Study on the characteristic properties of Gambian traditional fermented milk

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ガンビアの伝統的な発酵乳の特性に関する研究

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General Introduction

The Gambia is the smallest country on the African continent, with a total area of 11,000 square kilometers and a population of approximately 2.4 million. It is surrounded on all sides by Senegal except the West by the Atlantic Ocean. It is about 450 km long from east to west, and has a coastline of 80 km to the west and a border of about 25 km at the eastern end of the country. It is made up of five administrative regions and two municipalities. The Gambian economy is mainly reliant on services, agriculture and tourism. About 70% of the country's labor force are employed in agriculture. Although agriculture being an important sector of the economy, farmers in The Gambia still practice subsistence agriculture. In the past two decades, crop production has declined due to many factors including government policies and drought linked to climate change. Meanwhile livestock production has been increasing at an uneven rate. Cattle, goats, sheep, poultry, horses and donkeys are bred as livestock in the country. The estimated populations of cattle, goats, sheep and poultry, which are the major livestock, are approximately 300,000, 250,000, 150,000, and 870,000, respectively. The country's major livestock products are milk, meat (beef, mutton, goat meat, poultry meat) and eggs with an estimated output of 76,000, 7,000, and 1,000 tons, respectively (FAOSTAT 2018). Among these livestock, cattle are especially essential for the rural communities in The Gambia as they served as sign of wealth (prestige), expressing of cultural heritage, source of income, meat, milk, draught power and farm manure for growing crops.

Like other West African countries, cattle rearing and milk production is mostly done by the pastoralists Fulani in The Gambia. Majority of them lead a nomadic life and has not modernized their livestock production. Whilst milking is mostly done by men, the preparation and selling of milk and dairy products is dominated by women. Cattle herders usually perform milking in the morning using bare hands in the open ranches. Milk is collected in vessels such as Calabash or wooden gourds depending on the choice and convenience of the farmer. The milk is filtered through either a sieve or a clean white cloth. Some fresh milk will be eaten or sold as it is. The rest of the raw milk is allowed to spontaneously ferment in a Calabash Gourd or plastic vessels for 24 hours. The completed Gambian traditional fermented milk can be described as a yoghurt-like product with a very thick consistency, sour taste accompanied by a creamy top layer with a pleasing aroma. Traditional FM have a unique name for each ethnic group, as The Gambia is a multi-ethnic and multilinguistic country. For instance, among the three major ethnic groups, it is named as "*Kosam*" in the Fula language, "*Sow*" in Wolof and "*Nonno*" in Mandinka. Fermented milk is also consumed without pasteurization, like fresh milk. Generally, Gambian traditional fermented milk is consumed with sugar.

Otherwise, it is either drank alone or in many cases accompanied by cereals like Chakiri or Churai Gerteh (Porridge made by mixture of wholegrain rice and powered peanut) or Mono (a watery Porridge made from millet flour).

Milk and fermented milk are popular with people of all walks of lives and ages in The Gambia, as it is well known to contain useful nutrients such as carbohydrates, fats, proteins and calcium, which are essential to consumers. However, consumers have little knowledge about the characteristics of milk and dairy products produced in The Gambia. Hempen *et al.* (2004) have reported that Gambian milk and dairy products are highly contaminated with coliform bacteria as well as pathogenic bacteria, which can pose severe health risks to consumers. Such contamination has been suggested that it is due to the poor sanitation of the bovine udder, milker's hands, milking environment, milking, transport and fermentation vessels. The FAO/WHO (WHO/FAO 2004) and International Dairy Federation (IDF 2019) recommend milk to be pasteurized in other to be safe for public consumption. However, milk and fermented milk produced in The Gambia are consumed without pasteurization, like many other African countries. Diarrheal disorders in children under the age of 5 have been frequently reported in The Gambia (Saha *et al*. 2013), suggesting that pathogenic microorganisms present in unpasteurized milk and fermented milk are one of the causes (Hempen et al. 2004).

On the other hand, fermentation has been practiced to extend the shelf life of food in many African countries for centuries. One of such fermented foods is traditional fermented milk, which has been handed down for generations in various parts of Africa. During the fermentation of milk, LAB ferments lactose into mainly lactic acid which lowers the pH by increasing in acidity. Subsequently organic acid produced by LAB inhibits the growth of pathogenic and spoilage bacteria (Rubak *et al.* 2020; Widodo *et al.* 2017). Some LABs have also been reported to produce antimicrobial compounds such as bacteriocins, diacetyl, ethanol, fatty acids and hydrogen peroxide (Hassan *et al.* 2020). In addition to these antibacterial effects, some LAB is also known to relate the formation of texture, taste and flavor of dairy products, and to function as probiotics (Terzić-Vidojević *et al*. 2020 and 2021). So far, various LABs have been isolated from traditional African fermented milk by spontaneous fermentation of raw milk (Jans *et al.* 2017). Some of these LAB have already been used as starters for producing fermented milk. For instance, *Lactococcus lactis*, *Lactobacillus bulgaricus* and *Streptococcus thermophilus* respectively isolated in Chadian, Egyptian and Ghanaian fermented milks, are universally adopted as starter cultures for producing safe fermented milk (Doutoum *et al.* 2013; El-Baradei *et al.* 2008; Obodai and Dodd, 2006). Although LAB has never been

isolated from Gambian traditional fermented milk, it may also contain beneficial LAB as in other traditional fermented milk.

Consequently, due to improve living standards, changes in life style and consumer choices, there has been a steady increase in demand for milk and dairy products recently. Moreover, due to increase awareness and concerns on food safety by consumers, there is an urgent need to comprehensively analyze and evaluate traditional Gambian fermented milk. Therefore, the aim of this research was as follows;

1. Clarification of the total microbial diversity of Gambian traditional fermented milk and to assess its microbiological quality and safety.
2. Isolation of lactic acid bacteria from Gambian traditional fermented milk and evaluation of their effect on the quality by analyzing the characteristics of LAB, mainly acid producing ability and bacteriocin-like inhibitory substance producing ability.
3. Characterization of bacteriocin produced by LAB isolated from Gambian traditional fermented milk.

In addition, this research also provides an opportunity to preserve microbial resources isolated from Gambian traditional fermented milk. The lactic acid bacteria isolated in this research may be able to be used as a starter culture in milk fermentation in The Gambia and elsewhere. Therefore, this study may serve as a principal reference point for research into Gambian fermented milk in generations to come.

CHAPTER 1

Profiling of bacterial communities in Gambian traditional fermented milk, "*Kosam*"

1.1 Introduction

Fermented milk (FM) including yoghurt has been one of the most consumed fermented foods around the world. As it is rich in beneficial lactic acid bacteria, FM is eaten for numerous other reasons including health. Some of the health properties of FM include prevention of hypertension, diarrhea, bacterial, fungal and respiratory infections. It also has anticarcinogenic properties, promotes bone health, have hypocholesterolemic, antioxidant, anti-allergenic and immunomodulatory effects among others (García-Burgos *et al.* 2020). FM provides consumers with a rich source of beneficial microorganisms such as lactic acid bacteria (LAB) in addition to improving flavor, taste and digestibility (Shori 2012). As milk is an important source of nutrients, in poorer parts of the world with many malnourished people such as Africa, it offers an alternative source of proteins and vitamins to consumers (Jans *et al.* 2017). However, an estimated 30% of the milk produced in Africa is lost due to inadequate equipment and storage facilities prior to reaching consumers. Consequently, imported dairy products account for quarter to half of all milk and milk products consumed in many African countries (Jans *et al.* 2017). In The Gambia, despite cows representing the largest portion of number of cattle, the country does not produce enough milk to meet local demand. Some of factors responsible for this include low milk yield by local cattle breeds and climate change. Thus, over the years, the country has become a net importer of milk and milk products. For instance, in 2019 alone, 7200 tons of dairy products were imported into The Gambia (WITS/World bank 2019). Due to increasing population and changes in lifestyle, the demand for milk and milk products is expected to continue to grow in The Gambia and the rest of Africa. Some of the imported dairy products, such as powered milk, is often made into yoghurt with the addition of starter culture in the form of tablets. Locally-produced milk is often consumed raw, as well as in the form of traditional FM to extend shelf life.

However, despite the long tradition of FM in the African continent, general knowledge of the unique aspects of the fermentative microbiota of FM products has only recently been studied (Jans *et al.* 2017). There are numerous studies on FM produced in several African countries (Jans *et al.* 2017; Parker *et al.* 2018). These reports reveal that the microbiota of FM is mainly influenced by geographical location, fermentation vessel,

fermentation conditions among others. For instance, the predominant LAB in Kenyan FM "*Amabere amaruranu*" is *S. thermophilus, Lactiplantibacillus plantarum, Lactobacillus delbrueckii subsp. bulgaricus, Leuconostoc mesenteroides* (Nyambane *et al.* 2014). Amasi, a South African FM is predominated by *Lactococcus lactis* followed by other strains such as *Enterococcus faecalis*, *Lactobacillus casei*, *Lactobacillus paracasei.* Other pathogenic bacteria include *Klebsiela*, *Escherichia coli*, *Kluyvera*, *Shigella* (Osvik *et al.* 2013). In addition, *Enterococcus* spp., *Limosilactobacillus fermentum*, *Lactiplantibacillus plantarum*, *Lactococcus lactis* subsp*. lactis, Pediococcus pentosaceus, Weissella confusa* were isolated from Malian fermented milk, Fènè (Wullschleger *et al.* 2013). Similarly, Cameroonian FM, Pendidam is predominated by *Lactobacillus delbrueckii*, *Lactobacillus helveticus*, *Lactobacillus fermentum*, *Lactiplantibacillus plantarum*, *Enterococcus faecalis*, *Enterococcus faecium*, *S. thermophilus* followed by *Lactobacillus casei*, *Leuconostoc mesenteroides*, *Leuconostoc paramesenteroides* (Jiwoua and Milliere, 1990). Meanwhile, *Streptococcus* sp. and *Lactobacillus* sp. were found to predominate naturally FM in Northern Senegal. Other pathogenic bacteria such as the genus *Bacillus*, *Clostridium*, *Enterococcus*, *Salmonella*, and *Shigella* were also detected (Parker *et al*. 2018).

At the moment, there is very scanty information about the microbiota of *Kosam*. Although there are reports of pathogens present in fermented milk from The Gambia, there is no literature regarding other microorganisms. An earlier study by Hempen *et al.* (2004) stated that there are pathogenic bacteria such as *Bacillus cereus*, *Clostridia*, *Listeria*, *Escherichia coli*, *Salmonella*, *Staphylococci* in FM produced in The Gambia. The consumption of dairy products from such raw milk can bring harmful health consequences (Oliver *et al.* 2005). Therefore, this chapter aims to elucidate the microbial profile of Gambian traditional FM and evaluate its safety.

1.2 Materials and Methods

1.2.1 Sampling of Gambian traditional FM

Sampling was performed in Bansang, Central River Region (CRR) and Soma, Lower River Region (LRR) of The Gambia (Figure 1-1). One sample of traditional Gambian FM was collected in each of the identified sampling regions from Fulani women in October 2019. The pH of the samples was measured at the sampling sites using a portable pH-meter (Essentials pH meter, Hydrogarden, Coventry, United Kingdom). The samples were aseptically collected and transferred into sterile 50 ml tubes then kept in cooler boxes (4-

10°C) and transported to the Central Veterinary Laboratory of the Department of Livestock Services, Abuko, The Gambia. The samples were kept in a refrigerator (4°C) immediately upon arrival at the laboratory and analyzed within 24 h.

1.2.2 Microbial enumeration

The number of LAB in the FM was enumerated using BCP plate count agar (Eiken Chemical Co. Ltd., Tochigi, Japan), whilst coliform bacteria and *E. coli* were enumerated using XM-G agar (Nissui Ltd., Tokyo, Japan).

One milliliter of FM was thoroughly mixed with 9 mL of sterile 0.85% NaCl solution and then diluted in serial. Aliquots (0.1 mL) of appropriate dilutions (10^{-4}-10^{-7} for LAB, 10^{-1}-10^{-3} for coliforms and *E. coli*) were mixed with the respective selective media. All samples were incubated under aerobic conditions at 35°C similar to the average temperature of Gambian weather at the time. After the incubation for 72 h, the yellow colonies on BCP plate count agar were enumerated as lactic acid bacteria. On the other hand, the blue and red colonies on XM-G agar were respectively counted as *E. coli* and coliform bacteria after the incubation for 24 h. Microbial counts were carried out in triplicate and expressed as colony forming units per milliliter of FM (log CFU/mL).

1.2.3 Isolation of lactic acid bacteria

The colonies on countable BCP plates were randomly picked up using sterile disposable needles (Watson, Kobe, Japan), inoculated into MRS agar in the cryotube vials (1 colony per vial) and incubated aerobically at 35°C for 48 h. After confirmation of growth, the samples were kept at 4°C and then transported to Japan by air. The samples were immediately stored at 4°C upon arrival in our university and applied to further analysis.

Each microbial culture in a cryotube vial was picked using sterile disposable needles, inoculated into 5 ml of MRS broth and incubated aerobically at 35°C for 24 h. After incubation, each culture was streaked on MRS agar plate and then incubated under the same conditions. Each single colony on MRS agar plate was picked up and sub-cultured into MRS broth. After cultivation, the bacterial cells were aseptically collected

by centrifuging the culture in a Tomy MX-300 centrifuge (Tomy digital biolology, Tokyo, Japan) at 5,000 g for 10 min at 4°C. After discarding the supernatant, the cells were suspended in 200 µl of MRS broth containing 15% glycerol, transferred into cryotube vials and stored at −80°C until use. Before use, these LAB isolates were inoculated into MRS broth and then incubated for 35°C for 24 h. The LAB isolates were then activated by sub-culturing two more times.

1.2.4 Identification of LAB isolates

The isolated strains were confirmed to be Gram-positive by Gram staining, and acid-producing on BCP agar medium. Genotypic identification of these isolates was performed by 16S rRNA gene sequencing. The total genomic DNA of the LAB was extracted from overnight cultures using Bactozol Bacterial DNA Isolation Kit (Molecular Research Center, Ohio, United States) following manufacturer's instructions. The DNA concentration was determined using Ultrospec 2000 UV/Visible Spectrophotometer (Pharmacia Biotech, New Jersey, United States).

