fermentations. Among all samples obtained from controlled fermentation, sample produced using the combination of the three starter bacteria procduced flavor that was rated next best to the naturally fermented soybean daddawa. Among the products of controlled fermentation, the texture of *Bacillus subtilis* fermented soybean daddawa was rated best and *Leuconostoc mesenteroides ssp cremoris* fermented soybean daddawa the least. The texture of naturally produced soybean daddawa was the most acceptable. There was no significant difference ($p>0.05$) between the over all acceptability of soybean daddawa obtained through mono and two cultures fermentation, except *Leuconostoc mesenteroides ssp cremoris*

fermented soybean daddawa which was rated lowest. The overall acceptability of soybean daddawa produced using the three starter bacteria was rated next best to naturally fermented soybean daddawa.

Table 4
Sensory Qualities of Soybean Daddawa Fermented with StarterCultures obtained from Spontaneously fermented Soybean Daddawa

| | Organoleptic attributes | | | | |
|---|---|---|---|---|---|
| Fermented Product | Colour | Stickiness | Flavour | Texture | General acceptability |
| A | $7.00^{a}$ | $5.75^{a}$ | $5.17^{b}$ | $5.92^{ab}$ | $5.50^{bc}$ |
| B | $6.42^{ab}$ | $6.0^{a}$ | $4.67^{b}$ | $5.42^{b}$ | $5.83^{bc}$ |
| C | $6.17^{ab}$ | $5.58^{a}$ | $4.75^{b}$ | $5.00^{b}$ | $5.17^{c}$ |
| D | $5.83^{ab}$ | $5.58^{a}$ | $5.33^{ab}$ | $5.58^{b}$ | $5.58^{bc}$ |
| E | $6.67^{a}$ | $5.92^{a}$ | $5.33^{ab}$ | $5.83^{ab}$ | $5.42^{bc}$ |
| F | $6.00^{ab}$ | $6.25^{a}$ | $5.00^{b}$ | $5.42^{b}$ | $5.83^{bc}$ |
| G | $6.42^{ab}$ | $5.92^{a}$ | $5.67^{ab}$ | $5.75^{b}$ | $6.67^{ab}$ |
| H | $5.17^{b}$ | $6.42^{a}$ | 6.67a | $6.92^{a}$ | $7.17^{a}$ |

Values are means scores (n=50)Along column, values with different superscripts are significantly different ($p < .05$)

A-Fermentation with *Bacillus subtilis*; B-Fermentation with *Staphylococcus xylosus*

C-Fermentation with *Leuconostoc mesenteroides ssp cremoris*

D-Fermentation with *Bacillus subtilis* and *Staphylococcus xylosus*

E-Fermentation with *Bacillus subtilis* and *Leuconostoc mesenteroides ssp cremoris*

F-Fermentation with *Staphylococcus xylosus* and *Leuconostoc mesenteroides ssp cremoris*

G-Fermentation with *Bacillus subtilis +Staphylococcus xylosus + Leuconostoc mesenteroides ssp cremoris*

H-Natural fermentation

## IV. DISCUSSION

In the previously reported studies, *Bacillus subtilis, B. licheniformis and B. pumilus* had been implicated to be playing a great role in soybean daddawa fermentation (Popoola and Akueshi, 1985; Omafuvbe et al., 2000, 2002; Sarka et al., 1993). In addition to these three organisms, Dakwa et al. (2005) reported the involvement of other *Bacillus* speciessuch as *B. cereus* and *B. firmus.Bacillus* spp. manly *Bacillus subtilis* have also been reported to be responsible for the traditional alkaline fermentation of several legumes and seeds in West Africa including the African locust bean seeds (Campbell-Platt, 1980; Antai and Ibrahim, 1986), melon seeds, *Citrullus vulgaris* Schrad (Odunfa, 1981), castor oil seeds, *Ricinus communis* (Odunfa, 1985), cotton seeds (Sanni and Ogbonna, 1990), sand African oil bean seeds, *Pentaclethra macrophylla* Bentham(Obeta, 1983; Njoku et al. 1990).

In addition to *Bacillus* spp., Popoola and Akueshi (1985), Omafuvbe et al. (2000), Edema and Fawole (2006) have equally reported that various species of *Staphylococcus* are also involved in both locust bean and soybean daddawa fermentation. Antai and Ibrahim (1986) report's on the isolation of two species of lactic acid bacteria, *Leuconostoc mesenteroides* and *L. dextranicus* in almost equal proportion with the *Bacillus spp* from naturally fermenting locust bean daddawa was dubbed an unusual finding. However, subsequent findings have suggested the involvement of lactic acid bacteria in both soybean and locustbean daddawa fermentation (Dakwa et al., 2005; Edema and Fawole, 2006).

