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Changes in intestinal florae and serum inflammation in rheumatoid arthritis rats and the effects of probiotics

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Abstract. – OBJECTIVE: The aim of this study was to explore the changes in intestinal florae and serum inflammation in rats with rheumatoid arthritis (RA), and to investigate the effects of probiotics.

MATERIALS AND METHODS: A total of 30 Sprague Dawley (SD) rats were randomly divided into three groups, namely, control group, model group, and probiotic group. The rats in the model group were prepared into models of collagen II-induced arthritis. Meanwhile, the rats in probiotic group were treated with probiotics for 6 weeks via intragastric administration in addition to the treatment in the model group. Next, the feces of rats in the control group, model group, and probiotic group were sampled to detect the composition of intestinal florae. In addition, peripheral blood was collected from rats to determine the changes in the content of inflammatory factors, including tumor necrosis factor-alpha (TNF-α), interleukin-6 (IL-6), and IL-1β through enzyme-linked immunosorbent assay (ELISA).

RESULTS: Compared with the control group, the levels of serum inflammatory factors TNF-α, IL-6 and IL-1β were significantly upregulated in the model group ($p<0.05$). This suggested successful modeling. However, they decreased notably in probiotic group when compared with the model group ($p<0.05$), indicating that probiotics could inhibit inflammatory response in rats. The levels of microbes *Bacteroidetes*, *Streptococcus* and *Clostridiales* were significantly higher in the control group ($p<0.05$). The levels of *Ruminococcaceae*, *Asoccbarobacler*, *Coriobacteriaceae*, and fecal anaerobic coryneform bacteria were remarkably higher in the model group ($p<0.05$). Meanwhile, the levels of *Porphyromonadaceae*, *Barnsiella*, *Actinobacteria*, *Alloscardovia*, *Bifidobacteria* and *Parabacteroides* were remarkably higher in probiotic group ($p<0.05$). The intestinal level of *Bacteroides* was the highest in rats of control group, which decreased significantly in the model group ($p=0.000$). However, the intestinal level of *Bacteroides* in probiotic group was overtly higher than that in the model group ($p=0.000$), whereas was lower than the control group. The intestinal level of *Bifidobacteria* in the model group was significantly lower than that in the control group ($p=0.024$). However, it was evidently higher in the probiotic group than that in both model group and control group ($p=0.000$). The intestinal level of *Asoccbarobacler* was remarkably higher in the model group than that in control group ($p=0.005$). However, it was lower in probiotic group than that in model group ($p=0.003$), showing the highest in model group. There was an evidently negative correlation between *Firmicuteria* and *Clostridium* ($r=-0.82$, $p=0.000$), and a positive association between *Firmicuteria* and *Bacteroides* ($r=0.77$, $p=0.000$). *Bacteroides* was negatively correlated with *Clostridium* ($r=-0.89$, $p=0.002$) and Enterococcus ($r=-0.63$, $p=0.021$). In addition, *Enterococcus* had a highly positive correlation with *Clostridium* ($r=0.6$, $p=0.001$).

CONCLUSIONS: Evident changes in intestinal florae and serum inflammation are detected in rats with RA, and such changes can be partially reversed by probiotics.

Key Words:
Rheumatoid arthritis (RA), Intestinal florae, Probiotics, Serum inflammation.

Introduction

Rheumatoid arthritis (RA) is an autoimmune disease with complex and unknown pathogenesis. It often affects the surrounding joints of patients, whose incidence rate is relatively high in women[1,2]. For RA, inflammation in synovial tissues of the joints is often severe, chronic, and persistent. This may result in symmetrical and long-term arthritis mainly in facet joints[3,4]. In severe cases, articular cartilage and sclerostin are damaged, with severely damaged joint structure. Eventually, joint deformities or even dysfunction may occur. RA is characterized by local and systemic

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inflammatory responses. Meanwhile, the severity of inflammatory response is positively correlated with disease condition. Therefore, monitoring the serum inflammation level of patients is of great significance for judging the development and treatment efficacy of RA[5]. Currently, studies have found that the composition and proportion of intestinal microbes mainly affect the normal physiological process of the digestive system. They have also been proven to play a vital role in the progression of diseases[6,7]. Besides, intestinal microbes can affect both the protective function of the normal intestinal barrier, as well as the digestion and absorption of food[8]. In addition to the digestive system, intestinal microbes are capable of affecting the body's immune level, thereby affecting the development of immune diseases[9]. Probiotics, a beneficial component of intestinal microbes, exert an important regulatory effect on the development of diseases[10].

