Study on the Mechanism of Lactobacillus Rhamnosus Probio-M9 Combined With Antibiotics on Staphylococcus Aureus Mastitis in Rat

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Abstract

This study aims to investigate the potential anti-inflammatory mechanism of *Lactobacillus rhamnosus* Probio-M9 (Probio-M9) combined with antibiotics. Probio-M9, a probiotic strain previously isolated from the milk of healthy women. The Illumina Hiseq platform performs metagenomic sequencing on faeces samples at various time points; UPLC-Q-TOF/MS is used to detect a large number of endogenous metabolites in the serum of rats with mastitis induced by *Staphylococcus aureus*. The results show that the Probio-M9 combined with antibiotics increases beneficial intestinal bacteria (*Lactobacillus murinus*, *Lactobacillus reuteri*) and positively regulates metabolites (linoleic acid, glycerophospholipids, arachidonic acid, Sphingolipids. etc.), reduce inflammation in the body to play a protective role against mastitis. This research provides a new research method to relieve mastitis, and also provides a theoretical basis for the development and utilization of Probio-M9.

Key Points

- 1 Explain the mechanism of Probio-M9 combined with antibiotics on mastitis from the perspective of metabolomics
- 2 Explain the mechanism of Probio-M9 combined with antibiotics on mastitis from the perspective of Metagenomics
- 3 Provides a new research method for the Probio-M9 combined with antibiotics to relieve mastitis provides a theoretical basis for the development and utilization of Probio-M9

Introduction

Mastitis is not only refers to the local inflammation of breast tissue, but also includes inflammation of various breast physiological anatomical structures such as the nipple, acinus, and breast duc (Contreras et al. 2011). Mastitis is not only a common disease in humans, but also a huge threat to many mammals. Globally, approximately 10% of breast-feeding women suffer from mastitis (Cha et al. 2011). Its clinical symptoms include local pain, redness and swelling, which have a great impact on lactating mothers and children. Mastitis of dairy cow is one of the most serious diseases (Bar et al. 2008), which causes huge economic losses to dairy farms. Microbial infection is the main cause of mastitis, including *Staphylococcus aureus*, *Escherichia coli*, *Streptococcus dysgalactiae* and *Streptococcus uberis*, etc (Monistero et al. 2018). Over the years, antibiotics are commonly used to treat bovine mastitis (Nahum et al. 2006). However, abuse of antibiotics has led to the emergence of resistant strains and the presence of drug residues and has increased the difficulty of curing this disease (Normark et al. 2002). In addition, antibiotics kill most of the microbes that are present in the digestive tract, leading to imbalances in the gut microbiome and destruction of the ecosystem that are normally present in the gut.

Probiotics are considered as microbial feed supplements that can benefit the host (Reid 2005), when sufficient numbers of living bacteria reach the host gut and colonize, they alter the balance of the

intestinal flora and have health effects on the host (Vila et al. 2010). Recently, probiotics supplementation has been proposed as an alternative approach to ameliorate mastitis. These have demonstrated promising therapeutic effect in some animal and human clinical trials (Forkus et al. 2017; Ran et al. 2019; Fernandez et al. 2016; Klostermann et al. 2008; Bouchard et al. 2015; Souza et al. 2015). However, probiotics have a longer treatment period and well designed and conducted studies are needed before evidence-based recommendations can be made for use of probiotics in the treatment or prevention of mastitis. *Lactobacillus rhamnosus* M9 (Probio-M9), isolated from the milk of healthy women, has exhibited promising gastrointestinal fluid tolerance and good bile salt tolerance (Liu et al. 2020). This strain possibly could also improve the diversity of intestinal flora and enhance immunity. Studies have shown that it can improve the immune response function of the body and reduce the degree of inflammation by Oral Probio-M9 (Xu et al. 2021). Moreover it has a prevention and treatment effect on breast inflammation (Yang et al. 2021), and has a good therapeutic effect on inflammatory bowel disease and rat breast cancer (data not published).

