
原 著 論 文

Antibacterial and Antibiofilm Effects of Reuterin-Related Compounds to Periodontopathic Bacteria

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Abstract : OBJECTIVE: *Lactobacillus reuteri* is one of the probiotics that possesses preventive effects on oral infections including dental caries and periodontal disease. Reuterin is a bacteriocin-like compound produced by *L. reuteri* and plays an important role in the probiotic effects. However, it is difficult to obtain reuterin due to the general lability of its aldehyde moiety. Therefore, fourteen stable reuterin-related compounds (RRCs) were chemically synthesized. Here, we investigated the effects of RRCs on periodontopathic bacteria.

MATERIALS AND METHODS: The antibacterial activity of RRCs on pathogenic microorganisms including periodontopathic bacteria was determined. Moreover, the effects of RRCs on biofilm formation by *Fusobacterium nucleatum* were examined by a crystal violet biofilm formation assay. Cytotoxicity of RRCs was evaluated by a lactate dehydrogenase assay using oral keratinocytes.

RESULTS: Among synthesized RRCs, RRC-04, -05, -09, -10, -12, -13, and -14, exhibited antibacterial activities against periodontopathic bacteria. RRCs, except for RRC-06, -07, -08, and -11, significantly suppressed biofilm formation to 60-80 % of the control. Most RRCs, except for RRC-12 and -13, were not cytotoxic to human oral keratinocytes.

CONCLUSIONS: The synthesized RRCs can be potent novel oral care products for the prevention of periodontitis without adverse effects.

Introduction

Periodontitis is a chronic inflammatory condition initiated by a microbial biofilm formed in the periodontal pocket called "dental plaque" and leads to destruction of the supporting tissues of the teeth and finally to tooth loss¹⁾. Furthermore, the association of oral microflora with systemic diseases, such as cardiovascular disease and complications during pregnancy,

has been reported^{2, 3)}. Periodontopathic bacteria, such as *Porphyromonas gingivalis*, *Prevotella intermedia*, *Tannerella forsythia*, *Aggregatibacter actinomycetemcomitans*, and *Treponema denticola* are detected in high frequency in subgingival dental plaque⁴⁾. *Fusobacterium nucleatum*, which is also the most abundant Gram-negative organism in dental plaques and closely associates with periodontal pathogens,

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plays a central role as physical bridges to mediate the co-aggregation of bacterial cells and promotes the anaerobic microenvironment⁴. These facts suggest that suppressing of dental plaque formation and development is important to prevent periodontitis.

Various approaches have been applied to prevent periodontitis effectively. Tooth brushing and tongue scraping mechanically remove microorganisms. Mouthrinses and toothpastes containing antibacterial compounds, such as chlorhexidine (CHX), cetylpyridinium chloride (CPC), and triclosan, are commonly used for preventing the growth of periodontopathic bacteria. However, currently used antibacterial agents induce adverse effects, such as extrinsic brown staining of teeth and restorations, destruction of mucous membranes, burning sensation, and mouth irritation⁵.

Probiotics, live microorganisms that confer health benefits on the host when administered in appropriate amounts, are also natural substances expected to become oral care products⁶. *Lactobacillus reuteri* is used as a probiotic and has been extensively examined for its prevention of oral infections including dental caries⁷, periodontal disease^{8,9}, *Candida*-associated stomatitis¹⁰, and halitosis¹¹. It has been suggested to induce the production of antibacterial substances, such as hydrogen peroxide, acids, and the bacteriocin-like compound reuterin (3-Hydroxypropionaldehyde, 3-HPA)¹², and colonize the oral cavity¹³; however, its mechanisms of action remain unclear. Reuterin is water-soluble, active over a wide pH range, and exhibits antibacterial activity against various Gram-positive and -negative bacteria^{14,15} including enteric pathogens¹⁶. It is not yet known whether reuterin is effective against oral infections because it is rapidly converted into various compounds including HPA hydrate, HPA dimer, and acrolein. Kang tried to chemically synthesize reuterin, however, reported that there were difficulties associated with obtaining reuterin due to the general lability of its aldehyde moiety¹⁷. In the present study, hence, various stable reuterin-related compounds (RRCs) including bisulfite adducts, oximes, and hydrazones were chemically synthesized. We hypothesized that these RRCs might have inhibitory effects on periodontopathic bacteria since *L. reuteri* has been reported to have preventive effects on periodontal disease. To prove this hypothesis, antibacterial and antibiofilm activities of these RRCs against oral bacteria, particularly periodontopathic bacteria and cytotoxicity to human cells were evaluated.

