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Extracts of Drynariae Rhizoma Promote Bone Formation in OVX Rats through Modulating the Gut Microbiota

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Abstract:
Drynariae Rhizoma (DR) has been commonly used as a preventive and therapeutic agent for bone diseases. However, its pharmacological mechanisms have not been fully elucidated. Here, we aimed to investigate the effects of DR in a bilateral ovariectomized (OVX) rat model and explore the correlation with the gut microbiome. We established an OVX rat model and treated with different doses of DR (DR-L, 0.27 g/kg/day; DR-M, 0.81 g/kg/day; DR-H, 2.43 g/kg/day) through intragastric administration for 12 weeks. Results showed that DR alleviated body weight, moderated bone microstructure, and promoted the expression of bone formation related factors in OVX rats, in which DR-H behaved the most significant effects among the three doses. Furthermore, the effects of DR on promoting bone formation were correlated to the changes in microbial richness and the restorations of several genera, among which Ruminiclostridium and Ruminococcaceae_UCG_007 were positively correlated with the bone formation related factors, and both of them enriched in DR-H group as biomarker. Moreover, CMP-legionaminate biosynthesis I might be a crucial pathway of DR regulates gut microbiota, and the content of serum short-chain fatty acids in OVX rats were regulated by DR. Our results demonstrated that DR promoted bone formation in OVX rats, and it was related to the regulation of the gut microbiota structure.

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Abstract

Drynariae Rhizoma (DR) has been commonly used as a preventive and therapeutic agent for bone diseases. However, its pharmacological mechanisms have not been fully elucidated. Here, we aimed to investigate the effects of DR in a bilateral ovariectomized (OVX) rat model and explore the correlation with the gut microbiome. We established an OVX rat model and treated with different doses of DR (DR-L, 0.27 g/kg/day; DR-M, 0.81 g/kg/day; DR-H, 2.43 g/kg/day) through intragastric administration for 12 weeks. Results showed that DR alleviated body weight, moderated bone microstructure, and promoted the expression of bone formation related factors in OVX rats, in which DR-H behaved the most significant effects among the three doses. Furthermore, the effects of DR on promoting bone formation were correlated to the changes in microbial richness and the restorations of several genera, among which *Ruminiclostridium* and *Ruminococcaceae_UCG_007* were positively correlated with the bone formation related factors, and both of them enriched in DR-H group as biomarker. Moreover, CMP-legionaminate biosynthesis I might be a crucial pathway of DR regulates gut microbiota, and the content of serum short-chain fatty acids in OVX rats were regulated by DR. Our results demonstrated that DR promoted bone formation in OVX rats, and it was related to the regulation of the gut microbiota structure.

Keywords: osteoporosis; *Drynaria fortune*; Polypodiaceae; extracts of Drynariae Rhizoma; ovariectomied rats; bone formation; gut microbiota

Introduction

Osteoporosis (OP) is a widespread systemic bone disease that is characterized by a reduction in bone mass, damage to the microarchitecture of bone tissue, increased fragility of bones, and frequent fractures. It is estimated that the global prevalence of OP and osteopenia is 19.7% and 40.4% [1], respectively, with one in two postmenopausal women likely to experience an osteoporotic fracture during their lifetime [2]. Abnormal bone metabolism is the underlying cause of OP, which closely correlated with insufficient bone formation. At present, the anti-resorptive agents are the most commonly used, while bone formation related drugs are limited. Traditional Chinese medicine (TCM), which has been used for thousands of years, has unique advantages in the treatment of OP according to growing evidence. TCM is reported to have dual efficacy in promoting bone formation and inhibiting bone resorption, with higher safety and lower toxicity [3,4]. Thus, it is worthwhile to promote TCM as an alternative medicine for OP and to investigate the mechanisms underlying its promotion of bone formation.

Drynariae Rhizoma (DR) is obtained from the dried rhizome of *Drynaria fortune*, Polypodiaceae. It primarily composed of flavonoids and polyphenols, such as kaempferol, lignan, and naringenin [5,6]. In TCM clinics, DR is commonly used in Chinese medicine prescriptions in the form of water extracts for the prevention and treatment of OP, as well as in dietary supplements like YIN-YANG-HUO-GU-SUI-BU capsules (National Healthy Food Index No.: G20140729, China) and so on. Studies have reported that the water extracts of DR could moderate the bone microstructure in the osteoporotic rat model, which was related to the regulation of metabolites or AGE-RAGE signaling pathway [7,8]. Additionally, DR extracts could promote the proliferation and osteogenic differentiation of osteoblast [9,10]. However, there are

limited reports of DR on bone formation in OVX model animals, and its related mechanism requires further investigation.

