International Journal of Microbiology and Biotechnology
2016; 1(1): 16-24
http://www.sciencepublishinggroup.com/j/ijmb
doi: 10.11648/j.ijmb.20160101.13

Identification and Traceability of Microflora Contaminating the Different Stages of the Traditional Method of Shea Butter Processing

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To cite this article:

Esiegbuya Daniel Ofeoritse, Okungbowa Francisca Iziegbe. Identification and Traceability of Microflora Contaminating the Different Stages of the Traditional Method of Shea Butter Processing. *International Journal of Microbiology and Biotechnology.* Vol. 1, No. 1, 2016, pp. 16-24. doi: 10.11648/j.ijmb.20160101.13

Received: October 9, 2016; **Accepted:** November 2, 2016; **Published:** December 20, 2016

Abstract: The study into the traceability of the microflora contaminating the different stages of the traditional method of Shea butter processing was carried out with the aim of identifying the points of microflora contamination and also to track the movement of the associated microflora along the different stages of Shea butter processing into the final product. The microflora associated with the samples collected from the various stages of Shea butter processing from some villages in Kwara State was determined using the International Confectionery Association (ICA) specifications. The schematic flowchart of the Shea butter processing was used as a technique to track the associated microflora from their points of contamination to the final product. The results revealed the presence of fungi such as *A. flavus, A. niger* (aggregates) and *Penicllium* sp as well as bacteria such as *Salmonella parathyphi, Enterobacter* sp., *Escherichia coli* and *Klebsiella* sp. The points of entrance of these pathogens into the different processing stages include the use of microbe-infected Shea kernels, water source and sources of cross-contamination. The results also showed that the different microflora could move along the different processing stages into the Shea butter. This was as a result of below standard processing practices. The traceability results also revealed a mean microbial load of 100 cfu/2g for the Shea kernels collected from Apaola, Ilota and Futu villages while that of the grounded kernels were 40,45 and 50 cfu/2g for Apaola, Ilota and Futu villages, respectively. The increase in the mean microbial load of the kneaded paste when compared with the grounded kernels was attributed to introduction of water and due to certain other activities of the processors. The isolation of microbes from the processed Shea butter obtained from the three study locations was largely as a result of sources of cross-contamination from the processing environment. The results of this study indicate that purity of water, clean processing environment, clean working utensils, use of non-microbe infested Shea kernels and hygienic condition of processors must be regarded as critical factors in the processing of good quality Shea butter.

Keywords: Bacteria, Cross-contamination, Fungi, Shea Butter, and Traceability

1. Introduction

According to Tall (2001) traceability is the ability to trace and follow a food through all its stages of production and distribution. When considering a product, traceability can relate to the origin of material and parts, processing history and distribution and location of the product after delivery.

The Tracefish project (2001) identified two types of traceability: internal and chain traceability. Internal traceability is within one company and relates to data about raw materials and processes to the final product before it is delivered. Chain traceability is focused on the information about the product from one link in the chain to the next, it

describes what data are transmitted and received, and how. Olsen (2001) explained that chain traceability is between companies and countries and depends on the presence of internal traceability in each link.

It was mentioned that there are increasing demands for traceability throughout the food chain. The root causes of many of the recent food safety problems have been found in the primary production sector, although the problems are manifested at the other end of the chain (finished products). To determine the causes of the problems and taking remedial action, it is important to trace the processing of food right from the raw material stage to the finished product. This will enhance the process of withdraw or recall all the unsafe products produced. With chain traceability in place, these tasks can be done efficiently and with minimal commercial disturbance. Without chain traceability, whole sectors of the food industry may have to be closed down on a precautionary basis and the costs can be ruinous (Denton 2001; Tracefish, 2001).

Research on traceability in the fisheries chain has been ongoing for a few years in Europe. The research efforts have mainly been focused on the logistics of the products to ensure that products can be linked to their source while also protecting products of declared origin (both geographical and production system). Research on sophisticated molecular biology techniques as tools to verify the authenticity of species and for tracing contamination of products has also been the focus of research (Börresen, 2003).