In this study, therefore, the models of collagen II-induced arthritis were first established in rats. After treatment with probiotics, the changes in the composition of intestinal microbes in rats were determined. Meanwhile, the content of inflammatory factors tumor necrosis factor-alpha (TNF-α), interleukin-6 (IL-6) and IL-1β was detected. Our study aimed to investigate the changes in intestinal florae and serum inflammation in rats with RA, and to explore the effects of probiotics.

Materials and Methods

Grouping and Modeling

A total of 30 adult female rats weighing about 250 g were enrolled in this study. All rats were randomly divided into control group (n=10), model group (n=10), and probiotic group (n=10). The rats in control group received no treatment. In the model group, the rats were modeled *via* collagen-induced arthritis (CIA) method. Specifically, bovine CII was dissolved in acetic acid and added with IFA in drops to be prepared into emulsion. Subsequently, the emulsion was intracutaneously injected into the rats from multiple spots on the back and tail root and unilateral foot pads of rats, to induce inflammation responses. In the probiotic group, the rats were successfully modeled *via* CIA and treated with probiotics (containing *Bifidobacterium*, *Lactobacillus acidophilus*, and *Enterococcus* triple viable, NEMANS) through gavage at 1×10^7 CFU per day. After successful modeling, feces were collected from rats in each group. After the experiment was completed, all rats were sacrificed to collect peripheral blood. This investigation was approved by the Animal Ethics Committee of Jinzhou Medical University Animal Center.

Detection of Intestinal Microbes in Rats

Feces in control group, model group, and probiotic group were first sampled and cryopreserved in a liquid nitrogen tank. Next, they were sent to Shanghai Biotechnology Co., Ltd. (Shanghai, China) for analysis of intestinal microbes. After microbial genomic DNA extraction, amplification, database creation and labeling, high-throughput sequencing was performed using Illumina MiSeq and Ion PGM. Finally, the species and relative abundance of microbes in all rat fecal samples were determined.

Bioinformatics Analysis

The Galaxy website (http://huttenhower.sph.harvard.edu/galaxy/) was adopted for bioinformatics analysis on intestinal florae in control group, model group, and probiotic group. Briefly, after uploading the data on intestinal florae in each group, the LEfSe method was selected for analysis. After data transformation by LEfSe and calculation by LDA, the composition of florae in each group was obtained and visualized.

Measurement of Inflammatory Molecule Levels

The changes in the levels of TNF-α, IL-6, and IL-1β in rats of control group, model group and probiotic group were determined strictly according to the instructions of enzyme-linked immunosorbent assay (ELISA, R&D Systems, Minneapolis, MN, USA). Briefly, peripheral blood of rats was centrifuged at 3000 rpm for 5 min. The upper serum was collected for detection, with 3 replicates in each group. Absorbance at 450 nm was detected by a micro-plate reader (Bio-Rad, Hercules, CA, USA). Finally, the absorbance was converted to the actual concentrations of TNF-α, IL-6 and IL-1β by standard curves. The average sensitivity of the test was <0.42 pg/mL, and the inter-batch coefficient of variation was 5.4%.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 23.0 (IBM Corp., Armonk, NY, USA) was used for statistical processing. The differences between two groups were analyzed by using the Student's *t*-test. Comparison between multiple groups was done using One-way ANOVA

