Archives of Oral Biology 98 (2019) 243–247

Contents lists available at ScienceDirect

Archives of Oral Biology

journal homepage: www.elsevier.com/locate/archoralbio

Effects of *Lactobacillus salivarius* WB21 combined with green tea catechins on dental caries, periodontitis, and oral malodor

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ARTICLE INFO

Keywords:
Dental caries
(–)-Epigallocatechin gallate
Lactobacillus salivarius
Oral malodor
Periodontitis

ABSTRACT

Objective: To evaluate the combined use of *Lactobacillus salivarius* WB21 and (–)-epigallocatechin gallate (EGCg) for oral health maintenance.

Design: The effects of *L. salivarius* WB21 on growth of *Streptococcus mutans*, the insoluble glucan produced by *S. mutans*, and on growth of *Porphyromonas gingivalis* were evaluated *in vitro*. In addition, the susceptibility of five oral pathogenic bacteria and *L. salivarius* WB21 to EGCg, the inhibiting effect of EGCg on methyl mercaptan, and the effects of *L. salivarius* WB21 and EGCg in combination on growth of *P. gingivalis* were examined.

Results: *Lactobacillus salivarius* WB21 showed concentration-dependent inhibition of the growth of *S. mutans*. Addition of *L. salivarius* WB21 inhibited production of the insoluble glucan by *S. mutans* ($p < 0.001$). A filtrate of *L. salivarius* WB21 culture solution inhibited growth of *P. gingivalis* ($p < 0.001$ *vs.* control), and this effect was enhanced when it was used in combination with EGCg ($p < 0.001$ *vs.* the addition of *L. salivarius* WB21). In addition, EGCg directly inhibited methyl mercaptan in a concentration-dependent manner ($p < 0.001$). Concerning bacterial susceptibility to EGCg, growth of *P. gingivalis*, *Prevotella intermedia*, and *Fusobacterium nucleatum* was inhibited at 2.5 mg/mL of EGCg, while that of *L. salivarius* WB21 was inhibited at 25 mg/mL EGCg.

Conclusions: Our results imply that *L. salivarius* WB21 may be useful for controlling dental caries, periodontitis, and oral malodor. In addition, the effects of *L. salivarius* WB21 on periodontitis and oral malodor may be synergistically enhanced by use in combination with EGCg.

1. Introduction

Major diseases of the oral cavity, such as dental caries, gingivitis, periodontitis, and oral malodor, are caused by dental plaque or tongue coating, which are recognized as oral biofilms. Therefore, continuous and regular disruption of these biofilms is imperative for prevention and management of oral diseases. Mechanical plaque control using a toothbrush, interdental brush, dental floss, or tongue scraper is the first line of prevention. Chemical plaque control, such as mouthrinse and dentifrice, used in addition to mechanical oral hygiene procedures, is helpful in reducing oral infectious diseases. Many studies have supported significant plaque reduction by the use of chemical plaque control measures (Fedorowicz, Aljufairi, Nasser, Outhouse, & Pedrazzi, 2008; Figuero et al., 2017). However, the side effects and safety of these measures are often of concern. For example, chlorhexidine, the bactericidal agent that has been most studied and is recognized as the most effective for inhibition of plaque and prevention of gingivitis, periodontitis, and oral malodor, has several adverse effects, including extrinsic tooth staining, calculus build up, transient taste disturbance, and effects on the oral mucosa (Gürgan, Zaim, Bakirsoy, & Soykan, 2006; James et al., 2017). In addition, it has recently been suggested that triclosan may be hazardous to human health. The US Food and Drug Administration (FDA) named triclosan for toxicological evaluation in the National Toxicology Program in 2008. Although toothpaste and

Abbreviation: EGCg, (–)-epigallocatechin gallate

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https://doi.org/10.1016/j.archoralbio.2018.11.027
Received 26 July 2018; Received in revised form 27 November 2018; Accepted 27 November 2018

mouthwash are considered separately, the FDA decided to recommend changing consumer use of triclosan-containing body-wash products due to insufficient safety evidence (FDA, 2016).

