THE BIODIVERSITY and DESCRIPTION of MICROBIOTA IN TRADITIONALLY FERMENTED MILK PRODUCTS: a study in rural South Africa

Animal Ethics approval number: 018_033

by

Pieter Johannes de Waal

Student number: DWLPIE002

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Supervisor:

Professor Michael Levin (michael.levin@uct.ac.za)

Collaborator:

Centre of Proteomic and Genomic Research, Cape Town, South Africa:

Professor Shane Murray (shane.murray@cpgr.org.za)

Bioinformatician:

Katie Lennard (katieviljoen@gmail.com)

Department of Allergology, Division of Paediatrics, University of Cape Town

University of Cape Town

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Declaration

I, Pieter Johannes de Waal, hereby declare that the work on which this dissertation is based is my original work (except where acknowledgements indicate otherwise) and that neither the whole work nor any part of it has been, is being, or is to be submitted for another degree in this or any other university.

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Name: Pieter Johannes de Waal

Student number: DWLPIE002

Signature: Signed by candidate

Date: 5 May 2020

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Abstract

The rapid rise in allergic diseases has been linked to urbanization and Westernization. Recent observational studies indicate a significantly lower prevalence of allergic disease in children exposed to farming environments during the ante- and postnatal period. Consumption of unpasteurized and fermented cow's milk have been hypothesized as independent protective factors against allergy. Lack of microbial diversity and low levels of lactic acid producing bacteria (LAB) in infant diets may be predisposing factors to developing atopic eczema, allergic sensitisation and asthma. In South Africa, rural communities with a low prevalence of allergy consume unpasteurized and traditional fermented milk products on a regular basis. The objective of this study was to characterize and compare the microbiome of differently sourced cow's milk samples. Raw, unpasteurized cow's milk was collected from farms in an urban and rural setting, respectively. Another sample, collected from a cow on a rural farm, was left to naturally ferment (*amasi*) while three different brands of commercially fermented milk samples were obtained from a local retail shop. The variable V3 and V4 regions of the 16S rRNA gene were amplified and diversity and abundance plots were constructed and analyzed. Clear differences in the diversity and abundance of especially LAB in the differently sourced samples were demonstrated. Urban, and rural fresh cow's milk samples were the most diverse, and commercially bought products, the least. The commercially fermented products were similarly dominated by LAB, belonging to the phylum *Firmicutes* (more than 98% abundance) and the phylum *Proteobacteria* (less than 2% abundance). The homemade fermented milk (*amasi*) comprised approximately 50% *Firmicutes* and approximately 50% *Proteobacteria*. At the family member level, *Leuconostocaceae* dominated in all three the commercially bought samples. At the species level, *Lactococcus lactis* (AB100803) dominated in all the milk products, with less abundance in the fresh cow's milk samples. *Lactobacillus paracasei* (D79212) and *Streptococcus infantis* (AY485603) were abundant in *amasi* and absent in the commercially fermented products. Statistically significant difference between fermented and unfermented cow's milk samples at species level were demonstrated. *Lactococcus chungangensis* (EF694028), *Leuconostococcus mesenteroides* (AB023247) and *Leuconostococcus pseudomesenteroides* (AB023237) were abundant in the commercially fermented products, but absent in *amasi*. Important pathogens were identified in fresh cow's milk and *amasi*. We concluded that commercially fermented milk, although of low diversity, may be utilized as a safe allergy protective weaning food in infant diets. The microbiome of homemade *amasi* is more diverse than commercially fermented products and important allergy protective lactic acid producing organisms were identified in this study. However, the safety of *amasi* remains a concern. This information can be used in future research to produce important allergy protective 'starter cultures' and to appropriately shape the gut microbiome early in life.

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Chapter 1: INTRODUCTION AND LITERATURE REVIEW

The prevalence of IgE-mediated allergic diseases increased dramatically after the Second World War – it occurred too rapidly to be explained by genetic influence alone. For instance, a sudden rise in allergy has been demonstrated in individuals from African descent (with a low prevalence of allergic disease in their countries of origin), migrating to western parts of the world.[1] More so, it is estimated that up to 23% of children suffer from asthma and/or other allergic disease, without any first degree relative with allergy.[2] Westernization and the loss of protective environmental factors against allergy are being explored worldwide. Research is beginning to focus on antenatal and early life environmental exposures, leading to epigenetic 'programming' of the immune system in protecting against allergy.

In parallel, a rural versus urban gradient in allergy prevalence has been observed in children worldwide. Data from cross sectional studies, mostly Europe, indicate an allergy protective effect provided by ante- and postnatal farm-environment exposure. Important information comes from the GABRIEL Advanced Surveys (GABRIELA) and the Allergy and Endotoxin (ALEX) studies.[3,4] The GABRIELA group studied children from rural Switzerland, Austria and Germany and found that raw milk consumption was inversely associated with asthma (adjusted odds ratio [aOR], 0.59; 95% CI, 0.46-0.74), atopy (aOR, 0.74; 95% CI, 0.61-0.90), and hay fever (aOR, 0.51; 95% CI, 0.37-0.69). This protective effect was independent of other environmental exposures on farms. Interestingly, boiled farm milk did not protect against allergy and asthma.[5] In the ALEX cohort, unpasteurized milk consumption during pregnancy and in the first year of life, were also independently associated with significant lower prevalence of asthma (0.8% vs. 11.8%), allergic rhinitis (0.8% vs. 16.0%) and atopic sensitisation (8.2% vs. 32.9%) in children. In the Prevention of Allergy-Risk Factors for Sensitisation in Children Related to Farming and Anthroposophic Lifestyle (PARSIFAL) study and GABRIELA surveys, the allergy protective effect of drinking raw milk was found to be independent of family history of allergy. More so, raw milk consumption also significantly protected children against allergy, even if they were not exposed to a farm environment.[6]

From South Africa, similarly to European studies, the protective effect of rural environmental exposure against allergy was demonstrated. In the South African Food Sensitisation and Food Allergy (SAFFA) study, children (aged 1 to 3 years of age) living in an urban environment had higher rates of allergic diseases compared to their rural counterparts. In this study, the prevalence of food allergy in rural and urban black Africans were reported as 0.5% (95% CI, 0.1% to 1.8%) and 2.7% (95% CI, 1.5% to 4.5%; P=.006) respectively. Furthermore, statistically significant lower rates of asthma (1.0% vs.

6.0%), atopic dermatitis (1.8% vs. 19.6%), allergic rhinitis (3.3% vs. 16.0%) and skinprick test proven aeroallergen sensitisation (3.7% vs. 12.8%) were demonstrated between the two population groups.[7–9] By studying a single ethno-linguistic group in urban and rural settings respectively, the SAFFA study further highlighted the non-genetic influence of urbanization on allergy prevalence.

Homemade traditionally fermented milk, (isiZulu - '*amasi*'), is consumed daily by certain rural African communities. Previous knowledge also indicates that unpasteurized bovine milk (isiZulu - '*ubisi*'), is consumed more regularly by traditional rural communities.

The SAFFA study surprisingly highlighted an important finding: although the intake of fermented milk is thought to be a rural tradition with more rural black Africans consuming *amasi* during pregnancy, significantly more urban black African children (between 1 and 3 years of age) were regularly (>4 times per week) exposed to *amasi* compared to rural children of the same age. Furthermore, the consumption of fermented milk in urban black African children was associated with lower rates of allergic rhinitis (16.8% vs. 31.3%; $p<0.001$), atopic dermatitis (21.3% vs. 28.6%; $p=0.01$) and self-reported asthma (5.3% vs. 11.5%; $p=0.003$) compared to children who did not consume fermented milk. The consumption of fresh unpasteurized cow's milk was also associated with lower rates of atopic dermatitis in urban black Africans.[9]

The association of the intestinal microbiome and allergic sensitisation, asthma and eczema, have been highlighted in an extensive review by Zimmerman *et al*.[10] The role of early diversification of the infant's gut microbiome and the abundance of LAB are some of the anti-allergy role players being explored.[11–13] The composition of an infant's gut microbiome is highly dependent on the pre- and postnatal exposome. Geographical location, diet, outdoor and indoor environmental exposures (e.g. pets, microbes, dust, mode of delivery and antibiotics) are some important influential factors being investigated.[14] Furthermore, clear evidence now exists, indicating that changes to the composition and response of the human gut microbiome can be induced by the intake of specific fermented milk products.[15,16]

New 16S rRNA metagenomics techniques allow researchers to use culture independent methods to describe the human microbiome. These studies can be applied to study the microbiome in food products (e.g. fresh and fermented milk). Metagenomic studies targets the prokaryotic 16S ribosomal RNA gene. This gene is approximately 1500 base pairs long and contains nine hypervariable regions, interspersed between conserved regions. Compared to traditional culture-based identification methods, hypervariable regions are highly species specific and allow for a more comprehensive identification and description of taxa and organism function. On the other hand, conserved regions of the RNA gene, can be used as primer binding site for PCR-amplification.[17] Hypervariable loci are amplified by multiple polymerase chain reaction (PCR) steps with the subsequent formation of index reads. These are

demultiplexed while similar nucleotides are clustered to form Operational Taxonomic Units (OTUs). Traditionally, a pragmatic 97% cut-off value in similarity of these nucleotides, is used.[18] Computer software programs are utilized to generate taxonomy data. These are then compared with existing global databases to identify (and quantify) the microbes within a sample (*alpha diversity*) and to compare the microbiome of different samples with each other (*beta diversity*).[19,20]

The aim of this study was to identify the microbiome present in differently sourced cow's milk samples, using 16S rRNA metagenomic analysis. The influence of milk fermentation on the microbiome (at the species level) was also explored. To date, this is the first study from South Africa to use metagenomic techniques in comparing the microbiome of raw cow's milk (collected from suburban and rural farms) to that of home and commercially fermented milk products.

Chapter 2: METHODS

2.1 Ethical and safety considerations

This study received ethical approval from the Animal Research Ethics Committee (AREC) of the University of Cape Town (AREC approval number: 018_033). Permission under Section 20 of the Animal Diseases Act (Act No 35 of 1984) was obtained from the Department of Agriculture, Forestry and Fisheries to collect, safely transport, and store milk samples for analysis. All samples were discarded immediately after analysis. A registered and contracted waste disposable company was used by the analysing laboratory (Centre of Proteomics and Genomic Research (CPGR), Cape Town). A certificate was obtained from Environmental Affairs and Development Planning, Western Cape Government, certifying that CPGR is registered as a 'Hazardous Waste Generator'. Concerning the rural samples, a letter from the State Vet was obtained, confirming that the Mthatha State Veterinary Area (OIE code 706), particularly the Mqanduli Area (OIE Local Municipality Code 757), was not placed under quarantine. The area was declared free from Bovine Brucellosis and Tuberculosis for the past 12 months. No harm was inflicted on the animals while fresh samples were collected. Normal lactating practices, as per usual routine on the farms, were followed. Samples were collected by the farmer or farmworkers themselves, to ensure that the animals were not stressed at the time of milking.

2.2 Sample collection and delivery

2.2.1 Rural fresh cow's milk samples

During March 2019 fresh cow's milk samples were collected from three different farms in the Mqanduli rural area. Mqanduli is a town in the OR Tambo District Municipality, located in the Eastern Cape province of South Africa. This location was chosen because of preexisting knowledge, specifically patient profile, obtained during the aforementioned SAFFA study. This population lives closely to nature. Cattle farming, milking and consumption of unpasteurized cow's milk form part of their daily activity. After verbal informed consent was obtained from the owner of each farm, the animals were randomly chosen by the farmer of each farm and milked in a kraal. Sterile powder and latex free gloves (NUZONE®, Adventa Health), to prevent human microbiome contamination, were used by the farmers while milking was done by the farmers themselves. Before milking commenced, the cows and the udders were declared in a 'heathy state' by each farmer. To ensure daily milking routine and procedures were followed, farmers were not instructed on how to milk the cows. Two samples (three milliliters

each) of hind-milk were collected from each farm at ambient temperatures for that day. Milk was collected directly from the teats into sterile, screw top urine sample containers and sealed immediately after collection. The udders were not cleaned or washed prior to collection, as these procedures were not included in the normal milking routine on the farm. For overnight storing, samples were frozen shortly after collection in a household freezer. Samples were then transported by road to the nearest airport, after which they were transported by air to Cape Town. For transport, samples were kept in cryoboxes to ensure frozen delivery at the laboratory. After DNA-extraction, only two of the three samples passed the quality control (QC) step. These samples were labelled as *Rural fresh cow's milk 2* and *Rural fresh cow's milk 3*. (Picture 1 and picture 2)

2.2.2 Home fermented (*amas*i) milk samples

To produce traditionally fermented milk (*amasi*), rural folk leave unpasteurized milk for 3 to 5 days to naturally ferment at room temperature in plastic containers. At the same time of our rural fresh milk collection (March 2019), one sample of milk (about two hundred millilitres) was delivered to us by a farmer in the Mqanduli district, from a separative farm as where the other rural milk samples were collected. This sample of unpasteurized milk, delivered to us in a plastic cooldrink bottle, was kept unfrozen and sealed while it was transported. It was left to stand for 5 days at ambient temperature in Cape Town to allow natural fermentation. After this, it was transferred into two separate sterile urine sample containers (about three milliliters of fermented milk each), before it was delivered unfrozen to the laboratory. This sample was labelled as *Home fermented milk (amasi)*.

2.2.3 Urban fresh cow's milk samples

In May 2019, from a randomly chosen suburban farm at the outskirts of Cape Town, fresh urban cow's milk samples were collected. This farm is located in the suburb of Philippi (about twenty kilometres southeast of Cape Town). The owner sells unpasteurized, cooled down fresh milk to local customers. The cows on this farm are routinely immunised and considered 'healthy', as declared by the owner of the farm. These cows are milked by hand in a stable and no lactation equipment or machines are used. This farm is also frequently inspected during routine health inspector visits. For our study, the udders and teats of cows were cleaned by water, as per routine milking procedure on this farm. Sterile latex and powder free gloves (NUZONE®, *Adventa Health*) were used by the farmworker before milking commenced. This prevented human microbiome contamination of the samples. Milk was sampled from 3 different cows (each cow was sampled in duplicate). Each sample, about three milliliters of hind-milk, were collected directly into separate sterile, screw on urine sample containers at ambient temperature.

Samples were sealed, and frozen in a household freezer, before they were delivered to the laboratory the next day. Frozen transportation of these samples was ensured by using cryoboxes. The duplicate samples were pooled by the laboratory to be analyzed as three fresh urban cow's milks samples. These samples were labelled as *Urban fresh cow's milk 1*, *Urban fresh cow's milk 2* and *Urban fresh cow's milk 3*. (Picture 3)

2.2.4 Commercially fermented milk samples

In May 2019, three different commercially fermented bottled milk products (Othando®, Amyoli® and Maas®) were bought at a local retail store, near the laboratory in Cape Town. One bottle from each brand was randomly chosen from the store and immediately transported to the laboratory. In the store, these products are normally kept unfrozen but refrigerated. They were bought before their 'sell by' dates. One sample of each of the brands were poured directly from the container bottles upon arrival at the laboratory for analysis. These samples were labelled as *Com-othando*, *Com-maas* and *Com-amyoli*.

Samples were analyzed by the Centre for Proteomic and Genomic Research, Upper Level, St Peter's Square, Corner Anzio and Main Road, Observatory, Cape Town, South Africa. All milk samples were frozen to below minus 20°C upon receival at the laboratory. The project was conducted under the project identity number: 1161NGS_A_LEVIN_MIL.

Picture 1:
One of the rural farms where milk was collected. These communities live close to nature and farm animals. Nowadays, the blue containers (on the righthand side of the picture), replace traditional calabashes and clay pots for home fermentation of unpasteurized milk.

Picture 2:
The farmworker from one of the rural farms, catching the cows inside the kraal, to be milked for our study.

Picture 3:
The suburban farm, near Cape Town where our urban cow's milk samples were collected with Table Mountain in the background.

2.3 Sample preparation and laboratory analysis

DNA was extracted from the milk samples using the ZymoBIOMICS® DNA Miniprep Kit (Zymo Research, Irvine, CA, USA).

The variable V3 and V4 regions of the 16S rRNA gene were amplified from 2.5 ng to 25 ng of purified DNA by limited cycle PCR and barcoded for multiplexing using the Nextera® XT Index kit (Illumina, USA) and KAPA HiFi DNA Polymerase (Roche®, Pleasanton, CA, USA), according to Illumina's 16S sample preparation guide. The nine milk product samples, a positive control (ZymoBIOMICS® Microbial Community DNA standard (Zymo Research)) and a negative control (DNA suspension buffer) were included in library preparation.

The size of the libraries was verified using an Agilent® 2100 Bioanalyzer (Agilent, Santa Clara, CA, USA). Library concentration was evaluated using the KAPA Illumina Library Quantification Kit (Roche). The libraries were sequenced on MiSeq at the Centre for Proteomic and Genomic Research (Cape Town, South Africa), using a MiSeq v2 reagent kit (Illumina®) to produce paired-end 250 base pair reads. Indexed reads were demultiplexed and individual FASTQ files were generated from each sample. Reads were filtered and trimmed and amplicon sequence variants (ASVs) generated using the dada2 pipeline and the Refseq-RDP database for taxonomic annotation.

2.4 Statistical analysis

Illumina MiSeq read quality assessment and taxonomic profiling were performed on a high performance computer cluster using a custom Nextflow pipeline, available at: https://github.com/h3abionet/16S-rDNA-dada2-pipeline.

Quality was assessed with FastQC and MultiQC, primers were removed by trimming the first 17 and 21 bp from the start of the 251bp forward and reverse reads, respectively (--trimFor 17 and --trimRev 21).[21,22] Default settings were used for the remainder of the pipeline, which uses the DADA2 method to group reads into amplicon sequence variants (ASVs).[23] The main difference compared to the older operational taxonomic unit (OTU) clustering methods is that the DADA2 method detects exact ASVs, which unlike OTUs consist of a single unique sequence as opposed to a cluster of closely related (most commonly 97% identical) sequences. This is made possible by DADA2's error correction capabilities, which relies on sequence quality information to build a machine learning error model, alternating info on sample composition and error rates until convergence. This allows assignment of all relevant reads to an error-corrected sequence. Taxonomic assignment was performed against the RefSeq-RDP 16S database (v3 May 2018).[24]

Downstream statistical analyses were performed in R, using the packages phyloseq for alpha and beta diversity analyses, MetagenomeSeq for differential abundance testing, vegan for principal coordinates analysis (PCoA) and NMF for annotated heat maps.[25–28] For PCoA and heatmaps raw reads were standardized so that all samples had equal total read counts. An abundance filter was applied to remove ASVs with less than 10 counts in less than 10% of samples or that made up less than 0.1% of the total read count for a given sample, leaving 599 of the original 2952 ASVs. For the heatmap samples were clustered using complete linkage clustering of the Bray-Curtis dissimilarity matrix. Per-sample genus- and phylum-level bar plots were constructed using the bar plots function available in the public Github gist https://gist.github.com/kviljoen/97d36c689c5c9b9c39939c7a100720b9, excluding low-abundance taxa for ease of interpretation.

Differences in microbial compositions between fermented versus unfermented milk samples were assessed using the MetagenomeSeq MRfulltable function, applied to raw reads merged at the lowest available taxonomic level; a custom filter was applied to identify high quality, significant features, as implemented in the super.fitZig.kv function, which can be found in the aforementioned Github gist. Taxa were deemed significantly different (in terms of abundance and/or absence/presence) between fermented versus unfermented samples if they exhibited a fold change (beta coefficient) of ≥ 1.5 and had an adjusted p-value of ≤ 0.05 and if at least one of the two groups compared had $\geq 60\%$ of samples with the given ASV/taxon, or, if the result of Fisher's exact test was significant (after multiple-testing correction by the Benjamini-Hochberg method.[29] ASVs were first merged at the lowest available taxonomic level (e.g. for ASVs with *Lactobacillus* as the lowest available taxonomic annotation counts were summed, while ASVs with additional species-level annotation, e.g. *Lactobacillus acidophilus*, were summed at the species level instead). This taxonomic merging was performed using the tax_glom.kv function available in the aforementioned Github gist.

Chapter 3: RESULTS

3.1 Operational Taxonomic Units (OTUs)

The Illumina 16S metagenomics pipeline on the MiSeq Reporter software was used to calculate OTUs. The number of OTUs listed in each commercial fermented sample's text file were: 9, 12 and 13 (average of 11.3), while the homemade *amasi* sample had 44 OTUs listed. The number of OTUs found in the rural fresh cow's milk 2 sample was 83, and 609 in the rural fresh cow's milk 3 sample (average OTUs in rural fresh cow's milk samples was 346). The number of OTUs found in the urban fresh cow's milk 1 sample was 521, with 583 in the urban fresh cow's milk 2 sample and 557 in urban fresh cow's milk 3 (average OTUs in urban fresh cow's milk samples was 553.6). Overlapping OTUs indicate a high degree of convergence between OTUs leading to a decrease in microbial diversity within a sample. There were 317 overlapping OTUs within the urban fresh cow's milk group and 27 overlapping OTUs within the rural fresh cow's milk group. These data are summarized in table 1, figure 1 and figure 2.