PCR amplification was carried out in a 50 µl reaction volume consisting of the following; 5 µl of 10× Ex Taq buffer, 4 µl of 2.5 mM dNTPs and 0.25 µl of Taq polymerase (Takara, Shiga, Japan), 2 µl of each primer (10 µm) and 5 µl (5 ng) DNA template and ddH_2O. The 16S-derived universal bacterial primers 27F (5`-AGAGTTTGATCCTGGCTCAG-3`) and 1406R (5`-ACGGGCGGTGTGTAC-3`) were used to amplify the 16S rRNA gene at approximately 1300 bp. PCR was performed on Bioer thermal cycler (LifeEco, Winnipeg, Canada) with the following conditions: initial denaturation at 95°C for 3 min, 30 cycles of denaturation at 94°C for 30 sec, annealing at 60°C for 45 sec and extension at 72°C for 90 sec and final extension at 72°C for 5 min. Subsequently 9 µl of the PCR product were used to assessed the quality of amplified DNA using 1.5% (w/v) agarose gel electrophoresis; TAE 1 × buffer (40 mM Tris, 20 mM Acetate, 1 mM EDTA, pH 8.6). After the completion of the electrophoresis, the gels were stained with ethidium bromide (50 µg/ml) in 1×TAE (Wako, Nippon Gene) for 15 min and rinsed in running tap water for 10 min. The gels were then visualized using ATTO Printgraph (ATTO, Tokyo, Japan) to confirm the presence of amplicons.

The PCR products were purified using Fast Gene Gel/PCR Extraction kit (Nippon Genetics, Tokyo, Japan) according to manufacturer`s instructions. The samples were then prepared in a 0.2 ml 8-strip PCR tubes (Nippon Genetics, Tokyo, Japan) in a maximum volume of 20 µl consisting of 80-120 ng of DNA template, 6.4 pmol of primer and ddH_2O. The sequencing of purified products was performed at the Eurofins

Genomics Inc. (Tokyo, Japan). The sequences derived from the isolates were searched using the BLAST search tool https://www.ddbj.nig.ac.jp on DNA Data Bank of Japan (DDBJ) to identify LAB species.

1.2.5 Metagenomic analysis

In order to profile the microbiota in traditional Gambian FM, total DNA was directly extracted from the two samples of FM collected in CRR and LRR using ZymoBIOMICS DNA miniprep kit (Zymo Research, Irvine, United States) in accordance with the manufacturer's instructions. Illumina sequencing analysis for 16S rRNA gene profiling was conducted at Bioengineering Lab. Co. Ltd. (Sagamihara, Kanagawa, Japan). The full-length cDNA synthesis, cDNA amplification and short read sequencing library preparation were completed using the LoopSeq to obtain a comprehensive metagenomics of all bacteria present in Gambian traditional FM. The DNA was purified using M Pure-12 bacterial DNA Extraction Kit (MP Biomedicals, Santa Ana, California, United States) according to the manufacturer's instructions. The concentration of the extracted DNA solution was quantified using Synergy H1 (BioTek Instruments, Winooski, Vermont, Untied States) and QuantiFluor dsDNA System (Promega Co., Madison, Wisconsin, United States). The amplicon library was prepared using the LoopSeq 16S Microbiome SSC 24plex Kit (Loop Genomics, San Jose, California, United States) according to the protocol, vlp3. The concentration of the prepared library was measured using QuBit dsDNA HS Kit (Thermo Fisher Scientific, Waltham, Massachusetts, United States). The library quality was evaluated using Bioanalyzer and High Sensitivity DNA Kit (Agilent Technologies, Santa Clara, California, USA). Pair-end sequencing (2x150 bp) was carried out using the Illumina NextSeq system (Illumina, San Diego, California, United States).

1.2.6 Data Analysis

The data obtained from the sequencing were analyzed as follows. The distribution of samples and the creation of long-read contigs was performed in the LoopSeq cloud system. Contigs with a length of more than 1300 bases were filtered from the synthesized long-read contigs using the Biopython for sequence filtering. The sequences filtered for clustering were then imported into Qiime2 and clustered with a threshold value of 99% by applying the vsearch plugin. The Silva138 database was used as a reference for lineage estimation. The maximum hit sequences were set to 5 using the feature-classifier priming (classify_consensus_blast) of Qiime2, and lineage estimation was performed with the top hit strains being supported by the majority of sequences.

1.3 Results

1.3.1 pH and viable counts

Table 1-1 shows the pH and viable counts of Gambian traditional FM. The pH of FM sampled at CRR (CRR-FM) was 4.7, total counts of LAB was were 8.27±0.08 log CFU/mL. On the other hand, the pH of FM sampled at LRR (LRR-FM) was 5.4 and the LAB numbers were 7.21±0.09 log CFU/mL. The numbers of coliform bacteria and *E. coli* on the XM-G agar in LRR-FM were 5.73±0.17 log CFU/mL and 4.82±0.13 log CFU/mL, respectively. However, coliform bacteria and *E. coli* were not detected in CRR-FM.

1.3.2 Isolation and Identification of LAB in Gambian traditional FM

A total of 134 isolates were identified by 16S rRNA gene sequencing (Table 1-2). Sixty-seven isolates were detected in the CRR-FM and were classified as follows; *Lactobacillus delbrueckii* (35 isolates), *Streptococcus lutetiensis* (13 isolates), *Limosilactobacillus fermentum* (11 isolates), *Lactococcus lactis* (5 isolates), and *Lacticaseibacillus paracasei* (1 isolate), *Lactiplantibacillus plantarum* (1 isolate), and *Leuconostoc mesenteroides* (1 isolate). On the other hand, sixty-seven isolates were detected in the LRR-FM which were classified as *Lactococcus lactis* (57 isolates), *Enterococcus italicus* (7 isolates), and *Enterococcus faecalis* (3 isolates). *Lb. delbrueckii* was the predominant LAB in FM from CRR, whilst *Lc. lactis* dominated in LRR-FM. The 16S rRNA sequencing revealed that CRR-FM was more diverse in viable LAB. Seven-different LAB species were detected in the CRR-FM whilst only three different species were detected in FM collected in the Lower River Region.

1.3.3 Metagenomic analysis

DNA extracted from FMs was used as a template for 16S metagenomics analysis to describe the bacterial diversity. The microbiota of CRR-FM consisted of 19 different bacterial genera (Figure 1-2). *Lactobacillus* was the dominant genus consisting 34.3% of the total bacterial community in CRR-FM followed by *Streptococcus* (27.2%)*, Lactococcus* (14.3%) and *Acetobacter* (11.2%). Other genera such as *Klebsiella* consisting 4.0%*, Enterococcus* and *Kurthia* both 1.2% were also detected in the CRR-FM. Meanwhile, twenty different bacterial genera were detected in the LRR-FM. The most predominant genus detected in LRR-FM was *Lactococcus* consisting of 36.4%, followed by *Lactobacillus* with 29.3% of total detected

bacterial microbiota. In addition, coliforms *Klebsiella* and *Enterococcu*s, consisting of 14.4% and 8.9% respectively were more abundant in LRR-FM.

Figure 1-3 shows the microbial species in each FM. Forty-eight species were detected among the two FM samples. *Streptococcus lutetiensis* (21.6%) was the most predominant species in CRR-FM, followed by *Lb. delbrueckii* (13.9%)*, Lc. lactis* (12.1%) and *Lb. helveticus* (9.1%). In addition, there was noticeable presence of acetic acid bacteria including *Acetobacter ghanensis* consisting of 4.0%, *Acetobacter tropicalis* makes 2.7%, *Acetobacter pasteurianus* 1.9%, *Acetobacter orientalis* and *Acetobacter senegalensis* both consisting just 1.0% of species detected. Some pathogenic bacteria such as *Klebsiella pneumoniae* (3.3%), an opportunistic pathogen, was also detected. In LRR-FM, *Lc. lactis* (32.0%) was the predominant species followed by *Lb. delbrueckii* (20.2%) followed by *Enterococcus sulfureus* 7.4%. Among the other 27 species detected, there was an abundance of *K. pneumoniae* consisting of 13.3% of total the microbiota.

1.4 Discussion

This research presents the first total bacterial profile of Gambian traditional FM, *Kosam*. Gambian traditional FM was dominated by LAB as revealed by both the culture-dependent and culture-independent methods. *Lc. lactis* and *Lb. delbrueckii* predominate lactic acid bacterial species in Gambian FM followed by *S. lutetiensis*. Similarly, microbial analysis of Sudanese FM, *Rob*, revealed the presence of *Lb. fermentum*, *Streptococcus salivarius*, *Lc. lactis subsp. lactis* and *Lb. acidophilus* (Abdelgadir et al. 2001). Parker *et al.* (2018) reported that "*lait caille*" a FM produced in Senegal, an immediate neighbor to The Gambia, was dominated by *Streptococcus* spp. and *Lactobacillus* spp. followed by low percentage of *Weisella, Enterococcus*, *Leuconostoc*, *Vagococcus*, and *Pediococcus*. The major functions of *Lactococcus, Lactobacillus* and *Streptococcus* are the production of lactic acid, hydrolysis of casein, lipolysis of fat and fermentation of citric acid (Zhong *et al.* 2016). Accordingly, these LAB species may also have a significant influence on the texture and flavor of Gambian traditional FM. Milk composition and source, bacterial composition in cases inoculum is used, fermentation vessels and abiotic factors are some of the determinants of bacterial community of naturally FM (Parker *et al.* 2018). Therefore, one or all of these factors may have influenced the presence of the microbiota found in Gambian FM. The microbiota of FM greatly influences the shelf life, safety and general product characteristics.

In the culture-dependent method, no coliforms were detected in the CRR-FM sample with a pH value of 4.7, contrastingly the LRR-FM sample with a pH of 5.4 contained high numbers of coliforms and *E. coli.* In the culture-independent method, coliforms were detected in both FM whilst *E. coli* was not detected. There is no contradiction in these results because the number of LAB in LRR-FM (7.21 log CFU/mL) is approximately thirty times as that of coliforms and two hundred times more than of *E. coli* (4.82 log CFU/mL). *E. coli* were reported to be inhibited by low pH resulting from organic acids (e.g. lactic acid and acetic acid) produced by LAB (Suiryanrayna & Ramana 2015). In addition, a combination of multiple factors including low pH and the production of bacteriocin, hydrogen peroxide, and ethanol by LAB was reported to have an inhibitory effect on *E. coli* in FM (Yilma *et al.* 2015). According to Mathara *et al*. (2004), *Maasai* traditional FM with pH values more than 4.5 tended to contain relatively high numbers of *Enterobacteriaceae*. Therefore, the difference in the ratio of acid-producing LAB between these FMs may affect their pH values, *E. coli* and coliform counts.

Among the isolates from LRR-FM, seven strains were identified as *E. italicus* and three strains were identified as *E. faecalis*. *E. italicus* was previously detected during fermentation of "*nunu*", a Ghanaian FM produced from unpasteurized raw milk (Akabanda *et al.* 2013). According to Gaaloul *et al.* (2014), some *Enterococcus* have the ability to contribute to the process of milk fermentation and its aromatic smell. On the other hand, the presence of *enterococci* in raw milk is mainly due to cross-contamination from human or animal feces, water, environment, or milking equipment resulting from poor hygiene and handling conditions (McAuley *et al.* 2015). Therefore, it is necessary to search for the origin of these bacteria and further evaluate their safety.

As revealed by the metagenomic analysis, the genus *Klebsiella* consisted of 14.4% of total detected bacterial microbiota in LLR-FM and 4.0% in CRR-FM. As an opportunistic bacterial pathogen, *Klebsiella* is responsible for causing mastitis in cows and has previously been isolated in milk samples. Therefore, its presence in milk is mainly caused by contamination through infected cows (Langoni *et al.* 2015; Massé *et al.* 2020; Munoz *et al.* 2007; Podder *et al.* 2014). *K. pneumoniae* detected in the LRR-FM by the metagenomic analysis is one of the highly pathogenic species of the genera *Klebsiella*. It is known to cause infection in humans. Therefore, *Klebsiella* spp. coupled with other pathogens may make consumers health risky by intake of FM because coliforms containing *Klebsiella* spp. was detected in LRR-FM by the culture method (Massé *et al.* 2020).

Meanwhile the genera *Acetobacter* consisting of *A. ghanensis, A. pasteurianus, A. senegalensis* and *A. tropicalis* were more abundant in CRR-FM than in LRR-FM. The presence of *Acetobacter* spp. was previously reported in Mongolian and Indian natural FMs (Oki *et al.* 2014; Shangpliang *et al.* 2018). In particular, *Acetobacter* spp. such as *A. pasteurianus*, *A. lovaniensis*, and *A. syzygii* was found to be predominant in some Indian naturally FM products. Nakasaki *et al.* (2008) have reported that acetic acid bacteria grow better in acidic condition as it can hardly grow from neutral and alkaline conditions. In this study, CRR-FM was more acidic than LRR-FM. Thus, the acidity of FM could affect to the abundance of *Acetobacter* genera.

The diversity of the microbiota in some traditional African FM was reported in previous studies. It is suggested to be influenced by geographical location, temperature, humidity, source of milk, fermentation vessels, fermentation condition, starting material among others (Jans *et al.* 2017). Therefore, the difference in the microbiota between the FM sampled at the two regions could also be attributed to multiple factors, as in FM from other African countries and elsewhere. For the first time in this study, LAB strain was isolated and identified from Gambian traditional FM. It may be possible to elucidate the mechanism of bacterial flora formation and develop safer FM that inherits the tradition, by investigating the effects of the addition of these LAB on the bacterial flora of FM.

1.5 Summary

This research aims clarify the microbiological characterization of Gambian traditional fermented milk, "*Kosam*". Fermented milk (FM) samples were collected at two regions (Central River Region: CRR, Lower River Region: LRR) in The Gambia in 2019. The microbiota of these samples was analyzed by culture-dependent methods and Illumina sequencing. The number of lactic acid bacteria LAB in FM from CRR and LRR was 8.27±0.08 log CFU/mL and 7.21±0.09 log CFU/mL, respectively. Whilst no coliforms and *Escherichia coli* were detected in FM from CRR, that from LRR contained 5.73±0.17 log CFU/mL of coliforms and 4.82±0.13 log CFU/mL of *E. coli*. The dominant viable LAB in FM from CRR was *Lactobacillus delbrueckii*, followed by *S. lutetiensis*, while that from LRR was *Lc. lactis*. The metagenomic analysis also revealed that these species were dominant in Gambian traditional FM. Furthermore, it also revealed the possibility of the presence of pathogens such as *Klebsiella* spp. This study can enhance the knowledge concerning Gambian FM and contribute to the elucidation of microbial communities.

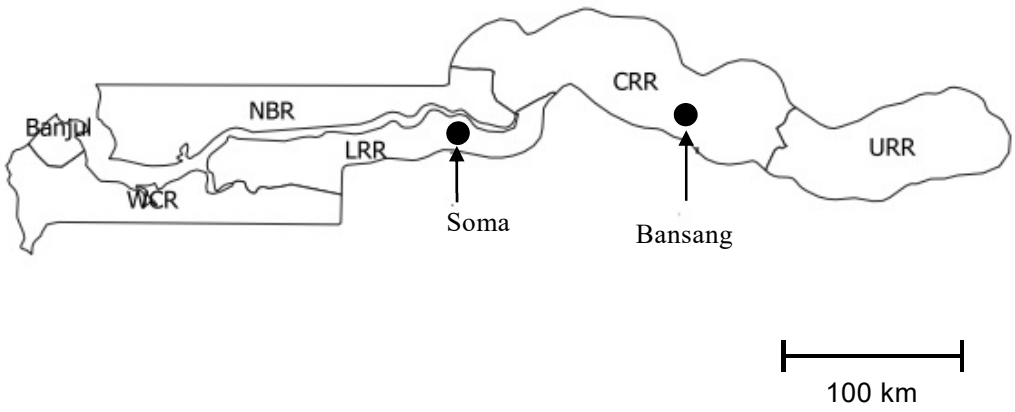

Figure 1-1. Sampling locations in The Gambia.

