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Bacterial microbiota composition of fresh unpasteurized cow's milk and home-made and commercially available fermented milk products

To the Editor,

The anti-allergic properties of raw and fermented cow's milk are being explored globally.[1,2]

Traditionally fermented milk (isiXhosa—"*amasi*") is consumed regularly by rural South African communities. To produce *amasi*, unpasteurized milk is left for three to five days to naturally ferment at room temperature. Commercially fermented *amasi* (CFA) is produced from pasteurized milk reseeded by microorganisms (commercially available starter cultures) and allowed to ferment. In South Africa, the process of commercial milk pasteurization is standardized and comprised heating to 72°C for at least 15 s and not longer than 25 s followed by rapid cooling.

The South African Food Sensitisation and Food Allergy (SAFFA) study demonstrated that children living in an urban environment had significantly higher rates of allergic diseases compared to their rural counterparts, and the consumption of fermented milk was associated with lower rates of allergic rhinitis, atopic dermatitis, and self-reported asthma.[3] Lactic acid–producing bacteria produce important by-products and end-products during milk fermentation which may contribute to their anti-inflammatory and anti-allergic properties.[4] In this study, we characterize and compare the bacterial microbiota in raw cow's milk (collected from urban and rural farms) and home-made and commercially fermented milk products by high-throughput 16S rRNA gene amplicon sequencing. This study received ethical approval from the University of Cape Town (animal ethics: 018_033).

Rural fresh cow's milk samples were collected from three farms in rural South Africa. Urban samples were collected from a farm in Cape Town. Before milking commenced, the cows and the udders were declared in a "heathy state" by each farmer. The udders were not cleaned or washed prior to milking, as these procedures were not included in the normal milking routine on the farms. Three samples from urban farms and two samples from rural farms were frozen within 1 hour of collection and transported frozen to the laboratory. A further sample of milk obtained from a separate farm in the rural area was sealed and left for five days at ambient temperatures to allow it to ferment naturally into *amasi*. Three different brands of commercially fermented *amasi* were obtained. All samples were analyzed by the Centre for Proteomic and Genomic Research (CPRG), Cape Town, South Africa.

DNA was extracted from the milk samples using the ZymoBIOMICS® DNA Miniprep Kit (Zymo Research). The V3-V4 variable region of the 16S rRNA gene was amplified from 2.5 ng to 25 ng of purified DNA by 25 cycles of PCR and barcoded for multiplexing using the Nextera® XT Index kit (Illumina) and KAPA HiFi DNA Polymerase (Roche®). The nine milk product samples, a positive control (ZymoBIOMICS® Microbial Community DNA standard), and a negative control (DNA suspension buffer) were included in the library preparation. The size of the libraries was verified using an Agilent® 2100 Bioanalyzer (Agilent). Library concentration was evaluated using the KAPA Illumina Library Quantification Kit (Roche). The libraries were sequenced on an Illumina MiSeq sequencer at the CPRG using MiSeq Reagent Kit v2 (Illumina®) to produce paired-end 250 base pair reads.

Illumina MiSeq read quality assessment and taxonomic profiling were performed on a high-performance compute cluster using a custom Nextflow pipeline [https://github.com/h3abionet/TADA], implementing FastQC[5] and MultiQC[6] for quality control, dada2[7] for ASV prediction, and the RefSeq-RDP 16S database (v3 May 2018) for taxonomic annotation.[8] All downstream analyses were performed in R, with custom functions [https://gist.github.com/kviljoen/97d36c689c5c9b9c39939c7a100720b9]. Taxa (merged at the lowest available taxonomic level, tax_glom.kv function) 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 ≤.05 and if at least one of the two groups compared had ≥60% of samples with the given amplicon sequence variant (ASV)/taxon or if the result of Fisher's exact test was significant (after multiple-testing correction by the Benjamini-Hochberg method), using the R package metagenomeSeq and custom function super.fitZig.kv.

