SOLID STATE FERMENTATION OF PLANT PROTEIN MEALS USING *Lactobacillus acidophilus* FOR IMPROVING FEED VALUE

Abstract

Usage of some legumes and oil seed meal as fishmeal substitute is hampered by low protein content and anti nutritional factors (ANF). Inclusion of some exogenous enzyme cocktail like phytase, xylanase can reduce some ANF but is costly. Solid state fermentation of the plant proteins is affordable and could be useful in upgrading the protein content, elevating the nutrient and mineral status and eliminating ANF from ~~plant based~~plant-based feed ingredients. We therefore extracted *Lactobacillus acidophilus* from intestine of adult African catfish. Extracted *L. acidophilus* was cultured at 37°C for 48hrs in molarhilton broth. Approximately 10g of the bacteria broth containing 9.4 log 10 colony forming unit (CFU) per ml was mixed with 200g meals of ~~–~~bambaranut meal and African yam beans meal placed in a brown bottom flask. The ground meals and bacteria mixtures were fermented for 72 hours. Temperature was maintained at 28.6°C to 34°C. The pH of the mixtures was measured everyday and the fermenting mixture was regularly stirred. Fermentation was stopped after 72hrs and the meals were subjected to proximate analysis. Protein content of the meals significantly increased (P<0.05) as follows: BNM, 24.82±0.15% to 40.37±0.27% and AYB, 23.65±0.07 % to 34.56±1.36 %. Lipid content of meals significantly increased (P<0.05) as follows BNM, 7.11±0.01 to 14.29±0.05% and AYB, 2.96±0.45% to 5.76±0.09%. There was general decrease in composition of carbohydrate and ANFs were drastically reduced or completely eliminated from the meals.

Key Words: Solid State fermentation, Lactobacillus, Anti nutritional factor, sesame seed, African yam beans and bambaranut meal

INTRODUCTION

Solid state fermentation is a bioprocess where microbial organism undertakes fermentation of substrate matrix in absence of ~~free flowing~~free-flowing water [1, 2, 3]. Although abundant water is absent in solid state fermentation the substrate must have enough water to sustain growth of microbes [4]. Based on the nature of substrate used solid state fermentation can be classified into two, those cultivated on natural material and inert materials [5]. Solid state fermentation is becoming more important because of bioactive compound or secondary metabolites produced in the process [6, 7, 8]. Solid sate fermentation has been used in reduction of ~~non starch~~non-starch polysaccharides and α-galactosides of soybean meal [9]. It has also been used in degrading glucosinolate ~~-~~in rapeseed meal [10]. Solid state fermentation could produce enzyme like phytase [4], xylanase [11], ~~-~~glucanases and xylanase ~~-~~[12], from the bioprocess of the microbe on the substrate matrix. These enzymes have immense application in feed industry. African yam beans (AYB) *Sphenostylis stenocarpa* is a neglected legume belonging to the family *Papilionacea*, subfamily *Leguminosae* [13]. African yam beans are cultivated in Western, Central and Eastern Africa. AYB is proteinous and the protein content is about 21-24% [14, 15]. Africa yam beans have been included in feed of African catfish with mixed results. Bambaranut (*Voandzeia subterranea*) is a ~~proteinous~~proteinoid legume belonging to the family Fabaceae. Bambaranut has always been regarded as of African origin therefore a C4 plant [16, 17]. But analysis of naturally occurring stable isotopes of δ^{13}C and δ^{15}N showed that Bambaranut is ~~actually a~~a C3 plant like soybean [18]. ~~Consequently~~Consequently, it could be that bambaranut was introduced by early explorers or is an outlier in the C4, C3 plant continuum. The crude protein content of bambaranut is 24–28 % [16, 17, 19]. The crude lipid content of Bambaranut is about 12–18 % [20, 17, 21]. Bambaranut is a good substitute of soybean in the diets of African catfish. Bambaranut also has lesser content of ANF like phytate than soybean [22]. Substitution of fishmeal with solid state fermented bambaranut meal (BNM) in the diets of African catfish *C. gariepinus* produced faster growth rate of the fish than the unfermented BNM [23]. Lactic acid bacteria LAB and carnobacterium species occurs as normal flora within the intestine of most healthy fish [24, 25]. The application of LAB in fermentation of feed products enhances the palatability and microbiological safety [26].

