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Unveiling the Microbial Symphony of Amasi: A Targeted Metagenomic 16S rRNA, ITS, and Metabolites Insights Using Bovine and Caprine Milk

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Abstract: Amasi, a traditional fermented milk produced in Southern Africa, is associated with several health benefits, such as probiotic activities, immune system modulation, and pharmacological (antimicrobial, antitumor and antioxidant) potential. This study investigated the microbial diversity in Amasi (produced from cow's and goat's milk) through targeted metagenomic bacterial 16S rRNA and fungal ITS sequencing, the metabolic functional prediction of Amasi samples using the Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) and profiled amino acids constituents using Liquid Chromatographic-Mass Spectrophotometry (LC-MS). The results obtained revealed Firmicutes, Bacteroidetes, and Proteobacteria as the most prevalent bacterial phyla, with *Lactococcus* and *Lactobacillus* being the most abundant genera. On the other hand, Ascomycota, Basidiomycota, and Mucoromycota were the main fungal phyla, while *Aspergillus*, *Kazachstania*, and *Debaryomyces* spp. dominated the fungal genera. Also, *Pseudomonas* spp., *Bacillus* spp., *Clostridium* spp., *Cronobacter* spp., *Alternaria* spp., *Diaporthe* spp., and *Penicillium* spp. were the probable pathogenic bacteria and fungi genera found, respectively. *Atopobium*, *Synechococcus*, and *Parabacteroides* were found less often as rare genera. It was found that the amino acid and drug metabolism pathway prediction values in Amasi samples were significantly higher ($p < 0.05$) than in raw cow and goat milk, according to the inferred analysis (PICRUSt). The amino acid validation revealed glutamine and asparagine values as the most significant ($p < 0.05$) for Amasi cow milk (ACM) and Amasi goat milk (AGM), respectively. Comparatively, ACM showed more microbial diversity than AGM, though there were relative similarities in their microbiome composition. PICRUSt analysis revealed significant metabolites in the two Amasi samples. Overall, data from this study showed heterogeneity in microbial diversity, abundance distributions, metabolites, and amino acid balance between raw cow/goat milk and Amasi samples.

Keywords: Amasi; LC-MS; microbial diversity; probiotics; 16S rRNA; ITS metagenomics; PICRUSt

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1. Introduction

Fermented milk products are increasingly consumed globally because of their unique health benefits such as probiotic activities, immune system modulation, and biological and or pharmacological potentials including antimicrobial, antitumor, and antioxidant activity [1,2]. Fermented foods are considered functional food based on the mentioned scientific findings and various scientific studies continue to explore all the health benefits present in them [3,4]. Fermented milk products are made from an array of animal milk such as camel, buffalo, sheep, yak, horse, and donkey [5], but raw cow milk and goat milk remain the most popular milk used in domestic fermentation [5]. While cow milk is mostly used for both milk and meat production [6], goat milk is used due to its better mineral availability, improved digestibility and presence of more balanced fat and protein profiles when it is fermented [6,7].

Amasi is a traditional South African fermented dairy product usually made at the household level from unpasteurized milk [8]. Although starter cultures have been used to process Amasi, together with some other additions like skim milk powder and gelatin to enhance the nutritional, functional, and textural properties [9], the traditionally produced Amasi is mostly preferred especially among rural consumers who see the process as a means of upholding the African heritage [8,10,11]. The processing method used for fermentation remains a critical step in the processing of Amasi, as it influences its sour taste, nutritional component and distinct characteristics compared to other related dairy-fermented products [8,9].

Probiotics primarily contain the bacteria genera *Lacticaseibacillus*, *Leuconostoc*, *Pediococcus*, *Bifidobacterium*, and *Enterococcus*. However, the most used species as probiotics are *Lacticaseibacillus*, *Acidophilus*, *Bifidobacterium* spp., and *Lacticaseibacillus* casei [2]. Interestingly, yeast and fungi also contribute to food fermentation, possessing probiotic properties just like bacteria. Examples include Saccharomyces cerevisiae, *Saccharomyces boulardii*, *Kluyveromyces marxianus* and *Pichia kudriavzevii* [3,12]. While many commercially produced fermented dairy products use a specific starter culture to deliver desirable organoleptic and quality attributes to the product [13], traditional fermentation is a treasured cultural method that easily preserves leftover milk similarly enhancing distinctive ways of food consumption. It is worth mentioning that Amasi is endowed with many probiotic bacterial species [8].

The identification of bacteria and fungi communities in Amasi from both culture methods and metagenomic approaches (such as 16S rRNA) has been submitted in the literature [8]. However, very little information is available on the yeast and fungal community based on culture-independent methods especially when comparing the microbiota and mycobiota communities. Furthermore, little or no information is available on Amasi made with goat milk using culture-independent methods. Furthermore, the nature of traditionally fermented milk varies depending on the geographical location and the local indigenous microflora [14], necessitating a comprehensive microbiome report on Amasi.

Notwithstanding the afore mentioned, it is important to state that the available literature on Amasi has not focused on the intrinsic metabolites of microbiota responsible for their functionality. While these microbiotas can be studied using an online metabolite prediction database [15], validation of these metabolites produced by microbes during the traditional fermentation process is important and needed for scientific applications, hence the use of PICRUSt in this study. Furthermore, the role of amino acids in microorganisms extends beyond their role as building blocks of protein synthesis. They can also play a role in metabolic processes, survival, and virulence. Therefore, it is important to investigate the presence and validate these amino acids in Amasi. Surprisingly, based on the author's knowledge, limited information about amino acids in Amasi is available in the literature.

Hence, this study hopes to provide information on the microbial diversity comparison between Amasi produced from cow and goat milk, the metabolites profiling by PICRUSt, and the amino acids present in the final product.

This study investigated the microbial (bacterial and fungal) diversity of Amasi made from cow and goat milk using the metagenomics 16S rRNA and the internal transcribed spacer (ITS 1 and 2) approaches, while the effective PICRUSt was used to predict the metabolism of the microbiota present in Amasi, and the liquid chromatographic-mass spectrometry method was used for the validation of the amino acids that are present in the predicted metabolites that are present in Amasi samples.

2. Materials and Methods

2.1. Study Design and Sample Collection

Raw, unpasteurized goat and cow milk obtained from farms that provide direct consumer supply was used in this study. Three samples each from both cow and goat were collected in covered plastics from Khulusani Farms, Howick, Kwa-Zulu Natal Province, South Africa (Coordinates: −29.487152503271606 N, 30.21611986622293 E). The milk samples were transported on dry ice to the laboratory where they were kept frozen (−25 °C) until they were needed for analysis.

2.2. Preparation of Amasi-Fermented Product

Amasi was produced from cow and goat milk following the method of Kayitesi, Behera [10] with some modifications. The milk samples were allowed to thaw at room temperature (25 ± 2 °C) and were left to ferment naturally for 48 h in a plastic container. A muslin cloth was used to cover the fermenting substrate at room temperature (25 ± 2 °C). The plastics for fermentation were left on the bench during fermentation, with limited oxygen exposure, as no inoculum was added. All samples were packaged in an airtight container and kept frozen (−25 ± 2 °C) until needed for analysis.

2.3. pH and Acidity Determination

The pH (PH-200F, Chemistore, Kempton Park, South Africa) and acidity in terms of the lactic and acetic acids of Amasi samples were carried out according to the method described by Li [16].

2.4. Deoxyribose Nucleic Acid Extraction (DNA), Polymerase Chain Reaction (PCR) Amplification and Sequencing

Genomic DNA was extracted from the Amasi samples using the DNeasy Power Kit (Qiagen, San Diego, CA, USA) for liquid foods according to the manufacturer's instructions. The quality of the extracted DNA obtained in each case was analyzed using a Nanodrop spectrophotometer (NanoPhotometer, NP80 Mobile, Implen, München, Germany) and 2% agarose gel electrophoresis [17]. The 16S ribosomal DNA sequencing (16S rRNA) gene was amplified with primers targeting the hypervariable region V4 (forward primer: 5′ GTGYCAGCMGCCGCGGTAA 3′; reverse primer: 5′ GGACTACHVGGGTWTCTAATCC 3′) for bacteria and the internal transcribed spacer (ITS 1 and 2) for fungi. The PCR primers 515/806 with a barcode on the forward and reverse primer for fungi (ITS1F 5′ CTTGGTCATTTAGAGGAAGTAA 3′, ITS1R GCTGCGTTCTTCATCGATGC) were used. These were run in triplicates over 30–35 cycles using the HotStarTaq Plus Master Mix Kit (Qiagen, USA) [17]. Both the 16S and ITS reverse primers contained the barcode sequence. The V4 region of 16S rRNA and the ITS1 region of the ITS1 genes were amplified in 96-well microtiter plates. The reaction mixture consisted of 50 ng template DNA and a total volume of 50 mL using the HotStarTaq Plus Master Mix Kit from Qiagen. The barcoded reverse primers were obtained from www.mrdnalab.com (accessed

on 11 October 2021) (Shallowater, TX, USA). The cycling parameters included an initial denaturation at 95 °C for 5 min followed by 30 cycles of denaturation at 95 °C for 30 s, annealing at 53 °C for 40 s and elongation at 72 °C for 60 s. Finally, there was a 10 min extension at 72 °C. The PTC-100 thermal controller (BIO-RAD, Hercules, CA, USA) was used for all the reactions. A negative control without a template was included for each barcoded primer pair.

After amplification, the PCR products were subjected to a 2% agarose gel electrophoresis to determine the success of amplification and the relative intensity of bands (SYBR Safe, Invitrogen Co., Carlsbad, CA, USA). Multiple samples were pooled together in equal proportions based on their molecular weight and DNA concentrations. Pooled samples were purified using calibrated Ampure XP beads. The purified PCR product in each case was used to prepare the Illumina DNA library before sequencing at MR DNA (www.mrdnalab.com, Shallowater, TX, USA) on an Illumina MiSeq platform.

2.5. Bioinformatics Analyses

Quantitative Insights into Microbial Ecology 2 (QIIME2) and PICRUSt Analysis of 16S rRNA and ITS Sequences

Raw 16S rRNA sequences were processed on QIIME 2 software (v2023.2) [18]. Sequences were aligned and <150 bp, as well as ambiguous base pairs, were removed. Quality filtering was performed using a maximum expected error threshold of 1.0 and dereplicated. The dereplicated or unique sequences were denoised and the identified errors were removed, followed by chimera removal, to give a denoised sequence. Amplicon sequence variants (ASVs) were defined by clustering at 100% similarity. Final ASVs were further processed for taxonomic assignment (Greengenes database, v13.5) [19,20], with the alpha and beta diversities calculated with QIIME2. The functionality of the 16S rRNA bacteria was analyzed using PICRUSt (version 12.0) and pathways for metabolic functions were extracted from the clusters of orthologous groups (COG) database [15]. Phylogenetic and diversity analyses were performed against a curated database that was derived from the National Centre for Biotechnology Information (NCBI) (www.ncbi.nlm.nih.gov) (McDonald et al., 2012 [19]). Both the 16S rRNA and ITS gene data were given accession numbers with Bioproject number PRJNA1096667 at the GenBank database.

2.6. Sample Preparation, Extraction, and Untargeted Liquid Chromatography-Mass Spectrometry (LC-MS) for Amino Acids Determination

2.6.1. Sample Preparation

Amasi samples and raw milk were concentrated, freeze-dried (ZZKD, Manteste equipment, Zhengzhou, China), and stored at −80 °C for analysis. A precise weight of 2 g was placed in a centrifuge tube and sealed with methanol and formic acid. Amasi milk samples were weighed (LBX 6, Supplywise, Boksburg, South Africa), followed by the addition of methanol and formic acid, vortexed (S1-0286, Electro Scientific, Singapore), and extracted in an ultrasonic bath (SUS304, Masiye Labs, Alberton, South Africa). The supernatant was centrifuged (CF0201003, Afrimedics, Johannesburg, South Africa) at 14,000 rpm for 5 min, to obtain a clear supernatant for analysis.

2.6.2. Extraction

Two grams of Amasi milk samples were accurately weighed into a 50 mL centrifuge tube with a screw cap. Methanol, 15 mL at 50%, and 1% formic acid were added, and the tubes were tightly capped. Thereafter, the samples were vortexed for 1 min, followed by extraction in an ultrasonic bath for 1 h. Two ml of the sample was then withdrawn and centrifuged at 14,000 rpm for 5 min. The clear supernatant was then transferred into 1.5 mL glass vials for analysis [21].

