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ORIGINAL ARTICLE

The Effect of Parabiotic Reuterin on the Expression of Genes Involved in *Candida albicans* Biofilm Formation: An *Ex vivo* Study

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ABSTRACT

Candida albicans has a number of properties, including resistance to various antimicrobial agents, which allow it to survive in the root canals. *Lactobacillus reuteri* plays a role in maintaining oral health through interactions with the oral microbiome. *L. reuteri* has potential as a preventive and therapeutic agent against inflammatory diseases. **Objective:** The aim of this study was to investigate the effect of irrigation with a reuterin-containing solution on *BCR1, ACE2, EFG1,* and *TEC1* gene expression in *C. albicans* root canal biofilms. **Methods:** *L. reuteri* was cultured in MRS broth and incubated anaerobically for 24 hours at 37°C. *C. albicans* was cultured in Sabouraud dextrose broth at 37°C for 48 hours. A total of 24 single-rooted premolar teeth were standardized and inoculated with *C. albicans* before irrigation with 50 µg/mL reuterin as a single, independent variable (Indonesian strain), 50 µg/mL reuterin Prodentis (a strain combination of *L. reuteri* DSM 17938 and *L. reuteri* ATCC PTA 5289), 2.5% sodium hypochlorite as positive control, and saline as negative control. A real-time quantitative polymerase chain reaction (RT-qPCR) assay was used to detect the expression of *BCR1, ACE2, EFG1,* and *TEC1* in *C. albicans* root canal biofilms. **Results:** Reuterin significantly reduced the expression of *BCR1* and *ACE2* genes, which play a role in *C. albicans* biofilm formation, at the biofilm maturation stage ($P < 0.05$). Reuterin also affected the expression of the *EFG1* and *TEC1* genes, although the effect was not significant. **Conclusion:** A reuterin isolate of *L. reuteri* exhibits antibiofilm activity against the expression of *C. albican*s genes involved in biofilm formation. Reuterin has potential as an irrigation agent in the treatment of root canals. Further research is needed to shed light on the effectiveness of reuterin against the expression of genes that play important roles in the formation of *C. albicans* biofilms.

Key words: biofilm, *Candida albicans*, *Lactobacillus reuteri,* reuterin, root canal

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INTRODUCTION

Dental caries and periodontal disease are common in the general population. Worldwide, approximately 2.43 billion adults (i.e., 36% of the total population) have dental caries. In 2018, 45.3% of people in Indonesia experienced dental and oral health problems, with caries present in 88.8% of the population (average decayed, missing, and filled teeth (DMFT) index is 7.1).[1] Host-related factors related to tooth enamel structure, immunological responses to cariogenic bacteria, and salivary composition, influence dental caries formation.[2] Dental caries (tooth decay) is a tooth dysfunction mainly caused by bacterial oral colonization with the ability to produce acids from fermenting carbohydrates that can cause prolonged period of low pH, resulting in demineralization. Untreated caries can lead to pulp infection and a need for endodontic treatment. *Enterococcus faecalis* and *Candida albicans* are frequently linked to root canal infections, with the latter the most commonly isolated fungal species from infected root canals.[3] New microbial detection methods have shed light on microbial species associated with endodontic infections, including the roles of *E. faecalis* and *C. albicans* in treatment-resistant infections.[4]

Although fungi are found in primary root canal infections, they appear to be more common in root canals with failed endodontic treatment, with

C. albicans found in 7–18% of cases of apical periodontitis.[5,6] *C. albicans* is characterized by hyphal formation and thigmotropism, which allows it to penetrate deep into dentin and the root canal, where it is resistant to conservative root canal treatment.[4] Previous studies reported that inadequate cleaning and irrigation of the root canal system contribute to treatment failure of periapical lesions.[6,7]

