Effect of Starter culture on Anti-Nutritional Factors and Shelf Life of Fermented Smoked Cassava (Pupuru): An African Fermented Staple

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Abstract: Lactic acid bacteria (LAB) are widely employed in food fermentation processes for the biosynthesis of certain important products or metabolites which helps in breaking down anti-nutritional factors in some food. This study investigated the role of LAB in extending the shelf life and reduction in anti- nutritional factors of 'pupuru' produced with selected LAB strain with the best technological properties. Fresh cassava tubers (*Manihot esculenta* Crantz) were purchased at Oja Tun-tun, Ile-Ife, Nigeria. The cassava tubers were washed with distilled water to remove adhered surface soil particles, peeled, chopped and thoroughly washed with sterile distilled water. Two LAB strains with desirable technological properties were selected as starter for this study. The cassava chips were divided into three portions for fermentation (one was fermented with *L. plantarum* only, the second was co-fermented with *L. plantarum* and *L. fermentum* and the third portion was fermented spontaneously that is without starter culture Changes in pH, titratable acidity, and hydrogen cyanide were monitored using standard procedures during fermentation. Reduction in anti nutrient and microbial load of the starter- produced and spontaneously- produced 'Pupuru' samples were determined. Lactic acid, hydrogen peroxide and diacetyl production by the isolates ranged from 0.2365-0.6418, 0.0008-0.0014, and 0.0962-0.2217 g/L respectively. There was a reduction in the hydrogen cyanide content of '*Pupuru*' samples as fermentation progressed (0.13-0.00 mg/100g). Cyanogenic glycosides reduced from 0.87-0.02 mg/100g, phytate from 65.00-31.67 mg/100g and protease inhibitor from 0.47-0.00 mg/100g. The microbiological monitoring of the quality assessment showed that 'Pupuru' produced through combined starter had a consistent microbiological quality during storage and the microbial load was within acceptable levels when compared with spontaneously fermented ones. The study concluded that combination of *Lactobacillus plantarum* and *L. fermentum* could be used as starter to reduce the anti-nutritional factors and assess the microbiological quality of smoked fermented cassava ('Pupuru') during storage.
Keywords: Anti-nutritional factors, Enzyme, Storage, Cassava, Lactic acid bacteria

INTRODUCTION

Cassava is a starchy root crop and a major source of food security in Africa because of its ability to grow in low-quality soil, its resistance to drought and disease, and its flexible cultivation cycle (Sanni *et al*., 2009; McNulty and Oparinde, 2015). Cassava has been reported to be the fourth most important food crop in developing nations after rice, wheat and maize/corn (Johnson *et al*., 2005). Cassava has also been reported to be world's third largest source of carbohydrates for human food (Nassar and Ortiz, 2010). Cyanide is the most toxic factor restricting the consumption of cassava roots and leaves; indeed, this is the main reason why cassava is not commonly consumed in Western countries (Montagnac *et al*., 2009). Cassava contains cyanogenic glucosides that are toxic for humans and which can lead to serious health disorders. The crop contains potentially toxic levels of cyanogenic glucosides in form of linamarin (95% of total cyanogen content) and lotaustralin (5%). Linamarin is present in all cassava tissues and it is synthesized from the amino acid valine. A great number of recent studies reported about many biotechnological approaches to improve the safety and quality of cassava products (Santana *et al*., 2002; Onitilo *et al*., 2007; Shittu *et al*., 2007) the effect of different processing modalities of the roots on the level of toxic substances and functional properties has been also assessed (Udensi *et al*., 2005). Boiling, steaming, baking and frying, drying, fermentation, steam distillation, starch production, as well as combination of more than one of these methods have been implemented in order to reduce the cyanide levels.

LAB have been used as food and feed preservatives for centuries, and bacteriocin-producing LAB could replace chemical preservatives for the prevention of bacterial spoilage and the outgrowth of pathogenic bacteria in food products (Yi-sheng Chen *et al*.,2010). In dairy and food industries, lactic acid bacteria(LAB) and related organisms have been used as starter culture and they plays very imperative role in fermentation. They may reduce anti nutritional quality, improve nutritional, organoleptic and shelf-life characteristics in diverse fermented foods and beverages (Shah and Prajapati, 2013; Capozzi *et al*., 2012).