The gut microbiota is a collective term used to describe the normal microorganisms present in the human intestine, including probiotic, neutrophilic, and pathogenic bacteria. The stability of species structure is essential for maintaining normal physiological activities. Once disrupting, such as increasing harmful bacterial proportions, it may lead to various diseases, including OP [11,12]. Studies have demonstrated that there are significant changes in the diversity and abundance of gut microbiota in patients with OP, and these changes are believed to affect the bone metabolic via regulation of the immune system, endocrine system, and calcium plasma absorption [13]. Thus, the gut microbiota represents a crucial factor in the regulation of bone metabolic homeostasis.

In this study, we observed the bone microstructure changes in OVX rats after treated with water extracts of DR, as well as some bone formation related factors. Next, we also explored the changes in the species and the abundance of gut microbiota by 16S rDNA high-throughput sequencing, expecting to provide a new perspective of DR in the treatment of OP.

Results

The chemical components of DR were analyzed by HPLC and the content of naringin was evaluated, which was found to be 3.99 mg/g (**Fig. 1A, 1B**).

Subsequently, the effects of DR in OVX rats were evaluated. The body weight of OVX group increased significantly over time compared with the Sham group, while DR treatment group could significantly alleviate this change (**Fig. 1C**). Furthermore, the

uterus weight and index were lower in OVX group than in Sham group, while DR treatment group had no influence in OVX rats (**Fig. 1D, 1E**).

To further evaluate the effect of DR on the bone microstructure in OVX rats, micro-CT and HE staining were performed. Results showed that the femur trabeculae of OVX rats appeared irregular, porous, and fractured, with significantly lower BMD, BV/TV, Tb.N and higher Tb.Sp compared to the Sham group. DR treatment groups significantly increased the BMD (P<0.05) in OVX rats. DR-H significantly increased the BV/TV, and significantly decreased the Tb.Sp compared to OVX rats (P<0.05). Besides, different dose of DR slightly increased Tb.N and Tb.Th in OVX rats, but with no significance (**Fig. 2A**). Moreover, HE staining revealed that OVX rats exhibited a higher degree of trabecular rarefaction and irregularity compared to Sham rats. While DR treatment group ameliorated these changes, in which DR-H presented the most significant effect (**Fig. 2B, 2C)**.

Figure 1. The effects of DR on body weight and uterus status in OVX rats (A) HPLC chromatogram of naringin; (B) HPLC chromatogram of water extracts of DR; (C) Change of body weight in rats (n = 6), the raw data represent replicate values; (D) Uterus morphology; (E) Uterus weight and index (n = 4), the raw data represent replicate values. *P < 0.05, **P < 0.01, ns: not significant, analyses were carried out twice and comparable results were obtained.

Figure 2. DR ameliorated bone microstructure in OVX rats (A) Micro-CT of the femur (n = 3), the raw data represented replicate values; (B) HE staining of the femur, scale bar: 50 μm or 200 μm; (C) HE staining of the lumbar vertebrae, scale bar: 50 μm or 200

μm. *P < 0.05, **P < 0.01, ns: not significant, analyses were carried out twice and comparable results were obtained.

Subsequently, we also investigated the alternation of bone metabolism factors in rats. The results revealed that the content of E2, OCN, ALP and Ca ion in serum was significantly lower in OVX group than Sham group, and DR treatment group could effectively reverse these trends (P<0.05) (**Fig. 3A, 3B**). But DR treatment group did not have a significant effect on TRAP in OVX rats (**Fig. 3A**), as well as the content of P ion (**Fig. 3B**).

To further explore the effect of DR on the bone formation, the changes bone formation related factors were detected. The protein expression of ALP, COL1A1 and RUNX2 was significantly lower in OVX group than Sham group, while DR treatment group could significantly increase the expression of ALP, BMP-2, COL1A1 and RUNX2 in OVX rats (P<0.05) (**Fig. 3C**). Additionally, we also evaluated the mRNA expression. It demonstrated that DR-M and DR-H significantly improved the mRNA expression of all these bone formation related factors in OVX rats; and DR-L significantly increased the mRNA expression of *bmp-2*, *col1a1*, and *runx2* in OVX rats (P<0.05) (**Fig. 3D**). IHC staining revealed that COL1A1 and RUNX2 were sparsely distributed in OVX rats, while different doses of DR could effectively modulate them in OVX rats (**Fig. 3E, 3F**). These findings suggested that DR-H may provide more benefits in terms of bone formation.

Figure 3. The expression of bone formation related factors in different groups (A) Content of E2, OCN, ALP and TRAP in serum (n = 4); (B) Content of Ca and P ion in serum (n = 4); (C) Cropped blots showed the protein expression of bone formation

related factors in different groups (n = 3), and the full-length blots were presented in Supplementary File 1; (D) mRNA expression of bone formation related factors in different groups (n = 3); (E-F) IHC results of COL1A1and RUNX2 in femur (n = 3), scale bar: 50μm or 100 μm. *P < 0.05, **P < 0.01, ns: not significant, analyses were carried out twice and comparable results were obtained.

