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ORIGINAL PAPER

# Metagenomic analysis reveals the influences of milk containing antibiotics on the rumen microbes of calves

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**Abstract** Milk containing antibiotics is used as cost-effective feed for calves, which may lead to antibiotic residues-associated food safety problems. This study aims to investigate the influence of antibiotics on rumen microbes. Through metagenomic sequencing, the rumen microbial communities of calves fed with pasteurized milk containing antibiotics (B1), milk containing antibiotics (B2) and fresh milk (B3) were explored. Each milk group included calves in 2 (T1), 3 (T2) and 6 (T3) months of age. Using FastQC software and SOAPdenovo 2, the filtered data, respectively, were performed with quality control and sequence splicing. Following KEGG annotation was conducted for the uploaded sequences using KAAS software. Using R software, both species abundance analysis and differential abundance analysis were performed. In the B1 samples, the species abundance of *Bacteroidetes* gradually decreased along with the extension of feeding time, while that of *Fibrobacteres* gradually increased. The species abundances of *Proteobacteria* ($p$ value = 0.01) and *Spirochaetes* ($p$ value = 0.03) had significant differences among T1, T2 and T3 samples. Meanwhile, only the species abundance of *Spirochaetes* ($p$ value = 0.04) had significant difference among B1, B2 and B3 samples. Cell cycle involving *GSK3β*, *CDK2* and *CDK7* was significantly enriched for the differentially expressed genes in the T1 versus T2 and T1 versus T3 comparison groups. Milk containing antibiotics might have a great influence on these rumen microbes and lead to antibiotic residues-associated food safety problems. Furthermore, *GSK3β*, *CDK2* and *CDK7* in rumen bacteria might affect milk fat metabolism in early growth stages of calves.

**Keywords** Newborn calf · Rumen microbial community · Milk containing antibiotics · Abundance analysis · Differential analysis

## Introduction

Many kinds of microbes have the abilities of multi-drug resistance and broad-spectrum resistance (Davies and Davies 2010). Smillie et al have observed 42 common antibiotic resistance genes between livestocks and human beings, as well as 43 antibiotic resistance genes across geographic borders, indicating that antibiotic resistance genes can result in severe transnational consequences for human microbiome is globally spread (Smillie et al. 2011). Bovine rumen is inhabited by a large amount of microbiota (such as fungi, bacteria, archaea and protozoa) which contribute to degrade plant materials into digestible compounds (including bacterial proteins and volatile fatty acids) (Brulc et al. 2009). Therefore, rumen microbiota is important for the quality and production of meat and milk and consequently is also critical for human (Stevenson and Weimer 2007; Sundset et al. 2009; Welkie et al. 2010). At present, antibiotics have become the most economical and effective products for treating pneumonia, mastitis, hysteritis and other diseases in dairy cows (Goshen and Shpigel 2006; Katsuda et al. 2009; Barlow 2011). However, the milk produced by cows treated with antibiotics may have remaining antibiotics, which can lead to antibiotic residues-associated food safety problems (Oliver et al. 2011). For milk

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containing antibiotics has been used as cost-effective feed resource for newborn calves, it is necessary to explore the influence of antibiotics on rumen microbes.

Recently, bovine rumen microbiota has been extensively researched based on metagenomic sequencing. For example, Singh et al have analysed the microbial diversity in buffalo rumen using metagenomic sequencing and identified the typically microbial genes including bacterial virulence genes and antibiotic resistance genes (Singh et al. 2012). Using pyrosequencing of 16S rRNAs, quantitative real-time PCR and denaturing gradient gel electrophoresis, bacterial communities adhered to the rumen epithelial of 8 cattle were analysed in the transition process of forage to concentrated diet, in the process of acidosis, and when recovered (Petri et al. 2013). Pandya et al characterize the bacterial communities in rumen of 3 adult Surti buffaloes and differentiate 42 operational taxonomic units; additionally, the high coverage of 16S rRNA libraries (94.76%) suggests that sequences in the libraries can represent most of the bacterial diversity in rumen (Pandya et al. 2010). Through whole-genome shotgun and pyrosequencing of 16S rRNA, Li et al characterize the rumen microbiota of calves fed with milk replacer and identify 170 bacterial genera including 45 genera in the core microbiome of pre-ruminant calves (Li et al. 2012). Besides, the bacterial populations in rumen of 16 lactating cows have been studied by pyrosequencing, and different samples show 51% similarity in bacterial taxa (Jami and Mizrahi 2012). Nevertheless, the influence of milk containing antibiotics on rumen microbiota of newborn calves has not been explored yet.

To investigate the differences of microbial communities in the rumen of calves fed with pasteurized milk containing antibiotics (B1), milk containing antibiotics (B2) and fresh milk (B3), species abundance analysis and differential abundance analysis of the microbial community in ruminal fluids were conducted. This study may provide scientific basis for the rational use of milk containing antibiotics and calves breeding.

