***Lactobacillus intestinalis* YT2 restores the gut microbiota and improves menopausal symptoms in ovariectomized rats**

E.Y. Lim^{1,2#}, E.-J. Song^{1#}, J.G. Kim³, S.Y. Jung^{1,2}, S.-Y. Lee^{1,2}, H.S. Shin^{1,2}, Y.-D. Nam¹ and Y.T. Kim^{1,2*}

¹Division of Food Functionality, Korea Food Research Institute, Wanju-gun 55365, Republic of Korea; ²Department of Food Biotechnology, Korea University of Science and Technology, Daejeon 34113, Republic of Korea; ³Food Functional Evaluation Support Team, Technical Assistance Center, Korea Food Research Institute, Wanju-gun 55365, Republic of Korea; ytkim@kfri.re.kr; #these authors contributed equally

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RESEARCH ARTICLE

Abstract

There are many studies focusing on the alleviation of menopausal symptoms; however, little is known about the role of gut microorganisms in menopausal symptoms. Ovariectomized (OVX) rats were administered a novel strain (YT2) of *Lactobacillus intestinalis* (a species with significantly reduced abundance in OVX rats) and the potential probiotic effect on the improvement of menopausal symptoms was evaluated. Of note, the gut microbial composition completely shifted after ovariectomy in rats. Treatment with *L. intestinalis* YT2 significantly alleviated menopausal symptoms, such as increased fat mass, decreased bone mineral density, increased pain sensitivity, depression-like behaviour, and cognitive impairment. Additionally, the administration of *L. intestinalis* YT2 restored the intestinal microbial composition, including an increased *Firmicutes/Bacteroides* ratio. *L. intestinalis* YT2 also promoted gut barrier integrity by increasing the mRNA levels of tight junction-related markers. In conclusion, *L. intestinalis* YT2 treatment alleviated menopausal symptoms via the modulation of the gut microbiota. Importantly, these results suggest that *L. intestinalis* YT2 should be considered as a therapeutic probiotic agent for menopausal women.

Keywords: menopause, ovariectomy, probiotics, *Lactobacillus*, gut barrier

1. Introduction

Menopause is a natural process, which is a result of decreased synthesis and secretion of oestrogen in the ovaries. Natural menopause usually occurs at around 50 years of age; the number of women undergoing early menopause is also increasing (Gold, 2011). Menopause-related sex hormone deficiency is associated with various menopausal symptoms, including sexual function alterations, metabolic changes, musculoskeletal and external changes, hot flashes, sleep disturbances, cognitive impairment, depression, anxiety, and migraines (Monteleone *et al.*, 2018). Of note, the symptoms continue for about 5-10 years after the onset of menopause and deteriorate the quality of life. Hormone replacement therapy is the first-line of treatment to improve menopause-related symptoms (Hlatky *et al.*, 2002). However, long-term use of hormone replacement therapy requires caution

because it can increase the risk of coronary heart disease, venous thromboembolism, and breast cancer (Rymer *et al.*, 2003). Considering that the current treatments are not always effective or recommended, alternative treatment options are needed for menopausal women.

Intestinal microorganisms, comprising about 10 trillion bacteria, have been studied extensively; the compositional imbalance of intestinal microorganisms, called dysbiosis, is associated with the pathogenesis of immune and metabolic diseases (Boulange *et al.*, 2016). Studies have also been conducted on the relationship between intestinal dysbiosis and the menopausal period. An increased ratio of *Firmicutes/Bacteroidetes* (F/B) was found in the gut microbiota of postmenopausal women in comparison to that in pre-menopausal women, which is consistent with the results from ovariectomized (OVX) rat models

(Santos-Marcos *et al.*, 2018; Zhang *et al.*, 2017). Of note, the attenuation of menopausal symptoms was associated with changes in gut microorganisms. For instance, increased body weight and elevated F/B ratio were normalised after treatment with grape seed extracts containing proanthocyanidin in an animal model (Jin *et al.*, 2018). Therefore, the alleviation of menopausal symptoms through the modulation of gut microbial dysbiosis is a promising strategy.

Probiotics were defined as ‘live microorganisms which when administered in adequate amounts confer a health benefit on the host’ by the FAO/WHO in 2001. Probiotics have the potential to prevent or treat various health problems, such as inflammatory bowel disease, colitis, asthma, autoimmune diseases, obesity, diabetes, and cardiovascular disease via the control of host-gut microbial interactions (Kim *et al.*, 2019). Recently, many studies have been conducted on the use of probiotics in the alleviation of menopausal symptoms. Postmenopausal women taking *Lactobacillus rhamnosus* GR-1 and *Lactobacillus reuteri* RC-14 showed an improved vaginal flora (Petricevic *et al.*, 2008). Moreover, the administration of probiotic mixtures containing *Bifidobacterium breve*, *Bifidobacterium longum*, *Lactobacillus acidophilus*, *Lactobacillus bulgaricus*, *Lactobacillus casei*, *L. rhamnosus*, and *Streptococcus thermophilus* for six months led to decreased serum bone metabolism biomarkers, including bone-specific alkaline phosphatase and collagen type 1 cross-linked C-telopeptide in a human trial (Jafarnejad *et al.*, 2017). *L. acidophilus* ATCC 4356 was also shown to inhibit osteoclastogenic Th17 cells and increase bone mineral density (BMD) in OVX animals (Dar *et al.*, 2018). However, the mechanism behind the probiotics-induced alleviation of menopausal symptoms has not yet been fully elucidated.

The previous studies have employed *in vitro* screening to identify the probiotics able to improve menopausal symptoms. This approach lacks a strategy for accurate target selection, often resulting in only partial and mild improvements. Therefore, here we aimed to demonstrate the broad improvement of menopausal symptoms, such as increased fat mass, decreased BMD, pain sensitivity, depression-like behaviour, and cognitive impairment after administration of *Lactobacillus intestinalis* YT2, which was selected as a probiotic target by exploring the gut microbial composition of OVX animals based on next-generation sequencing.

