

# *Lactobacillus helveticus* (ATCC 27558) upregulates *Runx2* and *Bmp2* and modulates bone mineral density in ovariectomy-induced bone loss rats

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**Purpose:** Osteoporosis is one of the major health concerns among the elderly population, especially in postmenopausal women. Many menopausal women over 50 years of age lose their bone density and suffer bone fractures. In addition, many mortality and morbidity cases among the elderly are related to hip fracture. This study aims to investigate the effect of *Lactobacillus helveticus* (*L. helveticus*) on bone health status among ovariectomized (OVX) bone loss-induced rats.

**Methods:** The rats were either OVX or sham OVX (sham), then were randomly assigned into three groups, G1: sham, G2: OVX and G3: OVX+*L. helveticus* (1 mL of 10<sup>8</sup>–10<sup>9</sup> colony forming units). The supplementation was force-fed to the rats once a day for 16 weeks while control groups were force-fed with demineralized water.

**Results:** *L. helveticus* upregulated the expression of *Runx2* and *Bmp2*, increased serum osteocalcin, bone volume/total volume and trabecular thickness, and decreased serum C-terminal telopeptide and total porosity percentage. It also altered bone microstructure, as a result increasing bone mineral density and bone strength.

**Conclusion:** Our results indicate that *L. helveticus* attenuates bone remodeling and consequently improves bone health in OVX rats by increasing bone formation along with bone resorption reduction. This study suggests a potential therapeutic effect of *L. helveticus* (ATCC 27558) on postmenopausal osteoporosis.

**Keywords:** *Lactobacillus helveticus*, bone mineral density, bone loss, osteoporosis, ovariectomized rats

## Introduction

Osteoporosis is a prevalent skeletal disease which is mainly related to aging and menopause. The risk of osteoporosis due to estrogen deficiency in menopausal women is high<sup>1</sup> and closely related to the imbalance of bone metabolism. This is accompanied by greater bone resorption rather than bone formation which increases fragility and fracture risk.<sup>2</sup>

One of the important risks for those with osteoporosis is fracture risk since 51% of total disability worldwide was reported as a result of fracture.<sup>3</sup> In this regard, many menopausal women over 50 years of age suffer from osteoporosis due to the reduction of bone density and bone micro-architecture.<sup>4</sup> Since one-third of a woman's life span is spent in the menopausal condition,<sup>5</sup> reduction of the adverse effect of menopause in women's health is vital.

One of the most common therapies for osteoporosis is bisphosphonates such as alendronate,<sup>6,7</sup> risendronate<sup>8</sup> and zoledronic acid.<sup>9,10</sup> However, this therapy causes some

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side effects including atypical subtrochanteric fractures<sup>11</sup> and osteonecrosis of the jaw.<sup>12</sup> The other therapy for osteoporosis is hormone replacement therapy (HRT).<sup>13</sup> Some studies show a strong effect of HRT in reducing fracture risks among postmenopausal women;<sup>14</sup> however, no effect of HRT to reduce fracture risk among postmenopausal women also has been reported.<sup>15</sup> It seems HRT is mostly useful for treating low bone density rather than treating postmenopausal women with a stabilized osteoporosis.<sup>16</sup> HRT, like bisphosphonates, also has some side effects and can cause different types of cancer including breast<sup>17</sup> and ovarian cancers.<sup>18</sup> Besides that, long-term usage of HRT has been shown as one of the causes of stroke.<sup>19</sup> Therefore, prolonged HRT for osteoporosis treatment is not recommended. In addition to this, reduction of bone density occurs more rapidly if HRT is stopped compared to normal reduction of bone density in menopausal women suffering from osteoporosis.<sup>20</sup> Thus, due to the several adverse effects of HRT, women prefer to have nonhormonal treatments to manage their postmenopausal osteoporosis.<sup>21</sup>

In this regard, the effect of beneficial microbes on different organs and in reducing the risk of getting different diseases is of growing interest.<sup>22,23</sup> Due to this, probiotics are considered as an alternative osteoporosis treatment and have shown inhibitory effects on osteoclastic bone resorption and/or properties of osteoblastic bone formation.<sup>24</sup> However, most osteoporosis therapies prevent bone loss by inhibiting bone resorption,<sup>22</sup> while increasing the bone formation and bone volume (BV) is also required for osteoporosis treatment. Probiotics, in this regard, have been shown to change both osteoclastic and osteoblastic activities.

