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Oral Administration of Mice with Cell Extracts of Recombinant *Lactococcus lactis* IL1403 Expressing Mouse RANKL

Abstract

Receptor activator of NF-kB ligand (RANKL) is known to play a major role in bone metabolism and the immune system, and its recombinant form has been expressed in bacterial systems for research since the last two decades. However, most of these recombinant forms are used after purification or directly using living cells. Here, there were cell extracts of recombinant *Lactococcus lactis* expressing mouse RANKL (mRANKL) used to evaluate its biological activity in mice. Mice were divided into three groups that were fed phosphate-buffered saline (PBS), WT_CE (wild-type *L. lactis* IL1403), and mRANKL_CE (recombinant *L. lactis* expressing mRANKL). The small intestinal transcriptome and fecal microbiome were then profiled. The biological activity of mRANKL_CE was confirmed by studying RANK-RANKL signaling in vitro and in vivo. For small intestinal transcriptome, differentially expressed genes (DEGs) were identified in the mRANKL_CE group, and no DEGs were found in the WT_CE group. In the PBS vs. mRANKL_CE gene enrichment analysis, upregulated genes were enriched for heat shock protein binding, regulation of bone resorption, and calcium ion binding. In the gut microbiome analysis, there were no critical changes among the three groups. However, *Lactobacillus* and *Sphingomonas* were more abundant in the mRANKL_CE group than in the other two groups. Our results indicate that cell extracts of recombinant *L. lactis* expressing mRANKL can play an effective role without a significant impact on the intestine. This strategy may be useful for the development of protein drugs.

Keywords: Cell extracts, RANKL, *L. lactis*, transcriptome, microbiome

Introduction

The production of recombinant proteins has been the foundation of the industrial and pharmaceutical biotechnology for the past 30 years (Puetz and Wurm, 2019). There are a wide variety of cell factories, such as mammalian cell lines, insect cells, whole plants, yeast, and bacteria (Ferrer-Miralles and Villaverde, 2013). *L. lactis* subsp. lactis IL1403 is a laboratory strain that is widely used in recombinant DNA technology (Pedersen et al., 2005). *L. lactis* is generally recognized as safe (GRAS) by the Food and Drug Administration (FDA) (Song et al., 2017). Moreover, *L. lactis* is a popular microbial factory system owing to the wealth of genetic knowledge about it, and several existing recombinant protein expression systems (Linares et al., 2010). With the emergence of safety issues related to live microbial cells, including living modified organisms (LMOs), there is increasing interest in probiotic cell components and metabolites (Teame et al., 2020). To date, there have been few studies on the oral administration of bacterial cell extracts as delivery vectors containing recombinant proteins.

Microfold (M) cells are specialized immune cells located in gut-associated lymphoid tissue (GALT), and play an essential role in the initiation of the intestinal immune response that transports luminal antigens through the intestine toward GALT (Foussat et al., 2001). Many previous studies have shown that the RANKL can induce the differentiation of M cells (Kanaya et al., 2012; Knoop et al., 2009; Kunisawa et al., 2008). Knoop et al. (2009) showed that purified recombinant mouse RANKL produced by a recombinant *Escherichia coli* differentiated M cells in the small intestine of RANKL-null mice (Knoop et al., 2009). In addition, Kim et al. (2015) showed that oral administration of recombinant *L. lactis* secreting mouse RANKL significantly increased the number of mature M cells in the mouse small intestine (Kim et al., 2015). These studies demonstrated that recombinant RANKL produced by bacteria is biologically active.

With the advent of next-generation sequencing (NGS) technology, it is easy to determine gene

expression levels in the tissues or gut microbiomes. Many studies have focused on the profiling gene expression in diseases, and also profiled the alteration of gut microbiome in diseases or determined the effect of consuming live probiotics, but the alteration of gene expression and gut microbiome after intake of bacterial cell extracts or recombinant proteins is not clear yet.

In this study, the effect of the cell extracts of recombinant *L. lactis* expressing mRANKL were evaluated on small intestinal gene expression and gut microbiome in mice, to determine the usefulness of combinations of cell extracts and recombinant proteins for the development of protein drugs.

Materials and Methods

Bacterial strains and growth conditions

Wild-type *L. lactis* IL1403 was used as the host strain for recombinant protein production, and grown in M17 medium (MBcell, Republic of Korea) supplemented with 5 g/L of glucose (M17G). Recombinant *L. lactis* IL1403 was grown in M17G media with chloramphenicol (5 μg/mL) and erythromycin (5 μg/mL) at 30°C.

Gene synthesis and plasmid construction

Mouse RANKL sequence was used in Knoop and Jeong-in Kim's studies (Kim et al., 2015; Knoop et al., 2009). To secrete the target protein, the signal peptide of USP45 (van Asseldonk et al., 1993) was added to the N-terminus, and to detect the expressed protein, a his-tag (his6x) was added to the C-terminus of the target gene. The designed amino acid sequence was codon-optimized using DNAWorks v3.2.4 (Hoover and Lubkowski, 2002) based on the *L. lactis* IL1403 codon usage table. The insert fragment was synthesized by Macrogen Inc. (Republic of Korea). The insert fragment was shown in Fig. S1. The plasmid vector pILPtuf.Mb was used as the backbone (Kim et al., 2009). Insert and vector were ligated at the *NdeI* and *XhoI* restriction

sites and transformed into wild-type *L. lactis* IL1403 competent cells. Vector construction is shown in Fig. 1A.

Physiological characterization of *L. lactis* strains

Wild-type and recombinant *L. lactis* strains were cultured in 50 mL of M17G broth without and with antibiotics, respectively. The optical density (OD) of each sample was measured at a wavelength of 600 nm every 1 h for 12 h and 24 h after inoculation.

Confirmation of the expression of mRANKL

The expression of mRANKL from recombinant *L. lactis* was measured by SDS-PAGE and western blotting. Wild type and recombinant *L. lactis* were cultured in M17G broth without and with antibiotics at 30°C for 10 h, respectively. For preparation of cell extracts, 10 mL of cultured cells were harvested by centrifugation at 13,500 rpm for 1 min and lysed using a bead beater with 0.5 g sterilized glass beads (0.5 mm) and 200 µL 1× phosphate buffered saline (PBS). The secreted proteins were collected from 10 mL of cultured cell-free supernatant (0.2 µm filtered) through trichloroacetic acid (TCA) precipitation, and dissolved in 200 µL 1× PBS for SDS-PAGE and western blot analysis. To quantify the amount of mRANKL produced, 18 kDa commercial recombinant His-tagged human calmodulin (MERCK, Darmstadt, Germany) was used for construction of the standard curve using amounts of 1.5, 1 and 0.5 µg.