This research is aimed at analyzing the nutritional effects of separately fermenting bambaranut meal (BNM) and African yam beans (AYB) meal with *Lactobacillus acidophilus* using solid state techniques.

MATERIALS AND METHODS

African yam beans: Grains of African yam beans (AYB) were purchased from open grain market at Enugu Nigeria. The grains were sorted to remove unwanted particles and stones. Sorted AYB were then autoclaved at 100^{o}C for 15mins, cooled and then cracked in a mill. The seed coats were removed after the cracking and the seed were ground to dust using a hammer mill. The ground meals were stored in air tight container till used within 24hrs.

Bambaranut meal: Bambaranut meal was produced from bambara groundnut purchased from open grains market in Enugu Nigeria. The grains were carefully ~~sorted~~sorted, and bad grains and stones were removed. The grains were washed with clean water and dried at 55^{o}C for 1h. The bambaranut were then autoclaved at 100^{o}C for 5 mins. After autoclaving the seed were cooled and cracked in a hammer mill and the grains were milled to dust, so as to pass a ~~40 mesh~~40-mesh sieve and stored in air tight container for use within 24hrs.

Micro organism used and ~~solid state~~solid-state fermentation

The *Lactobacillus acidophilius* used in this experiment were extracted from the gut of matured African catfish *Clarias gariepinus.* Mature African catfish of weight 865g and length 68cm were stocked at 2 fish per 35 litre glass ~~aquarium~~aquaria. The catfish was sacrificed with a gentle blow on the head. The stunned fish was ~~dissected~~dissected, and the gut was divided into foregut, mid gut and hind gut. The gut was cut open horizontally and 5g of the intestine piece was cut and minced in a test tube with distilled water making it up to 1ml. The 1ml stock solution was mixed with 9mls of distilled water to give a 1:10 dilution. The mixture was vortex for 5mins. This same procedure was carried out for intestinal samples from mid gut and hind gut. The stock solution was diluted with sterile 0.1% peptone water up to 10^{-6} according to [27]. 1m of the stock dilution was spread using pour plate techniques, on two replicate plates of nutrient agar, tryptic soy agar plates (TSA; MERCK, ~~-~~GERMANY). MacConkey agar and Eosin methylene blue agar, were added to determine the total bacterial counts, using sterile glass spreader. The agar plates were incubated at 36^{o}C for 48hrs. Plates were read after incubation by considering and selecting those plates containing between 30-300. The counting was done using and illuminated colony counter. The isolation of identified colonies was done by sub culturing of representative samples on freshly prepared plates. The plates were incubated at 37^{o}C for 48 hours. The colonies were subculture in tryptic soy agar plates (TSA; Merck, Germany) to obtain pure cultures. Bacterial isolates were subjected to morphological and biochemical characterisation of the sub cultured based on Gram staining techniques according to the Bergey's manual of determinative bacteriology [28, 27]. Morphological characteristics examined ~~color~~colour, edge, elevation, shape and arrangement of microorganisms. Microorganisms were examined under slide was made in oil immersion after Gram staining. The biochemical tests carried out in characterisation of the microbes were catalase test, coagulase test, motility test, oxidase test after [29]; sugar fermentation test and Voges –Proskauer test [30]. Extracted *L. acidophilus* was cultured at 37°C for 48hrs in Mueller Hinton broth. The fermentation was done in triplicates. The grinded plant protein meals (bambaranut meal, sesame seed meal and African yam beans meal) were weighed and 200g, separated for the experiment. The `grinded meals were placed in a brown bottom flask and 10g of the bacteria (*L. acidophilus*) broth containing 9.4 log 10 colony forming unit ~~-~~(CFU) per ml was mixed with the meals. The mixtures were fermented for 72 hours. The temperature was regularly checked and recorded. The temperature of the mixture ranged from 28.6°C to 34°C. The temperature of the fermented meal fluctuated constantly from 28.6°C to 34°C through the period of ~~solid state~~solid-state fermentation. The mixtures were stirred according to methods stated in Enyidi and Etim [23]. The pH of the mixtures was measured everyday using a pH meter. The fermentation was arrested after 72hrs and the plant protein meals were subjected to proximate analysis to determine the effects of the ~~solid state~~solid-state fermentation of the nutritional quality of the meals.

