ORIGINAL ARTICLE

Quantitative and qualitative analysis of goat's milk antibiofilm against *Streptococcus mutans* and *Candida albicans*: a laboratory experiment

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Received: 13 February 2025
Revised: 24 June 2025
Accepted: 01 July 2025
Published: 31 July 2025
DOI: 10.24198/pjd.vol37no2.61674

p-ISSN 1979-0201
e-ISSN 2549-6212

Citation:
Chismirina, S., Vernanda, E., Dericco D., Sungkar S., Rezeki, S., Said SM. Quantitative and qualitative analysis of goat's milk antibiofilm against Streptococcus mutans and Candida albicans: a laboratory experiment. Padj J Dent, July 2025; 37(2): 131-141

ABSTRACT

Introduction: *Streptococcus mutans* and *Candida albicans* interact in dental plaque biofilm to form a synergistic caries process. As a result, preventing tooth cavities necessitates disrupting the interaction between these two bacteria. This can be accomplished by giving anti-biofilm substances such as goat's milk, which contains lactoferrin, lactoperoxidase, and lysozyme. *Candida albicans* in dental biofilms promotes more violent caries than biofilms generated only by *Streptococcus mutants*. This study aimed to analyze the antibiofilm of goat's milk against *Streptococcus mutans* and *Candida albicans* biofilm masses quantitatively and qualitatively. **Methods:** The type of study used was an experimental laboratory with a Post-Test Control Group Design. The study was conducted utilizing the Biofilm Coverage Rate (BCR) and the Biofilm Assay to asses characteristics of the quantitative concentration of biofilm and the SDS-PAGE technique to observe the biofilm protein composition qualitatively. **Results:** The treatment group's BCR and Biofilm Assay concentrations were 0.45 ± 0.2 lower than the negative control group's 0.78 ± 0.25. Protein profile bands of *S. mutans* and *C. albicans* biofilms exposed to goat's milk ranged from 14.4 to 116 kDa. **Conclusion:** Goat milk exhibits anti-biofilm action against *Streptococcus mutans* and *Candida albicans* biofilms.

KEYWORDS

Biofilm, *Streptococcus mutans*, *Candida albicans*, *Biofilm Coverage Rate* (BCR), protein profile

INTRODUCTION

The primary cause of toothache and tooth loss is recognized to be dental caries, a disease that frequently affects teeth.[1] By preserving the minerals in teeth and managing oral biofilm, this condition can be avoided. Due to the potential for bacterial resistance from improper and prolonged use of antibiotics, efforts to reduce oral biofilm while treating dental caries have not been entirely embraced. As such, numerous studies are still being conducted today to identify substitute

materials for controlling oral biofilms. Utilizing organic animal components, like goat milk, is one such alternative.[2,3]

Planktonic bacteria and fungi form multicellular aggregates called biofilms. Biofilms create microbial communities with intricate three-dimensional structures.[4] Microorganisms form biofilms to defend themselves against antimicrobials and environmental changes (predation), such as chemical exposure, physical stress, and radiation, which increase their pathogenicity.[4,5] Microorganisms can be found throughout the oral cavity, including the tongue, cheeks, palate, gingival sulcus, and tonsils. These microorganisms form a biofilm on the surfaces of the teeth and oral soft tissue mucosa, known as oral biofilm.[6-8]

Streptococcus mutans is one type of bacteria found in oral biofilms.[9,10] It is the primary etiological agent in the production of dental caries, with dental plaque serving as its primary home on the surface of the tooth.[8] The oral cavity fungus *Candida albicans*, which serves as an agent of oral candidiasis infection, grows in part due to the molecular involvement of these bacteria. Due to *S. mutans' capacity* to form acidic environments, colonization of these fungi can rise. Moreover, *S. mutans* also promotes the development of *C. albicans* biofilms in alkaline circumstances. One of the disorders that might result from these two types of bacteria interacting in the mouth cavity is dental caries.[11-12]

For a long time, those who are allergic to cow's milk have used goat's milk instead.[13–15] Goat milk contains various active components, such as lysozyme, lactoperoxidase, and lactoferrin, that have the potential to be used as an alternative antibiotic and antifungal.[13,16–18] Enzymatic hydrolysis of goat's milk yielded many bioactive peptides, including antioxidants, antimicrobial peptides, and angiotensin-converting enzyme (ACE) inhibitors.[18–21] The study was conducted to assess the anti-biofilm agent characteristics of goat's milk against *Streptococcus mutans* and *Candida albicans* biofilms, both quantitatively and qualitatively, using the above description.