MATERIALS AND METHODS

Sample collection and processing of cassava

Fresh cassava tubers (*Manihot esculenta* Crantz) were purchased from Oja Tun-tun, Ile-Ife, Osun State. The cassava tubers were washed with distilled water to remove adhered surface soil particles. The tubers were peeled, chopped and thoroughly washed with sterile distilled water. Two kilograms of peeled, chipped and thoroughly washed cassava was submerged in two litres of sterile distilled water in a sterile 5 litres Erlenmeyer flask and surface sterilized with 3% hydrogen peroxide for ten minutes and rinsed with sterile distilled water severally and was then inoculated aseptically with standardized 2ml of the starters (3.0 x10^8cfu/ml). Fermentation was allowed for 120 hours. The starter fermented cassava mash was removed from water into a clean sterile muslin cloth bag to drain-off the water. It was then milled aseptically and moulded using sterile containers into balls and dried in an oven at 60°Cor 5 days. The outer part of the balls was scrapped off with sterile knife and the remaining was milled into *'Pupuru'* powder.

Selection of Starter

Lactobacillus plantarum (B2) and *Lactobacillus fermentum* (A1) were selected based on their ability to produce enzymes such as Rhodanase which helps in breaking down of cyanide in cassava, amylase, alpha galactosidase and invertase, and antagonistic activity as possible candidates for production of starter-induced 'Pupuru'.

Studies on the microbiological quality assessment of Spontaneous and Starter-mediated 'pupuru' during storage

The freshly produced 'pupuru' (spontaneous and starter-mediated) were stored at ambient temperature 30±2°C for 3 months in sterile plastic container with covers. During the storage period, samples were withdrawn for viable microbial count every 2 weeks for 3 months (Shinde, 2002).

Evaluation of Changes in the Total Bacteria Count, Coliform and Fungal Count during storage

The total bacteria count, coliform and fungi count were determined following standard methods using nutrient agar, MacConkey agar and potato dextrose agar respectively. Ten gram of *'Pupuru'* samples were weighed and transferred into 90ml of Maximum recovery diluents (MRD) and serially diluted up to 10^{-5}. Exactly 1 mL of appropriately diluted *'Pupuru'* samples were pour plated on nutrient agar, MacConkey agar and Potato dextrose agar for enumeration of total bacteria count, coliform and fungi respectively. The plates for viable and coliform counts were incubated at 37° C for 24h, and fungi plates were incubated at ambient temperature for 72h after incubation the plates were observed and colonies were enumerated and expressed as cfu/ml for bacteria and sfu/ml for fungi

Determination of Anti- nutritional Factors

Determination of hydrogen cyanide (HCN) content

This is determined by alkaline picrate colorimetric method of Kobawila *et al.* (2005). Two grammes of 'Pupuru' sample was dispersed in 50ml of distilled water in a 25ml conical flask. An alkaline picrate paper was hung over the sample mixture and the blank in their respective flasks.

The set up were incubated overnight and each picrate paper is eluted or dipped into a 60ml of distilled water. A standard cyanide solution was prepared and diluted to a required concentration. The absorbance of the eluted sample solutions were measured with colorimeter at 540nm wavelength with the reagent blank at zero.

The cyanide content was determined by the formula shown below:

$$\text{HCN}\frac{\text{mg}}{\text{kg}} = 100 \times au \times C \times D \ldots\ldots Eqn\ 1$$

W as

Where W =weight of sample analyzed; au = absorbance of test sample

as = absorbance of standard HCN solution; C = concentration of the standard in mg/d

D = dilution factor where applicable;

Determination of phytate content

Phytate in 'Pupuru' sample was determined using the Bipyrimidine colorimeter method described by Onwuka (2005). A weighed sample (2g) was soaked in 50mL of 0.2N HCl solution and shaker for 30min in a (XYT-2 model). It was filtered to obtain the extract. A portion of the extract (0.5mls) was dispensed into a test tube and 1ml of acidified ferrous ammonia sulphite solution was added to it. The tube was stoppered and boiled in water bath for 30min. It was then cooled in ice water for 15min and allowed to reach room temperature. The mixture was centrifuged at 3000 rpm for 5min and the supernatant was collected for analysis. Exactly 1mL of the supernatant was mixed with 1.5ml of 2.2 Bipyridine solutions. Meanwhile a standard solution of phytate was prepared and diluted to different concentration (mg/100g). Exactly 1mL of the standard solution was treated the same way as the sample extract as described above. The absorbance of the standard and the sample were read in a spectrophotometer at a wavelength of 519nm. A reagent blank was used to set the instrument at zero. The formula below was used to calculate the phytate content.

$$\%\text{phytate} = 100 \text{ x au x c x Vt } \ldots\ldots \text{Eqn } 2$$

W as 100 Va

Where au = Absorbance of sample; as = Absorbance of standard solution

c = Concentration of the standard; Vt = Total volume of extract

Va = Volume of extract analyzed.

