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Kuri, Victor

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Contact at The University of Plymouth:

Dr. Victor Kuri
v.kuri@plymouth.ac.uk

University of Plymouth. School of Biological and Marine Sciences,
PSQA410, Drake Circus, Plymouth, PL4 8AA United Kingdom
Tel: +44 (0)1752 5 84600 (Ext.84638); Fax +44 (0)1752 584605
http://www.plymouth.ac.uk/staff/vkuri

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Evaluation of the microbial community, acidity and proximate composition of akamu, a fermented maize food

ABSTRACT

BACKGROUND: *Akamu* is a lactic acid fermented cereal-based food that constitutes a major infant complementary food in most West Africa countries. Identity of LAB population from DGGE analysis and conventionally isolated LAB and yeasts from traditionally fermented *akamu* were confirmed by PCR-sequencing analysis. The relationship between pH, acidity and lactic acid levels and proximate composition of the *akamu* samples were investigated.

RESULTS: The LAB community in the *akamu* samples were mainly Lactobacilli species and included *Lactobacillus fermentum, Lb. plantarum, Lb. delbrueckii* subsp*. bulgaricus* and *Lb. helveticus* except for *Lactococcus lactis* subsp*. cremoris*. Identified yeasts were: *Candidia tropicalis*, *C. albicans, Clavispora lusitaniae and Saccharomyces paradoxus.* Low pH ≤3.95±0.01 was accompanied by high lactic acid concentrations (43≥LA≥84 mmol kg^{-1}). The protein (31.88±0.08 - 74.32±1.36 g kg^{-1}) and Lipid (17.74±0.79 - 36.83±0.77 g kg^{-1}) content had negative correlation with carbohydrate content of 897.48 - 926.20 g kg^{-1} (of which ≤ 1 g kg^{-1} were sugars). Ash was either not detected or in trace amounts ≤ 4 g kg^{-1} with energy level of 17.29±0.29 - 18.37±0.08 KJ g^{-1}.

CONCLUSION: The *akamu* samples were predominately starchy foods and had pH < 4.0 due to the activities of the fermentative LABs.

Keywords: *Akamu*, Lactic acid bacteria, yeasts, acidity, proximate composition

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INTRODUCTION

Traditionally fermented cereal foods constitute important part of peoples diet in most African countries and cereals are one of the most important sources of dietary protein, carbohydrates, minerals, vitamins and fibre worldwide.[1] The fermentation process involves the conversion of organic substrate into more desirable substances through the action of enzymes or microorganisms under controlled conditions. [2] The fermenting microbial population implicated in most cereal fermentations are mainly lactic acid bacteria: *Lactobacillus, Leuconostoc, Lactococcus, Pediococcus* and *Weisella* species and yeasts: *Candidia, Saccharomyces, Geotrichum, Kluyveromyces* and *Pichia* species. [3-15] Identification of these microorganisms in past decades relied on culture dependent techniques that utilised the phenotypic properties of the microorganisms. Conventional microbiological techniques may be simple to perform it lack discriminatory potency and reproducibility at species level. Selected growth conditions affect the cell's morphological characteristics and selection of only a small fraction and organisms of interest do not give the true representation of the complex ecosystem.[16-18] Rapid, sensitive and reliable molecular methods based on direct analysis of DNA in the environment, requiring no cell culture and enabling detection of individual species as well as overall profiling of structural changes in a microbial community with time have evolved. These culture-independent approaches include polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) and PCR-temporal temperature gradient gel electrophoresis (PCR-TTGE).[19, 20] Although selective selection of nucleic acid and amplification of a particular region of the rDNA aimed at identification of specific bacterial community may sometimes present a limitation to the broad knowledge of the entire population. For explicit and reliable species identifications, some authors use a polyphasic approach involving both phenotypic and genotypic methods to establish microbial diversity in some fermented cereal and legume foods.[14, 18, 21-23]

Akamu is a traditional lactic acid fermented cereal-based meal, made from maize (*Zea maize*), sorghum or millet. [23-25] The traditional processing technique of *akamu* involves steeping the grain in excess water for 2-3 days, washing, wet milling and sieving and allowing extracted solid to sediment overnight. In the liquid menstruum, activities of various microorganisms associated with the raw material and utensils takes place to give the product its characteristic taste and flavour. The product *(akamu*) varies in colour from white to yellow or dark brown depending on the variety of the cereal used. Addition of an equal part of boiling water to the fermented slurry with vigorous stirring yields a nearly gelatinized lump less porridge. The porridge forms an integral part of adult main meals or food for convalescents in most African countries and plays important role in the nutrition of infants and young children as a complementary food when diluted to a thinness of 8-10% of the total solids.[25, 26] The fermented slurry when cooked with water produces a stiff gel called *akidi* that serves as convenient food for travellers. [27]

Although, maize has been reported to constitute an important source of protein (90-130 g kg^{-1}) and energy in developing countries [28], various processing methods influences its protein content and quality and loss of other nutrients.[3, 29, 30] Aminigo and Akingbala,[31] attributed over 50% loss of protein and lipid in market samples *ogi* to processing methods. In a study by Antai and Nzeribe,[32] sieving of maize mash was particularly implicated in protein loss and complete absence of ash and fibre.

This study was therefore focused on (A) determining the lactic bacteria population in traditionally fermented *akamu* samples obtained from different parts of Rivers State in Nigeria by evaluating their DGGE banding patterns and identifying the bacteria by sequencing excised DGGE bands. The identity of conventionally isolated LAB and yeasts were confirmed by using direct PCR and sequence analysis. (B) Establishing the relationship between the pH, acidity and proximate composition of the *akamu* samples.