Results showed that the fresh cow's milk samples (both urban and rural) had significantly higher numbers of amplicon sequence variants (ASVs) and identified taxa compared to *amasi* and CFA. All three CFA samples appeared similar and compared to *amasi* had lower numbers of ASVs and merged taxa. CFA had the lowest Simpson alpha diversity. The Shannon alpha diversity was high in the three urban fresh cow's milk samples and low in all the CFA samples. The Shannon alpha diversity of the two rural fresh cow's milk samples was markedly dissimilar (Figure 1). Principal coordinate analysis, based on Bray-Curtis distances, was used to examine the dissimilarities between different cow's milk samples' microbiota communities. The four differently sourced milk groups (rural fresh, urban fresh, *amasi*, and CFA) were strikingly dissimilar. The home-fermented *amasi* and the two rural fresh milk samples were uniquely dissimilar from each other and from all the other milk samples (Figure 2).

All three CFA products were dominated at the phylum level by lactic acid–producing bacteria, belonging to the *Firmicutes* (>98% abundance) and the *Proteobacteria* (<2% abundance). The *amasi* sample comprised approximately 50% *Firmicutes* and approximately 50% *Proteobacteria*. Rural fresh milk 1 was comprised almost completely of *Proteobacteria* with small percentages of *Bacteroidetes*, *Firmicutes*, *Candidatus*, and *Saccharibacteria* (Figure S1).

All three CFA products appeared remarkably similar at genus level, with very low richness, comprising of mainly *Lactococcus* (>75% relative abundance) and *Leuconostococcus* (24% relative abundance). The *amasi* sample appeared to have higher richness than commercially fermented milk, but less than the fresh milk samples. In *amasi*, *Lactococcus* had the highest relative abundance, but the genus *Leuconostococcus* was absent. Furthermore, in *amasi*, the genera *Kluyvera*, *Citrobacter*, *Streptococcus*, *Lactobacillus*, and *Salmonella* were present (Figure S2).

Although lactic acid–producing bacteria were identified in the fermented milk products, the presence of these organisms in the fresh milk samples was inconsistent. In *amasi* and in the CFA products, *Lactococcus lactis* was the most abundant organism. *Lactobacillus paracasei* was abundant in *amasi*, but almost absent in all the other milk samples (Figure 3).