Probiotics are live microorganisms which confer a health benefit on a host when consumed in adequate amounts.<sup>25</sup> Probiotics, after consumption, normally lead to changes in host gut microbiota (GM).<sup>26</sup> Probiotics perform their function by manipulating intestinal microbiota and stimulating proliferation and differentiation of epithelial cells, leading to an improved immune system.<sup>27</sup> T cells have a critical role in immune response and can also affect bone remodeling.<sup>28</sup> T cell deficiency in mice has been shown to increase osteoclast (Oc) number and reduce bone mass.<sup>29</sup> Indeed, activation of T cells involves the autoimmune system, and in consequence reducing inflammation, bone loss, and osteoporosis.<sup>30</sup> Beneficial microbes in this regard reduce the expression of pro-inflammatory cytokines in the jejunum and ileum and activate the immune system, finally inhibiting reduction of bone mass.<sup>23</sup>

Bone is persistently remodeled by osteoblasts (Obs), the bone formation component, and Ocs, the bone resorption

component.<sup>31</sup> Regulation of bone metabolism is managed by complex mechanisms.<sup>32</sup> In this regard,<sup>33</sup> osteoblast regulators *Osterix*,<sup>34</sup> *Bmp2*,<sup>35</sup> *Runx2*<sup>36</sup> and Oc regulator *RANKL*<sup>37</sup> have shown some regulatory effects on bone metabolism. Therefore, our aim was to investigate the effects of *Lactobacillus helveticus* on *Runx2* and *Bmp2* expression and bone metabolism, and to evaluate bone mineral density (BMD) in bone loss-induced rats resulting from ovariectomy.

## Materials and methods

### Animal design and their intake

Twenty-four mature Sprague–Dawley rats, aged 10 weeks, with a body weight range of 280–290 g were enrolled in this research. The body weight-matched rats were randomly divided into three groups: G1: sham, G2: ovariectomized (OVX) and G3: OVX+*L. helveticus*, with eight rats in each group. Each rat was placed in a separate normal plastic cage in a controlled environment animal facility at 22°C±2°C with a 12-hour light/dark cycle and allowed to acclimatize for 2 weeks. The animals were fed with a standard normal chow pellet (Ridley Agri Products, Sydney, NSW, Australia) and ad libitum water access. The composition of rat chow pellet is shown in Table 1. The study was approved by the Animal Care and Use Committee (ACUC) of the Faculty of Medicine and Health Sciences, University Putra Malaysia (UPM), with approval number of UPM/FPSK/PADS/BR-UUH/00483. All the research was performed in the animal house of the Faculty of Medicine and Health Sciences, UPM, and guidelines for the use of animals were strictly followed (Universiti Putra Malaysia code of practice for the care and use of animals for scientific purposes).

### Surgical procedure

Surgical OVX was performed for G2 and G3 and sham ovariectomized for G1. The rats were anesthetized with an intraperitoneal injection using a combination of xylazine and ketamine with a dosage of 12/80 mg per kg rat body weight.<sup>38</sup>

**Table 1** Nutrients composition of rat chow diet

Standard rat chow diet ingredient	
Crude protein	21.0%
Crude fiber	7.0%
Crude fat	3.0%
Moisture	13.0%
Ash	8.0%
Calcium	1.0%
Phosphorus	0.5%
Nitrogen-free extract	46.5%

Following anesthesia, the rats were shaved on left and right laterals with an electric razor, and then hair was removed from part of the skin. The area was then washed and disinfected with povidone-iodine and ethanol 70% using gauze bandage. Two incisions 1 cm long were made on each of the caudal parts of the dorsolateral area of the rats using a sharp scalpel after lifting the skin with a tom-forceps. Muscles were separated along their fibers and ovaries were located, double-ligated and removed. After the removal of ovaries, the skin was closed in two layers with absorbable sutures. Then, the animals were covered with paper and located separately in a clean normal cage (one animal/cage) and left for 2 weeks to recover. After two weeks, the animal was transferred to their cages separately for the entire study.

### *L. helveticus* cultivation

*L. helveticus* (ATCC 27558) was commercially purchased as the lyophilized form from the American Type Culture Collection (ATCC). Then, based on the protocol of the company, the bacteria were revived three continuous times in de Man, Rogosa and Sharpe (MRS) broth. After that, the bacteria were subcultured in MRS agar several times with the aim of refreshing. Then, the bacteria from MRS agar were inoculated again to MRS broth to get ready for force-feeding. The bacterial concentration in colony forming units (CFU) was selected based on a previous study.<sup>39</sup> The rats in G3 were then force-fed with 1 mL of  $10^8$ – $10^9$  CFU of *L. helveticus* in phosphate buffer saline daily via oral gavage for 16 weeks; sham and OVX groups were force-fed with 1 mL of demineralized water.

### Animal euthanasia and sample collection

One day before culling, the animals were transferred to a metabolic cage individually and left overnight, and then fresh fecal samples were collected the next day in order to enumerate lactobacillus colonies. Then, the rats were subsequently anesthetized with a combination of xylazine and ketamine, and blood was collected via cardiac puncture. After that the rats were immediately euthanized and sacrificed, and then their femurs were removed gently. The left femurs were preserved in 10% buffered formalin for 24 hours, and then the solution is changed to 70% ethanol, and right femurs were snap-frozen and stored at  $-80^{\circ}\text{C}$  until the assessment day. The micro-CT ( $\mu$ -CT) analysis was done on the left femur of the animal; after that, a breaking force was applied to measure the stress of the bone on the same femurs; the proximal left femur was used for histology analysis and the distal left femur was used to measure Ca, Mg and Zn content of the femurs. The right femur was used for RNA isolation.