EXPERIMENTAL

Akamu samples

Samples of *akamu* were obtained from 5 different locations in Rivers State, Nigeria; Mile 3 Diobu (M1, M2 and M3); Emohua (E1, E2 and E3), Rumuokoro (R1 and R2), Aluu (A1) and Worgi (W1). The samples were all *akamu* made from yellow variety of maize except for sample E1 and W1 which were of the white variety

Microbial analysis

The lactic bacteria population in the *akamu* samples were determined by evaluating the DGGE banding patterns of the entire DNA from the *akamu* samples. A second step was to establish the identity of bacterial DNA on selected bands according to pre-established criteria by sequencing excised DGGE bands. In order to obtain viable bacterial cells for further studies, culture-dependent techniques were employed and the identity of the isolates were confirmed using direct PCR and sequencing analysis.

Microbial isolation

Ten grams of sample were homogenised with 90 ml of PBS in a stomacher lab-blender (400, Seward, UK) for 1 min and serially diluted (10^{-1} to 10^{-8}) in the same diluent following the procedure described by Harrigan.[33] 100 µl of the dilutions were spread-plated on appropriate microbial media for each microorganism. The isolation of LAB was on de Man, Rogosa and Sharpe (MRS) agar incubated at 37^{o}C in 35-37 mm Hg of CO_2 for 24-48 h. Yeasts and moulds was isolated on Rose Bengal Chloramphenicol Agar (RBCA) incubated at 25^{o}C for 72 h. MacConkey agar was used for Enterobacteriacae and Yersinia selective agar for *Yersinia*, these were incubated at 30^{o}C for 24-48 h. Taylor's xylose lysine desoxycholate (XLD) agar, Baird-Parker's agar with egg yolk supplement, Listeria selective agar and Tryptone Bile X-Glucuronide (TBX) agar were used for *Salmonella* and *Shigella, Staphylococcus aureus*, *Listeria monocytogenes* and *Escherichia coli* respectively with incubation for 48 h at 37^{o}C.

Maintenance and storage of culture

Microbial colonies selected randomly from plates of highest dilutions of MRS and RBCA were purified by streaking onto the same media. Purity of the isolates were checked by streaking again and sub culturing on fresh agar plates of the isolation media, followed by microscopic examinations. The purified LAB colonies were sub cultured into MRS broth incubated at 37^{o}C for 24 h and the yeast were grown in Malt Extract (ME) broth at 25^{o}C for 48 h. The cells were harvested by centrifugation, maintained in glycerol media and stored in liquid nitrogen at -80^{o}C until required for further analysis. All media used were from Oxiod, UK.

Phenotypic characterisation

Cell morphology of all isolates was determined using standard compound microscope (Medilux-12, Japan). LAB isolates were Gram-stained and tested for catalase production and other phenotypic properties such as carbon dioxide production from glucose, ammonia production from arginine, growth at different temperatures (15 and 45^{o}C) and production of dextran from sucrose as well as the ability to grow in different concentrations (40 and 100 g L^{-1}) of sodium chloride in MRS broth, following the methods of Harrigan [33] and Schillinger and Lücke. [34] Sugar (arabinose, cellobiose, esculine, fructose, galactose, glucose, lactose, maltose, mannitol, mannose, melezitose, mellibiose, raffinose, rhamnose, ribose, salicin, sorbitol, sorbose, sucrose, tetrahose and xylose) and starch fermentation patterns of the LAB isolates were determined by the method of Schillinger and Lücke. [34] Yeast isolates were characterised by Gram-staining, ascospore and ballistospore production, growth in ethanol, resistance to cycloheximide and sugar fermentation as described by Harrigan. [33] Inferences were based on triplicate experiments. The isolated LAB and yeasts were presumptively identified based on their phenotypic characteristics in comparison to known microorganisms using the scheme by Buchanan and Gibbons (Bergey's manual of determinative bacteriology)[35], Schillinger and Lücke[34], Wood and Holzapfel[36], Harrigan [33], Barnett and Pankhurst[37] and Guillermond[38].

Genotypic characterization

DNA extraction

Total DNA as extracted from the *akamu* samples using a DNeasy Mericon Food Kit (QIAGEN, UK) according to the manufacturer's protocol. DNA of pure microbial cultures: LAB and yeasts isolated from M3 and W1 samples were extracted from overnight broth cultures of the microorganisms by using the

Genthra Puregene Yeast/Bacteria Kit according to the manufacturer's protocol. The DNA purity was estimated by absorbance at 260 nm in a NanoVue plus spectrophotometer (GE Healthcare UK Ltd). The DNAs were stored at – 20^{o}C until required for use.

PCR amplification and purification

The 16S rDNA region of the *akamu* LAB community was amplified with the primers pairs P1 and P2, P3 and P2 as described by Muyzer et al.,[20] while the isolated LAB and yeasts 16S rDNA and 28S rDNA were amplified using primer pairs 27f and 1492r [39] and NL1 and NL4 [40] respectively. The PCR amplification reaction mixture was prepared by using MyTaq Mix (Bioline Ltd, UK) according to the manufacturer's standard protocol. Amplification was performed in an automated thermocycler (Techne TC-512, Scie-Plas Ltd) with initial denaturation step at 95^{o}C for 1 min, followed by 30 cycles of denaturation for 15 s at 95^{o}C, annealing for 15 s at 55^{o}C and extension for 10 s at 72^{o}C and final annealing at same temperature for 7 min. Aliquot of 2 µl of amplification products were resolved by electrophoresing in agarose gel in 1x TAE buffer (12 g L^{-1}) stained with SYBR safe for 20 min. PCR products were purified using SureClean column-free PCR clean-up (Bioline, UK) according to the manufacturer instruction. The successfully amplified product of the pure LAB and yeast DNAs were sent for sequencing while products of *akamu* DNAs were further analysed by denaturing gradient gel electrophoresis.

DGGE analysis and excision of DNA fragments

Perpendicular electrophoresis was performed with the PCR products (20 µL) at 60°C using 2.8-4.2 mol L^{-1} and 0.16-0.24 kg L^{-1} of urea and formamide mix gradient increasing in the direction of electrophoresis at 250 V in 1 × Tris-acetate-EDTA (TAE) buffer for 17 h in a DGGE-2001 apparatus (CBS Scientific

Co., CA). After electrophoresis, the gels were stained with SYBR gold (Qiagen, UK) for 20 min and subsequently photographed using a Bio-Rad Imager System equipped with a Gel Doc XR camera and Quantity-One software (Bio-Rad Inc., Hercules, CA). DGGE fragments were excised with sterile pipette and eluted in 20 µl of molecular water overnight at 4^{o}C. One microliter of the eluted DNA was re-amplified by using the same primers and PCR condition described earlier.