2.6.3. Liquid Chromatograph Mass Spectrometry Analysis

In this study, a Waters Synapt G2 Quadrupole time-of-flight mass spectrometer was used and a Waters Acquity ultra-performance liquid chromatograph for high-resolution UPLC-MS (Waters, Milford, MA, USA) analysis, capturing ultraviolent (UV) and the mass spectrometry (MS) spectra simultaneously through photodiode Array detector and electrospray ionization, was also used

The MS parameters were adjusted for maximum sensitivity and resolution, and data were collected using scanning in MSE and resolution modes. Leucine enkaphalin was used as the reference mass, and sodium formate was calibrated. Separation was achieved on a Waters HSS T3 column. The injection volume was 2 µL, with solvents A and B containing 0.1% formic acid and acetonitrile. The gradient was linear, ranging from 100% solvent A to 28% B, then B increased to 40%, followed by washing and re-equilibration. A calibration curve was generated using catechin standards, and data were analyzed with MSDIAL and MSFINDER [21,22].

2.7. Statistical Analyses

The R software (version 4.4.0) was used for ASVs clustering [23]. Bacterial and fungal communities were compared and presented with ggplot2 (v3.2.0) and phyloseq (v1.44.0) packages [24]. The Shannon index and richness were estimated where applicable with PERMANOVA and ANOSIM tests for statistical significance in the measures. Statistically, significant variation (where applicable) was set at $p < 0.05$ and one-way analysis of variance (ANOVA) was performed on the predicted metabolites and the amino acids. The relationship between the milk samples was further confirmed with a heatmap and principal component analysis (PCA) plot which was generated using an online web tool ClustVis (https://biit.cs.ut.ee/clustvis/) (accessed on 25 December 2023). The *t*-test was used for the amino acids analysis and the *p*-values were evaluated using the statistical package for social sciences (SPSS) (IBM SPSS Statistics 29.0.0.0).

3. Results

3.1. Physicochemical Properties of Traditionally Fermented Amasi

Table 1 shows the physicochemical properties of the cow and goat milk Amasi samples. There are significant differences ($p < 0.05$) in overall acidity, with notable increases from raw to fermented Amasi. The transition from raw cow milk (RCM) (0.009) to Amasi cow milk (ACM) (0.011) and the increase from raw goat milk (RGM) (0.008) to Amasi goat milk (AGM) (0.012) illustrates the cumulative impact of fermentation on acidity levels. Statistically significant ($p < 0.05$) variations in acetic acid concentrations were noticed, with RCM (0.16) rising to ACM (0.42) during fermentation. Nonetheless, RGM (1.8) exhibits a notable reduction to AGM (0.16), indicating divergent fermentation profiles or microbial activity in goat milk. The conversion from raw to fermented milk leads to a significant increase in lactic acid concentration. For instance, RCM (0.42) considerably rises to ACM (1.05), and RGM (0.04) to AGM (0.99). The pH values in both raw and Amasi-fermented samples indicate a significant increase ($p < 0.05$). Raw cow milk (RCM, 6.68) and raw goat milk (RGM, 6.60) both exhibited a significant decrease ($p < 0.05$) in pH during fermentation to Amasi-fermented cow milk (ACM, 4.36) and Amasi-fermented goat milk (AGM, 4.43), indicating an increase in acidity after fermentation.

Table 1. Physicochemical analysis of Amasi-fermented milk.

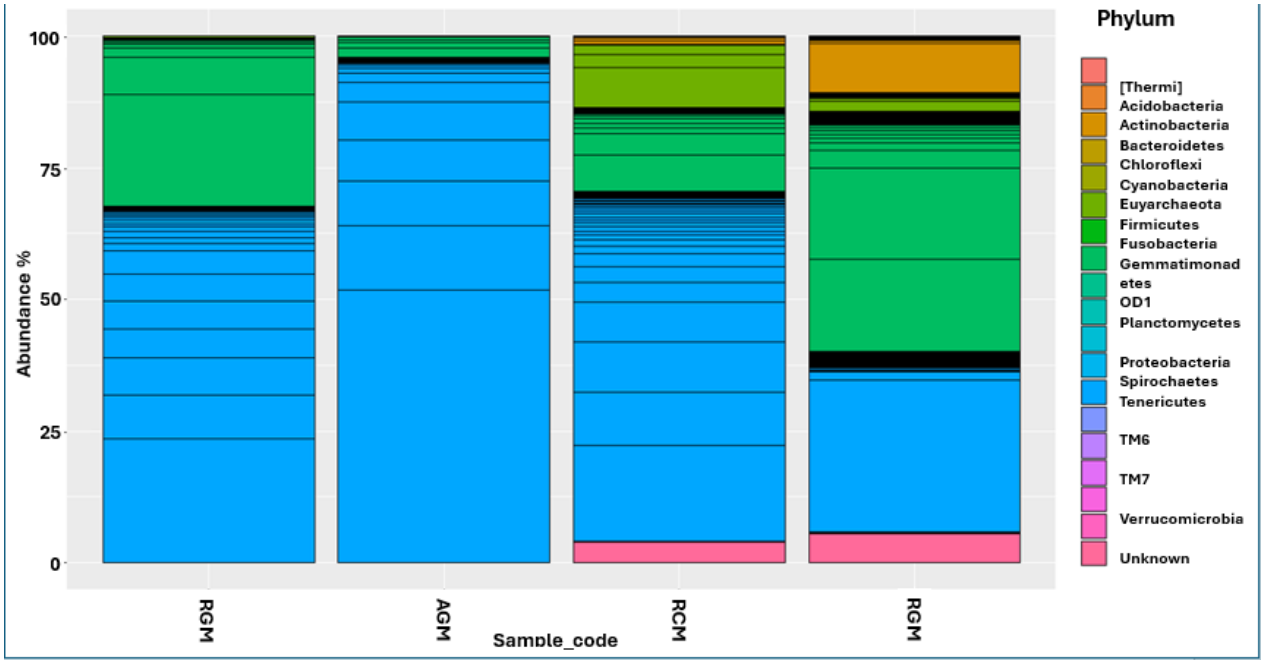
Sample_Codes	pH	Temperature	Lactic Acid (%)	Acetic Acid (%)	Total Acidity (%)
RCM	6.68 **	26.6 **	0.42 *	0.16 *	0.009 **
ACM	4.36 *	25.9 *	1.05 **	0.42 *	0.011 *
RGM	6.60 **	24 *	0.04 *	1.8 **	0.008 **
AGM	4.43 *	23.3 *	0.99 **	0.16 *	0.012 *

RCM—raw cow milk, ACM—Amasi-fermented cow milk, RGM—raw goat milk, and AGM—Amasi-fermented goat milk. Data were analyzed by one-way ANOVA with Tukey post-test. Asterisks (* and **) indicates a statistically significant difference when compared within groups, ** $p < 0.05$.

3.2. The Bacterial Composition of Amasi

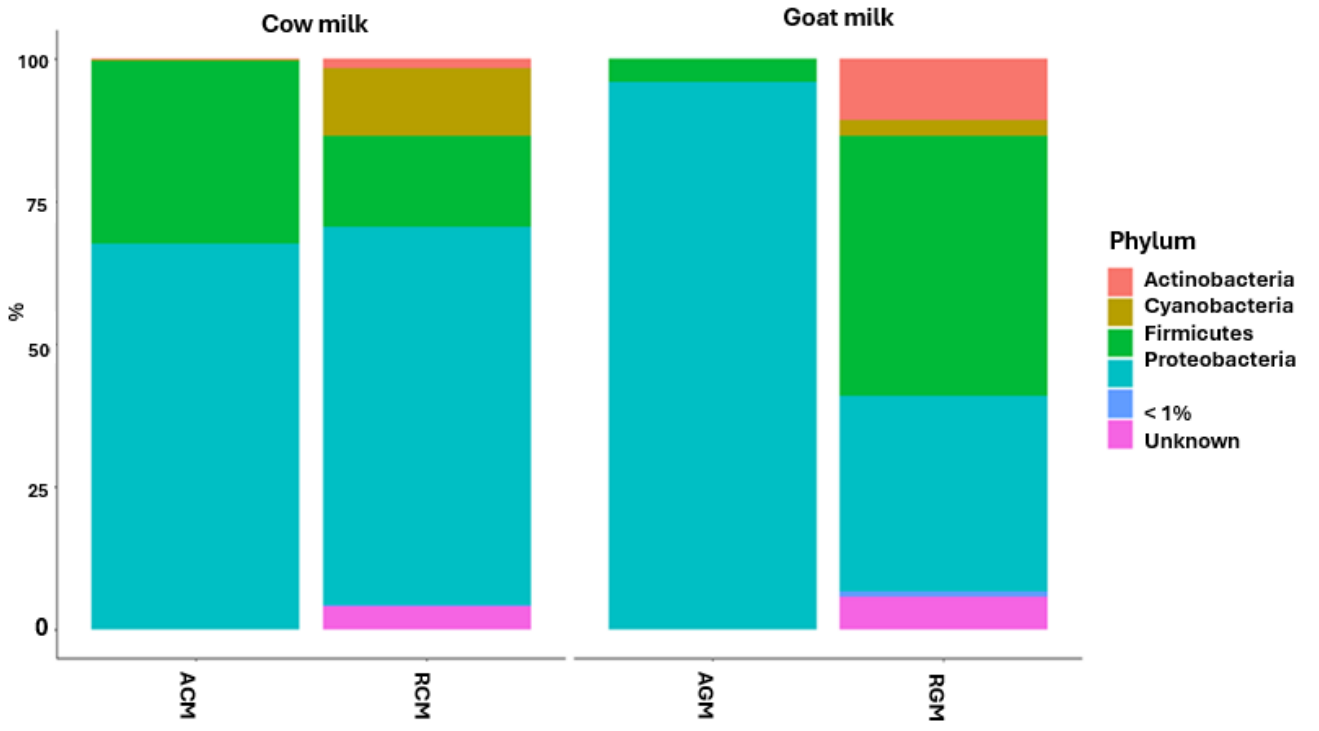
The Amasi milk variants generated 59,418,924 sequence reads with an 847 taxonomy classification after clustering to ASVs. The taxa were from 4 samples that had 14 sample variables with 842 internal nodes.

At the phylum level, the dominant bacterial communities in ACM were Proteobacteria (65%), Firmicutes (35%), and Actinobacteria (2.5%). In contrast, RCM exhibited Firmicutes (67.9%) as the most dominant phylum, followed by Proteobacteria (20.8%), Bacteroidetes (7.1%), and Actinobacteria (1.2%) (Figure 1A). In RGM, Proteobacteria (71.1%) surpassed Firmicutes (27.4%) in abundance, with Bacteroidetes (0.4%) and Actinobacteria (0.4%) showing the lowest abundance. AGM was characterized by the presence of Firmicutes (71.4%), Bacteroidetes (25.5%), Actinobacteria (1.9%), and Proteobacteria (1.0%) as the major phyla (Figure 1A,B). Overall, at the phylum level, the bacterial population or abundance decreased in both ACM and AGM, whereas RCM and RGM exhibited a similar but relatively varied abundance of the main bacterial phyla. The rare genera identified by the ASV included *Atopobium*, *Synechococcus*, *Parabacteroides*, and the *5-7N15* bacteria (Supplementary Table S1).