METHODS

The laboratory experimental study with a post-test control group design was conducted at the Research Laboratory, Faculty of Dentistry, USK, Banda Aceh. The goat milk was obtained from Etawa crossbreed goats from a milk-producing goat farm in Rumpet village, Barona Jaya District, Aceh Besar. *S. mutans* and *C. albicans* isolates used in this study were clinical isolates, respectively, from patients diagnosed with caries and candidiasis at the Dental and Oral Hospital, USK.

Streptococcus mutans clinical isolates from caries patients were identified microbiologically and molecularly using the PCR method, with primers that tracked the *gtf* gene, and were grown on TYS20B media using the T streak technique. The media contained 200 g sucrose (Merck, Germany), 40 g Trypticase Soy Agar (Merck, Germany), 10 g yeast extract (Gibco™, USA), 5 g Bacto Agar (Oxoid, UK), and 4 mg/0.004 g Bacitracin (200 UI) (Sigma Aldrich, USA). The cultivated media for *S. mutans* was then incubated for 2x24 hours at 37^0C in an anaerobic incubator (Oxoid HP0011A, USA). Apart from that, C. *albicans,* which was also isolated from patients diagnosed with candidiasis and was identified molecularly using a specific primer mixture of PsVIc to detect DNA topoisomerase II, was grown on Sabouraud Dextrose Agar (SDA) (Merck, Germany) media and incubated in an incubator for 24 hours.

Gram staining was used to determine that the colonies grown on each medium were not contaminated. Gram staining began with taking one loop of each isolate and depositing it on a glass slide. Then, the glass was passed over a Bunsen flame on the bottom area where there was no specimen about 10-20 times to connect the two types of bacteria to the glass preparation.[18,22]

Bacterial and fungal colonies mounted on various glass slides were dripped with crystal violet and left for one minute. The slide was then cleaned with running

water for about five seconds. After that, iodine fluid was poured, followed by a minute incubation at room temperature, before rinsing the slide under running water for five seconds. The decolorizer solution was placed on the prepared glass for 8-10 seconds, then it was rinsed again. After that, the specimen was dripped with safranin for one minute before being rinsed under running water. The water-absorbent paper was then used to remove surplus water from the slide. In the final step, the dry glass slide was covered with a cover glass and drizzled with emersion oil before being examined under a microscope.

The *S. mutans* and *C. albicans* suspensions were prepared separately, beginning with the isolation of *S. mutans* and *C. albicans* with a loop needle, then placing them in the respective media in the form of liquid NaCl (Merck, Germany) in the amount required, and finally homogenizing with a vortex. After homogenization, turbidity was measured using McFarland 0.5 (Himedia, India), which is equivalent to the estimated amount of suspension, 1.5 x 10^8 CFU/ml. If the suspension was too turbid, additional NaCl was added to correct it; if the suspension was still less turbid than McFarland, more inoculum was added.[22,23]

The next step was to perform an in vitro anti-biofilm test utilizing the Biofilm Assay technique. This in vitro antibiofilm test was performed on a 96-well microtiter plate (Iwaki, Japan), with each sample from each group repeated three times. The first step was to centrifuge the suspension of *S. mutans* and *C. albicans*, which had been equalized with McFarland 0.5, for 10 minutes at 1000 rpm. The microtiter plate was coated with 200 µl of *S. mutans* suspension and 200 µl of *C. albicans* suspension in each well. The plate was then closed and incubated for 1 day (24 hours). After incubation, bacterial cells that did not form a biofilm on the bottom of the plate were removed.