In a similar development, report on the alkaline fermentation of seeds of *Carthormion altissimum* also implicated species of *Bacillus* and *Staphylococcus* as the predominant fermenting organisms (Popoola et al., 2006). In that study, the involvement of these organisms was attributed to their proteolytic activity. Because of the uncertainty surrounding the source of organisms involved in the solid state fermentation of African leguminous and oil seeds into condiments, the fermentation at various times have been attributed to chance or described as spontaneous (Popoola et al., 2006). Considering the pre- fermentation treatment such as prolonged boiling of the dehulled soybean seeds, the organisms that mediated soybean daddawa fermentation may have come from air, plantain leaves and perhaps from human contact at the onset of fermentation.

In the present study, isolation of *Bacillus subtilis* from naturally fermenting soybean daddawa and its successful subsequent use in both monoculture and mixed culture fermentation of soybean daddawa is in agreement with earlier *Bacillus*-fermented soybean studies. The isolation of *Staphylococcusxylosus* and *Leuconostoc mesenteroides* ssp *cremoris* and their subsequent use in both monoculture and mixed culture fermentation of soybean daddawa in the present report, is introducing a new dimension to soybean fermentation. Suffice to say that the involvement of *Staphylococcus* and lactic acid bacteria in daddawa fermentation is unpopular as very few studies have investigated their possible use as starter cultures in daddawa fermentation. However, the results of the present work have evidently established that these two groups of organisms are also potential candidate microorganisms to be considered alongside with *Bacillus subtilis* in the optimization attempt of soybean daddawa production.

In the present study, pronounced interspecies differences in the proteolytic activities against casein were demonstrated by various species of *Bacillus* and *Staphylococcus*. The trend observed for amylolytic activity against starch was also similar to that observed for proteolytic activity. All the microorganisms screened for both proteolytic and amylolytic activities had clearing zones around their colonies. *Bacillus* isolates exhibited largest clearing zones on

both casein and starch agar when compared to those exhibited by *Staphylococcus* isolates. The ability of some *Bacillus subtilis* to degrade casein is well known (Vuillemard et al., 1985; Szczesna and Galas, 2000). The observed relatively higher proteolytic and amylolytic activities of *Bacillus* isolates in the present study may have been responsible for its popular use in many previous optimization attempts (Sarkar et al., 1993; Suberu and Akinyanju, 1996; Owens et al., 1997; Omafuvbe et al., 2002). However, it is also noticeable that *Staphylococcus xylosus* SAU3 exhibited higher proteolytic and amylolytic activities than some *Bacillussubtilis* strains LB5 and SS5. This is a possible indication of the former's ability to undertake biotransformation that is similar to that mediated by *B.subtilis.*

Of all *Staphylococcus* isolates obtained from naturally fermenting soybean daddawa, only one strain (*S. sciuri*) produced coagulase enzyme. However, this is of public health concern. The occurrence of coagulase positive *Staphylococcus* in spontaneously fermenting daddawa underscores the need for a careful selection of starter cultures for the production of indigenous food which has hitherto been produced through natural process. Earlier, Holzapel (2002) had opined that the potential attributes of the use of starter cultures include increased toxicological safety.

The isolation of both homo and heterofermenter lactic acid bacteria (LAB) (*Lactococcus hordinae* and *Leuconostoc mesenteroides* ssp *cremoris* respectively) from the naturally fermenting soybean daddawa is further lending credence to earlier reports of Antai and Ibrahim (1986) and Edema and Fawole (2006) which were earlier described as unusual findings. Heterofermenter LAB results in significant amounts of other end products such as ethanol, acetate and carbon dioxide in addition to lactic acid. Hence, the production of wide arrays of metabolic end products may play a significant effect in the generation of heterogenous flavour compounds, an attribute that would be beneficial for the production of a more acceptable condiment- whose primary function is flavour enhancement of soup.

