Clostridium Butyricum Alleviates the Gut Microbiota Alteration Induced Bone Loss after Bariatric Surgery by Promoting Bone Autophagy

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d) Abbreviations:  
BD-bone density  
BV/TV- bone volume over total volume
C. butyricum- Clostridium Butyricum

DXA- Dual Energy X-Ray Absorptiometry

GI- gastrointestinal

QIIME- Quantitative Insights into Microbial Ecology

RYGB- Roux-en-Y gastric bypass

SG- Sleeve gastrectomy

SMI- stricture model index

Tb.Th- trabecular thickness

Tb.N- trabecular number

Tb.Sp- trabecular sepacing

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Abstract

Bariatric surgery is the most common and effective treatment for severe obesity, while these bariatric procedures always display detrimental effects on bone metabolism with underlying mechanisms. This study aims to investigate the skeletal response to bariatric surgery, and explore whether *Clostridium butyricum* (*C. butyricum*) alleviates the gut microbiota alteration induced bone loss after bariatric surgery. Consequently, male SD rats respectively received Roux-en-Y gastric bypass (RYGB) and Sleeve gastrectomy (SG) surgery followed with body weight recording. The bone loss after bariatric surgery was further determined by Dual Energy X-Ray Absorptiometry (DXA), micro-CT measurement, histological analyses and western-blot. Besides, 16S rDNA gene sequencing was performed to determine the gut microbiota alteration after surgery, and intervention with fecal microbiota from RYGB donor was conducted in obese SD rats followed with *C. butyricum* administration. Accordingly, rats in RYGB and SG group maintained sustained weight loss, while DXA and Micro-CT measurement further demonstrated a significant bone loss after bariatric surgery. Besides, histological and western-blot analyses validated enhanced osteoclastogenesis, inhibited osteoblastogenesis and defective autophagy after surgery. The 16S rDNA gene sequencing suggested significant alteration of gut microbiota composition in RYGB group, and intervention with fecal microbiota from RYGB donor further determined that this kind of alteration contributed to the bone loss after RYGB group. Meanwhile, *C. butyricum* might protect against this postoperative bone loss by promoting osteoblast autophagy. In summary, this study
suggests novel mechanisms to clarify the skeletal response to bariatric surgery, and provide potential candidate for the treatment of bone disorder among bariatric patients.

Significance Statement

The significance of this study is the discovery of obvious bone loss and defective autophagy after bariatric surgery. Besides, it is revealed that gut microbiota alterations could be the reason of impaired bone mass after bariatric surgery. Furthermore, *C. butyricum* could alleviate the gut microbiota alteration induced bone loss after bariatric surgery by promoting osteoblast autophagy.

Key words: bariatric surgery; bone loss; autophagy; gut microbiome; *Clostridium butyricum*

Introduction

Obesity has been a global epidemic with increasing prevalence in adults, adolescents and even children (Pizzorno, 2016). Individuals with severe obesity always suffer from several health complications and even have shorter life expectancy than normal people (Pucci and Batterham, 2019). Nowadays, bariatric surgery such as sleeve gastrectomy (SG) and Roux-en-Y gastric bypass (RYGB) has been regarded as the most effective treatment for people suffering obesity (Rahman and Azagury, 2017). Specifically, RYGB directly regulates the gastrointestinal (GI) anatomy and nutrient flow (Pucci and Batterham, 2019), while SG mainly implicates with the resection of gastric fundus (Bredella et al., 2017). Though both of them could achieve the weight
loss, RYGB is reported to bring about greater body weight loss and lower risk of having diabetes in the future (Salminen et al., 2018). More importantly, it is documented that adverse outcome with significant bone loss will arise after the surgery of RYGB or SG despite on the supplementation of calcium and vitamin D (Scibora et al., 2015; Pizzorno, 2016; Bredella et al., 2017). Especially, people having RYGB surgery have shown an obvious alteration in the contents of bone turnover markers (Ivaska et al., 2017; Kim et al., 2020), which further validates the bone loss after bariatric surgery by bone remodeling mechanisms. However, the loss of bone mass in people having SG surgery is reported to be relative less than that in RYGB group (Bredella et al., 2017).

Recently, growing evidence has shown that gut microbiota is closely related to the pathogenesis of several disorders, such as the Alzheimer’s and Parkinson’s disease (Compare et al., 2016; Sun and Shen, 2018). More interestingly, gut microbiota is further reported to have close connection with bone metabolism, and earliest researches have demonstrated that the overgrowth of gut microbiota might elevate the risk of generating osteoporosis (Chen et al., 2017). As for the potential mechanisms by which gut microbiota mediates bone metabolism, it is believed that gut microbiome may induce the formation of bone via promoting the synthesis of insulin-like growth factor (IGF)-1, or affect the process of vitamin D metabolism (Pacifici, 2018). Besides, it is also documented that gut microbiota might participate in the regulation of bone metabolism through immune system (Chen et al., 2017). More importantly, bariatric surgery has been documented to significantly influence
several compositions of gut microbiota (Shao et al., 2017), and we thus wonder that this kind of gut microbiome alteration might contribute to the impaired bone metabolism after bariatric surgery (Yu, 2014).

