High-throughput sequencing analysis of bacterial diversity in raw and pasteurized goat milk

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Abstract

The aim of this study was to evaluate the microbial composition of both raw and pasteurized goat milk using high-throughput DNA sequencing. This analysis revealed that the dominant phylum found in the raw milk was *Proteobacteria*, and the dominant genus was *Kluyvera*; *Proteobacteria* and *Kluyvera* constituted up to 67.66% and 28.85% of the total bacteria population, respectively. The microorganisms in goat milk predominantly consist of Gram-negative bacteria. Notably, *Akkermansia* and *Faecalibacterium* were identified in goat milk for the first time. In addition, the results also indicate that some bacteria in pasteurized goat milk may exist in a viable but nonculturable (VBNC) state. This study provides a theoretical basis that may aid the community in better understanding bacterial diversity in goat milk. The results of this study will help us to improve the quality and safety of goat milk.

Importance The microbial diversity in goat milk and pasteurized goat milk at different refrigeration stages was described. Several bacterial species that have not previously been reported in goat milk were identified, including many VBNC bacteria. The findings provided the necessary microbial information for quality and safety of goat milk and dairy products.

Key words: Goat milk, high-throughput sequencing, bacterial diversity, cold storage

Introduction

Dairy products play an important role in the daily diet of humans with multifarious products including milk, yoghurt and cheese available for consumption. Goat milk contains an abundance of nutrients that are easily digested and absorbed

(Park et al., 2007). In recent years, there has been a growing interest in goat milk because of its medical and nutritional benefits, especially for people who are allergic to cow milk. Goat milk also contains many beneficial bacteria, especially lactic acid bacteria (LAB), which have been touted as suitable effectors of goat milk fermentation reactions (Fernanda et al., 2016; García et al., 2014). However, different species of lactic acid bacteria have different functions and these species are known to play decisive roles in the quality of dairy products (Montel et al., 2014). It is hoped that future research investigating the composition of lactic acid bacterial populations in goat milk can help us to better understand the fermentation of dairy products.

In general, pasteurization uses the application of heat to reduce the microbial load in raw milk. However, several studies have reported that pasteurized milk can only be stored for 3 to 10 days at refrigerated storage conditions (Petrus et al., 2010; Fan et al., 2016). A previous study by Fonseca et al. (2013) revealed that heat-treated goat milk should not be kept in cold storage for more than 3 days (4°C); refrigeration for longer than 3 days can affect the shelf-life of milk powder. Thermoduric bacteria are considered to be ubiquitous microorganisms in pasteurized milk (Ternström et al., 1993). In addition, plate-counting methods have been used to show that the prevalence of psychrophilic bacteria in pasteurized milk increases during refrigerated storage and these bacteria can produce heat-resistant proteolytic enzymes and lipases (Meunier-Goddik, L and Sandra, S. 2011, Angelidis et al., 2016), which can lead to reduced dairy product and milk shelf-lives (Doyle et al., 2017). Moreover, it is difficult to observe some of the changes that occur in relative bacterial abundances

due to difficulties associated with cultivation using plate-counting methods during cold storage.

From the perspective of food quality and storage time, identification of the microbial populations in goat milk is necessary for the safety of milk products. It is difficult to determine the entire bacterial composition of milk using culture-dependent methods; this is especially true for bacteria that exist in a VBNC state (Paszyńska-Wesołowska and Bartoszcze, 2009; Kibbee and Örmeci, 2017). Recent, high-throughput sequencing strategies have made it possible to identify many of the afore-mentioned bacteria at subdominant levels. These methodologies have been used to detect microorganisms in dairy products thereby helping us to better understand the diversity and dynamics of native microbial populations. Only a limited number of studies have reported the bacterial diversity of goat milk in China. Moreover, the composition and associated co-occurrences of microbial populations in pasteurized goat milk during cold storage (about 4°C) have not been investigated. In the current study, the primary aim was to determine the bacterial diversity in raw goat milk as well as in pasteurized goat milk at different stages of refrigeration using high-throughput sequencing. This study assessed bacterial diversity in goat milk and provides a basis for further analysis of goat milk.

Materials and methods

Sample collection

The goat milk samples were obtained from a goat farm with 200 Guanzhong goats. The goat farm is located at the Animal Husbandry Research Institute of

Guangxi Zhuang Autonomous Region in China. All animal experiments were performed in line with experimental animal administration regulations of Guangxi University. All goats were fed uniformly (peanut vine, elephant grass and 2 kg of complete feed, twice a day); the feed did not contain antibiotics, and all breasts of goats were healthy throughout the entire lactation period. All goat milk samples were collected during the fifth month of lactation. Milk samples were collected after teat ends had been disinfected with 70% ethyl alcohol. Raw goat milk was immediately placed into sterilized cone bottles; the samples were subsequently placed in an ice box until they were analyzed in the laboratory. The SCC of samples was below 200,000 cells/mL. The average fat and protein contents in raw goat milk were 3.87 g/100 mL and 3.16 g/100 mL, respectively. Raw goat milk was sterilized by pasteurization (at 72°C for 15 s), and the pasteurized goat milk samples were immediately placed into an ice box cooling to 4°C. Next, the pasteurized milk samples were stored at 4°C for 5 and 10 d before freezing at −80°C. To facilitate DNA extraction, the afore-mentioned goat milk samples were defrosted at 4°C.