Metagenomics, which describes the study of microbial diversity, population structure, evolutionary relationships, functional activity of microbial samples by retrieving genetic information from microbial populations of environmental samples without the need for preselection through culture (Chan et al. 2003). Metabolomics as a new omics platform which is used to study microbial and plant, mammalian, and environmental systems (Fiehn et al. 2000). In mammals, these applications include the study of human diseases (Roberson 2007), including the diagnosis of clinical diseases, the investigation of pathological mechanisms, and the discovery of new therapeutic targets.

- In order to reveal the therapeutic mechanism of oral Probio-M9 combined with antibiotics on mastitis, combined with the analysis results of rat intestinal microbes and blood non-targeted metabolomics, explore the therapeutic mechanism of Probio-M9 combined with antibiotics on mastitis, which provides the future application of Probio-M9 combined with antibiotics scientific basis.

Materials And Methods

Bacterial culture and preparation of bacterial liquid

- According to the method of newbould, it has been widely used in the cultivation and preparation of *S.aureus* isolated from bovine mastitis. Briefly, preserved *S. aureus* ATCC29740 was inoculated into Tryptone Soy Broth for activation. It was transferred three times in succession and each time it was cultured at 37°C for 24h. Colonies on the plates were counted after the cultivation. The activated *S.aureus* ATCC29740 was centrifuged for 5 min (3500 r/min) after washing with PBS. Afterwards, it was diluted in PBS to the required concentration, and kept in a refrigerator at 4°C.

Animals and treatment

- Female Wistar (SPF) rats were bought from the Charles River Laboratories, Beijing, China. Laboratory temperature is $25^{\circ}\text{C} \pm 2^{\circ}\text{C}$ relative humidity is $55 \pm 5\%$ and a 12-hour light-dark cycle, provide food and water for animals. After 7 days of adaptation, pregnant rats (300–350 g) were divided into antibiotics treated group (ATG, $n = 12$) and Probio-M9 combined with antibiotics (AMTG, $n = 12$), the ATG groups are feeding Cefalexin (21 mg/kg) for 3 days after infecting *S.aureus* ATCC29740 in mammary glands of rats, the AMTG groups are feeding Cefalexin (21 mg/kg), the Probio-M9 (4×10^9 CFU) are feeding after 2h.

Mouse mastitis model of infection

- Wistar lactating rats (7–10 days after birth of offspring), take out offspring 1–2 hours before inoculation of mammary gland, and anesthetize the lactating rats with ether. Two groups were inoculated in the experiment, and each group had 12 lactating rats. Turn the rat over, sterilize the fourth pair of nipples and the surrounding area with 70% ethanol, then clamp one nipple with fine forceps and locate the duct hole. Slowly inject 100 μL (1×10^6 CFU) of *S.aureus* through a syringe. The needle must be inserted no more than about three to four millimeters deep into the milk duct. Repeat the process for the inoculation of the second gland and then place the mouse in a newly replaced cage to reduce possible contamination. Three days after the challenge, all rats were killed and their blood and stool were collected. A total of 48 samples of stool and serum were collected at specific time points (before gavage of Probio-M9 in ATG/AMTG and after the rats were sacrificed)

Rat stool metagenomic sequencing

DNA extraction, metagenomic sequencing and quality control

- Metagenomic DNA was extracted from 100 mg stool using the QIAamp Fast DNA Stool Mini Kit (Qiagen, Hilden, Germany). The DNA quality was examined by agarose gel electrophoresis and a nanodrop spectrophotometer (260/280 nm ratio). Shotgun metagenomic sequencing was performed using an Illumina HiSeq 2500 instrument. Libraries were constructed by DNA fragments of ~ 300 bp length; pairedend reads were generated using 150 bp in both forward and reverse directions.
- Illumina's Hiseq 2500 sequencing platform was used for metagenomic sequencing of rat fecal DNA. The generated metagenomic reads were processed with the KneadData quality control pipeline (<http://huttenhower.sph.harvard.edu/kneaddata;v0.7.5>), which used Trimmomatic (a flexible trimmer for Illumina sequence data) to filter out low-quality reads of less than 60 nt in length. Rats contaminating reads were removed by Bowtie2 (v2.3.5.1) (Benson et al. 2010).