2. Materials and methods

Animals

Female Sprague-Dawley (SD) rats were purchased from Samtako (Gyeonggi-do, Korea) and divided into 10–11 rats per cage after surgery. All rats were housed under 12 h light/dark cycles (07:00 lights on, 19:00 lights off) in an air-conditioned room at 22 ± 1 °C and $55 \pm 5\%$ humidity. All animals were fed with the laboratory rodent diet 5010 (PMI Nutrition International, St. Louis, MO, USA) and were supplied food and drinking water *ad libitum*. The animals were adapted to the laboratory environment for at least 2 weeks (hereafter week and weeks are abbreviated as wk). All animal experiments were approved by the Korea Food Research Institutional Animal Care and Use Committee (KFRI-M-14003).

Experimental design

To establish a menopause model and select probiotic candidates, 10-wk-old SD female rats were ovariectomized (OVX). The normalisation of the gut microbiota was performed for 4 or 6 wk using random rotation before OVX. After normalisation, rats were randomly assigned to one of two groups: (1) sham-operated (n=11) and (2) OVX (n=11). Rats (190–250 g) were anesthetized under isoflurane (2%) during surgery. Both ovaries were removed in the OVX group and ovaries were left intact in the sham-operated group. All the rats were sacrificed 18 wk after surgery. An overview of the experimental schedule is depicted in Figure 1A.

To assess the efficacy of *L. intestinalis* YT2 treatment on OVX rats, rats were randomly divided into six experimental groups: (1) sham-operated + phosphate buffered saline (PBS) (vehicle, n=10); (2) OVX + PBS (n=10); (3) OVX + 17β -oestradiol (20 μ g/kg, i.p, n=10); (4) OVX + VSL#3 (10^9 cfu/ml, n=10); (5) OVX + *L. intestinalis* type strain (10^9 cfu/ml, n=11); and (6) OVX + *L. intestinalis* YT2 (10^9 cfu/ml, n=11). OVX was performed as described above. All samples except for 17β -oestradiol were dissolved in PBS and administered orally three times per wk; 17β -oestradiol was dissolved in distilled saline with 1% dimethyl sulfoxide (DMSO). Treatment started one wk after surgery and lasted for 18 wk. An overview of the experimental schedule is depicted in Figure 1B.

Serum oestradiol analysis

Blood samples were collected from the abdominal aorta 18 wk after ovariectomy. Blood samples were centrifuged at 4 °C for 15 min at $1000 \times g$, and serum was isolated. The serum oestradiol levels were determined via radioimmunoassay (RIA) using the Coat-a-Count kit (Diagnostic Products, Los Angeles, CA, USA).

Figure 1. Experimental schedules. (A) The gut microbiota was normalised for 4 wk before ovariectomy (OVX), and the rats were maintained for 18 wk after OVX. (B) Scheme of the behavioural tests including the von Frey filament test, forced swim test (FST), and passive avoidance test (PAT), and fat mass and bone mineral density (BMD) measurement. The gut microbiota was normalised for 6 wk before OVX, and the rats were maintained for 18 wk after OVX.

Dual-energy X-ray absorptiometry

The body compositions of the rats were measured using the dual X-ray absorptiometry method (pDEXA, Norland) (Heiss *et al.*, 2012). The rats were anesthetized with a constant flow of 2% isoflurane in oxygen/nitrous oxide mixtures. After calibration, each rat was placed on the platform in the prone position, and the whole body was scanned. Rats were scanned 8, 12, and 16 wk after ovariectomy. The fat mass and BMD were estimated using this method.

Metagenomic DNA extraction

Faecal samples (0.5 g) were collected at 0, 1, 3, 6, 10, 14, 18 wk after ovariectomy and used to extract metagenomic DNA. The samples were homogenised using LN2 and incubated with STES buffer overnight at 60 °C for cell lysis. Then, metagenomic DNA was extracted using the phenol-chloroform-isoamyl alcohol method (Zhang *et al.*, 2006). Crude DNA was incubated with 10 µl (100 mg/ml) of RNase for 1 h to eliminate RNA. Additionally, DNA was purified using the G-spin Genomic DNA Extraction kit (iNtRON Biotechnology, Seongnam, Korea), and quantitative/qualitative analysis of the purified DNA was performed by nanodrop/agarose gel electrophoresis. To assess the efficacy of *L. intestinalis* YT2 treatment on OVX rats, faecal samples (0.5 g) were collected 18 wk after ovariectomy, and DNA extraction was performed as described above.

16S rRNA gene amplification and sequencing analysis

The V1-V2 regions of the 16S rRNA gene were amplified using universal primers (9F, 5'-GAGTTTGATCATGGCTCAG-3'; 356R, 5'-TGCTGCCTCCCGTAGGAGT-3) in the context of each purified DNA sample. For the sample sorting after the sequencing process, an eight-nucleotide barcode sequence was added to the -5' of the primer. Amplicon sequencing libraries were constructed using the Ion Plus Fragment Library kit (Thermo Fisher Scientific, Waltham, MA, USA) and quantified using high-sensitivity DNA chips and the Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA). Template amplification/enrichment was performed using the Ion Torrent OneTouch 2 System; libraries were sequenced using a 318D sequencing chip and the Ion Torrent PGM as per the manufacturer's instructions.

Bioinformatics analysis

The Mothur program was used to process the reads with the following additional pre-processing steps (Schloss *et al.*, 2009). After quality control, sequences were realigned with the SILVA compatible alignment database (Quast *et al.*, 2012). Operational taxonomic units (OTUs) defined by a 3% distance level were phylogenetically classified using a modified bacterial RDP II database (<http://www.microgator.org>).