*Clostridium butyricum* (*C. butyricum*) is a strict gram-positive anaerobic bacterium widely distributing in the gut of organisms including human and animals. The non-toxigenic strain of *Clostridium butyricum* has been used as probiotics to treat digestive system disease in Asia (Li et al., 2018a) by changing microbiota composition in digestive tract. Recently, researchers further suggest the beneficial effect of *Clostridium butyricum* on bone related disease, and it has been proved that oral administration of ID-CBT5101 (tyndallized *Clostridium butyricum*) can relieve the osteoarthritis symptoms in monosodium iodoacetate treated rats by regulating the expressions of inflammatory cytokines and bone metabolism factors (Sim et al., 2018). Consequently, the purpose of this study is to investigate whether intestinal flora changes contribute to the bone loss after bariatric surgery and explore if *Clostridium butyricum* could alleviates the gut microbiota alteration induced bone loss after bariatric surgery with specific mechanisms.

**Materials and methods**

**Ethical statement**

The experimental protocol was approved by the Animal Ethics Committee of China Medical University (Approval code: No. 2019099), and followed the National Institutes of Health guide for the care and use of Laboratory animals. All effort has
been made to minimize the suffering of the animals.

**Bariatric Surgical protocol**

Male SD rats (8 weeks old) were housed at the temperature of 25±1°C and humidity of 44% to 55%. All rats were allowed to get *ad libitum* access to water and food with a 12-hour light and dark cycle. Rats were anesthetized by 3% pentobarbital sodium after the fasting overnight and divided into 4 groups (n=6 per group): SGS (SG sham), SG, RYGBS (RYGB sham) and RYGB group. The SG, RYGB and their sham surgery were conducted according to the previously published researches (Bruinsma et al., 2015; Kaufman et al., 2019; Ying et al., 2019). Afterwards, body weight change of each group was monitored every week for consecutive 10 weeks. At the end of experiment, rats were sacrificed along with the collection of fecal, serum and limb bone tissue in each group.

**16S rDNA gene sequencing**

The fecal samples from each group were collected and stored at -80 °C. The DNA from different samples was then extracted using the DNA extraction kit (D4015, Omega, USA) according to the manufacturer’s instructions. Basically, the V3-V4 region of 16S rDNA genes was amplified with the performance of polymerase chain reaction (PCR). The primers used for PCR were listed as follows:

Forward primer 341F: 5’-CCTACGGGNGGCWGCAG-3’;

Reverse primer 805R: 5’-GACTACHVGGGTATCTAATCC-3’;

The PCR product was then confirmed and purified for further sequencing. Accordingly, samples were sequenced on an Illumina NOvaSeq platform based on the
manufacture’s recommendation, and the analyses on the 16S rDNA gene sequences were performed using the Quantitative Insights into Microbial Ecology (QIIME) to illustrate the α diversity and β diversity of gut microbiota. Briefly, the alpha diversity indicated the species complexity among one sample, while the β diversity analysis was applied to assess the differences among different samples in species complexity. Furthermore, the alterations of bacteria taxa from phylum to genus were identified in different group of fecal sampling.

**Fecal Microbiota intervention and Clostridium butyricum administration**

Male specific pathogen free (SPF) SD rats (4 weeks old) were adaptively fed for a week and randomly divided into 3 groups (n=6 per group), including the sham donor (sham D), RYGB donor (RYGB D) and RYGB D+C. butyricum (CB) group. The SG group was dismissed owing to the results of 16S rDNA sequencing. During experimental modeling, fecal from sham and RYGB donor was administrated to the pseudo germ-free obese rats (achieved by high fat diet and antibiotic treatments) by coloclysis with indicated amount of fecal suspension (1.6 ml/kg). Besides, dissolution of clostridium tyriformis capsule in phosphate buffer saline (PBS) was performed to prepare *Clostridium butyricum*, and it was further administrated to RYGB D+CB group of rats by coloclysis at the concentration of 2.5×10⁸ CFU/kg after RYGB fecal microbiota intervention. Afterwards, body weight of each group was recorded once a week for consecutive 10 weeks and sampling of serum and limb bone was collected for further study at the end of experiments.
ELISA

The content of carboxy-terminal opeptide of type I collagen (CTX-I) and osteocalcin (OCN) in serum was determined by ELISA according to the manufacturer’s instructions (USCN Life Science, Wuhan, China).

Dual Energy X-Ray Absorptiometry (DXA)

The bone density (BD) of whole body was measured using Dual Energy X-Ray Absorptiometry (DXA). In details, measurement of BD was made with a Dual energy x radio bone density analyzer (GE Healthcare, Waukesha, WI, USA), and the testing of quality control model was firstly conducted for blanking. Afterwards, experimental rats were anaesthetized with 3.0% pentobarbital sodium solution and then placed onto the machine for DXA scanning using the standard procedures based on the manufacturer’s instruction. Each rat received three times of DXA scans and rat was repositioned by the same operator for each measurement. Moreover, BD was calculated as previously published (Hussain and Han, 2019).