## Materials and methods

### Sample collection, DNA extraction and next-generation sequencing

Animal procedures were approved by the Ethical Committee of Livestock Research Institute of Heilongjiang Bayi Agricultural University. A total of 54 newborn calves (without significantly different birth weights) were selected from spark dairy farm in Daqing City. The experiments continued for 6 months. During the study period, the calves had different milks before 2 months of age, and then were weaned and fed with the same amount of granules under the same feeding conditions. Through puncturing, the ruminal fluids were extracted from calves fed with pasteurized milk containing antibiotics, milk containing antibiotics and fresh milk. Each milk group included calves in 2 months of age (T1), 3 months of age (T2) and 6 months of age (T3). The calves in different ages in each milk group had 6 repeats. The milk containing antibiotics was produced by cows treated with intravenous drip of 500 ml Nacl and 2 packages of ceftiofur sodium (Zhengzhou Bairui Animal Pharmaceutical Co., Ltd, Zhengzhou, China; 0.5 g/package) once a day. The filtered ruminal fluids extracted from calves of the same age in each milk group were mixed and stored in liquid nitrogen for following sequencing. Based on the manufacturer's instructions, genomic DNA (gDNA) in ruminal fluids was isolated by the TIANamp Stool DNA Kit (Tiangen, Beijing, China). Subsequently, the sequencing library was constructed according to the manufacturer instruction of library preparation kits (New England Biolabs, Inc., Beverly, MA, USA). In addition, the library was performed with paired-end sequencing based on the platform of Illumina NextSeq 500 (Illumina, CA, USA). The sequencing data were uploaded to the public National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov/) database (Wheeler et al. 2007), and the accession number was SRP075724.

### Data filtering, quality control and sequence splicing

Using cutadapt-1.2.1 software (https://pypi.python.org/pypi/cutadapt/1.2.1) (Martin 2011), adapters were removed from the raw data. Then, the data were performed with quality screen, with average mass fraction ≥Q20 (5-bp non-overlap window) as the threshold. To filter out the fragments of host genome, the clean reads were mapped to the host genome by Burrows–Wheeler Aligner (BWA) software (http://bio-bwa.sourceforge.net) (Li and Durbin 2009). Moreover, FastQC software (http://www.bioinformatics.babraham.ac.uk/projects/fastqc) (Andrew 2010) was applied to perform quality control for the data. Additionally, the preprocessed data were spliced by SOAPdenovo 2 (http://soap.genomics.org.cn/soapdenovo.html) (Luo et al. 2012) to obtain contigs and scaffolds.

### KEGG annotation

Kyoto Encyclopedia of Genes and Genomes (KEGG, http://www.genome.jp/kegg) database can be utilized for the annotation and classification of metabolic pathways from cellular processes, human diseases, metabolism, environmental information processing, organismal systems and genetic information processing aspects (Kanehisa et al. 2012). Using KEGG Automatic Annotation Server (KAAS,

**Table 1** Results of data filtering

| Sample | Library name | Percentage of high-quality reads (%) | Length of high-quality data (bp) | Percentage of the length of high-quality data (%) |
|---|---|---|---|---|
| B1T3 | D14253 | 96.75 | 9,796,171,940 | 96.83 |
| B1T2 | D14252 | 97.24 | 9,380,113,407 | 97.3 |
| B1T1 | D14251 | 97.11 | 7,926,712,911 | 97.14 |
| B2T3 | D14256 | 97.65 | 9,477,269,827 | 97.7 |
| B3T3 | D14259 | 96.51 | 10,518,005,171 | 96.63 |
| B3T2 | D14258 | 96.89 | 9,865,097,391 | 97.03 |
| B2T2 | D14255 | 97.31 | 8,663,251,669 | 97.37 |
| B3T1 | D14257 | 96.87 | 8,937,621,291 | 97.03 |
| B2T1 | D14254 | 96.72 | 9,533,449,649 | 96.91 |

http://www.genome.jp/tools/kaas) software (Moriya et al. 2007), KEGG annotation was performed for the uploaded gene sequences. Metagenomes for prokaryotes were selected, and other parameters were set as defaults.

### Abundance analysis

Using BLAST (McGinnis and Madden 2004), the filtered contigs were aligned to the sequences of bacteria, fungi, archaea and viruses which were extracted from the NCBI nucleotide (nt) database (version: 2014-10 -19). The *e* value $\leq 1e-5$ was taken as the cut-off criterion. By the locally adaptive clustering (LAC) algorithm (Parvin et al. 2011), the taxonomic rank before the first branch was selected as the species annotation information of a certain sequence. Combined with the statistical information of sequences in the level of phylum, ANOVA test (Campbell and Lele 2013) and Tukey HSD multiple comparison test (Conagin et al. 2008) of species abundance were performed among multiple groups using R software package. The species with $p$ value <0.05 had significant differences among the groups. Principal components analysis (PCA) is a multivariate statistical technique that reduces dimensionality and retains variation in data (Ringnér 2008). Using R software package (Mevik 2007), PCA was performed based on the species abundance in the level of genus.

### Differential analysis

After the data were normalized, the differentially expressed genes (DEGs) in the T1 versus T2, T1 versus T3, B1 versus B2 and B1 versus B3 comparison groups were identified by paired $t$ test command (Zhou and Wang 2007) in R software package. The genes with $p$ value <0.05 and $|\log_2$ fold change (FC)$| > 1$ were taken as DEGs. Finally, the DEGs in different comparison groups were performed with KEGG pathway enrichment analysis, respectively.

## Results

### Data analysis

After the raw data were preprocessed, more than 96% high-quality reads were selected (Table 1). Then, the reads were performed with quality control, and the results of base mass distribution, base content distribution, GC content distribution and sequence base quality indicated a high quality. Through sequence splicing, a total of 3,498,534 contigs and 262,753 scaffolds were obtained.

### KEGG annotation

KEGG annotation was performed for the uploaded gene sequences, and the results are shown in Fig. 1. The enriched terms were mainly associated with cellular processes, environmental information processing, genetic information processing, human diseases, metabolism and organismal systems.

