Electronic Journal of Biotechnology 51 (2021) 67–78

Contents lists available at ScienceDirect

Electronic Journal of Biotechnology

journal homepage:

Research Article

Using PacBio sequencing to investigate the effects of treatment with lactic acid bacteria or antibiotics on cow endometritis

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ARTICLE INFO

Article history:
Received 28 April 2020
Accepted 26 February 2021
Available online 11 March 2021

Keywords:
Antibiotics
Cattle
Cow
Endometritis
l6S rRNA
Lactic acid bacteria
PacBio sequencing
Single-molecule real-time sequencing
Uterine mucus

ABSTRACT

Background: Endometritis is the most common disease of dairy cows and traditionally treated with antibiotics. Lactic acid bacteria can inhibit the growth of pathogens and also have potential for treatment of endometritis. Using PacBio single-molecule real-time sequencing technology, we sequenced the full-length l6S rRNA of the microbiota in uterine mucus samples from 31 cows with endometritis, treated with lactic acid bacteria (experimental [E] group) and antibiotics (control [C] group) separately. Microbiota profiles taken before and after treatment were compared.

Results: After both treatments, bacterial species richness was significantly higher than before, but there was no significant difference in bacterial diversity. Abundance of some bacteria increased after both lactic acid bacteria and antibiotic treatment: *Lactobacillus helveticus*, *Lactococcus lactis*, *Lactococcus raffinolactis*, *Pseudomonas alcaligenes* and *Pseudomonas veronii*. The bacterial species that significantly decreased in abundance varied depending on whether the cows had been treated with lactic acid bacteria or antibiotics. Abundance of *Staphylococcus equorum* and *Treponema brennaborense* increased after lactic acid bacteria treatment but decreased after antibiotic treatment. According to COG-based functional metagenomic predictions, 384 functional proteins were significantly differently expressed after treatment. E and C group protein expression pathways were significantly higher than before treatment ($p < 0.05$).

Conclusions: In this study, we found that lactic acid bacteria could cure endometritis and restore a normal physiological state, while avoiding the disadvantages of antibiotic treatment, such as the reductions in abundance of beneficial microbiota. This suggests that lactic acid bacteria treatment has potential as an alternative to antibiotics in the treatment of endometritis in cattle.

How to cite: Yang L, Huang W, Yang C, et al. Using PacBio sequencing to investigate the effects of treatment with lactic acid bacteria or antibiotics on cow endometritis. Electron J Biotechnol 2021:51. https://doi.org/10.1016/j.ejbt.2021.02.004

1. Introduction

Endometritis in cattle is caused by bacterial infection of the uterus after parturition [1]. The incidence of endometritis in cattle is 9.4–67% in Europe and North America, and the rate is 20–50% in China [2]. Without timely and effective treatment endometritis has significant negative effects on milk production and fertility of infected cows [3].

In otherwise healthy cows, the uterus destroys invading pathogens gradually over time and the cows recover completely. However, if the cow is already in poor health and the immune defense systems are weakened, then bacteria will multiply causing severe endometritis and pathological changes [4,5]. According to a variety of clinical features, endometritis can be divided into three types: acute, chronic and recessive. Acute endometritis is characterized by its quick onset, significant clinical symptoms, temperatures in excess of 40°C and depressed/declining ruminant ability. Most cows with acute endometritis frequently display an abnormal urination posture and eliminate foul secretions from the vagina. Clinical symptoms of chronic endometritis are mild, and there are no particular clinical symptoms in recessive cows, but, in both cases, it is always associated with infertility [6]. At present, rectal palpation is the most direct method to diagnose clinical endometritis in cattle. When there is inflammation of the endometrium, the uterus increases in size dramatically; a decrease

Peer review under responsibility of Pontificia Universidad Católica de Valparaíso
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https://doi.org/10.1016/j.ejbt.2021.02.004
0717-3458/

or fluctuation in plumpness, elasticity and contractility can be felt. Sick cows are seen to attempt valsalva maneuver and they eliminate inflammatory secretions from the vagina.

Some studies indicate that the key feature that marks bacterial reproductive tract diseases is the sharp decrease in abundance of *Lactobacillus* species, which are anaerobes and facultative anaerobes [7]. According to the microecological balance theory, we can hypothesize that endometritis in cattle could be prevented or cured by inoculating the uterus with live probiotics, such as *Lactobacillus* species. The theory is that these probiotics are antibacterial and prevent the development of pathogenic bacteria. Up to now, antibiotics and traditional Chinese medicine are generally used to cure clinical endometritis in cattle, but the results are often unsatisfactory [8]. For example, the endometrium can be strongly stimulated resulting in a severe decline in productivity [9]. In addition, the negative effects to the breeding industry are significant due to the common overuse of antibiotics resulting in antibiotic residues, increasing pathogen resistance and microbiota dysbiosis. Relative to antibiotic therapy, microbial preparations (probiotics) for treatment of endometritis have great promise as they are unlikely to lead to resistance, have noresidues, and could improve the natural defensive power of the cow's uterus.

High-throughput sequencing has been used to survey environmental microbiota and is capable of identifying microorganisms quickly and accurately, even organisms that cannot be cultured or are in low abundance [10,11]. The third-generation sequencer, PacBio single-molecule real-time (SMRT) sequencing platform, is an advanced DNA sequencing platform, which has the advantage of high throughput and the production of long reads. The technology is able to generate microbiota profiles at the species level to high taxonomic resolution, based on full-length 16S rRNA gene sequences [12]. PacBio SMRT sequencing reduces fuzzy classification, authenticates more extensive species diversity, describes microbiota more accurately and has a higher resolution ratio of phylogeny than 454 pyrosequencing and the Illumina sequencing platform [13]. The microbiota composition of the genital tract of humans and animals has been studied by second-generation sequencing technology. However, second-generation sequencing technology can only produce short sequence reads, resulting in low taxonomic resolution and failure to identify the causative agents of mastitis. Recently PacBio SMRT sequencing has successfully discovered some uncommon bacteria species, a large number of previously unidentified species, and species previously identified as unculturable [14,15]. Evidence increasingly indicates that microbial communities in similar environments have similar functions, even though individual species within the microbial community that are performing those functions, may be quite different [16,17,18]. Based on the results of prokaryotic 16S rRNA high-throughput sequencing, the community function or phenotype of prokaryotic microorganisms has been predicted by Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) [19]. The PICRUSt method: combines the measured microbial genome 16S rRNA sequences to predict the metabolic function spectrum of the corresponding bacterial and archaea community; connects the species to gene function; and is easier, faster and cheaper than metagenomic analysis. After metagenomic prediction by PICRUSt, functional annotation can be done using software and databases such as Kyoto Encyclopedia of Genes and Genomes (KEGG), Clusters of Orthologous Groups of proteins (COG) and protein families database (Pfam), to give biological meaning to the genetic information. The COG database can classify gene products based on a variety of protein sequence comparisons of multiple biological attributes. COG is an important tool for microbial genomics' functional annotation and the main platform for newly sequenced genomics' functional annotation and evolution studies. It can predict the possible biochemical activity and cellular function of the genome, statistically analyze all identified proteins, and provide a quick way to describe the functional characteristics of a microorganism or a community of microbes [20,21,22].

In this study we extracted microbial genomic DNA from uterine mucus samples from 31 cows that had either been treated with LAB or antibiotics. We amplified the bacterial 16S rRNA gene and sequenced it using third-generation PacBio SMRT sequencing to compare variation in the microbiota before and after LAB or antibiotic treatment. Full-length PacBio SMRT sequencing data of the processed 16S rRNA gene were used for metagenome prediction using the PICRUSt tool, and functional annotation of the protein-coding genes was done in combination with the COG database to evaluate the effect of LAB on endometritis and explore the mechanism of action. The purpose of the study was to provide the theoretical basis for replacing antibiotics with microbial preparations to treat endometritis in cattle.