DNA extraction

Good quality DNA is important for valid analysis of goat milk microbial diversity. Goat milk samples (20 mL) were concentrated by centrifugation for 10 min at 12,000 × *g* at 4°C. The aqueous and fatty layers were removed and discarded. Cell pellets were washed with 0.8% NaCl solution and centrifuged at 12,000 × *g* for 10 min at 4°C. Total genomic DNA was extracted using the food DNA Kit according to the manufacturer's instructions. The purity and yield of the extracted DNA were

111 determined with a Qubit® dsDNA BR Assay Kit in accordance with the
112 manufacturer's instructions; the integrity of the extracted DNA was determined by
113 agarose gel electrophoresis (using a 1% agarose gel).

114 **High-Throughput Sequencing and Bioinformatics Analysis**

115 The afore-mentioned DNA extracts were sequenced following amplification of the V3
116 and V4 regions of 16S rRNA genes using the universal forward primer 338F
117 (5'-ACTCCTACGGGAGGCAGCAG-3') and the universal reverse primer 806R
118 (5'-GGACTACHVGGGTWTCTAAT-3'). The reverse primer contained a set 6-bp
119 barcode. Genomic DNA samples (30 ng) and corresponding fusion primers were used
120 to perform the PCRs. The PCRs were performed as follows: 95°C for 3 min followed
121 by 30 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 45 s; a final extension step
122 of 72°C for 10 min was also performed. Amplified PCR products were purified with
123 Agencourt AMPure XP magnetic beads and dissolution in Elution Buffer was
124 performed to construct a DNA Library. The concentration and range of the library
125 were analyzed using an Agilent 2100 Bioanalyser according to the manufacturer's
126 instructions. The qualified library was sequenced using an Illumina HiSeq 2500
127 platform (Fadrosh et al., 2014), and the sequencing type was PE 300. Clean data were
128 obtained by processing the raw data using the Windows discard low quality approach,
129 while low-quality data were removed. According to the barcode and primers, the
130 allowable number of mismatches between barcode sequences and reads was 0 bp.
131 Paired-end reads were assembled using FLASH (Magoc and Salzbert, 2011) software
132 to generate the raw tags. The effective tags were clustered using USEARCH (Edgar,

2013) software to generate operational taxonomic units (OTUs) based on 97% threshold identity. The taxonomic annotation was performed using the RDP classifier (Wang et al., 2007) at the phylum and genus level. Alpha diversity was analyzed using Chao1 richness; Shannon, observed species and Good's coverage indices were calculated by mothur (Schloss et al., 2009) software. The high-throughput sequencing data generated were deposited in the NCBI database (Accession number: SRP 219141).

Results

High-Throughput Sequencing of Amplicons

Using high-throughput sequencing, a total of 1,199,746 raw reads were obtained from 9 samples; after filtering, 1,127,473 clean reads were generated. The rarefaction curve (Figure 1) revealed that sequencing data resulted in sufficient coverage, suggesting that the data were reliable for further analyses. The rank curve (Figure 1) showed that the abundance in the samples decreased during prolonged cold storage. The Chao1, Simpson, observed species, Shannon and ACE diversity indices of each group are shown in Table 1.

Bacterial composition of raw goat milk

The bacterial diversity of the raw goat milk was defined at both phylum and genus levels by high-throughput sequencing (Table 2, Figure 2). The sequences corresponded to 5 distinct phyla: *Proteobacteria*, *Firmicutes*, *Deinococcus-Thermus*, *Bacteroidetes* and *Actinobacteria* were detected in the raw goat milk. The results revealed that phylum *Proteobacteria* was the dominant phylum in raw goat milk

