Microbial evaluation of fermented milk and millet beverage (brukina) produced in Ghana by culture-independent technique.

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Abstract

Fermented food products have been reported to have great nutritional value however artisanal fermentation practices can comprise on food safety and quality. Brukina, a fermented milk and millet smoothie was used as a model to determine the influence of these fermentation practices on the bacterial populations present in the sample. Using 16S rRNA sequencing technique, it was observed that the bacterial populations were assigned to five main phyla; *Firmicutes*, *Proteobacteria*, *Deinococcus-Thermus*, *Actinobacteria* and *Bacteriodetes* with *Firmicutes* making up greater than 90% of the bacterial communities present. Most of the *Firmicutes* were lactic acid bacteria of the genera Lactobacillus, Streptococcus, Leuconostoc, Lactococcus and Weisella. Pathogenic genera such as Enterobacter, Yersinia and Sarcina were detected in the samples but in low abundance of phylum *Proteobacteria* and *Firmicutes* were also detected in the samples but in low abundance. Since these pathogenic genera are mainly environmental pathogens or associated with the human gut it suggest that adherence to safety protocol could limit their presence in food samples and improve on food safety.

Keywords: Fermented, Millet, Milk, Lactobacillus, Streptococcus, *Firmicutes*.

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Introduction

Fermented milk products have been consumed across different cultures for thousands of years. Although originally developed as a means of preserving the milk, it is now known that fermentation of milk improves the organoleptic properties of the milk while enhancing the health benefits of the milk [1]. Additionally fermented milk is a source of probiotic organisms which help maintain the health of the gastrointestinal tract, enhance the immune system, improves metabolism and maintain urogenital health [2,3]. Cereal based beverages are another class of functional foods which are a good source of probiotics. Fermentation of cereals leads to a reduction in the total carbohydrate content while increasing the bioavailability of certain B vitamins and amino acids [4,5]. It's therefore not surprising that these fermented cereals have been reported to have several health benefits [6].

Brukina, a fermented milk and millet smoothie is produced by mixing milled steamed millet with fermented milk and sugar. The mixture of milk and cereal in this product suggests that it will be a good source of essential nutrients and probiotics to support growth and development. This is particularly important in the African region where malnutrition and hunger is very prominent thus; brukina represents a cost-effective meal that can provide the essential nutrient required for growth and development [7]. Infact proximate analysis of brukina samples has shown that it has high nutritional content however; concerns on food quality and safety have been raised [8].

In the production of brukina, milk is usually fermented spontaneously or in some cases by back slopping with previously fermented milk. Since milk left at ambient conditions is a good environment for the proliferation of bacteria due to its high nutrient content and near neutral pH, it is thought that such uncontrolled fermentation procedure employed could support the proliferation of certain pathogenic strains [9]. Additionally, due to limited regulation on the food safety practices of most of the food producers in the informal sector, some of these vendors work under unsanitary conditions do not practice good personal hygiene or use non-potable water for the production. Despite these safety concerns there is scarcity of data on the microbial content of this beverage. The few studies that have looked at the microbial contents have however focused on looking at the presence of specific pathogenic strains using culture-dependent techniques and biochemical test. These studies have shown the presence of *E. coli* O157:H7 and *S. aureus* which are common enteric bacteria in the sample [10]. The use of these culture-dependent methods however, does not provide information on the total microbial community present in the sample.

Recent advancement in molecular biology has provided us with the means to determine a plethora of information on the microbial composition of specimen from different location using culture-independent techniques. These techniques amongst others provide information on low abundance microorganisms as well as uncultivable organisms present in a sample [11]. The aim of this study therefore was to characterize

the bacterial communities present in brukina samples obtained from different vendors in the Ho Municipality of Ghana using culture independent techniques in order to determine their role on food safety and quality.

Methodology

Source of sample and preparation

The brukina sample used in the study was obtained from five different vendors within the Ho municipality in Ghana. Samples were pulled together for downstream analysis. Method used in the commercial production of brukina is highlighted in (Figure 1).

DNA isolation

Collected samples were homogenized using a blender. Samples were then centrifuged at 16000g for 10 mins and the supernatant removed. This step was repeated four times to pellet enough cells for DNA isolation. Pellets were then lysed using bead beating and DNA isolation carried out using guanidine thiocyanate silica column based purification method as previously described .

PCR amplification and sequencing

The V4 variable region of the 16S rRNA gene was amplified using the following primers: forward primers GTGCCAGCMGCCGCGGTA and the reverse primers GGACTACHVGGGTWTC TAAT as previously reported. Samples were quantified on the BioRad MyiQ and subsequently sequenced on the Illumina Next Seq 500 sequencer.

