Effects of *Lactobacillus reuteri* MG5346 on RANKL-induced osteoclastogenesis and ligature-induced experimental periodontitis rats

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Running Title: *Lactobacillus reuteri* MG5346 on bone absorption

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Abstract

Effects of culture supernatants of *Lactobacillus reuteri* MG5346 (CS-5346) on receptor activator of nuclear factor-kappa B ligand (RANKL)-induced osteoclastogenesis were examined. CS-MG5346 treatment up to 400 μg/mL significantly reduced tartrate-resistant acid-phosphatase (TRAP) activity, the phenotype biomarker of osteoclast, without affecting cell viability. CS-MG5346 inhibited the expression of osteoclast specific transcriptional factors (c-fos and nuclear factor-activated T cells c1) and their target genes (*TRAP, cathepsin, and matrix metallo-proteinase-9*) in a dose-dependent manner ($p < 0.05$). The administration of *L. reuteri* MG5346 (2×10^8 CFU/day) for 8 wks significantly improved furcation involvement, but no difference was observed in alveolar bone loss in ligature-induced experimental periodontitis rats. The elevated RANKL/osteoprotegerin ratio, the biomarker of periodontitis, was significantly lowered in the gingival tissue by administration of *L. reuteri* MG5346 ($p < 0.05$). *L. reuteri* MG5346 showed excellent stability in simulated stomach and intestinal fluids and did not have antibiotic resistance. Based on the results, *L. reuteri* MG5346 has the potential to be a promising probiotic strain for oral health.

Keywords: *Lactobacillus reuteri* MG5346, culture supernatant, osteoclastogenesis, osteoclast specific gene expression, ligature-induced experimental periodontitis

Introduction

Gingivitis and periodontitis are common chronic inflammatory diseases and these periodontal diseases occur in about 20-50% of the world's population. Moreover, periodontal diseases increase the risk of systemic diseases, such as cardiovascular disease (Nazir, 2017). The surgical intervention and antibiotic treatment may not be sufficient to control periodontitis because of the complex etiology involved in oral microbiota (Gatej et al., 2017).

Probiotics are defined as safe live microorganism exerting health benefits and disease prevention when consumed in adequate amounts (Pinero and Stanton, 2007). The composition of oral microbiota was significantly different between healthy and periodontitis patients, and adequate oral lactobacilli, such as *Lactobacillus paracasei* and *Lactobacillus plantarum,* were able to reduce the occurrence of dental caries by inhibiting the growth and colonization of cariogenic bacteria (Ko˜ll-Klais et al., 2005). *Lactobacillus casei* 393 and *Lactobacillus plantarum* B719-fermented milk increased osteoblast activity and prevented bone loss in ovariectomized rats (Kim et al., 2009; Lee et al., 2020). In addition to probiotics, postbiotics refer to non-viable bacterial components or metabolites of probiotics, mitigate various inflammatory diseases, such as inflammatory bowel disease, rheumatoid arthritis, and obesity (Bungau et al., 2021; Cristofori et al., 2021).

As an ongoing effort to develop oral probiotics, *Lactobacillus reuteri* MG5346 (**MG5346**) has been selected from our preliminary screening study. *L. reuteri* is a Gram-positive bacterium that inhabits various locations in the human body, including the gastrointestinal tract, urinary tract and skin (Mu et al., 2018). *L. reuteri* showed an immune modulation effect by inducing anti-inflammatory regulatory T cells while reducing pro-inflammatory cytokines (He et al., 2017; Hsieh et al., 2016). *L. reuteri* lozenges helped to treat chronic periodontitis as an adjuvant

treatment and delayed recolonization for up to 6 months in the follow-up study (Tekce et al., 2015). In our previous study, probiotic culture supernatant (**CS**) inhibited both *Streptococcus mutans*-induced biofilm formation and receptor activator of the nuclear factor κB ligand (**RANKL**)-induced osteoclast formation (Jung et al., 2021). The inhibitory activity of CS on biofilm formation and osteoclastogenesis varied depending on the probiotic strain. These results suggested that the efficacy of oral probiotics, such as *L. reuteri,* are also strain-specific.

The objective of the present study was to examine the efficacy of MG5346 as an oral probiotic. To achieve this goal, the effects of the CS-MG5346 on RANKL-mediated osteoclastogenesis were analyzed. In addition, the effect of MG5346 administration on alveolar bone loss and tissue damage was evaluated using ligature-induced periodontitis rats.

Materials and methods

Materials

Dulbecco's modified Eagle's medium (DMEM), α-minimum essential Eagle's medium (α-MEM), penicillin-streptomycin solution, and fetal bovine serum (FBS) were purchased from Welgene, Inc. (Gyeongsan, Korea). TaqMan Gene Expression Master Mix, TaqMan probes (5'-fluorescein based reporter dye; 3'-TAMRA quencher), and High-Capacity RNA-to-cDNA Kit were purchased from Applied Biosystems (Foster City, CA, USA). RANKL was purchased from purchased ProSpec (Rehovot, Israel). All other reagents used in the experiment were purchased from Sigma-Aldrich Inc. (St. Louis, MO, USA).

Preparation of CS-MG5346

MG5346 was originally isolated from fermented foods. CS-MG5346 was prepared by the method described previously (Jung et al., 2021) and kindly provided by Mediogen (Jecheon, Korea).

Gastrointestinal tolerance of MG5346

The gastrointestinal tolerance of MG5346 was determined by the method of Tokatlı et al. (2015) with slight modification. MG5346 was harvested (3,460×g, 10 min) after being cultured in MRS media at 37℃ for 24 h. The MG5346 pellets were washed twice with sterile saline solution (0.85% NaCl, w/v) and resuspended to 10^7-10^8 CFU/mL in simulated gastric fluid (**SGF**; 3 g/L of pepsin in sterile saline solution, pH 2.5) or simulated intestinal fluid (**SIF**; 1 g/L of pancreatin, 0.3% bile salt in sterile saline solution, pH 8.0). The survival rate of MG5346 was determined after incubation at 37℃ for 4 h in SGF and 6 h in SIF, respectively. The viable cells were counted on MRS agar and expressed by the following formula:

$$\text{Survival rate (\%)} = \frac{\text{Log CFU of survived viable cells}}{\text{Log CFU of initial inoculated cells}} \times 100$$

Antibiotic susceptibility

The antibiotic susceptibility of MG5346 was determined by the minimum inhibitory concentration (MIC) test strip method described previously (Jung et al., 2022).

