

Metagenomic reveals succession in the bacterial community and predicts changes in raw milk during refrigeration

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Abstract

In this study, metagenomics was used to analyze microbial succession and predict changes in raw milk during 6 days of storage at 4 °C, aimed at determining how microorganisms drive the deterioration of refrigerated raw milk. The microbial community in raw milk changed significantly with an extension of refrigeration time ($P < 0.01$). The dominant bacterial genera gradually evolved from *Acinetobacter*, *Streptococcus*, and *Anaplasma* to *Flavobacterium*, *Pseudomonas*, and *Lactococcus*. KEGG annotation results indicated that the main role of the bacterial community included amino acid biosynthesis, carbon metabolism, and functioning as an ABC transporter. Additionally, lipid, amino acid and carbohydrate metabolism pathways were significantly expressed at the beginning, middle, and end of refrigeration, respectively. Refrigeration time is an important factor affecting the composition of the microbial flora in raw milk. The results of this study illustrate the role of microorganisms in the deterioration of refrigerated raw milk.

Introduction

Based on the physiological state of cows and the hygiene levels of their surroundings, raw milk has a certain abundance of initial microorganism, such as *Pseudomonas*, *Flavobacterium*, *Serratia*, *Acinetobacter*, *Leuconostoc*, *Lactococcus*, *Streptococcus*, *Bacillus*, and among others (Porcellato et al., 2018; Tilocca et al., 2020; von Neubeck et al., 2015; Yuan et al., 2017). Before milk is processed, refrigeration can effectively reduce microbial growth, although complete growth inhibition cannot be achieved (O'Connell et al., 2016). In particular, psychrophilic microorganisms in raw milk may continue to thrive even at low temperatures. Some of them produce heat-resistant alkaline proteases, lipases, or phospholipase (Fox et al., 1976; Ribeiro Junior et al., 2018), which may lead to gelation, rancidity, and shortening of the shelf life of dairy products (Quigley et al., 2013). Others may produce biofilms, which complicate the process of sterilization and enhance the thermal stability of enzymes through the formation of extracellular polymeric substances (Hahne et al., 2019). Of late, researchers worldwide have been focusing on microbial biodiversity to determine and characterize the dominant spoilage species, especially psychrophilic microorganisms in raw milk (Porcellato et al., 2018; Yuan et al., 2017). Studies show that the diversity of psychrophilic microorganisms is significantly different and influenced by geographical and seasonal factors and storage temperature (Bonizzi et al., 2009; O'Connell et al., 2016; Porcellato et al., 2018). Spoilage, however, is the end result of the gradual deterioration of food. Typically, raw milk is refrigerated for several days before processing (1–5 days) (Vithanage et al., 2016; von Neubeck et al., 2015; Zhang et al., 2020), during which microbes transform the nutrients in milk into metabolites that serve as substrates to support the growth of other microbes. Eventually, this transformation leads to the deterioration of raw milk. A gradual evolution in the type and number of microorganisms present in raw milk ultimately leads to the spoilage of refrigerated raw milk. Unfortunately, only a few studies have explored the relationship between microbial community succession and changes in milk composition during cold storage. To the best of our knowledge, there are no reports on how microorganisms drive the deterioration of refrigerated raw milk.

Among the various sequencing technologies, 16S rRNA profiling has been widely used in dairy microbiology. Although this technique is popular in terms of accuracy in sequencing, it has certain limitations in determining the microbial community at the species level. Metagenomics can be used to annotate microorganisms at the species or even at the strain level and is suitable in not only analyzing the composition of bacterial communities, but also in obtaining the functional information of bacteria, which can provide important information on the mechanism by which microorganisms affect the quality of raw milk.

In this study, using the Illumina HiSeq metagenomics sequencing platform, the microbial composition and dominant microbial succession of raw milk refrigerated at 4°C for 6 days were analyzed. The functional annotations of the microorganisms in raw milk were also obtained. The results from our study will help provide an in-depth theoretical

basis for the quality control of raw milk and also serve as a theoretical reference for the adjustments in refrigeration technology.

Materials And Methods

Sample Collection, and Bacterial Count

Raw milk samples were collected in December from a dairy farm in Yinchuan, Ningxia, China. The somatic cell number of raw milk was $1.76 \times 10^6 \pm 0.09 \text{ mL}^{-1}$, the total bacterial count was $0.78 \times 10^5 \pm 0.05 \text{ cfu mL}^{-1}$, fat content was $4.29 \pm 0.04 \text{ g} \cdot 100 \text{ mL}^{-1}$, protein content was $3.75 \pm 0.04 \text{ g} \cdot 100 \text{ mL}^{-1}$, and lactose content was $4.95 \pm 0.02 \text{ g} \cdot 100 \text{ mL}^{-1}$. The milk samples were transported over ice to our laboratory within 2 hours and refrigerated at 4°C for 2 hr, 1 day, 2 days, 3 days, 4 days, 5 days, and 6 days.

The total bacterial count (TBC) was determined by referring to the Chinese National Standard GB 4789.2-2016, using plate count agar (PCA) and culturing for $48 \pm 2 \text{ hr}$ at 37 °C. Psychrotrophic bacterial count (PBC) was determined by referring to the Chinese Agricultural Industry Standard NY/T 1331-2007, using psychrophilic bacteria count agar (MPC) and culturing for 10 days at 6.5 °C. The coliform bacterial count was determined by referring to the Chinese National Standard GB 4789.3-2016, using eosin methylene blue agar (EMB) and culturing for $24 \pm 2 \text{ hr}$ at 37 °C. The counts of lactic acid bacteria were determined by referring to the Chinese National Standard GB 4789.35-2016, using MRS agar for culture at $72 \pm 2 \text{ hr}$ at 37 °C.

DNA Extraction

Using metagenomics, we selected suitable sample sites from 16S amplicon analysis results for further analysis. Based on the previous results of 16S rRNA profiling, the significant turning time points in phylum abundance were determined to be 2 hr, 1 day, 3 days, 4 days, and 6 days; these were selected for metagenomics analysis and numbered A0, A1, A3, A4, and A6, respectively. Raw milk was pretreated before DNA extraction. Briefly, homogenized raw milk samples (10 mL) were centrifuged at $5000 \times g$ for 10 min at 4 °C and the supernatant and fat were removed. Next, 3 mL of sterile water was added and the bacterial pellet was mixed using a vortex mixer. Further, the samples were centrifuged at $5000 \times g$ for 5 min at 4 °C to remove the supernatant and fat, and 2 mL of phosphate-buffered saline was added to the residue and mixed well. This pretreatment procedure was performed three times for each sampling point (A0, A1, A3, A4, A6). Using the DNeasy Power Food Microbial Kit (100) (Qiagen, Hilden, Germany), DNA was extracted from 15 processed samples and numbered A01, A02, A03, A11, A12, A13, A31, A32, A33, A41, A42, A43, A61, A62 and A63. In addition, a nucleic acid test and 1% agarose gel electrophoresis were performed to determine DNA integrity. After passing this test, the DNA was stored at -20 °C until subsequent analysis.

Metagenomic Sequencing

Using Covaris M220 (Gene Company Limited, Shanghai, China), the DNA extract was fragmented for the construction of a paired-end library. The library was constructed using NEXTFLEX® Rapid DNA-Seq (Bioo Scientific, Austin, TX, USA). Paired-end sequencing was performed on Illumina HiSeq Xten (Illumina Inc., San Diego, CA, USA) at Majorbio Bio-Pharm Technology Co., Ltd. (Shanghai, China) using HiSeq X Reagent Kits (www.illumina.com) according to the manufacturer's instructions. Sequence data associated with this project have been deposited in the NCBI Short Read Archive database (Accession Number: PRJNA726626).

Adapter sequence were stripped from the 3' and 5' end of paired-end Illumina reads using SeqPrep (<https://github.com/jstjohn/SeqPrep>). Low-quality reads (length < 50 bp, or with a quality value < 20, or having N bases) were removed using Sickle (<https://github.com/najoshi/sickle>). Reads were aligned to the cow genome

published in the NCBI and Ensembl, and any hit associated with the reads was removed using BWA (<http://bio-bwa.sourceforge.net>). Metagenomics data were assembled using MEGAHIT (<https://github.com/voutcn/megahit>). Contigs with lengths of over 300 bp were selected for the final assembling.