Biological plaque control using living microorganisms, or probiotics, has been proposed as an alternative to chemical plaque control. The mechanism of this procedure is not bactericidal activity, but by antibacterial activity and additional functions such as changes in immune function, competition for nutrients, and alternation of environmental conditions in the mouth (Gungor, Kirzioglu, & Kivanc, 2015). Probiotics have traditionally been used to treat diseases related to the gastrointestinal tract, and recently investigation of the effects of probiotic bacteria on oral health has become an important research subject (Gungor et al., 2015). Several clinical trials have reported that regular consumption of *Lactobacillus salivarius* WB21 reduces periodontitis and oral malodor (Iwamoto, Suzuki, Tanabe, Takeshita, & Hirofuji, 2010; Shimauchi et al., 2008; Suzuki et al., 2012, 2014). A randomized clinical trial using *L. salivarius* WB21-containing oil drops in patients with periodontal disease for 2 weeks (Suzuki et al., 2012) showed a significant reduction in bleeding on probing in the experimental group compared to the placebo group ($p < 0.01$). A 14-day, double-blind, placebo-controlled, randomized crossover trial of tablets containing *L. salivarius* WB21 in patients with oral malodor showed a significant reduction in the concentration of volatile sulfur compounds ($p = 0.019$) and the average probing pocket depth ($p = 0.001$) in the probiotic period compared to the placebo period (Suzuki et al., 2014). A quantitative analysis found significantly lower levels of ubiquitous bacteria ($p = 0.003$) and *Fusobacterium nucleatum* ($p = 0.020$) in the probiotic period. Concerning dental caries, the oral intake of *L. salivarius* WB21-containing tablets decreased levels of mutans streptococci compared to placebo tablets, and did not affect other caries risk factors, including salivary pH, salivary flow, and buffering capacity (Nishihara, Suzuki, Yoneda, & Hirofuji, 2014). Thus, clinical trials have revealed the benefits of *L. salivarius* WB21 in oral health control, although the functions of this organism *in vitro* have not yet been clarified.

One difference between strategies using probiotic bacteria and those using bactericidal agents may be the time required to obtain beneficial effects or subjective improvement of clinical conditions. Green tea, a traditional drink in Japan and China, has been recognized as healthful and functional food. The tea catechin (–)-epigallocatechin gallate (EGCg) exhibits antibacterial and deodorizing activities (Fournier-Larente, Morin, & Grenier, 2016; Yasuda & Arakawa, 1995). Combination of catechin and lactic acid bacteria should result in both a probiotic effect on the oral environment and rapid inhibition of oral malodor, if the concentration of catechin that kills pathogenic bacteria without killing lactic acid bacteria can be determined.

In this study, the potential actions of *L. salivarius* WB21 on dental caries, periodontal disease, and oral malodor were evaluated *in vitro*. In addition, the effect of *L. salivarius* WB21 and catechin in combination on the growth of *Porphyromonas gingivalis* was evaluated.

2. Materials and methods

2.1. Bacterial strains

Lactobacillus salivarius strain WB21 was provided by Wakamoto Pharmaceutical Co. *Streptococcus mutans* JCM 5705 and MT8148 (JCM 5175), *Porphyromonas gingivalis* JCM 8525, *Fusobacterium nucleatum* JCM 8532 and *Aggregatibacter actinomycetemcomitans* JCM 8577 were provided by the RIKEN BRC through the National Bio-Resource Project of the Ministry of Education, Culture, Sports, Science and Technology/Agency for Medical Research and Development, Japan.

2.2. Inhibition of S. mutans growth by L salivarius WB21

To evaluate inhibition of the growth of *S. mutans* JCM 5705 and MT 8148 by *L. salivarius* WB21, bacteria were co-cultivated anaerobically using 10 mL fresh GAM broth (Nissui, Tokyo, Japan) supplemented with 0.7% glucose-containing suspensions of *S. mutans* (10^7 CFU/mL) and *L. salivarius* WB21 (0, 10^1, or 10^3 CFU/mL) at 37 °C. Counts of *S. mutans* in the culture medium were determined on GAM agar plates supplemented with 10 μg/mL bacitracin at 16, 24, and 40 h.