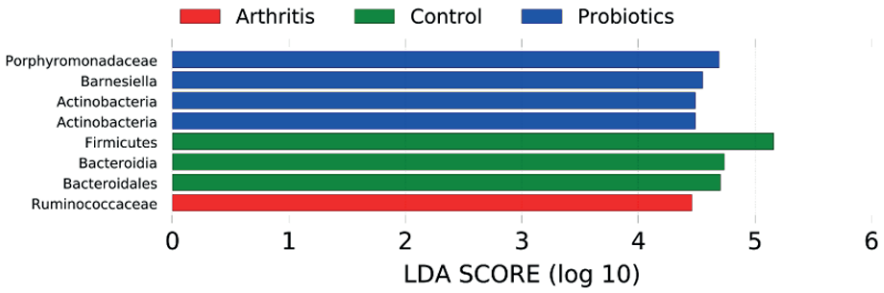

Figure 1. LDA scores of intestinal florae in control group, model group and probiotic group.

test, followed by post-hoc test (Least Significant Difference). Pearson method was selected for correlation analysis. $p<0.05$ was considered statistically significant.

Results

Serum Levels of Inflammatory Factors in Control Group, Model Group and Probiotic Group

As shown in Table I, the levels of serum inflammatory factors TNF-α, IL-6, and IL-1β were significantly higher in the model group than those in the control group ($p<0.05$). This implied successful modeling. However, they were overtly lower in probiotic group than those in the model group ($p<0.05$), suggesting that probiotics could repress inflammatory response in rats.

Composition of Intestinal Florae in Control Group, Model Group and Probiotic Group

The analysis of intestinal florae in control group, model group, and probiotic group (Figures 1 and 2) showed that in the control group, the microbes of *Bacteroidetes*, *Streptococcus* and *Clostridiales* were significantly higher ($p<0.05$). In the model group, *Ruminococcaceae*, *Asoccbarobacler*, *Coriobacteriaceae*, fecal anaerobic coryneform bacteria were significantly higher ($p<0.05$). Meanwhile, probiotic group displayed remarkably

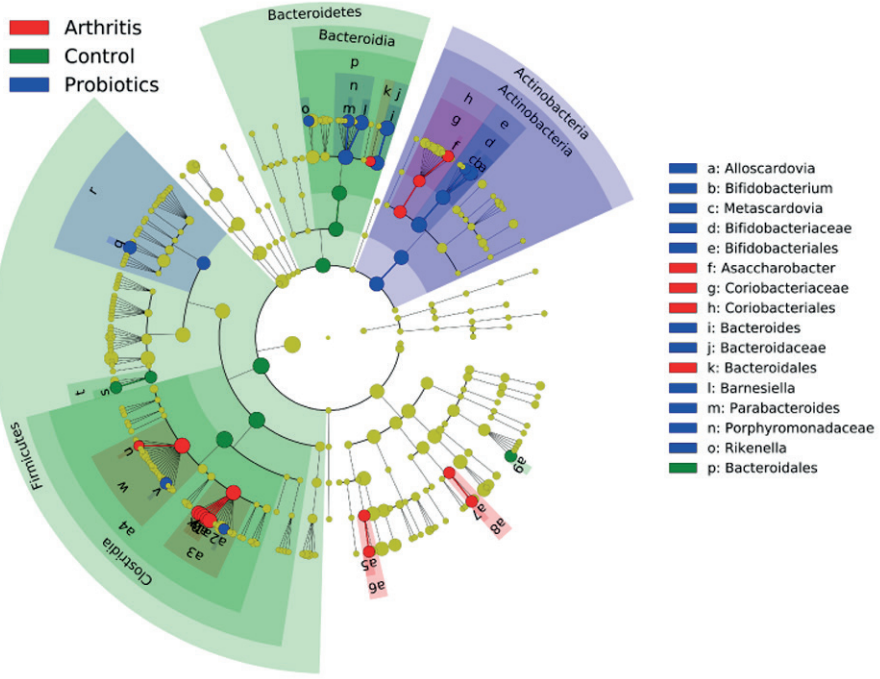

Figure 2. LEfSe analysis of intestinal florae in control group, model group and probiotic group.

Table I. Serum inflammatory factor levels in each group.

	n	IL-6 (ng/L)	IL-1β (ng/L)	TNF-α (ng/L)
Control group	10	7.73±1.23	6.32±0.89	18.24±2.15
Model group	10	25.34±2.24[a]	14.25±2.12[a]	56.14±4.27[a]
Probiotic group	10	11.24±2.12[b]	7.24±1.24[b]	33.64±4.21[b]

Note: [a]$p<0.05$ *vs.* control group, [b]$p<0.05$ *vs.* model group in the *t*-test.

