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METAGENOMIC TRACKING OF MICROBIAL CONSORTIA OF CASSAVA FLAKES (*garri*)

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ABSTRACT

The affirmation of several cross-sectional studies on the vulnerability of cassava flakes commonly called '*garri*' to microbial attack has long been documented. However, longitudinal data on metagenomic tracking of microbial consortia of this important staple food are scarce. Hence, this study was aimed at tracking the microbial consortia of *garri*. A total of eight samples (four each from both Nigeria and Republic of Benin markets) were randomly collected aseptically using pre-sterilized aluminum pans and processed through a metagenomic approach, while both the chemical and proximate components of *garri* were assessed following standard techniques. The analysis of the taxonomic consortia of *garri* reveals the predomination of bacteria (99.82 and 99.81% for samples from Nigeria and Republic of Benin, respectively) while the remaining sequences matched with the Archae (0.07%), fungi (0.09%) and protozoa (0.09%). A large proportion of the sequences were unclassified at the phylum level (approximately 84.10 and 86.2% for Nigerian and Beninese samples, respectively). The reads of cassava flakes metagenome of both Nigeria and Republic of Benin exhibited analogous level of average GC content with sequence count of between 187773-213444 for samples from Nigeria and 157784-198763 for samples from Republic of Benin. The functional characteristics of the inhabiting metagenomes were found containing the genes encoding for adhesins, bacteriocins, resistance to antibiotics, toxic chemicals as well as toxins and superantigens. Both the chemical and the proximate compositions of the examined *garri* samples, though exhibited significant disparity, but without any apparent variation in the patterns of metagenomic data. Our findings however revealed bacteria as the major contaminants of these cassava food products.

Keywords; Metagenomics, Microorganisms, Cassava flakes (*garri*), Proximate composition

INTRODUCTION

For more than three decades now, contamination of the most popular cassava food products in West Africa including Nigeria has become a topical issue eliciting great deal of public concern (Thomas *et al.,* 2012; Aguoru *et al.,* 2014; Lawali *et al.,* 2015; Okafor *et al.,* 2018). This cassava food product known as *garri* in several West African states has been reported to be variously contaminated by different xerophilic moulds in Nigeria (Egbuobi *et al.,* 2015; Lawali *et al.,* 2015; Orpin *et al.,* 2020), Kenya (Gacheru *et al.,* 2016; Orpin *et al.,* 2020), Liberia (Awoyale *et al*., 2017) and other African countries. Several other studies also documented avalanche of bacteria in this important cassava food product (Egbuobi *et al.,* 2015; Gacheru *et al.,* 2016; Okafor *et al.,* 2018).

In Nigeria, practices associated with *garri* processing, production and post processing such as spreading on the floor, mats and hauling in open bowls in market places may exacerbate microbial contamination and their subsequent proliferation (Ogiehor and Ikenebomeh, 2005; Thomas *et al.,* 2012; Gacheru *et al.,* 2016). These microbial contaminants may serve as vehicle for food borne diseases (Omar *et al*., 2003; Majumdar *et al.,* 2018), while mycotoxigenic fungi may be responsible for substantial effects in stored food stuffs including discolouration, losses in nutritional value, production of off-odours, deterioration in technological quality and contamination with mycotoxins (Basilico *et al.,* 2001; Magnolia *et al.,* 2006; Orpin *et al.,* 2020).

Despite the ubiquitous reports of microorganisms in this food, there is still paucity of information on the tracking of microbial consortia of these important cassava food products using metagenomic technique. This study was therefore aimed at investigating the microbiome and the functional gene profile of cassava flakes from some southern-western states of Nigeria and Republic of Benin. Our main questions however were (i) what are the microbial consortia of cassava flakes present in the sample

collection sites? (ii) what are the relative distribution of these microbial consortia at taxonomic level and (iii) what are the functional characteristics of the inhabiting microbial metagenomes?

MATERIALS AND METHODS

Sampling and Samples Collection

A total of eight samples (four each from four selected markets in Ogun State, Nigeria and another four from Ipobe, Ketou, Cove and Bohicon in Republic of Benin were used for this study (Table 1). The collection of samples was done in accordance with the specification of the International Commission for Specification for Foods (Anderson, 2018). Sampling was performed in March 2016 at various times in that month.