The result from possible antagonistic assay between the three organisms used as starter cultures in the present study depicted that the three starter cultures were not antagonistic to one another. This attribute qualified them to be used in multiple culture fermentation system. The present observation issimilar to that of Sarkar et al. (1993), who reported that *Enterococcus faecium* had no detectable effect on the growth of *Bacillus subtilis* in the controlled fermentation of soybean to produce *kinema* (a popular Asian condiment made from soybean). While conceptualizing the use of multiple starter cultures for the optimization attempts of indigenous flavouring food condiments, Achi (2005a) stated that the use of mixture of microorganisms with complimentary physiological and metabolic properties seems to be the best approach for obtaining a product with the nutritional and sensory properties desired. It is no doubt that the present work is a step in this direction. This could not be plainer considering the enzyme profiles of the three starter cultures employed in the present study. The three starter organisms have the following enzymatic activities in common: alkaline and acid phosphatase, esterase and esterase lipase, leucine arylamidase and naphthol-AS-BI-phosphohydrolase. However, the three organisms also differed in their significant contribution of a particular enzyme activity. For instance, *Bacillus subtilis* had significant acid phosphatase, leucine arylamidase, α-chymotrypsin and α-glucosidase activities while *Staphylococcus xylosus* possessed the highest trypsin and β-galactosidase activities. The enzymatic activities that were most pronounced and peculiar to *Leuconostoc mesenteroides* ssp *cremoris* include leucine arylamidase and alkaline phosphatase. It is evidently clear that the use of a single culture cannot generate such arrays of enzyme activities like the ones furnished by the starter organisms used in the present work.

Among the controlled fermentation, *B. subtilis* as a monoculture was unique in producing daddawa that was rated to be more acceptable even than the naturally fermented samples in term of colour attribute. This unique feature of *B. subtilis* has earlier been reported by Kolapo (2008). Similar result has been reported in kinema (Sarkar, 2000) and soybean daddawa (Omafuvbe et al., 2002) fermentations. However, in term of overall acceptability, the effect of multiple culture fermentation of soybean daddawa as shown in the present study has led to the production of daddawa that was next rated to naturally fermented samples. This is most likely to be consequent upon wide arrays of enzymes profile and the synergism among the three starters used. This holds a great promise for subsequent industrialization of soybean daddawa production as naturally fermentation of soybean daddawa is now nearly replicated in a controlled setting.

## REFERENCES


[1]. Achi, O..K. (2005a). Traditional fermented protein condiments in Nigeria. *African Journal of Biotechnology*.4(13):1612-1621

[2]. Achi, O.K. (2005b). The upgrading of traditional fermented foods through biotechnology. *African Journal of Biotechnology*.4:375-30

[3]. Adedeji, B.S., Ezeokolib O.T., Ezekiel C. N., Obadinaa, A.O, Somorin Y.M. Sulyokd, M, Adelekeb, R.A., Warth B., Nwangburukah, C.C. Omemui, A.M., Oyewolea, O.B. and Krska R. (2017). Bacterial species and mycotoxin contamination associated with locust bean,melon and their fermented products in south-western Nigeria. *International Journal of Food Microbiology* 258: 73–80

[4]. Afolabi F. T. and Abdulkadir M. 2016 Biochemical Changes and Sensory Evaluation of Soy Iru Produced Using Starter Culture. *British Microbiology Research Journal* 14(6): 1-10

[5]. Antai, S.P. and Ibrahim, M.H. (1986). Microorganism associated with African Locust Bean (*Parkia filicoidea* Welw.) Fermentation for `Dawadawa' production. *Journal of.Applied Bacteriology*. 61: 145-148.

[6]. Campbell-Platt, G. (1980). African locust bean (Parkia species) and its West African fermented food products, Dawadawa. *Ecology of Food and Nutrition.* 9:123-132

[7]. Dakwa S; Sakyi-Dawson, E; Diako C.; Annan, N.T.; Amoa-Awua, W.K. (2005). Effect of boiling and roasting on the fermentation of soybeans into dawadawa (soy- dawadawa). *International Journal of Food Microbiology* 104:69-82

[8]. Edema , M.O. and Fawole, O. 2006. Evaluation and Optimization of Critical Control Points in the Production of Iru. *Research Journal of Microbiology* 1(6): 503-511

[9]. Harrigan, W.F and McCance, M.E. (1976). Laboratory Methods in Food and Dairy Microbiology. Academic Press, London, UK, pp452

[10]. Holzapfel, W.H. (2002). Appropriate starter technologies for small scale fermentation in developing countries. *International Journal of Food Microbiology*. 78:119-131

[11]. Kolapo, A.L. (2008). Microbiological, deterioration and preservative studies on soybean daddawa-A condiment. MSc Thesis. University of Agricuture, Abeokuta. Nigeria. 108pp.

[12]. Kolapo, A.L., Popoola, T.O.S. and Sanni, M.O (2007a). Evaluation of Biochemical Deterioration of Locust bean Daddawa and Soybean Daddawa- Two Nigerian Condiments. *American Journal of Food Technology*. 2(5): 440-445

[13]. Kolapo, A.L.,. Popoola, T.O.S; Sanni, M.O.and Afolabi, R.O. (2007b). Preservation of soybean Daddawa condiment with Dichloromethane Extract of Ginger. *Research. Journal of .Microbiology*. 2(3): 254-259

[14]. Niba, L.L. (2003). The relevance of biotechnology in the development of functionsl foods for improved nutritional and health quality in developing countries. *African Journal of Biotechnology*. 2(12): 631-635

[15]. Njoku, H.O.; Ogbulie, J.N.and Nnubia, C. (1990). Microbiological study of the traditional processing of African oil bean (Pentaclethra macrophylla) for production of ugba. *Food Microbiology*. 7,13– 26.