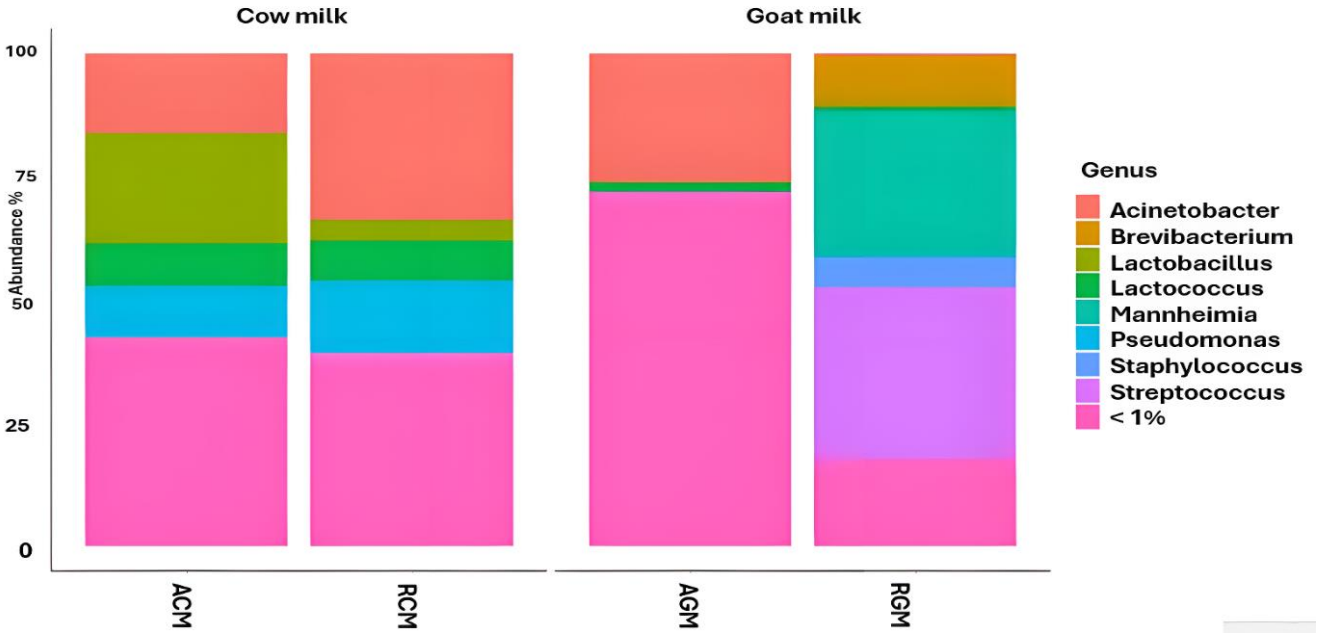


(**A**)

Figure 1. *Cont.*



(**B**)



(**C**)

Figure 1. (**A**–**C**) Bacteria relative abundance in Amasi cow and goat milk samples and top five most abundant phyla from Amasi cow and goat milk based on phylum (**A**,**B**) and genus (**C**) levels. RCM—raw cow milk, ACM—Amasi cow milk, RGM—raw goat milk, and AGM—Amasi goat milk. One percent indicates the rare taxa in each group, with a median relative abundance < 1%.

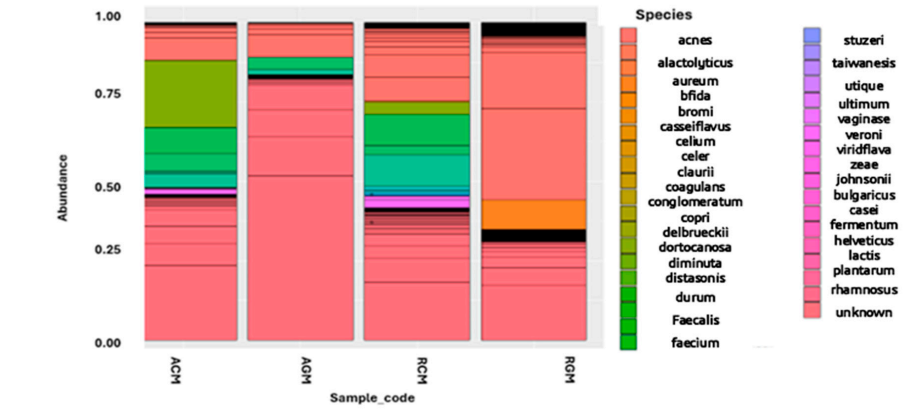
Regarding the relative abundance analyses at the genus level, ACM and AGM account for roughly 255 genera at the genus level, and 80% of all these genera are connected to the dairy microbiota (Figure 1C) (Supplementary Table S2). The overall four most abundant genera are *Lactobacillus* spp. (13.6%), *Lactococcus* spp. (7.6%), *Acinetobacter* spp. (5.6%), and *Mannheimia* spp. (3.8%). The most abundant genera for ACM were *Acinetobacter* spp. (31.6%), *Lactobacillus* spp. (20.2%), *Proteus* spp. (17.3%), and *Prevotella* spp. (12.7%). AGM

on the other hand revealed *Lactobacillus* spp. (42.9%), *Prevotella* spp. (25.5%), *Lactococcus* spp. (24.6%), and *Facklamia* spp. (3.32%) as most dominant (Supplementary Table S2). It is worth noting that the *Lactobacillus* genus was overhauled and divided into close to thirty genera in 2020, and bacterial database updates are still in progress. The prevalence of probiotics-related bacteria was found in milk variants used in this study, and they shared the same similarity for both ACM and AGM. While the *Lactobacillus* and *Lactococcus* genera increased for AGM, the genus *Acinetobacter* decreased in the ACM sample. RGM in terms of genus showed a relative abundance similar to the ACM, *Lactobacillus* and the *Acinetobacter* were common in both ACM and AGM, although, the abundance of these two genera was much higher in cow milk compared to goat milk. The RGM genera had a high abundance of *Streptococcus* (0.1%), *Brevibacterium* (0.07%), and *Pseudomonas* species (0.1%). However, *Lactobacillus* was very low and surprisingly a high count of the *Mannheimia* species was noticed in RGM (Supplementary Table S2).

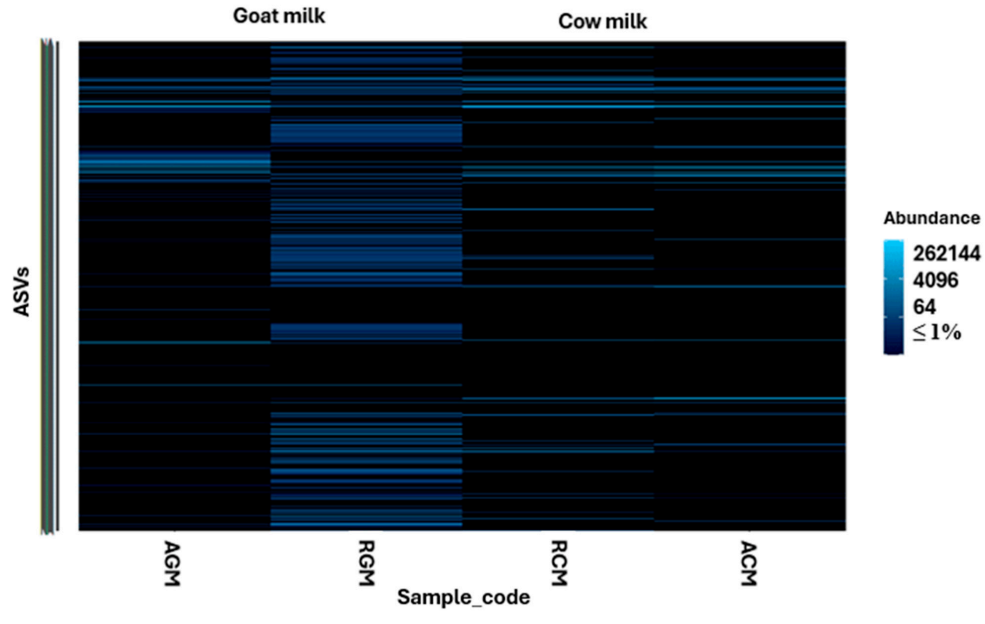
The data obtained regarding the species level showed there was an increase in the abundance of probiotic bacteria (Supplementary Table S3) in Amasi milk variants. Figure 2A shows the species level and the most abundant species for each sample. The overall predominant species are the *Lactobacillus kefiranofaciens* (Lk) (7.56%), *Lactococcus lactis* (4.6%), *Lactobacillus hamsteri* (3.31%), and *Lactobacillus raffinolactis* (3.07%). *Lactobacillus kefiranofaciens* was widely distributed as a core bacterial species in all the samples. ACM has 5.1% of Lk which was less when compared with RCM (5.93%). ACM also has *Acinetobacter lwoffii* (31.6%), *Streptococcus thermophilus* (1.07%), and *Lactobacillus hamsteri* (0.93%) which were increased compared to RCM which have a decreased relative abundance in *Acinetobacter lwoffii* (2.43%), *Lactobacillus raffinolactis* (1.32%), and *Lactococcus lactis* (1.25%). The common core bacteria species for AGM are similar to that of ACM, thus a relative abundance in *Lactobacillus raffinolactis* (24.46%) and *Lactobacillus hamsteri* (2.83%). ACM also has *Streptococcus thermophilus* (0.16%) which is one of the core bacteria species presents in Amasi milk variants. RGM surprisingly has *Acinetobacter guillouiae* (23.72%) as the highest core bacteria, and this bacterial species is also present in AGM and ACM but in small levels (Supplementary Table S3).

The relative abundance was depicted with the heatmap (Figure 2B), and the abundance of each bacterial taxa is represented by a specific color in the figure. The dark color indicates low abundance, and the lighter color (light blue color) indicates a high abundance of bacterial taxa for the Amasi milk variants. The Venn diagrams (Figure 2C) showed the genus abundance relationships between the core bacteria ASVs present in cow and goat milk Amasi and their relative abundance. The Venn diagram depicted the total number of core ASVs of the most abundant genus present in both cow and goat milk. Only 4 ASVs and 24 core ASVs were present in cow milk samples alone, while only 1 core ASV was present in the goat milk samples.

At the phylum level, the unique phyla in RCM were Acidobacteria and TM7, and ACM samples were unique in Chloroflexi while Gemmatimonadetes was only found in RGM samples. There was no exclusive phylum identified in AGM samples; however, Actinobacteria, Bacteroidetes, Cyanobacteria, and Firmicutes were common to both ACM and AGM (Supplementary Table S1).



(**A**)



(**B**)

(**C**)

Figure 2. (**A**–**C**) Bacteria relative abundance in Amasi cow and goat milk based on species (**A**), and the hierarchical clustering of core ASVs in the four experimental groups, divided by quadrants (**B**). The black background represents 0 counts, whereas the light blue color indicates higher counts for that taxonomic unit in a specific sample. Venn diagram of core bacterial ASVs present in cow and goat milk Amasi (**C**). RCM—raw cow milk, ACM—Amasi cow milk, RGM—raw goat milk, and AGM—Amasi goat milk. One percent indicates the rare taxa in each group, with a median relative abundance < 1.

At the genus level, six exclusive genera were found in ACM: *Leucobacter*, *Planomicrobium*, *T78*, *Gordonia*, *Termicanus*, and *Cryocola*. Among these, *Leucobacter* was among the top 20 most abundant genera. RCM exhibited 37 exclusive genera, with *Acetobacter* and *Streptococcus* being unique. RGM had 14 exclusive genera, with only *Propionibacterium* being unique. AGM had two exclusive genera, *Inquilinus* and *Alloiococcus*, neither of which appeared in the unique genera list. The common genera to all samples are the *Acinetobacter*, *Lactobacillus*, *Proteus*, *Prevotella*, *Mannheimia*, *Staphylococcus*, *Lactococcus*, *Brevundimonas*, *Corynebacterium*, *Brevibacterium*, *Jeotgalicoccus*, *Clostridium*, and *Pseudomonas*. *Shewanella*, *Selenomonas*, *Anaerococcus*, *Bacillus*, *Sediminibacterium*, *5-7N15*, *Peptoniphilus*, *Leuconostoc*, *Dorea*, *Enterococcus*, and *Fusobacterium* were all exclusive to RGM and RCM alone (Supplementary Table S2). The species level has seven common bacteria species: *Lactobacillus kefiranofaciens*, *Lactococcus raffinolactis*, *Lactococcus lactis*, *Brevundimonas diminuta*, *Streptococcus thermophilus*, *Lactobacillus hamsteri*, and *Brevibacterium aureum* across all four samples (Supplementary Table S3). RCM exhibits 15 exclusive species, including *Acinetobacter lwoffii*, *Lactobacillus johnsonii*, *Streptococcus alactolyticus*, and *Acinetobacter guillouiae*. These species occur in higher counts in RCM. Additionally, species such as *Pythium ultimum*, *Lactobacillus rhamnosus*, *Roseomonas mucosa*, *Lactobacillus bulgaricus*, *Ruminococcus bromii*, *Lactobacillus* delbrueckii, *Atopobium vaginae*, *Brachybacterium conglomeratum*, and *Psychrobacter pulmonis* are exclusively found in RCM. Interestingly, there are no species exclusively present in both ACM and AGM when compared. A similar trend was observed for RCM and RGM, where all species found in RCM and RGM are also found in either ACM and AGM or both. Notably, probable pathogenic species present in ACM and AGM fermented products include *Pseudomonas* spp. (47%), *Bacillus* spp. (0.2%), *Clostridium* spp. (1.1%), and *Cronobacter* (0.27%) (Supplementary Table S3).

