Global Scientific JOURNALS

GSJ: Volume 8, Issue 2, February 2020, Online: ISSN 2320-9186

www.globalscientificjournal.com

Microbiological and Shelf Stability Studies of Fermented Bambara Groundnut (*Vigna subterranean* (L) *Verdc.*) Flour

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Abstract

Microbial load of Bambara groundnut after cooking prior to fermentation, the fermenting medium of 24, 48 and 72 hours of the fermentation period and the fermented flour was determined to ascertain the microbial succession during bioprocessing. The analysis indicates that the raw material after thermal treatment had no microbial growth while the steep water after 72 hours of fermentation ranged from $1.2x10^3$ to $3.8x10^3$, $2.9x10^3$ to $4.8x10^3$ and $1.0x10^3$ to no-growth for bacterial, viable cell and mold counts, respectively. Real-time storage was used to determine the effect of fermentation on the shelf stability of the bioprocessed flour during storage period of 360 days and analyzed for fat, protein, fiber and ash; as well as for colonies of molds, insect infestation and physical changes at intervals of 60 days. At 360 days, fat increased from 4.18 to 7.22% and protein decreased from 22.18 to 19.70%, while fiber and ash was unaffected when compared to initial content. Mold count ranged from $1.34x10^3$ to $1.63x10^3$ and no-growth to $0.38x10^3$ for raw and bioprocessed samples respectively. No presence of insects or their eggs was observed. Parameters such as hand feel and color observation was employed to determine for physical changes and no color change occurred in both samples. However, the raw sample showed slight formation of lumps at 150 days of storage and this became more evident at the end of the study period; thus no such changes was observed in the bioprocessed sample.

Keywords: Shelf-life, Bioprocess, Non-spontaneous, Bambara groundnut, Flour

INTRODUCTION

Food processing makes use of various unit operations and technologies to convert relatively bulky, perishable and typically inedible raw materials into more useful shelf-stable and palatable foods or beverages. Processing contributes to food security by minimizing waste and losses in the food chain and by increasing food availability and marketability. Food is also processed in order to improve its quality and safety. Food safety is a scientific discipline that provides assurance that food will not cause harm to the consumer when it is prepared and/or eaten according to its intended use. Hence, biotechnology as applied to food processing in most developing countries makes use of microbial inoculants to enhance properties such as shelf-life and nutritional value of foods. The process whereby microorganisms and their enzymes bring about these desirable changes in food materials is known as fermentation.

Technologies applied in the processing of food must assure the quality and safety of the final product. Safe food is food in which physical, chemical or microbiological hazards are present at a level that does not present a public health risk. Safe food can therefore, be consumed with the assurance that there are no serious health implications for the consumer (Codex Alimentarius Commission, 2009). A range of technologies is applied at different levels and scales of operation in food processing across the developing world. Conventional or "low-input" food processing technologies undertake fermentation in most of its processes. However, the use of fermentation in most traditional food processing has increased the utilization of most indigenous crops in the developing regions of the world.

Bambara groundnut (*Vigna subterranean* (L)) is a highly nutritive but underutilized grain legume indigenous to West and Central Africa. Quaye and Kanda (2004) provided the following nutritional breakdown: carbohydrates: 54.5 - 69.3%, protein 17 - 24.6% and fat 5.3 - 7.8%. The nut can be ground into flour and blend into many customary dishes. Despite the almost total scientific neglect, the crop still makes a great contribution to the diet of many parts of Africa (National Research Council, 2006).

Many underutilized crops like Bambara groundnut has been identified to have the potential to contribute towards improving food security and owing to its outstanding qualities, there is need to research new ways to effectively harness the potentials of this crop and use it in various food applications. Although it has been established from literatures that fermentation improves nutrient bioavailability, elimination of antinutrients and increased production or creation of phytonutrients in foods; no such advance has been made to investigate this benefits on extension of shelf-life in Bambara groundnut as it is presently underutilized irrespective of its nutritive values and potentials in new product designs.

