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(Research Paper)

Evaluating the impact of Leech Saliva on *Streptococcus mutans* and *Streptococcus sobrinus*

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Abstract

Leech saliva, containing over 100 biologically active proteins, includes the antimicrobial protein destabilase. Functioning in a manner similar lysozyme, destabilase degrades bacterial cell walls. Tooth decay, caused by *Streptococcus mutans* and *S. sobrinus*, necessitates their elimination for prevention and treatment. The effect of leech saliva from different groups on *S. mutans* and *S. sobrinus* was investigated. Saliva samples were collected from three leech groups salivated at one, two and three months after feeding. Additionally, the antibacterial properties of destabilase were investigated through bioinformatics and molecular docking. Our study revealed an increase in salivary protein concentration with the duration of starvation, reaching 769 µg/ml after three months. Among the various samples, the saliva concentration of leeches that had undergone three months of starvation exhibited the most potent antibacterial properties. Specifically, it exhibited an antibacterial percentage of 35.3% against *S. mutans* and 42.6% against *S. sobrinus* at a concentration of 2 mg/ml. The concentration of 2 mg/ml in the three-month saliva sample also exhibited the highest anti-biofilm percentage against both bacteria, with values of 31.6% for *S. mutans* and 44.2% for *S. sobrinus*. Given the global challenge of tooth decay, leech saliva especially destabilase, shows potential for preventing and treating this common dental condition. A significant concentration of destabilase, an antimicrobial protein present in the saliva of leeches, was identified. Also, a novel strategy was proposed to inhibit the formation of biofilms on *S. mutans* and *S. sobrinus*, which are commonly associated with tooth decay.

Keywords: Leech saliva, Destabilase, Bioinformatics, Tooth decay.

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1. Introduction

Leech therapy, a complementary treatment that uses leeches, has been shown to be effective in various fields. This treatment involves medicinal leeches from various species, with the most commonly used being *Hirudo medicinalis*, which extracts blood and delivers saliva with therapeutic properties. It is effective in treating a range of conditions such as cardiovascular diseases, diabetes complications, deep vein thrombosis, tinnitus, acute and chronic ear infections, as well as in plastic, restorative, and microsurgical purposes. Other species of leeches such as *Hirudo orientalis*, *H. verbana*, and *H. manilensis* are also used for medical purposes (1, 2).

The leech that lives in Iran with the scientific name *H. orientalis* is one of the medicinal leech species that looks similar to *H. medicinalis*, and systematic molecular studies also confirm this issue (3). The differences between these two species are in the distribution of pigments on the dorsal and ventral surfaces (4). Leeches are naturally distributed across most Iranian provinces owing to their four seasons. The Caspian Sea shoreline provides the most suitable habitat for this species due to favorable weather conditions (3).

During the feeding process (blood-feeding), leeches secrete a complex mixture of bioactive and medicinal substances in the wound (5). There are many proteins in leech saliva, but only a few of them have been identified (6). The presence of more than 100 proteins with a molecular weight between 10 and 97 kilodaltons has been detected in leech saliva. Studies have shown that *H. medicinalis* and *H. orientalis* are closely related in terms of the composition of saliva secreted from the salivary glands, which is consistent with the data on the phylogenetic relationship between these two medicinal leech species (7). Out of the more than 100 bioactive substances found in leech saliva, over 20 proteins have been identified. These proteins have various biological functions such as dilation of blood vessels, inhibition of platelet aggregation, anti-coagulation, anti-inflammatory, analgesic, and antimicrobial activities (8).

Leech saliva's antimicrobial properties make it valuable for treatment. So far, several proteins have been isolated from leech saliva that are responsible for this function, the most important of which is called destabilase (1). Destabilase is a multifunctional protein with fibrinolytic, thrombolytic, muramidase, isopeptidase, and antibacterial activities. The molecular weight of this protein was measured by an electrophoresis device to be 12.3 kilodaltons (9). Until now, three isoforms of this enzyme have been identified, which are coded by a family consisting of three genes, Ds1, Ds2, and Ds3. The characteristics of each of the isoforms have not been described in a pure form because destabilase can only be isolated from leech saliva as part of a complex with other proteins. Moreover, its synthetic production is inefficient. The degree of homology between different isoforms of destabilase is between 66 and 87% (10).

After searching the analog of destabilase protein the available data showed a clear homology between residues 2–16 of destabilase and 4–19 of *Asterias rubens* lysozyme, which led to the question of whether destabilase has lysozyme activity (11). Destabilase protein has glycosidase activity, which is very similar to egg white lysozyme, lysozyme found in human saliva, and tear fluid. Destabilase digests beta 1 to 4 glycosidic bonds in peptidoglycans in the bacterial cell wall and induces bacterial cell lysis. This activity is present in all three protein isoforms. The conducted studies showed that the antimicrobial activity of destabilase is not only related to the glycosidase enzyme activity because the lysozyme activity in the protein is sensitive to heat and depends on the proper folding of the molecule. However, it seems that there is a second antimicrobial function associated with this protein, which is related to the non-enzymatic components of destabilase. Even in its denatured form shows a dose-dependent bacteriostatic effect on *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Escherichia coli* (1, 9).

The use of leeches in dentistry has also been reported. In 1817, Thomas Bell treated oroantral fistula, which was accompanied by swelling of the face, by placing six leeches on the patient's face. In general, leeches have been used to treat toothache, gingivitis, and tooth abscesses. This solution provided rapid pain relief (11, 12). Tooth decay is

known as one of the most infectious diseases worldwide that affects all ages, ethnicities and genders. Accumulation of bacteria on the tooth surface (dental plaque biofilm) is the main precursor of caries. The properties of bacteria in biofilm are unique, therefore the effect of antibacterial agents may be different between immobile bacteria in biofilm and bacteria in suspension (13).

The main bacteria responsible for tooth decay are typically referred to as mutans streptococci. *Streptococcus mutans* and *S. sobrinus* are among the main bacteria that are active in tooth decay. *S. mutans* exists in the oral flora and is capable of fermenting carbohydrates into organic acids. These acids can decrease the pH of the mouth and increase the solubility of tooth enamel ultimately leading to tooth decay (14). Also, these bacteria produce large amounts of polysaccharides such as dextran and levan from sucrose, which play a very important role in tooth decay (15). The presence of *S. mutans* in dental caries is more common than in *S. sobrinus* (14).