For the disclosure of the effect of probiotics on gut microbiota, sequence quality control, taxonomy assignment, and phylogenetic insertion were performed using QIIME2 pipelines (Bolyen *et al.*, 2018). The SILVA database was used for the taxonomy assignment (Quast *et al.*, 2012). To compare groups of samples based on the Weighted UniFrac distance matrices, principal coordinate analysis (PCoA) was performed using QIIME2 and plotted using R. To detect the differential distribution of taxa in the different groups, linear discriminant analysis effect size (LEfSe) was performed using the online galaxy tool, version 1.0 (<http://huttenhower.sph.harvard.edu/galaxy/root>).

All raw sequences obtained in this study were uploaded and made available through the DDBJ database under the accession numbers DRA008693 and DRA009363.

Bacterial strains and culture

L. intestinalis YT2 (KCCM11812P), a novel functional probiotic candidate, and the *L. intestinalis* type strain (KCTC 5052) were cultivated in MRS broth (BD Difco, Sparks, MD, USA) at 37 °C under anaerobic conditions. A multi-strain probiotic with bone health-improving characteristics, VSL#3 (Li *et al.*, 2016), was purchased (VSL pharmaceuticals, Fort Lauderdale, FL, USA) and used as the positive control.

Behavioural tests

The von Frey filament test was used to measure mechanical allodynia (Chaplan *et al.*, 1994). Nylon von Frey monofilaments (Stoelting, Wood Dale, IL, USA) were used to assess the sensitivity of the plantar surface of the hind paw to increasing force (2–60 g). Rats were acclimated for 15 min on an elevating wire mesh grid under a plexiglass cage. To assess the time-course of mechanical allodynia, mechanical withdrawal threshold (MWT) was measured 4, 8, 12, and 16 wk after surgery.

To measure depression-like behaviour, a forced swim test (FST) was employed as previously described (Detke *et al.*, 1995); two sessions were conducted. 24 h before testing, animals were forced to swim for 15 min in a transparent cylinder (50 cm height, 20 cm diameter) filled with 23–25 °C water to a depth of 30 cm for adaptation. In the second session, rats were videotaped for 6 min, and the immobility time was analysed by a SMART video tracking system (SMART v3.0, Panlab SL, Barcelona, Spain). Rats were considered immobile when they were floating in the water without struggling and made simple movements to keep their heads above the water. During the FST, rats will initially try to get out of the cylinder but will eventually exhibit immobility, which means behavioural despair.

For the evaluation of the memory function, the step-through passive avoidance test was carried out as previously described with minor modifications (Yamada *et al.*, 1999). The apparatus for this test comprised a light and dark compartment shuttle box separated by a guillotine. In the training session, rats were placed in the light compartment, and when they entered the dark chamber, the door was closed and an electrical foot shock (0.3 mA, 3 s) was delivered through floor grids. In the retention session, 24 h after the training session, rats were placed in the light compartment and the time to enter into the dark compartment was measured. The cut-off latency time was measured for 300 s and no electric shock was delivered.

In vitro gut barrier function assay

Caco-2 cells (KCLB 30037.1) were obtained from the Korean Cell Line Bank (KCLB) and cultured in DMEM containing 1 g/l glucose and supplemented with 10% FBS, 1% NEAA, 100 U/ml penicillin, and 100 µg/ml streptomycin at 37 °C in humidified air with 5% CO₂. The cells were seeded at a density of 2×10⁵ cells/ml onto 12-transwell or 24-well plates (Costar, Corning, NY, USA) and allowed to grow for 3 wk; the culture medium was replaced every 2–3 days. Transepithelial electrical resistance (TEER) values were measured to check the integrity of Caco-2 cell monolayers using a Millicell-ERS device (Millipore, Bedford, MA, USA) as previously described (Shin *et al.*, 2013).

Ovalbumin (OVA) was measured by ELISA as previously described (Shin *et al.*, 2013). Immunoplates were coated with anti-OVA primary antibodies (ab17290; Abcam, Cambridge, UK) at 4 °C overnight. After PBST (0.05% Tween) washing, the coated plates were blocked with 1% bovine serum albumin for 1 h, and the supernatant was incubated for 2 h at room temperature. The plates were washed three times with PBST, and then incubated with HRP-conjugated anti-OVA secondary antibodies (ab20415, Abcam) for 1 h. The 3,3',5,5'-tetramethylbenzidine (TMB) solution was added into each well in the dark. Finally, absorbance was measured at 450 nm after the reaction was stopped with 2 N sulfuric acid solution.

Real-time reverse-transcription PCR

To measure the expression of tight junction proteins, real-time (RT) PCR was conducted as previously described (Shin *et al.*, 2013). Briefly, total RNA was extracted from Caco-2 cells using the RNeasy® Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol. cDNA synthesis and removal of genomic DNA were performed using the QuantiTect Reverse Transcription Kit (Qiagen) according to the manufacturer's instructions. RT-PCR reactions were performed in a Rotor-Gene Q system at 95 °C for 5 min followed by 40 cycles of 95 °C for 5 s and

60 °C for 10 s. The expression levels were normalised to those of the housekeeping genes, β -actin and GAPDH. The specific primer pairs used are listed in Table 1.

Biochemical measurements

Blood samples were collected from the abdominal aorta and the biochemical parameters were measured 18 wk after surgery; the serum levels of triglycerides (TG), glucose (GLU), total cholesterol (TCHO), high-density lipoprotein cholesterol (HDL), alkaline phosphatase (ALP), glutamic oxaloacetic transaminase (GOT), and glutamic pyruvic transaminase (GPT) were measured using an automated analyser (Fuji Dri-Chem, Fujifilm, Japan).

