Brief Report

DOI: 10.5582/bst.2024.01037

Vitamin C alleviates rheumatoid arthritis by modulating gut microbiota balance

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SUMMARY Rheumatoid arthritis (RA) is a systemic autoimmune disease characterized by chronic and symmetric in-flammation. Our previous research revealed an imbalance in the gut flora of RA patients and showed that certain gut microbiota can accelerate RA progression by enhancing vitamin C degradation. However, it is unclear whether vitamin C supplementation could improve the gut microbiota to prevent the development of arthritis by interfering with the gut-joint axis. In this work, we aimed to evaluate the effects of vitamin C in regulating the gut microbiota and to elucidate its potential role in the onset and progression of RA in a mouse model, thus providing a basis for the development of new intervention strategies and treatments for RA. In this study, collagen-induced arthritis (CIA) mouse models, biochemical, histological and 16S rRNA microbiological methods were used to investigate the role and possible mechanism of vitamin C in rheumatoid arthritis. The results showed that treatment of CIA mice with vitamin C effectively rescued the gut mi-crobiota imbalance and suppressed the inflammatory response associated with RA, and effectively alleviated arthritis symptoms in mice in which levels of the pro-inflammatory cytokines IL-6 and TNF-α were specifi-cally reduced. In conclusion, our results demonstrate the potential of vitamin C as a potential therapeutic choice for RA.

Keywords rheumatoid arthritis, vitamin C, gut microbiota, intestinal dysbiosis

1. Introduction

Rheumatoid arthritis (RA) is a chronic autoimmune disease that affects 0.5% to 1% of the population, making it the second leading cause of disability in China (*1*). It is characterized by symmetric arthritis and progressive destruction of the synovium and articular cartilage (*2*). While the primary treatments for RA, including glucocorticoids (GCs), disease-modifying antirheumatic drugs (DMARDs) and biologics, can provide some relief of symptoms, they also have several drawbacks, including limited distribution in inflamed joints and potential toxic side effects (*3*).

Recent research has shown a link between gut microbiota and several extra-intestinal tissue diseases, including RA (*4*) . The gut microbiota plays an important role in maintaining immune homeostasis in the body and has been implicated in the pathogenesis and progression of RA (*4,5*) . Given the critical role of the gut microbiota in the development and progression of RA, there is a need to further investigate innovative treatment strategies that address and promote a healthy gut microbiota balance (*6*) . Such approaches may lead to more effective and less toxic treatments for this debilitating disease.

In a previous study, we found that the degradation of vitamin C by the gut microbiota in RA patients was positively correlated with the pro-inflammatory cytokines TNF-α and IL-6. This suggests that the gut microbiota may contribute to the progression of RA by promoting vitamin C degradation (*7*). However, there is currently no evidence to support the use of vitamin C supplementation to restore gut microbiota balance and prevent RA exacerbation *via* the gut-joint axis.

In this study, we supplemented vitamin C in a CIA (collagen-induced arthritis) mouse model to evaluate its

regulatory effect on gut microbiota and RA development. Our findings suggest that vitamin C supplementation may provide a non-toxic and potentially side-effect-free intervention strategy for RA.

2. Materials and Methods

2.1. Establishment of a collagen-induced arthritis (CIA) mouse model

Seven-week-old male DBA/1 mice, weighing 20 g, were housed in an ultra-clean animal laboratory (SPF grade) with a humidity of 55% and a temperature of 26°C. The animal study protocol was approved by the Institutional Animal Care and Use Committee of the Institutional Review Board of the Shandong Research Center for Medicinal Biotechnology (2020S307). Mice were maintained according to institutional guidelines.

CIA models were established as described previously (*8*). Mice were immunized with collagen II and randomly divided into three groups: (*i*) the CIA-VC group, consisting of mice successfully induced with CIA and treated daily with 100 mg/kg vitamin C for 6 weeks (n = 6); (*ii*) the CIA group, consisting of mice induced with CIA (n = 6); and (*iii*) the control group (n = 6). Both the CIA and control groups received 0.9% normal saline.

2.2. Assessment of arthritis severity in CIA

To continuously monitor the progression of arthritis in the animals, the severity of arthritis was scored after the first immunization using an established grading system as described previously (*9*). The arthritis score for each mouse was determined by summing the scores of all four paws, and the average score for each treatment group was calculated. Scoring of arthritis severity in each category was performed independently by two observers to ensure objectivity.