Gene Prediction

MetaGene (<http://metagene.cb.k.u-tokyo.ac.jp/>) was used to predict the open reading frames (ORFs) from the assembled contigs. Using the NCBI translation table (<http://www.ncbi.nlm.nih.gov/Taxonomy/taxonomyhome.html/index.cgi?chapter=tgencodes#SG1>), the predicted ORFs with a length of 100 bp or over were retrieved and translated into amino acid sequences. The predicted gene sequences were clustered using CD-HIT (<http://www.bioinformatics.org/cd-hit/>), and the longest gene sequences of each type were selected to construct non-redundant gene catalogs. Reads after quality control were mapped to the representative sequences using SOAPaligner (<http://soap.genomics.org.cn/>) (parameter: 95% identity), and the gene abundance in the corresponding samples were evaluated using the RPKM (Reads Per Kilobase Million) method. The calculation method for the combination of three samples in the group was to determine the average value.

Taxonomy and Functional Annotation

The sequences of the non-redundant gene catalog were aligned to the NCBI NR database and the Kyoto Encyclopedia of Genes and Genomes (KEGG) database using BLASTP (Version 2.2.28+, <http://blast.ncbi.nlm.nih.gov/Blast.cgi>) with an e-value of $1e^{-5}$ for taxonomic annotation and KEGG annotation.

Statistical Analysis

The bacterial counts of raw milk were calculated and are reported as the average value \pm standard deviation. R Programming Language was used to calculate the P-value and plot Venn diagrams, column diagrams, box-plots, and principal coordinates analysis (PCoA) based on Bray-Curtis dissimilarity. Kruskal–Wallis H test was used to determine significant differences between samples. LEfSe analysis with an all-against-all multi-group comparison strategy was used to evaluate the functions that had significant differences in sample division.

Results And Discussion

Bacterial Count and Nutrient Analysis of Raw Milk

The TBC, PBC, coliform bacteria count, and lactic acid bacteria count of raw milk increased significantly during refrigeration at 4 °C after 6 days ($P < 0.001$) (Fig 1a). The average (\pm SD) TBC increased from $3.71 \pm 0.06 \log_{10} \text{ cfu mL}^{-1}$ to $6.14 \pm 0.05 \log_{10} \text{ cfu mL}^{-1}$, PBC increased from $4.27 \pm 0.06 \log_{10} \text{ cfu mL}^{-1}$ to $8.19 \pm 0.03 \log_{10} \text{ cfu mL}^{-1}$, coliform bacteria count increased from $1.06 \pm 0.10 \log_{10} \text{ cfu mL}^{-1}$ to $3.63 \pm 0.14 \log_{10} \text{ cfu mL}^{-1}$, and lactic acid bacteria count increased from $3.14 \pm 0.10 \log_{10} \text{ cfu mL}^{-1}$ to $5.01 \pm 0.02 \log_{10} \text{ cfu mL}^{-1}$.

The changes in fat, protein, and lactose content of raw milk during refrigeration at 4 °C after 6 days are presented in Fig 1b. The fat content decreased significantly from $4.29 \pm 0.04 \text{ g} \cdot 100 \text{ mL}^{-1}$ at 2 hr to $3.59 \pm 0.04 \text{ g} \cdot 100 \text{ mL}^{-1}$ on day 6. The protein content decreased gradually from $3.75 \pm 0.04 \text{ g} \cdot 100 \text{ mL}^{-1}$ at 2 hr to $3.22 \pm 0.11 \text{ g} \cdot 100 \text{ mL}^{-1}$ on day 6. The lactose content declined slightly during refrigeration after 6d and ranged from $4.95 \pm 0.02 \text{ g} \cdot 100 \text{ mL}^{-1}$ to $4.75 \pm 0.01 \text{ g} \cdot 100 \text{ mL}^{-1}$.

Metagenomic Sequencing and Quality Control

The results in Table 1 show a total of 663,909,326 raw reads generated from metagenomic sequencing, and that the average length of each sample is 151 bp. After processing for quality, the clean reads accounted for more than 98% of the raw reads, and the average number of optimized sequences for each sample after removing the host sequences was 3,491,874. Genome assembly calculations suggested that the average contig of each sample was 967,826. The max and min contig lengths, N50 and N90, were used to evaluate the assembly quality. Gene catalog construction revealed a total of 101960 non-redundant genes.

Table 1 Metagene sequencing and assembly information

Sample	Number of raw reads	Raw base (bp)	Clean reads (%)	Number of contigs	Max contig Length [bp]	Min contig Length [bp]	N50(bp)	N90(bp)	Number of ORFs
A01	46594904	7035830504	98.56	1185084	12260	300	535	340	468210
A02	42830370	6467385870	98.51	1036717	15958	300	522	336	406453
A03	41555626	6274899526	98.45	986607	14493	300	517	335	387369
A11	41214226	6223348126	98.49	895047	286562	300	505	333	350953
A12	42810966	6464455866	98.54	984225	166712	300	518	335	387670
A13	42821540	6466052540	98.54	913929	175323	300	511	334	361932
A31	42850376	6470406776	98.52	961986	335253	300	522	335	388361
A32	42813426	6464827326	98.80	966010	233984	300	520	336	391163
A33	46130794	6965749894	98.61	1105472	262628	300	533	338	448883
A41	47490840	7171116840	98.63	1097905	312185	300	527	337	447196
A42	43345866	6545225766	98.53	957042	240800	300	510	334	383853
A43	44451870	6712232370	98.70	984696	243417	300	511	334	401414
A61	50212154	7582035254	98.84	1014507	334568	300	518	336	422810
A62	41881336	6324081736	98.56	640605	303714	300	484	326	267961
A63	46905032	7082659832	98.68	787569	290884	300	489	328	325677

Change in Raw Milk Using Metagenomics

The Venn diagram (Fig 2) shows that a total of 699 bacteria are annotated at the genus level and 2,058 at the species level. With an extension in refrigeration time, the variety of bacteria increased. Based on PCoA (Fig 3), the differences between groups (A0, A1, A3, A4, A6) were greater than that within groups at both genus and species level, which suggested that the bacterial community in raw milk changed significantly during cold storage ($P < 0.01$).

The relative bacterial abundances at the genus level in refrigerated raw milk are shown in Fig 4a. A total of 14 bacterial genera were annotated with a relative abundance greater than 0.01. The dominant genera in the A0 sample were *Acinetobacter* (66.81%), *Streptococcus* (11.53%), and *Anaplasma* (8.80%). Compared to that in A0, the relative abundance of *Streptococcus* and *Anaplasma* in A1 decreased to 0.41% and 0.32% respectively. Correspondingly, the

relative abundance of *Acinetobacter* increased to 83.87% and was the most dominant genus in A1. The dominant genera in A3 were *Acinetobacter* (54.20%), *Flavobacterium* (18.26%), and *Pseudomonas* (13.57%). Compared to that in A3, the relative abundance of *Pseudomonas* decreased to 3.97% in A4 and the dominant genus changed to *Acinetobacter* (59.32%), *Flavobacterium* (14.82%), and *Lactococcus* (10.67%). Otherwise, *Lactococcus* (53.16%) was observed as the most abundant genus in A6, followed by *Acinetobacter* (23.18%) and *Flavobacterium* (5.37%). Moreover, other bacteria with lesser abundance, such as *Carnobacterium*, *Serratia*, *Enterococcus*, *Lactobacillus*, *Brochothrix*, *Clostridium*, *Mycobacterium*, and *Bacillus* were annotated. Kruskal–Wallis H test showed statistically significant ($P < 0.05$) differences between the refrigeration time groups in all of the above bacterial genera (Fig 5a). Briefly, the dominant bacterial genera gradually evolved from *Acinetobacter*, *Streptococcus*, and *Anaplasma* to *Flavobacterium*, *Pseudomonas*, and *Lactococcus* during the six days in the refrigerated raw milk.

At the species level, a total of 20 species were annotated (Fig 4b) with a relative abundance greater than 0.01. The top 3 dominant species in the relative abundance of each sample were defined. They were *Acinetobacter* sp. TTH0-4 (41.91%), *Anaplasma phagocytophilum* (8.60%), and *Streptococcus pneumoniae* (6.06%) in A0; *Acinetobacter* sp. TTH0-4 (73.02%), unclassified *Acinetobacter* (4.65%), and *Acinetobacter baumannii* (2.54%) in A1; *Acinetobacter* sp. TTH0-4 (41.63%), *Flavobacterium frigidarium* (15.24%), and *Pseudomonas fluorescens* (9.77%) in A3; *Acinetobacter* sp. TTH0-4 (45.63%), *Flavobacterium frigidarium* (12.18%), and *Lactococcus piscium* (8.05%) in A4; and *Lactococcus piscium* (35.13%), *Acinetobacter* sp. TTH0-4 (18.21%), and *Lactococcus raffinolactis* (12.90%) in A6. In addition, other species with low abundances, such as *Acinetobacter johnsonii*, *Acinetobacter bohemicus*, *Lactococcus lactis*, *Carnobacterium maltaromaticum*, and *Brochothrix thermosphacta* were also annotated. Kruskal–Wallis H test showed statistically significant ($p < 0.05$) differences between the refrigeration time groups in all above bacterial species (Fig 5b). With the extension in refrigeration time, the dominant species of raw milk gradually evolved from *Acinetobacter* sp. TTH0-4, *Anaplasma phagocytophilum*, and *Streptococcus pneumoniae* to *Flavobacterium frigidarium*, *Pseudomonas fluorescens*, *Lactococcus piscium*, and *Lactococcus raffinolactis*.