Statistical analysis

All data are expressed as the mean $\pm$ standard error of the mean (SEM). Statistical significance was determined using the unpaired t-test for two-group comparison and the one-way ANOVA followed by Tukey's post hoc analysis for multiple comparisons. Statistical analyses were performed using GraphPad Prism 5 (GraphPad Software, Inc., San Diego, CA, USA). $P < 0.05$ was considered statistically significant.

3. Results

Establishment of the menopause rat model and selection of probiotic candidates

Bilaterally OVX rats were used to establish the menopausal model; rats were distributed into two groups based on their body weight before OVX. A significant increase in body weight was observed from the first wk after surgery. The body weight gradually increased until the experimental endpoint (18 wk); the average body weights of sham-operated and OVX rats were 327.6 $\pm$ 5.0 and 414.5 $\pm$ 11.3 g, respectively (Figure 2A). Additionally, the serum oestradiol concentration was significantly decreased in the OVX group at 18 wk (Figure 2B). The BMD was also reduced in the OVX group (Figure 2C). Importantly, PCoA plotting using the Weighted Unifrac distance matrix revealed a clear

separation of gut microbial composition between the sham-operated and OVX groups starting from 10 wk after surgery (Figure 2D and Supplementary Figure S1). Of note, the compositional differences in the gut microbiota of sham-operated versus OVX rats at the species level were explored in-depth, 18 wk after surgery (Table 2). Interestingly, the abundance of *L. intestinalis* was decreased in OVX versus sham-operated rats. It is not unreasonable to consider that the decreased abundance of this microorganism might be associated with OVX-induced menopausal symptoms; therefore, we hypothesised that the restoration of *L. intestinalis* in the gut could promote the alleviation of menopausal symptoms. To test this hypothesis, we isolated *L. intestinalis* YT2 from faecal samples and evaluated its potential as a probiotic agent to attenuate the menopausal symptoms in OVX rats. Of note, the *L. intestinalis* type strain was used as a control to compare the strain difference effects on the menopausal symptoms in OVX rats.

Effect of Lactobacillus intestinalis YT2 on the gut microbiota

When the gut microbiota of each group of OVX rats was analysed 18 wk after treatment, the alpha diversity indexes showed no significant difference (Supplementary Figure S2). However, PCoA based on Weighted UniFrac distance matrices (Figure 3) revealed a clear separation between samples from sham-operated and OVX rats treated with PBS. It is noteworthy that the cluster of samples from OVX rats treated with *L. intestinalis* YT2 was clearly distinguished from that of OVX rats treated with PBS group, clustering together with the samples from sham-operated rats. Stacked bar charts showing the average relative abundance of prevalent bacteria at the phylum and family levels in all groups were also generated (Supplementary Figure S3A and S3B). The F/B ratio increased after ovariectomy; additionally, at the family level, the proportion of *Lachnospiraceae* and *Ruminococcaceae* increased and the proportion of *Muribaculaceae* decreased after ovariectomy. Of note, oestradiol did not alter the composition structure, while treatment with probiotics tended to recover the ovariectomy-induced dysbiosis. Additionally, the microorganisms significantly different between OVX-

Table 1. Table of primer sequences.

Gene	Forward primer	Reverse primer	Genebank No.
Claudin-1	CCAGTCAATGCCAGGTACGAAT	TTGGTGTGGGTAAGAGGTTGTT	AF134160
Occludin	ACAGAGCAAGATCACTATGAGACA	TGTTGATCTGAAGTGATAGGTGGA	NM_002538
ZO-1	TGGTGTCTACCTAATTCAACTCA	CGCCAGCTACAAATATTCCAACA	ZM_175610
JAM-1	AACACACTGGGACATACACTTGT	CGATGAGCTTGACCTTGACCT	AF111713
β -actin	TCATGAAGTGTGACGTGGACATC	TGCATCCTGTGCGCAATG	NM_001101

Figure 2. (A) Body weight of sham-operated and ovariectomized (OVX) rats was measured every 2 wk for 18 wk. Body weight was significantly increased 2 wk after OVX. (B) Serum oestradiol concentration estimated by ELISA; 18 wk after OVX, the concentration was significantly reduced compared to that in the sham-operated group. (C) Bone mineral density (BMD) measured using DEXA. BMD was significantly reduced 18 wk after OVX. (D) 16S rRNA amplicon analysis of gut microbiota between sham-operated and OVX group via PCA plotting. All data are expressed as the mean $\pm$ standard error of the mean. ### $P < 0.001$ compared with the sham-operated group; Student's unpaired t -test.

Table 2. The difference of closest¹ bacteria between sham-operated and ovariectomized (OVX) rats.

Closest bacteria	Sham		OVX		P -values ²
	Mean (%)	STDV (%)	Mean (%)	STDV (%)	
<i>Clostridium disporicum</i>	11.1	6.98	1.56	2.23	0.005
<i>Lactobacillus murinus</i>	9.82	5.01	29.8	14.06	<0.001
<i>Eubacterium tenue</i>	4.18	2.41	1.42	1.66	0.013
<i>Lactobacillus intestinalis</i>	3.16	3.2	0.08	0.15	0.026
<i>Clostridium</i> sp. ID4	0.98	1.08	0.03	0.06	0.038
<i>Prevotella</i> sp. DJF B116	0.58	0.81	2.89	4.29	0.045
<i>Ruminococcus bromii</i>	0.14	0.11	0.92	1.08	0.008
<i>Clostridium</i> sp. BG-C36	0.12	0.13	0.01	0.03	0.034
<i>Papillibacter cinnamivorans</i>	0.1	0.12	0.58	0.88	0.040
<i>Clostridiaceae</i> bacterium SH032	0.06	0.05	0.01	0.03	0.041
<i>Ruminococcus</i> sp. CO47	0.04	0.05	0.15	0.13	0.006
<i>Ruminococcus</i> sp. ZS2-15	0.03	0.07	0.44	0.54	0.006
<i>Clostridium indolis</i>	0.03	0.07	0.43	0.53	0.007
<i>Prevotellaceae</i> bacterium DJF VR15	0.02	0.04	0.18	0.25	0.019

¹ Closest means best hit in the database by taxon assignment.