2.3. Micro-computed tomography (micro-CT)

Micro-computed tomography (Micro-CT) (Quantum GX, Perkin Elmer, USA) was used to scan and reconstruct the three-dimensional structure of the hind paw joint. The settings were 209m, 90kV X-ray tube volt-age, 160uA current and 3 minutes scan time. The angle of the X-ray scan was rotated 180 degrees. The resolution is 2μm and the field of view is 12.8 mm × 12.8 mm.

2.4. Hematoxylin and eosin (HE) staining

H&E stained sections were used to evaluate the degree of cartilage degeneration and synovial invasion.Tissue samples were fixed in 4% paraformaldehyde solution for 24 hours. After fixation, the tissues were embedded in paraffin and cut into 5μm slices. The slices were then incubated at 65°C for 4 hours and then dehydrated through a gradient of ethanol. The sections were then stained with haematoxylin for 5 minutes. After a short differentiation step in 1% hydrochloric acid-alcohol for 2 seconds, the sections were incubated in ammonia water for 2 minutes and stained with eosin for 1 minute. Finally, the sections were dehydrated, cleared and mounted in neutral resin. The sections were examined by light microscopy (Olym-pus Corporation, Tokyo, Japan).

2.5. Enzyme-linked immunosorbent assay (ELISA)

Mouse plasma was collected and centrifuged (3,000 g, 15 min) for serum collection. The levels of in-flammation were determined by ELISA (mlbio, Shanghai, China), total necrosis factor-α (TNF-α) and inter-leukin-6 (IL-6), strictly following the reagent manufacturer's protocols. Absorbance was measured at 450 nm.

2.6. Faecal sample collection and DNA extraction

Faecal samples were collected at the end of the experiment and stored at -80°C for extraction of total faecal DNA (Bacterial DNA Kit, TIANGEN), with the tube containing PBS as an environmental control. Only samples with sufficient bacteria had sufficient bacterial DNA content (≥ 10 ng) for high-throughput sequencing of the bacterial 16S rRNA gene.

2.7. 16S rRNA gene sequencing and analysis

The V1-V2 regions of the 16S rRNA gene were sequenced on the Illumina Hiseq 2500 (Illumina Inc., San Diego, CA, USA). Using split_libraries.py in QIIME (v.1.9.1) to trim raw reads for adapter and primer sequences, paired-end sequences were then joined using FLASH with default parameters. In addition, chimeric sequences were identified and removed using de novo chimera detection in USEARCH (v.6.1). Finally, the resulting sequences were clustered into operational taxonomic units (OTUs) for subsequent analysis. The UCLUST algorithm was used to analyze and determine the community composition of each group at multiple levels of classification, including phylum, family and genus. Rarefaction analysis based on Mothur (v.1.21.1) was used to determine diversity indices, including Chao 1, ACE and Shannon diversity indices. All statistical analyses were performed using the R stats package. Linear discriminant analysis effect size (LEfSe) analysis was performed to identify biomarkers of high-dimensional gut bacteria. Species with significant differences in abundance between different groups were detected using non-parametric Kruskal-Wallis rank sum, consistency of differences was tested using Wilcoxon rank sum, followed by linear discriminant analysis (LDA) was used to estimate the effect size of each distinctly abundant taxa.

2.8. Statistical analysis

GraphPad Prism software version 9.0 and R software version 4.1.3 were used for statistical analysis and presentation. Pearson correlations and Mantel tests were analyzed using the packages 'linkET 0.0.7.4' and 'ggplot2 3.3.3'. A significance level of $p < 0.05$ was considered statistically significant. Data are presented as mean ± standard error of the mean (SEM) or mean ± standard deviation (SD). Statistical significance be-tween groups was assessed using unpaired t-tests and one-way analysis of variance (ANOVA) for the indicators. Corrected p-values were used to adjust for multiple testing.