Table 1: Number of OTUs in different milk samples	
Rural fresh cow's milk 3	609
Urban fresh cow's milk 2	583
Urban fresh cow's milk 3	557
Urban fresh cow's milk 1	521
Rural fresh cow's milk 2	83
Home fermented milk (*amasi*)	44
Commercially fermented milk products (average between the three samples)	11

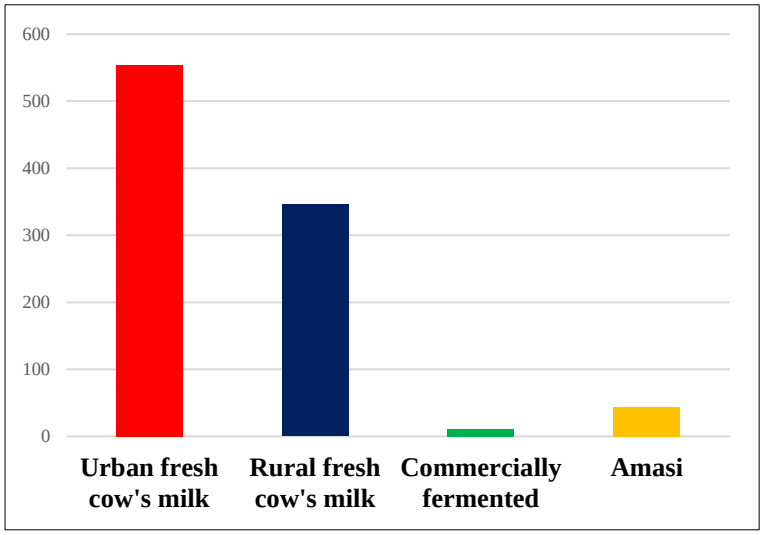


Figure 1: Bar graph of averaged OTUs in urban and rural milk samples.

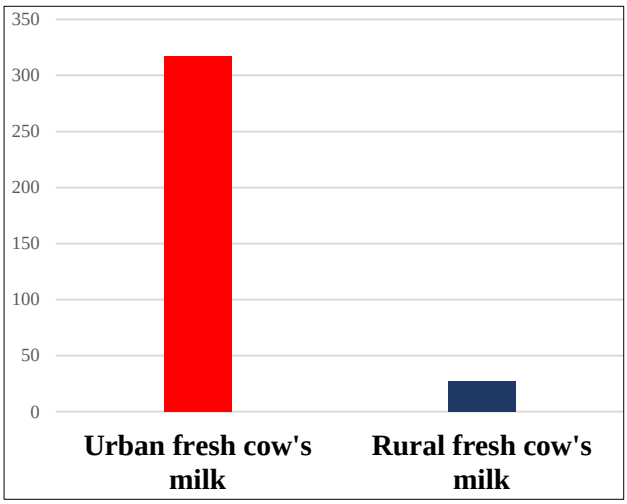


Figure 2: Bar graph of overlapping OTUs in urban and rural milk samples.

3.2 Alpha- and beta diversity

3.2.1 Simpson alpha diversity

The Simpson alpha diversity index was high in urban fresh cow's milk. The three samples were clustered together on the Simpson alpha diversity plot. The commercially fermented milk had the lowest Simpson alpha diversity and were relatively dissimilar from each other. The Simpson alpha diversity of the two rural fresh cow's milk samples, was also high and relatively dissimilar from each other (figure 3).

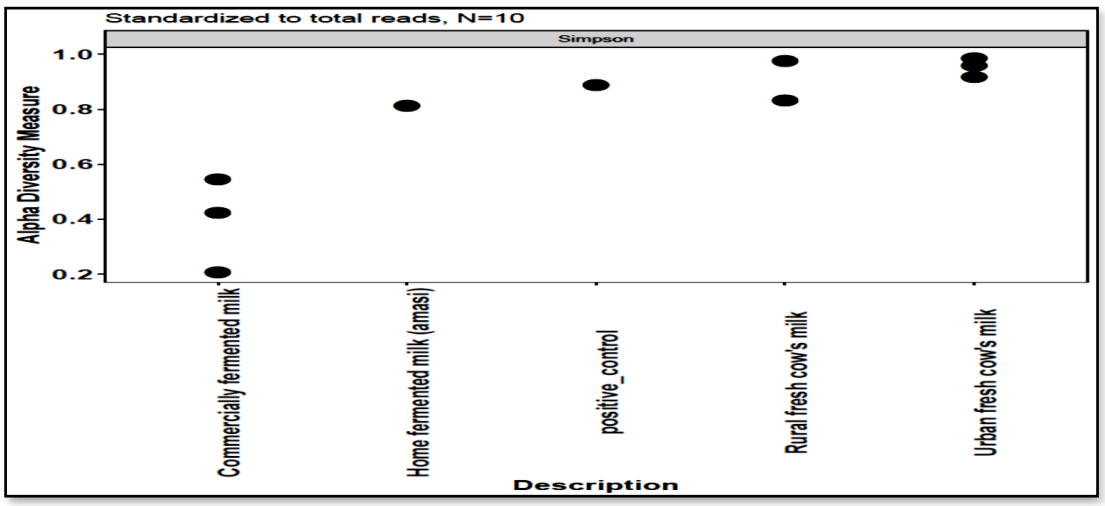


Figure 3:
Simpson alpha diversity of different cow's milk samples. (Positive control: ZymoBIOMICS® Microbial Community DNA standard (Zymo Research)).

3.2.2 Shannon alpha diversity

The Shannon alpha diversity were high in the three urban fresh cow's milk samples. The commercially fermented milk samples were very similar and low in diversity. The Shannon alpha diversity of the two rural fresh cow's milk samples, was markedly dissimilar (figure 4).

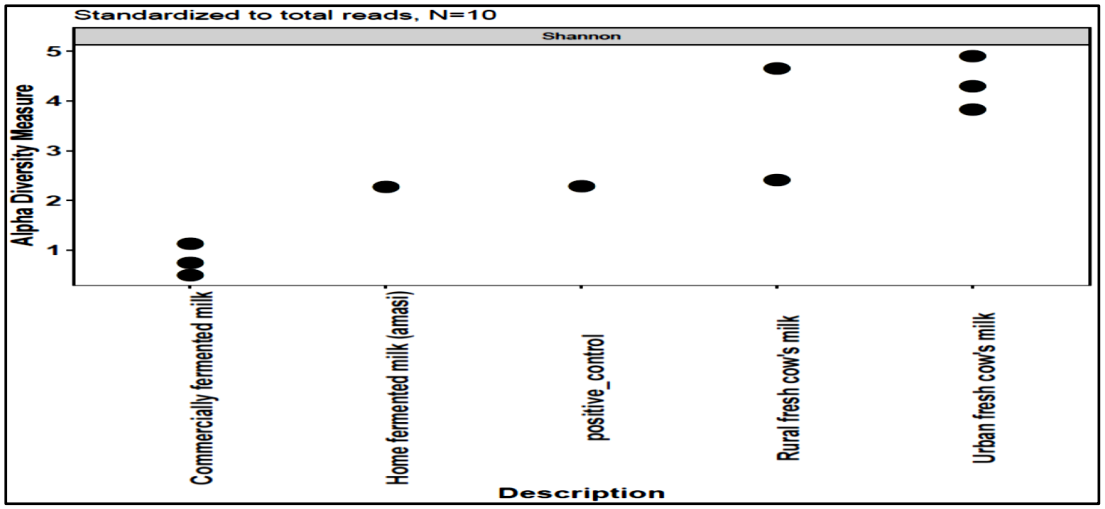


Figure 4:
Shannon alpha diversity of different cow's milk samples. (Positive control: ZymoBIOMICS® Microbial Community DNA standard (Zymo Research)).

3.2.3 Principle coordinates analysis (PCoA)

Principal coordinates analysis was used in calculating the Bray-Curtis distance between different cow's milk samples. The four differently sourced milk groups (rural fresh, urban fresh, *amasi*, and commercially fermented) were strikingly dissimilar from each other. The three commercially fermented products had almost no dissimilarity from each other, but as a group, were markedly dissimilar from the other milk samples. The three urban fresh cow's milk samples were also remarkably dissimilar from all the other samples. The home fermented milk sample *amasi* and the two rural fresh milk samples, were uniquely dissimilar from each other and from all the other milk products groups (figure 5).

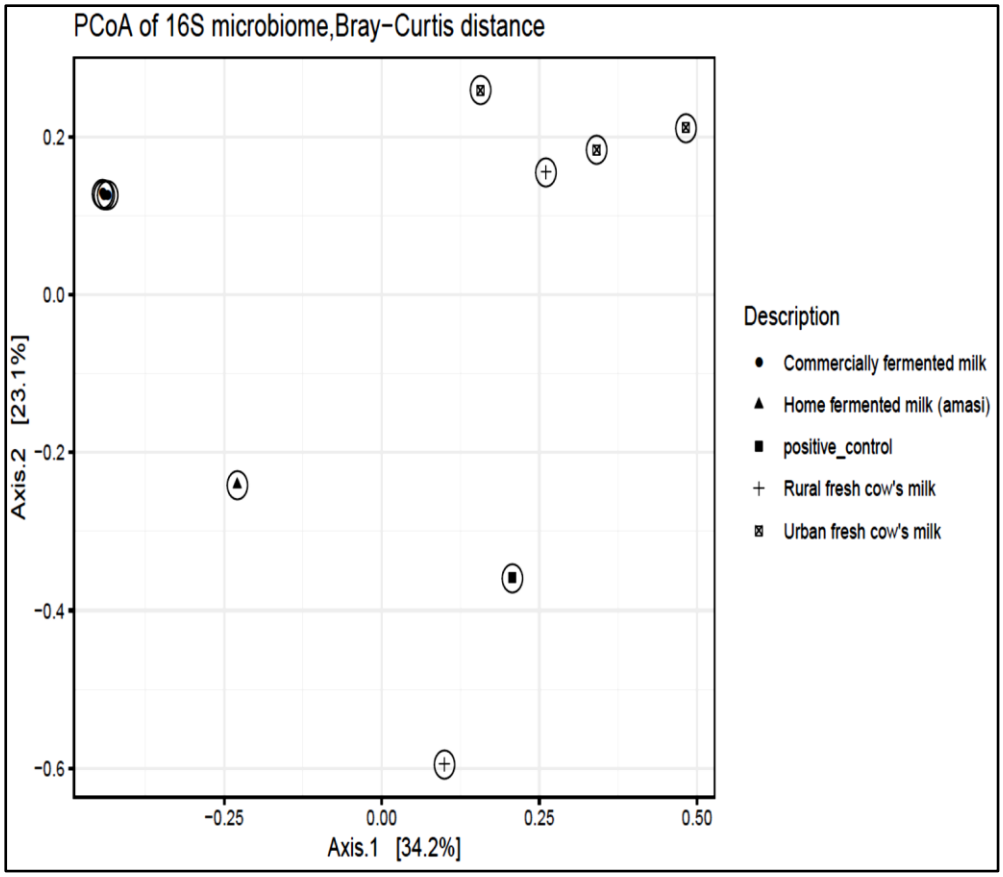


Figure 5:
Principle coordinates analysis (PCoA) of different cow's milk samples.

3.3 Phylum identification

The commercially fermented products were similarly dominated by LAB, belonging to the phylum *Firmicutes* (more than 98% abundance) and the phylum *Proteobacteria* (less than 2% abundance). The *amasi* sample comprised approximately 50% *Firmicutes* and approximately 50% *Proteobacteria*. Rural fresh milk 1 comprises almost completely *Proteobacteria* with small percentages belonging to the phyla *Bacteroidetes*, *Firmicutes* and *Candidatus_Saccharibacteria*. Rural fresh milk 2 appeared remarkably similar at phylum level to the urban fresh cow's milk samples, comprising *Firmicutes, Actinobacteria, Proteobacteria, Bacteroidetes* with small percentages belonging to the phylums *Chloroflexi* and *Candidatus_Saccharibacteria*. (Figure 6)

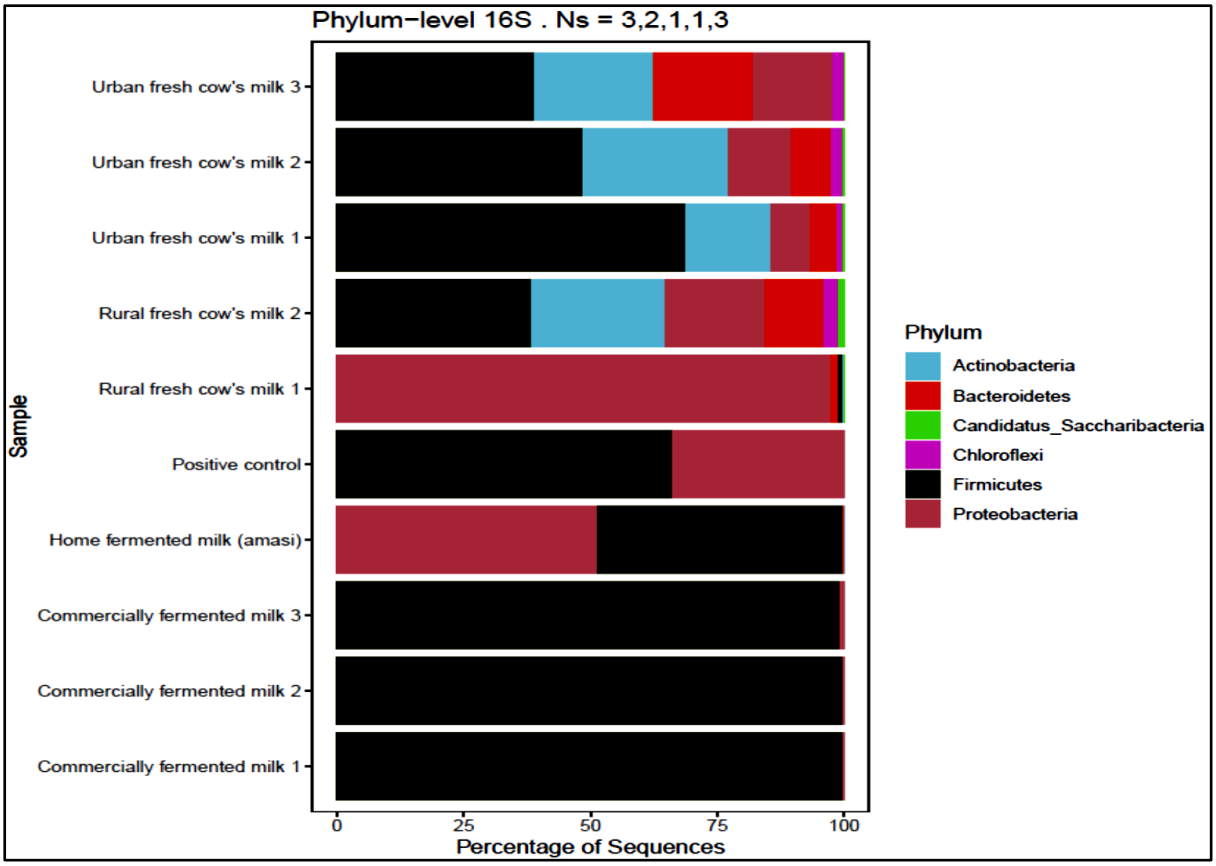


Figure 6:
Bar plot of relative abundance of organisms in the different cow's milk samples at phylum level.

3.4. Genus identification (figure 7)

Commercially fermented products (Othando®, Amyoli® and Maas®)

These products appeared remarkably similar at genus level, with the genus *Lactococcus* (more than 75%). The genus *Leuconostococcus*, was present to a lesser extent.

Amasi

This sample appeared more diverse than commercially fermented milk, but less diverse than the fresh milk samples. The genus *Lactococcus* dominated. The genus *Leuconostococcus* was absent. The genus *Kluyvera* and *Citrobacter* were present in almost equal percentages. The genus *Streptococcus*, *Lactobacillus* and *Salmonella*, although less abundant than other organisms at the genus level, were present in almost equal amounts.

Rural fresh cow's milk

The two rural fresh cow's milk samples were markedly dissimilar at the genus level. *Salmonella, Citrobacter, Kluyvera* and *Pseudomonas* dominated in rural fresh cow's milk 1, while *Lactobacillus, Lactococcus* and *Bacillus* dominated in rural fresh cow's milk 2.

Urban fresh cow's milk

Urban fresh cow's milk 1 were dominated by the genus *Staphylococcus* and *Lactobacillus*. Urban fresh cow's milk sample 2, were dominated (in almost equal amounts) by *Lactococcus, Anoxybacillus, Rothia* and *Lactobacillus*. Urban cow's milk 3 appeared more evenly distributed at genus level with *Lactobacillus, Escherichia/Shigella, Faclamia, Psychrobacter, Corynebacterium, Dietzia, Listeria, Rothia* and *Romboutsia* present in almost equal amounts.

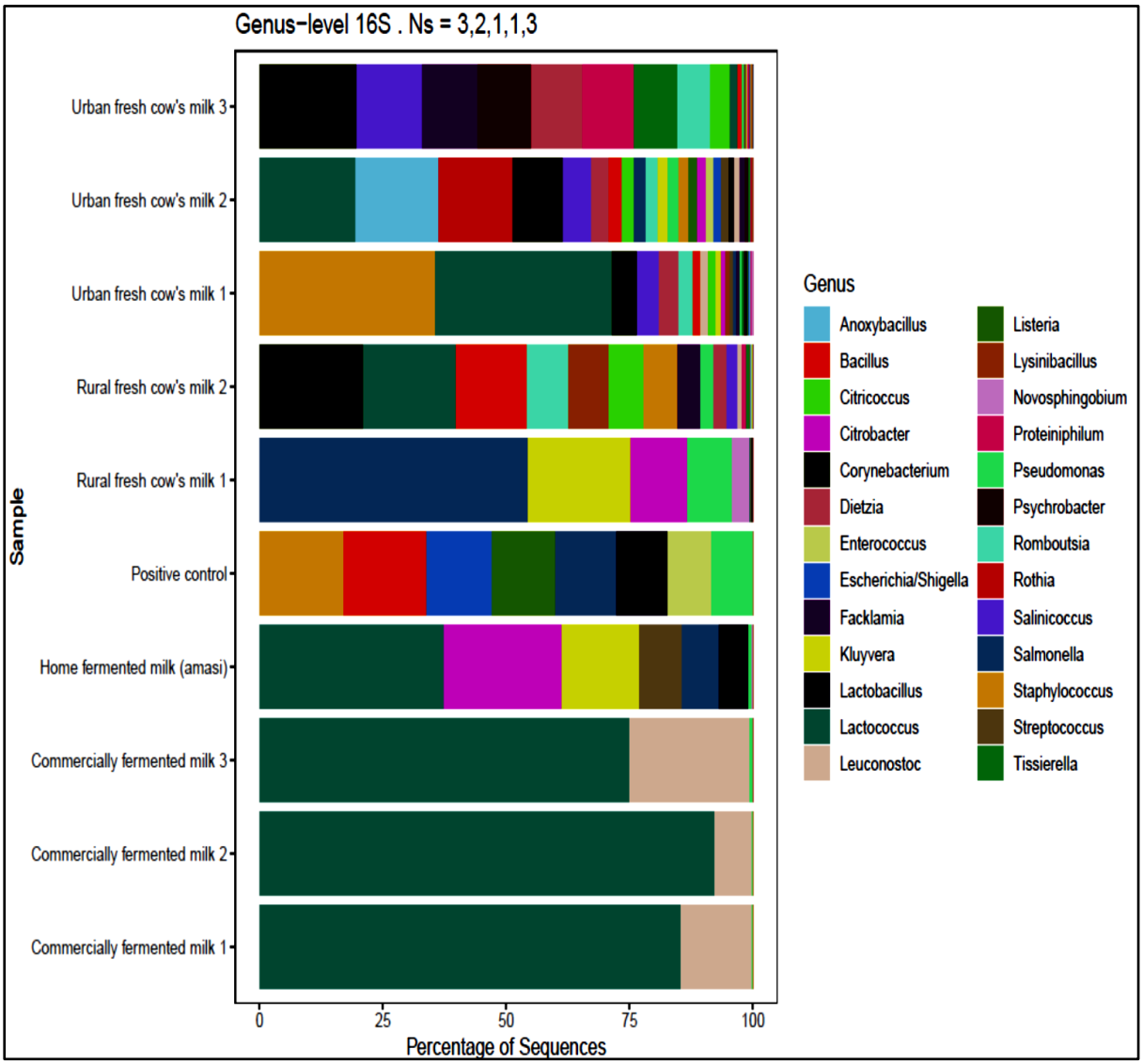


Figure 7:
Bar plot of relative abundance of organisms in the different cow's milk samples at genus level.

3.5 Bray-Curtis distance (merged taxa)

Merged taxa were identified, using the Bray-Curtis distance of abundance. Heatmaps were then constructed at both the family and the species level.

3.5.1 Family merged taxa (figure 8)

Streptococcaceae dominated uniformly in the commercially fermented milk products but also occurred in high abundance in *amasi*. *Leuconostocaceae* was abundant in the commercially fermented products. This family was absent in *amasi*. Furthermore, *Staphylococcaceae* was absent in the commercially fermented products and *amasi* samples, while of low abundance in all the fresh milk samples. *Enterobacteriaceae* was abundant in one of the rural fresh cow's milk and the *amasi* sample and of low abundance in all the urban fresh cow's milk samples. It was absent in the commercially fermented products. *Pseudomonadaceae* had low abundance in urban fresh cow's milk, *amasi*, and commercially fermented samples – it was abundant in rural fresh cow's milk. *Lactobacillaceae* had uniquely high abundance in *amasi*, while being absent in commercially fermented products. It was of low abundance to completely absent in all the other milk samples. Important findings at the family member level are summarized in table 2.

Table 2: Family member merged taxa

	Commercially fermented milk	***Amasi***	**Urban fresh cow's milk**	**Rural fresh cow's milk**
Leuconostocaceae	Abundant	Absent	Low abundance	Low abundance - absent in one sample
Streptococcaceae	Dominated	Abundant	Low abundance	Low abundance
Staphylococcaceae	Absent	Absent	Low abundance	Low abundance
Enterobacteriaceae	Absent	Abundant	Low abundance	Abundant in one sample - low abundance in the other sample.
Pseudomonadaceae	Low abundance	Low abundance	Low abundance	Abundant
Lactobacillaceae	Absent	Abundant	Low abundance – absent in one sample	Low abundance - absent in one sample.