The plate was then washed with 500 µl of PBS (Merck, Germany) using multi-channel Eppendorf equipment and shaker for 15 minutes. Next, 200 µl of pasteurized goat's milk were put into each well (pasteurization was done using the Low Temperature Long Time (LTLT) method at 63^0C for 30 minutes using a water bath). The plate was then closed and incubated for 24 hours to produce a response between goat's milk and antigens (*S. mutans* and *C. albicans*). After 24 hours, all wells were washed with PBS, shaken, and dried with an absorbent tissue.

After staining with 50 µl of 0.1% crystal violet and incubating for 20 minutes, the sample was then washed three times with PBS to eliminate any unabsorbed crystal violet by the *S. mutans* and *C. albicans* biofilms. The wells were rinsed with 200 µl of 98% alcohol, incubated for 10 minutes, washed again with 96% alcohol, incubated for another 10 minutes, and dried using absorbent tissue. The final step is to read the findings using an ELISA reader (Thermo Scientific™, USA) set to 620 nm optical density.

The final step of this study was to use the SDS-PAGE technique to examine the protein profiles of *S. mutans* and *C. albicans* biofilms after being exposed to goat milk. The prepared samples were dissolved in 5% SDS solution (Merck, Germany) at a 1:5 ratio and heated at 85°C for 60 minutes. The mixture was centrifuged at 4230 rpm for about 5 minutes. The centrifugation supernatant was collected and combined with the sample buffer at a 1:1 ratio. The following step was to cook it in hot water for around two minutes.[24,25]

The negative control group received a sample volume of 4 µL, whereas the treatment and positive control groups received 6 µL. To compare samples, 4 µL of Excel Band was utilized as a protein standard with low molecular weight (LMW) (Sigma Aldrich, USA). The next step of electrophoresis was carried out at a voltage of 70 V for 3 hours until the bromophenol blue (Merck, Germany) had almost reached the bottom of the gel.

The gel staining stage was then carried out for 15 minutes with a dye solution, followed by a 15-minute wash, and was repeated four times. The gel was let to sit overnight, rinsed with distilled water, and stored. GelAnalyzer 2010a was used to assess the SDS-PAGE results.[25,26] The research findings were analyzed using the Statistical Package for the Social Sciences (SPSS) version 26 software.

An ANOVA test was conducted on the mass concentration of *S. mutans* and *C. albicans* biofilms after exposure to goat's milk, while the protein profile was described descriptively.

RESULTS

The morphology of *S. mutans* colonies cultivated on TYS20B media resembled circular colonies with flat edges, yellowish white, and a typical yeast scent, as shown in Figure 1A. Meanwhile, Gram staining confirmed that the colonies were purple, coccus-shaped (round), and organized in a chain, as illustrated in Figure 1B.

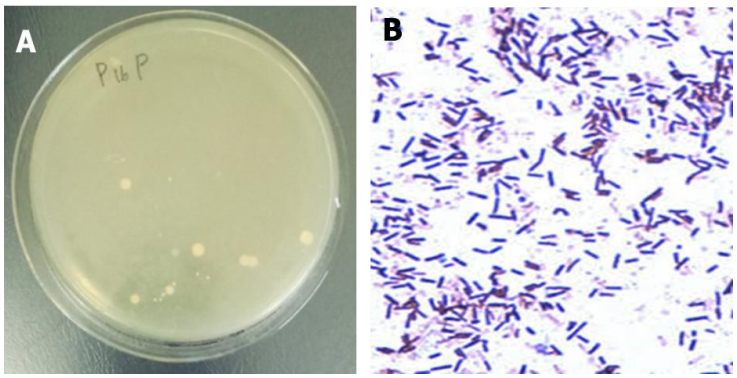

Figure 1: *Streptococcus mutans* colony morphology on TYS20B (A) and Gram staining results (B)

Meanwhile, *C. albicans* culture on SDA media produced colonies that were white to cream in color, smooth with a slightly convex surface, and had a yeast-like scent, as illustrated in Figure 2A. Gram staining on *C. albicans* under a light microscope revealed round to subspherical colonies with purple buds, blastoconidia, and pseudohyphae, as shown in Figure 2B.