Due to the presence of various compounds with many applications in leech saliva, many researchers have conducted studies on the properties and characteristics of leech saliva. Considering the importance of preventing and treating tooth decay, in this research we intend to investigate the effect of leech saliva, which contains a protein with lysozyme-like properties, on *S. mutans* and *S. sobrinus* bacteria, which are one of the main causes of tooth decay.

2. Materials and methods

2.1. Feeding and maintenance of leeches in the laboratory

A total of 30 specimens of the cosmetic leech species *H. orientalis* were acquired for the study. These leeches were subsequently housed in plastic containers made of polyethylene terephthalate (PET) and filled with chlorine-free water (16). The containers were then placed in an incubator with adjustable temperature control, set at 25 °C to facilitate leech acclimatization prior to feeding (17).

Throughout this acclimation period, the water in the containers was replaced every two days to maintain optimal conditions for the leeches. Sterile blood was used as the feeding source for the leeches, and the feeding sessions were carefully prepared and monitored.

2.2. Categorization of leeches

In our study, three cohorts, each comprising five leeches, were investigated. Subsequent to their feeding, the first group was observed after one month, the second after two months, and the third one after three months. The leeches were housed in a controlled laboratory environment, and samples of their saliva were collected. To maintain leech viability, the temperature was systematically decreased by 1 °C each day until reaching the optimal temperature of 18 °C, at which point they were maintained. Furthermore, two large 3-liter containers of chlorine-free water were used for their habitat.

2.3. Leeches' salivation

To collect saliva from leeches, a method involving the stimulation and elevation of their digestive system was implemented. Initially, 0.05 grams of menthol powder was dissolved in 100 ml of water in a beaker. A leech was then introduced into the beaker containing the menthol solution, where it was left for a period of 15 to 20 seconds. Subsequently, the leech was transferred to another flask containing 20 ml of physiological serum to facilitate the secretion of saliva. This process was repeated sequentially for each of the five leeches in the group, with each leech being moved to a separate tank once its saliva was collected. It should be emphasized that the use of menthol-induced stimulation for leech saliva extraction was a novel approach and was employed for the first time in this study.

After obtaining saliva samples from all leeches, the physiological serum mixed with the leeches' saliva was transferred to a 50 ml falcon and centrifuged at 4 °C and 8241 g for 10 min (18). The resulting supernatant was then passed through a 0.45 µm syringe filter to ensure purity. The filtered saliva was frozen at −20 °C for 24 h, and then lyophilized using a freeze dryer at −40 °C under vacuum. The

resulting lyophilized saliva was stored at −20 °C for future applications. Additionally, a portion of the filtered liquid was kept at 4 °C in a vial for protein quantification using the Bradford assay.

2.4. Bradford protein assay

To prepare Bradford test reagent, begin by weighing out 10mg of Coomassie Brilliant Blue dye and placing it in an Erlenmeyer flask. Next, mix the dye by adding 5 ml of 95% ethanol, followed by the gradual addition of 10 ml of 85% phosphoric acid. Ensure that the dye is completely dissolved before increasing the volume of the solution to 100ml with distilled water.

Due to the light sensitivity of Coomassie Brilliant Blue dye, it is crucial to prepare the reagent in a dark room and store it in an opaque container to prevent degradation. The prepared solution can be stored at room temperature for up to one month, but for longer-term storage, it should be refrigerated at 4 °C. These steps will ensure the stability and reliability of Bradford test reagent for accurate protein quantification in biochemical assays (19).

2.5. Sketching the standard Bradford curve

To prepare a standard calibration curve, a precise measurement of 1 mg of Bovine Serum Albumin (BSA) was dissolved in 1 ml of deionized water. Subsequently, 12 protein concentrations were meticulously prepared by diluting the basic BSA solution with varying amounts of deionized water, resulting in concentrations spanning from 0 to 1 mg/ml.

Using a 96-well plate, 10 µl of each protein concentration was carefully dispensed into individual wells, with three replicates for each concentration. Following this, 200 µl of Bradford reagent was added to each well, and the absorbance was measured at a wavelength of 595 nm using a multimode reader (20). The average absorbance value from the three replicates for each concentration was utilized to construct a standard curve, which graphically represents the relationship between the concentration of proteins and their optical absorption (Figure 1).

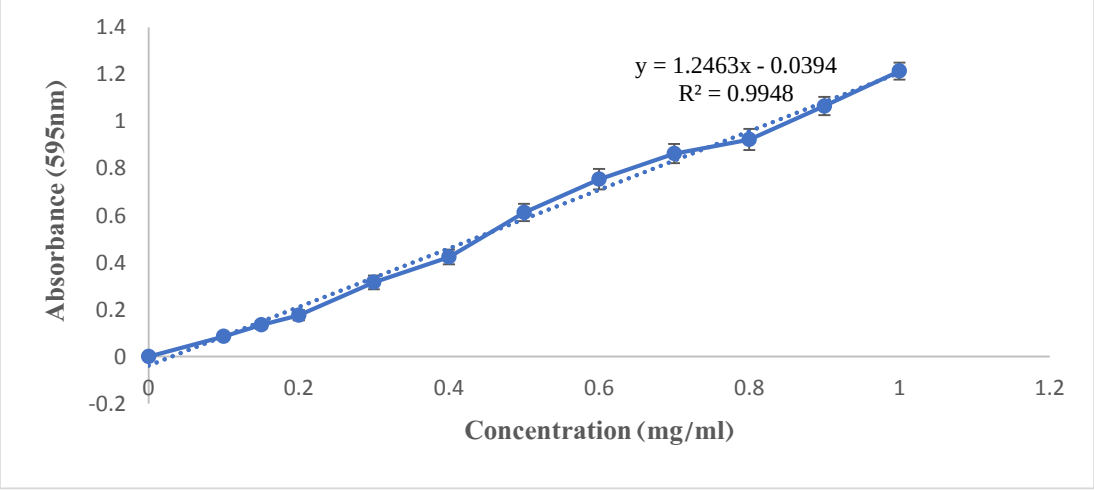

Figure 1. Bradford assay standard curve.