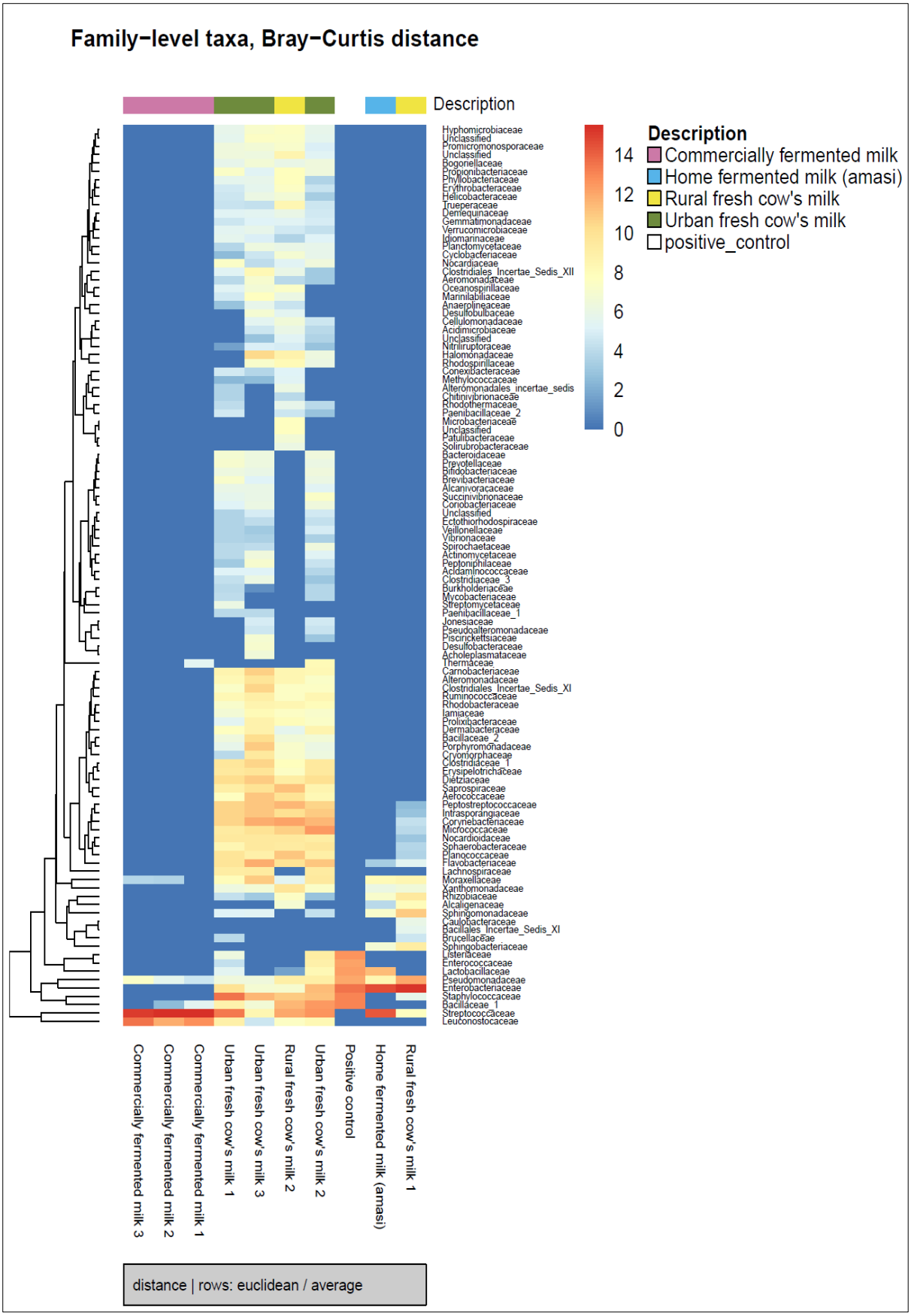


Figure 8:
Family member merged taxa (Bray-Curtis distance).

3.5.2 Species merged taxa (figure 9)

Rural fresh cow's milk 2 and all three the urban fresh cow's milk samples were more diverse than the other samples. Rural fresh cow's milk 1 and *amasi* appeared similar in diversity while the commercially fermented milk samples were the least diverse and contained highly similar species.

Lactococcus lactis (AB100803) was the most abundant organism in *amasi* and in the commercially fermented products, and less abundant in the fresh milk samples. *Leuconostococcus mesenteroides* (AB023247), *Leuconostococcus pseudomesenteroides* (AB023237) and *Lactococcus chungangensis* (EF694028) were abundant in all three the commercially fermented samples. These were absent in *amasi* and had low abundance to complete absence in the fresh milk samples.

Salmonella enterica (AE006468) was abundant in *amasi* and in one of the rural fresh cow's milk samples. It was of low abundance in the other fresh cow's milk samples. It was absent in the commercially fermented products.

In *amasi*, *Pseudomonas*, *Citrobacter*, *Kluyvera cryocrescens* (AF310218), *Enterobacteriaceae,* and *Raoultella ornithinolytica* (U78182) were abundant. *Pseudomonas* and *Citrobacter* were also abundant in one of the rural fresh cow's milk samples. *Streptococcus infantis* (AY485603) was absent in the commercially fermented milk, abundant in *amasi*, and of low abundance in the fresh milk samples. *Bacillus* was of low abundance in the fresh milk samples, and absent in the commercially fermented products and *amasi*.

Staphylococcus epidermidis (D83363) and *Anoxybacillus* were abundant in one of the urban fresh cow's milk samples. *Lactobacillus paracasei* (D79212) was abundant in *amasi*, but almost completely absent in all the other milk samples. *Rothia endophytica* (KC806052) was uniquely abundant in one of the urban fresh cow's milk samples and had low to no presence in all the other milk samples.

Prevotella copri (AB064923) and *Prevotella* (unspecified) were absent in all the milk samples, except in urban fresh cow's milk, where they were of low abundance.

Numerous other organisms, mostly occurring in low abundance, were identified in fresh cow's milk, and not in the fermented products. Important findings at the species level are summarized in table 3.

Table 3: Species merged taxa

	Commercially fermented milk	***Amasi***	**Urban fresh cow's milk**	**Rural fresh cow's milk**
Lactococcus lactis (AB100803)	Most abundant organism	Most abundant organism	Less abundant	Less abundant
Leuconostoccus mesenteroides (AB023247), *Leuconostococcus pseudomesenteroides* (AB023237) and *Lactococcus chungangensis* (EF694028)	Abundant	Absent	Low abundance to absent	Low abundance to absent
Salmonella enterica (AE006468)	Absent	Abundant	Low abundance	Abundant in one sample – low abundance in the other sample
Pseudomonas, *Citrobacter*, *Kluyvera cryocrescens* (AF310218), *Enterobacteriaceae*, *Raoultella ornithinolytica* (U78182)	Pseudomonas - low abundance. Other species absent.	Abundant	Low abundance	*Pseudomonas* and *Citrobacter* abundant in one sample – absent in the other sample
Streptococcus infantis (AY485603)	Absent	Abundant	Low abundance	Low abundance
Bacillus	Absent	Absent	Low abundance	Low abundance - absent in the other sample
Staphylococcus epidermidis (D83363)	Absent	Absent	Abundant only in one urban sample	Absent
Anoxybacillus	Low abundance in one sample	Absent	Abundant in one sample – low abundance in the other sample	Low abundance – absent in the other sample
Lactobacillus paracasei (D79212)	Absent	Abundant	Low abundance in only one sample – absent in the other samples	Low abundance - absent in the other sample
Rothia endophytica (KC806052)	Absent	Absent	Abundant in one sample. Low abundance to absent in the other samples.	Absent
Prevotella copri (AB064923) and *Prevotella* (unspecified)	Absent	Absent	Low abundance	Absent

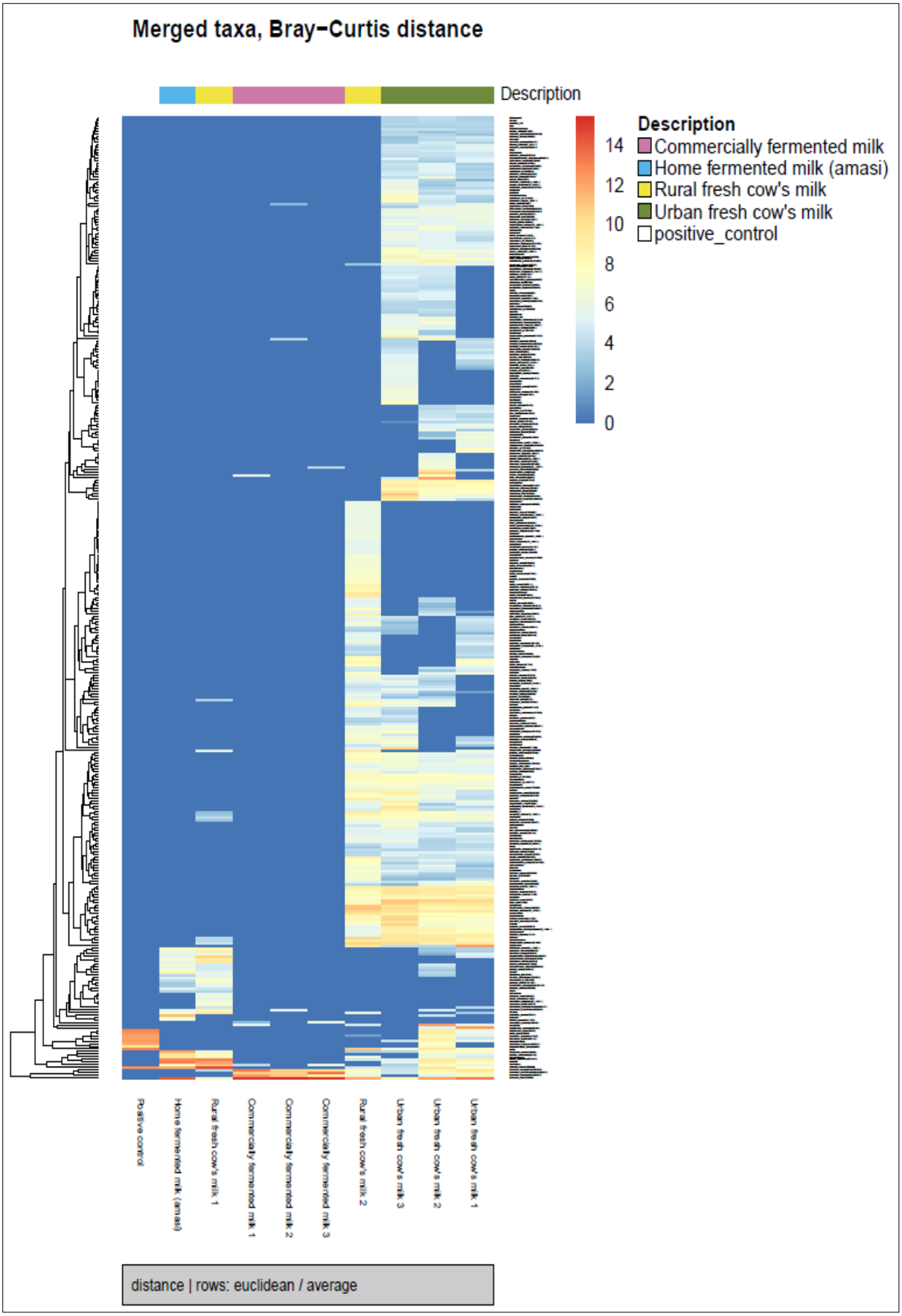


Figure 9:
Species level merged taxa (Bray-Curtis distance).

3.6 Fermented versus unfermented milk samples

Species level comparison was performed between fermented (home and commercially) versus unfermented milks. Because of the relatively small N used in this study, all fermented milk samples (*fermented group*) were combined for statistical comparison against the unfermented milk samples (*unfermented group*). Taxa were deemed significantly different if they had at least a compared 1.5-fold change between the two groups (*fermented* versus *unfermented*) and an adjusted p-value of 0.05 or less and if at least one of the two groups compared had 60% or more of a given ASV/taxon, or, if the result of the Fisher's exact test was significant. A heatmap was constructed, illustrating organisms, some down to species level, with statistically significant occurrence between the two groups of milk (figure 10).

Some of these findings, are summarized in table 4.

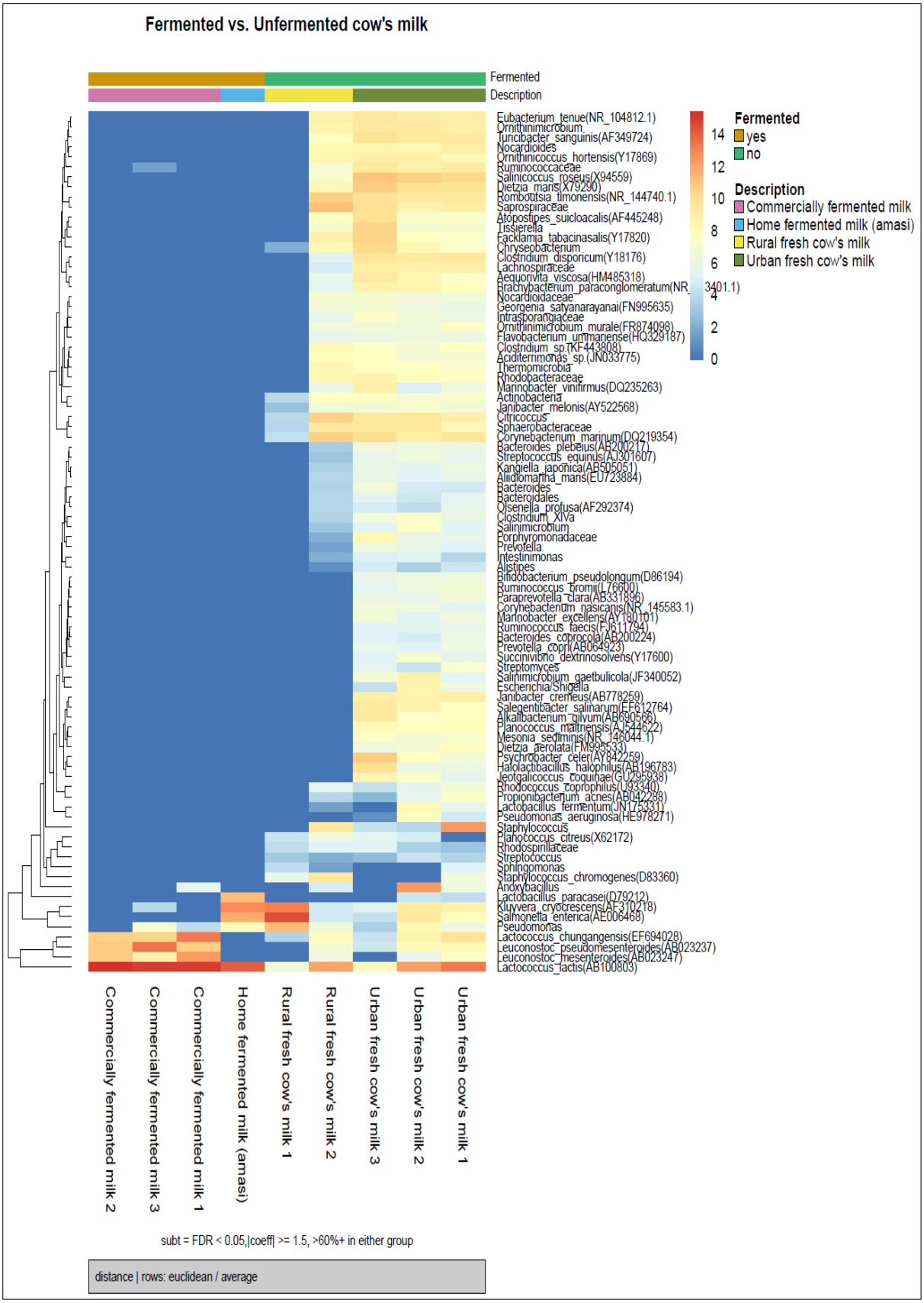


Figure 10:
Differential relative abundance testing of organisms (mostly down to species-level) in the fermented versus unfermented sample groups.

Results for differential abundance testing clearly show significant difference in *Lactococcus* and *Leuconostococcus* species between the fermented and unfermented milk groups.

Furthermore, from the heatmap, the fresh urban cow's milk samples were strikingly more even (closeness in number of each species compared to each other), contributing to a higher diversity compared to rural and fermented milk samples. There are several taxa that uniformly dominated in commercially fermented milk, contributing to its unevenness and decreased diversity.

Lactococcus lactis (AB100803) dominated in the fermented samples, including *amasi*. *Lactococcus chungangensis* (EF694028) had strikingly similar abundance in the commercially fermented products. It was absent in *amasi* and were exceptionally low to absent in the fresh cow's milk products. *Leuconostococcus mesenteroides* (AB023247) and *Leuconostococcus pseudomesenteroides* (AB023237) were absent in *amasi*. *Lactobacillus paracasei* (D79212) was abundant in *amasi*, with low to no occurrence in all the other samples. *Prevotella copri* (AB064923) was present in only the urban cow's milk samples, with complete absence in fresh rural, commercially fermented and *amasi* samples.

Potential milk pathogens were identified. *Salmonella enterica* (AE006468) was present in high numbers in *amasi*, and less so, in fresh cow's milk (urban and rural). It was absent in commercially fermented products. *Escherichia/Shigella* (unidentified species) were present in only urban fresh samples and absent in rural and fermented milk samples.

Table 4: Species level comparison of fermented and unfermented milk products

	Unfermented milk	**Fermented milk**
Diversity	Increased	Decreased
Lactococcus lactis (AB100803)	Present	High abundance (dominated)
Lactococcus chungangensis (EF694028)	Present	Commercial products: high abundance. A*masi*: absent
Leuconostococcus mesenteroides (AB023247) and *Leuconostococcus pseudomesenteroides* (AB023237)	Present	Commercial products: high abundance. *Amasi*: absent
Lactobacillus paracasei (D79212)	Urban fresh: present. Rural fresh: absent.	Commercial products: absent. A*masi*: high abundance
Prevotella copri (AB064923)	Urban fresh: present. Rural fresh: absent.	Absent
Salmonella enterica (AE006468)	Present.	Commercial products: absent. *Amasi*: present
Kluyvera cryocrescens (AF310218)	Present.	Commercial products: absent. *Amasi*: high abundance.
Escherichia/*Shigella* (unidentified species)	Urban fresh samples: present. Rural fresh samples: absent	Absent

Chapter 4: DISCUSSION

4.1. The human gastrointestinal microbiome: an early window of opportunity for allergy prevention

In 1908, Ilya Ilyich Mechnikov (1845 – 1916), a Russian zoologist, received the Nobel Prize for his work done on natural immunity. He recognized fermented dairy products and other intentionally fermented foods as beneficial to human health. He is now considered as the 'father' of the probiotic theory. The word '*probiotic*' is derived from combining the Latin preposition '*pro*' (for) and the Greek adjective '*bios*'(life). In 2001, almost a century later, the World Health Organization (WHO) redefined probiotics as: 'live microorganisms which, when administered in adequate amounts, confer a health benefit on the host'.[30]

Early life microbiome composition and the microbiome-immune interaction are nowadays considered as one of the main contributors towards not only short term but also long-term health. It is recognized as a key role player in the Developmental Origins of Health and Disease (DOHaD).[31–33] Allergic disease is one of many conditions currently being investigated to have its origin determined by early-life microbiome influences.

One of the most extensively investigated human microbiome niches is found in the gut. Being highly sensitive to its environment, early life influences, including antenatal exposures, mode of delivery, early infant diet, exposure to oral antibiotics and environmental exposures, can cause dysbiosis (imbalance in a microbial ecosystem), can lead to a dysregulated immune system and ultimately, in the genetically susceptible individual, to allergy.[10,34,35] These influences, through an optimized diet, can be utilized early in life to shape a well-balanced gastrointestinal microbiome.[36,37] The human gut microbiome is furthermore a very dynamic ecosystem with an enormous inter-personal compositional variation. It also varies significantly at a population level, with exposure to human breastmilk being one of the most important drivers of variation and diversity early in life.[38,39] However, the role of other milk sources (e.g. bovine fresh milk and fermented milk) in shaping the gut microbiome, cannot be overlooked, as many children from certain population groups are exposed to these from a very young age. Our study investigated the microbiome of cow's milk, either consumed unprocessed or as a fermented product and frequently being introduced before the age of one year in certain population groups with a low prevalence of allergy.

Research shows that between the age of 2- and 3 years, the human gastrointestinal microbiome starts to resemble an 'adult-state', with the phyla *Firmicutes* and *Bacteroidete*s dominating.[40] Looking at the bar

plot from our study (figure 6), these two phyla were present in all the fresh cow's milk samples - *Firmicutes* dominated.

Zimmerman *et al.* report neonates and children with low intestinal microbiome diversity to have a higher allergic sensitisation and eczema rate. In this systematic review, eczema was more common in children with a low abundance of intestinal *Bacteroidetes* at 1 month of age and *Proteobacteria* at 12 months of age.[10] Studies also associate a lower abundance of intestinal *Proteobacteria* at an early age with early life allergic sensitisation.[41] In the Copenhagen Prospective Study on Asthma in Childhood (COPSAC) study, Bisgaard *et al*. illustrated an inverse relationship between both 1 and 12-month old children's gut bacterial diversity with allergic sensitization, allergic rhinitis, and peripheral blood eosinophilia risk. However, this inverse relation could not be demonstrated for asthma or atopic dermatitis risk.[42] In the Canadian Health Infant Longitudinal Development (CHILD) study, a subset of children with low gut microbiota richness, lower abundance of *Bacteroidaceae* and higher abundance of *Enterobacteriacea*, were associated with food sensitisation.[43] In our study, *amasi* comprises 50% *Proteobacteria* and 50% *Firmicutes* at the phylum level. Worth noting, there was almost a complete loss of *Proteobacteria* in the commercially fermented products, consisting of almost 100% *Firmicutes*.