### Abundance analysis

In the level of phylum, the relative species abundances of the samples are exhibited in Fig. 2. In the samples, the species abundances of *Bacteroidetes* (mean abundance = 69.8%), *Firmicutes* (mean abundance = 9.8%), *Proteobacteria* (mean abundance = 9.2%), *Fibrobacteres* (mean abundance = 4.9%) and *Actinobacteria* (mean abundance = 2.8%) were relatively higher in the level of phylum. In the B1T1, B1T2 and B1T3 samples, the species abundances of *Bacteroidetes* and *Firmicutes* in the level of phylum gradually decreased along with the extension of feeding time, while those of *Proteobacteria*, *Fibrobacteres* and *Actinobacteria* were just the opposite. In the B2T1, B2T2 and B2T3 samples, the species abundances of *Proteobacteria* and *Verrucomicrobia* in the level of phylum

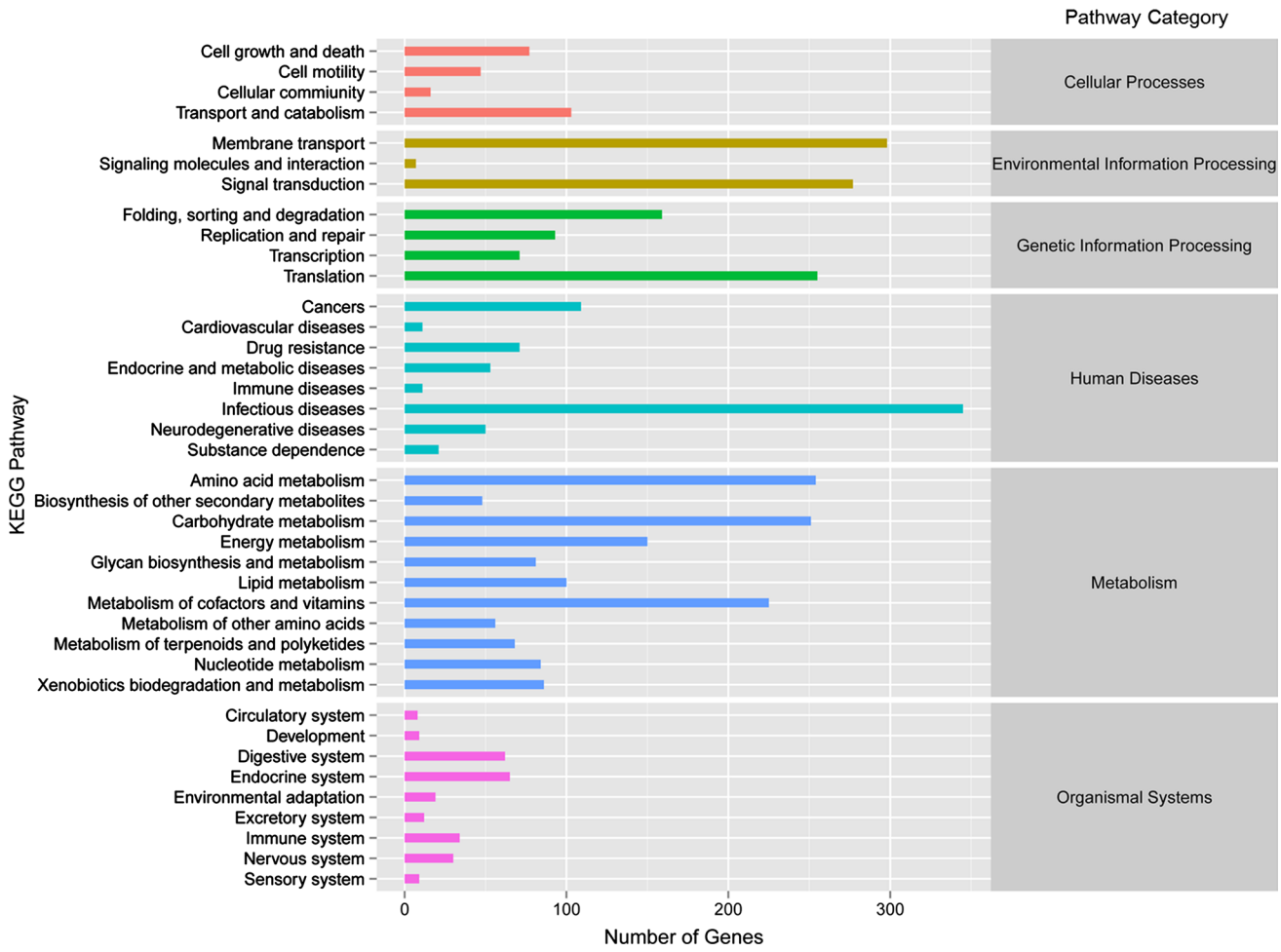


**Fig. 1** Results of Kyoto Encyclopedia of Genes and Genomes (KEGG) annotation performed for the uploaded gene sequences. *x* axis represents "Number of Genes;" *y* axis indicates "KEGG Pathway." The broad classification represents "Pathway Category"

gradually increased along with the extension of feeding time, while those of other species had no rules. In the B3T1, B3T2 and B3T3 samples, the species abundance of *Proteobacteria* in the level of phylum gradually increased along with the extension of feeding time; however, those of other species had no rules. In general, B3T1 sample with 11,754,882 species and B2T1 sample with 11,753,187 species had relatively higher species abundances in the level of phylum, which might be caused by that microbe inherent in B1 had been killed by pasteurization.

In the level of genus, the species abundances of *Prevotella*, *Bacteroides*, *Fibrobacter*, *Barnesiella* and *Alistipes* in all of the samples were relatively higher. In the B1T1, B1T2 and B1T3 samples, the species abundance of *Fibrobacter* in the level of genus gradually increased along with the extension of feeding time, while those of *Prevotella*, *Bacteroides* and *Barnesiella* were just the opposite. In the B2T1, B2T2 and B2T3 samples, the species abundance of *Fibrobacter* also gradually increased along with the extension of feeding time. However, the species abundance of *Fibrobacter* gradually decreased along with the extension of feeding time in the B3T1, B3T2 and B3T3 samples (Fig. 3).