Osteoclast differentiation from RAW 264.7 cells

The murine RAW 264.7 cell line was purchased from the American Type Culture

115 Collection (ATCC, Manassas, VA, USA). The cells were cultured in DMEM supplemented
116 with 10% FBS and penicillin-streptomycin (100 untis/mL) at 37℃ in a 5% CO_2 humidified
117 atmosphere. Osteoclastogenesis was induced by replacing the α-MEM medium with the
118 medium containing RANKL (100 ng/mL) and M-CSF (50 ng/mL). Cytotoxicity of CS-
119 MG5346 was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium
120 bromide (MTT) assay (Lee and Imm, 2017).

121

122 **Tartrate-resistant acid phosphatase (TRAP)-positive activity**

123 RAW 264.7 cells were seeded in 96-well plates at a density of 3×10^3 cells/well for 24
124 h and were cultured in the presence of RANKL (100 ng/mL), M-CSF (50 ng/mL), and CS-
125 MG5346 for another 7 days. The cells were lysed using 0.05% Triton X-100/saline solution
126 and were dispersed in 50 mM citrate buffer (pH 4.7) containing 10 mM sodium tartrate and 10
127 mM *p*-nitrophenylphosphate. TRAP activity was determined according to the method of Kim
128 et al. (2019)

129

130 **Animal experiment**

131 Animal experiments were conducted according to the guideline of the Institutional
132 Animal Care and Use Committee (approval number: KNOTUS 21-KE-032). Male Sprague-
133 Dawley rats (6 wks old; Orient Bio, Korea) were housed in an animal facility and maintained
134 under the conditions of a 12-h light-dark cycle at 23 ± 3℃, 55 ± 15% humidity. After
135 acclimation for 7 days, rats were randomly divided into three groups: 1) untreated control (n =
136 10), 2) ligature + vehicle (n = 10), 3) ligature + MG5346 (2×10^8 CFU/day; n = 10). The

ligature was placed around the right second molar of the mandible using a sterile 4-0 silk under anesthesia with zoletil 50 (Virbac, Carros, France) and xylazine (Rompun, Bayer AG, Leverkusen, Germany). Maintenance of the ligature was checked regularly during the entire experimental period (8 wks).

Alveolar bone loss and tissue damage measurement using micro-CT analysis

Mandibular jaws of all rats were scanned using a micro-CT (vivaCT 80, Scanco Medical, Switzerland) after 8 wks of ligation induction and MG5346 administration. The cement–enamel junction (CEJ)–alveolar bone crest (ABC) distance and degree of maxillary molar furcation involvement were used as an index of alveolar bone loss and periodontal tissue damage.

Quantitative real-time PCR (qRT-PCR)

Total RNA was extracted using NucleoZOL reagent (Macherey-Nagel, Düren, Germany), and qRT-PCR analysis was performed using cell lysates (nuclear factor-activated T cells c1 [**NFATc1**], TRAP, c-Fos, TRAP, and cathepsin K) and rat gingival tissue (RANKL and osteoprotegerin [**OPG**]) as described previously (Jung et al., 2022). The relative expression of osteoclast-specific transcriptional factor and target genes were analyzed using the following probes: β-actin (Mm00607939_s1), TRAP (Mm00475698_m1), cathepsin K (Mm00484039_m1), NFATc1 (Mm00479445_m1), c-Fos (Mm00487425_m1), RANKL (Rn00589289_m1), and OPG (Rn00563499_m1). qRT-PCR was performed using the StepOne Plus Real-Time PCR System (Applied Biosystems), and the expression of target genes was

normalized to the housekeeping gene, β-actin.

Statistical analysis

All analytical experiments were performed in triplicate, and SPSS Statistics (SPSS 26; SPSS, Inc., Chicago, IL, USA) software was used for statistical analysis. Data were expressed as mean ± standard deviation (SD). Significant differences ($p < 0.05$) were assessed using a one-way analysis of variance (ANOVA), followed by Duncan's post-hoc test.

Results and Discussion

Gastrointestinal tolerance of MG5346

The acid resistance and bile salt tolerance of probiotics are the most important requirements to ensure health benefits to the host (Tokatlı et al., 2015). As shown in **Table 1,** MG5346 showed 91% and 92% survival rates in SGF and SIF, respectively. *L. reuteri* is one of the few resident *Lactobacillus* species found in various sites in the human body human body, including the gastrointestinal tract (Valeur et al., 2004). This high adaptability of *L. reuteri* might be related to its tolerance in the gastrointestinal environment. Chen et al. (2019) reported that *L. reuteri* WHH1689 contained various stress-resistant genes related to acid (FoF1-ATP synthase and the sodium proton antiporter) and bile (choloylglycine hydrolase and inorganic pyrophosphatae) tolerance.

Antibiotic resistance of MG5346

The antibiotic resistance of probiotics is a principal safety consideration because

probiotics can be a source of transferable resistance genes to pathogens (Li et al., 2020). Thus, the MIC of eight antibiotics against MG5346 was determined. MG5346 showed much lower MIC values for eight antibiotics than corresponding cut-off MIC values. This result indicates that MG5346 does not have antibiotic resistance. According to the report of Jose et al. (2015), probiotics generally show resistance to vancomycin, ciprofloxacin, gentamicin and streptomycin. Similar to the result of this study, 32 representative *L. reuteri* strains did not have any transferable or acquired antibiotic resistance. In addition, they did not show virulence potential in the gelatinase activity, and hemolysis test (Singh et al., 2012).