155 samples, with more than 67.66% of the total population consisting of *Proteobacteria*
156 (Table 2, Figure 2A). At the family level, *Enterobacteriaceae* was the predominant
157 family, accounting for 49.29% of all bacteria (data not shown). Genus *Kluyvera* was
158 the dominant genus in raw goat milk, representing approximately 28.85% of the total
159 population (Table 2, Figure 2B).
160 The most abundant genera *Kluyvera*, *Aquabacterium*, *Pseudomonas*, *Burkholderia*,
161 *Thermus* and *Acinetobacter* detected in goat milk were Gram-negative. Indeed,
162 Gram-negative bacteria accounted for more than 82% of the total population in raw
163 goat milk (Figure 4).
164 We also identified several bacterial genera that had not previously been reported in
165 raw goat milk. These genera included *Faecalibacterium* and *Akkermansia*.
166 In this study, the hygienic safety status of raw goat milk was also assessed. Several
167 pathogens, including *Shigella*, *Staphylococcus* and *Serratia* were identified in the raw
168 milk. Probiotics including *Lactobacillus*, *Lactococcus*, *Bifidobacterium*, *Weissella* and
169 *Enterococcus* were also identified. This analysis revealed the identity of some LAB at
170 the species level: *Lactobacillus_helveticus* (0.07%), *Lactobacillus_gasseri* (0.009%),
171 *Lactobacillus_xiangfangensis* (0.005%), *Lactobacillus_casei* (0.01%),
172 *Lactobacillus_iners* (0.009%), *Lactobacillus_pobuzihii* (0.01%),
173 *Lactobacillus_paralimentarius* (0.01%), *Lactobacillus_ruminis* (0.001%),
174 *Lactobacillus_versmoldensis* (0.0003%), *Lactobacillus_delbrueckii* (0.004%),
175 *Lactococcus_lactis* (0.25%), *Lactococcus_chungangensis* (0.01%),
176 *Bifidobacterium_merycicum* (0.006%), *Bifidobacterium_pseudolongum* (0.02%),

Bifidobacterium_psychraerophilum (0.01%), *Weissella_paramesenteroides* (0.04%), and *Enterococcus_faecalis* (0.01%). These results are important for the future production of probiotic milks.

Bacterial composition of pasteurized milk

The bacterial community of pasteurized goat milk was analyzed at the genus level at different stages of 4°C storage (Figure 3). At the phylum level, there was no significant change in bacterial diversity (data not shown). In this current study, taxonomic analysis revealed that, at the genus level, the predominate genera in pasteurized goat milk stored for 5 d was similar to that for raw goat milk (Figure 3). The relative abundance of *Acinetobacter* in pasteurized goat milk was similar to that of raw goat milk (3.78 vs 4.08%) after 5 d of storage. Following 10 d of storage, an increase in the relative abundance of *Acinetobacter* was observed in pasteurized goat milk (3.78 vs 10.00%); *Acinetobacter* became the dominant genus at d 10 (Figure 3). A relatively low abundance of *Meiothermus* was observed in raw goat milk, whereas the *Meiothermus* population in pasteurized goat milk appeared to increase gradually during cold storage (0.005 vs 8.68%). Similar results were observed for *Sphingomonas* and *Staphylococcus* (Figure 3). By contrast, the proportion of other genera (those present in pasteurized goat milk in addition to the afore-mentioned genera) gradually decreased in pasteurized goat milk stored between 5 d and 10 d (Figure 3). Furthermore, the prevalence of Gram-negative, obligate aerobes significantly increased following storage for 5 d to 10 d (20.49 vs 35.90%, Figure 4). Correlation between the microbial genus composition of goat milk during cold storage

To better understand the abundances and relationships between dominant species (more than 1% of total bacterial composition) during cold storage, a Spearman's correlation heatmap was generated for the dominant species (Figure 5). The results revealed that *Acinetobacter_pittii* was positively correlated with *Burkholderia multivorans* (R=0.67, *P*=0.04). *Acinetobacter lwoffii* was positively correlated with *Sphingomonas oligophenolica* (R=0.85, *P*=0.003) and *Meiothermus silvanus* (R=0.76, *P*=0.016). Meanwhile，*Acinetobacter lwoffii* and *Sphingomonas oligophenolica* were negatively correlated with *Burkholderia multivorans* (R=0.88, *P*=0.001 and R=0.86, *P*=0.002, respectively). *Geobacillus stearothermophilus* was positively correlated with *Aquabacterium parvum* (R=0.83, *P*=0.005).

Discussion

In this current study, the bacterial diversity of raw goat milk and the effect of cold storage on the bacterial diversity of pasteurized goat milk from Guangxi, China was investigated using a high-throughput sequencing strategy. The results of this analysis revealed that 5 distinct phyla (i.e., *Proteobacteria*, *Firmicutes*, *Deinococcus-Thermus*, *Bacteroidetes* and *Actinobacteria*) and 4 distinct genera were present (i.e., *Kluyvera*, *Geobacillus*, *Thermus* and *Pseudomonas*) in the raw milk of goats. Notably, the genera, *Akkermansia* and *Faecalibacterium*, were identified in raw goat milk for the first time. Furthermore, following prolonged storage under refrigerated conditions, the dominant genera were *Geobacillus* and *Kluyvera* after 5 d of storage while *Kluyvera*, *Acinetobacter* and *Meiothermus* were the dominant genera after 10 d.

In this study, the less prevalent genera in goat milk constituted a significant proportion

of the total bacterial population; this result is similar to results published in other reports (Kable et al., 2016; Quigley et al., 2013).