Materials and Methods

Synthesis of Reuterin-Related-Compounds (RRCs)

The structures of reuterin and all synthesized RRCs are shown in Figures 1a, 1c and 1e. Sodium 1,3-dihydroxypropane-1-sulfonate (RRC-01) was obtained

as follows: A solution of acrolein (2 mL, 30.0 mmol) and 1.5 M sulfuric acid (2.6 mL) in H₂O (8.6 mL) was stirred at 50 °C for 1.5 h. The reaction mixture was cooled to 0 °C and pH was adjusted to 6.8 by adding solid potassium carbonate. Ethanol (6 mL) and sodium metabisulfite (3.26 g, 17.2 mmol) were added and stirred at room temperature for 2 h. The reaction mixture was cooled to 0 °C, filtered, and concentrated *in vacuo*. The residue was suspended in methanol, filtered, and concentrated *in vacuo* to afford RRC-01 (447 mg, 8 %) as a white solid. The synthetic pathway of RRC-01 is shown in Figure 1b. The chemical structure of RRC-01 was determined by spectroscopic analysis (Figure 2a). The other bisulfite adducts (RRC-02 and -03) were obtained from the corresponding aldehydes in a similar manner.

3-(Benzyloxy)propanal oxime (RRC-04) was obtained as follows: A solution of hydroxylamine hydrochloride (397 mg, 5.71 mmol) and sodium acetate (468.8 mg, 5.71 mmol) in H₂O (1.5 mL) was added to a solution of 3-(benzyloxy)propanal (555 mg, 3.38 mmol) in ethanol (12 mL). The reaction mixture was stirred at room temperature for 2 h, and concentrated *in vacuo*. The residue was extracted with chloroform. The extract was dried over anhydrous magnesium sulfate and concentrated *in vacuo* to afford RRC-04 (603.6 mg, 99 %) as a white solid. The synthetic pathway of RRC-04 is shown in Figure 1d. The chemical structure of RRC-04 was determined by spectroscopic analysis (Figure 2b). Other derivatives (RRC-05, -06, -07, -08, -09, -10, -11, -12, -13, and -14) were obtained from the corresponding aldehydes and amines (hydroxylamine, *O*-benzylhydroxylamine, semicarbazide, and thiosemicarbazide) in a similar manner. The bisulfite adduct of aldehyde is typically crystalline and regenerates the aldehyde by hydrolysis in aqueous solution. Bisulfite adducts (RRC-01, -02, and -03) were dissolved in distilled H₂O, oxime (RRC-04, -05, -06, -07, -08) and hydrazone derivatives (RRC-09, -10, -11, -12, -13, and -14) were dissolved in dimethyl sulfoxide (DMSO).

Bacterial strains, culture media, and culture conditions

The bacterial strains used, their culture media, and culture conditions are shown in Table 1. Trypticase soy broth (TSB, Becton Dickinson, Sparks, MD, USA) supplemented with hemin (5 µg/mL) and menadione (0.5 µg/mL) was used in the biofilm formation assay. All bacteria were grown at 37 °C.

Antibacterial activity assay

The minimum inhibitory concentrations (MIC) of RRCs were assessed using a microbroth dilution method. Approximately 1 × 10⁶ colony forming unit (CFU) of a bacterial culture were inoculated into 100 µL of medium containing a two-fold serial dilution of RRCs in a 96-well