Although a majority of African food fermentation processes make use of spontaneous inoculation; there are major limitations which include their inefficiency, low yields of product and variable product quality. While spontaneous fermentations generally enhance the safety of foods owing to a reduction of pH, and through detoxification, in some cases there are safety concerns relating to the bacterial pathogens associated with the raw material or unhygienic practices during processing. Hence, the present study aims to evaluate the effect of non-spontaneous fermentation on the microbiological quality and shelf stability of Bambara groundnut flour as well as the proximate composition during storage.

MATERIALS AND METHODS

Pretreatment of Raw Material and Reactivation of Starter Culture - The Bambara groundnut was carefully cleaned and freed of all extraneous materials as well as damaged nuts prior to use. It was washed twice with ordinary water, rinsed with distilled water and cooked to softness as a pretreatment measure and to eliminate existing microflora before inoculation with starter cultures. Pure cultures of freeze dried *Lactobacillus plantarum* [NRRL B-4306] and *Lactobacillus fermentum* [NRRL B-1932] preserved in a dormant state by drying a heavy suspension of cells in sterile bovine serum was obtained from Agricultural Research Services Culture Collection, Bacterial Foodborne Pathogens and Mycology Research Unit; National Center for Agricultural Utilization Research of the United States Department of Agriculture, Peoria Illinois USA.

The freeze dried cells of *Lactobacillus plantarum* [NRRL B-4306] and *Lactobacillus fermentum* [NRRL B-1932] was brought to active state by growing in 25 ml Nutrient Broth and incubated in CO_2 enriched jars for 24 hours and centrifuged at 3600-x g for 15 min. The recovered cells were rinsed using 10 ml sterile distilled water and spine twice at 3600-x g for 15 min. After this, a 9 ml suspension of the cells was made using sterile distilled water. The suspensions were serially diluted and plated out on Plate Count Agar using the pour plate method. After 24 h incubation period in CO_2 enriched jars, the colonies on each plate of dilution factor was counted and the plate with approximately 10^6 cfu/ ml was noted and used at every inoculation of the fermentation process.

Production of Bioprocessed Flour - Twenty kilogram of cooked Bambara groundnut was submerged in 30 liters of sterile distilled water, inoculated with bacterial strains of *Lactobacillus plantarum* [NRRL B-4306] and *Lactobacillus fermentum* [NRRL B-1932] containing approximately 10^6 cfu/ml and allowed to stand for 3 days. The Bambara groundnut was dried using hot air oven set at 60^OC for 10 hours, ground and sieved to obtain the flour.

Shelf Stability Studies – Real-time storage was used to study the inoculated and uninoculated flour samples which was stored in an airtight polypropylene bag at ambient temperature and humidity for 360 days and analyzed at intervals of 60 days for fat, protein, fiber and ash. The stored flour was also observed for insect infestation and physical changes such as formation of lumps and color change.

Determination of Proximate Composition during Storage - The proximate composition of the inoculated and uninoculated flour was determined using the standard procedures of Association of Official Analytical Chemists (2010). Ash was determined by incineration (550°C) of known weights of the samples in a muffle furnace. Crude fat was determined by exhaustively extracting a known weight of sample in petroleum ether (boiling point, 40 to 60°C) in a soxhlet extractor. Protein (N × 6.25) was determined by the Kjeldahl method. Crude fiber was determined after digesting a known weight of fat-free sample in refluxing 1.25% sulfuric acid and 1.25% sodium hydroxide.

Microbiological Analysis - The microbial load of the fermenting medium of 24, 38 and 72 hours of the fermentation period was determined to ascertain the microbial status during fermentation with *Lactobacillus plantarum* [NRRL B-4306] and *Lactobacillus fermentum* [NRRL B-1932]. For determination of the number of viable cells in the fermenting medium, samples (~5 ml) were taken at different intervals from the top (1 cm below the surface) and bottom (1 cm above) of the mixture. The harmonized sample (~1 ml) was diluted with saline solution (9 ml) for pour plate count on Rogosa Agar medium for lactic acid bacteria and on Violet Red Bile Agar for *E. coli*. Lactic acid bacteria were incubated at 37°C for a period of 3 days in an anaerobic jar while *E. coli* was incubated at 37°C for a period of 24 h. Molds was isolated using a selective medium, Potato Dextrose Agar. Colonies of respective microbial types appearing in incubated plates were counted and expressed as colony forming units per gram (CFU/g).

