

Supplementary Materials

Synthetic_gene	----- ^{NdeI} <u>CATATG</u> AAGAAGAAGATCATCAGTGC ^{usp45} CAATTCTTATGTCAACCGTTATTTT	50
pILPtuf_mRANKL	AGACATTTT <u>CATATG</u> AAGAAGAAGATCATCAGTGC ^{usp45} CAATTCTTATGTCAACCGTTATTTT	60
	Vector *****	
Synthetic_gene	^{usp45} ATCTGCTGCCGCTCCATTGTCTGGTGTGTATGCTGATACACAAGATTCAAGTGGAGCTCC	110
pILPtuf_mRANKL	^{usp45} ATCTGCTGCCGCTCCATTGTCTGGTGTGTATGCTGATACACAAGATTCAAGTGGAGCTCC	120

Synthetic_gene	AGCTATGATGGAGGGATCATGGCTTGATGTTGCACAAAGAGGAAACCAGAAGCTCAACC	170
pILPtuf_mRANKL	AGCTATGATGGAGGGATCATGGCTTGATGTTGCACAAAGAGGAAACCAGAAGCTCAACC	180

Synthetic_gene	ATTTGCACATTTAACTATTAATGCCGCAAGTATCCCATCAGGATCACATAAAGTGACATT	230
pILPtuf_mRANKL	ATTTGCACATTTAACTATTAATGCCGCAAGTATCCCATCAGGATCACATAAAGTGACATT	240

Synthetic_gene	ATCAAGTTGGTACCATGATCGTGGTGGGCTAAAATCTCAAATATGACTCTTCAAATGG	290
pILPtuf_mRANKL	ATCAAGTTGGTACCATGATCGTGGTGGGCTAAAATCTCAAATATGACTCTTCAAATGG	300

Synthetic_gene	GAAATTACGTGTAATCAAGATGGTTTCTATTTATTTGTATGCTAATATTTGTTTTCGTCA	350
pILPtuf_mRANKL	GAAATTACGTGTAATCAAGATGGTTTCTATTTATTTGTATGCTAATATTTGTTTTCGTCA	360

Synthetic_gene	TCATGAGACTTCAGGTTCCAGTCCCAACGGATTATTTACAATGATGGTTTATGTTGTTAA	410
pILPtuf_mRANKL	TCATGAGACTTCAGGTTCCAGTCCCAACGGATTATTTACAATGATGGTTTATGTTGTTAA	420

Synthetic_gene	AACATCAATTAATAATACCATCTTCTCATAATTTAATGAAAGGTGGATCTACTAAAAATG	470
pILPtuf_mRANKL	AACATCAATTAATAATACCATCTTCTCATAATTTAATGAAAGGTGGATCTACTAAAAATG	480

Synthetic_gene	GTCTGGAATTCAGAATTCATTTTATTTCAATTAACGTTGGAGGGTTTTTAAATTACG	530
pILPtuf_mRANKL	GTCTGGAATTCAGAATTCATTTTATTTCAATTAACGTTGGAGGGTTTTTAAATTACG	540

Synthetic_gene	TGCTGGAGAAGAGATTTCTATTTCAGGTTCTTAATCCATCTTTATTAGATCCAGATCAAGA	590
pILPtuf_mRANKL	TGCTGGAGAAGAGATTTCTATTTCAGGTTCTTAATCCATCTTTATTAGATCCAGATCAAGA	600

Synthetic_gene	TGCTACTTACTTTGGGGCTTTCAAAGTTCAAGACATTGAT ^{his6x} CACCATCATCACCACCATTG	650
pILPtuf_mRANKL	TGCTACTTACTTTGGGGCTTTCAAAGTTCAAGACATTGAT ^{his6x} CACCATCATCACCACCATTG	660

Synthetic_gene	^{XhoI} ACTCGAG-----	657
pILPtuf_mRANKL	^{XhoI} ACTCGAGGGATCCAGGA	677