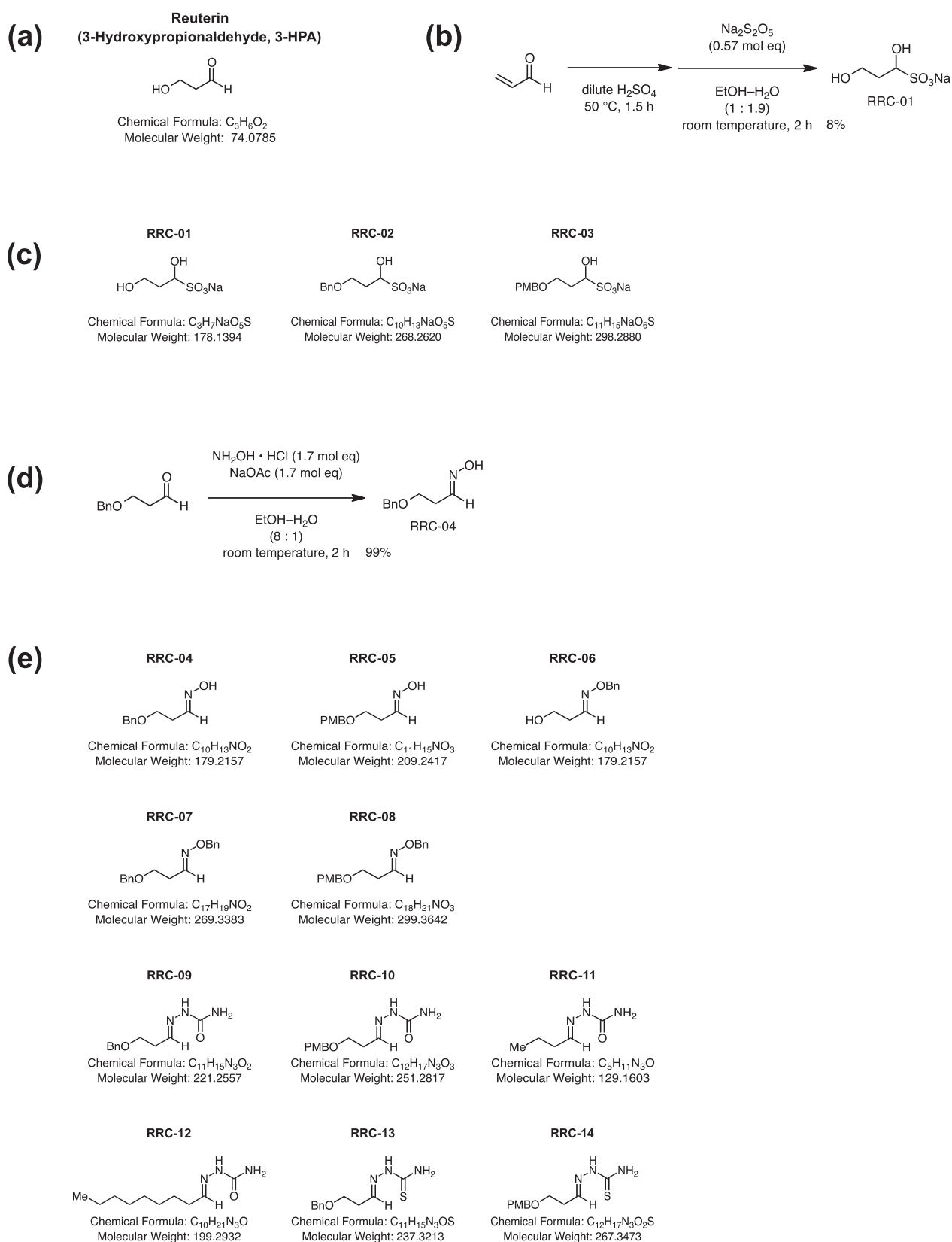


Fig. 1 Structures of reuterin and reuterin-related compounds (RRCs).

(a) Structure of reuterin. (b) The synthetic pathway of RRC-01. (c) Structures of bisulfite adducts (RRC-01, -02 and -03). (d) The synthetic pathway of RRC-04 (e) Structures of oximes (RRC-04, -05, -06, -07 and -08) and hydrazones (RRC-09, -10, -11, -12, -13 and -14).