Statistical analyses

- All statistical analyses were performed using the R software (v.4.0.2). Unless otherwise stated, data were expressed as mean $\pm$ SD. Wilcoxon test, t-test, and Kruskal-Wallis test were used to evaluate differences in the microbiome and metabolome between two groups.

Rat blood non-targeted metabolomics

Serum sample collection and preparation

- Serum samples were used 400 μ L of serum into a 2.0 mL centrifuge tube, add 1200 μ L of methanol according to the ratio of sample: methanol (1:3); shake for 15 s to mix samples and reagents, and operate at room temperature Precipitate the protein, centrifuge at 15,800 rpm for 15 min to remove the precipitated protein; take the supernatant and place in a vacuum rotary evaporator to dry completely; 400 μ L methanol aqueous solution (4:1) to reconstitute, 12000 rpm centrifugation for 15 min, and filter with 0.22 μ m membrane, Measured on the machine, all samples take 5 μ L and mix to make QC samples (Duarte et al. 2010).

Chromatographic separation and mass spectrometry

- Liquid separation is carried out in a chromatographic column: the column temperature is 35°C, and the flow rate is 0.32 mL/min. The autosampler maintains a low temperature of 4°C, the sample volume is 5 μ L, and the column equilibration time is 5 min. The jet ion source uses positive (ESI⁺) and negative ion (ESI⁻) modes. The mobile phase in ESI⁺ mode is 0.1% formic acid-water solution (A) and 0.1% formic acid-acetonitrile solution (B); elution conditions are 0–2 min: 98% A; 2–17 min: 98% A; 17–22 min: 98% B, 22–24 min: 98% B. In ESI⁻ mode, the mobile phase is 0.1% ammonium hydroxide (A) and acetonitrile solution (B). The gradient elution conditions are 0–1 min: 98% A; 1–15 min: 98% A; 15–20 min: 98% B, 20–22 min: 98% B. Agilent Acquity UPLC-Xevo G2 QTOF mass spectrometer in the positive and negative electrospray ion source. The first-level data collection is performed in ion mode, and the mass scan range is 50-1100 m/z (Sangster et al. 2006).

Data analysis and statistical analysis

- The MS data were processed by Progenesis QI software, baseline correction, peak identification, peak extraction, peak alignment, and normalization using default parameters; then the multi-dimensional statistical analysis is into SIMCA-P 14.1 (Umetrics, Umea, Sweden) software. Screen the differential metabolites with VIP > 1 and continue to one-dimensional statistical analysis, including t-test and multiple change analysis; the final screening also meets VIP > 1. The difference between $p < 0.05$ and Fold change > 2 conditions is analyzed for compound identification. Search the HMDB and METLIN databases based on the first-level atlas to obtain the identification results of differential metabolites. Application MetaboAnalyst 5.0 online software (<http://www.metaboanalyst.ca/faces/ModuleView.xhtml>) conducts pathway and enrichment analysis of differential metabolites.

Results

Metagenomic analysis of rat stool

Analysis of rat intestinal diversity

Diversity refers to a set of indices or metrics used to quantify the distribution of taxa abundance. Species diversity can be divided into Alpha (α), Beta (β) and Gama (γ) diversity. Among them, α diversity is mainly concerned with the number of species in a uniform local habitat, β diversity is concerned with the number of species in the same locality and different groups; γ diversity is concerned with the number of species in different localities and even in different climates (Sanchez et al. 2017). In this study, α diversity and was used to analyze the diversity of rat feces.

It can be seen from Fig. 1 that all samples have similar α diversity on day 0; the alpha diversity of the ATG group was significantly decreased (Two-sided Wilcoxonsigned-rank test, $p < 0.05$) on day 30, while the alpha diversity of the AMTG group was significantly increased (Two-sided Wilcoxonsigned-rank test, $p < 0.05$) on day 30. Which proved that the antibiotics intervention in rats of mastitis would lead to the decrease of intestinal diversity in rats, and after the intervention of Probio-M9 combined with antibiotics, it can increase the diversity and abundance of gut in rats.