Shea butter production in Nigeria consists of a series of stages of production which start with the use of Shea kernels. The Shea kernels undergo series of processing such as drying/roasting, grinding of the Shea kernels to consistent paste, kneading, boiling and scooping of liquid butter into a clean container. During these stages of production, the raw material and the different processing stages are usually exposed to external factors such as processing utensils/machine, processing water and exposure to human and environmental contact. Apart from exposure to these external factors, majority of the dried Shea kernels are usually microbe infested during drying and storage. The effect of these external factors and the use of these microbe infested Shea kernels contribute to the quality deterioration of the processed Shea butter.

Several effort have been made by researchers to improve on the quality of the Shea butter through proper postharvest handling of the Shea fruit, nuts and kernels. One of which includes the standard production process that controls the process of harvesting, de-pulping, boiling, drying, cracking, dehulling, heating, pressing and extraction has been proposed by Atehnkeng, *et al.,* (2014). The authors highlighted five critical points of microorganism's contamination of the Shea nuts and kernels in the production of Shea butter. The author pointed out that if the corrective measures are applied, there will be minimal contamination of the Shea products by aflatoxin and other polycyclic aromatic hydrocarbons. These points are at the point of collection of fallen fruits, boiling of dried nuts, drying of both boiled and Shea nut/kernels and roasting of crushed kernels. The preventive measures to exert control at these stages are to avoid delay in picking of fallen fruits, boiling of adequately dried fruits, avoid drying of boiled nuts and kernels on unhygienic surfaces and roasting of crushed kernels over naked flame. They also pointed out that aflatoxin contamination of the Shea nut/kernels was higher when compared to the obtained processed Shea butter. This they attributed to the heating of the Shea nut/kernels during processing which has destroyed some of the heat labile aflatoxin.

Obibuzor *et al,* (2013) also highlighted the effect of sprouted Shea nut on the quality depreciation of Shea butter especially with respect to free fatty acids, peroxide value and fatty acid composition. The workers observed that linoleic acid was mobilized during germination more than the other constituent fatty acids and the elemental composition was not generally affected. The overriding implication of their findings was that processing Shea kernel batch that has high ratio of germinated Shea nuts compared to ungerminated Shea nuts would result in low quality butter with significantly altered fatty acid composition. According to the workers in order to improve on the quality of Shea butter prompt sterilization and sorting out, the germinated kernel was needed.

Microbiological analysis of street vended Shea butter according to Esiegbuya *et al*. (2015) revealed the presence of some indicator microorganisms such as *E. coli, Micrococcus, P. aeroginosa, Enterobacter* spp, *Mucor* sp*, Penicillium* sp*., A. niger,* and *A. flavus*. The workers attributed the possible sources of contamination by these organisms to cross-contamination from the selling point. According to Makun *et al*. (2009, these organisms are of serious public health concern because of their ability to produce toxins.

The aim of this study was to contribute towards the microbial quality of processed Shea butter through the identification of the possible sources of microbial contamination across the different stages of processing and also track the movement of the associated microflora into the processed Shea butter.

2. Materials and Methods

2.1. Source of Shea Butter Samples

The Shea samples (Shea kernels, ground kernels, kneaded paste, Shea butter extract and water used for processing) used in this investigation was obtained from Apaola, Ilota and Futu villages of Kwara State.The samples were obtained from local Shea butter processors who adopted the local method of Shea butter production. The samples were collected in clean plastic containers.

2.2. Microbiological Analysis of the SHEA Samples

The microbiological specifications used in this study were

based on the International Confectionery Association (ICA) described in the deZaan Cocoa and Chocolate Manual (2009).

The microbiological analyses were done in order to determine:

1. The points of contamination and cross-contamination of the processing line
2. Monitor/tracking of the movement of the associated microbes

2.3. Preparation of Lactose and Peptone Broth Stock Solution

Thirteen grams each of lactose and peptone powder was weighed separately in a weighing balance and then transferred into conical flask containing 1,000 ml of demineralized water in a separate glass flasks. After which, 10mls each of the lactose and peptone broth was dispensed into four 100mls conicallabeling Shea kernels, grinded kernels, kneaded paste, and crude Shea butter extract (four labeled for lactose broth and four for peptone broth). The labeled lactose and peptone broth conical flasks were corked with cotton wool wrapped with aluminum foil. They were then sterilized in an autoclave at 121°C (250° F) for 30 minutes. It was allowed to cool to about 45°C (113° F) and the pH was checked to be 6.9. After sterilization two grams each of the collected samples was weighed into the labeled lactose and peptone broth conical flasks, it was then closed and shaken properly well. The suspension was allowed to stand for about 30 minutes before carrying out the serial dilution process.