Effects of CS-MG5346 on TRAP activity in RANKL-stimulated RAW 264.7 macrophages

RANKL mediates the conversion of hematopoietic precursors, such as monocytes and macrophages into osteoclasts with the cooperation of M-CSF (Kong et at., 1999). TRAP is highly expressed in response to the conversion of macrophages to multinucleated osteoclasts, and increased TRAP activity is often used as a phenotype marker for osteoclasts (Tanaka et al., 2005). TRAP activity was significantly increased by RANKL stimulation, while the addition of CS-MG5346 decreased TRAP activity in a dose-dependent manner (**Fig. 1A**). This suggests that CS-MG5346 is able to inhibit osteoclast formation. Britten et al. (2014) reported that *L. reuteri* ATCC 6475 released compounds inhibiting RANKL-induced osteoclastogenesis. Although the exact nature of the inhibitory compounds for osteoclastogenesis was not clarified, histamine might be associated with the suppression of osteoclast differentiation. Thomas et al. (2012) demonstrated that histamine released from *L. reuteri* inhibited TNF-α activity, which promotes osteoclastogenesis. In addition, CS-MG5346 did not show a cytotoxic effect in the MTT assay up to 400 μg/mL. Thus, a further experiment proceeded within this non-cytotoxic

concentration range (**Fig. 1B**).

Effect of CS-MG5346 on osteoclastogenesis-associated gene expression

Osteoclast differentiation requires transcription factors essential for the induction of target genes (Kim and Kim, 2014). The effects of CS-MG5346 on the gene expression of two key osteoclast-specific transcriptional factors (*c-Fos* and *NFATc1*) were analyzed. CS-MG5346 treatment significantly downregulated RANKL-mediated elevated *c-Fos* and *NFATc1* gene expression in a dose-dependent manner (**Fig. 2A** and **2B**). The binding of RANKL to RANK on the surface of osteoclast precursor cells recruits c-Fos at the early osteoclast differentiation stage, which in turn, activates NFATc1, a master regulator of osteoclastogenesis (Zhao et al., 2010). It has been reported that *c-Fos*-knock-out mice failed to undergo osteoclast differentiation (Wang et al., 1992). Thus, the downregulation of *c-Fos* and *NFATc1* can be a major contributor to the inhibition of osteoclastogenesis.

Stimulation of *NFATc1* promotes the expression of osteoclast-specific genes, such as *TRAP*, *cathepsin K*, and metallo-proteinase-9 *(MMP-9)*, that causing the degradation of bone extracellular matrix proteins (Asagiri, 2007; Sundaram et al., 2007). Consistent with these reports, CS-MG5346 treatment significantly suppressed the expression of TRAP, cathepsin K, and MMP-9 ($p < 0.05$; **Fig. 2C, 2D**, and **2E**). The mRNA levels of TRAP, cathepsin K, and MMP-9 showed a high correlation with the level of bone resorption in patients with osteoarthritis and osteoporosis (Logar et al., 2007).

Effects of MG5346 on alveolar bone loss and furcation involvement in ligature-induced periodontitis rat model

227 The mechanisms for the initiation and progression of periodontitis are still unclear and
228 a large number of oral bacteria are involved in etiology of chronic periodontitis (Graves et al.,
229 2008). The rat ligature model is one of the most frequently used non-primate animal
230 periodontitis models. The ligatures around teeth cause plaque accumulation and induce
231 periodontal inflammation and subsequent alveolar bone loss (Xu and Wei, 2006).

232 The effect of MG5346 administration for 8 wks on alveolar bone loss was determined
233 in ligature-induced experimental periodontitis rats. The periodontal destruction was observed
234 in both vertical (CEJ-ABC) and horizontal (furcation involvement) direction in multi-rooted
235 teeth (Pilloni and Rojas, 2018). Micro-CT tomography indicated that ligation significantly
236 increased both CEJ-ABC distance and furcation involvement. Although a decreasing tendency
237 was observed in the CEJ-ABC distance by *L. reuteri* MG5346 administration, there was no
238 significant difference between the ligation control and *L. reuteri* MG5346 group (1.60±0.22
239 vs.1.44±0.19). Conversely, furcation involvement was significantly decreased by administration
240 of MG5346 (0.58±0.15 vs.0.46±0.0.9; $p < 0.05$; **Fig. 3**). The prolonged inflammation by
241 periodontitis leads to bone resorption and furcation defect, and reduced furcation involvement
242 significantly reduces the risk of bone loss (Parihar and Katoch, 2015). The adjuvant use of *L.*
243 *reuteri* DSM 17938 (1 x 10^8 CFU/lozenge) for 21 days ameliorated chronic periodontitis by
244 reducing gingival inflammation and deep periodontal pockets in smokers (Theodoro et al.,
245 2019).

246

247 **Effects of MG5346 on RANKL and OPG gene expression in gingival tissue**

248 RANKL-RANK is a key pathway modulating the formation and differentiation of
249 osteoclasts. (Wada et al., 2006). OPG competitively binds to RANKL, subsequently interfering

250 with the binding of RANKL with RANK. The imbalance in RANKL/OPG led to increased
251 bone resorption (Boyce and Xing, 2008). Periodontal tissue was isolated from rats, and the
252 expression of *RANKL* and *OPG* was analyzed using qRT-PCR. The expression of *RANKL*
253 increased by ligation while it was decreased by MG5346 administration (**Fig. 4A**). The
254 expression of *OPG*, which was decreased by periodontitis induction was significantly
255 recovered by administration of MG5346 ($p < 0.05$; **Fig 4B**). The RANKL/OPG ratio in
256 MG5346-fed groups was close to the non-ligation control group (**Fig 4C**).