2. Material and methods

2.1. Ethics approval

The study protocol was reviewed and approved by the Ethics Committee of the Inner Mongolia University (Hohhot, China); the license number was SYXK (meng) 2014-0002. The use of experimental cows was permitted by the owner of the dairy.

2.2. Experimental materials

Uterine mucus samples were collected from 31 Holstein cows with acute endometritis (similar age, 2–4 d after delivery and less than 15 d postpartum) maintained in the Tianzhengzhongdi Ecology Ranch Ltd. in Datong City, Shanxi Province. The cows were divided randomly into two groups: the experimental group (n = 16 cows; E group) and the control group (n = 15; C group). The E group were treated with a LAB preparation while the C group were treated with a traditional antibiotic treatment. The health status of each cow was determined based on clinical symptoms, by professional veterinarians, on the 5th day after treatment. Uterine samples were collected from each cow on two occasions, once before treatment (day 0; ET1 [experimental group] and CT1 [control group]) and once after treatment (ET2 [experimental group] and CT2 [control group]) to provide 62 samples in total. No cows had a history of antibiotic use or any other medication known to influence the uterine mucus microbiota, in the three months before the study.

The LAB preparation was comprised of two isolates of *Lactobacillus plantarum* (A and B), and one isolate of *Lactobacillus casei* (C), and the three isolates were stored in the Key Laboratory of Dairy Biotechnology and Engineering, Ministry of Education, Inner Mongolia Agricultural University. The LAB were formulated in 2 g tablets each containing 1×10^{11} CFU viable bacteria/ tablet. The LAB tablets were retrieved from storage at −20°C and acclimated to ambient temperature for 1 h. For each tablet the LAB were suspended in 50 mL water with 20% glucose for introduction into each of the E group cows. The researcher used a shoulder-length plastic gloved hand in the rectum of each cow to hold the cervix and guide the stainless-steel insemination gun containing the LAB suspension, and perfused the uterus directly [23].

The antibiotic treatment was by intramuscular injection of Ceftiofur crystalline free acid injection (Zoetis Inc., Florham Park, US) at a dose of 1 mL per 20 kg weight into the neck; no more than 2 mL were injected per site.

2.3. Sample collection

Prior to sample collection the perineum of each cow was cleaned with 75% alcohol and then uterine mucus samples were collected using sterile cytobrushes [24], modified for use in large animals. Each cytobrush (approx. 3 cm in length), was threaded onto a solid stainless-steel rod (65 cm in length and 4 mm in diameter) and placed within a stainless-steel tube (50 cm in length and 5 mm in diameter), for passage through the cows' cervix. The instrument was placed in a sterile plastic sleeve to prevent vaginal contamination. The vulva was then cleaned and embrocated with wet towels and lubricating gel, and the instrument passed through the vagina into the external cervix. When the instrument entered into the base of the larger cervical horn, the stainless steel tube was retracted to expose the cytobrush. Endometrial cytology samples were collected by rotating the cytobrush in a clockwise direction when it was in contact with the uterine wall. The cytobrush was retracted into the stainless steel tube prior to removal from the uterus. The same clinician collected all the samples. After sample collection each cytobrush was placed immediately into a 2 mL sterile centrifuge tube and dipped with 1.5 mL non-enzyme water, frozen quickly in liquid nitrogen and stored at −20°C for rapid transfer to the laboratory of the Inner Mongolia Agricultural University where it was frozen at −80°C for further molecular biology research.

2.4. Clinical diagnosis

Five days after treatment with LAB or antibiotics, the veterinarian observed the clinical symptoms of each cow's uterus through palpation, including the size, texture, shape and contraction response of the uterus and the swelling of the cervix and vaginal mucosa. According to the veterinarian's clinical experiences, it was generally assumed that when the clinical symptoms disappeared completely, it was acceptable to say that the cow was completely cured, and that when the clinical symptoms had partly disappeared that the cow had improved and that the treatment was still effective.

Complete cure rate (%) = number of cows completely cured/number of treated cows × 100%

Effective treatment rate (%) = (number of cows completely cured + number of improved cows)/number of test cows × 100%

2.5. Sample preparation and DNA extraction for PacBio SMRT sequencing

Uterine mucus samples were thawed and DNA extracted using a DNA extraction kit (OMEGA Bio-tek Ink., Norcross, US) following the manufacturer's instructions. The integrity, purity and concentration of DNA samples was determined by 1% agarose gel electrophoresis and microscale ultraviolet spectrophotometer. High quality DNA, was stored at −20°C until evaluation and not longer than 1 month.

2.6. Amplification of full-length 16S rRNA and PacBio SMRT sequencing

Full-length sequences of bacterial 16S rRNA genes were selected as the target amplification fragment. The extracted DNA was used as the template and the 16S full-length universal primer was used for PCR amplification (upstream primers, 5′-AGAGTTT GATCMTGGCTCAG-3′, downstream primers, 5′-ACCTTGTTAC GACTT-3′). In order to distinguish different samples in the same library, an identification tag Barcode containing 16 bases was added to both ends of all primers. The whole PCR solution was prepared by mixing 1.5 μL of 20 ng/μL DNA, 1.5 μL of 10 μmol/L upstream primer, 1.5 μL of 10 μmol/L downstream primer, 25 μL of 5 U/μL polymerase mixture (KAPA HiFiTM HotStart ReadyMix PCR Kit, US), and 20.5 μL sterilized ultra-pure water. The droplets produced by each sample were transferred to a 96-well plate, and PCR amplification was done using the EvaGreen program: 95°C for 3 min, 98°C for 20 s, 62°C for 15 s, 72°C for 45 s, 30-cycles, followed by 72°C for 1.5 min. PCR products were purified by adding Gnome DNA Clean magnetic beads of equal volume. DNA concentration was measured by Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific, Waltham, MA). The samples with DNA concentrations greater than 20 ng/μL were used for constructing the DNA library (Pacific Biosciences SMRT bell™ Template Prep Kit 1.0), sequencing by PacBio SMRT RS Ⅱ instrument P6 - C4 reagent computer, and the movie times were 240 min by CCS mode.

2.7. Sequence processing

In order to extract high-quality sequences, the original sequence was quality controlled using RS_ReadsOfinsert. 1. The quality control standard was that the cycle times were no less than 5, the minimum prediction accuracy was 90, and the minimum and maximum inserted fragments were 1400 bp and 1800 bp, respectively [25]. After obtaining high-quality sequences, they were divided amongst the corresponding samples according to Barcode. After that, the Barcode and primers of each sequence were removed before subsequent analysis. The QIIME (V1.7.0) platform was used for subsequent bioinformatics analysis.

2.8. Bioinformatics analysis

Sequences were aligned by PyNAST. UCLUST was used to divide operational taxonomic units (OTUs) under the condition of 100% clustering of sequence identity to obtain representative sequences. After this, OTUs were classified under 97% similarity. The chimeric OTU sequences were removed by Vsearch [26,27,28,29]. The Ribosomal Database Project (RDP, Release 11.5), Greengenes (version 13.8) and Silva (version 128) databases were used to compare homology amongst representative sequences in OTUs, and from that the taxonomic status of each OTU was determined [30]. The sequence number of each sample was standardized and alpha diversity and beta diversity determined. The rarefaction curve and Shannon diversity curve were used to determine whether the sequencing depth represented the bacterial composition in the sample. The abundance and diversity of the corresponding flora in the samples was evaluated by calculating the OTU number index and the Shannon diversity index at the same sequencing depth.