WCR: Western Region, NBR: North Bank Region, LRR: Lower River Region, CRR: Central River Region, URR: Upper River Region, ●: Sampling location.

Table 1-1. Viable counts (log CFU/mL) and pH of Gambian fermented milk

Sample	LAB	Coliforms	*E. coli*	pH
CRR	8.27±0.08	n.d.	n.d.	4.7
LRR	7.21±0.09	5.73±0.17	4.82±0.13	5.4

n.d.: not detected

Table 1-2. Identification of LAB isolated from traditional Gambian fermented Milk

Species	**Number of strains**	
	CRR	LRR
Enterococcus faecalis	0	3
Enterococcus italicus	0	7
Lacticaseibacillus paracasei	1	0
Lactiplantibacillus plantarum	1	0
Lactobacillus delbrueckii	35	0
Lactococcus lactis	5	57
Leuconostoc mesenteroides	1	0
Limosilactobacillus fermentum	11	0
Streptococcus lutetiensis	13	0

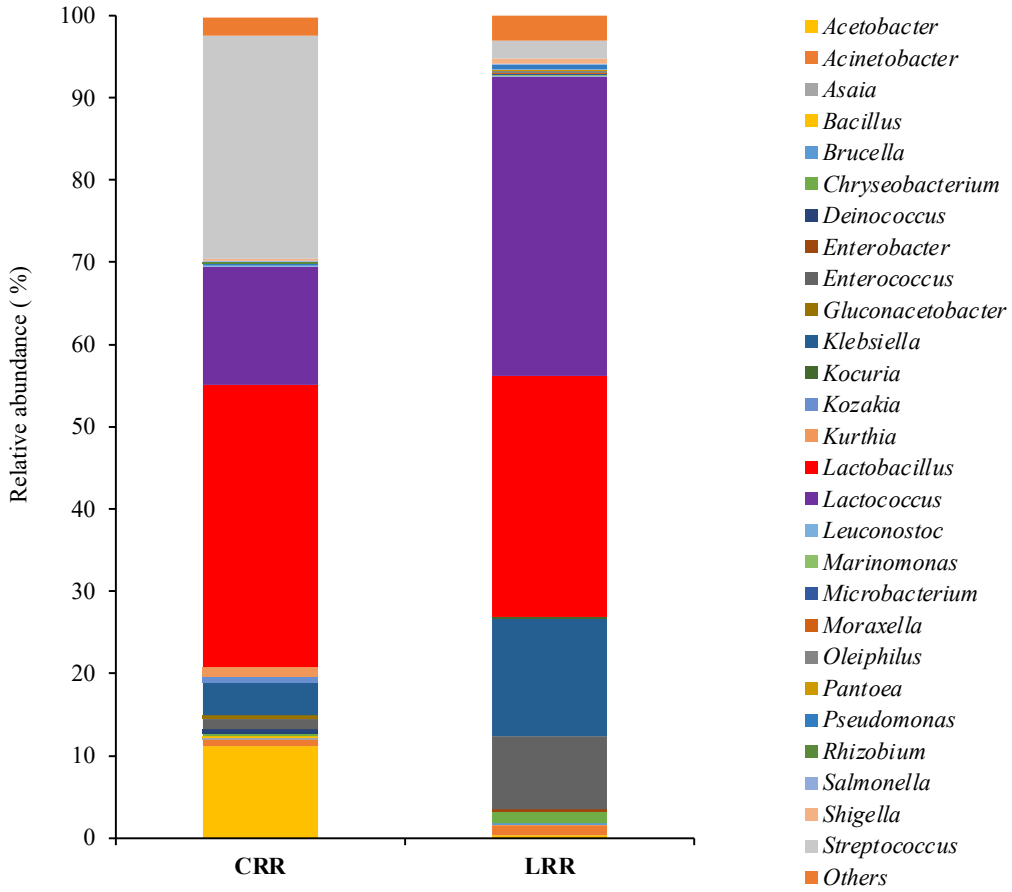

Figure 1-2. Relative abundance (%) of bacterial genera in Gambian fermented milk

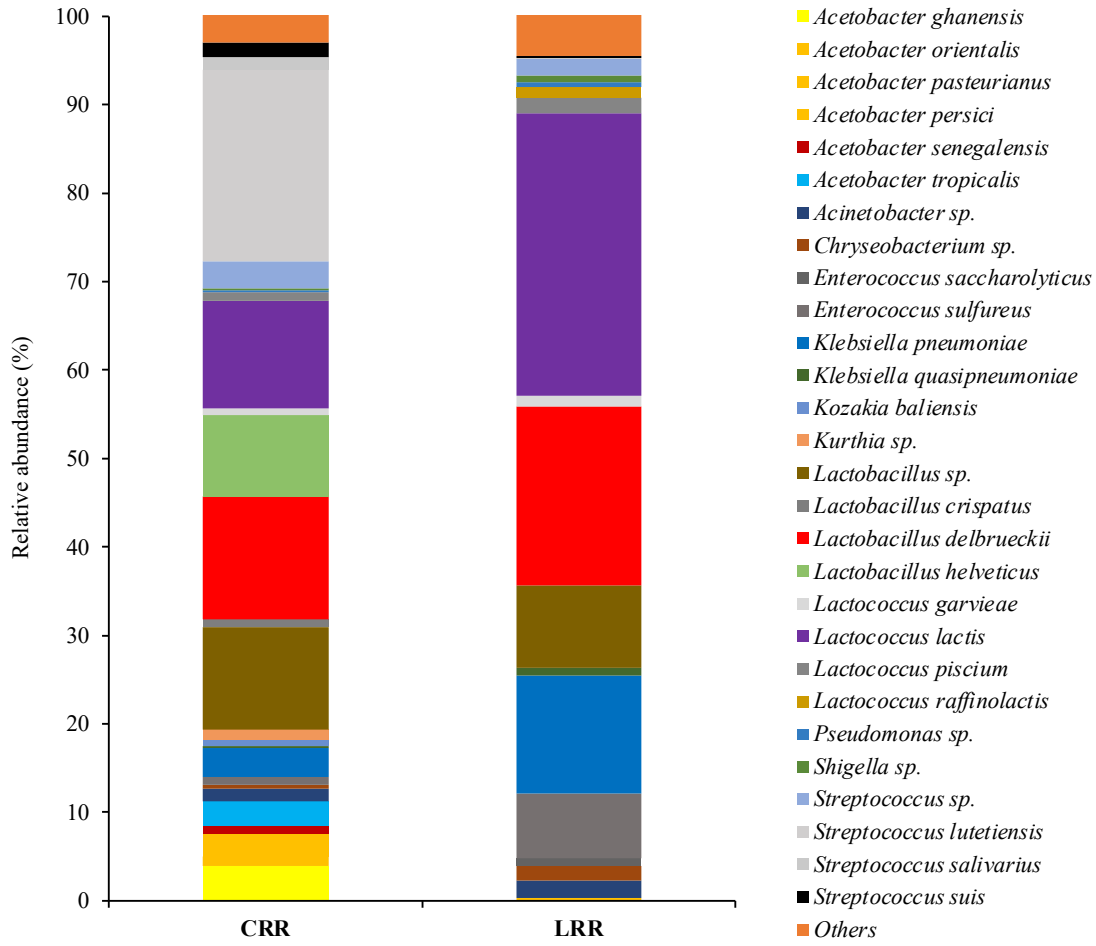

Figure 1-3. Relative abundance (%) of bacterial species in Gambian fermented milk. Only bacterial species with a relative abundance above 0.5% in at least one sample are shown.

CHAPTER 2

Characterization of LAB isolated from traditional Gambian fermented milk

2.1 Introduction

Fermented milk (FM) products containing yogurt are produced by fermenting milk with LAB. In the process of fermenting milk, LAB provides superior nutritional benefits to milk, such as prevention of diarrhea by decomposition of lactose, improvement of digestion and absorption by decomposition of protein, improvement of Ca absorption by lowering pH, and addition of vitamins. (Tomovska *et al*. 2006). In addition, some LAB have a probiotic function, which can be used in the production of fermented milk to further contribute to the health of consumers.

Screening for LAB from natural resources is one of the means to obtain LAB strains for manufacturing FM products. LABs for producing FM are usually selected based on their acid-producing ability and their influence on the taste, aroma, flavor and textural properties of the final product. There is a relationship between the acid-producing ability of LAB and the textural properties of FM. The coagulation of milk by LAB is related to the production of organic acids, mainly lactic acid by LAB during fermentation and the accompanying change in pH. The pH of milk is usually between 6.5-6.7. The decrease in pH due to fermentation reduces the electrostatic repulsion force between casein micelles. When the pH value reaches 5.5 to 5.0, the colloidal calcium phosphate (CCP) of casein micelles is solubilized, which changes the bond between caseins in the micelles and strengthens the hydrophobic interaction and hence form a gel. Furthermore, when the pH value reaches 4.6 or less, a stable gel is formed by isoelectric point precipitation of casein. Texture properties such as viscosity, smoothness, hardness, cohesiveness, stickiness and elasticity of FM thus formed influence consumers' product choices (Han *et al*. 2016; Mudgil. *et al*. 2017). Furthermore, the amount of lactic acid in FM at such pH contributes to not only the overall texture but also preservation of the products (Gastaldi *et al*. 1996).

The preserving property of products are not unique to lactic acid. Bacteriocins also inhibits the growth of other bacteria. Bacteriocins produced by LAB are classified into 5 classes based on the following; amino acid composition, molecular weight, mechanism of action, secretory mechanism, antibacterial spectrum, etc. Bacteriocins are often active at different pH values, are resistant to high temperatures, and exhibit a

broader inhibition spectrum (Shah and Dave 2002). The most representative bacteriocin produced by LAB is nisin, which is produced by *Lactococcus lactis* subsp, *lactis*. Nisin was approved for use in food in 1969 by the Joint FAO/WHO Expert Committee on Food Additives (JECFA). It is currently approved as "Generally Regarded as Safe" (GRAS) by the US Food and Drug Agency (FDA) for use in foods. Nisin possesses a broad spectrum of activity against Gram-positive bacteria such as *Bacillus* sp., *Clostridium* sp., *Lactobacillus* sp., *Lactococcus* sp., and *Listeria* sp. etc. (Fujita *et al.* 2007; Shah and Dave 2002; Younes *et al*. 2017). On the other hand, due to its broad-spectrum of activity, nisin-producing *Lactococcus lactis* subsp. *lactis* strains can be considered to influence the microbiome during the producing of fermented food (Abee *et al*., 1995).

In Chapter 2, LAB strains isolated from Gambian traditional FM presented in Chapter 1 were evaluated for acid production in reconstituted skimmed milk. In addition, the interactions among LAB strains were evaluated on the bases of bacteriocin-like inhibitory activity.

2.2 Materials and Methods

2.2.1 Acid production in milk

The acid-producing ability of LAB strains isolated from the Gambian traditional FM was evaluated using 10% reconstituted skimmed milk (RSM). Each LAB stock solution (10 µl) was inoculated into 5 mL of MRS broth (MRS; Oxoid Ltd., Basingstoke, UK) and incubated for 24 h at 35°C. In order to activate the cells, 50 µl (1%, v/v) of the culture was sub-cultured twice into the same medium and incubation conditions. Thereafter, the cells were aseptically collected by the centrifugation at 5,000 g for 10 min at room temperature using Tomy MX-300 centrifuge (Tomy digital biology, Tokyo, Japan). After removing of the supernatant, the cells were washed twice with 0.85% NaCl solution. The cells were then suspended in saline solution and adjusted to OD_{600} = 1.0 using ASUV1100 Spectrophotometer (AS ONE Co., Osaka, Japan). The suspension (50 µl) was inoculated into 5 ml of 10% RSM and incubated at 35°C for 24 h. After incubation, the pH of each culture was measured using a HM-41X pH meter (DKK-TOA Co., Tokyo, Japan). The measurement of pH was done in triplicate and the average pH was calculated.

2.2.2 Bacteriocin-like inhibitory activities among LAB strains

Twenty strains (9 species) were selected from 134 strains (9 species) isolated from the Gambian traditional FM. These 20 strains were used as indicator strains for estimating of bacteriocin-like inhibitory activity by the agar well diffusion assay (Yang *et al.* 2012). Each LAB stock solution (10 µl) was inoculated into 5 ml of MRS broth (Biokar diagnostic, Allone, France) and incubated for 24 hrs at 35°C. In order to activate the cells, 50 µl (1% v/v) of the culture was sub-cultured twice into the same medium and incubation conditions. Thereafter, the cells were aseptically collected by centrifugation at 5000 g for 10 min at room temperature. After removing of the supernatant, the cells were washed twice with 0.85% NaCl solution. The cells were then suspended in saline solution and adjusted to OD_{600} = 1.0. Then, the cell suspension was mixed at a ratio of 1% (v/v) with MRS agar kept at 50°C and then transferred into Petri dishes. After solidification, four wells (6 mm diameter) were cut in each plate using a cork borer (Nonika Rikaki, Tokyo, Japan). The preparation of cell-free supernatant (CFS) of all strains isolated from the Gambian traditional FM was performed as described below. Each stock culture solution (10 µl) was inoculated into 5 ml of MRS broth and incubated overnight at 35°C. After that, 50 µl of the overnight culture was sub-cultured into the same medium and incubation conditions. After incubation, the culture was centrifuged at 5000 g for 10 min and recovered the supernatant. The supernatant was neutralized by the addition of 1.0M NaOH and then sterilized by filtering through a 0.22 µm membrane filter unit (Advantec, Tokyo, Japan). The resulting solution was applied to bacteriocin-like inhibitory activity as a cell-free supernatant (CFS). The CFS (50 µl) was poured into the wells on the plates prepared as described above, and incubated overnight at 35°C. After the incubation, the formation of clear zones (halo) around the wells was observed.