2.4. Preparation of Sterile Water Blank for Microbiological Analysis of Water Samples

Ten millilitres of distilled water was pipetted into four McCartney bottles representing each village for the water source. The bottle was labeled S1 to S4. The bottles were then sterilized in an autocalve at 121°C for 15 minutes. After sterilization, I ml each of the water source representing each of the village was transferred into the McCartney bottles as stock solution. With a sterile pipette, 1ml each was transferred from the stock bottles into the bottles labelled S1 to S4 containing nine millilitres of sterilized distilled water for serial dilution preparation of the water sample.

2.5. Serial Dilution

McCartney bottles divided into eight groups label101 to 105 and the stock bottle labeling Shea kernels, grinded kernels, kneaded paste, crude Shea butter extract and the identified sources of cross-contamination were prepared in triplicates for the lactose and peptone broth. They were sterilized in an autoclave at 121°C for 15 minutes. With a sterile pipette, 1ml each was transferred from the stock bottles into the bottles labelled 10^1 to 10^6 containing nine millilitres of sterilized distilled water for serial dilution preparation.

2.6. Method of Inoculation

The pour plate method of inoculation technique was used in the isolation of the microorganisms associated with the Shea butter and water samples. One ml each of the serial dilution prepared samples was pipette with the aid of a syringe and was transferred into the corresponding labeled Petri dishes. Nine ml of molten prepared potato dextrose agar (PDA) and nutrient agar (NA) was dispensed into the Petri dishes.

2.7. Isolation of Airborne Microbes

The airborne microbe was isolated by exposing an already prepared potato dextrose agar on a Petri dish at the processing environment for fifteen minutes. After which, the Petri dish was incubated in an incubator.

2.8. Molecular Identification of Fungal Isolates

Molecular identification of the fungal isolates was carried by the International Mycological Institute, Surrey, UK. Molecular identification was carried out on the samples using nucleic acid as a template. A proprietary formulation [microLYSIS®-PLUS (MLP), Microzone, UK)] was subjected to the rapid heating and cooling of a thermal cycler, to lyse cells and release deoxyribonucleic acid (DNA). Following DNA extraction, Polymerase Chain Reaction (PCR) was employed to amplify copies of the ITS fragment of rDNA in vitro for filamentous fungi. The quality of the PCR product was assessed by undertaking gel electrophoresis. PCR purification step was carried out to remove unutiliseddNTPs, primers, polymerase and other PCR mixture compounds and obtain a highly purified DNA template for sequencing. This procedure also allowed concentration of low yield amplicons. For all samples, sequencing reactions were undertaken using BigDye® Terminator v3.1 kit from Applied Biosystems (Life Technologies, UK) which utilises fluorescent labelling of the chain terminator ddNTPs, to permit sequencing. Removal of excess unincorporated dye terminators was carried out to ensure a problem-free electrophoresis of fluorescently labelled sequencing reaction products on the capillary array AB 3130 Genetic Analyzer (DS1) DyeEx™ 2.0 (Qiagen, UK) modules containing prehydrated gel-filtration resin were optimized for clean-up of sequencing reactions containing BigDye® terminators. Dye removal was followed by suspension of the purified products in highly deionisedformamide Hi-Di™ (Life Technologies, UK) to prevent rapid sample evaporation and secondary structure formation. The samples were loaded onto the AB 3130 Genetic Analyzer and sequencing undertaken to determine the order of the nucleotide bases, adenine, guanine, cytosine, and thymine in the DNA oligonucleotide. Following sequencing, identification was undertaken by comparing the sequences obtained with those available at the European Molecular Biology Laboratory (EMBL) database via the European Bioinformatics Institute (EBI).

