Vol 11, Special Issue 1, 2019

INTERNATIONAL JOURNAL OF APPLIED PHARMACEUTICS

INNOVARE ACADEMIC SCIENCES Knowledge to Innovation

ISSN - 0975-7058

Research Article

EFFECT OF *VEILLONELLA INFANTIUM* ON BIOFILM FORMATION OF ORAL STREPTOCOCCUS

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Received September 20, 2018, Revised and Accepted Desember 22, 2018

ABSTRACT

Objective: Therefore, we aimed in this study to evaluate the effect of *Veillonella infantium* on the biofilm formation of oral *Streptococcus* species.

Methods: Dual-species biofilm was formed between *V. infantium* and oral *Streptococcus* using the wire method, and it was then incubated at 37°C under anaerobic condition for 5 days. Biofilm formation was determined by measuring the DNA concentration. Single species biofilm of oral *Streptococcus* was generated under the same conditions and was used as a control group.

Result: The presence of *V. infantium* decreased the biofilm formation of *Streptococcus mutans*, where, in contrast, the formation of biofilm in *Streptococcus sanguinis* was increased by the presence of *V. infantium* ($p<0.05$).

Conclusion: The presence of *V. infantium* decreased the biofilm formation of *S. mutans*.

Keywords: Biofilm, *Veillonella infantium*, *Streptococcus mutans*, *Streptococcus sanguinis.*

 DOI: http://dx.doi.org/10.22159/ijap.2019.v11s1.18458

INTRODUCTION

More than 700 bacterial species have been identified as existing in the oral cavity [1]. Interactions between those microorganisms are found biofilm on the dental surface, which is known as dental plaque. The presence of biofilm in the oral cavity could impair oral health [2]. Approximately 65% of bacterial infectious disease is associated with bacterial biofilm. Biofilm plays a role on the development of oral infectious disease, such as caries [3].

Biofilm is a community of bacteria that is embedded in an extracellular polymeric matrix and is attached to a substratum or surfaces, or each bacterium of the community is attached to each other [4]. Biofilm formation consists of several stages initiated by the attachment of an initial colonizer such as *Streptococcus* species, on pellicle-coated dental surfaces, followed by an early colonizer such as *Veillonella* species. After the settlement of the early colonizer, the maturation process occurs followed by dispersion of mature cells to other surfaces to begin the cycle of biofilm formation [3,5].

Streptococcus species is an initial colonizer in the biofilm formation and is found on 60–90% of subgingival plaque in the first 24 h of bacterial colonization [6]. *Streptococcus* catabolizes carbohydrates into short-chain organic acids, such as lactic acid and pyruvic acid [7]. The acid product of *Streptococcus* can decrease the pH in the oral cavity, leading to the demineralization in the formation of caries. The end product of *Streptococcus* could be utilized as the nutrient source to other bacteria such as *Veillonella*. Oral *Veillonella* is known as an early colonizer that interacts with *Streptococcus* in the early stage of biofilm formation [8].

Oral *Veillonella* is a Gram-negative, strictly anaerobe constituting 5% of initial dental plaque biomass [9]. Seven species of *Veillonella* have been identified in the oral cavity: *Veillonella atypica*, *Veillonella denticariosi*, *Veillonella dispar*, *Veillonella Parvula*, *Veillonella rogosae*, *Veillonella tobetsuensis*, and *Veillonella infantium* [10-12]. *V. infantium* was identified from the T11011-4 strain isolated from the biofilm existing in 10-year-old children in Thailand who all had good oral hygiene status. The colony was round in shape with a smooth surface, opaque grayish white, 0.5–2 mm in diameter, without hemolysis on brain heart infusion (BHI) blood agar, and showed basic fuchsin decolorization on *Veillonella* selective media [12].