### Blood biochemical analysis

The collected blood was transferred into plain tubes and centrifuged at 3,000 rpm for 10 minutes at  $4^{\circ}\text{C}$  to isolate serum for biochemical assay. Calcium and magnesium in the serum were analyzed using a Roche Cobas® C-311 Japan analyzer (Hitachi Ltd., Tokyo, Japan). Osteocalcin (OC) and C-terminal telopeptide (CTX) were analyzed using standard commercial ELISA kits; OC was analyzed using Rat-MID™ OC EIA (IDS, Fareham, UK) and CTX was analyzed using RatLaps™ EIA (IDS).

### Micro-CT analysis

Images of the left femurs were obtained using  $\mu$ -CT (SkyScan, 1176; Bruker microCT, Kontich, Belgium) at a resolution of 35  $\mu\text{m}$ , filter 0.5, exposure 100, voltage 40, and current 100 A to measure BMD ( $\text{g}/\text{cm}^3$ ) and various bone structures. To get the images, the left femurs were wrapped in a moistened tissue paper and placed in the  $\mu$ -CT scan device. The images were then reconstructed using NRecon software version 1.6.3.3 and analyzed with SkyScan CT analyzer software version 1.9.1. Then, BMD ( $\text{g}/\text{cm}^3$ ) of the femurs was calculated by the formula which was created by the analyzer. In addition to this, BV/total volume (TV) percentage, trabecular thickness (Tb.Th; mm), trabecular number (Tb.N;  $\text{mm}^{-1}$ ), trabecular separation (Tb.Sp; mm) and total porosity percentage (pro.tot) of the femurs were measured with the software. All values are reported as mean of cortical and trabecular bones. The correction of beam hardening was conducted using a metal filter during the scanning and reconstructing to improve the quality of the  $\mu$ -CT images. Calibration of the  $\mu$ -CT analyzer was carried out by phantoms with known density of calcium hydroxyapatite, size 4 mm in pairs. A concentration of  $0.25 \text{ g}/\text{cm}^3$  was regarded as lower density and  $0.75 \text{ g}/\text{cm}^3$  as higher density.

### Stress of the femur

The stress of the left femur was measured using a three-point bending method from the center of the femur using a universal testing machine (8874; Instron Ltd., High Wycombe, UK) equipped with 5 kN load transducer. The procedure was based on the work of Goda et al.<sup>40</sup> Briefly, the midpoint of the femur was marked, and then a breaking force was applied. After that, inside and outside radii were measured to calculate the area moment of inertia. Medullary canal diameter was calculated by subtracting the thickness of the medial and lateral walls from the diameter of the diaphysis. Moment was calculated by applied force of the machine multiplied by support points, and then divided by 4. Finally, the stress of the femur was calculated by multiplying the moment to

the farthest point in the cross section to the neutral axis, and then divided by the moment of inertia.<sup>41</sup>

## Bone histomorphometric measurements

Bone histomorphometric measurements were done as recommended by the American Society of Bone Mineral Research Histomorphometry Nomenclature Committee (ASBMR).<sup>42</sup> The secondary spongy area of the left femurs, 1 mm from the lateral cortex and 3–7 mm from the lowest point of the growth plate, was used for histomorphometric purposes.<sup>42</sup> As a brief explanation for the sample preparation, the left femurs were decalcified in 5% nitric acid for 24 hours. Then, the decalcified samples were transferred to an automated vacuum tissue processor (Leica ASP 300; Leica, Wetzlar, Germany). After that, the samples were embedded in paraffin histology wax and then sectioned at 6 µm thickness using a microtome (Leica). Finally, the samples were stained with hematoxylin and eosin (H&E) and observed under a light microscope (Olympus BX51TRF-CCD Microscope, Tokyo, Japan). After that, osteoclast surface/bone surface (OcS/BS), osteoblast surface/bone surface (ObS/BS), eroded surface/bone surface (ES/BS), osteoid surface/bone surface (OS/BS) and osteoid volume (OV)/BV ratios were measured using Weibel's technique.<sup>43</sup>

## Calcium, magnesium and zinc content of the femur

The calcium, magnesium and zinc content of the left femurs was determined by atomic absorption spectrophotometry (novAA<sup>®</sup> 400; Analytik Jena, Jena, Germany) according to the procedure of Harrison et al<sup>44</sup> with slight modification. Distal left femurs were dried at 105°C for 24 hours. Then, they were placed in a muffle furnace at 550°C for another 24 hours to get the ash samples. The ashes were then crushed and hydrolyzed with 6 M HCl. Finally, the solution was used to determine the mineral content of the femurs. Calcium was determined at a wavelength of 422.7 nm and lamp current 4.0 mA, magnesium was determined at a wavelength of 285.2 nm and lamp current 2.0 mA and zinc was determined at a wavelength of 213.9 nm and lamp current 2.0 mA.