Determination of protease inhibitor

Egg albumin 2% solution and 0.1% solution of Bromelain, both in pH 7 phosphate buffer, were prepared. Exactly 5 mL of the egg albumin substrate and 1 mL of the Bromelain enzyme were incubated at 55°C for 10 min. About 5 mL of 10% Trichloroacetic acid (TCA) was added to stop the reaction. The precipitate was filtered off with Whatman No. 1 filter paper and the absorbance of the filtrate was measured at 280 nm on Spectrophotometer. The entire procedure was repeated but incubating with the enzyme and substrate mixture, i.e. 1 ml of the extract of the material for protease inhibitor determination labelled (As). The absorbance of the filtrate was measured at 280 nm. This was denoted Ai.

$$\%\ \text{protease inhibitor} = \frac{\text{AS} - \text{Ai}}{\text{AS}} \times 100 \ \ldots\ldots Eqn\ 3$$

Where AS= Absorbance of sample

Ai= Absorbance of blank/initial

Statistical Analysis

The data obtained in this study were subjected to analysis of variance and Duncan multiple range of variables using the SARS statistical software.

RESULTS

Changes in rhodanase production in fermenting cassava inoculated with single and combined starter cultures during fermentation.

Rhodanase production increased in the fermenting cassava as fermentation progressed.

It was noted that cassava inoculated with combined starter has higher rhodanase production than the singly inoculated cassava and the spontaneously fermented cassava. It was also observed that there was peak production of this enzyme on day 5. Changes in the rhodanase production are shown in Table 1.

Table 1: Rhodanase Production by the Starters during Fermentation

	Fermentation period (Days) / rhodanase enzyme production			
Sample code (RU/Min)	**Day 0**	**Day 2**	**Day3**	**Day 5**
P1	*13.85±0.88^{c+}	39.26±6.40^{b}	47.35±3.25^{a}	3.36±2.17^{d}
P2	27.65±8.71^{c}	39.51±3.91^{b}	83.28±5.81^{a}	9.75±1.59^{d}
P3	44.44±0.65^{c}	46.76±1.23^{c}	95.03±2.29^{a}	64.78±7.35^{b}

*Values are mean of three replicates ± standard error
P1= Spontaneously Fermented 'Pupuru'
P2= 'Pupuru' Fermented with *L. plantarum*
P3='Pupuru' Fermented with *L. plantarum* and *L. fermentum*
+ Values with the same superscript are not significantly different and the values are compared within each row

Table 2: Enzyme Produced by the Lactic Acid Bacteria Isolates

Lab Isolates	Amylase (mg/mL)	Invertase (mg/mL)	Alpha galactosidase (mg/mL)
A3	*1.205±0.01^{b+}	0.350±0.01^{d}	1.910±0.01^{c}
A1	1.010±0.01^{c}	0.430±0.01^{c}	1.970±0.01^{b}
B2	1.310±0.01^{a}	0.485±0.01^{a}	1.980±0.00^{a}
G1	1.030±0.01^{b}	0.210±0.01^{e}	1.150±0.00^{d}
C3	1.055±0.01^{b}	0.450±0.00^{b}	1.150±0.00^{d}
E5	0.910±0.01^{c}	0.470±0.01^{a}	1.755±0.01^{c}
B1	0.955±0.01^{c}	0.140±0.01^{d}	1.960±0.01^{b}

*Values are Mean of three replicates ± Standard Deviation
+ Values with different superscript are significantly different and the values are compared with each column

Changes in the cyanide content in cassava inoculated with single and combined starter cultures during fermentation

Changes in the cyanide content in fermenting cassava inoculated with single and combined starter cultures of LAB are presented in Table 3. Cyanide content of the fermenting cassava reduced drastically from day 3 and no cyanide was detected in the fermenting cassava for 'Pupuru' with combined starter on day 7.