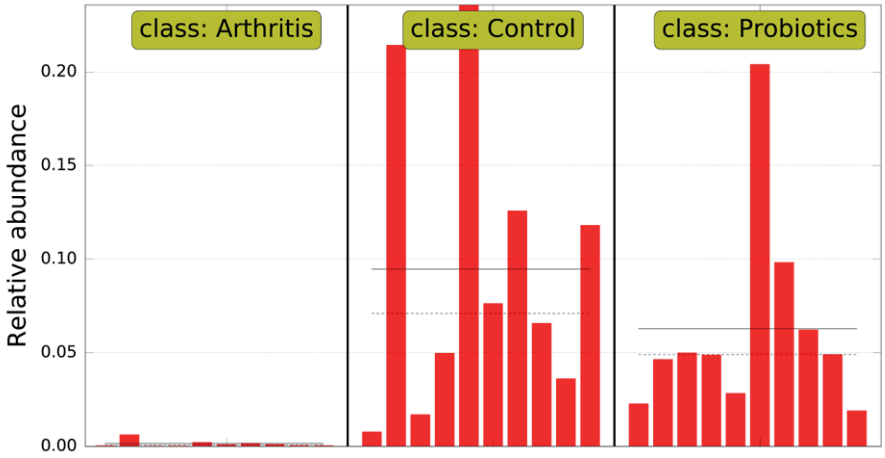

Figure 3. Changes in *Bacteroides* level in control group, model group and probiotic group.

higher levels of *Porphyromonadaceae*, *Barnsiella*, *Actinobacteria*, *Alloscardovia*, *Bifidobacteria*, and *Parabacteroides* ($p<0.05$).

Changes in Bacteroides in Control Group, Model Group and Probiotic Group

The changes in *Bacteroides* level in control group, model group, and probiotic group were shown in Figure 3. Intestinal level of *Bacteroides* was the highest in the control group, which decreased significantly in the model group ($p=0.000$). However, intestinal level of *Bacteroides* in probiotic group was overtly elevated when compared with model group ($p=0.000$), which was still lower than the control group.

Changes in Bifidobacteria in Control Group, Model Group and Probiotic Group

Intestinal level of *Bifidobacteria* was relatively low in control group, which was the lowest in model group ($p=0.024$) and the highest in probiotic group ($p=0.000$) (Figure 4).

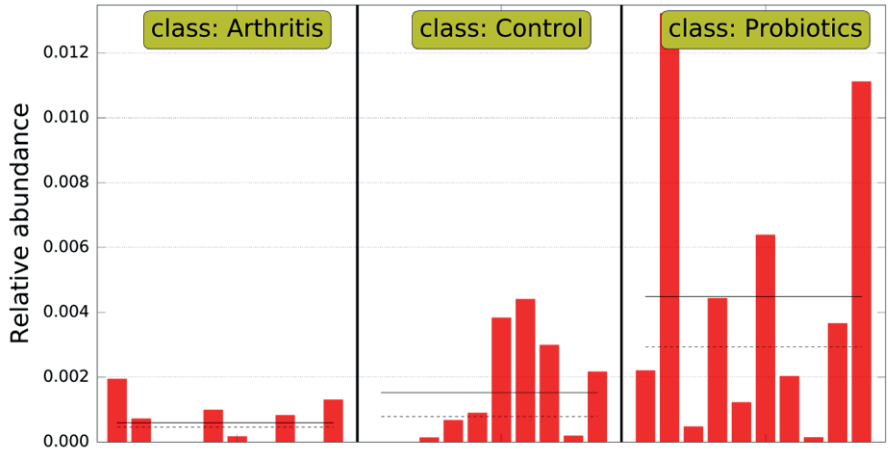

Figure 4. Changes in *Bifidobacterium* level in control group, model group and probiotic group.

