Weissella cibaria CMU exerts an anti-inflammatory effect by inhibiting *Aggregatibacter actinomycetemcomitans*-induced NF-κB activation in macrophages

MIN-JEONG KIM[1*], YONG-OUK YOU[2*], JOO-YI KANG[2], HYUN-JIN KIM[3**] and MI-SUN KANG[4**]

Departments of [1]Convergence Technology for Food Industry and Dental Research Institute, [2]Oral Biochemistry and [3]Oral Anatomy and Dental Research Institute, School of Dentistry, Wonkwang University, Iksan, Jellabukdo 54538; [4]Research and Development Department, Research Institute, OraPharm Inc., Seoul 04782, Republic of Korea

Received January 14, 2020; Accepted May 15, 2020

DOI: 10.3892/mmr.2020.11512

Abstract. Periodontitis is a chronic inflammatory disease caused by various periodontal pathogens. *Weissella cibaria* CMU (oraCMU) is a probiotic that promotes oral health. However, its anti-inflammatory effects against periodontal pathogens have not yet been investigated. The present study evaluated the anti-inflammatory effects of live oraCMU against stimulation with the formalin-inactivated periodontal pathogen *Aggregatibacter actinomycetemcomitans* in RAW 264.7 macrophages. Cell viability was analyzed by the MTS assay in a dose-dependent manner (at multiplicities of infection of 0.1, 1, 10, 100 and 1,000). Nitric oxide (NO) was monitored using the Griess test. The mRNA expression of proinflammatory cytokines such as interleukin (IL)1β and IL6 was assessed by reverse transcription-quantitative PCR. Western blotting was used to examine the effects of oraCMU on the phosphorylation of NF-κB inhibitor α (IκBα) and IκBα kinase (IKK), the nuclear translocation of the NF-κB subunit p65 and the expression of inducible NO synthase (iNOS). Live oraCMU had no cytotoxic effects on RAW 264.7 macrophages. In *A. actinomycetemcomitans*-stimulated RAW 264.7 macrophages, oraCMU reduced NO production by suppressing iNOS expression and downregulating the mRNA expression of proinflammatory cytokines in a dose-dependent manner. IKK phosphorylation and IκBα degradation were dose-dependently inhibited by oraCMU and the nuclear translocation of p65 via the canonical NF-κB pathway was simultaneously reduced. The results indicated that oraCMU possessed anti-inflammatory activity associated with the inhibition of NF-κB signal activation in response to periodontal pathogens. This suggests that oraCMU is a beneficial anti-inflammatory probiotic that can aid in the maintenance of oral health.

Correspondence to: Dr Mi-Sun Kang, Research and Development Department, Research Institute, OraPharm Inc., 905-ho, 9-16 Yeonmujang-5-gil, Seongdong-gu, Seoul 04782, Republic of Korea
E-mail: jieenkang@orapharm.com

Professor Hyun-Jin Kim, Department of Oral Anatomy and Dental Research Institute, School of Dentistry, Wonkwang University, 460 Iksandaero, Iksan, Jellabukdo 54538, Republic of Korea
E-mail: khjin1005@wku.ac.kr

[*,**]Contributed equally

Key words: probiotics, inflammation, periodontal, NF-κB, macrophage

Introduction

Inflammation occurs in defense against various external insults and metabolic products, and results in erythema, edema, fever, pain and dysfunction due to the activation of numerous inflammatory mediators (1). It is a local protective response to injury and infection; however, excessive or persistent inflammation leads to chronic inflammatory diseases, including periodontitis (2). Periodontitis is a common disease of the oral cavity that involves chronic inflammation of the supporting tissue around the teeth; it is characterized by alveolar bone destruction and high concentrations of periodontal bacteria (3). Therefore, regulation of the inflammatory response in host cells has been proposed as a method for controlling the progression of periodontitis (4). Gram-negative bacteria, including *Aggregatibacter actinomycetemcomitans*, *Porphyromonas gingivalis* and *Tannerella forsythia*, are well-known periodontitis-associated pathogens (5); they stimulate periodontal cells to produce various inflammatory cytokines, such as interleukin (IL)1β and IL6, and induce cellular immune inflammatory responses, thereby destroying periodontal tissue (6). In particular, *A. actinomycetemcomitans*, a facultative anaerobic, gram-negative, rod-shaped bacterium, is a major causative agent of localized aggressive periodontitis (7,8). It expresses various virulence factors, including a powerful leukotoxin, lipopolysaccharide (LPS), cell surface-associated materials, enzymes and less well-defined virulence factors that modulate the activity of host defenses (9-12).

Among immune cells, macrophages play a pivotal role in inflammation by releasing inflammatory mediators, including proinflammatory cytokines (13). Macrophages are activated by periodontal pathogens and induce inflammatory responses through the NF-κB pathway (14). NF-κB, a transcription factor, is normally sequestered in the cytoplasm in a complex with NF-κB inhibitor α (IκBα) (15). Periodontal pathogens activate IκBα kinase (IKK)α and IKKβ via phosphorylation, allowing them to phosphorylate IκBα. This disrupts the stable complex between NF-κB and IκBα, enabling the translocation of the NF-κB p65 subunit into the nucleus to activate the transcription of proinflammatory genes (15). Consequently, inducible nitric oxide synthase (iNOS) is expressed, and nitric oxide (NO) and proinflammatory cytokines (16), including IL1β and IL6, are secreted to induce inflammatory responses (17). Thus, inhibiting NF-κB activation is an important therapeutic goal for various inflammatory diseases.

Inflammation is involved in the pathological processes of a number of diseases. Recently, periodontal pathogens have been implicated in systemic conditions, including cardiovascular diseases, premature birth and Alzheimer's disease (18-20). Therefore, substances capable of modulating the expression of various inflammatory mediators in response to these pathogens are promising candidate treatments to prevent and suppress not only periodontitis but also systemic disease. Various antibiotics, including minocycline, doxycycline, metronidazole and tetracycline, have been used to treat periodontal inflammation (21). However, as these drugs have a number of adverse effects, including hypersensitivity, and can result in antibiotic resistance, the development of alternative therapeutic agents is actively under way (22).