It has been reported that six oral *Veillonella* species such as *V. atypica*, *V. denticariosi*, *V. dispar*, *V. parvula*, *V. rogosa*, and *V. tobetsuensis* affected the formation of four oral *Streptococcus* biofilm, *Streptococcus mutans*, *Streptococcus salivarius*, *Streptococcus sanguinis*, and *Streptococcus gordonii*, synergistically and antagonistically [9]. However, the effect of *V. infantium* on the biofilm formation of oral *Streptococcus* had not been investigated.

METHODS

Bacterial strains and growth conditions

S. mutans ATCC 25175 and *S. sanguinis* 10556 were cultured on TY agar (TY Bacto Tryptic Soy Broth and Bacto Yeast Extract) under anaerobic conditions (N_2:CO_2:H_2, 80:10:10) at 37°C for 3 days. *V. infantium* T11011-4 was cultured on BHI blood agar (bacto BHI) supplemented with 5% defibrinated sheep blood and 2% sodium lactate under anaerobic conditions at 37°C for 5 days.

Biofilm formation using the wire method

Biofilm formation was performed using commonly utilized cobalt-chrome alloy wires that were 0.9 mm in diameter and 9 cm in length (TECHNOFLEX, Rocky Mountain Morita Corporation, Tokyo, Japan). The wires were sterilized in an autoclave after affixing a rubber plug and flexible tubing (TYGON Saint-Gobain, Malvern, PA, USA) to the wire ends.

About 1 mL of a suspension of each culture of *Streptococcus* spp., the turbidity of which was determined by measuring the absorption at OD_{660}:0.5 (5×10^7 CFU/ml), was inoculated into a test tube of 4 ml of BHI broth containing 1% sucrose. Next, a wire treated with artificial saliva (Saliveht; TEIJIN, Osaka, Japan), was inserted into the test tube as a base for biofilm formation. After incubation under anaerobic conditions at 37°C for 1 day, the wire with the *Streptococcus* biofilm was transferred into suspensions of *V. infantium* cultures, the turbidity of which was also been determined by measuring the absorption at OD_{660}:0.5 (0.5×10^7 CFU/ml) and test tubes containing the same media were supplemented with 2% sodium lactate. The cocultures were

Table 1: DNA concentration of dual-species biofilm formation of *S. mutans*, *S. sanguinis*, and *V. infantium* and control group on days 1, 3, and 5 (ng/µl)

Bacterial Combination of Biofilm	Mean±standard deviation of DNA concentration (ng/µl)					
	Control day 1	S+VI day 1	Control day 3	S+VI day 3	Control day 5	S+VI day 5
S. mutans+V. infantium	0.217±0.050	0.213±0.101	0.322±0.070	0.458±0.162	0.736±0.408	0.623±0.206
S. sanguinis+V. infantium	0.021±0.030	1.163±0.505	0.116±0.164	1.902±0.625	0.364±0.090	3.548±2.256

S. mutans: Streptococcus mutans, V. infantium: Veillonella infantium, S. sanguinis: Streptococcus sanguinis

incubated in anaerobic conditions at 37°C for 1 day to generate mixed biofilms containing *Streptococcus* spp. and *V. infantium*. The media in the test tubes were replaced with fresh media every day for 5 days. Biofilm containing *S. mutans* and *S. sanguinis* alone was generated on wires under the same conditions and was used as a control.

DNA extraction and quantification

The biofilms on the wires were removed using flexible tubing and were collected in a sterilized tube. DNA was extracted using phenol-chloroform and ethanol precipitation. Briefly, biofilm cells were dispersed with BioMasher after mixing with saline EDTA. Sodium dodecyl sulfate (SDS) 20% was added to the tube and then heated on HeatLock at 60°C for 10 min. Phenol-chloroform was added and homogenized with vortex, and the tube was centrifuged at 4°C and 15,000 rpm for 5 min. The aqueous layer was collected and moved to another tube using an Eppendorf pipette. Proteinase-K 50 µg/ml and 0.5% SDS were added to the tube. The tube was then heated at 56°C for 1 h. Ethanol precipitation was performed by adding 99.5% ethanol, 5 M sodium chloride, and glycogen 10 µg/ml to the tube containing aqueous phase, and the tube was stored at 4°C overnight. The next day, the tube was centrifuged at 4°C and 8500 rpm for 10 min. The supernatant was removed and the formed pellet was dissolved by adding TE solution. The genomic DNA was stored at −20°C until further analysis. The DNA concentration of each fraction was determined with a Qubit Fluorometer using Qubit dsDNA BR Assay Kit (Invitrogen), according to the manufacturer's instructions. At least 2 µl of each sample was used to measure DNA concentration.