3. Results and Discussion

3.1. Vitamin C balances the immune response and reduces arthritis progression in CIA mice

Clinical scores were measured every four days during the study. The CIA mice developed severe arthritis with paw thickness increasing continuously until day 12 after booster immunisation, and the clinical scores increased rapidly in the late period (Figure 1A and 1B). The late-stage progression of altered arthritic symp-toms in mice treated with vitamin C was similar to that observed in CIA mice, albeit with significantly less severe arthritis compared to CIA mice (see Figure 1A and 1B). In addition, serum pro-inflammatory cyto-kines were essential indices to assess arthritis activities, and the relative levels of serum pro-inflammatory cytokines were measured by ELISA (Figure 1C). The concentrations of serum TNF-α and IL-6 in the CIA group were significantly higher than those in the control group, while oral administration of vitamin C effec-tively restored the levels of TNF-α and IL-6 in early immunization compared with CIA ($p < 0.01$) (Figure 1C). Overall, oral administration of vitamin C during early immunization rebalances the immune response and partially rescues the arthritis phenotype.

3.2. Vitamin C attenuated cartilage destruction and inflammation in joint tissue

Imaging studies revealed a higher degree of cartilage damage and bone erosion in the interphalangeal joint of the paws of CIA mice, whereas VC-treated CIA mice showed reduced levels of bone erosion and destruction as shown by micro-CT images (Figure 2A). Therefore, these findings suggest that early administration of vitamin C is effective in delaying disease onset and preventing joint destruction.

In addition, we used histological H&E staining to assess the effect of vitamin C on joint pathology and cartilage destruction. Significant inflammatory cell infiltration and cartilage destruction, granulation tissue prolif-eration, lymphocyte infiltration and chronic inflammation were observed in all CIA mouse groups, whereas the vitamin C group showed few of these symptoms and had significantly lower histological and cartilage destruction than the CIA group (Figure 2B). Taken together, these results suggest that early administration of vitamin C is effective in delaying disease onset and preventing joint destruction. In conclusion, the above re-sults demonstrate that vitamin C supplementation is effective in ameliorating the symptoms and phenotype of RA in CIA mice.

3.3. Vitamin C altered gut microbial diversity and composition in CIA mice

To gain insight into the effect of vitamin C on arthritis-induced dysbiosis, faeces were analysed to compare their microbial composition. Alpha diversity was assessed

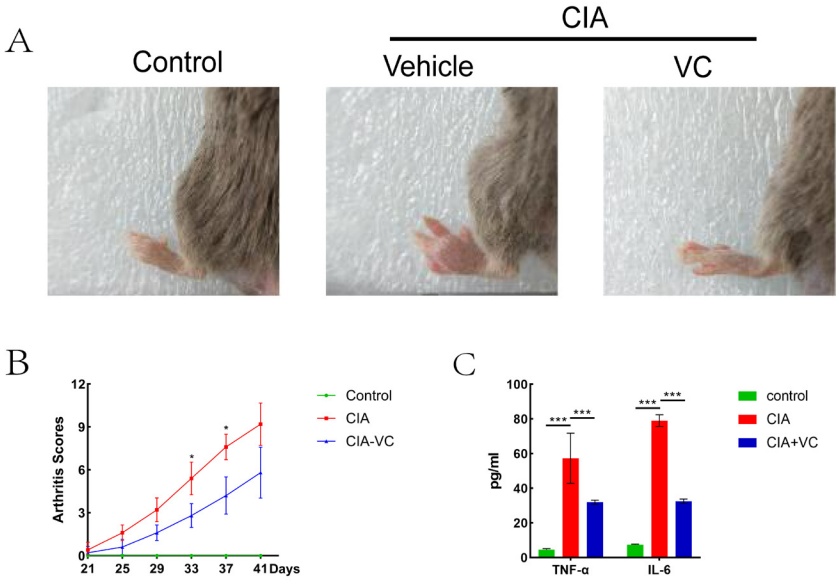

Figure 1. Vitamin C supplementation attenuated the progression of rheumatoid arthritis in CIA. (A) Representative images of paws in each group prior to sacrifice. **(B)** Arthritis scores of CIA were monitored every four days. **(C)** The levels of pro-inflammatory factors, IL-6 and TNF-α, in the serum of mice were de-termined by ELISA. Values are expressed as mean ± SD. One-way ANOVA between groups. (ns: not sig-nificant, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

