

Isolation, characterization and storage of probiotics associated with the fermentation of Bambara groundnut bran

Abstract

The research was carried out to isolate, characterize, and store lactic acid bacteria (LAB) from bambara groundnut bran. The debranned Bambara groundnut were fermented for four days, and TTA (Total Titrable Acidity), pH and microbial load determination were carried out for every 24 hours of fermentation. Eighteen acid-producing cultures were isolated from these samples, and isolates were divided into classes first by phenotype. Phenotypic and biochemical characteristics led to identification of seven bacterial groups (1 to 7). *Lactobacillus plantarum* was the most abundant type of LAB distributed in the isolates of fermented bambara groundnut bran, followed by *Lactobacillus acidophilus* which was the most abundant LAB found in two of the isolates and *Lactobacillus esenteroides* was found in the remaining two isolates. These results suggest that various LAB are distributed in bambara groundnut bran and *L. plantarum* was the most abundant LAB found in this study. The microorganisms isolated were then freeze dried using a freeze drier and then kept at a low temperature in the refrigerator so as to preserve/store the organisms for further processes. This report thereby shows that bambara groundnut bran can be used for nutraceuticals instead of disposing them as waste.

Keywords: bambara groundnut, bran, lactic acid, bacteria, fermentation, isolate

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Introduction

Isolation refers to the separation of a strain from a natural, mixed population of living microbes as present in the environment.¹ Bacterial isolation, purification and identification are the first steps to bacteriological studies. Isolation is done to obtain pure bacterial cultures. Pure culture is essential in the study of the morphology, physiology, biochemical characteristics, and susceptibility to antimicrobial agents of a particular bacterial strain.¹ The formulation of the dietary supplements, functional foods or herbal products into marketed medicinal products is known as “nutraceuticals”; a term which combines “nutrition” and “pharmaceuticals”.² Among various nutraceuticals of major importance in diseases prevention is probiotics.³ Probiotics are live microorganisms which when administered in adequate amounts confer a beneficial health benefit on the host.⁴ Probiotics can be delivered commercially either as nutritional supplements, pharmaceuticals or foods. A large number of probiotic products are available in the market in the form of milk, drinking and frozen yoghurts, probiotic cheeses, ice creams, dairy spreads and fermented soya products. Also, special freeze-dried pharmaceutical dietary preparations are available in the form of tablets.⁵ Together with prebiotics, probiotics are often consumed as functional foods, demonstrated to be effective for the treatment or control of several diseases. Prebiotic substances, such as lactulose, lactitol, xylitol, inulin and certain non-digestive oligosaccharides, selectively stimulate the growth and activity of, for example, bifidobacteria in the colon (Zubillaga and Roberfroid, 2010). Most widely and commercially used probiotic species are *Lactobacillus* (*L. acidophilus*, *L. casei*, *L. fermentum*, *L. gasseri*, *L. johnsonii*, *L. lactis*, *L. paracasei*, *L. plantarum*, *L. reuteri*, *L. rhamnosus*, *L. salivarius*), *Bifidobacterium* (*B. bifidum*, *B. breve*, *B. lactis*, *B. longum*), *Streptococcus* (*S. thermophilus*) species, yeasts and molds (*Saccharomyces boulardii*).

Probiotic microorganisms have been studied as successful ingredients in functional food segment. The popularity of probiotic

foods is due to its numerous health benefit attributes to its intake.⁶ A number of health benefit of probiotic products have been proposed including antimicrobial, antimutagenic, anticarcinogenic, antihypertensive properties, reduction of allergic symptoms, reduction of diarrhea and stimulation of the immune system.⁷⁻¹⁰ To achieve the benefit from their injection, the probiotics microorganisms must reach the intestine alive and in sufficient concentration, surviving the harsh conditions found during the flow through the gastrointestinal tract (Hassan, *et al.*, 2012). A minimum daily intake of 8.9 log of colony forming unit (CFU) or 6-7 log cfu/g or 1ml of food is generally recommended.¹¹ The survival of probiotic microorganisms in food products is strongly influenced by pH and post-acidification, which may occur during refrigerated storage of fermented products. Other factors such as production of hydrogen peroxide, oxygen level, temperature and food matrix also affect the microbial viability.¹²