2.3 Results

2.3.1 Lactic acid production by LAB

The lactic acid production in milk ability of LAB strains isolated from Gambian traditional FM was assessed based on changes in pH of RSM. As shown in Table 2-1, 80% of *Lc. lactis* isolates, which were predominant among the isolates from FM, were unable to lower the pH of RSM below 6.0. On the other hand, 82.9% of *Lb. delbrueckii* isolates could lower the pH of RSM below 6.0. In addition, all *S. lutetiensis* isolates could lower the pH of RSM below 5.0. The ratio of isolates that reduced the pH of RSM to less than 6.0 are 79.1% (53 isolates) and 16.4% (11 isolates) in CRR-FM and LRR-FM isolates, respectively. Among the CRR-FM

isolates, strains that reduced the pH of RSM to less than 5.0 were one *Leu. mesenteroides* strain, two *Lb. fermentum* strains, sixteen *Lb. delbrueckii* strains and thirteen *S. lutetiensis* strains. On the other hand, only three *Lc. lactis* strains among the LRR-FM isolates could reduce the pH of RSM to less than 5.0.

2.3.2 Bacteriocin-like inhibitory activities among LAB isolates

As shown in Table 2-2, *S. lutetiensis* strains showed bacteriocin-like inhibitory activity in 15 of 20 strains (5 of 9 species) used as indicators. Similarly, *Lc. lactis* strains and *Lb. delbrueckii* strains showed activity against 7 strains (3 species) and 6 strains (2 species), respectively. In contrast, *E. faecalis* G2049, *Lc. lactis* G2053, *Lb. fermentum* G3049, *Lb. plantarum* G3037, and *Leu. mesenteroides* G3061 were not inhibited by all other LAB strains isolated from Gambian FM.

Compared the proportion of strains with bacteriocin-like inhibitory activity, all of *E. faecalis* strains showed the bacteriocin-like inhibitory activity against three *Lb. delbrueckii* strains as indicators. *Lb. fermentum* had a high proportion of strains with bacteriocin-like inhibitory activity, as 7-8 of the 11 strains showed activity against *Lb. delbrueckii* strains. Of the 13 strains of *S. lutetiensis*, 7-8 strains showed activity against *E. italicus*, 5-9 strains against *Lc. lactis*, 6-8 strains against *Lb. delbrueckii*, and 3 strains against *Lb. paracasei*, respectively. On the other hand, the proportions of *Lb. delbrueckii* strains and *Lc. lactis* strains with bacteriocin-like inhibitory activity were as low as 1-2 strains per 35 strains and 1-5 strains per 62 strains, respectively.

2.4 Discussion

Evaluating acid production by LAB based on FM samples, most of LAB (79.1%) isolated from CRR-FM can decrease the pH of skimmed milk to less than 6.0, while most of LAB (77.6%) isolated from LRR-FM cannot. This result does not contradict that the pH of CRR-FM and LRR-FM were 4.7 and 5.4, respectively. The pH reduction by the production of organic acids (e.g. lactic acid and acetic acid) in FM products was reported to cause the inhibition of *E. coli* and other coliforms. Mathara *et al*. (2004) has reported that the Maasai traditional FM in Kenya with pH values more than 4.5 tended to contain relatively high numbers of Enterobacteriaceae. In addition, a combination of factors including low pH and the production of bacteriocin, hydrogen peroxide, and ethanol by LAB was reported to have an inhibitory effect on *E. coli* in FM (Yilma *et al.* 2015). Therefore, the proportion of strains that can produce acid in milk was considered

to affect the number of coliforms and *E. coli* in Gambian FM.

The majority (80.6%) of the *Lc. lactis* strains isolated most from the Gambian FM were unable to lower the pH of RSM below 6.0. *Lc. lactis*, which is used in the production of dairy products, can generally produce lactic acid in milk and reduce the pH of milk. Li *et al*. (2020) reported that 75% of *Lc. lactis* strains isolated from various dairy products reduced the pH of skim milk to 4.5 within 24 h. On the other hand, *Lc. lactis* is divided into three subspecies namely *Lc. lactis* subsp. *lactis*, *L. lactis* subsp. *cremoris*, and *Lc. lactis* subsp. *hordniae* (Parapouli *et al*. 2013). Among these three subspecies, except *Lc. lactis* subsp. *hordinae*, most of *Lc. lactis* subsp. *cremoris* and *Lc. lactis* subsp. *lactis* strains can metabolize lactose to produce lactic acid. In addition, lactose-negative mutants of *Streptococcus lactis* (old name of *Lc. lactis*) have been reported to occur spontaneously in broth cultures (Mckay *et al*. 1972). Furthermore, non-dairy or wild *Lactococcus* isolates have been shown to take longer to coagulate milk than dairy strains, based on the acidification profiles in milk (Mckay *et al*. 1972; Cavanagh *et al*. 2015). Therefore, the less or no lactose metabolization may be characterizations of *Lc. lactis* strains isolated from the Gambian FM.

Among *Lb. delbrueckii* strains isolated from CRR-FM, 16 strains (46%) were coagulated by lowering the pH of skim milk to less than 5.0. *Lb. delbrueckii* subsp. *bulgaricus* is utilized as a starter culture for yogurt and has a significant impact on the acidity, flavor and texture properties of dairy products (Crow and Curry, 2002). Therefore, some of *Lb. delbrueckii* subsp. *bulgaricus* isolated in this study (only species level is shown in Table 1-2) may be used as a starter for producing FM. Similarly, 13 strains of *S. lutetiensis* isolated from CRR-FM could also reduce the pH of skim milk to less than 5.0. The genus *Streptococcus* includes not only species known as pathogens such as *Streptococcus pyogenes*, *Streptococcus pneumoniae*, and *Streptococcus agalactiae*, but also species such as *S. thermophilus* that have been used in the production of yogurt together with *Lb. delbrueckii* subsp. *bulgaricus*. *S. lutetiensis* is isolated from various sources such as infant feces (Suzuki 2011), milk and dairy products. *S. lutetiensis* is suggested to be rarely associated with bovine mastitis, but has good adaptability to bovine mammary cells or tissues and potential to spread within a dairy herd (Chen *et al*. 2021). In addition, although there are few cases, *S. lutetiensis* has been reported to be associated with infectious diseases (Yamamoto *et al*. 2021; Yu *et al*. 2021). On the other hand, *S. lutetiensis* isolated from Wagyu (Japanese beef) cattle milk has been reported to produce a large amount of acid and diacetyl-acetoin, which has influence on the quality of FM (Tsuda and Kodama 2021). Therefore, *S. lutetiensis* strains isolated in this study may also be utilized in the production of dairy products, although it is necessary to confirm the safety sufficiently.

Bacteriocin-like inhibitory activity tests between the isolated strains revealed that growth inhibition was observed between not only the species but also the strains in the same species. Among total sixty-two strains of *Lc. lactis*, eleven strains isolated from LRR-FM showed bacteriocin-like inhibitory activity against *Lb. delbrueckii* subsp. *delbrueckii* G3055. In addition, three *E. faecalis* strains isolated from LRR-FM inhibited the growth of *Lb. delbrueckii* subsp. *delbrueckii* G3055, *Lb. delbrueckii* subsp. *indicus* G3060, and *Lb. delbrueckii* subsp. *lactis* G3050. Some strains of *Lc. lactis* and *E. faecalis* were reported to produce bacteriocins such as nisin and enterocin respectively in skimmed milk (Penna *et al*. 2005; Jozala *et al*. 2007; Izquierdo *et al*. 2009). As described in Chapter 1, *Lb. delbrueckii* was detected in CRR-FM in the culture-dependent method while none was detected in LRR-FM. On the other hand, *Lb. delbrueckii* were detected in both CRR-FM and LRR-FM in the culture-independent method. These results may show that bacteriocin-like inhibitory substances produced by some strains of *Lc. lactis* and *E. faecalis* during fermentation are a cause of *Lb. delbrueckii* mortality. Therefore, the detection of *Lb. delbrueckii* in the culture-independent method may have been caused by the amplification of DNA extracted from the dead cells.

There are few reports on the bacteriocin-like inhibitory activity of *S. lutetiensis*, which was isolated from CRR-FM. In this study, some *S. lutetiensis* strains inhibited not only the growth of *E. italicus*, *Lc. lactis*, *Lb. delbrueckii*, and *Lb. paracasei* as indicators of different species, but also that of the same species strains. Similar growth inhibition was also confirmed in some *Lc. lactis* and *Lb. delbrueckii* strains. This phenomenon is common because bacteriocins are known to inhibit the growth of some closely related species. For instance, Batdorj *et al*. (2005) demonstrated that bacteriocin extracted from *E. durans* inhibited the growth of same species. In another study, Yang *et al*. (2012) reported that some species of *Lactobacillus sakei* were also inhibited by bacteriocin isolated from same species. However, it is important to note that the effectivity of a bacteriocin (in a medium) is influenced by many factors including pH, temperature, metabolic characteristics, availability of oxygen, source of bacteriocin, presence of other substances like metallic ions etc (Matevosyan *et al*. 2019, Marianelli *et al*. 2010, Parada *et al*. 2007, Xi *et al*. 2018). Regarding *S. lutetiensis*, the strains isolated from infant feces have been reported to inhibit the growth of different strains (Suzuki 2011). These facts may indicate that some of *S. lutetiensis* strains have a relatively broad antibacterial activity.

This study revealed that LAB strains isolated from the Gambian FM have varying milk fermentability ability and antimicrobial activity. These facts showed some strains might have contributed to the biodiversity of Gambian FM, as lactic acid and bacteriocin can influence the microbiota of fermented milk (Shah and Dave

2002). By selecting the strains in consideration of the acid-producing ability of the strains and the inhibitory activity among the strains, it may be possible to produce safe FM products that follow the Gambian traditional FM.

2.5 Summary

In this study, LAB isolates from Gambian traditional FM were characterized on the basis of acid production in reconstituted skimmed milk (RSM) and their bacteriocin-like inhibitory activity among isolates. Among the strains derived from CRR-FM and LRR-FM, the proportions of the strains in which the pH of RSM was lowered to 6.0 or less were 79% and 16%, respectively. The majority of *Lc. lactis* strains isolated from both Gambian FM could not decrease the pH value of RSM to less than 6.0. On the other hand, among LAB isolated from CRR-FM, some of *Lb. delbrueckii*, *Leu. mesenteroides*, L*imosilactobacillus fermentum*, and *Streptococcus lutetiensis* strains could reduce the pH to less than 5.0. These results suggested that the proportion of strains that can produce acid in milk affected pH and the number of coliforms and *E. coli* in Gambian FM.

A bacteriocin-like inhibitory activity assay among isolates was performed by the agar well diffusion assay. *S. lutetiensis* strains showed bacteriocin-like inhibitory activity in 15 of 20 strains isolated from Gambian FM (5 of 9 species), suggesting that they have a relatively wide range of antibacterial activity. Similarly, *Lc. lactis* strains and *Lb. delbrueckii* strains showed the activity against 7 strains (3 species) and 6 strains (2 species), respectively. All of *E. faecalis* strains showed the bacteriocin-like inhibitory activity against three *Lb. delbrueckii* strains as indicators. In contrast, *E.faecalis*, *Lb.plantarum*, *Lb.fermentum*, *Lc.lactis*, and *Leu. mesenteroides* were not inhibited by all other LAB strains isolated from Gambian FM. These results suggest that the bacteriocin-like active substances produced by LAB strains are involved in the formation of the microbiota of Gambian FM.

Table 2-1. pH of reconstituted skimmed milk fermented by LAB isolates for 24 h

Region	Species	pH range			
		6.5-6.0	5.9-5.5	5.4-5.0	*<5.0*
CRR	*Lacticaseibacillus paracasei*	-	1	-	-
	Lactiplantibacillus plantarum	-	1	-	-
	Lactobacillus delbrueckii	6	9	4	16
	Lactococcus lactis	4	1	-	-
	Leuconostoc mesenteroides	-	-	-	1
	Limosilactobacillus fermentum	4	5	-	2
	Streptococcus lutetiensis	-	-	-	13
LRR	*Enterococcus faecalis*	3	-	-	-
	Enterococcus italicus	6	1	-	-
	Lactococcus lactis	46	6	2	3

Table 2-2. Bacteriocin activity of LAB strains isolated from Gambian FM

Indicator strains	*E. faecalis*	*E. italicus*	*Lb. paracasei*	*Lb. plantarum*	*Lb. delbrueckii*	*Lc. lactis*	*Leu. mesenteroides*	*Lb. fermentum*	*S. lutetiensis*
E. faecalis G2049	0/3	0/7	0/1	0/1	0/35	0/62	0/1	0/11	0/13
E. italicus G2024	0/3	0/7	0/1	0/1	0/35	0/62	0/1	0/11	7/13
E. italicus G2035	0/3	0/7	0/1	0/1	0/35	0/62	0/1	0/11	8/13
Lc. lactis G2053	0/3	0/7	0/1	0/1	0/35	0/62	0/1	0/11	0/13
Lc. lactis G2062	0/3	0/7	0/1	0/1	0/35	0/62	0/1	0/11	6/13
Lc. lactis G2085	0/3	0/7	0/1	0/1	0/35	1/62	0/1	0/11	8/13
Lc. lactis G3004	0/3	0/7	0/1	0/1	0/35	0/62	0/1	0/11	5/13
Lc. lactis subsp. *lactis* G2079	1/3	1/7	0/1	0/1	0/35	6/62	0/1	0/11	9/13
Lc. lactis subsp. *lactis* G2096	0/3	0/7	0/1	0/1	0/35	0/62	0/1	0/11	6/13
Lb. delbrueckii subsp. *delbrueckii* G3055	3/3	0/7	0/1	1/1	1/35	11/62	1/1	8/11	7/13
Lb. delbrueckii subsp. *indicus* G3038	0/3	0/7	0/1	0/1	1/35	0/62	0/1	7/11	7/13
Lb. delbrueckii subsp. *indicus* G3060	3/3	1/7	0/1	0/1	1/35	5/62	0/1	7/11	8/13
Lb. delbrueckii subsp. *lactis* G3047	0/3	0/7	0/1	1/1	1/35	1/62	1/1	7/11	8/13
Lb. delbrueckii subsp. *lactis* G3050	3/3	0/7	0/1	1/1	2/35	3/62	1/1	8/11	6/13
Lb. fermentum G3049	0/3	0/7	0/1	0/1	0/35	0/62	0/1	0/11	0/13
Lb. paracasei G3016	0/3	0/7	0/1	0/1	1/35	2/62	0/1	0/11	3/13
Lb. plantarum G3037	0/3	0/7	0/1	0/1	0/35	0/62	0/1	0/11	0/13
Leu. mesenteroides G3061	0/3	0/7	0/1	0/1	0/35	0/62	0/1	0/11	0/13
S. lutetiensis G3005	0/3	0/7	0/1	0/1	0/35	0/62	0/1	0/11	9/13
S. lutetiensis G3015	0/3	0/7	0/1	0/1	0/35	0/62	0/1	0/11	9/13

* All strains were given a unique identification number before stocking

CHAPTER 3

Purification and characterization of a bacteriocin from *Streptococcus lutetiensis* G3067

3.1 Introduction

Every year about 600 million people fall sick and over two-third of that number die annually from the consumption of contaminated food (Lee and Yoon 2021). Food preservatives are effective in reducing such microbial hazards in foods. In recent years, as consumers and producers have become more aware of food safety, there have been concerns about the health effects of traditional chemical preservatives. Therefore, there is increase interest in natural antimicrobial additives (Zhu *et al*. 2000). LAB is well-known to produce several antimicrobial compounds such as lactic acid, acetic acid, ethanol, carbon dioxide, diacetyl, hydrogen peroxide and bacteriocins (Hassan *et al*. 2020, Matevosyan *et al*. 2019). Among these antimicrobial compounds, bacteriocins are proteins mostly secreted by certain LAB that demonstrate the antimicrobial activity against other species of bacteria (Abanoz and Kunduhoglu 2018; Arakawa *et al*. 2009; Ye *et al*. 2021). The type of bacteriocin differs depending on the producer LAB and its antibacterial spectrum differs depending on the type of bacteriocin (Arakawa *et al*. 2009).