Validation of the biological activity of recombinant mRANKL in vitro

RAW 264.7 cells were seeded on cell culture dishes in Dulbecco's modified Eagle's medium (DMEM) (CAPRICORN, Germany) with 10% fetal bovine serum (FBS) (CAPRICORN, Germany) and 1% penicillin and streptomycin (P/S) at a density of 2×10^6 cells/mL. After seeding for 4 h, the medium was changed to DMEM with 10% FBS, 1% P/S, and 30 ng/mL macrophage colony-stimulating factor (M-CSF) with crude cell extracts of wild-type or

recombinant *L. lactis*, that were prepared as described above. The group treated with 1× PBS as control was named PBS, and groups treated with cell extracts from wild type *L. lactis* IL1403 and mRANKL producing *L. lactis* were named WT_CE and mRANKL_CE, respectively. According several dose test, finally, the cell extracts containing 90 ng/mL of mRANKL used in this study, and the same amount of cell extracts of wild-type *L. lactis* IL1403 were used. Commercial mouse RANKL (Abcam, Republic of Korea) was added to the medium at a concentration of 60 ng/mL.

After 72 h of treatment, the medium was replaced with fresh media, and the cells were incubated for another 72 h. Total RNA was extracted using TRIzol® (ThermoFisher, Republic of Korea) according to the manufacturer's instructions, and cDNA was synthesized using PrimeScript™ RT reagent Kit (TAKARA, Japan). qRT-PCR was conducted using TB Green® Premix Ex Taq™ (Tli RNaseH Plus, TAKARA, Japan) with specific primers TRAP-F:5′-GCGACCATTGTTAGCCACATACGG-3′; TRAP-R:5′-CGCCCAGGGAGTCCTCAGATCCAT-3′ and primers for the housekeeping gene GAPDH-F:5′-AACTTTGGCATTGTGGAAGGGCTC-3′; GAPDH-R:5′-AAGGCCATGCCAGTGAGCTTC-3′. mRNA levels were normalized to those of GAPDH (Kim et al., 2015).

Animal experiments

Four-week-old BALB/c mice were randomly assigned to the three groups (PBS, WT_CE, and mRANKL_CE). Before oral administration, 300 μL of neutralizing reagent (1.5% NaH2CO3) was administered to prevent gastric acidity. After 30 min, 100 μL 1 × PBS was fed to the PBS group, and the cell extracts from 2.5×10^8 CFU wild type *L. lactis* IL1403 and cell extracts containing mRANKL (4.2 μg) from 2.5×10^8 CFU of recombinant *L. lactis* were fed to WT_CE and mRANKL_CE groups, respectively. All groups were fed for seven consecutive days and

sampled on the eighth day (Fig. S2).

Validation of the biological activity of recombinant mRANKL in vivo

Peyer's patches of small intestine samples were extracted to determine the abundance of mature M cells by measuring the GP2 mRNA expression level. Isolation of RNA and synthesis of cDNA was same as above method. qRT-PCR was conducted using TB Green® Premix Ex Taq™ (Tli RNaseH Plus, TAKARA, Japan) with M cell-specific primers GP2-F:5′-GATACTGCACAGACCCCTCCA-3′; GP2-R:5′- GCAGTTCCGGTCATTGAGGTA-3′ (Kusunose et al., 2020), and primers for the housekeeping gene GAPDH-F:5′-AACTTTGGCATTGTGGAAGGGCTC-3′; GAPDH-R:5′-AAGGCCATGCCAGTGAGCTTC-3′. mRNA levels were normalized to those of GAPDH.

RNA extraction and sequencing

Nine ileum samples of the same size (1 cm) were extracted from the same position (distal ileum). Total RNA was isolated from the tissues using the Maxwell (Promega) method. One milligram of total RNA was processed to prepare the mRNA sequencing library using the MGIEasy RNA Directional Library Prep Kit (MGI), and sequencing was performed using the MGIseq system.

Bioinformatic analysis of the transcriptome

After a quality check, raw reads were trimmed to 100 bp prior to mapping to the mouse reference genome GRCm38/mm10 using HiSat2 (version 2.1.0) (Pertea et al., 2016). Following read alignment, counts assigned to features were computed using the featureCounts (version 2.0.3) (Liao et al., 2014). Differentially expressed genes (DEGs) were identified using the DESeq2 (v1.24.0) (Love et al., 2014). A gene was considered to be a DEG with a fold change>1.45 and adjusted p value<0.1 using the Benjamini-Hochberg method (Benjamini and

Hochberg, 1995). Gene Ontology (GO) enrichment analysis was performed using in-house Perl scripts. The significantly enriched GO terms were determined by Fisher's exact test with $p<0.05$ and odds ratio>1.

16S rRNA amplicon sequencing

Genomic DNA was extracted from fecal samples using a NucleoSpin Soil kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's instructions. DNA samples (5 ng) were used to amplify the 16S ribosomal RNA V4 region using Takara Ex-taq DNA polymerase (Takara Bio, Shiga, Japan) with universal primer sets (Forward:5′-GGACTACHVGGGTWTCTAAT-3′ and R:5′-GTGCCAGCMGCCGCGGTAA-3′) (Han et al., 2018). After amplification, all the samples were normalized to 50 ng per sample. A DNA library was then constructed and sequenced using the Illumina MiSeq platform (Illumina Inc., San Diego, CA, USA), generating 2×300 bp paired-end reads.

Bioinformatic analysis of the gut microbiome

To analyze the gut microbiome, de-multiplexed and pre-processed sequence reads were imported into Quantitative Insights Into Microbial Ecology (QIIME2, version 2021.2) (Bolyen et al., 2019). Barcode and primer removal, quality control, amplicon sequence data correction, and de-replication, were performed using the DADA2 (Callahan et al., 2016). Feature tables and representative sequence files were merged for downstream analysis using QIIME2. Taxonomic classification was performed using the SILVA 132 database with 99% identity, based on the V4 16S region. All classification was performed within QIIME2 and was assigned using the naïve Bayesian algorithm available in the sklearn python library. For phylogenetic diversity analysis, alpha and beta diversities were calculated using the q2-diversity plugin, and included Faith's phylogenetic diversity and weighted and unweighted UniFrac distances. Differential abundance analysis of microbiota was performed using an in-house Perl script. We

considered a p value<0.05 to indicate statistical significance.

Calcium assay

Serum samples were collected on the eighth day, and concentration was measured using a Calcium Colorimetric Assay Kit (BioVision Inc., USA).

Statistical analysis

Statistical analysis was performed using an in-house Perl script and R (version 4.1.0) language. One-way analysis of variance (ANOVA) followed by Tukey's post-hoc test was used for determining statistical significance.

Results

Expression of mRANKL from recombinant *L. lactis*

To confirm the expression of mRANKL, intracellular and secreted proteins were precipitated and analyzed by western blotting. No mRANKL was detected in the wild-type *L. lactis* samples. In recombinant *L. lactis*, intracellular and secreted mRANKL proteins were detected with expected size of 23.86 and 20.88 kDa, respectively (Fig. 1B). According to the standard curve, recombinant *L. lactis* produced 2.1 μg/mL of mRANKL in intracellular fraction (Fig. S3).

Physiological characterization of wild type and recombinant *L. lactis*

To examine the physiological characteristics of wild type and the recombinant *L. lactis*, patterns of growth were measured. As shown in Fig. S4, recombinant *L. lactis* showed a slightly delayed growth rate compared to the wild type; after 10 h though, these two strains showed similar growth patterns.

Validation of the biological activity of recombinant mRANKL in vitro

To validate the biological activity of mRANKL, RAW 264.7 cells were used to observe the stimulation of RANK-RANKL signaling. As shown in Fig. S5, treatment with culture media containing commercial RANKL and mRANKL_CE significantly ($p<0.05$) increased TRAP mRNA expression levels compared with those in the PBS and WT_CE groups.