Over the last few years, researchers have reported the benefits of using probiotics to maintain oral health (23,24). Probiotics are living microorganisms that confer health benefits on their host organisms when consumed in appropriate amounts (25). Effects on oral conditions, such as dental caries, periodontitis and bad breath, have been reported for a few probiotics, including *Streptococcus salivarius*, *Lactobacillus reuteri* and *Weissella cibaria* (26-28). In particular, *W. cibaria* was first classified in a taxonomic study in 2002 and has been denoted as the dominant species in fermented foods, including kimchi (29). Notably, some *W. cibaria* strains are reported to possess stronger immunomodulatory activity than the commercially available strain (30,31).

The *W. cibaria* Chonnam Medical University (CMU) strain (oraCMU) was isolated from the oral cavity and is an effective oral care probiotic (32). OraCMU inhibits the growth of periodontal pathogens and the production of proinflammatory cytokines, including IL6 and IL8, in oral epithelial cells (33). Furthermore, oraCMU was recently reported to reduce periodontal tissue destruction by regulating the production of inflammatory cytokines in a periodontitis mouse model (34).

However, the mechanism by which oraCMU inhibits inflammation caused by periodontal pathogens has yet to be elucidated. The purpose of the present study was to investigate the effects of oraCMU on the production of inflammatory mediators in response to the periodontal pathogen *A. actinomycetemcomitans* in RAW 264.7 macrophages and explore its molecular mechanism of action.

Materials and methods

Bacterial strains and sample preparation. A. actinomycetemcomitans (ATCC 33384, American Type Culture Collection) was provided by the Laboratory of Oral Biochemistry (School of Dentistry, Wonkwang University, Korea) and oraCMU was provided by OraPharm Inc. *A. actinomycetemcomitans* was grown anaerobically (85% N_2, 10% H_2 and 5% CO_2) in tryptic soy broth supplemented with yeast extract (1 mg/ml) and 10% horse serum (HyClone; Cytiva) at 37˚C. Bacteria in the logarithmic growth phase were used in the experiments. The bacteria were harvested, washed three times with phosphate-buffered saline (PBS) and resuspended in PBS in 10-fold concentrated volumes. To obtain inactivated *A. actinomycetemcomitans*, the bacteria were exposed to 0.5% formalin for 30 min at 4˚C and inactivation was confirmed by agar plating. The optical density (OD) of the formalin-inactivated bacterial suspension was measured at 600 nm on a microplate reader (Sunrise™; Tecan Group, Ltd.) and the suspension was diluted to an OD of 1, which corresponded to $1x10^8$ colony forming units (CFUs)/ml. To evaluate its potential anti-inflammatory effects, oraCMU was grown in DeMan, Rogosa and Sharpe (MRS) broth (Difco; BD Biosciences) at 37˚C for 16 h under aerobic conditions. OraCMU was subcultured twice in MRS broth before each experiment. Bacterial cultures were harvested, washed twice with PBS and then resuspended in antibiotic-free Dulbecco's modified Eagle's medium (DMEM; Gibco; Thermo Fisher Scientific, Inc.) containing 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.).

Cell culture. The RAW 264.7 macrophage line (TIB-71, ATCC) was maintained in DMEM supplemented with 10% FBS and 1% antibiotic-antimycotic solution (Gibco; Thermo Fisher Scientific, Inc.) at 37˚C in 5% CO_2. The cells were subcultured and plated at 80% confluency. Antibiotic-free DMEM medium was used for the coculture of RAW 264.7 macrophages and live oraCMU.

Bacterial infection. To prepare live oraCMU, bacterial cultures were harvested, washed twice with PBS and resuspended in antibiotic-free DMEM medium. The OD was measured at 600 nm and the suspension was diluted to obtain an OD of 0.5, which corresponded to $5x10^8$ CFU/ml. For each experiment, RAW 264.7 macrophages were seeded in 24-well plates at $5x10^5$ cells/well. After 24 h, the medium was removed and the macrophages were incubated with various doses of live oraCMU [multiplicities of infection (MOIs) of 0.1, 1 and 10] in antibiotic-free DMEM medium and $1x10^7$ CFU/ml *A. actinomycetemcomitans* cells, and the cocultures were incubated at 37˚C in 5% CO_2. For mRNA analysis, oraCMU and *A. actinomycetemcomitans* were added for 4 or 6 h. For western blotting, $1x10^6$ cells/ml were seeded in 60-mm dishes and oraCMU and *A. actinomycetemcomitans* were added for 10 min, 30 min, 1 h or 16 h.

Cell viability assays. The cytotoxicity of live oraCMU was measured using the MTS assay (CellTiter 96® Aqueous One Solution Cell Proliferation Assay kit; Promega Corporation). RAW 264.7 macrophages were seeded in a 96-well culture plate at $1x10^5$ cells/well and incubated overnight at 37˚C in

5% CO_2. Then, cells were treated with various concentrations of oraCMU (MOIs =0.1, 1, 10, 100 and 1,000). After incubation for 24 h at 37˚C, the media was changed to remove almost all oraCMU and only macrophages were left in each well. After adding only DMEM, MTS was added to each well at a 1:5 ratio and the plate was incubated at 37˚C and 5% CO_2 for 2 h. The absorbance was measured at 490 nm on a microplate reader.

NO quantification assays. RAW 264.7 cells were seeded at 5x10^5 cells/well in 24-well culture plates. After 24 h at 37˚C, the cells were treated with *A. actinomycetemcomitans* and various concentrations of oraCMU (MOIs =0.1, 1 and 10). Following incubation for 24 h at 37˚C, the supernatants were assessed by mixing with the same volume of Griess reagent (Promega Corporation). The absorbance was measured at 540 nm on a microplate reader and the nitrite concentration was calculated using a sodium nitrite calibration curve.