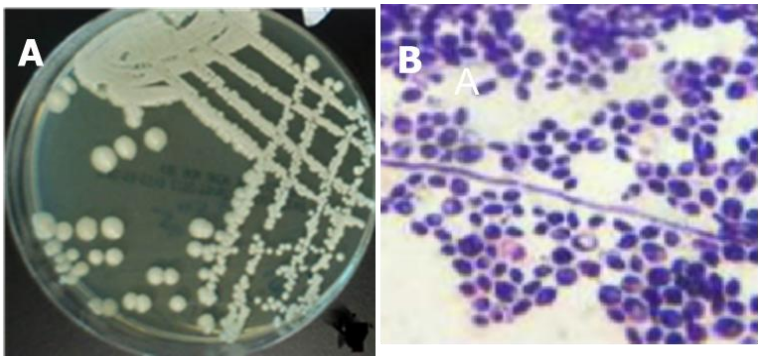

Figure 2: **Morphology of *Candida albicans* colonies (A. On SDA media; B. Gram staining results)**

Meanwhile, the results of microscopic images of *S. mutans* and *C. albicans* biofilm masses were obtained using a Nikon Ei microscope paired with an Optilab Plus digital camera. The collected results were then evaluated with Image J software, as shown in Figure 3.

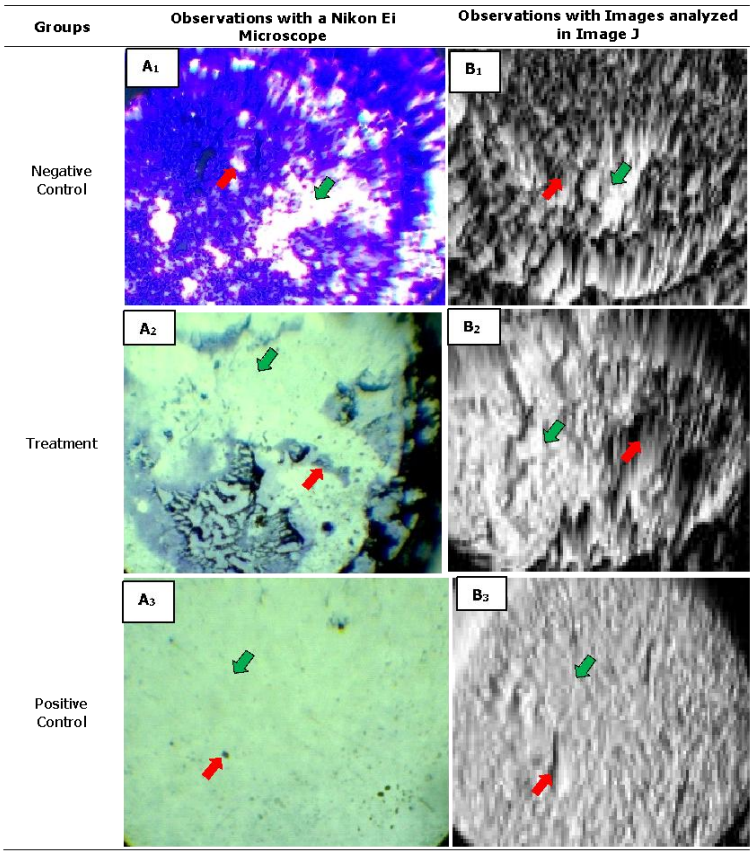

Figure 3: Results of microscopic image of *Streptococcus mutans* and *Candida albicans* biofilm mass (A1-A3. Using a Nikon Ei microscope; B1-B3. Image J; green arrow shows well base; and red arrow shows biofilm mass)

According to Figure 3, the more hills in the image, the greater the biofilm mass coverage. There were several hills in the negative control group, indicating that the bulk of biofilm cover was greater than in the other groups. Meanwhile, the treatment group generated fewer hills than the negative control group but more than the positive group. This demonstrates that the mass of biofilm cover was larger than the positive control (amoxicillin) but lower than the negative control. The positive control group had fewer slopes than the other groups, which was interpreted as the lowest biofilm coverage period. The results of the Biofilm Coverage Rate (BCR) concentration calculation using Image J and ANOVA analysis with a significance level of $p<0.05$ can be seen in Table 1.