We investigated the gut microbiota composition using 16S rDNA sequencing. After removing unqualified sequences, an average of 67,082 effective tags were obtained, and each samples obtained an average of 30,285 feature numbers for an average of 443 feature sequences. The venn diagram of feature sequences from each group is shown in **Fig. 4A**.

Alpha diversity analysis was conducted to describe the composition of the bacteria in different samples, including the low abundance feature coverage (Goods coverage), the species richness (Chao and Observed species), as well as the species diversity (Simpson and Shannon indices). As indicated in **Table 2**, the Goods coverage value of gut microbiota was greater than or equal to 0.97 in each group, indicating that the abundance of features obtained by sequencing was credible, and the sequencing depth satisfied the subsequent bioinformatics analysis. Chao, Observed species and Simpson analysis showed no significant difference among each group. Interestingly, the Shannon index was significantly improved in OVX rats compared to Sham rats, while DR-L and DR-H could significantly reverse it in OVX rats.

Table 2. Summary of the alpha diversity statistical analysis in different groups

Group	Goods coverage	Chao	Observed species	Simpson	Shannon
Sham	1.00±0	396.71±185.3	388.67±172.33	0.98±0.01	7.01±0.32
OVX	1.00±0	537.12±30.91	527.67±25.33	0.99±0	7.60±0.08*
DR-L	1.00±0	389.67±110.00	384.67±70.33	0.98±0.01	7.15±0.31$^{\#}$

DR-M	1.00±0	498.10±58.56	488.67±52.33	0.99±0	7.45±0.20
DR-H	1.00±0	397.86±95.86	393.00±91.00	0.98±0.01	6.89±0.17$^{\#\#}$

*P < 0.05 vs Sham; $^{\#}P$ < 0.05 vs OVX, $^{\#\#}P$ < 0.01 vs OVX.

Furthermore, the beta diversity of UniFrac-based PCoA was used to evaluate a distinct clustering pattern of gut microbiota structure among each group, as depicted in **Fig. 4B**. The results showed a clear separation between the Sham and the other groups in the gut microbiota structure, while a clear separation was also observed between the OVX and DR-H groups.

Figure 4. Venn diagram of feature sequences (A) and beta diversity analysis (B) of gut microbiota

At the phylum level, there were 16 phyla detected in all groups, among which the *Firmicutes*, *Bacteroidetes*, and *Proteobacteria* were the dominant phyla. Compared with the Sham group, the abundance of *Firmicutes* was increased, while *Bacteroidetes* and *Proteobacteria* were decreased in OVX group, resulting in an obviously increase in the ratio of *Firmicutes*/*Bacteroidetes*. Conversely, different doses of DR treatment group decreased the abundance of *Firmicutes* and increase the abundance of *Bacteroidetes*, resulting in decreasing the ratio of *Firmicutes*/*Bacteroidetes* in OVX rats. Furthermore, the DR-M and DR-H treatment increased the abundance of *Proteobacteria* in OVX group (**Fig. 5A**).

At the genus level, there were 257 genera detected in all groups, among which the *Muribaculaceae_unclassified*, *Lactobacillus*, *Firmicutes_unclassified*, *Lachnospiraceae_NK4A136_group*, and *Ruminococcaceae_UCG-014* were the dominant genera. Compared with the Sham group, the abundance of

Muribaculaceae_unclassified, *Lactobacillus*, and *Lachnospiraceae_NK4A136_group* were decreased, while the abundance of *Firmicutes_unclassified* and *Ruminococcaceae_UCG-014* were increased in OVX group. Conversely, different doses of DR treatment group increased the abundance of *Muribaculaceae_unclassified* and *Lactobacillus*, and decreased the abundance of *Lachnospiraceae_NK4A136_group* in OVX rats. Additionally, DR-H could decrease the abundance of *Ruminococcaceae_UCG-014*, and DR-L and DR-M decreased the abundance of *Firmicutes_unclassified* in OVX rats (**Fig. 5B**).

Kruskal-Wallis test was executed to detect genera among different groups. Results showed that the abundance of *Ruminococcaceae_UCG-007* and *Ruminiclostridium* were significantly decreased in OVX group, while the *Prevotellaceae_Ga6A1_group* was significantly increased compared with the Sham group. Meanwhile, different doses of DR treatment group decreased the abundance of *Peptococcaceae_unclassified* in OVX rats. Furthermore, the abundance of *Prevotellaceae_NK3B31_group*, *Ruminococcaceae_UCG-007*, and *Ruminiclostridium* were increased in DR-H group, as well as the *Ruminococcaceae_UCG-007* in DR-M group, and *Prevotellaceae_NK3B31_group*, *Prevotellaceae_Ga6A1_group* and *Bacteroides* in DR-L group (**Fig. 5C**). The flora distribution and different abundance (Kruskal-Wallis test, $P<0.05$) of gut microbiota of rats at the class, order, family and species level were showed in **supplementary figure 1**.