The formation of a *C. albicans* biofilm can be divided into four basic phases, based on in vitro studies: (1) attachment and colonization of yeast-shaped (nearly spherical) cells to the host surface; (2) growth and proliferation of yeast-forming cells to allow formation of a base layer of anchoring microcolonies; (3) growth of pseudohyphae (ellipsoid cells joined end to end) and broad hyphae (chains of cylindrical cells), along with the production of extracellular matrix material; and finally (4) dispersal of yeast cells from biofilms, leading to the formation of new biofilms.[8] Many *C. albicans* genes are involved in biofilm formation. These genes encode transcription factors and protein kinases.[9] A large transcriptional network comprising six major transcriptional regulators (Efg1, Tec1, Bcr1, Ndt80, Brg1, and Rob1), each of which is required for biofilm development, controls *C. albicans* biofilm formation.[8,10] Transcriptional regulators are specific DNA-binding proteins that regulate transcription of DNA. Thirty genes of transcriptional regulators are involved in the attachment stage of *C. albicans*, of which *BCR1*, *ACE2*, *SNF5*, and *ARG81*, are required for biofilm formation.[10] The expression of *BCR1* and *ACE2* in the adherent phase of *C. albicans* indicates that these genes are required for cell-substrate adherence in biofilm formation.[11] The gene *BCR1* acts as main regulator for hyphae to adhere to each other in biofilm formation.[10] *ACE2* is known to affect adherence, biofilm formation, and hyphal morphogenesis. *EFG1* and *TEC1* are major activators of hyphae development.[11]

Probiotics contribute to oral health and a healthy microbial balance through interactions with oral microbes. Thus, probiotics are used to combat biofilm formation in the oral cavity.[12] The most common probiotic genera are *Bifidobacterium* and *Lactobacillus*. Some probiotic bacteria produce acid from carbohydrate fermentation, which results in a low pH environment, and some lactic acid bacteria produce hydrogen peroxide and bacteriocins harmful to pathogens.[13] *Lactobacillus reuteri* produces antimicrobial compounds, including organic acids, ethanol, and reuterin.[14] Due to its antimicrobial activity, *L. reuteri* inhibits the colonization of pathogenic microbes and remodels the composition of commensal microbiota in the host.[14] In spite of the literature review, to the best of the author's knowledge, there were no documented study that showed reuterin as parabiotic compound that express anti-fungal properties. Using sterilized premolar obtained from healthy patients as ex-vivo model[15], the aim of this preliminary study was to investigate the effect of the reuterin-containing irrigation solution on biofilm formation by *Candida albicans* by analyzing the mRNA expression of selected biofilm-associated genes.

METHODS

Tooth sample preparation

Premolars (N = 24) that had been excised at the cementoenamel junction were prepared with a K-file #15. Cleaning and shaping were performed using ProTaper Ni-Ti instruments (Dentsply Maillefer, Ballaigues, Switzerland), and the entire root canal was enlarged to F2 size. [15] One of the root canals of each premolar was closed using putty silicone impression material, and the root surface was covered with varnish. Thus, a biofilm would form only inside the root canal of the tooth.

Microbiological cultures

C. albicans ATCC 10281 was thawed from -80°C and was cultured in a microcentrifuge tube containing 10 mL of nutrient broth media and incubated aerobically for 24 hours at 37°C. The optical density of the culture was adjusted to 1.5×10^8 CFU/mL or 0.5 McFarland. *L. reuteri* LC 382415 (Indonesian strain) and L. reuteru ProDentis were cultured in de Man, Rogosa and Sharpe (MRS) broth and incubated anaerobically for 24 hours at 37°C.

Reuterin isolation

Reuterin isolation was adapted from Widyarman & Theodorea in 2021.[16] To make 100% reuterin, *L. reuteri* cells were collected by centrifugation at 5,000 g for 15 minutes at 20°C, followed by rinsing with phosphate-containing saline (PBS, pH 7.4) and centrifugation at 5,000 g for 15 minutes. Subsequently, the cells were resuspended to 1.5×10^{10} CFU/mL in 300 mM glycerol solution and incubated for 3 hours at 37°C, anaerobically. They were then re-centrifuged at 5,000 g for 15 minutes, and the supernatant was filtered using a 0.22 µm filter. After isolation, supernatant was considered as pure reuterin and the reuterin concentration was measured using Bradford assay.

Root canal inoculation

Each prepared tooth sample was placed in a microcentrifuge tube, with the root positioned at the bottom of the tube. The tooth was inoculated with *C. albicans* at 1.5×10^8 CFU/mL concentration and incubated aerobically at 37°C for 24 hours. Subsequently, the root canal was irrigated with PBS solution.