Validation of the biological activity of recombinant mRANKL in vivo

Cell extracts containing recombinant mRANKL were orally administered to mice for seven consecutive days, and the expression of mature M cells marker in Peyer's patches was analyzed by qRT-PCR. As shown in Fig. S6, the GP2 mRNA expression levels in the mRANKL_CE group were significantly ($p<0.05$) higher than those in the PBS and WT_CE groups. In addition, body weight gain was measured between day 1 and day 8 of the experiment, and the results showed that oral administration of WT_CE and mRANKL_CE had no significant effect on body weight (Table S1). Our results showed that recombinant mRANKL from recombinant *L. lactis* is biologically active and does not cause significant changes in body weight.

Gene expression profiling

To compare the gene expression between the PBS, WT_CE, and mRANKL_CE groups, RNA-Seq analysis of the mouse small intestine was performed. The WT_CE and mRANKL_CE groups were compared with the PBS group, with a cut-off fold change>1.45 and adjusted p value<0.1. No DEGs were identified between the PBS and WT_CE groups. Between the PBS and mRANKL_CE groups, 63 DEGs, including 53 upregulated and 10 downregulated DEGs, were identified (Fig. 2 and Table S2). Between the WT_CE and mRANKL_CE groups, 192 DEGs were identified, including 169 upregulated and 23 downregulated DEGs (Table S2). These results indicate that cell extracts from wild-type *L. lactis* IL1403 had no effect on mouse small intestinal gene expression, and only mRANKL had an effect.

GO analysis of DEGs

To analyze the gene enrichment of DEGs, GO analysis were performed with a threshold of $p<0.05$ and odds ratio>1. Between the PBS and mRANKL_CE groups, 49 upregulated and 9 downregulated DEGs were annotated with GO terms. The upregulated GO terms included 'de novo' protein folding ($p<0.001$), response to unfolded protein ($p<0.001$), regulation of bone resorption ($p<0.01$), heat shock protein binding ($p<0.001$), and calcium ion binding ($p<0.05$) (Table 1 and S3). Between the WT_CE and mRANKL_CE groups, 159 upregulated and 22 downregulated DEGs were annotated with GO terms. The upregulated GO terms included calcium ion binding ($p<0.001$), calmodulin binding ($p<0.001$), and 'de novo' protein folding ($p<0.05$) (Table 2 and S3).

Gut microbial diversity

To compare the gut microbial diversity, the alpha and beta diversities of the three groups from normalized microbiome sequencing reads were investigated. For alpha diversity, three indices including observed features, Shannon and Faith's phylogenetic diversity (Faith PD) were measured. None of the three indices showed any significant differences among the three groups (Fig. S7). For beta diversity, principal coordinate analysis (PCoA) of unweighted and weighted UniFrac distances was performed. There were no significant differences among the three groups (Fig. S8).

Gut microbial composition

To compare the differences in major gut microbial taxa among the three groups, the microbial composition in these three groups were examined. The overall microbial composition in the gut was not significantly different among the three groups. However, the genera *Lactobacillus* ($p<0.05$), *Sphingomonas* ($p<0.01$) and *Acinetobacter* ($p<0.01$) differed significantly (Fig. 3 and

241 Table S4). Moreover, there was no significant difference among the three groups in the genus
242 *Lactococcus* (Fig. 3 and Table S4). These results showed that feeding cell extracts with or
243 without mRANKL did not have a significant effect on the gut microbiome.

244 **Calcium assay**

245 After RANKL administration, serum calcium levels were measured to compare the changes in
246 calcium concentration among the three groups. There was no significant difference in serum
247 calcium levels among the three groups (Fig. S5).

248

249 **Discussion**

250 Many recombinant microorganisms are widely used in food, chemical, and pharmaceutical
251 industries today (Khan et al., 2016). The direct use of LMOs poses potential risks to the
252 environment, humans, and animals (Prakash et al., 2011).

253 LAB are traditionally used in fermentation and food preservation, and are recognized as safe
254 for consumption. In particular, *L. lactis* has been engineered as a live vehicle for the delivery
255 of DNA vaccines and production of therapeutic biomolecules (Tavares et al., 2020). In bacterial
256 cell extracts study, cell extracts from recombinant *L. lactis* expressing SARS-CoV-2 spike
257 protein were used to oral immunize mice, and antigen-specific antibodies were produced from
258 immunized mice (Xuan et al., 2022). *L. lactis* IL1403 is a representative laboratory strain that
259 can be used to produce recombinant heterologous proteins (Tavares et al., 2020), and there is
260 no evidence to date that metabolites of *L. lactis* IL1403 are toxic to experimental animals.
261 Moreover, use of cell-free extracts removes the risk of LMOs being released into the
262 environment. Our results demonstrated that cell extracts containing mRANKL from
263 recombinant *L. lactis* IL1403 can differentiate RAW 264.7 cells into osteoclast-like cells (Fig.

S5) and increase the number of mature M cells in mice (Fig. S6). In addition, in the transcriptome analysis, no DEGs were found between PBS and WT_CE groups; while DEGs were found between PBS and mRANKL_CE groups. These results indicate that wild-type *L. lactis* IL1403 has no effect on mouse small intestinal transcriptome, whereas mRANKL does have an effect. Moreover, RANKL not only affects the differentiation of M cells, but also affects other RANKL signal-responsive cells (Kukita and Kukita, 2013). Knoop et al. (2009) demonstrated not only GALT expressed RANK in mice (Knoop et al., 2009). Heat shock proteins are a family of proteins produced by cells in response to stress, and they positively regulate osteoclastic bone resorption through the RANKL-RANK signaling pathway (Hang et al., 2018). In this study, genes associated with heat shock protein binding and regulation of bone resorption were found to be upregulated in the mRANKL_CE group. Calcium signaling plays a significant role in osteoclastogenesis; the RANKL receptor utilizes calcium signaling to drive osteoclast differentiation (Komarova et al., 2003), and genes associated with calcium ion binding were found to be upregulated in the mRANKL_CE group. Additionally, *SLIT3* was found to be co-upregulated in the mRANKL_CE group compared to its expression in the PBS (adjusted $p<0.1$) and WT_CE (adjusted $p<0.1$) groups (Table S2). *SLIT3* expression increases during osteoclast differentiation (Koh, 2018). In addition, bone remodeling-associated genes *ZBTB16* (vs. PBS, adjusted $p<0.001$; vs. WT_CE, adjusted $p<0.01$) and *ZBTB40* (vs. PBS, adjusted $p<0.01$; vs. WT_CE, adjusted $p<0.1$) were found to be co-upregulated in the mRANKL_CE group (Table S2) (Felthaus et al., 2014; Twine et al., 2016). However, no study so far has investigated whether an increase in RANKL levels in the intestine causes bone resorption. Since the cell extracts of the host producing recombinant proteins do not appear to have any effect on experimental animals, this strategy may be an alternative to the use of live cells. The well-known signal peptide USP45 is located at the upstream of the target protein and its secretion is relatively low. Improving the secretion yield of target proteins in cell-free culture

supernatant, can be a good alternative to the direct use of LMOs.