Statistical analysis

The experiment was conducted in triplicate, and the obtained data were expressed as mean±standard deviation (SD). Statistical significance was determined using a parametric independent t-test and non-parametric Mann–Whitney U-test for the comparison between the control and experimental groups on days 1, 3, and 5 of incubation. $p<0.005$ was considered to be statistically significant.

RESULTS

Table 1 and Figs. 1 and 2 show the amount of biofilm formed by *S. mutans* and *S. sanguinis* with *V. infantium* after 5 days, including control, as determined from the results of DNA concentration measurement. The combination of *V. infantium* and *S. mutans* decreased the amount of biofilm formation after 5 days of incubation as compared with the control group. Alternatively, the presence of *V. infantium* increased the amount of the biofilm formation in combination with *S. sanguinis* on days 1, 3, and 5 of incubation as compared with control group.

Biofilm formed by *S. mutans* in combination with *V. infantium* was slightly lower than that in the control group on day 1 and then increased on day 3 of incubation and was higher than the amount of biofilm in the control group. A lower amount of biofilm formed by *S. mutans* in combination with *V. infantium* was observed after 5 days of incubation compared with the control group, although it was not statistically significant ($p>0.05$). When *V. infantium* was combined with *S. sanguinis*, biofilm formation on days 1, 3, and 5 of incubation was significantly higher than biofilm formation in the control groups with $p=0.003$, 0.004, and 0.002, respectively. An increasing amount of biofilm formation over time was also observed in the combination of *S. sanguinis* and *V. infantium* on days 1, 3, and 5 of incubation.

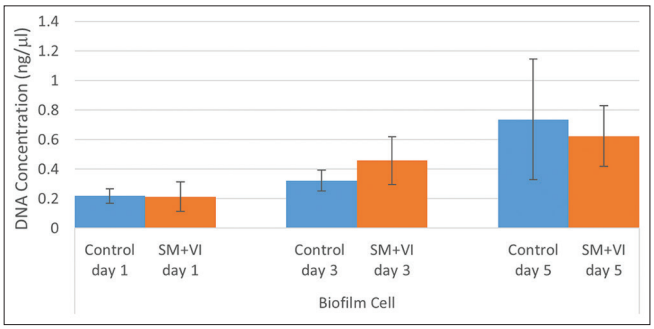

Fig. 1: Dual-species biofilm formation of *Streptococcus mutans* and *Veillonella infantium* dual species compared with *S. mutans* single species (control) based on DNA concentration

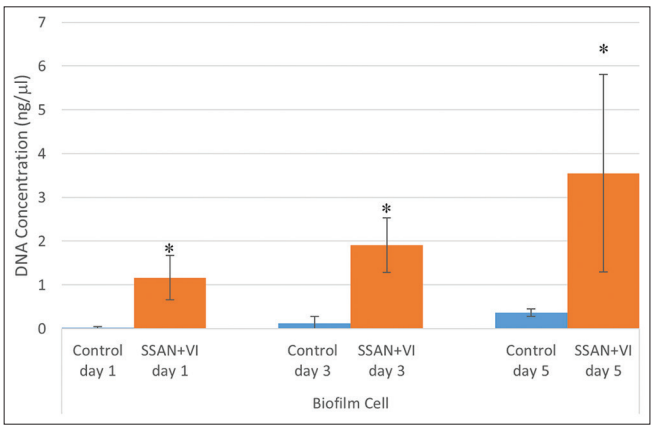

Fig. 2: Dual-species biofilm formation of *Streptococcus sanguinis* and *Veillonella infantium* compare with *S. sanguinis* single species (control) based on DNA concentration