## RNA isolation and quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

Total RNA was extracted from the right frozen femurs using a HiYield<sup>®</sup> Total RNA Mini Kit (Real Biotech, Taipei,

Taiwan) using the manufacturer's protocol. Extracted RNA was only used if the absorbance ratio at A260/230 and A260/280 was between 1.8 and 2.0 with specific bands on gel electrophoresis.<sup>45</sup> Then, cDNA was synthesized from 1 µg of RNA from each sample in a total 20 µL reactions using an i-script cDNA synthesis kit (Bio-Rad). PCR reagent was prepared from cDNA in a reaction mixture containing 3 µL of cDNA in a total volume of 25 µL with 2× SYBR<sup>®</sup> Green I Hot-Start. Amplifications were then placed in a thermal cycler (Eppendorf, Hamburg, Germany) with initial denaturation at 95°C for 15 minutes, followed by 40 cycles at 95°C for 15 seconds, 58.6°C for 40 seconds and 68°C for 20 seconds. The data were then calculated as fold changes compared to the control group, relative to the housekeeping gene. The primers were designed based on sequences obtained from the ENA and GenBank (<http://www.ncbi.nlm.nih.gov/tools/primer-blast>) and supplied by Helix Biotech Ltd. (Richmond, BC, Canada) (Table 2) and were diluted to a final concentration of 100 µL with nuclease-free water.

## Enumeration of *Lactobacillus* in the feces

Fresh rat feces were diluted and cultivated in MRS agar, then incubated for 48 hours at 37°C. Then, lactobacilli were enumerated using a colony counter (Stuart Scientific, Staffordshire, UK) and reported as log<sub>10</sub> CFU/g.

## Statistical analysis

The univariate procedure of SAS software (version 8.2, SAS Institute Inc., Cary, NC, USA) was used for the normality test. Then, data were analyzed by one-way analysis of variance (ANOVA) with PROC MIXED in SAS. The results were then reported as mean±SEM and differences between the groups were analyzed using a post hoc Duncan's test. Microbial data were converted to log<sub>10</sub> and reported on a wet weight basis. Significance was considered at  $P < 0.05$ .

**Table 2** List of genes and primer sequences

Genes	Accession number	Primer sequences (5→3)
Runx2	NM_053470	F: GCGTCCTATCAGTTCCCAAT R: ATCAGCGTCAACACCATCAT
Bmp2	NM_017178	F: CAGGTCTTTGCACCAAGATG R: GCTGGACTTAAGACGCTTCC
GAPDH (housekeeping)	NM_017008	F: TCAAGAAGGTGGTGAAGCAG R: AGGTGAAGAATGGGAGTTG

**Abbreviations:** Bmp2, bone morphometric protein; Runx2, runt-related transcription factor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.



## Results

### Effect of *L. helveticus* on serum biomarkers

Table 3 shows the analysis of biochemical assays on the serum. The OVX group showed a significantly lower level of OC and a higher level of CTX in the serum ( $P=0.0001$ ) compared to other groups. However, the group of rats supplemented with *L. helveticus* showed a significantly increased level of serum OC ( $P=0.0001$ ) and a decreased level of serum CTX ( $P=0.0001$ ). These results indicate that the OVX+*L. helveticus* group had more bone formation and less bone resorption activity than the OVX group. *L. helveticus* had no significant effects on serum Ca and Mg levels.

### Effect of *L. helveticus* on BMD and trabecular structure changes

Table 4 presents BMD and trabecular structure changes and Figure 1 shows reconstructed cross-sectional CT scan images, 1 cm above the lateral condyles of the femur. The OVX group showed a significantly lower BMD of the femur ( $P<0.05$ ) than the other groups; however, the OVX+*L. helveticus* group showed a higher BMD of the femur which was nearest to the sham group ( $P<0.05$ ). Tb.Th showed a higher value in sham and a lower value in OVX groups ( $P<0.05$ ), while the *L. helveticus*-fed group showed a significant increase of Tb.Th compared to the OVX group ( $P<0.05$ ). *L. helveticus* also affected the total porosity percentage and BV/TV of the femur. In this regard, *L. helveticus* significantly increased BV/TV and decreased total porosity percentage of the femur compared to the OVX group ( $P<0.05$ ). Tb.N and Tb.Sp were not affected by *L. helveticus*.

**Table 3** Effect of *L. helveticus* on serum biomarkers

Serum parameters	Sham	OVX	OVX+ <i>L. helveticus</i>	P-value
OC (ng/mL)	144±2.2 <sup>a</sup>	77±6.3 <sup>b</sup>	118±6.4 <sup>c*</sup>	0.0001
CTX (ng/mL)	174±14.7 <sup>a</sup>	391±22.6 <sup>b</sup>	195±7.7 <sup>c*</sup>	0.0001
Ca (mmol/L)	2.30±0.13	2.24±0.10	2.23±0.07	0.96
Mg (mmol/L)	0.98±0.07	0.90±0.10	0.93±0.04	0.73

**Notes:** Ten-week-old female Sprague–Dawley rats were supplemented with 1 mL *L. helveticus* ( $10^8$ – $10^9$  CFU) once a day during 16 weeks, starting 2 weeks after surgery, while sham and OVX groups received 1 mL of demineralized water. At the end of the study, serum of the rats was analyzed for OC, CTX, Ca, and Mg. Values are presented as mean±SEM ( $n=8$  in each group). <sup>a,b,c</sup>Values with different letters are significantly different at  $*P<0.05$  based on one-way ANOVA, followed by Duncan post hoc test. a: different from b; c, b: different from a; and c, c: different from a and b.