In South Africa, the gut microbiome of rural children (aged 12 to 36-months) has been shown to be more diverse compared to urban children, possibly explaining the lower prevalence of atopic dermatitis. More so, the enrichment of *Prevotella copri* in the gut microbiome of rural black South African (Xhosa) children, were also found to be inversely related to atopic dermatitis.[44] In an earlier study by De Filippo *et al*. a difference between the fecal microbiota of European and rural Burkina Faso children was demonstrated. This study concluded that rural African children's gut microbiome were enriched with the phylum *Bacteroidetes* and depleted in the phylum *Firmicutes*. The authors mentioned the genus *Prevotella* and *Xylanibacter*, uniquely abundant in rural African children. The importance of a unique set of genes, present in these organisms, capable of hydrolysis of cellulose and xylan, to produce short-chain fatty acids (SCFA), were highlighted.[45] However, our study failed to demonstrate a significant abundance of the phylum *Bacteroidetes* and the genus *Prevotella* in any of the milk samples obtained from a rural environment.

Manipulating the gut microbiome through dietary interventions, to achieve the desired health outcome, is certainly worth exploring. Veiga *et al*. demonstrated a clear change in the gut microbiome of adult subjects with inflammatory bowel syndrome (IBS), by introducing a fermented milk product, containing dairy starters and *Bifidobacterium animalis*.[16] An increased production of colonic SCFA and improvement in patient-related IBS symptoms were detected in patients receiving the fermented milk product. Unfortunately, reproduction of similar interventional studies involving probiotics and other

dairy products to manipulate the gut microbiome in allergy prevention, have been published anecdotally.[46–50]

4.2 Describing the microbiome: the importance of Operational Taxonomic Units (OTUs), microbiome diversity and dissimilarity

Conventional metagenomic microbiome analysis amplifies the hypervariable regions (typically V1 to V3 or V3 to V5) of the prokaryotic 16S ribosomal RNA (16S rRNA) gene and uses these gene sequences for taxonomical identification. Similar gene sequences are clustered together to form OTUs, allowing the reduction of millions of sequence-reads to more statistical interpretable numbers. Every OTU represents a single 97% similarity in genetic sequence.[18,20,51] However, due to the possible overestimation of gene sequence similarity at this cut-off value, criticism about the conventional 97% threshold value, has been published.[51] In our study, the variable V3 and V4 regions of the 16S rRNA gene were amplified after DNA-purification. The 97% threshold overestimation error was eliminated by using DADA2's error correction capabilities. This ensures DADA2 detection of exact amplicon sequence variants (ASVs), which unlike OTU calculation, consist of a single unique sequence as opposed to a cluster of closely related sequences. Our research demonstrated that the average OTU of urban fresh cow's milk was the highest of all the milk samples. Commercially fermented milk had the lowest average OTU. Home fermented milk had a strikingly low number of OTUs (figure 1). However, urban fresh cow's milk had more overlapping OTUs than rural fresh cow's milk, contributing to an increased diversity in the rural fresh milk samples (figure 2).

The early life human gut microbiome richness, abundance and diversity, and how these pertain to especially allergy prevention, are being explored extensively.[2,11,40,52,53] One common theme emerges from the literature: a diverse gut microbiome protects against allergy. In our study we used popular microbiome diversity measurements, namely the Simpson and Shannon indexes to determine species alpha diversity within our different samples. These indexes are quantitative mathematical measurements and are based on richness (number of species present in a sample) and evenness (relative distribution of each species in a sample).[11,54,55]

In our study, the Simpson index (considered to give more statistical weight to dominant species) was the highest in urban and rural fresh cow's milk samples. The commercially fermented milk products had the lowest Simpson index (figure 3). The relatively similar alpha diversity between all three the urban fresh cow's milk samples are worth noting. These samples were taken from three cows grazing on the same suburban farm.

One disadvantage of the Simpson index is rarer species, with only a few representatives in a sample, might be missed. Therefore, we added the Shannon alpha diversity index to also account for evenness of species in each sample. This confirmed the high diversity of urban and rural fresh cow's milk in our samples. The two rural fresh cow's milk samples differ substantially from each other in terms of Shannon index measurement (figure 4). These were taken from two different rural farms, relatively far apart. In addition, the rural cows were milked differently from the urban cows, with no cleaning of the cow's udders prior to milking. Thus, environmental contamination of milk samples is more likely to occur with milk obtained from the rural environment, adding to diversity within these samples. The lowest diversity was detected in the processed, commercially fermented milk products. Environmental influences (e.g. grazing patterns and udder health) and the effect of milk processing on the composition of the microbiome will be discussed later.

For measuring dissimilarity (beta diversity) between the sample groups, a Bray-Curtis principle coordinate plot (Principle Coordinates Analysis (PCoA)) was performed after raw reads were standardized. This ensured all samples had equal total read counts (figure 5). PCoA is an ordination statistical method and commonly used in microbiome studies to measure the Bray-Curtis distance between samples. It serves as a form of non-linear multidimensional statistical reduction. It also helps to identify outliers in a data set when evaluated on scatterplots.[20] The immediate striking absence of dissimilarity of the microbiome of the three commercially fermented milk samples is apparent. The three urban fresh cow's milk samples also appeared less dissimilar. The two rural fresh cow's milk samples were strikingly dissimilar from each other and differed remarkably from the urban fresh cow's milk samples. This reiterates the variability of microbiome composition of cow's milk if collected from different farms (different herds of cattle) and if different milking techniques were used, allowing higher levels of environmental contamination.

Although small in sample numbers, it is clear from our study that the milk microbiome from herds of cattle grazing close together and staying on the same farm, appeared remarkably similar in terms of alpha diversity. When looking at beta diversity, fresh cow's milk samples, *amasi*, and commercially fermented products were all highly dissimilar from each other.

4.3 Human breastmilk microbiome: complexity and the role of breastfeeding in allergy prevention

When exploring the possible anti-allergy effects of bovine milk, it may be a good idea to investigate the probiotic 'golden standard', as determined by human breastmilk, first. Breastmilk contains

approximately 10^3 to 10^5 live organisms per milliliter. Although numerous health-promoting and anti-infective properties of breastmilk are well known, the exact role of the human breastmilk microbiome and its immune-modulatory mechanisms involved in allergy protection, are less clear. More so, whether breastfeeding and the duration thereof, prevents allergy, is not known. In a systematic review and meta-analysis, Lodge *et al*. indicated a reduction of asthma risk in children 5 to 18 years of age, only in children exposed to prolonged periods of breastfeeding. A reduction in eczema risk in children aged 2 years and younger was also reported if they were exclusively breastfed for 3 to 4 months (OR 0.74; 95% CI, 0.57-0.97). The protection against asthma and eczema were found particularly in low- to medium-income countries. However, breastfeeding was not shown to be protective against food allergy. The authors concluded studies generally to be of low methodological quality.[56] Furthermore, although limited, evidence exists that ω-3 polyunsaturated fatty acids (n-3 PUFAs) in breastmilk decrease the odds of eczema and other atopic diseases in childhood.[57]

Unfortunately, many factors, are known to cause variations in the breastmilk microbiome composition, making clear, scientific conclusions difficult. For instance, a higher abundance of *Lactobacilli* has been reported in breastmilk from Finnish-, Taiwanese-, Chinese-, and South African women who gave birth via cesarean section. However, higher breastmilk *Lactobacilli* abundance was reported in Taiwanese women who gave birth via normal vaginal delivery, illustrating the inconsistency of microbiome study findings across the globe.[58,59] Transition from colostrum (more diverse microbiome) to mature breastmilk (less diverse microbiome), the infant's oral microbiome, maternal nutritional status, diet, postnatal psychological stress, and body mass index have also been shown to alter the milk microbiome.[60,61] Maternal mastitis (apart from the influence of antibiotic administration during active infection) has been shown to decrease the breastmilk microbiome diversity, change its composition at the phylum level, and decrease the abundance of anaerobic breastmilk organisms.[62] The influence of maternal Human Immune Deficiency Virus (HIV) status on breastmilk, has also been extensively studied in the African and South African context.[63–65] Reverse causation and selection bias (because of the inability to randomize patients to breastfeeding) complicate breastmilk research even more. Despite all these, many authors are of opinion that the perfect microbiome 'cocktail' of a healthy breastfeeding mother, is determined independently from environmental influence.[59,66]

The gut microbiome of breast and formula-fed infants differ remarkably. The gut microbiome of breastfed infants is dominated by *Bifidobacteria* and *Lactobacillus* species while formula-fed infants have a more diverse microbiome, which resembles an ‘adult’ state. In our study, *Bifidobacteria* and *Lactobacillus* were of low abundance, to completely absent in fermented and fresh cow’s milk samples.

Compared to formula-fed infants, breastfed infants have lower concentrations of fecal short-chain fatty acids (SCFA) and higher concentrations of fecal lactate. Especially stool acetate levels were found to be higher in exclusively breastfed infants, compared to formula-fed infants.[67,68] Research highlights the role of human milk oligosaccharides (HMOs), contributing to an important difference between the gut microbiome of breast and formula-fed infants.[69] HMOs are complex indigestible sugars (short-chain and long-chain oligosaccharides present in a unique ratio within breastmilk). They provide energy to colonocytes, thereby promoting gut mucosal integrity. They also serve as an energy substrate for fermentation by especially anaerobic bacteria, present in the gut.[48,52,68,70,71] HMOs furthermore serve as an antiadhesive agent – inhibiting pathogen adhesion to gastrointestinal mucosal surfaces.[72] Their uniqueness in breastmilk is largely attributed to the genetic influence of the human secretor fucosyltransferase2 (FUT2) and Lewis (FUT3) genes. Regulation of HMOs takes place by the glycosyl-transferases enzymes, found within the human breast.[69,73] In recently published results of the Canadian Healthy Infant Longitudinal Development (CHILD) cohort, 3-month old children who developed atopy later in life, had deficiencies in HMO enzyme encoding-genes and lower gut butyrate (a by-product of fermentation) levels.[74] The allergy protective role of butyrate and other SCFAs in the human gut, will be discussed later.

There are, without any doubt, still many unanswered questions regarding the breastmilk microbiome and its allergy protective properties. Nevertheless, the World Health Organization (WHO) and the European Academy of Allergy and Clinical Immunology (EAACI) still recommend exclusive breastfeeding for the first 4 to 6 months of life and promote combined multifaceted (dietary and environmental) interventions to prevention allergy.[33,75–77]

4.4 The microbiome of human breastmilk and bovine milk: similar or not?

The nutritional differences between human and cow's milk have been well described, with probably the protein content (casein and whey fractions) of each species' milk being uniquely adapted to suit the requirements of the suckling. Human milk contains considerably less protein than ruminant milk. Lactoferrin, α lactalbumin, and lysozyme are major anti-infective role-players found in breastmilk. However, lactoferrin and lysozyme only occur in trace amounts in ruminant milk. Therefore, substituting human milk with unmodified cow's milk early in life carries major infection and nutritional risks. Early life gut immaturity with the inability to handle high protein and lactose concentrations (present in cow's milk) are other concerns.[78]

Human milk and milk from ruminants are far from sterile and the origin of each individual's microbiome, multifactorial.[79] As mentioned before, the gut microbiome of healthy human individuals consists of mainly four phyla: *Bacteroidetes* and *Firmicutes,* while *Proteobacteria* and *Actinobacteria* are less commonly encountered.[80] Interestingly, the urban fresh cow's milk samples collected in our research, consistently comprised a mixture of *Firmicutes*, *Actinobacteria, Bacteroidetes,* and *Proteobacteria,* while the fermented products were dominated by the phylum *Firmicutes* (figure 6) – the same abundant phyla described in the healthy human gut.

At the genus level, human and cow's milk seem to differ more substantially. Most next-generation sequencing studies report the presence of a 'core set 'of microbiota present in healthy human breastmilk – this microbiome is not population or individual-specific and not influenced by confounding factors. These organisms belong to the genera: *Streptococcus*, *Staphylococcus, Propionibacterium,* and *Pseudomonas.*[60] Other studies also highlight the abundance of *Lactobacillus* and *Bifidobacterium spp*.[73,81] The most common LAB generally found in raw cow's milk, belongs to the genera *Lactococcus*, *Lactobacillus*, *Leuconostococcus*, *Streptococcus*, and *Enterococcus*. The presence of these genera was also highlighted in our study. Compared to studies on human breastmilk, our study identified the presence of *Lactobacillus* in *amasi* and all urban fresh cow's milk samples. *Lactobacillus* was absent in the commercially fermented products (figure 7).

Human and bovine milk microbial communities are without any doubt, remarkably diverse and dynamic, making the identification of specific organisms (down to strain, species, and even genus level) exceedingly difficult. Enormous research work (from 38 countries) has been published by Togo *et al*., illustrating the presence of more than 800 bacterial species in human breastmilk. *Staphylococcus aureus, Staphylococcus epidermidis, Streptococcus agalactiae, Propionibacterium acnes, Enterococcus faecalis, Bifidobacterium breve, Escherichia coli, Streptococcus sanguinis, Lactobacillus gasseri,* and *Salmonella enterica* were the most prominent species.[82] In our cow's milk analysis, *Staphylococcus epidermidis* (D83363) was abundant in only one fresh urban cow's milk sample and *Salmonella enterica* (AE006468) was abundant only in *amasi*. In global comparative research, common microbiotica taxa, shared between human and cow's milk are: *Staphylococcus, Streptococcus, Pseudomonas, Bacteroides, Bifidobacterium, Corynebacterium, Enterococcus* and *Propionibacterium.*[83] From our study, *Streptococcus infantis* (AY485603) and *Pseudomonas* were abundant in *amasi*.

Remarkable similarities between the influencing factors on the microbiome of human and bovine milk are being described. In humans, the *entero-mammary pathway* explains how organisms (mainly anaerobes) from a mother's gastrointestinal system, are also present in her breastmilk and ultimately

become available for 'seeding' of the infant's gut during the first few months of life. For instance, the presence of *Enterobacteriaceae* in breastmilk is not regarded as a consequence of environmental contamination, but rather a consequence of maternal gut transportation of these organisms into breastmilk.[59] This process is believed to be facilitated by dendritic cells (DCs) and macrophages, acting as microbial transporters from the gastrointestinal lymphoid tissues to the mammary glands.[84] Also, retrograde flow of breastmilk occurs during breastfeeding, suggesting an interplay between the infant's oropharyngeal and the lactating mother's mammalian microbiome.[85] The *entero-mammary pathway*, the retrograde flow of milk during sucking and the suckling's oropharyngeal microbiome have also been shown to influence the milk microbiome in bovines (figure 11).[86] The abundance of the family *Enterobacteriaceae*, especially in rural fresh cow's milk and *amasi*, may reiterate the presence of an *entero-mammary pathway* in shaping the bovine milk microbiome of our samples (figure 9 and table 2).

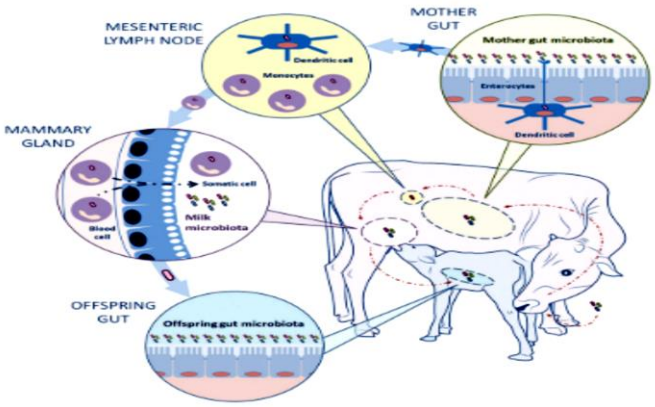


Figure 11:
The entero-mammary pathway's contribution to the bovine milk microbiome (from Addis *et al*.[86]).

Maternal health and diet, mastitis, geographical location, lactation stage, and antibiotic exposure are multifactorial intrinsic and environmental factors having dynamic influences on the breast milk microbiome. The effect of breastmilk refrigeration on the microbiome has also been published. Giribaldi *et al*., in line with previous research, demonstrated that milk refrigeration, over 4 days, at mean temperatures of 6.8°C plus/minus 1.1°C, had no effect on lysozyme-, lactoferrin-, IgA, *Enterococci* and other LAB concentrations in human milk. A reduction in coagulase-positive organisms was reported.[87] However, concerns about the growth of harmful bacteria during cold storage, especially psychrophilic microorganisms (organisms thriving at temperatures of 20°C or below e.g. *Acinetobacter*, *Aeromonas* and *Pseudomonas* spp.) were also highlighted.[88] The effect of milk processing, refrigeration, intrinsic, and environmental factors, as well as seasonal variation, have also been shown to dramatically influence the milk microbiome of ruminants.[89]

Striking similarities in immunological markers, cytokines, and other immune molecules are found between human- and bovine milk. Some of these include transforming growth factor β (TGF-β), interleukin 10 (IL-10), and soluble cluster of differentiation-14 (s-CD14) molecules. Interestingly, previous studies found higher concentrations of TGF-β (with isoforms TGF-β1 and TGF-β2) in breastmilk from mothers living in a farm environment.[90] In human studies, TGF-β induces IL-10, which leads to the conversion of naïve peripheral T cells into FoxP3+ regulatory T cells. IL-10 further leads to the formation of allergen-specific IgG4 (considered a marker of allergen tolerance) and inhibition of T-helper 2 cell proliferation.[91] The 100% homology between human- and bovine milk TGF-β, may suggest a similar immune function.[92] A major resemblance (about 80%) between human- and bovine IL-10, is also worth mentioning.[93] Even more intriguing, the presence of bio-active exosomal microRNAs (miRNA), identified in high amounts in both human- and bovine milk, may suggest a similar epigenetic modulation of the immune system.

The immunological importance of PUFAs (found in both breast- and bovine milk) and conjugated linoleic acids in protecting against eczema and asthma have been highlighted.[94,95] Furthermore, although in much lower concentrations and less extensively researched than HMOs, bovine milk oligosaccharides (BMOs) have also been identified in cows' milk as an important energy substrate for LAB in the bovine gut.[86]

To summarise, although quite different in nutritional composition, the overlap between human- and bovine milk microbiota, at a functional level, is worth appreciating. Similarities in fermentative LAB, as well as similarities in immunological markers and cytokines, may indicate a closer relationship between us and ruminants than previously appreciated. The influence these organisms have on thymic maturation and the development of FoxP3+ and Treg cells via kynurenine and tryptophan production are some exiting new immunomodulatory and allergy protective mechanisms being investigated.[76,95,96]

4.5 The allergy protective characteristics of raw cow's milk: more than just the microbiome

The interaction of raw cow's milk immune-constituents with the human immune system are more complex than previously thought.[97] Compelling evidence from cross-sectional European studies, identifies raw cow's milk exposure, both ante- and postnatally, as an independent protective factor against allergy. To highlight this, further evidence of the allergy protective effect of unprocessed milk consumption comes from the GABRIELA study, where raw milk consumption was associated with a reduction in asthma and atopic sensitisation risk of up to 50%. Interestingly, the GABRIELA study

found an inverse relation between asthma (not atopy) and the heat-labile whey proteins in raw milk.[5] During the first year of life, consumption of unprocessed cow's milk was also protective against rhinitis, respiratory infections and otitis in the rural PASTURE birth cohort.[98]

Globally, the majority of studies found cow's milk to have no inflammatory to clear anti-inflammatory effects in humans.[99] Bordoni *et al.* concluded in a large meta-analysis that dairy products indeed have anti-inflammatory effects and reassured that low-fat dairy products were not found to be pro-inflammatory.[100]

The immune-modulating effects of raw cow's milk have been well documented.[101] For instance, bovine IgG has also been found to block IgE-mediated mast cell activation.[91] The protein component of cow's milk consists of casein (about 80%; heat stabile), whey (about 20%; heat labile), and bovine serum. The whey component comprises α lactalbumin, β lactoglobulin, immunoglobulins, lactoferrin, enzymes, serum albumin (BSA), and important cytokines. In the GABRIELA survey, an inverse association between asthma and α lactalbumin, β lactoglobulin, and BSA were demonstrated.[5] Lactoferrin, a whey protein, has be found to stimulate the production of IL-10 and TGF-β. Furthermore, lactoferrin is an iron-binding glycoprotein and promotes the intestinal growth of organisms with low iron requirements (e.g. SCFA producing *Bifidobacteria* and *Lactobacilli*). Lactoferrin has also been shown to have antimicrobial properties.[92]

In the PASTURE cohort, higher levels of ω-3 PUFAs (more abundant in milk with a high-fat content), were found to be anti-inflammatory. Higher milk fat levels were also found to have a protective effect on milder asthma.[94] From the Netherlands, the PIAMA birth cohort indicated a protective effect on recent asthma at 3 years of age when full cream milk, daily versus rarely (aOR, 0.59; 95% CI, 0.40 - 0.88) and butter, daily versus rarely (aOR, 0.28; 95% CI, 0.09 - 0.88) were consumed.[102] Together with ω-3 PUFAs, ruminant trans-fatty acids (vaccenic and rumenic acid), have been demonstrated to provide additional protection against especially atopic dermatitis, and to a lesser extent, atopy. These conjugated linoleic acids (CLAs) are produced by biohydrogenation, a process that occurs in the rumen of animals, by bacteria converting unsaturated fatty acids to saturated fatty acids.[95] In human breastmilk, vaccenic and rumenic acid are derived mainly from dietary fat intake, with only a small amount being produced by the human breast itself.[103]

SCFAs (butyrate and propionate) are found at high levels in bovine milk. They are mainly produced by lactic acid bacteria in the gut during the fermentation of fiber and have been shown to operate on an epigenetic level by inhibiting histone deacetylation. This leads to the proliferation of regulatory T cells, increased IL-10 production and FoxP3+ expression.[91] In a subgroup of the PASTURE cohort, children

with the highest levels of stool propionate or butyrate, at 1 year of age had lower food and/or inhalant sensitisation by 6 years of age. Higher levels of butyrate were also associated with a lower trend in asthma, allergic rhinitis, and food allergy risk.[104]

The microbial content, being highly dependent on the exposure from the environment, contributes to the lipopolysaccharide (LPS) and other endotoxin levels of raw cow's milk. However, older data from the PASTURE study did not find any difference in farm versus non-farm endotoxin levels in raw milk.[105] LPS is the major component of the outer membrane of Gram-negative bacteria and interact with the human innate immune system.[106] By recognizing LPS and other bacteria endotoxins, the cluster of differentiation-14 (CD14) receptor (found in both human and bovine milk) has been demonstrated to act as an important pathogen recognition receptor (PRR).[107] Bieli *et al.* found an inverse relation (independent of farm exposure) between polymorphisms in the CD14 promotor gene and asthma, allergic rhinoconjunctivitis, and wheezing. This may partially explain a genetic origin in farm milk protection against allergy.[108]

On an epigenetic level, miRNAs, are involved in the demethylation of FoxP3+ associated thymic regulatory T cell maturation, and a decreased prevalence of atopic sensitisation and asthma in children.[109] Multiple other candidate genes (including the interleukin 13-gene, responsible for eosinophil activation and survival) targeted by interfering miRNAs, have been published.[110] The important role of epigenetic influences, was further highlighted in a pilot study by Michel *et al*. Cord blood DNA from European farmers' and non-farmers' children, illustrated statistically significant difference in hypermethylation of asthma genome-wide association study (GWAS) genes.[111]

It is clear that the microbiome alone does not fully explain the anti-allergy properties of unpasteurized cow's milk. Multiple unaltered immunological constituents, present in raw milk, seem to operate not only on genetic but also on an epigenetic level and may complement the microbiome in allergy protection. These mechanisms are far from being understood and should be important aspects explored in future research.