The most abundant phyla observed in raw goat milk were similar to those published in previous studies (Zhang et al., 2017). Conversely, the predominant genera observed in raw goat milk in this study differed from those identified in other studies. McInnis et al. (2015) reported that the most abundant genera in raw goat milk were *Micrococcus*, *Rhodococcus*, *Stenotrophomonas*, *Pseudomonas* and *Phyllobacterium*; these results were not consistent with our research. Meanwhile, previous research revealed that the genus *Pseudomonas* was abundant in goat milk (Scatamburlo et al., 2015). In this current study, genus *Kluyvera* constituted a significant proportion of the total bacterial population in raw goat milk. These differences in the associated abundances could be related to many factors, including lactation stage, feed, weather environment, health of the animal, and farm management practices (Callon et al., 2007).

In our study, the predominant genera (i.e., *Kluyvera*, *Thermus*, *Aquabacterium*, *Pseudomonas*, *Burkholderia* and *Acinetobacter*) observed in raw goat milk were Gram-negative bacteria. Dalmasso et al. (2017) studied the bacterial diversity of donkey milk and reported that, similar to our study, the dominant genera *Pseudomonas*, *Ralstonia*, *Cupriavidus*, *Acinetobacter*, *Citrobacter* and *Sphingobacterium* were also Gram-negative bacteria. Gram-negative bacteria are usually considered a major cause of milk spoilage and poor hygiene (Ercolini et al., 2009; Neugebauer and Gilliland, 2005). Nevertheless, some Gram-negative bacteria may play a positive role in the sensory characteristics of milk (Delbès-Paus et al.,

2012). Larpin-Laborde et al. (2011) also reported that some Gram-negative bacteria could have potential applications in cheese-manufacturing technologies. However, little is currently known about the role of Gram-negative bacteria in associated manufacturing strategies. Hence, the role of Gram-negative bacteria in milk merits further study.

The genera *Thermus*, *Burkholderia* and *Aquabacterium* are usually found in hot springs, soil, and water, and are therefore considered environmental microorganisms. In addition, the genera *Akkermansia* and *Faecalibacterium* are generally considered gut microbes. *Akkernansia* is considered to be a potentially protective intestinal bacterium (Arias et al., 2017). *Akkermansia* is associated not only with the intestinal health of obese and diabetic individuals but is also known to promote the therapeutic effects of tumor PD-1 (Reunanen et al., 2015; Routy et ai., 2018). In a recent study, *Akkermansia* was shown to promote intestinal mucosal immunity homeostasis (Ottman et al., 2017). The species *Faecalibacterium* could play an important role in gut homeostasis, and has been shown to exhibit anti-inflammatory activity (Sokol et al., 2009). The effects of these microbes in goat milk on human health remain to be elucidated. Nevertheless, this study will provide us with a platform to identify new functional microorganisms that have not yet been discovered.

Our study also revealed high bacterial diversity in pasteurized goat milk. The rarefaction curve and rank abundance curve (Figure 1) confirmed that the bacterial diversity of pasteurized goat milk decreased during cold storage.

It is widely perceived that pasteurization is sufficient to eliminate the threat of

265 psychrophilic bacteria. Psychrophilic bacteria exhibit proteolytic and lipolytic
266 enzymatic activities, and therefore can reduce the shelf-life of milk products.
267 However, the study revealed that the prevalence of *Acinetobacter* can increase during
268 5 to 10 d of refrigeration (Figure 3). The authors speculate that some bacteria (i.e.,
269 *Acinetobacter*) that are supposed to be eliminated by pasteurization are likely to
270 survive and may be in a damaged and/or VBNC state. *Acinetobacter* and
271 *Pseudomonas* are psychrophilic bacteria which increase in prevalence during
272 refrigeration. Our study revealed that the genus *Acinetobacter* increased in prevalence
273 following storage for 10 d. This finding is similar results published by Raats et al.
274 (2011) where *Acinetobacter* was the predominant genus after cold incubation for 54 h.
275 Conversely, the genus *Pseudomonas* gradually decreased during prolonged storage.
276 This result differs from the results of a study published by Porcellato et al (2018)
277 where genus *Pseudomonas* was abundant following cold storage.
278 Researchers have suggested that the microbial composition of milk changes and
279 affects the quality of milk during cold storage (De Jonghe et al., 2011; von Neubeck et
280 al., 2015). The correlation analysis revealed the relationships among the dominant
281 bacteria in pasteurized goat milk during refrigerated storage (Figure 5); this analysis
282 indicated that there were different interdependence relationships among the
283 microorganisms in goat milk. During prolonged cold storage, *Acinetobacter*
284 populations play a key role in maintaining the interrelationships between
285 microorganisms. The existence of dominant species leads to a negative correlation
286 between microorganisms in goat milk (Figure 5).