Spearman's correlation analysis was carried out to investigate the association between the different genera and bone metabolism factors in serum. The results showed that *Peptococcaceae_unclassified* was negatively correlated with OCN, ALP, and Ca ion. *Ruminococcaceae_UCG-007* was positively correlated with OCN but negatively correlated with P ion. *Ruminiclostridium* and *Prevotellaceae_NK3B31_group* were

positively correlated with ALP and Ca ion (**Fig. 5D**). Therefore, we speculated that *Ruminococcaceae_UCG-007*, *Ruminiclostridium*, and *Prevotellaceae_NK3B31_group* may be the potential targets of DR to promote bone formation.

Figure 5. Composition and abundance of gut microbiota in different groups rats (A) Composition of gut microbiota at the phylum levels; (B) Composition of gut microbiota at the genus levels; (C) Abundance of gut microbiota at the genus levels. (D) Spearman's correlation of the abundant genus with bone metabolism factors. The data are expressed as means ± SD ($n = 3$ in each group). *$p < 0.05$; **$p < 0.01$.

Combining the previous results, DR-H presented the most significant effects on bone formation. To get further insight into the underlying mechanisms of DR-H, LEfSe and PICRUSt2 analysis were conducted among Sham, OVX, and DR-H groups. LEfSe analysis was utilized to explore the potential biomarker that was significant enriched in the bacterial community, while PICRUSt2 was used to analyze the KEGG pathways and assess the function of variational gut microbiota.

The results of the LEfSe analysis indicated that the genus and species levels exhibiting significant difference among different groups. (Linear discriminant analysis score > 2.5). Specifically, at the genus level, *Quinella* enriched in Sham group, while *Ruminiclostridium*, *Coriobacteriales_unclassified*, and *Ruminococcaceae_UCG_007* enriched in DR-H group; at the species level, *Quinella_unclassified* enriched in Sham group, *Negativibacillus_unclassified* enriched in OVX group, while *Ruminiclostridium_unclassified*, *Coriobacteriales_unclassified*, and *Ruminococcaceae_UCG_007_unclassified* enriched in DR-H group (**Fig. 6A**).

The PICRUSt2 analysis revealed that sixteen metabolic pathways were disordered in OVX group compared with Sham group. Notably, the relative abundance of CMP-legionaminate biosynthesis I was significantly increased in OVX group, while this trend could be reversed by DR-H treatment. Therefore, we inferred that CMP-legionaminate biosynthesis I might be a crucial pathway of DR regulates gut microbiota (**Fig. 6B**).

Figure 6. Advanced analysis of LEfSe (A) and function prediction (B) of gut microbiota

We also investigated the serum short-chain fatty acids (SCFA) levels in OVX rats treated with DR (**Fig. 7**). The results indicated a significant reduction in serum SCFA concentrations in OVX rats ($P<0.05$), which was notably ameliorated by DR intervention ($P<0.01$). Notably, both acetic acid and butyric acid levels were significantly diminished ($P<0.05$) in the serum of OVX rats. However, treatment with both DR-H and DR-M significantly enhanced the serum acetic acid levels ($P<0.05$). Furthermore, DR-H was also effective in elevating the serum butyric acid levels in OVX rats ($P<0.01$).

Figure 7. Concentrations of SCFA in serum of rats

Discussion

OP is a significant public health concern affecting the elderly population, and its prevention and treatment remain worldwide challenges. Current drugs used for OP have remarkable efficacy [14], but exist some limitations, including poor tolerability and

significant adverse effects [15,16]. Consequently, it needs to develop new drugs with fewer adverse effects. DR has been used as a kidney-nourishing herb to treat bone diseases and as a primary component of many healthcare products. DR and its active ingredients have been shown to exhibit skeletal protective effects in glucocorticoid, and OVX-induced models [7,17,18]. However, the specific mechanism underlying its promotion of bone formation remains poorly understood.