Table 1: Longitude and Latitude of the Sample Collection Sites

Sample code	Collection sites	Country	Longitude	Latitude
SNN1	Obantoko	Nigeria	7º 11' 8" N	3º 22' 36" E
SNN2	Fibigbade	Nigeria	6º 56' 47" N	3º 55' 35" E
SNN3	Ajaka	Nigeria	6º 51' 7" N	3º 38' 13" E
SNN4	Oja odan	Nigeria	6º 53' 46" N	2º 50' 15" E
RPB1	Ipobe	Rep. of Benin	6º 98' 32" N	2º 66' 36" E
RPB2	Ketou	Rep. of Benin	7º 36' 03" N	2º 60' 40" E
RPB3	Cove	Rep. of Benin	7º 21' 89" N	2º 33' 94" E
RPB4	Bohicon	Rep. of Benin	7º 17' 98" N	2º 07' 14" E

SNN= Samples from Nigeria, RPB= Samples from Republic of Benin
Each sample was collected in duplicate per collection site and analyzed separately.

Chemical and Proximate Analyses of *Garri*

The chemical analysis of *garri* was determined using the method described by Nout *et al.* (1989) while the proximate analysis was estimated following the recommended method of the Association of official Analytical chemists as described by Ajifolokun and Adeniran (2018)

DNA Extraction and Metagenomic Sequencing

Total DNA from cassava flakes was extracted using the Norgen's food DNA isolation kit (Norgen Biotek Corp., Thorold, ON, Canada) according to the manufacturer's protocol, from approximately 500 mg of cassava flakes. DNA quality was checked using 1% agarose gel electrophoresis and quantified using 2100 Bioanalyzer (Agilent technologies, USA). DNA samples were sequenced using the ion proton platform with chip PI and Ion PI tempted OT2 200v3 and Ion PI sequencing 200v3 (Life technologies, USA) according to manufacturer's instruction.

Sequence and Statistical Analysis

The raw sequence reads were filtered and trimmed using the PRINSEQ software (Schmieder and Edwards, 2011). Microbial composition analysis was performed using the MG-RAST best hit classification tool, where reads were compared to the SSU-SILVER (non-redundant) database using a minimum identity of 80%, maximum e-value of $1e^{-5}$ and a minimum alignment length of 60, measured in base-pair to generate their taxonomic profiles (Quast *et al.,* 2013). Functional classification was done using the MG-RAST hierarchial classification tool based on KEGG orthology (KO) (Kanehisa *et al.,* 2004 and SEED subsystems (Overbeek *et al.,* 2005). The data was compared to each database using a maximum e-value of $1e^{-5}$, a minimum identity of 80%, and a minimum alignment length of 20, measured in amino-acids to generate functional profiles. The proximate and the chemical composition of *garri* obtained from Nigeria were individually pooled together relative to those collected from Republic of Benin and subsequently analyzed using student t test.

RESULTS

The proximate composition of *garri* reveals significant disparity in the mean moisture, ash, crude fibre, carbohydrate, dry matter, protein and

fat contents while both titrable acidity and pH also shows apparent statistical variation. The samples however show good representation of carbohydrate (75.68 ± 2.36% for Nigerian samples and 77.38 ± 0.42% for Beninese samples) and dry matters. Both samples were consequently found to be very poor in fat and protein contents (Table 1).

The analysis of the taxonomic consortia of cassava flakes (*garri*) revealed that this food was dominated by bacteria, 99.82 and 99.81% for samples from Nigeria and Republic of Benin respectively. Other matches include Archae (0.07%), fungi (0.09%) and protozoa (0.09%) among samples collected (Figure 1). The microbial compositions contained 10 different phyla and three other phyla namely *Ascomycota, Ciliophora* and *Euryarchaeota.* A large proportion of the sequences were unclassified at the phylum level (approximately 84.10 and 86.2% for Nigerian and Beninese samples, respectively). The relative abundance of the organisms revealed significant statistical variation between bacterial abundance and other organisms (Figure 2). The reads of cassava flakes metagenome of both Nigeria and Republic of Benin exhibited analogous level of average GC content with sequence count of between 187773-213444 for samples from Nigeria and 157784-198763 for samples from Republic of Benin (Table 2). The functional characteristics of microbial metagenomes inhabiting cassava flakes in Nigeria and Republic of Benin determined by the classification of predicted functional genes revealed the presence of adhesins, bacteriocins, resistance to antibiotics and toxic chemicals as well as toxins and super antigens (Table 3).