Micro-CT measurement

The microstructure of trabecular bone was determined by Micro-CT (Skyscan1174 X-Ray Microtomograph, Bruker, Belgium) as previously described (Luo et al., 2016). In particular, the trabecular bone region of tibia was firstly defined at about 1.5 mm away from the growth plate of proximal tibia and scanned by a micro-CT system with high resolution for totally 125 slices. Besides, scan resolution was set as 12 μm. Subsequently, the software in the Micro-CT system including the RO-I, N-Recon and CT-AN were used to measure the parameters of trabecular bone.
microstructure including the bone volume over total volume (BV/TV), trabecular thickness (Tb.Th), trabecular number (Tb.N), trabecular separation (Tb.Sp) and structure model index (SMI).

Hematoxylin and eosin (H&E)-staining

Bone tissue samples from the left lower limbs were fixed in 10% formalin for 24 hours followed with paraffin embedding. The fixed bone tissue was sectioned at the thickness of 5 μm. Sections were subsequently stained with hematoxylin (H8070, Solarbio) and eosin (A600190, Sangon biotech) after being deparaffinized and rehydrated. Following the dehydration in a graded series of ethanol and xylene, images of sections were captured by microscope at the magnification of 200 (OLYMPUS, BX53, Japan).

Alkaline phosphatase (ALP) staining

Bone tissue samples from the left lower limbs were fixed and paraffin embedded as described in the method of H&E staining. After slicing and dewaxing, ALP staining was performed on sections using the ALP detection kit (DE0001, LEAGENE, Beijing) according to the manufacture’s instruction. The hematoxylin (H8070, Solarbio) staining was further conducted for 5 minutes followed with image capture. Particularly, images were obtained by microscope at the magnification of 200 (OLYMPUS, BX53, Japan).

TRAP staining

Sections were prepared as previously described (Luo et al., 2016). More specifically, they were fixed, paraffin embedded, and sliced at the thickness of 5 μm.
After being deparaffinized and rehydrated, TRAP staining was further performed on sections in accordance to the instructions of manufacturer (387A-1KT, Sigma, USA). Subsequently, sections were counterstained with hematoxylin (H8070, Solarbio) for another 3 minutes. Images were next captured using the microscope at the magnification of 200 (OLYMPUS, BX53, Japan), and TRAP positive cells were defined as osteoclasts.

**Immunohistochemical staining**

Sections were obtained as described in the method of H&E staining, and incubated with the 0.1 M citrate antigen retrieval solution for 10 minutes to get antigen restored. After additional rinsing in PBS, sections were subjected to incubation of 3% H$_2$O$_2$ (10011218, Sinopharm Chemical Reagent) at room temperature for 15 minutes. The incubation of normal goat serum (SL038, Solarbio) was further performed at room temperature for another 15 minutes to guarantee the specificity of staining. Subsequently, sections were treated with the primary antibodies against RANKL (D263508-0025, Sangon Biotech, Shanghai), OPG (D221075-0025, Sangon Biotech, Shanghai) and Beclin 1 (A7353, Abclonal, Wuhan) at the dilution ratio of 1:50 at 4 °C overnight. Next, sections were subjected to the treatment of HRP conjugated secondary antibody (#31460, 1:500, Thermofisher, USA) for 1 hour at 37 °C. The staining of DAB and hematoxylin was further performed followed with image capture by microscope (OLYMPUS, BX53, Japan).

**Sirius Red staining**

Sections were prepared as previously described. The Sirius Red staining was
performed with the incubation of saturated Sirius red picric acid (G1471, Solarbio, China) for 5 minutes followed with sealing. Images of the pathological sections were captured using the microscope at the magnification of 200 (OLYMPUS, BX53, Japan), suggesting the changes in the content of bone collagen.

**Western-blot analysis**

The extraction of total protein from bone tissue samples were performed using RIPA lysis buffer containing PMSF (1mM) followed with protein quantification. Equal amount of protein samples were loaded on the sodium dodecyl sulfate polyacrylamide gel electrophoresis for separation and then transferred to a PVDF membrane. The membrane was incubated with primary antibodies against LC3II/I (1:1000, A19665, Abclonal, Wuhan), p62 (1:1000, A19700, Abclonal, Wuhan) and Beclin 1(1:1000, A7353, Abclonal, Wuhan) at 4 °C overnight after being blocked with 5% skim milk for 1 hour. The PVDF membrane was subsequently rinsed and then treated with the indicated secondary antibody for 1 hour at room temperature. Finally, the bands of indicated proteins were visualized by enhanced chemiluminescence substrate (PE0010, Solarbio, China) and their abundance was analyzed by Gel-Pro-Analyzer (Media Cybernetics, Bethesda, MD, USA). Internal reference was GAPDH.

**Statistical analysis**

The results were presented as Mean ± SD and analyzed by GraphPad Prism. Student t test was used to analyze the difference between just two groups. Comparisons between groups were made by using one-way ANOVA followed by
Tukey’s tests. Any value of $p$ less than 0.05 were considered statistically significant.