With $p$ value <0.05 as threshold, variance analysis of species abundance was performed among multiple groups. The species abundances of *Proteobacteria* ($p$ value = 0.01) and *Spirochaetes* ($p$ value = 0.03) had significant differences among T1, T2 and T3 samples. Meanwhile, only the species abundance of *Spirochaetes* ($p$ value = 0.04) had significant difference among B1, B2 and B3 samples. Additionally, the result of PCA showed that the samples could be significantly divided into 3 parts according to feeding time (Fig. 4).

## Differential analysis

There were 318 and 1398 DEGs in the T1 versus T2 and T1 versus T3 comparison groups, respectively. A total of 9 and 33 pathways, respectively, were significantly enriched for

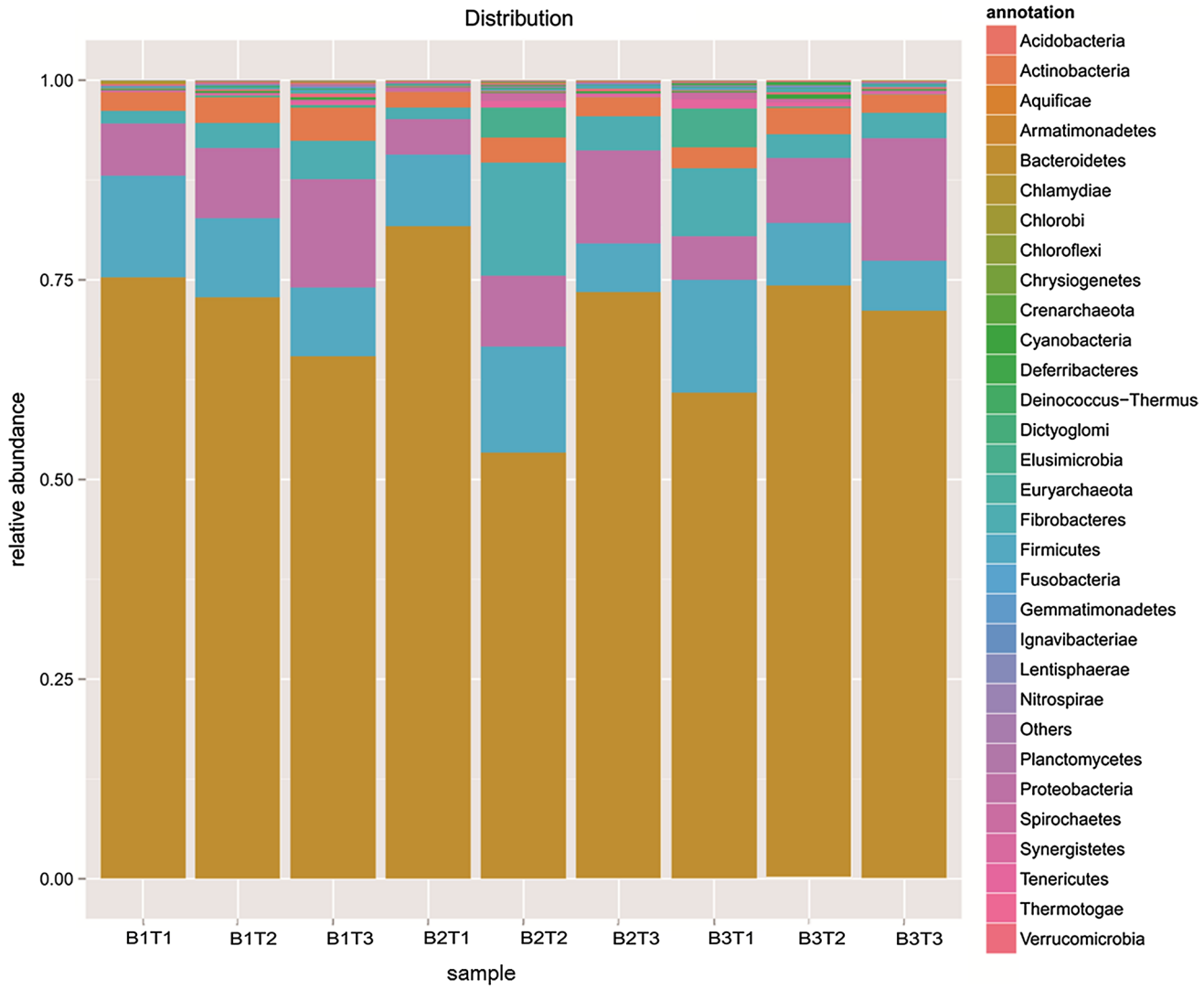


**Fig. 2** Relative species abundances of the samples in the level of phylum. *x* axis represents sample, and *y* axis indicates relative abundance

the DEGs in the T1 versus T2 and T1 versus T3 comparison groups, including 2 common pathways of cell cycle (Fig. 5; which involved glycogen synthase kinase-3β, *GSK3β*; cyclin-dependent kinase 2, *CDK2*; and cyclin-dependent kinase 7, *CDK7*) and biosynthesis of amino acids (Fig. 6). A total of 30 and 24 DEGs were identified for the B1 versus B2 and B1 versus B3 comparison groups, respectively. There, respectively, were 9 and 1 pathways significantly enriched for the DEGs in the B1 versus B2 and B1 versus B3 comparison groups. However, no common pathways were found for the DEGs in the two comparison groups.