Our results indicated that DR treatment group significantly decreased the body weight and increased estradiol levels in serum in OVX rats, showing the estrogen-like effect in OVX rats. The estrogenic effects of phytoestrogen-containing drugs or selective estrogen receptor modulators are always a concern due to their potential to stimulate the growth of reproductive tissues, such as the breast and uterus [19,20]. Our results proved that DR did not exhibit any stimulatory effects on uterus in OVX rats, which was correspond to a previous study that DR only exerted estrogenic effects in a tissue-selective manner, such as bone [21]. Next, micro-CT and HE staining results indicated that DR significantly moderated bone mass and bone microstructure. Furthermore, the content of OCN, ALP and Ca ion was significantly up-regulated with DR treatment group, while TRAP has no significant difference. In addition, DR treatment group increased the expression of bone formation related factors, as shown by IHC staining, western blot, and RT-PCR. Our results suggest that extracts of DR significantly stimulated bone formation in a dose-dependent manner, with the highest efficacy observed at the DR-H dosage. These findings indicate that DR has obvious effects on bone formation in OVX rats.

Gut microbiota is the primary members of the intestinal microecology which interacting with organs and systems of the body mainly through SCFA and inorganic s alts [12]. Studies have shown that the structural changes of gut microbiota are closely

related to the development of OP [22,23]. In OP patients or animal models both found an increase of the ratio of *Firmicutes*/*Bacteroidetes*. Conversely, several probiotics have been shown to have a protective effect, indicating that the gut microbiota is an important target for the treatment of OP [24,25]. Until now, increasing evidences suggested that the anti-OP effect of TCM is closely related to the gut microbiota. For instance, *Eucommia ulmoides* leaf extract could alter the composition of gut microbiota and increase the ratio of *Firmicutes*/*Bacteroidetes* to *ameliorates osteoporosis* [26]; Xian-Ling-Gu-Bao capsule regulates the gut microbiota to further control lipid and bile acid metabolism, stimulating the increasment of bone mass in OVX rats [27].

Our results revealed that the water extracts of DR led to a significant improvement in the ratio of *Firmicutes*/*Bacteroidetes* and the abundance of *Muribaculaceae_unclassified* and *Lactobacillus* in OVX rats. Prior research has demonstrated that *Muribaculaceae_unclassified* is associated with the regulation of lipid metabolism and inflammation, and its abundance being reduced in animal models related to adiposity [28], ulcerative colitis [29], and diabetic kidney disease [30]. Interestingly, *Muribaculaceae_unclassified* was positively correlated with the increasement of butyrate production, while butyrate could stimulate bone formation via T regulatory cell-mediated regulation of WNT10B expression [31]. Our previously study revealed that ovariectomy could lead to abnormal fat metabolism and weight gaining [32], whereas DR could significantly reduce body weight, promote bone formation, increase the abundance of *Muribaculaceae_unclassified* in this study. Therefore, we speculated that *Muribaculaceae_unclassified* might be one of the mechanisms of DR regulating bone formation. Of course, further exploration is required to validate this hypothesis. As for the *Lactobacillus*, several strains including *Lactobacillus brevis* AR281 [33], *Lactobacillus rhamnosus* GG [34], and *Lactobacillus*

casei [35] have been demonstrated to promote osteogenesis or attenuate osteoclastogenesis in OVX rats. Moreover, *Ruminococcaceae_UCG-007* was decreased in DR-H and DR-M group when contrasted to OVX rats, which was similar with Chinese medicine preparation You-gui pill in the treatment of OP rats [36]. In addition, *Peptococcaceae_unclassified* was significantly decreased in DR groups. It was reported that abundance of the family *Peptococcaceae* was decreased in high fat and cholesterol diet-induced hypercholesterolemia mouse [37]. It was worth noting that the abundance changes of differential genera such as *Ruminococcaceae_UCG-007* or *Ruminiclostridium* showed not a typical dose-effect relationship with drug dose of DR, and there were several reasons for this. For one thing, different dosage of a drug may affect the gut microbiota differently. A low dose may only affect specific bacteria, while a high dose may have a broader effect on the flora [38]. For another, the gut microbiota is composed of thousands of different bacteria, and the complex interactions among them may tend to maintain the structural and functional stability of the intestinal microenvironment, resisting the changes in flora abundance brought about by different dose of drugs [39].

We also performed spearman correlation analysis between the different gut microbiota community at genus level and bone metabolism factors in serum. We concluded that *Ruminococcaceae_UCG-007*, *Ruminiclostridium* and *Prevotellaceae_NK3B31_group* was positive correlation with the bone formation related factors, while *Peptococcaceae_unclassified* was negative correlation. Our results were accordance with a recent study that *Ruminiclostridium* was positive correlation with Ca ion in OVX rats [40]. Furthermore, LEfSe analysis also demonstrated that *Ruminiclostridium* and *Ruminococcaceae_UCG_007* were enriched in DR-H group. There results suggested the anti-OP effect of DR may correlate with the

improvement of *Lactobacillus* in gut microbiota, and *Ruminococcaceae_UCG-007*, *Peptococcaceae_unclassified* and *Ruminiclostridium* may be the other potential targets.