Data Analysis - Data generated were analyzed using one-way analysis of variance and mean separation was done by Duncan's new multiple range test and paired t-tests. Significant difference was accepted at $p < 0.05$.

RESULTS

Real-time storage studies was used to demonstrate the stability of the flour samples for a period of 360 days (12 months) at ambient temperature and humidity in a pack similar to one which can be used for the finished product for commercial purposes. After storage in an airtight polypropylene bag, inoculated and uninoculated Bambara groundnut flour was analyzed at intervals of 60 days for crude fat, crude protein, crude fiber and total ash; as well as for colonies of molds, insect infestation and physical changes and the results presented in Tables 1 and 2.

Table 1: Effect of storage on proximate composition of bioprocessed and raw flour

Storage (Days)	Fat (%)		Protein (%)		Fiber (%)		Ash (%)	
	RBG	NFBG	NFBG	FBG	NFBG	FBG	NFBG	FBG
60	6.70±0.4^{c}	8.79±0.1^{b}	20.30±.1^{ab}	19.70±0.2^{a}	6.60±07	3.96±0.4	3.50±0.2	3.20±0.4
120	5.90±0.3^{b}	8.5±0.3^{b}	19.78±0.4^{a}	19.60±0.6^{a}	--	--	--	--
180	5.50±0.8^{b}	8.50±0.1^{b}	19.23±0.7^{a}	19.65±0.5^{a}	--	--	--	--
240	5.09±0.1^{b}	8.12±0.3^{b}	21.04±0.3^{b}	19.58±0.5^{a}	--	--	--	--
300	4.70±0.3^{a}	7.50±0.4^{a}	21.88±0.2^{b}	19.69±0.1^{a}	--	--	--	--
360	4.18±0.5^{a}	7.22±0.8^{a}	22.18±0.3^{bc}	19.70±0.4^{a}	--	--	--	--

Values are means ± SD (n = 3).
Values on the same row with different superscripts are significantly ($p < 0.05$) different.
--: No difference, NFBG: Non-fermented Bambara Groundnut, FBG: Fermented Bambara Groundnut

Table 2 showed results for analysis of mold count, insect infestation and physical changes. Thus, the study observed no significant difference for both inoculated and uninoculated Bambara groundnut flour samples for the study duration with regards to color when compared to the initial color of the samples before storage. Parameters such as hand feel and color observations was employed to determine if physical changes occurred in the study samples. However, the uninoculated flour showed slight formation of lumps at 180 days of storage and this became more noticeable at the end of the study period; thus no such changes was observed in the fermented Bambara groundnut flour sample.

Table 2: Effect of storage on colonies of mold, insect infestation and physical changes of non-fermented and fermented flour

Storage Period (Days)	Mold Count (CFU/g)		Insect Infestation		Physical Changes	
	NFBG	FBG	NFBG	FBG	NFBG	FBG
60	$1.34x10^{3}$	<10	--	--	=	=
120	$1.38x10^{3}$	<10	--	--	=	=
180	$1.47x10^{3}$	<10	--	--	= +	=
240	$1.48x10^{3}$	$1.0x10^{1}$	--	--	= +	=
300	$1.56x10^{3}$	$1.0x10^{1}$	--	--	= ++	=
360	$1.63x10^{3}$	$1.5x10^{1}$	--	--	= ++	=

Values are mean of triplicate determinations
-- No Infestation; = No Color Change; + Slight Formation of Lumps; ++ Noticeable Formation of Lumps; NFBG: Non-fermented Bambara Groundnut; FBG: Fermented Bambara Groundnut

The microbial load of the Bambara groundnut (*Vigna subterranean* (L)) after thermal treatment prior to fermentation, the fermenting medium of 24, 48 and 72 hours of the fermentation period

and the fermented flour was determined to ascertain the microbial succession during bioprocessing and the results presented in Table 3. The analysis indicates that the raw material after cooking had no microbial growth while the steep water after 72 hours of fermentation, showed the highest microbial count of bacterial and viable cell counts while mold was greatly suppressed.