**Results**

*Effect of bariatric surgery on body weight*

As shown in Figure 1A, male SD rats respectively received Roux-en-Y gastric bypass (RYGB) and Sleeve gastrectomy (SG) surgery followed with body weight recording (Figure 1B), and it was indicated that baseline of body weight of different groups has no significant difference before the surgery. Besides, it was found that body weight in each group was gradually decreased in the first 3 weeks after operation and steadily elevated in the rest of 7 weeks, while rats in RYGB group gained significant body weight loss since the 2\textsuperscript{nd} week after surgery when compared with RYGBS group (RYGBS, 2\textsuperscript{nd} week, $511.57\pm19.99$ vs RYGB, 2\textsuperscript{nd} week, $418.92\pm11.47$, $P<0.0001$). Meanwhile, the difference between SGS and SG group became significant since the 4\textsuperscript{th} week after surgery (SGS, 4\textsuperscript{th} week, $532.02\pm41.97$ vs SG, 4\textsuperscript{th} week, $478.3\pm31.8$, $p<0.05$). Furthermore, it was determined that both RYGB and SG surgery could result in sustained and significant body weight loss when compared with their sham group at the 10\textsuperscript{th} week after surgery (RYGBS, $649.73\pm25.16$ vs RYGB, $502\pm16.13$, $p<0.0001$; SGS, $634\pm50.06$ vs SG, $554\pm35.56$, $p<0.05$). However, rats in RYGB group achieved greater body weight loss than that in SG group after the surgery (RYGB, $502\pm16.13$ vs SG, $554\pm35.56$, $p<0.05$).

*Effect of bariatric surgery on bone turn over markers*

As shown in Table 1, the content of carboxy-terminal opeptide of type I collagen (CTX-I) and osteocalcin (OCN) was determined by ELISA. In particular, CTX-I is an
important marker for bone resorption while OCN is for bone formation. It was indicated that CTX-I production was significantly elevated after RYGB or SG surgery. However, the OCN content was significantly reduced in RYGB or SG group of rats.

Effect of bariatric surgery on bone metabolism in rats

The bone density (BD) of whole body was analyzed using dual-energy X-ray absorptiometry (DXA). As shown in Figure 2A, rats received RYGB surgery showed significant lower BD when compared with their sham group. The trabecular bone microstructure of tibia in each group was determined by Micro-CT in Figure 2B, and it was proved that RYGB and SG group of rats both showed a lower BV/TV, Tb.N and higher Tb.Sp than that in their sham group. Nevertheless, no significant difference was found in the alteration of Tb.Th. Besides, the SMI was determined to be significantly elevated in both RYGB and SG group. Result of H&E staining further indicated the significant pathological changes in the bone tissue of RYGB and SG rats (Figure 2C). In addition, activities of osteoblast and osteoclast were respectively measured by ALP and TRAP staining, and it was determined that RYGB and SG group showed the significantly decreased area with ALP staining and remarkably increased area with TRAP staining when compared with their sham group (Figure 2D-E). Analysis of Sirius red staining indicated a significant decrease in bone collagen fiber content with less red staining, which further validated the impaired bone metabolism in rats after bariatric surgery (Figure 2F). Furthermore, the expression levels of osteoclastogenesis related protein RANKL and anti-osteoclastogenic related factor OPG were determined by immunohistochemical
staining. It was indicated that significant up-regulated expression of RANKL and down-regulated OPG abundance was found after the bariatric surgery (Figure 2G).

Effect of bariatric surgery on osteoblast autophagy in rats

The western-blot analyses (Figure 3A) indicated a significant decrease in LC3II/I and Beclin 1 expression after the bariatric surgery. However, the protein level of p62 was found to be significantly elevated in both RYGB and SG rats. We thus believed that bariatric surgery could result in the defective bone autophagy in rats. It should be noted that osteoclasts/osteoblasts/osteocytes mediated autophagy all have been documented to implicates with the occurrence of bone loss, and they have specific distributions in bone tissue based on their functions. As a consequence, immunohistochemical staining of Beclin 1 was further performed to identify the bone cell type that mediated autophagy in this study. As shown in Figure 3B-C, though positive staining of beclin 1 could be found in osteoblasts, osteoclasts and osteocyte, beclin-1 positive osteoblasts were found to be significantly decreased in bone tissues of RYGB/SG group, which suggests that bariatric surgery could lead to defective osteoblast autophagy in rats.

Effect of bariatric surgery on gut microbiome in rats

The 16S rDNA gene sequencing was performed to investigate the alteration in gut microbiome of SD rats after bariatric surgery. Sequences with 97% similarity were assigned to the same operational taxonomic units (OTUs) by Vsearch. As shown in Figure 4A, the shared and exclusive OTUs among samples were presented as a Venne diagram to suggest the similarities and overlaps between the gut microbiome of
different groups. The shannon index in Figure 4B was analyzed to show the alpha diversity of gut microbiota. It was proved that the shannon index was significantly reduced in RYGB group when compared with its sham group, indicating that RYGB significantly reduce the species diversity within samples. Besides, beta diversity was further calculated by PCoA analysis of unweighted UniFrac distance using QIME software, and we found that there was obvious distance between RYGB/SG and their sham group (Figure 4C), suggesting the reasonable grouping and sample credibility in this study. Furthermore, the microbial community structure of gut microbiota at phylum and genus levels was indicated as heatmap analysis according to their relative mean abundance (Figure 4D and 4F). It was revealed that the relative abundance of bacterial phyla and genus was differentially regulated in RYGB group rather than SG when compared with their sham group. More specifically, relative mean abundance of top 4 bacterial phyla including the *Firmicutes*, *Bacteroidetes*, *Proteobacteria* and *Verrucomicrobia* was separately analyzed (Figure 4E). We found that the percentage of *Firmicutes* in RYGB group was apparently lower than that in its sham group (RYGBS, 82.37±4.31 vs RYGB, 16.82±10.37, p<0.001), while the proportions of *Bacteroidetes*, *Proteobacteria* and *Verrucomicrobia* were found to be significant higher than that in RYGBS group (p<0.001). At the genus level, the top 8 bacterial genera were respectively assessed and we found 4 of them (*Porphyromonadaceae_unclassified*, *Bacteroides*, *Escherichia* and *Akkermansia*) to be differentially up-regulated and others (*Ruminococcaceae unclassified*, *Lachnospiraceae unclassified*, *Ruminococcus* and *Firmicutes unclassified*) to be
down-regulated (Figure 4G) in RYGB group.