Reverse transcription-quantitative (RTq)-PCR. Total RNA was extracted with TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol, and quantified spectrophotometrically. First-strand cDNA was synthesized from 1 µg of RNA using PrimeScript RT Reagent kit (Takara Bio, Inc.). RT-qPCR was performed on a GeneAmp PCR system 2400 (Applied Biosystems; Thermo Fisher Scientific, Inc.) using the AccuPower PCR PreMix kit (Bioneer Corporation). Each RT-PCR reaction used 0.5 µM of each primer. Each cycle consisted of denaturation at 94˚C (30 sec), annealing at 55˚C (30 sec) and extension at 72˚C (60 sec). The primer sequences were as follows: IL6 forward, 5'-GATGGATGCTACCAAACTGGA-3' and reverse, 5'-TCTGAAGGACTCTGGCTTTG-3' (142 bp); IL1β forward, 5'-GAAAGACGGCACACCCACCCT-3' and reverse, 5'-GCTCTGCTTGTGAGGTGCTGATGTA-3' (166 bp); and β-actin forward, 5'-CATCACTATTGGCAACGAGC-3' and reverse, 5'-GACAGCACTGTGTTGGCATA-3 (159 bp). The number of PCR cycles for IL6, IL1β and β-actin were 25, 25 and 28, respectively. β-actin was used as an internal control. The amplified cDNA products were resolved on 1.5% agarose gels. The sizes of the amplified DNA fragments were identified by comparison with a SolGent 100 bp Plus DNA Ladder (SolGent Co., Ltd.). Bands were detected using an Azure cSeries (Azure Biosystems, Inc.). Densitometry was performed using ImageJ 1.52a software (National Institutes of Health) and normalized to the untreated control group. Quantitative amplification of cDNA was conducted in a StepOnePlus™ Real-Time PCR System (Applied Biosystems; Thermo Fisher Scientific, Inc.) with PowerSYBR Green PCR Master Mix (Applied Biosystems; Thermo Fisher Scientific, Inc.). The RT-qPCR conditions were as follows: Incubation for 5 min at 95˚C, followed by 30 cycles of denaturation for 15 sec at 95˚C, annealing for 15 sec at 60˚C and extension for 15 sec at 72˚C. Relative mRNA levels were calculated using a standard curve generated from cDNA dilutions. The $2^{-\Delta\Delta Cq}$ method was used to calculate relative gene expression using quadruplicate measurements, with β-actin as an internal control (35).

Western blot analysis. Cytosolic protein extracts from RAW 264.7 cells were prepared with PhosphoSafe Protein Extraction Reagent (Novagen, Inc.), according to the manufacturer's protocol. Isolation of nuclear fractions from RAW 264.7 cells was performed using a nuclear extraction kit (Cayman Chemical Company). Total protein was quantified using a bicinchoninic acid protein assay (Pierce; Thermo Fisher Scientific, Inc.) at a wavelength of 562 nm, and 15 µg cytosolic protein/lane and 10 µg nuclear protein/lane were resolved by SDS-PAGE on 10% gels and transferred to Protran nitrocellulose membranes (Whatman plc; Cytiva). Membranes were blocked with 10 mM Tris-buffered saline with 0.1% Tween-20 (TBST) containing 5% skimmed milk for 1 h at 25˚C, followed by incubation with primary antibodies overnight at 4˚C with gentle shaking. The antibodies used were as follows: phosphorylated (p-)IκBα kinase (IKK)α/β (cat. no. 2697; 1:1,000; Cell Signaling Technology, Inc.), IKKα (cat. no. 2682; 1:1,000; Cell Signaling Technology, Inc.), IKKβ (cat. no. 2678; 1:1,000; Cell Signaling Technology, Inc.), p-IκBα (cat. no. 2859; 1:1,000; Cell Signaling Technology, Inc.), IκBα (cat. no. 9242; 1:1,000; Cell Signaling Technology, Inc.), NF-κB p65 subunit (cat. no. 8242; 1:1,000; Cell Signaling Technology, Inc.), iNOS (cat. no. 13120; 1:1,000; Cell Signaling Technology, Inc.) and proliferating cell nuclear antigen (PCNA; cat. no. sc-56; 1:1,000; Santa Cruz Biotechnology, Inc.). A mouse monoclonal primary antibody against β-actin (cat. no. A5441; 1:5,000; Sigma-Aldrich; Merck KGaA) was used as a loading control. The blots were washed in TBST and then incubated for 2 h at room temperature with horseradish peroxidase (HRP)-conjugated anti-rabbit (cat. no. 7074; 1:2,500; Cell Signaling Technology, Inc.) or anti-mouse IgG (cat. no. sc-516102; 1:2,500; Santa Cruz Biotechnology, Inc.). The blots were subsequently washed with TBST and protein bands were visualized with HRP Substrate Luminol Reagent (EMD Millipore) and imaged on a Chemiluminescent Western Blot Imaging System (Azure Biosystems, Inc.). Densitometry of western blot bands was performed using ImageJ 1.52a software (National Institutes of Health). The detected bands were quantified using ImageJ and normalized to the untreated control group.

Statistical analysis. Statistical analyses were performed using SPSS v17.0 (SPSS, Inc.). Data are presented as the mean ± standard deviation of the mean. The Kruskal-Wallis test followed by Dunn's post hoc test was used to compare the different groups. $P<0.05$ was considered to indicate a statistically significant difference. Each experiment was performed three times.

Results

Cytotoxic effects of oraCMU on RAW 264.7 macrophages. To assess the cytotoxicity of live oraCMU, its effects on the viability of RAW 264.7 macrophages were examined. No cytotoxic effects were detected after 24 h of treatment at various concentrations (Fig. 1).

Inhibitory effects of oraCMU on NO production and iNOS expression. To investigate whether oraCMU possesses anti-inflammatory effects against *A. actinomycetemcomitans* in RAW 264.7 macrophages, its effects on NO production were examined. After 24 h of *A. actinomycetemcomitans* treatment, NO release was higher compared with that in the untreated controls (Fig. 2A). Treatment with oraCMU significantly decreased *A. actinomycetemcomitans*-induced NO production in a dose-dependent manner. Changes in NO production can

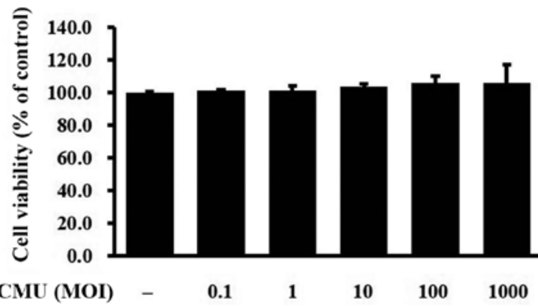

Figure 1. OraCMU does not affect the viability of RAW 264.7 macrophages. Data represent the mean ± standard deviation of three independent experiments. OraCMU, *Weissella cibaria* CMU; MOI, multiplicity of infection.