4.6 Consumption of raw cow's milk: immune protective, but what are the health risks?

Consumption of raw, unprocessed milk can be hazardous to human health. Due to this, raw milk consumption, especially in children, cannot be recommended in global nutritional recommendations.[59,66,81] This is certainly also applicable to a developing country, like South Africa, where malnutrition and other immunosuppressive conditions are common.

Bovine mastitis, a common inflammatory infection of the mammary gland tissue, has been shown to have a major impact on the cow's milk microbiome and is an important source of milk pathogens. More so, the prevalence of subclinical mastitis (inflammation of the udder *without* clinical signs) is much higher and of greater health concern. When compared to milk produced from cows with mastitis, Oikonomu *et al.* described the presence of the genera *Fecalibacterium*, unclassified *Lachnospiraceae*, *Aeribacillus* and *Propionibacterium* as 'core' microbiota present in milk obtained from non-mastitic udders. *Streptococcus uberis* and *Staphylococcus aureus*, were furthermore present in milk from healthy udders, indicating the possibility that these bacteria could be part of the normal flora found on the udder's skin or inside the gut of healthy cows. Some of the most common pathogens identified in clinical bovine mastitis were: *Escherichia coli*, *Klebsiella spp*, *Streptococcus uberis*, *Trueperella pyogenes*, *Streptococcus agalactiae*, and *Staphylococcus aureus*.[112] It is speculated that a dysbiosis in udder microbiome rather than the primary invasion by a pathogen, may lead to mastitis.[113] In their study, Faletin *et al*. determined by pyrosequencing, that the alpha diversity was reduced and severe alterations in the milk microbiome taxonomic profiles were present, even if there was a distant history of clinical mastitis. The contribution of antibiotics (given during active mastitis) to these alternations, is unclear.[114]

In our study, layman's physical inspection of the cow's udder (by the farmers milking the cows), was the only screening done to exclude active mastitis before sampling commenced. It was also impossible for us to determine whether subclinical mastitis was present. Our study also did not calculate somatic cell count per milliliter of milk, which is commonly used to determine the presence of an inflammatory response in udders.[86] The presence of *Escherichia*/*Shigella*, in especially our urban fresh cow's milk samples (figure 7) was worrisome. The abundance of other pathogen species, namely *Salmonella enterica*, *Pseudomonas*, and *Kluyvera cryocrescens*, in rural fresh cow's milk and *amasi* (figure 10), are other concerns. Noteworthy, these organisms, at the species level, were absent in the commercially fermented products (figure 10 and table 4) and brought us to the conclusion that these are safer for human consumption than *amasi* and fresh cow's milk.

Unimmunized cows in rural areas carry a higher risk of being infected with *Brucella*, *Salmonella*, and other pathogens. Bovine tuberculosis is reported at significantly higher rates in cows from developing countries.[115] Despite this, the aforementioned SAFFA study (conducted in the same location where we collected our urban fresh cows' milk), indicated traditionally fresh milk consumption from about 6-months of age. Surprisingly, in the SAFFA-study study, urban children were more exposed to unpasteurized milk cow's milk than rural children.[7] In our study, before we collected any rural cow's milk, we obtained clarification from a state vet, declaring the area free from Bovine Brucellosis and Bovine Tuberculosis for the past 12 months.

4.7 Environmental factors altering the microbiome of cow's milk

Apart from udder health, numerous other environmental factors have been described that can dramatically influence the milk microbiome. The type of the cow's breed, the udder's skin microbiome, feces, sheds, grazing (indoor versus outdoor) patterns and milking practices have been highlighted. Furthermore, contamination from humans (while milking), milking machines, transport by tanker trucks, storage facilities, refrigeration, water- and even air contamination, have been identified as influencing factors.[81] The seasonal influence and the influence of geographic locational of cattle are also described.[116] Kable *et al*. published results from the USA, indicating species diversity and median OTUs in their milk samples to be higher when milk was collected in the spring. The richness of the phylum *Firmicutes* was also lower when samples were collected in the spring while the phylum *Bacteroidetes* was significantly richer in milk collected during the autumn.[117] Undesirably high numbers of *E. coli*, were highlighted in regularly consumed milk collected from peri-urban farms in the Free State, South Africa. These counts were significantly lower, when milk was collected during the winter season.[118] From Shanghai, Li *et al*. demonstrated the month to month variation of the milk microbiome and determined temperature and humidity to be the most influential factors at the phylum level.[119] In a study from Italy, raw cow's milk, collected in the summer or autumn, had higher levels specific *Lactobacilli*, with antifungal properties.[120] Furthermore, significant between-breed differences in the microbiome were demonstrated in milk collected from Indian Holstein Friesian and Rendena cows. These differences remained significant, even if these cows were raised on the same farm and under the same conditions.[121]

Our rural and urban milk samples were collected during the South African autumn (March to May). The rural samples were collected from a coastal area in the Eastern Cape, considered more humid than Cape Town, where the urban samples were collected. Similar grazing and milking patterns on the same urban farm may explain the striking similarity (e.g. overlapping OTUs) between the three urban cows' milk samples. Both the urban and suburban cows, in this study, were free, outdoor, wild grass grazers, which received no special diet. The rural cattle were of mixed breed, while the urban cattle were of indigenous South African Friesian breed.

As highlighted in human breastmilk studies, more research is needed to study the effect of these confounding factors down to species level. To complicate matters even further, milk processing has also been shown to dramatically influence the microbiome and may even hamper some of the health-promoting properties of raw cow's milk. The influence of milk processing therefore warrants further discussion.

4.8 Milk processing and its milk altering properties

To qualify as a 'probiotic', a high number of viable organisms must reach the duodenum after oral ingestion. Many probiotic organisms do not survive a low PH environment in the stomach, oxygen exposure during refrigeration, and heat exposure during milk processing.[122] In the milk industry, milk is typically stored at temperatures of 4°C, before transport takes place to dairy plants.[92] Although processing makes milk safer for human consumption, evidence exists that these processes hamper some of the health-promoting and allergy protective characteristics of dairy products.

Milk undergoes various processing methods before it is commercially sold. On arrival at the dairy plant, fresh milk is centrifuged, thereby separating the milk fat, and leaving skimmed milk. These are then recombined to form semi-skimmed milk (about 2% fat) and full fat (>3% fat) milk.[92,123] Heat treatment normally follows this process and is classified based on the temperature level, time of heat exposure, and subsequent cooling. Pasteurization (71°C to 74°C for 15 to 40 seconds), sterilization (110°C to 120°C for 10 to 20 minutes) and ultra-high temperature (UHT) processing (135°C to 145°C for 0.5 to 4 seconds) are commonly used. High-temperature Short-Time (HTST), also known as flash pasteurization, uses temperatures of 71.5°C to 74 °C, for about 15 to 30 seconds, followed by rapid cooling to about 4 °C. HTST pasteurization is particularly used to kill bacteria and can keep milk fresh for up to 2 weeks, without refrigeration. It is then sold commercially as "pasteurized milk". UHT, less commonly used, can extend the shelf-life of milk for up to 9 months. These kinds of milk are sold as "ultra-pasteurized".[92]

Milk is an emulsion and therefore requires homogenization, either before or after heat treatment. Homogenization is a physical process and entails heating, and passing milk under high pressure, through a tiny orifice, after which it is rapidly cooled. This leads to the breakdown of fat globules and by increasing their density, allows milk fat to stay integrated as an emulsion, rather than rising to the surface as cream. Reduction in the size of milk fat globules leads to an increase in the total droplet surface area with the subsequent inclusion of casein proteins on the fat droplet surface - possibly a cause for cow' milk allergy.[124]

Apart from killing harmful organisms, numerous studies have been published on the unwanted effects of heat, homogenization, centrifuging, and pasteurization on raw milk. Lower levels of especially ω-3 polyunsaturated fatty acids and heat-sensitive bioactive whey proteins (e.g. lactoferrin, lactadherin, α lactalbumin, β lactoglobulin, and bovine serum albumin) were reported after heat treatment. When denaturized by heat (generally accepted to occur at temperatures of above 60°C), these proteins lose

their immunological and anti-infective properties. The potential loss of miRNAs during heat treatment and storage of cow's milk is another concern.[92,125,126] Heating also affects immunoglobulin-, TGF-β2 and IL-10 levels.[92] The importance of these cytokines in immune tolerance induction, has already been highlighted.

Evidence also exists on how milk processing can influence the microbiome of raw cow's milk. For instance, lower lactose levels (in processed milk) decrease levels of certain advantageous organisms.[101] Denaturation of lactoferrin (an iron-binding protein), may prevent the growth of health-promoting bacteria with low iron requirements in the gut (e.g. *Lactobacilli* and *Bifidobacteria*).[92] Our study may reiterate this, with *Lactobacilli* absent in the commercially processed (fermented) milk samples but present in the fresh cow's milk samples and in *amasi*.

Sipka *et al*. found unprocessed milk to harbor not only more bacteria but also higher levels of LPS, which may partially explain the allergy protective trend seen when raw cow's milk is consumed regularly.[127] From the GABRIELA study, the authors found total bacterial counts to be higher in unheated versus heated (pasteurized) milk.[5] Interestingly, no effect of total bacterial counts on asthma and atopy were demonstrated.[91,105,127]

As previously mentioned, from our study, a remarkable decrease in both alpha and beta biodiversity were detected in processed (commercially fermented) milk compared to the fresh unpasteurised samples. All three the commercially fermented samples appeared remarkably similar in diversity indexes. When compared to fresh and home fermented milk, a decrease in diversity could be noticed from the phylum down to species level. However, the commercially fermented milk samples, appeared 'safer' for human consumption, compared to *amasi* and fresh cow's milk.

The need for minimally processed, yet safe milk, are apparent. Unfortunately, immunologically unaltered milk after commercial heat processing, does not seem to exist. Milk fermentation, another form of milk processing, may partially overcome this problem. Although decreasing the diversity of the raw milk microbiome, fermentation still has the benefit of harnessing (even enhancing) its anti-allergic properties. The benefits of fermented milk, either homemade or commercially prepared will be discussed below.

4.9 Fermented milk and its allergy protective characteristics

In traditional African communities, milk fermentation is probably the oldest and most accepted method to prevent spoilage. Unpasteurized milk from cattle, known as *ubisi* is obtained by manual lactation of cows. After traditional fermentation, it is consumed by South African and Zimbabwean rural populations as a nutritiously rich staple food, known as *amasi*. Traditionally, calabashes and clay pots were used as containers. These might have been smoked before the addition of milk, to prevent mold growth.[128–130] Interestingly, in our study, rural folk told us that modern, ordinary plastic containers largely replaced traditional calabashes and clay pots for home fermentation (picture 1). These containers are left to stand indoors, at ambient temperatures (>20°C) for 3 to 5 days. During the fermentation period, the separated thin, watery liquid is periodically removed from the top layers of milk in the container while new freshly lactated, unpasteurized milk is continuously added. Traditionally, the fermentation duration and temperature can be modified, to produce a desired taste and consistency. The thick layer at the bottom of the container will be the final product after fermentation.

Microorganisms actively involved in milk fermentation are LAB. These are found naturally in human breastmilk and milk from ruminants (including cows). LAB are Gram-positive, anaerobic, or facultative aerobic cocci or rods, which produce important by products and end products during the catabolism of carbohydrates (complex milk sugars).[131] Broadly, depending on their production of end products during fermentation, LAB can be divided into homofermentative, heterofermentative, and facultative heterofermentative organisms.

Homofermentative LAB, utilize the Embden Meyerhof pathway, to produce lactic acid as a by-product during the fermentation of milk lactose. *Lactococcus* spp., are examples of homofermentative LAB, and are commonly used in commercial dairy starter cultures. Strains commonly used in yogurt production, include rods (*Lactobacillus delbruckii* subsp. *bulgaricus*, *Lactobacillus acidophilus*) and cocci (*Streptococcus salivarius* subsp. *thermophilus*). Examples of thermophilic LAB (organisms that thrive at higher temperatures i.e. between 41°C and 122°C) include *Lactobacillus helveticus* and are also homofermentative.

Heterofermentative LAB produce lactic acid, acetic acid, ethanol, methanoic acid, and carbon dioxide during fermentation. These organisms include *Leuconostococcus* spp. (cocci) and *Lactobacillus brevis*, *Lactobacillus fermentum*, and *Lactobacillus reuteri* (rods). Heterofermenters are generally mesophilic and can grow at a wide range of temperatures (2°C to 53°C).[132] Other *Lactobacillus* species are "facultative" heterofermentative, meaning they produce end-products such as lactic acid, acetic acid,

and carbon dioxide, like heterofermentative bacteria, or only lactic acid (depending on the type of sugars and substrate available). (Figure 12).

Some LAB can produce gas from other substrates including citrate, gluconate, and certain amino acids. Citrate fermenters can be used as a dairy starter formula to provide flavor. These include *Leuconostococcus mesenteroides* subsp. *cremoris* and *Lactococcus lactis* subsp. *lactis* biovar *diacetylactis* - commonly used in buttermilk.

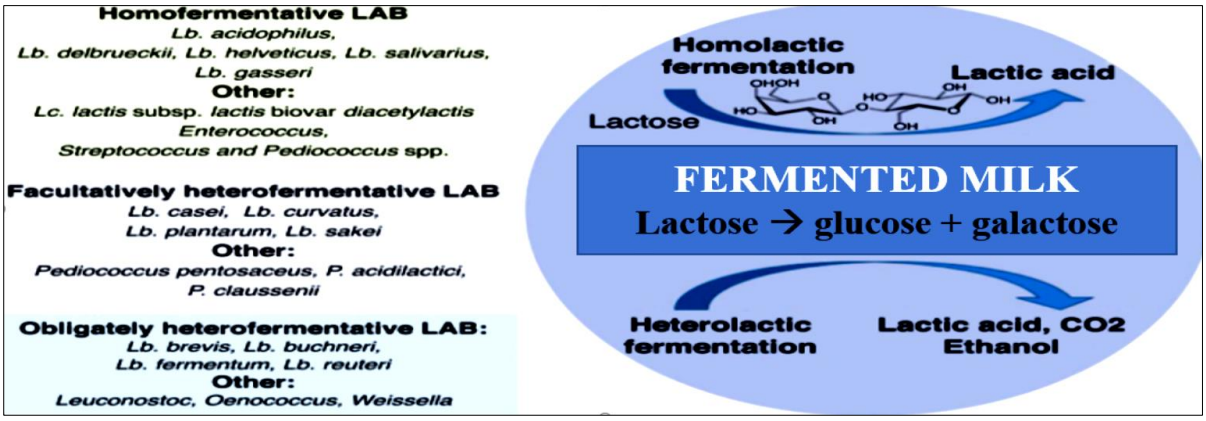


Figure 12:
Heterogeneous fermentation processes and end-products produced by LAB (from Macor *et al*.[133]; Lb. – *Lactobacillus*, Lc. – *Lactococcus*.).

In our study, at the genus level, *Lactococcus* was abundant in all the fermented products, more so in commercially fermented milk. *Leuconostococcus* dominated in the commercially fermented products but was absent in *amasi*. At the species level, *Lactococcus lactis* (AB100803) were most abundant in both commercially fermented milk and *amasi*, with extraordinarily little presence in the fresh milk samples. *Leuconostococcus mesenteroides* (AB023247), *Leuconostococcus pseudomesenteroides* (AB023237), and *Lactococcus chungangensis* (EF694028) dominated the commercially fermented milk, with no presence in *amasi*, probably indicating the abundance of these organisms in commercial dairy starter cultures. Noteworthy, *Lactobacillus paracasei* (D79212) and *Streptococcus infantis* (AY485603) were abundant in *amasi*, while being absent in commercially fermented milk.

Flourishing LAB enhance the flavour of milk, while the fermentation process adds nutritional quality, preserves (by lowering its PH to below 4), detoxifies (e.g. inhibits aflatoxins from molds), and has antibiotic properties (e.g. against Gram-negative pathogens).[134–136] The presence of large amounts of yeasts, found in African fermented products, with a possible synbiotic effect between these and LAB, needs future exploration.[137] In older studies, the nutritional properties of fermented and raw milk, are similar. Additionally, LAB produces lactate and lowers the PH with the provision of subsequent anti-

infective properties.[138,139] Compelling evidence for the health benefits of fermented food products around the world, has led to the inclusion of fermented foods in global food guidelines.[140,141] In the South African SAFFA study the regular consumption of commercially fermented milk, was protective against asthma, atopic dermatitis and allergic rhinitis in urban children, but not in rural children.[9]

Probiotics most extensively evaluated in allergy prevention include the genus *Bifidobacteria* and certain *Lactobacilli* species.[52] Apart from our *amasi* sample, *Lactobacillus* was of low abundance to completely absent in all our other milk samples. Furthermore, *Bifidobacteria (Bifidobacterium pseudolongum* (D86194)) was present in low abundance in fresh milk and absent in all the fermented milk samples (figure 9). This may indicate an important need to investigate other, possibly unique South African species in allergy protection, apart from those being highlighted in mostly European research.

As mentioned before, during the fermentation process, important by-products are produced. Some of these by-products include short- and long-chain fatty acids, vitamins, and amino acids.[142] We are just at the beginning stages of exploring how these products interact with the immune system in allergy protection. For instance, long-chain fatty acids (especially ω-3 fatty acids) have been demonstrated to interact with various innate gastrointestinal receptors to increase IL-10 (an immunomodulatory cytokine). In a systematic review and meta-analysis by Vahdaninia *et al*., pooled analysis did indicate a protective effect of ω-3 fatty acids on allergic sensitisation to egg and peanut in the offspring when administered in pregnancy and to the infant after birth.[143]

SCFAs (acetate and butyrate), inhibits mucosal-associated invariant T-cells (MAIT-cells) – important innate immune cells responsible for producing the pro-inflammatory cytokine IL-17. Another SCFA, propionate, interacts with mucosal dendritic cells in the gut, leading to the deviation away from a Th2-cytokine milieu. Vitamin B9 and the amino-acid tryptophan, have been found to upregulate anti-allergic Treg cells in the intestinal lumen. Tryptophan simultaneously suppresses Th2-cells (an important driver of allergic inflammation).[48,80,144] (Figure 13).

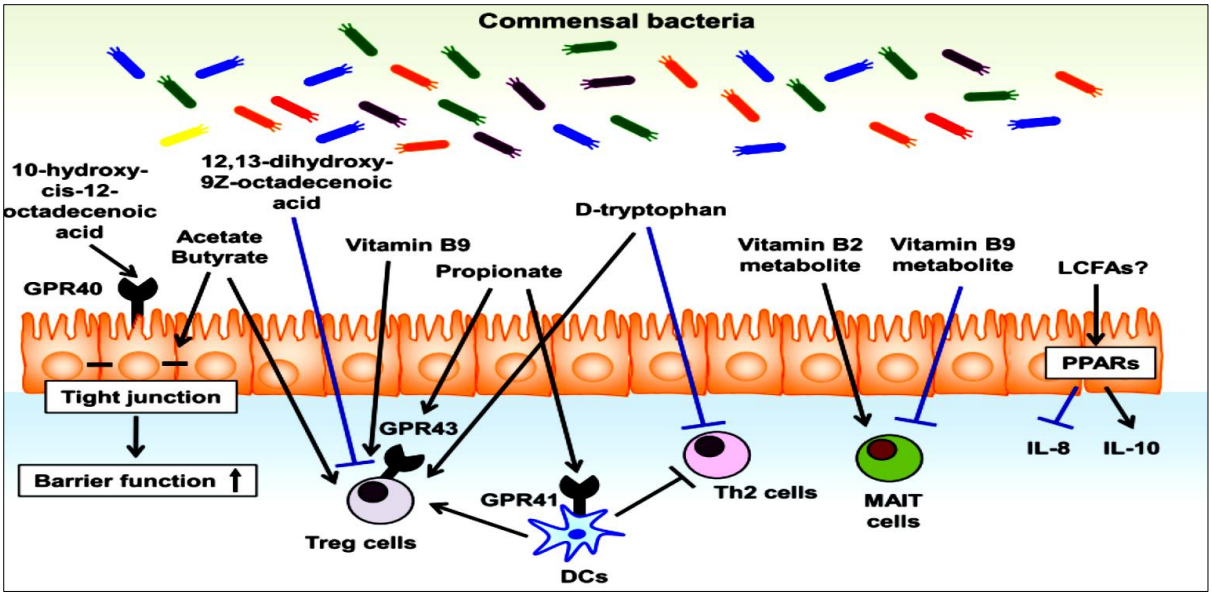


Figure 13:
The potential immune mechanisms of favorable gut microbiota involved in milk fermentation and allergy protection – from Hirata *et al*.[48] (LCFA - long-chain fatty acids; IL – interleukin; GPR - G protein-coupled receptor; DCs – dendritic cells; Treg cells – regulatory T cells; MAIT cells – mucosa-associated invariant T cells; PPAR - peroxisome proliferator-activated receptor).