Recently, some studies have used postbiotics replace the live bacteria to improve the intestinal environment; postbiotics are defined as “non-viable bacterial products or metabolic products from microorganisms that have biological activity in the host” (Nataraj et al., 2020; Siciliano et al., 2021). Postbiotics contain a wide range of molecules, including peptidoglycans, surface proteins, cell wall polysaccharides, secreted proteins, bacteriocins, and organic acids, which can have positive effects on the host, including immunomodulatory, antitumor, antimicrobial, and barrier preservation effects (Nataraj et al., 2020; Teame et al., 2020). In our study, bacterial crude cell extracts had no adverse effect on the host, but exogenous proteins had favorable effects. Hence, exogenous protein can be produced by probiotics, and the cell extract can then be used for therapeutic or other purposes. Also, the crude cell extracts can be used directly, without the need for purification (Taghinezhad et al., 2021).

There have been many recent studies on the host-microbiome interactions in postbiotics (Nataraj et al., 2020; Peluzio et al., 2021; Siciliano et al., 2021; Teame et al., 2020). However, there have been no studies on the state of intestinal microbiome after intake of live recombinant LAB or its cell extracts. In this study, NGS were used to characterize how cell extracts of wild-type or recombinant *L. lactis* affected the gut microbiome. Although there was no significant change in the gut microbiome, the abundances of several genera were significantly different (Fig. 3 and Table S4). RANKL stimulates the differentiation of monocyte / macrophage precursor cells into osteoclasts, and overexpression of RANKL resulting in bone erosion in rheumatoid arthritis (RA) (Tanaka, 2019). The abundance of *Lactobacillus* is significantly higher in patients with RA than in healthy controls (Li et al., 2021). Our results also showed that the abundance of *Lactobacillus* was significantly ($p<0.05$) higher in the mRANKL_CE group. The abundance of *Sphingomonas* was also significantly ($p<0.01$) higher in the

mRANKL_CE group. Eriksson et al. (2022) showed that *Sphingomonas* abundance was positively correlated with RA (Eriksson et al., 2022). Another RA-related genus, *Bacteroides*, showed decreased abundance in the mRANKL_CE group, although the difference was not significant. In addition, the abundance of *Bacteroides* was decreased in patients with RA compared with its abundance in in the healthy controls (Wang et al., 2022). These results may indicate changes in abundances of specific microbes caused by excessive RANKL in the intestine. One diagnostic marker in RA patients is a higher serum calcium concentration than that in the healthy controls (Tawfik et al., 2019). In addition, an increase in RANKL levels in the bone microenvironment leads to bone resorption and increased calcium release (Ono et al., 2020). Our result indicated no significant differences in serum calcium levels among the three groups (Table S5). Moreover, the abundance of *Lactococcus*, which was used as the host for the production of mRANKL, was not significantly different among the three groups (Fig. 3). This indicates that the cell extracts of *L. lactis* did not elicit an immune response, which may be because *Lactococcus* is a natural inhabitant of the mouse gut. Our results indicate that the cell extracts of *L. lactis* did not have a significant impact on the mouse gut microbiome.

Conclusion

In summary, the cell extracts containing mRANKL from recombinant *L. lactis* are biologically active both in vitro and in vivo, and the cell extracts from wild-type *L. lactis* IL1403 did not affect intestinal transcriptome. In addition, there was no significant change in the gut microbiome after administration of cell extracts of wild-type *L. lactis* IL1403 or recombinant *L. lactis*. This strategy could potentially be used for the development of protein drugs.

Ethical Statement

The animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC approval number: KW-190605-1) at Kangwon National University.

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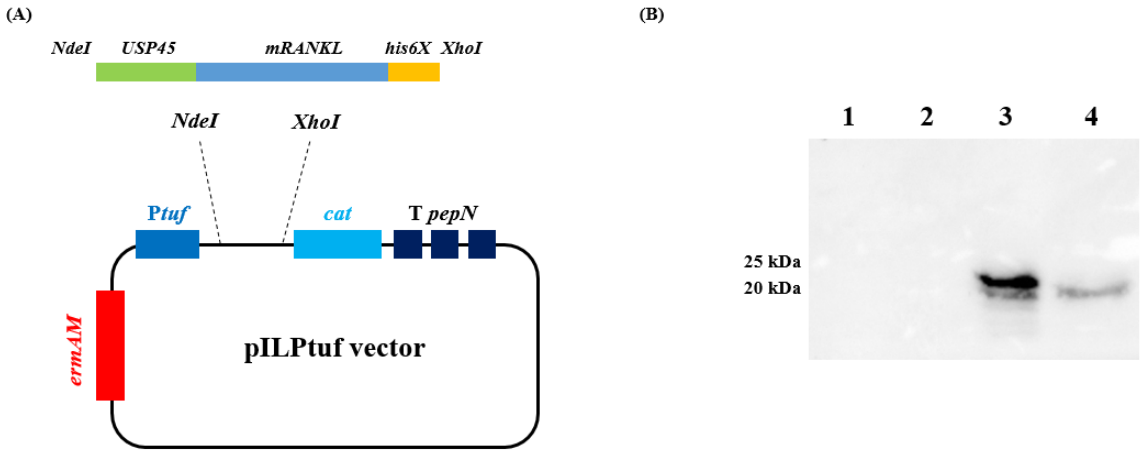

468

469 **Figure 1. Schematic diagram for construction of recombinant mRANKL expression**

470 **vector system and detection of target gene expression. (A)** Plasmid vector system

471 (modified from [EB Kim et al., 2009]). **(B)** Western blot for detecting mRANKL from cell

472 extracts (intracellular) and cell-free culture supernatants (extracellular), lane 1: Cell extracts

473 of *L. lactis* IL1403; lane 2: Cell-free culture supernatant of *L. lactis* IL1403; lane 3: Cell

474 extracts of recombinant *L. lactis* IL1403; lane 4: Cell-free culture supernatant of recombinant

475 *L. lactis* IL1403.