KEGG Function Annotation Analysis

To better understand the importance of the role of microbiota in raw milk samples, the levels of various metabolic pathways were determined. Based on the pathway classification statistics histogram (Fig 6), all functions were classified as metabolism (63.68%), genetic information processing (10.94%), environmental information processing (10.11%), cellular processes (5.90%), human diseases (5.42%), and organismal systems (3.85%), among which the function of metabolism was found to be the most abundant. The heatmap (Fig 7) of the top 50 functions of total abundance at the KEGG pathway level 3 showed that the biosynthesis of amino acids (ko01230), ABC transporters (ko02010), and carbon metabolism (ko01200) were the most represented pathways in all samples. Moreover, using LEfSe analysis (Table 2), we studied the functions related to lipid, amino acid, and carbohydrate metabolism, which we were interested in. The results indicated that pyruvate metabolism (ko00620) and fatty acid biosynthesis (ko00061) pathways were significantly enriched after one day of refrigeration. Glycine, serine, and threonine metabolism (ko00260), as well as alanine, aspartate, and glutamate metabolism (ko00250) pathways were significantly enriched after 3 days of refrigeration. Lastly, after refrigeration for 6 days, glycolysis/gluconeogenesis (ko00010) and amino sugar and nucleotide sugar metabolism (ko00520) pathways were significantly enriched.

Table 2 LEfSe analysis of functions

KEGG level3	Group	LDA value	P value	KEGG level2
Biosynthesis of amino acids	A6	4.02	0.01	Global and overview maps
ABC transporters	A6	4.03	0.02	Membrane transport
Carbon metabolism	A6	3.72	0.01	Global and overview maps
Two-component system	A1	3.72	0.01	Signal transduction
Purine metabolism	A4	3.63	0.03	Nucleotide metabolism
Ribosome	A1	3.55	0.01	Translation
Quorum sensing	A6	3.98	0.01	Cellular community-prokaryotes
Pyrimidine metabolism	A6	3.62	0.01	Nucleotide metabolism
Oxidative phosphorylation	A0	3.65	0.02	Energy metabolism
Pyruvate metabolism	A1	3.42	0.03	Carbohydrate metabolism
Glycolysis/Gluconeogenesis	A6	3.56	0.01	Carbohydrate metabolism
Bacterial secretion system	A1	3.39	0.01	Membrane transport
Sulfur metabolism	A1	3.72	0.03	Energy metabolism
Amino sugar and nucleotide sugar metabolism	A6	3.63	0.01	Carbohydrate metabolism
Aminoacyl-tRNA biosynthesis	A6	3.14	0.01	Translation
Homologous recombination	A6	3.45	0.04	Replication and repair
Cysteine and methionine metabolism	A6	3.26	0.01	Amino-acid metabolism
Carbon-fixation pathways in prokaryotes	A1	3.39	0.01	Energy metabolism
2-Oxocarboxylic acid metabolism	A1	3.24	0.01	Global and overview maps
Glycine, serine, and threonine metabolism	A3	3.24	0.01	Amino-acid metabolism
Peptidoglycan biosynthesis	A6	3.51	0.01	Glycan biosynthesis and metabolism
Fatty-acid metabolism	A1	3.30	0.01	Lipid metabolism
Glyoxylate and dicarboxylate metabolism	A1	3.36	0.01	Carbohydrate metabolism
Mismatch repair	A6	3.32	0.02	Replication and repair
Propanoate metabolism	A1	3.37	0.01	Carbohydrate metabolism
Alanine, aspartate, and glutamate metabolism	A3	3.20	0.02	Amino-acid metabolism
Phenylalanine, tyrosine, and tryptophan biosynthesis	A6	3.26	0.01	Amino-acid metabolism
Methane metabolism	A1	3.19	0.01	Energy metabolism
Citrate cycle (TCA cycle)	A1	3.19	0.01	Carbohydrate metabolism
DNA replication	A6	3.42	0.01	Replication and repair

The correlation between the dominant microorganisms and functional metabolic pathways was analyzed (Fig 8). The results indicated that lipid metabolism was significantly correlated with *Acinetobacter* ($P < 0.001$), amino acid metabolism was significantly correlated with *Pseudomonas* ($P < 0.01$), and carbohydrate metabolism was significantly correlated with *Lactococcus* ($P < 0.01$).

On the basis of the above results, it could be explained that the lipid metabolism pathway of raw milk after refrigeration for 1 day was the most represented pathway and was significantly related to *Acinetobacter*. The amino acid metabolism pathway was the most represented pathway after refrigeration for 3 days and was significantly related to *Pseudomonas*. After 6 days of refrigeration, the carbohydrate metabolism pathway was the most represented pathway and was significantly related to *Lactococcus*.

Discussion

Several studies show that the TBC and PBC of raw milk are increased with an extension of refrigeration (Boubendir et al., 2016; Paludetti et al., 2018; Rasolofo et al., 2010) and these findings were consistent with the results of our study. O'Connell et al. (2016) demonstrated that after 4 days of refrigeration, the TBC of raw milk increased; but this increase was not significant ($P > 0.09$). On the other hand, they report that the PBC of raw milk increased remarkably ($P < 0.02$). In our study, both the TBC and PBC in raw milk increased significantly after 4 days of refrigeration at 4°C. The discrepancy between these findings may be related to the initial microbial load and composition in the raw milk samples. These influencing factors include somatic cell counts (SCCs), endogenous lysozyme system, as well as environmental factors, such as cow skin, the milking equipment used, feed, and soil.

Studies have shown that to maintain normal growth and metabolism of microorganisms at conditions of low temperature, fatty acids on the cell membranes can prevent cell damage by modifying lipids or synthesizing new lipids (Stanislava., 2017; Chintalapati et al., 2004). The decrease rate in lipid content in the early stages of cold storage (2 hr – 1 days) was significantly lower than that in the middle and late stages of cold storage (3–6 days), which may be due to microbial adaptation to the cold environment and lipid synthesis. Milk fat is composed of triglycerides, phospholipids, and sterols. Triglycerides can be degraded into free fatty acids by lipase produced by microorganisms, where phospholipids are decomposed by phospholipase and may be the reason for the decrease in fat levels in raw milk during late refrigeration. The decrease in protein content may be due to endogenous plasmin and microbial production of heat-resistant protease to decompose casein in raw milk. Lactose can be degraded to produce glucose and galactose, and glucose and galactose can also be synthesized under the action of enzymes. Additionally, lactose can also be produced by the action of 3-oxidation-reductase. *Lactobacillus* also plays a role in lactose decomposition. Therefore, the decrease in lactose content during refrigeration may be related to the interaction of endogenous substances and the microbial decomposition of raw milk.

The taxonomy annotation results showed that the microbial composition in raw milk changed significantly during refrigeration ($P < 0.01$). During the entire storage period, *Acinetobacter* was the dominant genus. Similar to that in our study, Hahne et al. (2019) used 16S rRNA sequencing technology to analyze the microbial diversity of refrigerated raw milk and determined that *Acinetobacter* was the genus with the highest abundance. In our study, during the early stage of refrigeration (2 hr-1 day), the relative abundance of *Acinetobacter* increased significantly (66.81–83.87%), which may be owing to its tolerance of low temperature (Chiesa et al., 2020; Gurung et al., 2013). In particular, *Acinetobacter* sp. TTH0-4, which was found in our study as the most abundant strain at the species level has not yet been studied in raw milk. It has been reported that this strain was isolated from the permafrost region of Qinghai Tibet Plateau (Zhang et al., 2016); therefore, it is inferred that the detection of this strain in raw milk may be related to the nature and

bacterial composition of the soil in the grazing pastures. Meanwhile, the relative abundance of *Streptococcus* decreased significantly after 1 day of refrigeration, likely because of their sensitivity to antimicrobial peptides, such as lactoferrin, lysozyme, and peroxide in raw milk (Adduci et al., 2019; Lara-Aguilar & Alcaine, 2019). Anaplasmosis is an infectious disease that has a huge impact on the dairy industry. *Anaplasma* has been widely studied and reported as a pathogen that mainly thrives in blood cells (Tumwebaze et al., 2020). In the current study, the relative abundance of *Anaplasma* decreased remarkably after a day of refrigeration, indicating that the low temperature at which raw milk was stored was may unsuitable for its growth. Similarly, using nested PCR and sequence analysis, Dela et al. (2019) also detected *Anaplasma* in raw milk. *Anaplasma phagocytophilum*, annotated in this study, has been reported to be present in fresh and healthy goat milk by Zhang et al. (2016). It is the most common species in *Anaplasma* (Ben et al., 2018). Whether the live organism can be separated or cultured from milk remains to be further studied.