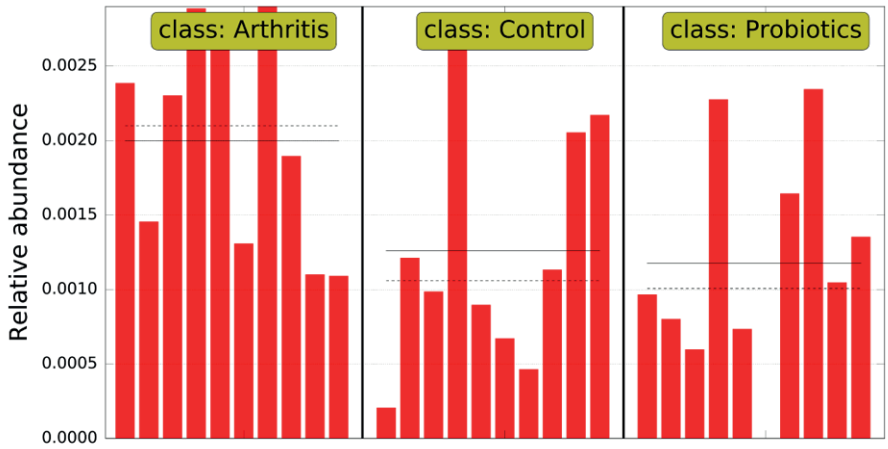

Figure 5. Changes in *Asoccbarobacler* level in control group, model group and probiotic group.

Changes in Asoccbarobacler in Control Group, Model Group and Probiotic Group

Intestinal level of *Asoccbarobacler* was low in control group, which was significantly higher in model group than that in control group (p=0.005). However, it was significantly lower in the probiotic group than that in the model group (p=0.003) (Figure 5).

Correlation Analysis of Intestinal Florae in Control Group, Model Group and Probiotic Group

Firmicuteria showed a clearly negative correlation with *Clostridium* (r=-0.82, p=0.000) and a significantly positive association with *Bacteroides* (r=0.77, p=0.000). *Bacteroides* were negatively associated with *Clostridium* (r=-0.89, p=0.002) and *Enterococcus* (r=-0.63, p=0.021). However, *Enterococcus* was highly positively correlated with *Clostridium* (r=0.6, p=0.001) (Figure 6).

Discussion

RA is a disease characterized by systemic immune response. It mainly leads to varying degrees of damage to human joints[11-13]. RA can even affect the severity of systemic inflammation, involving the cardiovascular system and the respiratory system (such as inducing inflammatory diseases of arteriolitis, interstitial lung disease, and pleurisy). The incidence of RA is relatively high in middle-aged women. Currently, the exact pathogenesis of RA is unknown. Multiple studies have found that RA may be attributed to heredity (HLA-DR), infection and trauma[14]. The severity of inflammation in the body is one of the main factors affecting the progression of RA. As a result, RA is mainly treated by controlling inflammatory response in patients (for instance, the application of glucocorticoids and non-steroidal anti-inflammatory drugs)[15]. Therefore, discovering strategies that can relieve inflammatory response of RA is of great importance for promoting the development and clinical application of therapeutic drugs for the disease.

Intestinal microbes is a flora system colonizing in intestinal tissues of the body. It consists of many microorganisms mainly distributed at the end of the colon, including *Ruminococcaceae*,

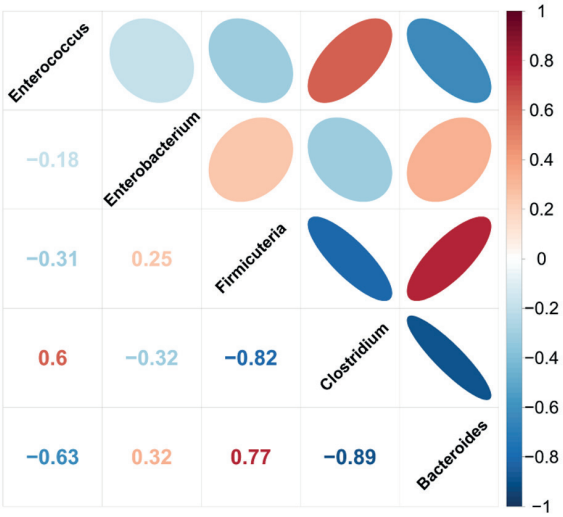

Figure 6. Correlation analysis of intestinal florae in control group, model group and probiotic group.