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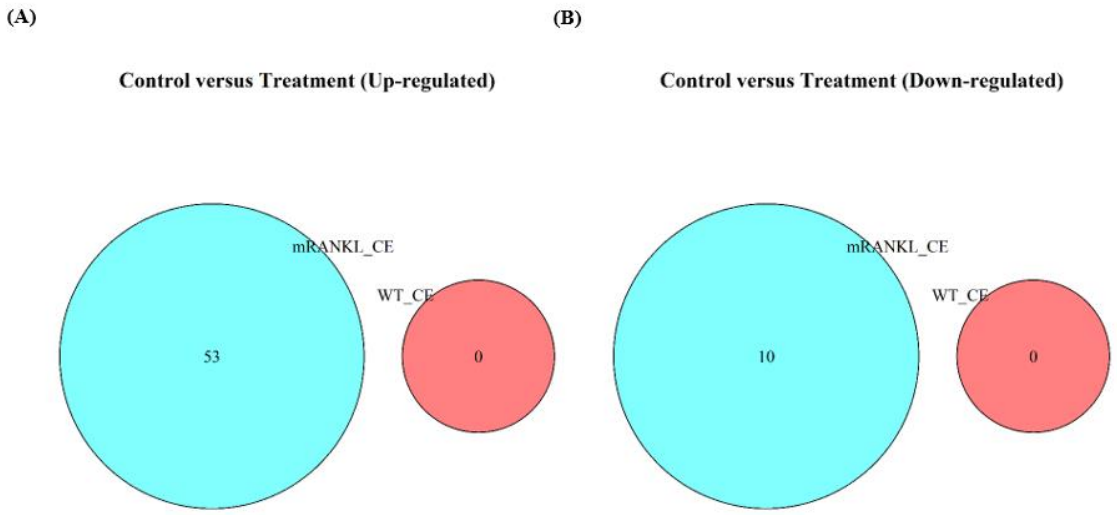
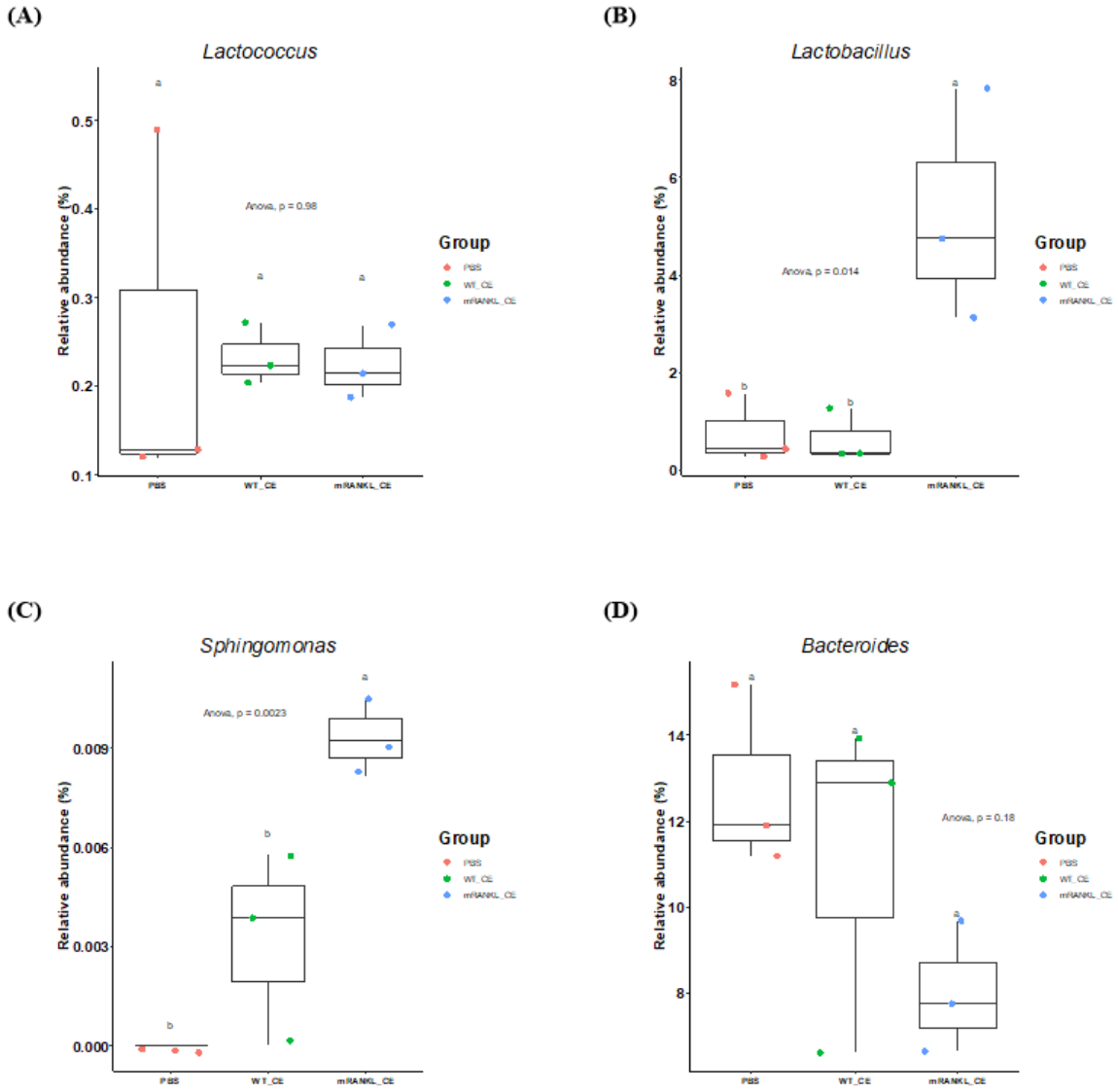


Figure 2. Venn diagram of co-up and co-downregulated genes. (A) Upregulated genes that control (PBS) versus treatment (WT_CE and mRANKL_CE). **(B)** Downregulated genes that control (PBS) versus treatment (WT_CE and mRANKL_CE).



482

483

484 **Figure 3. The relative abundance (%) of genus among that PBS, WT_CE and**

485 **mRANKL_CE three groups. (A)** *Lactococcus*. **(B)** *Lactobacillus*. **(C)** *Sphingomonas*. **(D)**

486 *Bacteroides*. For significance tests, a one-way analysis of variance (ANOVA) followed by