Proximate analysis

The crude protein analyses dried samples were done by Kjeldahl method using Tecator kjeltec model 1002 system with block digestion plus steam distillation. The crude protein was calculated as %N x 6.25. The total lipids of the fermented meals were analyzed by chloroform-methanol extraction at a ratio of 2:1 [31, 32, 21]. Moisture content of the feeds was determined by oven drying feed samples at 105°C. Ash content was determined by incineration samples in a muffle furnace at 550°C for 24 hrs. The ash % was weight of ash/weight of sample x 100. The energy value was measured using a bomb calorimeter and expressed in kcal.

Anti nutritional factors

The phytate was measured after [33]. The phytic acid of the raw and fermented meal variants were analysed.

Mineral composition

The metal contents of the meals were measured by weighing 2.0 g of the meals mixing this with the digesting mixture made of 1ml of 30 % hydrogen peroxide (H_20_2) and 6 ml of concentrated nitric acid (HNO3). The mixture was placed in a microwave set at 70^{o}C till digestion was over. The digested samples were filtered using what-man filter paper, the filtrate was diluted with distilled water in a 250ml volumetric flask. Resultant solution ~~were~~was analysed for metals using Atomic Absorption Spectrophotometer (UNICAM 939) that is connected to MS Window application software.

Calculations and statistical analysis

The mean values of the proximate analysis from the three plant protein meals were subjected to ~~one way~~one-way analysis of variance (ANOVA). Pair wise independent t test was carried out to examine significant differences between the proximate analyses of fermented and ~~non fermented~~non-fermented variants of each plant protein meal.

Process flow chart for production of solid state fermented African yam beans meal (AYBM) for improved feed production

African yam beans seed purchased

↓

Sorting and removal of unwanted material and bad seed

↓

Washing of seed with clean water

↓

Drying of seed at 55°C

↓

Milling of seed to dust in hammer mill

↓

Sieving of meal and removal of particles of hard seed coat

↓

Cooling of AYBM at room temperature

↓

Mixing with *L. acidophilus* broth

↓

Fermented for 72 hours

↓

Drying of seed at 55°C

↓

Milling of meal in attrition mill

↓

Solid state fermented AYBM

↓

Storage in cool dry place

Process flow chart for production of solid state fermented -Bambaranut meal for improved feed production