2.9. Statistical analysis and visualization

The structural differences in microbial communities amongst different samples were calculated by principal coordinates analysis (PCoA) of unweighted and weighted UniFrac distances. The Wilcoxon rank sum test and non-parametric Kruskal–Wallis were used to test for significant differences between the two groups and amongst multiple groups of samples, respectively. The false positive rate of multiple tests was corrected using the method of Benjamini and Hochberg [31]. Spearman Rank correlation analysis was used to determine the correlation in the core intestinal microbiota. R language (V3.2.5) and Origin 8.6 were adopted to achieve data analysis and visualization.

2.10. Functional metagenomic prediction by PICRUSt

In order to elucidate the functions of microbiota in cow uterine mucus further, we used PICRUSt software to predict and analyze the functional metagenome [32], and annotated the gene and genome databases with the COG database. COG annotation: http://www.ncbi.nlm.nih.gov/COG.

2.11. Nucleotide sequence accession numbers

All sequencing data generated in this study have been uploaded to the MG-RAST database under the project number mgp 91849 (http://www.mg-rast.org).

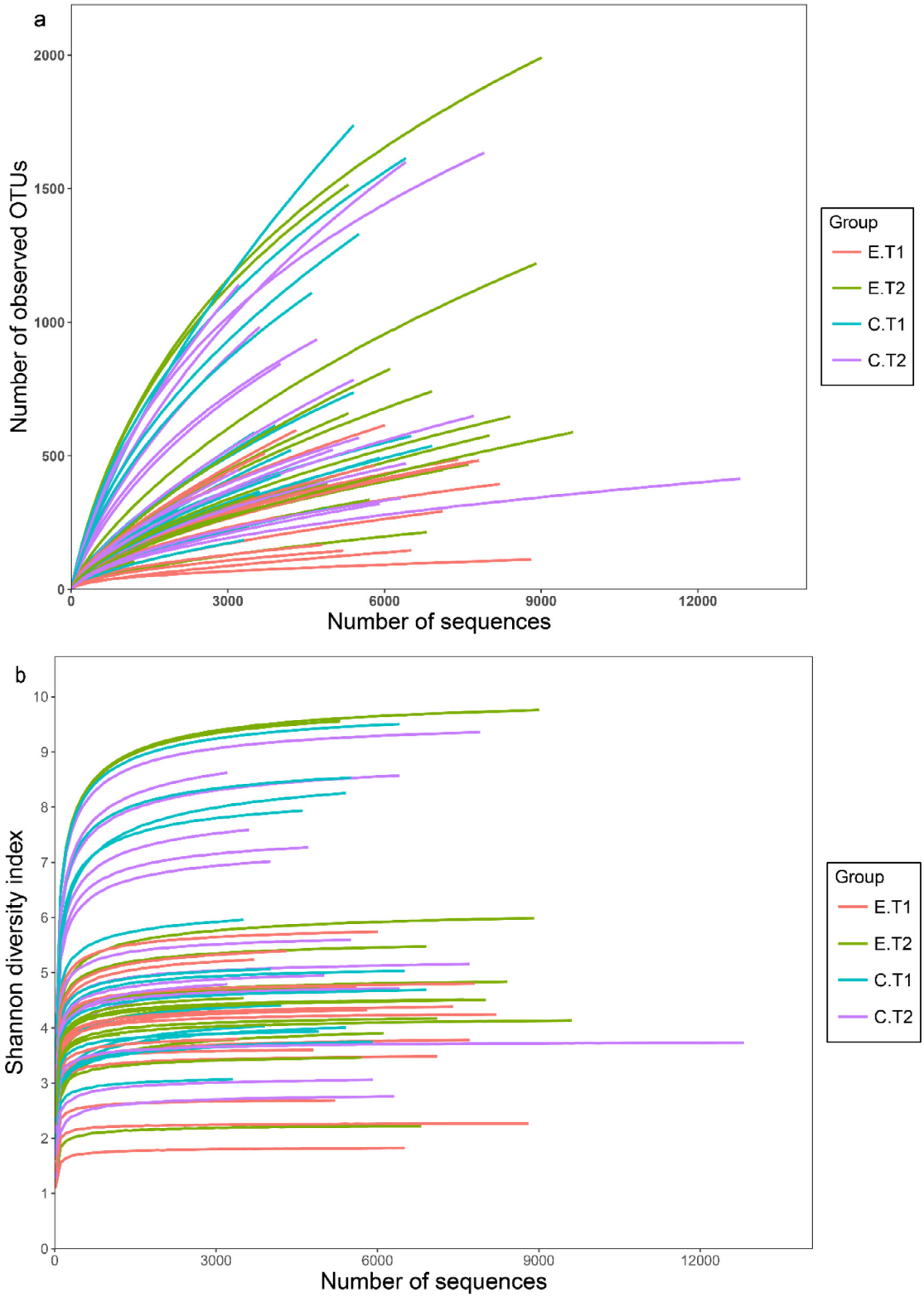

Fig. 1. α-diversity amongst different samples. Rarefaction (a), Shannon diversity (b).

3. Results

3.1. Clinical effects

Following LAB treatment ten of the 16 cows in the E group were completely cured. Their systemic symptoms had disappeared; the size, texture and shape of the uterus had returned to normal, the contraction response was obvious, the swelling of the cervix and vaginal mucosa had disappeared, and no inflammatory secretions were found. The symptoms of a further two cows had partially disappeared; the size, texture and shape of the uterus had returned to normal, the contraction response was relatively obvious, the swelling of the cervix and vaginal mucosa had disappeared, but there were still some inflammatory secretions. The symptoms of a further four cows had been alleviated; the size, texture and shape were restored to some extent, the contraction response was still weak, they still had congestion and there were still some inflammatory secretions outside the cervix. The complete cure rate of the E group of cows receiving LAB was 62.5%, and the effective cure rate was 75%.

Following antibiotic treatment, eight of the 15 cows in the C group were completely cured. Their systemic symptoms had disappeared; the size, texture and shape of the uterus had returned to normal, the contraction response was relatively obvious, the swelling of the cervix and vaginal mucosa had disappeared, and no inflammatory secretions were found. The symptoms of a further two cows had partially disappeared; the size, texture and shape of the uterus had returned to normal, the contraction response was relatively obvious, the swelling of the cervix and vaginal mucosa had disappeared, but there were still some inflammatory secretions. The symptoms of a further five cows had been alleviated; the size, texture and shape of the uterus was restored to some extent, the contraction response was still weak, they still had congestion and some inflammatory secretions were still found outside the cervix. The complete cure rate of the C group of cows was 53.3%, and the effective cure rate was 66.7%.

3.2. 16S rRNA full-length sequencing

After PacBio SMRT sequencing, 371,765 high-quality 16S rRNA sequences were obtained from across all samples, with an average sequencing quantity of 5996 sequences per sample. After 100% similarity cluster analysis, 158,615 representative reads were obtained. Furthermore, 13,067 OTUs were obtained under 97% similarity.

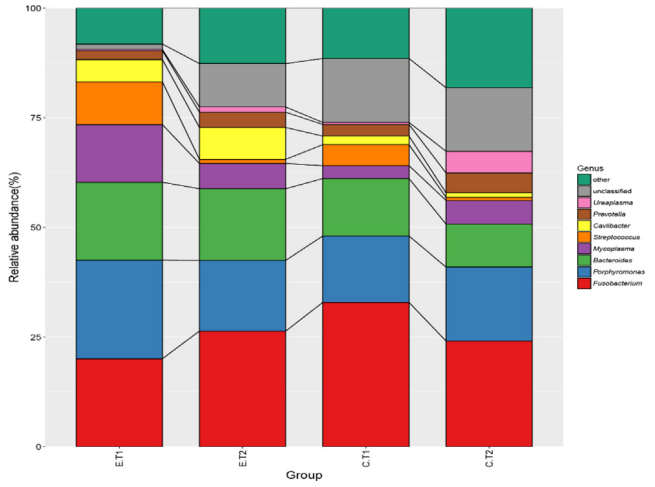

Fig. 2. Compositions of uterus mucus microbiota at the genus level from different groups.