Table 1. Anova Test Results Calculating BCR Concentration Using Image J

Group	BCR Concentration Calculation Results Using Image J				*p* Value
	Test			X ± SD	
	1	2	3		
C-	60,1	68	73,5	67,1 ± 7,4	0,000
T	46,4	58,3	59,1	54,6 ± 8,4	
C+	48,5	34	43,5	42,0 ± 8,8	

Note: C- (Aquadest); T (Goat Milk); C+ (Amoxicillin)

The results of statistical analysis show a p-value = 0.000, which means there is a significant difference in the amount of biofilm mass coverage between all groups. Meanwhile, the results of significance between groups using the LSD advanced test can be seen in Table 2.

Table 2. LSD Test Results BCR Calculation Between Groups After Giving Test Materials

Testing	Group	Comparison	*p* Value	Conclusion
Calculation of BCR Concentration	C-	C+	0,000	Significant
		T	0,004	
	T	C-	0,004	
		C+	0,003	
	C+	C-	0,000	
		T	0,003	

Table 2 shows significant ($p<0.05$) differences between the negative control, treatment, and positive control groups. This illustrates that providing goat's milk affects biofilm creation, as indicated by a decrease in biofilm mass coverage, even though the positive control had less biofilm mass.The results of calculating the mass concentration of biofilm using a ELISA Reader at a wavelength or Optical Density (OD) of 620 nm and ANOVA analysis can be seen in Table 3 below.

Table 3. Anova Test Results Calculating Biofilm Mass Concentration using ELISA Reader

Group	Biofilm Mass Concentration Calculation Results using ELISA Reader (CFU/ml)				*p* Value
	Test			X ± SD	
	1	2	3		
C-	0,61	1	0,75	0,78 ± 0,25	0,000
T	0,42	0,46	0,46	0,45 ± 0,2	
C+	0,36	0,42	0,35	0,38 ± 0,7	

The results of statistical analysis show that the p value = 0.000, which means there is a significant difference in wavelength between all groups. Meanwhile, the results of significance between groups using the LSD advanced test can be seen in Table 4.

Table 4. LSD Test Results Calculation of Biofilm Mass Concentration Using ELISA Reader

Testing	Groups	Comparison	*p* Value	Conclusion
Calculation of Biofilm Mass Concentration using ELISA Reader	C-	K+	0,000	Significant
		KP	0,001	Significant
	T	K-	0,001	Significant
		K+	0,460	Not Significant
	C+	K-	0,000	Significant
		KP	0,460	Not Significant

Table 4 demonstrates substantial differences ($p<0.05$) between the negative control group, treatment group, and positive control group. Meanwhile, the treatment group showed no significant difference from the positive control group ($p>0.05$). Figure 4 shows the results of detecting the protein profiles of *S. mutans* and *C. albicans* biofilms using the SDS-PAGE method.

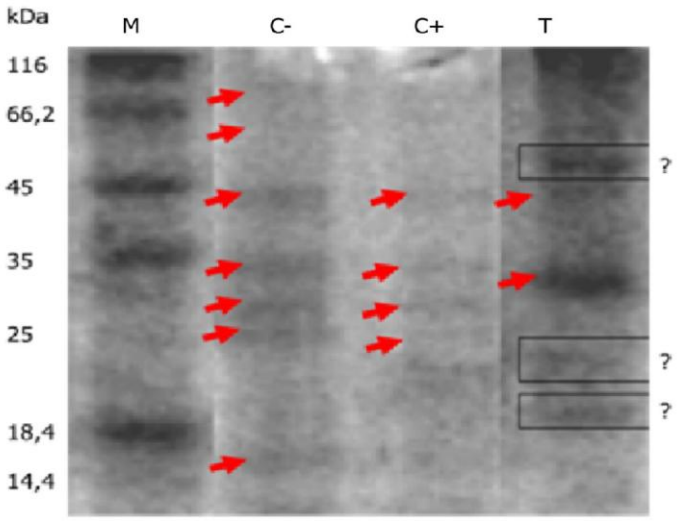

Figure 4. The protein patterns of *Streptococcus mutans* and *Candida albicans* biofilms (M is the marker; C- is the negative control group; C+ is the positive control group; and T is the treatment group)

Figure 4 indicates that the negative control group's protein profile has the most protein bands. This image also shows two protein bands, with molecular weights of 45 kDa and 35 kDa, which belong to the three groupings. However, in the treatment group, three protein bands were absent from the other two groups: one with a molecular weight of 45-66.2 kDa and two with a molecular weight of 18.4-25 kDa.