## Discussion

After preprocessing, more than 96% high-quality reads were identified from the raw data. Through sequence splicing, a total of 3,498,534 contigs and 262,753 scaffolds were obtained. The result of PCA showed that the samples could be significantly divided into 3 parts according to feeding time. The species abundances of *Fibrobacteres* and *Fibrobacter* were relatively higher in the samples. The phylum *Fibrobacteres* consists of one genus (*Fibrobacter*) and two species (*Fibrobacter intestinalis* and *F. succinogenes*) and is known for degrading cellulosic plant biomass in the gut of herbivore, suggesting that cellulose degradation may be a potential characteristic of the phylum (Ransom-Jones et al. 2012, 2014; Rosenberg 2014). As effective cellulolytic bacteria, the members of *Fibrobacteres* act in rumen function and serve as promising sources of novel enzymes that can be used for bioenergy applications (Jewell et al. 2013). Previous study detects relatively more members of the phylum *Fibrobacteres* in cellulolytic ecosystems through the 16S rRNA-based environmental

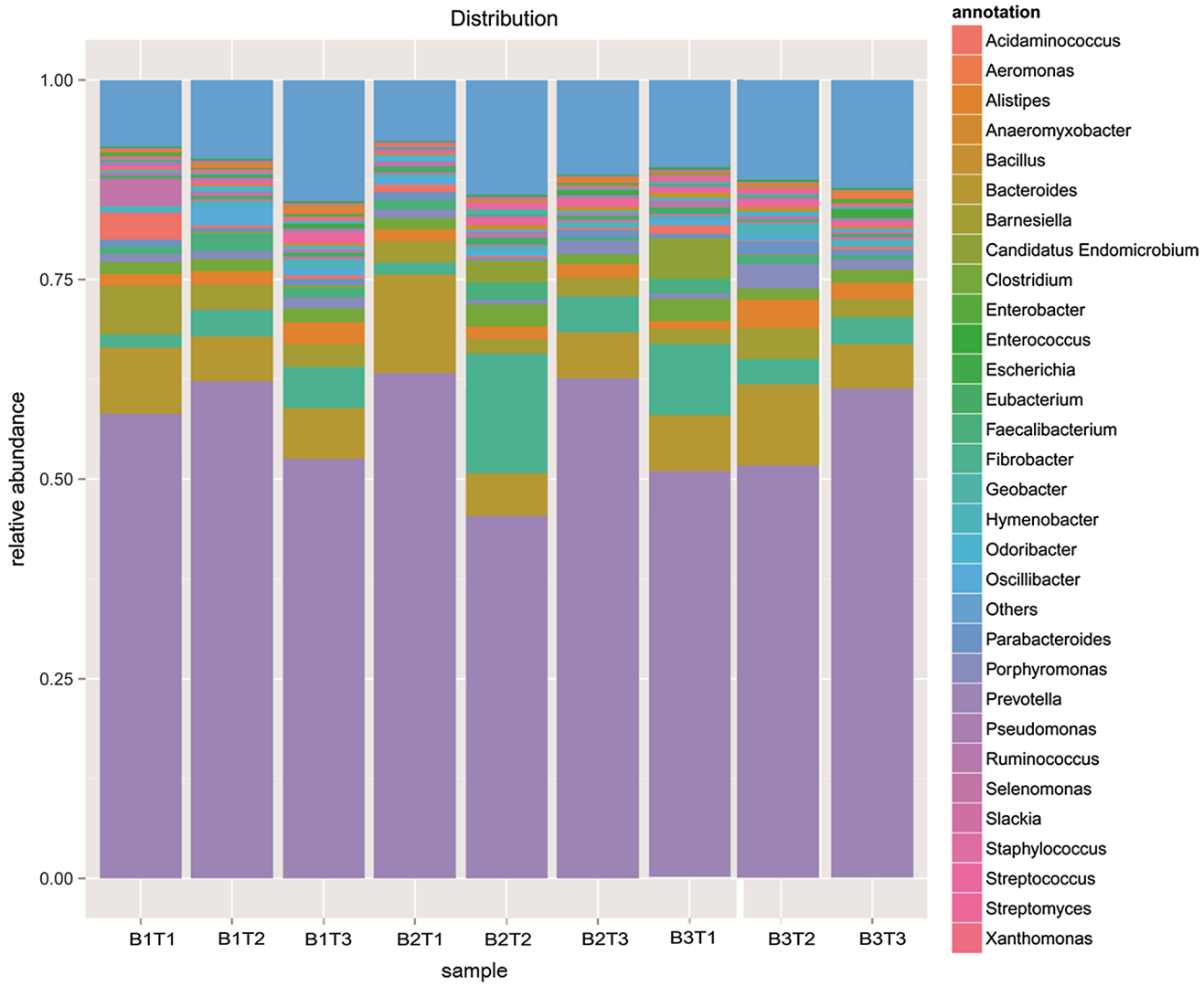


**Fig. 3** Relative species abundances of the samples in the level of genus. *x* axis represents sample, and *y* axis indicates relative abundance