In this experiment, Rarefaction Curve and Shannon Curve were used to evaluate the sequencing volume and the biological diversity of microbiota at the 97% similarity level (Fig. 1). The Rarefaction Curve and Shannon Curve of all samples tended to be horizontal with increasing sequencing amount, which indicated that, although it was possible to find new bacterial germline types, the current sequencing amount met the requirements for subsequent bioinformatics analysis and effectively revealed the composition of the main flora in the samples (Fig. 1a, 1b).

3.3. Bacterial composition of uterine mucus from cows

A total of 472 bacterial genera were identified at the genus level in the 62 uterine mucus samples from 31 cows, amongst which eight bacterial genera with an average relative content of more than 1% were identified (Fig. 2): *Fusobacterium* (25.78%), *Porphyromonas* (17.71%), *Bacteroides* (14.32%), *Mycoplasma* (6.90%), *Streptococcus* (4.09%), *Caviibacter* (3.91%), *Prevotella* (3.16%) and *Ureaplasma* (1.68%). These genera accounted for 77.55% of all sequences.

In the 62 uterine mucus samples from 31 cows, a total of 691 bacterial species were identified. Amongst these, there were 12 species with an average relative content of more than 1% (Fig. 3a): *Fusobacterium necrophorum* (24.74%), *Porphyromonas levii* (13.37%), *Bactericoides heparinolyticus* (8.02%), *Bacteroides pyogenes* (3.92%), *Caviibacter abscessus* (3.91%), *Mycoplasma bovigenitalium* (3.44%), *Streptococcus dysgalactiae* (2.80%), *Mycoplasma californicum* (2.70%), *Porphyromonas somerae* (2.55%), *Ureaplasma diversum* (1.68%), *Streptococcus uberis* (1.20%), and *Bacteroides fragilis* (1.12%). These species accounted for 69.45% of all sequences.

After treatment with LAB, the abundance of *Mycoplasma californicum, Mycoplasma bovigenitalium, Streptococcus dysgalactiae, Bacteroides pyogenes* and *Porphyromonas levii* decreased in the uterine mucus of cows. In contrast, the abundance of *Ureaplasma diversum, Caviibacter abscessus, Bacteroides heparinolyticus* and *Fusobacterium necrophorum* increased. After antibiotic treatment, the abundance of *Streptococcus dysgalactiae, Porphyromonas somerae, Bacteroides pyogenes, Bacteroides heparinolyticus* and *Fusobacterium necrophorum* decreased in the uterus mucus. In contrast, the abundance of *Bacteroides fragilis, Ureaplasma diversum* and *Porphyromonas levii* increased.

In ET1 and ET2 samples, the abundance of *Bacteroides fragilis* and *Streptococcus uberis* were always low. In the ET2 samples, the abundance of *Ureaplasma diversum* increased and *Mycoplasma californicum* decreased suddenly compared with the ET1 samples. In CT1 and CT2 samples, the abundance of *Streptococcus dysgalactiae* and *Mycoplasma bovigenitalium* were always low. In the CT2 group, the abundance of *Ureaplasma diversum* increased and *Streptococcus uberis* decreased suddenly compared with CT1 samples.

Bacterial species composition and relative abundance varied significantly amongst the different uterine mucus samples (Fig. 3b).

3.4. Comparison of bacterial community structure in uterine mucus samples

This study conducted principal coordinates analysis (PCoA) based on unweighted UniFrac distance and weighted UniFrac distance (Fig. 4a, 4b). The results were highly significantly different based on the unweighted UniFrac distance ($p < 0.01$), while the results were not significantly different based on the weighted UniFrac distance ($p > 0.05$). The results showed that there was little difference between the two groups in the composition of microbiota with high abundance, but there was a great difference in the composition of microbiota with low abundance.

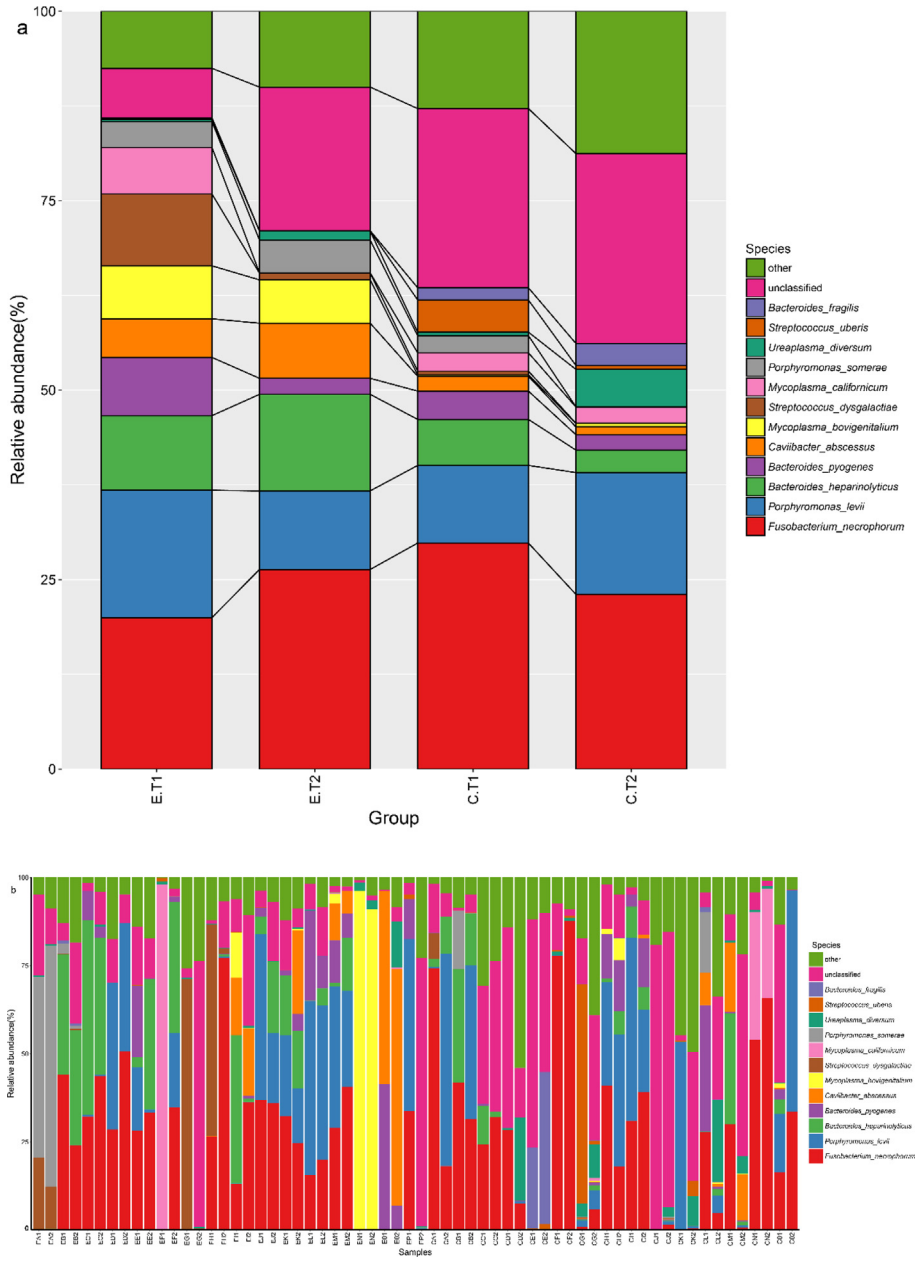

Fig. 3. Composition of uterus mucus microbiota at the species level from different groups (a). Composition of uterus mucus microbiota at species level from different samples (b).