During the middle period of cold storage (3–4 days), it is possible that the accumulation of metabolites had changed the nutrient composition of raw milk. Thus, *Flavobacterium* and *Pseudomonas* may have obtained the requisite nutrients for propagation, which resulted in an increase in their relative abundance. In addition to amylase, lipase, and catalase, *Flavobacterium* can also produce phospholipase C, which can degrade the phospholipids of the globular membrane of milk fat and increase the sensitivity of triglycerides to lipolysis (Waśkiewicz & Irzykowska, 2014), thereby causing bitterness in milk (Capodifoglio et al., 2016). In this study, *Flavobacterium frigidarium* was found to be the most abundant strain of *Flavobacterium*. To the best of our knowledge, this finding has not yet been reported in raw milk. *Pseudomonas* is a ubiquitous microorganism in raw milk that has been widely studied because of its ability to produce thermostable protease, lipase, and biofilms. In particular, *Pseudomonas fluorescens* has been the most studied microbe in this genus. It can produce alkaline metalloproteinase APRX, which can degrade κ -casein and result in the gelatinization of dairy products during storage. Moreover, *Pseudomonas* can use the nutrients in raw milk and continue to flourish (Colantuono et al., 2020; Meng et al., 2018; Zhang et al., 2020).

During the last phase of cold storage (6 d), *Lactococcus* was found to be the most dominant bacteria in the milk samples. This genus can produce L-lactic acid, which can increase the acidity of raw milk and inhibit the growth of non-acid-resistant microorganisms. In this study, *Lactococcus piscium* was the species with the highest relative abundance in the *Lactococcus* genus. Carraro et al. (2011) also detected the presence of *Lactococcus piscium*, but its effect in raw milk has not been clearly reported. Moreover, *Lactococcus raffinolactis* was found to be the third dominant species based on relative abundance after 6 days of refrigeration. Previous studies suggest that *Lactococcus raffinolactis* has higher growth requirements and depends on minerals and amino acids in the medium; it can ferment the lactose present in milk (Kimoto-Nira et al., 2012), which is closely related to changes in milk quality.

We performed the KEGG function annotation to explore the effect of microbial metabolism on raw milk. The results showed that the relative abundance of amino acid biosynthesis and carbon metabolism pathways were relatively high, which indicated that microorganisms grow and propagate mainly by the metabolism of nitrogen and carbon sources in raw milk. Besides, ABC transporters also had a high relative abundance. A study by Qu et al. (2020) indicates that ABC transporters can promote the transmembrane transport of nutrients and exhaust unnecessary exogenous substances and secondary metabolites in cells in time, thereby reducing the cell load and promoting growth. These findings suggested that the microorganisms in refrigerated raw milk could regulate membrane permeability through ABC transporters to improve substrate utilization in raw milk, thus enhancing the adaptability of microorganisms to changes in the milk environment.

The synthesis of fatty acids requires a rich supply of carbon sources. Alvarez & Steinbuchel (2002) suggested that the gram-negative oleaginous *Acinetobacter* species are capable of accumulating free fatty acids and triacylglycerols. The relative abundance of *Acinetobacter* increased after the refrigeration of raw milk for one day, which was significantly correlated with lipid metabolism. Hence, we deduced that *Acinetobacter* may influence the lipid composition of raw milk

in the early stages of refrigeration. Studies show (Liu & Huo, 2001; Li, 2013; Ding et al. 2020) that change in the flavor of raw milk is related to the synthesis and metabolism of amino acids. In this study, the amino acid metabolism pathways were enriched after raw milk was refrigerated for 3 days, and were significantly related to the relative abundance of *Pseudomonas*, which increased during this period. It is well known that *Pseudomonas* can cause milk spoilage through proteolytic metabolism. The carbohydrate metabolism pathways were significantly enriched six days after refrigeration. During this period, *Lactococcus* was found to be dominant, which was significantly related to carbohydrate metabolism. It hydrolyzes lactose to yield glucose and galactose, which are converted into lactic acid or other organic acids by glycolysis, subsequently increasing the acidity of raw milk. If the milk acidity is too high, casein and lactoglobulin precipitate, resulting in the clotting of raw milk and separation of whey.

Studies show that several products in a particular pathway can serve as reactants for other pathways (Wang & Xie, 2020). Carbohydrate, amino acid and lipid metabolism influence each other with a change in the microbial composition and abundance. Eventually, the complex metabolic system comprising raw milk microbiota will be formed.

Conclusion

Using metagenomic sequencing, we studied the changes in raw milk with respect to the microbial community and its metabolic potential during storage at 4°C. With an extension in storage time, the bacterial count, microbial community composition, and the predicted metabolic pathways of microbiota changed significantly, indicating that refrigeration time was an important factor determining microbial composition. Taxonomy annotation showed high microbial diversity in raw milk during refrigeration and the microbial succession was remarkable. At the genus level, the dominant bacteria gradually evolved from *Acinetobacter*, *Streptococcus*, and *Anaplasma* to *Flavobacterium*, *Pseudomonas*, and *Lactococcus*. At the species level, *Acinetobacter* sp. TTH0-4, *Flavobacterium frigidarium*, and *Lactococcus raffinolactis* have not been previously reported in raw milk, and warrant further study. Functional annotation showed that amino acid biosynthesis, carbohydrate metabolism, and ABC transport were the three functions with the highest relative abundance, enabling microorganisms to effectively utilize carbon and nitrogen sources in raw milk during refrigeration and enhancing their environmental adaptability. Moreover, lipid metabolism pathways were significantly enriched after one day of refrigeration, which may be related to the effect of *Acinetobacter*. Amino acid metabolism pathways were significantly enriched after refrigeration for 3 days, which may be related to the hydrolysis of proteins by *Pseudomonas* to yield amino acids. Carbohydrate metabolism pathways were significantly enriched after refrigeration for 6 days, which may be related to the decomposition of lactose by *Lactococcus*. The results from our study could serve as a starting point for further research on raw milk and likely shed light on the techniques of preservation and processing of milk and dairy products.

Declarations

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Data Availability The datasets generated during the current study are available from the corresponding author upon reasonable request.

Competing Interests The authors declare no competing interests.

Author's contribution details Rong Guo performed the research, analyzed most of the data and wrote the main manuscript text. Ning Ju developed the initial idea for the study and contributed to the text polish. Yuanyuan Wang, Meng Gou and Puyu Li analyzed parts of the data and prepared figure 1-8 and table 1-2. Yulong Luo gave guidance on data analysis methods. All authors reviewed the manuscript and agreed on the order of authorship of this manuscript.