**Abbreviations:** OVX, ovariectomized; OC, osteocalcin; CTX, C-terminal telopeptide; CFU, colony forming units; SEM, standard error from mean; *L. helveticus*, *Lactobacillus helveticus*.

**Table 4** Effect of *L. helveticus* on micro-CT scan analysis of the femur

Parameters	Sham	OVX	OVX+ <i>L. helveticus</i>	P-value
BV/TV (%)	76±6.30 <sup>a</sup>	49±5.7 <sup>b*</sup>	57±6.6	0.05
Porosity (%)	25±5.3 <sup>a</sup>	53±4.6 <sup>b*</sup>	42±6.6	0.02
Tb.Th (mm)	7.6±0.5 <sup>a</sup>	5.3±0.4 <sup>b*</sup>	5.8±0.5	0.03
Tb.Sp (mm)	5.2±0.4	6.4±0.6	6.1±0.4	0.69
Tb.N (mm <sup>-1</sup> )	0.10±0.00	0.09±0.01	0.09±0.00	0.95
BMD (g.cm <sup>-3</sup> )	1.07±0.02 <sup>a</sup>	0.76±0.06 <sup>b*</sup>	0.91±0.05 <sup>a</sup>	0.003

**Notes:** Ten-week-old female Sprague–Dawley rats were supplemented with 1 mL *L. helveticus* ( $10^8$ – $10^9$  CFU) once a day during 16 weeks, starting 2 weeks after surgery, while sham and OVX groups received 1 mL of demineralized water. At the end of the study, dissected femurs were analyzed with micro-CT scan analyzer. Values are presented as mean±SEM analysis of cortical and trabecular femur ( $n=8$  in each group). <sup>a,b</sup>Values with different letters are significantly different at  $*P<0.05$  based on one-way ANOVA, followed by Duncan post hoc test. a: different from b.

**Abbreviations:** OVX, ovariectomized; BV/TV, bone volume/total volume; Tb.Th, trabecular thickness; Tb.Sp, trabecular separation; Tb.N, trabecular number; BMD, bone mineral density; CFU, colony forming units; SEM, standard error from mean; *L. helveticus*, *Lactobacillus helveticus*.

### Effect of *L. helveticus* on stress of the femur

Femur breaking forces are shown in Figure 2. The significantly highest and lowest stress of femurs were reported in the sham and OVX groups, respectively ( $P<0.05$ ). *L. helveticus* group showed increasing the stress of the femur compared to the OVX group ( $P<0.05$ ).

### Effect of *L. helveticus* on Ob, Oc, OS, ES and OV

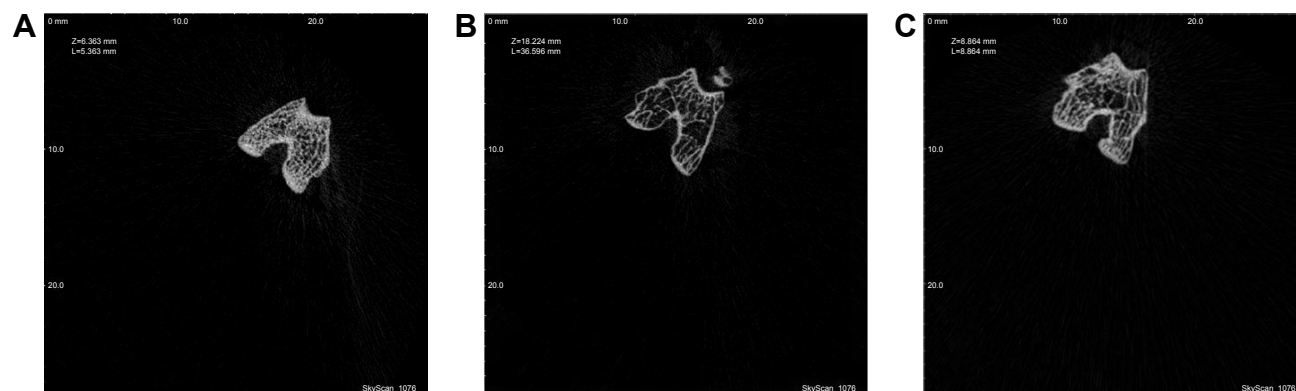
ObS/BS, OS/BS and OV/BV were significantly lower and ES/BS and OcS/BS were significantly higher in the OVX group ( $P=0.0001$ ) compared to the other groups. However, OV+*L. helveticus*-fed group had higher ObS/BS, OS/BS and OV/BV and lower OcS/BS and ES/BS compared to the OVX group ( $P=0.0001$ ), as shown in Table 5.