3.5. Shared OTUs and core OTUs in uterine mucus samples

The common OTUs (that appeared in two or more samples) and unique OTUs (that appeared in only one sample) in the different groups were analyzed statistically. The results showed that 937 OTUs were distributed in all samples, from ET1, ET2, CT1 and CT2 groups. (Fig. 5). In addition to the common OTUs, the microbiota in the uterine mucus of each group had its unique OTUs. There were 187 unique OTUs in the ET1 samples, and the annotation results were *Phascolarctobacterium faecium, Porphyromonas levii, Parvimonas micra, Bacteroides heparinolyticus, Streptococcus dysgalactiae* and *Bacteroides pyogenes*. There were 193 unique OTUs in the ET2 samples, and the annotation results were *Fusobacterium necrophorum*, *Acinetobacter lwoffii, Porphyromonas levii*, *Bacteroides heparinolyticus*, *Lactococcus lactis*, *Ureaplasma diversum*, *Pseudomonas fragi* and *Enhydrobacter aerosaccus.* There were 208 unique OTUs in the CT1 samples, and the annotation results were *Phascolarctobacterium faecium*, *Porphyromonas levii*, *Parvimonas micra*, *Bacteroides heparinolyticus* and *Bacteroides pyogenes*. There were 343 unique OTUs in the CT2 samples, and the annotation results were *Lactococcus lactis*, *Fusobacterium necrophorum*, *Bacteroides heparinolyticus*, *Pseudomonas fragi*, *Porphyromonas levii*, *Ruminobacter amylophilus*, *Pseudomonas veronii*, *Comamonas jiangduensis, Parasutterella secunda, Lactococcus raffinolactis, Acinetobacter lwoffii* and *Pseudomonas alcaligenes*.

3.6. Differences in bacteria from uterine mucus samples

The Wilcoxon rank sum test was used to determine the significance of differences in the microbiota and different bacterial abundance in uterine mucus samples from different groups at the species level (Table 1, Table 2). In the tables, mean value means

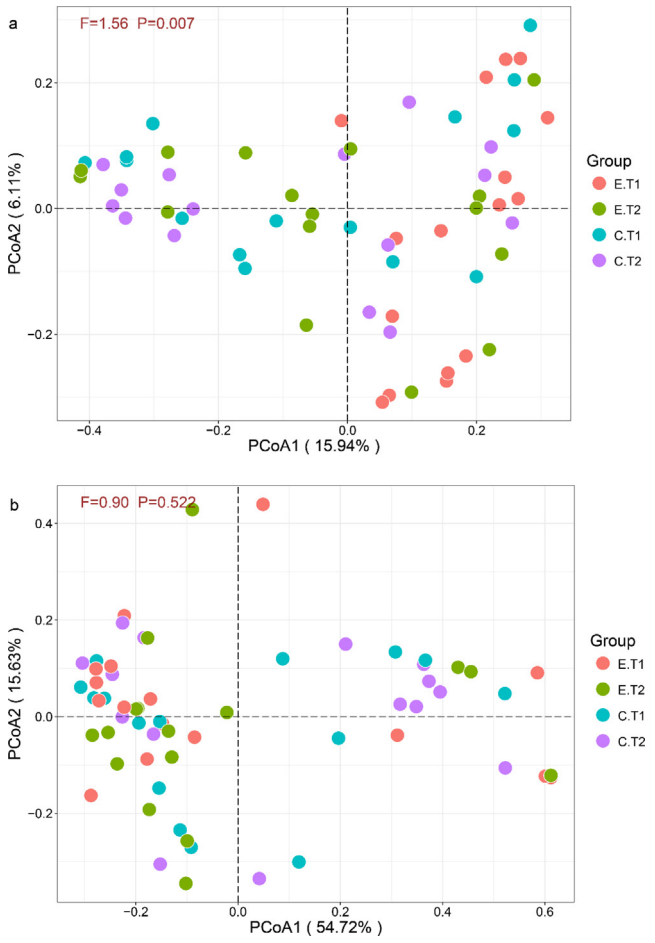

Fig. 4. Analysis of bacterial composition in uterus mucus based on unweighted UniFrac (a) and weighted UniFrac (b).

average value and SEM means standard deviation. We could compare the changes in the abundance of bacterial species between 2 groups by mean value, such as ET1 and ET2, and CT1 and CT2. When the *p*-value was less than 0.05, the differences between the two groups were significant. When the *p*-value was less than 0.01, the differences between the two groups were highly significant.

There were significant differences between ET1 samples and ET2 samples in the relative abundances of 33 species ($p < 0.05$) and the relative abundance of 15 species were highly significantly different between ET1 and ET2 samples ($p < 0.01$). In particular, *Anaeroplasma abactoclasticum* and *Faecalibacterium prausnitzii* were only present in the ET2 samples and *Lactobacillus helveticus*, *Lactococcus raffinolactis*, *Luteococcus japonicus* (amongst others) were significantly more abundant in ET2 samples than in ET1 samples. In contrast, *Streptococcus uberis* was significantly less abundant in ET2 samples than ET1 samples.

There were significant differences between CT1 samples and CT2 samples in the relative abundance of 23 species ($p < 0.05$) and the relative abundance of six species was very highly significantly different ($p < 0.01$). In particular, *Bifidobacterium animalis, Bifidobacterium breve* and *Bifidobacterium pseudocatenulatum* amongst others were significantly more abundant in CT2 samples than in CT1 samples. In contrast, *Campylobacter corcagiensis*, *Staphylococcus equorum* and *Treponema brennaborense* were significantly less abundant in CT2 samples than in CT1 samples.

Some bacterial species increased in abundance both after treatment with LAB and after treatment with antibiotics, and included *Lactobacillus helveticus, Lactococcus lactis, Lactococcus raffinolactis, Pseudomonas alcaligenes* and *Pseudomonas veronii.* No species significantly decreased in abundance after either LAB or antibiotic treatment.

3.7. Functional metagenomic prediction

The COG database is a tool to analyze the function and evolution of proteins at the genome level. It is established based directly on