DISCUSSION

Streptococcus mutans and *C. albicans* were identified and confirmed as the starting point for this in vitro study (Figure 1 and Figure 2). The characteristics of the two bacteria are revealed by observations of colonies growing on selective media for each bacterium, as well as Gram staining data. On TYS20B medium, *S. mutans* colonies were round with flat edges, yellowish white, and had a typical yeast aroma, whereas *C. albicans* colonies on SDA media were white to cream-colored, smooth with a slightly convex surface, and had a yeast-like smell. Meanwhile, Gram staining revealed a purple color, indicating that *S. mutans* and *C. albicans* had cell walls covered with thick peptidoglycan, allowing them to retain the crystal violet dye during the staining procedure. These findings originate from earlier research projects that *C. albicans* appear purple when Gram staining is used.[27–29]

In this study (Figure 3), the negative control created the greatest hills between the positive control and the treatment. The treatment group that received goat milk had fewer hills than the negative control group. These findings are consistent with the BCR concentration calculation, which demonstrates that the goat's milk treatment group had lower mass coverage than the negative control group (Table 1 and Table 2). This study is in accordance with a study by Magac et al.;[30] this occurs because goat milk includes bioactive substances with bactericidal and bacteriostatic properties, such as lactoperoxidase, thiocyanate, hydrogen peroxidase, lactoferrin, and lysozyme.[30-34]

Lactoperoxidase is the most prevalent enzyme in goat milk, and its existence is stimulated by thiocyanate ions and hydrogen peroxidase, both of which are naturally present in goat milk. This enzyme is critical for guarding against a variety of diseases, including bacteria and fungi. Lactoperoxidase has bacteriostatic qualities that are thought to limit the growth of the bacteria in this study, *S. mutans* and *C. albicans*, by causing these two microbes to remain stationary in the dental biofilm. Aside from that, this enzyme is also bactericidal, capable of

changing microbial metabolism and causing changes in various microbial cell structures such as cell walls, active and passive transport systems, glycolytic enzymes, and nucleic acids, which causes microorganisms to die and disrupts the ability of microorganisms to reproduce.[34-38]

The positive control group had lower biofilm mass coverage than the treatment and negative controls. This arises because amoxicillin belongs to the beta-lactam antibacterial class. Beta-lactams work by attaching to penicillin-binding proteins, which prevent transpeptidation and activate autolytic enzymes in the bacterial cell wall. This mechanism lyses the cell wall, killing the bacterial cell. According to the study's findings, amoxicillin outperformed the treatment group because its antibacterial mechanism directly damages the biofilm by targeting the cell wall, whereas lactoperoxidase's action as an anti-bacteriostatic and antibacterial depends on factors such as the type of microorganism, the type of electron donor in the membrane protein, pH, temperature, incubation time, and age of microbial cell density. Aside from microbial factors, the antimicrobial activity of lactoperoxidase is influenced by characteristics in the goat that produces milk, such as nutrition, age, and type of goat. Amoxicillin was chosen as a positive control in this study because it has an antibacterial activity that can interfere with microbe biofilm development and is commonly used as an antibiotic to treat dental and oral infections.[37,39,40]

To determine goat's milk's ability to prevent biofilm, the mass concentration of biofilm was estimated using an ELISA Reader at OD 620 nm. The resulting results are consistent with the BCR concentration calculations (Table 3 and Table 4). The data revealed substantial differences across all groups, indicating that adding goat's milk to the suspension of *S. mutans* and *C. albicans* altered biofilm formation. The findings of this study are supported by Azizkhani et al. (2020); who found that goat's milk has an antibacterial impact against Staphylococcus aureus and an antifungal effect against *Aspergillus niger*. This antibacterial activity is consistent with previous researchers who found that goat's milk includes many bioactive peptides and lactoperoxidase enzymes that activate antibacterial activities.[31,34,41-43]