Root canal irrigation

The samples (N = 24) were divided into four treatment groups: (1) saline water (negative control), (2) 2.5%

sodium hypochlorite (positive control), (3) 50 µg/mL (w/v) of reuterin ProDentis, and (4) 50 µg/mL (w/v) of reuterin. The samples were irrigated with 5 mL of irrigation solution using a disposable plastic syringe and irrigated again 5 minutes later. The irrigation procedure was repeated twice. The irrigated tooth were then left at 37°C for 5 minutes (estimated common time for one-time irrigation treatment) and 30 minutes (estimated common total time for a whole endodontic treatment), respectively, in the pooled respective used irrigation solution. Subsequently, the samples were irrigated with 5 mL of PBS. The PBS containing biofilms were collected in airtight sterile tubes and vortexed. The teeth were removed and the PBS containing biofilms were centrifuged at 5000 g for 5 minutes at 4°C. The pellet containing treated biofilms were collected and transferred into a new sterile 1.5 ml microcentrifuge tube, followed by dissolving it with 1 ml PBS.

Bacterial colony counting

The PBS containing biofilms were diluted at a ratio of 1:1000 for each sample group. From each dilution, 50 µL was removed using a micropipette and smeared on Saboraud's Dextrose Agar (SDA) (Oxoid, Hampshire, UK), followed by incubation at 37°C (95% humidity) for 24 hours, aerobically. After colonies had formed, the number of colonies in each group was counted.

RNA extraction

The irrigated samples were centrifuged at 5,000 rpm for 10 minutes. The supernatant was discarded, and the pellet (precipitate) was homogenized in 1,000 µL of GENEzol™ reagent (Geneaid, Taipei, Taiwan) according to the manufacturer's instruction, followed by incubation for 5 minutes at room temperature (approximately 26 °C). Then, 200 µL of chloroform were added, vortexed, and incubated for 3 minutes at room temperature. The sample was then re-centrifuged. Next, RNA was dissolved in the aqueous phase and transferred into a different tube. RNA was precipitated from the aqueous phase by adding isopropranol to the aqueous phase, then mixed by inverting the tube several times, and incubated for 10 minutes at room temperature. The samples were centrifuged at 12–16,000 x g for 10 minutes at 4ºC to form a tight RNA pellet. The supernatant was carefully removed and discarded. Then, 1 ml of 70% ethanol was added to wash the RNA pellet, followed by brief vortexing. The sample was centrifuged at 12–16,000 x g for 5 minutes at 4ºC. After that, the supernatant was carefully removed with a pipette without contacting the RNA pellet. The RNA pellet was air-dried for 5-10 minutes at room temperature. Finally, the RNA was resuspended in nuclease-free water, incubated at 60°C for 10 minutes, and then stored at -80°C. The extracted RNA was quantified using Invitrogen™ high-sensitivity RNA Qubit 3.0 Fluorometer Kit (Thermo Fisher Scientific, Waltham Massachusetts), with the threshold minimum of 2 µg for cDNA synthesis. cDNA conversion was done using ReverTra AceTM qPCR RT Master Mix with genomic DNA remover (Toyobo, Osaka, Japan) according to the manufacturer's instructions. The cDNA was quantified using an Invitrogen™ high-sensitivity DNA Qubit 3.0 Fluorometer (Thermo Fisher Scientific, Waltham, Massachusetts).

Real-Time quantification PCR

RT-qPCR amplification and detection were performed using qPCR master mix. Table 1 details the components of the master mix.

5x HOT FIREPol EvaGreen® qPCR Mix Plus (Solis Biodyne, Tartu, Estonia) was used to detect amplification of the target gene. Actin was used as a housekeeping gene for normalization purposes. RT-qPCR was performed using cDNA samples as template. The RT-qPCR assay was performed using the primers shown in Table 2.[15,16] The conditions were as follows: initial denaturation at 95°C for 5 minutes, 40 cycles of denaturing at 95°C for 1 minute, and annealing at 58°C for 1 min. The relative quantification of gene expression was calculated using double delta cycle threshold formula ($2^{-\Delta\Delta CT}$).

Table 1. The components of the master mix used in the qPCR test

Components	Volume
5x HOT FIREPol EvaGreen® qPCR Mix Plus	4 µl
Primer forward	1 µl
Primer reverse	1 µl
DNA template	2 µl
Nuclease-free water	12 µl
Total	**20 µl**

Statistical Analysis

All data was analyzed using one way ANOVA test. A p-value of less than 0.05 was considered statistically significant. Statistical analysis was performed using SPSS Statistics for Windows software v. 26 (IBM, Armonk, NY).