Bambara ground nut seed purchased

↓

Sorting and removal of unwanted material and bad seed

↓

Washing of seed with clean water

↓

Drying of seed at 55°C

↓

Milling of seed to dust in hammer mill

↓

Sieving of meal and removal of particles of hard seed coat

↓

Cooling of bambaranut meal at room temperature

↓

Mixing with *L. acidophilus* broth

↓

Fermented for 72 hours

↓

Drying of seed at 55°C

↓

Milling of meal in attrition mill

↓

Solid state fermented BNM

↓

Storage in cool dry place

RESULTS

The results of pair wise independent t test analysis of proximate content of bambarant meal shows that there are significant differences between the proximate composition of fermented and ~~non fermented~~non-fermented bambaranut meal. The proximate compositions of the raw bambaranut meal are tabulated in Table 1. The proximate compositions of bambaranut meal were generally increased after the four days of fermentation. Protein content of the fermented meal (40.37±0.27%) (means ±SD) was significantly higher than the raw meal (24.82±0.15%) ($P<0.05$) table 2. The lipid content of the BNM was significantly increased from 7.11±0.01% of the raw BNM to 14.29±0.05% of the fermented BNM ($P<0.05$). Conversely, the carbohydrate content of the fermented BNM (20.65±0.27 %) was much lower than the content of the raw BNM 54.59±0.06% (Table 2). Crude fibre of the raw BNM was 7.62±0.15% but this was reduced to 2.41±0.06 in the fermented BNM. Moisture content of the raw BNM was significantly increased after the ~~solid state~~solid-state fermentation. Moisture content increased from 9.15±0.06% of the raw BNM to 16.26±0.59% of the fermented BNM ($P<0.05$). ~~Consequently~~Consequently, dry matter of the fermented BNM, 83.74±0.58 was lower than that of the raw BNM 90.8±0.01. There was however no difference in the dry matter of the fermented and raw BNM ($P>0.05$~~).There~~). There was however a significant increase in the ash content of the fermented BNM 9.53±0.03% compared to the raw BNM 4.52±0.03% ($P<0.05$).

Copper, sodium, iron and zinc. Raw bambaranut meal is a good source of calcium. The calcium content of raw bambaranut meal was 244.5 ± 0.06 mg/100g. Solid state fermentation of BNM significantly ($P<0.05$) elevated the calcium content to 400.06±0.12mg/100g. Phosphorous composition of raw BNM was 74.56±0.78, while fermented BNM had phosphorous content of 140.56±0.56mg/100g (Table 3). ~~Similarly~~Similarly, there was significant increase in the potassium content of the fermented meal. The raw BNM had potassium content of 182.09±0.08mg/100g while the fermented had 203.67±0.05 mg/100g. The magnesium (Mg) content of the BNM was not much affected by the ~~solid state~~solid-state fermentation. The Mg content of the raw BNM was 134.05±0.58 mg/100g but after fermentation the Mg value was significantly increased to 183.47±0.13mg/100g ($P<0.05$). The copper content of raw BNM was 3.89±0.78mg/100g but this was doubled 6.23 ± 0.89mg/100g in the solid state fermented BNM (Table 3). Raw BNM has low content of sodium 19.98±0.56mg/100g. Solid state fermentation of BNM significantly ($P<0.05$), increased the sodium content to 29.09±0.08mg/100g. Conversely, the iron content of the raw BNM was very low 1.57±0.07mg/100g. The iron content of the fermented BNM 1.54±1.23mg/100g was not significantly different from the raw BNM ($P>0.05$). Zinc content of raw BNM was 20.81±0.03mg/100g, but fermentation of BNM did not produce any significant increase on the zinc 20.88±0.87mg/100g. Raw BNM had phytate content of

0.87±0.06mg/100g. After the solid statesolid-state fermentation of BNM, phytic acid was not detectable from the meal (Table 2). The analysis of tannins in BNM showed that raw BNM had 16.73± 0.06 mg/100g of tannin. However, after solid state fermentation the tannins were nonno detectable (Table 3).

Table 1. The proximate composition of raw bambaranut meal and African yam beans used in solid state fermentation

Parameters	Bambaranut	African yam beans	$FLSD_{0.05}$
Protein	24.82±0.15[a]	18.61±0.39[c]	0.1747
Lipid	7.11±0.01[b]	5.19±0.03[c]	0.18808
Carbohydrate	54.59±0.06[a]	56.49±0.49[a]	0.14325
Crude fiber	7.62±0.15[a]	7.61±0.02[a]	0.18487
Moisture	9.15±0.06[b]	9.83±0.05[a]	0.21777
Dry matter	90.8±0.01[ns]	90.17±0.05[ns]	0.89255
Ash	4.52±0.03[c]	4.93±0.04[b]	0.14897
Phytic acid	0.87±0.06[c]	1.02±0.09[b]	0.15712
Energy	12627.34±58.36[a]	12543.66±31.91[c]	0.09812