The protein profile of *S. mutans* and *C. albicans* biofilms exposed to goat's milk was analyzed using the SDS PAGE technique and LMW markers (Excel Band, Taipei) with molecular weights ranging from 14.4 to 116 kDA (Figure 4). Figure 4 shows the protein profile of the virulence factors that *S. mutans* and *C. albicans* have after being exposed to goat's milk. The protein profile indicates that goat's milk has an antibiofilm impact due to its antibacterial and antifungal properties, which cause protein degradation in *S. mutans* and *C. albicans* biofilms. Because there were no anti-biofilm chemicals to suppress protein production of microbe virulence factors, the negative control group had the most abundant protein bands.[40-43]

The positive control group had fewer protein bands than the negative control group due to amoxicillin's activity, which interfered with the action of *S. mutans*' virulence factors. In a previous study by Magac et al.,[43] *S. mutans* has three main pathogenic components that play an important role in biofilm formation: antigen I/II (190 kDa), glucosyltransferase (140 kDa), and glucan-binding protein (60 kDa).[43,44] The description of this group's protein profile shows that the protein bands are below 60 kDA, indicating that they are *C. albicans* protein bands with a low molecular weight, specifically below 60 kDa. The treatment group contains the fewest protein bands; nonetheless, there are three protein bands that the other two groups do not have: one band with a molecular weight between 45-66.2 kDa and two bands with a molecular weight between 18.4-25 kDA. These bands are assumed to be protein bands from milk's bioactive components, specifically albumin (molecular weight 66.0 kDa) and type 3 casein (molecular weight 23-30 kDa).[45,46]

The limitation of this study was that pasteurization of goat's milk was done conventionally by boiling it in a water bath. Goat's milk's main component is

protein, which is easily damaged by heat; consequently, for further study, the milk pasteurization process should be carried out using modern pasteurization equipment. Further study can be conducted based on the findings of this study, including a description of the microscopic structure of the tooth surface after exposure to goat's milk, given that goat's milk contains antibacterial compounds as well as minerals.

CONCLUSION

Goat milk reduces the concentration of *S. mutans* and *C. albicans* biofilms, according to BCR concentration calculations using Image J, biofilm concentration calculations using ELISA Reader, and protein profiles examined with SDS PAGE. As a result, goat's milk has the potential to act as a quantitative and qualitative antibiofilm agent against biofilms of *S. mutans* and *C. albicans*. Consequently, this study suggests that the utilization of animal products, specifically goat's milk, which has been demonstrated to disrupt the biofilms of S. mutans and C. albicans, presents an opportunity to prevent dental caries. Additionally, the findings of this study pave the way for further in vitro, in vivo, and in situ research into the effects of goat's milk on oral homeostasis.

Acknowledgement: Our gratitude to LP2M USK
Author Contributions: Conceptualization, SC and SS.; methodology, SR, DD.; software, SC and EV.; validation, SC., SS, and SR.; formal analysis, SC and DD.; investigation, SC, DD, and EV.; resources, SC and SS.; data curation, SC; writing original draft preparation, SC; writing review and editing, SC and SMS.; visualization, SC and DD.; supervision, SC, SMS, and SS.; project administration, SC, SS, DD, EV; funding acquisition, SC. All authors have read and agreed to the published version of the manuscript." Please turn to the CRediT taxonomy for the term explanation. Authorship must be limited to those who have contributed substantially to the work reported.
Funding: This research was conducted all thanks to the funding of LP2M, Universitas Syiah Kuala, Number: 170/UN11/SPK/PNBP/2021.
Institutional Review Board Statement: Not applicable" for studies not involving humans or animals
Informed Consent Statement: This article does not contain any studies with human or animal subjects that require permission from ethics committees or other institutions.
Data Availability Statement: Data supporting reported results can be found in the results section of the article manuscript.
Conflicts of Interest: The authors declare no conflict of interest.

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