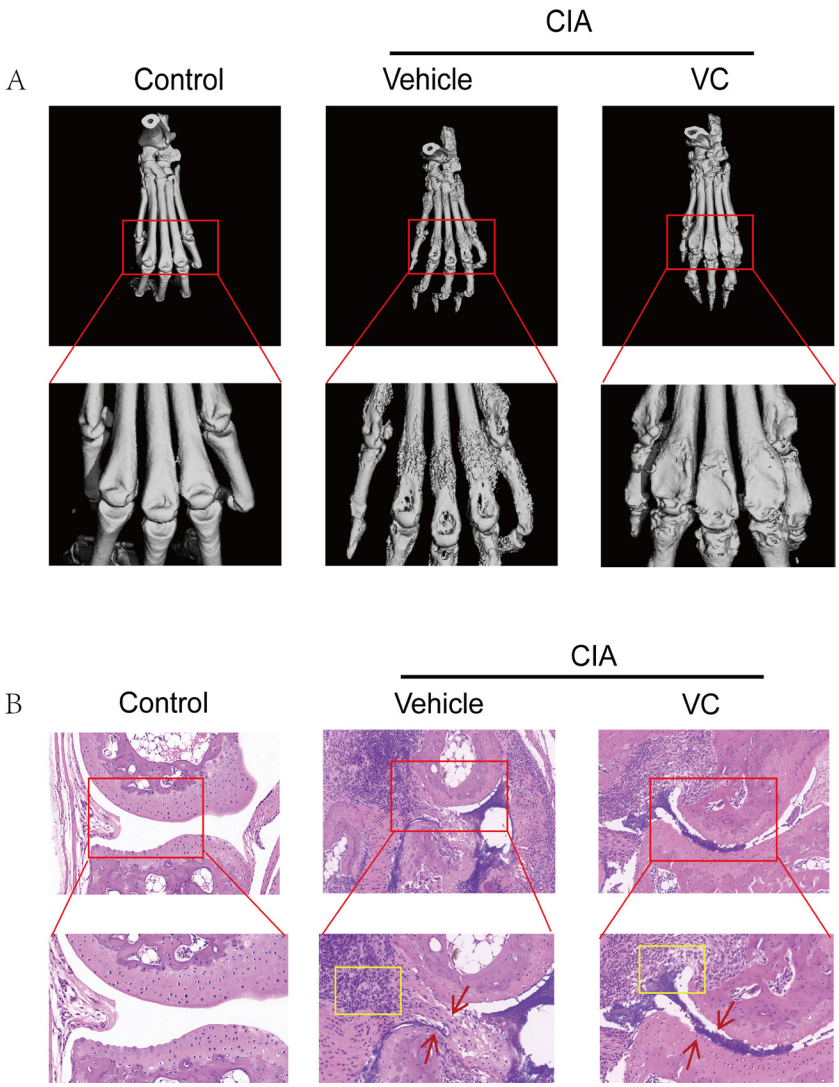

Figure 2. Vitamin C supplementation ameliorates joint pathology and cartilage destruction of rheuma-toid arthritis in CIA. (A) Representative micro-CT images of hind paws and interphalangeal joints. **(B)** Rep-resentative histological images of H&E stained interphalangeal joint showing pathological changes including synovial proliferation and joint destruction.

using Shannon, Richness, ACE and Chao indices. Compared to control mice, the diversity indices of ACE and Chao were significantly decreased in CIA mice, and the other two alpha diversity indices (Richness and Shannon) were also significantly altered. Compared to the model group, Chao1 and ACE indices were significantly higher in the early vitamin C intervention group (CIA-VC group), and Richness and Shannon also increased (Figure 3A). Vitamin C treatment was able to modestly restore gut microbial diversity in CIA mice to near normal levels, thereby reducing gut damage.

Among the dominant phyla, Firmicutes and Proteobacteria were slightly downregulated in the CIA group, while Bacteroidetes were enriched (Figure 3B). Compared to the CIA group, the proportion of Bacteroidetes and Tenericutes was reduced in the vitamin C treatment group, while that of Firmicutes was significantly increased (Figure 3B).

At the family level, Bacteroidaceae, Muribaculaceae and Lachnospiraceae were dominant in both control and CIA model groups, Lachnospiraceae and Bacteroidaceae were decreased, Muribaculaceae and Helicobacteraceae were increased in CIA mice, and the relative abundance of Bacteroidaceae and Muribaculaceae was decreased in the vitamin C treatment group, whereas Lachnospiraceae and Helicobacteraceae were upregulated (Figure 3C). At the genus level, Bacteroides and Odoribacter were significantly more abundant in CIA mice than in control mice. After vitamin C intervention, Bifidobacterium, Kineothrix and Helicobacter in-creased while Odoribacter and Phocaeicola decreased (Figure 3D).