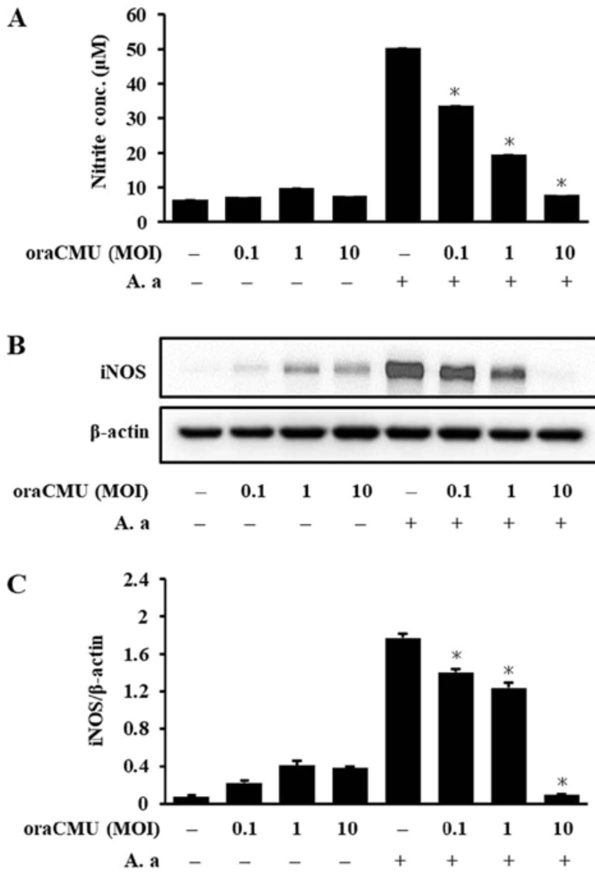

Figure 2. OraCMU suppresses NO production and iNOS expression in *A. actinomycetemcomitans*-induced RAW 264.7 macrophages. (A) NO levels and (B) iNOS expression levels in *A. actinomycetemcomitans*-induced RAW 264.7 macrophages. Similar data were obtained in three independent experiments. (C) Relative quantification of iNOS levels normalized to β-actin. Data are presented as the mean ± standard deviation of three independent experiments. *P<0.05 vs. *A. actinomycetemcomitans*-treated group. OraCMU, *Weissella cibaria* CMU; NO, nitric oxide; iNOS, inducible nitric oxide synthase; MOI, multiplicity of infection.

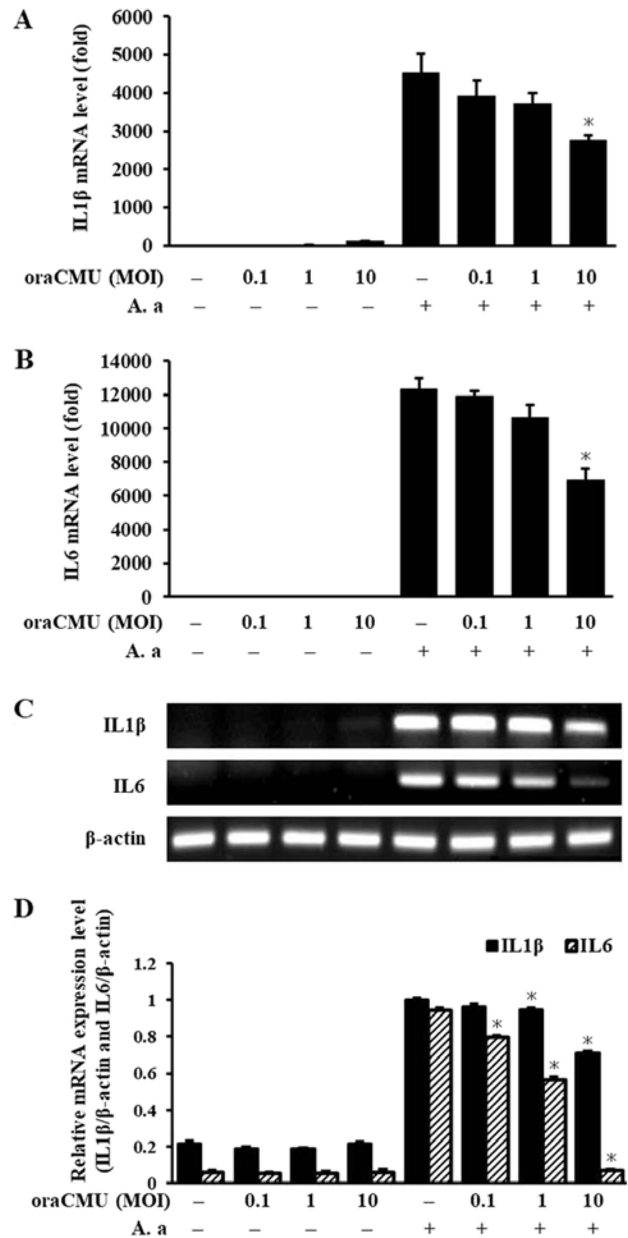

Figure 3. OraCMU downregulates proinflammatory cytokine expression in *A. actinomycetemcomitans*-induced RAW 264.7 macrophages. Expression levels of (A) IL1β and (B) IL6 mRNA, normalized to β-actin. (C) Agarose gel electrophoresis showing the relative mRNA expression level of IL1β and IL6 following oraCMU treatment. (D) Relative quantification of IL1β and IL6 expression levels normalized to β-actin. Data are presented as the mean ± standard deviation of three independent experiments. *P<0.05 vs. respective *A. actinomycetemcomitans*-treated group. OraCMU, *Weissella cibaria* CMU; IL, interleukin; MOI, multiplicity of infection.

be attributed to changes in iNOS expression (30). Treatment with *A. actinomycetemcomitans* significantly increased iNOS expression in RAW 264.7 macrophages and oraCMU treatment significantly decreased iNOS expression in a dose-dependent manner (Fig. 2B and C).

Inhibitory effects of oraCMU on the mRNA expression of proinflammatory cytokines. To determine whether oraCMU modulates the mRNA expression of proinflammatory cytokines, RAW 264.7 macrophages were incubated with *A. actinomycetemcomitans* and various concentrations of oraCMU. IL6 and IL1β increased significantly with *A. actinomycetemcomitans* treatment and were significantly decreased at higher doses of oraCMU (Fig. 3).

