

Optimization of Salivary *Streptococcus mutans* culture: A Technical Note

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Abstract

Introduction: Accurate quantification of *S. mutans* in Saliva serves as a critical biological determinant for assessing caries risk. This technical note details an optimized and standardized protocol for the selective isolation and quantification of *S. mutans* from stimulated salivary samples. **Materials and Methods:** Stimulated saliva was collected and subjected to serial dilution using sterile phosphate-buffered saline. Mitis Salivarius Bacitracin agar used to grow the bacteria under aerobic conditions. **Results:** The protocol yielded colonies with characteristic *S. mutans* blue to creamy white, granular "frosted glass" colonies, with strong adherence to the agar. The present protocol was found to be optimal for achieving countable plates and maintaining a homogeneous distribution, which is essential for a reliable colony count. **Conclusion:** By optimizing procedural variables like dilution factors and media preparation, this approach ensures reliable quantification suitable for clinical and epidemiological research.

Keywords: *Streptococcus mutans, Mitis Salivarius Bacitracin agar, Saliva, Caries Risk Assessment, Colony Forming Units, Microbiology Optimization.*

Introduction

Streptococcus mutans (*S. mutans*) account for approximately 30% of dental plaque microflora. It is a primary etiological agent in the development of dental caries, largely due to its acidogenicity and aciduric nature, which enables it to thrive in the low pH environments it creates.¹ This bacterium ferments various carbohydrates into lactic acid, significantly lowering the local pH and creating a selective pressure that favors its own survival and growth while inhibiting competing, less aciduric species.^{2,3} This acid production capacity is crucial for the

demineralization of tooth enamel, a hallmark of caries progression.^{4,5} Consequently, accurate and efficient cultivation of *S. mutans* from salivary samples is critical for both diagnostic applications, such as assessing caries risk, and for research aimed at understanding its pathogenic mechanisms and developing targeted interventions.¹ Despite its well-established role, optimizing culture conditions for *S. mutans* from complex salivary microbiomes remains a technical challenge, given the heterogeneity in strain attributes and the presence of numerous co-existing oral streptococci and other microbial species.⁶ Therefore, developing a standardized

and optimized culture method is essential for reliable quantification and characterization of *S. mutans* from salivary samples, which is a critical indicator for assessing an individual's caries risk.⁷ A crucial aspect of this optimization involves the inclusion of human saliva in growth media, as it has been shown to significantly modify the growth and competitive behaviors of oral streptococci, thereby mimicking the natural oral environment more accurately.⁸ Our objective was to develop an optimized protocol for the selective culturing and accurate quantification of *Streptococcus mutans* from stimulated salivary samples, addressing key challenges such as strain heterogeneity and interference from co-existing oral streptococci, and the need for reliable caries risk assessment.

Methodology

Preparation of Culture Plates

The initial step involved preparing Mitis Salivarius Bacitracin agar plates using Nutriselect™ Mitis Salivarius Agar and Bacitracin A VETRANAL®, analytical standard, for the culture media. Ninety grams of Nutriselect™ Mitis Salivarius Agar powder were added to 1000 mL of Millipore water. A magnetic stirrer with heating was used to dissolve the powder completely. The mixture was placed in a glass bottle, loosely covered with sterile gauze, and secured with autoclave indicator tape prior to sterilization. The solution was autoclaved at 121°C for 15 minutes to ensure sterility. After the cycle was complete, it was cooled to 50°C, with the temperature carefully monitored using infrared thermometry to prevent premature solidification. Once the desired temperature was reached, 2 units of Bacitracin A VETRANAL® powder (equivalent to 0.04 mg/mL per liter of medium) were added. The bacitracin was first dissolved in 20 mL of Millipore water, which was then poured into the bottle containing the medium to ensure uniform distribution. Once mixed well, the tempered medium was poured into sterile Petri dishes (20–25 mL per plate) and

allowed to solidify on a level surface to ensure uniform thickness. The medium solidified at room temperature before the plates were stored at 4°C until use. All preparation steps were performed in a laminar flow hood to maintain aseptic conditions throughout the process. The culture plates were used within one week of preparation, inverted, and stored at 4°C to minimize condensation and preserve selective properties.

Saliva collection and transport

To obtain accurate and representative samples, stimulated salivary collection was performed using a standardized paraffin wax chewing method for 1 min. Participants were asked not to swallow the saliva, and the expectorated sample was collected in a sterile container. The samples were immediately placed in medical thermos at 4°C for transportation to the laboratory, minimizing microbial growth and degradation of salivary components prior to processing. Upon arrival, samples were processed within one hour to preserve the viability and integrity of the microbial population.

Salivary dilution, Inoculation and Incubation

Salivary samples were subjected to serial dilution using sterile phosphate-buffered saline (PBS; pH 7.0). A dilution of up to 10⁻⁸ was prepared to obtain countable colony-forming units (CFUs). A volume of 0.1 mL from the selected dilution was inoculated onto MSB agar plates and evenly spread using a sterile L-shaped spreader. Prior to inoculation, plates were allowed to equilibrate to room temperature under aseptic conditions.