Effect of C. butyricum and RYGB fecal microbiota intervention on body weight in pseudo germ-free obese rats

Considering the significant alteration of gut flora in RYGB group rather than SG group, RYGB fecal microbiota intervention was performed followed with the C. butyricum administration on pseudo germ-free obese rats (Figure 5A). The body weight change was recorded every week after the fecal microbiota intervention. Accordingly, rats in RYGB D group showed a lower body weight from the 14th week, and C. butyricum intervention significantly protected against this kind of body weight loss since the 16th week (Figure 5B).

Effect of RYGB fecal microbiota intervention and C. butyricum administration on bone turnover markers

As shown in Table 2, content of carboxy-terminal opeptide of type I collagen (CTX-I) and osteocalcin (OCN) was determined by ELISA. It was indicated that the CTX-I production was significantly elevated after RYGB fecal microbiota intervention and reduced after C. butyricum administration. However, the OCN content was determined to be significantly reduced in RYGB D group while elevated after C. butyricum administration.

Effect of the C. butyricum on RYGB fecal microbiota intervention induced impaired bone metabolism in pseudo germ-free obese rats

The bone density after the RYGB fecal microbiota intervention was determined by dual-energy X-ray absorptiometry (DXA). It was indicated that rats in RYGB D
group possessed significantly reduced BD when compared with sham group. Besides, BD was significantly elevated after the *C. butyricum* administration (Figure 6A). The microarchitectural bone parameters including the BV/TV, Tb.Th, Tb.N, Tb.Sp and SMI were assessed using the in vitro micro-CT. As shown in Figure 6B, rats in RYGB D group showed a much lower, BV/TV, Tb.N, and Tb.Th along with higher Tb.Sp when compared with sham group. The significantly elevated SMI further confirm that the RYGB fecal microbiota intervention induced bone loss in obese rats. Moreover, treatment of *C. butyricum* was found to significantly reverse the effect on BV/TV, Tb.Th and SMI, suggesting that *C. butyricum* might protect against the bone loss in RYGB D rats. The H&E staining was performed to detect the pathological alteration in bone tissues after the RYGB fecal microbiota intervention, and we determined that *C. butyricum* could significantly alleviated the pathological changes in RYGB D group (Figure 6C). The reduced osteoblast activity and elevated osteoclast activity were found after the RYGB fecal microbiota intervention according to the ALP and TRAP staining (Figure 6D-E), while administration of *C. butyricum* significantly compensated this effect on the contrary. The Sirius Red staining in Figure 6F suggested a loss of bone collagen content after the RYGB fecal microbiota intervention, but *C. butyricum* treatment significantly increased the collagen content on the contrary. The osteoclastogenesis related protein RANKL and anti-osteoclastogenic factor OPG were further analyzed by immunohistochemical staining. Results in Figure 6G indicated a significant augment in RANKL expression and attenuation in OPG expression after RYGB fecal microbiota intervention.
However, *C. butyricum* treatment displayed an inverse effect on these alterations.

*Effect of the C. butyricum on RYGB fecal microbiota intervention induced* osteoblast *in pseudo germ-free obese rats*

The western-blot analysis in Figure 7A-C determined the down-regulated protein levels of LC3II/I and Beclin1 as well as the up-regulated p62 expression in bone tissue after the RYGB fecal microbiota intervention, which suggested the defective bone autophagy. Moreover, *C. butyricum* treatment could promote the autophagy with the reversal of these changes. In line with Figure 3B, immunohistochemical staining of Beclin 1 was further performed to identify the bone cell type that mediated autophagy in this study. As shown in Figure 7D-E, though positive staining of beclin1 could be found in osteoblasts, osteoclasts and osteocyte, beclin-1 positive osteoblasts were found to be significantly decreased in bone tissues of RYGB D group and increased after *C. butyricum* treatment, which suggests that *C. butyricum* might alleviate the gut microbiota alteration induced bone loss after bariatric surgery by promoting osteoblast autophagy