surveys (Ransom-Jones et al. 2014). Physiological and ecological characterization shows that *F. succinogenes* is an important cellulolytic microbe and plays an essential role in fibre digestion in the rumen (Kobayashi et al. 2008; Shinkai et al. 2010). *F. succinogenes* degrades plant cell walls via arabinofuranosidase(s), esterases, glucanases and xylanases, opening its way through the complex matrix of hemicellulose and cellulose (Jun et al. 2007). The phylum *Bacteroidetes* includes 4 classes (*Bacteroidia*, *Cytophagia*, *Flavobacteria* and *Sphingobacteria*), and approximately 7000 species, in particular *Flavobacteria* is the largest class which comprises of more than four times of species than other classes (Whitman et al. 2012). Members of the phylum *Bacteroidetes* are the major microbes in the gastrointestinal tract and are increasingly considered as special decomposers of high molecular weight organic matter, such as carbohydrates and proteins (Thomas et al. 2011; Fernández-Gómez et al. 2013). In the B1T1, B1T2 and B1T3 samples, the species abundance of *Bacteroidetes* gradually decreased along with the extension of feeding time, while that of *Fibrobacteres* was just the opposite. These were consistent with the results in the level of genus. These declared that *Bacteroidetes* and *Fibrobacteres* contributed to digestion in the rumen, and *Bacteroidetes* was more sensitive to pasteurized milk containing antibiotics.

The β-lactam antibiotics (such as clavulanate, carbapenem, cephalosporin, nocardicin, monobactam and penicillins) are appropriate for the therapy of bacterial

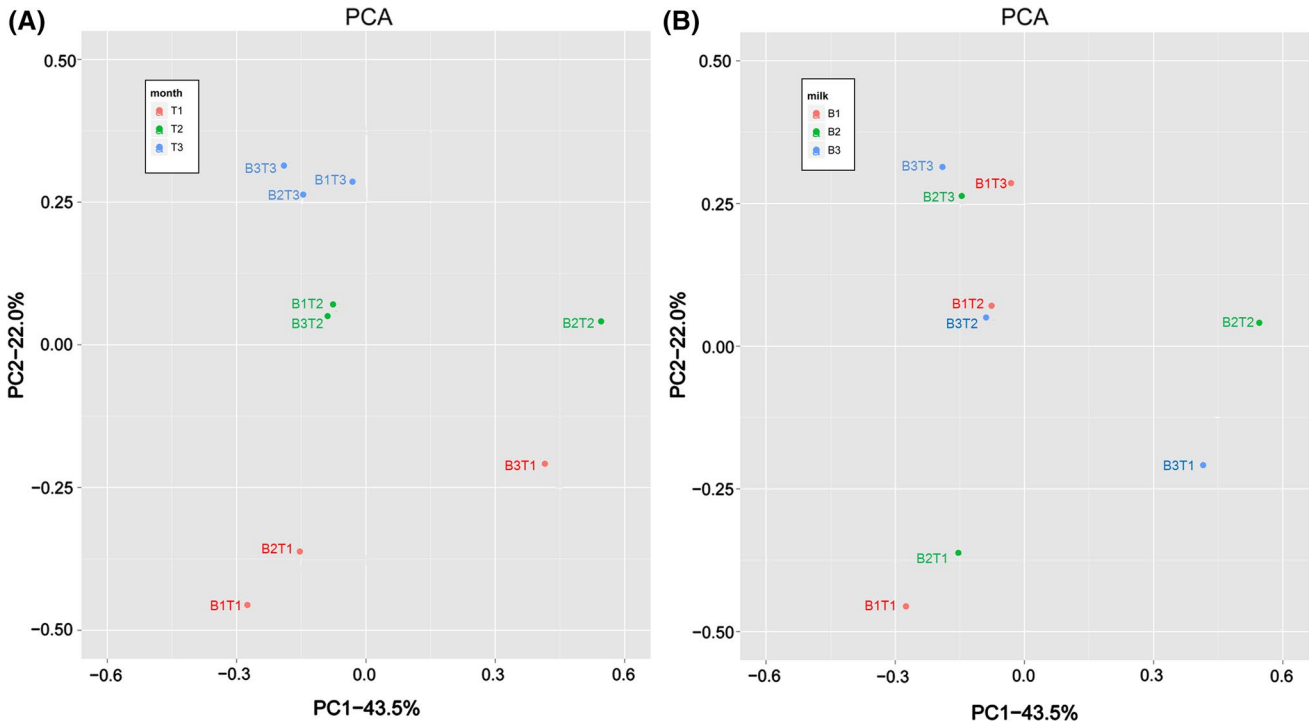


**Fig. 4** Results of principal components analysis (PCA) show that the samples can be significantly divided into 3 parts according to month (**a**), but not milk (**b**)

infection for they have unparalleled broad antibacterial spectrum and clinical safety (Testero et al. 2010). Due to the appearance and fast spreading of antibiotic-resistant pathogens, the issue of antibiotic resistance has attracted much attention (Aminov and Mackie 2007). Bacteria can acquire resistance to β-lactam antibiotics through utilizing β-lactam-insensitive cell wall transpeptidases, producing β-lactam-hydrolyzing β-lactamase enzymes, or expulsing β-lactam molecules from Gram-negative cells (Wilke et al. 2005). The antibiotic resistances are reported to mainly be acquired resistance, resulting by antibiotic resistance genes transferred from other taxonomically and ecologically distant bacteria (Aminov and Mackie 2007). The phylum *Proteobacteria* contains most diverse phylogenetic lineage (such as *Alphaproteobacteria*, *Betaproteobacteria*, *Gammaproteobacteria*, *Deltaproteobacteria* and *Epsilonproteobacteria*), which possesses excessive metabolic diversity and has important biological significance (Kersters et al. 2006). *Spirochaetes* are spiral-shaped, Gram-stain-negative and motile cells, and most *Spirochaetes* have periplasmic flagella containing organelles of motility (Paster 2010). Anaerobic *Spirochaetes* belonging to the genus *Brachyspira* have been regarded as key pathogens of pig gut for long, but they are increasingly considered as causes of disease in other animals (Hampson and Ahmed 2009). The species abundances of *Proteobacteria* ($p$ value = 0.01) and *Spirochaetes* ($p$ value = 0.03) had significant differences among T1, T2 and T3 samples. Meanwhile, only the species abundance of *Spirochaetes* ($p$ value = 0.04) had significant difference among B1, B2 and B3 samples and was more abundant in B2 samples, suggesting that the abundant *Spirochaetes* contributes to the antibiotic resistance. Thus, milk containing antibiotics might have influence on rumen microbial community and might lead to antibiotic residues-associated food safety problems through affecting pathogenic *Spirochaetes*.