487 Tukey's post-hoc test were used.

488

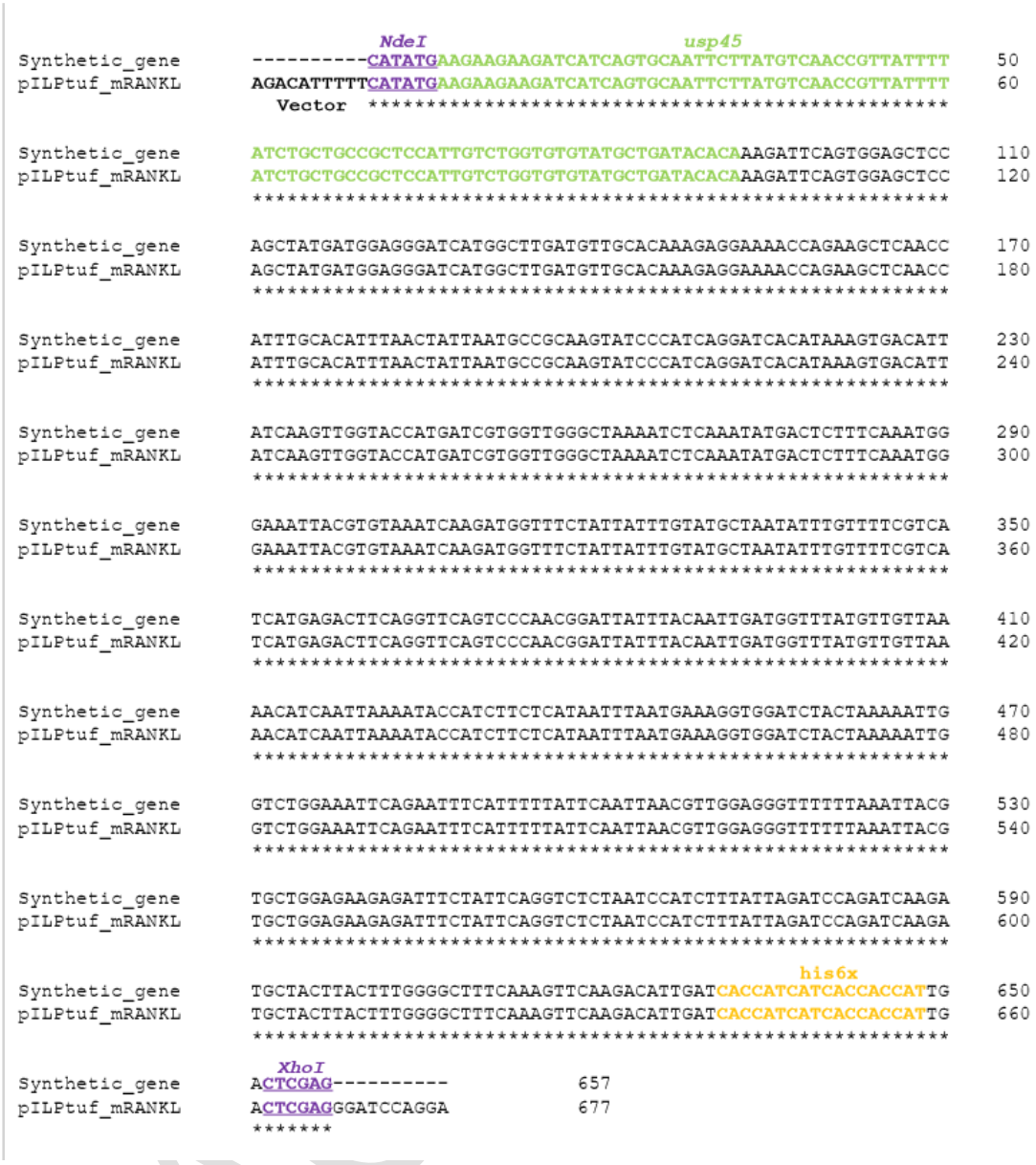


Figure S1. Sequence alignment between reference (synthetic gene) and pILPtuf.mRANKL. pILPtuf.mRANKL: Vector backbone (1-10bp), *NdeI* site (11-16bp), start codon (14-16bp), *usp45* (17-103bp), mRANKL protein (104-640bp), his6x (641-658bp), stop codon (659-661bp), XhoI (662-667bp), vector backbone (668-677bp).

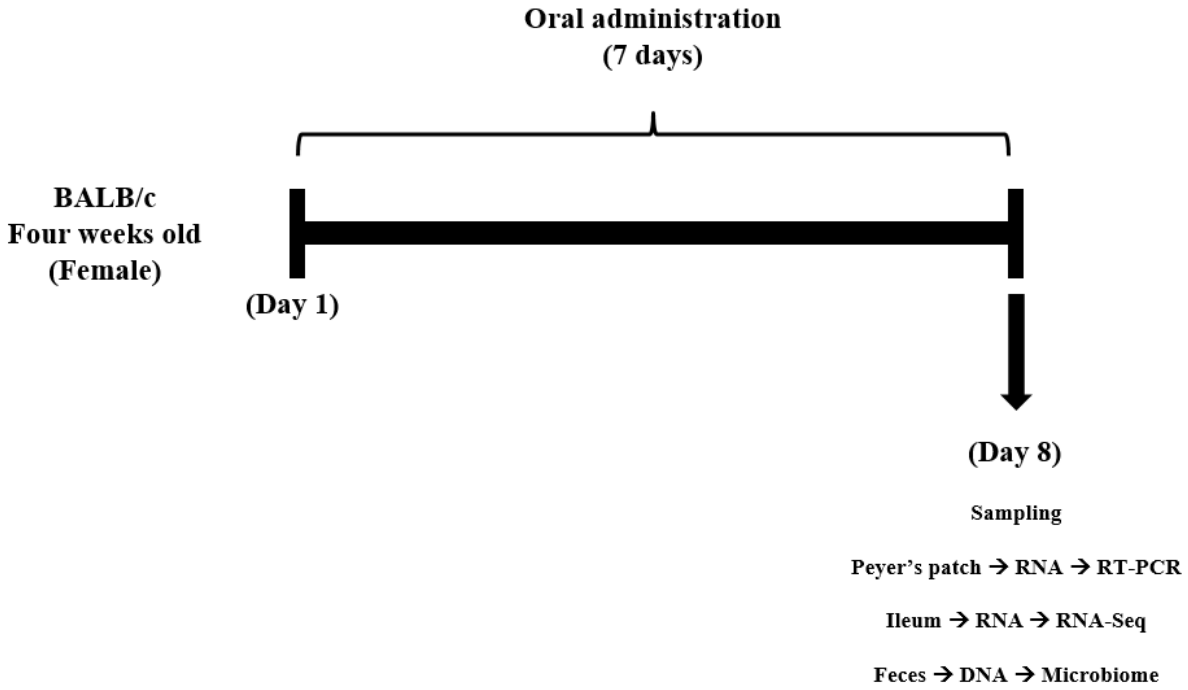
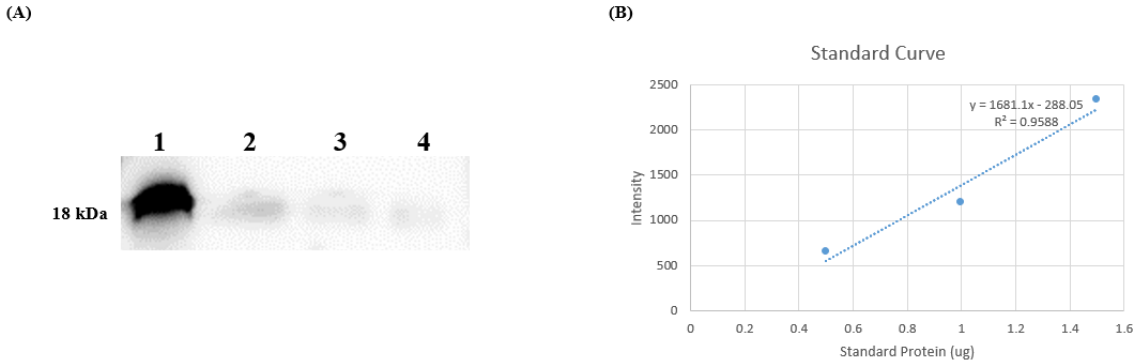


Figure S2. Validation of cell extracts containing mRANKL from recombinant *L. lactis*.

Schematic view of treatment and sampling schedule.