Sequence analysis

Amplified products were sent to GATC Biotech Ltd, London, UK, for sequencing. The nucleotide sequences were submitted to the BLAST search programme of the National Centre for Biotechnology Information (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to determine the closest known microorganisms.

Physico-chemical analysis

pH and Titratable acidity

The pH of 1 g of *akamu* sample mixed with 10 ml of sterile distilled water was determined with a pH meter (Accumet^{R} AB10, Singapore). The pH was calibrated against standard buffer solutions (Fisher Scientific, UK) at pH 4 and 7. The amount of acid as total titratable acidity (TTA) produced in the fermentation was determined by modifying the method according to Annan et al., [41] One gram of each sample was mixed with 10 ml of sterile distilled water and titrated against 0.1 mol L^{-1} NaOH with phenolphthalein as indicator. Results were expressed as lactic acid in g kg^{-1}.

Organic acids and sugar analysis

About 0.5±0.01 g of the solid samples were dispersed in 1 ml of distilled water and centrifuged at 13 000 x *g* for 20 min (Sanyo-MSE Micro Centaur Centrifuge, Alconbury, UK). To 100 µl of the sample supernatant in 400 µl of Milli-Q water (Millipore Corp., Bedford, MA, USA) was added 20 µl of 92 mmol L^{-1} H_2SO_4. This was filtered through MF-millipore microfiltre (0.20 µm pore size) into vials and sealed with crimp cap (11mm, Ruber/PTFE, Fisher Scientific, UK). Thereafter, the organic acid compositions of the samples (in triplicate) were analysed by the method of Niven et al.,[42] using high performance liquid chromatography (HPLC), Gynkotek (Dionex Corp., Sunnyvale, CA, USA).

Proximate analysis

Proximate analyses were carried out on all *akamu* samples except W1, using standard methods.[43] Moisture content was calculated after drying at 105°C to constant weight in an air oven (Thermo Scientific-UT 6200, Germany). Ash was determined gravimetrically after incineration in a muffle furnace (Carbolite AAF-11/18, UK) for 24 h at 550°C. Lipids were estimated by exhaustive extraction of known weight of samples with petroleum ether using rapid soxhlet extraction apparatus (Gerhardt Soxtherm SE- 416, Germany). A bomb Calorimeter (PARR 1356, Japan) was used to determine gross energy (MJ kg^{-1}). Determination of protein was by Kjeldahl method. After the digestion of the sample in Kjeldatherm digestion unit (Gerhardt, Germany), nitrogen was assayed in a Vapodest distillation unit with the aid of Vapodest manager computer programme. The efficiency of the Nitrogen values were corrected with acetanilide values and multiplied by the factor of 6.25 to obtain the protein value. Carbohydrate content was determined by difference.

Statistical analysis

Statistical analysis of the data was carried out using Minitab (Release 16.0) Statistical Software English (Minitab Inc. UK). Statistical differences were evaluated by analysis of variance (ANOVA) under general linear model and

Tukey pairwise comparisons at 95% confidence level. Based on the sample origin, correlations between pH, TTA and lactic acid concentration employed the first-order partial correlation coefficient, while relationship between the proximate variables utilised multiple regressions and correlations. Correlation between each pair of variables was determined while holding constant the values of other variables. This was to eliminate any effect of the interaction of the variable held constant on the relationship between the other two correlated variables.[44]

RESULTS AND DISCUSSION

Microbial Identification

The amplified DNA template from the samples appeared as single bands on the polyacrylamide gel electrophoresis as shown in the example in Figure 1. Single band is usually the design for successful PCR. The mixture of sequences of the various lactic acid bacteria community in the DNA template of the *akamu* samples were separated out as bands in the parallel denaturing gel gradient (Figure 2). Each pattern from the PCR products of the different *akamu* samples produced up to 10 to 20 bands. The relative intensity of each band and its position suggested the common occurrence of LAB of the same or very closely related strains in the *akamu* samples. Matching of the relative band positions of previously identified LAB isolates with the samples' band patterns allowed a presumptive identification of the most likely organisms present in the samples. This was against the backdrop that DNA fragment with identical base-pair sequences would have identical melting temperatures and thus stop migrating at a particular position once that temperature was reached. [20] Evidenced in this study was the identification of the excised DGGE fragments 2 and 3 as *Lb. plantarum* and *Lb. fermentum* and their respective band positions matched the band position of pure *Lb. plantarum* and *Lb. fermentum* cultures. Bands that appeared in all or 90% of the samples were assumed to be linked to the predominant species.

In Figure 2, the PCR products obtained for the pure cultures migrated over a narrow region of the DGGE gel in comparison to the *akamu* samples and with all the pure cultures having multiple bands on the DGGE gel. Different species may yield PCR products that co-migrate in DGGE gels which could pose an inherent bias on the identification processes. [45] Fragment 4 had the same homology of 100% for *Lb. delbrueckii* subsp*. bulgaricus* and *Lb. helveticus.* This suggested the closeness of these 2 strains with similar DNA regions. This ambiguity however can be resolved with the use of complementary technique such as Randomly Amplified Polymorphic DNA fingerprinting as was the case in the study by Omar and Ampe. [22]