² Difference of closet bacteria between sham and OVX. Results from Wilcoxon rank-based test.

Figure 3. PCoA plot using Weighted UniFrac distance matrices showing the different gut microbial community structures in the treatment compared to sham-operated and ovariectomized (OVX) rats treated with phosphate buffered saline (PBS). S = sham-operated; P = OVX with PBS; E = OVX with oestradiol; V = OVX with VSL#3; T = OVX with the *Lactobacillus intestinalis* type strain; Y = OVX with *L. intestinalis* YT2.

treated and -untreated rats were identified through LEfSe (Supplementary Figure S4). The five most impacted microorganisms, whose relative abundance was recovered after the administration of probiotics were *Bacteroidetes*, *Firmicutes*, *Muribaculaceae*, *Ruminiclostridium* 5, and *Ruminococcaceae* UCG-013 (Supplementary Figure S3C).

Effect of *Lactobacillus intestinalis* YT2 on the body weight, fat mass, and average intake of food and water

Next, the effect of *L. intestinalis* YT2 on menopausal symptoms was examined in OVX rats; oestradiol (17β -oestradiol) and VSL#3, a probiotic mixture of eight different bacterial strains, were used as positive controls. All treatments, except oestradiol did not prevent the OVX-induced body weight gain (Figure 4A). In fact, as shown in Figure 4B and C, treatment with oestradiol and *L. intestinalis* YT2 significantly increased food intake versus that in OVX rats treated with PBS group ($P < 0.01$); of note, the water intake was also increased in *L. intestinalis* YT2-treated OVX rats. As expected, a significant increase in the fat mass (291.1%) was observed following OVX (Figure 4D); however, treatment with *L. intestinalis* YT2 significantly inhibited this increase by 31.8%, despite the increased food and water intake ($P < 0.05$).

Effect of *Lactobacillus intestinalis* YT2 on the bone mineral density

The results of the femoral BMD are presented in Figure 5. The BMD was significantly decreased in OVX versus sham-operated rats 8 wk after surgery ($P < 0.0001$). Importantly, the BMD was significantly increased in OVX rats treated with oestradiol and microorganisms compared to that in OVX rats treated with PBS 8 wk after surgery. Of note, at 12 and 16 wk, OVX rats treated with *L. intestinalis* showed the highest BMD.

Effect of *Lactobacillus intestinalis* YT2 on the pain sensitivity, depression-like behaviour, and cognitive impairment

Next, the effects of *L. intestinalis* YT2 on pain sensitivity, depression-like behaviour, and cognitive impairment were investigated. The hind paw MWT, was used to measure pain sensitivity. As shown in Figure 6A and B, the MWT values were significantly increased after treatment with oestradiol and microorganisms in comparison to those in OVX rats treated with PBS at 4, 8, 12, and 16 wk after surgery. Notably, the OVX rats treated with *L. intestinalis* YT2 showed increased MWT to a level similar to that in

Figure 4. Effect of *Lactobacillus intestinalis* YT2 on the body weight, food intake, water intake, and fat mass in ovariectomized (OVX) rats. (A) Body weight was measured every wk for 16 wk after OVX. (B) Food intake and (C) water intake are expressed as the percentage of body weight (BW) (% g/body weight/wk) and measured for 10–16 wk. (D) The fat mass 16 wk after OVX was measured using DEXA. The sham-operated (S) and OVX groups were orally treated with distilled water, and the oestradiol treatment group was injected intraperitoneally. P = OVX with phosphate buffered saline (PBS); E = OVX with oestradiol; V = OVX with VSL#3; T = OVX with the *L. intestinalis* type strain; Y = OVX with *L. intestinalis* YT2. All data are presented as the mean $\pm$ standard error of the mean. ## $P < 0.01$, and ### $P < 0.001$ compared with the sham-operated group. *, $P < 0.05$, **, $P < 0.01$, and ***, $P < 0.001$ compared with OVX rats treated with PBS; one-way ANOVA followed by Tukey's post hoc analysis.

Figure 5. Effect of *Lactobacillus intestinalis* YT2 on bone mineral density (BMD) in ovariectomized (OVX) rats. The BMD values were measured using DEXA, 8, 12, and 16 wk after OVX. The OVX-induced BMD loss was reversed in OVX rats treated with microorganisms. S = sham-operated; P = OVX with phosphate buffered saline (PBS); E = OVX with oestradiol; V = OVX with VSL#3; T = OVX with the *L. intestinalis* type strain; Y = OVX with *L. intestinalis* YT2. Values are expressed as the mean $\pm$ standard error of the mean. ## $P < 0.01$, and ### $P < 0.001$ compared with the sham-operated group. *, $P < 0.05$, **, $P < 0.01$, and ***, $P < 0.001$ compared with OVX rats treated with PBS; one-way ANOVA followed by Tukey's post hoc analysis.

