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Intermittent fasting promotes repair of rotator cuff injury in the early postoperative period by regulating the gut microbiota

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ABSTRACT

Background: The repair of rotator cuff injury is affected by lifestyle and metabolic factors. Intermittent fasting (IF) can promote repair of damaged tissue by regulating intestinal flora, which provides an idea of therapy for rotator cuff injury. The aim of this study was to investigate the effects of fasting on rotator cuff repair after injury, and the role of intestinal flora or a single strain in this process.

Methods: Mice underwent rotator cuff injury were treated with intermittent fasting or fed *ad libitum*. Fasting began one month before surgery and continued until euthanasia. Fresh feces were collected at 2 weeks before surgery, on the day of surgery, and 2, 4, 8 weeks postoperatively for 16S rRNA microbiome sequencing. Supraspinatus tendon-humerus (SSTH) complex was collected at 2, 4 and 8 weeks after surgery. Live *parabacteroides distasonis* (*Parabacteroides distasonis*) was used for repair of rotator cuff injury, with equal amount of pasteurized *P. distasonis* (KPD) or sterile anaerobic phosphate buffer saline (PBS) as control. Biomechanical, radiological, histological analysis were used to assess the effect of rotator cuff repair.

Results: Biomechanical, radiological and histological analysis indicated that intermittent fasting significantly promoted the repair of rotator cuff injury in the early postoperative period ($P < 0.05$), but significantly inhibited the repair of rotator cuff injury at 4 weeks postoperatively ($P < 0.05$). 16S rRNA Microbiome sequencing result showed that *P. distasonis* was the species with the most obvious changes in intestinal flora of mice after fasting. The results of tensile test, X-ray analysis and histological analysis indicated that the live *P. distasonis* (LPD) significantly impaired the biomechanical properties, bone regeneration and fibrocartilage regeneration of enthesis postoperatively ($P < 0.05$).

Conclusion: Intermittent fasting promoted repair of rotator cuff injury in the early postoperative period by regulating the gut microbiota, in which *P. distasonis* played an important role.

The translational potential of this article: Intermittent fasting (IF) may be a beneficial lifestyle for the repair of rotator cuff injury in the early postoperative period in clinical, and the influence of a certain strain on the repair of rotator cuff injury may also provide an idea for the treatment of rotator cuff injury in the future.

1. Introduction

Rotator cuff tear is one of the common sports system injuries, shoulder pain and dysfunction are common manifestations clinically, which brings suffering to patients and imposes a heavy economic burden on society. Although surgical repair brings good clinical results for rotator cuff injuries, the incidence of re-tear after surgery is as high as 5%–34% [1]. Studies have shown that the repair of rotator cuff injury is affected by various factors, including the age of patient, tear size, muscle quality, smoking, diabetes and so on [2–4]. Moreover, lifestyle and metabolic factors also play an important role in rotator cuff disease [5].

Recent studies have shown that individuals were able to perform higher levels of physical and cognitive function when they were exposed to relatively scarce food. Intermittent fasting (IF) is a common pattern of fasting, which has been shown to enhance energy metabolism, promote repair of damaged tissue and improve a range of age-related disorders,

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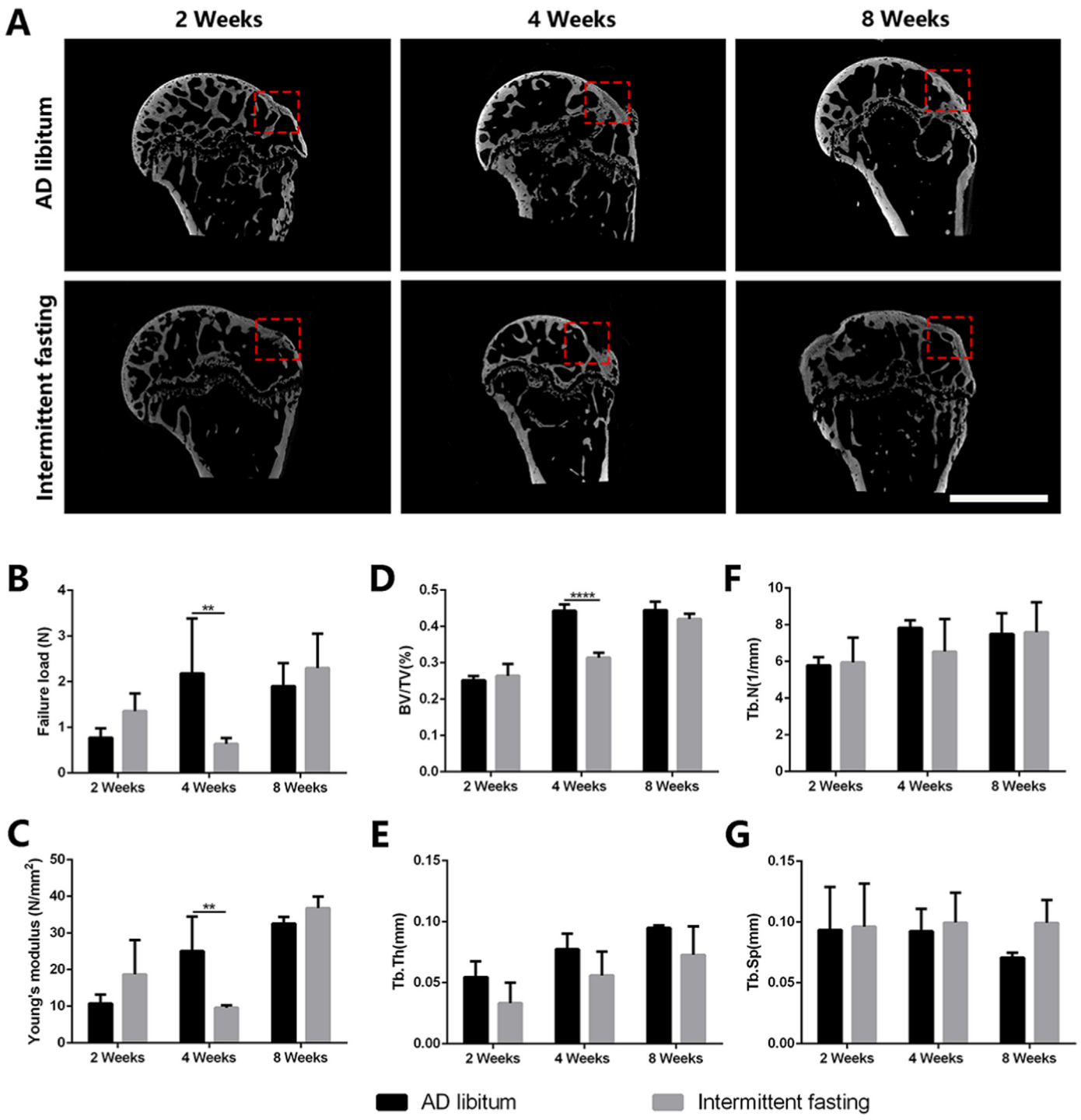