**Discussion**

It has been suggested that increased skeletal fragility and higher risk of fracture are closely related to the bariatric surgery (Nakamura et al., 2014), and prospective studies have indicated that significant bone loss is always accompanied with this kind of surgery related weight loss, proving that bariatric surgery has a profound effect on
bone outcomes (Scibora, 2014). In this study, it was determined that bariatric surgery could cause significant bone loss based on the DXA and micro-CT analysis. Moreover, the acceleration of TRAP/CTX-I-mediated bone resorption and the impaired ALP/OCN-mediated bone formation was also found after the bariatric surgery, which confirms the defective bone metabolism after the bariatric surgery. Actually, changes in bone metabolism after bariatric surgery are not surprising. It is reported that bariatric surgery such as RYGB and SG surgery both greatly reduce the intragastric volume, the amount of food intake and weaken the function of digestive tract, which will further accounts for the restricted absorption of vitamins and calcium afterwards. Besides, it should be noted that alterations in vitamins and calcium absorption has shown essential effect on bone metabolism (Panda et al., 2004). More interestingly, this study further demonstrated the defective bone autophagy together with bone loss after bariatric surgery. Of note, osteoclasts/osteoblasts/osteocytes mediated autophagy all have been documented to participate in the occurrence of bone loss and they have specific distributions in bone tissue based on their functions. Specifically, it is documented that osteoclasts are group of multinuclear cells that are located at the interface of bone tissue to absorb bone and finally leave a single marrow cavity (Cappariello et al., 2014; Juan et al., 2018). Osteoblasts are always characterized with the location on periost as closely arranged mononuclear cells (Roeder et al., 2016; Juan et al., 2018). As for osteocytes, they mainly distribute within mineralized matrix with the most abundant cell population (Kalajzic et al., 2013). As a consequence, immunohistochemical staining of Beclin 1 was further performed to identify the bone
cell type that mediated autophagy in this study, and it is validated that osteoblasts are mainly responsible for this kind of disorder. Actually, it has been reported that rapamycin-induced autophagy could significantly relieve the severity of senile osteoporosis (Luo et al., 2016), which suggests the beneficial effect of promoted autophagy on abnormal bone loss. Moreover, the osteoblast mediated defective autophagy has also been determined to cause obvious bone loss and results in the impairment of bone homeostasis (Li et al., 2018b), which is consistent with our findings.

Though cause of bone loss after bariatric surgery is multifactorial, it has been suggested that gut microbiome changes might be related to the bone loss after bariatric surgery (Stein and Silverberg, 2014; Ibáñez et al., 2019). Thus, 16S rDNA gene sequencing was further performed in this study with significant alterations observed in gut microbiome of RYGB group when compared to its sham group. As we know, our gastrointestinal tract has the greatest amounts of microorganisms that are tightly associated with regulation of human nutrition, metabolism and immune system function. More importantly, bariatric surgery is a basic method about anatomic rearrangements in gastrointestinal tract and this kind of alteration might further result in the changes of gut microbial community (Anhe et al., 2017). Besides, strong evidence has shown that gut microbiome displays a profound effect on bone homeostasis (Pacifici, 2018). It is reported that microbiota implicates with the regulation of bone growth and bone mass acquisition through multiple mechanisms. For example, it is documented that gut microbiota affects the absorption of calcium
and phosphate by influencing the fermentation of lactose and fructose (Daguet et al., 2016). Meanwhile, gut microbiota could promote the formation of bone via inducing the generation of a critical factor IGF-1 to maintain the bone health (Yan et al., 2016). Additionally, microbiota might affect the levels of vitamin D metabolites or steroid hormones to influence the bone cell function (Charles et al., 2015). In this study, fecal microbiota intervention was conducted using the fecal samples from RYGB donors to investigate whether gut microbiome changes contribute to the bone loss after bariatric surgery. According to the results of DXA and Micro-CT measurements, it was figured out that RYGB fecal microbiota intervention could result in the significant bone loss in experimental rats when compared to its sham group. Moreover, significantly up-regulated osteoclast activity (termed as TRAP staining) and down-regulated osteoblast activity (termed as ALP staining) were also determined in RYGB D group of rats, suggesting the impairment of bone remodeling after fecal microbiota intervention. It is well established that bone is a dynamic tissue with two important processes including the remodeling and modeling to maintain bone homeostasis and regulate bone metabolism (Raggatt and Partridge, 2010). Especially, impairments of these two processes can cause diseases like osteoporosis. It should be noted that bone remodeling is mainly responsible for the removal and repair of destructive bones with the assistance of osteoblasts and osteoclasts (Kylmaoja E Fau - Nakamura et al., 2016). Generally, it is tightly coordinated by the osteoclast-mediated bone resorption and the subsequent osteoblast-modulated bone formation. Osteoblasts are group of bone-forming cells with several critical roles in bone remodeling such as the
regulation on osteoclastogenic factors, generation of bone matrix proteins and the mineralization of bone (Karsenty, 2008), while osteoclasts mainly contribute to the bone resorption. More importantly, osteoblasts and osteoclasts could communicate with each other via indicated pathways. For instance, osteoblasts have been reported to affect formation, differentiation and apoptosis of osteoclasts through the OPG/RANKL/RANK signaling (Chen et al., 2018). Previous studies have indicated that RANKL could function as an activator of osteoclastogenesis, while OPG inhibits it (Bonnet et al., 2019). In accordance with this study, significantly elevated RANKL expression and reduced OPG abundance were determined after RYGB fecal microbiota intervention, further validating the acceleration of osteoclastogenesis in RYGB D group. In general, the present findings indicate that gut microbiota alteration accounts for the bone loss after the bariatric surgery and enrich the mechanism of abnormal bone metabolism after the surgery.