Horizontal community structure of intestinal bacterial species in rats

- The relative levels of specific bacterial species in rat stool samples in this study are shown in Fig. 2. On day 0, the composition of the feces of the rats in each group was similar, mainly *Lactobacillus*(60–70%), *Bacteroides* (10–15%) and *Lachnospiraceae* (5–10%); when the rats breast tissue was challenged, the abundances of *Lactobacillus*, *Bacteroides* and *Lachnospiraceae* all declined in ATG group, the *Lactobacillus murinus* (10.2%), *Lactobacillus johnsonii* (8.4%), *Lactobacillus reuteri* (9.4%), *Lactobacillus animalis* (0.6%) *Parasutterella excrementihominis* (1.1%), *Enterococcus faecalis* (0.1%) *Escherichia coli* (1.2%), *Bacteroides massiliensis* (1.1%) were significantly reduced on the 30th. While in the AMTG group, *Lactobacillus murinus* (13.4%), *Lactobacillus johnsonii* (9.3%), *Lactobacillus reuteri* (13.4%), *Lactobacillus animalis* (1.2%), *Parabacteroides graminis* (1.3%), *Bacteroides fragilis* (2.4%) significantly increased, while *Alipis shahii* (2.6%), *Brevibacterium massiliense* (1.1%), *Enterococcus faecalis* (1.0%), *Desulfovibrio desulfuricans* (1.8%) and decreased significantly. The results are consistent with the diversity results. These results showed that antibiotic treatment did not change the intestinal flora of rats with mastitis, while the combination of Probio-M9 and antibiotics significantly changed the intestinal flora of rats with mastitis, up-regulating the beneficial bacteria and potential beneficial bacteria, down-regulating the harmful bacteria and opportunistic pathogenic bacteria, and improving the intestinal diversity.

Path analysis

- In this study, MetaCyc database was used to conduct difference pathway analysis of metacomgenome. As shown in the Fig. 3, most of the differential pathways were significantly upregulated in the ATG and AMTG groups on day 30. Compared with the ATG group, the common upregulated pathway in AMTG were glycolysis, superpathway of purine nucleotide salvage, superpathway of L-alanine biosynthesis.

Non-targeted Metabonomics analysis of rat blood

Multivariate statistical analysis

- Unsupervised statistical model Principal Component Analysis (PCA), Which is a multivariate statistical analysis method. Partial least square discriminant analysis (PLS-DA) is a supervised statistical method of discriminant analysis, which uses partial least squares regression to establish a model of the relationship between metabolite expression and sample category, so as to realize the prediction of sample category (Thomas et al. 2016). The PL-SDA scores of the two groups of serum samples were shown in Fig. 4.

ATG AMTG

In this study, the PLS-DA model of the AMTG group and ATG group was established for 30 days, The R^2X of the positive ion (A) was 0.935 and Q^2 was 0.958, and the R^2X of the negative ion (B) was 0.913 and Q^2 was 0.897. It shows that the model has good predictive ability. As can be seen from Fig. 4, the samples of the two groups are distributed far apart and concentrated in their respective regions, indicating that the content of the two groups of samples is significantly different.

Differential metabolite identification

According to the VIP value obtained by the PLS-DA model, the influence intensity and explanatory ability of the expression pattern of each metabolite on the classification and discrimination of each group of samples were measured, and the different metabolites with biological significance were mined.

Metabolites with both multidimensional statistical analysis $VIP > 1$ and univariate statistical analysis $P < 0.05$ were selected as the metabolites with significant differences, as shown in Table 1.

Compared with ATG, 29 significantly different metabolites were significantly changed with AMTG. The results show that Probio-M9 combined with antibiotics regulate the metabolic disorder caused by rats mastitis to a certain extent, so as to achieve the purpose of treating mastitis.