Fig. 1. Intermittent fasting improves biomechanical properties and bone regeneration at the enthesis of ratator cuff (A) Representative X-ray analysis images of the proximal humerus in the AD libitum group and the intermittent fasting group at postoperative week 2, 4 and 8. The dotted box is the ROI. Scale bar = 125 μm. **(B&C)** Results of biomechanical testing in the AD libitum group and the intermittent fasting group at postoperative week 2, 4 and 8. **(D–G)** Quantitative analysis of the ROI. BV/TV, bone volume fraction; Tb.N, trabecular number; Tb.Th, trabecular thickness; Tb.Sp, trabecular separation. $^{****}P < 0.0001$, $^{**}P < 0.01$.

etc [6–9]. In this process, the gut flora plays an important role. It has been shown that IF could confer protection in central nervous system autoimmunity, alleviate diabetes-induced cognitive impairment, promote white adipose browning and prevent retinopathy by modulating the gut microbiota [10–13].

In recent years, the gut microbiome has been recognized as an important role on human health [14,15]. There is a lot of evidence suggested that the change of intestinal microbiota composition may influence the progression of joint disease [16–20]. *Parabacteroides distasonis* (*P. distasonis*) is one of the core members in the intestinal flora of human [21]. Correlation analysis have shown that the content of *P. distasonis* in the gut microbiome has a significant correlation with obesity, non-alcoholic fatty liver disease, diabetes mellitus, colitis and other diseases [22–25].

In this study, we investigated the effect of fasting on rotator cuff repair after injury, and the role of intestinal flora or a single strain in this process.