Table 2: Chemical and Proximate Composition of *Garri* Sampled from Nigeria and Republic of Benin

Sample code	MC(%)	Ash content (%)	CF(%)	CHO(%)	DM (%)	PC (%)	FTC (%)	TA (%)	pH
SNN	9.36±1.40^{a}	1.58±0.19^{c}	1.66±0.03^{e}	75.68±2.36^{g}	91.15±2.15^{i}	1.20±0.05^{k}	1.06±0.06^{m}	0.23±012^{o}	5.43±0.92^{q}
RPB	9.21±0.71^{b}	1.46±0.13^{d}	1.73±0. 09^{f}	77.38±0.42^{h}	89.89±1.49^{j}	1.16±0.06^{l}	1.13±0.03^{n}	0.35±010^{p}	5.20±0.41^{r}

MC= moisture content, CF= Crude fibre, CHO= Carbohydrate, DM= Dry matter, PC= Protein content, FTC= Fat content, TA= Titrable acidity %= Percentage, SNN= Samples from Nigeria, RPB= Samples from Republic of Benin, Values expressed in Mean±SD followed by different letters are statistically different ($P < 0.05$).

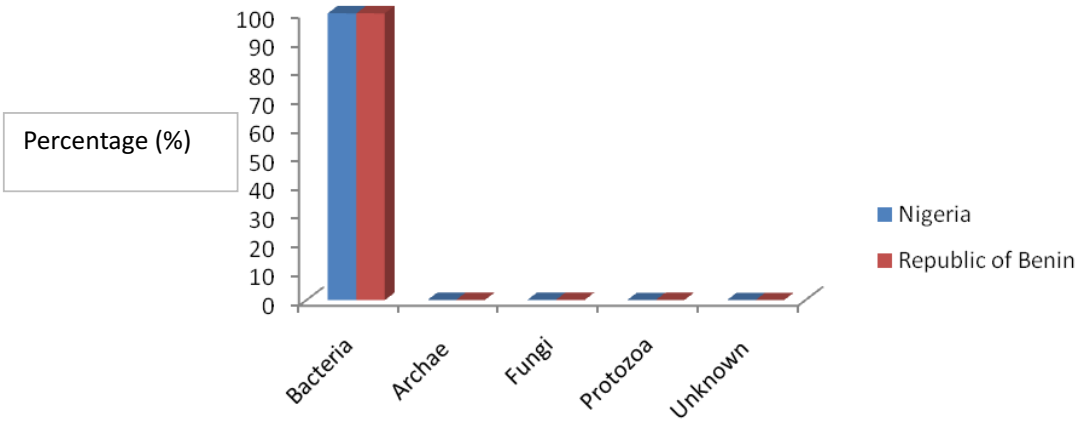

Figure 1: Taxonomic Classification of Microbial Consortia by Kingdom

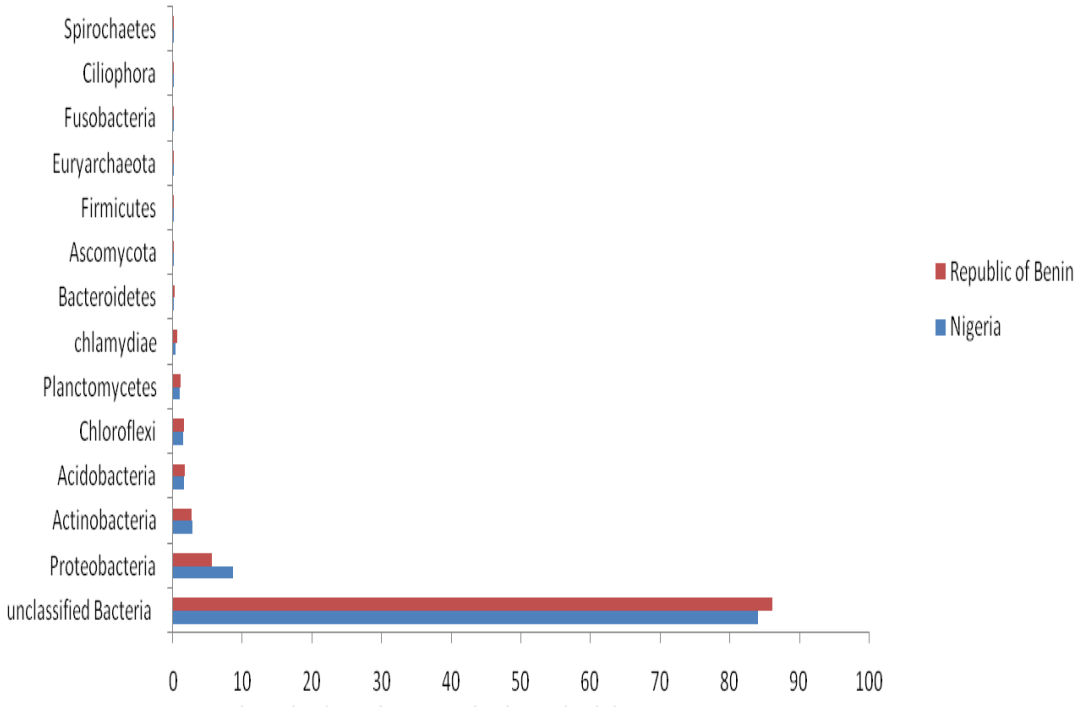

Figure 1: Relative Abundance of Organisms' Classification by Phylum (%)

Table 3: DNA Sequence Read Metrics of the Eight Metagenomic Samples from *Garri*

Metagenome		Sequences Count	Sequences Count Post Quality Control	Average GC content	Reads Average Length
Nigeria	SNN1	187773	145210	66±2%	95bp
	SNN2	200773	182311	65±4%	95bp
	SNN3	195773	152114	64±2%	95bp
	SNN4	213444	178111	66±3%	95bp
ROB	RPB1	196674	162181	59±10%	110bp
	RPB2	198763	142263	56±11%	110bp
	RPB3	157784	111102	53±10%	115bp
	RPB4	163463	142301	54±10%	120bp

ROB= Republic of Benin, SNN1-4= Samples from Nigeria, RPB1-4= Samples from Republic of Benin, bp =base pair, %= percentage

Table 4: Functional Characteristics of Microbial Metagenomes Inhabiting *Garri* in Nigeria and Republic of Benin (%)

Functional category	Nigeria	Republic of Benin
Adhesion	0.00013	0.00010
Bacteriocins	0.0011	0.0001
Resistance to antibiotics and toxic compounds	0.0123	0.0011
Toxins and super antigens	0.003	0.0012

DISCUSSION