Enterococci, and *Lactobacilli*[16]. They play a crucial role in maintaining the normal physiological barrier and metabolic level of human intestinal tract. Meanwhile, the changes in the composition and abundance of florae may lead to or affect the development of various diseases[17]. Currently, it has been detected that such diseases as primary liver cancer[18] and diabetes mellitus[19] are associated with the changes in the composition of intestinal florae. Intestinal microbes can affect the local microenvironment of the digestive system. They may also change the level of systemic immunity and inflammation, eventually affecting the progression of autoimmune diseases. The alteration of intestinal florae may increase the permeability of intestinal wall. This can also allow harmful substances to enter the circulatory system through the intestinal cavity, thereby facilitating the development of diseases. As beneficial components in the intestinal tract, probiotics play a vital role in regulating the composition of florae and the stability of internal environment[20]. In this study, experimental rats were prepared into models of collagen II-induced arthritis (model group) and treated with probiotics for 6 weeks (probiotic group). The results revealed that serum level of inflammatory factors TNF-α, IL-6a, and IL-1β increased significantly in the model group when compared with those in the control group ($p<0.05$). This indicated successful modeling in rats. However, they markedly declined in probiotic group compared with those in the model group ($p<0.05$), implying that probiotics could suppress the inflammatory response in rats. These results demonstrated the effectiveness of CII in constructing a rat model of collagen-induced arthritis. Our findings also manifested that probiotics might be of great significance in the treatment of RA. The specific mechanisms were further explored in subsequent experiments in this study.

Based on the detection results of the changes of intestinal florae in control group, model group and probiotic group, the possible specific mechanism of probiotics in the treatment of RA was discovered. The analysis of intestinal florae denoted that the control group exhibited higher levels of *Bacteroidetes*, *Streptococcus* and *Clostridiales*. Model group had higher levels of *Ruminococcaceae*, *Asoccbarobacler*, *Coriobacteriaceae*, and fecal anaerobic coryneform bacteria. Meanwhile, probiotic group displayed higher levels of *Porphyromonadaceae*, *Barnsiella*, *Actinobacteria*, *Alloscardovia*, *Bifidobacteria* and *Parabacteroides*. Intestinal level of *Bacteroides* was the highest in the control group, which decreased significantly in the model group ($p=0.000$). However, intestinal level of *Bacteroides* in probiotic group was overtly elevated when compared with the model group ($p=0.000$), which was still lower than control group. Intestinal level of *Bifidobacteria* was low in control group, showing the lowest in model group ($p=0.024$) and the highest in probiotic group ($p=0.000$), respectively. Intestinal level of *Asoccbarobacler* was low in control group, which was significantly higher in the model group than that in the control group ($p=0.005$). However, it was significantly lower in probiotic group than that in the model group ($p=0.003$). The above results suggested that RA might cause great changes in the composition of intestinal florae. Meanwhile, these changes could be partially restored after treatment with probiotics, thereby increasing the content of beneficial components in intestinal florae (such as *Barnsiella*, *Alloscardovia*, and *Bifidobacteria*). This might be the mechanism by which probiotics improved RA development.

Further correlation analysis of intestinal microbes revealed that *Firmicuteria* showed a significantly negative correlation with *Clostridium* ($r=-0.82$, $p=0.000$) and a notably positive association with *Bacteroides* ($r=0.77$, $p=0.000$). *Bacteroides* was negatively associated with *Clostridium* ($r=-0.89$, $p=0.002$) and *Enterococcus* ($r=-0.63$, $p=0.021$). However, *Enterococcus* was highly positively correlated with *Clostridium* ($r=0.6$, $p=0.001$).

Conclusions

The novelty of this study was that in the case of RA, the composition and regulation of intestinal microbes form a complex network. Meanwhile, microbes may have a close correlation with each other and jointly regulate the progression of RA. The specific mechanism will be further studied in future researches, since it has great value in clinic.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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