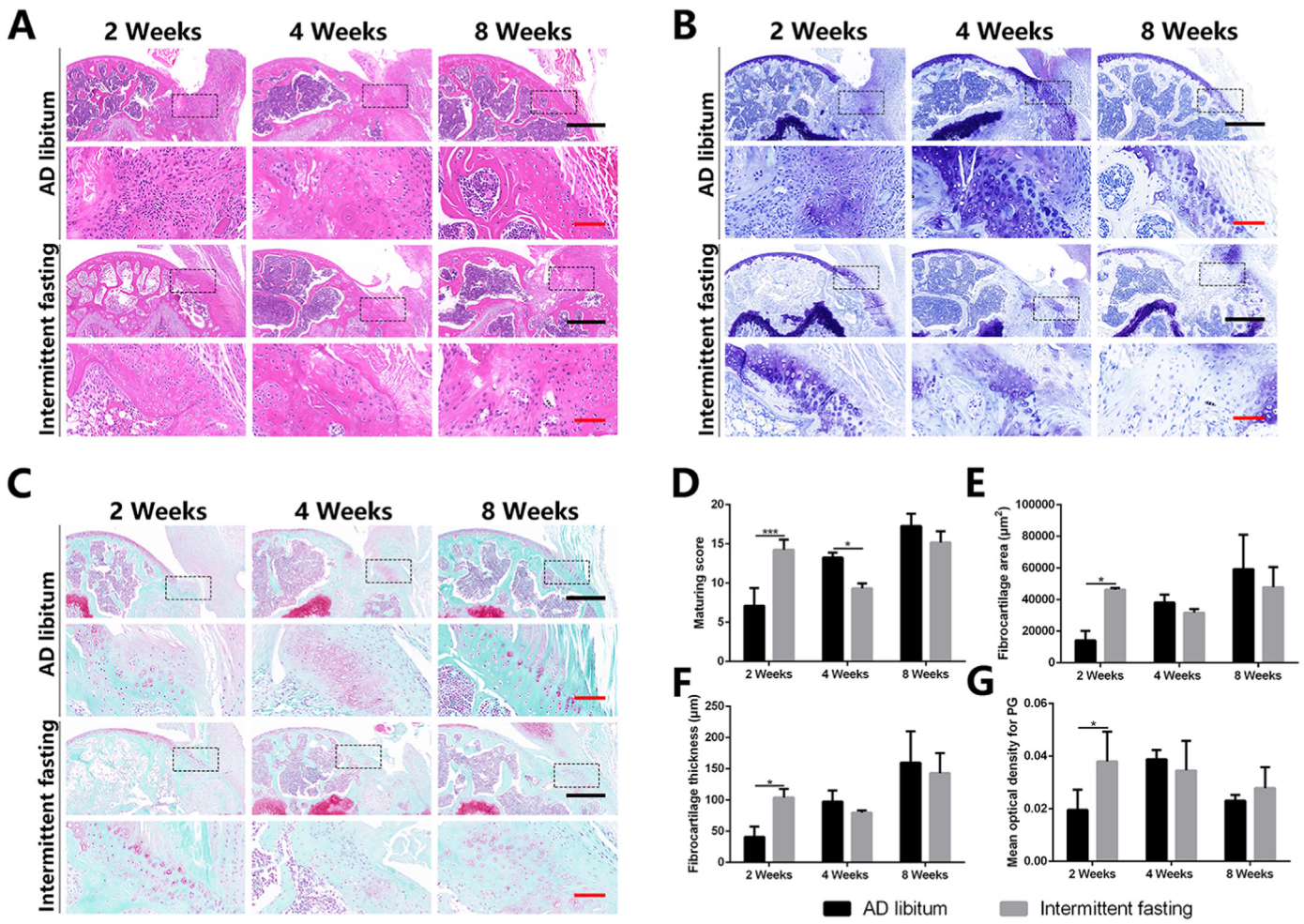

Fig. 2. Intermittent fasting improves histomorphology of the regenerated tissue at the enthesis of ratator cuff (A) Representative hematoxylin and eosin (H&E) staining images of regenerated enthesis and **(D)** quantitative analysis of maturing score **(B)** Representative toluidine blue staining images of regenerated enthesis, and **(E&F)** quantitative analysis of fibrocartilage area and thickness **(C)** Representative safranin O/fast green (SO/FG) staining images of regenerated enthesis and **(G)** quantitative analysis of mean optical density for proteoglycans (PG). The dotted box is the regenerated enthesis. Black scale bar = 100 μm; red scale bar = 20 μm $^{***}P < 0.001$, $^{*}P < 0.05$.

2. Materials and methods

2.1. Study design

A total of 150 male C57BL/6 mice (12 weeks-old, 24±3g) were used in this study. Sixty mice were randomly divided into two groups, then fed *ad libitum* with a standard diet or treated with intermittent fasting, which underwent rotator cuff repair surgery after fasting for one month (n = 30). Another ninety mice underwent rotator cuff repair surgery were randomly divided into three groups, and treated with live *P. distasonis* (LPD), pasteurized *P. distasonis* (KPD) or an equivalent volume of sterile anaerobic phosphate buffer saline (PBS) (n = 30). Feces samples (n = 6) from the fasting group and the control group were collected in sterile cryopreserving tubes at 5 time points: 2 weeks before surgery, on the day of surgery, and 2, 4, 8 weeks postoperatively. 2 to 4 fresh feces were collected from each mouse, transported in liquid nitrogen and preserved in - 80 °C for further 16S rRNA Microbiome sequencing. The supraspinatus tendon-humerus (SSTH) complex were collected at 2, 4 and 8 weeks after surgery for a series of subsequent evaluations (n = 10), including biomechanical, radiological, histological assessment.

2.2. Animal model and surgery procedure

The C57BL/6 mice underwent rotator cuff repair surgery on the left upper limb, according to a previous protocol [26–28]. After intraperitoneal injection of 0.3% pentobarbital sodium (0.6 ml/20 g) for anesthetization, a longitudinal skin incision was created on the shoulder. Transverse the deltoid, expose the supraspinatus tendon (SST), thread the tendon with 6–0 absorbable polydioxanone suture (PDS) (Ethicon) in a figure of eight pattern. Then the SST was severed with a sterile blade from the attachment point on the humerus head, and the fibrocartilage layer at the enthesis was removed. Next, use a 30-G needle to create a bone tunnel across the humeral head. The sutures with the tendon was then passed through the tunnel and fastened. The last, the deltoid muscle and skin were sutured successively. Penicillin G was given every day for three days after surgery.

2.3. Treatment of animals

To test the effect of fasting on rotator cuff injury healing, sixty mice underwent surgery were randomly divided into two groups (n = 30 per group). The mice in the control group were fed *ad libitum* with a standard diet, and the mice in the fasting group were treated with periodic fasting, i.e. a cycle of 11 days [8,12,29], with fasting treatment on day 1, 3 and 5, and normal diet for the rest of the time. Fasting began one month before surgery and continued until euthanasia. The timeline of fasting in relation to surgery and sampling was illustrated by a diagram as shown in Fig. S1.