2.6. Measurement of the proteins in leech saliva

Following the salivation stage, the solution underwent filtration to determine the concentration of leech saliva proteins. 10 µl samples were transferred to the wells of a 96-well plate with six replicates. Then, 200 µl of Bradford reagent was added to each well, and the absorbance was measured at a wavelength of 595 nm. The average absorbance of six replicates of each sample was used to calculate the protein concentration of that

sample. By drawing a standard curve using the obtained line equation, the protein concentration of each leech saliva sample was ultimately determined (21).

2.7. Measuring the antibacterial properties of leech saliva using optical absorption

The strains of *S. mutans* and *S. sobrinus*, identified as PTCC1683 and PTCC1601, respectively, were obtained from the Scientific and Industrial Research Organization of Iran. They were meticulously prepared and stored at -80 °C for preservation. The antibacterial properties of leech saliva were investigated by using a Muller Hinton culture medium (22).

Initially, an 18-h culture of *S. mutans* and *S. sobrinus* bacteria was cultivated in Mueller-Hinton broth, after which a 0.5 McFarland standard was prepared from the bacterial suspension. Subsequently, various concentrations (0.25, 0.5, 1, and 2 mg/ml) of lyophilized saliva from four types of leeches were prepared. Then, 100 µl of the different saliva concentrations from the three groups of leeches were dispensed into the wells of a 96-well plate in triplicate. Each well then received 100 µl of the 0.5 McFarland bacterial suspension, and the mixture was thoroughly pipetted to ensure a proper combination of saliva and bacteria. Positive control wells were included to monitor bacterial growth, with 100 µl of 0.5 McFarland suspension used in triplicate for this purpose. Additionally, negative control wells containing 100 µl of the culture medium in triplicate were employed to confirm the absence of contamination. After inoculation, the plates were incubated at 37 °C. Following a 24-h incubation period, the optical absorbance of the wells was measured at a wavelength of 630 nm using an ELISA reader under sterile conditions. The collected data was then analyzed utilizing Excel software to assess the impact of leech saliva concentrations on bacterial growth.

2.8. Biofilm assay

A nutrient-broth medium was used to prepare a bacteria culture, with a McFarland standard of 0.5 established. The study utilized 48 plates to evaluate biofilm and experimental feasibility. Each well was inoculated with 400 µl of 0.5 McFarland bacterial suspension and 100 µl of leech saliva prepared at final concentrations of 0.25, 0.5, 1, and 2 mg/ml within a total volume of 500 µl. To provide the bacteria with suitable nutritional conditions for biofilm formation, lyophilized saliva was dissolved in a sterile liquid culture medium. The positive control involved adding 400 µl of 0.5 McFarland bacteria with 100 µl of culture medium, while the negative control consisted of pouring 500 µl of culture medium into the wells. To increase the test accuracy, each saliva sample and the positive and negative controls were poured into the wells with three repetitions. The plates were then placed in an incubator shaker at 80 revolutions per minute and heated for 48 h at 37 ˚C. After 48 h, the bacterial biofilm was stained and measured.

2.8.1. Bacterial biofilm staining and assay steps

The initial step in the experimental procedure involved emptying and washing the wells three times with phosphate buffer to remove any planktonic cells present. Then, the plates were stabilized by incubating them in a 60-degree oven. Subsequently, 250 µl of 0.1% crystal violet dye was added to each well and allowed to incubate for 10 minutes. The plates were then washed, and 400 µl of 95% ethanol was added to each well and left at room temperature for 30 minutes. The ethanol solution containing crystal violet was then transferred from each well and pipetted into corresponding wells on another plate.

Subsequently, the optical absorbance of the wells was measured at a wavelength of 595 nm to analyze the collected data. To determine the inhibition percentage of each saliva sample, the following equation was applied:

$$\text{Biofilm inhibition (\%)} = \frac{(\text{OD of untreated control}) - (\text{OD of treated sample})}{\text{OD of untreated control}} \times 100$$

This method allowed for the quantification of the inhibitory effects of the saliva samples on the experimental system, providing valuable insights into their potential antimicrobial activities (23).

2.9. SEM imaging of tooth surface biofilm

A Scanning Electron Microscope (SEM) technique was utilized to examine the bacterial biofilm formation on tooth surfaces with and without leech

saliva. Several healthy molars were collected from dental clinics in Isfahan city. As dental plaques form on the enamel surface of teeth in the oral cavity, the tooth enamel surface was used to simulate these conditions during the experiment. The Dental Materials Research Center cut the collected teeth using a toothed cutter. Incisions were made therefore, only tooth enamel was included. The tooth slices were autoclaved to sterilize and added to the wells under sterile conditions. The remaining steps were similar to the biofilm test before staining.

2.9.1. Sample preparation for SEM imaging

After 48 h, the contents of the wells were removed using a sampler to prepare the sample for SEM imaging. To remove the planktonic cells, phosphate buffer was added to the wells three times using a sampler and then removed. Next, the dental slices were then fixed with a 2.5% glutaraldehyde solution for 3 h to stabilize the biofilm on the tooth surface. To dehydrate the biofilm, 30%, 50%, and 70% ethanol solutions were prepared and each was added to the wells containing tooth clippings for 10 min. Finally, 96% ethanol was added to the wells in two 10-min periods. After removing the ethanol solution, the dental slices were placed under sterile conditions in a hood for 24 h to dry (24).

2.10. Inspecting the antibacterial properties of destabilase using molecular docking

2.10.1 Collecting the 3D structure of destabilase and its ligand

The three-dimensional structure of the destabilase protein in leech saliva has not yet been reported in the Protein Data Bank (PDB). Therefore, it is important to predict its tertiary structure. To achieve this aim, the protein sequence was extracted from the NCBI database, and the I-TASSER server was used to predict the protein tertiary structure. As previously mentioned, destabilase has lysozyme-like activity. Lysozyme is an enzyme that hydrolyzes beta 1 to 4 bonds between N-acetylmuramic acid and N-acetylglucosamine in the peptidoglycan of bacteria cell walls (25). Hence, a NAM-NAG-NAM trisaccharide was employed as a ligand for docking. The complex structure of this ligand, which was previously bound with lysozyme in PDB, was preserved for further analysis.

2.10.2. Preparation of the structure of protein and its ligand for docking

The ligand structure received from Gallus gallus (PDB ID: 9LYZ) was found to be associated with multiple non-relevant chains. To isolate the desired structure, we utilized ConTEXT software, a text editor, to remove these superfluous chains. The edited structure was then saved in PDB format for further analysis.