References

1. Adduci, F., Elshafie, H. S., Labella, C., Musto, M., Freschi, P., Paolino, R., Ragni, M., & Cosentino, C. (2019). Abatement of the clostridial load in the teats of lactating cows with lysozyme derived from donkey milk. *Journal of Dairy Science*, *102*, 6750–6755. doi:10.3168/jds.2019-16311
2. Alvarez, H. M., & Steinbuchel, A. (2002). Triacylglycerols in prokaryotic microorganisms. *Applied Microbiology and Biotechnology*, *60*, 367–376. doi:10.1007/s00253-002-1135-0
3. Ben, M., Belkahia, H., & Messadi, L. (2018). *Anaplasma* spp. in North Africa: A review on molecular epidemiology, associated risk factors and genetic characteristics. *Ticks and Tick-Borne Diseases*, *9*, 543–555. doi:10.1016/j.ttbdis.2018.01.003
4. Bonizzi, I., Buffoni, J. N., Feligini, M., & Enne, G. (2009). Investigating the relationship between raw milk bacterial composition, as described by intergenic transcribed spacer-PCR fingerprinting, and pasture altitude. *Journal of Applied Microbiology*, *107*, 1319–1329.
5. Boubendir, A., Serrazanetti, D. I., Hamidechi, M. A., Vannini, L., & Guerzoni, M. E. (2016). Changes in bacterial populations in refrigerated raw milk collected from a semi-arid area of Algeria. *Annals of Microbiology*, *66*, 777–783.
6. Capodifoglio, E., Vidal, A. M. C., Lima, J. A. S., Bortoletto, F., D'Abreu, L. F., Goncalves, A. C. S., Vaz, A. C. N., Balieiro, J. C. D., & Netto, A. S. (2016). Lipolytic and proteolytic activity of *Pseudomonas* spp. isolated during milking and storage of refrigerated raw milk. *Journal of Dairy Science*, *99*, 5214–5223.
7. Carraro, L., Maifreni, M., Bartolomeoli, I., Martino, M. E., Novelli, E., Frigo, F., Marino, M., & Cardazzo, B. (2011). Comparison of culture-dependent and -independent methods for bacterial community monitoring during Montasio cheese manufacturing. *Research in Microbiology*, *162*, 231–239.
8. Chiesa, L. M., DeCastelli, L., Nobile, M., Martucci, F., Mosconi, G., Fontana, M., Castrica, M., Arioli, F., & Panseri, S. (2020). Analysis of antibiotic residues in raw bovine milk and their impact toward food safety and on milk starter cultures in cheese-making process. *Lwt-Food Science and Technology*, *131*, 109783.
9. Colantuono, A., D'Incecco, P., Fortina, M. G., Rosi, V., Ricci, G., & Pellegrino, L. (2020). Milk substrates influence proteolytic activity of *Pseudomonas fluorescens* strains. *Food Control*, *111*, 106073.
10. Dela Cruz, A. P., Galay, R. L., Sandalo, K. A. C., Pilapil-Amante, F. M. I. R., & Tanaka, T. (2019). Molecular detection of *Anaplasma* spp. in blood and milk of dairy cattle in the Philippines. *Turkish Journal of Veterinary & Animal Sciences*, *43*, 540–545.
11. Ding, R., Liu, Y., Yang, S., Liu, Y., Shi, H., Yue, X., Wu, R., & Wu, J. (2020). High-throughput sequencing provides new insights into the roles and implications of core microbiota present in pasteurized milk. *Food Research International*, *137*.
12. Fox, C. W., Chrisope, G. L., & Marshall, R. T. (1976). Incidence and identification of phospholipase C-producing bacteria in fresh and spoiled homogenized milk. *Journal of Dairy Science*, *59*, 1857–1864.
13. Gurung, M., Nam, H. M., Tamang, M. D., Chae, M. H., Jang, G. C., Jung, S. C., & Lim, S. K. (2013). Prevalence and antimicrobial susceptibility of *Acinetobacter* from raw bulk tank milk in Korea. *Journal of Dairy Science*, *96*, 1997–2002.
14. Hahne, J., Isele, D., Berning, J., & Lipski, A. (2019). The contribution of fast growing, psychrotrophic microorganisms on biodiversity of refrigerated raw cow's milk with high bacterial counts and their food spoilage potential. *Food Microbiology*, *79*, 11–19.
15. Kimoto-Nira, H., Aoki, R., Mizumachi, K., Sasaki, K., Naito, H., Sawada, T., & Suzuki, C. (2012). Interaction between *Lactococcus lactis* and *Lactococcus raffinolactis* during growth in milk: Development of a new starter culture.

Journal of Dairy Science, 95, 2176–2185.

17. Kosa, M., & Ragauskas, A. J. (2011). Lipids from heterotrophic microbes: advances in metabolism research. *Trends Biotechnol*, 29, 53–61.
18. Lara-Aguilar, S., & Alcaine, S. D. (2019). Lactose oxidase: A novel activator of the lactoperoxidase system in milk for improved shelf life. *Journal of Dairy Science*, 102, 1933–1942.
19. Li, H. W. (2013). Review on sensory evaluation of milk. *Inner Mongolia Petrochemical Industry*, 39, 40–43.
20. Liu, F., & Huo, G. C. (2001). Management of milk flavor through the manipulation of rumen microorganism. *Journal of Dairy Science and Technology*, 24, 14–17.
21. Meng, L., Liu, H. M., Dong, L., Zheng, N., Xing, M. R., Zhang, Y. D., Zhao, S. G., & Wang, J. Q. (2018). Identification and proteolytic activity quantification of *Pseudomonas* spp. isolated from different raw milks at storage temperatures. *Journal of Dairy Science*, 101, 2897–2905.
22. O'Connell, A., Ruegg, P. L., Jordan, K., O'Brien, B., & Gleeson, D. (2016). The effect of storage temperature and duration on the microbial quality of bulk tank milk. *Journal of Dairy Science*, 99, 3367–3374.
23. Paludetti, L. F., Kelly, A. L., O'Brien, B., Jordan, K., & Gleeson, D. (2018). The effect of different precooling rates and cold storage on milk microbiological quality and composition. *Journal of Dairy Science*, 101, 1921–1929.
24. Porcellato, D., Aspholm, M., Skeie, S. B., Monshaugen, M., Brendehaug, J., & Mellegard, H. (2018). Microbial diversity of consumption milk during processing and storage. *International Journal of Food Microbiology*, 266, 21–30.
25. Qu, J., Chen, T., Yao, M., Wang, Y., Xiao, W., & Li, B. (2020). ABC transporter and its application in synthetic biology. *Journal of Bioengineering*, 36, 1754–1766.
26. Quigley, L., O'Sullivan, O., Stanton, C., Beresford, T. P., Ross, R. P., Fitzgerald, G. F., & Cotter, P. D. (2013). The complex microbiota of raw milk. *FEMS Microbiology. Review*, 37, 664–698.
27. Rasolofo, E. A., St-Gelais, D., LaPointe, G., & Roy, D. (2010). Molecular analysis of bacterial population structure and dynamics during cold storage of untreated and treated milk. *International Journal of Food Microbiology*, 138, 108–118.
28. Ribeiro Junior, J. C., de Oliveira, A. M., Silva, F. G., Tamanini, R., de Oliveira, A. L. M., & Beloti, V. (2018). The main spoilage-related psychrotrophic bacteria in refrigerated raw milk. *Journal of Dairy Science*, 101, 75–83.
29. Salgado C A., de Almeida F A., Barros E., Baracat-Pereira M C., Baglinière F., & Vanetti M C D. (2021). Identification and characterization of a polyurethanase with lipase activity from *Serratia liquefaciens* isolated from cold raw cow's milk. *Food Chemistry*, 337, 127954.
30. Stanislava K. (2017). Role of fatty acids in cold adaptation of Antarctic psychrophilic *Flavobacterium* spp. *Systematic and Applied Microbiology*, 40, 329–333.
31. Tilocca, B., Costanzo, N., Morittu, V. M., Spina, A. A., Soggiu, A., Britti, D., Roncada, P., & Piras, C. (2020). Milk microbiota: characterization methods and role in cheese production. *Journal of Proteomics*, 210, 103534.
32. Tumwebaze, M. A., Lee, S. H., Moumouni, P. F. A., Mohammed-Geba, K., Sheir, S. K., Galal-Khallaf, A., Latif, H. M. A., Morsi, D. S., Bishr, N. M., Galon, E. M., Byamukama, B., Liu, M. M., Li, J. X., Li, Y. C., Ji, S. W., Ringo, A. E., Rizk, M. A., Suzuki, H., Ibrahim, H. M., & Xuan, X. N. (2020). First detection of *Anaplasma ovis* in sheep and *Anaplasma platys*-like variants from cattle in Menoufia governorate, Egypt. *Parasitology International*, 78, 102150.
33. Vithanage, N. R., Dissanayake, M., Bolge, G., Palombo, E. A., Yeager, T. R., & Datta, N. (2016). Biodiversity of culturable psychrotrophic microbiota in raw milk attributable to refrigeration conditions, seasonality and their spoilage potential. *International Dairy Journal*, 57, 80–90.

34. von Neubeck, M., Baur, C., Krewinkel, M., Stoeckel, M., Kranz, B., Stressler, T., Fischer, L., Hinrichs, J., Scherer, S., & Wenning, M. (2015). Biodiversity of refrigerated raw milk microbiota and their enzymatic spoilage potential. *International Journal of Food Microbiology*, *211*, 57–65.
35. Wang, X. Y., & Xie, J. (2020). Assessment of metabolic changes in *Acinetobacter johnsonii* and *Pseudomonas fluorescens* co-culture from bigeye tuna (*Thunnus obesus*) spoilage by ultra-high-performance liquid chromatography-tandem mass spectrometry. *Lwt-Food Science and Technology*, *123*, 109073.
36. Waśkiewicz, A., & Irzykowska, L. (2014). *Flavobacterium* spp. - characteristics, occurrence, and toxicity. In C. A. Batt and M. L. Tortorello (Eds.), *Encyclopedia of Food Microbiology (Second Edition)* (pp. 938–942). Oxford: Academic Press.
37. Yuan, L., Sadiq, F. A., Liu, T. J., Flint, S., Chen, J. C., Yang, H. Y., Gu, J. S., Zhang, G. H., & He, G. Q. (2017). Psychrotrophic bacterial populations in Chinese raw dairy milk. *Lwt-Food Science and Technology*, *84*, 409–418.
38. Zhang, C. Y., Bijl, E., Muis, K. E., & Hettinga, K. (2020). Stability of fat globules in UHT milk during proteolysis by the AprX protease from *Pseudomonas fluorescens* and by plasmin. *Journal of Dairy Science*, *103*, 179–190.
39. Zhang, D., Palmer, J., Teh, K. H., & Flint, S. (2020). Identification and selection of heat-stable protease and lipase-producing psychrotrophic bacteria from fresh and chilled raw milk during up to five days storage. *Lwt-Food Science and Technology*, *134*, 110165.
40. Zhang, G. S., Chen, T., Chang, S. J., Zhang, W., Wu, X. K., Wu, M. H., Wang, Y. L., Long, H. Z., Chen, X. M., Wang, Y., & Liu, G. X. (2016). Complete genome sequence of *Acinetobacter* sp TTH0-4, a cold-active crude oil degrading strain isolated from Qinghai-Tibet Plateau. *Journal of Biotechnology*, *226*, 54–55.
41. Zhang, Y., Lv, Y. L., Cui, Y. Y., Wang, J. H., Cao, S. X., Jian, F. C., Wang, R. J., Zhang, L. X., & Ning, C. S. (2016). First molecular evidence for the presence of *Anaplasma* DNA in milk from sheep and goats in China. *Parasitology Research*, *115*, 2789–2795.