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

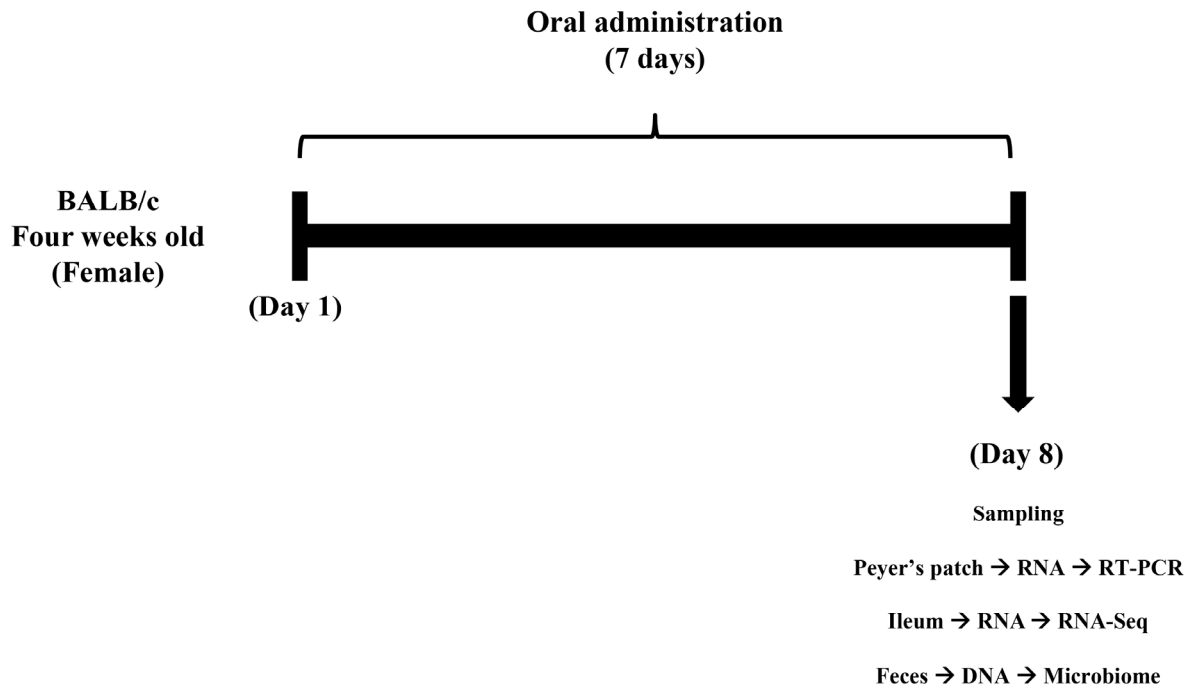


Fig. S2. Validation of cell extracts containing mRANKL from recombinant *Lactococcus lactis*. Schematic view of treatment and sampling schedule. mRANKL, mouse receptor activator of NF- κ B ligand.

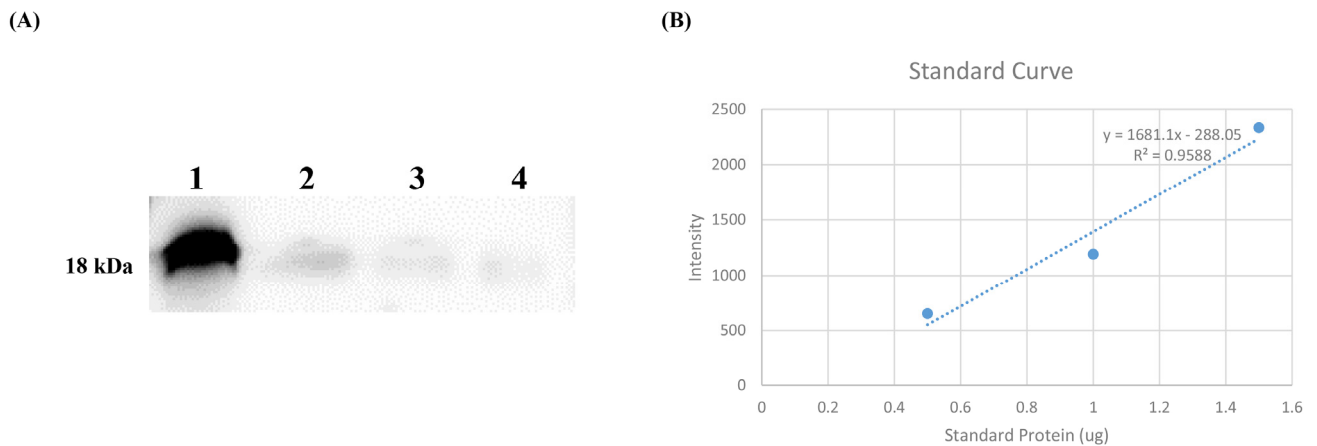


Fig. S3. Production yield of mRANKL from recombinant *Lactococcus lactis*. (A) Lane 1: Cell extracts of mRANKL (23.86 kDa) expressing recombinant *L. lactis*; Lane 2–4: Calmodulin (18 kDa) 1.5, 1, and 0.5 μ g, respectively. (B) Standard curve of commercial calmodulin according to protein amount and western blotting intensity. mRANKL, mouse receptor activator of NF- κ B ligand.