Probiotics have been used for centuries in food fermentation and dairy products are the main carriers of probiotic bacteria to human, as these products provide a suitable environment for probiotic bacteria that support their growth and viability.¹³ The world-wide probiotic foods are milk based and very few attempts are made for the development of probiotic foods using other fermentation substrates such as legumes. With an increase in the consumer vegetarianism in the developing countries coupled with the low purchasing power of low income people especially in sub-Saharan Africa to consume much dairy product needed for probiotics, there is also a demand for the vegetarian probiotic products. Nondairy probiotic products have shown a big interest among vegetarians and lactose intolerance customers. A potential source of vegetarian probiotics are legumes due to their large distribution and important nutritive value. Bambara groundnut (*Vigna subterranean*) is an indigenous African crop known to have been domesticated in West Africa from its presumed wild ancestor (Ferry, 2002). Bambara groundnut contains about 61.3% carbohydrate, 20.7% protein and 6.0% oil,¹⁴ and is used as main food,

snacks, relish and medicine, and has high ceremonial value.¹⁵ Despite its high and balanced protein content, Bambara groundnut remains under-utilized because it takes a long time to cook and contains anti-nutritional factors such as tannins and trypsin inhibitors, and it has poor milling characteristics, as it does not dehull easily.¹⁶

In order to maximize the utilization of the Bambara groundnut, the bran was then subjected to fermentation process to enhance the quality parameters and functional properties of the raw material. Fermentation with well-characterized starter cultures, yeast or Lactic Acid Bacteria (LAB), is a potential means to improve the palatability and process ability of brans and whole-meal flours.¹⁷ Furthermore, bran fermentation could assist in the management of indigenous microbes and improvement of the microbiological safety of bran. Therefore, this research was carried out to isolate, characterize and store Lactic Acid Bacteria from the bran of Bambara groundnut instead of disposing it as waste.

Materials and methods

Dried Bambara groundnut was obtained from Institute of Tropical Agriculture, Ibadan, Nigeria. The cleaned sample of Bambara groundnut was de-branned by removing the outer part of the legume. This was done by soaking the seed in water for 30 minutes to enable easy removal of the seed coat which was done manually.

Bran fermentation

45g of the powdered bran was measured using an analytical balance into a sterile container and was mixed with 200ml of distilled water and the sterile container was covered, this was done in four different sterile container to allow fermentation to be done for 4days (24hr at 20 or 32°C for each container). Spontaneous fermentations were carried out. Fresh samples were taken from each fermented bran for microbiological analyses. In addition, samples were also taken for later measurements of pH and total titratable acidity (TTA) and fermented bran samples were later freeze-dried for analysis of bioactive compounds. Fermentations were done in duplicate and performed twice (four times altogether) (Figure 1).

Determination of pH

The pH meter was first calibrated using buffer solution of pH 4 and pH 7 in order to determine the accuracy of the pH meter to be used. 90ml of the fermented substrate was measured into a beaker and 10ml of distilled water was added to it, the pH meter was dipped into the measured substrate, switched on and allowed to be stabilized before the result displayed on the meter was taken and recorded.¹⁸

Determination of total titratable acidity (TTA)

This was done by the methods described by Antony and Chandra, 1997¹⁹ which was later modified by Ferrati et al.²⁰ The burette was rinsed and set up on the retort stand and fixed tightly and carefully. 10mls of the fermented substrate was measure into a conical flask and 90ml of distilled water was added to it. 0.1M NaOH was run into the burette to fill the burette to any mark, 2 drops of phenolphthalein indicator were carefully dropped into the substrate in the beaker and shaken. The initial volume of the alkaline in the burette was noted. The alkali was run briskly into the beaker containing the substrate and indicator which was consistently shaken until there was a sharp change of the substrate to pink. The volume of the alkali in the burette at this point was noted to enable the determination of the volume of

base (titre value of alkali) used. TTA was expressed as the amount of NaOH used (ml).