257 Although the modulation of bone metabolism by administration of probiotics has been
258 reported (Jung et al., 2022; Yousf et al., 2015; Britton et al., 2014), the evidence is still
259 inconclusive. Hu et al. (2021) reported that extracellular vesicles (**EVs**) released from *L. reuteri*
260 might be involved in the mitigation of periodontitis. The administration of EVs from *L. reuteri*
261 BBC3 exerted an anti-inflammatory effect in lipopolysaccharide-stimulated chicken
262 macrophages and improved intestinal injury in chickens. Alternatively, the ability of probiotics
263 to modulate C-X-C motif chemokine (**CXCL8**) was suggested as a potential mechanism of
264 probiotic immune modulation (Mendi et al., 2016). CXCL8 is a chemokine released from
265 various cell types, such as gum epithelial cells, and it recruits neutrophils to the site of infection
266 (Yamamoto and Aizawa, 2021). *Porphyromonas gingivalis*-mediated CXCL8 inhibition
267 reduced the host immune response and enhanced periodontal tissue damage (Sochalska and
268 Potempa 2017). Probiotics, such as *L. rhamnosus* ATCC 9595, *L. casei* 324 m, and *L. reuteri*
269 upregulated CXCL8 gene expression and counteracted *P. gingivalis*-mediated CXCL8
270 suppression (Albuquerque-Souza et al., 2021; Allaker and Stephen, 2017; Mendi et al., 2016).
271 Oral administration of *L. reuteri* tablet significantly reduced proinflammatory cytokine levels
272 (TNF-α, IL-1β, and IL-17) in 18 out of 24 patients with chronic periodontitis. The clinical

indices, such as bleeding index, periodontal probe depth, and clinical adhesion level, were also significantly improved (Szkaradkiewicz et al., 2014).

Conclusion

Probiotics are generally regarded as safe, except for specific health conditions, such as patients with immune-compromisation. The development of oral probiotics/postbiotics offers valuable options to prevent or alleviate periodontitis. Based on results in osteoclastogenesis and the ligature-induced periodontitis rat model, MG5346 can be a promising probiotic strain for oral health. Carefully designed clinical studies are required to warrant the efficacy of oral probiotics/postbiotics.

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Conflicts of Interest

YongGyeong Kim and Chang-Ho Kang are employees of Mediogen. Industry employees are involved in the study probiotic characterization, but they did not play a role in other data collection, analyses, or interpretation of data, writing of the manuscript, or in the decision to publish the results.

IRB/IACUC approval

Animal experiments were carried out after approval from the Institutional Animal Care and Use Committee (KNOTUS 21-KE-032).

Author Contribution

Conceptualization: Imm J-Y and Kang C-H, Data curation: Jeong Y-J, Jung J-I, and Kim Y, Investigation: Jeong Y-J, Jung J-I, and Kim Y, Writing - original draft: Jeong Y-J and Kim Y, Writing - review & editing: Jeong Y-J, Kim Y, Kang C-H, and Imm J-Y.

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Table 1. Survival rate of *Lactobacillus reuteri* MG5346 under simulated gastrointestinal conditions

Strain	Initial count[a] (log CFU/mL)	Survival in SGF[b]		Survival in SIF[c]	
		log CFU/mL	%	log CFU/mL	%
L. reuteri MG5346	7.55 ± 0.19	6.85 ± 0.07	90.74	6.94 ± 0.03	91.87

The results are expressed as means ± SD. [a]Initial counts evaluated at 0 h. [b]Survival rate in simulated gastric fluid (SGF, pH 2.5) was determined at 37℃ after 4 h. [c]survival rate in simulated intestinal fluid (SIF, pH 8.0) was determined at 37℃ after 6 h.

437 **Table 2. MIC of antibiotics for *Lactobacillus reuteri* MG5346**

Antibiotics	Microbiological cut-off values (mg/L)	
	EFSA	*L. reuteri* MG5346
Ampicillin	2	0.06
Chloramphenicol	4	1
Clindamycin	4	<0.016
Erythromycin	1	0.03
Gentamycin	8	0.38
Kanamycin	64	8
Streptomycin	64	4
Tetracycline	32	2

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439 MIC (minimum inhibitory concentration) indicates the lowest concentration of antibiotic that
440 prevents visible bacterial growth. Antibiotic resistance was determined according to the
441 European Food Safety Authority (EFSA) guidelines.

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Figure captions

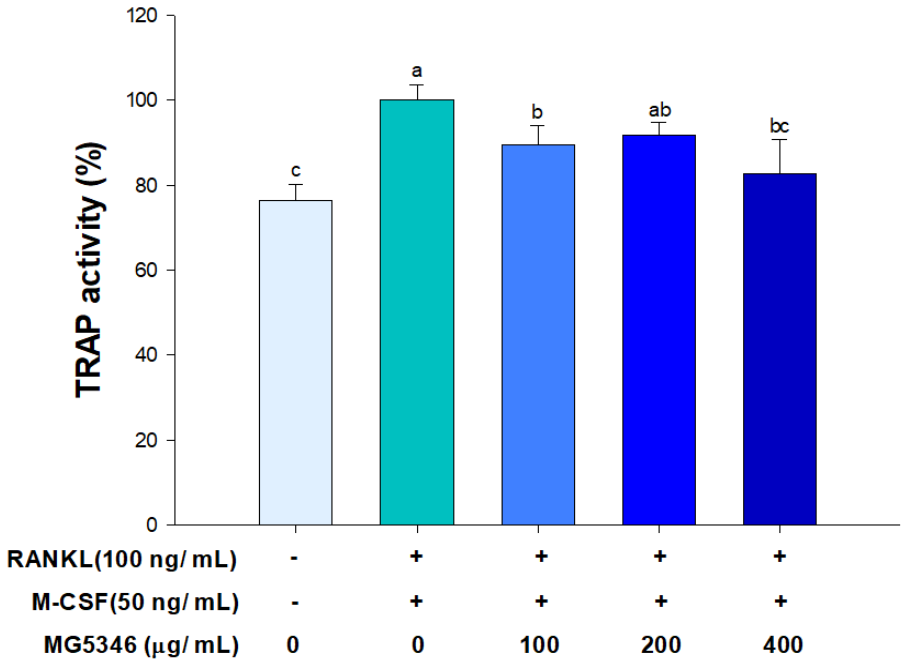
Fig. 1. Effects of CS-MG5346 on (A) TRAP activity and (B) cell viability in RANKL-stimulated RAW 264.7 macrophages. CS-MG5346, culture supernatant of *Lactobacillus reuteri* MG5346; TRAP, tartrate-resistant acid-phosphatase; RANKL, receptor-activator of nuclear factor-kappa B ligand. Different letters indicate significant differences at $p < 0.05$.