Figure 6. Effect of *Lactobacillus intestinalis* YT2 on mechanical pain sensitivity, depression-like behaviour, and cognitive function in ovariectomized (OVX) rats. (A) The mechanical withdrawal threshold of the plantar surface of the rat hind paw was measured using von Frey filaments before (baseline), and 4, 8, 12, and 16 wk after surgery. (B) The mechanical withdrawal threshold of the plantar surface of the rat hind paw was measured using von Frey filaments 16 wk after surgery. OVX-induced mechanical allodynia was significantly attenuated in OVX rats treated with *L. intestinalis* YT2. (C) The depression-like behaviour was measured using the forced swim test 18 wk after OVX. The immobility time was significantly reduced after treatment with *L. intestinalis* YT2. (D) Cognitive impairment was measured using the passive avoidance test. The latency time to enter the dark chamber was significantly increased after treatment with *L. intestinalis* YT2, 18 wk after OVX. Microorganisms were orally administered. S = sham-operated; P = OVX with phosphate buffered saline (PBS); E = OVX with oestradiol; V = OVX with VSL#3; T = OVX with the *L. intestinalis* type strain; Y = OVX with *L. intestinalis* YT2. All data are expressed as the mean $\pm$ standard error of the mean. # $P < 0.05$, and ### $P < 0.001$ compared with the sham-operated group. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ compared with OVX rats treated with PBS; one-way ANOVA followed by Tukey's post hoc analysis.

the oestradiol-treated group. The immobility time in the forced swim test was measured to evaluate depression-like behaviour. The increased immobility time induced by OVX was significantly reversed in all treatment groups (Figure 6C). Again, treatment with *L. intestinalis* YT2 reduced the immobility time to a level similar to that of the oestradiol-treated group. The effect of *L. intestinalis* YT2 on cognitive function was also examined using the passive avoidance test (Figure 6D). OVX caused impairment of learning and memory function, as indicated by a decreased of the latency time to enter the dark compartment. Importantly, the latency time was significantly increased in the *L. intestinalis* YT2-treated group, with a latency time similar to that of the sham-operated group.

Effect of *Lactobacillus intestinalis* YT2 on the serum biochemical markers

The effects of *L. intestinalis* YT2 on the levels of serum biochemical markers are shown in Table 3. Compared with those in the sham-operated group, the levels of triglycerides (TG), alkaline phosphatase (ALP), glutamic pyruvic transaminase (GPT), total cholesterol (TCHO), and high-density lipoprotein cholesterol (HDL) were significantly increased in OVX rats treated with PBS ($P < 0.01$, versus sham-operated group); interestingly, the administration of *L. intestinalis* YT2 prevented the increase in the levels of TG and ALP ($P < 0.05$). The serum levels of glucose (GLU) and glutamic oxaloacetic transaminase (GOT) were also increased in OVX rats treated with PBS (without statistical significance). However, the increased

Table 3. Menopause related serum parameter of all groups.^{1,2}

Serum parameter ³	Sham-operated	Ovariectomized (OVX)				
	PBS	PBS	Estradiol	VSL#3	<i>L. intestinalis</i> Type strain	<i>L. intestinalis</i> YT2
TG (mg/dl)	93.6±12.1	141.8±14.3 [#]	107.2±11.3	65.1±5.4 ^{***}	77.0±12.7 ^{**}	54.2±3.8 ^{***}
ALP (U/l)	226.9±17.7	422.6±39.3 ^{###}	381.1±33.9	362.7±35.2	279.0±28.1 ^{**}	318.4±22.2 [*]
GLU (mg/dl)	156.9±4.2	163.9±8.9	158.7±4.0	153.0±6.5	136.5±4.3 [*]	139.9±5.9 [*]
GOT (U/l)	102.1±5.1	90.4±4.3	81.0±7.1	95.4±7.1	78.0±4.5	93.0±6.6
GPT (U/l)	33.1±2.4	40.7±1.5 [#]	40.0±3.7	31.0±2.3 ^{**}	31.6±2.2 ^{**}	36.2±2.3
TCHO (mg/dl)	94.6±5.1	118.9±7.6 [#]	96.0±4.7 [*]	124.3±6.1	118.6±4.4	117.4±5.5
HDLC (mg/dl)	62.0±3.2	77.3± 3.5 ^{###}	66.6±3.3 [*]	79.8±3.2	80.4±1.9	77.7±3.0

¹ Serum parameters in sham, OVX with phosphate buffered saline (PBS), oestradiol, VSL#3, *Lactobacillus intestinalis* type strain, and *L. intestinalis* YT2 for 18 wk.

² Data presented as mean ± standard error of the mean (n=10-11). # *P*<0.05, ## *P*<0.01, and ### *P*<0.001 as compared with the sham-operated group. * *P*<0.05, ** *P*<0.01, and *** *P*<0.001 as compared with OVX rats treated with PBS.

³ TG = triglycerides; ALP = alkaline phosphatase; GLU = glucose; GOT = glutamic oxaloacetic transaminase; GPT = glutamic pyruvic transaminase; TCHO = total cholesterol; HDLC = high-density lipoprotein cholesterol.

levels of GLU were significantly reversed in OVX rats treated with *L. intestinalis* YT2 (*P*<0.05).

Effect of *Lactobacillus intestinalis* YT2 on the barrier function in monolayers of Caco-2 cells

To understand whether *L. intestinalis* YT2 enhances the function of the gut barrier, we used an *in vitro* system based on epithelial Caco-2 cells. *L. intestinalis* YT2 significantly increased the TEER value compared to that in the non-treated group, 3 h after treatment (Figure 7A). In line with this, ovalbumin (OVA) permeation was considerably decreased 3 h after *L. intestinalis* YT2 treatment, suggesting an enhancement of the barrier function (Figure 7B).

Besides, the expression level of representative genes of tight junctions such as *claudin-3*, *claudin-4*, and *ZO-1* was significantly increased after *L. intestinalis* YT2 treatment (Figure 7C).