Table 1. The changing trend of significantly different metabolites in the positive ion and negative ionmode with two groups

NO.	tR/min	KEGG	Metabolite	Ion source
1	2.952616667	C02455	1-phenethylamine	ESI+
2	19.840	C00157	Phosphatidylcholine	ESI+
3	7.35915	C00422	Leukotriene A4	ESI+
4	8.959333333	C04483	sphinganine	ESI+
5	5.807	C09547	Bethanidine	ESI+
6	7.61465	C11002	3b,12a-Dihydroxy-5a-cholanoic acid	ESI+
7	7.61465	C11135	Hyodeoxycholic acid	ESI+
8	8.1484	C1120	Sphingosine1-phosphate	ESI+
9	3.272916667	C13809	2(N)-Methyl-norsalsolinol	ESI+
10	72.541	C00093	Glycerophosphate	ESI+
11	6.604	C14774	Glycochenodeoxycholic acid	ESI+
12	5.2943	C14812	Taurochenodeoxycholic acid	ESI+
13	0.724616667	C01712	Taurine	ESI-
14	9.23678	C1595	Linoleate	ESI-
15	0.781783333	C17737	pantothenic acid	ESI+
16	0.504466667	C05462	Ribonolactone	ESI+
17	8.845733333	C07663	A-12(13)-EpODE	ESI-
18	6.66835	C08153	17,18-epoxy-5,8,11,14-eicosatetraenoic acid	ESI-
19	6.803416667	C08277	12-Ketodeoxycholic acid	ESI+
20	7.61465	C08821	Isohyodeoxycholic acid	ESI+
21	5.807	C09547	Bethanidine	ESI-
22	7.61465	C11002	3b,12a-Dihydroxy-5a-cholanoic acid	ESI-
23	7.61465	C11135	Hyodeoxycholic acid	ESI+
24	6.604	C14774	Glycerophosphocholine	ESI+
25	3.272916667	C13809	2(N)-Methyl-norsalsolinol	ESI-
26	1.222716667	C14475	Nicotinamide	ESI-
27	6.604	C14774	Glycochenodeoxycholic acid	ESI+

NO.	tR/min	KEGG	Metabolite	Ion source
28	7.60035	C02165	Leukotriene B4	ESI+
29	10.29528	C14823	8(S)-HPETE	ESI+

- **Analysis of serum differential metabolite pathway**
- The metabolic pathways of 29 significantly different metabolites were analyzed by Metabo Analyst website (<https://www.metaboanalyst.ca/MetaboAnalyst/home.xhtml>). The results showed that the major metabolic pathways were sphingolipid metabolism, linoleic acid metabolism, Glycerophospholipid metabolism, phenylalanine metabolism, Primary bile acid biosynthesis, arachidonic acid metabolism (Fig. 5). According to the analysis. It is suggested that the therapeutic mechanism of Probio-M9 on *S.aureus*-induced mastitis may be through regulating the metabolic disorder caused by mastitis, and by regulating the significantly different metabolites to restore it to normal, thereby playing the role of treating mastitis.