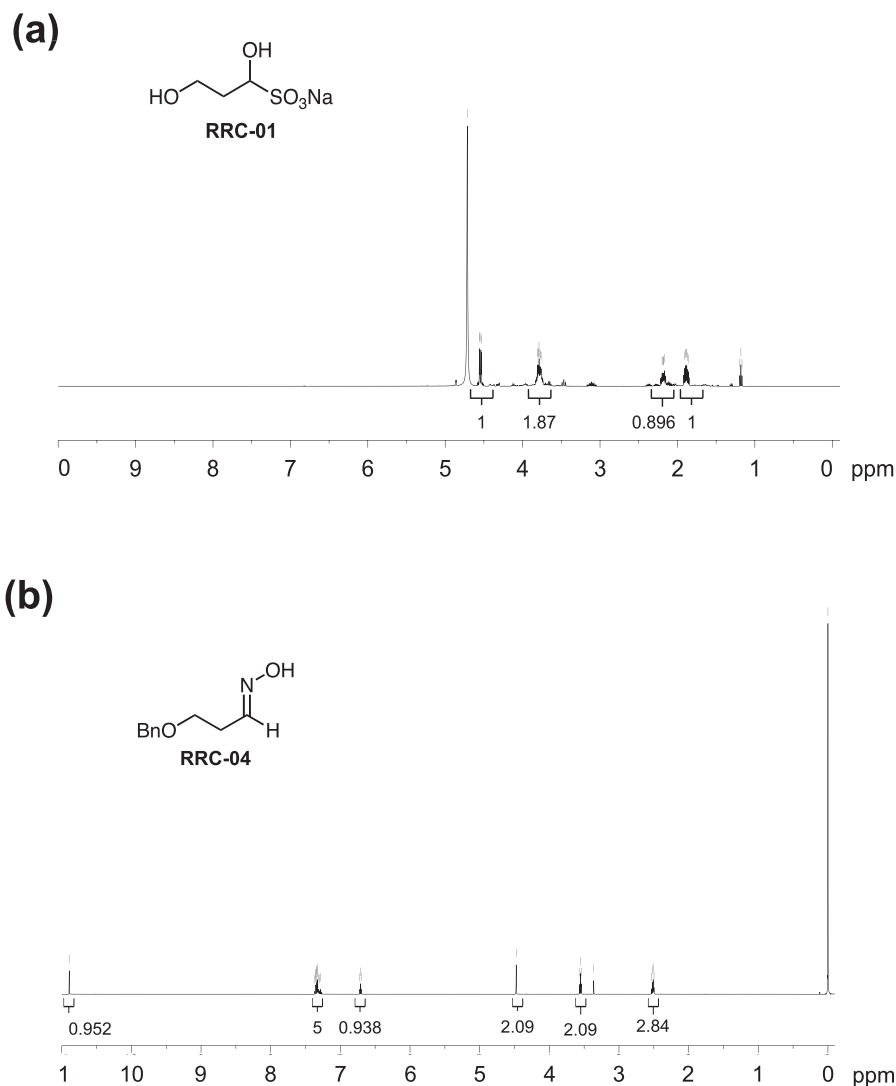


Fig. 2 $^1\text{H-NMR}$ spectra of RRC-01 (a), and RRC-04 (b).

culture plate (#92906, TPP, Trasadingen, Switzerland). *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Escherichia coli*, and *Candida albicans* were aerobically incubated for 20 h. *Streptococcus mutans*, *A. actinomycetemcomitans*, and *P. intermedia* were anaerobically incubated for 24 h. *F. nucleatum* and *P. gingivalis* were anaerobically incubated for 48 h. MICs were defined as the lowest concentration showing no bacterial growth.

Biofilm formation assay

A crystal violet biofilm formation assay was performed as previously described¹⁸. A total of 5×10^6 CFU of *F. nucleatum* were inoculated into 150 μL of broth containing each RRC (32 or 16 $\mu\text{g}/\text{mL}$) in a 96-well plate (#650185, CellStar, Greiner-bio-one, Frickenhausen, Germany). Bacteria were anaerobically incubated at 37 $^\circ\text{C}$ for 24 h. After incubation,

the biofilms formed were washed with purified H_2O twice and stained with 150 μL of 0.1 % crystal violet at room temperature for 10 min. After washing, the stained biofilm was extracted with 150 μL of ethanol, and the absorbance of the extracts was measured at 595 nm using a microplate reader (model 680; Bio-Rad Laboratories, Hercules, CA, USA). The number of bacterial cells was also measured by using an ATP assay (BacTiter-GloTM Microbial Viability Assay, Promega, Madison, WI, USA). This assay verifies the number of bacterial cells by a luminescent signal, which is proportional to the amount of ATP in bacterial cells. ATP assay can be used for reliably detecting and quantifying bacterial cell numbers^{19,20}.

Cytotoxicity assay

The cytotoxicity of RRCs was evaluated using a lactate

Table 1 Bacterial strains, culture media and conditions in this study

Bacterial strain	Source	Culture medium and culture condition
<i>S. aureus</i> (MSSA) BCL1	Clinical isolates	Muller-Hinton Broth (MHB, Becton Dickinson, Sparks, MD, USA) supplemented with 25 µg/mL CaCl ₂ , 12.5 µg/mL MgCl ₂ and 2 % NaCl, cultured aerobically
<i>S. aureus</i> (MRSA) BCL2	Clinical isolates	MHB supplemented with 25 µg/mL CaCl ₂ , 12.5 µg/mL MgCl ₂ and 2 % NaCl, cultured aerobically
<i>Candida albicans</i> CAD1	Clinical isolates	Sabouraud dextrose medium (Nissui, Tokyo, Japan) composed of 10 g/L peptone and 40 g/L glucose, cultured aerobically
<i>E. coli</i> ATCC23053	Type strain	MHB supplemented with 50 µg/mL CaCl ₂ , 25 µg/mL MgCl ₂ , cultured aerobically
<i>P. aeruginosa</i> PAO1	Type strain	MHB supplemented with 50 µg/mL CaCl ₂ , 25 µg/mL MgCl ₂ , cultured aerobically
<i>S. mutans</i> MT8148	Clinical isolates	Brain Heart Infusion (BHI, Becton Dickinson), cultured anaerobically
<i>P. intermedia</i> 163	Clinical isolates	Todd Hewitt Broth (THB, Becton Dickinson) supplemented with 5 µg/mL hemin, 0.5 µg/mL menadione and 1 % yeast extract, cultured anaerobically
<i>A. actinomycetemcomitans</i> Y4	Type strain	THB supplemented with 5 µg/mL hemin, 0.5 µg/mL menadione and 1 % yeast extract, cultured anaerobically
<i>P. gingivalis</i> ATCC33277	Type strain	BHI supplemented with 5 µg/mL hemin and 0.5 µg/mL menadione, cultured anaerobically
<i>F. nucleatum</i> JCM8532	Type strain	BHI supplemented with 5 µg/mL hemin and 0.5 µg/mL menadione, cultured anaerobically