4. Discussion

In this study, we used the OVX rat model, which mimics the conditions of postmenopausal women (Jin *et al.*, 2018; Zhang *et al.*, 2017). OVX rats showed significant body weight gain, a low serum oestradiol concentration, and the loss of femoral BMD compared to those in the sham-operated group. Moreover, we found that the gut microbiota of the sham-operated and OVX groups had

Figure 7. Effect of *Lactobacillus intestinalis* YT2 on the permeability of Caco-2 cell monolayers. (A) Transepithelial electrical resistance (TEER) values and (B) ovalbumin (OVA) influx were measured as indices of barrier function. (C) The mRNA expression of tight junction-related genes was detected by real-time RT-PCR. All data are expressed as the mean ± standard error of the mean. * *P*<0.05, and ** *P*<0.01 compared with the OVA group; Student's unpaired t-test.

distinct compositional structures 10 wk after surgery. Among the microorganisms that significantly decreased 18 wk after OVX, *L. intestinalis* YT2 was selected and isolated as a probiotic candidate to alleviate menopausal symptoms. Indeed, *L. intestinalis* YT2 treatment in OVX rats relieved menopausal symptoms, such as increased fat mass, decreased BMD, pain sensitivity, depression-like behaviour, and reduced memory function without influencing the body weight. Additionally, *L. intestinalis* YT2 treatment also restored the OVX-induced gut microbial dysbiosis and gut permeability alterations. Treatment of *L. intestinalis* YT2 showed better effects on restoring gut microbiota and relieving menopausal symptoms, such as increased fat mass, decreased BMD, and increased pain sensitivity than *L. intestinalis* type strain.

The composition of the gut microbiota is altered following the changes in the levels of female sex hormones, and the recovery of gut microbial dysbiosis is considered as one of the treatment targets to alleviate menopausal symptoms (Jin *et al.*, 2018; Santos-Marcos *et al.*, 2018; Zhang *et al.*, 2017). In fact, the comparison of the overall composition of the gut microbiota in women before and after menopause showed large differences at the phylum and genus levels. In postmenopausal women, the proportion of *Firmicutes* and the ratio of F/B were higher than those in premenopausal women (Santos-Marcos *et al.*, 2018). Importantly, similarly to the clinical context, gut microbial dysbiosis was observed in OVX animals, and the F/B ratio was also increased (Jin *et al.*, 2018; Zhang *et al.*, 2017). Interestingly, the F/B ratio was also reported to increase in obese people and obesity is one of the menopausal symptoms. Curcumin and grape seed extract administration reduced the F/B ratio and weight gain caused by OVX (Jin *et al.*, 2018; Zhang *et al.*, 2017). In line with the above, our result showed an increase in the F/B ratio in OVX rats, and its recovery after *L. intestinalis* YT2 treatment. Since a reduction in the fat mass was also observed, we suggest that *L. intestinalis* YT2 recovers both the F/B ratio and obesity in OVX rats.

Pain, depression, cognition, and bone loss were all associated with gut microbial dysbiosis. Increased mechanical hyperalgesia and altered gut microbiota profiles were found in a chemotherapy-induced neuropathic pain model; remarkably, the increased pain sensitivity was attenuated in both germ-free and antibiotic-treated mice (Shen *et al.*, 2017). In addition, there is a negative correlation between *Faecalibacterium* and depressive symptoms in patients with major depressive disorder (Jiang *et al.*, 2015). Cognitive impairment and gut microbiota alterations were also identified in a high-fat diet-induced obesity model and were improved after teasaponin treatment (Wang *et al.*, 2017). BMD was also significantly related to changes in the gut microbiota in older adults with osteoporosis and osteopenia (Das *et al.*, 2019). In addition, an increase in the relative abundance of *Muribaculaceae* (previously known as S24-7)

was identified in the OVX model, similar to the observed in a previous report (Choi *et al.*, 2017), and the abundance of *Muribaculaceae* was restored after *L. intestinalis* YT2 treatment. The mechanism of gut dysbiosis in the OVX model was not proposed or investigated in-depth, but considering the association between gut microbiota and the symptoms of menopause, the recovery of dysbiosis following *L. intestinalis* YT2 treatment may have played a role in the regulation of menopausal symptoms.

Dysbiosis plays a pivotal role in increased gut permeability (Boulange *et al.*, 2016). Disruption of the intestinal epithelium barrier results in a leaky gut that allows harmful substances to pass through mucosal tissues, leading to a number of diseases including inflammatory bowel disease, celiac disease, and type 1 diabetes (Groschwitz and Hogan, 2009). In fact, the restoration of gut permeability is a possible mechanism to explain the impact of *L. intestinalis* YT2 on menopausal symptoms in OVX rats. In fact, associations between gut permeability and menopause were previously disclosed in animal models (Li *et al.*, 2016). Additionally, the gut permeability was also associated with menopausal symptoms, such as obesity (Boulange *et al.*, 2016), depression (Arseneault-Breard *et al.*, 2012), cognitive function (Jang *et al.*, 2018), pain sensitivity (Zhou *et al.*, 2009), and bone loss (Li *et al.*, 2016). For instance, obesity was associated with a leaky gut as well as with alterations in the composition of the gut flora; increased leakage of bacteria and bacterial products is a risk factor of obesity-associated diseases, including cardiovascular diseases and type 2 diabetes (Boulange *et al.*, 2016). Depression and gut permeability also showed a positive association in both preclinical and clinical studies (Arseneault-Breard *et al.*, 2012; Leclercq *et al.*, 2014). Further, probiotics restored gut barrier integrity and relieved the depressive-related behaviour (Arseneault-Breard *et al.*, 2012). Memory impairment occurred together with gut leakiness in a colitis model (Jang *et al.*, 2018). Furthermore, visceral and thermal pain sensitivity were positively correlated with gut permeability in irritable bowel syndrome patients (Zhou *et al.*, 2009). In the OVX model, bone loss and increased gut permeability were previously identified, and these symptoms were prevented by probiotics (Li *et al.*, 2016). Caco-2 cells are widely used to measure gut permeability and efflux liability *in vitro* (Shin *et al.*, 2013). Here, in the context of this *in vitro* system, *L. intestinalis* YT2 restored the gut permeability and significantly increased the expression of tight junction genes, such as *claudin-3*, *claudin-4*, and *ZO-1*. Therefore, *L. intestinalis* YT2 may impact menopausal symptoms via gut permeability restoration.