Consistent with the changes in diversity at both phylum and family level, the microbial composition changed after CII or vitamin C intervention. The domain phyla were Bacteroidetes and Firmicutes, followed by Pro-teobacteria and Tenericutes in all groups (Figure 3E). LEfSe was used to identify the major bacterial markers responsible for the observed dissimilarity between groups. The LDA bars of taxa with differential abundance after conventionalisation with the microbiome showed that there were five, five and fourteen dominant bacterial biomarkers in the control, CIA and CIA-VC treatment groups, respectively. Among the identified markers, the relative abundance of a well-known probiotic Lactobacillales was significantly enriched in the control

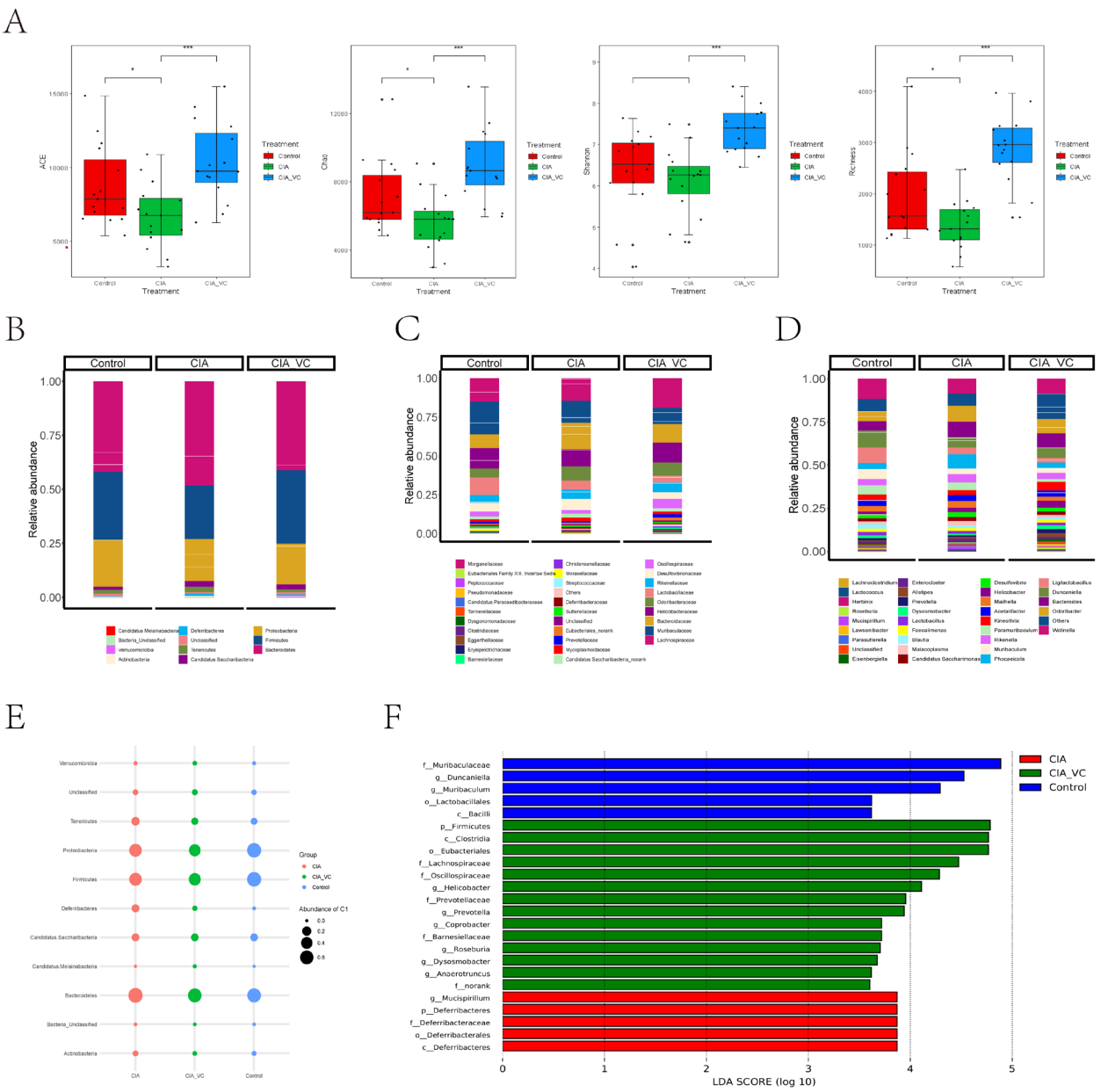

Figure 3. Effects of vitamin C in gut microbiota regulation on diversity and abundance. **(A)** Alpha di-versity analysis of faecal microbiota OTU at different taxonomic ranks including ACE, Chao, Shannon and Richness indices. The gut microbial composition profiles. **(B)** The relative abundance and bacterial taxonomic analysis of major differentiated gut taxa at the phylum level. **(C)** The relative abundance and bacterial taxo-nomic analysis of key differentiated gut taxa at the family level. **(D)** The relative abundance and bacterial taxonomic analysis of key differentiated gut taxa at the family