Figures

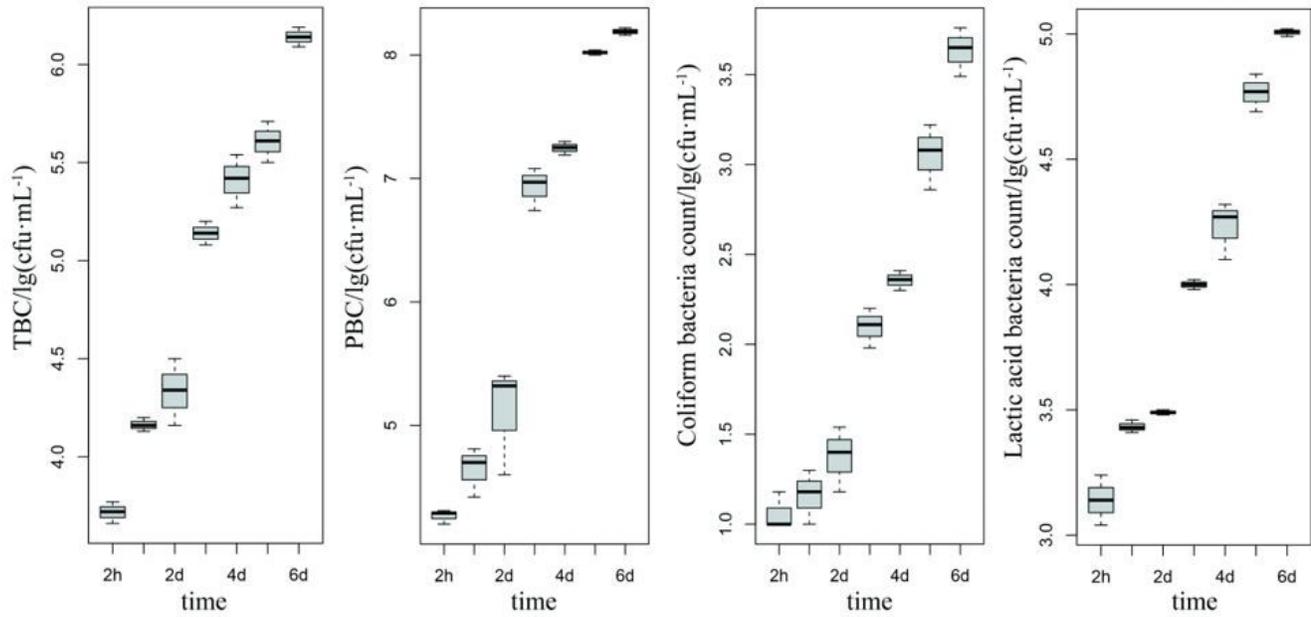


Fig 1a

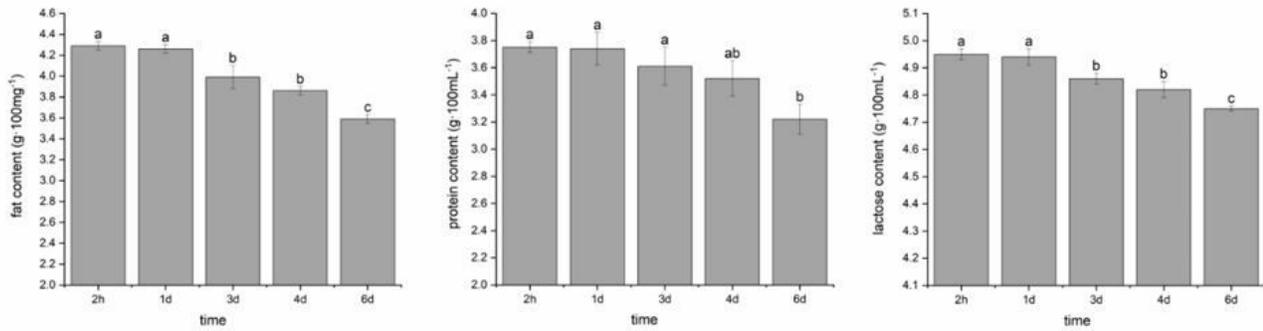


Fig 1b

Figure 1

1a. Box-plots of TBC, PBC, coliform bacteria count, and lactic acid bacteria count of raw milk during refrigeration at 4 °C for 6 days

1b: Chart-plots of fat, protein, and lactose content of raw milk during refrigeration at 4 °C for 6 days

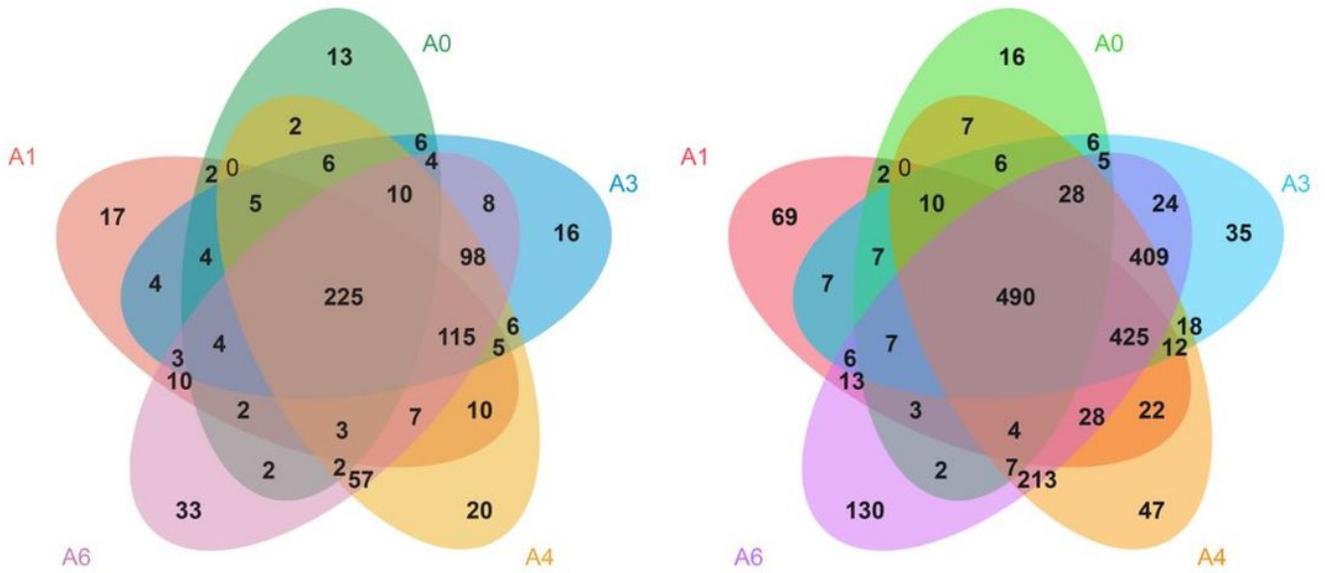


Figure 2

Venn diagrams of bacterial composition at the genus (left) and species (right) level