Inhibitory effects of oraCMU on NF-κB activation. As the NF-κB pathway plays an important role in the transcriptional activation of proinflammatory factors, the effects of oraCMU on NF-κB activation were next assessed by examining p65 levels

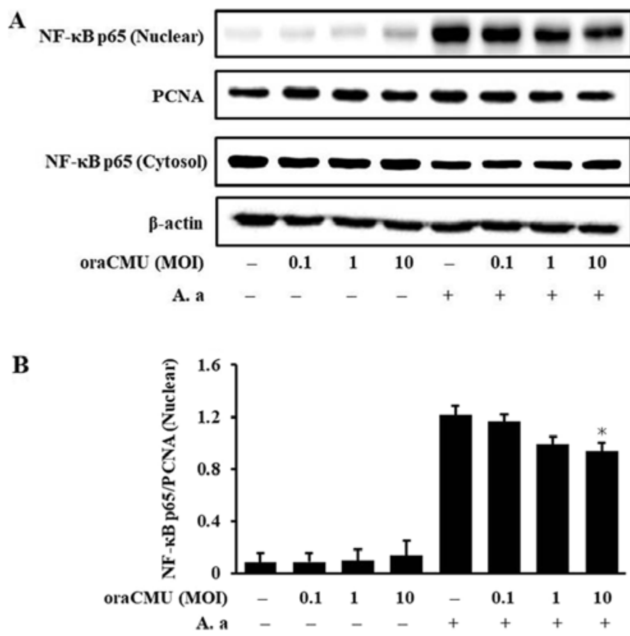

Figure 4. OraCMU inhibits p65 nuclear translocation in *A. actinomycetemcomitans*-induced RAW 264.7 macrophages. (A) Expression levels of p65 in cytosolic and nuclear extracts. PCNA and β-actin were used as nuclear and cytosolic loading controls, respectively. (B) Relative quantification of nuclear p65. Data are presented as the mean ± standard deviation of three independent experiments. *P<0.05 vs. *A. actinomycetemcomitans*-treated group. OraCMU, *Weissella cibaria* CMU; PCNA, proliferating cell nuclear antigen; MOI, multiplicity of infection.

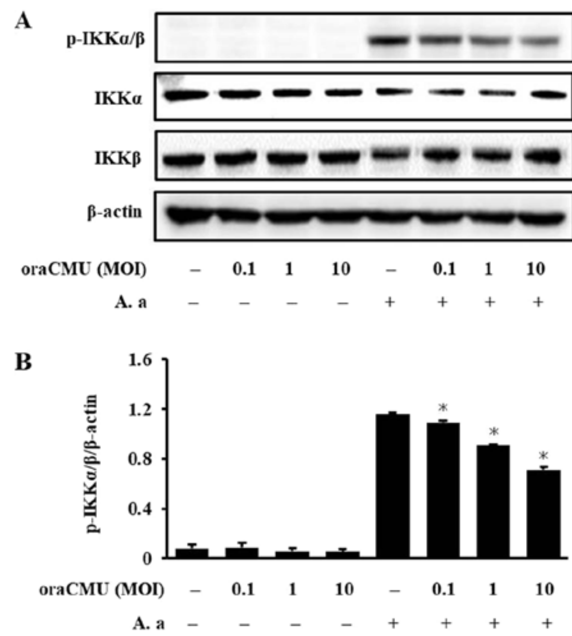

Figure 6. OraCMU inhibits IKKα/β phosphorylation in *A. actinomycetemcomitans*-induced RAW 264.7 macrophages. (A) Expression levels of p-IKKα, p-IKKβ, IKKα and IKKβ. (B) Relative quantification of p-IKKα/β. Data are presented as the mean ± standard deviation of three independent experiments. *P<0.05 vs. *A. actinomycetemcomitans*-treated group. OraCMU, *Weissella cibaria* CMU; IKK, IκBα kinase; p-, phosphorylated; MOI, multiplicity of infection.

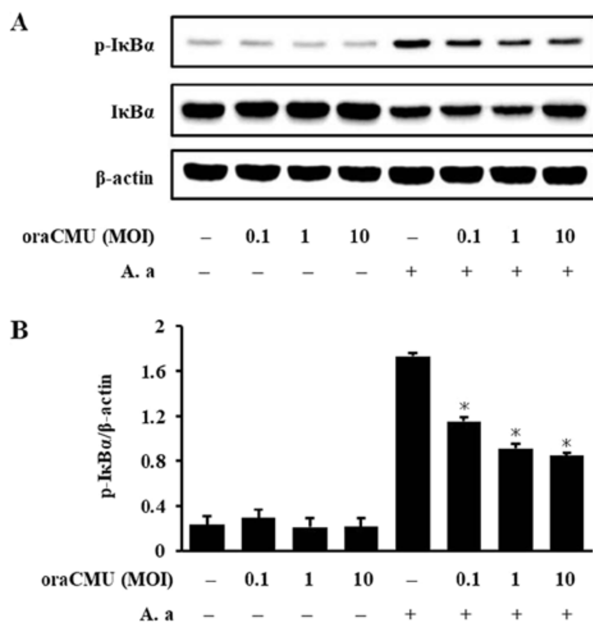

Figure 5. OraCMU inhibits IκBα phosphorylation in *A. actinomycetemcomitans*-induced RAW 264.7 macrophages. (A) Expression levels of p-IκBα and IκBα in cytosolic extracts. (B) Relative quantification of IκBα phosphorylation. Data are presented as the mean ± standard deviation of three independent experiments. *P<0.05 vs. *A. actinomycetemcomitans*-treated group. OraCMU, *Weissella cibaria* CMU; IκBα, NF-κB inhibitor α; p-, phosphorylated; MOI, multiplicity of infection.

in cytosolic and nuclear extracts from macrophages treated with *A. actinomycetemcomitans* and oraCMU for 30 min or 1 h. *A. actinomycetemcomitans* alone resulted in increased nuclear p65, whereas oraCMU treatment significantly inhibited the nuclear accumulation of p65 at high doses (Fig. 4).

Inhibitory effects of oraCMU on A. actinomycetemcomitans-induced IκBα and IKKα/β phosphorylation. As IκBα and IKKα/β are important regulators of NF-κB activation, their activation after oraCMU treatment of *A. actinomycetemcomitans*-stimulated macrophages were evaluated. After 30 min, OraCMU significantly inhibited IκBα phosphorylation in a dose-dependent manner (Fig. 5). In addition, after 10 min, oraCMU dose-dependently inhibited IKKα/β phosphorylation (Fig. 6).