There were 318, 1398, 30 and 24 DEGs in the T1 versus T2, T1 versus T3, B1 versus B2 and B1 versus B3 comparison groups, respectively. Cell cycle (which involved *GSK3β*, *CDK2* and *CDK7*) and biosynthesis of amino acids were the two common pathways, which were significantly enriched for the DEGs, respectively, in the T1

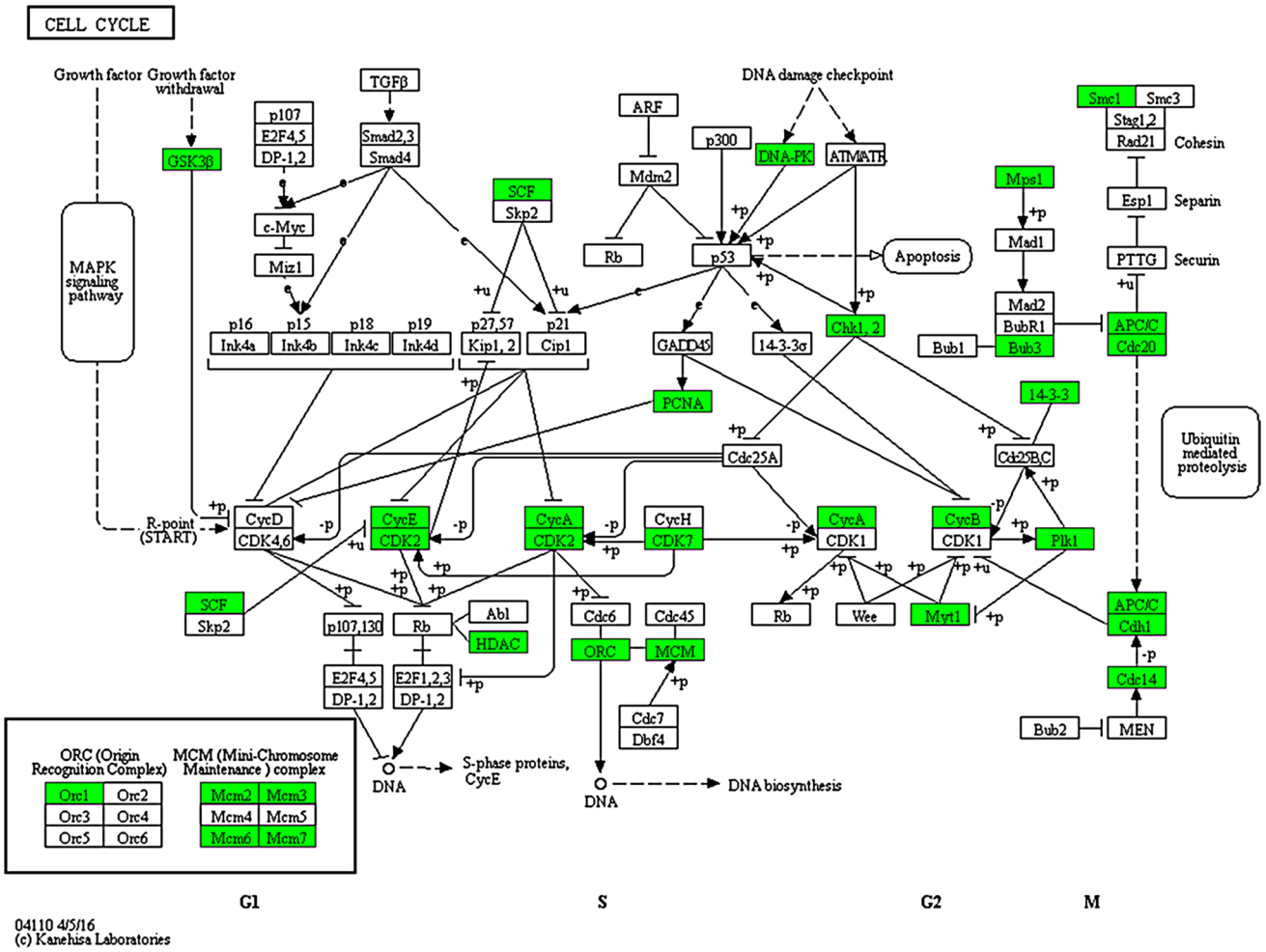


**Fig. 5** Pathway map of cell cycle (which involved glycogen synthase kinase-3β, *GSK3β*; cyclin-dependent kinase 2, *CDK2*; and cyclin-dependent kinase 7, *CDK7*). The *green rectangles* represent down-regulated genes in the T1 versus T2 or T1 versus T3 comparison group (color figure online)

versus T2 and T1 versus T3 comparison groups. Reportedly, GSK3β could mediate milk synthesis and the proliferation of mammary epithelial cells in dairy cow (Zhang et al. 2014). In addition, phosphorylation of GSK3 by the prolactin receptor (PRLR) is highly associated with milk production during lactation (Shi et al. 2016). These suggest the involvement of GSK3β in cell cycle and proliferation regulation. Unfortunately, expression of GSK3β in rumen tissue has not yet been reported. CDK2 plays an important role in cell growth and division (Lu et al. 2012). Reduced activity of CDK2 induces the G1 cell cycle arrest of *trans-10* conjugated linoleic acid (CLA), which presents less than 1% in the CLA isomer in milk fat that is generated by rumen bacteria (Rosbergcody et al. 2007). CDK7 is another CDKs family member relating to cell cycle regulation (Fisher 2005). Based on our study, the three above genes were all differentially expressed among three stages in rumen bacteria fed with different milk. These collectively hint that rumen bacteria might influence the milk fat metabolism via cell cycle regulation, in the early growth stages (2–6 months) of calves, whatever the milk feeding is.