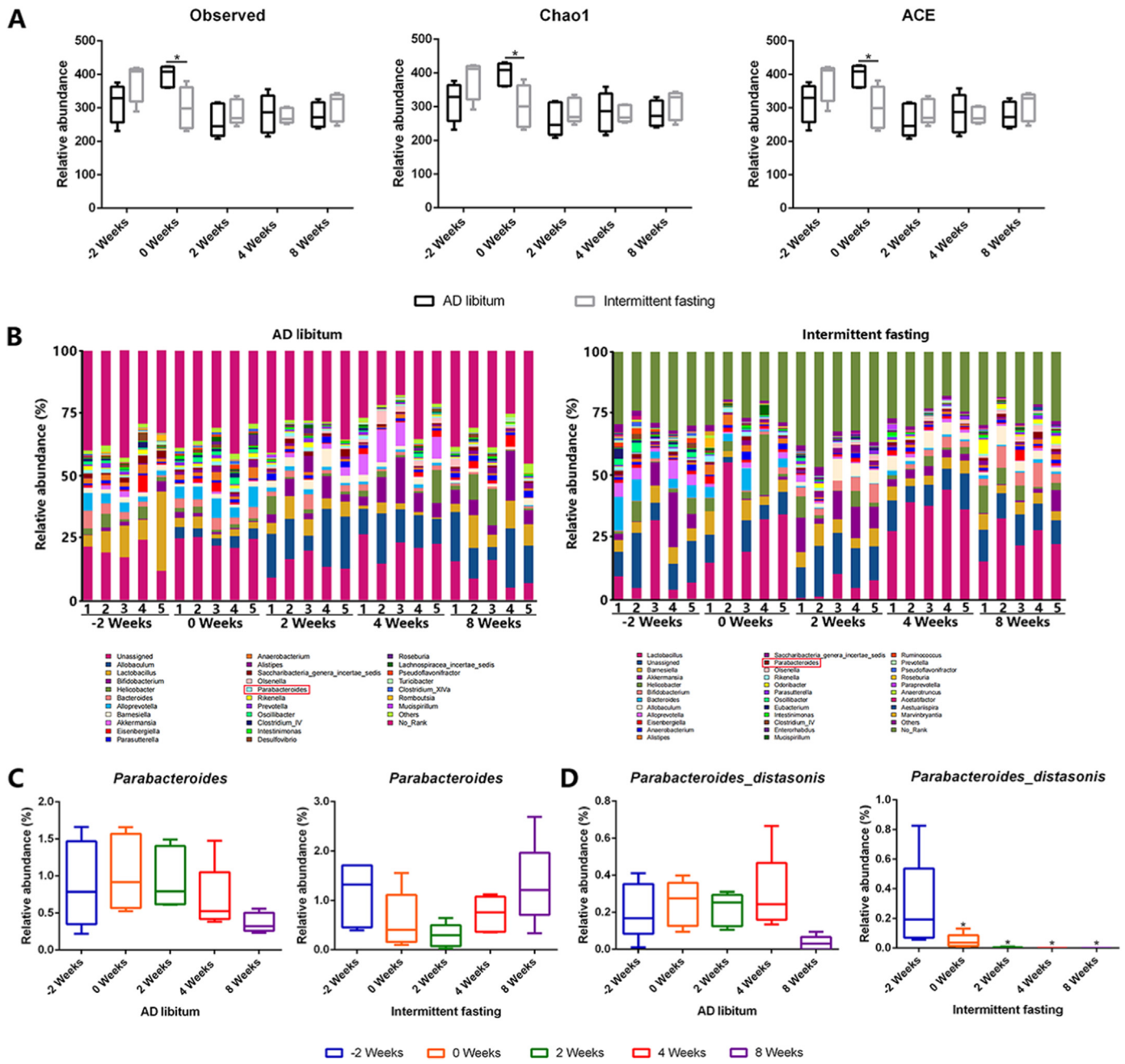

Fig. 3. Intermittent fasting alters composition of the gut microbiome in mice (A) Observed number of OTU and estimated OTU (Chao1 and ACE) of the fecal microbiota in the AD libitum group and the intermittent fasting group **(B)** Relative abundance of the fecal microbiota at the genus level in the AD libitum group and the intermittent fasting group. **(C&D)** Relative abundance of the genus Parabacteroides **(C)** and the species *P. distasonis* **(D)** of the fecal microbiota in the AD libitum group and the intermittent fasting group. $^{*}P < 0.05$.

For the efficiency of *P. distasonis* on the repair of rotator cuff injury, we divided ninety mice that had rotator cuff surgery into three groups (n = 30 per group). One treatment group was given LPD at 6×10^7 cfu in 0.2 ml phosphate buffer solution (PBS) per mouse orally, the other treatment group was given an equal amount of KPD (keeping the LPD at 65 °C for 30 min), and the vehicle group was given an equivalent volume of sterile anaerobic PBS. The gavage treatment of LPD or KPD were given twice a week after operation.

2.4. 16S rRNA microbiome sequencing

The DNA were extracted from 60 fecal samples collected above with the QIAamp Fast DNA Stool Mini Kit according to the manufacturer's instructions. The bacterial V3–V4 region of 16S ribosomal RNA was amplified with the primer: 341F: 5′-CCTACGGGNGGCWGCAG-3′ and 805R: 5′-GACTACHVGGGTATCTAATCC-3′. The library was constructed with the amplified PCR products. After amplification and enrichment, high-throughput second-generation sequencing was performed by Miseq (Illumina, CA, USA). Then the obtained sequence was compared with a specific database to confirm the species. Through OUT cluster analysis, the composition of microorganisms in the sample and the relative abundance of different microorganisms could be known.