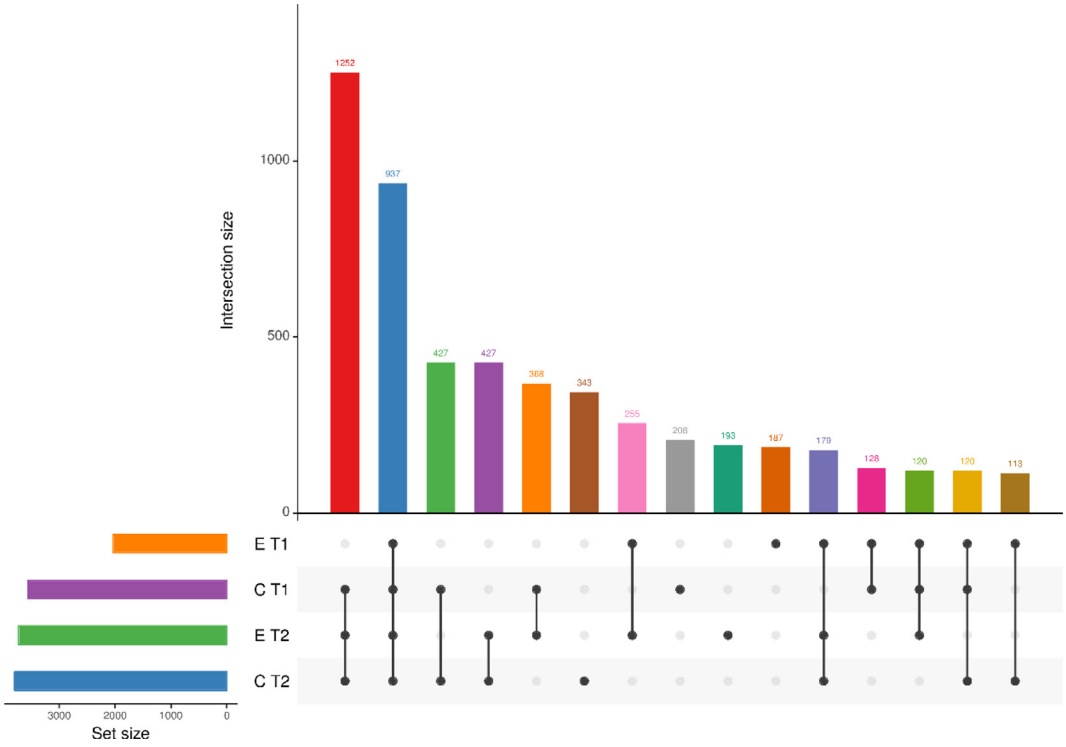

Fig. 5. Shared OTUs and core OTUs in uterus mucus from cows in different groups.

homologous genes, which usually encode the key regulatory proteins, enzymes or coenzymes necessary for life's activities. From the sequencing data of the 62 uterine mucus samples 4792 kinds of functional proteins or enzymes were predicted and annotated (COG), amounting to 116351220 in total from 23 categories. In the E and C groups, there were 384 kinds of functional proteins that were significantly differently expressed after the treatments were applied. There were nine categories at the primary functional

Table 1
Comparative analysis of abundance of bacterial species in ET1 and ET2.

Species	Mean value		SEM		*p*-value
	ET1	ET2	ET1	ET2	
Alloprevotella_rava	0.005032968	0.078543481	0.002371	0.044115914	0.019946889
Anaerocella_delicata	0.004435294	0.1193463	0.003676394	0.086537248	0.019343223
Anaeroplasma_abactoclasticum	0	0.020399582	0	0.010013951	0.003532381
Anaeroplasma_varium	0.002503125	0.034921538	0.00173269	0.01634789	0.017092621
Butyrivibrio_crossotus	0.002276085	0.061769348	0.001617438	0.030637214	0.025519679
Caproiciproducens_galactitolivorans	0.001443085	0.046832199	0.001443085	0.026178879	0.007788009
Clostridium_lactatifermentans	0.002311985	0.074395902	0.001634104	0.041195751	0.003207675
Comamonas_jiangduensis	0.003590766	0.022182646	0.002101169	0.006337595	0.014337481
Eubacterium_coprostanoligenes	0.005877631	0.113459764	0.002866139	0.06245614	0.037859095
Eubacterium_siraeum	0.001443085	0.031789072	0.001443085	0.016522967	0.016243892
Faecalibacterium_prausnitzii	0	0.007912256	0	0.002373464	0.003532381
Lactobacillus_helveticus	0.00264707	0.036592343	0.001472958	0.010786085	0.003457004
Lactococcus_lactis	0.011702117	0.114842113	0.003689317	0.041015318	0.036551675
Lactococcus_raffinolactis	0.019226674	0.056613749	0.013071396	0.020880389	0.007845234
Luteococcus_japonicus	0.000947113	0.011881812	0.000947113	0.003170555	0.00266616
Monoglobus_pectinilyticus	0.000947113	0.058460788	0.000947113	0.031061626	0.014169257
Oscillibacter_valericigenes	0.013090521	0.258216139	0.007327545	0.132816407	0.025372054
Paludibacter_propionicigenes	0.002886169	0.126397745	0.002886169	0.0681217	0.006755861
Parasutterella_secunda	0.002390198	0.164187553	0.001672508	0.083551904	0.002090104
Phascolarctobacterium_succinatutens	0.012786507	0.239301425	0.010105615	0.126278711	0.007593542
Pseudomonas_alcaligenes	0.00967542	0.044582588	0.005565361	0.012654911	0.0222668
Pseudomonas_veronii	0.032954195	0.094695684	0.019808457	0.030560103	0.028134361
Romboutsia_ilealis	0.001821626	0.01721045	0.001821626	0.00868809	0.021206914
Ruminobacter_amylophilus	0.003637045	0.161569276	0.002113032	0.085082914	0.014337481
Ruminococcus_champanellensis	0.000947113	0.051448127	0.000947113	0.024770672	0.005052223
Sphingobacterium_multivorum	0.001821626	0.010685537	0.001821626	0.003894898	0.021206914
Staphylococcus_equorum	0.002881667	0.016242902	0.002045615	0.005215477	0.009873609
Streptococcus_salivarius	0.002222617	0.02989326	0.002222617	0.011739462	0.000668533
Streptococcus_uberis	0.166281901	0.001007415	0.096192981	0.001007415	0.004355386
Treponema_brennaborense	0.001666222	0.025602371	0.001666222	0.012499143	0.025093539
Treponema_bryantii	0.000792042	0.030652062	0.000792042	0.012389305	0.025093539
Vampirovibrio_chlorellavorus	0.006258195	0.139878167	0.004402824	0.081284809	0.040718508
Victivallis_vadensis	0.001660942	0.080504876	0.001136027	0.044661906	0.006653952
unclassified	6.5218945	18.95103707	1.693397129	5.899967459	0.041830595

Table 2
Comparative analysis of abundance of bacterial species in CT1 and CT2.

Species	Mean value		SEM		*p*-value
	CT1	CT2	CT1	CT2	
Aeromonas_salmonicida	0.001699813	0.026569866	0.001699813	0.014868401	0.031254946
Atopostipes_suicloacalis	0.001699813	0.039518086	0.001699813	0.021748864	0.015336786
Bifidobacterium_animalis	0.005099439	0.287101564	0.005099439	0.184965015	0.004479444
Bifidobacterium_breve	0.001699813	0.163356184	0.001699813	0.107536551	0.031254946
Bifidobacterium_pseudocatenulatum	0.003399626	0.047878608	0.003399626	0.025152132	0.031254946
Campylobacter_corcagiensis	0.074877097	0.001677149	0.038662304	0.001677149	0.027122184
Comamonas_jiangduensis	0.013194013	0.078157526	0.010191943	0.037458611	0.006451566
Comamonas_piscis	0.001115947	0.013324465	0.001115947	0.004760643	0.013226601
Enhydrobacter_aerosaccus	0.018507442	0.202751289	0.012577008	0.108505924	0.008382836
Lactobacillus_helveticus	0.037920256	0.250929086	0.033781856	0.146988567	0.024375492
Lactobacillus_plantarum	0.011505923	0.073780568	0.008531926	0.03144687	0.016083759
Lactococcus_lactis	0.038014284	0.417424338	0.014099832	0.175227181	0.007099135
Lactococcus_raffinolactis	0.065247244	0.235891122	0.057434196	0.121246494	0.009841848
Leuconostoc_mesenteroides	0.00814121	0.054186398	0.005633584	0.024727179	0.033543342
Paracoccus_marcusii	0.010329059	0.019546651	0.008565109	0.006471421	0.04269745
Pseudomonas_alcaligenes	0.04753341	0.217699209	0.042329213	0.079464915	0.009586432
Pseudomonas_fragi	0.089651526	0.90418369	0.07748915	0.545520917	0.008904085
Pseudomonas_veronii	0.126509052	0.451225217	0.107783196	0.165144168	0.020625025
Serratia_marcescens	0.016152478	0.054676493	0.013527499	0.019404407	0.016083759
Staphylococcus_equorum	0.035696073	0.033155479	0.035696073	0.013082076	0.027022031
Staphylococcus_petrasii	0.007915199	0.021693064	0.00681111	0.00627548	0.015328
Stenotrophomonas_terrae	0.005099439	0.015176132	0.005099439	0.005839451	0.023544527
Treponema_brennaborense	0.041774693	0.00515406	0.017854088	0.004179823	0.038278838