Fig. 2. Effects of CS-MG5346 on gene expression of (A) c-fos, (B) NFATc1, (c) TRAP, (D) cathepsin K, and (E) MMP-9 in RANKL-stimulated RAW 264.7 macrophages. CS-MG5346, culture supernatant of *Lactobacillus reuteri* MG5346; NFATc1, nuclear factor-activated T cells c1; TRAP, tartrate-resistant acid-phosphatase; MMP-9, matrix metallo-proteinase-9; RANKL, receptor-activator of nuclear factor-kappa B ligand. Different letters indicate significant differences at $p < 0.05$.

Fig. 3. Effects of MG5346 on CEJ-ABC distance and furcation involvement in experimental periodontitis rats. (A) Indication of cemento enamel junction-alveolar bone crest (CEJ-ABC) and furcation involvement, (B) representative image of untreated control group, (C) representative image of ligature control group, (D) representative image of ligature + *Lactobacillus reuteri* MG5346 group, (E) CEJ-ABC distance, and (F) furcation involvement. The cement–enamel junction (CEJ)–alveolar bone crest (ABC) distance and degree of maxillary molar furcation involvement were used as an index of alveolar bone loss and periodontal tissue damage. Bars with different letters indicate significant differences at $p < 0.05$.

Fig. 4. Effects of MG5346 on gene expression of (A) RANKL, (B) OPG, and (C) RANKL/OPG ratio in gingival tissue of experimental periodontitis rats. MG5346, *Lactobacillus reuteri* MG5346; RANKL, receptor activator of nuclear factor-kappa-B ligand; OPG, osteoprotegerin, Bars with different letters indicate significant differences at $p < 0.05$.

Fig. 1 **(A)**


TRAP activity (%)
120
100
80
60
40
20
0
a
b
ab
bc
c
RANKL(100 ng/ mL) - + + + +
M-CSF(50 ng/ mL) - + + + +
MG5346 (μg/ mL) 0 0 100 200 400

(B)

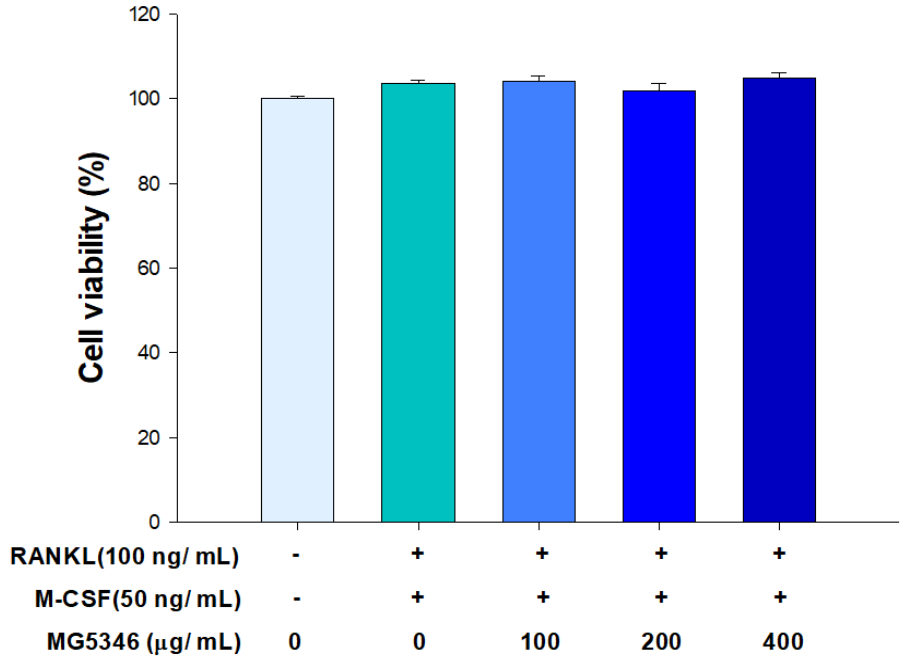
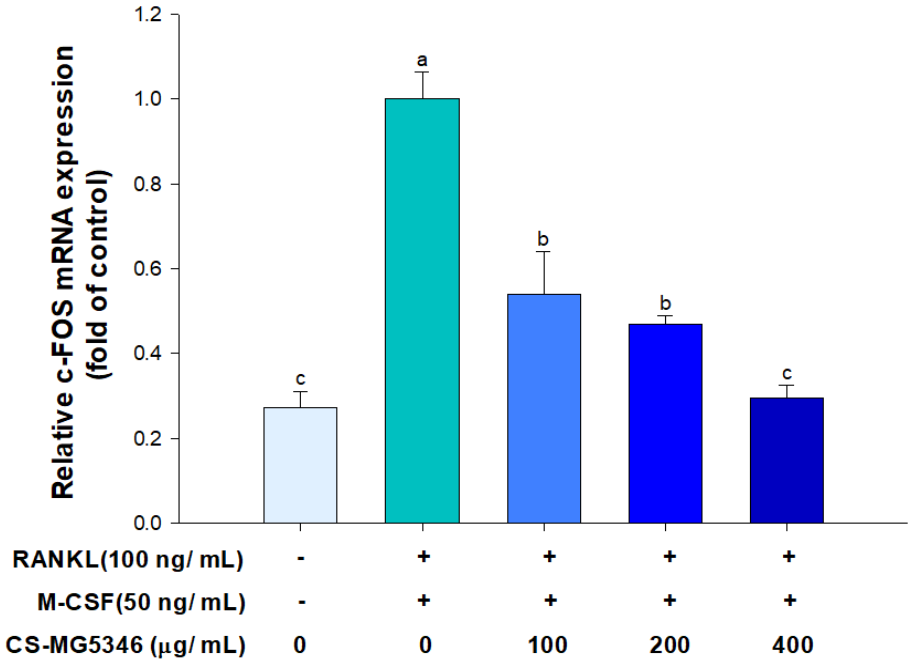