2.5. Culture and identification of P. distasonis

P. distasonis (ATCC BAA-1295, USA) was cultured in an anaerobic

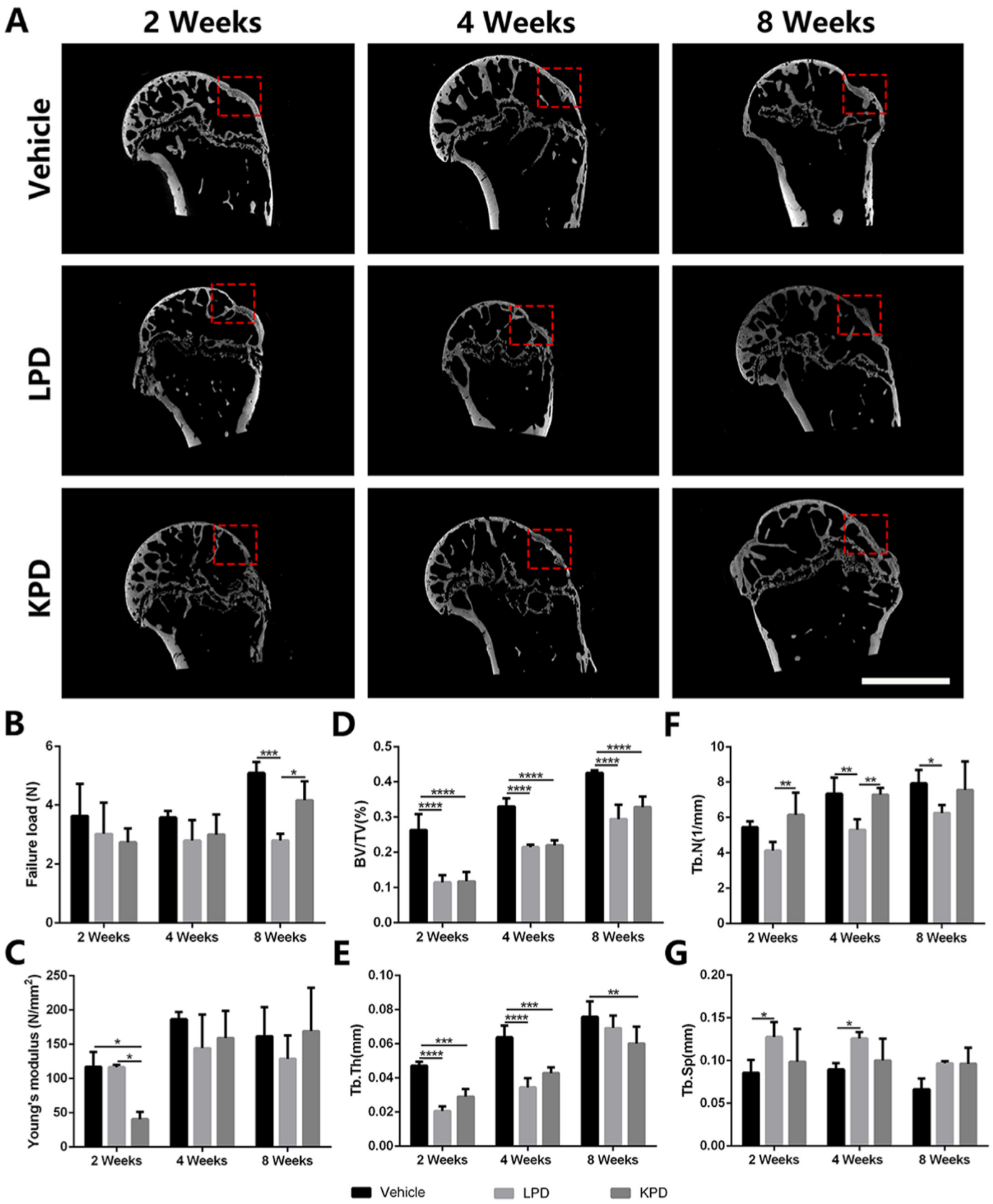

Fig. 4. Mice treated with *P. distasonis* impair biomechanical properties and bone regeneration at the enthesis of ratator cuff (A) Representative X-ray analysis images of the proximal humerus in the vehicle, the LPD- and KPD-treated group at postoperative week 2, 4 and 8. The dotted box is the ROI. Scale bar = 125 μm. **(B&C)** Results of biomechanical testing in the vehicle, the LPD- and KPD-treated group at postoperative week 2, 4 and 8. **(D–G)** Quantitative analysis of the ROI. BV/TV, bone volume fraction; Tb.N, trabecular number; Tb.Th, trabecular thickness; Tb.Sp, trabecular separation. $^{****}P < 0.0001$, $^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$.