Table 3: Hydrogen Cyanide Content of Raw Cassava, Spontaneously Fermented, Starter-mediated 'Pupuru' Samples

	Fermentation period (Days) / Hydrogen cyanide content			
Sample code	**Day 0**	**Day 2**	**Day 3**	**Day 5**
P1	*0.12±0.01^{a+}	0.07±0.01^{b}	0.04±0.01^{c}	0.01±0.00^{c}
P2	0.12±0.00^{a}	0.09±0.00^{b}	0.05±0.00^{b}	0.02±0.01^{b}
P3	0.13±0.00^{a}	0.07±0.00^{b}	0.02±0.00^{b}	0.00±0.00

*Values are Mean of three replicates ± Standard Error ND=Not Detected
P1= Spontaneously Fermented 'Pupuru'
P2= 'Pupuru' Fermented with *L. plantarum*
P3='Pupuru' Fermented with *L. plantarum* and *L. fermentum*
+ Values with the same superscript are not significantly different and values are compared within each row

Anti-Nutritional Composition of Raw Cassava, Spontaneous and Starter-induced 'Pupuru'

Values obtained from the anti-nutritional analysis of the samples showed that protease inhibitor was not detected in 'Pupuru' with combined starter. The highest concentration of all the anti-nutrient components was detected in the raw cassava with values of (65.00mg/100g) for phytate (0.87mg/100g) for protease inhibitor and (0.47mg/100g) for cyanogenic glycosides while starter culture fermented 'Pupuru' and spontaneously fermented *'Pupuru'* shows a relatively lower concentration for the anti-nutrients. The detail of this is shown in Table 4

Table 4:Anti-nutrients Content of Raw Cassava, Spontaneous and Starter Induced 'Pupuru'

Parameters (mg/100g)	**Raw cassava**	**P1**	**P2**	**P3**
Phytates	*65.00±5.00^{a+}	35.00±5.00^{c}	40.00±5.00^{b}	31.67±2.89^{d}
Cyanogenic glycosides	0.87±0.01^{a}	0.04±0.01^{b}	0.03±0.01^{b}	0.02±0.01^{b}
Protease inhibitors	0.47±0.01^{a}	0.02±0.01^{b}	0.03±0.01^{b}	0.00±0.00

*Values are mean of three replicates ± standard deviation
ND=Not Detected
P1= Spontaneously Fermented 'Pupuru'
P2= 'Pupuru' Fermented with *L. plantarum*
P3='Pupuru' Fermented with *L. plantarum* and *L. fermentum*
+ Values with different superscript are significantly different and comparism was made within each row

Changes in Viable Count of Spontaneous and Starter-Induced 'Pupuru' Samples during storage

Results showed no growth of coliform bacteria throughout the 3 months of monitoring. Total bacteria and fungi count for P1 and P2 samples increased at the end of the second month in which spontaneously fermented sample (P1) had the highest count of $7.20x10^3$ cfu/ml and $5.70x10^3$ sfu/ml at the end of the third month. Table 5 shows the details of the microbiological monitoring of the shelf life during storage.

Table 5: Monitoring of the microbial load of Spontaneous and Starter-Induced 'Pupuru' during storage

Month	P1			P2			P3		
	Bacterial count (cfu/ml)	Coliform count (cfu/ml)	Fungal count (sfu/ml)	Bacterial count (cfu/ml)	Coliform count (cfu/ml)	Fungal count (sfu/ml)	Bacterial count (cfu/ml)	Coliform count (cfu/ml)	Fungal count (sfu/ml)
1	$2.00x10^3$	0.00	$1.20x10^3$	$1.60x10^3$	0.00	1.10x103	$1.40x10^3$	0.00	$1.00x10^3$
	$2.50x10^3$	0.00	$1.15x10^3$	$1.70x10^3$	0.00	$1.25x10^3$	$1.45x10^3$	0.00	$1.15x10^3$
2	$3.20 x10^3$	0.00	$2.00x10^3$	$1.80x10^3$	0.00	$2.00x10^3$	$1.65x10^3$	0.00	$1.20x10^3$
	$4.25 x10^3$	0.00	$3.30x10^3$	$2.50x10^3$	0.00	$2.50x10^3$	$1.80x10^3$	0.00	$1.60x10^3$
3	$6.52x 10^3$	0.00	$5.20x10^3$	$5.80x10^3$	0.00	$4.75x10^3$	$3.20x10^3$	0.00	$2.30x10^3$
	$7.20x10^3$	0.00	$5.70x10^3$	$6.25x10^3$	0.00	$5.45x10^3$	$3.80x10^3$	0.00	$2.95x10^3$