dehydrogenase (LDH) assay. RT-7, an immortalized human oral keratinocyte cell line, was cultured in keratinocyte-SFM (Gibco BRL, Gaithersburg, MD, USA) as described previously²¹). Confluent RT-7 cell monolayers in a 24-well plate were cultured with RRCs (100 µg/mL) at 37 °C for 24 h. As a positive control, RT-7 cells were treated with 0.1 % Triton X-100 at room temperature for 10 min. As commercial disinfectants, 0.01 % CPC and 0.01 % CHX were employed. LDH released into the culture medium was measured using a LDH cytotoxicity assay kit (Cayman Chemical, Ann Arbor, MI, USA) according to the manufacturer's instructions. Absorbance was measured at 490 nm by a microplate reader (Infinite® 200 PRO, Tecan, Männedorf, Switzerland).

Statistical analysis

All experiments were conducted in triplicate and statistical

analyses were performed using the unpaired Student's *t*-test. Differences were considered significant when probability values were less than 5 %.

Results

Antibacterial activities of RRCs

Reuterin produced by *L. reuteri* is extremely unstable due to the general lability of its aldehyde moiety. Fourteen stable RRCs instead of reuterin, therefore, were chemically synthesized by replacement of the aldehyde moiety with bisulfite adducts, oximes, or hydrazones via the shown pathway to improve the lability (Figures 1b and 1d). Although the structure of reuterin was difficult to be identified by ¹H NMR analysis¹⁷), the chemical structures of RRCs were confirmed by spectroscopic analysis including ¹H NMR analysis (Figures 1c, 1e, 2a, and 2b), indicating that these

Table 2 Antibacterial activity of RRCs

RRCs	<i>E. coli</i>	<i>P. aeruginosa</i>	<i>C. albicans</i>	<i>S. aureus</i> (MRSA)	<i>S. aureus</i> (MSSA)	<i>E. coli</i>
01	>512	>512	>512	>512	>512	>512
02	>512	>512	>512	>512	>512	>512
03	>512	>512	>512	>512	>512	>512
04	>512	>512	>512	>512	>512	>512
05	>512	>512	>512	>512	>512	>512
06	>512	>512	>512	>512	>512	>512
07	>512	>512	>512	>512	>512	>512
08	>512	>512	>512	>512	>512	>512
09	>512	>512	>512	>512	>512	>512
10	>512	>512	>512	>512	>512	>512
11	>512	>512	>512	>512	>512	>512
12	>512	>512	256	512	512	>512
13	>512	>512	>512	>512	>512	>512
14	>512	>512	>512	>512	>512	>512

RRCs	<i>S. mutans</i>	<i>A. actinomycetemcomitans</i>	<i>F. nucleatum</i>	<i>P. gingivalis</i>	<i>P. intermedia</i>
01	>512	>512	>512	>512	>512
02	>512	>512	>512	>512	>512
03	>512	>512	>512	>512	>512
04	>512	128	256	128	64
05	>512	256	256	128	64
06	>512	256	512	256	256
07	>512	>512	>512	>512	>512
08	>512	>512	>512	>512	>512
09	>512	128	128	128	32
10	>512	256	256	128	32
11	>512	512	>512	>512	512
12	>512	>512	128	256	64
13	256	128	128	64	16
14	256	128	128	64	32

(µg/mL)

RRCs are stable. The antibacterial activities of RRCs against various bacteria are shown in Table 2. Most RRCs exhibited no antibacterial activities against general pathogenic microorganisms, such as *E. coli*, *P. aeruginosa*, *C. albicans*, and *S. aureus* (methicillin-resistant *S. aureus*; MRSA and methicillin-sensitive *S. aureus*; MSSA). RRC-12, however, showed antibacterial activity against *C. albicans* (256 µg/mL) as well as MRSA and MSSA (512 µg/mL). RRC-04, -05, -06, -09, -10, -12, -13, and -14 exhibited antibacterial activities against periodontopathic bacteria, including *A.*

actinomycetemcomitans (128-256 µg/mL), *F. nucleatum* (128-512 µg/mL), *P. gingivalis* (64-256 µg/mL), and *P. intermedia* (16-256 µg/mL). RRC-13 and -14 were active against the cariogenic bacterium, *S. mutans* (256 µg/mL). RRC-01, -02, -03, -07, -08, and -11 had no antibacterial activity against any of the bacteria tested.