RESULTS

Colony counts

The *ex vivo* colony counting method was used to determine the number of *C. albicans* cells after treatment with *L. reuteri* reuterin isolate. Based on the colony count, the reuterin (50 µg/mL (w/v)) treatment decreased root canal biofilm formation. (Fig. 1,2). Reuterin was significantly effective in reducing the number of *C. albicans* at both treatment times (i.e., 5 and 30 minutes).

Table 2. Primer sequences

Genes	Primer Sequence
BCR1 [17]	*forward*: 5'-CTTCAGCAGCTTCATTAACACCTA-3' *reverse*: 5'-AATGGGTGAATAAATCCCTCCCTAA-3'
ACE2[18]	*forward*: 5'-AGAATTGACCGTTGTCCGTGTAAG-3' *reverse*: 5'-AATGGGTGAATAAATCCCTCCCTAA-3'
EFG1[18]	*forward*: 5'-TGCCAATAATGTGTCGGTTG-3' *reverse*: 5'-CCCATCTCTTCTACCACGTGTC-3'
TEC1[19]	*forward*: 5'-TTTTCTATTCTAACCACCCTCTGC -3' *reverse*: 5'-CCCGCCTTGCCCCTCTT-3'
House - keeping gene: *ACT1*[20]	*forward*: 5'-TTTCATCTTCTGTATCAGAGGAACTTATTT-3' *reverse*: 5'-ATGGGATGAATCATCAAACAAGAG-3'

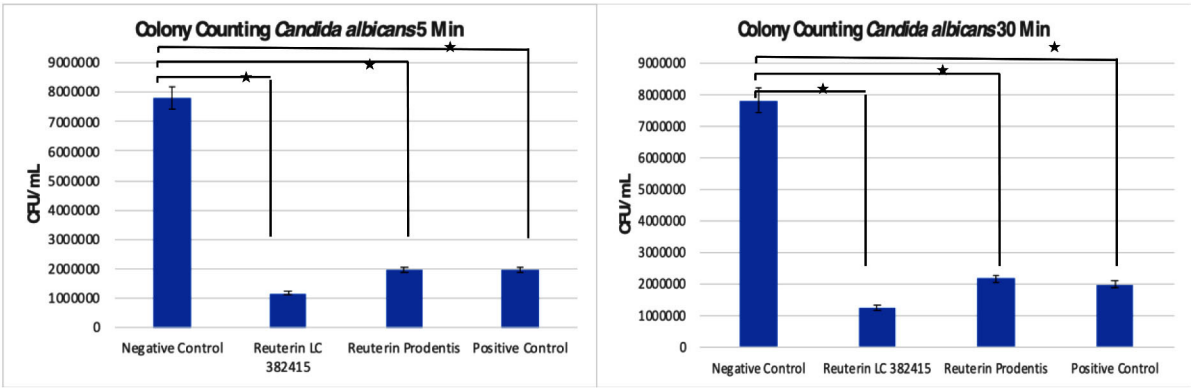

Figure 1. The viability of *Candida albicans* biofilm in root canal after treatment with saline water (negative control), 50µg/mL (w/v) of reuterin LC 382415, 50 µg/mL (w/v) of reuterin ProDentis, and 2.5% sodium hypochlorite (positive control) with 5- and 30-minute treatment time