As described in Chapter 2, several strains of *Lc. lactis* and *S. lutetiensis* have bacteriocin-like activity. Although *Lc. lactis* is well-known to produce the most representative bacteriocin, nisin, bacteriocin production has also been reported in LAB belonging to the genus *Enterococcus*, *Lactobacillus*, *Lactococcus*, and *Streptococcus*, etc. *Streptococcus equinus* and *Streptococcus gallolyticus* isolated from the rumen of animals have been reported to produce bovicin HC5 and bovicin 255 (De Sousa *et al*. 2021; Joachimsthal *et al*. 2010; Whitford *et al*. 2001). However, there are no reports of bacteriocin-like substances (BLS) produced by *S. lutetiensis*. Therefore, this chapter aims to clarify the characteristics of BLS produced by *S. lutetiensis* isolated from traditional Gambian fermented milk.

3.2 Materials and Methods

3.2.1 Screening for BLS synthesizing *S. lutetiensis* strains

Thirteen strains of *S. lutetiensis* isolated from Gambian traditional FM were estimated for bacteriocin-like inhibitory activity against *Lb. delbrueckii* subsp. *bulgaricus* NBRC13953^{T}. A stock culture solution (10 µl) of each strain was inoculated into 5 mL of MRS broth and incubated at 35°C overnight. After that 50 µl of the overnight culture was sub-cultured in the same medium at 35°C for 24 h. The culture was centrifuged at 15,000 g for 10 min using a Tomy MX-300 centrifuge (Tomy Digital Biolology Co. Ltd. Tokyo, Japan) and then recovered the supernatant. The supernatant was neutralized by the addition of 1.0 M NaOH and then sterilized by the filtration using a 0.22 µm membrane filter unit (Advantec, Tokyo, Japan) to achieve a cell-free supernatant (CFS). *Lb. delbrueckii* subsp. *bulgaricus* NBRC13953^{T} as an indicator was incubated at 35°C overnight in MRS broth. An aliquot (150 µl) of the overnight culture was transferred into Petri dishes, poured and mixed 15 mL of MRS agar kept at 50°C. After solidification, four wells (6 mm diameter) were cut off on each plate using a cork borer (Nonika Rikaki, Tokyo, Japan). Thereafter, 50 µl of CFS of each LAB strain was poured into a well and incubated overnight at 35°C. After incubation, the size of the clear zone (halo) around the well was measured by a digital caliper (AS ONE Co., Osaka, Japan). CFS from five strains (G3032, G3034, G3056, G3066, and G3067) could be observed to form halo. Among them, *S. lutetiensis* G3067 was selected based on the size of the halo and applied as follows.

3.2.2 Separation of BLS produced by *S. lutetiensis* G3067

S. lutetiensis G3067 strain isolated from Gambian FM was used as the BLS-producing strain. The preculture under the same conditions mentioned in 3.2.1 was inoculated into 1.2 L of MRS broth at a ratio of 1% (v/v). After incubation, the culture was removed the cells by the centrifugation and recovered the supernatant. The supernatant was neutralized by 1.0 M NaOH, transferred into a separating funnel and then added an equal volume of chloroform. After shaking sufficiently, the mixture was allowed to settle for an hour that resulted in the formation of two distinct layers. The lower chloroform layer was recovered, concentrated to dryness using a rotary evaporator and then dissolved in 40 ml of distilled water. The solution was added grounded ammonium sulfate in small portions with continuous stirring and reached 50% on the final saturated concentration of ammonium sulfate. Insoluble components were recovered by the centrifugation at 5 000 g for 5 min at room temperature, re-dissolved in 5 ml of distilled water, and dialyzed in a SpectraPor

Dialysis membrane (Repligen Co. California, USA) for 48 h. After the dialysis, the sample was evaporated to dryness using a rotary evaporator, re-dissolved in 5 ml distilled water and applied to the following experiments as a crude BLS.

The crude BLS was separated by anion exchange chromatography using a Toyopearl DEAE-650M column (16 mm x 15 cm, Tosoh Co., Tokyo, Japan). The crude BLS fraction was applied to the column, which had been equilibrated with 20 mM phosphate buffer (pH 7.0). Elution was done first with 100 mL of buffer and then with a liner gradient of NaCl from 0 to 1.0 M in the same buffer in a total volume of 400 mL at a flow rate of 1.0 mL/ min. Fractionation was done by 10 ml each, using the CHF100AA fraction collector (Advantec, Tokyo, Japan). Each fraction was monitored at 220 nm and 280 nm by ASUV-1100 Spectrophotometer (AS ONE Co., Osaka, Japan). Fractions with absorption of two wavelengths were pooled, concentrated using a rotary evaporator and dialyzed as mentioned above. After dialysis, each fraction was dissolved in 5 ml of distilled water and subjected to a bacteriocin-like inhibitory activity assay.

A bacteriocin-like inhibitory activity of each fraction was estimated by the agar well diffusion method according to 3.2.1. The bacteriocin titer was determined and expressed as arbitrary units (AU/ml) using the formula: AU/mL= D1000/V (D: dilution factor, V: the volume of each solution (50 µl). One AU was defined as the reciprocal of the highest serial two-fold dilution showing a clear zone of growth inhibition of the indicator strain.

3.2.3 High performance Liquid Chromatography (HPLC)

HPLC analysis of a BLS fraction was performed on a Shimaldzu LC10-AD pump using a TSKgel G2000 PW (7.8 mm I.D. × 30 cm, pore size 12 µm, Tosoh Co., Tokyo, Japan) connected with the guard column. Elution was done using distilled water at 40°C with a flow rate of 1 mL/min. The detection was performed by the absorbance at 220 nm using a Shimadzu SPD-10A UV/VIS detector.

3.2.4 Tricine SDS-PAGE

Tricine SDS PAGE was performed using P-T16.5S p-PAGEL precast gel (ATTO Co., Tokyo, Japan) according to a manufacture's protocol. Electrophoresis was done using AE-1415 EzRun T buffer (ATTO

Co., Tokyo, Japan) as a running buffer at a constant voltage of 15 V for 30 min and 30 V during the rest of the separation. The gel was stained with Coomassie Brilliant Blue G-250.

3.2.5 Enzyme treatment of BLS

The BLS produced by *S. lutetiensis* G3067 was investigated for its sensitivity to proteolytic enzyme. Proteinase K (4 µl) was mixed with 100 µl of the peak1 fraction on the anion exchange chromatography, and 10 mM sodium phosphate buffer (pH 6.5, 96 µl). After incubation at 37°C for 1 hr, a bacteriocin-like inhibitory activity was conducted according to 3.2.1. A mixture with the treatment solution without proteinase K was used as a control.

3.2.6 Thermal stability

The peak1 fraction on the anion exchange chromatography, which has a bacteriocin activity, was used for the evaluating of thermal stability. An aliquot (100 µl) was subjected for 30 min to heat treatment at 40°C, 50°C, 60°C, 70°C, 100°C and 121°C, respectively. After cooling, it was estimated an inhibitory activity by the agar well diffusion method according to 3.2.1. An unheated sample was used as a control.

3.2.7 Effect of metal ions

The effect of metal ions on the bacteriocin activity was evaluated by mixing 90 µl of the peak 1 fraction with 10 µl of 50 mM of each metal ion ($MgCl_2$, $FeSO_4$, $CaCl_2$, $ZnSO_4$, $MnSO_4$, or $CuSO_4$). A mixture with distilled water was prepared as a control. Each mixture was conducted as bacteriocin activity according to 3.2.1.

3.2.8 Statistical analysis

Statistical significance between control and test group was determined by a paired t-test using MS Excel 2019. All measurement data are expressed as means ± standard deviations. A significant difference was considered when p value was <0.05.

3.3 Results

3.3.1 Purification of bacteriocin

The CFS produced by *S. lutetiensis* G3067 was obtained as described above. The bacteriocin produced by *S. lutetiensis* G3067 was separated from the CFS by three- steps: chloroform extraction, ammonium sulfate precipitation and anion exchange chromatography. By the chloroform extraction, the activity of BLS increased from 20 AU/mL to 400 AU/mL, the yield was 66.7% (Table 3-1). Furthermore, by the ammonium sulfate precipitation, the activity of BLS increased from 400 AU/mL to 800 AU/mL, the yield was 33.3%. The ammonium sulfate precipitate was separated by anion exchange chromatography into two peaks: a unadsorbed fraction (peak 1) and a 0.2 M NaCl eluted fraction (peak 2) (Figure 3-1). Peak 2 fraction was dialyzed, concentrated and evaluated for bacteriocin activity, but showed no activity. On the other hand, the activity of BLS of peak1 was 800 AU/mL, did not change and the yield decreased to 16.7%. This peak 1 fraction eluted as a single peak at 13 minutes on high performance liquid chromatography using gel filtration column (Figure 3-2). The fraction was detected as a broad band at the position between 1.0 and 3.5 kDa on Tricine SDS-PAGE (Figure 3-3). The estimated molecular weight was calculated about 3.0 kDa on the basis of the mobility. In addition, the fraction was inactivated by treatment with proteinase K (data not shown), like many other bacteriocins. Therefore, this fraction was named bacteriocin G3067.

3.3.2 Effects of heat treatment and metal ions on the activity of bacteriocin G3057

In the thermal stability experiments, after treatment at 40°C, 50°C and 60°C for 30 min, the activity of bacteriocin G3067 did not change significantly compared with the unheated sample, and the antibacterial activity remained unchanged (Figure 3-4). After treatment at 70°C, 100°C and 121°C for 30 min, the activity of bacteriocin G3067 increased significantly compared with the unheated sample. In particular, after 30 min of treatment at 100°C, the diameter of the inhibition zone increased 1.5 times as that of unheated sample. On the other hand, the addition of metal ions did not cause a noticeable change in the activity of bacteriocin G3067 (Figure. 3-5).

3.4 Discussion

Bacteriocins have attracted increased attention in the food industry due to their ability to inhibit and control the growth of microorganisms including food spoilage and foodborne pathogenic bacteria. This study aims to characterize the bacteriocin produced by *S. lutetiensis* isolated from traditional Gambian FM. The fraction with bacteriocin-like activity prepared from the supernatant of *S. lutetiensis* G3067 culture was low molecular weight and inactivated by protease treatment. These results show that the active component is a protein (peptide). Therefore, this component was clarified to be bacteriocin and named bacteriocin G3067.

Bacteriocin G3067 was characterized by not only remarkable thermal stability but also increase of the activity by heating. Many bacteriocins have been reported to be inactivated or reduced in activity by heat treatment (Li *et al.* 2021; Hassan *et al.* 2020; Hwanhlem *et al.* 2013; Abanoz and Kunduhoglu 2018; Yang *et al.* 2012). In the other hand, some researchers have also demonstrated that some bacteriocins maintain their antimicrobial activity after being subjected to heat treatment (Girma and Aemiro 2021; Lasik-Kurdyś and Sip 2019; Ren *et al.* 2018). Bacteriocins that are known have thermostability includes plataricins, nisin, pediocin, brevican 37 and lactacin F (Navarro *et al.* 2000). Some of the reasons for thermotolerance of some bacteriocins are due to low molecular weight and chemically diverse secondary structures (or lack of tertiary structures) or the presence of covalent bonds that help stabilize the protein globular structure among others (Girma and Aemiro 2021; Lasik-Kurdyś and Sip 2019; Ren *et al.* 2018). For widespread use in the manufacture of foods, bacteriocins are better to have higher heat stability. Therefore, bacteriocin G3067 is considered to have one of the properties that can be utilized in food production in terms of thermal stability.

Divalent metal ions have been reported so far to increase or decrease the activity of some bacteriocins (Du *et al.* 2022; Sudha and Aranganathan 2021; Zhang *et al.* 2018). However, the activity of bacteriocin G3067 was not affected by the addition of divalent metal ions. This result suggests that the activity of bacteriocin G3067 remains even in foods containing various metal ions such as dairy products. However, it will be necessary to investigate the influence of the concentration dependence of metal ions in order to use it in foods in the future.

Since most bacteriocins are produced during LAB growth, the pH of the medium has a significant effect on bacteriocin production (Parente *et al.* 1994). The addition of metal cations to the medium has also been reported to affect the production of bacteriocins (Arakawa *et al.* 2009; Matevosyan *et al.* 2019). In this

study, such factors affecting the production of bacteriocin by *S. lutetiensis* were not investigated. Since the amount of bacteriocin produced in this study is small, it will be necessary to investigate the better culture conditions in order to further analyze the characteristics of the bacteriocin.

3.5 Summary

A bacteriocin produced by *S. lutetiensis* G3067 isolated from Gambian FM was purified and characterized. The cell-free supernatant (CFS) prepared from MRS broth culture of *S. lutetiensis* G3067 was subjected to three preparation steps: chloroform extraction, ammonium sulfate precipitation, anion exchange chromatography. Bacteriocin-like inhibitory activity assay was performed by an agar well diffusion method using *Lb. delbrueckii* subsp. *delbrueckii* NBRC13953^{T} as an indicator strain. By these purification steps, bacteriocin activity increased from 20 AU/mL in CFS to 800 AU/mL in Peak 1 on anion exchange chromatography, with a yield of 16.7%. Peak 1 fraction was inactivated by proteinase K and eluted as a single peak on HPLC. In addition, it was detected as a broad band at the position between 1.0 and 3.5 kDa on Tricine SDS-PAGE, and its molecular weight was estimated 3.0 kDa. These results revealed that this component was a bacteriocin. This bacteriocin, named bacteriocin G3067, had thermal stability against the heat treatment at 40°C, 50°C and 60°C, for 30 min. Furthermore, after treatment at 70°C, 100°C and 121°C for 30 min, its activity increased significantly compared with the unheated sample. In addition, an activity of bacteriocin G3067 was not changed by the addition of divalent metal ions. These properties suggest that bacteriocin G3067 may be available for food production.