The alpha diversity metrics were presented using the observed richness, Shannon, and Simpson metrics (Figure 3A). The observed metrics based on the abundance showed a significant difference ($p < 0.05$) between RGM and AGM. For the cow milk, there was a much closer relationship in the observed genus and species from both RCM and ACM. The Shannon diversity indicates the uniformity of the genera within the samples and the disparity in the number of taxonomic groups. RCM (3.05) had the highest index, and AGM had the lowest (1.75) ($p < 0.05$). The ACM and RGM are in the middle to signify a close relationship in terms of taxonomic distribution. The same trend was highlighted for the Simpson diversity except for RGM and ACM which displaced each other compared to Shannon diversity in the Amasi milk variants (Figure 3A). The beta diversity index measures the similarity and the dissimilarity of species. Based on the results obtained (Figure 3B), the beta diversity based on Non-metric Multidimensional Scaling (NMSD) (not normalized to one) showed AGM with the lowest score of −6.0 and RCM with the highest score of 0.21, which is statistically significant ($p < 0.05$). RCM and ACM had values in between the two (Figure 3B). The updated taxonomy correction for the bacterial genus and species mentioned in this study can be found in the List of Prokaryotic Names with Standing in Nomenclature, LPSN (https://lpsn.dsmz.de/).

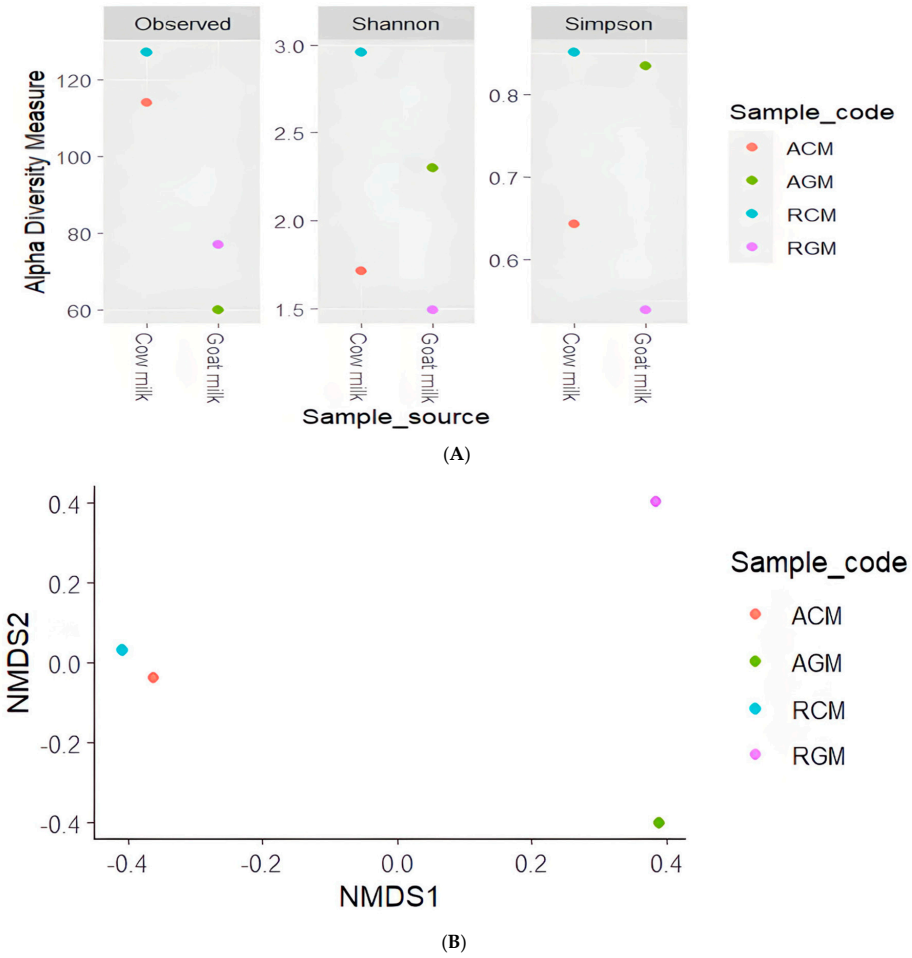


Figure 3. (**A**,**B**) Alpha diversity and beta diversity measure bacteria abundance in Amasi. RCM—raw cow milk, ACM—Amasi-fermented cow milk, RGM—raw goat milk, and AGM—Amasi-fermented goat milk.

3.3. Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) of Fermented Amasi

The functionalities of the 16S rRNA bacteria using PICRUSt and pathways for metabolic functions were extracted from the database of Clusters of Orthologous Groups of proteins (COG) and the result was categorized into different metabolic pathways (Supplementary Table S6). The relative abundance of the metabolic pathways were categorized into different metabolic pathways including amino acid metabolism (AAs), carbohydrate metabolism, energy metabolism, lipid metabolism, nucleotide metabolism, xenobiotics biodegradation metabolism (XB), metabolism of cofactors and vitamins (CVs), degradation of aromatic compounds (DA), metabolism of terpenoids and polyketides (TP) (diseases, drugs pathway and development, etc.) significantly increased ($p < 0.05$) for Amasi samples compared to both raw cow and goat milk (Figure 4A–C). The fermented milk samples (ACM and AGM) showed a significant increase ($p < 0.05$) in the metabolites that relate to nutrition, energy, diseases, and drug discovery pathways (Figure 4A). Generally, ACM showed a significant increase ($p < 0.05$) in most of the identified pathways compared to AGM. ACM produced the highest AAs and had the lowest XB metabolism (Figure 4A). The carbohydrates, glycans, nucleotides, and vitamin pathways also showed a significant increase ($p < 0.05$) in ACM when compared to AGM (Figure 4A). Importantly, the drug development pathways, chemical structures, and disease metabolites increased in the fermented products but more significantly increased ($p < 0.05$) in ACM than in AGM (Figure 4C). AGM had the highest metabolites in the nucleotides, CVs, and the AAs

pathways, respectively, and had a significant increase ($p < 0.05$) when compared to RGM (Figure 4A–C). RCM has increased metabolites in terms of predicted metabolites quantity as the functional classification showed that ACM has a significant increase ($p < 0.05$) compared to RGM. The most abundant class of the predicted metabolites in the overall samples are the nutrition-related metabolites such as AAs, carbohydrates, lipids, and energy. The fermented samples also showed a significant increase ($p < 0.05$) in the diseases and drug-related pathways (Figure 4C). RCM showed a more significant increase ($p < 0.05$) in metabolites than RGM, while ACM also had more metabolites than RGM. The heatmap (Figure 4D) shows the trend of metabolite distribution in all the samples where darker and lighter color shades represent higher and lower concentrations of the predicted metabolites, respectively.

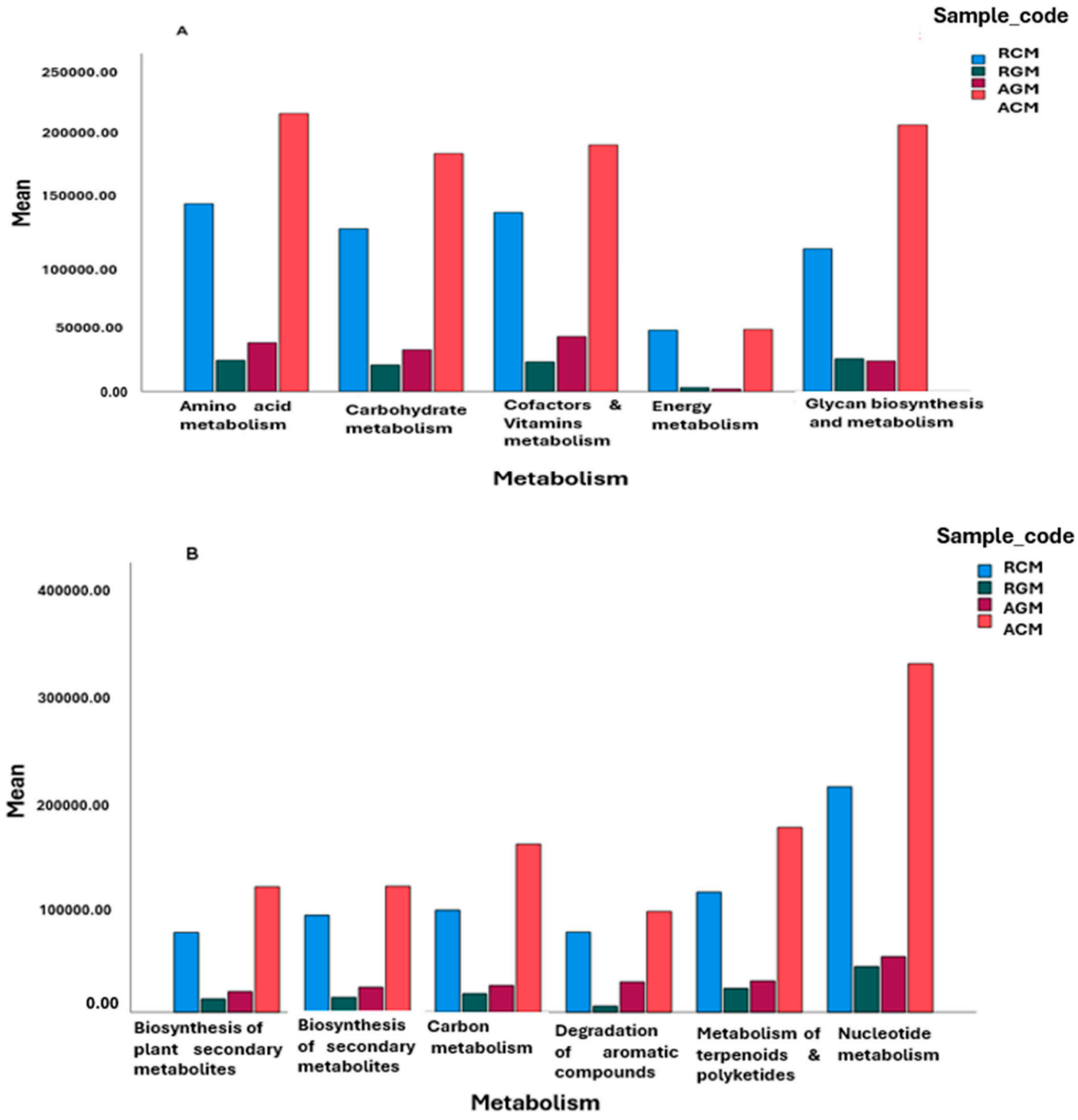
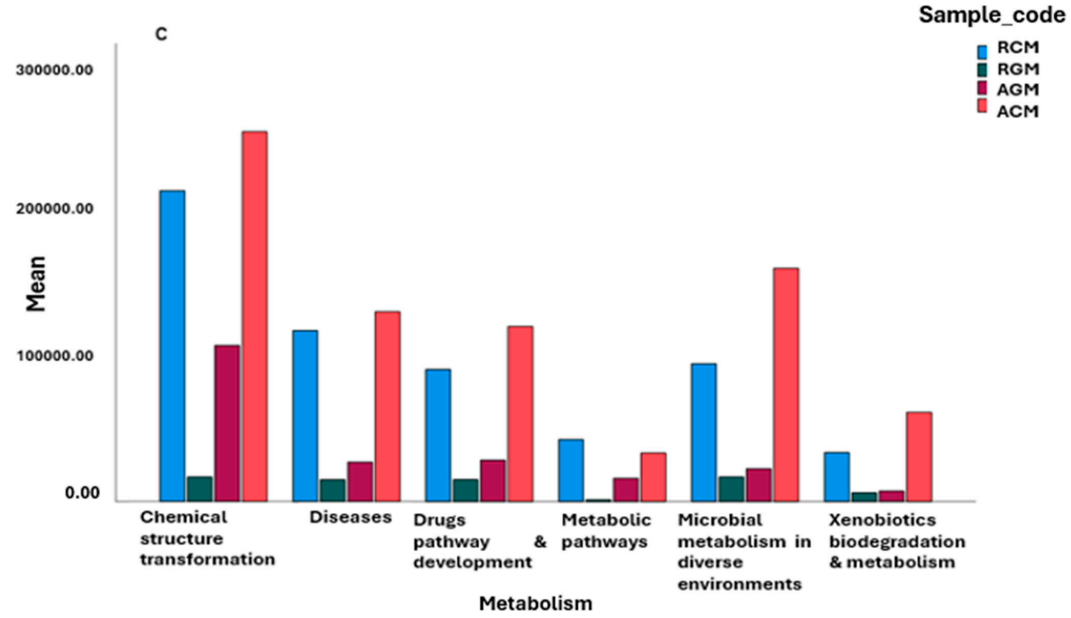
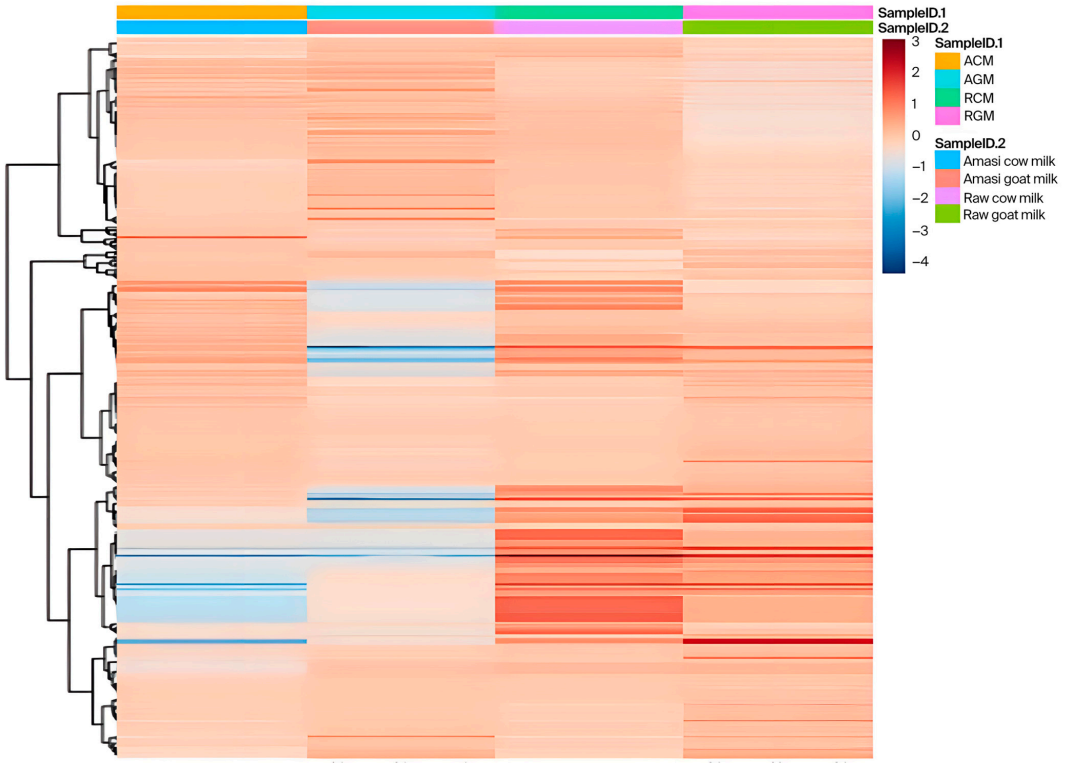