2.9. Analytical Profile Index for Bacteria Identification

The bacteria identification was carried out at the Anaerobe Laboratory, Molecular Biology and Biotechnology Division, Nigerian Institute of Medical Research, Yaba Lagos. This was done using Analytical Profile Index (API) 20A system according to the method of (Murray *et al.,* 1985). The procedure involved 21 tests for the biochemical identification of the isolates and the test strips consisted of 20 microtubes containing dehydrated substrates. Each Inoculum was prepared inone ampoule of the API medium. A sterile swab was used to harvest all the growth obtained on tryptose soy plates and, with the ampoule held upright, the organism, was emulsified to obtain a final turbidity equal to 3 McFarland standards. During inoculation the API strip was removed from its packing and bent so that it fitted into an empty Petri dish. Sterile pipette was used to inoculate the strip with the suspension in the ampoule avoiding the formation of air bubbles. The indole tube was slightly under filled and overlaid with mineral oil. The base and the lid of the Petri dish was sealed together with two strips of adhesive tape and the setting placed in an incubator and incubated for 24 hrs hours at 36^{o}C.The reduction of nitrates was additionally carried out by adding two drops of potassium nitrate (6 mg/ml). After 24 hours of incubation the microtube for nitrate was overlaid with Nit 1 and Nit 2 reagent (ref. 70440 and 70450) respectively.

After incubation the strips were observed and the result recorded by referring to the API Index table and all the reactions requiring no addition of reagents recorded on the result sheet.

Carbohydrate test: One drop of bromocresol purple (BCP) reagent was added into all micro tubes containing carbohydrates. A positive result was denoted by yellow or yellow green colouration while no colour change denotes negative.

Indole test: A drop of xylene reagent added to the micro tube was mixed using an applicator stick and left for 2-3 minutes followed by a drop of paradimethylaminobenzaldehyde (HER reagent) and results observed after 5 minutes. A colour change to red was recorded as positive on the result sheet.

For Identification, the result sheet reproduced the outline of API 20 A with its 20 tests strip plus catalase reaction and three morphological characteristics, SPOR for spore, GRAM for gram and COCCO for coccus. The tests divided into three groups had values designated as 1, 2, or 4 assigned to them. The numbering that corresponded to positive reactions with each group was given eight digit numbers from the numerical profile and the numerical profile was looked up in the analytical profile index.

2.10. Test Reaction

The color reactions for amino acid decarboxylations (ADH through ODC) and carbohydrate fermentations (GLU through ARA).

The amino acids tested were (in order) arginine, lysine and ornithine. Decarboxylationwas shown by an alkaline reaction (red colour of the particular pH indicator used)

The carbohydrates tested were glucose, mannitol, inositol, sorbitol, rhamnose, sucrose, melibiose, amygdalin and arabinose. Fermentation is shown by an acid reaction (yellow color of indicator).Hydrogen sulfide production (H2S) and gelatin hydrolysis (GEL) result in a black color throughout the tube. A positive reaction for tryptophan deaminase (TDA) gives a deep brown color with the addition of ferric chloride; positive results for this test correlate with positive phenylalanine and lysine deaminase reactions which are characteristic of *Proteus*, *Morganella* and *Providencia*.

2.11. Identification of E. coli

From each of the positive tubes identified above, a drop was transferred into a 5 ml test tube of trypton water and incubated at 44°C for 24 hours. A drop of Kovac's reagent was then added to the tube of trypton water. All tubes that showed a red ring colour development after gentle agitation denoted the presence of indole and recorded as presumptive for thermotolerantcoliforms (*E. coli*). Counts per 1 ml were calculated from Most Probable Number (MPN) tables (Hood *et al*, 1983).

2.12. Identification of Other Microorganisms

Colonies from the total viable count were subcultured until pure cultures were obtained. These were examined by their colonial and cell morphology, Gram reaction and other biochemical tests. Identification of species was carried out by assaying cultures in Analytical Profile Index (API) galleries; API 20E (BioMérieux, Marcy L'Etoile, France).