### Effect of *L. helveticus* on Ca, Mg and Zn content of the femur

*L. helveticus* in this study failed to have any significant effects on bone mineral content including Ca, Mg and Zn, as shown in Table 6.

### Effect of *L. helveticus* on expression of *Runx2* and *Bmp2*

The mRNA expression of *Runx2* and *Bmp2* was lower in the OVX group compared to the sham group ( $P<0.05$ ). However, OVX+*L. helveticus*-fed group had increased mRNA expression of *Runx2* and *Bmp2* and was nearest to the sham group ( $P<0.05$ ; Figure 3A and B).



**Figure 1** Reconstructed cross-sectional CT-scan images of the femur. (A) Sham, (B) OVX, (C) OVX+*L. helveticus*.

**Notes:** Ten-week-old female Sprague–Dawley rats were supplemented with 1 mL *L. helveticus* ( $10^8$ – $10^9$  CFU) once a day during 16 weeks, starting 2 weeks after surgery, while sham and OVX groups received 1 mL of demineralized water. At the end of the study, the cross-sectional images of the femur were performed using micro-CT and reconstructed using NRecon software version 1.6.3.3. The pictures display the reconstructed images from 1 cm above the lateral condyles of the femur.

**Abbreviations:** CFU, colony forming units; OVX, ovariectomized; *L. helveticus*, *Lactobacillus helveticus*.

## Lactobacillus enumeration in the feces

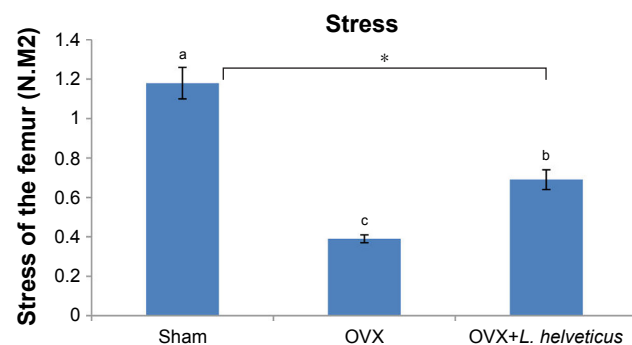
The total lactobacillus count from animal feces is presented in Figure 4. At the end of the experiment, the number of lactobacillus colonies increased significantly in the feces of rats fed with *L. helveticus* compared to the sham and OVX groups ( $P<0.05$ ).

## Discussion

Beneficial bacteria supplementation affects the GM such as by increasing the population of bacteria in the host, which in turn affect the host's health.<sup>28</sup> Similarly, in the current study, we showed that the amount of lactobacillus in rat feces increased via feeding of *L. helveticus*. This means the

bacteria remain alive after eating and passage from the gastrointestinal (GI) tract. Considering this aspect helps justify the better function of lactobacillus on the host. Generally, probiotics modulate GM, influence different target organs and alter the host's functions.<sup>46</sup> Previously, it has been shown that a lack of GM leads to elevated bone mass; however, colonization of mice with normal GI microbes normalized bone mass.<sup>47</sup> In this regard, beneficial microbes stimulate endocrine cells which are located in the gut and improve the immune system.<sup>46</sup> Since stem cells in the bone marrow generate T cell precursors, the immune system interacts with these pathways.<sup>48</sup>

Estrogen deficiency leads to changes in immune status via increases of inflammatory factors and, therefore, decreases



**Figure 2** Effect of *L. helveticus* on stress of the femur.

**Notes:** Ten-week-old female Sprague–Dawley rats were supplemented with 1 mL *L. helveticus* ( $10^8$ – $10^9$  CFU) once a day during 16 weeks, starting 2 weeks after surgery, while sham and OVX groups received 1 mL of demineralized water. At the study, the stress of the femur was tested using the three-point bending method in the center of the femur with a universal testing machine. Values are presented as mean of each group in column graph with standard error bar from mean ( $n=8$  in each group). <sup>a,b</sup>Values with different letters are significantly different at  $*P<0.05$  based on one-way ANOVA, followed by Duncan post hoc test. a: different from b and c; b: different from a and c; c: different from a and b.

**Abbreviations:** CFU, colony forming units; OVX, ovariectomized; *L. helveticus*, *Lactobacillus helveticus*.

**Table 5** Effect of *L. helveticus* on histomorphometric measurements of the femur

Parameters (%)	Sham	OVX	OVX+ <i>L. helveticus</i>	P-value
ObS/BS	53±0.4 <sup>a</sup>	37±0.4 <sup>b,*</sup>	46±0.5	0.0001
OcS/BS	23±0.5 <sup>a</sup>	28±0.4 <sup>b,*</sup>	25±0.4	0.0001
OS/BS	24±0.2 <sup>a</sup>	18±0.2 <sup>b,*</sup>	23±0.3	0.0001
OV/BV	13±0.3 <sup>a</sup>	7±0.2 <sup>b,*</sup>	10±0.2	0.0001
ES/BS	29±0.1 <sup>a</sup>	38±0.3 <sup>b,*</sup>	34±0.1	0.0001

**Notes:** Ten-week-old female Sprague–Dawley rats were supplemented with 1 mL *L. helveticus* ( $10^8$ – $10^9$  CFU) once a day during 16 weeks, starting 2 weeks after surgery, while sham and OVX groups received 1 mL of demineralized water. At the end of the study, percentage of ObS/BS, OcS/BS, ES/BS, OS/BS and OV/BV were measured from histology slide (H&E) method of dissected femur. Values are presented as mean±SEM ( $n=8$  in each group). <sup>a,b</sup>Values with different letters are significantly different at  $*P<0.05$  based on one-way ANOVA, followed by Duncan post hoc test. a: different from b.