Proximate compositions were measured in percentage (%) but energy was measured in kcal. Means not followed by same superscript are significantly different $P<0.05$, values are means ±SD

Table 2. Proximate composition of solid state fermented bambaranut meal and African yam beans

Parameters	Bambaranut	African yam beans	FLSD 0.05
Protein	40.37±0.27[a]	29.85±0.51[c]	0.11480
Lipid	14.29±0.05[b]	9.00±0.33[c]	0.23079
Carbohydrate	20.65±0.27[b]	29.86±1.03[a]	0.12735
Crude fiber	2.41±0.06[b]	2.00±0.01[c]	0.14420
Moisture	16.26±0.59[b]	18.83±0.90[a]	0.18095
Dry matter	83.74±0.58[a]	81.17±0.90[b]	0.15113
Ash	4.53±0.03[a]	3.13±0.08[c]	0.23358
Phytic acid	nd	nd	
Energy	13631.01±59.11[b]	13547.66±32.85[c]	0.05812

Proximate compositions were measured in percentage (%) but energy was measured in kcal. Means not followed by same superscript are significantly different $P<0.05$, values are means ±SD

Table 3. Minerals and anti-nutritional factors of Raw and Fermented Bambaranut meal

Parameters in	Raw -bambaranut	Fermented bambaranut
Trypsin inhibitor	6.56±0.02^{a}	3.29±0.04^{b}
Tannins	16.73± 0.06	nd
Calcium	8+.5 ±0.06^{a}	14.06±0.12^{b}
Phosphorous	74.56±0.78^{b}	140.56±0.56^{a}
Potasium	182.09±0.08^{b}	203.67±0.05^{a}
Copper	3.89±0.78^{b}	6.23±0.89^{a}
Sodium	19.98±0.56^{b}	29.09±0.08^{a}
Iron	1.57±0.07^{ns}	1.54±1.23^{ns}
Zinc	20.81±0.03^{ns}	20.88±0.87^{ns}
Energy	12627.34±58.36^{b}	13631.01±59.11^{a}

Means not followed by same superscript are significantly different P<0.05

Values are mean ±SD

Table 4 Minerals and anti-nutritional factors of Raw and Fermented African yam bean

Parameters	Raw African yam beans	Solid state fermented African yam beans
Oxalic acid	2.40— ±0.01	nd
Trypsin inhibitor	5.98 ±0.07	nd
Calcium	228.78±0.67^{ns}	231.6±0.07^{ns}
Phosphorous	24.06±0.09^{b}	57.94±0.04^{a}
Potassium	24.98±1.08^{b}	30.34±1.23^{a}
Magnesium	40.40 ±0.43^{b}	54.45±0.34^{a}
Copper	2.32±1.24ns	2.65±0.07ns
Sodium	348.39±0.07^{b}	398.56±0.08^{a}
Iron	11.32±0.9ns	11.33±0.56ns
Zinc	7.09±0.21ns	6.04±1.02ns
Energy	12550.55±0.26^{b}	14550.55±0.26^{a}

Means not followed by same superscript are significantly different P<0.05,

Values are means ±SD

Trypsin inhibitors contained in the raw BNM was 6.56±0.02mg/100g. ~~Similarly~~Similarly, the content of trypsin inhibitors in the raw BNM was 6.56±0.02mg/100g, while it was significantly reduced (P<0.05) to merely 1.29±0.04 mg/100g in

The energy value of the BNM showed a significant increase from 12627.34±58.36 kcal of raw BNM to 13631.01±59.11kcal (Table 3) of FBNM. Fermentation significantly increased the protein content of AYB from 23.65±0.07% of raw AYB to 34.56±1.36% of fermented variant (Table 4). Lipid content of AYB were also increased from 2.96±0.45% (raw AYB) to 5.76±0.09% (fermented AYB). The carbohydrate content of the AYB was reduced by fermentation to to 4.21±0.07% (Table 4). The mineral content of AYB increased after solid state fermentation compared to the raw AYB (Table 4). Conversely ANF like trypsin inhibitors, phytic acids and oxalic acid were drastically reduced or ~~non detectable~~non-detectable (Table4). The energy content of the meals also increased from 12550.55±0.26 Kcal of raw AYB to 14550.55±0.26kcal in the fermented variant.