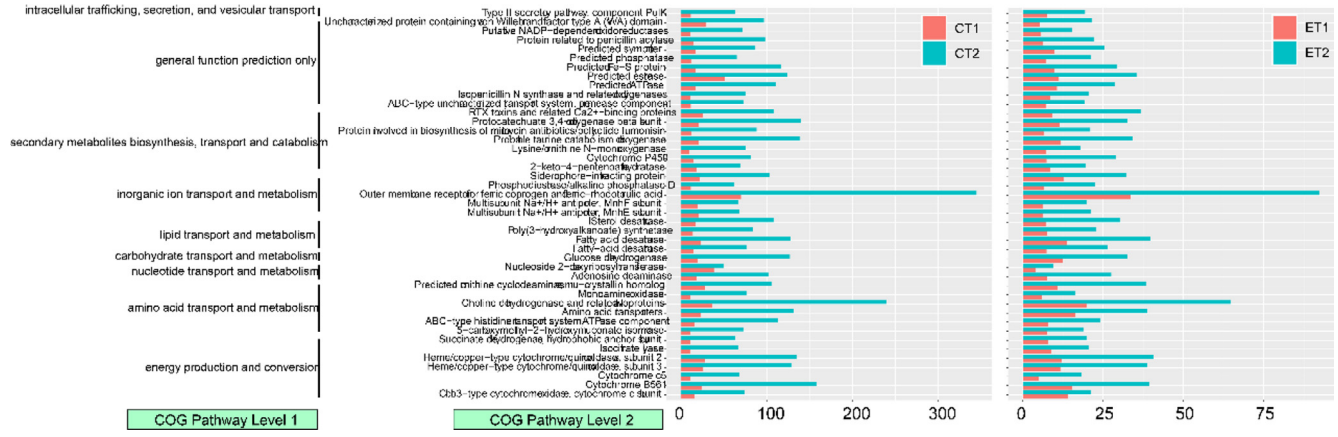

Fig. 6. Metabolic pathways from different specific COG annotation and the number of corresponding proteins.

level and 43 categories at the secondary functional level. Expression of protein pathways after treatment were significantly higher than before treatment ($p < 0.05$, Fig. 6).

4. Discussion

Endometritis is a common reproductive system disease of dairy cows. Not only does it affect the normal physiological function of cows, but it also leads to long-term infertility, or even lifelong infertility, and significant economic losses to the dairy cattle breeding industry. To date pathogens isolated from the genital tracts of cows with endometritis include mycoplasma, viruses, bacteria, fungi and parasites. The main pathogens were bacteria, including *Fusobacterium necrophorum*, *Bacteroides species*, *Escherichia coli*, *Streptococcus hemolyticus*, *Proteusbacillus vulgaris*, *Pseudomonas species* and *Clostridium species* and they are strongly associated with endometritis in cattle [33]. Rare species and species that cannot be cultured also exist in the cow genital tract and can harm their health [34]. As a result, full identification of the microbiota associated with the genital tract of cattle could underpin the reproductive health of cows and provide the understanding necessary to prevent and cure reproductive tract diseases.

In this study, we used the PacBio SMRT third-generation sequencing technology to describe the bacterial community composition of uterine mucus samples from cows with endometritis before and after treatment with LAB or antibiotics. We achieved this by measuring the full length microbial 16S rRNA gene. The majority of previous studies have evaluated the microbial composition of dairy products using second generation sequencing technology, but the read length is short; this reduces their accuracy often only allowing identification to genus level rather than species level. Using PacBio SMRT sequencing, rare species and previously unculturable microorganisms can be identified [35] and were also found in our study.

In this study, the symptoms of most sick cows improved following treatment, many returning to a healthy physiological status. More recovered following LAB treatment than antibiotic treatment. Currently, endometritis in cattle is usually treated with antibacterial anti-inflammatory products to accelerate uterine contraction and the removal of uterine contents [36]. There have been no previous reports on the treatment of acute endometritis with LAB. Reports on LAB and endometritis in dairy cows have generally focused on clinical evaluation methods after infection, and the screening of LAB affecting cow endometritis [37,38,39,40].

Defense mechanisms in the reproductive tract include the anatomical structure, endocrine regulation and microecological flora in the reproductive tract. The vaginal wall is composed of mucous membranes, muscular layers and fibrous tissue. Although there is no gland in the vaginal mucosa, the mucus secreted by the cervix keeps the vagina moist, which is a favorable environment for microbial growth. Under the action of oestrogen, vaginal epithelial cells proliferate in the bottom layer and gradually evolve into middle and surface cells. The surface cells proliferate and keratinize. Glycogen storage is abundant in these cells.

After ovulation, under the action of progestin, epithelial cells shed, glycogen is released, cervical mucus and endometrium is stripped and bleeding occurs which provides all the nutrient sources required for bacterial growth. Cervical mucus contains sodium chloride, glycoprotein, and cervical orifice oxygen partial pressure, which are the conditions suitable for the growth of anaerobic bacteria. Anaerobes are the main microbiota of the reproductive tract of healthy women, and are 5–10 times more abundant than aerobes and facultative anaerobes added together. Amongst the anaerobes, the detection rate of *Lactobacillus* was highest, followed by *Streptococcus*, *Peptostreptococcus*, *Enterococcus*, *Corynebacterium*, and *Bacteroides*, but there are no uniform reports about the detection of other bacteria [41].

Some microorganisms in the reproductive tract have antagonistic relationships, while others have symbiotic relationships. They colonize at the vaginal mucosa in a hierarchical and orderly manner, and form a biofilm that maintains the balance of microbiota in the reproductive tract. However, some bacteria are conditioned pathogens and if these overgrow, they break the balance of microbiota and cause reproductive tract infection [42]. Some studies have reported that reproductive tract infections were commonly associated with a decrease in the number of *Lactobacillus* [43]. Although no specific pathogenic bacterial species were isolated from patients with genital tract infections, the proportion of some species of bacteria had changed, such as *Escherichia coli*, *Bacteroides* and *Staphylococcus aureus*. Therefore, researchers speculate that dysbiosis of the bacterial community was the key cause of reproductive tract infection [44]. Our study in cattle rather than humans, is consistent with this as the proportions of different bacterial species changed in the genital tracts of diseases cows after treatment. Specifically, the relative abundances of various bacterial species were significantly higher than before treatment, but bacterial diversity was not changed. The abundance of *Ureaplasma diversum* in both E and C groups increased after treatment. Therefore, we hypothesize that endometritis directly affects *Ureaplasma diversum* in the reproductive system of cows. After intervention with LAB, the abundance of *Mycoplasma californicum* significantly reduced, which indicated that LAB might inhibit the growth of *Mycoplasma californicum*. After antibiotic treatment, the abundance of *Strepto-*

coccus uberis significantly decreased, while the abundance of *Bacteroides fragilis* significantly increased, indicating that antibiotics do help to restore the cow's uterus to a healthy state. This result was consistent with previous reports that *Bacteroides* was the main bacterial genus in the reproductive tract of healthy women [42]. Therefore, from our study, we find that reducing the abundance of *Mycoplasma californicum* and *Streptococcus uberis* is an effective treatment of endometritis.