2.3. Inhibition of the insoluble glucan produced by S. mutans by L. salivarius WB21

Inhibition of the insoluble glucan produced by *S. mutans* JCM 5705 and MT 8148 by *L. salivarius* WB21 was evaluated. *S. mutans* (10^7 CFU/mL) and L. *salivarius* WB21 (0 or 10^7 CFU/mL) were cultured in 5 mL fresh GAM broth supplemented with 2.0% sucrose at 37 °C for 24 h at a 30° angle. After incubation, the culture medium was centrifuged at 1570 × *g* for 20 min and the supernatant was discarded. The pellet was washed twice with 5 mL phosphate-buffered saline (PBS) followed by centrifugation at 1570 × *g* at 4 °C for 20 min. Subsequently, 5 mL 1 N NaOH was added to the pellet for suspension, and the insoluble glucan was obtained as a supernatant by centrifugation (1570 × *g* at 4 °C for 20 min). The amounts of each fraction of insoluble glucan were measured by absorbance at 492 nm using the phenol-sulfuric acid method (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956). Each sample (200 μL) was reacted with 5% phenol (200 μL) and sulfuric acid (1 mL) for 20 min at room temperature. Glucose dilution (0–200 μg/mL) was used to plot a standard curve.

2.4. Susceptibility of oral pathogenic bacteria and L. salivarius WB21 to EGCg

The susceptibility of *P. gingivalis* ATCC 33277, *Prevotella intermedia* ATCC 25611, *F. nucleatum* JCM 8532, *A. actinomycetemcomitans* JCM 8577, *S. mutans* JCM 5705, and *L. salivarius* WB21 to EGCg (Sunphenon EGCg-OP, Taiyo Kagaku Co., Tokyo, Japan) was examined. GAM broth supplemented with hemin (5 μg/mL) and vitamin K (1 μg/mL) was prepared to test the susceptibility of *P. gingivalis* to EGCg. In contrast, GAM broth supplemented with 0.7% glucose was prepared to test the susceptibility of *L. salivarius* WB21 to EGCg. Unsupplemented GAM broth was used to test the susceptibility to EGCg of the other bacteria. The minimum inhibitory concentration was determined by incubating each bacterium in medium with 25, 10, 5, 2.5, 1, 0.5, 0.25, and 0.1 mg/mL EGCg.

2.5. Inhibition of P. gingivalis growth by L. salivarius WB21 and EGCg

P. gingivalis ATCC 33277 and JCM 8525 were cultivated on *Brucella* agar plates (Becton Dickinson, Le Pont-de-Claix, France) supplemented with 5% defibrinated horse blood, hemin (5 μg/mL), and vitamin K (1 μg/mL) at 37 °C anaerobically for 40 h, and bacterial cells were suspended in sterile physiological saline to an optical density at 600 nm (OD_{600}) of 1.0. *L. salivarius* WB21 was cultivated in GAM broth supplemented with 0.7% glucose at 37 °C anaerobically for 24 h. The culture supernatant was collected by centrifugation (10,000 × *g* at 4 °C for 20 min) and sterilized using a sterile membrane filter (pore size, 0.22 μm; Millipore, Billerica, MA, USA). To evaluate the effects of *L. salivarius* WB21 and EGCg on the growth of *P. gingivalis*, the filtrate of the *L. salivarius* WB21 culture solution was added at a final concentration of 50% to the liquid culture medium for *P. gingivalis* (*i.e.*, GAM broth supplemented with 0.7% glucose, 5 μg/mL of hemin, and 1 μg/mL of vitamin K), and EGCg was added to the culture medium at 1 mg/mL. A 0.1-mL *P. gingivalis* cell suspension was incubated for 6 h in 10 mL culture medium containing EGCg, the filtrate of the *L. salivarius* WB21 culture, or a combination of EGCg and the *L. salivarius* WB21 culture filtrate, and viable *P. gingivalis* cells were counted on agar plates.

2.6. Inhibition of methyl mercaptan by EGCg

The reaction mixture consisted of 170 µL 10 ppm methyl mercaptan standard solution (WAKO, Osaka, Japan), 300 µL 0.2 M PBS (pH 7.5), and 30 µL EGCg solution (at final concentrations of 300, 150, 75, or 0 ppm). Following incubation at 37 °C for 10 min, the gas in the headspace was adsorbed to an adsorbent and analyzed using gas chromatography (Agilent 6890/5973 GC/MSD System, Agilent Technologies, Santa Clara, CA, USA).