499

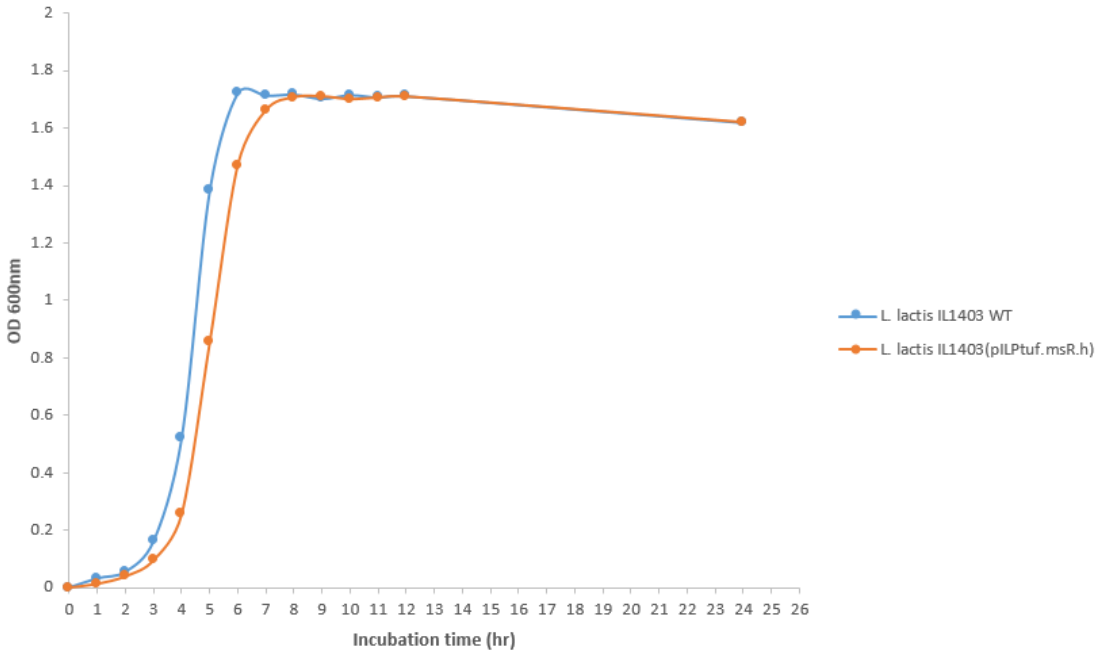
500 **Figure S3. Production yield of mRANKL from recombinant *L. lactis*. (A)** Lane 1: Cell

501 extracts of mRANKL (23.86 kDa) expressing recombinant *L. lactis*; Lane 2-4: Calmodulin

502 (18 kDa) 1.5, 1 and 0.5 µg, respectively. **(B)** Standard curve of commercial calmodulin

503 according to protein amount and western blotting intensity.

504



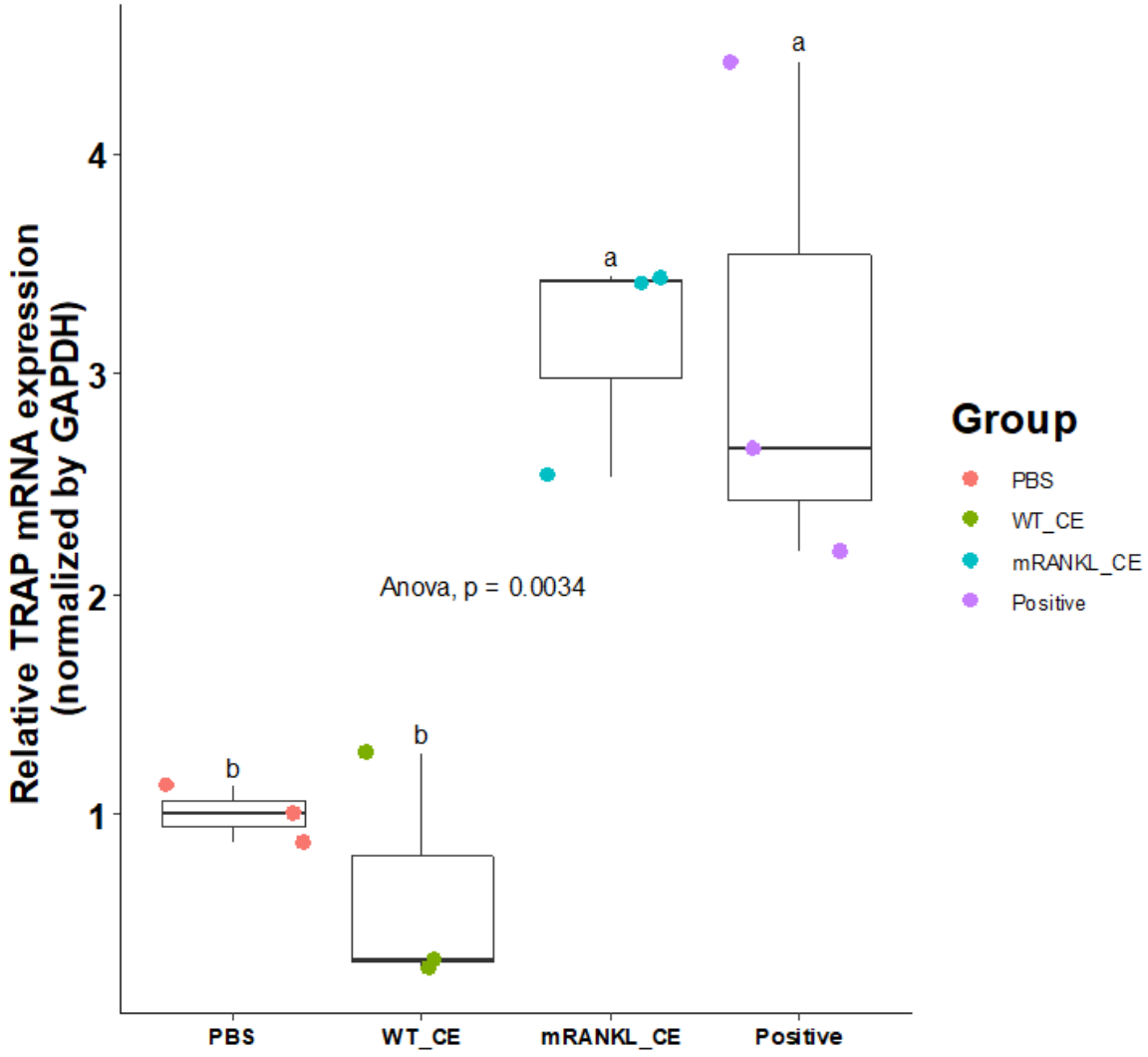
505

506 **Figure S4. Physiological characterization of recombinant *L. lactis* IL1403.** Growth of wild

507 type and recombinant *L. lactis* IL1403 were traced by measuring OD value at wavelength of

508 600 nm.

509



510

511 **Figure S5. qRT-PCR analysis of RANK-RANKL signaling-related gene expression to**