4.10 Traditionally fermented milk products in Africa and South Africa

In an extensive review of African fermented dairy products, Jans *et al*. highlight the dominant *Lactococcus lactis*, *Streptococcus infantarius* subsp. *infantarius* (Sii), *Lactobacillus spp*. and yeasts in raw and fermented milk products. In this review, a novel Sii, with several unique African variants, were highlighted. The pheno- and genotypical characteristics enables African Sii to survive environments, otherwise favorable for pathogens. This review also highlights the safety and importance of potentially including African Sii lineages in future starter cultures for milk fermentation.[115] Although *Lactococcus lactis* (AB100803) was the most abundant organism in our *amasi* sample, we did not detect any *Streptococcus infantarius* in our study.

Other well-documented studies from the African continent, come from the Kenyan product, *kule naoto*, produced from raw cow's milk fermentation in traditional calabashes. At the genus level, *Lactobacillus* dominated in *kule naoto*, while *Lactococcus*, *Leuconostococcus*, and *Enterococcus* were also reported in high numbers. Dominant species included: *L. fermentum*, *L. paracasei*, and *L. acidophilus*. *Kule naoto* was regarded as safe because of the absence (< log 2.0/ml) of *Enterobacteriaceae* in a low PH (<4.5) environment.[131] From Ghana, the traditionally fermented milk product *nunu*, yielded *Lactobacillus fermentum* as the dominant LAB throughout the fermentation process, while *Lactobacillus plantarum* and *Leuconostococcus mesenteroides* were found to play an important role

during the first few hours of fermentation.[145] When comparing *kule naoto* with *amasi* from our study, *Lactobacillus* was only found in small numbers. Furthermore, in contrast with *kule naoto*, *Enterobacteriaceae* was abundant in *amasi*. *Leconostococcus* and *Enterococcus* were almost absent in *amasi*. However, as with *kule naoto*, *L. paracasei* was abundant in *amasi*. When comparing *amasi* with *nunu*, *Leuconostococcus mesenteroides* was absent in our *amasi* sample.

Research on LAB in *amasi* has been published before. From South Africa and Namibia, an earlier study by Beukes *et al.* investigated *amasi* by using culture-dependent methods. The genera *Leuconostococcus*, *Lactococcus*, and *Lactobacillus* were abundant. The dominant species were *Lactococcus lactis* subsp. *lactis* and *Leuconostococcus mesenteroides* subsp. *dextranicum*. Potential pathogens were *Staphylococcus aureus*.[129] In another study, from the EkuPindiseni Community, KwaZulu-Natal Province, *amasi* was collected during the South African mid-summer month of December, and analyzed by using 16S rRNA clone library and Denaturing Gradient Gel Electrophoresis. In this study, *Lactococcus*, *Lactobacillus*, and *Leuconostococcus* were major genera identified, while *Lactococcus lactis*, *Enterococcus faecalis*, *Lactobacillus casei*, *Lactobacillus paracasei*, *Lactobacillus plantarum*, and *Leuconostococcus pseudomesenteroides*, were the most abundant species present.[146] These findings were relatively similar to ours, concerning *Lactococcus*, *Lactococcus lactis*, *Lactobacillus paracasei*, and *Leuconostococcus pseudomesenteroides* abundance. However, the genus *Lactobacillus* and *Leuconostococcus* were of less abundance in our *amasi* sample.

Although daily consumption of homemade fermented milk is reported worldwide, the safety of this remains a concern. This is probably the biggest reason why these products are not sold commercially without industrialized milk processing.[147,148] Growth inhibition of the pathogens *Escherichia coli* and *Salmonella enteritidis* have previously been shown to occur in *amasi*.[149] However, in our study, although no *Escherichia*/*Shigella* were present in *amasi*, the high abundance of the species *Salmonella enterica* (AE006468) could be potentially hazardous. Evivie *et al.* stress that LAB do not eliminate harmful pathogens to a satisfactory 'safe for human consumption' level and safety cannot be assured by milk fermentation.[122,150]

The abundance of the genus *Citrobacter* and other potential pathogens (e.g. *Kluyvera cryocresence*) in unpasteurized milk were noted by authors from Zimbabwe.[151] From our study, *Citrobacter* and *Kluyvera cryocresence*, naturally occurring in water, soil and sewage, were also present in *amasi*. Possible water contamination from inside the container used for fermentation, should be considered. The cows' udder may also be contaminated with soil or sewage. It should be noted that the aim of our study was not to analyze fermented milk collected from a sterile environment but to ensure a representative sample of milk was obtained by using traditional milking and fermentation processes.

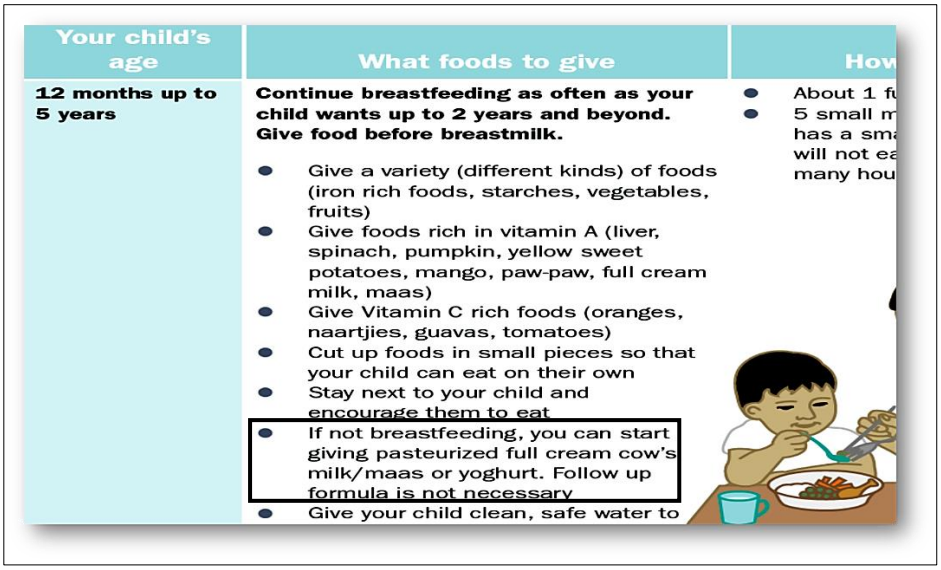
Our only pre-requisite for this study was to prevent human microbiome contamination and hence sterile gloves were worn when the milk samples were collected.

In summary, although some similarities exist, the above highlights the dissimilarities between traditionally fermented milk products from different rural communities, even if they were collected from the same continent. These dissimilarities become even more apparent when these products are compared to commercially fermented milk. These findings further highlight the diverse outcome in microbiome studies published in the literature, even within the same country, making it difficult to make firm conclusions. However, the message remains clear, traditionally fermented products contain pathogens and cannot be promoted as safe.

4.11 *Amasi*, Maas and other South African commercially fermented products

Since the 1980s, Maas® has been commercially produced by dairy companies, by using either 'in container' or 'tank' fermentation. Standard mesophilic starter cultures, typically including *Lactococcus lactis* subsp. *lactis*, *Lactococcus lactis* subsp. *cremonis* and *Leuconostococcus mesenteroides* subsp. *cremonis* are used for this purpose.[129] A landmark change from the 2001 South African Food-Based Dietary Guidelines, was published in 2012, with the inclusion of the recommendation: "*have milk, maas or yogurt every day*".[152] Maas® was included mainly because of its health-promoting fat content, low lactose concentration, and associated energy-dense properties - factors important for addressing malnutrition, lactose intolerance, and non-communicable diseases, especially in developing countries, like South Africa. It has a shelf life of about 21 days but should be kept refrigerated, as per manufacture's recommendation. This may not be possible for all socioeconomic and rural population groups in South Africa, where access to electricity is not always within reach.

In the recently updated "Road to Health"-booklet for South African children, the inclusion of maas in the weaning diet of non-breastfeeding children from 12-months of age, is recommended. From other parts of the world, fermented milk was also highlighted as a complementary feed, in as young as 3-months of age.[138]



Picture 4 and 5:
Full cream Maas®, sold at local retail stores in South Africa. A snapshot from the recently updated South African Road to Health-booklet.

An important aim of our study was to compare the microbiome (at species level) of fermented versus unfermented milk products, to indicate whether fermentation (either commercially or home fermented) had a significant influence on the occurrence of certain taxa. We chose three commercially fermented products namely, Othando®, Amyoli®, and Maas® because these are readily available in all urban and rural shops in South Africa. Furthermore, the South African Maas® product was studied before. Only taxa with ≥ 60% abundance in either (or both) of the two groups were compared in our statistical analysis. *Lactococcus lactis* (AB100803) in the fermented group, reached the highest statistical difference between the two groups. Furthermore, in the fermented group, the abundance of *Lactococcus chungangensis* (EF694028), *Leuconostococcus mesenteroides* (AB023247), *Leuconostococcus pseudomesenteroides* (AB023237) and *Lactobacillus paracasei* (D79212) were also statistically different from the unfermented milk group, but less so than *Lactococcus lactis* (AB100803). *Salmonella enterica* (AE006468), *Anoxybacillus*, and *Staphylococcus* in unfermented milk were also of differential importance. Some of these findings are summarized in table 4. Numerous other organisms, reaching statistical differences in abundance between the two groups, are illustrated on the heatmap in figure 10. Some of these taxa include important pathogens. The three commercially fermented products appeared to be free of well-described human pathogens.

In a South African case study on commercial maas, Du Plooy *et al*. published interesting data, obtained from the South African consumer database, product-related database Ipsos-Markinor (2014), and the Target Group Index database (2014). At the time of the study, about 80 different maas products were available commercially, with only about 10% of South African households, consuming maas daily.[153]

These surveys indicated that 90% of maas consumers were black, adults, and from the lowest socio-economic income groups in South Africa.

Chelule *et al*. published important reasons for rural caregivers of children under 5 years of age not giving fermented milk products. Some of these reasons include: no information on fermented products was conveyed to them by community healthcare workers, lack of knowledge on how to prepare traditionally fermented products, lack of knowledge on the nutritional and health benefits of fermented products, and concerns about safety and taste (sourness). Although well known to them (used by their parents and grandparents), these products are no longer uniformly or regularly prepared and consumed.[154] Another South African study highlighted South African dieticians not adhering to especially the updated "maas' food guidelines, leading to poor patient enforcement to consume these products regularly.[155]

Changing times and modernization should be considered when constructing feeding policies. Paradigm shifts in population characteristics are noted globally, even in our study. An example from our study is the apparent change in the method of preparing home fermented milk in plastic containers as supposed to clay pots or calabashes. Home fermentation is also time-consuming. Knowledge is therefore lost with urbanization and modernization, preventing younger generations from continuing with an ancient rural tradition of fermenting milk at home.[156] Based on these findings, ideally future recommendations should be based on research harvested from studies like ours, to design starter cultures and to produce an accessible 'dry' probiotic, which is unable to spoil. The role of starter cultures and enzymes for safer milk fermentation are extensively reviewed by Bezie *et al*. These starter cultures typically include *Lactococcus lactis*, *Lactobacillus* species, *Streptococcus thermophilus*, *Bifidobacterium* species, and *Leuconostococcus* species, which can be added to pasteurized milk and left to ferment at controlled temperatures and for a certain amount of time, to produce the desired end product. Special growth media (not milk) should be used when developing these starter cultures, mainly to prevent potential harmful organisms (e.g. bacteriophages) from thriving.[157]

4.12 What about viruses, fungi, and other organisms present in bovine milk?

If there is insufficient exposure to a variety of microbes, the human immune system, according to the '*old friends hypothesis*', will fail to mature, leading to hypersensitivity towards often harmless, every day encountered stimuli. This led to the appreciation of organisms (bacteria, viruses, fungi, parasites, and other unicellular eukaryotes) involved in 'training' the immune system to develop tolerance.[158]

Apart from bacteria (the '*bacteriome*'), the most abundant organism in the human microbiome, are viruses (the '*virome*') which outnumber bacteria in the gut. Although lower in abundance, other inhabitants, across different human body sites, are fungi (the '*mycobiome*') and helminths (the '*macrobiome*').[159] Based on metagenomic studies, the human '*virome*', mainly consists of bacteriophages, with *Caudovirales* (*Siphoviridae*, *Myoviridae*, and *Podoviridae*) and *Microviridae*, the most well described. We currently know very little of the intriguingly complex interaction of these organisms with one another and the human immune system.

Probably one of the most puzzling '*friend or foe*' phenomena, is the human immune system's response towards exposure to parasites and anthelminthic treatment.[160] From West Africa (considered to be an endemic area of parasitic infection), Gabonese children, carrying this infection, had lower rates of skinprick test reactivity to house dust mites, compared to controls. Studies conducted in Brazilian and Ecuadorian children also reported lower allergy occurrence with co-existing parasitic infection.[161] On the other hand, in a study from South Africa (the SAFFA-study), children between the age of 1 and 3 years old, and regularly dewormed, had significantly lower rates of atopic dermatitis, allergic rhinitis, and self-reported asthma.[7]

Parasites co-evolved with humans and while evidence exists of human parasitic infection leading to innate immune system stimulation and production of FoxP3+ expression in naïve CD4+ T cells, other well-described immune features, including the production of a pro-allergic Th2-mediated immune profile upon exposure to parasites, do exist.[162] Interesting to note, parasites are also known to produce SCFAs (e.g. acetate) - the immunological importance of this by-product has already been highlighted before. The complex microbiota-parasitic cohabitation, with a possible bidirectional influence on one another in the human gut, has been explored. For example, studies have been published, indicating *Lactobacillu*s may promote helminth infection.[159] However, the role of host-specific commensal protozoa (e.g. *Entamoeba histolytica*) found largely in relation to parasites, is uncertain.[163] The particular state of symbiosis versus dysbiosis of all these collective gut inhabitants, seems to be an important factor in determining whether their existence will be pro- or anti-inflammatory.

Approximately 10^{15}, predominantly dsDNA viruses, inhabit the healthy human gut. Plant-derived RNA viruses (believed to have their origin exclusively from the diet), also inhabit the gut and are less well researched.[159,164] The human '*virome*', comprises largely viruses that parasitise bacteria (bacteriophages), and use it for its replication. Much like the microbiome, the human '*virome*' seems to decrease in diversity from birth to about 2 years of age, with a shift from *Caudoviridae* to an increased *Microviridae* abundance.[159] In the adult steady-state, this remains highly stable in each individual.[165] A complex viral-bacterial-immune interaction on the gut mucosal surface exists. For instance, viruses can

bind to bacterial structures (e.g. flagellin or pili) or molecules (e.g. LPS) and lead to synergistic induction of immune tolerance cytokines (e.g. IL-10). LAB may also protect against viral invasion, by not only lowering the environmental PH through the production of lactic acid but also by producing reactive oxygen species and defensins. *Bifidobacterium breve* has been shown to induce interferon-gamma, tumor necrosis factor-alpha and interleukin 4, aimed against combatting viruses.[166] The innate immune system seems to play an important role in either limiting or promoting viral growth and virulence.[164,167] These findings open a new world of opportunity for harnessing viruses to create a favourable microbial gut ecosystem and to use bacteriophages to deliver an 'on-site' immunomodulation treatment through the gastrointestinal system.

Of all the '*biomic*' research, the least is known about the human gut '*mycobiome*'. Described as the 'rare biosphere', studies found diverse fungal communities in all sections of the human gut. Although some fungi, especially *Candida albicans,* are described as commensal flora, constant attempts from other organisms (especially favourable bacteria) are made to keep fungi at bay in the gut. The *Lactobacillus-Candida* antagonism describes the phenomenon of *Candida*, preventing *Lactobacillus* growth (and *vice versa*) after, for example, oral antibiotic exposure.[168] Protective mechanisms of commensal microbiota against *Candida albicans* gut invasion, are believed to be mediated through short- and medium-chain fatty acids, as well as gas (e.g. hydrogen and methane) production during natural fermentation.[169]

The most extensively investigated yeast in food and beverages is *Saccharomyces cerevisiae* (used in brewing, winemaking, and baking i.e. brewer's yeast and baker's yeast). Yeasts are not normally added to starter cultures for commercial fermentation of milk and are thought to originate from environmental contamination (e.g. industrial equipment and air). Pasteurization has furthermore been shown to dramatically lower yeast counts in commercially prepared milk. However, natural fermentation, leads to increased numbers of yeasts, indicating a possible symbiotic relation between yeasts and LAB in a low PH environment.[170] Yeasts have also been shown to be relatively resistant to sanitizers and cleaning agents used in commercial dairy plants. Interestingly, from the African continent, *S. cerevisiae* was the most dominating yeast in Ghana's *nunu* fermented milk product. Although small in numbers, a study from Italy, illustrated the absence of *S. cerevisiae* in raw cow's milk, even after they received feeds with yeast supplementation. This indicates that *S. cerevisiae* may find raw cow's milk, an unfavorable habitat.[171]

Yeasts support LAB by lowering the environmental PH and through the production of CO2 and essential growth metabolites (e.g. vitamins and organic acids). They also prevent spoilage by filamentous fungi and bacteria (e.g. *Escherichia coli, Staphylococci, Vibrio cholera, Clostridium difficile,* and *Salmonella*). Furthermore, in the traditionally fermented milk product *kule naoto,* from the

Maasai in Kenya, the numbers of yeasts and *Enterobacteriaceae* were found to be inversely related.[131,145] *Enterobacteriaceae* was uniquely abundant in our *amasi* sample and completely absent in commercially fermented milk. It was furthermore abundant in one of the rural fresh cow's milk samples.

Katakura *et al*. described *Lactococcus lactis* IL1403 surface proteins, involved in recognizing yeast membrane mannan, and thereby promoting adhesion of LAB to yeasts.[172] Research by Xie *et al*. reported proteins on the cell surface of *Lactobacillus paracasei* H9 and polysaccharides in the cell wall of *Saccharomyces cerevisiae* involved in co-aggregation of these two organisms, thereby enhancing the probiotic potential of *Lactobacillus paracasei.* The yeast *Williopsis saturnus* var. *saturnus* was also shown to enhance the survival of *Lactobacillus burgaris* and *Lactobacillus rhamnosus* in fermented milk.[173]

Our study did not include the identification and description of viruses, helminths, fungi, and yeasts. The contribution these organisms play in shaping the microbiome of cow's milk and the human gut after milk ingestion, needs future exploration. More specifically, the interaction between LAB and *S. cerevisiae* in milk seems noteworthy.

4.13 Bovine milk research: potential problems, pitfalls, and future research

Studying a highly bioactive and complex substance, like cow's milk, is difficult, and the interpretation of research results should appreciate the influential role played by intrinsic and environmental factors. Global standardization of milk specimen collection, storing of samples, the time between sample collection and analysis, DNA extraction, PCR bias, trustworthy bioinformatic pipelines, and quality of existing databases are some major concerns in current microbiome research. The importance of underestimating the contribution of unassigned taxa, found in studies, is also highlighted.[20,174] Some microorganisms are resistant to cell lysis (e.g. Gram-positive bacteria), applied during the DNA extraction process, and may, therefore, be absent in the final taxonomic assignment. Despite these potential pitfalls, the concordance rate between conventional culture-positive identification techniques and metagenomic studies, are reported to be high (91.8%, with a sensitivity rate of 52.7%).[175]

DNA cross-contamination (e.g. with human commensals or instrument mismatch, known as 'index hopping') between samples with low microbial biomass, is another concern. This can be partially eliminated by using standardized mock communities as positive controls during analysis. Positive controls further enable the monitoring of DNA extraction efficiency and determine the lower limit of organism detection.[176] In our analysis, numerous quality control steps were included (as per the

Illumina®-instrument workflow recommendation). We used the standardized '*ZymoBiomics*' product as a positive control. A 'no template' (DNA suspension buffer) was used as a negative control to identify potential laboratory contamination. Furthermore, the use of sterile gloves during sample collection eliminated possible human DNA cross-contamination. There were also no deviations from the study plan and analysis workflow, ensuring standardization and reliability of our results.

Our study had small numbers - the main reason being cost constraints. The inclusion of more samples will add value to future research. In developing countries, like South Africa, the cost of microbiome analysis and accessibility to metagenomic laboratories, are huge hurdles in conducting routine microbiome research. Keeping samples frozen, for long-distance transport to laboratories, is another practical problem, especially if these samples were collected at distant, rural sites, like ours.

Dust and other environmental microbial contamination from milking stables and farms should be accounted for in bovine milk research as they considerably influence the microbiome of cow's milk. Furthermore, grazing and feeding influences, undetected illnesses (i.e. subclinical mastitis), antibiotic use, breed and age of the animals, lactation stage, geographic location, temperature, the season of sample collection and the influence of other animals (i.e. pets, goat, pigs, sheep) present on the farm, are other well-described microbiome research confounders.[86,116,177] Further research is needed to determine how these confounders influence the allergy protective LAB in raw and fermented cow's milk.

The importance of organism underrepresentation in final data analysis should be accounted for. The fact that an organism occurs in low abundance in a sample, does not mean it has less important functions in a specific microbial ecosystem. A better understanding of the '*virome*', '*mycome*', '*macrobiome*' and their influence on bacteria, is also warranted. Whole-genome DNA-sequencing ('shotgun metagenomics') of bacterial, viral and fungal communities, partially overcome these problems and eliminate conventional 16S ribosomal PCR amplicon bias. *Metatranscriptomics*, *metaproteomics*, and *metametabolomics* enable us to study the functions of a specific microbial niche as a whole, rather than just naming organisms present therein.[13,86,178] In microbiome data analysis, the use of adjusted p-values and the Fisher's exact test (after multiple-testing correction) help to inflate taxonomic data, thereby ensuring the inclusion of taxa that would have been deemed non-significant by conventional statistical methods.