2.7. Statistical analyses

All examinations were repeated at least three times for reproducibility. The statistical analyses were performed using StatLight (Yukms Co., Ltd., Kawasaki, Japan). Inhibition of the insoluble glucan produced by *S. mutans* was evaluated by Aspin-Welch's *t*-test or Student's *t*-test following the F test. The results of other examinations were evaluated by the Tukey-Kramer test following the Bartlett test. *P*-values < 0.05 were considered statistically significant.

3. Results

3.1. Inhibition of S. mutans growth by L. salivarius WB21

Inhibition of *S. mutans* growth by *L. salivarius* WB21 was concentration-dependent (Fig. 1). The growth of *S. mutans* JCM 5705 was significantly decreased at 16 h in the co-culture media with both 10^1 and 10^3 CFU/mL *L. salivarius* WB21 compared to the negative control ($p < 0.05$ and $p < 0.001$, respectively), and was entirely inhibited at 40 h in the co-culture medium with 10^3 CFU/mL *L. salivarius* WB21 ($p < 0.05$). Although the growth of *S. mutans* MT 8148 in medium containing 10^3 CFU/mL *L. salivarius* WB21 was also entirely inhibited at 40 h ($p < 0.05$), no significant decrease was evident in the co-culture experiment with 10^1 CFU/mL *L. salivarius* WB21.

3.2. Inhibition of the insoluble glucan produced by S. mutans by L. salivarius WB21

The insoluble glucans produced by *S. mutans* JCM 5705 and MT 8148 decreased after co-culture for 24 h with LsWB21 (Fig. 2). There was a significant difference compared to the negative control ($p < 0.001$). Although inhibition of the growth of *S. mutans* at 24 h by *L. salivarius* WB21 was strain-dependent, inhibition of the insoluble glucan of the two strains was similar at 94.2% for *S. mutans* JCM 5705 and 85.4% for *S. mutans* MT 8148.

3.3. Susceptibility of oral pathogenic bacteria and L. salivarius WB21 to EGCg

Table 1 shows the minimum inhibitory concentrations of EGCg against oral pathogenic bacteria and *L. salivarius* WB21. Growth of *P. gingivalis* ATCC 33277, *P. intermedia* ATCC 25611, and *F. nucleatum* JCM 8532 was inhibited by 2.5 mg/mL of EGCg. In contrast, the susceptibility of *L. salivarius* WB21 was the lowest among the organisms examined in this study; growth of *L. salivarius* WB21 was inhibited by 25 mg/mL of EGCg.

3.4. Inhibition of the growth of P. gingivalis by L. salivarius WB21 and EGCg

Growth of *P. gingivalis* ATCC 33277 and JCM 8525 was not significantly inhibited by addition of EGCg to the medium, compared to the negative control (Fig. 3). In contrast, addition of *L. salivarius* WB21 culture solution filtrate to the medium inhibited growth of *P. gingivalis* ATCC 33277 and JCM 8525 by 14.1% and 15.1%, respectively. Furthermore, addition of both *L. salivarius* WB21 and EGCg to the medium resulted in a synergistic action, with growth of the *P. gingivalis* strains inhibited by 30.6% (ATCC 33277) and 23.3% (JCM 8525). These results differ significantly from those obtained using the control condition, EGCg alone, and the *L. salivarius* WB21 culture filtrate alone ($p < 0.001$).

3.5. Inhibition of methyl mercaptan by EGCg

Decreased methyl mercaptan was observed for all EGCg concentrations (75–300 ppm) tested (Fig. 4), and there were significant differences compared to the untreated control ($p < 0.001$).

4. Discussion

Lactobacillus salivarius WB21 inhibited both the growth of *S. mutans* and production of the insoluble glucan generated by *S. mutans*. The growth of two *S. mutans* strains at 40 h was entirely inhibited by co-culture with *L. salivarius* WB21; however, the inhibition rates at 24 h