level. **(E)** The bubble plot shows domain phy-la in all groups. **(F)** Histogram of linear discriminant analysis (LDA) showing significant differences in gut microbiota abundance between groups, LDA score > 3.5 and $p < 0.05$.

group (Figure 3F). Meanwhile, among the markers affected by vitamin C intervention, Lachnospiraceae, Barnesiellaceae, Oscillospiraceae and Prevotel-laceae were dominant families considered as candidates for next-generation probiotics (NGPs), whereas Mucispirillum and Deferribacteraceae were more abundant in CIA mice. These changes indicated that the gut microbiota in CIA mice tended to restore the normal flora balance after vitamin C administration.

3.4. Correlation between pro-inflammatory cytokines and microbial communities in CIA mice

To verify the correlation between pro-inflammatory cytokines and microbial communities, and to identify potential co-abundance and co-exclusion interactions in microbial communities, we correlated taxonomic community composition with that of inflammatory factors at the family level. Manteltest correlation showed that Muribaculaceae was the strongest correlate of both IL-6 and TNF-α, being positively associated with IL-6 and TNF-α ($p < 0.05$). Lachnospiraceae was also positively associated with IL-6 ($p < 0.05$), while no statistically significant correlation was found for TNF-α. Furthermore, Pearson's r showed that there was

