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Research Article

Effect of Bacterial Metabolites From Good Oral-Hygiene to Biofilm Formation From Poor Oral-Hygiene

[1]Sarah Athiyyahmaulidya Refyan, [1,2*]Endang Winiati Bachtiar, [1]Ratna Ramadhani, [1,2]Boy Muchlis Bachtiar, [3]Wahyu Sulistiadi

[1]Department of Oral Biology, Faculty of Dentistry, Universitas Indonesia, Depok, Indonesia
[2]Oral Science Research Center, Faculty of Dentistry, Universitas Indonesia, Salemba, Indonesia
[3]Department of Health Administration and Policy, Faculty of Public Health, Universitas Indonesia, Depok, Indonesia

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KEYWORDS

Biofilm metabolites, biofilm mass, cell viability, oral hygiene.

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ABSTRACT

Introduction: Microbial variations and oral hygiene (OH) status of an individual are related to the oral biofilm formation. The metabolite of microorganisms influences biofilm formation. This research aims to analyse the effect of bacterial isolates' metabolites from a good OH individual on the in vitro biofilm of bacterial isolates from a poor OH individual.

Materials and Methods: Spent medium of bacteria isolated from a good OH individual tongue swab that contains different protein and nitrate concentrations was treated in vitro with biofilm from a poor OH individual tongue swab to evaluate the cell viability and in vitro biofilm mass under aerobic conditions. The methods used include the Bradford test, Griess test, Crystal Violet test, and Total Plate Count.

Results and Discussions: There were significant differences in the cell viability of bacteria isolated from poor OH individual treated by spent medium isolated from good OH individual with different concentrations of protein and nitrate (p value <0.05), as well as biofilm mass of the sample that was treated with spent medium containing different nitrate concentration (p value < 0.05).

Conclusion: The protein and nitrate content in the spent medium from a good OH tongue swab can influence cell viability and in vitro biofilm mass from a poor OH individual tongue swab.

Corresponding Author:
Endang Winiati Bachtiar
Department of Oral Biology, Faculty of Dentistry
Universitas Indonesia, Depok, Indonesia
Email: endang04@ui.ac.id

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INTROODUCTION

The formation of the oral microbiome begins as early as the onset of growth and is influenced by several factors, including oral hygiene (OH), diet, systemic health, and genetics.[1,2] As the biofilm matures, bacteria in its deeper layers experience a decrease in oxygen concentration, which triggers an increase in nitric oxide (NO) production. This NO may cause cell injury and stimulate the production of degradative enzymes.[3] Several factors affect NO synthesis, such as genetics, dietary patterns, oxygen levels, and oral hygiene.[4,5] Consequently, an individual's oral hygiene status can impact the microbial composition and metabolism within the oral cavity. Therefore, this study aims to analyze the effect of metabolites from bacterial isolates obtained from individuals with good oral hygiene on the in vitro biofilm formed by bacterial isolates from individuals with poor oral hygiene.

MATERIALS AND METHODS

This was a laboratory comparative experimental analytic study with two samples. Oral hygiene status was evaluated through the subject's anamnesis, followed by assessment of oral hygiene using the debris index (DI) and calculus index (CI) on six predetermined tooth surfaces. The scores were then summed to calculate the simplified Oral Hygiene Index (OHI-S).

The tongue swab samples were collected from the dorsum to the anterior one-third of each subject's tongue. Swabbing was performed 2–3 times using sterile cotton buds, which were then placed in sterile 1.5 ml Eppendorf tubes containing phosphate-buffered saline (PBS) and phenylmethylsulfonyl fluoride (PMSF).

Two types of tongue swab samples were distinguished: those from individuals with an OHI-S score of 0–1.2, categorized as good oral hygiene (OH), and those from individuals with an OHI-S score of 1.3–6.0, categorized as moderate or poor OH. The samples were then centrifuged to separate the supernatant and pellet and diluted using the serial dilution method.