Cell viability (%)
120
100
80
60
40
20
0
RANKL(100 ng/ mL) - + + + +
M-CSF(50 ng/ mL) - + + + +
MG5346 (μg/ mL) 0 0 100 200 400

Fig. 2 **(A)**


Relative c-FOS mRNA expression
(fold of control)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
a
b
b
c
c
RANKL(100 ng/ mL) - + + + +
M-CSF(50 ng/ mL) - + + + +
CS-MG5346 (μg/ mL) 0 0 100 200 400

(B)

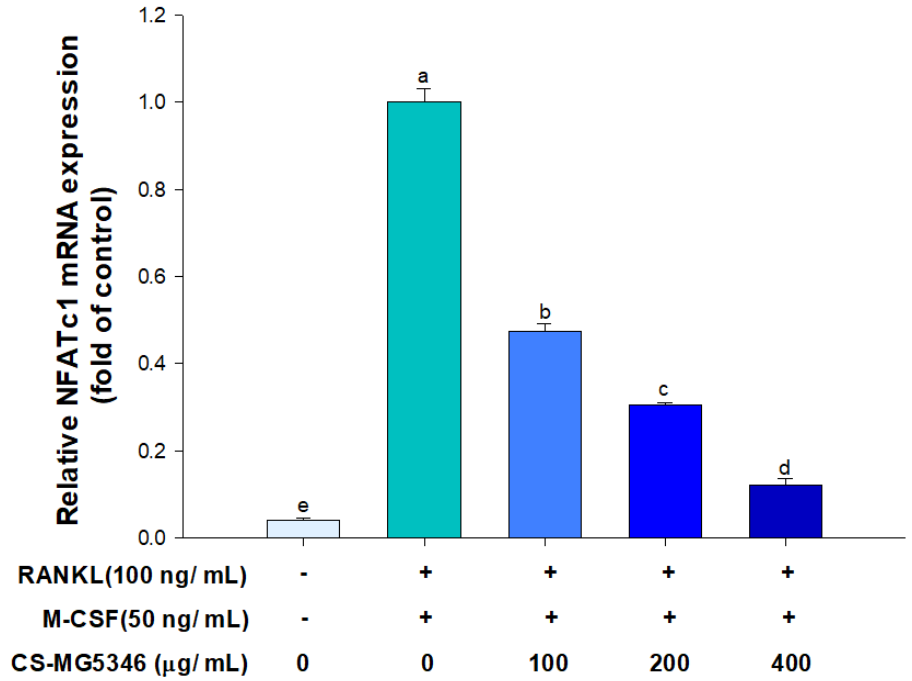
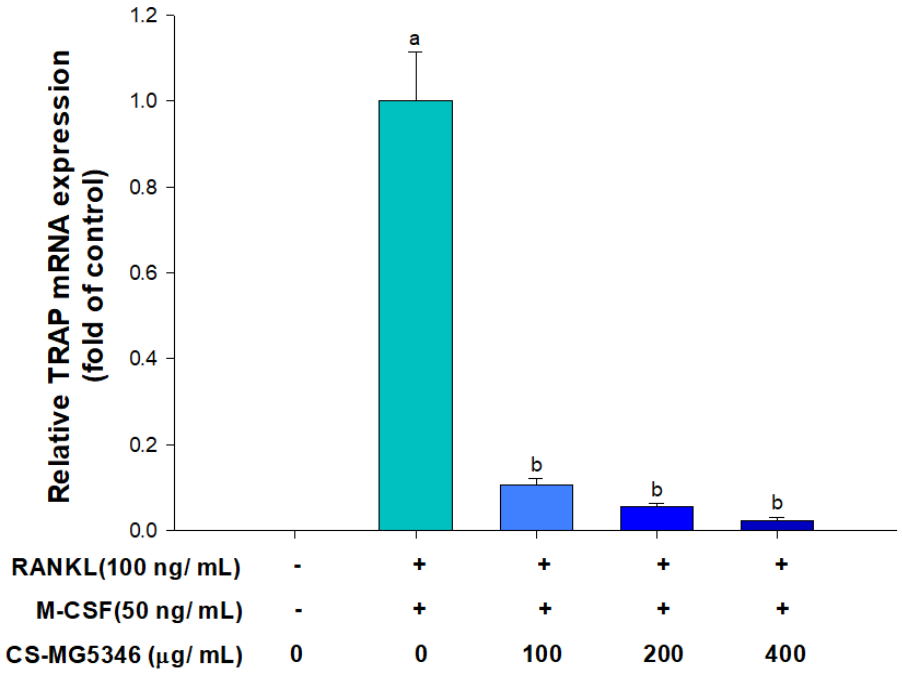

Relative NFATc1 mRNA expression
(fold of control)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
a
b
c
d
e
RANKL(100 ng/ mL) - + + + +
M-CSF(50 ng/ mL) - + + + +
CS-MG5346 (μg/ mL) 0 0 100 200 400

Fig. 2-continued (C)



(D)