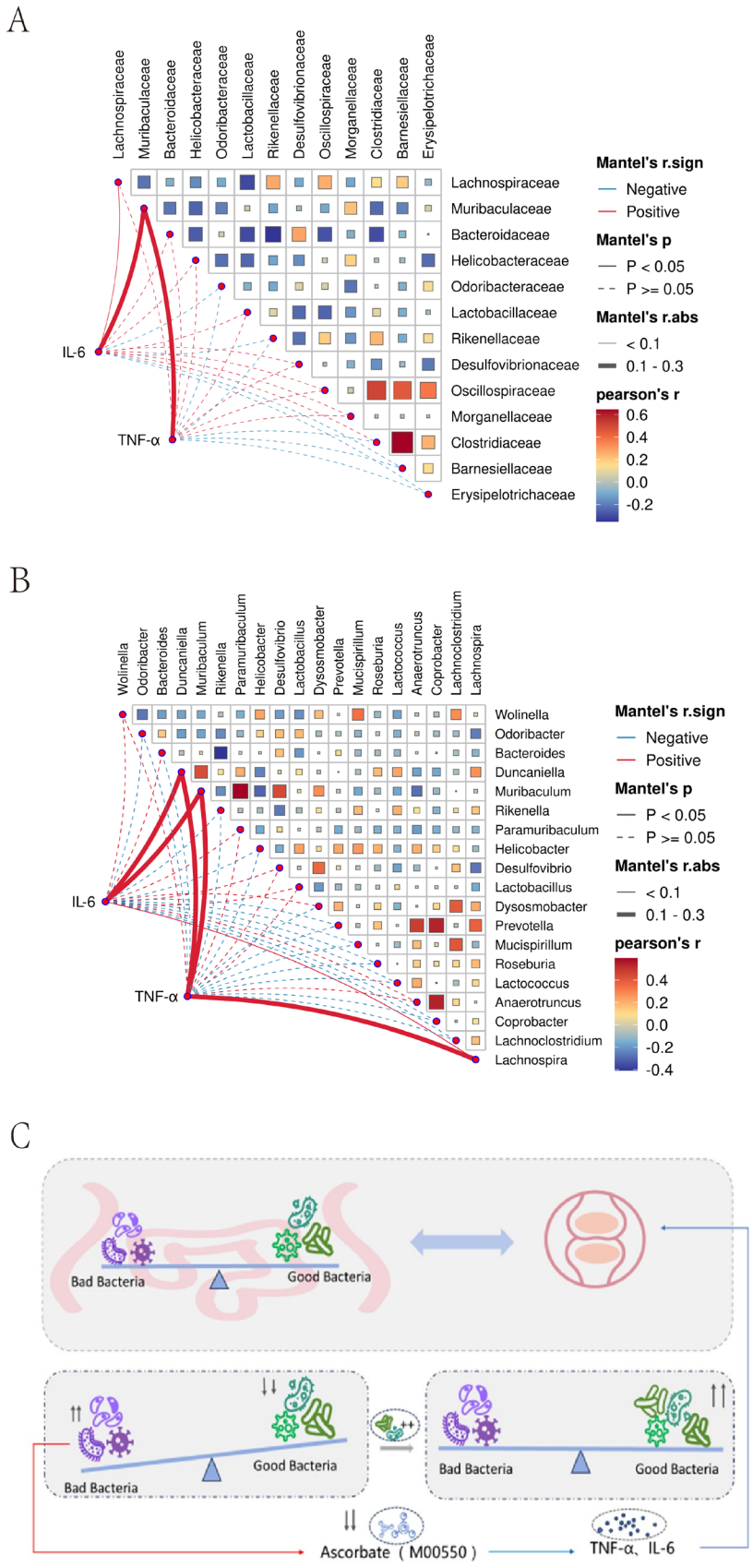

Figure 4. The correlation analysis between gut microbiota composition and pro-inflammatory factors. (A) The correlation analysis between gut microbiota composition at the family level and pro-inflammatory factors, and **(B)** the correlation analysis between gut microbiota composition at the genus level and pro-inflammatory factors.The community composition was related to each pro-inflammatory factor by partial Mantel tests. Edge width corresponds to Mantel's r statistic for the corresponding distance correlations, red is a positive correlation and blue is a negative correlation. Data are presented as mean ± SEM, $p < 0.05$ was considered significant. Pairwise comparisons of microbial components are shown, with a colour gradient in-dicating Spearman's correlation coefficients. **(C)** Graphical summary of this study. Based on the gut-joint axis interactions, ascorbic acid alleviated RA symptoms by regulating and balancing the gut microbiota.

a strong positive correlation between Clostridiaceae and the relative content of Barnesiellaceae, Oscillospiraceae was positively associated with the relative abundance of Clostridiaceae, Barnesiellaceae and Erysipelotrichaceae, while Bacteroidaceae was negatively correlated with Clostridiaceae, Oscillospiraceae, Rikenellaceae and Lactobacillaceae (Figure 4A).

At the genus level, both Muribaculum and

Duncaniella were strongly correlated with IL-6 and TNF-α, there are positive correlations between Muribaculum and Duncaniella with IL-6 and TNF-α ($p < 0.05$), as well as Lachnospira was positively associated with IL-6 and TNF-α, while IL-6 was only weakly correlated, except for Muribaculum, Lachnospira and Duncaniella, the correlations were not statistically significant with other genera. In addition, there was a strong negative correlation between Rikenella and the relative abundance of Bacteroides, Coprobacter was positively correlated with Prevotella and Anaerotruncus, Muribaculum was positively associated with the relative abundance of Paramuribaculum (Figure 4B).

Our previous study showed that the gut microbiota may promote RA progression by increasing the break-down of ascorbate (vitamin C) and may provide a potential approach to prevent the development of arthritis by interfering with the gut-joint axis (*7*). The aim of this study was to determine whether vitamin C supplementation could reverse these pathological processes, which remains to be investigated. In this study, we demonstrated the regulatory effects of oral vitamin C supplementation on the structure of the gut flora in CIA mice. We preliminarily confirmed that vitamin C effectively suppressed various RA phenotypes in the CIA model, as evidenced by the reduction of joint swelling, articular cartilage and bone destruction, and inflammatory cell infiltration. These data provide further evidence for the therapeutic potential of vitamin C in the treatment of RA.