Table 3-1. Purification yield rate of bacteriocin of *Streptococcus lutetiensis* G3067

Purification step	**Volume (mL)**	**Titer (AU/mL)**	**Total activity (AU)**	**Yield (%)**
CFS	1200	20	24000	100
Chloroform Extraction	40	400	16000	66.7
Ammonium sulfate precipitation	10	800	8000	33.3
Anion-exchange chromatography (Peak 1)	5	800	4000	16.7

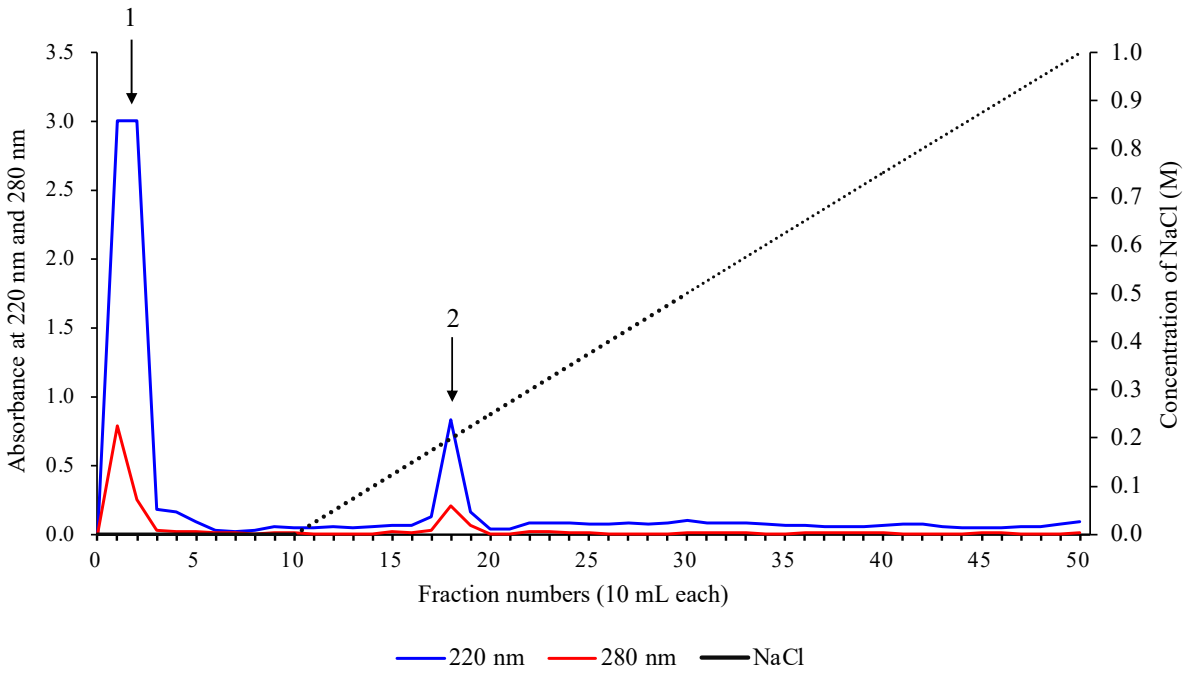

Figure 3-1. Anion exchange chromatogram of ammonium sulfate precipitate prepared from CFS of *S. lutetiensis* G3067

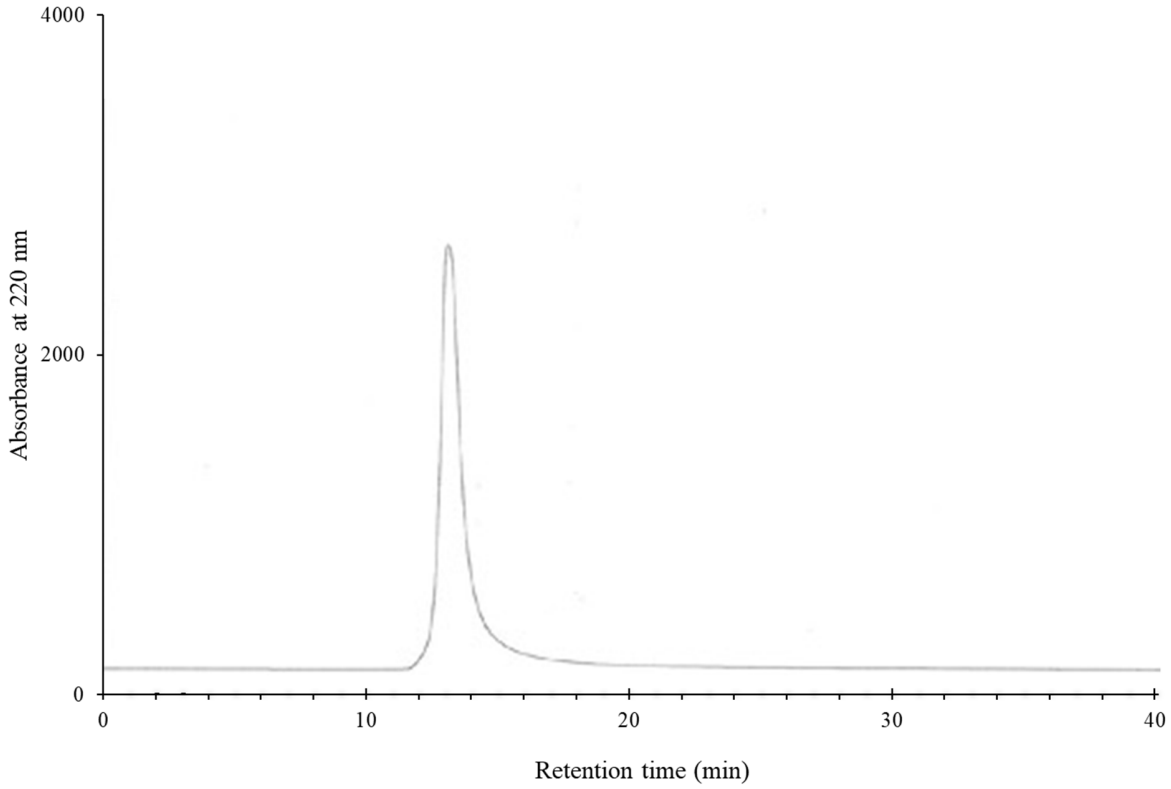

Figure 3-2. HPLC of peak 1 component on anion exchange chromatograph

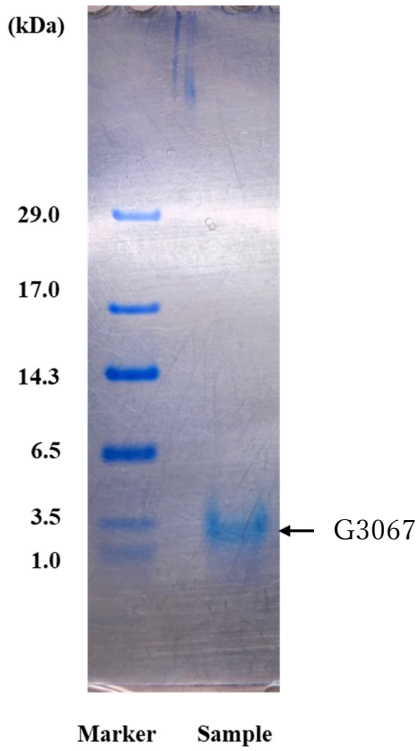

Figure 3-3. Tricine-SDS-PAGE of bacteriocin produced by *S. lutetiensis* G3067

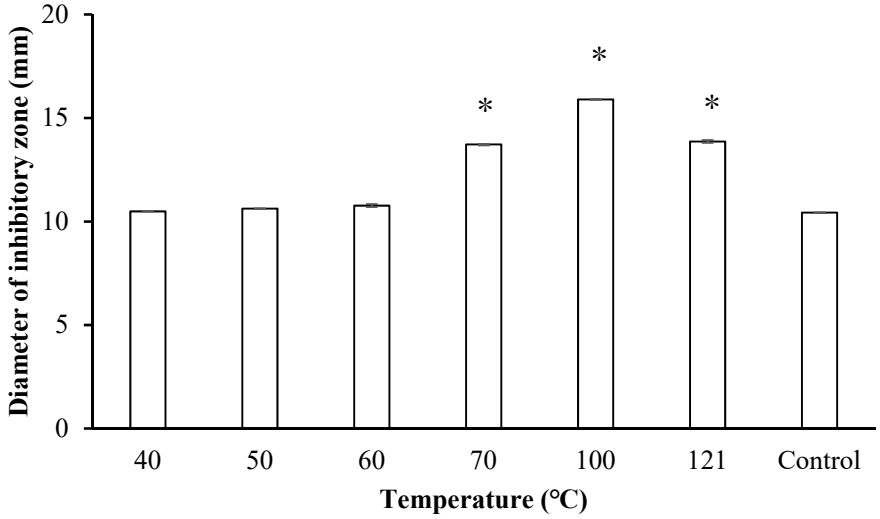

Figure 3-4. Heat stability of bacteriocin G3067.

*<0.05

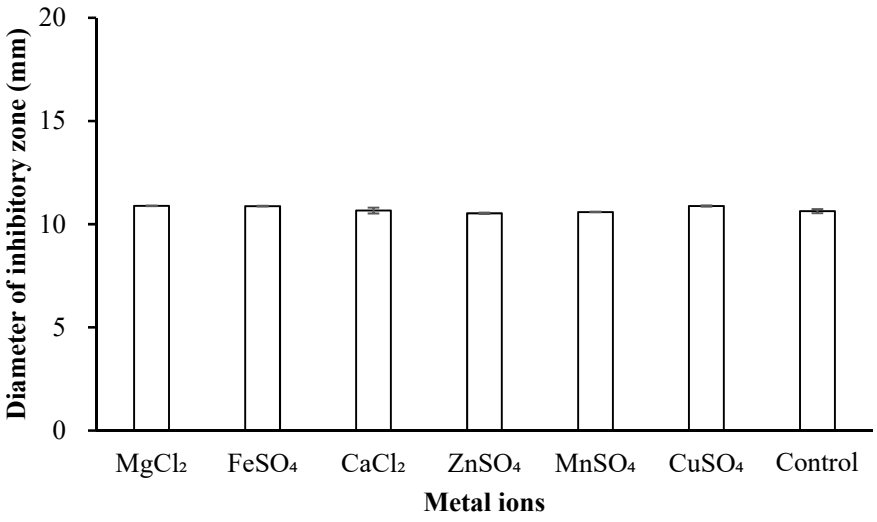

Figure 3-5. Effect of metal ions on the activity of bacteriocin G3067

General Discussion

In The Gambia, milk is one of the most popular foods and is consumed either in fresh raw milk or fermented form as there are no dairy plants in the country yet. Cattle farmers in The Gambia are yet to adopt modern dairy farming techniques and do not have adequate refrigeration facilities. Therefore, milk is often rapidly spoiled or unsafe for consumption. It has been well verified that the intake of such milk can cause many problems for human health, especially for babies and people with weak immunity.

Fermentation has also been a common method for storing milk as food for centuries, as it can prolong the shelf life of milk, even on the African continent. In the past, there have been several studies on the microbiology of FM products in many other African countries such as Burkina Faso (Savadogo *et al.* 2004), Egypt (Ayad *et al.* 2004), Ethiopia (Yilda *et al.* 2015), Ghana (Akabanda *et al.* 2010 and 2013), Morocco (Hamama, 1992), Nigeria (Obi & Ikenebomeh 2007; Uzeh *et al.* 2006; Atanda and Ikenebom 1989), Senegal (Parker *et al.* 2018), South Africa (Beukes *et al.* 2001), Sudan (Abdelgadir *et al.* 1998 and 2001, Mohammed *et al.* 2011), Tanzania (Isono *et al.* 1994), Kenya (Nyambane *et al.* 2014; Mathara *et al.* 2004), Zimbabwe (Feresu and Muzondo 1990; Gadaga *et al.* 1999). Most of these reports illustrate the microbial diversity and the presence of foodborne pathogens among African fermented milks. Also, regarding Gambian FM, Hempen *et al.* (2004) reported that the abundance of pathogens such as *Bacillus cereus*, *Clostridia* spp., *Listeria* spp., *Escherichia coli*, *Salmonella* spp., *Staphylococci* spp. was as high as that of raw milk. However, they did not investigated microorganisms other than pathogenic bacteria including LAB and its microbiota has not been clarified in detail. Therefore, this study aimed to elucidate the microbial profile of Gambian traditional FM.

This study suggested that various pathogens might still be present in Gambian traditional FM, 20 years after the report by Hempen *et al.* (2004). Therefore, it is important to publicize the importance of pasteurization in order to ensure that Gambian consumers can safely consume milk and dairy products. However, this requires a long time to educate producers and consumers. As one of the methods for producing safe FM during less period, the addition of LAB as a starter into the milk can be considered. Some LAB can suppress the growth of harmful microorganisms such as *E. coli* and coliform bacteria by producing lactic acid and acetic acid. Not only were *Lb. delbrueckii* and *S. lutetiensis* the dominant species in Gambian FM, but some of them can decrease the pH of the milk below 5. If beneficial strains with acid-producing ability and antibacterial activity against pathogens can be selected from these strains and used for the production of

fermented milk, FM products will be safer than the conventional FM. Furthermore, FM produced using these LAB strains is suggested to have a taste, flavor and texture similar to those of conventional FM, since the origin of strain is Gambian traditional FM.

This is the most comprehensive study of Gambian traditional FM so far. The information on the total microbiota of traditional Gambian fermented milk has revealed its safety level. The study also provided an opportunity to preserve LABs isolated from Gambian FM as microbial resources. Furthermore, in this study, the bacteriocin produced by *S. lutetiensis* G3067 was characterized and suggested the possibility to use as a natural preservative. These findings will become important not only for Gambian politicians and bureaucrats to consider laws and regulations, but also to promote safe dairy products eating habits for the general public in The Gambia.