3. Results

The results of the identification of sources of contamination and traceability of microbes for the local method of Shea butter processing in Apaola, Ilota and Futu villages of Kwara State shown on Figure 1-3, revealed three major points of microbial contamination of the local method of Shea butter processing. These include the use of unsorted microbe's infected Shea kernels from the field, the water used in processing and the sources of cross-contamination from the processing environment.

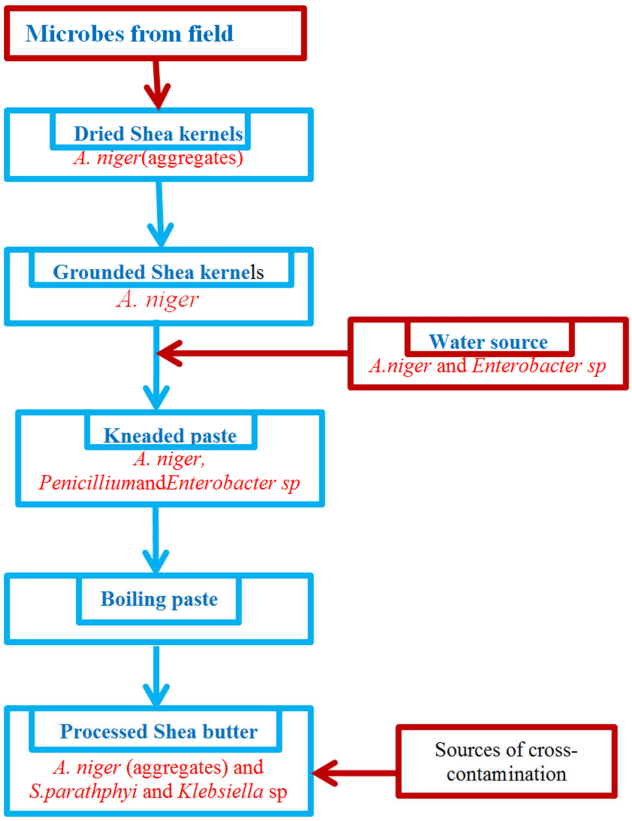

Figure 1. *Identification of source of contamination and traceability system of the local method of Shea butter processing in Apaola village.*

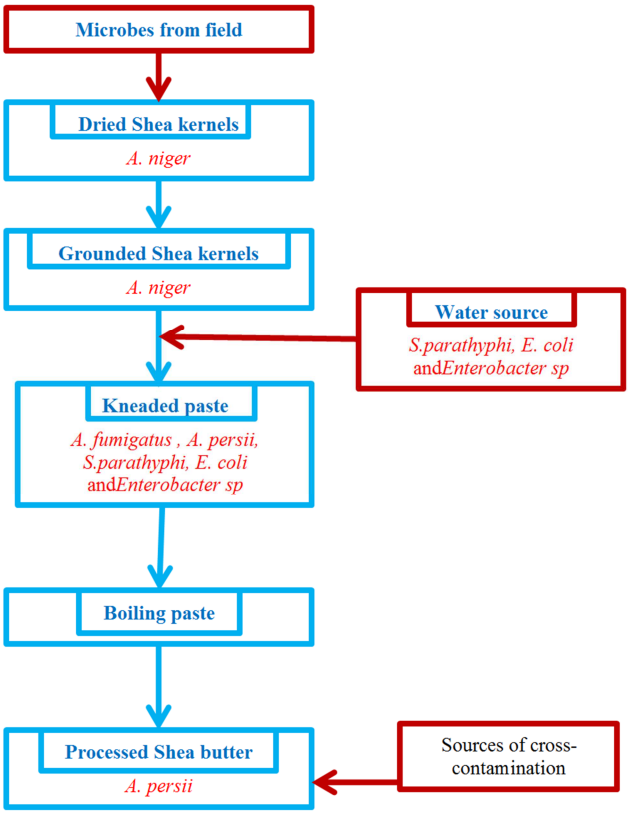

Figure 2. *Identification of source of contamination and traceability system of the local method of Shea butter processing in Ilota village.*