In Table 1, the NCBI blast search revealed that the LAB community in the *akamu* samples were all of the genera Lactobacilli with a strain of *Lactococcus lactis* subsp*. cremoris* from the genus Lactococcus. The identified Lactobacilli were: Obligate homofermentative *Lb. delbrueckii* subsp*. bulgaricus*, *Lb. helveticus*, *Lb. acidophulis*; facultative homofermenters: *Lb. plantarum*, *Lb. rhamnosus*, *Lb. casei*, *Lb. salivarius,* and obligate heterofermenters: *Lb. fermentum* and *Lb*. *reuteri.* Maize been the only substrate for the fermented *akamu* samples, all the samples had the *Zea mays* chloroplast (fragment 17). The common occurring bands (fragment 3, 4 and 5) in Figure 2 were identified as *Lb. fermentum, Lb. delbrueckii* subsp*. bulgaricus* or *Lb. helveticus* and *Lb. plantarum,* hence suggesting the predominance of the LAB in the *akamu* samples. The results of this study were in agreement with reports in the literature on the predominance of Lactobacillus spp: *Lb. fermentum*, *Lb.*

plantarum and *Lb. acidophilus* in fermented cereal foods [10, 11, 15, 46, 47]. The predominance of *Lactococcus lactis* subsp. *lactis* was reported in fermented cereal beverages. [9, 48] Literature reports also confirmed the isolation of *Lb. rhamnosus, Lb. casei* and *Lb. delbrueckii* from different kinds of cereal products. [11, 49, 50] The isolation and occurrence of *Lb. helveticus* in maize fermentation seemed unique. Although, a commercial strain of *Lb. helveticus* ATCC15009 has been employed in the fermentation of sour dough bread. [51] During the culture-dependant isolation procedure, the growth of Enterobacteriacae, *Yersinia, Staphylococcus aureus, Salmonella*, *Shigella*, *Listeria* and *Escherichia coli* were not found in the *akamu* samples. The phenotypic characteristics of the isolated LABs and their genomic identity were shown in Table 2 and 3. The DGGE profile confirmed the presence of the isolated LABs in the *akamu* samples. However, *Lb. reuteri*, *Lb. salivarius* and *Lb. delbrueckii* subsp. *bulgaricus* were not isolated using culture-dependent techniques a confirmation of the limitations of the culture-dependent methods. [18, 52] Although, not all the DGGE bands were excised and some fragments were not successfully sequenced which could account for the absence of *L. acidophilus* in the DGGE profile.

The phenotypic characteristics of the isolated yeasts and their genotypic identity were shown in Table 4 and 5. The identified yeasts were *Candidia albicans, C. tropicalis*, *Clavispora lusitaniae and Saccharomyces paradoxus.* Nout et al., [53] had demonstrated that *C. albicans* can grow very well in fermented porridges with pH ≤4.0. As a dimorphic yeasts that inhabits the mucosal surfaces of the oral and vaginal cavities and the gastro-intestinal tract of humans, [54] the presence of *C. albicans* in the *akamu* samples suggested contamination from handlers. *C. tropicalis* has been associated with the fermentation of cereal gruel, beverages and dough.[7, 23, 46, 55] Recently, Mukisa et al.,[56] identified *Clavispora lusitaniae* from *obushera a* Ugandan fermented sorghum and millet beverage. Reports from literature on the association of *Saccharomyces paradoxus* in maize fermented food seemed scarce, making it an addition to the knowledge of the kinds of yeasts that could be found in fermented cereal.

The presumptive identification of the isolated LAB and yeasts based on their phenotypic characteristics in comparison to known microorganisms showed some variations from the genotypic identification. Although, some of the isolates had same identity with both methods, identification based on biochemical test was more related to metabolic functions than genetic closeness.

pH, titratable acidity (TTA), organic acid and sugar concentrations

The pH, titratable acidity and lactic acid concentrations of the various *akamu* samples and the resulting mean for each location were shown in Table 5. The samples pH, TTA and lactic acid concentration were in the ranged of 3.22 - 3.95, 6.01 – 15.91 g kg^{-1} and 43.10 – 84.29 mmol kg^{-1}. There was no significant difference observed among the 4 different locations, although variations were observed amongst the individual samples. This probably points at some similarity in the fermentation processes with the predominance of lactic acid producing bacteria. Partial correlation coefficient between the variables pH, TTA and lactic acid concentrations and their respective P-values obtained under 95% confidence interval were as follows: pH and TTA at a constant lactic acid level ($r_{it.l}$) = 0.78 (0.01); pH and lactic acid at a constant TTA ($r_{il.t}$) = -0.68(0.04) while TTA and lactic acid with pH been held constant ($r_{tl.i}$) = 0.61(0.08). Lactic acid concentrations had no correlation with the titratable acidity as the probability of 0.08 was an indication of zero correlation at P≤0.05.

This implies that some other metabolites of the fermentation may have added to the titratable acidity.

Sugars (glucose and maltose) were detected in some of the samples at concentrations below 3 mmol kg^{-1}. Glucose levels of 2.24±0.45 and 2.48±0.45 mmol kg^{-1} were detected in samples E2 and R1 respectively while samples M3 and A1 had maltose concentrations of 1.26±0.05 and 0.44±0.05 mmol kg^{-1} respectively. For products suspected to be 3 to 5 days old, the sugars may have been converted to mainly lactic acid resulting in high lactic acid contents and undetectable or low sugar levels (<3 mmol kg^{-1}). The acetic acid level of 79.44±0.87 mmol kg^{-1} detected in only sample M3 suggested the activities of heterofermenters and evidenced in this study was the distinct identification of two strains of *L. reuteri* (band fragment 9 and 18 of Figure 2) from this sample.

Proximate composition

The proximate composition of the samples was presented in Table 6. The moisture content varied from 465.83±0.19 to 513.89±0.20 g kg^{-1}. Traditionally *akamu* is stored in homes under excess water and is usually decanted daily and replaced with fresh water. Water decanting in *akamu* for sale is followed by squeezing out of excess water using muslin bags to obtain the cake that is moulded into balls of different sizes for sale. The observed moisture content in this study was higher than the ranges of 63 -109 g kg^{-1} reported in literature for similar fermented cereal products.[30, 31, 57-59] Variations in moisture could be a function of the amount of water squeezed out from the slurry before wrapping in cellophane bags for sale. Moisture levels are likely to influence the stability of food products. However, the pH (3.22 - 3.95) of the samples were able to check the growth of undesirable microorganisms evidenced in by absences of

Enterobacteriacae, *Yersinia, Staphylococcus aureus, Salmonella*, *Shigella*, *Listeria* and *Escherichia coli* in this study.