Abstract (要約)

牛乳と発酵乳は，有用な栄養素を含んでいることから，ガンビアのあらゆる階層の人々に人気があります。しかしながら，ガンビアの発酵乳（FM）に *Bacillus* 属，*Clostridia* 属，*Listeria* 属，*Escherichia* 属，*Salmonella* 属およびブドウ球菌などの病原体が存在しているという報告はあるものの，ガンビアの伝統的な発酵乳の微生物叢に関する知見はほとんどありません。近年，ガンビアでは 5 歳未満の子供の下痢性疾患が頻繁に報告されており（Saha *et al*。2013），低温殺菌されていない牛乳と発酵乳に存在する病原性微生物が原因の 1 つであることが示唆されています。消費者や生産者はこのような問題だけでなく，ガンビアで生産される牛乳や乳製品の特性についてもほとんど知識がありません。そこで本研究では，ガンビアの伝統的な FM「Kosam」の微生物プロファイルを解明し，その安全性を評価することを最初に目的としました。

発酵乳試料には 2019 年にガンビアの 2 つの地域（中央川地域：CRR、下川地域：LRR）で収集したものを用いました。発酵乳の菌叢解析は，培養法とイルミナシーケンスによって行いました。CRR および LRR の発酵乳中の乳酸菌（LAB）の数は，それぞれ 8.27±0.08 log CFU/mL および 7.21±0.09 logCFU/mL でした。大腸菌群と大腸菌は CRR の発酵乳（CRR-FM）では検出されませんでしたが，LRR の発酵乳（LRR-FM）には 5.73±0.17 log CFU/mL の大腸菌群と 4.82±0.13 logCFU/mL の大腸菌が検出されました。CRR-FM の主要な LAB は *Lactobacillus delbrueckii* であり，次いで *Streptococcus lutetiensis* でした。また，LRR-FM では *Lactococcus lactis* でした。イルミナシーケンスによるメタゲノム解析においても，これらの種がガンビアの伝統的発酵乳中で優勢であることを明らかとなりました。さらに，本解析により *Klebsiella* 属などの病原体の存在の可能性も明らかとなりました。

今後，ガンビアで安全な発酵乳を製造するために，ガンビアの伝統的な FM から分離された LAB は，還元脱脂乳（RSM）の pH への影響および分離株間のバクテリオシン様阻害活性を基に特徴付けられました。 CRR-FM および LRR-FM からの LAB 分離株のうち，35℃で 24 時間のインキュベーション後に RSM の pH を 6.0 以下に低下させた菌株の割合は，それぞれ 79%および 16%でした。両方のガンビア発酵乳から分離された *Lc. lactis* の大部分は，RSM の pH 値を 6.0 未満に下げることができませんでした。一方，CRR-FM から分離された LAB では，*Lb. delbrueckii*, *Leu. mesenteroides*, *Limosilactobacillus fermentum*,および *Streptococcus lutetiensis* の一部の菌株が，pH を 5.0 未満に下げることができました。CRR-FM と LRR-FM の pH はそれぞれ 4.7 と 5.4 であり，ミルクで酸を生成できる菌株の割合が発酵乳の pH に影響を与えることが示唆されました。

さらに, pH の低下はガンビアの FM の大腸菌群と大腸菌の数にも影響を与えることも示唆されました。

分離株間のバクテリオシン様阻害活性アッセイは，寒天ウェル拡散法によって行いました。ガンビアの発酵乳から分離された 9 菌種 20 菌株を指標として用いました。 *S. lutetiensis* 菌株は，15 菌株（5 種）に対してバクテリオシン様阻害活性を示し，比較的広範囲の抗菌活性を持っていることが示唆されました。同様に，*Lc. lactis* 株と *Lb. delbrueckii* 菌株は，それぞれ 7 菌株（3 種）と 6 菌株（2 種）の指標菌に対して活性を示しました。すべての *E.faecalis* 株が，指標として用いた 3 菌株の *Lb. delbrueckii* に対してバクテリオシン様阻害活性を示しました。対照的に，指標として用いた *E. faecalis*，*Lb. plantarum*，*Lb. fermentum*，*Lc. lactis* および *Leu. mesenteroides* は，ガンビア FM から分離された他のすべての LAB 株によって阻害されませんでした。以上のことから，LAB 菌株が産生するバクテリオシン様活性物質がガンビアの発酵乳の微生物叢の形成に関与していることが示唆されました。

比較的広範囲の抗菌活性を有した *S. lutetiensis* 菌株の中から，より高いバクテリオシン様活性を有する菌株として *S. lutetiensis* G3067 を選抜しました。さらに，この菌株によって産生されたバクテリオシンを精製し，その特徴解析を行いました。*S. lutetiensis* G3067 の MRS 培養液から調製した無細胞上清（CFS）を，クロロホルム抽出，硫酸アンモニウム沈殿および陰イオン交換クロマトグラフィーの 3 つの調製ステップに供しました。バクテリオシン様阻害活性アッセイは，*Lb. delbrueckii* subsp. *delbrueckii* NBRC13953^{T} を用いた寒天ウェル拡散法により行いました。これらの精製ステップにより，バクテリオシン活性は CFS の 20 AU/mL から，陰イオン交換クロマトグラフィーのピーク 1 で 800 AU/ mL に増加し，その収率は 16.7%でした。ピーク 1 画分は，プロテイナーゼ K によって不活化され，HPLC で単一のピークとして溶出されました。また，トリシン SDS-PAGE 上でで 1.0～3.5 kDa の位置にブロードなバンドとして検出され，移動度から分子量は 3.0 kDa と推定されました。これらの結果から，この成分がバクテリオシンであると確認され，バクテリオシン G3067 と命名しました。このバクテリオシンは，40℃，50℃および 60℃で 30 分間の熱処理に対して熱安定性を有していました。また，70℃，100℃および 121℃で 30 分間処理により, その活性は非加熱サンプルと比較して有意に増加しました。このバクテリオシン G3067 の活性は，二価の金属イオンの添加によって影響を受けませんでした。これらの特性は，バクテリオシン G3067 が食品加工に利用できる可能性があることが示唆されました。

この研究はガンビア FM に関する知見を高め，微生物群集の解明に貢献しました。 また，ガンビア FM から分離された LAB 株を微生物資源として保存する機会も提供しました。 これらの知見は，ガンビアの政治家や官僚が法規制を検討するためだけでなく，ガンビアの一般市民が安全な乳製品を食べることを促進するためにも重要になるでしょう。

References

Abanoz HS & Kunduhoglu B. 2018. Antimicrobial activity of a bacteriocin produced by *Enterococcus faecalis* KT11 against some pathogens and antibiotic-resistant bacteria. Korean Journal for Food Science of Animal Resources. 38:1064–1079.

Abdelgadir WS, Ahmed TK, & Dirar HA. 1998. The traditional fermented milk products of the Sudan. International Journal Food Microbiology. 44: 1–13.

Abdelgadir WS, Hamad SH, Moller PL, & Jakobsen M. 2001. Characterization of the dominant microbiota of Sudanese fermented milk Rob. International Dairy Journal. 11: 63-70.

Abee T, Krockel L, & Hill C. 1995. Bacteriocins: modes of action and potentials in food preservation and control of food poisoning. International Journal of Food Microbiology. 28:169–185.

Akabanda F, Owusu-Kwarteng J, Tano-Debrah K, Glover RLK, Nielsen DS, & Jespersen L. 2013. Taxonomic and molecular characterization of lactic acid bacteria and yeasts in nunu, a Ghanaian fermented milk product. Food Microbiology. 34: 277-283.

Akabanda F, Owusu-Kwarteng, Glover RLKJ, & Tano-Debrah K. 2010. Microbiological characteristics of Ghanaian traditional fermented milk product, *Nunu*. Nature and Science. 8: 178–187.

Arakawa K, Kawai Y, Nishimura J, Kitazawa H, & Saito T. 2009. Negative effect of divalent metal cations on production of gassericin T, a bacteriocin produced by *Lactobacillus gasseri*, in milk-based media. International Dairy Journal. 19: 612-616.

Atanda OO & Ikenebom MJ. 1989. Effect of heat treatments on the microbial load of nono. Letters in Applied Microbiology. 9:233-235.

Ayad E, Nashat S, El-Sadek N, Metwaly H, & El Soda M. 2004. Selection of wild lactic acid bacteria isolated from traditional Egyptian dairy products according to production and technological criteria. Food Microbiology. 21: 715-725.

Batdorj B, Dalgalarrondo M, Choiset Y, Pedroche J, Métro F, Prévost H, Chobert JM, & Haertlé T. 2006. Purification and characterization of two bacteriocins produced by lactic acid bacteria isolated from Mongolian airag. Journal of Applied Microbiology. 101: 837-848.

Beukes EM, Bester BH & Mostert JF. 2001. The microbiology of South African traditional fermented milk. International Journal Food Microbiology. 63: 189-197.

Bintsis T. 2018. Lactic acid bacteria as starter cultures: An update in their metabolism and genetics. AIMS microbiology. 4: 665–684.

Cavanagh D, Fitzgerald GF & McAuliffe O. 2015. From field to fermentation: the origins of *Lactococcus lactis* and its domestication to the dairy environment. Food Microbiology. 47: 45-61.

Chen P, Qiu Y, Liu G, Li X, Cheng J, Liu K, Qu W, Zhu C, Kastelic JP, Han B & Gao J. 2021. Characterization of *Streptococcus lutetiensis* isolated from clinical mastitis of dairy cows. Journal of Dairy Science. 104: 702-714.

Crow V & Curry B. 2002. *Lactobacillus* spp./*Lactobacillus delbrueckii* Group. Encyclopedia of Dairy Sciences. 3: 1696-1697.

De Sousa BL, Azevedo AC, Oliveira IMF, Bento CBP, Santana MF, Bazzolli DMS, & Mantovani HC. 2021. PCR screening reveals abundance of bovicin-like bacteriocins among ruminal *Streptococcus* spp. isolated from beef and dairy cattle. Journal of Applied Microbiology. 4: 1695-1709.

Delgado-Fernández P, Moreno FJ, Corzo N, & Nöbel S. 2020. Physical properties of synbiotic yogurts as affected by the acidification rate. International Dairy Journal. 105: 1046665. doi.org/10.1016/j.idairyj.2020.104665

Doutoum AA, Tidjani A, Balde M, Tidjani SMT, Abdelaziz Arada I, Sylla KSB, Seydi MG, & Toguebaye BS. 2013. Identification of lactic bacteria of milk quail in Chad. Journal of Applied Biology and Biotechnology. 1(4): 7–15.

El-Baradei G, Delacroix-Buchet A, & Ogier JC. 2008. Bacterial biodiversity of traditional Zabady

fermented milk. International Journal of Food Microbiology.121: 295–301.

FAO/WHO. 2004. Code of hygienic practice for milk and milk products. CAC/RCP 57-2004.

FAOSTAT, Food and Agricultural Organization of the United Nations, Rome, Italy. Food Balances (2004-2018). https://www.fao.org/faostat/en/#data/FBS. Accessed December 10th, 2021.

Feresu SB & Muzondo MI. 1990. Identification of some lactic acid bacteria from two Zimbabwean fermented milk products. World Journal Microbiology and Biotechnology. 6: 178- 186.

Fujita K, Ichimasa S, Zendo T, Koga S, Yoneyama F, Nakayama J, & Sonomoto K. 2007. Structural analysis and characterization of lacticin Q, a novel bacteriocin belonging to a new family of unmodified bacteriocins of gram-positive bacteria. Applied Environmental Microbiology. 73: 2871-2877.

Gaaloul N, Ben Braiek O, Berjeaud JM, Arthur T, Cavera VL, Chikindas ML, Hani K, & Ghrairi T. 2014. Evaluation of antimicrobial activity and safety aspect of *Enterococcus Italicus* GGN10 strain isolated from Tunisian bovine raw milk. Journal of Food Safety 34: 300-311.

Gadaga TH, Mutukumira AN, & Narvhus JA. 1999. Feresu S B. A review of traditional fermented foods and beverages of Zimbabwe. International journal of Food Microbiology. 53: 1-11. doi.org/10.1016/S0168-1605(99)00154-3

García-Burgos M, Moreno-Fernández J, Alférez MJM, Díaz-Castro J, & López-Aliaga I. 2020. New perspectives in fermented dairy products and their health relevance. Journal of Functional Foods. 72:1-11.

Gastaldi E, Lagaude A, & Tarodo de La Fuente G. 1996. Micellar transition state in casein between pH 5.5 and 5.0. Journal of Food Science. 61: 59–64.

Girma, A & Aemiro A. 2021. Antibacterial Activity of Lactic Acid Bacteria Isolated from Fermented Ethiopian Traditional Dairy Products against Food Spoilage and Pathogenic Bacterial Strains. Journal of Food Quality. doi.10.1155/2021/9978561.

Hamama A. 1992. Moroccan traditional fermented dairy products. In: Ruskin, F.R. (Ed.) Applications of biotechnology to traditional fermented foods. National Academy press, Washington DC. 75-79.

Han X, Yang Z, Jing X, Yu P, Zhang Y, Yi H, & Zhang L. 2016. Improvement of the texture of yogurt by use of exopolysaccharide producing lactic acid bacteria. Biomed Research International. 2016: 1945675. dx.doi.org/10.1155/2016/7945675

Hassan MU, Nayab H, Rehman TU, Williamson MP, Haq KU, Shafi N, & Shafique F. 2020. Characterisation of bacteriocins produced by *Lactobacillus* spp. isolated from the traditional Pakistan yoghurt and their antimicrobial activity against common foodborne pathogens. Biomed Research International. 2020: 8281623. https://doi.org/10.1155/2020/8281623

Hempen M, Unger F, Münstermann S, Seck MT, & Niamy V. 2004. The hygienic status of raw and sour milk from smallholder dairy farms and local markets and potential risk for public health in The Gambia, Senegal and Guinea. Animal Health Research Working Paper 3. International Trypanotolerance Centre. 1-54.

Hwanhlem N, Biscola V, El-Ghaish S, Jaffrès E, Dousset X, Haertlé T, H-Kittikun A, & Chobert JM. 2013. Bacteriocin-producing lactic acid bacteria isolated from mangrove forests in southern Thailand as potential bio-control agents: purification and characterization of bacteriocin produced by *Lactococcus lactis* subsp. *lactis* KT2W2L. Probiotics Antimicrobial Proteins. 4: 264-278.

IDF. 2019. The technology of pasteurisation and its effect on the microbiological and nutritional aspects of milk. Bulletin of the IDF N° 496 2019:

Isono Y, Shingu I, & Shimizu S. 1994. Identification and Characteristics of lactic acid bacteria isolated from Masaï fermented milk in Northern Tanzania. Bioscience Biotechnology Biochemistry. 58: 660-664.

Izquierdo E, Wagner C, Marchioni E, Aoude-Werner D, & Ennahar S. 2009. Enterocin 96, a novel class II bacteriocin produced by *Enterococcus faecalis* WHE 96, isolated from Munster cheese. Appllied Environmental Microbiology. 75: 4273-4276.

Jans C, Meile L, Kaindi DWM, Kogi-Makau W, Lamuka P, Renault P, Kreikemeyer B, Lacroix C, Hattendorf J, Zinsstag J, Schelling E, Fokou G, & Bonfoh B. 2017. African fermented dairy products - Overview of predominant technologically important microorganisms focusing on African *Streptococcus infantarius* variants and potential future applications for enhanced food safety and security. International Journal of Food Microbiology. 250: 27-36.

Jiwoua C & Milliere JB. 1990. Lactic flora and enterococci of fermented milk (Pindidam) produced in Adamaoua (Cameroon). Lait. 70: 475–486.

Joachimsthal EL, Reeves RKH, Hung J, Nielsen, LK, Ouwerkerk D, Klieve AV, & Vickers CE. 2010. Production of bacteriocins by *Streptococcus bovis* strains from Australian ruminants. Journal of Applied Microbiology. 108: 428-436.