Vitamin C, also known as ascorbic acid, is one of the most effective antioxidants (*10*). Previous studies have evaluated the effects of vitamin C as an antioxidant supplement intervention on the levels of plasma inflammatory molecules and disease severity in RA patients (*11*). This study has shown the noteworthy effect of vitamin C on the gut microbiome, particularly in terms of microbial alpha diversity. Other data also confirmed the effect of high-dose vitamin C to improve both alpha and beta diversity of the bacterial community, including an increase in Collinsella, which is a producer of SCFAs such as butyrate and propionate (*12-14*). This suggests that vitamin C may be a potential modulator of the gut microbial community (*13*). The results in the mice model reliably validated the regulatory effect of vitamin C on the balance of gut microbiota. Lachnospiraceae which exhibited high abundance in CIA mice with vitamin C supplementation, was also specifically enriched in the intestinal tract of CIA mice after vitamin C intervention. Lachnospiraceae, Oscil-lospiraceae, Morganellaceae, Barnesiellaceae, and Clostridiaceae were enriched in CIA-VC compared to CIA. Among these families, Muribaculaceae and Lachnospiraceae were revealed to enhance gut microbiota metabolite production, such as SCFAs and polyamines (15). The Lachnospiraceae family, consisting of Lachnospira, Fusicatenibacter, Roseburia, and Lachnoclostridium, is able to produce SCFAs such as acetic acid, which play important roles in kidney protection, including anti-inflammatory, anti-atherosclerosis, and anti-oxidation effects (*16,17*). Other studies have also confirmed the anti-inflammatory properties of SCFAs (including valerate, butyrate, propionate, and acetate), produced by the gut microbiota (*18,19*). Most of these microbes listed above, which are positively associated with vitamin C, are considered as candidate probiotics for producing SCFAs (*20*). SCFAs are revolved in the body's redox process, regulating intestinal balance and improving intestinal function (*20-22*). Together, these microbes may serve as a mediator for vit-amin C to exert its anti-inflammatory effects on RA pathology. In this study, vitamin C intervention was found to effectively inhibit various RA phenotypes in the CIA model, as evidenced by reductions in joint swelling, articular cartilage and bone destruction, and infiltration of inflammatory cells. Importantly, vitamin C could effectively ameliorate the inflammatory phenotype in CIA model mice, downregulating IL-6 and TNF-α, suggesting the previous findings that vitamin C regulates immune response and further showing that inflammatory cytokines are decreased in mice gavaged with vitamin C (*23,24*).

In view of the above results, vitamin C intervention alleviates RA symptoms and also improves the composition of the gut microbiota. It is reasonable to conclude that vitamin C increases the abundance of potential probiotics in the gut associated with RA, such as Muribaculaceae and Lachnospiraceae, regulate the composition of the gut flora in patients, which could achieve the goal of reducing disease-related inflammatory factors, alleviating symptoms and slowing disease progression (Figure 4C). These findings support the prevailing idea that vitamin C can be used as an adjuvant therapy for RA.

Notably, these results were obtained in an experimental mouse model of arthritis, therefore, in-depth studies in RA patients are urgently needed to verify our findings and to assess whether this approach would dramatically improve the prognosis of patients. To elucidate their potential role in the onset and progression of RA, the precise molecular mechanisms underlying the preventive and therapeutic effects of vitamin C and the new probiotics, such as the Muribaculaceae identified in the gut, deserve further experimentation. This will facilitate the development of a novel adjunctive treatment option for RA.

Funding: This research was supported by Natural Science Foundation of China (No. 82003766), Taishan Scholars Pro-gram (NO. tsqn202211219), the Key Research and Development project of Shandong Province (No. 2021ZDSYS27) and Jinan Clinical Medical Science and Technology Innovation Plan (No. 202019037).

Conflict of Interest: The authors have no conflicts of interest to disclose.

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Received February 26, 2024; Revised April 1, 2024; Accepted April 7, 2024.

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Released online in J-STAGE as advance publication April 10, 2024.