Microorganisms from the tongue swab of an individual with good oral hygiene (OH) were cultured on BHI agar medium (HiMedia, Kennett Square, USA). Ten microliters (µl) of the sample were inoculated onto the medium and spread using a sterile spreader. The culture was then incubated for 24 hours at 37°C under aerobic conditions. Next, the microorganisms cultured on BHI agar were inoculated into tubes containing 5 ml of BHI broth medium using a heat-sterilized inoculation loop and incubated for 24 hours at 37°C under aerobic conditions. The tubes were then centrifuged at 3000 rpm for 15 minutes at 4°C to separate the supernatant from the pellet. The supernatant was collected, filtered, and used as spent medium containing microbial metabolites. This procedure was repeated twice to collect two tubes of spent medium.

At the end of the procedure, 1% KNO_3 solution (filtered) was added to one of the spent media samples. Protein concentration was evaluated using the Bradford assay, and nitrate concentration was assessed by the Griess assay, both with established standard reference curves.

Biofilm formation was performed using a 96-well plate arranged in a two-column design (duplo) with predetermined rows and columns. Each well was filled with BHI broth and a diluted bacterial isolate from the tongue swab of a poor oral hygiene (OH) individual, then incubated for 3 hours. After this initial incubation, each well was treated with spent medium from the tongue swab of a good OH individual at protein and nitrate concentrations of 5% and 10%, followed by incubation for 24 hours at 37°C. Biofilms grown without the addition of any spent medium from good OH individuals served as the control group.

BHI broth from each well was discarded, and the wells were rinsed with sterile PBS to remove planktonic bacteria. The biofilm adhering to the well walls was then carefully scraped off. The scraped biofilm was suspended in PBS and transferred into 1.5 mL Eppendorf tubes, followed by serial dilution to achieve a 10^{-6} concentration. Then, 10 µl of the diluted biofilm suspension was inoculated onto BHI agar plates and incubated for 24 hours at 37°C. After incubation, the colonies were counted to

determine the number of colony-forming units per milliliter (CFU/ml).

BHI broth from each well was discarded, and the wells were rinsed with sterile PBS and then dried. Crystal violet solution was diluted to a 1% concentration and added to each well. The wells were then incubated at room temperature for 15 minutes. After incubation, the crystal violet solution was discarded, and microscopic photographs were taken to examine biofilm formation. Next, each well was filled with 96% alcohol, and the absorbance was measured at 600 nm using a microplate reader (AccuReader M965/M965+; Nangang, Taipei, Taiwan) to obtain the optical density (OD) value.

Data analysis in this study was conducted using Prism 10.1.1 software (GraphPad Software, Inc.). The normality of the data was assessed using the Shapiro-Wilk test, as the sample size was less than 50. Homogeneity of variance was evaluated with Levene's test. Comparative analysis between groups was performed using ANOVA for normally distributed (homogeneous) data and an independent parametric T-test for non-homogeneous data, to examine differences in cell viability and biofilm mass in the samples.

RESULTS AND DISCUSSION

The Bradford test showed that the spent medium from tongue swab bacterial isolates of individuals with good oral hygiene (OH) in the presence of KNO3 (0.87 µg/ml; n=1) had a higher protein concentration compared to the spent medium without KNO3 (0.3785 µg/ml; n=1). KNO3, which consists of K^+ and NO_3^- ions, may serve as a source of nitrogen that influences and increases the protein concentration.[6]

The Griess test showed that the spent medium from a tongue swab bacterial isolate of a good oral hygiene (OH) individual containing KNO3 (n=1) had a nitrate concentration of 0.0575 µM/mL, while the spent medium without KNO3 (n=1) had a nitrate concentration of 0.046 µM/mL. The Griess test indicates that the spent medium from the good OH individual containing KNO3 has a higher nitrate concentration compared to that without KNO3. This higher nitrate concentration is influenced by the $NO3^-$ ion in the spent medium, which promotes increased production of nitrite ($NO2^-$) through the reduction of $NO3^-$. The nitrite then reacts with the diazotization reagent to form a stable azo compound, allowing the elevated nitrite concentration to be measured.