2.10.3. Molecular docking

The protein-ligand docking process was conducted using the SwissDock server. Firstly, the 3D structure of the destabilase protein was predicted and optimized in PDB format along with the ligand structure. This file was then uploaded to the server, and a docking request was initiated. Finally, the results obtained from the interaction between the protein and the ligand were analyzed. To visualize the docking results and protein-ligand interactions, BIOVIA Discovery Studio software was used.

2.11. Data analysis

Using the data obtained from the results of the tests, the corresponding graphs were plotted in the Excel program, and the average and standard deviation were calculated for all the data. SPSS statistical analysis software and one-way analysis of variance were used to compare the data and determine the significance of their differences. Also, the significant difference in average data was considered at $P < 0.05$ level.

3. Results and discussion

3.1. Concentration of leech saliva proteins

Utilizing the graph generated and the equation derived from Fig 1, the protein concentration in the saliva of various leech groups can be accurately quantified. Fig 2 illustrates a positive relationship between the hunger levels of leeches and the concentration of saliva samples, indicating that as hunger intensifies, so does the protein concentration in the saliva.

3.2. Antibacterial effect of leech saliva

Fig 3 and 4 show the growth chart of *S. mutans* and *S. sobrinus* respectively. Analysis of these graphs reveals that the peak growth of bacteria occurred at 24 h post-incubation. Subsequently, as a result of nutrient depletion and bacterial accumulation, there was a gradual decline in bacterial growth.

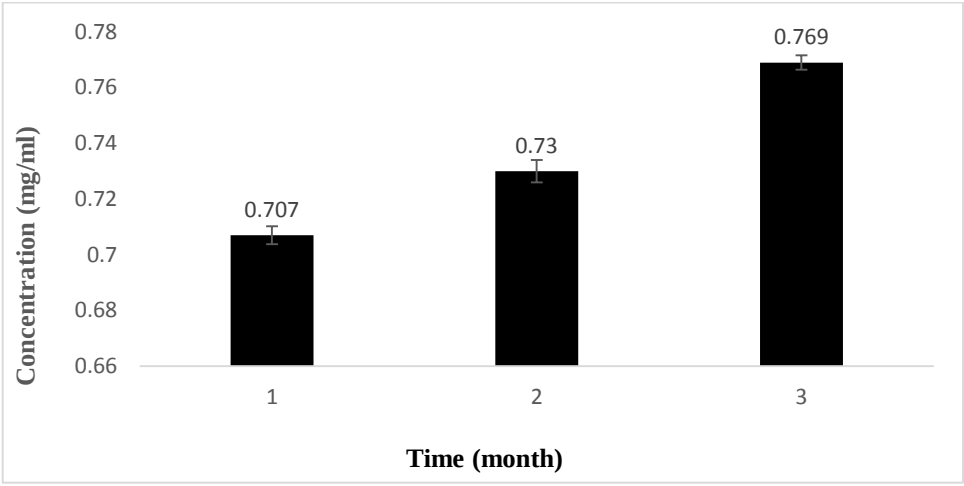

Figure 2. Concentration of saliva samples. A significant difference (P<0.05) was observed in these concentrations among the time points.

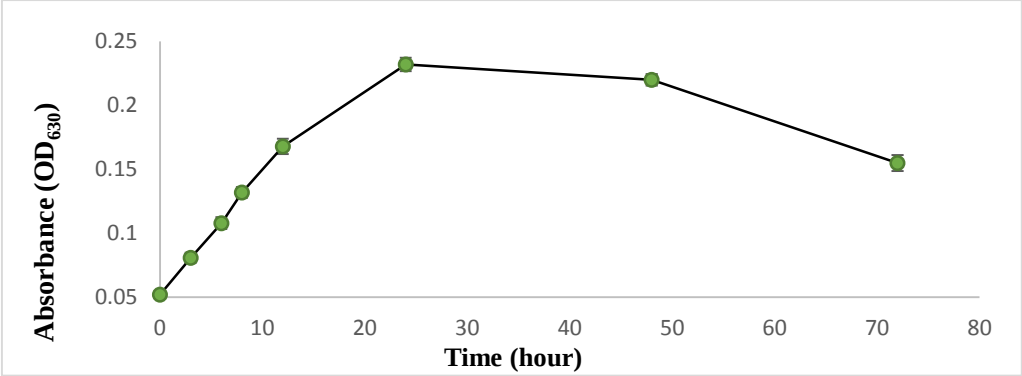

Figure 3. Growth curve of *S. mutans*.

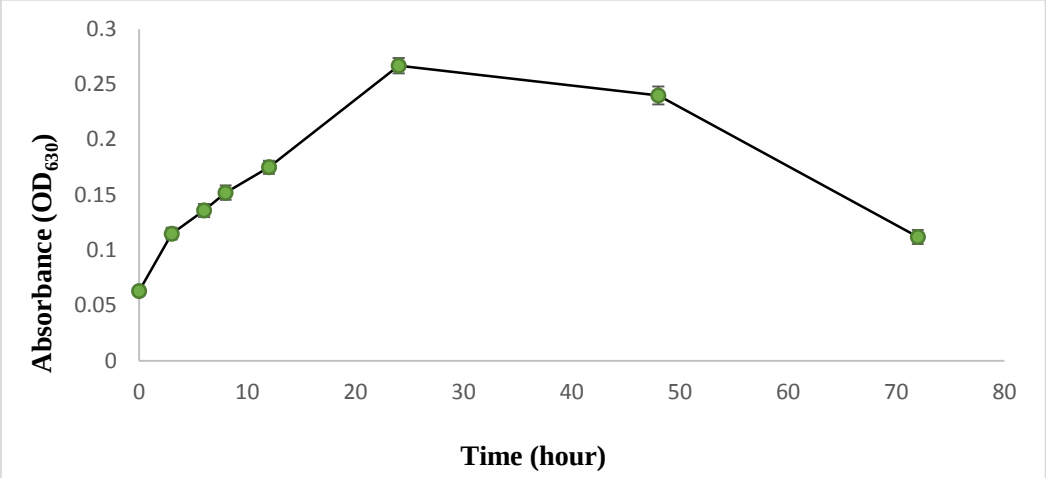

Figure 4. Growth curve of *S. sobrinus.*

In Fig 5 and 6, the results of calculating the antibacterial percentage of different saliva samples are presented. Notably, an increase in the starvation period of leeches corresponds to a higher antibacterial effect. Furthermore, it was observed that the antibacterial activity increased with the concentration of saliva in each sample. Specifically, the sample containing 2 mg/ml of salivary secretions from leeches starved for three months exhibited the highest antibacterial percentage compared to other samples, demonstrating values of 35.3% for *S. mutans* and 42.6% for *S. sobrinus.*