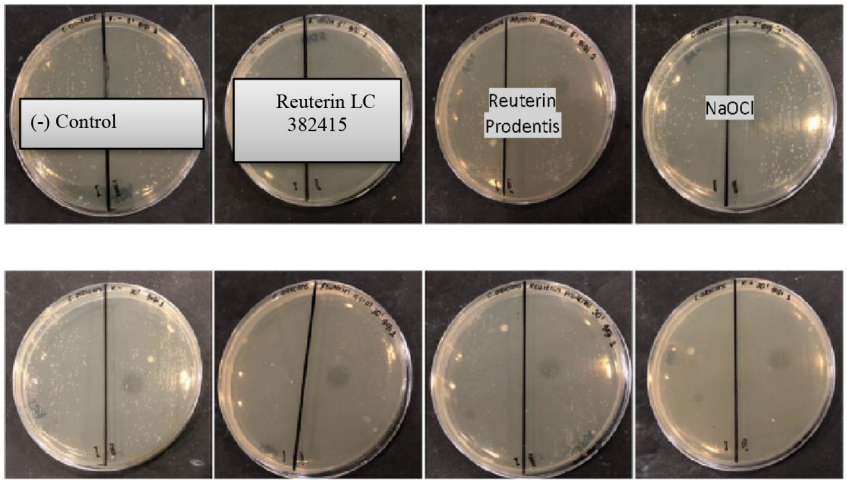

Figure 2. Colony counts of *Candida albicans* biofilm after treatment with saline water (negative control), 50µg/mL of reuterin LC 382415, 50 µg/mL of reuterin ProDentis, and 2.5% sodium hypochlorite (positive control) on SDA after 5- and 30-minute treatment time

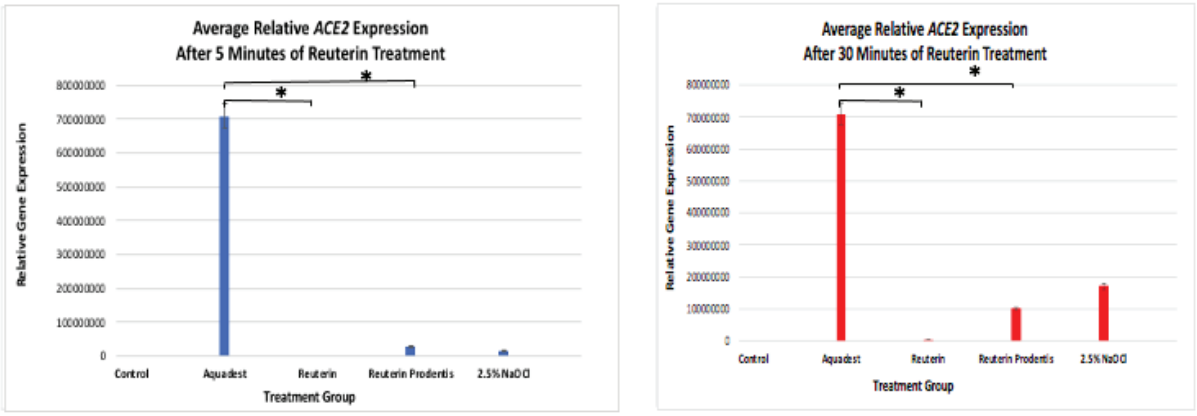

Figure 3. Results of the real-time PCR assay of the impact of the reuterin (50 µg/mL) treatment on *ACE2* gene expression and *Candida albicans* biofilm formation ($2^{-\Delta\Delta Ct}$)

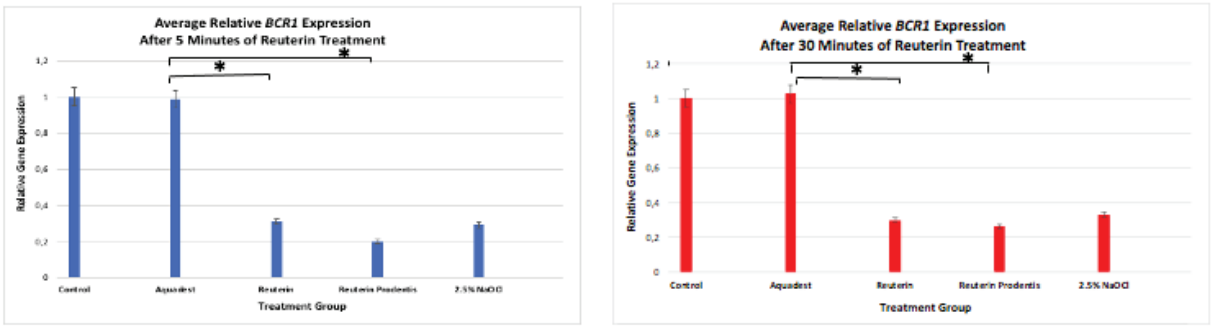

Figure 4. Results of the real-time PCR assay of the impact of the reuterin (50 µg/mL) treatment on *BCR1* gene expression and *Candida albicans* biofilm formation ($2^{-\Delta\Delta Ct}$)