Taxonomic annotation and reference database generation

The Illumina BCL2FASTQ algorithm was used for demuliplexing and base calling. Reads were filtered using an average Q-score>30 after which pair-end reads were assembled together and clustering done with Swarm alogorithm. A real biological sequence was defined as the most abundant sequence per cluster and this was assigned the count of all reads in the cluster. Chimera was removed from representative reads from the entire cluster using VSEARCH algorithm. Reads were then aligned against a hand-curated database of target 16S rRNA gene sequences and taxonomic annotations derived from version 123 of the SILVA database. The relative abundance of each taxon was determined by taking the ratio of the count linked to those taxa to the total number of filtered reads.

Discussion

Globally, consumption of unsafe food results in approximately 420,000 deaths annually and is the cause of more than 200 diseases ranging from diarrhoea to cancers [12]. Food safety and quality is therefore an important public health issue since it affects people of all ages, gender and socioeconomic status. The role of microbes in food safety has been widely studied over years and it's been established that these microbes have a dual role in terms of food safety. Whereas some microbes produced mycotoxins and biogenic amines which results in food spoilage, others produce antioxidants and bacteriocin which helps to improve the self-life of food products [13,14]. Hence a detailed understanding of the microbial communities present in a food sample is important in promoting food safety.

In the current study 16S rRNA sequencing technique was used to determine the bacterial communities present in brukina, a fermented milk and millet smoothie. At the phylum level, *Firmicutes* was the most abundant phylum followed by Proteobacteria. Other phyla seen were *Deinococcus Thermus*, *Bacteriodetes* and *Actinobacteria* (Figure 2). The observation of these five bacterial phyla in this sample is similar that reported previously when fresh camel milk from Mongolian was evaluated for its microbial communities but their study results showed a high percentage of *Proteobacteria* than *Firmicutes* [15].

The phylum *Firmicutes* includes Lactic Acid Bacteria (LAB) family such as Lactobacillus, Streptococcus, Leuconostonoc and Weisella which have identified as probiotic bacteria populations [16] LAB have also been documented to improve food safety by inhibiting the growth of food spoilage microbes, enriching the nutritional value of food and improving the organoleptic properties of food [17,18]. The results of this study show that the high *Firmicutes* seen was mainly due to the high prevalence of LAB such as Lactobacillus and Streptococcus (Figure 3). Other LAB such as Leuconostoc, Weissella and Lactococcus were detected in low abundance (Table 1). In addition to this bacterial strain the phyla *Firmicutes* also contain genera such as Clostridium, Bacillus, Staphylococcus which contain pathogenic species that have been associated with foodborne illnesses [19]. These common foodborne pathogens were not detected in this study however, Sarcina (Table 1) seen in this study has been reported to be associated with gastric stasis [20] Geobacillus and Anoxybacillus which were observed in our study (Table 1) are examples of thermophilic bacillus that have been reported to be associated with milk. They can secrete enzymes that can affect the nutritional value of the food by affecting oxidation while at the same time form biofilms on production surfaces which can promote further contamination of dairy products [21,22].

The phylum *Proteobacteria* was the next abundant phyla. This phylum was represented by Acetobacteraceae, Moraxellaceae, Pseudomonadaceae and Moraxellaceae which contain many enviromental pathogens and are occasionally the cause of human infection [23]. Common foodborne pathogens in this phyla such as Salmonella spp, Shigella spp and E. coli were not detected in the samples analyzed however, Enterobacter and Yersinia which contain pathogenic species were detected [24] (Table 1). Given that traditional fermentation is often done in the open air without the adherence to food safety practices, it is not surprising that these microbial populations often associated with the environment and human gut can be detected in the samples. This implies that the use of proper food hygience protocols may limit the levels of these

organisms in the sample and improve on its safety and quality [25].