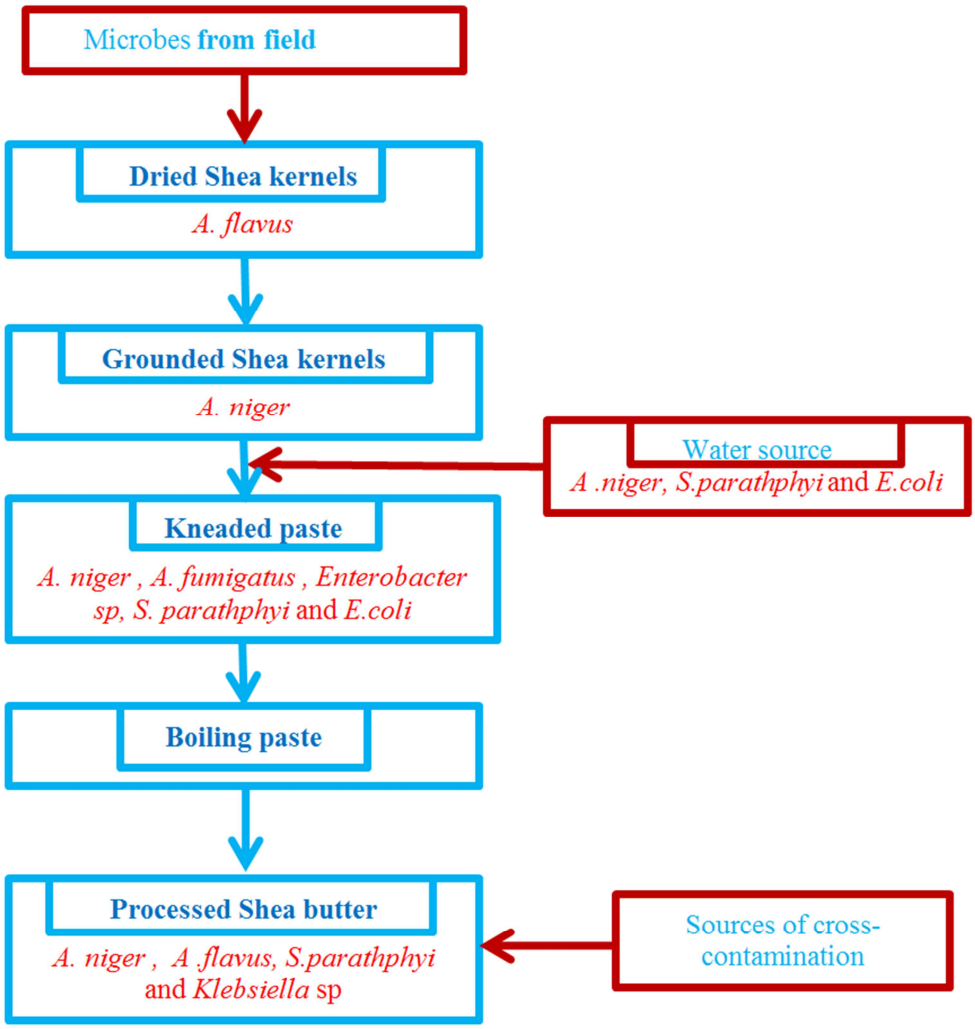

Figure 3. *Identification of source of microbial contamination and traceability system of the local method of Shea butter processing in Futu village.*

The microorganisms associated with the use of unsorted microbes infected Shea kernels were mainly Aspergillus spp. The detected Aspergilus spp. from the Shea kernels was traced through the different processing stages to the kneaded paste using their macroscopic, microscopic and molecular features. The addition of water to the grounded Shea kernels to form a paste contributed to increase in the microbial diversity of the processing stage when compared to the previous stage (grounded kernel). The microbes introduced as a result of water addition were Salmonella parathyphi, Enterobacter sp. and Escherichia coli. No microbe was isolated from the boiling stage (boiled scooped paste). There was however, the presence of microbes in the processed butter.

The isolation of A. niger (aggregate),A. niger, A. flavus, S. parathyphi and Klebsiella sp. from the processed Shea butter from the three villages came were from the sources of cross-contamination identified on Table 1.