Discussion

OraCMU is the first commercialized oral care probiotic in Korea (32) that can help prevent bad breath and dental caries (28,36,37). It inhibits the Fusobacterium nucleatum-induced increase of the proinflammatory cytokines IL6 and IL8 in oral epithelial cells (33) and has antimicrobial activity against various representative periodontal pathogens, including *A. actinomycetemcomitans* (38). Therefore, oraCMU may aid in preventing periodontal disease. However, its inhibitory effects on proinflammatory cytokine expression in macrophages stimulated with *A. actinomycetemcomitans*, a periodontal pathogen, have not yet been reported. To the best of our knowledge, the present study is the first to elucidate the mechanism by which *W. cibaria* inhibits inflammatory cytokine expression after infection with periodontal pathogens.

The present study evaluated whether live oraCMU had inhibitory effects on the inflammation induced by

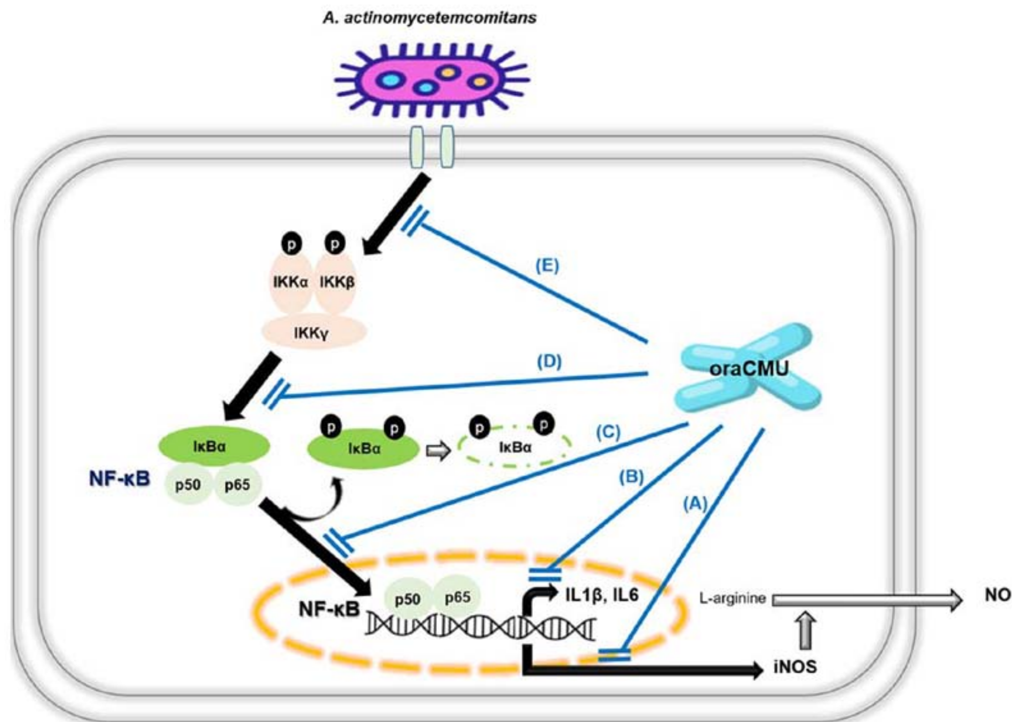

Figure 7. Model of the mechanism of the anti-inflammatory effect of oraCMU in *A. actinomycetemcomitans*-infected RAW 264.7 macrophages. The results suggest that oraCMU: (A) Suppresses iNOS expression and NO production; (B) inhibits IL1β and IL6 mRNA expression; (C) inhibits p65 nuclear translocation; (D) inhibits IκBα phosphorylation; and (E) inhibits IKKα and IKKβ phosphorylation. OraCMU, *Weissella cibaria* CMU; iNOS, inducible nitric oxide synthase; NO, nitric oxide; IL, interleukin; IκBα, NF-κB inhibitor α; IKK, IκBα kinase.

formalin-inactivated *A. actinomycetemcomitans* in RAW 264.7 cells. The *A. actinomycetemcomitans*-induced inflammatory response was characterized by increased NO production and increased iNOS, IL1β and IL6 expression; oraCMU decreased the levels of these proinflammatory mediators. To exclude the possibility that cytotoxicity caused by live oraCMU infection was responsible for the inhibition of the proinflammatory mediators, the viability of oraCMU-infected cells was tested. No obvious cytotoxic effects were detected at any MOI used, consistent with the results of our previous study (33).

NO is an important biomarker of the inflammatory response and is regulated by iNOS (16). The iNOS enzyme cannot be detected under normal conditions but is induced through NF-κB activation, leading to excessive NO production. Excessive NO leads to the upregulation of other proinflammatory cytokines and can cause malfunctions ranging from severe cellular damage to inflammatory disorders (39). Thus, regulating iNOS expression is an important strategy in the development of inflammatory disease therapies. The present study found that oraCMU significantly decreased *A. actinomycetemcomitans*-induced NO production and downregulated iNOS expression in a dose-dependent manner, suggesting that oraCMU acts as an anti-inflammatory regulator.

Madeira *et al* (40), found that *A. actinomycetemcomitans* LPS plays an important role in alveolar bone loss. They also demonstrated that it can induce NO production in murine macrophages. The present study used inactivated *A. actinomycetemcomitans* as a trigger instead of its LPS and found that live oraCMU decreased *A. actinomycetemcomitans*-induced NO production in RAW 264.7 cells by inhibiting iNOS at the mRNA level. Similarly, Yu *et al* (31), reported that treatment with heat-inactivated *W. cibaria* JW15 decreased NO production in RAW 264.7 cells upon LPS stimulation, which was attributable to downregulated iNOS expression.

NF-κB regulates the expression of iNOS and other proinflammatory factors (16,17). In the present study, *A. actinomycetemcomitans* stimulation increased p65 levels in the nucleus; this was inhibited by high doses of oraCMU, suggesting that it can inactivate NF-κB. In addition, it inhibited IκBα and IKKα/β phosphorylation in a dose-dependent manner. These results suggested that oraCMU blocks the expression of proinflammatory mediators by inhibiting the classical NF-κB pathway.