Besides, PICRUSt2 analysis showed that CMP-legionaminate biosynthesis I might be an important pathway which DR regulated bone formation through affecting gut microbiota in OVX rats. Research indicated that this pathway is involved in the biosynthesis of sialic acids, and they have been found to play a critical role in the virulence of pathogenic organisms by enabling them to evade host immune responses and invade cells [41]. In particular, the metabolite sialic acid-like 5,7-diacetamido-3,5,7,9-tetradeoxy-nonulosonate, which is a product of CMP-legionaminate biosynthesis I, could be incorporated into the glycoconjugates of virulence-associated cell surface, such as lipopolysaccharides (LPS), capsular polysaccharides, pili, and flagella [42]. LPS, in turn, is implicated in bone destruction caused by inflammatory [43 ,44], above all suggesting a potential link between CMP-legionaminate biosynthesis I pathway and bone metabolic.

Previous research has established that SCFA may enhance calcium absorption through several mechanisms, such as the regulation of pH value, and the expressions of Calbindin D9k, TRPV6, and VDR [45]. Our results showed that DR increased acetic acid and butyric acid, and the resulting increase in SCFA may be an important mechanism for promoting bone formation.

All in all, the purpose of this study was to examine the bone formation effect of DR and its relationship with the gut microbiota in OVX rats. Specifically, we found that DR alleviated body weight, moderated the bone microstructure, and promoted the expression of bone formation related factors in femur, in which DR-H behaved the most significant effects among the three doses. The effect of DR was correlated with the changes in microbial abandance and the restorations of several genera, among which

Ruminiclostridium and *Ruminococcaceae_UCG_007* were positively correlated with the bone formation related factors, and both of them enriched in DR-H group as biomarkers. Furthermore, CMP-legionaminate biosynthesis I pathway and SCFA production might be involved in the process of DR-H regulating bone formation.

Conclusions

The present study demonstrated that extracts of DR promoted bone formation in OVX rats, and the mechanism of which might be related to regulate the composition and function of the gut microbiota. These findings offer valuable insights into the underlying mechanisms of DR in the treatment of OP, and provide a new theoretical foundation for its clinical application.

Materials and Methods

Preparation of water extracts of DR

Four kilogram of DR was obtained from Bei Jing Shen Gang Company (batch number 1905036), and it meets the quality standard of pharmacopoeia of People's Republic of China (2020 Edition) [46]. The DR extraction process involved twice reflux-extracting with thirty-two or twenty-eight kilogram of distilled water each time at 100 °C [46]. The resulting extracts were combined and concentrated using rotary evaporation at 60 °C to achieve a final concentration of 1 gram of DR extract per milliliter. The concentrated extract was then cooled to room temperature and stored at -80 °C for further use.

High Performance Liquid Chromatography (HPLC) detection of water extracts of DR

The whole process was following the pharmacopoeia of the People's Republic of China (2020 Edition), and naringin standards (batch number B21594, purity greater than 98%) obtained from Shanghai Yuanye Bio-Technology Co., Ltd. Initially, 10.0 mL of water extracts of DR was freeze-dried using an ALPHA 2-4 LD Plus freeze-dryer, yielding 1.40 grams of powder. Subsequently, 0.025 grams of the powder was dissolved in 10 mL of methanol, and ultrasonic-assisted extraction was carried out at 300 watts for 30 minutes at 40 °C with the missing solution replenished. An Agilent/1260 Infinity II HPLC-diode array detector (HPLC-DAD) system (Agilent, Palo Alto) was employed to detect the content of the water extracts of DR. Lichrospher-C_{18} columns (250 mm × 4.6 mm, 5 μm; Jiangsu Hanbang Science & Technology Co., Ltd) were used. The mobile phase consisted of water, methanol, and acetic acid with a proportion of 65, 35, and 4 for 20 minutes, and the column temperature was maintained at 30 °C. The DAD detector was set at 283 nm to monitor elutes. Additionally, a standard curve of naringin with different concentrations (36, 72, 108, 144, and 180 mg/L) was established with the same detecting condition. The presence of naringin was confirmed by comparing retention times and ultraviolet spectra with reference standards, and its quantification was calculated upon the corresponding standard curve based on the peak areas of the samples.