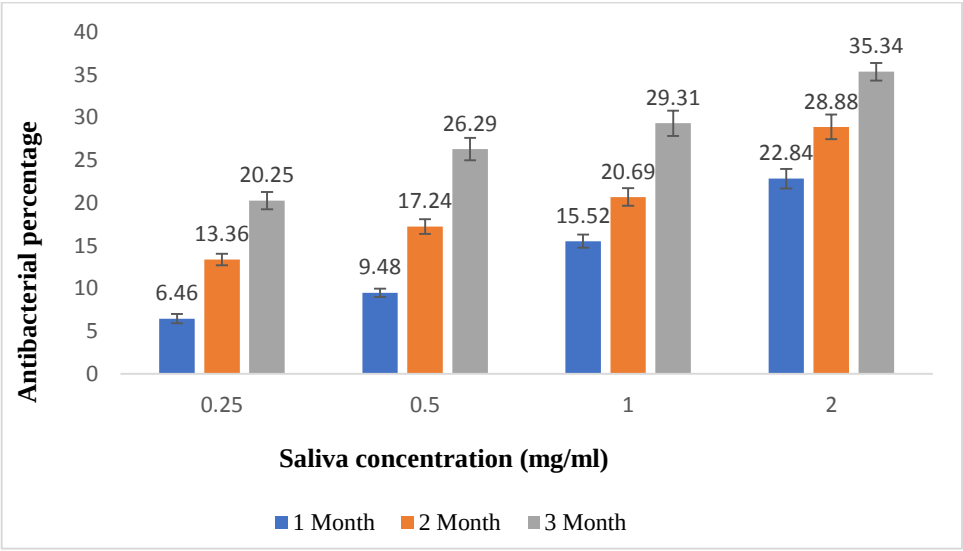

Figure 5. Antibacterial effect of different leech saliva samples on *S. mutans.* A significant difference (P<0.05) was observed in the antibacterial percentage values among different saliva concentrations across all samples.

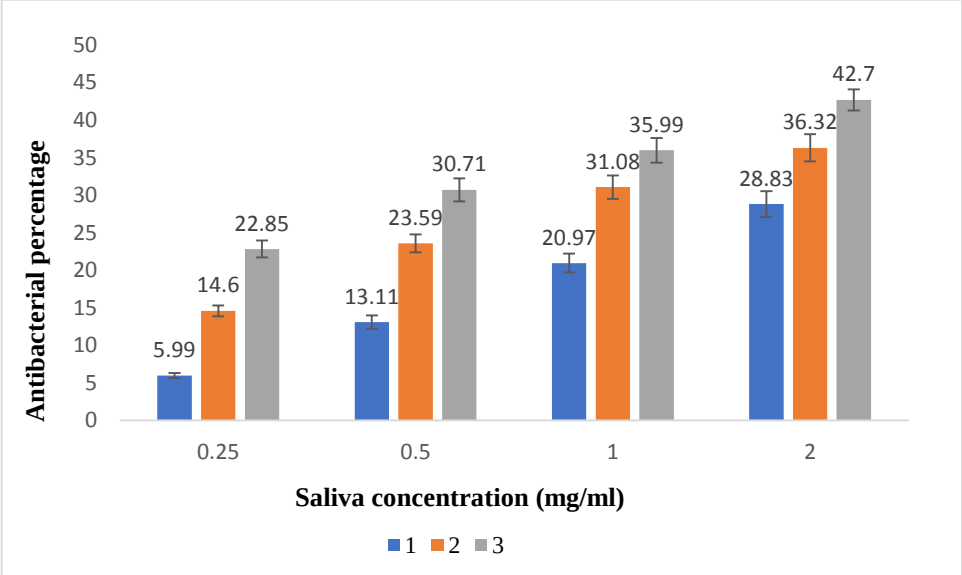

Figure 6. Antibacterial effect of different leech saliva samples on *S. sobrinus.* A significant difference (P<0.05) was observed in the antibacterial percentage values among different saliva concentrations across all samples.

3.3. Antibiofilm effect of leech saliva

Fig 7 and 8 illustrate the inhibitory effect of various saliva samples on the biofilm formation by *S. mutans* and *S. sobrinus*, respectively. The anti-biofilm activity of each sample demonstrates a positive correlation with increasing concentration. The most significant anti-biofilm percentage observed for both bacterial strains is associated with a concentration of 2 mg/ml from the three-month saliva sample, exhibiting values of 31.6% against *S. mutans* and 44.2% against *S. sobrinus*.

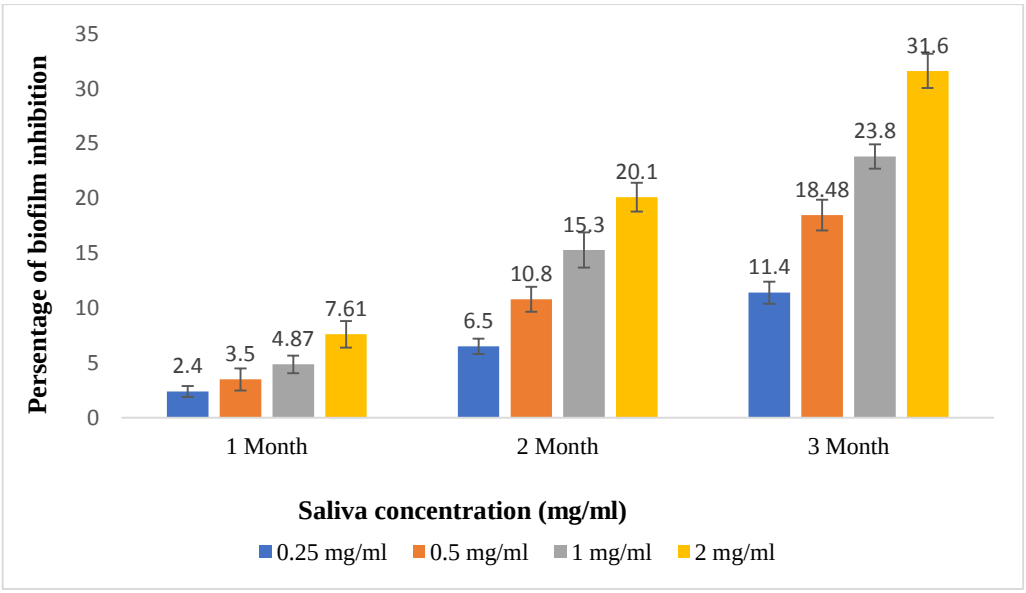

Figure 7. Antibiofilm effect of different leech saliva samples on *S. mutans.* A significant difference (P <0.05) was observed in the percentage of inhibition among different saliva concentrations across all time points.