Optical density (OD) analysis shows that the OD value of the spent medium from a good oral hygiene (OH) tongue swab bacterial isolate containing KNO3 (n=1) was lower than that of the medium without KNO3 (n=1). The OD values of the spent medium from good OH samples with 5% protein concentration, with and without KNO3, were 0.168 and 0.353, respectively. For samples with 10% protein concentration, the OD values with and without KNO3 were 0.1145 and 0.182, respectively. The in vitro biofilm mass of both groups treated with spent medium from a good OH individual, at 5% and 10% protein concentrations without KNO3, was higher compared to the control group that was not treated with spent medium. The differences in protein concentration affect the attachment process during the initial phase of biofilm formation. Some proteins play a role in cell communication, enabling cells to attach to one another. Therefore, it can be concluded that higher protein concentration promotes biofilm development, resulting in increased biofilm mass.[7,8]

The results of the crystal violet test show that the OD values of both treated groups were higher compared to the control group. The OD value (mean±standard deviation (SD)) of 5% and 10% were 0.08±0.02 and 0.106±0.02, respectively, compared to the control group, 0.069±0.01. Between the two tested groups, the lowest OD value was shown by the in vitro biofilm from a poor OH individual that was treated by spent medium from a good OH individual, which contains 5% protein concentration. However, there are no significant differences in the mass of in vitro biofilm from the poor OH sample that was treated with spent medium from the good OH sample with protein concentrations of 5 and 10% compared to the control group, with the p value 0.5663 and 0.082, respectively. There were significant differences in the viability of the cells of in vitro biofilm from poor OH individuals based on the differences in protein concentration of the spent medium from good OH individuals. Some proteins play a role in the growth and

viability of the cell. Microorganisms in the biofilm were aggregated in an EPS matrix that contains protein. Higher protein concentration may increase the number of microorganisms that are attached to the biofilm matrix.[9] However, the present results do not share the same principle and may be caused by the duplicate TPC test, so that the replication number did not represent the ability of cell growth based on different protein concentrations of the spent medium.

Total plate count results (Figure 1) show that the growth of microorganisms that had been incubated for 24 hours tended to decrease as the protein concentration of spent medium from a good individual sample increased. The number of colonies from the sample treated with spent medium from a good OH sample with 5% protein concentration was higher than control group. The TPC values (mean±SD in CFU/ml) of 5% protein-treated group and the control group were 1950±70.71 and 331.25±114.34, respectively. On the other hand, the number of microorganism colonies treated with the spent medium of the good OH sample with 10% protein concentration was lower compared to the control. The TPC values (mean±SD CFU/ml) of 10% of protein protein-treated group and control group were 312.5±265.17 and 331.25±114.34, respectively. There were significant differences in the viability of the cells of in vitro biofilm from poor OH samples based on those that were treated with spent medium from good OH samples containing 5% and 10% protein concentration compared to the control group, with the p value of 0.0063 and 0.0121, respectively.

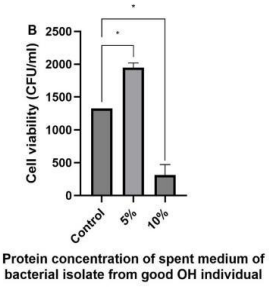

Figure 1. Total plate count (TPC) in CFU/ml of in vitro biofilm of bacterial isolate from poor OH individual treated with the spent medium of bacterial isolate from good OH individual with 5% and 10% of protein concentration. Asterisks denote statistically significant differences between the cell viability determined by total plate count, $p < 0.05$.

Crystal violet results (Figure 2) show a decrease in the growth of in vitro biofilm from the poor OH sample as the nitrate concentration of spent medium from the good OH sample increased. OD value of the in vitro biofilm from a poor OH sample treated with spent medium from a good OH sample with 5% nitrate concentration was higher than control group. The OD values (mean±SD) of 5% nitrate-treated and control groups were 0.122±0.06 and 0.049±0.01, respectively. In contrast, the OD value from in vitro biofilm from a poor OH sample that was treated with spent medium from a good OH sample with 10% nitrate concentration was lower than control group. The OD values (mean±SD) of 5% nitrate-treated and control groups were 0.132±0.00 and 0.049±0.01, respectively. Statistically, there were significant differences in 5% and 10% nitrate concentrations of spent medium from the good OH sample to in vitro biofilm mass of the poor OH sample compared to the control group, with p p-value of 0.0001 for both concentrations.

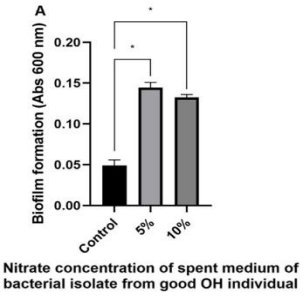

Figure 2. Optical density (OD) value of in vitro biofilm mass of bacterial isolate from poor OH individual treated with the spent medium of bacterial isolate from good OH individual with 5% and 10% nitrate concentration. Asterisks denote statistically significant differences between the biofilm formation determined by crystal violet assays, $p < 0.05$.