The proximate analysis confirmed that *akamu* is predominantly a starchy food with carbohydrate composition over 900 g kg^{-1} (of which <1 g kg^{-1} were sugars). About 65 - 75% of the cereal grain composition have been reported to be carbohydrates [60] and the traditional technique of *akamu* production, which involves several washing steps, wet milling and sieving invariably, yielded a starchy product with very low levels of other nutrients. This was evidenced by the negative correlation between carbohydrate and the other nutrient variable in the samples as follows: -0.984(0.016), -0.994(0.006) and -1 with protein, lipid and ash respectively). The observed lipid levels (21.25±0.00 - 36.83±0.08 g kg^{-1}) were similar to values reported in literature [28, 30-32, 61] but lower than values reported by Egounlety et al., [57] Osundahunsi and Aworh [62] and Oluwamukomi et al. [59] The lipid distribution in maize kernel is such that 760-830 g kg^{-1} are found in the germ with 130-150 g kg^{-1} in the aleurone layer. [63] The removal of the germ and aleurone layers during processing may have contributed to the low lipid content and absence or trace amount of ash respectively. Low lipid level may also be due to the oxidation of fatty acids by the microorganism to obtain energy for metabolic activities as reported by Oyarekua.[64] Low lipid levels are however desirable for product storage stability.

The protein and energy values ranged between 31.88±0.08 - 74.32±1.36 g kg^{-1} and 17.29±0.29 - 18.37±0.08 KJ kg^{-1} respectively. These were consistent with literature reports. According to the report of the Joint WHO/FAO/UNU Expert consultation [65] the daily protein and energy requirement for an infant female of 0.5 years old that is involved in moderate physical activity level were 1.12 g kg^{-1} and 340 KJ kg^{-1} respectively. This implies that for the same infant with an average weight of 7.34 kg WHO/FAO/UNU [28] the protein and energy values obtained in this study would meet about 39 - 90% and 0.69 - 0.73% of the protein and energy requirements respectively. Consequently, the infant would require about 111 - 258 and 136 - 144 g of *akamu* to meet the minimum daily protein and energy requirements respectively. However, higher quantities may be required as the process of the porridge preparation involves cooking and diluting the infant food with large quantities of water to obtain thin gruel.

In conclusion, this study revealed that *akamu* samples as sold in the market were predominately starchy foods and had pH < 4.0 due to the activities of the fermentative LABs with common occurrence of *Lb. fermentum, Lb. plantarum, Lb. delbrueckii* subsp*. bulgaricus* and *Lb. helveticus*. Although yeasts were conventionally isolated and later identified, establishment of the total yeast population and their relative role in the fermentation could constitute a different study. Evaluation of the fermentation characteristics of the microbial isolates would enhance the choice of starter culture for controlled fermentation that could assure the product quality and safety.

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Patience C. Obinna-Echem[1, 2]*, Victor Kuri[1] and Jane Beal[1]

1 Contact: Victor Kuri, School of Biomedical and Biological Sciences,

University of Plymouth, PL4 8AA, Plymouth, United Kingdom

e-mail: v.kuri@plymouth.ac.uk

2: Obinna-Echem, Patience Chisa, Rivers State University of Science and

Technology, P.M.B 5080, Port Harcourt, Rivers State, Nigeria

E-mail: chisanupat@yahoo.com

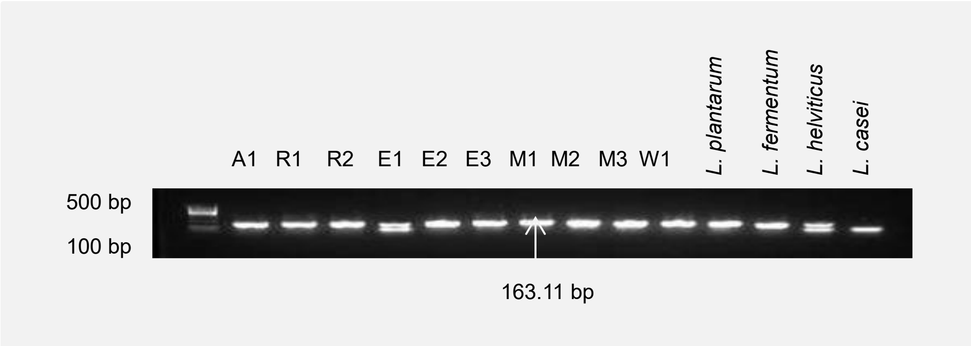

Figure 1 PCR amplified product of DNA templates of the *akamu* samples and pure bacteria cultures.

The alphabets with numbers were representations of *akamu* samples based on their origin: Aluu (A1), Emohua (E1-E3), Rumuokoro (R1 and R2), Mile 3 (M1-M3) and Worgi (W1).