Unfortunately, no strong evidence currently exists to promote any of the 'on the market' probiotics to prevent allergy, less so, to treat it. Although limited evidence exists for *Lactobacillus rhamnosis* (HN001) in preventing atopic dermatitis, the beneficial effect of probiotics seems species and even

strain-dependent, warranting intensive future research.[46,179] Guidelines from all major global allergy and immunology societies (including the European Academy of Asthma and Clinical Immunology and the World Allergy Organization), do not recommend probiotics to prevent allergy. Although conditionally, the World Allergy Organization does favour the use of probiotics in pregnancy, lactation, and infancy when a family history of allergy is present.[180] This beneficial effect seems pronounced when probiotics are administered with prebiotics, but the exact strain, dose, route of administration, and duration of treatment, are still unknown.[52] Most microbiome research publications describe their findings largely at the phylum, some at the genera level. As was done by our study, future research should involve 'deeper diving', describing these organisms down to species, and even strain level.

Major future strides are being made in harnessing LAB for its health-promoting properties and to deliver it in a safe form at a population level. The Yoba for Life foundation used dried seed cultures of *Lactobacillus rhamnosus GG*, with an adjuvant culture of *Streptococcus thermophilus* C106, to be used with milk, vegetables, and cereals, in combatting diarrheal disease in Africa.[181] The principles of these initiatives may also be replicated in the future to preventing allergic diseases. An important publication warranting future exploration, by Nishino *et al*., describes culture-independent density gradient centrifugation to recover the majority of viable LAB from yogurt, and indicated this as an affordable and quick method of recovering these organisms from fermented milk products, while preserving their physiological and health-beneficial functions.[182] Future research should be aimed at developing and promoting the use of these products in urban, westernized communities. These aims were already identified in a Joint FAO/WHO Workshop, held in Pretoria, South Africa several years ago.[156] The development of appropriate starter formulas, combined with prebiotics via microencapsulation techniques, and delivering them orally, will not only extend the shelf life of these products but will also ensure safety.[183–185] These products may bring us closer to a much-needed and allergy protective 'farm capsule'.

4.14 Conclusions

The microbiome of raw cow's milk and its interaction with the human immune system, especially in allergy protection, seem more complicated than previously thought. Multiple environmental- and intrinsic bovine factors play a role in shaping this microbiome. In our study, bacteria, present in unpasteurized rural, urban, and home fermented milk, as well as organisms in pasteurized commercially fermented products were investigated. These milk samples differed markedly depending on the source, possibly due to factors involved in their collection, storage, and fermentation techniques. At the species level, fermented milk comprised a statistically significant different microbiome than unpasteurized

milk, regardless of the source (pasteurized or not; homemade or mass-produced). The microbiota composition of different brands of commercially available fermented pasteurized milk was highly consistent and contained LAB postulated to protect against allergy. Urban and rural fresh cow's milk samples were the most diverse, and commercially bought products, the least. At the family member level, *Leuconostocaceae* dominated in all three the commercially bought samples. At the species level, *Lactococcus lactis* (AB100803) dominated in all the milk products. *Leuconostococcus mesenteroides* (AB023247), *Leuconostococcus pseudomesenteroides* (AB023237), and *Lactococcus chungangensis* (EF694028) were abundant in all three the commercially fermented samples. These were absent in *amasi* and had low abundance in the fresh milk samples. *Amasi* had higher diversity than commercially available fermented milk, with the unique abundance of *Streptococcus infantis* (AY485603). Important pathogens were identified in fresh cow's milk and *amasi*. Ingestion of unpasteurized milk, whether fermented or not, may, therefore, have adverse health outcomes.

It is currently unknown which species and strain of LAB may have an allergy protective effect. Furthermore, these organisms and their metabolic by-products, maybe allergy protective on a genetic as well as an epigenetic level. Further research should be aimed at harnessing findings from research like ours, in designing a safe synbiotic. In the meantime, commercially fermented milk, although of low diversity, may be utilized as an allergy protective weaning food in infant diets, especially in westernized and modern urban populations. The ingestion of these products should therefore not be restricted to rural and traditional African communities, but to all of society.

5. REFERENCES

1. Silverberg JI, Simpson EL. Associations of childhood eczema severity: A US population-based study. Dermatitis. 2014;25(3):107–14.
2. Vuitton DA, Dalphin JC. From Farming to Engineering: The Microbiota and Allergic Diseases. Engineering. 2017;3(1):98–109.
3. Genuneit J, Büchele G, Waser M, Kovacs K, Debinska A, Boznanski A, et al. The GABRIEL Advanced Surveys: Study design, participation and evaluation of bias. Paediatr Perinat Epidemiol. 2011;25(5):436–47.
4. Riedler J, Braun-Fahrländer C, Eder W, Schreuer M, Waser M, Maisch S, et al. Exposure to farming in early life and development of asthma and allergy: a cross-sectional survey. Lancet. 2001;358(9288):1129–33.
5. Loss G, Apprich S, Waser M, Kneifel W, Genuneit J, Büchele G, et al. The protective effect of farm milk consumption on childhood asthma and atopy: the GABRIELA study (e764). J Allergy Clin Immunol. 2011;128(4):766–73.
6. Waser M, Michels KB, Bieli C, Flöistrup H, Pershagen G, Von Mutius E, et al. Inverse association of farm milk consumption with asthma and allergy in rural and suburban populations across Europe. Clin Exp Allergy. 2007;37(5):661–70.
7. Botha M, Basera W, Facey-Thomas HE, Gaunt B, Gray CL, Ramjith J, et al. Rural and urban food allergy prevalence from the South African Food Allergy (SAFFA) study. J Allergy Clin Immunol. 2019;143(2):662-668.e2.
8. Allen KJ, Koplin JJ. What can urban/rural differences in food allergy prevalence tell us about the drivers of food allergy? J Allergy Clin Immunol. 2019;143(2):554–6.
9. Levin ME, Botha M, Basera W, Facey-Thomas HE, Gaunt B, Gray CL, et al. Environmental factors associated with allergy in urban and rural children from the South African Food Allergy (SAFFA) cohort. J Allergy Clin Immunol. 2020;145(1):415–26.
10. Zimmermann P, Messina N, Mohn WW, Finlay BB, Curtis N. Association between the intestinal microbiota and allergic sensitization, eczema, and asthma: A systematic review. J Allergy Clin Immunol. 2019;143(2):467–85.
11. Huang YJ, Marsland BJ, Bunyavanich S, O'Mahony L, Leung DYM, Muraro A, et al. The microbiome in allergic disease: Current understanding and future opportunities—2017 PRACTALL document of the American Academy of Allergy, Asthma & Immunology and the European Academy of Allergy and Clinical Immunology. J Allergy Clin Immunol. 2017;139(4):1099–110.

12. Marco ML, Heeney D, Binda S, Cifelli CJ, Cotter PD, Foligné B, et al. Health benefits of fermented foods: microbiota and beyond. Curr Opin Biotechnol. 2017;44:94–102.
13. Ghosh T, Beniwal A, Semwal A, Navani NK. Mechanistic Insights Into Probiotic Properties of Lactic Acid Bacteria Associated With Ethnic Fermented Dairy Products. Front Microbiol. 2019;10:502. doi:10.3389/fmicb.2019.00502.
14. Sbihi H, Boutin RC, Cutler C, Suen M, Finlay BB, Turvey SE. Thinking bigger: How early life environmental exposures shape the gut microbiome and influence the development of asthma and allergic disease. Allergy. 2019;74(11):2103-15.
15. Volokh O, Klimenko N, Berezhnaya Y, et al. Human Gut Microbiome Response Induced by Fermented Dairy Product Intake in Healthy Volunteers. Nutrients. 2019;11(3):547. doi:10.3390/nu11030547.
16. Veiga P, Pons N, Agrawal A, et al. Changes of the human gut microbiome induced by a fermented milk product. Sci Rep. 2014;4:6328. doi:10.1038/srep06328.
17. Hornung B, Martins Dos Santos VAP, Smidt H, Schaap PJ. Studying microbial functionality within the gut ecosystem by systems biology. Genes Nutr. 2018;13:5. doi:10.1186/s12263-018-0594-6.
18. Mysara M, Vandamme P, Props R, et al. Reconciliation between operational taxonomic units and species boundaries. FEMS Microbiol Ecol. 2017;93(4):fix029. doi:10.1093/femsec/fix029.
19. Cao Y, Fanning S, Proos S, Jordan K, Srikumar S. A Review on the Applications of Next Generation Sequencing Technologies as Applied to Food-Related Microbiome Studies. Front Microbiol. 2017;8:1829. doi:10.3389/fmicb.2017.01829.
20. Goodrich JK, Di Rienzi SC, Poole AC, Koren O, Walters WA, Caporaso JG, et al. Conducting a microbiome study. Cell. 2014;158(2):250–62.
21. Andrews S. FastQC: a quality control tool for high throughput sequence data [Internet]. 2010 [cited 2020 Mar 15]. Available online at: http://www.bioinformatics.babraham.ac.uk/projects/fastqc
22. Ewels P, Magnusson M, Lundin S, Käller M. MultiQC: Summarize analysis results for multiple tools and samples in a single report. Bioinformatics. 2016;32(19):3047–8.
23. Callahan BJ, McMurdie PJ, Rosen MJ, Han AW, Johnson AJ, Holmes SP. DADA2: High-resolution sample inference from Illumina amplicon data. Nat Methods. 2016;13(7):581-583. doi:10.1038/nmeth.3869
24. Alishum A. DADA2-formatted 16S rRNA gene sequences for both bacteria & archaea (Version 3) [Internet]. 2019 [cited 2020 Mar 15]. Available online at: https://zenodo.org/record/3266798#.XhxpjpMzZTY

25. McMurdie PJ, Holmes S. phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data. PLoS One. 2013;8(4):e61217. doi:10.1371/journal.pone.0061217.
26. Paulson JN, Stine OC, Bravo HC, Pop M. Differential abundance analysis for microbial marker-gene surveys. Nat Methods. 2013;10(12):1200-1202. doi:10.1038/nmeth.2658.
27. Oksanen AJ, Blanchet FG, Kindt R, Legendre P, Minchin PR, Hara RBO, Simpson GL, Solymos P, Stevens MHH, Wagner H. 2016. Package "vegan." R Foundation for Statistical Computing, Vienna, Austria.
28. Gaujoux R. 2014. Generating heatmaps for nonnegative matrix factorization. R Foundation for Statistical Computing, Vienna, Austria.
29. Benjamini Y, Hochberg Y. Controlling the False Discovery Rate: A Practical and Powerful Approach to Multiple Testing. Journal of the Royal Statistical Society. Series B (Methodological). 1995;57(1):289-300.
30. Mcguire MK, Mcguire MA. Human Milk : Mother Nature's Prototypical Probiotic Food? Adv Nutr. 2015;6:112–23.
31. Lacagnina S. The Developmental Origins of Health and Disease (DOHaD). Am J Lifestyle Med. 2020;14(1):47–50.
32. Arrieta MC, Stiemsma LT, Amenyogbe N, Brown EM, Finlay B. The intestinal microbiome in early life: health and disease. Front Immunol. 2014;5:427. doi:10.3389/fimmu.2014.00427.
33. Victora CG, Bahl R, Barros AJD, França GVA, Horton S, Krasevec J, et al. Breastfeeding in the 21st century: Epidemiology, mechanisms, and lifelong effect. Lancet. 2016;387(10017):475–90.
34. De Filippo C, Cavalieri D, Di Paola M, Ramazzotti M, Poullet JB, Massart S, et al. Impact of diet in shaping gut microbiota revealed by a comparative study in children from Europe and rural Africa. Proc Natl Acad Sci U S A. 2010;107(33):14691–6.
35. Tamburini S, Shen N, Wu HC, Clemente JC. The microbiome in early life: Implications for health outcomes. Nat Med. 2016;22(7):713–22.
36. Reynolds LA, Finlay BB. Early life factors that affect allergy development. Nat Rev Immunol. 2017;17(8):518–28.
37. Stiemsma LT, Turvey SE. Asthma and the microbiome: defining the critical window in early life. Allergy Asthma Clin Immunol. 2017;13:3. doi:10.1186/s13223-016-0173-6.
38. Falony G, Joossens M, Vieira-Silva S, Wang J, Darzi Y, Faust K, et al. Population-level analysis of gut microbiome variation. Science. 2016;352(6285):560–4.
39. Milani C, Duranti S, Bottacini F, et al. The First Microbial Colonizers of the Human Gut: Composition, Activities, and Health Implications of the Infant Gut Microbiota. Microbiol Mol Biol Rev. 2017;81(4):e00036-17. doi:10.1128/MMBR.00036-17.

40. Clemente JC, Ursell LK, Parfrey LW, Knight R. The impact of the gut microbiota on human health: An integrative view. Cell. 2012;148(6):1258–70.
41. Nakayama J, Kobayashi T, Tanaka S, Korenori Y, Tateyama A, Sakamoto N, et al. Aberrant structures of fecal bacterial community in allergic infants profiled by 16S rRNA gene pyrosequencing. FEMS Immunol Med Microbiol. 2011;63(3):397–406.
42. Bisgaard H, Li N, Bonnelykke K, Chawes BLK, Skov T, Paludan-Müller G, et al. Reduced diversity of the intestinal microbiota during infancy is associated with increased risk of allergic disease at school age. J Allergy Clin Immunol. 2011;128(3):646–652 e1-5.
43. Azad MB, Konya T, Guttman DS, Field CJ, Sears MR, Hayglass KT, et al. Infant gut microbiota and food sensitization: Associations in the first year of life. Clin Exp Allergy. 2015;45(3):632–43.
44. Mahdavinia M, Rasmussen HE, Botha M, Binh Tran TD, Van den Berg JP, Sodergren E, et al. Effects of diet on the childhood gut microbiome and its implications for atopic dermatitis. J Allergy Clin Immunol. 2019;143(4):1636-1637.e5.
45. De Filippo C, Cavalieri D, Di Paola M, Ramazzotti M, Poullet JB, Massart S, et al. Impact of diet in shaping gut microbiota revealed by a comparative study in children from Europe and rural Africa. Proc Natl Acad Sci U S A. 2010;107(33):14691–6.
46. Wickens K, Stanley TV, Mitchell EA, Barthow C, Fitzharris P, Purdie G, et al. Early supplementation with Lactobacillus rhamnosus HN001 reduces eczema prevalence to 6 years: Does it also reduce atopic sensitization? Clin Exp Allergy. 2013;43(9):1048–57.
47. Canani RB, Sangwan N, Stefka AT, Nocerino R, Paparo L, Aitoro R, et al. Lactobacillus rhamnosus GG-supplemented formula expands butyrate-producing bacterial strains in food allergic infants. ISME J. 2016;10(3):742–50.
48. Hirata S, Kunisawa J. Gut microbiome, metabolome, and allergic diseases. Allergol Int. 2017;66(4):523–8.
49. Glatz M, Jo JH, Kennedy EA, Polley EC, Segre JA, Simpson EL, et al. Emollient use alters skin barrier and microbes in infants at risk for developing atopic dermatitis. PLoS One. 2018;13(2):e0192443.
50. Ozdemir O. Role and Use of Probiotics in Allergic Diseases: Review of the Literature. Istanbul Med J. 2018;19(2):95–104.
51. Batista D, Carvalho AP, Costa R, Coutinho R, Dobretsov S, Verstraete W, et al. Comparative integrated omics: identification of key functionalities in microbial community-wide metabolic networks. NPJ Biofilms Microbiomes. 2002;57(6):10–3.
52. Bridgman SL, Kozyrskyj AL, Scott JA, Becker AB, Azad MB. Gut microbiota and allergic disease in children. Ann Allergy, Asthma Immunol. 2016;116(2):99–105.

53. Ver Heul A, Planer, J. Kau AL. The Human Microbiota and Asthma. Clinic Rev Allerg Immunol. 2019;57**:**350–363. doi.org/10.1007/s12016-018-8719-7.
54. Hill MO. Diversity and evenness: a unifying notation and its consequences. Ecology. 1973;54(2):427–32.
55. Hurlbert SH. The nonconcept of species diversity: A Critique and alternative parameters. Ecology. 2012;52(4):577–86.
56. Lodge C, Tan D, Lau M, Dai X, Tham R, Lowe A, et al. Breastfeeding and asthma and allergies: A systematic review and meta-analysis. Acta Paediatr Int J Paediatr. 2015;104:38–53.
57. Waidyatillake NT, Dharmage SC, Allen KJ, Lodge CJ, Simpson JA, Bowatte G, et al. Association of breast milk fatty acids with allergic disease outcomes—A systematic review. Allergy Eur J Allergy Clin Immunol. 2018;73(2):295–312.
58. Kumar H, du Toit E, Kulkarni A, et al. Distinct Patterns in Human Milk Microbiota and Fatty Acid Profiles Across Specific Geographic Locations. Front Microbiol. 2016;7:1619. doi:10.3389/fmicb.2016.01619.
59. McGuire MK, McGuire MA. Got bacteria? The astounding, yet not-so-surprising, microbiome of human milk. Curr Opin Biotechnol. 2017;44:63–8.
60. Ruiz L, García-Carral C, Rodriguez JM. Unfolding the Human Milk Microbiome Landscape in the Omics Era. Front Microbiol. 2019;10:1378. doi:10.3389/fmicb.2019.01378.
61. Browne PD, Aparicio M, Alba C, et al. Human Milk Microbiome and Maternal Postnatal Psychosocial Distress. Front Microbiol. 2019;10:2333. doi:10.3389/fmicb.2019.02333.
62. Jiménez E, De Andrés J, Manrique M, Pareja-Tobes P, Tobes R, Martínez-Blanch JF, et al. Metagenomic analysis of milk of healthy and mastitis-suffering women. J Hum Lact. 2015;31(3):406–15.
63. Liu J, Williams B, Frank D, Dillon SM, Wilson CC, Landay AL. Inside Out: HIV, the Gut Microbiome, and the Mucosal Immune System. J Immunol. 2017;198(2):605–14.
64. Claassen-Weitz S, Gardner-Lubbe S, Nicol P, et al. HIV-exposure, early life feeding practices and delivery mode impacts on faecal bacterial profiles in a South African birth cohort. Sci Rep. 2018;8(1):5078. doi:10.1038/s41598-018-22244-6.
65. González R, Mandomando I, Fumadó V, Sacoor C, Macete E, Alonso PL, et al. Breast milk and gut microbiota in African mothers and infants from an area of high HIV prevalence. PLoS One. 2013;8(11):e80299.
66. Yu JE, Miller RL. Got milk? Understanding the farm milk effect in allergy and asthma prevention. J Allergy Clin Immunol. 2016;137(6):1707–8.
67. Le Hurou-Luron I, Blat S, Boudry G. Breast- v. formula-feeding: Impacts on the digestive tract and immediate and long-term health effects. Nutr Res Rev. 2010;23(1):23–36.

68. Bridgman SL, Azad MB, Field CJ, et al. Fecal Short-Chain Fatty Acid Variations by Breastfeeding Status in Infants at 4 Months: Differences in Relative versus Absolute Concentrations. Front Nutr. 2017;4:11. doi:10.3389/fnut.2017.00011.
69. Munblit D, Peroni DG, Boix-Amorós A, et al. Human Milk and Allergic Diseases: An Unsolved Puzzle. Nutrients. 2017;9(8):894. doi:10.3390/nu9080894.
70. Bode L. Human milk oligosaccharides: Prebiotics and beyond. Nutr Rev. 2009;67(Suppl. 2):S183–91.
71. Marcobal A, Sonnenburg JL. Human milk oligosaccharide consumption by intestinal microbiota. Clin Microbiol Infect. 2012;18(Suppl. 4):S12–5.
72. Bode L. The functional biology of human milk oligosaccharides. Early Hum Dev. 2015;91(11):619–22.
73. Le Doare K, Holder B, Bassett A, Pannaraj PS. Mother's Milk: A Purposeful Contribution to the Development of the Infant Microbiota and Immunity. Front Immunol. 2018;9:361. doi:10.3389/fimmu.2018.00361.
74. Cait A, Cardenas E, Dimitriu PA, Amenyogbe N, Dai D, Cait J, et al. Reduced genetic potential for butyrate fermentation in the gut microbiome of infants who develop allergic sensitization. J Allergy Clin Immunol. 2019;144(6):1638-1647.e3.
75. Nieto A, Wahn U, Bufe A, Eigenmann P, Halken S, Hedlin G, et al. Allergy and asthma prevention 2014. Pediatr Allergy Immunol. 2014;25(6):516–33.
76. van den Elsen LWJ, Garssen J, Burcelin R, Verhasselt V. Shaping the Gut Microbiota by Breastfeeding: The Gateway to Allergy Prevention? Front Pediatr. 2019;7:47. doi:10.3389/fped.2019.00047.
77. Guaraldi F, Salvatori G. Effect of breast and formula feeding on gut microbiota shaping in newborns. Front Cell Infect Microbiol. 2012;2:94. doi:10.3389/fcimb.2012.00094.
78. Howcroft R, Eriksson G, Lidén K. The Milky Way: The implications of using animal milk products in infant feeding. Anthropozoologica. 2012;47(2):31–43.
79. Moossavi S, Azad MB. Origins of human milk microbiota: new evidence and arising questions. Gut Microbes. 2019. doi:10.1080/19490976.2019.1667722.
80. Shi N, Li N, Duan X, Niu H. Interaction between the gut microbiome and mucosal immune system. Mil Med Res. 2017;4:14. doi:10.1186/s40779-017-0122-9.
81. Quigley L, O'Sullivan O, Stanton C, Beresford TP, Ross RP, Fitzgerald GF, et al. The complex microbiota of raw milk. FEMS Microbiol Rev. 2013;37(5):664–98.
82. Togo AH, Grine G, Khelaifia S, et al. Culture of Methanogenic Archaea from Human Colostrum and Milk. Sci Rep. 2019;9(1):18653. doi:10.1038/s41598-019-54759-x.
83. Oikonomou G, Addis MF, Chassard C, et al. Milk Microbiota: What Are We Exactly Talking About?. Front Microbiol. 2020;11:60. doi:10.3389/fmicb.2020.00060.