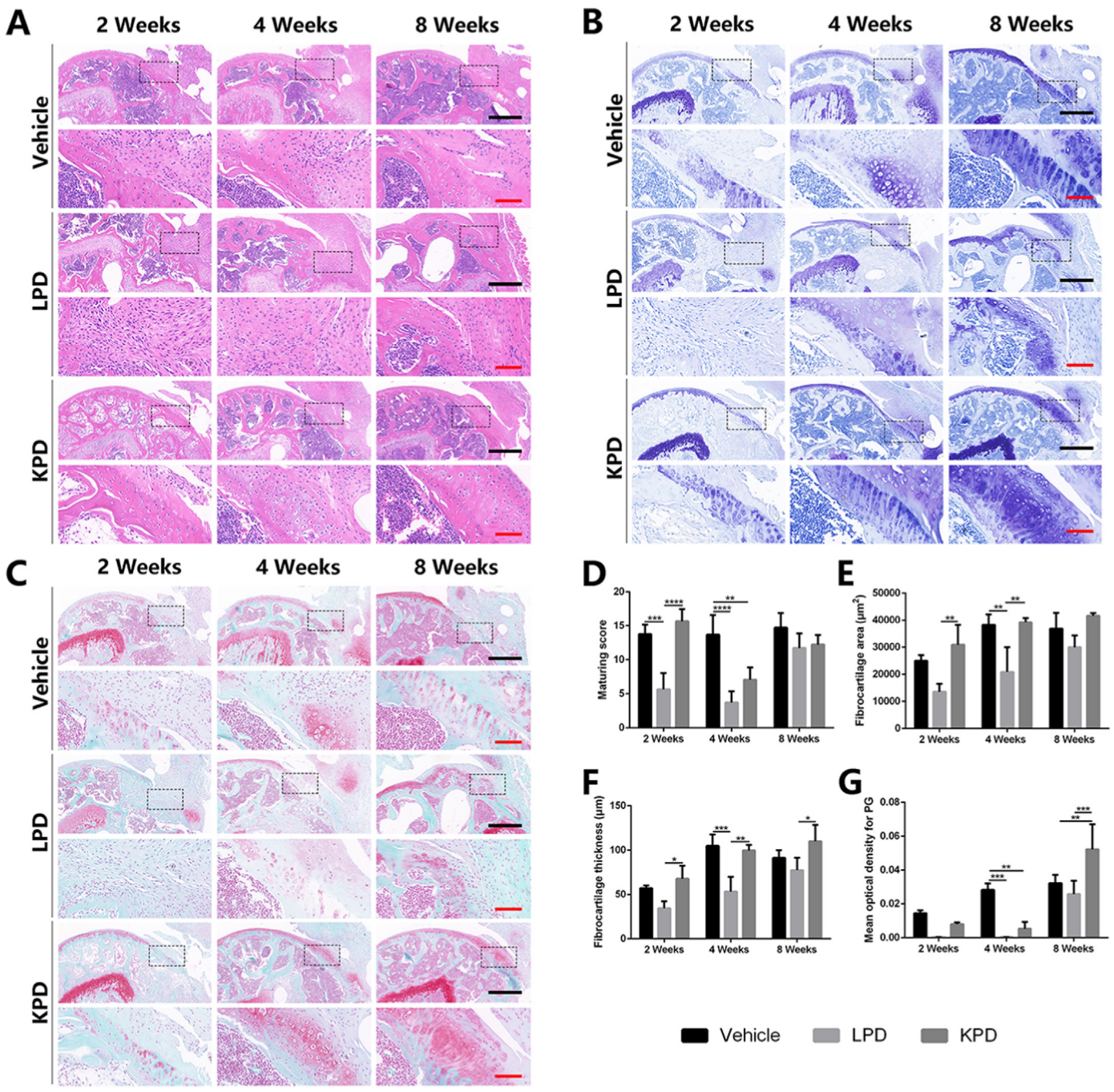

Fig. 5. Mice treated with *P. distasonis* show poor quality of rotator cuff healing (A) Representative hematoxylin and eosin (H&E) staining images of regenerated enthesis and **(D)** quantitative analysis of maturing score **(B)** Representative toluidine blue staining images of regenerated enthesis, and **(E&F)** quantitative analysis of fibrocartilage area and thickness **(C)** Representative safranin O/fast green (SO/FG) staining images of regenerated enthesis and **(G)** quantitative analysis of mean optical density for proteoglycans (PG). The dotted box is the regenerated enthesis. Black scale bar = 100 μm; red scale bar = 20 μm ****$P < 0.0001$, ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$.

environment at 37 °C in trypticase soy broth (TSB) with shaking constantly. Then the cultured *P. distasonis* was characterized. Firstly, the bacterial pellet was collected at 2000g for 10 min, then the total DNA was extracted and polymerase chain reaction (PCR) amplification of *P. distasonis* DNA was performed with the primer: F: 5′-GGA-CACGTCCCGCACTTTAT-3′; R: 5′-TTCTGAGAGGAAGGTCCCCC-3′. Next, the amplified products were carried out by Sanger sequencing, and the sequence was verified by NCBI blast.

2.6. Biomechanical test

The collected fresh SSTH tissue samples at 2, 4 and 8 weeks post-operatively were subjected to a biomechanical testing machine (Instron, USA). Excess ligaments and muscles around the SSTH tissue samples were carefully removed and immersed in 0.9% saline solution. Prior to the test, the supraspinatus muscle and tendon were wrapped in gauze and clipped to one end of the stretcher fixation device, while the humerus was

clipped to the other end. The stretching began when the initial displacement and load were zero, then the failure load and Young's modulus were recorded. All samples were failed at the SST attachment site, and no specimens were excluded (n = 4).

2.7. 3D imaging analysis

The specimens were fixed in 4% paraformaldehyde for 48 h and washed with phosphate buffer solution. Then the newly formed bone at the enthesis of the specimens were observed by X-ray microscopy (ZEISS Xradia 410 Versa). Scan parameters were as follows: resolution, 3.25 μm; beam energy, 18 KeV; exposure time, 600 ms; distance, 10 cm. Drangon software was used to reconstruct the 3-dimensional image. After circling the region of interest (ROI), which indicated the region of bone within the humeral head near the attachment of the tendon, we used VG Studio software to analysis the bone volume fraction (BV/TV), trabecular number (Tb.N), trabecular thickness (Tb.Th), and trabecular separation (Tb.Sp) of the ROI (n = 3 or 4).

2.8. Histological analysis

The collected postoperative specimens were fixed in 4% paraformaldehyde at 4 °C for 48 h, decalcified in 10% Ethylene Diamine Tetraacetic Acid (EDTA), dehydrated with graded alcohol and paraffin embedded. Each slide of specimens was 5-μm thickness, hematoxylin-eosin (H&E), toluidine blue (TB) and safranin O/fast green (SO/FG) staining were performed to observe the general morphology, the regenerated cartilage of healing tissue. To quantitatively evaluate the maturity of healing bone tendon, we used a modified maturing scoring system for tendon-to-bone according to a previously published method [28,30]. The scoring items include cellularity, continuity, bone ingrowth, fibrocartilage cells, tidemark, and the rating scale is 1–4. The scoring process was carried out in a blinded manner. The area and thickness of fibrocartilage layer and the mean optical density of proteoglycans (PG) were quantitatively measured according to a previously established protocol [31–34]. Briefly, the area of the fibrocartilage layer refers to the region between the proximal boundary of humerus and the distal boundary of SST. The length of the fibrocartilage layer is the distance between the upper and low point in the middle region of the fibrocartilage region. The thickness of the fibrocartilage layer was calculated by the fomula: Thickness = Area/Length. The area of proteoglycans was measured by Image-Pro Plus (IPP) (version 6.0), and the ratio of PG area to the total area of fibrocartilage layer is the mean optical density of PG (n = 3).

2.9. Statistical analysis

GraphPad Prism 6 was used for statistical analysis. All quantitative data were presented as mean ± standard deviation (SD), and $P < 0.05$ was considered to be statistically significant. Two-way analysis of variance with Bonferroni *post hoc* test was used to analyze the difference.

3. Results

3.1. Intermittent fasting improves biomechanical properties during rotator cuff repair in mice

Tensile test was performed to evaluate the effect of intermittent fasting on the biomechanical properties of repaired rotator cuff. As shown in Fig. 1B and C, the failure load and Young's modulus in the intermittent fasting group was higher than that in the AD libitum group at 2 and 8 weeks postoperatively, but significantly lower than that in the AD libitum group at 4 weeks postoperatively ($P < 0.05$).