Animal treatments

Thirty three-month-old female Sprague Dawley rats were obtained from Guangdong Provincial Laboratory Animal Public Service Center (SCXK （Yue） 2018-0034) and raised at Boji Medical & Technological Co. Ltd. (SYXK(Yue)2017-0134) under specific-pathogen-free facilities with controlled temperature (23 ± 1 °C), humidity (50 ± 10%), a 12-hour light/dark cycle, and free

access to food and water. After one week adaptation phase, rats were divided into five groups: a sham-operated (Sham, *n*=6) group, an ovariectomized (OVX, *n*=6) group, an DR treatment group with high-dose (DR-H; 2.43 g/kg/day, *n*=6) group, an DR treatment group with middle-dose (DR-M; 0.81 g/kg/day, *n*=6) group, and an DR treatment group with low-dose (DR-L; 0.27 g/kg/day, *n*=6) group. Bilateral ovariectomy surgery was performed to stimulate bone mass loss following the protocol described in previous studies [32]. Briefly, rats were anesthetized by 2% sodium pentobarbital through intraperitoneal injection with the dosage of 0.2 mL per 100 g. Then, laparotomy was carried out after rats were unconscious, followed with bilateral oophorectomy on the rats in OVX and DR treatment group, while equivalent volume of fat near the ovary were removed in the Sham group. According to the Pharmacopoeia of the People's Republic of China (2020 Edition) [46], the recommended dosage of DR decoction piece for human consumption is 9 grams, which was used as the middle-dose in this study. Based on the body surface area conversion of clinical doses, the dose of intragastric administration of DR for each group were one-third and three times the dose of DR-M, respectively [47,48]. Our protocol abided the guidelines established by the National Institutes of Health and Boji Medical & Technological Co. Ltd. Animal Care Committee (Approval number: IACUC-N2040-PD, November 23th, 2020). After 12 weeks treatment, all rats were anesthetized by 2% sodium pentobarbital. After rats were unconscious, blood from the abdominal aorta was collected and quiescence in room temperature for two hours, centrifuged at 1500 rpm for 15 minutes, then extracted the supernatant. Also, the bone tissues such as femurs and lumbar vertebrae were obtained for further analysis.

Micro-computed tomography (micro-CT) images of femurs

The femurs were collected and fixed in 4% paraformaldehyde, then subjected to scanning using a Hiscan XM Micro-CT (Suzhou Hiscan Information Technology Co., Ltd). The parameters were established at 60 kV and 133 μA, with images captured at a resolution of 50 μm. A 0.5° rotation step through a 360° angular range, with 50 ms exposure per step. The reconstructed images were analyzed using Hiscan Reconstruct software and Hiscan Analyzer software (Version 3.0, Suzhou Hiscan Information Technology Co., Ltd) to analyze the data of bone mineral density (BMD), bone volume fraction (BV/TV), trabecular thickness (Tb.Th), trabecular number (Tb.N), and trabecular separation (Tb.Sp).

Hematoxylin and eosin (HE) and immunohistochemical (IHC) analysis

The femurs and lumbar vertebrae were collected and subsequently fixed with a 4% paraformaldehyde solution. Then, decalcified with a 10% ethylenediaminetetraacetic acid solution at room temperature for one month. After that, the dehydrated samples were embedded in paraffin and then sectioned about 5 μm, which were subsequently dehydrated with an alcohol gradient and embedded in paraffin. A portion of the sections were stained with HE, while others were reserved for IHC analysis, as described in a previous study [47]. Briefly, the sections were prepared, stained, counterstained, dehydrated, hyalinized, and mounted, following which the antibody against collagen type I alpha 1 (COL1A1, 72026, Cell Signaling Technology) and runt-related transcription factor 2 (RUNX2, ab76956, Abcam, China) were diluted at a ratio of 1:100. All images were captured by a microscope (Zeiss, AXIO; Carl Zeiss Microscopy GmbH).

Enzyme linked immunosorbent assay (ELISA) assay

The content of estradiol (E2, Enzyme-linked Biotechnology), osteocalcin (OCN, E nzyme-linked Biotechnology), tartrate-resistant acid phosphatase (TRAP, Enzyme-linked Biotechnology) and short-chain fatty acids (SCFA, Bioroyee Biotech) in serum was analyzed by ELISA kit, and the processes following the manufacturer's instructions.

Inorganic ion assay

The content of calcium (Ca) and phosphorus (P) ions in serum was analyzed by using the Ca and P assay kit (GM1125 and GM1127, Servicebio) according to the manufacturer's instructions. and the quantitatively analysis based on a biochemical analyzer (Chemray-800, Rayto).

Alkaline phosphatase assay

The content of alkaline phosphatase (ALP) in serum was evaluated in an ALP analysis kit (A059-2, Nanjing Jiancheng Biological Engineering Research Institute). Briefly, serum samples were prepared, and related reagents were added, then detected at 520 nm in microplate reader (Biotek, Epoch) according to the instructions provided by the manufacturer.