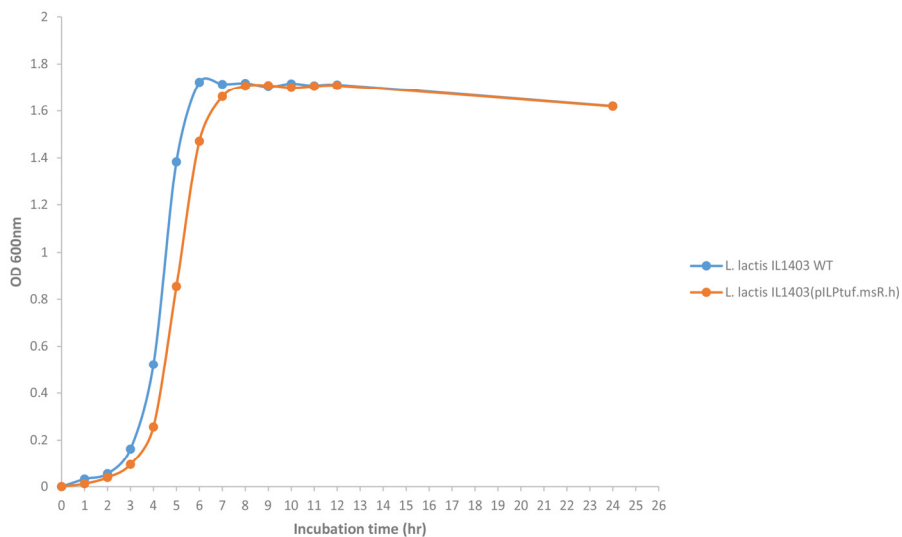


Fig. S4. Physiological characterization of recombinant *Lactococcus lactis* IL1403. Growth of wild type and recombinant *L. lactis* IL1403 were traced by measuring OD value at wavelength of 600 nm. OD, optical density.