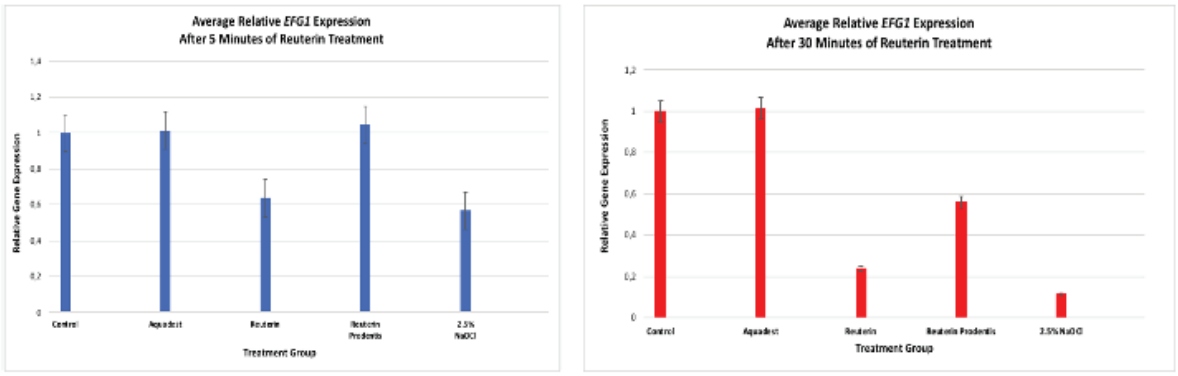

Figure 5. Results of the real-time PCR assay of the impact of the reuterin (50 µg/mL) treatment on *EFG1* gene expression and *Candida albicans* biofilm formation ($2^{-\Delta\Delta Ct}$)

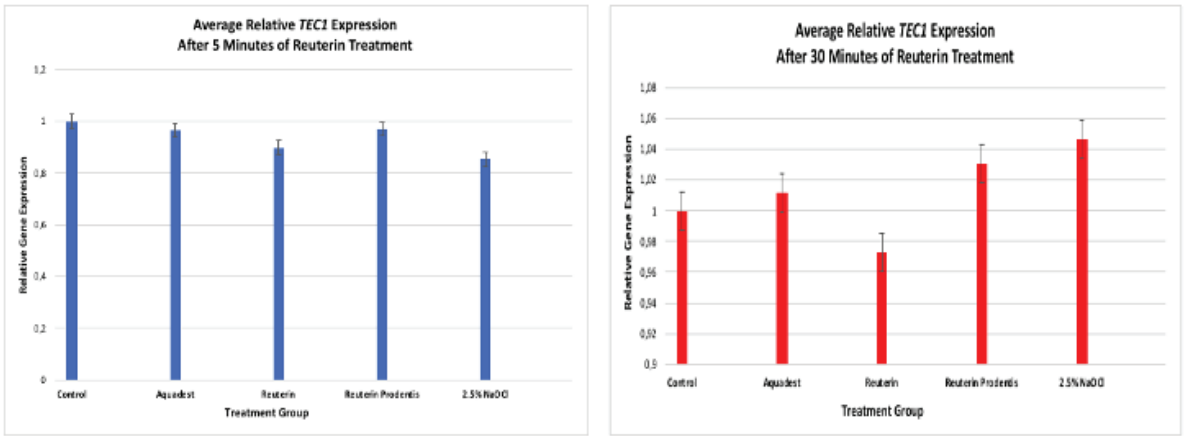

Figure 6. Results of the real-time PCR assay of the impact of the reuterin (50 µg/mL) treatment on *TEC1* gene expression and *Candida albicans* biofilm formation ($2^{-\Delta\Delta Ct}$)

Real-Time quantification PCR
The real-time qPCR method was used to evaluate the effect of the reuterin irrigant treatment (50 µg/mL for 5 or 30 minutes) on *EFG1, BCR1, ACE2,* and *TEC1* mRNA expression (Fig 3-6). The results revealed a decrease in the expression of the *ACE2* and *BCR1* genes at both treatment times.