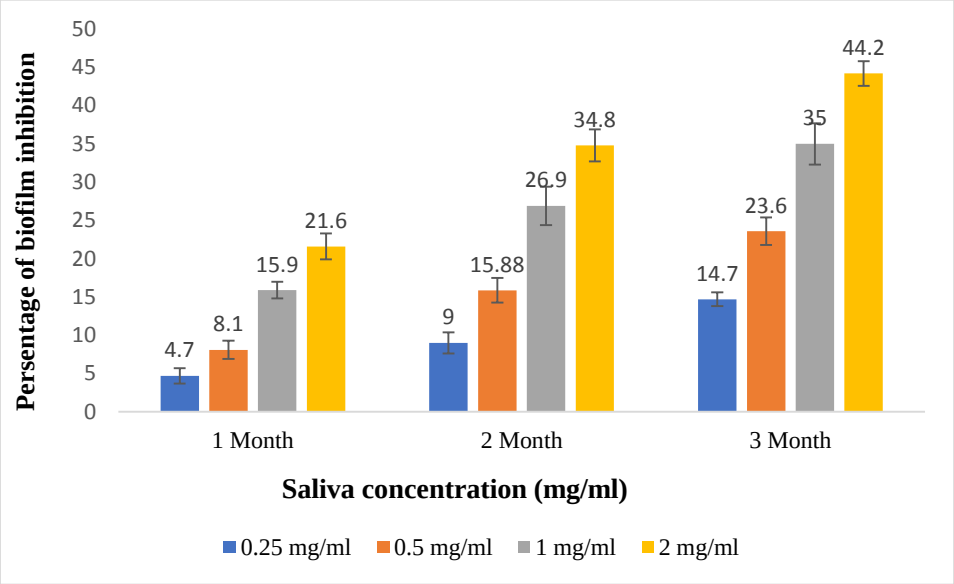

Figure 8. Antibiofilm effect of different leech saliva samples on *S. sobrinus.* A significant difference (P <0.05) was observed in the percentage of inhibition among different saliva concentrations across all time points.

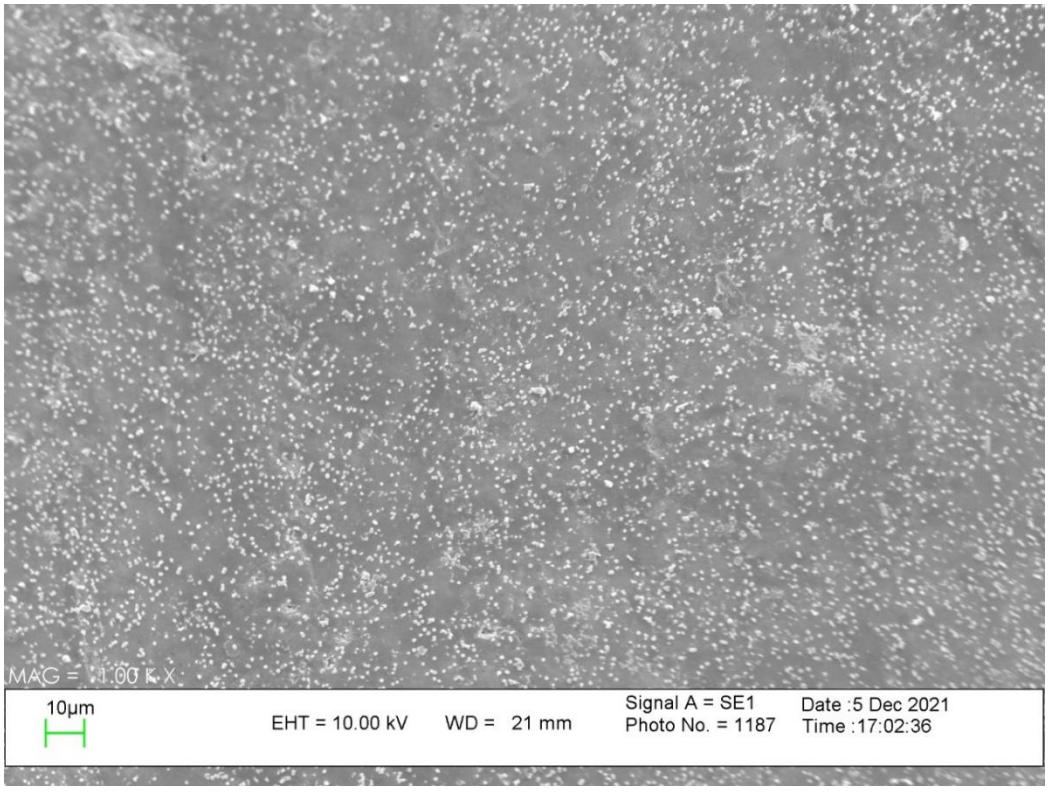

Figure 9. SEM image of *S. sobrinus* biofilm formation on tooth surface.