Western blot

Total protein was extracted by RIPA lysis buffer (P0013B, Beyotime) and separated by 10% SDS-PAGE electrophoresis, followed by transfer protein onto PVDF membranes. Then incubated with primary antibodies, including anti-ALP (GTX42809, GeneTex, anti-bone morphogenetic protein-2 (BMP-2, ab225898, Abcam, USA), anti-COL1A1 (72026, Cell signaling technology), anti-RUNX2 (ab192256, Abcam), and

anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH, 5147S, Cell signaling technology), all at a dilution of 1:1000 and at 4 °C overnight. The next day, incubated the membranes with HRP-conjugated secondary antibodies at a dilution of 1:2500 (7074P2, Cell signaling technology) for 1 hour at room temperature. The signals were visualized using a Bio-rad apparatus, and band intensities were determined using Image J software and normalized to GAPDH.

Real-time quantitative polymerase Chain reaction (RT-PCR)

Total RNA was extracted by Trizol reagent (15596-026, Takara), and the quality and quantity of the extracted RNA were evaluated by nanodrop 2000. One point five micrograms of total RNA with were then reverse transcribed into cDNA using a cDNA synthesis kit (638313, Takara). SYBR Green mix (A304-01, Genestar) was used to amplify, and data were calculated using the formula of $2^{-\Delta\Delta ct}$ with GAPDH as an internal control. The prime sequences were listed in **Table 1**.

Table 1. The primer sequences

Gene name		5′-3′ sequence
Alpl	Forward:	GACAAGAAGCCCTTCACAGC
	Resverse:	ACTGGGCCTGGTAGTTGTTG
Bmp-2	Forward:	GCCATCGAGGAACTTTCAGA
	Resverse:	TGTTCCCGAAAAATCTGGAG
Col1a1	Forward:	ACGTCCTGGTGAAGTTGGTC
	Resverse:	TCCAGCAATACCCTGAGGTC
Runx2	Forward:	AACAGCAGCAGCAGCAGCAG
	Resverse:	GCACGGAGCACAGGAAGTTGG
Gapdh	Forward:	GACATGCCGCCTGGAGAAAC
	Resverse:	AGCCCAGGATGCCCTTTAGT

16S rDNA sequencing

The DNA from various fecal samples was extracted using the CTAB extraction method in accordance with the manufacturer's instructions (Ansiang, L-AX0699). The complete 16S rDNA gene was amplified using the primers 27F: 5'-AGRGTTYGATYMTGGCTCAG-3' and 1492R: 5'- RGYTACCTTGTTACGACTT-3', each tagged with a specific barcode corresponding to the respective sample. PCR amplification was carried out, and the resultant products were verified using 2% agarose gel electrophoresis. The purified products were obtained using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences), in accordance with the manufacturer's instructions. The amplicon pools were quantified using the TM-ST kit (Promega), and libraries were prepared for sequencing. The libraries were prepared with the Pacific Biosciences TM Template Prep kit 1.0 (PacBio) and sequenced on a Sequel II platform (LC-Bio Technology Co., Ltd., Hangzhou).

Gas chromatography detection (GC)

The GC analysis was performed on trace 1310 gas chromatograph (Thermo Fisher Scientific, USA). The GC was fitted with a capillary column Agilent HP-INNOWAX (30 m × 0.25 mm ID ×0.25 μm) and helium was used as the carrier gas at 1 mL/min. Injection was made in split mode at 10:1 with an injection volume of 1 μL and an injector temperature of 250℃. The temperature of the ion source and MS transfer line were 300℃ and 250℃, respectively. The column temperature was programmed to increase from an initial temperature of 90℃, followed by an increase to 120℃ at 10℃/min, and to 150℃ at 5℃/min, and finally to 250℃ at 25℃/min which was maintained for 2 min.

Statistical analysis

The data presented in this study are either in replicated values or as mean ± standard deviation. The normality of the data was assessed using Graphpad Prism 8.0 (GraphPad software), and differences between groups were evaluated by one-way analysis of variance (ANOVA). A *P* value less than 0.05 was considered to indicate a significant difference.

The 16S rDNA sequencing data were analyzed using the following methods: Circular Consensus Sequence (CCS) reads were generated from raw subreads using SMRT Link (v6.0) with the parameters set to minPasses = 3 and minPredictedAccuracy = 0.99. Lima (v1.7.1) was employed to differentiate CCS reads from different samples, while cutadapt (v1.9) was used to identify primers. CCS reads ranging from 1200 bp to 1650 bp in length were retained after length filtration. After dereplication and filtering of chimeric sequences using DADA2, feature table and feature sequence were obtained. Normalized alpha diversity and beta diversity were calculated by randomly matching the same sequences. The alpha diversity was used to analyze the complexity of species diversity in a sample via six indices, including Chao1, Observed_features, Goods coverage, Shannon, Simpson, and Pielou_e. All these indices were calculated using QIIME2. Beta diversity was also calculated using QIIME2. The annotated Amplicon Sequence Variants were aligned with the SILVA database (release 138). Other diagrams were generated using R packages.

Conflict of interest statement

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Declaration of competing interests

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