The proinflammatory cytokines IL1β and IL6 are representative diagnostic markers that provide information about the progression of periodontal disease (17). Their expression is higher at sites of periodontal inflammation and is closely associated with the clinical severity of periodontitis. IL6 secretion, stimulated by exposure to IL1β, is involved in the periodontal tissue destruction that occurs in periodontitis (17). In the present study, live oraCMU displayed dose-dependent anti-inflammatory activities in macrophages activated by *A. actinomycetemcomitans* by inhibiting NF-κB signaling (Fig. 7). Heat-inactivated *W. cibaria* JW15 has also been shown to suppress IL1β and IL6 expression; moreover, mechanistically, its anti-inflammatory properties are mediated by mitogen-activated protein kinase signaling and result in NF-κB inhibition (31).

Probiotics are viable microorganisms that have a number of health benefits, which includes regulating intestinal microbial balance and exerting immune-modulating effects on the host through colonization of the intestinal microflora (25). Since the

composition of bacterial surface molecules, including amino acid residues, disaccharide ratio and differences in cross-link type, are different between microbes, microbe-mediated immune responses are probiotic strain-specific (23-25). The present study simultaneously inoculated macrophages with formalin-inactivated *A. actinomycetemcomitans* and live bacteria oraCMU, and then confirmed the mechanism of action of *A. actinomycetemcomitans*-induced inflammatory cytokine expression via cell signaling. Probiotics and periodontal pathogens were evaluated by direct contact with macrophages.

Metabolites and altered surroundings produced by probiotics can affect the inhibition of inflammatory cytokine expression in macrophages, but do not exert inhibitory effects due to the use of inactivated *A. actinomycetemcomitans*. Since oraCMU is commercially used as a living bacterium, the live bacterium oraCMU was used in this study. As oraCMU is an anaerobic bacterium, it was expected to be effective because it can grow well under anaerobic conditions when it enters the periodontal pocket.

Previous studies on *W. cibaria* strain derived from Kimchi, a fermented food, have focused on intestinal immunity (30,31). However, previous studies have reported that probiotics work in the oral cavity and have beneficial effects (24-28). The present study is novel in that the *W. cibaria* strain, derived from the saliva of children with healthy mouths, was investigated for the prevention of proinflammatory responses by *A. actinomycetemcomitans* and for its cellular signaling mechanism.

In conclusion, the present study demonstrated the anti-inflammatory effects of the oral cavity-derived probiotic oraCMU, indicating its usefulness as a prophylactic oral probiotic. OraCMU inhibited proinflammatory signaling in *A. actinomycetemcomitans*-induced macrophages by blocking NF-κB activation, resulting in decreased phosphorylation of IKKs and IκBα, decreased translocation of p65 to the nucleus and decreased expression of iNOS, IL1β and IL6. Although further *in vivo* research will be required to confirm the anti-inflammatory effects of this strain, these results provide molecular evidence for the immunomodulatory effects of oraCMU. Overall, the findings of present study indicated that oraCMU could be used to develop oral care probiotics that can aid in the prevention of periodontal disease. The effects of live probiotics were investigated in the present study. Comparative studies on live and dead oraCMU in a further study might be meaningful and should be considered in further research.

Acknowledgements

Not applicable.

Funding

The present study was supported by the Basic Science Research Program of the National Research Foundation of Korea (NRF) funded by the Ministry of Education (grant no. 2017R1D1A1B03030952).

Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

MJK and JYK performed most of the experiments. YOY and HJK analyzed the data. HJK and MSK designed the study and wrote the manuscript. All authors read and approved the final manuscript, and agree to be accountable for all aspects of the research and ensure that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

MSK is employed by OraPharm Inc. The other authors declare that they have no competing interests.

References

1. Wojdasiewicz P, Poniatowski ŁA and Szukiewicz D: The role of inflammatory and anti-inflammatory cytokines in the pathogenesis of osteoarthritis. Mediators Inflamm 2014: 561459, 2014.
2. Kinane DF, Stathopoulou PG and Papapanou PN: Periodontal diseases. Nat Rev Dis Primers 3: 17038, 2017.
3. Cochran DL: Inflammation and bone loss in periodontal disease. J Periodontol 79 (Suppl): 1569-1576, 2008.
4. Silva N, Abusleme L, Bravo D, Dutzan N, Garcia-Sesnich J, Vernal R, Hernández M and Gamonal J: Host response mechanisms in periodontal diseases. J Appl Oral Sci 23: 329-355, 2015.
5. Sánchez GA, Acquier AB, De Couto A, Busch L and Mendez CF: Association between Aggregatibacter actinomycetemcomitans and Porphyromonas gingivalis in subgingival plaque and clinical parameters, in Argentine patients with aggressive periodontitis. Microb Pathog 82: 31-36, 2015.
6. Graves D: Cytokines that promote periodontal tissue destruction. J Periodontol 79 (Suppl): 1585-1591, 2008.
7. Gholizadeh P, Pormohammad A, Eslami H, Shokouhi B, Fakhrzadeh V and Kafil HS: Oral pathogenesis of Aggregatibacter actinomycetemcomitans. Microb Pathog 113: 303-311, 2017.
8. Cheng Z, Meade J, Mankia K, Emery P and Devine DA: Periodontal disease and periodontal bacteria as triggers for rheumatoid arthritis. Best Pract Res Clin Rheumatol 31: 19-30, 2017.
9. Brage M, Holmlund A and Johansson A: Humoral immune response to Aggregatibacter actinomycetemcomitans leukotoxin. J Periodontal Res 46: 170-175, 2011.
10. Singh S, Bhatia R, Singh A, Singh P, Kaur R, Khare P, Purama RK, Boparai RK, Rishi P, Ambalam P, *et al*: Probiotic attributes and prevention of LPS-induced pro-inflammatory stress in RAW264.7 macrophages and human intestinal epithelial cell line (Caco-2) by newly isolated Weissella cibaria strains. Food Funct 9: 1254-1264, 2018.
11. Weidenmaier C, Kristian SA and Peschel A: Bacterial resistance to antimicrobial host defenses--an emerging target for novel antiinfective strategies? Curr Drug Targets 4: 643-649, 2003.
12. Nau GJ, Richmond JF, Schlesinger A, Jennings EG, Lander ES and Young RA: Human macrophage activation programs induced by bacterial pathogens. Proc Natl Acad Sci USA 99: 1503-1508, 2002.
13. Turner MD, Nedjai B, Hurst T and Pennington DJ: Cytokines and chemokines: At the crossroads of cell signalling and inflammatory disease. Biochim Biophys Acta 1843: 2563-2582, 2014.
14. Choi EY, Jin JY, Lee JY, Choi JI, Choi IS and Kim SJ: Anti-inflammatory effects and the underlying mechanisms of action of daidzein in murine macrophages stimulated with Prevotella intermedia lipopolysaccharide. J Periodontal Res 47: 204-211, 2012.