Our culture-independent analyses revealed a low proportion of *Sphingomonas* and *Meiothermus* in raw goat milk, whereas a significantly greater proportion of *Sphingomonas* and *Meiothermus* were observed in pasteurized goat milk during cold storage (Figure 3). *Sphingomonas* spp. are phylogenetically related to *Pseudomonas* spp., and represent a new type of microbial resource. A Spearman's correlation heat map showed that *Sphingomonas* and *Meiothermus* were positively correlated with *Acinetobacter* spp. (Figure 5), and these bacteria increase during cold storage. However, the effect of these microbes in pasteurized goat milk on the hygienic quality and shelf-life of goat milk is still unknown.

It is generally considered that LAB are the dominant bacteria in milk from several animals. The relatively low abundance of LAB observed in this study is consistent with a study published by Cavallarin et al. (2015). In this study, members of the *Lactococcus* (0.26%) genus were more prevalent than those off the *Lactobacillus* genus (0.14%); this result was not consistent with the Setyawardani et al. (2011) report. Some LAB in raw goat milk were detected at the species level. LAB in milk have shown potential in the production of natural antimicrobials for the improvement of human and animal health (Quigley et al., 2013). Recently, Perna et al. (2015) observed that a LAB strain isolated from cow's milk had a positive effect on the fermentation of milk. In another study, Jeronymo-Ceneviva et al. (2014) isolated a new probiotic bacterium from dairy products produced from buffalo milk. Previous studies have shown that goat milk can treat patients with milk allergies and gastrointestinal diseases (Haenlein et al., 2004). Our results provide a theoretical basis

for the isolation of beneficial bacteria in goat milk.

Conclusions

This study describes the bacterial diversity in goat milk as well as in pasteurized goat milk during refrigerated storage. The analysis revealed the presence of bacteria that had not been previously been detected. Furthermore, high-throughput DNA sequencing revealed the presence of probiotic and pathogenic strains in goat milk. This study also showed that microorganisms believed to be eliminated by pasteurization are likely to survive commercial pasteurization. Meanwhile, a Spearman's correlation analysis showed that some psychrophilic bacteria were positively correlated with *Sphingomonas* and *Meiothermus*; the effects of these microorganisms in goat milk remain unknown. Further studies should focus on the dynamic relationship between bacterial populations and goat milk composition as well as the isolation of beneficial bacteria from goat milk.

Conflict of interest

The authors declare that there is no commercial or associative interest that represents a conflict of interest in connection with the work submitted.

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492 **Figure legends**

493 **Figure 1**. Rarefaction curve and rank abundance curve of bacterial diversity in goat
494 milk. P_5= pasteurized goat milk samples stored for 5 d at refrigeration temperature;
495 P_{10} = pasteurized goat milk samples stored for 10 d at refrigeration temperature. OTU
496 = operational taxonomic units.

497 **Figure 2**. Relative abundance of the indigenous microflora (present above 0.1%)
498 phyla (A) and genera (B) in raw goat milk.

499 **Figure 3**. Relative abundance of the microflora (present above 0.5%) at the genus
500 level in pasteurized goat milk at refrigeration temperature. P_5 = pasteurized goat milk
501 samples stored for 5 d. P_{10} = pasteurized goat milk samples stored for 10 d.

502 **Figure 4**. Relative abundance of the Gram-positive and Gram-positive bacteria
503 (present above 0.5%) at the genus level in pasteurized goat milk at refrigeration
504 temperature. P_5 = pasteurized goat milk samples stored for 5 d. P_{10} = pasteurized goat
505 milk samples stored for 10 d.

506 **Figure 5.** Correlations among predominant bacterial genera of pasteurized goat milk
507 during cold storage.

508 Table 1. Characteristics of high-throughput sequencing data

Sample	Raw reads	Clean reads	Observed species	Chao	Shannon	ACE
Raw milk	374,136	356,787	551	562.25	3.09	556.35
P_5[1]	402,612	385,036	526	541.44	3.17	533.95
P_{10}[2]	422,998	387,056	331	337.89	2.96	335.91

509 [1]P_5= Pasteurized goat milk samples were stored for 5 d at refrigeration temperature.

510 [2]P_{10}= Pasteurized goat milk samples were stored for 10 d at refrigeration temperature.

511

512 Table 2. Prevalence of bacterial phyla and genera detected in raw goat milk by

513 high-throughput sequencing

Phylum	Mean	Genus	Mean
Proteobacteria	67.66	*Kluyvera*	28.85
Firmicutes	18.56	*Geobacillus*	9.30
Deinococcus-Thermus	8.50	*Thermus*	8.49
Bacteroidetes	3.30	*Pseudomonas*	8.28
		Acinetobacter	4.08
		Shigella	1.93
		Aquabacterium	1.02
		Burkholderia	1.18
		Streptococcus	1.06