In conclusion, a total of 3,498,534 contigs and 262,753 scaffolds were obtained. *Bacteroidetes* and *Fibrobacteres* contributed to digestion in the rumen, and *Bacteroidetes* was more sensitive to pasteurized milk containing antibiotics. Besides, milk containing antibiotics had influence on rumen microbial community and might lead to antibiotic residues-associated food safety problems through affecting pathogenic *Spirochaetes*. Furthermore, *GSK3β*, *CDK2*

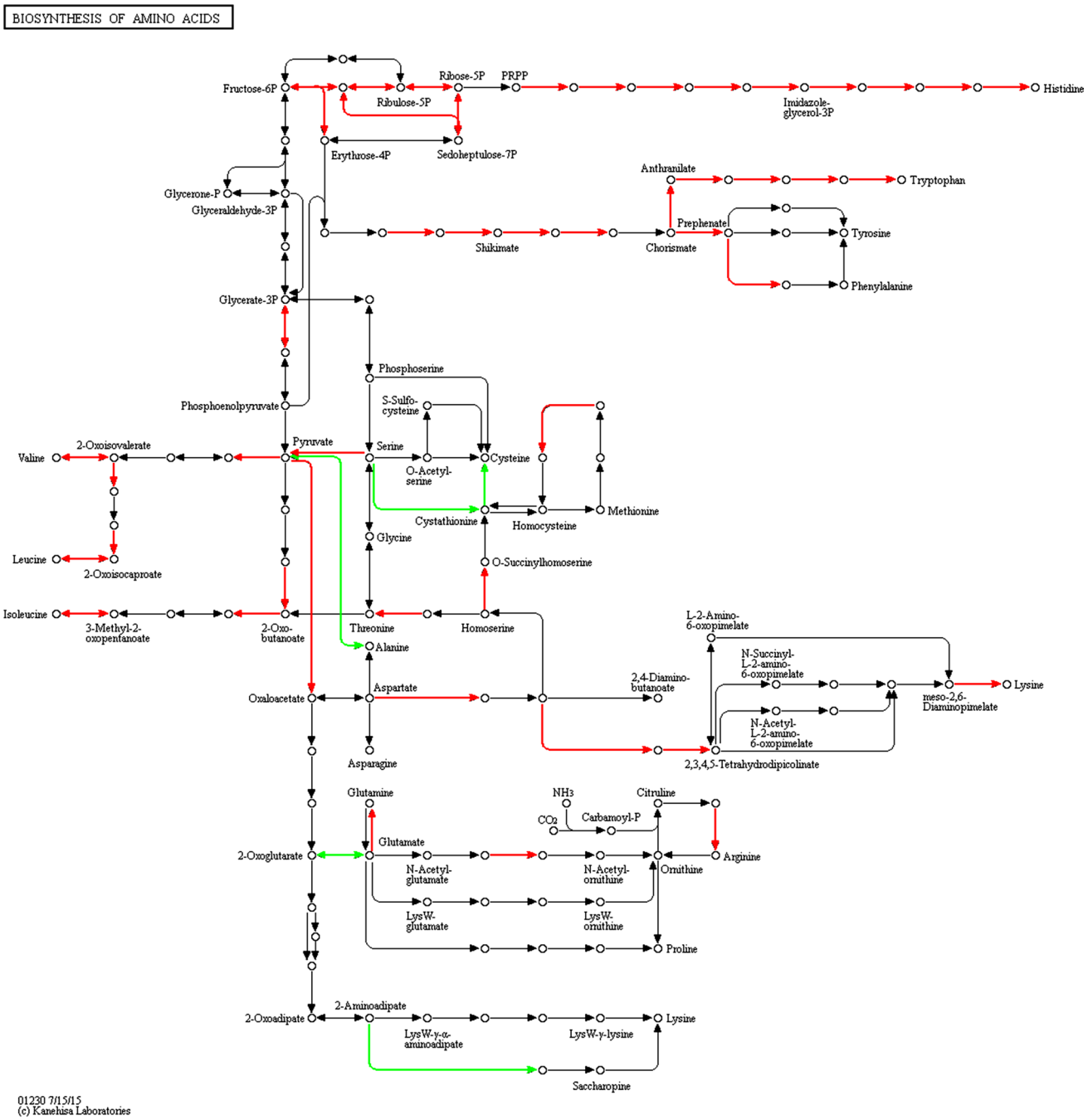


**Fig. 6** Pathway map of biosynthesis of amino acids. The *red* and *green rectangles,* respectively, represent up- and down-regulated genes in the T1 versus T2 or T1 versus T3 comparison group

and *CDK7* in rumen bacteria might affect the cell cycle of milk fat metabolism in early growth stages (2–6 months) of calves, regardless of different milk feedings.

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**Compliance with ethical standards**

**Conflict of interest** The authors declare that they have no conflict of interest.

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