The differential composition of microorganisms can be used as a disease marker; therefore, comparing the gut microbiome between normal and disease models can lead to the discovery of new therapeutic approaches (Bo *et al.*, 2020; Boulange *et al.*, 2016; Qiu *et al.*, 2013;

Schott *et al.*, 2018; Takahashi *et al.*, 2016). For example, *Bifidobacterium pseudolongum* was significantly reduced in the gut of obese mice compared to lean mice, and the administration of *B. pseudolongum* to high-fat diet-induced obese animals resulted in a decrease in body mass, plasma TG, gross energy intake, and visceral fat (Bo *et al.*, 2020; Schott *et al.*, 2018). Furthermore, a reduced abundance of *Faecalibacterium prausnitzii* was observed in patients with Crohn's disease (Takahashi *et al.*, 2016). Importantly, the oral administration of *F. prausnitzii* (ATCC 27766) significantly reduced the severity of TNBS-induced colitis and the secretion of inflammatory cytokines, in a mouse model of human Crohn's disease (Qiu *et al.*, 2013). In the current study, significant changes in various microorganisms were observed during the establishment of the OVX model. Microorganisms with a significantly reduced relative abundance in OVX group were considered as potential probiotic candidates: *Lactobacillus*, *Clostridium*, and *Eubacterium*. Of the potential candidates, *L. intestinalis* YT2 was finally selected because the *Lactobacillus* and *Bifidobacterium* genera are commonly used as probiotics and are known to be safe (Collins *et al.*, 2017). Of note, previously the administration of *Lactobacillus* spp. improved menopausal symptoms in clinical and animal experiments (Collins *et al.*, 2017; Dar *et al.*, 2018; Petricevic *et al.*, 2008). *L. acidophilus* ATCC 4356 and *L. paracasei* DSM13434 reduced the bone loss and increased bone resorption in OVX mice (Collins *et al.*, 2017; Dar *et al.*, 2018). Additionally, *L. rhamnosus* GR-1 and *L. reuteri* RC-14 restored the vaginal flora in postmenopausal women in a randomised, double-blind, placebo-controlled study (Petricevic *et al.*, 2008).

L. intestinalis, are Gram-positive bacteria isolated from the intestines of mice and rats, resistant to acid and bile salts, which support their potential as probiotics (Fujisawa *et al.*, 1990; Jena *et al.*, 2013). To the best of our knowledge, our study is the first try to evaluate the probiotic effects of *L. intestinalis* in OVX-induced menopausal symptoms. However, a previous study reported conflicting effects of *L. intestinalis* on depressive behaviour (Wang *et al.*, 2020). The analysis of gut microbiota suggested that the abundance of *L. intestinalis* and *L. reuteri* was higher in antibiotic-treated mice with faecal microbiota transplantation from mice subjected to chronic social defeat stress (CSDS) versus control mice. Curiously, the oral administration of the two microorganisms induced depressive behaviour in antibiotic-treated mice. On the other hand, several studies have shown that *L. reuteri* decreased depressive behaviour in CSDS and OVX mice (Sovijit *et al.*, 2021; Xie *et al.*, 2020). In our study, 16S rRNA analysis showed a decreased abundance of *L. intestinalis* in OVX versus sham-operated animals. Importantly, we were able to show that the oral administration of *L. intestinalis* improved OVX-induced depressive behaviour in rats, probably via the restoration of the gut microbiota. Further studies are required to

identify the probiotic effects of *L. intestinalis* in various animal models of depression. Recently, *L. intestinalis* was involved in the production of equol, a daidzein metabolite that relieves menopausal symptoms (Heng *et al.*, 2019). Therefore, the production of equol may be one of the possible mechanisms behind the relief of the menopausal symptoms observed here.

We suggest that *L. intestinalis* YT2 relieves menopausal symptoms in OVX rats without affecting the oestrogen levels; in fact, oestrogen administration impacts body weight as shown in our study. Although we suggest that *L. intestinalis* YT2 relieves menopausal symptoms via the restoration of intestinal dysbiosis and permeability, the mechanisms behind have not been fully elucidated. Therefore, further studies are needed to identify the mechanism of the role of *L. intestinalis* YT2 in menopause.

5. Conclusions

In conclusion, we show that *L. intestinalis* YT2 treatment recovered gut dysbiosis in OVX rats and improved menopausal symptoms such as increased fat mass, pain sensitivity, depressive-related behaviour, cognitive impairment, and bone loss. Of note, *L. intestinalis* YT2 seems to promote the general improvement of menopausal symptoms as well as specific ones. Moreover, the efficiency of the probiotic treatment was improved by selecting candidate strains based on gut microbial dysbiosis. Taken together, these findings strongly suggest that *L. intestinalis* YT2 could be considered as a potential therapeutic tool for improving menopausal symptoms.

Supplementary material

Supplementary material can be found online at <https://doi.org/10.3920/BM2020.0217>.

Figure S1. 16S rRNA amplicon analysis of gut microbiota between sham-operated and OVX group via PCA plotting.

Figure S2. Alpha diversity in different groups according to 3 algorithms.

Figure S3. Effect of the administration of *Lactobacillus intestinalis* YT2 on the gut microbiota.

Figure S4. Histograms of linear discriminant analysis effect size (LEfSe).

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Conflict of interest

The authors declare no conflicts of interest.

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