**Abbreviations:** OVX, ovariectomized; ObS/BS, osteoblast surface/bone surface; OcS/BS, osteoclast surface/bone surface; ES/BS, eroded surface/bone surface; OS/BS, osteoid surface/bone surface; OV/BV, osteoid volume/bone volume; CFU, colony forming units; SEM, standard error from mean; *L. helveticus*, *Lactobacillus helveticus*; H&E, hematoxylin and eosin.

**Table 6** Effect of *L. helveticus* on Ca, Mg and Zn content of the femur

Ca, Mg and Zn content of the femur (mg/L)	Sham	OVX	OVX+ <i>L. helveticus</i>	P-value
Ca	492±2.3	484±1.3	487±2.0	0.06
Mg	33±0.4	32±0.2	33±0.2	0.09
Zn	17±1.4	14±0.6	14±0.8	0.12

**Notes:** Ten-week-old female Sprague–Dawley rats were supplemented with 1 mL *L. helveticus* ( $10^8$ – $10^9$  CFU) once a day during 16 weeks, starting 2 weeks after surgery, while sham and OVX groups received 1 mL of demineralized water. At the end of the study, Ca, Mg and Zn content of the femur were analyzed. Values are presented as mean±SEM (n=8 in each group).

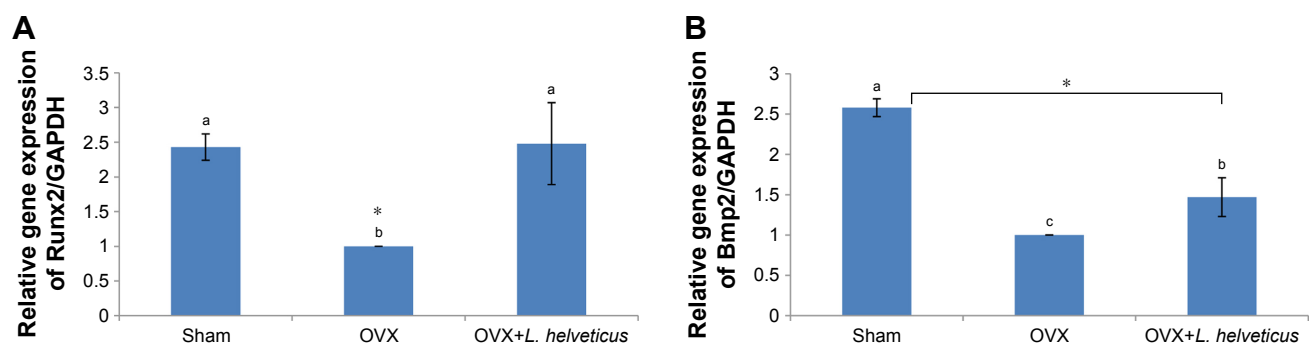
**Abbreviations:** OVX, ovariectomized; CFU, colony forming units; SEM, standard error from mean; *L. helveticus*, *Lactobacillus helveticus*.

the osteoprotegerin level.<sup>49</sup> Indeed, probiotics have the capacity to increase immune system function by attenuating the elevation of inflammatory cytokines, which is shown in OVX rats,<sup>50</sup> or via the production of bioactive peptides and consequent increase of absorption of minerals such as calcium,<sup>23</sup> which leads to calcium accumulation in bones and thereby preventing deterioration of bone mass. However, this study failed to detect a significant increase of minerals in the serum and bone accumulation, but the changes of Ob and Oc activity as well as the modulation of bone remodeling were unblemished.

OVX caused elevation of CTX in the serum of the rats; however, those OVX rats fed with *L. helveticus* did not show elevated CTX in the serum. This means that *L. helveticus* caused a reduction in the level of CTX in the serum, and since CTX is a bone resorption indicator, it could be the consequence of bone resorption activity reduction occurring in the *L. helveticus*-fed group. In contrast, OC in the serum was increased with *L. helveticus*, suggesting that bone