Table 3: Microbial counts of Bambara groundnut before, during and after fermentation

Microbial Counts	Pre-Fermentation	Fermentation			Post-Fermentation
		24h	48h	72h	
Mold Count (CFU/g)	NG	$1.0x10^3$	<10	<10	<10
Bacterial Counts (CFU/g)	NG	$1.2x10^3$	$2.0x10^3$	$3.8x10^3$	$0.4x10^3$
Total Viable Cells (CFU/g)	NG	$2.9x10^3$	$3.6x10^3$	$4.8x10^3$	$0.8x10^3$

Values are mean of triplicates

DISCUSSION

The study for effect of fermentation on proximate composition of the flour samples observed that crude fat decreased during the storage period for both study samples but was observed greater in non-fermented Bambara groundnut (NFBG) flour. The decrease in NFBG was observed as early as 60 days of storage while for fermented Bambara groundnut (FBG) flour the decrease became significant at 210 days of storage. This decrease may be attributed to the lipolytic activity of enzymes i.e. lipase and lipoxidase. The lipolytic activity was lower in the fermented flour probably because of the actions of the lactic acid bacteria used in the fermentation which might have slowed the enzymatic activities.

Leelavathi *et al.* (1984) also reported a decrease in crude fat of whole wheat flour during storage. Rehman and Shah (1999) who studied biochemical changes in wheat flour during storage; noted that whole grain flour storage is accompanied by a cascade of biochemical changes that lead to reduced flour functionality and they opinioned that the most unstable components in whole grain flour are the lipids and lipid degradation is the predominant cause of the loss of flour functionality during storage. Lipids begin to break down in whole grain flour by hydrolytic rancidity, which can be followed by oxidative rancidity. These changes can occur enzymatically or non-enzymatically and affect flour quality, the researchers noted. Their report further stated

that lipid oxidation during storage of whole wheat flour is a much slower process than lipid hydrolysis. This is because, unlike lipase, lipoxygenase exhibits very little activity at moisture contents typically found during storage, and because whole wheat flour contains high levels of protective antioxidants. Despite being a slower process than lipid hydrolysis during storage, lipid oxidation can contribute substantially to loss of product quality. The report also noted that oxidation of lipids can lead to a decrease in nutritional quality and consumer acceptability of whole grain flour and whole grain flour-based products. Lipid oxidation reduces nutritional quality through loss of essential fatty acids, although more significantly, reduced nutritional quality is affected through co-oxidation of other flour components.

The results pertaining to the crude protein content showed no significant changes for the fermented flour sample during storage period. Hence, a decreasing effect with storage was observed in non-fermented flour. This could be attributed to the drying treatment given to the FBG sample which is presumed to contain a less moisture content than the NFGB samples. Thus, higher moisture content in foods is said to favor proteolytic activity. The decline in crude protein for NFBG was observed as early as 60 days of storage. After 120 days of storage, the crude protein of the NFBG sample increased again and this could be attributed to microbial growth. These results are in close agreement with the results obtained by Upadhyay (1994) who observed a decrease and subsequent increase in crude protein of whole wheat flour during storage and attributed the later increase to insect infestation and microbial growth.