Effects of RRCs on biofilm formation by *F. nucleatum*

The effects of RRCs on biofilm formation by *F. nucleatum*, a key bacterium in dental plaque formation, were investigated.

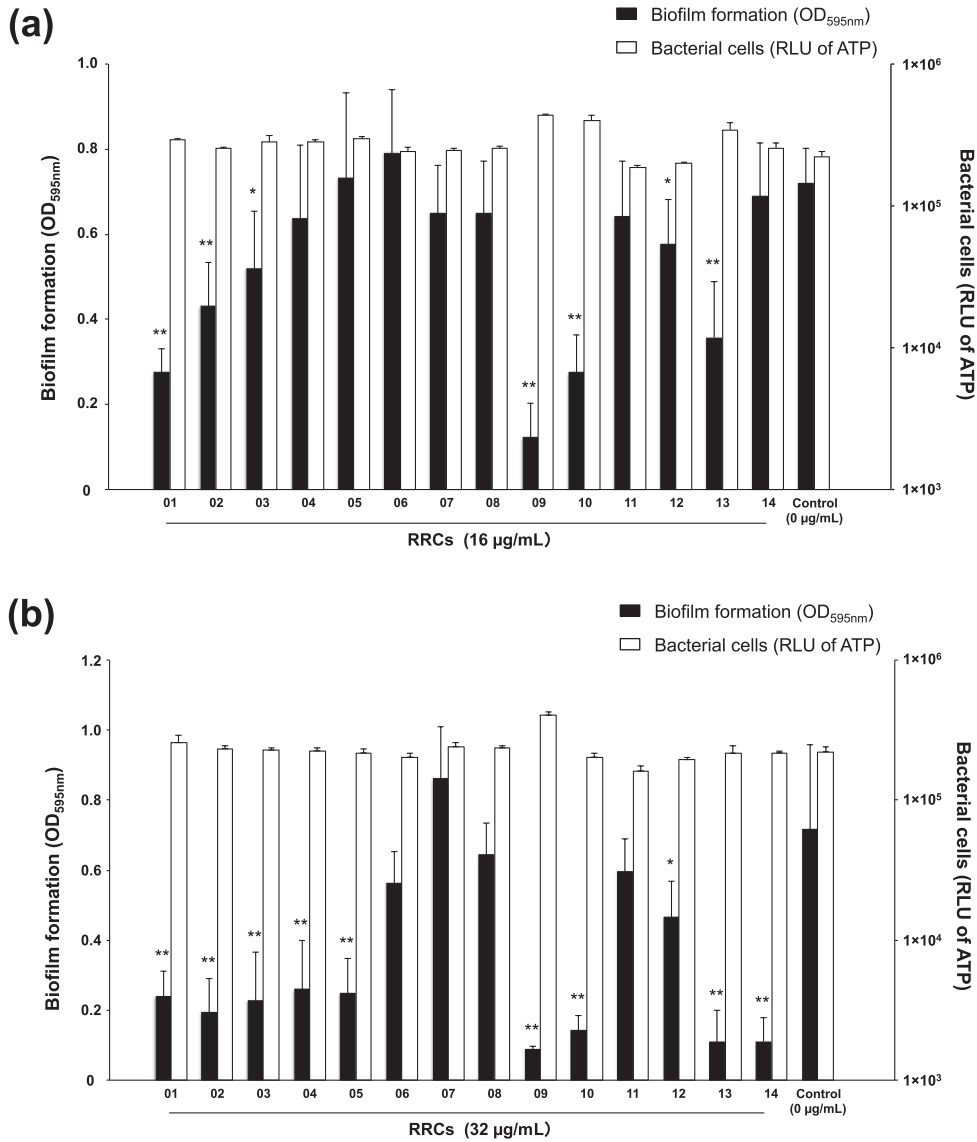


Fig. 3 Effects of RRCs on biofilm formation by *F. nucleatum*.

F. nucleatum (5×10^6 CFU/mL) was incubated anaerobically with each RRC in a 96-well plate at 37 °C for 24 h. After incubation, the biofilms that formed were stained with crystal violet and the absorbances of the ethanol extracts of these biofilms were measured. The cell number of *F. nucleatum* was examined using an ATP assay. The relative luminescence unit was represented as RLU. Effects of each RRC treatment at 16 (a) and 32 µg/mL (b) on biofilm formation are shown. Data represent the mean ± SD of 3 independent experiments. * $p < 0.05$ and ** $p < 0.01$ significantly different from the control (without RRCs).