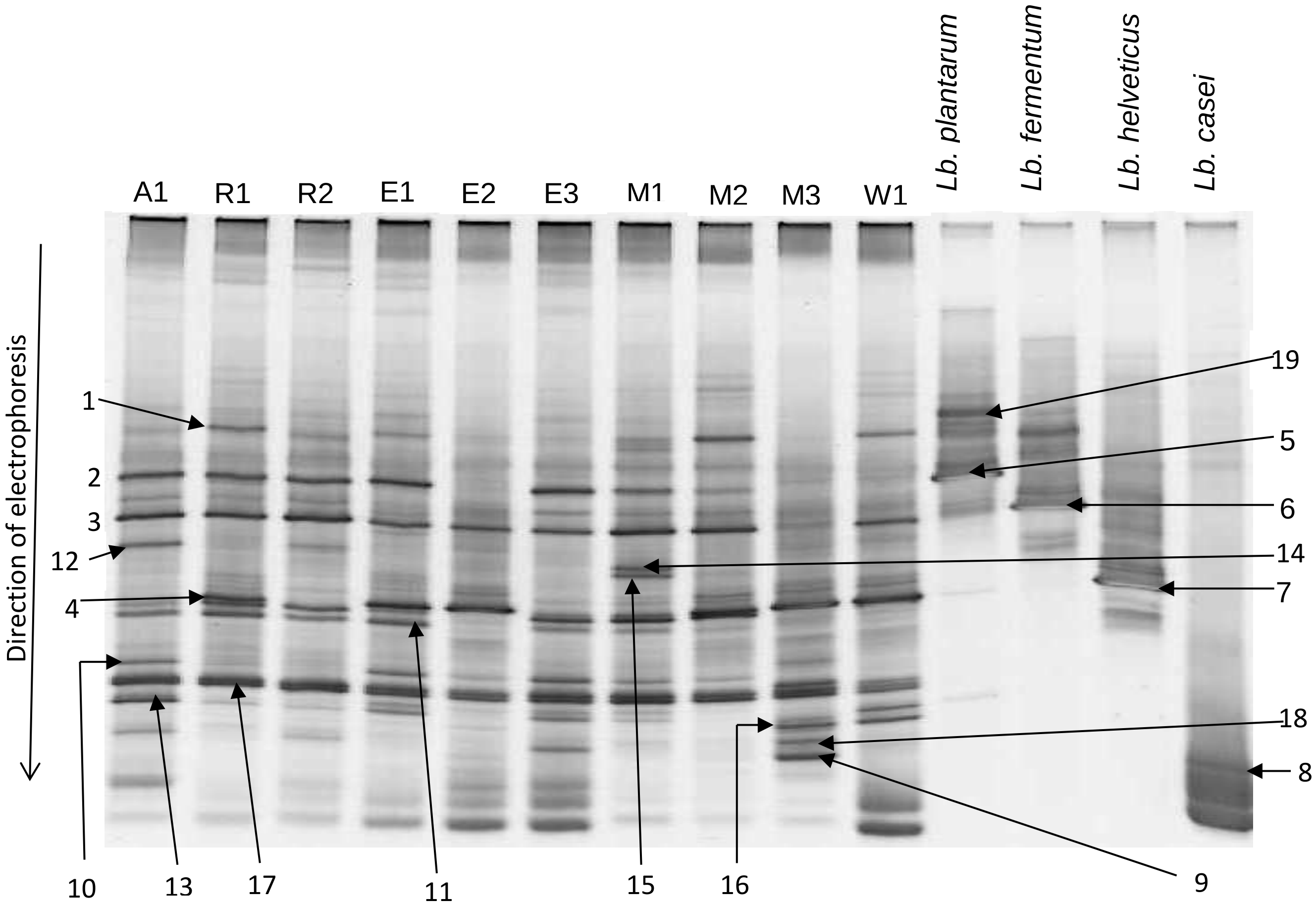

Figure 2 DGGE analysis of natural LAB population in spontaneously fermented *akamu* samples.
The alphabets with numbers were representations of *akamu* samples based on their origin: Aluu (A1), Emohua (E1-E3), Rumuokoro (R1 and R2), Mile 3 (M1-M3) and Worgi (W1).
The last four wells were the PCR product of pure LAB cultures.
The numbers 1-19 represented the fragments that were excised for sequencing.

Table 1 Identities of bands obtained from DGGE analysis of the *akamu* LAB community

*Excised band fragment No.	†Sequence length	Closest relative	‡Maximum identity (%)	Accession Number
2	92	*Lb. plantarum* WCFS1	96	NC_004567.2
3	133	*Lb. fermentum* CECT 5716	100	NC_017465.1
4	121	*Lb. delbrueckii* subsp. *bulgaricus* 2038 *Lb. helveticus* H10	100	NC_017469.1 NC_017467.1
5	126	*Lb. plantarum* WCFS1 *Lb. plantarum* JDM1	98	NC_004567.2 NC_012984.1
7	111	*Lb. delbrueckii* subsp. *bulgaricus* 2038 *Lb. helveticus* H10	100	NC_017469.1 NC_017467.1
8	142	*Lb. rhamnosus* ATCC 8530 *Lb. casei* LC2W	99	NC_017491.1 NC_017473.1
9	100	*Lb. reuteri* SD2112	95	NC_015697.1
15	104	*Lb. salivarius* CECT 5713	95	NC_017481.1
16	123	*Lactococcus lactis* subsp. *cremoris* NZ9000	100	NC_017949.1
17	74	*Zea mays* chloroplast	100	NC_001666.2
18	108	*Lb. reuteri* SD2112	96	NC_015697.1

*Band excised from DGGE gel shown in Figure 2.
†The number of nucleotides obtained from sequencing of the excised band fragments
‡Percentage sequence homology of the nucleotides in the sequence of the DGGE excised fragment and that of the closest relative found in the GenBank.

Table 2 Phenotypic characteristics and genomic identity of LAB isolated from a selected *akamu* Sample (W1)