Figure 4. *Cont.*





(**D**)

Figure 4. (**A**–**D**): Functional prediction of metabolism (**A**,**B**) disease and drug pathways (**C**) and the heatmap metabolites distribution (**D**). The lighter color represents 0 counts, whereas the dark color shade indicates higher counts for that taxonomic unit in a specific sample. RCM—raw cow milk, ACM—Amasi-fermented cow milk, RGM—raw goat milk, and AGM—Amasi-fermented goat milk. Bars indicate the mean ± SD. Data were analyzed by one-way ANOVA with Tukey post-test. Asterisk indicates a statistically significant difference when compared within groups, $p < 0.05$.

3.4. The Fungal Composition of Amasi

The fungal composition of Amasi and the raw milk samples (cow and goat) were also investigated using the same milk samples. The ITS 1 and 2 regions generated 546,778 reads with 403 taxonomy classifications from six samples with 14 variables.

Figure 5A illustrates the fungal composition at the phylum level across all samples. Ascomycota dominates (71.3%), followed by Basidiomycota (18.4%), Mucoromycota (1.7%), and Rozellomycota (0.25%). At the genus level, *Aspergillus* (12.9%), *Kazachstania* (5.7%), *Debaryomyces* (4.7%), *Kluyveromyces* (4.7%), and *Penicillium* (3.7%) are most abundant. Noteworthy species include *Aspergillus penicillioides* (5.7%), *Debaryomyces prosopidis* (4.7%), *Kluyveromyces marxianus* (3.7%), *Kazachstania turicensis* (3.23%), *Kazachstania diaspora* (2.2%), and *Candida sake* (1.74%) (Figure 5A).

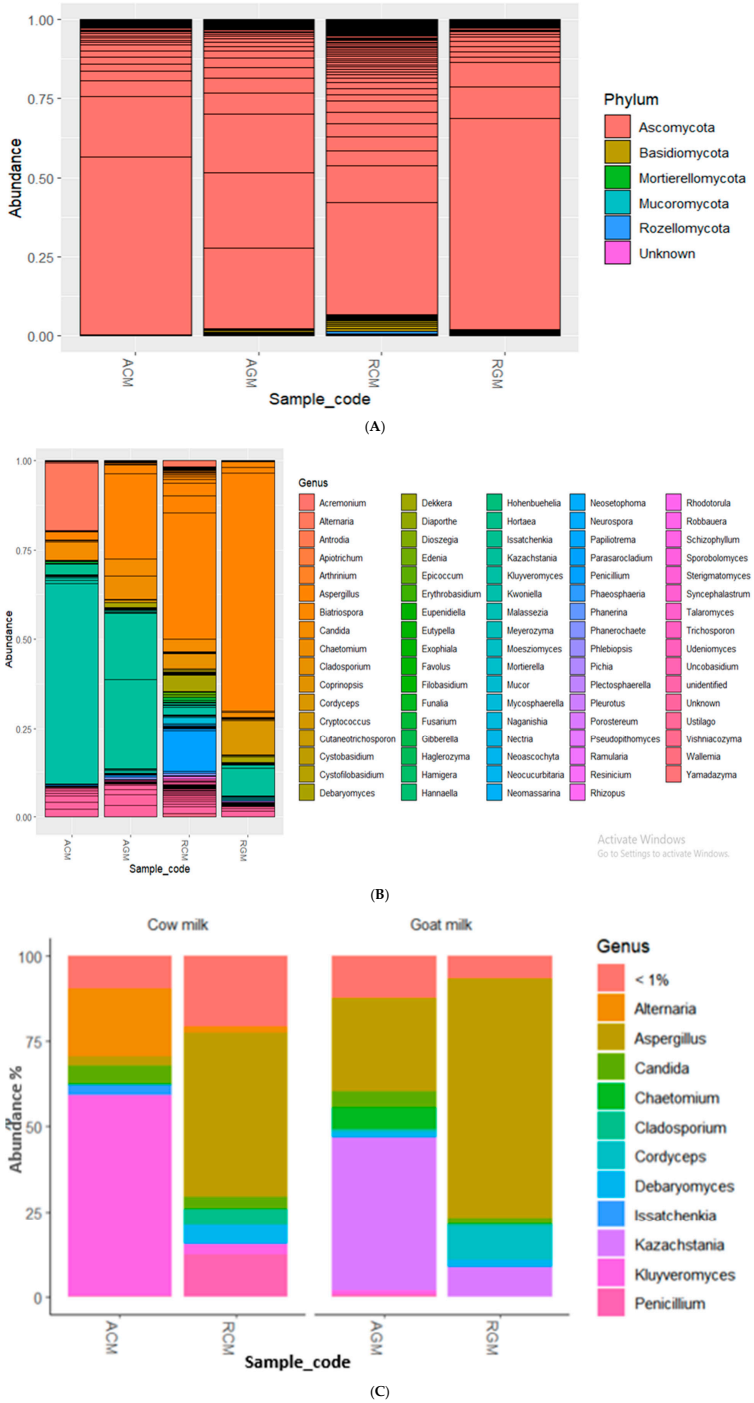


Figure 5. *Cont.*

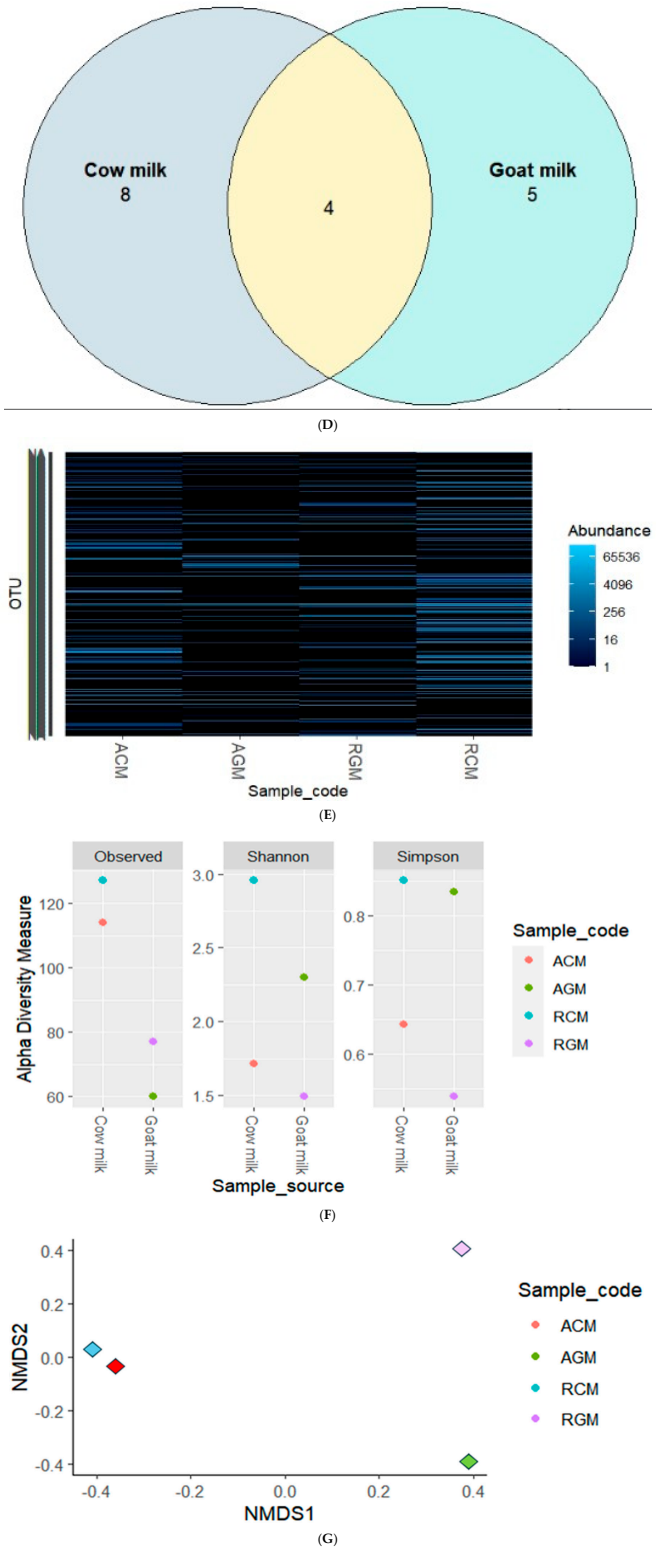


Figure 5. (**A**–**G**) Relative abundance of fungi in Amasi based on phylum (**A**), genus (**B**), the Venn diagram of core fungal ASVs present in Amasi (**C**), and the hierarchical clustering of core ASVs in the four experimental groups (Heatmap), divided by quadrants (**D**). The black background represents 0 counts, whereas the light blue color indicates higher counts for that taxonomic unit in a specific sample. Alpha diversity measures fungal abundance in Amasi-fermented milk. Beta diversity measures fungal abundance in Amasi. RCM—raw cow milk, ACM—Amasi-fermented cow milk, RGM—raw goat milk, and AGM—Amasi-fermented goat milk. One percent indicates the rare taxa in each group, with a median relative abundance < 1.