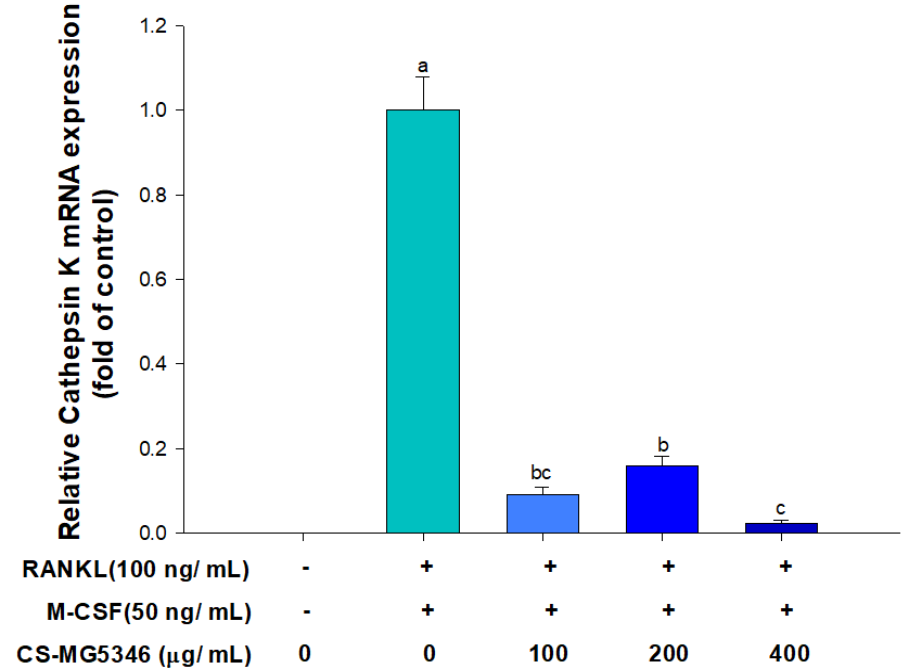


Fig. 2-continued (E)