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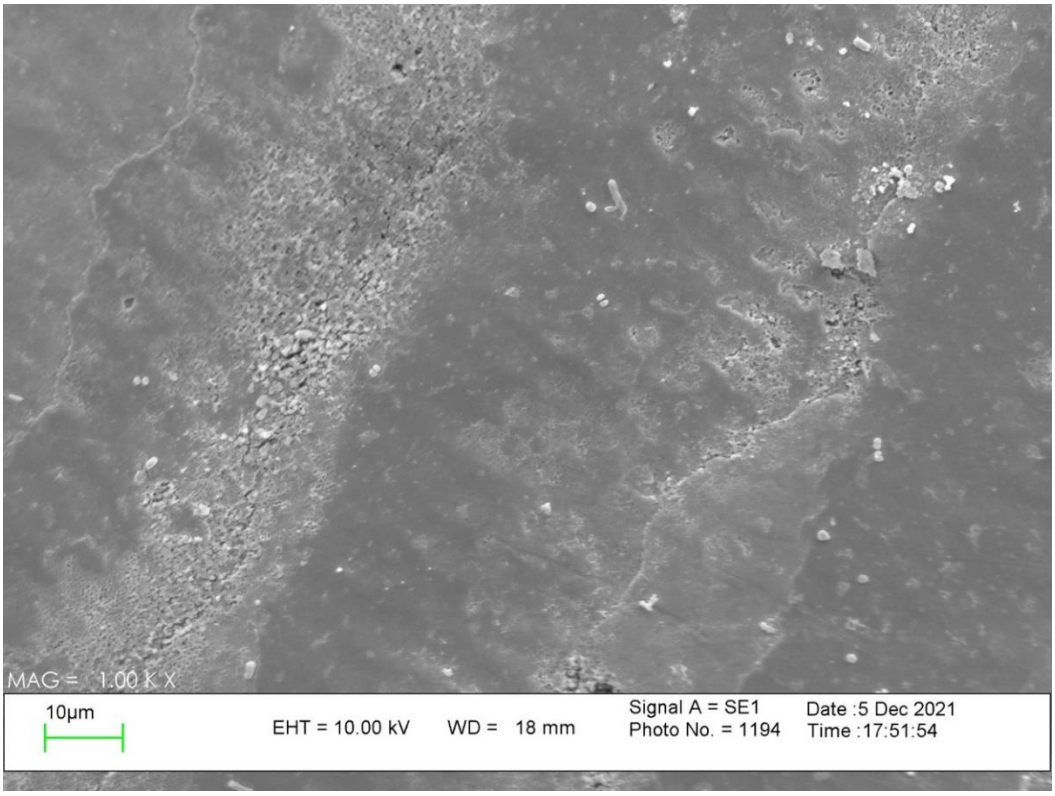

Figure 10. SEM image of *S. sobrinus* biofilm formation on the tooth surface in the concentration of 2 mg/ml of three-month saliva.

Based on the biofilm test results, it was found that the concentration of 2 mg/ml of saliva collected over three months showed the highest percentage of inhibition in biofilm formation on *S. sobrinus*. Consequently, this specific sample and concentration were selected for SEM imaging. Fig 9, the SEM image shows the control sample showing *S. sobrinus* biofilm formation on the tooth surface. In contrast, Fig 10 displays the SEM image of *S. sobrinus* biofilm formation on the tooth surface in the presence of 2 mg/ml leech saliva collected after three months of starvation. A comparison of these images indicates a significant decrease in the ability of *S. sobrinus* to form biofilms in the presence of leech saliva.

3.4. Results related to bioinformatics studies

The protein sequence of destabilase from *H. medicinalis*'s saliva was retrieved from the NCBI database (accession number: AAA96144). This protein consists of 136 amino acids and is represented in FASTA format as follows:

>AAA96144.1 destabilase I

MIIAIYVSLALLIASVEVNSQFTDSCLRCICKV
EGCDSQIGKCGMDVGSLSCGPYQIKKPYWID
CGKPGGGYESCTKNKACSETCVRAYMKRYG
TFCTGGRTPTCQDYARIHNGGPRGCKSSATV
GYWNKVQKCLR

The I-TASSER server generates numerous structural models for a given sequence but reports a maximum of five models. Typically, the first model exhibits the best quality. In the case of destabilase, the first model was selected for subsequent docking studies. Fig 11 displays the initial model suggested by I-TASSER, along with the structure of the ligand employed in the analysis.

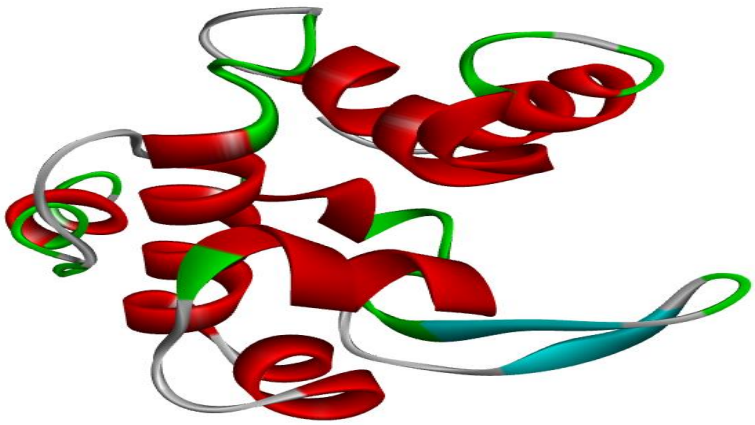

Figure 11. 3D structure of the destabilase protein proposed by the I-TASSER server.

3.5. Molecular docking results

Recent research indicates that the docking energy between destabilase and its ligand performs optimally, with a value of -308.71 kcal/mol. These results strongly support the idea that destabilase has antibacterial properties, consistent with previous laboratory experiments. It seems that both the binding energy and the ligand are well-matched for interacting with the bacterial cell wall. Interestingly, Fig 12 provides a visual representation of the docking between the destabilase ligand.

Using BIOVIA Discovery Studio software, docking analysis was performed to explore the interaction between a destabilase protein and its ligand. Our findings revealed a hydrogen bond formation involving arginine 136, cysteine 134, and glutamine 132 of destabilase during the docking. Fig 13 provides additional details on other amino acids participating in this interaction.

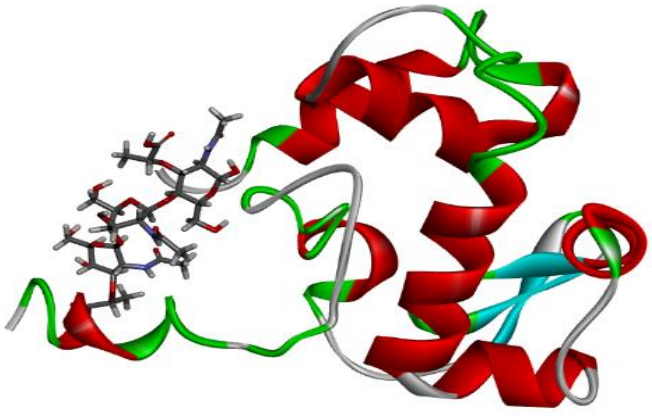

Figure 12. The docking of the destabilase ligand.