The identification of the fungal isolates was confirmed by the Commonwealth Mycological Institute (CMI), UK and were assigned identification numbers as shown in Table 2.

The Analytical profile index (API 20 E kit) was used to identify the bacteria isolates as shown on Table 3.

Table 1. *Identification of fungi associated with some of the sources of cross-contamination of the Shea butter processing line.*

Sources of cross	**Kwara State**		
Contamination	**Futu**	**Apaola**	**Ilota**
Dirty utensils	*Penicilium*sp	*Aspergillus*sp	*Penicilium*sp
	Yeast cells	*A.flavus*	*A. niger*
	Yeast cells		
String stick	*A. flavus*	*A. flavus*	*A. niger*
	Yeast cells	Yeast cells	
Shea waste	*Aspergillus*sp		
	Yeast cells		
Skimmed butter	*Aspergillus*sp	*Aspergillus*sp	
	Yeast cells	*Penicillium*sp	
Surrounding air	*Aspergillus*sp	*Aspergillus*sp	*Aspergillus*sp

Table 2. *Identified fungal isolates.*

Fungus	**CMI code**
Aspergllus niger	502033
Aspergillus niger (aggregate)	503859
Penicillium sp	502028
Aspergillus flavus	503857

Table 3. *Biochemical characteristics of the bacterial isolates from source of water used in Shea butter processing.*

Biochemical test	isolates			
	Salmonella parathyphi	***Enterobacter sp.***	***Klebsiella sp.***	***E.coli***
Lactose	-	+	+	+
Glucose	+	+	+	+
H_2S	-	-	-	-
Gas	+	+	+	+
Motility	+	+	-	-
Indole	-	-	+	+
Urease	-	-	+	-
Catalase	-	+	+	+
Oxidase	-	-	-	-

The result of the mean microbial load across the different stages of Shea butter processing shown on Figure 4, revealed a microbial load of 100 cfu/2g for the Shea kernels collected from Apaola, Ilota and Futu villages, while the grounded kernels has a mean microbial load of 40, 45 and 50 cfu/2g from Apaola, Ilota and Futu villages respectively. There was an increase in the mean fungi load of the kneaded paste which was close to that of the Shea kernels when compared to the grounded kernels. This was attributed to introduction of water and activities of the processors. No microbial load were recorded in the boiling paste while the mean microbial load obtained in the processed Shea butter from Apaola, Ilota and Futu villages was 4, 4 and 6 cfu/2g, respectively.

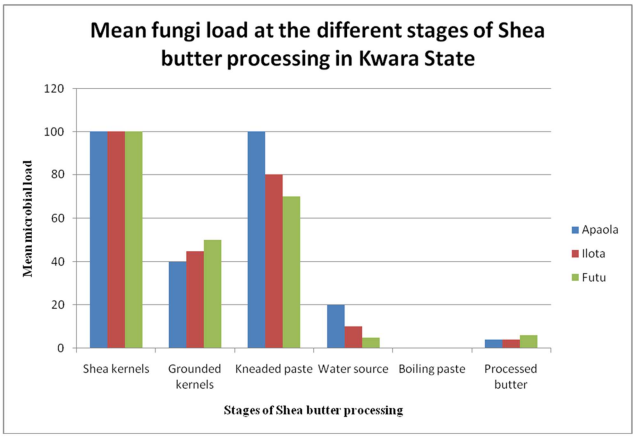

Figure 4. *Mean fungal load at the different stages of Shea butter processing in Kwara State.*

The results of the percentage occurrence of the fungal isolates across the various locations in Kwara State (Figure 5) revealed the percentage occurrence and prevalence of *A. niger* to be higher and more spread within the three villages when compared to the other fungi isolates. While that of the bacteria in Figure 6 shows *Salmonella parathyphi, Enterobacter* sp. and *Klebsiella* sp. to be more spread when compared to *Escherichia coli.*