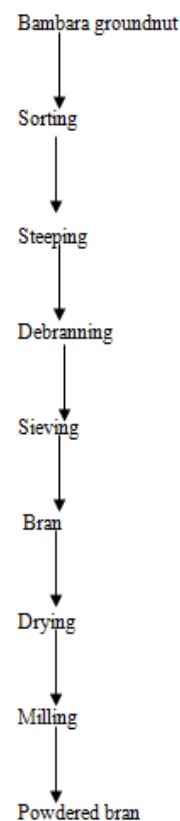


Figure 1 Flowchart showing the debranning of Bambara Groundnut Analysis

Preparation of culture media and diluent

The culture media, each in the powdered form were reconstituted with distilled water according to the manufacturers' instructions. Proper dissolution was achieved by placing the flask in a hot plate at 50°C for 4 min. They were sterilized by autoclaving at 121°C and 15mmHg for 15minutes and cooled to 45°C before dispensing into sterile Petri dishes and left to gel. The diluent was prepared by adding 0.85g NaCl to 100ml of distilled water and 9ml each was measured into dilution bottles which were covered tightly and sterilized in an autoclave at 121°C and 15mmHg for 15minutes and cooled to 45°C.

Isolation of microorganisms

Isolation of microorganisms was done by method described by Harrigan and McCance²¹ After sterilization of agars and diluents, the workbenches were sterilized using ethanol and cotton wool and a spirit lamp was lighted, placed on the work bench to create an aseptic environment. The sterile Petri dishes were arranged and labeled on the sterilized work bench, the cooled dilution bottles were also placed on the work bench and serial dilution was done at 3-fold dilutions (10^0 , 10^{-1} , 10^{-2} , and 10^{-3}). 1ml of dilution factor 10^{-2} was inoculated via pour plate method on MRS agar by adding 0.3ml of lactic acid. The Petri dishes containing the substrate and medium were incubated in an anaerobic jar which was put into an incubator at 37°C for 48hours. Discrete colonies from each plate were sub cultured. The pure isolates gotten were preserved in a prepared broth bottle containing nutrient agar before biochemical tests were further carried out.

Identification and characterization of isolates

Colonies were selected randomly and were characterized using morphological characterization and biochemical tests such as gram stain, catalase, and sugar fermentation tests. Bacterial isolates were identified with reference to Cowan and Steel's Manual for the Identification of Medical bacteria (Cowan, 1985) and Bergey's manual of determinative Bacteriology.²² The bacterial isolates identification was based on colony morphology, cultural characteristics and biochemical tests using the methods described by Cheesebrough.²³

Morphological characterization

The Morphological characterization observed include the shape, colour, size and surface.

Biochemical characterization

Distinct pure colonies observed to be dominant were checked for gram reactions using microscopic examination for cell morphology.

Gram stain

The method used was that described by Harrigan and McCance²⁴ A smear of the test organism was made on a grease free glass slide and heat fixed. The smear was stained with crystal violet for 1 minute, rinsed with water and excess water was drained off. The slide was flooded with lugol's iodine for 1 minute and it was rinsed with running water. The smear was decolorized with absolute alcohol over it until blue colouration ceased to leave the smear, it was rinsed immediately with water and drained off excess water for 5–10 seconds. The slide was counterstained with 0.5% safranin for 1 minute, the slide was rinsed with water and allowed to dry. Immersion oil was added and was observed under the microscope using the oil immersion(x100) objective lens. Gram positive bacteria retained the colour of the primary stain which is purple. Gram negative bacteria take the colour of the counter stain (safranin, neutral red, dilute carbolfuchsin) which is red or pink.

Spore stain

The malachite green staining method was used. The staining was carried out as described by Harrigan and McCance²⁴ Smears of the pure isolates were made on grease-free glass slide and heat fixed. The slides were flooded with 5% v/v malachite green solution. The slides were flamed in such a way that the stain steamed but did not boil. The slides were then allowed to stand for 5 minutes. The stain was then washed out in running tap water. The smears were counter stained with safranin for 30 seconds. This was stained with safranin for were blotted, dried and examined under the oil immersion objective. The spores stained green while vegetative cells stained red.

Motility test

This test was used to determine if the isolates were motile. Motility test is usually used to differentiate motile organisms from non-motile ones. For this test, the hanging drop technique was employed and the technique was carried out as described by Harrigan and McCance²⁴ Vaseline jelly was rubbed around the cavity of a hanging drop slide. A drop of peptone water containing the pure culture was placed on a cover slip. The hanging drops slide was then placed over the drop of peptone water in such a way that the center of the depression lies over the drop. The slide was quickly inverted and viewed under the microscope, using oil immersion objective.