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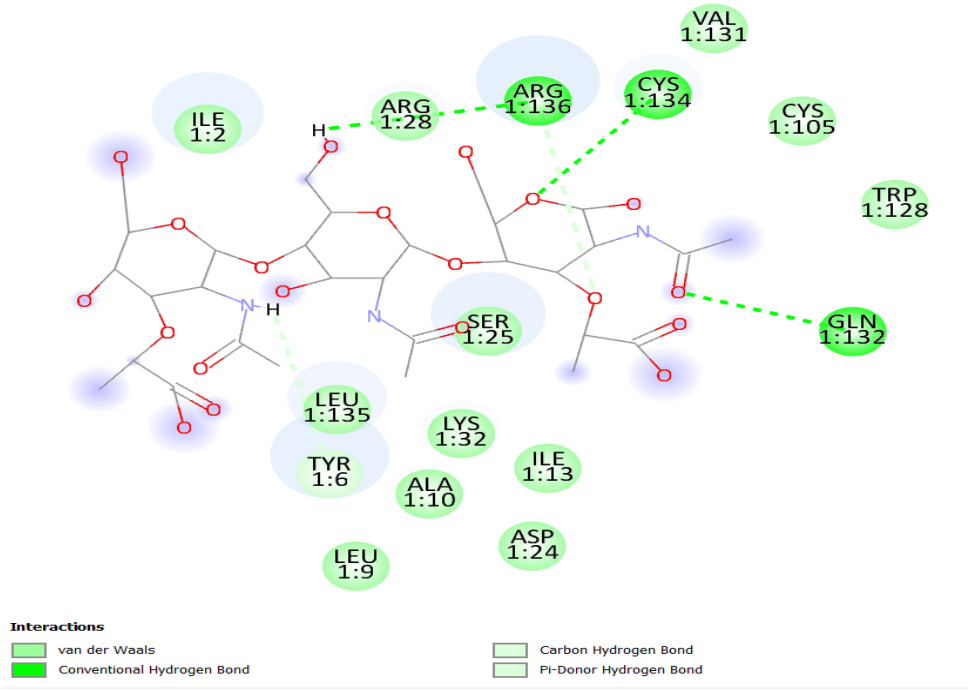

Figure 13. A two-dimensional view of the interaction between destabilase and ligand, highlighting the involved amino acids.

4. Discussion

In a study conducted in Malaysia focusing on indigenous leech species, it was observed that following 20 weeks of starvation, the protein concentration in the crude saliva extract measured at80 µg/ml, which subsequently decreased to 62.6 µg/ml after 23 weeks (26). In contrast, our research revealed a consistent increase in saliva protein concentration to the duration of starvation, recording concentrations of 707 µg/ml after one month, 730 µg/ml after two months, and 768 µg/ml after three months. These findings provide valuable insights into the physiological response of leeches to hunger and underscore the importance of further investigation into this intriguing phenomenon. Alaama *et al.* demonstrated the synthesis of high protein concentrations (105-91 µg/mL) in leech saliva is influenced by the duration of fasting, with the highest concentrations observed after a fasting period of 12-15 weeks. Low protein concentrations are associated with absent bands in the electrophoretic analysis of the saliva. Additionally, leeches can regain approximately 42% of their original protein concentration within four weeks of fasting following their first meal. Therefore, it is evident that the duration of starvation plays a crucial role in determining the protein concentration of leech saliva, which in turn affects its therapeutic effectiveness (27).

Abdualkader *et al.*, conducted a study aimed at evaluating the antibacterial effect of leech saliva on five different bacterial strains: *Bacillus cereus*, *S. aureus*, *P. aeruginosa*, *E. coli*, and *Salmonella typhi*. Utilizing antibiogram discs and preparing dilutions, the researchers determined the minimum concentration required to inhibit bacterial growth. Results indicated that the extracted saliva effectively inhibited the growth of *S. typhi* and *E. coli*, albeit with smaller inhibition zones compared to conventional antibiotics such as ciprofloxacin. Through dilution of leech saliva, the researchers were able to inhibit the growth of *S. aureus* and *E. coli*. Overall, the study suggested that leech saliva shows promise as an antibacterial agent (26). The in vitro conditions have shown that the growth of *S. aureus* can be effectively inhibited by the saliva of leeches, potentially through a significant reduction in the level of TNF-α (28).

In a study conducted by Ojo *et al.*, it was found that the salivary extract of leeches contains a significant amount of oleic acid (33.9%) and

palmitic acid (22.6%). This fatty acid profile was shown to possess strong anti-tubercular properties and bactericidal effects against *Mycobacterium tuberculosis* at a concentration of 50% (v/v) (29). Building upon these findings, Babayi *et al.*, suggest that silver nanoparticles, when combined with leech salivary extract, may synergistically treat infectious diseases caused by *P. aeruginosa* and *Klebsiella* species. This proposed treatment method could be implemented through the agar well diffusion technique (30).

Notably, the present study represents the first investigation into the antibacterial properties of destabilase using molecular docking. A prior study by Pushkaran *et al.* focused on lysozyme, examining the resistance mechanism of *S. aureus* through molecular docking (31). In our current study, we utilized egg white lysozyme and a similar ligand for the docking analysis.

5. Conclusion

The highest protein concentration in leech saliva can be obtained by collecting it three months after feeding the leeches. Leech saliva possesses antibacterial properties and can prevent the formation of bacterial biofilms. The increase in protein concentration in saliva is directly correlated with the strength of these properties. Studies have shown that leech saliva can inhibit the growth of *S. mutans* and *S. sobrinus*, major contributors to tooth decay. Additionally, leech saliva is effective in preventing the formation of biofilms

Several proteins in leech saliva contribute to its antibacterial properties, with destabilase standing out for its lysozyme-like activity against bacteria. Bioinformatics research suggests that destabilase plays a significant role in reducing bacterial growth and preventing biofilm formations. Given the prevalence of tooth decay, leech saliva and its destabilase component could potentially serve as a promising solution for preventing and treating this common dental issue.

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