Biofilm formation and the amount of ATP, reflecting bacterial numbers, after a 24-h culture are shown in Figure 3. The growth of *F. nucleatum* was similar in the presence (16 and 32 µg/mL) and absence of RRCs. RRC-01, -09, and -10 at 16 µg/mL significantly suppressed biofilm formation to 60-70 % of the control (Figure 3a). RRC-01, -02, -03, -04, -05, -09, -10, -13, or -14 at 32 µg/mL significantly suppressed biofilm formation to 70-80 % of the control (Figure 3b).

Cytotoxicity of RRCs against human oral keratinocytes

The cytotoxicity of RRCs (100 µg/mL) was examined against human oral keratinocytes by measuring LDH release from RT-7 cells. RRC-01, -02, and -03 were not cytotoxic (Figure 4). RRC-12 released approximately two-fold more LDH than the positive control (Triton X-100) and RRC-12 and -13 released significantly more LDH than DMSO. RRC-04, -05, -06, -07, -08, -09, -10, and -11 released similar amounts of LDH to 0.01 % CHX or 0.01 % CPC.

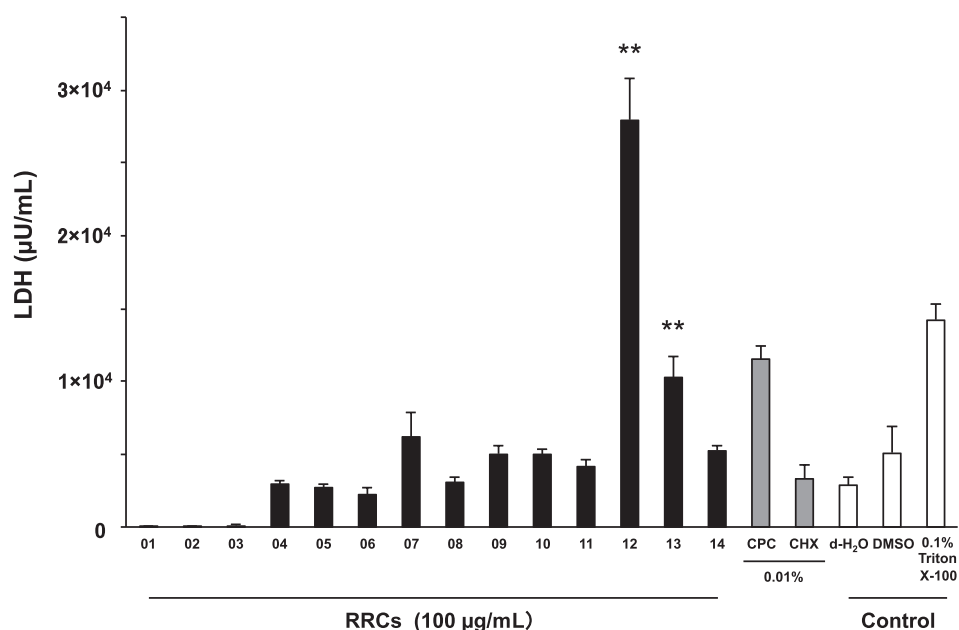


Fig. 4 Cytotoxicity of RRCs against human oral keratinocytes.

Human oral keratinocytes, RT-7 cells, were treated with RRCs (100 µg/mL). Cytotoxicity was assessed by a lactate dehydrogenase (LDH) assay. As a positive control, RT-7 cells were treated with 0.1 % Triton X-100 at room temperature for 10 min. As a negative control, RT-7 cells were treated with distilled H₂O or DMSO. As commercial disinfectants, 0.01 % cetylpyridinium chloride (CPC) and chlorhexidine (CHX) were used. Data represent the mean ± SD of 3 independent experiments. ** $p < 0.01$ significantly different from the control (DMSO).

Discussion

L. reuteri is one of the probiotics that possesses preventive effects on oral infections including dental caries and periodontal disease in clinical experiments⁷⁻⁹. These reports showed improving of periodontal status such as probing depth and gingival index through the effects of *L. reuteri* on decreased number of cariogenic and periodontopathic bacteria. In these reports, they did not examine the colonization of *L. reuteri* in the oral cavity. The difficulty of colonization of *L. reuteri* in the oral cavity was pointed out by the previous findings; i) colonization of *L. reuteri* was not observed in saliva and subgingival pockets after administration of tablets containing *L. reuteri*¹³, and ii) the uptake of *L. reuteri* for 2 weeks was insufficient for the permanent colonization of *L. reuteri* in the oral cavity²². These cumulating findings suggest that the effects of *L. reuteri* for preventing oral infections may be independent on the colonization of *L. reuteri* in the oral cavity. *L. reuteri* produces antibacterial substances such as hydrogen peroxide, acids, and the bacteriocin-like compound reuterin¹². Among the antibacterial substances from *L. reuteri*, reuterin exhibits antibacterial activity against various Gram-positive and -negative bacteria^{14, 15} including enteric pathogens¹⁶, suggesting that reuterin could play an important role in the probiotic effects of *L. reuteri*. Reuterin is, however,