Catalase test

This test was used to demonstrate which of the isolates could produce the enzyme catalase that release oxygen from hydrogen peroxide. This test is usually used to differentiate other catalase positive organism from catalase negative (Barker, 1976). The method employed here was that described by Jagbir²⁵ A loop of the pure colony was transferred into a plain, clean glass slide. The sample was then mixed with a drop of 3% v/v hydrogen peroxide. It was observed for effervescence, immediate bubbling indicates that the organism is catalase positive.

Sugar fermentation test

Each of the isolates was tested for its ability to ferment a given sugar with the production of acid and gas or acid only. Since most bacteria especially Gram negative bacteria utilize different sugars as source of carbon and energy with the production of both acid and gas, or acid only the test is used as an aid in their differentiation. The growth medium used was peptone water and the method used was that described by Grujot²⁶ Peptone water was prepared in a conical flask and the indicators phenol red was added. The mixture was dispensed into test tubes containing Durham tubes. The tubes with their content were sterilized by autoclaving at 121°C for 15 minutes. 1% solution of the sugar was prepared and sterilized separately at 115°C for 10 minutes. This was then aseptically dispensed in 5ml aliquot volume into the tubes containing the peptone water and indicator. The tubes were inoculated with young culture of the isolates and incubated at 37°C. Acid and gas production or acid only were observed after about 24 hours of incubation. Acid production was indicated by the change of the medium from pale red to yellow colour, while gas production was indicated by the presence of gas in the Durham's tubes. The control tubes were not incubated Prescott et al.²⁷

Preparation of Fresh Broth and Freeze Drying

After biochemical test, the pure isolates gotten were sub cultured in a fresh prepared broth (according to the manufacturer's instructions) bottle containing nutrient agar and was incubated at 37°C for 24 hours. After incubation, the sample bottles containing broth and the bacteria were transferred into a freezer to freeze and then loaded in a freeze drier for proper and complete drying. This was done by the method described by Oetjen et al.²⁸ The samples were frozen below the critical temperature of the formulation and were then followed by primary drying, where the chamber pressure was lowered, the shelf temperature usually increased and the unbound water removed by sublimation. Finally, a secondary drying step was done to remove the bound water by desorption and the samples were gradually brought back to ambient temperature (Figure 2).

Results and discussion

Determination of pH, total titrable acidity (TTA) and microbial load

For pH, there is increment in the pH level as the fermentation days increases and the TTA also increases as the fermentation days increases. One hundred and twelve (112) LAB colonies were isolated from the spontaneous fermentations in all the plates for 4 days (in duplicates each day), 45 colonies for day 1, 35 colonies for day 2, 25 colonies for day 3 and 12 colonies for day 4. The result is being represented in Table 1.

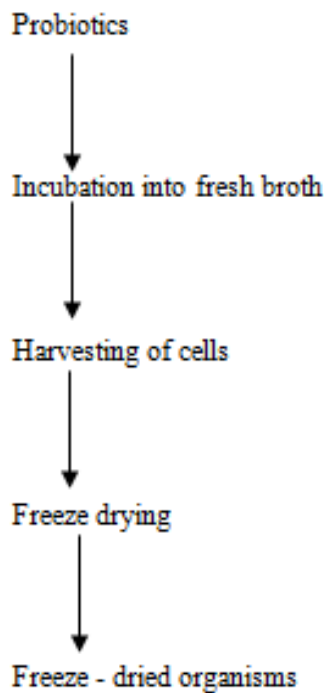


Figure 2 Flowchart showing the processing of freeze-dried organisms.
Table 1 pH, TTA and microbial results during fermentation for 4days

Analysis	BGB 1	BGB 2	BGB 3	BGB 4
pH	2.94	4.50	6.01	6.11
TTA	0.012%	0.011%	0.008%	0.0088%
Microbial load	45	35	20	12

KEYS

BGB 1: Bambara Groundnut Bran in sterile container at 24hours
 BGB 2: Bambara Groundnut Bran in sterile container at 48hours
 BGB 3: Bambara Groundnut Bran in sterile container at 72hours
 BGB 4: Bambara Groundnut Bran in sterile container at 96hours