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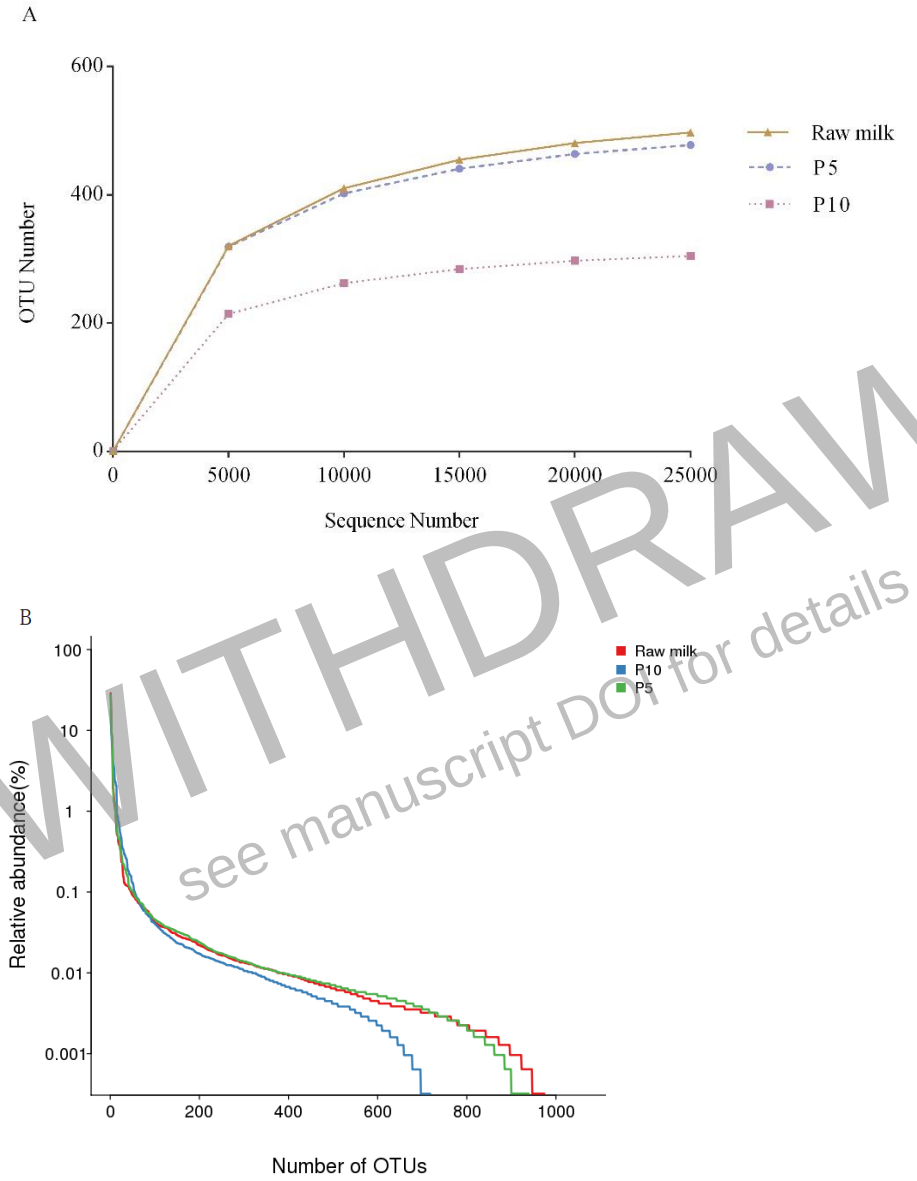

Figure 1.

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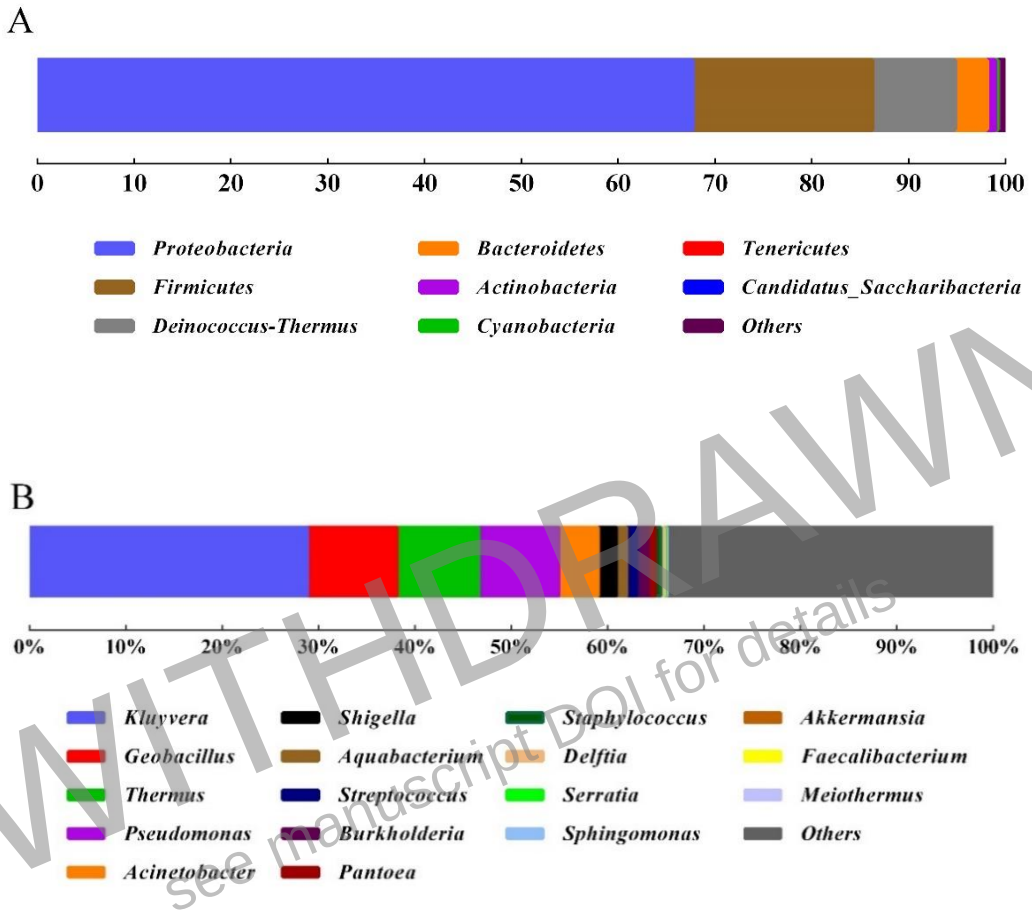