84. Rodríguez JM. The Origin of Human Milk Bacteria : Is There a Bacterial Entero-Mammary Pathway during Late Pregnancy and Lactation? Am Soc Nutr Adv Nutr. 2014;5:779–84.
85. Ramsay DT, Kent JC, Owens RA, Hartmann PE. Ultrasound Imaging of Milk Ejection in the Breast of Lactating Women. Pediatrics. 2004;113(2):361–7.
86. Addis MF, Tanca A, Uzzau S, Oikonomou G, Bicalho RC, Moroni P. The bovine milk microbiota: Insights and perspectives from -omics studies. Mol Biosyst. 2016;12(8):2359–72.
87. Giribaldi M, Ortoffi MF, Giuffrida MG, Gastaldi D, Peila C, Coscia A, et al. Effect of prolonged refrigeration on the protein and microbial profile of human milk. Int Dairy J. 2013;31(2):121–6.
88. Raats D, Offek M, Minz D, Halpern M. Molecular analysis of bacterial communities in raw cow milk and the impact of refrigeration on its structure and dynamics. Food Microbiol. 2011;28(3):465–71.
89. Hernell O. Human milk vs. cow's milk and the evolution of infant formulas. Nestle Nutr Work Ser Pediatr Progr. 2011;67:17–28.
90. Peroni DG, Piacentini GL, Bodini A, Pigozzi R, Boner AL. Transforming growth factor-β1 is elevated in unpasteurized cow's milk. Pediatr Allergy Immunol. 2009;20(1):42–4.
91. Sozańska B. Raw Cow's Milk and Its Protective Effect on Allergies and Asthma. Nutrients. 2019;11(2):469. doi:10.3390/nu11020469.
92. Abbring S, Hols G, Garssen J, van Esch BCAM. Raw cow's milk consumption and allergic diseases – The potential role of bioactive whey proteins. Eur J Pharmacol. 2019;843:55–65.
93. den Hartog G, Savelkoul HFJ, Schoemaker R, Tijhaar E, Westphal AH, de Ruiter T, et al. Modulation of human immune responses by bovine interleukin-10. PLoS One. 2011;6(3):e18188.
94. Brick T, Schober Y, Böcking C, Pekkanen J, Genuneit J, Loss G, et al. Ω-3 fatty acids contribute to the asthma-protective effect of unprocessed cow's milk. J Allergy Clin Immunol. 2016;137(6):1699-1706.e13.
95. Thijs C, Müller A, Rist L, Kummeling I, Snijders BEP, Huber M, et al. Fatty acids in breast milk and development of atopic eczema and allergic sensitisation in infancy. Allergy Eur J Allergy Clin Immunol. 2011;66(1):58–67.
96. Kemter AM, Nagler CR. Influences on allergic mechanisms through gut, lung, and skin microbiome exposures. J Clin Invest. 2019;129(4):1483–92.
97. Taponen S, McGuinness D, Hiitiö H, Simojoki H, Zadoks R, Pyörälä S. Bovine milk microbiome: a more complex issue than expected. Vet Res. 2019;50(1):44. doi:10.1186/s13567-019-0662-y.

98. Loss G, Depner M, Ulfman LH, Van Neerven RJJ, Hose AJ, Genuneit J, et al. Consumption of unprocessed cow's milk protects infants from common respiratory infections. J Allergy Clin Immunol. 2015;135(1):56-62.e2.
99. Knopfler M. How Compatible is Cow's Milk with the Human Immune System ? Sci J Lander Coll Arts Sci. 2016;9(2):182–90.
100. Bordoni A, Danesi F, Dardevet D, Dupont D, Fernandez AS, Gille D, et al. Dairy products and inflammation: A review of the clinical evidence. Crit Rev Food Sci Nutr. 2017;57(12):2497–525.
101. Van Neerven RJJ, Knol EF, Heck JML, Savelkoul HFJ. Which factors in raw cow's milk contribute to protection against allergies? J Allergy Clin Immunol. 2012;130(4):853–8.
102. Wijga AH, Smit HA, Kerkhof M, De Jongste JC, Gerritsen J, Neijens HJ, et al. Association of consumption of products containing milk fat with reduced asthma risk in pre-school children: The PIAMA birth cohort study. Thorax. 2003;58(7):567–72.
103. Mosley EE, McGuire MK, Williams JE, McGuire MA. Cis-9, Trans-11 Conjugated Linoleic Acid Is Synthesized from Vaccenic Acid in Lactating Women. J Nutr. 2006;136(9):2297–301.
104. Roduit C, Frei R, Ferstl R, Loeliger S, Westermann P, Rhyner C, et al. High levels of butyrate and propionate in early life are associated with protection against atopy. Allergy Eur J Allergy Clin Immunol. 2019;74(4):799–809.
105. Gehring U, Spithoven J, Schmid S, Bitter S, Braun-Fahrländer C, Dalphin JC, et al. Endotoxin levels in cow's milk samples from farming and non-farming families - The PASTURE study. Environ Int. 2008;34(8):1132–6.
106. Niebuhr M, Werfel T. Innate immunity, allergy and atopic dermatitis. Curr Opin Allergy Clin Immunol. 2010;10(5):463–8.
107. Zanoni I, Granucci F. Role of CD14 in host protection against infections and in metabolism regulation. Front Cell Infect Microbiol. 2013;3:32. doi:10.3389/fcimb.2013.00032.
108. Bieli C, Eder W, Frei R, Braun-Fahrländer C, Klimecki W, Waser M, et al. A polymorphism in CD14 modifies the effect of farm milk consumption on allergic diseases and CD14 gene expression. J Allergy Clin Immunol. 2007;120(6):1308–15.
109. Lluis A, Depner M, Gaugler B, Saas P, Casaca VI, Raedler D, et al. Increased regulatory T-cell numbers are associated with farm milk exposure and lower atopic sensitization and asthma in childhood. J Allergy Clin Immunol. 2014;133(2):551-559e10.
110. Kirchner B, Pfaffl MW, Dumpler J, Von Mutius E, Ege MJ. MicroRNA in native and processed cow's milk and its implication for the farm milk effect on asthma. J Allergy Clin Immunol. 2016;137(6):1893-1895.e13.

111. Michel S, Busato F, Genuneit J, Pekkanen J, Dalphin JC, Riedler J, et al. Farm exposure and time trends in early childhood may influence DNA methylation in genes related to asthma and allergy. Allergy Eur J Allergy Clin Immunol. 2013;68(3):355–64.
112. Oikonomou G, Machado VS, Santisteban C, Schukken YH, Bicalho RC. Microbial Diversity of Bovine Mastitic Milk as Described by Pyrosequencing of Metagenomic 16s rDNA. PLoS One. 2012;7(10):e47671.
113. Oikonomou G, Bicalho ML, Meira E, Rossi RE, Foditsch C, Machado VS, et al. Microbiota of cow's milk; distinguishing healthy, sub-clinically and clinically diseased quarters. PLoS One. 2014;9(1):e85904.
114. Falentin H, Rault L, Nicolas A, et al. Bovine Teat Microbiome Analysis Revealed Reduced Alpha Diversity and Significant Changes in Taxonomic Profiles in Quarters with a History of Mastitis. Front Microbiol. 2016;7:480. doi:10.3389/fmicb.2016.00480.
115. Jans C, Meile L, Kaindi DWM, Kogi-Makau W, Lamuka P, Renault P, et al. 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. Int J Food Microbiol. 2017;250:27–36.
116. Zhong Z, Hou Q, Kwok L, Yu Z, Zheng Y, Sun Z, et al. Bacterial microbiota compositions of naturally fermented milk are shaped by both geographic origin and sample type. J Dairy Sci. 2016;99(10):7832–41.
117. Kable ME, Srisengfa Y, Laird M, Zaragoza J, McLeod J, Heidenreich J, et al. The core and seasonal microbiota of raw bovine milk in tanker trucks and the impact of transfer to a milk processing facility. MBio. 2016;7(4):e00836-16.
118. De Beer H, Van Biljon A, Shale K. Microbial quality of milk, produced by small scale farmers in a peri-urban area in South Africa. African J Microbiol Res. 2010;4(17):1823–30.
119. Li N, Wang Y, You C, et al. Variation in Raw Milk Microbiota Throughout 12 Months and the Impact of Weather Conditions. Sci Rep. 2018;8(1):2371. doi:10.1038/s41598-018-20862-8.
120. Delavenne E, Mounier J, Déniel F, Barbier G, Le Blay G. Biodiversity of antifungal lactic acid bacteria isolated from raw milk samples from cow, ewe and goat over one-year period. Int J Food Microbiol. 2012;155(3):185–90.
121. Cremonesi P, Ceccarani C, Curone G, Severgnini M, Pollera C, Bronzo V, et al. Milk microbiome diversity and bacterial group prevalence in a comparison between healthy holstein friesian and rendena cows. PLoS One. 2018;13(10):e0205054.
122. Evivie SE, Huo GC, Igene JO, Bian X. Some current applications, limitations and future perspectives of lactic acid bacteria as probiotics. Food Nutr Res. 2017;61(1):1318034. doi:10.1080/16546628.2017.1318034.

123. Lamichhane P, Kelly AL, Sheehan JJ. Effect of milk centrifugation and incorporation of high-heat-treated centrifugate on the composition, texture, and ripening characteristics of Maasdam cheese. J Dairy Sci. 2018;101(7):5724–37.
124. Poulsen OM, Hau J, Kollerup J. Effect of homogenization and pasteurization on the allergenicity of bovine milk analysed by a murine anaphylactic shock model. Clin Exp Allergy. 1987;17(5):449–58.
125. Brick T, Ege M, Boeren S, et al. Effect of Processing Intensity on Immunologically Active Bovine Milk Serum Proteins. Nutrients. 2017;9(9):963. doi:10.3390/nu9090963.
126. Howard KM, Jati Kusuma R, Baier SR, Friemel T, Markham L, Vanamala J, et al. Loss of miRNAs during processing and storage of cow's (Bos taurus) milk. J Agric Food Chem. 2015;63(2):588–92.
127. Sipka S, Béres A, Bertók L, Varga T, Bruckner G. Comparison of endotoxin levels in cow's milk samples derived from farms and shops. Innate Immun. 2015;21(5):531–6.
128. Anukam KC, Reid G. African Traditional Fermented Foods and Probiotics. J Med Food. 2009;12(6):1177–84.
129. Beukes EM, Bester BH, Mostert JF. The microbiology of South African traditional fermented milks. Int J Food Microbiol. 2001;63(3):189–97.
130. Franz CMAP, Huch M, Mathara JM, Abriouel H, Benomar N, Reid G, et al. African fermented foods and probiotics. Int J Food Microbiol. 2014;190:84–96.
131. Mathara JM, Schillinger U, Kutima PM, Mbugua SK, Holzapfel WH. Isolation, identification and characterisation of the dominant microorganisms of kule naoto: The Maasai traditional fermented milk in Kenya. Int J Food Microbiol. 2004;94(3):269–78.
132. Ukeyima MT, Enujiugha, Sanni TA. Current applications of probiotic foods in Africa. African J Biotechnol. 2010;9(4):394–401.
133. Macori G, Cotter PD. Novel insights into the microbiology of fermented dairy foods. Curr Opin Biotechnol. 2018;49:172–8.
134. Chelule P, Mokoena M, Gqaleni N. Advantages of traditional lactic acid bacteria fermentation of food in Africa. Curr Res Technol Educ Top Appl Microbiol Microb Biotechnol. 2010;2:1160–7.
135. Baschali A, Tsakalidou E, Kyriacou A, Karavasiloglou N, Matalas A-L. Traditional low-alcoholic and non-alcoholic fermented beverages consumed in European countries: a neglected food group. Nutrition Research Reviews. Cambridge University Press. 2017;30(1):1–24.
136. Beermann C, Hartung J. Physiological properties of milk ingredients released by fermentation. Food Funct. 2013;4(2):185–99.
137. Narvhus JA, Gadaga TH. The role of interaction between yeasts and lactic acid bacteria in African fermented milks: A review. Int J Food Microbiol. 2003;86(1–2):51–60.

138. Branca F, Rossi L. The role of fermented milk in complementary feeding of young children: Lessons from transition countries. Eur J Clin Nutr. 2002;56:S16–20.
139. Paul Ross R, Morgan S, Hill C. Preservation and fermentation: Past, present and future. Int J Food Microbiol. 2002;79(1–2):3–16.
140. Chilton SN, Burton JP, Reid G. Inclusion of fermented foods in food guides around the world. Nutrients. 2015;7(1):390–404.
141. Bell V, Ferrão J, Fernandes T. Nutritional Guidelines and Fermented Food Frameworks. Foods. 2017;6(8):65. doi:10.3390/foods6080065.
142. Morrison DJ, Preston T. Formation of short chain fatty acids by the gut microbiota and their impact on human metabolism. Gut Microbes. 2016;7(3):189–200.
143. Vahdaninia M, Mackenzie H, Dean T, Helps S. ω-3 LCPUFA supplementation during pregnancy and risk of allergic outcomes or sensitization in offspring: A systematic review and meta-analysis. Ann Allergy, Asthma Immunol. 2019;122(3):302-313.e2.
144. Granier A, Goulet O, Hoarau C. Fermentation products: Immunological effects on human and animal models. Pediatr Res. 2013;74(2):238–44.
145. Akabanda F, Owusu-Kwarteng J, Tano-Debrah K, Glover RLK, Nielsen DS, Jespersen L. Taxonomic and molecular characterization of lactic acid bacteria and yeasts in nunu, a Ghanaian fermented milk product. Food Microbiol. 2013;34(2):277–83.
146. Osvik RD, Sperstad S, Breines E, Hareide E, Godfroid J, Zhou Z, et al. Bacterial diversity of amasi, a South African fermented milk product, determined by clone library and denaturing gradient gel electrophoresis analysis. African J Microbiol Res. 2013;7(32):4146–58.
147. Cissé H, Muandze-Nzambe JU, Somda NS, Sawadogo A, Drabo SM, Tapsoba F, et al. Assessment of safety and quality of fermented milk of camels, cows, and goats sold and consumed in five localities of Burkina Faso. Vet World. 2019;12(2):295–304.
148. Devirgiliis C, Barile S, Perozzi G. Metagenomic libraries from fermented dairy food products as a novel tool to improve food quality and safety. J Biotechnol. 2010;150:62–62.
149. Mufandaedza J, Viljoen BC, Feresu SB, Gadaga TH. Antimicrobial properties of lactic acid bacteria and yeast-LAB cultures isolated from traditional fermented milk against pathogenic Escherichia coli and Salmonella enteritidis strains. Int J Food Microbiol. 2006;108(1):147–52.
150. Borresen EC, Henderson AJ, Kumar A, Weir TL, Ryan EP. Fermented foods: patented approaches and formulations for nutritional supplementation and health promotion. Recent Pat Food Nutr Agric. 2012;4(2):134–40.
151. Gran HM, Wetlesen A, Mutukumira AN, Rukure G, Narvhus JA. Occurrence of pathogenic bacteria in raw milk, cultured pasteurised milk and naturally soured milk produced at small-scale dairies in Zimbabwe. Food Control. 2003;14(8):539–44.

152. Vorster HH, Wenhold F, Wright H, Wentzel-Viljoen E, Venter C, Vermaak M. “Have milk, maas or yoghurt every day”: a food-based dietary guideline for South Africa. S Afr J Clin Nutr. 2013;26(3)(Supplement):S57-65.
153. Du Plooy Z, Schönfeldt HC, Hall N. The role of traditional foods in food-based dietary guidelines – A South African case study on maas (cultured milk). Food Chem. 2018;238:22–8.
154. Chelule PK, Mokgatle MM, Zungu LI, Chaponda A. Caregivers’ Knowledge and Use of Fermented Foods for Infant and Young Children Feeding in a Rural Community of Odi, Gauteng Province, South Africa. Heal Promot Perspect. 2014;4(1):54–60.
155. Wenhold FAM, White Z. Dairy intake-related intentions, attitudes, subjective norms and perceived behavioural control of South African nutrition professionals. South African J Clin Nutr. 2017;30(2):27–33.
156. Reid G, Nduti N, Sybesma W, et al. Harnessing microbiome and probiotic research in sub-Saharan Africa: recommendations from an African workshop. Microbiome. 2014;2:12. doi:10.1186/2049-2618-2-12.
157. Bezie A, Regasa H. The Role of Starter Culture and Enzymes/Rennet for Fermented Dairy Products Manufacture-A Review. Nutr Food Sci Int J. 2019;9(2):555756.
158. Rook GAW, Brunet LR. Microbes, immunoregulation, and the gut. Gut. 2005;54(3):317–20.
159. Rowan-Nash AD, Korry BJ, Mylonakis E, Belenky P. Cross-Domain and Viral Interactions in the Microbiome. Microbiol Mol Biol Rev. 2019;83(1):e00044-18. doi:10.1128/MMBR.00044-18.
160. Mumcuoglu I. Interactions between Parasites and Human Microbiota. Eur J Ther. 2019;25(1):6–11.
161. Maizels RM, Mcsorley HJ, Smyth DJ. Helminths in the hygiene hypothesis: Sooner or later? Clin Exp Immunol. 2014;177(1):38–46.
162. Reynolds LA, Finlay BB, Maizels RM. Cohabitation in the Intestine: Interactions among Helminth Parasites, Bacterial Microbiota, and Host Immunity. J Immunol. 2015;195(9):4059–66.
163. Partida-Rodríguez O, Serrano-Vázquez A, Nieves-Ramírez ME, Moran P, Rojas L, Portillo T, et al. Human Intestinal Microbiota: Interaction Between Parasites and the Host Immune Response. Arch Med Res. 2017;48(8):690–700.
164. Scarpellini E, Ianiro G, Attili F, Bassanelli C, De Santis A, Gasbarrini A. The human gut microbiota and virome: Potential therapeutic implications. Dig Liver Dis. 2015;47(12):1007–12.
165. Faith JJ, Guruge JL, Charbonneau M, et al. The long-term stability of the human gut microbiota. Science. 2013;341(6141):1237439. doi:10.1126/science.1237439.

166. Domínguez-Díaz C, García-Orozco A, Riera-Leal A, Padilla-Arellano JR, Fafutis-Morris M. Microbiota and Its Role on Viral Evasion: Is It With Us or Against Us?. Front Cell Infect Microbiol. 2019;9:256. doi:10.3389/fcimb.2019.00256.
167. Robinson CM, Pfeiffer JK. Viruses and the Microbiota. Annu Rev Virol. 2014;1(1):55–69.
168. Huffnagle GB, Noverr MC. The emerging world of the fungal microbiome. Trends Microbiol. 2013;21(7):334–41.
169. Sam QH, Chang MW, Chai LY. The Fungal Mycobiome and Its Interaction with Gut Bacteria in the Host. Int J Mol Sci. 2017;18(2):330. doi:10.3390/ijms18020330.
170. Viljoen BC. The interaction between yeasts and bacteria in dairy environments. Int J Food Microbiol. 2001;69(1–2):37–44.
171. Grilli E, Tormo H, Fustini M, Deneufbourg C, Losio M, Formigoni A, et al. Is raw milk microbiota influenced by the use of live yeast (Saccharomyces cerevisiae) as ruminant feed additive? Int J Dairy Sci. 2016;11(3):124–9.
172. Katakura Y, Sano R, Hashimoto T, Ninomiya K, Shioya S. Lactic acid bacteria display on the cell surface cytosolic proteins that recognize yeast mannan. Appl Microbiol Biotechnol. 2010;86(1):319–26.
173. Hatoum R, Labrie S, Fliss I. Antimicrobial and probiotic properties of yeasts: from fundamental to novel applications. Front Microbiol. 2012;3:421. doi:10.3389/fmicb.2012.00421.
174. Amrane S, Lagier J-C. Metagenomic and clinical microbiology. Hum Microbiome J. 2018;9:1–6.
175. Abayasekara LM, Perera J, Chandrasekharan V, et al. Detection of bacterial pathogens from clinical specimens using conventional microbial culture and 16S metagenomics: a comparative study. BMC Infect Dis. 2017;17(1):631. doi:10.1186/s12879-017-2727-8.
176. Eisenhofer R, Minich JJ, Marotz C, Cooper A, Knight R, Weyrich LS. Contamination in Low Microbial Biomass Microbiome Studies: Issues and Recommendations. Trends Microbiol. 2019;27(2):105–17.
177. Kim D, Hofstaedter CE, Zhao C, et al. Optimizing methods and dodging pitfalls in microbiome research. Microbiome. 2017;5(1):52. doi:10.1186/s40168-017-0267-5.
178. Moya A, Ferrer M. Functional Redundancy-Induced Stability of Gut Microbiota Subjected to Disturbance. Trends Microbiol. 2016;24(5):402–13.
179. Forsberg A, West CE, Prescott SL, Jenmalm MC. Pre- and probiotics for allergy prevention: time to revisit recommendations? Clin Exp Allergy. 2016;46(12):1506–21.
180. Wang HT, Anvari S, Anagnostou K. The Role of Probiotics in Preventing Allergic Disease. Children (Basel). 2019;6(2):24. doi:10.3390/children6020024.

181. Kort R, Westerik N, Mariela Serrano L, et al. A novel consortium of Lactobacillus rhamnosus and Streptococcus thermophilus for increased access to functional fermented foods. Microb Cell Fact. 2015;14:195. doi:10.1186/s12934-015-0370-x.
182. Nishino T, Matsuda Y, Yamazaki Y. Separation of viable lactic acid bacteria from fermented milk. Heliyon. 2018;4(4):e00597. doi:10.1016/j.heliyon.2018.e00597.
183. Dianawati D, Mishra V, Shah NP. Survival of Microencapsulated Probiotic Bacteria after Processing and during Storage: A Review. Crit Rev Food Sci Nutr. 2016;56(10):1685–716.
184. Zhao W, Ho H-en, Bunyavanich S. The gut microbiome in food allergy. Ann Allergy, Asthma Immunol. 2019;122(3):276–82.
185. Sharma G, Im SH. Probiotics as a potential immunomodulating pharmabiotics in allergic diseases: Current status and future prospects. Allergy, Asthma Immunol Res. 2018;10(6):575–90.