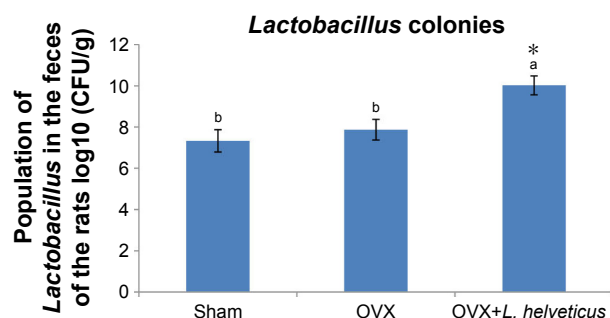
formation activity was increased via this bacterium. A possible improvement of bone formation activity is supported by upregulation of the two osteogenic genes measured in this study, *Runx2* and *Bmp2*. These two genes augment Ob functions; in fact, Ob differentiation is regulated by cytokines, a range of hormones and some transcription factors.<sup>51</sup> *Bmp* is one of the important keys to regulate the maintenance of postnatal bone and has an essential role in endochondral bone development.<sup>52</sup> *Bmp2*, a member of *Bmps*, has a key role in cell growth, embryogenesis and bone formation progress as well as repairing bone fractures,<sup>53</sup> and massively increases OC expression.<sup>54</sup> *Bmp2* performs its osteogenic action by activation of some signals such as Smad1/5/8 and transcript regulation of some other osteogenic genes such as *Runx2*.<sup>55</sup> *Runx2* regulates the expression of other osteoblastic genes such as collagen type 1.<sup>56</sup> Moreover, *Runx2* is a critical transcription factor for Ob differentiation.<sup>57</sup> This is well understood in *Runx2*-deficient rats which show a complete lack of mature Obs and bone formation.<sup>58,59</sup>

Collectively, the present results indicate that the effectiveness of *L. helveticus* on bone might be due to the attenuation of bone remodeling, specifically Ob proliferation and differentiation and increasing bone formation activity. This aspect which showed the protective effect of probiotics on bone via alteration of bone formation and resorption is confirmed by the Oc and Ob numbers on the bone surface. In the current study, ObS/BS, OS/BS and OV/BV were found to be increased; in contrast, OcS/BS and ES/BS were decreased by *L. helveticus* in OVX rats. As a result of increasing the number of Obs and osteoids, bone formation activity is increased and, in consequence, serum OC also increases. However, if the amount of Oc and eroded surface is decreased, it can

**Figure 3** Effect of *L. helveticus* on mRNA expression of (A) *Runx2*, (B) *Bmp2*.

**Notes:** Ten-week-old female Sprague–Dawley rats were supplemented with 1 mL *L. helveticus* ( $10^8$ – $10^9$  CFU) once a day during 16 weeks, starting 2 weeks after surgery, while sham and OVX groups received 1 mL of demineralized water. At the end of the study, the mRNA expression of *Runx2* and *Bmp2* was estimated by qRT-PCR analysis. Values are presented as mean of each group in column graph with standard error bar from mean (n=8 in each group). <sup>a,b</sup>Values with different letters are significantly different at \**P*<0.05 based on one-way ANOVA, followed by Duncan post hoc test. a: different from b and c; b: different from a and c; c: different from a and b.

**Abbreviations:** CFU, colony forming units; OVX, ovariectomized; qRT-PCR, quantitative reverse transcription-polymerase chain reaction; *Runx2*, runt related transcription factor 2; *Bmp2*, Bone morphometric protein 2; *L. helveticus*, *Lactobacillus helveticus*; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.



**Figure 4** Total *Lactobacillus* colonies in the fecal samples.

**Notes:** Ten-week-old female Sprague–Dawley rats were supplemented with 1 mL *L. helveticus* ( $10^8$ – $10^9$  CFU) once a day during 16 weeks, starting 2 weeks after surgery, while sham and OVX groups received 1 mL of demineralized water. At the end of the study, the amount of *Lactobacillus* was quantified in the feces. Values are presented as mean of each group in column graph with standard error bar from mean ( $n=8$  in each group). <sup>ab</sup>Values with different letters are significantly different at  $*P<0.05$  based on one-way ANOVA, followed by Duncan post hoc test. a: different from b.

**Abbreviations:** CFU, colony forming units; OVX, ovariectomized; *L. helveticus*, *Lactobacillus helveticus*.

be suggested that bone resorption activity is decreased and, following that, serum CTX is also decreased.<sup>60</sup>

As a result of increased bone formation and decreased bone resorption activity, the percentage of BV will be increased; in contrast, the percentage of total porosity will be decreased. Since BMD and bone strength are associated with an increase of BV and a reduction of porosity percentage, our results regarding improvement of BMD and bone strength along with a reduction of total porosity percentage which occurred in the *L. helveticus*-fed group can be confirmed. Thereby, *L. helveticus* treatment has the capacity to reduce fracture risk and/or prevent the femur from easily fracturing.<sup>61</sup>

## Conclusion

*L. helveticus* increases the expression of some osteoblastic genes and thereby bone formation activity. Therefore, by the attenuation of bone remodeling which is obtained via *L. helveticus* consumption, OVX rats can be protected from bone loss and fracture risk. These data suggest a potential therapeutic effect of *L. helveticus* on postmenopausal osteoporosis.

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## Disclosure

The authors report no conflicts of interest in this work.

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