Figure 2.

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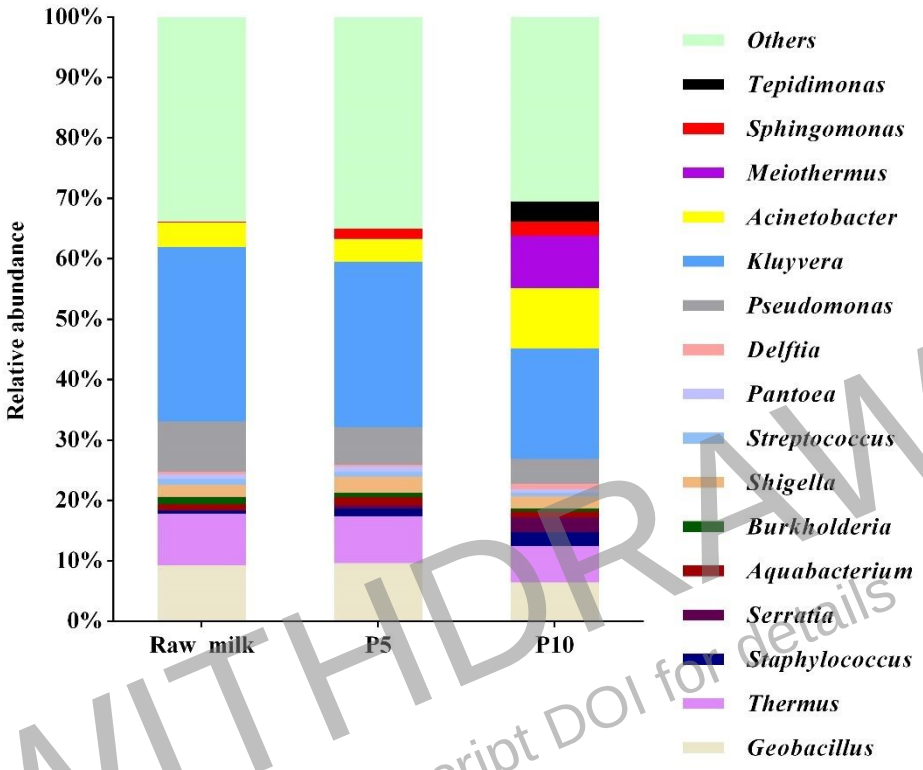

Figure 3.