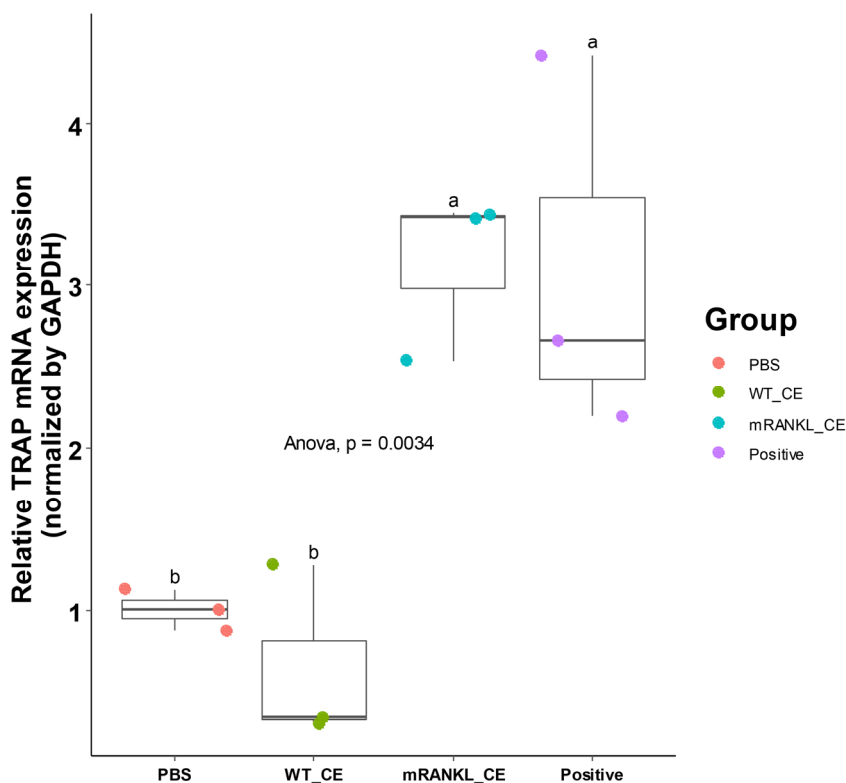


Fig. S5. qRT-PCR analysis of RANK-RANKL signaling-related gene expression to validate the functional activity of mRANKL in RAW 264.7 cells. TRAP was analyzed at day 6 after exposure media of PBS, WT_CE, mRANKL_CE (90 ng/mL) and commercial mouse RANKL (Positive, 60 ng/mL) to RAW 264.7 cells. For significance tests, a one-way analysis of variance (ANOVA) followed by Tukey’s post-hoc test were used. PBS, phosphate-buffered saline; WT_CE, wild-type *Lactococcus lactis* IL1403; mRANKL_CE, recombinant *L. lactis* expressing mouse receptor activator of NF- κ B ligand.

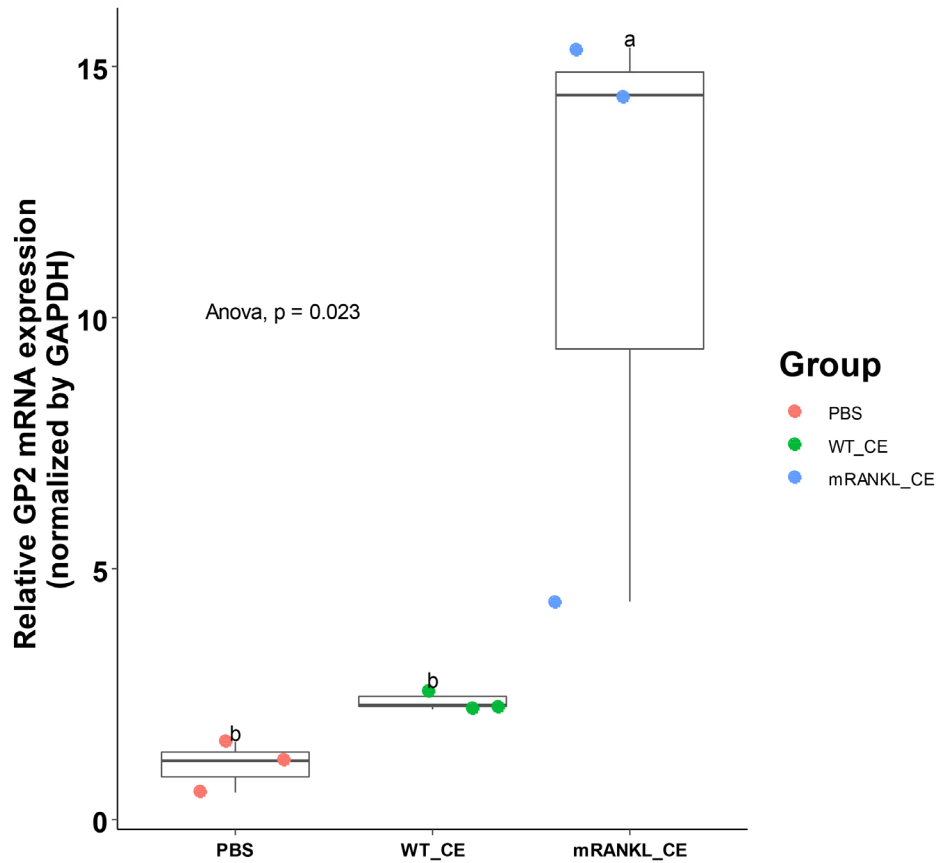


Fig. S6. qRT-PCR analysis of M cell marker that GP2 expression to validate the functional activity of mRANKL in mouse small intestine. GP2 was analyzed at day 8 after oral administration. For significance tests, a one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test were used. PBS, phosphate-buffered saline; WT_CE, wild-type *Lactococcus lactis* IL1403; mRANKL_CE, recombinant *L. lactis* expressing mouse receptor activator of NF- κ B ligand.