[16]. Obatolu V.A.; Okoruwa A. and Iken J.E. (2007) Improvement of home-made maize tortilla with soybean. *International Journal of Food Science and Technology* 42:420-426.

[17]. Obeta, J.A.N. (1983). A note on the microorganisms associated with the fermentation of seeds of the African oil bean tree (Pentaclethra macrophylla). *Journal of Applied Bacteriology*. 54:433– 435

[18]. Odunfa, S.A.(1981). Microbiology and amino acid composition of ogiri, a food condiment from fermented melon seeds. *Die Nahrung* 25: 811-816

[19]. Odunfa, S.A. (1985). Microbiological and toxicological aspects of fermentation of castor oil seeds for Ogiri production. *Journal of Food Science*. 50, 1958– 1959.

[20]. Ogbadu, C.O. and Okagbue, R.N. (1988). Bacterial fermentation of soybeans for daddawa production. *Journal of Applied. Bacteriology* 65:353-356

[21]. Okorie C.P. and Olasupo N. A. (2013) Controlled fermentation and preservation of UGBA –an indigenous Nigerian fermented food. *SpringerPlus* 2:470.

[22]. Omafuvbe B.O, Shonukan, O.O. and Abiose, S.H. (2000). Microbiological and Biochemical changes in the traditional fermentation of soybean daddawa – a Nigerian Food Condiment. *Food Microbiology* 17, 469-474.

[23]. Omafuvbe B.O, Shonukan, O.O. and Abiose, S.H. (2002).Fermentation of Soybean (*Glycine max*) for soy- daddawa production by Starter cutures of *Bacillus*. *Food Microbiology* 19, 561-566.

[24]. Ouoba, L.I.I; Rchinger, K.B.; Barkholt, V.; Diawara, B.; Traore, A.S and Jakobsen, M. (2003). Degradation of proteins during the fermentation of African locust bean (*Parkia biglobosa*) by strains of *Bacillus subtilis* and *Bacillus pumulus* for the production of Soumbala. J*ournal of Applied Microbiology* 94: 396-402

[25]. Owens, J.D.; Allagheny,N.; Kipping, G. and Ames, J.M. 1997. Formation of Volatiles compounds during Bacillus subtilis fermentation of soybeans. *Journal of Science, Food and Agriculture*. 74:132-140

[26]. Popoola, T.O.S and Akueshi, C.O (1985). Microorganisms associated with the fermentation of soybean for the production of Soybean `Daddawa' (A condiment). *Nigeria Food Journal*. 2: 194-196

[27]. Popoola, T.O.S.; Jolaoso, A.A.; Afolabi, R.O.and Akintokun, A.K. (2006). Microbiological studies on the production of Oso: A condiment made from fermenting seeds of *Cathormion altissimum. Journals of Herbs, Spices and Medicinal plants*. 12(1/2):129-138

[28]. Sanni, A.I., Ogbonna, D.N., (1990). The production of owoh- a Nigerian fermented seasoning agent from cotton seed (*Gossypium hirsutum* L.). *Food Microbology*. 8: 23– 229.

[29]. Sarkar, P.K.; Cook, P.E. and Owens, J.C 1993. *Bacillus* Fermentation of Soybean. *World Journal of Microbiology and Biotechnology*.9: 295-299

[30]. Sarkar, P.K.(2000). Kinema: microbiological and biochemical changes accompanying fermentation. 11th World Congress of Food Science and Technology: Proceedings of Pre-Congress Internet Conference, May 8-Dec 31. Seoul, Korea. P. 75-76

[31]. Skerman, V.B.D (1967). A guide to the Identification of Genera. Williams and Wilkins. Baltimore.

[32]. Suberu, H.A. &Akinyanju, J.A (1996). Starter culture for the production of Soyiru. *World Journal. Of Microbiology and Biotechnology*12 :403-404

[33]. Szczesna, M. and Galas, E. (2000). Protein hydrolysis by immobilized *Bacillus subtilis*cells. In Foods Biotechnology ed. Bielecki, S.,Tramper, J. and Polak, J. pp. 177–185. Amsterdam: Elsevier.

[34]. Vuillemard, J.C.; Amiot, J. and Gauthier, S. (1985). Evaluation de lactivite´ proteolytique des bacte´ries par une technique de diffusion sur plaque. *Microbiologie–Aliments–Nutrition* 3, 327–332.