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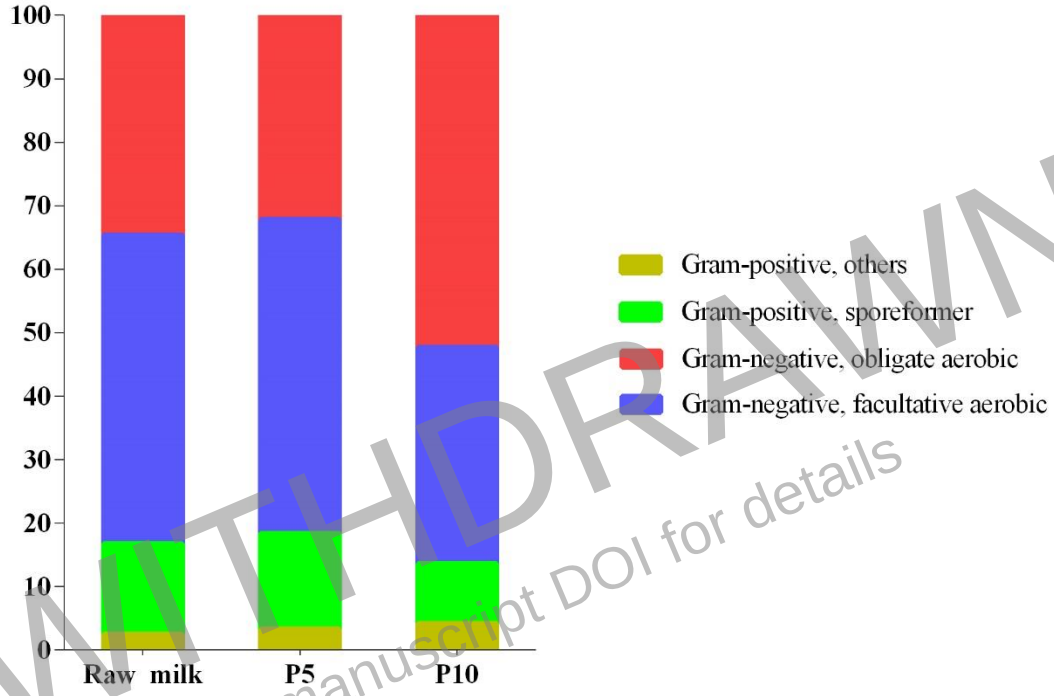

Figure 4.

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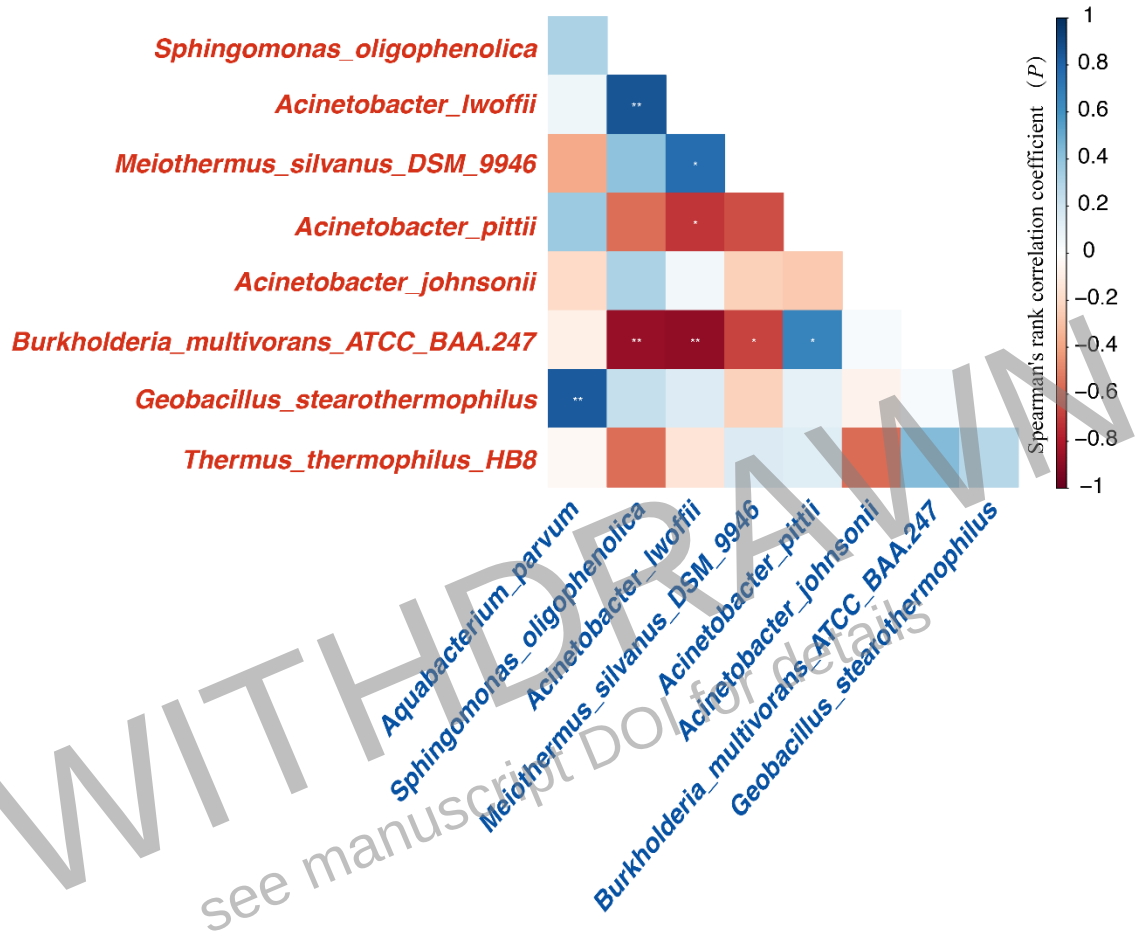

Figure 5.

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