Discussion

Metagenomic analysis of rat stool

- The human intestine is one of the densest and most diverse microbial communities (Turnbaugh et al. 2006). The intestinal microbiome includes a large number of organisms such as bacteria, viruses, fungi, archaea and protozoa. *Bacteroides* are potential settlers of the colon and account for the main part of the intestinal flora. These gram-negative obligate anaerobes play multiple roles in the human intestinal flora and are the main players in maintaining the intestinal microbial food web. *Bacteroides* have been proven to be symbiotic bacteria, not only acting as a "provider" for the host and other microorganisms living nearby, but also helping the host by providing many health benefits. The research results (Rettig et al. 2014) show that the by-products of *Bacteroides fragilis* in the metabolic process have significantly improved the barrier function of epithelial cells and mucus, have significant antibacterial properties, can inhibit the adhesion of intestinal pathogens in the body, and improve the intestinal microenvironment of the body stability. *Lactobacillus* is a type of probiotic that exists in the human body. It has the functions of regulating intestinal flora, enhancing immunity, protecting gastric mucosa, improving intestinal function, defecating, preventing diarrhea, promoting digestion, anti-tumor, anti-oxidation (Spinler et al. 2014). *Lactobacillus reuteri* mainly exists in the intestines of vertebrates and mammals, and is harmless to humans and animals. By competing for resources with harmful bacteria attached to the intestines, it can reduce the colonization of harmful bacteria, maintain the homeostasis of intestinal flora, and balance the composition of intestinal flora. *Lactobacillus reuteri* can improve the unusual anti-inflammatory molecule NGF and inhibits the translocations of NF- κ B into the nucleus (Ayala et al. 2018). In this study, the content of *Bacteroides fragilis*, *Lactobacillus reuteri* and other *Lactobacillus* are increased significantly after Probio-M9 intervention.

- *Enterococcus faecalis* and *Escherichia coli* were significantly reduced in the AMTG group in this study. *Enterococcus* is widely found in nature and is a normal flora in the intestinal tract of humans and animals. *Enterococcus*, as an important pathogen, can cause urinary tract infections, skin and soft tissue infections, and more serious abdominal infections, sepsis, meningitis and other diseases (Sánchez et al. 2020). *Escherichia coli*, widely exists in the intestinal tract of humans and animals. When the intestinal flora is dysregulated or the body's immune system is disturbed, *Escherichia coli* will become pathogenic bacteria and cause intestinal diseases or other diseases of organizational organs (Martinson et al. 2020). After the intervention of Probio-M9, The contents of *E. faecalis* and *E. Coil* are decreased significantly.
- Purine itself does not exist in nature, but its derivatives are widely present in nature. Adenine and guanine are important components of nucleic acid. Studies have found that disrupting the biosynthesis of purine in organisms will lead to the growth of a large number of pathogenic bacteria, such as *Escherichia coli*, *Bacillus anthracis*, *staphylococcus aureus*, etc. Mastitis can cause DNA damage of microorganisms in the body, such as superpathway of purine deoxyribonucleosides degradation, adenine and adenosine salvage III et al.) and down-regulate energy metabolism (e.g., glycolysis, lactose and galactose degradation pathways, etc.). After probiotic intervention, the DNA repair pathway of gut microbes can be up-regulated.

Non-targeted Metabonomics analysis of rat blood

After a breast infection with *Staphylococcus aureus*, the metabolic pathways of combination of Probio-M9 and antibiotics involved are shown in Fig. 5, the metabolites involved in the glycerophospholipid metabolism pathway, such as phosphocholine, glycerophosphocholine, and phosphatidylcholine, were all significantly affected. Phosphatidylcholine can inhibit lipid peroxidation to prevent cell membrane damage. It also has the effect of emulsifying and decomposing fats, reducing the content of neutral fats and removing peroxides (Barr et al. 2010). In addition, glycerophosphate is an important part of glycerolipid metabolism, and is related to other energy metabolism pathways. The results showed that when *S.aureus* stimulates breast tissue, the energy demand provided by fatty acid oxidation may increase, and the content of glycerophosphate, glycerophosphatecholine, phosphatidylcholine and phosphocholine increased significantly. After feeding Probio-M9, Glycerophosphoric acid and other metabolites are significantly reduced, thereby regulating the metabolic pathway of glycerophospholipids.