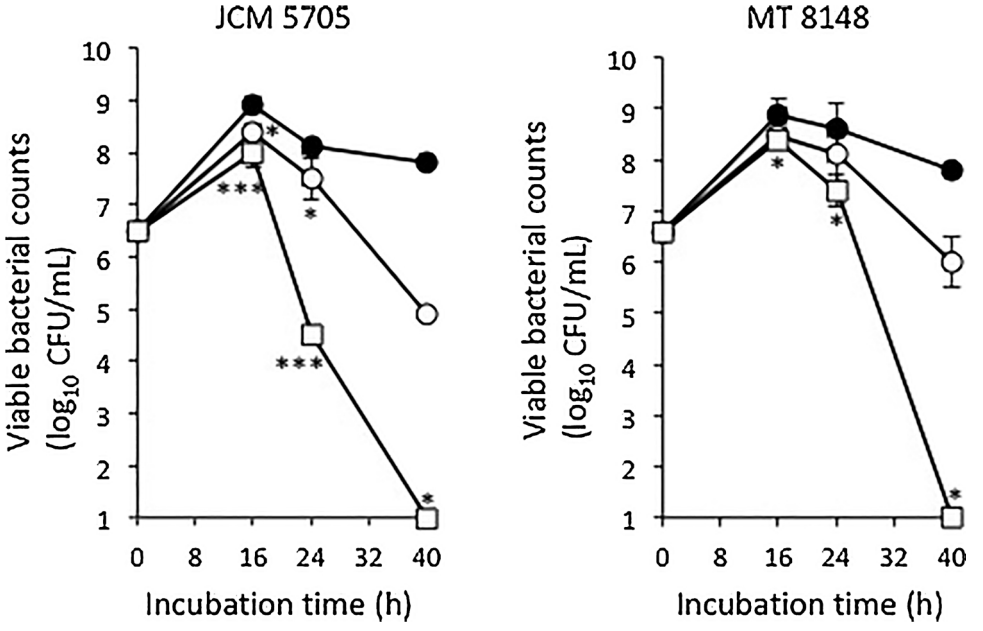

Fig. 1. Viable counts of *Streptococcus mutans* JCM 5705 and MT 8148 in mixed culture with *Lactobacillus salivarius* WB21. ●: number of *S. mutans* in monoculture, ◯ :number of *S. mutans* in mixed culture with 10^1 CFU/mL *L. salivarius* WB21, and □: number of *S. mutans* in mixed culture with 10^3 CFU/mL *L. salivarius* WB21.
*, *** Significant difference compared to monoculture (* $p < 0.05$ and *** $p < 0.001$).

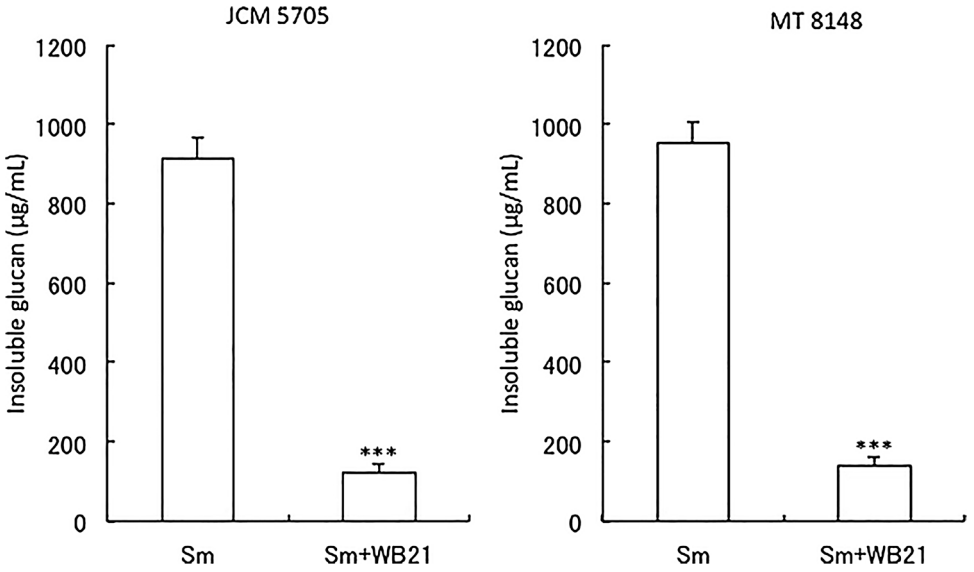

Fig. 2. The insoluble glucan produced by *S. mutans* JCM 5705 and MT 8148 in mixed culture with *L. salivarius* WB21. Sm: *Streptococcus mutans*, WB21: *L. salivarius* WB21.
*** Significant difference compared to monoculture ($p < 0.001$).

Table 1
Minimum inhibitory concentrations of EGCg against oral pathogenic bacteria and *Lactobacillus salivarius* WB21.