3.2. Intermittent fasting improves tissue regeneration during rotator cuff repair in mice

Bone regeneration at the enthesis after rotator cuff injury was shown in Fig. 1A. The BV/TV in the intermittent fasting group had a slight increase at postoperative week 2, but had a significant decrease at postoperative week 4 when compared to the control group ($P < 0.05$). For other evaluation parameters, including Tb.Th, Tb.N, Tb.Sp, there was no statistical significance ($P > 0.05$) (Fig. 1D–G).

The histomorphology of the healed rotator cuff was evaluated by HE staining, and representative images were shown in Fig. 2A. At 2 weeks postoperatively, the insertion site in the AD libitum group was mostly healed with fibrovascular granulation tissue. But in the intermittent fasting group, fibrocartilage formation was observed at the enthesis with no tidal line, and there were numerous vascular transects in the bone region. The maturity score in the intermittent fasting group was significantly higher than that in the AD libitum group at 2 weeks after surgery (Fig. 2D). At postoperative week 4, the formation of tidemark in the fibrocartilage layer were observed in both groups, but the tissue in the AD libitum group was more organized than that in the intermittent fasting group, and the maturity score in the intermittent fasting group was significantly lower than that in the AD libitum group (Fig. 2D). At 8 weeks postoperatively, the fibrocartilage layer at the bone tendon insertion were remodeled to appear more normal fibrocartilage zone, and the larger areas of fibrocartilage and more organized tissue could be observed in the AD libitum group, and the maturity score in the AD libitum group was higher than that in the intermittent fasting group with no statistical significance (Fig. 2D).

The regeneration of fibrocartilage layer during the repair of rotator cuff was evaluated. TB staining, safranin O/fast green staining were used for the assessment of the fibrocartilage area and thickness, the proteoglycan deposition respectively. As shown in Fig. 2B, E and 2F, at postoperative week 2, the fibrocartilage area and thickness in the intermittent fasting group was significantly larger than that in the AD libitum group, and there was no significant difference at postoperative week 4 and 8. In the same way, the mean optical density of proteoglycan in the intermittent fasting group was significantly higher than that in the AD libitum group at 2 weeks postoperatively, and there was no significant difference at postoperative week 4 and 8 (Fig. 2C and G).

3.3. Intermittent fasting alters composition of the gut microbiome in mice

To explore the most significant microbe species that mediates the effect of intermittent fasting on rotator cuff repair, feces samples collected before and after surgery were used for 16S rRNA Microbiome sequencing. The results showed that intermittent fasting resulted in a significant decrease of community richness in fecal microbiota after fasting for one month, that is, the day of surgery, including the observed number of operational taxonomic units (OTU) and estimated OTU [Chao1 and ACE (abundance-based coverage estimator)] (Fig. 3A). The relative abundance of the microbial community composition at genus level between the AD libitum group and the intermittent fasting group were shown in Fig. 3B. In both the AD libitum group and the intermittent fasting group, there was no significant difference in the relative abundance of *Parabacteroides* with the prolongation of fasting time (Fig. 3C). However, in the intermittent fasting group, the content of *P. distasonis* decreased gradually with the prolongation of fasting time, and began to be significantly different after fasting for one month. And the amount of *P. distasonis* remained roughly constant over time in the AD libitum group (Fig. 3D). Therefore, we finally determined that *P. distasonis* was the species with the most obvious changes in intestinal flora of mice after fasting. The identification result of culture strain was *P. distasonis* as shown in Fig. S2, the sequence reading data.

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GAACCTTCATCCCTCACGCGACTTGGCTGGTTCAGCCTCTCGGC-CATTGACCAA-TATTCCTCACTGCTGCCTCCCGTAGGAGTTTGGTCCGTGTCTCAGTAC-CAATGTGGGGGACCTTCCTCTCAGAA. The Sanger sequence map and demonstration results of sample sequence in NCBI were shown in Figs. S2A and S2B.

3.5. Mice treated with P. distasonis impair biomechanical properties of repaired rotator cuff

We then explored the effect of *P. distasonis* on rotator cuff repair by gavage treatment of live or pasteurized *P. distasonis*, as well as an equivalent volume of sterile anaerobic PBS.

Tensile test was performed to assess the impact of *P. distasonis* on the biomechanical properties of repaired rotator cuff. As shown in Fig. 4B and C, at 8 weeks postoperatively, the failure load in the LPD-treated group was significantly lower than that in the vehicle group, and there was also significant difference between the LPD- and KPD-treated group. At postoperative week 2, the Young's modulus in the KPD-treated group was significantly lower than that in the vehicle group and the LPD-treated group. The above results indicated that the treatment of LPD can impair biomechanical properties during rotator cuff repair in mice.

3.6. Mice treated with P. distasonis show poor quality of rotator cuff healing

X-ray results indicated the impact of *P. distasonis* on the bone regeneration after rotator cuff injury (Fig. 4A). At 2, 4 and 8 weeks postoperatively, BV/TV in the LPD- and KPD-treated group were both significantly lower than that in the vehicle group. At 2 and 4 weeks postoperatively, Tb.Th in the LPD- and KPD-treated group was significantly lower than that in the vehicle group, and at postoperative week 8, there was also a significant difference between the vehicle group and the KPD-treated group ($P < 0.05$). At postoperative week 4 and 8, Tb.N in the LPD-treated group was significantly lower than that in the vehicle group. And Tb.Sp in the LPD-treated group was significantly higher than that in the vehicle group at postoperative week 2 and 4 ($P < 0.05$) (Fig. 4D–G).