The use of metagenomics for understanding the microbial diversity in food has become a powerful tool for deciphering the level of food contamination in order to determine their safety level without any pre-cultivation experiments (Miller *et al.,* 2013; Forbes *et al.,* 2017; Gruetzke *et al.,* 2019). Our study was the first to track the microbial consortia of cassava flakes (*garri*) in some selected markets in both Nigeria and the Republic of Benin using shotgun metagenomics. The significant variation observed in the mean chemical and proximate compositions of the examined *garri* samples may not be unconnected to the variation in their methods of processing. This is because it had earlier been documented by Kavitha and Parimalavalli (2014) that processing methods influence the composition (both the chemical and proximate components) of cereals and legume flours. In another vein, Adane *et al.* (2013) observed that proximate and mineral composition of taro (*Colocasia esculenta* (L.) Schott) can be increased by optimization of their processing techniques.

The higher content of carbohydrate observed in this study however is a result of higher contents of sugar and starchy grains present in cassava roots that were subsequently released during rettting of cassava (Brauman *et al.,* 1996) while the lower level of proteins and fats may be ascribed to their low presence in the tuber which were then lost or washed off during processing (Oyewole and Odunfa, 1989; Oyewole and Afolami, 2001). It was hitherto found that despite the significant statistical variation in the chemical and proximate composition of this important staple food, metagenomic data of samples from both Nigeria and Republic of Benin follows the same pattern. This finding is an indication that the overall patterns of taxonomic and functional characteristics of microbial metagenomes inhabiting *garri* do not necessarily correlate with the nutritional and chemical composition of this food.