Table 3 Biochemical identification result for isolates on MRS plates

Biochemical Identification	Isolate 1	Isolate 2	Isolate 3	Isolate 4	Isolate 5	Isolate 6	Isolate 7
Morphology	Bacilli	Bacilli	Bacilli	Bacilli	Bacilli	Bacilli	Bacilli
Gram stain	Positive	Positive	Positive	Positive	Positive	Positive	Positive
Spore stain	Negative	Negative	Negative	Negative	Negative	Negative	Negative
Motility test	Negative	Negative	Negative	Negative	Negative	Negative	Negative
Catalase test	Negative	Negative	Negative	Negative	Negative	Negative	Negative

Conclusion

In this research, Lactic Acid Bacteria (LAB) was isolated, characterized, identified and stored from spontaneously fermented bambara groundnut bran. The results obtained shows that microorganisms associated with the fermented bambara groundnut

Identification on Isolates

Each of the isolates was subjected to Gram stain test and were examined under light microscope. Each of the isolates (7 from MRS) gave blue-purple color with staining; hence they all were Gram positive bacteria. The isolates from MRS plates were rod-like bacilli with long or rounded ends. Spore staining and motility test were negative for all isolates both on the MRS plates and NA plates. This implies none of the isolates formed spores and are non-motile. All of the isolates both on MRS and NA plates were tested for catalase. None of them showed catalase activity. This showed that they are catalase negative. On MRS plate for gas production, isolate 1 was negative for Glucose, Fructose, Lactose, Sucrose and Mannitol. Isolate 2 was negative for Fructose, Maltose, Sucrose and Mannitol. Isolate 3 was negative for Glucose, Fructose, Maltose, Lactose, Sucrose and Mannitol. Isolate 4 was negative for Mannitol only. Isolate 5 was negative for Glucose, Fructose, Maltose, Sucrose and Mannitol. Isolate 6 was negative for Glucose and Mannitol only.

For acid production, Isolate 1 was negative for Glucose, Sucrose and Mannitol. Isolate 2 was negative for none of the sugars. Isolate 3 was negative for Glucose, Fructose, Maltose, Lactose, Sucrose and Mannitol. Isolate 4 was negative for none of the sugars. Isolate 5 was negative for Glucose, Fructose, Maltose, Lactose, Sucrose and Mannitol. Isolate 6 was negative for Fructose, Maltose, Lactose and Mannitol. Isolate 7 was negative for Mannitol only. The results are shown in Table 2–4.

Table 2 Morphological characteristics of isolated organism

Morphological characteristics	Probable organisms
Purple, very short rod, irregular shape	<i>Lacobacillus mesenteroides</i>
Purple, short rod, regular shape	<i>Lacobacillu splantarum</i>
Purple, long rod, regular shape	<i>Lactobacillus acidophilus</i>
Purple, short rod, regular shape	<i>Lactobacillus plantarum</i>
Purple, short rod, regular shape	<i>Lactobacillus plantarum</i>
Purple, short rod, irregular shape	<i>Lactobacillus mensenteroides</i>
Purple, long rod, regular shape	<i>Lactobacillus acidophilus</i>

bran are bacteria that are probiotics which can beneficial to human and can then be used for nutraceuticals in the production of encapsulated products for the enhancement of immune system and proper functioning of the human GIT (Gastro Intestinal Tract).

Table 4 Carbohydrate fermentation result for isolates on MRS plates

Carbohydrates Fermentation	Isolate 1	Isolate 2	Isolate 3	Isolate 4	Isolate 5	Isolate 6	Isolate 7
Glucose	-	Gb	-	Gb	-	-	-
	-	As	-	As	-	As	Ab
Fructose	-	-	-	Gb	-	-	Gs
	As	As	-	Ab	-	-	Ab
Maltose	-	-	-	Gb	-	-	Gb
	As	-	-	Ab	-	-	Ab
Lactose	-	Gs	-	Gs	-	-	Gs
	As	As	-	As	-	-	Ab
Sucrose	-	-	-	Gb	-	-	Gs
	-	-	-	Ab	-	As	As
Mannitol	-	-	-	-	-	-	-
	-	As	-	As	-	-	-

KEYS

-, negative to the test

Gb, gas (Durham tube more than ¼ full)

Ab, acid, full color change Gs, slight gas (Durham tube less than ¼ full)

As, small amount of acid i.e indicator not fully changed in colour

Acknowledgements

None.

Conflicts of interest

The authors declare that there is no conflicts of interest.

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