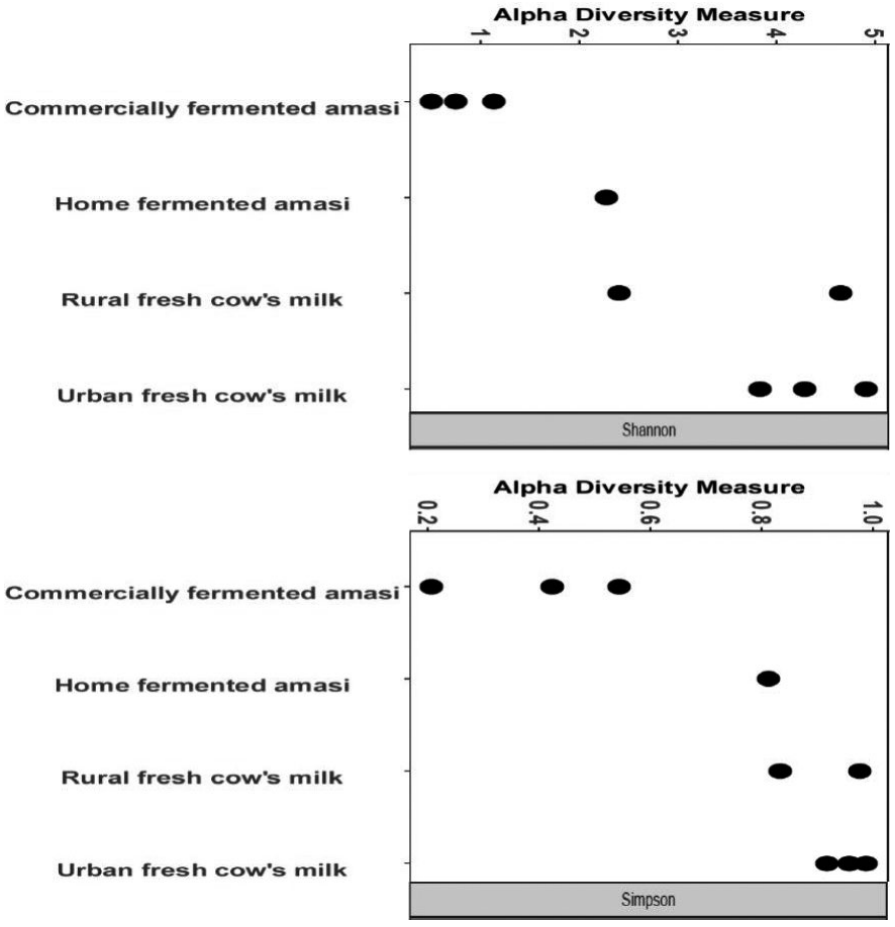

FIGURE 1 Simpson and Shannon alpha diversity of different cow's milk samples

Microbiota composition in fermented (*amasi* and all the combined CFA products) versus unfermented milk was compared. Because of the relatively small number of samples used in this study and the association between allergy protection and the consumption of fermented milk does not seem to be limited to *amasi* rather than CFA, all fermented milk samples were included for statistical comparison against the unfermented milk samples.

Results for differential abundance testing clearly show significant difference in *Lactococcus* and *Leuconostococcus* species between the fermented and unfermented milk groups. There are several taxa that uniformly dominated in CFA milk, contributing to its evenness and decreased diversity. *Lactococcus lactis* dominated in the fermented samples, including *amasi*. *Lactococcus chungangensis* had high abundance in all CFA products and was absent in *amasi* and present at exceptionally low levels in the fresh cow's milk products. *Leuconostococcus mesenteroides* and *Leuconostococcus pseudomesenteroides* were absent in *amasi*. *Lactobacillus paracasei* was abundant in *amasi*, with low to no occurrence in all the other samples (Figure S3).

Potential milk pathogens were also differentially abundant between fermented and unfermented milk. *Salmonella enterica* in addition to being present in all fresh cow's milk samples was also abundant in *amasi*, but absent in CFA. *Escherichia/Shigella* was present in urban fresh samples and absent in rural and fermented milk samples (Figure 3 and Figure S3).

In our study, the genus *Lactococcus* dominated all CFA samples and was also abundant in *amasi*. An important aim of our study was to compare the microbiota (at species level) of fermented versus unfermented milk to indicate whether fermentation (either commercially or home fermented) had significant influences on the occurrence of certain taxa. *Lactococcus lactis* in the fermented group reached the highest statistical difference between the two groups. *Lactococcus chungangensis*, *Leuconostococcus mesenteroides*, *Leuconostococcus pseudomesenteroides*, and *Lactobacillus paracasei* were also significantly more abundant in fermented milk. Potential human pathogens were identified in fresh cow's milk and *amasi*. Noteworthy, these organisms were absent in the commercially fermented products.

Our study was of small numbers and limited to bacteria without assessing viral, parasitic, or fungal differences. Concerns about contaminant DNA (eg, from the laboratory environment, laboratory technicians, and nucleic extraction kits) and cross-contamination (eg, DNA from other laboratory samples and sample runs) when analyzing low microbial biomass samples have been published.[9] These concerns may also be applicable to this study.