In this study, we identified an increased abundance of bacteria within the phyla *Proteobacteria, Actinobacteria* and *Acidobacteria* as the major contaminants of this important staple food in West Africa. *Proteobacteria* which represents the most predominant in this study is a major phylum of Gram-negative bacteria that include a wide variety of pathogenic genera, such as *Escherichia, Salmonella, Vibrio, Helicobacter, Yersinia, Legionellales* among others, signifying the consumption of this food as a potential source of human disease (Rizzatti *et al.,* 2017).

Actinobacteria, the second most prevalent phylum of Gram-positive bacteria, can be terrestrial or aquatic, are of great economic importance due to their contributions to soil systems. This implies the possibility of food contaminated via soil (Elshafei, 2017). Processes associated with *garri* processing such as spreading on the mats as well as on plastered surfaces might have exacerbated such contamination from soil (Ogiehor and Ikenebomeh, 2005; Thomas *et al.,* 2012; Okafor *et al.,* 2018).

Consequently, the representative sequence counts and the average GC contents observed in this study further reinforced that the analyzed samples were significantly rich in microbial contaminants. This is contingent upon the fact that the genomic DNA base composition is significantly associated with genome size and holocentric chromosomal structure (Smarda *et al.,* 2014; Thomas *et al.,* 2019). According to Smarda *et al.* (2014), these genomic DNA base compositions are also known to significantly affect genome functioning and species ecology. In this study, genes encoding adhesins, bacteriocins, resistance to antibiotics and toxic chemicals as well as toxins and super antigens were found from both the samples from Republic of Benin and Nigeria to affirm these functional characteristics as major intersecting factors of cassava flakes. Adhesins which was delineated as one of the functional metagenomes of cassava flakes are virulence factors produced by certain pathogenic microorganisms and are considered to play an essential role in disease pathogenesis by allowing bacteria to attach to host cells. Although many pathogenic bacteria express various kinds of adhesins, often they are encoded on the bacterial backbone DNA (Hallstrom and McCormick, 2015; Vance *et al.,* 2019). Bacteriocin genes observed in this study are produced by both Gram-positive (*Lactobacillus*, *Lactococcus*, *Streptococcus*, *Enterococcus*, *Leuconostoc*, *Pediococcus*,

and *Propioni bacterium*) and by Gram-negative bacteria (*Escherichia coli*, *Shigella*, *Serratia*, *Klebsiella* and *Pseudomonas*). The interest in them reflects potential application of the metabolites in medicine and as natural food conserving agents (Karpiński and Szkaradkiewicz, 2013; Silva *et al.,* 2018).

The availability of genes encoding resistance to antibiotics and toxic chemicals in the analyzed *garri* samples may be connected to the presence of certain antibiotics inactivating enzymes (Brandt *et al.,* 2017; Markley and Wencewicz, 2018). This latter structural change has been shown to reduce bacterial susceptibility to cationic antimicrobial peptides and polymyxin, and to contribute to increased pro-inflammatory signaling (Helander *et al*., 1995; Nummila *et al*., 1995; Gunn *et al*., 1998; Markley and Wencewicz, 2018; Ezadi *et al.,*2019). Consequently, the presence of toxins and super antigen encoding genes further suggest the possibility of this food serving as source of food poisoning especially if it is not properly prepared under good hygienic processes (Thomas *et al.,* 2012; He *et al.,* 2018). These observations however add to our microbiological results implying that the analyzed food is characterized by a core of microorganisms that is capable of evading host defenses and even certain antimicrobial drugs, thereby playing important role in their pathogenesis.

CONCLUSION

The results of the present study showed that bacteria are the major contaminants of *garri while* the functional characteristics of the inhabiting metagenomes were found containing the genes encoding for adhesins, bacteriocins, resistance to antibiotics, toxic chemicals as well as toxins and superantigens.

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