However, at the end of the storage period crude fiber and total ash were not affected during the study for both fermented and non-fermented Bambara groundnut flour. The results for colonies of mold during storage showed that the molds differed significantly with respect to both samples. Much greater mold counts were observed for the non-fermented Bambara groundnut flour during and after the storage period (Table 2). Many factors are presumed to contribute to the significant difference in mold counts of both samples and this includes the initial microbial load of the NFBG when compared to FBG which was cooked before fermentation. Another factor includes higher moisture content which favored mold growth in NFBG sample. Insect infestation in both samples was monitored by observing for insect eggs microscopically and for adults by visual examination. The results obtained with regard to insect infestation indicated no presence of

insects or their eggs (Table 2). It could be assumed that the airtight polypropylene bags provided adequate protection against insect infestation.

The analysis for microbial succession indicates that the raw material after thermal treatment had no microbial growth while the steep water after 72 hours of fermentation, showed the highest microbial count of bacterial and viable cell counts while mold was greatly suppressed. This may be as a result of the presence and activities of the starter culture used in the fermentation process. This was in line with the studies of Nout (1994) who observed a significant decrease in mold and metabolic detoxification of mycotoxins by *Rhizopus oryzae* and also stated that fermentation can minimize or prevent negative factors such as growth and metabolism of pathogenic and toxinogenic bacteria, i.e., bongrek acid and toxoflavin formation by *Pseudomonas cocovenenans.*

After fermentation, a similar analysis was carried out to determine the microbial load of the fermented flour. However, a drastic decrease of the microbial load was observed. This was in agreement with the work of Ashenafi and Busse (1989); the researchers studied the inhibitory effect of *Lactobacillus plantarum* on *Salmonella infantis, Enterobacter aerogenes* and *Escherichia coli* during tempeh fermentation. In 1992, Ashenafi and Busse also studied the growth of *Staphylococcus aureus* in fermenting tempeh made from various beans and its inhibition by *Lactobacillus plantarum*. Kingamkono *et al*. (1996) conducted a study that investigated the inhibition of different strains of *enteropathogens* in lactic fermenting cereal gruel, while, Karunaratne *et al.* (1990) investigated the inhibition of mold growth and aflatoxin production by *Lactobacillus* spp. This works portrays the ability of *Lactobacillus* species to inhibit other microorganisms when used in a fermentation process.

Notwithstanding the inhibitory ability of the *Lactobacillus species,* it might be sensible to say that overall reduction may also have occurred due to factors resulting from fermentation and drying after fermentation. Effect of pH, reduced water activity (a_w), extent of heat and period of exposure to heat during drying, as well as rinsing and subsequent removal by water, may be few of the reasons for the decrease in the microbial load of the bioprocessed Bambara groundnut flour. While a less significant growth was still observed in the flour after fermentation, it is assumed that this may have occurred during milling and sieving, either from persons or air from the surrounding environment which had access to the flour and possibly initiate contamination.

CONCLUSION

Starter culture improvement, together with the improvement and development of bioreactor technology for the control of fermentation processes in developed countries, has played a pivotal role in the production of high-value food products and food ingredients. These products are increasingly produced in more advanced developing economies, and are increasingly imported by less advanced developing countries, for consumption or as inputs for their food processing operations. Hence, there is need for the developing economies to harness the use of starter culture technologies in food fermentations as to ensure conformity to regulatory standards.

This study has characterized the microbiological quality and shelf-life of fermented Bambara groundnut flour in comparison to the non-fermented flour as well as the proximate composition of the samples during storage. Comparative analysis of food processing methods is of great importance as this can ensure effective measures for food security. Bambara groundnut should not only be seen and cultivated as subsistence crop; rather it should be seen as a crop that is relevant to food security. Thus, the knowledge from this study will help food manufacturers understand the effect of controlled fermentation on the keeping quality of the flour. Nonetheless, the study duration and parameters determined in these shelf-life studies can be helpful in determining storage potentials of this fermented Bambara groundnut flour for use in foods, however, actual shelf-life could be shorter or longer depending on temperature and humidity during storage.

Acknowledgement

The authors wish to acknowledge the Agricultural Research Services Culture Collection, Bacterial Foodborne Pathogens and Mycology Research Unit; National Center for Agricultural Utilization Research of the United States Department of Agriculture, Peoria Illinois USA for providing the bacterial culture used for this study.

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