Interestingly, the administration of C. butyricum after fecal microbiota intervention showed significant protective effect on these bone abnormalities, suggesting the critical role of C. butyricum on bone metabolism. To the best of my knowledge, C. butyricum belongs to the family of gram-positive obligate anaerobic bacillus, and it widely distributes in soil, animal and human intestines. Non-toxigenic strain of C. butyricum could be used as a probiotics in some Asia countries (Duncan Sh Fau - Hold et al., 2002), and it also has been proved to possess strong ability to alleviate intestinal flora disorders by promoting the growth of probiotics, such as Bifidobacterium, and Lactobacillus (Hagihara et al., 2018). Accordingly, probiotics
Lactobacillus acidophilus is documented to be able to inhibit the bone loss in osteoporotic mice by mediating the balance between Treg and Th17 cells (Dar et al., 2018). In addition, the beneficial effect of Bifidobacterium on bone metabolism also has been revealed in the model of experimental periodontitis via regulating the RANKL/OPG pathway (Oliveira et al., 2017). Moreover, C. butyricum has shown an important role in maintain the regular function of intestine epithelial barrier (Li et al., 2018a), which contributes a lot to the gut nutrition metabolism especially the absorption of calcium and vitamin D, and further implicates with regulation of bone health (Masuyama, 2014; Casselbrant et al., 2020). Besides, C. butyricum is also documented to display positive effects on bone metabolism via regulating gut microbiome and their metabolic short chain fatty acids (SCFAs), such as acetate, propionate, and butyrate (Zaiss et al., 2019). Actually, SCFAs have a momentous role in the regulation of musculoskeletal system, and some SCFAs is reported to regulate osteoclast differentiation and effectively prevent pathological alterations in bone mass (Montalvany-Antonucci et al., 2019). More importantly, the promoted osteoblast autophagy was also observed after the C. butyricum intervention in RYGB D group. Recent study has shown that osteoblast autophagy has been suggested as a potential therapeutic strategy against bone disorders, and it is documented that the enhanced autophagy of osteoblasts might alleviate remarkable bone loss by the mediation of SIRT1 and PI3K/AKT/mTOR signaling (Yang et al., 2019). However, effect of C. butyricum on autophagy has not been extensively explored, which further suggests the novelty of this study.
In summary, this study indicates a significant bone loss with defective autophagy after bariatric surgery. Besides, it is found that gut microbiota alterations could be one of the reasons of the impaired bone metabolism after the bariatric surgery. Furthermore, the \textit{C. butyricum} alleviates the gut microbiota alteration induced bone loss after the bariatric surgery by promoting bone autophagy.

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**Authors’ contributions:**

Participate in research design: Xueying Shang and Jian Du

Conducted Experiments: Xueying Shang, Xiaolei Zhang, Cen Du and Zhuoqi Ma

Performed data analysis: Shi Jin, Na Ao and Jing Yang

Wrote or contributed to the writing of manuscript: Xueying Shang and Jian Du

**Conflicts of Interests**

No author has an actual or perceived conflict of interest with the contents of this article

**References**


Bone 95:47-54.


Li H, Gong Y, Xie Y, Sun Q and Li Y (2018a) Clostridium butyricum protects the epithelial barrier by maintaining tight junction protein expression and regulating microflora in a murine model of dextran sodium sulfate-induced


Footnote

a) This research was funded by the Basic Research Project of the Educational Department of Liaoning Province [Grant No. JC2019022].
Legends

Table 1 Effect of bariatric surgery on bone turnover markers

All values are expressed mean ± SD (n = 6). Compared with RYGBS group: #P<0.05, ##P<0.01, ### P<0.001, #### P<0.0001; Compared with SGS group: *P < 0.05, **P < 0.01, ***P < 0.001, **** P<0.0001.

Table 2 Effect of RYGB fecal microbiota intervention and *C. butyricum* administration on bone turnover markers

All values are expressed mean ± SD (n = 6). Compared with sham D group: #P<0.05, ##P<0.01, ### P<0.001, #### P<0.0001; Compared with RYGB D group: *P < 0.05, **P < 0.01, ***P < 0.001, **** P<0.0001.

Figure 1 Body weight alterations after bariatric surgery. (A) Experimental designing was presented as flowchart. SD rats were randomized to have RYGB or SG surgery, and body weight alterations were measured every week (B). Data are expressed as mean ± SD of n=6. Compared with RYGBS group: #P<0.05, ##P<0.01, ### P<0.001, #### P<0.0001; Compared with SGS group: *P < 0.05, **P < 0.01, *** P<0.001, **** P<0.0001.