DISCUSSION

In this study, *C. albicans* in the root canals were treated with different concentrations of reuterin (25 and 50 µL) of reuterin-containing irrigant for different times (5 and 30 minutes) to determine its effectiveness against *C. albicans* biofilm formation. The result of the colony counting showed that the effectiveness of reuterin was similar to that of the positive control. In contrast, as shown by results of the post hoc test, there was a significant difference in colony count result in the negative control and reuterin treatment groups at both treatment times (5 minutes and 30 minutes). Thus, reuterin shows a good ability to inhibit the growth of *C. albicans* in root canals *ex vivo.*

Strains of *L. reuteri* are often used as probiotic agents due to the beneficial effects of the antimicrobial compounds they produce, including reuterin.[14] Previous research showed that reuterin exhibits broad-spectrum antibiotic properties and that it is effective against Gram-positive and negative bacteria, as well as fungi and protozoa.[21] In this study, the RT-qPCR showed that reuterin from *L. reuteri* exhibited antibiofilm activity against *C. albicans* biofilm formation, hence this result is in accordance with Rao et al. in 2013. [22] As shown by the RT-qPCR test, there was a decrease in the expression of *C. albicans* biofilm-related genes. The expression of the *BCR1* and *ACE2* genes decreased significantly at both treatment times. The results of the RT-qPCR test showed that the genes involved in the mechanism of inhibition of *C. albicans* biofilm formation in the reuterin-treated groups was downregulation of *BCR1* and *ACE2* expression. The *BCR1* is a major transcription factor, which plays an important role in *C. albicans* biofilm formation.[23] The *BCR1* transcription factor has long been used to determine the functional basis of pathogenesis traits. As transcription factors control target genes, defects in transcription factors can abolish the function of a specific gene.[24] Thus, the expression of the *BCR1* gene may depend on environmental conditions.[23] This idea is supported by the results of this study, which found a statistically significant decrease in the expression of the *BCR1* gene of reuterin isolate-treated, reuterin ProDentis-treated, and positive control in both treatment times.

EFG1 gene plays an important role in the development of hyphae in the human pathogen *C. albicans*.[21] The *EFG1* is a central regulator of transcriptional morphogenesis and metabolism in *C. albicans*. *EFG1* interacts directly with hyphal-specific promoters to activate transcriptional promoters during hyphal development.[25,26] The role of *EFG1* as a central regulator of transcription can explain the absence of significant changes in the expression of *EFG1* in the reuterin isolate-treated group, as shown in Figure 5.

The results showed that the effect of reuterin on *TEC1* gene expression was not significantly different from that of the negative control in terms of *C. albicans* biofilm formation (*p-value* > 0.05). The transcriptional regulator genes, including *TEC1* and *EFG1*, regulate the biofilm formation through hyphal elongation, synthesis and deposition of extracellular matrix (ECM) which function is to increase impermeability of the biofilm to low- and high-molecular weight molecules, as well as drug resistance.[26,27] It may be probable that *TEC1* gene regulations were not affected by the presence of reuterin. However, this statement needs further research for confirmation.

The results of this study point to a statistically significant decrease in *ACE2* gene expression. *ACE2* is required for hyphae formation and acts as a virulence factor that regulates the expression of genes involved in cell separation.[28] *ACE2* gene expression causes upregulation of the expression of many genes required for mitochondrial function, which is crucial to the fungal colonization and biofilm formation.[28] The expression of *ACE2* seems to be affected by the antimicrobial activity of reuterin-producing *L. reuteri*, which can eradicate bacterial and fungal colonization by changing the conditions of the environment.[14]

According to a recent report by the World Health Organization, probiotics dietary consumption of probiotics has beneficial effects for human health. when consumed in a sufficient amount. In terms of its probiotic activity, *L. reuteri* probiotics release antimicrobial compounds (e.g., lactic acid, bacteriocin, and hydrogen peroxide) and compete with pathogens, which induce an increased immune host response.[29] The results of this study showed that reuterin isolated from *L. reuteri* can reduce the expression of some major biofilm-related genes that regulates cell adherence and hyphal formation in *C. albicans* biofilms.

CONCLUSION

Reuterin isolated from *L. reuteri* Indonesian strain shows antibiofilm activity against *C. albicans* in root canals. Reuterin affected *C. albicans* biofilm formation by decreasing the expression of genes involved in biofilm formation, namely *BCR1* and *ACE2*, at the maturation stage. As shown by this *ex vivo* study, reuterin has potential properties to be used

as an alternative root canal irrigation agent during endodontic treatment. However, further studies are still warranted using other endodontic pathogens to confirm this result.

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