The consumption of fermented milk appears protective in our setting. Traditional home fermentation is time-consuming and reducing in frequency being supplanted by the ingestion of commercially fermented milk. Although low in diversity, commercially fermented milk appears safe and accessible to urban populations and its microbiota composition appears to be consistently abundant of lactic acid–producing bacteria. Because of its excellent nutritive properties, commercially sold *amasi* (and yogurt) is advised to children from one year of age in the recently updated South African food-based dietary guidelines.[10] Adoption of these guidelines might help to control the tendency of developing allergic diseases in populations undergoing urbanization.

KEYWORDS

allergy prevention, amasi, biodiversity, cow's milk, fermentation, microbiota, rural

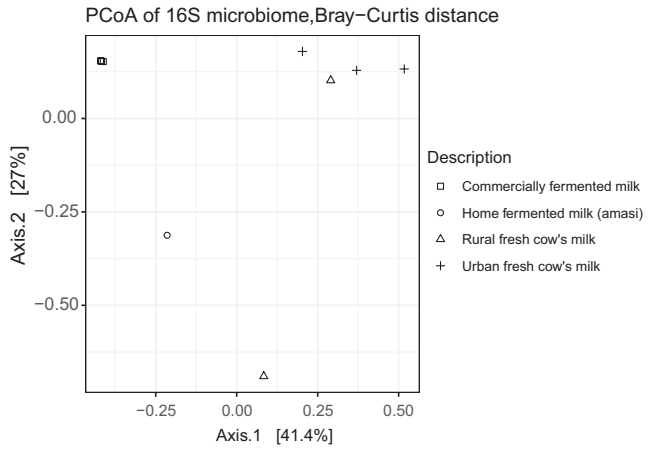

FIGURE 2 Principal coordinate analysis (PCoA) based on Bray-Curtis dissimilarity of different cow's milk samples

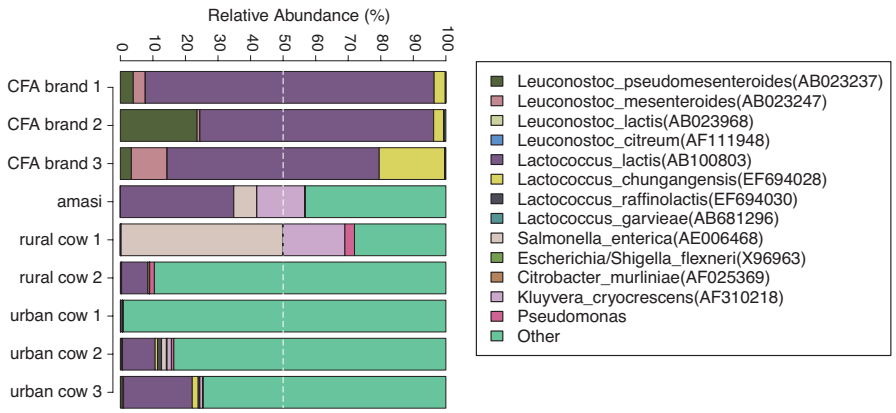

FIGURE 3 Composite bar graph of lactic acid–producing bacteria and pathogens with potential relevance to human health

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AUTHOR CONTRIBUTIONS

Pieter Johannes de Waal: Conceptualization (equal); Funding acquisition (equal); Investigation (equal); Methodology (equal); Project administration (equal); Writing-original draft (equal); Writing-review & editing (equal). **Shane Murray:** Formal analysis (equal); Investigation (equal); Methodology (equal); Writing-original draft (equal); Writing-review & editing (equal). **Katie Viljoen:** Data curation (lead); Formal analysis (lead); Investigation (equal); Methodology (equal); Software (lead); Visualization (lead); Writing-original draft (equal); Writing-review & editing (equal). **Jeanne Korsman:** Investigation (equal); Methodology (equal); Validation (equal); Writing-original draft (equal). **Michael Eliad Levin:** Conceptualization (lead); Investigation (lead); Methodology (lead); Project administration (lead); Supervision (lead); Validation (lead); Writing-original draft (lead); Writing-review & editing (lead).

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.