Jozala, AF, de Andrade MS, de Arauz LJ. Pessoa Jr A, Christina T, & Penna V. 2007. Nisin production utilizing skimmed milk aiming to reduce process cost. Applied Biochemistry and Biotechnology. 136-140: 515-528. doi.org/10.1007/s12010-007-9076-9

Langoni H, Guiduce MVS, Nóbrega DB, Da Silva RC, Richini-Pereira VB, Salina A, & Guimarães FDF. 2015. Research of *Klebsiella pneumoniae* in dairy herds. Pesquisa Veterinaria Brasileira. 35: 9-12.

Lasik-Kurdyś M & Sip A. 2019. Evaluation of the antimicrobial activity of bacteriocin-like inhibitory substances of enological importance produced by *Oenococcus oeni* isolated from wine. European Food Research and Technology. 245. 375–382.

Lee H, & Yoon Y. 2021. Etiological agents implicated in foodborne illness worldwide. Food Science of Animal Resources. 41: 1–7. doi.org/10.5851/kosfa.2020.e75

Li HW, Xiang, YZ, Zhang M, Jiang YH, Zhang Y, & Liu YY. 2021. A novel bacteriocin from *Lactobacillus salivarius* against *Staphylococcus aureus*: Isolation, purification, identification, antibacterial and antibiofilm activity. LWT-Food Science and Technology. 140: 110826. doi.org/10.1016/j.lwt.2020.110826

Li W, Ren M, Duo L, Li J, Wang S, Sun Y, Li M, Ren W, Hou Q, Yu J, Sun Z & Sun T. 2020. Fermentation characteristics of *Lactococcus lactis* subsp. *lactis* isolated from naturally fermented dairy products and screening of potential starter isolates. Frontiers in Microbiology. 11: 1794. doi.org/10.3389/fmicb.2020.01794

Marianelli C, Cifani N, Pasquali P. 2010. Evaluation of antimicrobial activity of probiotic bacteria against Salmonella enterica subsp. enterica serovar typhimurium 1344 in a common medium under different environmental conditions. Research in Microbiology. 161: 673-80.

Massé J, Dufour S, & Archambault M. 2020. Characterization of *Klebsiella i*solates obtained from clinical mastitis cases in dairy cattle. Journal of Dairy Science. 103: 3392-3400.

Matevosyan L, Bazukyan I, & Trchounian A. 2019. Comparative analysis of the effect of Ca and Mg ions on antibacterial activity of lactic acid bacteria isolates and their associations depending on cultivation conditions. Applied Microbiology Express. 9: 32. doi: 10.1186/s13568-019-0758-9

Mathara JM, Schillinger U, Kutima PM, Mbugua SK & Holzapfel WH. 2004. Isolation, identification and characterisation of the dominant microorganisms of *kule naoto*: the Maasai traditional fermented milk in Kenya. International Journal of Food Microbiology. 94: 269-278.

McAuley CM, Britz ML, Gobius KS & Craven HM. 2015. Prevalence, seasonality, and growth of *enterococci* in raw and pasteurized milk in Victoria, Australia. Journal of Dairy Science. 98: 8348-8358.

Mckay LL, Balwin KA, Zottola EA. 1972. Loss of lactose metabolism in lactic *streptococci*. Applied Microbiology. 23: 1090-1096.

Mohammed SAM, el Sanousi SM & El Zubeir IEM. 2011. A Review on the Sudanese traditional dairy products and technology. International Journal of Dairy Science. 6: 227-245.

Mudgil D, Barak S & Khatkar BS. 2017. Texture profile analysis of yogurt as influenced by partially hydrolyzed guar gum and process variables. Journal of Food Science and Technology. 54:.3810-3817.

Munoz MA, Welcome FL, Schukken YH, & Zadoks RN. 2007. Molecular epidemiology of two *Klebsiella pneumoniae* mastitis outbreaks on a dairy farm in New York State. Journal of Clinical Microbiology. 45: 3964-3971.

Nakasaki K, Yanagisawa M, & Kobayashi K. 2008. Microbiological quality of fermented milk produced by repeated-batch culture. Journal of Bioscience and Bioengineering. 105: 73-76.

Navarro L, Zarazaga M, Sáenz J, Ruiz-Larrea F & Torres C. 2000. Bacteriocin production by lactic acid bacteria isolated from Rioja red wines. Journal of Appllied Microbiology. 88.44-51.

Nyambane B, Thari WM, Wangoh J, & Njage PM. 2014. Lactic acid bacteria and yeasts involved in the fermentation of amabere amaruranu, a Kenyan fermented milk. Food Science and Nutrition. 2: 692-699.

Obi CN & Ikenebomeh MJ. 2007. Studies on the microbiology and nutritional qualities of a Nigerian fermented milk product (*Nono*). International Journal of Dairy Science. 2: 95-99.

Obodai M & Dodd CE. 2006. Characterization of dominant microbiota of a Ghanaian fermented milk product, nyarmie, by culture- and nonculture-based methods. Journal of Applied Microbiology. 100:1355–1363.

Oki K, Dugersuren J, Demberel S, & Watanabe K. 2014. Pyrosequencing analysis of the microbial diversity of airag, khoormog and tarag, traditional fermented dairy products of Mongolia. Bioscience of Microbiota, Food and Health. 33: 53-64.

Oliver SP, Jayarao BM, & Almeida RA. 2005. Foodborne pathogens in milk and the dairy farm environment: food safety and public health implications. Foodborne Pathogens and Disease. 2: 115–129.

Osvik RD, Sperstad S, Breines E, Hareide E, Godfroid J, Zhou Z, Ren P, Geoghegan, C, Holzapfel C, & Ringø, E. 2013. Bacterial diversity of AMasi, a South African fermented milk product, determined by clone library and denaturing gradient gel electrophoresis analysis. African Journal of Microbiology Research. 7: 4146–4158.

Parapouli M, Delbes-Paus C, Kakouri A, Koukkou AI, Montel MC, & Samelis J. 2013. Characterization of a wild, novel nisin a-producing *Lactococcus* strain with an *L. lactis* subsp. *cremoris* genotype and an *L. lactis* subsp. *lactis* phenotype, isolated from Greek raw milk. Applied Environmental Microbiology. 79: 3476–3484.

Parente E, Ricciardi A, & Addario G. 1994. Influence of pH on growth and bacteriocin production by *Lactococcus lactis* subsp. *lactis* 14ONWC during batch fermentation. Applied Microbiology and Biotechnology. 41: 388–394.

Parker M, Zobrist S, Donahue C, Edick C, Mansen K, Nadjari MHZ, Heerikhuisen M, Sybesma W, Molenaar D, Diallo AM, Milani P, & Kort R. 2018. Naturally fermented milk from northern Senegal: Bacterial community composition and probiotic enrichment with *Lactobacillus rhamnosus*. Frontiers in Microbiology. 9: 2218. doi.org/10.3389/fmicb.2018.02218

Penna, TCV, Jozala A, Novaes LCD, Pessoa A, & Cholewa O. 2005. Production of nisin by *Lactococcus lactis* in media with skimmed milk. Applied Biochemistry and Biotechnology. 121: 619-637.

Podder MP, Rogers L, Daley PK, Keefe GP, Whitney HG, & Tahlan K. 2014. *Klebsiella* species associated with bovine mastitis in Newfoundland. PLoS ONE 9:106518. doi: 10.1371/journal.pone.0106518.

Ren D, Zhu J, Gong S, Liu H, & Yu H. 2018. Antimicrobial characteristics of lactic acid bacteria isolated from homemade fermented foods. BioMed Research International. 5416725. doi: 10.1155/2018/5416725.

Rubak YT, Nuraida L, Iswantini D, & Prangdimurti E. 2020. Angiotensin-I-converting enzyme inhibitory peptides in milk fermented by indigenous lactic acid bacteria. Veterinary World. 13: 345-353.

Saha D, Akinsola A, Sharples K, Adeyemi MO, Antonio M, Imran S, Jasseh M, Hossain MJ, Nasrin D,

Kotloff KL, Levine MM, & Hill PC. 2013. Health care utilization and attitudes survey: understanding diarrheal disease in rural Gambia. American Journal of Tropical Medicine and Hygiene.1:13-20.

Savadogo A, Ouattara CAT, Savadogo PW, Baro N, Ouattara AS, & Traore AS. 2004. Microorganism involved in Fulani fermented milk in Burkina Faso. Pakistan Journal of Nutrition. 3: 134-139.

Shah NP & Dave R. 2002. Antimicrobial substances including bacteriocins produced by lactic acid bacteria. Bioscience and Microflora. 21:217-22.

Shangpliang HNJ, Rai R, Keisam S, Jeyaram K, & Tamang JP. 2018. Bacterial community in naturally fermented milk products of Arunachal Pradesh and Sikkim of India analysed by high-throughput amplicon sequencing. Scientific Reports 8. 1532. doi.org/10.1038/s41598-018-19524-6

Shori AB. 2012. Comparative study of chemical composition, isolation and identification of micro-flora in traditional fermented camel milk products: Gariss, Suusac, and Shubat. Journal of the Saudi Society of Agricultural Sciences. 11: 79-88.

Sudha SS & Aranganathan V. 2021. Experimental elucidation of an antimycobacterial bacteriocin produced by ethnomedicinal plant-derived *Bacillus subtilis* (MK733983). Archieves of Microbiology. 203: 1995-2006.

Suiryanrayna MV & Ramana JV. 2015. A review of the effects of dietary organic acids fed to swine. Journal of Animal Science and Biotechnology.6:45. doi.org/10.1186/s40104-015-0042-z

Suzuki C. 2011. Isolation, identification and characterization of lactic acid bacteria from infant feces. Annual report on Exploration and Introduction of Microbial. Genetic Resourses. 24:43–49.

Terzic´-Vidojevic´ A, Veljovic´ K, Popovic´ N, Tolinacˇki M, & Golic´ N. 2021. *Enterococci* from raw-milk cheeses: Current Knowledge on Safety, Technological, and Probiotic Concerns. Foods. 10: 2753. doi.org/10.3390/foods10112753

Terzic´-Vidojevic´ A, Veljovic´ K, Tolinacˇki M, Živkovic´ M, Lukic´ J, Lozo J, Fira Đ, Jovcˇic´ B, Strahinic´ I, Begovic´ J, Popovic N, Mijovic M, Kojic M, Topisirovic L, & Golic N. 2020. Diversity of non-starter lactic acid bacteria in autochthonous dairy products from western Balkan countries—Technological and probiotic properties. Food Research International. 136: 109494. doi.org/10.1016/j.foodres.2020.109494.

Tomovska J, Gjorgievski N, & Makarijoski, B. 2016. Examination of pH, titratable acidity and antioxidant activity in fermented milk. Journal of Materials Science and Engineering. 6: 326-333.

Tsuda H & Kodama K. 2021. Evaluating the technological properties of lactic acid bacteria in Wagyu cattle milk. Journal of Dairy Research. 88: 210-216.

Uzeh R, Ohenhen R, & Rojugbokan A. 2006. Microbiological and nutritional qualities of dairy products*:* *Nono* and *Wara*. Nature and Science. 4: 37-40.

Whitford MF, McPherson MA, Forster RJ, & Teather RM. 2001. Identification of bacteriocin-like inhibitors from rumen *Streptococcus* spp. and isolation and characterization of bovicin 255. Applied Environmental Microbiology. 67.2: 569-574.

Widodo H, Handaka R, Wahyuni E, & Taufiq T. 2017. The quality of fermented milk produced using intestinal-origin lactic acid bacteria as starters. International Food Research Journal. 24: 2371-2376.

WITS/WHO.2019.https://wits.worldbank.org/trade/comtrade/en/country/GMB/year/2019/tradeflow/Imports/partner/ALL/product/040299. Accessed March 28th, 2022.

Wullschleger S, Lacroix C, Bonfoh B, Sissoko-Thiam A, Hugenschmidt S, Romanens E, Baumgartner S, Traoré I, Yaffee M, Jans C, & Meile L. 2013. Analysis of lactic acid bacteria communities and their seasonal variations in a spontaneously fermented dairy product (Malian *Fènè*) by applying a cultivation/genotype-based binary model. International Dairy Journal. 29: 28–35.

Yamamoto K, Noriyasu H, Kushiyama C, Murata S, Watanabe M, Fujimoto T, & Ohkusu K. 2017. A case of infective endocarditis caused by *Streptococcus infantarius* that led to coronary artery embolism. The Japanese journal of medical technology. 66: 277-283.

Yang E, Fan L, Jiang Y, Doucette C, & Fillmore S. 2012. Antimicrobial activity of bacteriocin-producing lactic acid bacteria isolated from cheeses and yogurts. Applied Microbiology Express. 2: 48. doi: 10.1186/2191-0855-2-48

Ye P, Wang J, Liu M, Li P, & Gu Q. 2021. Purification and characterization of a novel bacteriocin from *Lactobacillus paracasei* ZFM54. LWT-Food Science and Technology. 143: 111125. doi.org/10.1016/j.lwt.2021.111125

Yerlikaya O. 2014. Starter cultures used in probiotic dairy product preparation and popular probiotic dairy drinks. Food Science and Technology. 34: 221-229.

Yilma Z, Loiseau G, & Faye B. 2015. Growth and Survival of *Eschericia coli* O157: H7 during the Manufacturing of Ergo and Ayib, Ethiopian Traditional Fermented Milk Products. Journal of Food and Dairy Technology. 4: 31-36.

Younes M, Aggett P, Aguilar F, Crebelli R, Dusemund B, Filipič M, Frutos MJ, Galtier P, Gundert-Remy U, Kuhnle G, Lambré C, Leblanc JC, Lillegaard I, Moldeus P, Mortensen A, Oskarsson A, Stankovic I, waalkens-Berendsen I, Woutersen R, & Gott D. 2017. Safety of nisin (E 234) as a food additive in the light of new toxicological data and the proposed extension of use. European Food Safety Authority Journal. 15: 5063. doi: 10.2903/j.efsa.2017.5063.

Yu AT, Shapiro K, Beneri CA, Wilks-Gallo LS. 2021. *Streptococcus lutetiensis* neonatal meningitis with empyema. Access Microbiology. 3:000264. doi:10.1099/acmi.0.000264

Zhang J, Yang Y, Yang H, Bu Y, Yi H, Zhang L, & Ai L. 2018. Purification and partial characterization of bacteriocin Lac-B23, a novel bacteriocin production by *Lactobacillus plantarum* J23, isolated from Chinese traditional fermented milk. Frontiers in Microbiology. 9:1-7.

Zhong Z, Hou Q, Kwok L, Yu Z, Zheng Y, Sun Z, Menghe B, & Zhang H. 2016. Bacterial microbiota compositions of naturally fermented milk are shaped by both geographic origin and sample type. Journal of Dairy Science. 99: 7832-7841.

Zhu WM, Liu W, & Wu DQ. 2000. Isolation and characterization of a new bacteriocin from *Lactobacillus gasseri* KT7. Journal of Applied Microbiology. 88:877-86.

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