In the case of RCM, Ascomycota (95%) and Basidiomycota (4.8%) dominate at the phylum level (Figure 5A) (Supplementary Table S4). The prominent genera are *Penicillium* spp. (29.8%), *Aspergillus* spp. (24.3%), *Debaryomyces* spp. (12%), and *Candida* spp. (6.1%). Notable species taxa include *Debaryomyces prosopidis* (12%), *Aspergillus penicillioides* (10.7%), *Candida sake* (6.1%), *Alternaria angustiovoidea* (4.9%), and *Cladosporium tenuissimum* (3.64%) (Figure 5B,C) (Supplementary Table S6). After RCM fermentation to Amasi (ACM), there is a noticeable shift in the phylum relative abundance. Ascomycota abundance in ACM increases from 99.7% to nearly 100%, while Basidiomycota abundance level decreases to 0.22% (Figure 5A; Supplementary Table S4). At the genus level, *Kluyveromyces* spp. (83.1%) dominates, followed by *Candida* spp. (5.58%), *Issatchenka* spp. (1.41%), and *Aspergillus* spp. (1.1%) which are the core genera present (Figure 5B,C; Supplementary Table S5). Species levels include *Kluyveromyces marxianus* (82.9%), *Candida sake* (5.2%), *Issatchenkia orietalis* (1.4%), *Aspergillus penicillioides* (0.96%), and *Candida inconspicua* (0.6%) (Supplementary Table S6).

For RGM, the phyla are Ascomycota (97.6%), Basidiomycota (1.3%), and Mucoromycota (0.3%) (Figure 5A; Supplementary Table S4). Compared to RCM, RGM is more diverse due to the presence of Rozellomycota. Notable genera in RGM include *Aspergillus* spp. (58.6%), *Cordyceps* spp. (14.8%), *Kazachastina* spp. (11.9%), *Debaryomyces* spp. (2.8%), and *Candida* spp. (2.6%) (Figure 5B,C; Supplementary Table S5). Species observed include *Aspergillus penicillioides* (56.2%), *Kazachanista unispora* (11.1%), *Debaryomyces prosopidis* (2.8%), *Aspergillus conicus* (2.3%), and *Candida sake* (2.1%) (Supplementary Table S6).

The Venn diagram for fungal ASV indicates the core/unique ASVs present in cow and goat milk Amasi (Figure 5D). The total core ASVs present in the samples were 17, 8 ASVs were common to cow milk and 5 ASVs were common to goat milk. However, four core ASVs were common to both cow, goat, and Amasi samples.

In terms of phyla level, five different phyla (Ascomycota, Basidiomycota, Mortierellomycota, Mucoromycota, and Rozellomycota) were present and there were no exclusive phyla to any of the samples. Mucoromycota is exclusive to ACM, RGM, and AGM and Rozellomycota is exclusive to both RGM and AGM (Supplementary Table S4).

At the genus level, 14 fungi were common to all samples, and *Aspergillus*, *Penicillium*, *Debaryomyces*, *Cladosporium*, *Candida*, and *Kluyveromyces* are among the unique fungal genera (Supplementary Table S5). The exclusive genera for ACM are *Filobasidium*, *Apiotrichum*, *Haglerozyma*, and *Diaporthe* but only *Parasarocladium* is present in RCM. RGM-exclusive genera are *Coprinopsis*, *Eutypella*, *Naganishia*, *Exophiala*, *Eupenidiella*, *Sporobolomyces*, *Kwoniella*, *Schizophyllum*, *Arthrinium*, *Pleurotus*, and *Syncephalastrum*. AGM has *Acremonium*, *Talaromyces*, and *Moesziomyces*. Furthermore, no genus is exclusive to both AGM and ACM only, likewise for RCM and RGM (Supplementary Table S5).

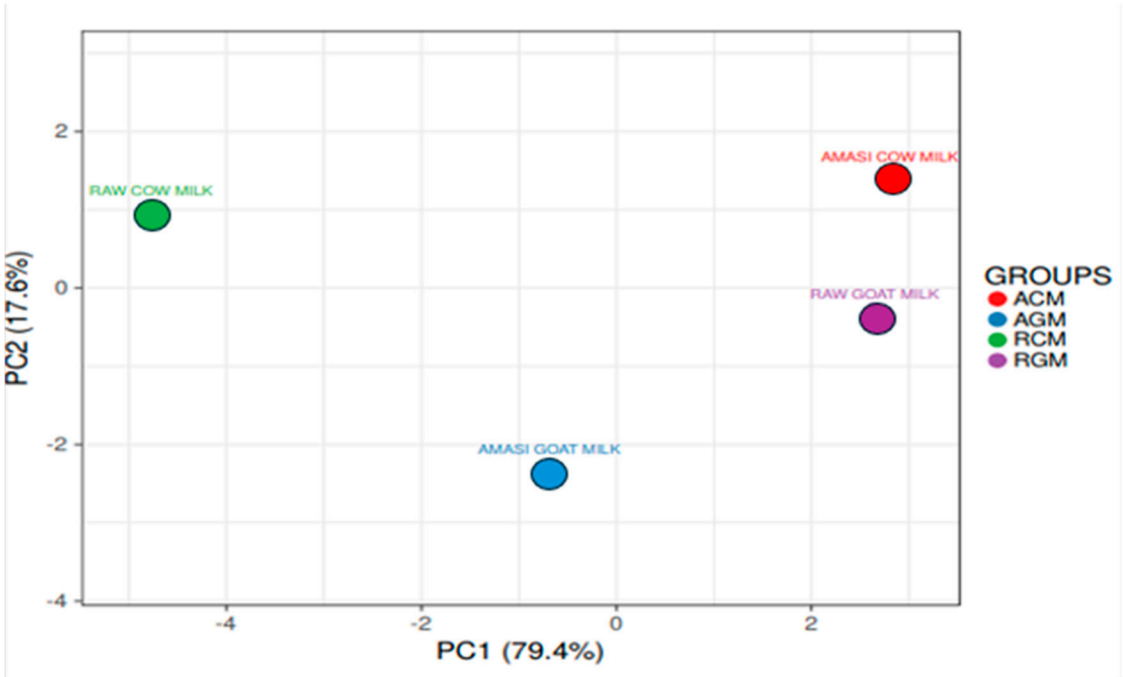
The fungal species that are common or can be found in all the samples are *Aspergillus penicillioides*, *Candida hyderabadensis*, *Candida sake*, *Debaryomyces prosopidis*, *Kluyveromyces marxianus*, *Malassezia restricta*, and *Mycosphaerella tassiana* (Supplementary Table S6). For RCM, the only exclusive fungal species is *Parasarocladium*, while ACM has *Apiotrichum laibachii*, *Diaporthe aseana*, *Filobasidium chernovii*, *Filobasidium magnum*, *Haglerozyma chiarellii*, *Pseudopithomyces rosae*, and *Vishniacozyma victoriae*. RGM has 16 exclusive fungal species, and it is the most diverse of all the samples. Examples includes *Arthrinium esporlense*, *Aspergillus sydowii*, *Cystobasidium fimetarium*, *Eupenidiella venezuelensis*, *Eutypella citricola*, *Exophiala bergeri*, *Hannaella kunmingensis*, *Hannaella oryzae*, *Hannaella sinensis*, *Kwoniella bestiolae*, *Naganishia albida*, *Papiliotrema flavescens*, *Pleurotus opuntiae*, *Pseudopithomyces karoo*, *Schizophyllum commune*, and *Sporobolomyces koalae*. In addition, AGM has nine exclusive species such as *Acremonium acutatum*, *Cladosporium sphaerospermum*, *Hannaella siamensis*, *Malassezia globosa*, *Moesziomyces aphidis*, *Papiliotrema rajasthanensis*, *Phanerochaete pseudomagnoliae*, *Talaromyces siglerae*, and *Talaromyces wortmannii*. Meanwhile, there are no species

that are exclusively present in both AGM and ACM samples, likewise in RCM and RGM (Supplementary Table S6).

The alpha diversity result for the fungal population in the milk samples was depicted with different metrics (Figure 5F). The observed, Shannon and Simpson's metrics are shown to have significant values ($p < 0.05$) when the mean of each sample ASVs was compared. The alpha diversity measure of richness observed in the fungal species showed a greater population or abundance in RCM compared with RGM. This was shown in the difference from 80% to 120% (Figure 5F). The Shannon metric showed similar trends to the observed richness, as RGM (1.5) and ACM (1.75) have close and less significant values ($p < 0.05$) compared to RCM (2.9) and AGM (2.25) which are significantly different ($p < 0.05$). The Simpson metrics which signify the evenness index showed equality of samples in terms of various fungal species present in our samples. AGM and RCM showed more closeness in terms of equitability, and they have a more significant difference ($p < 0.05$) when compared with ACM and RGM (Figure 5F). The beta diversity indicated similarities and dissimilarities in terms of species (Figure 5G) and our result was not normalized to one. RGM had the highest score (0.4) while AGM (−0.4) had the lowest score, ($p < 0.05$). ACM and RCM have their scores in between RGM and AGM (Figure 5G).

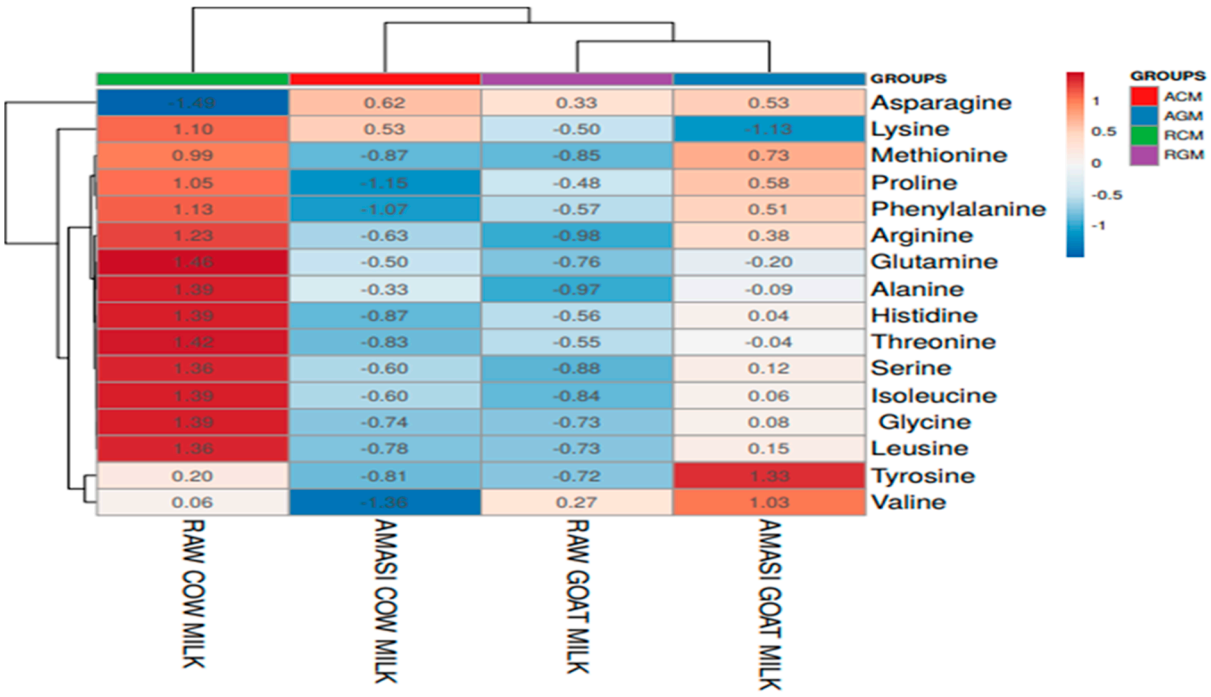
3.5. Validation of Amino Acids Present in Amasi Samples

The distribution of amino acids in the dairy products was investigated using principal component analysis (PCA) (Figure 6A) and the data points are represented in a two-dimensional space, with PC1 (Principal Component 1) on the *x*-axis and PC2 (Principal Component 2) on the *y*-axis. The numbers (−4, −2, 0, 2) on the *x*-axis represent the values of PC1, which accounts for 79.4% of the total variance counts. The numbers on the *y*-axis represent the values of PC2, which accounts for 17.6% of the total variance. The PCA plot helps visualize the similarity or dissimilarity between the different groups. Different milk types cluster together; the ACM (blue dots) and RCM (green dots) group closely together. Similarly, the distribution of the amino acids in AGM (orange dots) and RGM (red dots) groups are also clustered together.