P1= Spontaneously Fermented 'Pupuru'
P2= 'Pupuru' Fermented with *L. plantarum*
P3='Pupuru' Fermented with *L. plantarum* and *L. fermentum*

DISCUSSION

Lactic acid bacteria are active microorganisms in cassava fermentation, this process of fermentation helps in reducing the hydrogen cyanide, and other anti-nutritional content of cassava and prolong the shelf-life of the fermented products (Meryandini *et al*., 2011). The antimicrobial effect of LAB has been used by man through fermented foods for more than 10,000 years without any adverse effects (Soomro *et al*., 2002). And this enables man to fortuitously improve the shelf-life, safety and nutritional status of many foods. Starter cultures for indigenous fermented foods and beverages should be isolated from the products they are supposed to be used for and selected according to their technological properties (Glover *et al.,* 2005).

According to Ogunbanwo *et al.* (2004), LAB has the potential to inhibit the growth of pathogenic and spoilage bacteria and possibility exist for using them to improve the shelf life of different foods. Inhibitory activity of LAB has been reported to be due to a combination of many factors such as production of lactic acid which brings about reduction of pH of the fermentation medium (Adebayo-Tayo and Onilude, 2008) and production of inhibitory bioactive compounds such as hydrogen peroxide and bacteriocins which are responsible for most antimicrobial activity (Ogunbanwo, 2005). Lactic acid bacteria (LAB) play a major part in most fermentation processes, not only because of their ability to improve the flavour and aroma but especially for their preservative effects on food. The use of non-pathogenic microorganisms and/or their metabolites to improve microbiological safety and extend the shelf life of foods is defined as bio-preservation (Nath *et al*., 2013). Bio-preservation refers to extended storage life and enhanced safety of foods using the natural micro flora and (or) their antibacterial products. One of the most common forms of food bio-preservation is fermentation, a process based on the growth of microorganisms in foods, whether natural or added (Martinis *et al*., 2001).

The ability of LAB to produce alpha galactosidase which is very useful in digestion of bulky starchy food like cassava and breaking down of anti-nutritional factors present in the food has been assessed in the work of Jean *et al.* (2005) which stated that the importance of alpha galactosidase produced by LAB ingested to overcome host deficiency of the enzyme. All LAB isolated in this study produce alpha galactosidase enzyme in abundance, in which *L. plantarum* was the highest producer of the enzyme with concentration of 1.980mg/ml followed by *L. fermentum* having the value of 1.970mg/ml . This is in contrast with the work of Adeyemo *et al.* (2016) in which *L. brevis* was the highest producer of alpha galactosidase. Also the production of this enzyme enhanced the reduction of anti-nutritional factors present in cassava. This findings agrees with those of Taylor *et al.* (2007) and Adeyemo and Onilude (2013).

The reduction of the anti-nutrients of the 'Pupuru' samples with both single and combined starters compared to the spontaneously fermented sample (P1) could be as a result of increase in the production of alpha-galactosidase by the LAB used as starter culture during fermentation. Rodrigueze *et al.* (2008) stated that combination of fermentation-enzyme treatments were effective in diminishing tannin and phytate in fermented cassava. This was however corroborated by the work of Kayode *et al.* (2007) who attributed the reduction in phytate to metabolic activities of some lactic acid bacteria and yeasts. The reduction of the phytic acid in the *'Pupuru'* sample with combined starter (P3) will make nutritionally essential minerals available because phytic acid had been reported to interfere with Ca, Fe, Mg and Zn absorption as a result of its ability to chelate divalent cationic minerals (Wakil and Benjamin, 2015).