Histologically, the maturity score in the LPD-treated group was significantly lower than that in the vehicle group and KPD-treated group at 2 weeks postoperatively. And at postoperative week 4, the maturity score in the LPD- and KPD-treated group were both significantly lower than that in the vehicle group ($P < 0.05$) (Fig. 5D). At 2 weeks postoperatively, the insertion site in the LPD-treated group was mostly healed with fibrovascular granulation tissue. But in the vehicle and KPD-treated group, fibrocartilage formation could be observed at the enthesis. At postoperative week 4, only fibrous scar tissue could be observed at the bone tendon interface in the LPD-treated group, but the fibrocartilage layer in the vehicle and KPD-treated group could be observed. At 8 weeks postoperatively, the fibrocartilage layer appears at the bone tendon insertion with no tidal line in the LPD-treated group. And in the vehicle and KPD-treated group, the fibrocartilage layer showed a more organized and mature state (Fig. 5A).

The regeneration of fibrocartilage layer could be observed in Fig. 5B. At postoperative week 4, the fibrocartilage area and thickness in the LPD-treated group was significantly lower than that in the vehicle group, and there was also a significant difference between the LPD- and KPD-treated group at postoperative week 2 and 4 (Fig. 5E and F). At 4 weeks postoperatively, the mean optical density of proteoglycan in the LPD- and KPD-treated group were both significantly lower than that in the vehicle group. To my surprise, the mean optical density of proteoglycan in the KPD-treated group was significantly higher than that in the vehicle group and LPD-treated group at 8 weeks postoperatively (Fig. 5C and G).

4. Discussion

The repair of rotator cuff injury is affected by various factors, including lifestyle and metabolic factors [5]. In this study, We evaluated the effect of periodic fasting on the repair of rotator cuff injury, and explored the role of intestinal flora in this process. The results have shown that intermittent fasting can promote repair of rotator cuff injury in the early postoperative period, and the intestinal flora especially a single species *P. distasonis* played an important role in it, which provided a new idea for the therapy of rotator cuff injury.

As prior studies shown, people tend to show a higher level of cognition and physiology in a food-deprived or fasted state [6]. Fasting includes calorie restriction, time-restricted feeding, and intermittent fasting, of which the latter is a combination of the former two. In recent studies, intermittent fasting has been shown to enhance energy metabolism, promote repair of damaged tissue and improve a range of age-related disorders, etc [7–9]. In our study, intermittent fasting has been shown to improve biomechanical properties during rotator cuff repair, promote regeneration of bone and fibrocartilage layer, including the fibrocartilage area and thickness, as well as the deposition of proteoglycan in the early postoperative period, meanwhile maturity score of histomorphology of the healed rotator cuff has also been significantly improved in the early postoperative period. However, at 4 weeks postoperatively, the biomechanical properties, the regeneration of bone and the maturity degree of the regenerated enthesis were significantly worse after fasting than that in the AD libitum group. So why does this happen? Then we hypothesized that it may be the changes of intestinal flora by intermittent fasting that played an important role in rotator cuff healing.

As we know, the gut flora has been recognized as an important role on human health [14,15]. There is considerable evidence suggested that intestinal flora plays an important role in the protection of intermittent fasting against disease, such as autoimmune diseases, diabetes-induced cognitive disorders and so on [10–13], including the progression of joint disease [16–20]. In our study, we found that intermittent fasting could result in a significant decrease of community richness in fecal microbiota after fasting for one month, especially the change of *P. distasonis* was most obvious before and after fasting. Therefore, *P. distasonis* was used to study the effect on repairing rotator cuff injury. The results indicated that oral treatment of LPD can weaken the biomechanical properties during repair of rotator cuff injury. In addition, the treatment of LPD also impaired the bone regeneration at the enthesis of rotator cuff, as well as the regeneration of fibrocartilage layer and the maturity of rotator cuff tissue. These results suggested that LPD may impair rotator cuff repair at any stage post-operatively. Prior studies have shown that *P. distasonis* can alleviate obesity and obesity-related dysfunctions [22]. Oral administration of *P. distasonis* has been shown to ease colitis [23]. Diabetes and fatty liver disease were also considered to be related to *P. distasonis* [24,25]. In these studies, the role of *P. distasonis* was beneficial, contrary to the fact that *P. distasonis* was not beneficial to the repair of rotator cuff injury in this study. In our study, *P. distasonis* was significantly reduced after fasting for one month, which may be an important reason why intermittent fasting promotes the repair of rotator cuff injury in the early postoperative period. As to why intermittent fasting resulted in poor bio-mechanical and histological properties of the healing enthesis at 4 weeks after surgery, we suspected that some bacteria may have changed at 4 weeks postoperatively. As shown in Fig. S3, *Clostridium_sp._Culture-54* increased significantly in the intermittent fasting group at 4 weeks postoperatively, which may be the reason for poor rotator cuff healing at 4 weeks postoperatively after fasting, although no studies on *Clostridium_sp._Culture-54* have been reported. However, taking all the parameters measured into account, KPD treatment in mice did not show significant effect on inhibiting rotator cuff healing, which indicated that the cellular components of *P. distasonis* have little effect on the repair of rotator cuff injury.

There were still some limitations in this study. First, the abundance of *P. distasonis* was not measured after oral administration of LPD or KPD.

Second, the role of metabolites derived from the gut microbiota in the repair of rotator cuff was not studied, which will be researched in the future.

In conclusion, this study was the first time to investigate the effect of intermittent fasting on the healing of rotator cuff injury, and the role of intestinal flora in it, which provided a new therapeutic approach for the healing of rotator cuff injury.

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Authorship & conflicts of interest statement

D.-Q.X. and S.-S.X. conceived and designed this study. S.-S.X. wrote the manuscript. S.-S.X., C.-B.G., T.-M.H., Y.-Q.L. and F.-F.Y. did the experiments, analyzed the data and prepared the figures. The authors have no conflict of interests to declare.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jot.2022.09.006.

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