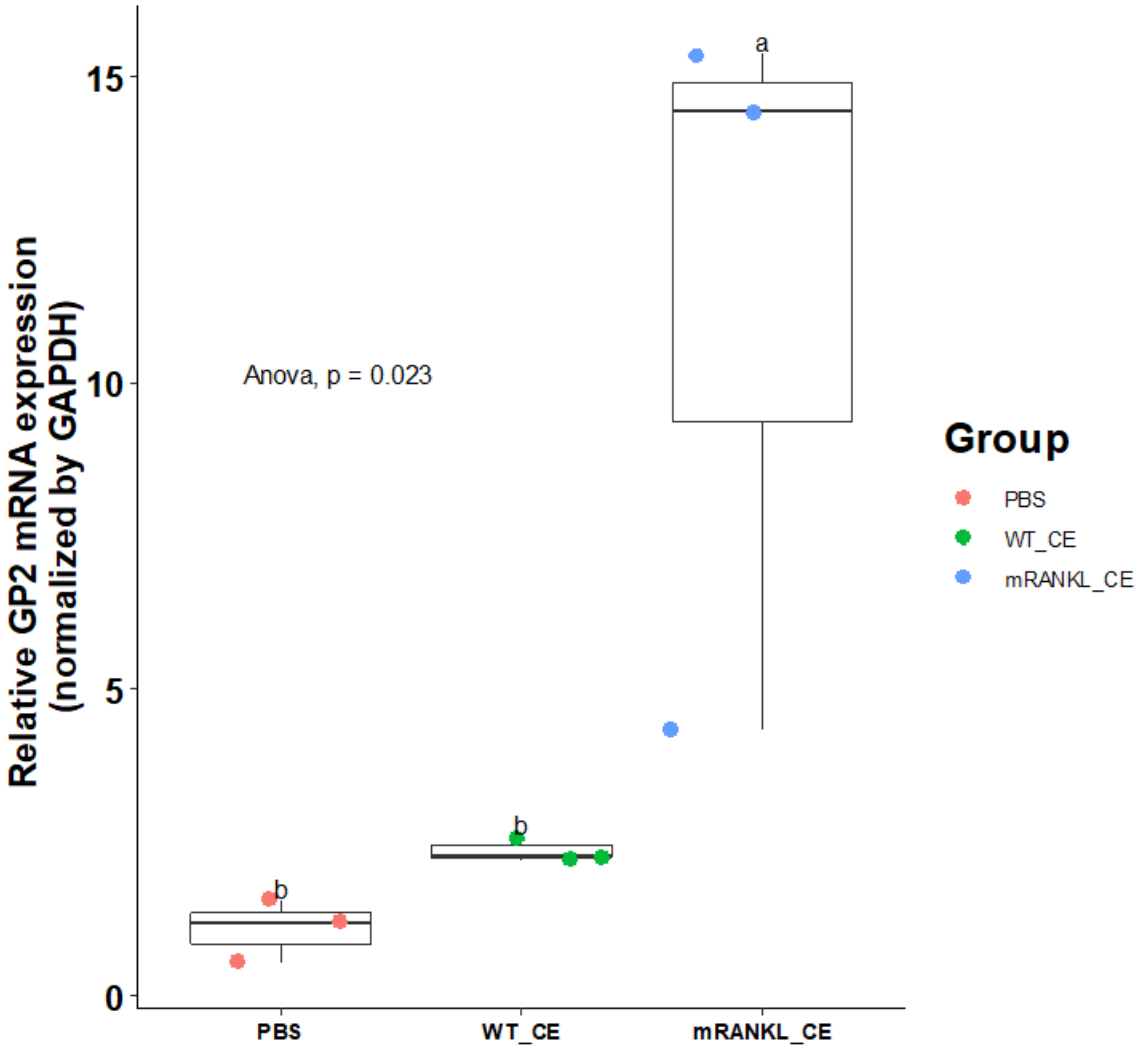
512 **validate the functional activity of mRANKL in RAW 264.7 cells.** TRAP was analyzed at

513 day 6 after exposure media of PBS, WT_CE, mRANKL_CE (90 ng/mL) and commercial

514 mouse RANKL (Positive, 60 ng/mL) to RAW 264.7 cells. For significance tests, a one-way

515 analysis of variance (ANOVA) followed by Tukey's post-hoc test were used.

516



517

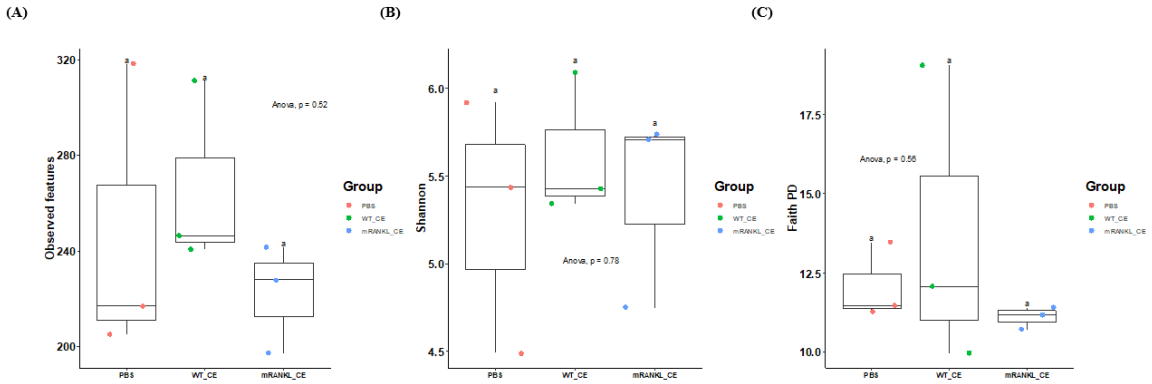
518 **Figure S6. qRT-PCR analysis of M cell marker that GP2 expression to validate the**

519 **functional activity of mRANKL in mouse small intestine.** GP2 was analyzed at day 6 after

520 oral administration. For significance tests, a one-way analysis of variance (ANOVA) followed

521 by Tukey's post-hoc test were used.

522



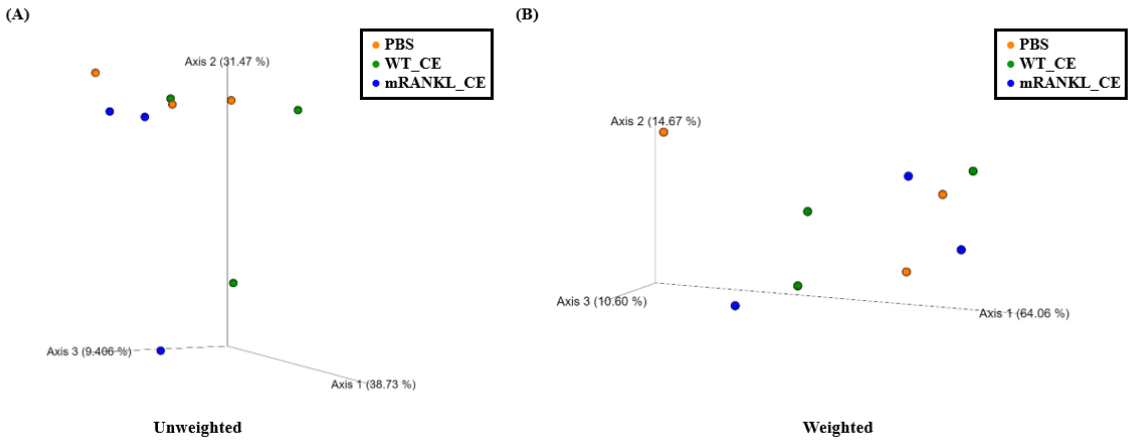
523

524 **Figure S7. Microbial diversity indices of PBS, WT_CE and mRANKL_CE groups. (A)**

525 Rarefaction analysis of observed features (Number of operational taxonomic units), **(B)**

526 Shannon index and **(C)** Faith's phylogenetic diversity (Faith PD).

527



528

529 **Figure S8. Principal coordinate analysis of the microbiota among PBS, WT_CE and**

530 **mRANKL_CE three groups. (A)** unweighted and **(B)** weighted based on UniFrac distances.

531 Subject color: orange, PBS (n = 3); green, WT_CE (n = 3); blue, mRANKL_CE (n = 3).

532