Strain	MIC (mg/mL)
Porphyromonas gingivalis ATCC 33277	2.5
Prevotella intermedia ATCC 25611	2.5
Fusobacterium nucleatum JCM 8532	2.5
Aggregatibacter actinomycetemcomitans JCM 8577	10
Streptococcus mutans JCM 5705	5
Lactobacillus salivarius WB21	25

differed between strains JCM 5705 (44.4%) and MT 8148 (14.0%). In contrast, inhibition by *L. salivarius* WB21 of the insoluble glucan produced by *S. mutans* at 24 h was similar at 94.2% (JCM 5705) and 85.4% (MT 8148). These results indicate that *L. salivarius* WB21 has the potential ability to inhibit production of insoluble glucan by *S. mutans*, and that the mechanism is not limited to growth suppression of *S. mutans*. A previous study of *Enterococcus faecium*, a lactic bacterium used as a probiotic in human systemic health, reported its inhibition of biofilm formation by *S. mutans* in a manner independent of growth inhibition of *S. mutans* (Suzuki et al., 2011). Another previous study identified a protein that inhibited streptococci biofilm formation produced by *E. faecium* (Kumada et al., 2009).

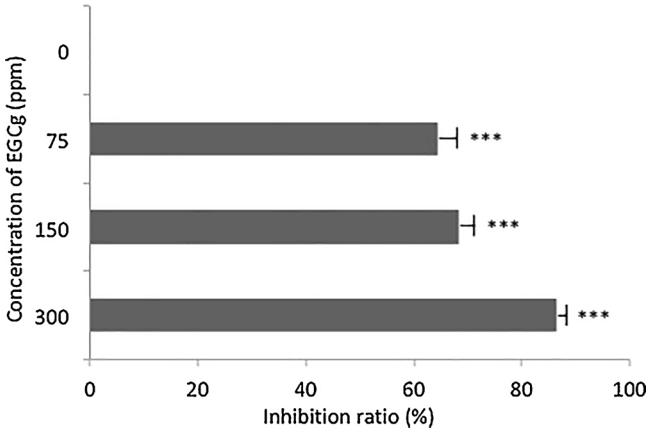

Fig. 4. Inhibition of methyl mercaptan by EGCg.
*** Significant difference compared to the negative control ($p < 0.001$).

Growth of *P. gingivalis* was strongly inhibited by co-culture with *L. salivarius* WB21, and was entirely inhibited at 6 h (data not shown). Similar phenomena were observed in our previous study using *E. faecium* WB2000 (Suzuki et al., 2016). It has been reported that low pH (≤6.0) and the presence of lactic acid (40–50 nmol/L) induce *P.*

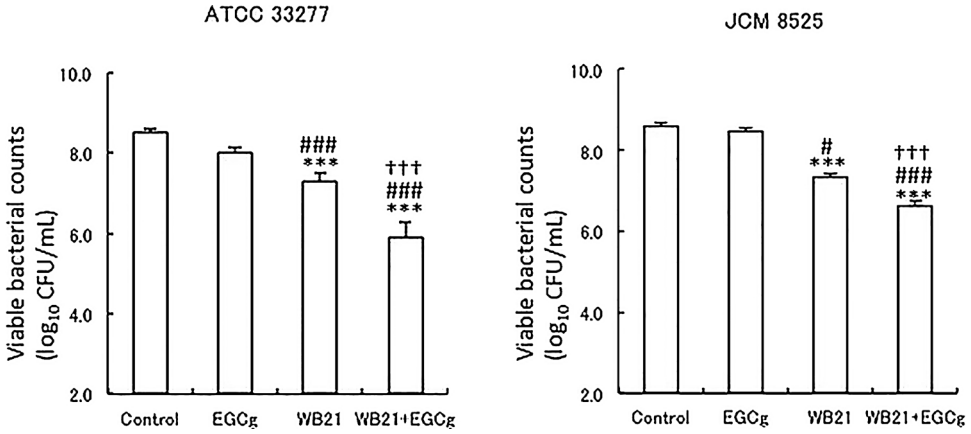

Fig. 3. Viable counts of *Porphyromonas gingivalis* ATCC 33277 and JCM 8525 following incubation with EGCg, the filtrate of *L. salivarius* WB21, and the combination of EGCg and the filtrate of *L. salivarius* WB21.
*** Significant difference compared to the control ($p < 0.001$).
#, ### Significant difference compared to EGCg (# $p < 0.05$ and ### $p < 0.001$).
††† Significant difference compared to the filtrate of *L. salivarius* WB21 ($p < 0.001$).