(**A**)

Figure 6. *Cont.*



(**B**)

Figure 6. (**A**,**B**) Principal component analysis of the amino acids distribution in Amasi milk variants. Samples with similar microbial compositions tended to be in the same area of the graph, while shapes were far apart from each other and represented samples with dissimilar amino acids (**A**). The hierarchical clustering of the amino acids distribution in the Amasi milk variants (heatmap) (**B**). RCM—raw cow milk, ACM—Amasi cow milk, RGM—raw goat milk, and AGM—Amasi goat milk.

The amounts of distinct amino acids in Amasi milk variants are depicted in the heatmap (Figure 6B). The relative concentrations of each amino acid in the various milk samples are displayed on the heatmap using color coding. The color scale goes from red, which denotes high levels, to blue, which indicates low levels of amino acid concentrations based on LCMS results.

Based on the heatmap Supplementary Table S1, we can observe that certain amino acids have higher levels in specific types of milk. For example, asparagine, lysine, methionine, proline, phenylalanine, and arginine are generally higher in ACM compared to RCM. Glutamine, alanine, histidine, threonine, and serine also show higher levels in ACM compared to RCM. Similarly, AGM generally has higher levels of asparagine, lysine, methionine, proline, phenylalanine, arginine, glutamine, alanine, histidine, threonine, serine, isoleucine, glycine, leucine, tyrosine, and valine compared to RGM. Overall, the heatmap shows the concentration distribution for amino acids in the milk variants.

Table 2 shows the pair comparison of amino acids present in the samples. There is a significant difference ($p < 0.05$) mostly in the distribution of amino acids across samples. The comparison between RCM/ACM and RGM/AGM showed a significant difference ($p < 0.05$) in all the 16 amino acids detected. RCM valine significantly decreased ($p < 0.05$) when compared with RGM valine; the same trend was also detected with RCM methionine and AGM methionine. For ACM/RGM, glycine, threonine, tyrosine, methionine, and leucine, significantly decreased ($p < 0.05$) while asparagine and alanine also followed the same trend.

Table 2. Pair comparison of amino acids of Amasi-fermented milk.

Amino Acids	RCM vs. ACM		RCM vs. RGM		RCM vs. AGM		ACM vs. RGM		ACM vs. AGM		RGM vs. AGM	
	$\bar{x}_d$ (S.E)	*p*-Value	$\bar{x}_d$ (S.E)	*p*-Value	$\bar{x}_d$ (S.E)	*p*-Value	$\bar{x}_d$ (S.E)	*p*-Value	$\bar{x}_d$ (S.E)	*p*-Value	$\bar{x}_d$ (S.E)	*p*-Value
Histidine	0.95 (0.03)	<0.001 *	0.82 (0.03)	<0.001 *	0.56 (0.03)	<0.001 *	−0.13 (0.03)	0.009 *	−0.38 (0.03)	<0.001 *	−0.26 (0.03)	<0.001 *
Arginine	0.50 (0.03)	<0.001 *	0.60 (0.03)	<0.001 *	0.23 (0.03)	<0.001 *	0.09 (0.03)	0.044 *	−0.27 (0.03)	<0.001 *	−0.37 (0.03)	<0.001 *
Serine	0.88 (0.03)	<0.001 *	1.00 (0.03)	<0.001 *	0.56 (0.03)	<0.001 *	0.12 (0.03)	0.011 *	−0.32 (0.03)	<0.001 *	−0.44 (0.03)	<0.001 *
Glycine	0.28 (0.03)	<0.001 *	0.28 (0.03)	<0.001 *	0.17 (0.03)	0.001 *	−0.002 (0.03)	1.000	−0.11 (0.03)	0.023 *	−0.11 (0.03)	0.025 *
Asparagine	−1.81 (0.03)	<0.001 *	−1.56 (0.03)	<0.001 *	−1.73 (0.03)	<0.001 *	0.25 (0.03)	<0.001 *	0.08 (0.03)	0.098	−0.17 (0.03)	0.001 *
Glutamine	2.77 (0.03)	<0.001 *	3.15 (0.03)	<0.001 *	2.35 (0.03)	<0.001 *	0.38 (0.03)	<0.001 *	−0.43 (0.03)	<0.001 *	−0.80 (0.03)	<0.001 *
Threonine	0.63 (0.03)	<0.001 *	0.55 (0.03)	<0.001 *	0.41 (0.03)	<0.001 *	−0.08 (0.03)	0.110	−0.22 (0.03)	<0.001 *	−0.14 (0.03)	0.005 *
Alanine	0.32 (0.03)	<0.001 *	0.44 (0.03)	<0.001 *	0.28 (0.03)	<0.001 *	0.12 (0.03)	0.013 *	−0.04 (0.03)	0.460	−0.16 (0.03)	0.002 *
Proline	1.23 (0.03)	<0.001 *	0.86 (0.03)	<0.001 *	0.26 (0.03)	<0.001 *	−0.37 (0.03)	<0.001 *	−0.97 (0.03)	<0.001 *	−0.59 (0.03)	<0.001 *
Lysine	0.17 (0.03)	0.002 *	0.48 (0.03)	<0.001 *	0.67 (0.03)	<0.001 *	0.31 (0.03)	<0.001 *	0.50 (0.03)	<0.001 *	0.19 (0.03)	<0.001 *
Tyrosine	0.37 (0.03)	<0.001 *	0.34 (0.03)	<0.001 *	−0.41 (0.03)	<0.001 *	−0.03 (0.03)	0.682	−0.78 (0.03)	<0.001 *	−0.75 (0.03)	<0.001 *
Methionine	0.41 (0.03)	<0.001 *	0.40 (0.03)	<0.001 *	0.06 (0.03)	0.265	−0.004 (0.03)	0.999	−0.35 (0.03)	<0.001 *	−0.34 (0.03)	<0.001 *
Valine	0.18 (0.03)	<0.001 *	−0.03 (0.03)	0.806	−0.12 (0.03)	0.011 *	−0.21 (0.03)	<0.001 *	−0.31 (0.03)	<0.001 *	−0.10 (0.03)	0.036 *
Isoleucine	0.81 (0.03)	<0.001 *	0.90 (0.03)	<0.001 *	0.54 (0.03)	<0.001 *	0.10 (0.03)	0.041 *	−0.27 (0.03)	<0.001 *	−0.36 (0.03)	<0.001 *
Leucine	1.33 (0.03)	<0.001 *	1.29 (0.03)	<0.001 *	0.75 (0.03)	<0.001 *	−0.03 (0.03)	0.668	−0.58 (0.03)	<0.001 *	−0.54 (0.03)	<0.001 *
Phenylalanine	1.00 (0.03)	<0.001 *	0.77 (0.03)	<0.001 *	0.28 (0.03)	<0.001 *	−0.23 (0.03)	<0.001 *	−0.72 (0.03)	<0.001 *	−0.49 (0.03)	<0.001 *

RCM—raw cow milk, ACM—Amasi-fermented cow milk, RGM—raw goat milk, and AGM—Amasi-fermented goat milk. Data were analyzed by one-way ANOVA with Tukey post-test; red numbers indicate statistically significant difference values when compared within groups, * $p < 0.05$. $\bar{x}_d$ = mean difference; S.E = standard error.

4. Discussion

The genetic diversity and safety of the conventional methods for making fermented goods from cow milk have previously been published; thus, it is reasonable to believe that the primary benefit of consuming dairy fermented milk products is primarily their probiotic content. This must be confirmed and reported [8,25,26].

In this study, we find that organic acids (lactic and acetic acids) that are produced during the fermentation process of Amasi lower the pH values of Amasi samples. Typically, this low pH causes the milk to coagulate and also prevents the growth of typical spoilage microbes that degrade the product as reported [8]. When compared with raw milk, the amount of lactose in Amasi is reduced during fermentation due to the conversion of lactose sugar to lactic acid by LAB, and lactose-intolerant individuals can usually tolerate this low amount of lactose [8]. In addition, the glycemic response, satiety, and appetite control properties can all benefit from the delayed stomach emptying caused by the low pH of fermented milk generally but also reported for Amasi [8,27]. The presence of these acids informed the probable presence of various health benefits and metabolites conferred on Amasi; this is in line with the reported work on Amasi and fermented dairy milk products [8,28].

The most abundant bacteria phyla commonly reported for both cow milk Amasi (ACM) and goat milk Amasi (AGM) are the Proteobacteria and the Firmicutes [8]. Relative nutrients and bacterial composition are dependent on animal health, milking equipment, environmental circumstances (temperature, humidity, etc.), and milking handlers, etc. These factors can have an impact on the nutritional quality and microbiota of raw milk [25,29]. Khulusani Farms in Howick, Kwa-Zulu Natal Province, South Africa, provided the raw milk used in this study, and this might have influenced the microbiota of the Amasi that was made as a result.

The unique genera, *Atopobium*, *Synechococcus*, *Parabacteroides*, and the *5-7N15* are rarely reported in fermented milk and to the author's knowledge, have never been reported in Amasi, but these are all anaerobic, Gram-positive, and mostly environmental bacterium species. The *Atopobium* species are related to the human vagina and they are mostly anaerobic and can be transferred to milk through human handling [30]. The *Synechococcus* and the *Parabacteroides* are associated with environmental water bodies and the *5-7N15*

bacteria have been found in certain goat and cow milk types, especially the raw milk type [31–33]. The incidence of these bacteria in our milk samples may be because of the raw milk used or the geographical location of the farm where our raw milk was purchased.

The genus, *Lactobacillus*, are fastidious Gram-positive bacteria that populate nutrient-rich habitats associated with food, plants, and mucosal surfaces of animals and humans [34]. They are one of the major taxa associated with probiotics. The relative abundance of *Lactobacillus* spp. in Amasi made from both raw cow and raw goat milk increased after fermentation as suggested by our result. The problem of pathogens in raw milk can never be over-emphasized; this study found the usual perpetrators as probable pathogens in both cow and goat milk. The presence of *Bacillus* spp., *Pseudomonas* spp., and *Clostridium* spp. is of economic importance and safety concern [35]. Raw milk has been implicated in infections relating to pathogenic diseases which can be transferred to humans; thus, the pasteurization of milk is still very important [6].

The relative abundance of bacteria in Amasi showed that spontaneous fermentation still plays a big role in the distribution of microbiota in dairy products. There is a change in diversity after fermentation of both Amasi samples (ACM and AGM) as they have less bacteria diversity compared to raw milk (RCM and RGM) [25]. The presence of *Acinetobacter*, *Lactobacillus*, *Lactococcus*, *Corynebacterium*, and *Brevibacterium* common to both ACM and AGM and the absence of any exclusive genera in both indicates similar diversity between ACM and AGM microbiota. These genera are known to possess probiotic strains and are linked with different health benefits [3].

Based on the species level, the bacterial counts showed an increase in possible probiotic bacteria, and this is expected from fermented milk products. Most bacteria species present in this study have been associated with dairy products [4]. The most abundant bacteria genera found in ACM and AGM are relatively similar and they are mostly different based on the relative abundance of the species from cow to goat milk. Although the species level may not be reliable when 16S sequencing is inferred in diversity studies, taxonomy was inferred using 100% amplicon sequencing variants (ASVs) for this study, thus we report the species level [15]. The *Mannheimia* spp. found in ACM and RCM are linked with dairy animals' throat diseases; this is a concern as raw milk has been implicated in the transfer of pathogens that cause diseases in humans [36]. This can also serve as a quality or safety concern in the consumption of raw milk. The RCM and the RGM are generally related based on the bacteria phylum and genus levels, but they are different in terms of the abundance of these bacteria [37]. The abundance level in terms of the ASVs composition as depicted by observed richness indicated that the fermentation process redistributed the microbiota of both ACM and AGM, and there is a significant increase ($p < 0.05$) in the bacteria counts after fermentation. Although RCM is much more diverse than RGM, the same trend was observed in the ACM and AGM. The alpha diversity based on the Shannon and Simpson metrics showed RCM has the highest diversity when compared with other samples indicating that fermentation redistributes the microbiome of the milk samples [25]. The beta diversity brought out the difference between milk samples based on the microbiota and this shows that bacteria diversity in this study is highly diverse with ACM and RCM being the most diverse. You, Yang [38] reported that fermented milk products are diverse in terms of bacteria composition and fermentation changes the population or abundance of certain species and this is noted in RCM and AGM. The probiotics abundance significantly increased during and after fermentation in both ACM and AGM; this is significant since fermented milk is consumed as a result of its probiotics content [8].