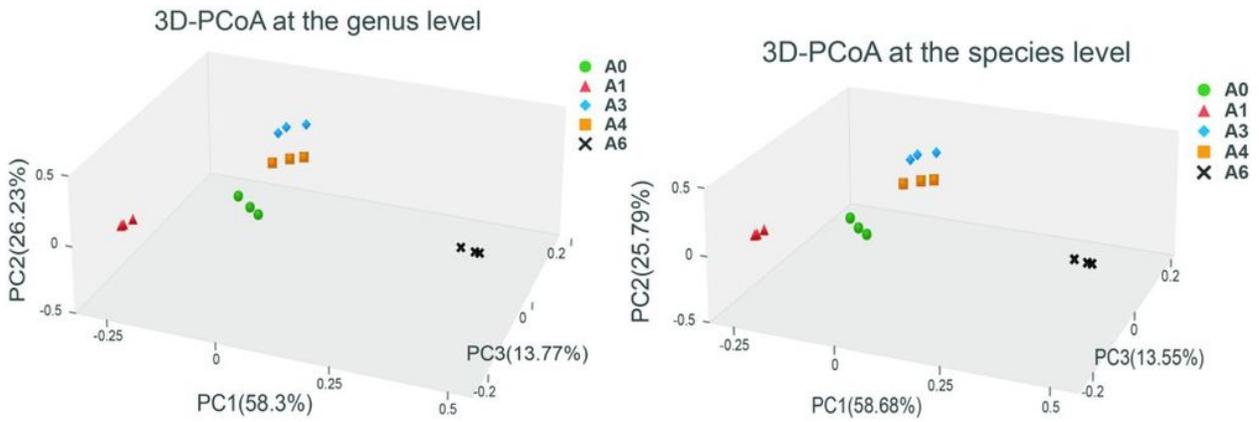


Figure 3

Principal coordinates analysis (PCoA) at the genus (left) and species (right) level