an intermediate metabolite that is rapidly converted into various compounds including HPA hydrate, HPA dimer, and acrolein. Chemically synthesized reuterin seems to be unstable because of the general lability of its aldehyde moiety¹⁷. Although we also tried to synthesize reuterin, it was unstable and easily degraded (data not shown). These findings suggest that there were difficulties associated with clinical applications of chemical synthesized reuterin. In the present study, fourteen stable RRCs instead of reuterin were chemically synthesized by replacing the aldehyde moiety with bisulfite adducts, oximes, or hydrazones to improve the lability. Although the structure of reuterin was difficult to be identified by ¹H NMR analysis¹⁷, the chemical structures of RRCs were clearly identified (Figures 2a and 2b), indicating that these RRCs are stable. The antibacterial activities of RRCs against periodontal pathogens were retained in stable storage (-20 °C) for several months (data not shown). The RRCs were composed of novel compounds (RRC-01, -02, -03, -05, -08, -10 and -14) and chemically-defined compounds (RRC-04, -06, -07, -09, -11, -12 and -13)^{23, 24} (Figures 1c and 1e). This is the first report showing the effects of RRCs against periodontopathic bacteria.

Microorganisms often form biofilms that are resistant to antibacterials and disinfectants and cause infectious

diseases²⁵). Fusobacteria play a central role as physical bridges to mediate the co-aggregation of bacterial cells and promote the anaerobic microenvironment. *F. nucleatum* is also the most abundant and a key bacteria in dental plaque formation and closely associates with other periodontal pathogens⁴). Therefore, the inhibition of biofilm formation by *F. nucleatum* is an effective strategy for preventing severe periodontal diseases. The biofilm formation of *F. nucleatum* was significantly suppressed by RRCs in the present study. RRCs suppressed biofilm formation by *F. nucleatum* without affecting bacterial cell numbers (Figure 3). The number of bacterial cells, including both planktonic cells in the medium and cells consisting of the biofilm, were measured by ATP assay. It is unclear how RRCs suppressed biofilm formation without affecting bacteria growth.

It is known that the factors, such as the attachment of bacteria to a surface, the exopolysaccharide (EPS) production and Quorum sensing (QS) system are involved in biofilm formation. In *F. nucleatum*, the mechanisms of the attachment and the EPS production are still unclear. QS system is famous as the regulator of biofilm formation without affecting bacterial growth in some bacteria²⁶). QS system is a bacterial cell-to-cell communication process that depends on bacterial population density and uses the signaling molecules called autoinducer such as *N*-acyl homoserine lacton²⁷). In *P. aeruginosa*, QS inhibitor was reported to reduce biofilm formation without affecting bacterial growth²⁸⁻³⁰). Asahi *et al.* demonstrated that an *N*-acyl homoserine lacton analog significantly inhibits biofilm formation of *P. gingivalis* at the concentration, which did not affect cell growth³¹). It was reported that autoinducer 2 (AI-2) plays important role in inter- and intraspecies interactions and biofilm formation, however, *F. nucleatum* biofilm formation is not affected by AI-2 inhibitors^{32, 33}). In *F. nucleatum*, the relationship between QS and biofilm formation is still unclear. The RRCs, thus, may inhibit biofilm formation of *F. nucleatum* by some unknown mechanisms.

In order to apply novel synthesized substances to clinical practice, consideration of their safety for humans is needed. As shown in Figure 4, most RRCs, except for RRC-12 and -13, were not cytotoxic to human oral keratinocytes. RRC-01, -02, and -03 were water-soluble and showed extremely low cytotoxicity, suggesting that they are appropriate compounds for oral care from the viewpoint of their safe usability.

In conclusion, we synthesized RRCs that exhibited antibacterial activity against periodontopathic bacteria and antibiofilm activity against *F. nucleatum*. It should be noted that most RRCs were not cytotoxic to human oral keratinocytes. RRCs, therefore, can be potent oral care products for the prevention of periodontal disease

without adverse effects. To apply RRCs for clinical use, the mechanisms of RRCs on antibacterial and antibiofilm activities against other oral pathogenic microorganisms should be clarified, and clinical trials should be performed.

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