As one of the most important beneficial bacteria in the reproductive tract and digestive tract of humans and animals, *Lactobacillus* species act in a number of ways: as biological barriers; through immunomodulatory, antitumor, anti-aging and antioxidant activity; and through other physiological functions. This maintains the microecological balance and plays an important role in preventing and treating reproductive tract diseases. Specifically, *Lactobacillus* species acts via three key mechanisms. Firstly, *Lactobacillus* species adhere to epithelial cells, forming placeholder protection and preventing the initial adhesion of pathogens. At present, the mechanism of *Lactobacillus* adhesion to vaginal epithelial cells is not well understood. It may be through intermolecular forces, electrostatic forces or brush bristles, or as a lipid wall acid, carbohydrate or the synergistic action of other substances [45]. Secondly, *Lactobacillus* species and other bacteria, such as *Staphylococcus epidermidis* and *Enterococcus*, maintain the pH value between 3.5 and 4.5 in the vaginas of healthy women, effectively inhibiting the reproduction of other pathogenic bacteria [46]. Boskey et al. [47] analyzed the number, growth rate and acid-producing capacity of *Lactobacillus* species in women's vaginas and found that *Lactobacillus* species had the ability to maintain the acidic environment [47,48]. Thirdly, *Lactobacillus* species can produce a variety of antibacterial substances, such as H_2O_2, which can kill pathogens directly or by oxidation.

In this study, some species significantly increased in abundance in diseased cows after LAB treatment, including *Lactobacillus helveticus, Lactobacillus raffinose, Iinsoluble anaerobic mycoplasma* and *Clostridium leptum* amongst others. However, pathogenic bacteria such as *Streptococcus bovis,* significantly decreased. The probable reason for this was that after treatment with LAB, *Lactobacillus* species adhered to the genital tract epithelial cells, blocked the adhesion of pathogens, reduced the probability of disease and achieved a therapeutic effect. Further speculation based on this would suggest that *Streptococcus bovis* might be the key causative pathogenic bacterium associated with cow endometritis.

After treatment with antibiotics, *Bifidobacterium* species increased markedly in abundance in our study; they included *Bifidobacterium animalis, Bifidobacterium breve* and *Bifidobacterium pseudocatenulatum. Bifidobacterium* species are beneficial bacteria. The possible reason for their increase following antibiotic treatment could be because cephalosporin antibiotics would have killed the pathogenic bacteria, such as *Campylobacter corcagiensis* and *Staphylococcus equorum*, allowing the genital tract to return to a normal physiological state and for the beneficial *Bifidobacterium* species to recolonize. The antibiotic used in this experiment was ceftiofur, which is a third-generation cephalosporin product. It had considerable antibacterial activity against Gram-positive bacteria, and a strong killing effect on Gram-negative bacteria, including *Enterobacter*, *Pseudomonas aeruginosa* and anaerobes [49]. Our results revealed that only the abundance of *Campylobacter corcagiensis, Staphylococcus equorum* and *Treponema brennaborense* was significantly reduced after treatment with ceftiofur. This suggests that the pathogenic bacteria causing cow endometritis in this study were likely to be *Campylobacter corcagiensis, Staphylococcus equorum* or *Treponema brennaborense*.

There were six species that increased in abundance after both LAB and antibiotic treatment: *Comamonas jiangduensis*, *Lactobacillus helveticus*, *Lactococcus lactis*, *Lactococcus raffinolactis*, *Pseudomonas alcaligenes* and *Pseudomonas veronii*. These species can, therefore, be considered as the typical microbiota of the cured cow genital tract in this study.

For cows at the early stage of postpartum lactation, immunity decreases due to calving and massive lactation, which is a severe test for the microbial community in the milk. This decrease in immunity following birth is also one of the reasons for the occurrence of cow endometritis [50]. Based on the known immune regulatory abilities of *Lactobacillus* in the animal genital tract, the increased abundance of *Lactobacillus helveticus* in cows that had been cured following treatment with LAB or antibiotics, indicates that *Lactobacillus helveticus* might be the key species responsible for immune regulation in the genital tract of healthy cows. The absence of species that decreased in abundance after both LAB and antibiotics treatment, might indicate that, although these treatments could cure cow endometritis, the effects on microbiota in the cow genital tract were different. For example, *Staphylococcus equis* and *Treponema brennaborense* increased after LAB treatment but decreased after antibiotic treatment. This might suggest that these two species were typical bacteria in healthy cow genital tracts that had recovered growth after LAB treatment, but that they were also sensitive to ceftiofur in this experiment, accounting for their decreased abundance in the antibiotic treatment. It also demonstrates that antibiotics might influence the normal microbiota of healthy cow genital tracts, reducing the species richness of microbiota and, therefore, having a negative role. We further suggest that *Campylobacter corcagiensis* might be an important contributory cause of cow endometritis in this study.

Understanding a particular microbial community and its gene function could reveal the relationship between environment and microorganisms and the interactions between them, thus providing valuable information for practical applications. Using high-throughput sequencing technology to sequence the 16S rRNA gene of samples is the best method for analysis of microbial diversity and community structure. When the gene components within samples have been sequenced then gene function can be determined by analysis of the metagenomic information further. However, to match the two kinds of sequencing data exactly is costly and time-consuming. Nevertheless, the development of bioinformatics software in recent years has made it possible to use 16S rRNA gene sequencing data to analyze the functions of the microbes concerned. PICRUSt software can use the 16S rRNA gene sequencing data to construct phylogenetic trees, reconstruct ancestral states based on the characteristics of known nodes, and then estimate the functional gene content of unknown nodes to make strong predictions of the metagenome inferred by 16S rRNA genes. Currently, PICRUSt has been widely used in humans [51,52,53,54,55], animals [56,57,58,59], intestinal, and pulmonary inflammation studies [60], dairy related studies [61,62], fermented foods [63,64], and environmental sciences [65,66]. Therefore, it is reasonable and feasible to apply PICRUSt to predict and analyze the metagenomic data from cow genital tract mucus samples. In this study, the amino acid sequences, which were inferred from cow genital tract mucus samples, were functionally annotated in the COG database. Thousands of functional proteins and enzymes were obtained. The expression pathways of many of these were up-regulated following LAB or antibiotic treatment of cow endometritis. We hypothesize that the effects of LAB or antibiotic treatments on protein expression pathways showed consistent trends suggesting that LAB treatments could replace antibiotic treatments for cow endometritis.

In conclusion, LAB treatments could cure cow endometritis, restore the uterus and microbiota to a normal physiological state and avoid the disadvantages of antibiotic treatment, such as the overall reduction in microbial abundance and the killing of microbes normally found in healthy cow genital tracts. In conjunc-

tion with PacBio SMRT third generation sequencing technology, the microbiota of cured cows with endometritis genital tracts could be extrapolated from those found in cows that had recovered following LAB or antibiotic treatment, such as *Clostridium tender*, *Mycoplasma californicum, Lactococcus lactis*. At the same time, potentially causative pathogenic bacteria could be identified, such as *Campylobacter corcagiensis*, *Mycoplasma californicum* and *Streptococcus uberis*, which provide a target for the treatment of cow endometritis. Through PICRUSt function prediction, the feasibility of LAB for treatment of cow endometritis was proved.

Conflicts of interest

The authors declare that they have no conflicts of interest.

Financial support

This research was supported by China Agriculture Research System, China (grant numbers CARS-36), and Inner Mongolia Science and Technology Plan (grant numbers 201802063).

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