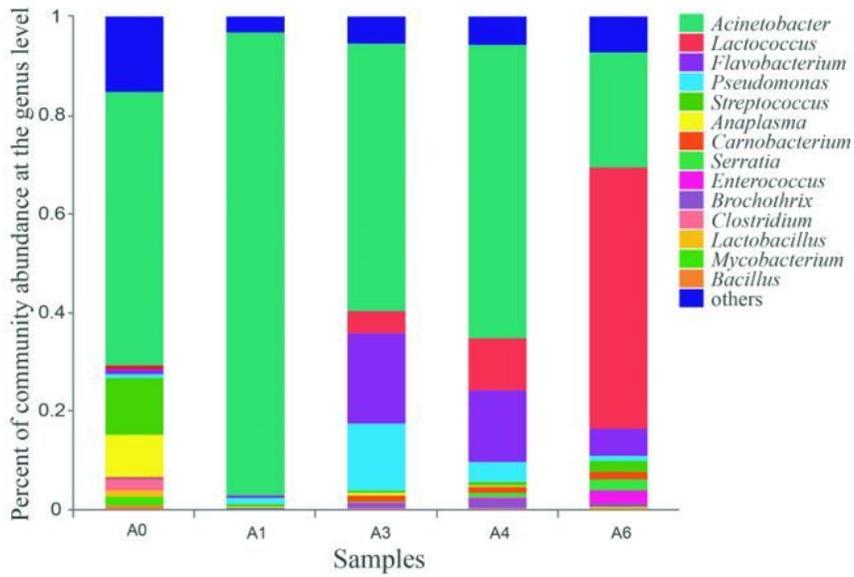


Fig 4a

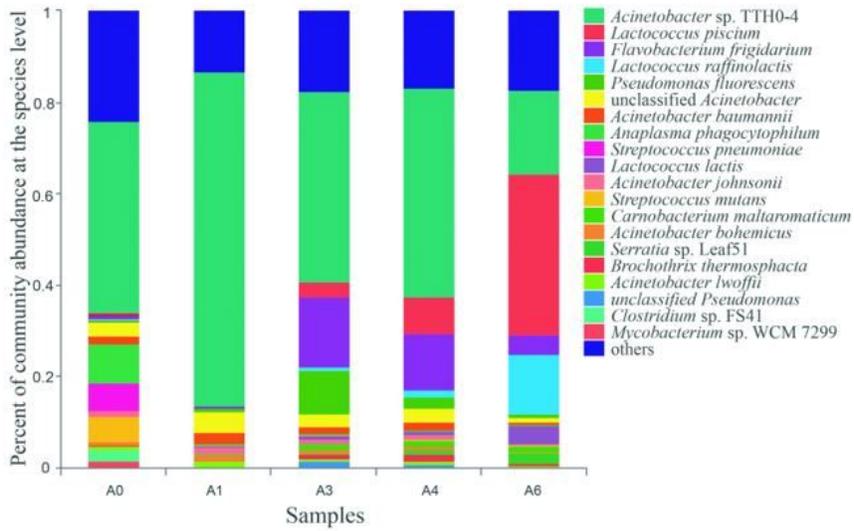


Fig 4b

Figure 4

4a. Bar chart of taxonomy annotation at the genus level

4b. Bar chart of taxonomy annotation at the species level

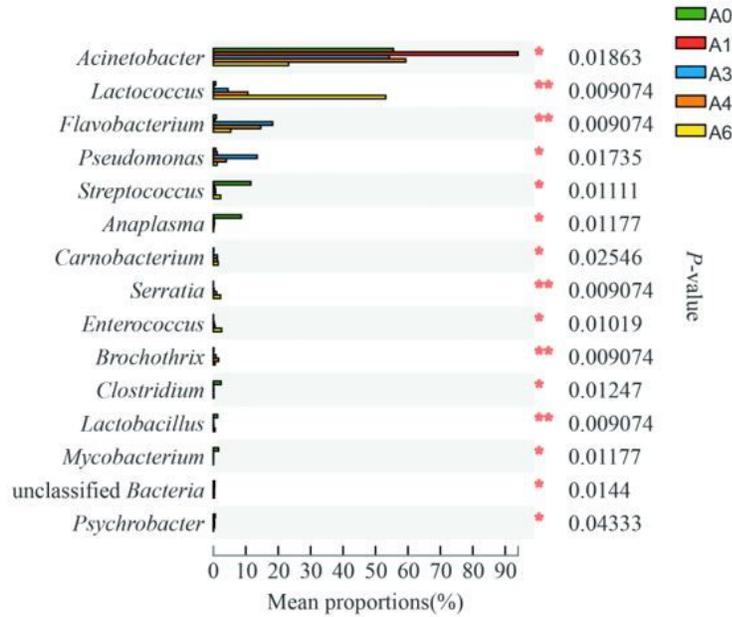


Fig 5a

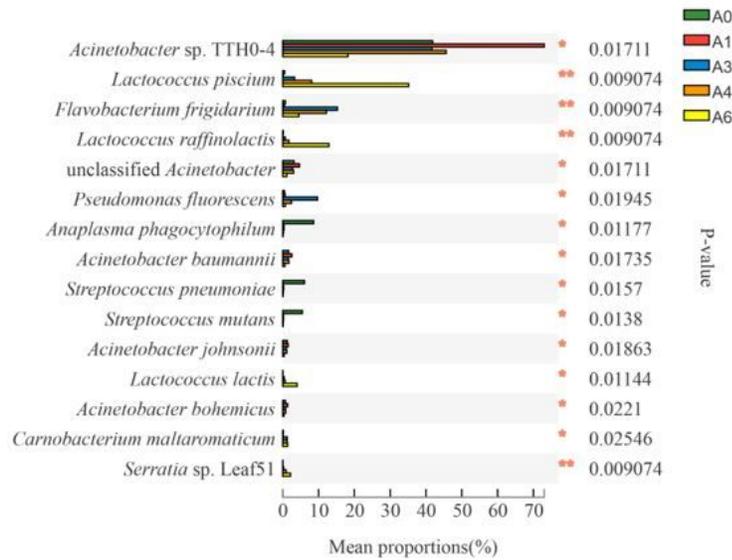


Fig 5b

Figure 5

5a. Kruskal-Wallis H test for bacterial composition at the genus level

5b. Kruskal-Wallis H test for bacterial composition at the species level

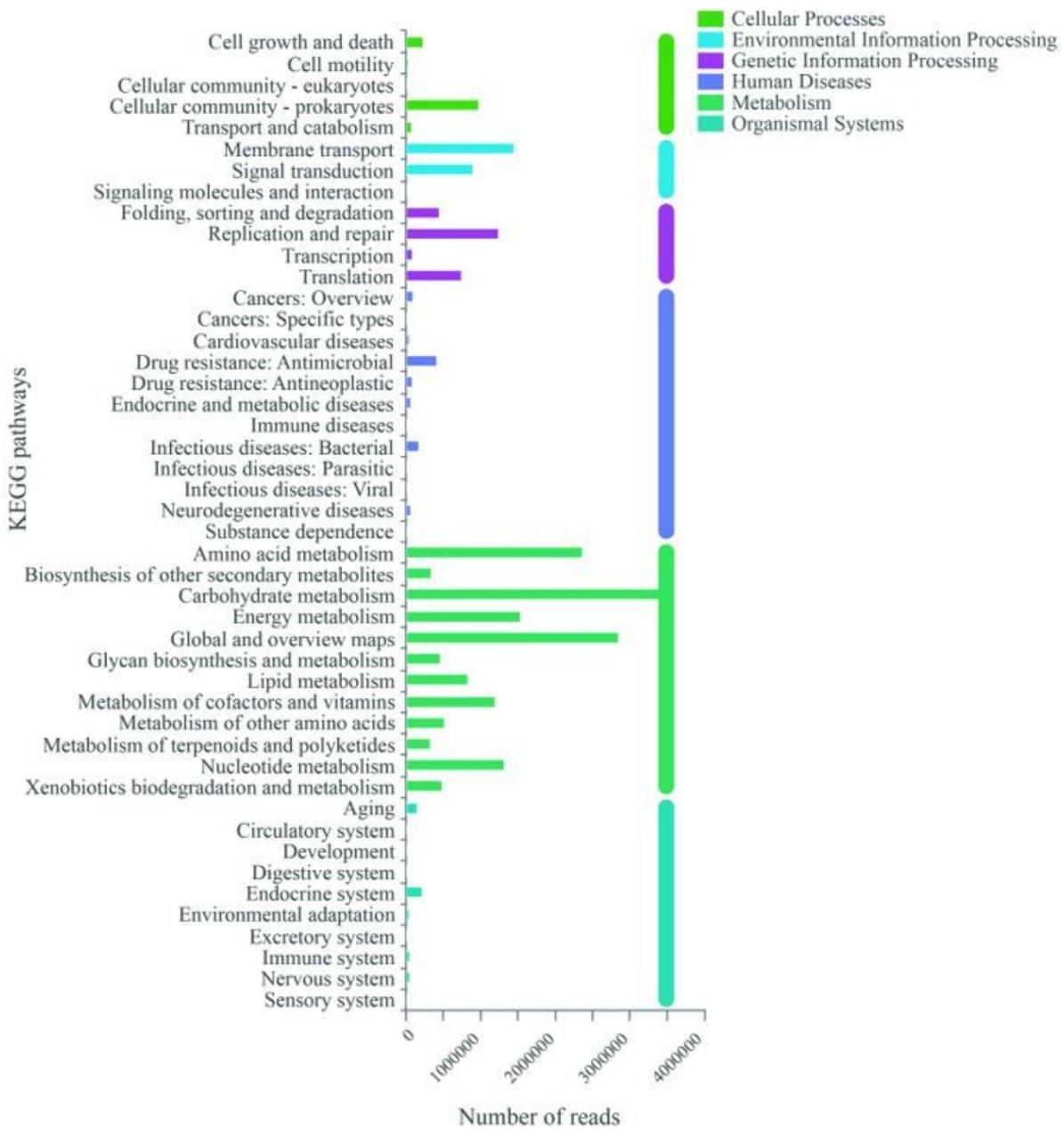


Figure 6

Histogram of pathway classification statistics

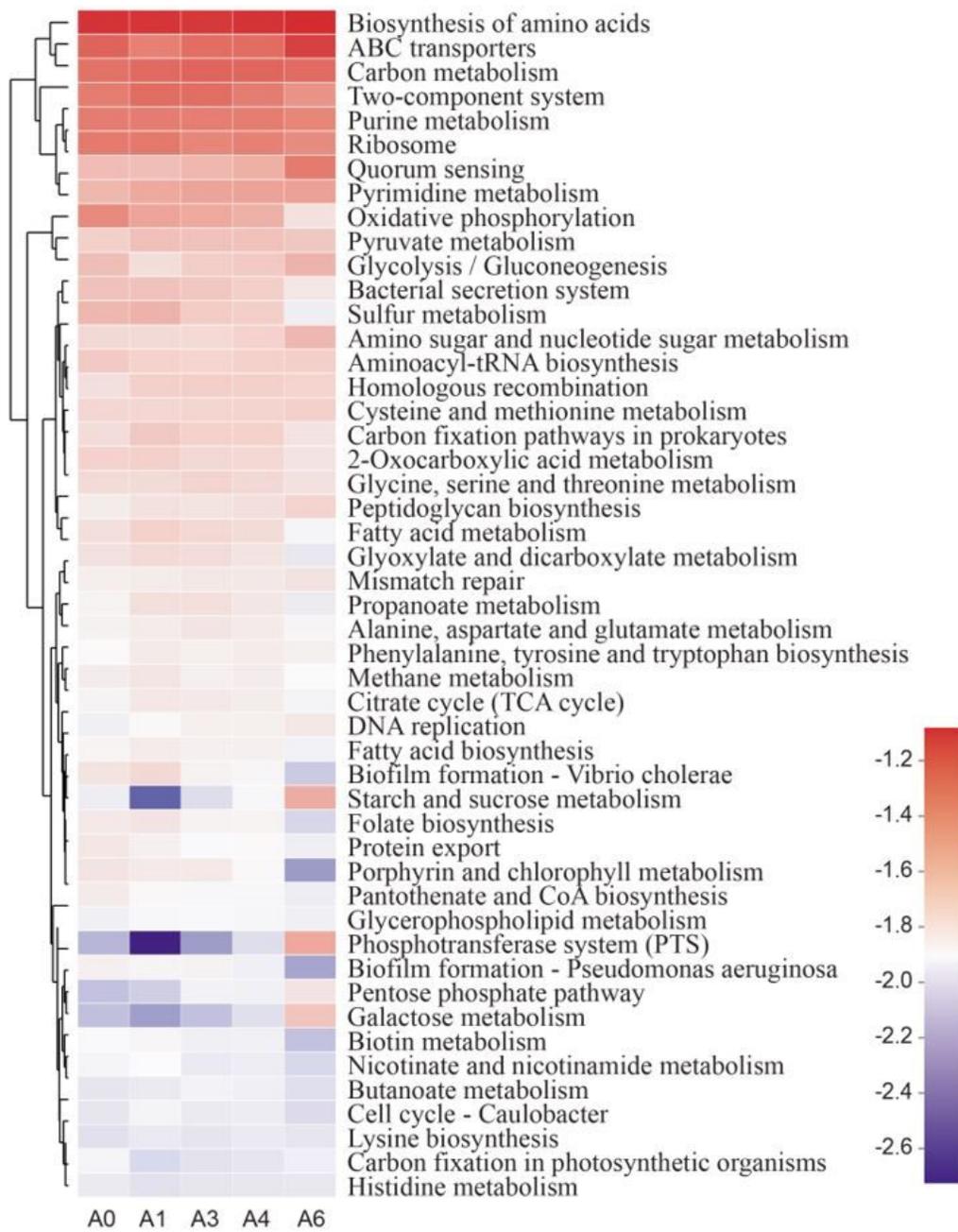


Figure 7

Heat map of KEGG pathway level 3

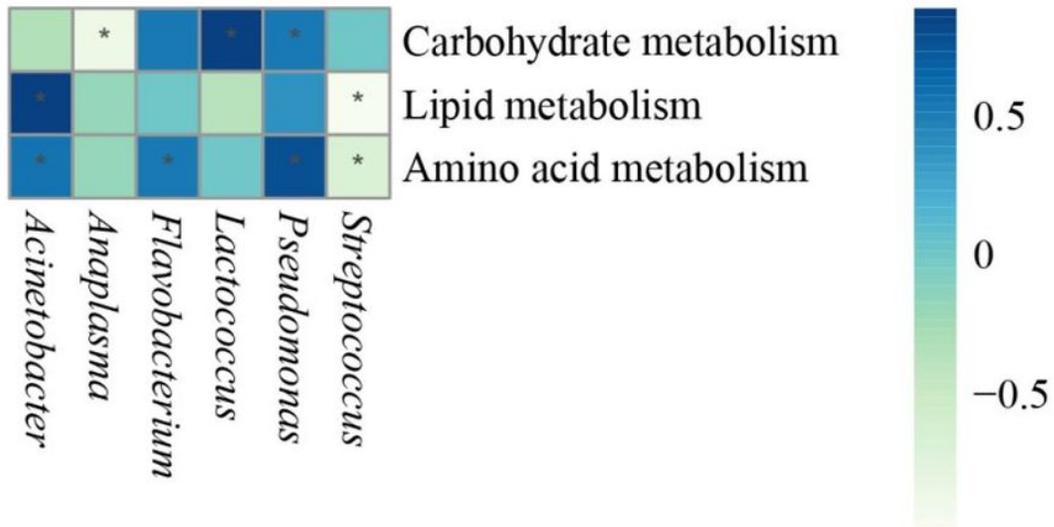


Figure 8

Correlation analysis between the primary metabolic functions and dominant microorganisms