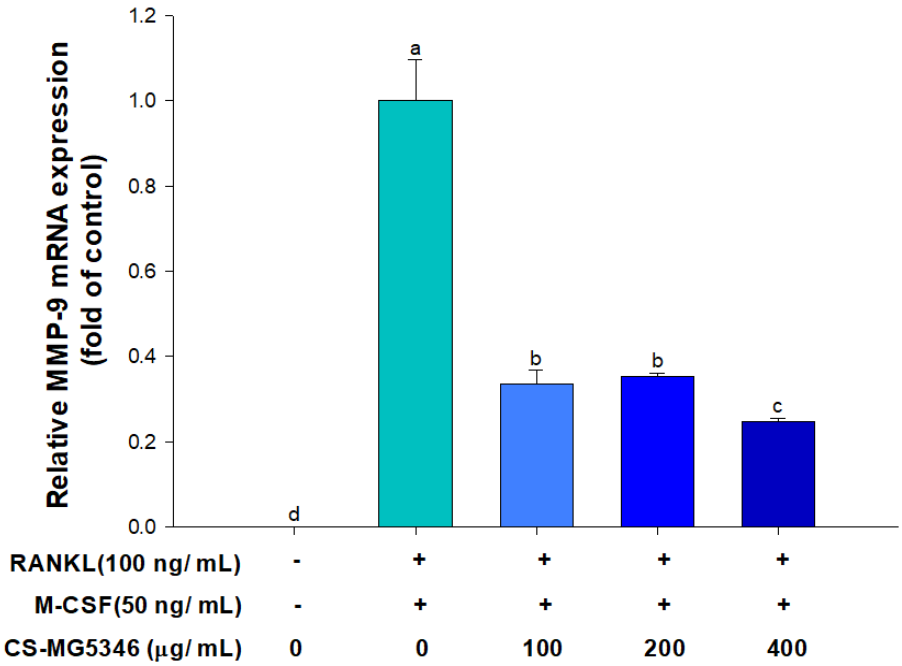


Fig. 3

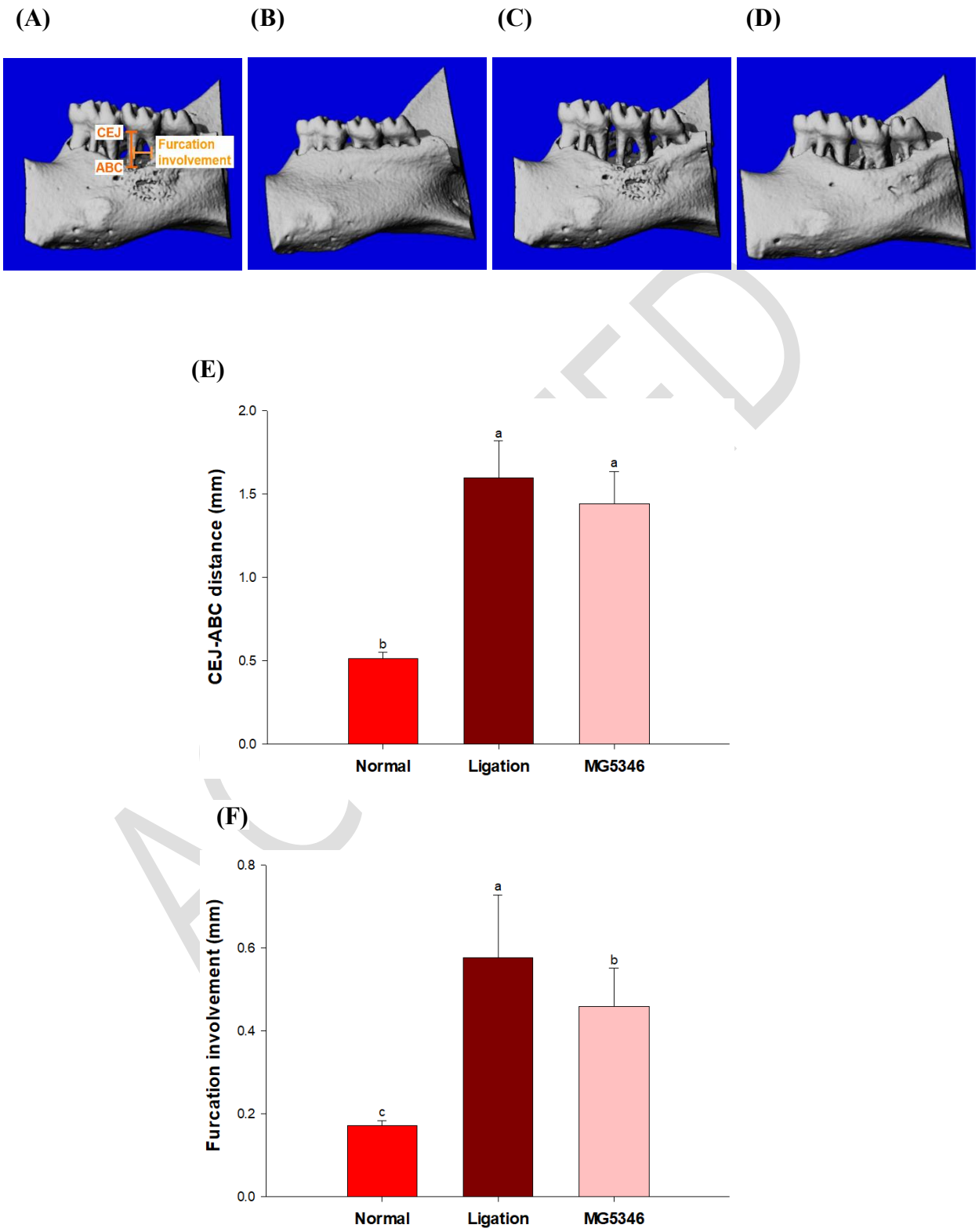
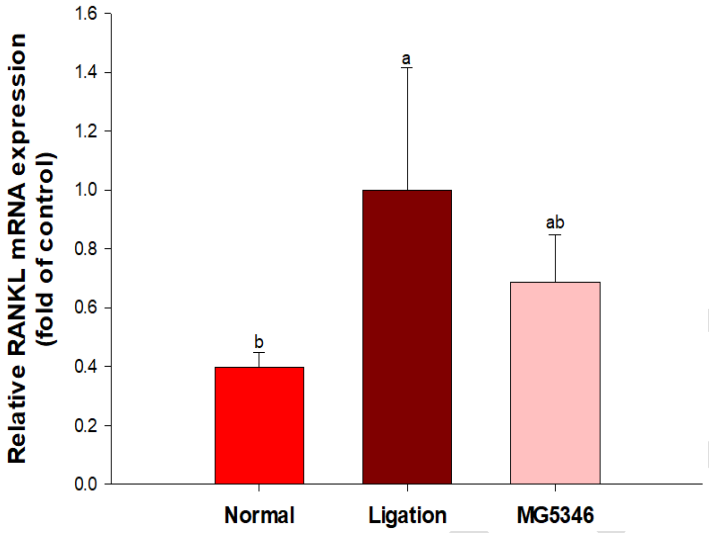
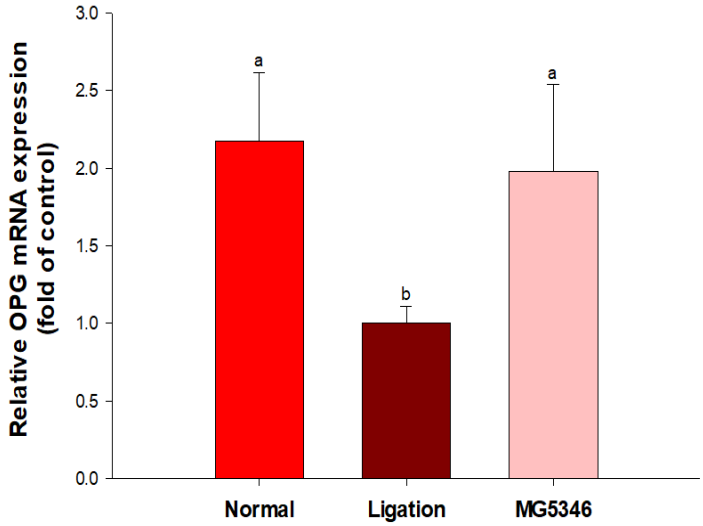

(A)
(B)
(C)
(D)
CEJ
ABC
Furcation involvement
(E)
CEJ-ABC distance (mm)
2.0
1.5
1.0
0.5
0.0
a
a
b
Normal
Ligation
MG5346
(F)
Furcation involvement (mm)
0.8
0.6
0.4
0.2
0.0
a
b
c
Normal
Ligation
MG5346

Fig. 4 (A)



(B)



591 **Fig. 4-continued (C)**

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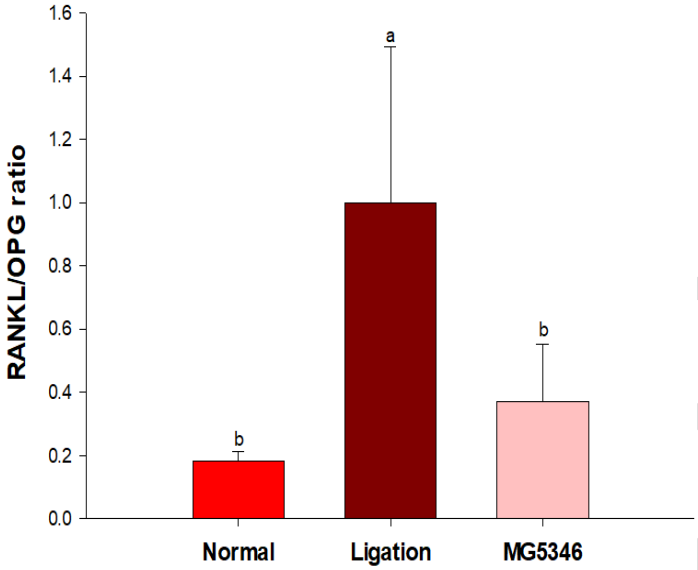

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