Most of the bacteria genera and species detected in this study, *Lactobacillus kefiranofaciens*, *Lactococcus raffinolactis*, *Lactococcus lactis*, *Streptococcus thermophilus*, *Acinetobacter lwoffii*, *Lactobacillus johnsonii*, *Streptococcus alactolyticus*, *Lactobacillus rhamnosus*, *Lactobacillus*

bulgaricus, and *Lactobacillus delbrueckii*, have been widely reported in Amasi [14,25] and these LAB species undergo proteolytic activities that are associated with flavors and beneficial metabolites production in Amasi [8]. Proteolytic activities are essential in the utilization of casein and the formation of metabolites that enhance the growth of other microorganisms [3,8]. Most of these probiotics' genera have also been related to many health benefits including gastrointestinal tract disease amelioration, obesity treatments, and immunodeficiency therapies [39–41]. For example, Lactobacillus kefiranofaciens, *Lactobacillus plantarum*, and *Lactococcus lactis* were found to reduce diarrhea infection by Clostridium difficile [40]. *Lactobacillus kefiranofaciens* M1 was administered to BALB/c mice and a subsequent EHEC infection, resolving infection-induced symptoms, bacterial translocation, renal damage, intestinal damage, and Stx penetration [42]. A probiotic cocktail containing *B. longum*, *B. infantis*, *Lactobacillus plantarum*, *L. acidophilus*, *L. casei*, *L. delbrueckii subsp. bulgaricus* and *Streptococcus thermophilus*, all found in Amasi samples, have been found to effectively treat celiac disease by hydrolyzing gliadin's toxic polypeptides and aiding digestion [42].

Functional genes of the bacteria taxa and their metabolism are present in the Amasi samples. The PICRUSt software was used to predict the functional genes of the bacteria present in all milk samples and their metabolic pathways [43]. In this study, it was found that spontaneous fermentation of raw milk increases the metabolism abundance (i.e., amino acid metabolism, carbohydrate metabolism, energy metabolism, nucleotide metabolism, metabolism of cofactors and vitamins, glycans metabolism and lipid metabolism) of milk samples' metabolites, which has been reported earlier [6]. The functional prediction of Amasi samples showed a significant increase in the metabolites abundance due to spontaneous fermentation [14]. Bacterial fermentation increases the metabolic activities of the organisms, thus, increasing the nutritional metabolites that characterize the change in appearance and taste of fermented milk. Chemical and structural changes from raw milk to fermented milk carried out by microbes show increased activity in terms of metabolites abundance which resulted in fermented milk with various health benefits [37,44].

Bacteria, fungi, and yeast are known microflora of milk, and they are known for the conversion of carbohydrates and proteins. Microbe fermentation causes milk macromolecules such as peptides, amino acids, fatty acids, and vitamins present in raw milk to undergo degradations that increase the metabolites abundance in fermented ACM and AGM [6]. However, ACM has a more significant increase ($p < 0.05$) in all the classes of predicted metabolic pathways compared to AGM. Since ACM has more microbiota based on the microbial counts of both 16S rRNA and the ITS 1 and 2 of this study, this result is consistent with the metabolites prediction by the PICRUSt tool [15]. Raw goat milk has been found to contain fewer metabolites due to fewer microbiota populations compared to raw cow milk which has a more diverse and larger population of microbiota [6].

The prediction tools for the metabolites database are based on the microbial counts or the taxonomy table that were inferred from the maker gene of an organism and the output is given based on the total ASVs present in a sample [15]. This means the more the abundance or the more the population of a sample is in terms of the inferred ASVs, the more the predicted quantity of metabolites from the microbiota present in the sample [15]. It has been found that oligosaccharides in milk are mainly fermented by probiotics which are found abundantly in Amasi samples [45].

This study explores the ITS 1 and 2 regions that investigate the fungal abundance of milk samples. To the best of the author's knowledge, this is the first time that the fungal community in Amasi made from cow and goat has been explored. The fungal population consists of yeast and mold and their abundance were reported. Based on the phyla level, RCM and RGM have similar fungi and yeast populations after fermentation. Compositional differences occurred during fermentation and RCM appears to be more fungal abundant

than RGM. The same trend was noticed in the Amasi samples (ACM and AGM). The presence of the phyla; Ascomycota, Basidiomycota, Mucoromycota, and Rozellomycota are associated with published work based on culture-independent methods and they are common in fermented foods [46,47]. The genus level also has the usual microbes present i.e., *Aspergillus* spp., *Kazachstania* spp., *Debaryomyces* spp., and *Kluyveromyces* spp. which are all associated with fermented foods [28]. This study is in line with reported work with species levels associated with traditional fermented milk products which include *Aspergillus penicillioides*, *Kluyveromyces marxianus*, and *Candida sake* all of which are common to both RCM and RGM [8,29]. The differences in genus level may be due to the initial microbiota of milk used in this study. Raw milk has been found to contain more diverse microbiota compared to pasteurized milk [48]. The species level for fungi increases based on the abundance data generated from the ASV table. The probiotic nature of the fungi in Amasi is poorly reported especially through culture-independent methods, thus, probiotics present in this present study will serve as references for further studies and comparisons.

The alpha diversity measure showed the distributions of the abundance with RCM having a much similar diversity compared to RGM, but the counts were relative. Both Amasi samples (ACM and AGM) showed an increase in observed richness after fermentation, thus, fermentation increases the abundance of fungal communities as shown in reported studies [28]. The Shannon and Simpson metrics also share similarities in result, as fermentation caused a shift in the abundance of the genus and species levels of fermented products. Beta diversity measures equity similarities and dissimilarity, thus, fungal species in Amasi samples are significantly different only based on the counts or abundance present in the raw milk and the fermented products. A study by Ghosh and Beniwal [28] showed a significant dissimilarity in traditional fermented milk in terms of their fungal genera and species after fermentation.

The most common fungal phyla (Ascomycota, Basidiomycota, Mucoromycota, and Rozellomycota) are the most reported and they form the core mycobiota of the fermented and raw milk samples [8]. Ascomycota is very diverse and has a large community (over 93,000 species) of fungi which are spread throughout the environment and fermented foods [17]. They form part of the endophytic organisms present in raw milk and their abundance increases during fermentation [28]. They are most responsible for the fungal diversity of fermented milk. Amasi-fermented milk is poorly reported in terms of the fungal phyla but many general species of fungi have been reported in the spontaneous Amasi [8]. In this study, the major fungi genera and species found in Amasi are *Aspergillus penicillioides*, *Debaryomyces prosopidis*, *Cladosporium tenuissimum*, *Candida sake*, and *Kluyveromyces marxianus* [4]. These genera also serve as the core mycobiota of Amasi, and they are present in all the dairy samples (RCM, ACM, RGM and AGM). They are widely reported as the common mycobiota of dairy fermented milk and they are explored in food industries for fermentation and probiotic properties [28]. The *Aspergillus species* are used in the production of certain carbohydrates such as amylase, maltase, and amyloglucosidase among others [49]. The identified yeast taxa are *Candida sake* and *Pichia kluyveri* (found only in RGM and AGM). *Alternaria*, *Diaporthe*, and *Penicillium* spp. found in the core taxa of Amasi samples are a concern as they are potential pathogenic species and may constitute safety concerns [50]. *Alternaria* spp. have been implicated in infant milk contamination that is formulated with vegetable oil. The *Alternaria* can be found in soil, air, fruits, and vegetables, and since our milk samples are raw milk, it is very probable that they are found in the milk samples [51]. There are over 70 toxins produced by several species of the *Alternaria* spp. and this is of economic importance and food safety concern [52].

In this study, 16 amino acids (histidine, arginine, serine, glycine, asparagine, glutamine, threonine, alanine, proline, lysine, tyrosine, methionine, valine, isoleucine, leucine, and

phenylalanine) were validated in the dairy samples (Table 2) through LCMS analysis. There are eight essential and non-essential amino acids present in our study. Compared to previous work based on raw milk (cow, goat, ewe, sheep, etc.), aspartic acid, tryptophan, and glutamic amino acids are absent in this study [53]. There are significant differences in most of the results of the amino acids and this showed that fermentation influences the protein and amino acids present in milk [53]. The amino acids included in fermented dairy products are derived from milk proteins. During fermentation, these proteins are broken down by lactic acid bacteria (LAB) into smaller peptides and amino acids. The flavor and aroma of fermented dairy products are enhanced by these amino acids. Bioactive peptides with a variety of health-promoting qualities are also produced by LAB. Amino acids in addition contribute significantly to the antioxidant activities of milk and fermented dairy products [54]. However, which specific amino acids end up in fermented dairy products depends on the type of starter cultures utilized and the fermentation procedure [53]. Generally, from our result, fermentation increases the amino acid content, for example, asparagine increased in ACM due to fermentation. The same trend was observed in glutamine and glycine for AGM. It was reported that glycine and tryptophan are higher in cow milk than in goat milk; this result corroborates this finding, although there is no detection of tryptophan in the milk variants [55]. RCM is high in serine, threonine, proline, lysine, and leucine, and the amino acid with the highest amount is glutamine. This substantiates the result of Landi, Ragucci and Di Maro [56], where glutamine is the highest in cow milk. Although goat milk reportedly has higher values in most of the essential amino acids, our result is contradictory as cow milk generally has more amino acids in terms of quantity. This may be because in this study, raw unpasteurized milk was used for analysis, and variations are mostly reported due to many factors ranging from animal breed, feed type, location, etc. [55,56]. RGM has higher asparagine compared to cow milk. The same trend in quantity of amino acids was observed in ACM and AGM but ACM is higher except in asparagine and tyrosine. The amino acids result also validates the amino acids metabolism in the PICRUSt metabolites prediction which are highly significant compared to other metabolisms. The amino acids are reduced significantly for Amasi samples after fermentation; this may be because of the microbial activities in the utilization of the amino acids in proteolytic activities. Proteolysis is the breakdown of proteins into peptides and amino acids by proteolytic enzymes, affecting the quality and properties of fermented products and generating metabolites [57]. The proteolytic activities have been shown in the literature to involve the probiotics using amino acids to produce metabolites such as bacteriocins and peptides [58]. These confer antibacterial and preservative properties in fermented milk [58]. This result from our study is important as it showed that Amasi has significant levels of amino acids that are needed for healthy food intake for both adults and small children.

5. Conclusions

This study highlighted the microbial diversity and heterogeneity across Amasi-fermented milk products. Data from the result revealed that metagenomic sequencing is expanding our understanding of low-complexity microbial communities. This present study has shown that there is a considerable diversity of microbial communities in Amasi made from raw cow and goat milk through spontaneous fermentation. At the bacterial phylum level, there was a similarity in microbial diversity between Amasi made from cow and goat milk as there is no exclusive microbiota for either of them as suggested by the result. The presence of *Mannheimia* spp. observed in the current study highlights the high probability of transfer of bacterial communities from the host animal to Amasi. The fungal community also has a similar diversity trend as fermentation increases the diversity of

fungal in fermented milk, including an increase in the probiotic fungal just as it is found in bacteria. The amino acids validation of Amasi showed an increased nutritional application of Amasi and this was corroborated by the metabolites functional prediction (PICRUSt) with increased amino acid metabolism function of Amasi and many more useful metabolites and this informed the reasons for many health benefits claimed by Amasi. Overall, the findings of this study indicated that both cow and goat milk Amasi have similar microbiota that are varied in terms of abundance of the microbiota and the mycobiota. The exposition of the functional metabolites' prediction of Amasi based on this study suggested research into the functionality or drug discovery studies for health benefits validation. The database used to resolve taxonomic sequences needs an update to accommodate new names of some probiotics which are reported. In addition, the analysis carried out in this study concluded that probiotic species are present in the Amasi samples, but it failed to inform about specific strains as probiotics are strain-specific in their probiotics ability.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/fermentation11010006/s1, Table S1: Relative abundance of bacteria at the taxonomic level phylum; Table S2: Relative abundance of bacteria at the taxonomic level genus; Table S3: Relative abundance of bacteria at the taxonomic level Species; Table S4: Relative abundance of fungi at the taxonomic level phylum; Table S5: Relative abundance of fungi at the taxonomic level genus; Table S6: Relative abundance of fungi at the taxonomic level Species. Table S7: The functional prediction of metabolisms and pathways as predicted by PICRUSt.

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