DISCUSSION

The previous study showed that six species of oral *Veillonella* species combined with four oral *Streptococcus* species synergistically and antagonistically affected the formation of dual-species biofilm [9]. Other experiments have shown that oral *Streptococcus* interacted with *V. atypica* through the mediation of adhesins [13]. In addition, one of the *Streptococcus* transcription factors, catabolite control protein A, is needed for interaction between *S. gordonii* and *V. atypica* [14]. In this study, the presence of *V. infantium* was able to decrease the biofilm formation of *S. mutans*. The presence of *V. infantium* was conversely able to increase the biofilm formation of *S. sanguinis* ($p<0.05$). *V. infantium* is shown to have an antagonistic and synergistic effect on the biofilm formation of *S. mutans* and *S. sanguinis*, respectively.

Coaggregation and coadhesion are mechanisms of bacterial interaction [15]. A previous study has shown that *V.parvul*a that was isolated from subgingival plaque coaggregated with S.sanguinis. However, in the same study, *V.parvula* was found not coaggregated with *S.salivarius* [16]. Another study has shown that *V. rogosae*, which is frequently isolated from tongue biofilm, coaggregated with *S. salivarius* [17]. Based on these findings, it could be stated that the coaggregation characteristic among

oral *Veillonella* species showed a selective interaction of compatible partners for biofilm formation. In this study, the combination of the dual species of *S. sanguinis* and *V. infantium* demonstrated a higher biofilm formation compared with the combination of *S. mutans* and *V. infantium*.

The presence of *V. infantium* resulted in higher and lower amounts of biofilm formation by *S. mutans* after 3 and 5 days of incubation, respectively, compared with the control group, which suggests that *V. infantium* would affect the amount of biofilm formation of *S. mutans* as the time of incubation increased. In this study, the presence of *V. infantium* increased the amount of biofilm formation of *S. sanguinis*. Another study has shown that the presence of *V. atypica*, *V. denticariosi*, *V. rogosae*, and *V. tobetsuensis* decreased the amount of biofilm formation of *S. sanguinis*. In the same study, the presence of *V. parvula* increased the amount of biofilm formed by *S. sanguinis* [9]. These results might suggest that *V. infantium* shares a similar characteristic with *V. parvula* in the formation of biofilm combined with *S. sanguinis*, synergistically. *S.sanguinis* was shown in order study to produce hydrogen peroxide, which was found to inhibit the growth of *S.mutans* [18]. These findings might suggest a similar antagonistic interaction between *S. sanguinis* and *V. infantium* on *S. mutans*.

CONCLUSION

The presence of *V. infantium* decreased the biofilm formation of *S. mutans*. However, an increasing amount of biofilm formation between *V. infantium* and *S. sanguinis* was observed as incubation time increase suggesting an antagonistic interaction between *V. infantium* and *S. mutans* and a synergistic interaction between *V. infantium* and *S. sanguinis*. Further studies are needed to evaluate the proportion of each species and the mechanism of interactions. Furthermore, an investigation of the effect of *V. infantium* on the biofilm formation of *S. mutans* and *S. sanguinis in vivo* is needed.

ACKNOWLEDGMENT

The authors are grateful to Professor Nakazawa Futoshi Ph.D., Mashima Izumi Ph.D., Miyakawa Hiroshi Ph.D., and Fujita Mari Ph.D., for the kind help and support throughout this study. The publication of this manuscript is supported by the Directorate of Research and Community Engagement of the Universitas Indonesia.

CONFLICTS OF INTEREST

There are no conflicts of interest to declare.

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