TPC results show consistent results with a decrease in cell viability as the nitrate concentration of spent medium from the good OH sample increased. The number of colonies in in vitro biofilm from a poor OH sample that was treated with spent medium from a good OH sample with nitrate concentrations of 5% and 10% was lower than the control group. The TPC values (mean±SD in CFU/ml) for 5% and 10% of nitrate-treated and control groups were 125±35.4, 75±35.4, and 2600±2392.0, respectively. Statistically, there were no significant differences in cell viability based on different nitrate concentrations in the

spent medium of the good OH sample compared to the control group. The p-values were 0.2398 and 0.2321 for the treated group with 5% and 10% of nitrate concentration compared to the control group, respectively.

The higher concentration of nitrate in a spent medium from a good OH individual induced a lower mass of in vitro biofilm from a poor OH individual. NO may promote biofilm dispersion and increase the formation of biofilm in several microorganisms. It can be assumed that the increase in biofilm mass was consistent with the increase in nitrate concentration, and it may have occurred due to aerobic conditions that did not support the production of NO, so that the supporting factors of biofilm dispersion were lower. The cell viability of in vitro biofilm from poor OH individuals decreased as the nitrate concentration of the spent medium increased. The lower NO concentration generally can increase viability and cell proliferation, whereas higher NO concentration may inhibit cell growth.[10]

Crystal Violet results show that the OD value of in vitro biofilm from poor OH isolate treated with spent medium from good OH sample that contained KNO_3 was higher than that without KNO_3. The OD values of in vitro biofilm treated with spent medium with and without KNO_3 were 0.082±0.05 and 0.089±0.02, respectively. Statistically, there were no significant differences (p value = 0.701) in the OD value of in vitro biofilm from poor OH samples treated with spent medium from good OH samples with and without KNO_3.

TPC results show that the number of colonies of in vitro biofilm from a poor OH sample treated with spent medium from a good OH sample that contained KNO_3 was higher than those without KNO_3. The TPC value (CFU/ml) of in vitro biofilm with and without KNO_3 was 1471.43±763.07 and 731.25±2192.57, respectively. There were no significant differences (p = 0.562) in the cell viability of in vitro biofilm from poor OH treated with spent medium from good OH sample with and without KNO_3.

The mass of in vitro biofilm from poor OH isolate that had been treated with spent medium from good OH individual that contained KNO_3 was higher than that without KNO_3. Nitrate ion supports the reduction process of nitrate, which results in NO that has a role in the biofilm dispersion process, while potassium ion contributes as a nutrient source for the living cells to maintain their physiological function, which can affect the process of biofilm formation.[11] The number of colonies of in vitro biofilm from poor OH individuals treated with spent medium from good OH individuals that contained KNO_3 was higher than those without KNO_3. K^+ and NO_3^- ions may increase NO metabolites that can be used as nutrient sources for the cell.

The limitation of this study was the lack of identification of bacterial species that may contribute to the metabolites production, as well as the bacterial species that were involved in the changes of cell viability and mass of in vitro biofilm. Furthermore, this study was performed with two replications, which might cause less homogeneous and representative results.

CONCLUSION

Based on the above results, it can be concluded that the increasing protein concentration of metabolites from a good OH individual may be able to increase the mass of the in vitro biofilm of the bacterial isolate from a poor OH individual. However, the increase in nitrate concentration of metabolites from a good OH individual was able to decrease the cell viability of the in vitro biofilm of the bacterial isolate from a poor OH individual. Moreover, nitrate ions in the metabolites of good OH individuals may also increase the cell viability and mass of the in vitro biofilm of the bacterial isolate from poor OH individuals.

Further evaluation should be performed by using a more replication method so that the mean value could be more homogeneous and representative. Besides, protein profile assay may be performed to evaluate the protein profile of metabolites from a good OH individual that affect cell viability and mass of in vitro biofilm. On the other hand, the species of microorganism that grows in a specific medium may be observed by using Real Time-Polymerase Chain Reaction (RT-PCR).

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