DISCUSSIONS

Solid state fermentation of BNM ~~-~~and AYB was useful in upgrading their nutritive values. Solid state fermentation process had been used for improvement of plant protein ingredients [2, 9, and 23]. The increase in protein content of the fermented BNM from initial value of 24.82±0.15% [34, 35], to 40.37± ~~-~~0.27%, is significant quality improvement. The protein increase could be because microbe used in the ~~solid state~~solid-state fermentation secreted proteins as the fermentation proceeded. This had been noted in a previous work [36]. Solid state fermentation had been noted to increase the protein contents of fermented meals like bambaranut meal [23]; rapeseed cake [10]; Soybean meal [10] and cassava meal [37]. Reduction in carbohydrate content of BNM could also be due to hydrolysis of sugars and amylolytic activities of the *L. acidophilus*. Microbial amylase activities within fish gut has been documented [38]. The reduction in sugar contents makes BNM more suitable as feed ingredients for carnivorous fish. Bambaranut meal is known to have about 50-58% carbohydrate [39, 35]. High content of carbohydrates could lead to ~~hyperglysaemia~~hyperglycaemia in carnivorous fish [40], high glycogen and elevated hepatosomatic index [41, 42, 43]. The reduction in sugar could also mean that BNM inclusion in the diets of any fish could lead to lesser deposit of fat in the fish. Carbohydrates gets converted and stored as fat in the body of fish. The lipid content of the BNM was doubled after fermentation. This suggests more energy value of the feed if fermented BNM is used in production. Fish use lipids for their energy needs, thereby sparing protein [44]. The lipid content of BNM in the research, 7.11±0.01 and 14.29±0.05 for raw and fermented respectively was in line with previous findings of between 3.11±0.01% to 9.0% [45]. The increase in lipid content of fermented BNM would be beneficial in feed formulation because the energy value of the feed would be increased. Fermentation of BNM reduced the crude fiber content from 7.62±0.15 to 2.41±0.06%. This is important attribute since most fish find it hard to digest fiber. In a previous research [46] noted that fermentation of bambaranut was more effective in reducing ANF than other processing methods. The complete removal of phytic acid is very significant since phytic acid is a major ANF present in plant protein meal [47, 48~~,~~]. The increase in the protein content of fermented AYB is very significant and in line with previous findings of Chikwendu et al. [49] and ~~[49] and 50]~~ Iyang and Zakari [50] on fermented AYB. Similar results were derived for fermented soybeans by Omafuvbe et al., [51], for rapeseed by Shi et al., [10] -and for bambaranut meal Enyidi and Etim [23]. The increase in protein content could be due to the increase in biomass of the bacteria agent of fermentation [2], ~~and also~~and due to the proteolytic action of the bacteria. African yam beans have high content of lysine and an increase in the protein content may also lead to increase in some essential amino acids. In a previous research Wang *et al*. [52], and Uckun *et al*. [53], noted that solid state fermentation of rapeseed meal with *Aspergillus* *oryzae* produced free amino acids, increasing protein value of fermented meal. There is little lipid contained in AYB but solid sate fermentation increased AYB lipids content. This could be because of the possible utilization of the AYB carbohydrate and production of fatty acids and as energy source [10].

CONCLUSIONS

Solid state fermentation is a good means of upgrading the nutritional values of plant protein meals. The reduction in carbohydrate content of the meals and the increase in energy level suggest that solid state fermented BNM and AYB could be good ingredients in diets of carnivorous fish. The upgrading of pant proteins using solid state fermentation could be easily applied in ingredient processing instead of dosing with micronutrients. Fermented plant proteins seem to be plausible choice ingredients in aquafeed manufacturing.

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