Phylum	Class	Order	Family	Genus
Actinobacteria 0.01%	*Actinobacteria* 0.01%	Micrococcales 0.01%	Microbacteriaceae 0.01%	Curtobacterium 0.01%
				Microbacterium <0.01%
	Coriobacteriia <0.01%	Coriobacteriales <0.01%	Coriobacteriaceae <0.01%	Parvibacter <0.01%
Bacteroidetes 0.04%	Flavobacteria 0.03%	Flavobacteriales 0.03%	Flavobacteriaceae 0.03%	Chryseobacterium 0.03%
	Cytophagia <0.01%	Cytophagales <0.01%	Cytophagaceae <0.01%	Flectobacillus <0.01%
	Sphingobacteriia <0.01%	Sphingobacteriales <0.01%	Sphingobacteriaceae <0.01%	Nubsella <0.01%
Deinococcus-Thermus 2.00%	Deinococci 2.00%	Thermales 2.00%	Thermaceae 2.00%	Thermus 2.00%
Firmicutes 94.72%	Bacilli 94.62%	Bacillales 1.28%	Bacillaceae 1.22%	Anoxybacillus 0.68%
				Exiguobacterium 0.05%
				Geobacillus 0.54%
			Staphylococcaceae 0.01%	Macrococcus 0.01%
		Lactobacillales 93.33%	Lactobacillaceae 61.00%	Lactobacillus 60.09%
			Leuconostoceae 0.05%	Leuconostoc 0.02%
				Weissella 0.02%
			Streptococcaceae 32.34%	Lactococcus <0.01%
				Streptococcus 32.31%
	Clostridia 0.11%	Clostridiales 0.10%	Clostridiaceae 0.10%	Anaerobacterium 0.07%
				Sarcina 0.03%
Proteobacteria 3.21%	*Alpha - proteobacteria* 0.62%	Rhodospirillales 0.53%	Acetobacteraceae 0.53%	Acetobacter 0.53%
				Gluconobacter 0.01%
		Sphingomonadales 0.08%	Sphinogomonadaceae 0.08%	Novosphingobium 0.04%
				Sphingobium 0.03%
			Erythrobacteraceae	Porphyrobacter <0.01%
				<0.01%
	Beta-proteobacteria 0.14%	Neisseriales 0.02	Chromobacteriaceae 0.02%	Vogesella 0.02%
		Burkholderiales 0.11%		Roseateles 0.01%
			Burkholderiaceae <0.01%	Pandoraea <0.01%
			Comamonadaceae 0.03%	Comamonas <0.01%
				Delftia <0.01%
				Pelomonas <0.01%
			Oxalobacteraceae 0.05%	Herbaspirillum <0.01%
				Massilia 0.04%
				Undibacterium <0.01%
	Gamma-proteobacteria 2.44%	Enterobacterales 0.43%	Enterobacteriaceae 0.41%	Enterobacter <0.01%
				Klebsiella 0.39%
				Kluyvera <0.01%
			Erwiniaceae 0.01%	Pantoea 0.01%
			Yersiniaceae <0.01%	Yersinia <0.01%
		Xanthomonadales 0.01%	Xanthomonadaceae 0.01%	Stenotrophomonas 0.01%
		Pseudomonadales 2.00%	Moraxellaceae 1.85%	Acinetobacter 1.85%
			Pseudomonadaceae 0.15%	Pseudomonas 0.15%

Table 1: *The in-depth taxonomical annotation of the different bacterial populations present in the brukina sample is shown in Table 1. The results show that 40 different genera belonging to 5 phyla were obtained from the sample analysed. Out of these, most of the genera (20) belonged to the phylum Proteobacteria however; most of these genera were in low abundance. The abundant genera, Lactobacillus and Streptococcus were however assigned to the phylum Firmicutes.*

Results

Results show that *Firmicutes* is the most abundant phyla in brukina making up greater than 90% of the total bacterial communities in the sample. This is followed by *Proteobacteria*

which makes up 3.2% and then *Deinococcus-Thermus* that makes up 2.00% of the sample. Other rare phyla detected in the sample are *Actinobacteria* and *Bacteriodetes*.

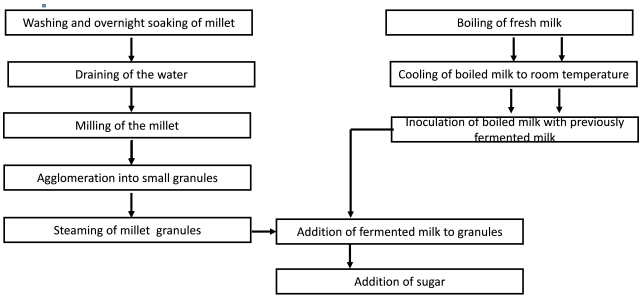

Figure 1: *Steps involved in the artisanal production of brukina.*

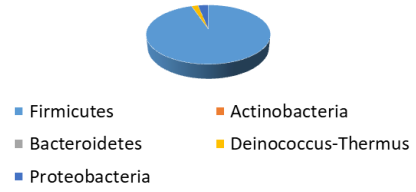

Figure 2: *Relative abundance of bacterial communities at the phylum level.*

The relative abundance of the different bacterial communities at the genus level is presented in Fig 3. *Lactobacillus* and *Streptococcus* (both of which are *Firmicutes*) made up 60.96% and 32.31% of the bacterial communities respectively. This was followed by *Thermus* which made up 2.00% of the bacterial communities and *Acinetobacter* which made up 1.85%. All other rare genus present in the sample represented only 2.86% of the bacterial population.

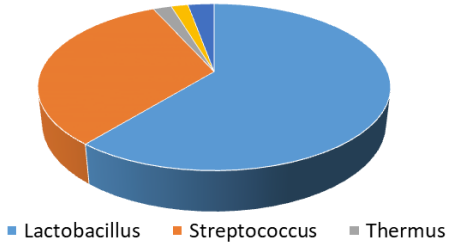

Figure 3: *Relative abundance of bacterial communities at the genus level.*

Conclusion

The results of this study show that brukina is predominantly composed of *Lactobacillus* and *Streptococcus* which have been shown to possess probiotic functions. Although potential pathogenic genera such as *Yersinia*, *Enterobacter* and *Sarcina* were detected in the sample they were present in low abundance. Since these are mainly environmental pathogens good hygiene practices may limit their presence in the food and improve on its safety.

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