Fatty acids play an important role in the body's biofilm synthesis, cell energy supply and signal transduction pathways effect. According to whether it needs to be taken from food, it can be divided into essential and non-essential fatty acids (Moyes et al. 2014). Among them, linoleic acid is a typical essential fatty acid. It is an important beneficial metabolite from bacteria. A series of positions and geometric isomers of linoleic acid constitute conjugated linoleic acid (Simopoulos 2008). Conjugated linoleic acid plays an important role in anti-cancer, anti-inflammatory, anti-atherosclerosis, regulating the immune system, antagonizing intestinal pathogenic bacteria and maintaining the homeostasis of intestinal flora. Arachidonic acid is an important precursor substance of eicosanoid, which can be

produced by linoleic acid; and eicosanoid is an inflammatory mediator, such as prostaglandins and leukotrienes, which can affect the body's inflammation and immune response (Brash 2001). The content of linoleic acid in the blood metabolites of the model group in this study was significantly reduced, while arachidonic acid and leukotrienes were significantly increased; pathogenic bacteria severely disrupted the body's fatty acid metabolism. After oral administration of probiotics, the content of linoleic acid increased significantly, while the content of arachidonic acid and leukotrienes decreased significantly.

Sphingolipids are an important component of biological membranes, which have the function of protecting intracellular substances and also play an important role in signal transduction of cell life activities (Hannun et al. 2018). Sphingosine-1-phosphate (S1P) is a kind of bioactive lipid with important physiological functions. It is widely present in blood, lymph, red blood cells, neutrophils, platelets and other body fluids and cells (Snider et al. 2009). S1P can be used as a second messenger to directly act on intracellular targets, or it can be transported outside the cell, a series of downstream signaling pathways are activated to produce important physiological functions and participate in various physiological functions (Biroulet et al. 2017). In this study, Probio-M9 can significantly regulate S1P and sphingosine in the blood, thereby achieving the purpose of treating mastitis.

- The Probio-M9, a probiotic isolated from healthy breast milk, can play a probiotic function in the intestines of humans and animals. Whether the Probio-M9 has a relieving effect on mastitis is still unclear. For this purpose, this study used fecal metagenomics and blood metabonomics methods to study the mechanism of Probio-M9 alleviating mastitis. The specific results are as follows: Through the analysis of rat fecal metagenomics, mastitis rat intestine Significant changes in the tract flora, harmful bacteria (*Enterococcus faecium*, *Escherichia coli*) increased, and beneficial bacteria (*Lactobacillus reuteri*, *Lactobacillus animal*) decreased, after the intervention of the Probio-M9, the beneficial bacteria increased, the harmful bacteria decreased, the microbial diversity and abundance of the rat's intestinal tract were also improved, and the overall stability of the intestinal flora was improved. The intervention of the Probio-M9 significantly reduced the significantly altered fatty acid content (arachidonic acid) caused by mastitis, and increased the content of major amino acid metabolites that maintain normal intestinal function, thereby promoting the body's nutrition substance absorption and enhancement of vitamin metabolism to reduce the harmful effects of mastitis on the body. The Probio-M9 combined with antibiotics plays a role in relieving mastitis by regulating the structure of the intestinal flora, regulating key metabolites in the body.

Declarations

Author Contributions

All of the authors have contributed substantially to the manuscript. Concept, drafting and interpretation—Heping Zhang, Ruibo Xu. Collection and assembly of data—All authors. Data analysis and interpretation: Ruibo Xu. All authors read and approved the final manuscript.

Consent for publication

All authors listed have read the complete manuscript and have approved submission of the paper.

Competing interests

The authors declare that they have no conflict of interest.

Ethics approval and consent to participate

Not applicable to humans. All animal experiments were conducted in accordance with the Inner Mongolia Agricultural University Division of Laboratory Animal Medicine and Institutional Animal Care and Use Committee.

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Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Figures

Figure 1

The alpha-diversity of fecal microbiota in rats Note: A: The distribution map of the shannon diversity index of the samples; B: The distribution map of the Simpson diversity index of the samples.

Figure 2

The relative abundance of bacteria in rats feces at species level at 30d

Figure 3

Different metabolic pathways of intestinal microbes in ATG and AMTG

Figure 4

The PLS-DA analysis between ATG and AMTG at 30 d Note: A-B: The positive and negative PLS-DA scores of the BG group and CG group in 30d (A: Positive ions, B: Negative ions);

A

B

Figure 5

KEGG pathway and enrichment analysis of blood differential metabolites Note: A: KEGG pathway; B: enrichment analysis