Code	Identity		Growth				Fermentation																				
			NaCl		Temp		Arginine		Glucose																		
	Genotypic	Phenotypic	40 g L^{-1}	100 g L^{-1}	15ºC	45ºC	Ammonia	Gas	Acid	Gas	Slime	Arabinose	Cellobiose	Fructose	Galactose	Lactose	Maltose	Mannitol	Mannose	Melezitose	Mellibiose	Raffinose	Ribose	Salicin	Sucrose	Tetrahose	Xylose
NGL1w	*Lb. fermentum*	*Lb. acidophilus*	+	-	-	+	-	+	+	-	+	-	-	-	-/+	+	+	-	+	n	+	+	-	-	+*	+	-
NGL2w	*Lb. fermentum*		+	-	-	+	-	+	+	-	-	+	-	+	-	-	-	-	-	n	-	+	-	-	+	-	-
NGL3w	*Lb. helveticus*	*Lb. fermentum*	n	-	-	+	+	-	+	+	+	-	-	-	-	-	-	-	+	n	-	-	-	-	-	+	-
NGL4w	*Lb. fermentum*		+	-	-	+	-	+	+	-	-	+	-	-	-	+	+	-	+	n	+	+	+*	-	+	+	-
NGL5w	*Lb. fermentum*	*Lb. salivarius spp. salivarus*	+	-	-	+	-	+	+	-	-	-	-	+	+*	+	+	-	+	n	+	+	+*	-	+	+	+
NGL6w	*Lb. fermentum*		+	-	-	+	-	+	+*	-	-	+	-	+	-	-	-	-	-	n	-	+	-	-	+	-	-
NGL7w	*Lb. fermentum*	*Lb. fermentum*	+	-	-	+	+	+	+	+	-	+	-	+	+	+	+	-	+	-	+	+	-	+*	+	+	+
NGL8w	*Lb. rhamnosus*	*Lb. plantarum*	+	-	+	-	-	-	+	-	-	-	+	+	+	-	+	+	+	+	-	-	+	+	+*	+	-
NGL9w	*Lb. casei*	*Lb. plantarum*	+	-	+	-	-	-	+	-	-	-	-	+	+	+	+	+	+	+	-	-	-	+*	+	+	-
NGL10w	*Lb. fermentum*	*Pediococcus halophilus*	+	+	-	-	-	-	+	-	-	-	-	+	-	+	-	-	-	n	-	-	-	n	+	-	+
NGL11w	*Lb. helveticus*	*Lb. acidophilus*	+	-	-	+	-	+	+	-	-	+	+	+	+	+	+*	-	+*	n	+	+	+	n	+	+	-
NGL12w	*Lb. fermentum*	*Lb. delbrueckii subs delbrueckii*	-	-	-	+	-	-	+	-	-	-	+	+	+	-	+	-	-	n	-	-	-	n	+	-	-
NGL13w	SN	*Lb. reuteri*	-	-	-	+	+	+	+	+	-	+	-	+	+	-	+	-	-	n	+	+	-	n	+	-	-

All isolate were Gram positive and catalase negative. Esculine, rhamnose, sorbitol, sorbose and starch were negative for NGL1w to NGL6w but not tested for others.
*Weak; n - Not tested. SN – Sequencing was not successful.
NGL2w, NGL4w and NGL6w were difficult to classify phenotypically

Table 3 Phenotypic characteristics and genotypic identity of LAB isolated from a selected *akamu* Sample (M3)

Code	Identity		Growth				Fermentation																				
			NaCl		Temp		Arginine		Glucose																		
	Genotypic	Phenotypic	40 g L^{-1}	100 g L^{-1}	15°C	45°C	Ammonia	Gas	Acid	Gas	Slime	Arabinose	Cellobiose	Fructose	Galactose	Lactose	Maltose	Mannitol	Mannose	Melezitose	Mellibiose	Raffinose	Ribose	Salicin	Sucrose	Tetrahose	Xylose
NGL1	*Lb. helveticus*	*Lb. jensenii*	-	-	-	+	+	-	+	-	-	-	+	-	-	-	+	-	+	-	-	-	+	+	+	+	-
NGL2	*Lb. acidophilus*	*Lb. acidophilus*	-	-	-	+	-	-	+	-	-	-	+	+	+	-	+	-	+	-	-	-	-	+*	+	+	-
NGL3	*Lb. helveticus*	*Lb. helveticus*	-	-	-	+	-	-	+	-	-	-	+*	-	+	-	+	-	-	-	-	-	-	-	+*	-	-
NGL4	*Lactococcus lactis* subsp. *lactis*	*Leuconostoc mesenteroides*	+	-	+	-	-	-	+	+	-	-	+	+	+	+	+	-	+	-	-	-	+	-	-	+	+
NGL5	*Lb. plantarum*	*Lb. plantarum*	+	-	+	-	-	-	+	-	-	-	+	+	+*	+	+	+	+	-	+*	+	+	-	+	+	-
NGL6	*Lb. acidophilus*	*Lb. acidophilus*	-	-	-	+	-	-	+	-	-	-	+	+	+	-	+	-	+	-	-	-	-	-	+	+	-
NGL7	*Lb. plantarum*	*Lb. plantarum*	+	-	+	-	-	-	+	-	-	-	+	+	+	+	+	+	+	+	+	+	-	-	+	+	-
NGL8	*Lb. helveticus*	*Lb. helveticus*	-	-	-	+	-	-	+	-	-	-	-	+	+	-	+	-	-	-	-	-	-	-	+*	-	-

All isolate were Gram positive and catalase negative
*Weak

Table 4 Phenotypic characteristics and genotypic identity of yeasts isolated from a selected *akamu* sample W1

Code		NGY1w	NGY2w	NGY3w
Identity	Genotypic	*Candida albicans*	*Clavispora lusitaniae*	*Saccharomyces paradoxus*
	Phenotypic	*Pichia membranaefaciens,*	*Kluyveromyces marxianus*	*Saccharomyces rouxii*
Morphology	Cell	Spherical and elongated, filled with vacuoles.	Cylindrical	Spherical and elongated, in chains
Cultural	RBCA	Round, smooth, white colonies	Pink butryous (rhizoid) colonies	Big pink umbonated colonies.
	Broth	Scum	Scum	Scum
Gram Stain		+	+	+
Ascospores		-	-	+ (deploids)
Ballistospores		-	-	-
Budding		+ No mycelia	+pseudomycelium	+
Resistance to cycloheximide		-	+	-
Ethanol Utilization		+	+	+
Fermentation and gas production	Cellobiose	-	-	-
	Fructose	-	+	-
	Galactose	-	-	-
	Glucose	-	+	+
	Inulin	-	-	-
	Lactose	-	-	-
	Maltose	-	-	-
	Mannose	-	+	+
	Mellibiose	-	-	-
	Raffinose	-	-	-
	Rhamnose	-	-	-
	Sucrose	-	+	+
	Tetrahose	-	-	-
	Xylose	-	-	-

Table 5 Phenotypic characteristics and genomic identity of the yeasts isolated from *akamu* sample M3