Figure 2 Effect of bariatric surgery on bone metabolism in rats. (A) The bone density of whole body was determined by using dual-energy X-ray absorptiometry (DXA). (B) The trabecular bone microstructure of tibia in each group was determined by Micro-CT with the analysis of BV/TV, Tb. Th, Tb. N, Tb.Sp and SMI. (C) The pathological alteration of each group was determined by H&E staining. Scale bar: 100 μm. The activities of osteoblast and osteoclast in each group were respectively
analyzed by ALP staining (D) and TRAP staining (E). Scale bar: 100 μm. (F) The content of bone collagen of each group was assessed by Sirius Red staining, scale bar: 100 μm. (G) The expression levels of RANKL and OPG was detected by immunohistochemical staining, scale bar: 50 μm. Data are expressed as mean ± SD of n=6. *P < 0.05, **P < 0.01, *** P<0.001, **** P<0.0001.

**Figure 3** Effect of bariatric surgery on osteoblast autophagy in rats. (A) The LC3II/I, p62 and Beclin 1 expression levels were analyzed by western blot. (B) The expression level of Beclin 1 in each group was determined by immunohistochemical staining. Black arrow: beclin-1 positive osteoblasts. Scale bar: 100 μm. (C) Quantitative analysis of beclin-1 positive osteoblasts. Data are expressed as mean ± SD of n=6. *P < 0.05, **P < 0.01, *** P<0.001, **** P<0.0001.

**Figure 4** Alterations of gut microbiome after bariatric surgery. (A) The venne diagram to show the similarities and overlaps between four groups. (B) The Shannon index to suggest the alpha diversity among different groups. (C) The unweighted PCoA plot analysis to indicate the β diversity in each group. (D) The difference between different phyla was presented by heatmap. (E) Relative abundance analysis of top 4 bacterial phyla. (F) The difference at genus level in different group was presented by heatmap. (G) Relative abundance analysis of top 8 differentiated bacterial genera. Data are expressed as mean ± SD of n=5. *P < 0.05, **P < 0.01, *** P<0.001, **** P<0.0001

**Figure 5** Body weight alterations after the RYGB fecal microbiota transplantation and C. butyricum intervention. (A) Experimental designing was...
presented as flowchart. Obese rats received RYGB fecal microbiota transplantation and *C. butyricum* intervention. The body weight changes were recorded every week after manipulation (B). Data are expressed as mean±SD of n=6. *P < 0.05, **P < 0.01, *** P<0.001, **** P<0.0001

**Figure 6** Effect of the *C. butyricum* on RYGB fecal microbiota transplantation induced impaired bone metabolism in pseudo germ-free obese rats. (A) The bone density of whole body was determined by using dual-energy X-ray absorptiometry (DXA). (B) The images of tibia trabecular microstructure with micro-CT analyses in different groups. (C) The pathological alterations in each group were determined by H&E staining. (D) The activity of osteoblast was presented as ALP staining, scale bar: 100 μm. (E-F) TARP and Sirius Red staining were respectively performed to analyze the activity of osteoclast and collagen content. Scale bar: 100 μm. (G) The expression levels of RANKL and OPG were determined by immunohistochemical staining. Scale bar: 50 μm. Data are expressed as mean±SD of n=6. *P < 0.05, **P < 0.01, *** P<0.001, **** P<0.0001.

**Figure 7** Effect of the *C. butyricum* on RYGB fecal microbiota transplantation induced defective osteoblast autophagy in pseudo germ-free obese rats. (A-C) the western-blot was conducted to measure the difference in protein levels of LC3II/I, p62 and Beclin1. (D) Immunohistochemical staining was performed to show the changes in Beclin 1 expression. Black arrow: Beclin-1 positive osteoblasts. Scale bar: 100 μm. (E) Quantitative analysis of Beclin-1 positive osteoblasts. Data are expressed as mean±SD of n=6. *P < 0.05, **P < 0.01, *** P<0.001, **** P<0.0001.
Table 1. Effect of surgery on the bone turnover markers

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<th>SGS</th>
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<td>OCN (pg/ml)</td>
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Note: All values are expressed mean ± SD (n=6). Compared with RYGBS group: 

#P<0.05, ##P<0.01, ### P<0.001, #### P<0.0001; Compared with SGS group: *P < 0.05,

**P < 0.01, *** P<0.001, **** P<0.0001.
Table 2. Effect of RYGB fecal microbiota intervention and *C. butyricum*
administration on bone turnover markers

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Note: All values are expressed mean ± SD (n= 6). Compared with Sham D group:

#P<0.05, ##P<0.01, ### P<0.001, #### P<0.0001; Compared with RYGB D group: *P<0.05, **P<0.01, *** P<0.001, **** P<0.0001.
Figure 1

A

Male SD rats (8 weeks old)

RYGB/SG surgery

Bone mass detection

Bone autophagy investigation

Gut microbiota analysis

Body weight recording once a week for 10 weeks

10th week

B

Body weight changes (g)

0 2 4 6 8 10

week

SGS

RYGBS

SG

RYGB
Figure 2
Figure 3
Figure 4
Figure 5

A

Male SPF SD rats (4 weeks old)
High fat diet and pseudo germ-free antibiotic treatments
Obese rats (8 weeks old)
Fecal Microbiota intervention from RYGB sham and RYGB group
C. butyricum (CB) treatment once a week for 10 weeks
18 weeks old
Bone mass detection
Bone autophagy investigation

B

Body weight change (g)

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Figure 5
Figure 6
Figure 7