Complete fermentation enhanced the effective exclusion of pathogenic microorganisms; reduction of anti-nutritional contents in fermented cassava and also increases the nutritional benefits. LAB has been used to reduce the microbial load of contaminating organisms and anti-nutritional composition of cassava, cereals, and legumes by the production of enzymes. Adeyemo and Onilude (2013) reported that *L. plantarum* isolates reduced the anti-nutritional factors in the fermented food as result of which the adequate nutritional composition of the food would be enhanced by the presence of LAB.

The anti-nutrient (Cyanogenic glycosides, phytates, and protease inhibitor) composition of the cassava fermented into 'Pupuru' was lower and significantly different from the raw cassava (P0). The anti-nutrients level of the 'Pupuru' sample obtained from cassava fermented with both *Lactobacillus fermentum* (A1) and *Lactobacillus plantarum* (B2) was however the lowest. It was noted that protease inhibitor was not detected in sample with combined starter (P3). Fermentation of cassava had been reported to significantly reduce the anti-nutrients level (Oboh *et al.*, 2002). Addition of starter cultures help a lot in the mode of reduction of cyanogenic glycosides and other anti-nutrient in cassava, it is worthy to note the higher rate of reduction in this anti-nutrient factors in 'Pupuru' sample (P3) with combined starter culture.

Cyanide in cassava is the most toxic factor restricting the consumption of cassava roots and leaves indeed; this is the main reason why cassava is not commonly consumed in Western countries (Montagnac *et al.*, 2009). Cassava contains cyanogenic glycosides that are toxic to humans and can lead to serious health disorders. Consumption of 50 to 100 mg/day of cyanide has been associated with acute poisoning and has been reported to be lethal for adults (Montagnac*et al.*, 2009a). Retting of cassava tubers allows softening of cassava roots for further processing and the reduction of the potentially toxic cyanogenic glycosides present in the roots of cassava (Oyewole, 2002).

Reduction in cyanide level of fermented cassava samples for 'Pupuru' production as well as increase in rhodanase enzyme produced by the starter cultures in the fermenting medium was monitored for five days. There was an increase in production of rhodanase enzyme by the starter cultures as fermentation progressed as well as a corresponding decrease in cyanide level of the fermenting cassava. The reduction in cyanide content could be attributed to catalytic effect of rhodanase enzyme produced by the starters in the fermenting medium and evaporation of Hydrogen cyanide during drying (Okpokiri *et al.*, 1995). There was an increase in rhodanase production on the fifth day which was confirmed by subjecting the starters to grow at different time interval and the result from the optical density of the isolate was highest for the organisms at day 5. It could be said that the optimum growth of the starters as well as rhodanase enzyme production is day 3 because enzyme production was highest on day three and a reduction was obtained thereafter this is in line with the report of Adeyemo et al. (2016) who also observe that the highest production of alpha-galactosidase enzyme was observed on day 3. The cyanide content of the 'Pupuru' sample with single starter (P2) reduces from 0.12mg/ml on day1 to 0.2mg/ml on day 5, combined starter samples (P3) also reduces from 0.12mg/ml on day1 to 0.2mg/ml on day 5 and no cyanide was detected on day 5. This supports the report of Fagbemi and Ijah (2005) that there could be over 200% reduction in cyanide content using starter cultures.

The 'pupuru' samples were stored for three (3) months and the microbial load in the samples were monitored. Samples with combined starter cultures (P3) showed an improvement over the single starter sample (P2) and spontaneously fermented 'pupuru' sample (P1). Although there was no coliform growth in all the three samples at the end of the three months used in monitoring but total heterotrophic bacteria count differs among the samples in which P1 had the highest heterotrophic and fungi count of$7.20x10^3$cfu/ml and $5.70x10^3$sfu/ml respectively. This result is comparable to those of Ogunbanwo *et al.* (2004) who use bacteriocin producing lactobacillus strains in extending shelf-life of fufu, a traditionally fermented cassava product during storage.

CONCLUSION

This study reveal a reduction in the antinutritional contents of cassava fermented into 'pupuru' using two different starters. 'Pupuru' can therefore be produced by the use of mixed starter using LAB with desirable technological properties since the 'pupuru' samples obtained from these starters have considerably low anti-nutrient . The cyanogenic glucosides levels in the 'pupuru' samples after fermentation is relatively very safe and within the acceptable limit of 10 mg HCN equivalent/Kg body weight recommended by FAO. Also, fermentation with these starter culture reduced the cyanide content to safe levels in all the 'Pupuru' samples.

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