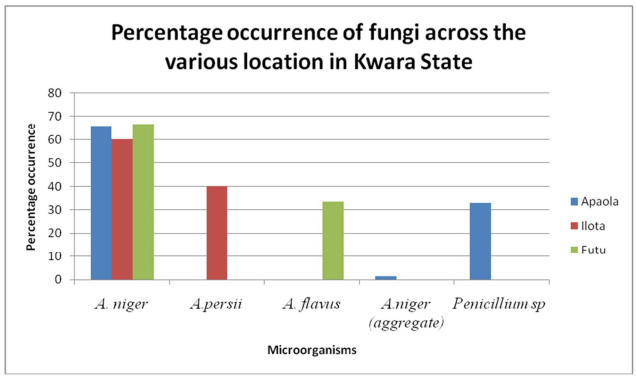

Figure 5. *Percentage occurrence of fungal isolates across the various locations in Kwara State.*

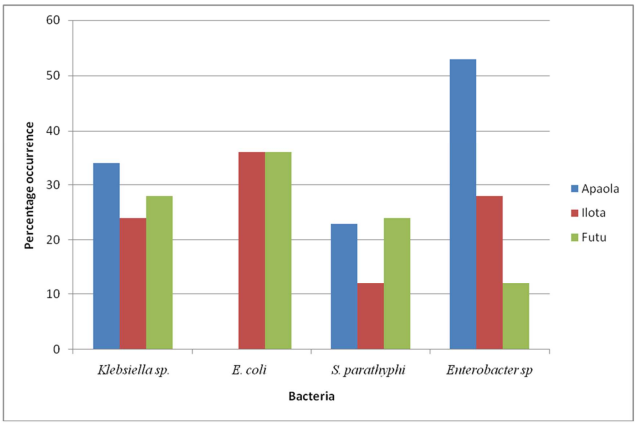

Figure 6. *Percentage occurrence of bacteria isolated from Kwara State.*

4. Discussion

Several authors have made contributions on Shea butter processing with special emphasis on the socio-economic status of women involved in the processing, identification of their means of livelihood of the processors, the existing processing methods, the level of production of Shea butter by the women processors, ascertaining their level of income and assessing the constraints encountered by the women respondents in Shea butter production. According to Ololade and Ibrahim (2014), some of the major constraints experienced by Shea butter processors include inadequate Shea nuts, involvement in non- farm activities, inadequate finance, inadequate extension services, high cost of equipment, inadequate credit facilities, inadequate source of water and un-coordinated marketing system. But the issue of the microbial contamination of the Shea butter during processing has not been addressed

The results from this study showed the sources of microbial contamination of the different stages of Shea butter processing from the three villages surveyed to be the use of microbes infected Shea kernels, water use in processing, and sources of contamination from the processing environment.

Tracking and monitoring the source of contamination is critical for problem identification and remediation Fong, *et al.* (2005). The traceability methods used for tracking the movement of associated the microflora from their points of

contamination to the processed Shea butter were the macroscopic, microscopic and molecular data for fungi and biochemical data for bacteria. This was enhanced by using the schematic flow chart of Shea butter processing

The presence of coliforms in the Shea butter samples indicates that the source of water use for processing by processors from the three villages is exposed to fecal contamination. According to Arizona Department of Environmental Quality (2010), *E. coli* and *Enterobacter* sp. are indicator microorganisms that are used to predict the presence of and/or minimize the potential risk associated with pathogenic microbes. This is because they are nonpathogenic, rapidly detected, easily enumerated, have survival characteristics that are similar to those of the pathogens of concern, and can be strongly associated with the presence of pathogenic microorganisms

The *Aspergillus* spp. associated with these sources of contamination along the processing line was similar to those previously reported by Esiegbuya *et al*. (2014) which were reported to have the ability to change the colour of Shea kernels thereby causing kernel discoloration and deterioration.

Aspergillus niger which was one of the dormant species isolated from this study, has been reported as a major contaminant of peanuts, corns, grains (Cheesborough, 2005) and most popular staple foods in Africa (Thomas *et al*., 2012). They are also known to produce secondary metabolites and causing of infections in immunocompromised individuals (Klich, 2002).

5. Conclusion

This study has shown that in order for the microbial quality of processed Shea butter to be improved on, factors such as purity of water, clean processing environment/working utensils, use of non-microbe infested Shea kernels and hygienic condition of processors must be regarded as critical factors in the processing of good quality Shea butter.

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