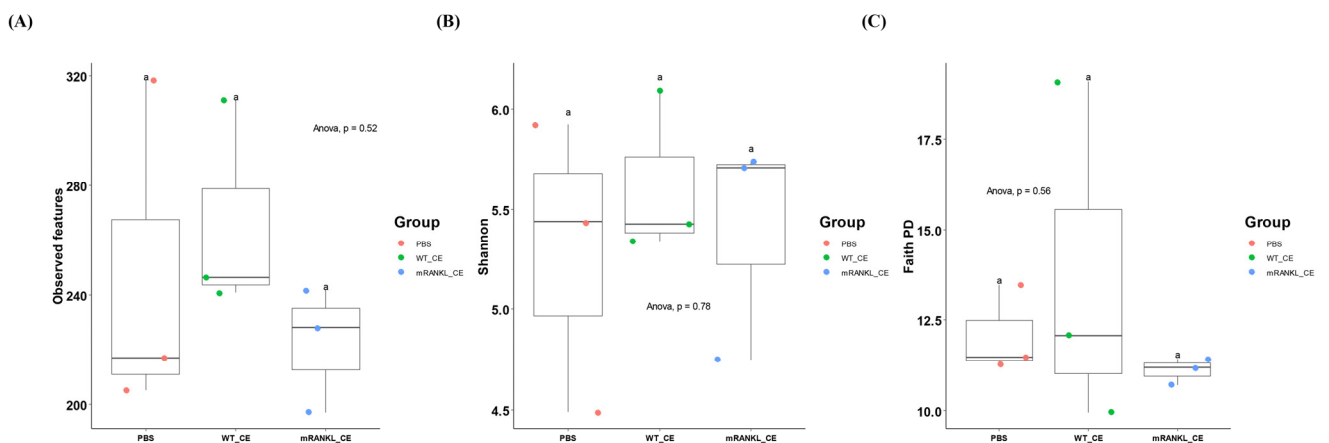


Fig. S7. Microbial diversity indices of PBS, WT_CE, and mRANKL_CE groups. (A) Rarefaction analysis of observed features (Number of operational taxonomic units), (B) Shannon index and (C) Faith's phylogenetic diversity (Faith PD). PBS, phosphate-buffered saline; WT_CE, wild-type *Lactococcus lactis* IL1403; mRANKL_CE, recombinant *L. lactis* expressing mouse receptor activator of NF- κ B ligand.

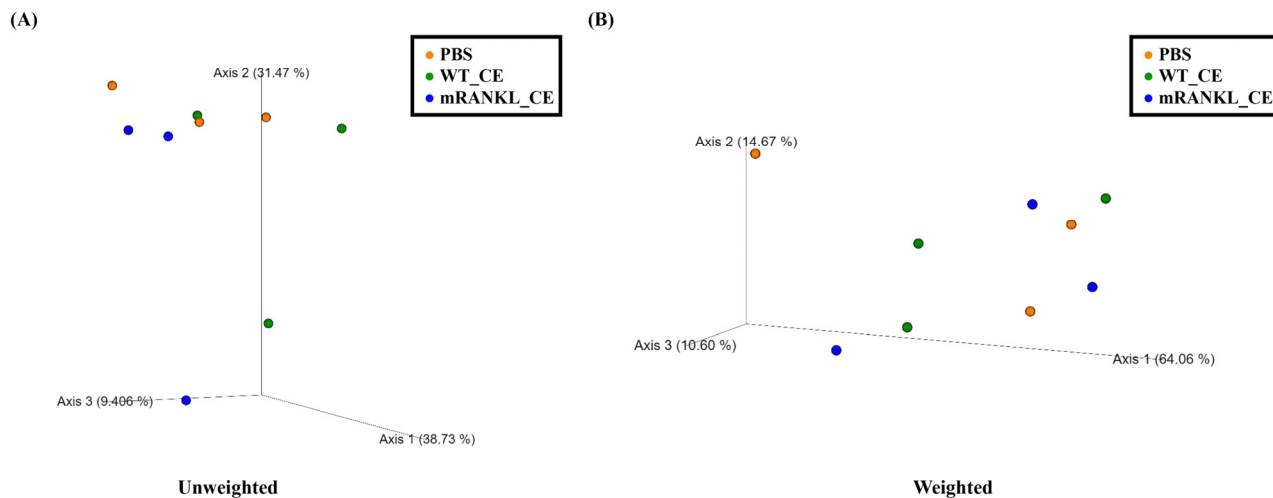


Fig. S8. Principal coordinate analysis of the microbiota among PBS, WT_CE, and mRANKL_CE three groups. (A) Unweighted and (B) weighted based on UniFrac distances. Subject color: orange, PBS (n=3); green, WT_CE (n=3); blue, mRANKL_CE (n=3). PBS, phosphate-buffered saline; WT_CE, wild-type *Lactococcus lactis* IL1403; mRANKL_CE, recombinant *L. lactis* expressing mouse receptor activator of NF- κ B ligand.