Code		NGY1	NGY2	NGY3	NGY4
Identity	**Genotypic**	*C. tropicalis*	*C. albicans*	*C. albicans*	*C. albicans*
	Phenotypic	*Sacch. cerevisia*	*C. tropicalis*	*Zygosacch. lactis*	*C. mecedomiensis*
Morphology	**Cell**	Spherical, ovoid	Cylindrical with rounded ends	Spherical	Elongated
	RBCA	Smooth dull cream domed	Umbonated with two zones of cream and pink colour	Round big pink umbonate colonies	Pink and rhizoid
Cultural	**MEA**	Smooth shiny cream and convex	Light grey to grey	Flat, smooth, round and greyish white	White butyrous with rough edges
	Broth	Clumped at top corner of conical flask	Sediment	Sediment	Film
Gram Stain		+	+	+	+
Ascospores		Ascus (2-4)	-	Conjugation, ascus (2)	-
Ballistospores		-	-	-	-
Budding		Budding No mycelia	Mycelium with blastospore	-	Multi-lateral budding
Cycloheximide resistance		-	-	-	-
Ethanol Utilization		-	+	+	+
Fermentation and gas production	**Cellobiose**	-	-	-	-
	Fructose	+	+	+	+
	Galactose	+	-	-	+
	Glucose	+	+	+	+
	Inulin	-	-	-	+*
	Lactose	-	-	-	-
	Maltose	+	+	-	-
	Mannose	+*	+	+	+
	Mellibiose	-	-	-	-
	Raffinose	-	-	-	+*
	Rhamnose	-	-	-	-
	Sucrose	+	+	+	+*
	Tetrahose	+	-	-	-
	Xylose	-	-	-	-

*Weak

Table 6 pH, titratable acidity and lactic acid levels of *akamu* samples obtained from Rivers State, Nigeria

Origin	Sample code	pH	*TTA ($g\ kg^{-1}$)	Lactic acid ($mmol\ kg^{-1}$)
Mile 3 Diobu		(3.66±0.15)	(11.11±2.50)	(62.61±8.23)
	M1	$3.56±0.03^{cd}$	$7.51±0.52^{cd}$	$51.51±1.37^{ef}$
	M2	$3.46±0.01^{b}$	$9.91±0.00^{ab}$	$78.68±6.29^{ab}$
	M3	3.95±0.01	15.91±0.52	$57.65±3.49^{de}$
Emohua		(3.46±0.04)	(7.91±0.96)	(64±11.90)
	E1	$3.42±0.01^{a}$	$9.01±0.00^{abc}$	$64.65±2.31^{cd}$
	E2	$3.44±0.01^{ab}$	$8.71±0.52^{bc}$	$84.29±3.02^{a}$
	E3	$3.53±0.01^{c}$	$6.01±0.52^{d}$	$43.10±1.79^{f}$
Rumuokoro		(3.29±0.06)	(9.46±1.05)	(69.71±0.15)
	R1	3.22±0.01	$8.41±0.52^{bc}$	$69.56 ±2.64^{bc}$
	R2	3.35±0.00	$10.51±0.52^{a}$	$69.85±2.00^{bc}$
Aluu	A1	(3.58±0.00)	($8.41±1.04^{bc}$)	($61.93±5.63^{cd}$)

*TTA was expressed as lactic acid equivalent
Values that share the same superscript in the same column do not differ significantly ($p \leq 0.05$).
Values for the individual samples were mean of triplicate determinations ± standard deviation.
Values in brackets were origin mean ± standard error of mean except for A1.
Means based on origin did not differ significantly ($p \leq 0.05$).

Table 7 Proximate composition (g kg^{-1}) and energy (KJ g^{-1}) values of *akamu* samples obtained from Rivers State, Nigeria

Origin	Sample codes	Moisture	Carbohydrates†	Protein	Lipids	Ash	Energy
Mile 3 Diobu		(474.29±1.01)	(913.60±79)	(55.16±4.15)	(27.93±0.46)	(3.31±0.66)	(17.80±0.07)
	M1	473.01±3.54^{c}	923.05	47.23±2.47^{c}	25.70±2.03^{cd}	4.02±0.03^{a}	17.91±0.04^{abc}
	M2	492.38±2.62^{b}	919.78	56.97±0.65^{b}	21.25±0.03^{e}	1.99±0.05^{b}	17.66±0.02^{bc}
	M3	457.47±1.22	897.98	61.27±1.24^{ab}	36.83±0.77	3.92±0.11^{a}	17.82±0.24^{abc}
Emohua		(493.56±1.10)	(915.60±1.14)	(56.52±12.7)	(26.19±0.10)	(1.34±0.67)	(17.94±0.24)
	E1	476.03±1.26^{c}	943.69	31.88±0.08	24.42±1.52^{de}	ND	17.54±0.07^{bc}
	E2	490.76±0.85^{b}	906.70	63.34±0.27^{a}	27.96±1.24^{bc}	2.02±0.01^{b}	18.37±0.08^{a}
	E3	513.89±1.99^{a}	897.48	74.32±1.36	26.19±0.84^{cd}	2.02±0.00^{b}	17.92±0.05^{abc}
Rumuokoro		(487.77±2.18)	(921.67±45)	(53.37±9.76)	(23.97±0.62)	(1.00±1.00)	(17.51±0.22)
	R1	465.83±1.90	917.13	63.13±0.33^{a}	17.74±0.79	2.02±0.01^{b}	17.29±0.29^{c}
	R2	509.49±4.02^{a}	926.20	43.61±3.31^{c}	30.19±0.79^{ab}	ND	17.72±0.05^{bc}
Aluu	A1	478.12±0.75^{c}	900.81	63.70±0.03^{a}	31.49±0.75^{a}	4.00±0.02^{a}	17.97±0.28^{ab}

*Values with same superscript in the same column do not differ significantly ($p \leq 0.05$).
Values for the individual samples are means of triplicate determinations ± standard deviation
Values in brackets are origin means ± standard error of mean except for A1. Means within origin are not significantly different ($p \leq 0.05$).
†Carbohydrate was obtained by difference