15. Liu T, Zhang L, Joo D and Sun SC: NF-κB signaling in inflammation. Signal Transduct Target Ther: doi: 10.1038/sigtrans.2017.23.
16. Aktan F: iNOS-mediated nitric oxide production and its regulation. Life Sci 75: 639-653, 2004.
17. Chen CC, Chang KL, Huang JF, Huang JS and Tsai CC: Correlation of interleukin-1 beta, interleukin-6, and periodontitis. Kaohsiung J Med Sci 13: 609-617, 1997.
18. Carrizales-Sepúlveda EF, Ordaz-Farías A, Vera-Pineda R and Flores-Ramírez R: Periodontal disease, systemic inflammation and the risk of cardiovascular disease. Heart Lung Circ 27: 1327-1334, 2018.
19. Parihar AS, Katoch V, Rajguru SA, Rajpoot N, Singh P and Wakhle S: Periodontal disease: A possible risk-factor for adverse pregnancy outcome. J Int Oral Health 7: 137-142, 2015.
20. Ganesh P, Karthikeyan R, Muthukumaraswamy A and Anand J: A potential role of periodontal inflammation in Alzheimer's disease: A Review. Oral Health Prev Dent 15: 7-12, 2017.
21. Da Rocha HA, Silva CF, Santiago FL, Martins LG, Dias PC and De Magalhães D: Local drug delivery systems in the treatment of periodontitis: A literature review. J Int Acad Periodontol 17: 82-90, 2015.
22. Blumenthal KG, Peter JG, Trubiano JA and Phillips EJ: Antibiotic allergy. Lancet 393: 183-198, 2019.
23. Gupta G: Probiotics and periodontal health. J Med Life 4: 387-394, 2011.
24. Saha S, Tomaro-Duchesneau C, Tabrizian M and Prakash S: Probiotics as oral health biotherapeutics. Expert Opin Biol Ther 12: 1207-1220, 2012.
25. Reid G, Jass J, Sebulsky MT and McCormick JK: Potential uses of probiotics in clinical practice. Clin Microbiol Rev 16: 658-672, 2003.
26. Burton JP, Drummond BK, Chilcott CN, Tagg JR, Thomson WM, Hale JDF and Wescombe PA: Influence of the probiotic Streptococcus salivarius strain M18 on indices of dental health in children: A randomized double-blind, placebo-controlled trial. J Med Microbiol 62: 875-884, 2013.
27. Szkaradkiewicz AK, Stopa J and Karpiński TM: Effect of oral administration involving a probiotic strain of Lactobacillus reuteri on pro-inflammatory cytokine response in patients with chronic periodontitis. Arch Immunol Ther Exp (Warsz) 62: 495-500, 2014.
28. Kang MS, Kim BG, Chung J, Lee HC and Oh JS: Inhibitory effect of Weissella cibaria isolates on the production of volatile sulphur compounds. J Clin Periodontol 33: 226-232, 2006.
29. Björkroth KJ, Schillinger U, Geisen R, Weiss N, Hoste B, Holzapfel WH, Korkeala HJ and Vandamme P: Taxonomic study of Weissella confusa and description of *Weissella cibaria* sp. nov., detected in food and clinical samples. Int J Syst Evol Microbiol 52: 141-148, 2002.
30. Park HE, Kang KW, Kim BS, Lee SM and Lee WK: Immunomodulatory potential of Weissella cibaria in aged C57BL/6J mice. J Microbiol Biotechnol 27: 2094-2103, 2017.
31. Yu HS, Lee NK, Choi AJ, Choe JS, Bae CH and Paik HD: Anti-inflammatory potential of probiotic strain Weissella cibaria JW15 isolated from Kimchi through regulation of NF-κB and MAPKs pathways in LPS-induced RAW 264.7 cells. J Microbiol Biotechnol 29: 1022-1032, 2019.
32. Jang HJ, Kang MS, Yi SH, Hong JY and Hong SP: Comparative study on the characteristics of Weissella cibaria CMU and probiotic strains for oral care. Molecules 21: 21, 2016.
33. Kang MS, Lim HS, Kim SM, Lee HC and Oh JS: Effect of *Weissella cibaria* on Fusobacterium nucleatum-induced interleukin-6 and interleukin-8 production in KB cells. J Bacteriol Virol 41: 9-18, 2011.
34. Kim JW, Jung BH, Lee JH, Yoo KY, Lee H, Kang MS and Lee JK: Effect of Weissella cibaria on the reduction of periodontal tissue destruction in mice. J Periodontol: doi:10.1002/JPER.19-0288.
35. Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the $2(-\Delta\Delta C(T))$ method. Methods 25: 402-408, 2001.
36. Kang MS, Chung J, Kim SM, Yang KH and Oh JS: Effect of Weissella cibaria isolates on the formation of Streptococcus mutans biofilm. Caries Res 40: 418-425, 2006.
37. Park HR, Kim HJ and Kang MS: Clinical studies on the dental caries prevention effects of the ability of Weissella cibaria CMU to adhere to the oral cavity. Indian J Public Health Res Dev 9: 1163-1169, 2018.
38. Asok A, Bhandary R, Shetty M and Shenoy MS: Probiotics and periodontal disease. Int J Oral Health Sci 8: 68-72, 2018.
39. Baron VT, Pio R, Jia Z and Mercola D: Early growth response 3 regulates genes of inflammation and directly activates IL6 and IL8 expression in prostate cancer. Br J Cancer 112: 755-764, 2015.
40. Madeira MF, Queiroz-Junior CM, Cisalpino D, Werneck SM, Kikuchi H, Fujise O, Ryffel B, Silva TA, Teixeira MM and Souza DG: MyD88 is essential for alveolar bone loss induced by Aggregatibacter actinomycetemcomitans lipopolysaccharide in mice. Mol Oral Microbiol 28: 415-424, 2013.

This work is licensed under a Creative Commons Attribution-NonCommercial-NoDerivatives 4.0 International (CC BY-NC-ND 4.0) License.