gingivalis death (Matsuoka, Nakanishi, Aiba, & Koga, 2004). In addition, the high growth rate of *L. salivarius* WB21 may also affect its rapid inhibition of *P. gingivalis* growth. The aim of the present study was to evaluate the effect of *L. salivarius* WB21 and EGCg in combination on the growth of *P. gingivalis*, and therefore both materials had to be prepared under conditions that did not kill *P. gingivalis*. Hence, the filtrate of the *L. salivarius* WB21 culture solution and 1 mg/mL of EGCg, which is below the minimum inhibitory concentration for *P. gingivalis*, were employed in the study. The results reveal a synergistic effect of the combination of *L. salivarius* WB21 and EGCg on the growth of *P. gingivalis*. Tea catechins can irreversibly damage the bacterial cytoplasmic membrane (Ikigai, Nakae, Hara, & Shimamura, 1993). EGCg generates hydrogen peroxide in the lipid layer of the bacterial cytoplasmic membrane, resulting in leakage of intracellular materials (Arakawa, Maeda, Okubo, & Shimamura, 2004). It is possible that EGCg damages the cell membrane of *P. gingivalis*, allowing the components of the *L. salivarius* WB21 culture medium greater opportunity to permeate into *P. gingivalis* cells.

There have been many reports on the antimicrobial and deodorizing actions of catechin (Fournier-Larente et al., 2016; Yasuda & Arakawa, 1995). In this study, we examined the susceptibility of oral pathogenic bacteria and *L. salivarius* WB21 to EGCg, taking into consideration the use of EGCg and *L. salivarius* WB21 in combination. The susceptibility tests revealed that among the bacteria examined *L. salivarius* WB21 had the strongest resistance to EGCg (25 mg/mL). In contrast, periodontopathic bacteria, including *P. gingivalis*, *P. intermedia*, and *F. nucleatum*, showed strong susceptibility to EGCg (2.5 mg/mL). Previous studies have reported that the minimum inhibitory concentration of EGCg for *P. gingivalis* ranged from 125 to 500 μg/mL (Fournier-Larente et al., 2016) and the minimum inhibitory concentration for *Solobacterium moorei*, which has also been recognized as a halitosis-associated organism, was 250 μg/mL (Morin et al., 2015). On the other hand, methyl mercaptan was inhibited by 86.3% with 300 ppm EGCg (0.3 mg/mL), which was the lowest minimum inhibitory concentration of the oral pathogenic bacteria. These results imply that the ability of live *L. salivarius* WB21 can be effectively elicited, in addition to a rapid deodorizing effect of EGCg, by using EGCg at concentrations below 25 mg/mL, which is the minimum inhibitory concentration for *L. salivarius* WB21.

In previous studies, continuous oral intake of *L. salivarius* WB21 inhibited oral malodor at 2 and 4 weeks (Iwamoto et al., 2010; Suzuki et al., 2014). In addition, improved clinical and bacterial parameters have been demonstrated, including bleeding on probing, probing pocket depth, numbers of *Tannerella forsythia* in subgingival plaque, and numbers of ubiquitous bacteria and *F. nucleatum* in saliva (Iwamoto et al., 2010; Mayanagi et al., 2009; Shimauchi et al., 2008; Suzuki et al., 2012, 2014). Although the immediate effect of *L. salivarius* WB21 on oral malodor has never been examined, its effect is considered weak compared to the bactericidal agents that are used for chemical plaque control. In the case of lactic acid bacteria, continuous administration is important to improve the microbial community and immune system. However, consumers tend to expect an immediate effect, especially on oral malodor. The results of this study imply that a product containing both EGCg and *L. salivarius* WB21 might both deodorize oral malodor and offer continuous oral health control. This hypothesis should be examined in future clinical trials. In addition, it might be necessary to explore the effect of combinations of *L. salivarius* WB21 and other probiotic bacteria to identify a more effective method to maintain oral health in the future.

Role of the funding source

Part of the current study was supported by a research grant of 5,000 USD from Wakamoto Pharmaceutical Co.

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