Incubation: After inoculation, the plates were incubated at 37°C for 48 hours under aerobic conditions. Since hypothesis testing was not a part of this standardization procedure, a power analysis and sample size calculation were not performed. However, 50 clinical samples from healthy young adult volunteers were used to validate the current protocol.

Results

Following incubation, colonies consistent with *Streptococcus mutans* were identified on Mitis Salivarius Bacitracin (MSB) agar based on characteristic morphological features. The colonies appeared blue to creamy white in color and exhibited a granular “frosted glass”

appearance. The circular colonies appeared to be strongly adherent or “rooted” into the agar surface and typically range from 0.5 mm to 2 mm in diameter. Some colonies were also surrounded by clear halo and glistening appearance (Figure 1).



Figure 1. *Streptococcus mutans* colonies

Discussion

The use of a 0.1 mL inoculum volume in the spread plate method was selected in accordance with established microbiological protocols for salivary *S. mutans* culture.^{9,10} This volume facilitates a quick surface absorption by the agar surface and segregated colonies. This also allows

for even distribution when using an L-shaped spreader.

An important consideration in microbiological enumeration is the achievement of a countable plate, typically defined as containing between 15 and 150 colony-forming units (CFUs).¹¹ In the present protocol, serial dilution was employed to

achieve this range, with a dilution of up to 10^{-8} found to provide optimal colony separation and countability in salivary samples. This step was critical in preventing confluent growth and ensuring accurate quantification of *S. mutans*. In a previous study Souror et al use 10^{-5} as their dilution factor which lead to confluent growth. Increasing the dilution factor to 10^{-8} facilitates more accurate count, especially while using a colony counter software.¹²

In the present study we have used significantly higher level of bacitracin concentration compared to the gold standard concentration utilized in the previous studies by Bansal et al and Zeng et al.^{7,13} This modification was done due to inhibit competing oral microflora identified during standardization procedure using the gold standard concentration. Additionally, several conventional protocols dictate utilization of anaerobic incubation or CO_2 enriched atmosphere to support the growth of *S. mutans*.¹² However, in the present study we relied on aerobic incubation and achieved reasonable growth of *S. mutans*. This modification makes the procedure more accessible for clinical dental settings where specialized anaerobic equipment may not be available.

The choice of diluent is another factor that can influence bacterial viability and recovery. While sterile water, saline, and buffered solutions have all been used in dilution protocols, buffered systems offer advantages in maintaining physiological pH and stabilizing microbial viability during processing.¹⁴ Buffered solutions help stabilize the microbial population, which is necessary for the accurate isolation and identification of *S. mutans* using selective media like Mitis Salivarius Bacitracin agar.¹ In this study, sterile phosphate-buffered saline (PBS; pH 7.0) was utilized, which provided a stable environment for the preservation of *S. mutans* during serial dilution and subsequent plating.

Overall, these methodological considerations contributed to improved consistency in colony isolation and enumeration, highlighting the

importance of optimizing seemingly minor procedural variables in salivary microbiological analysis.

Conclusion

Based on the results from this study, it can be concluded that effective isolation, infection control, better accessibility and visibility as well as improved treatment outcomes are the factors that reflect the attitude of dental practitioners towards the use of rubber dams.

References

1. Fang Y, Ma Q, Zhao W, et al. A novel selective medium Sucrose-Bacitracin agar 10 for accurate isolation and identification of *Streptococcus mutans*. *BMC Oral Health* . 2025;25(1). doi:10.1186/s12903-025-06675-y
2. Lemos JA, Palmer S, Zeng L, et al. The Biology of *Streptococcus mutans*. *Microbiology Spectrum* . 2019;7(1). doi:10.1128/microbiolspec.gpp3-0051-2018
3. Rodríguez ZG, Thome SKR, Castillejos RD, et al. Precision Pediatric Caries Diagnostics: Saliva-Check Mutans versus Culture for High-Density *Streptococcus mutans* Detection. *Discoveries* . 2025;13(4). doi:10.15190/d.2025.19
4. Matsui R, Cvitkovitch DG. Acid Tolerance Mechanisms Utilized by *Streptococcus Mutans*. *Future Microbiology* . 2010;5(3):403-417. doi:10.2217/fmb.09.129
5. Shen R, Tan YZ, Zheng J, et al. Research on the prevention of tooth demineralization and the effects and mechanisms of different mineralization solutions on the metabolism of *Streptococcus mutans*. *Frontiers in Oral Health* . 2025;6. doi:10.3389/froh.2025.1647945
6. Balolong MP, Mendoza MAF. Understanding Oral Diseases: Exploring Opportunities from Filipino Oral Microbiome Research. In: *IntechOpen eBooks* . IntechOpen; 2021. doi:10.5772/intechopen.94751
7. Zeng Y, Youssef M, Wang L, et al. Identification of Non-*Streptococcus mutans* Bacteria from Predente Infant Saliva Grown on Mitis-Salivarius-Bacitracin Agar. *Journal of Clinical Pediatric Dentistry* . 2020;44(1):28-34. doi:10.17796/1053-4625-44.1.5
8. Choi A, Dong K, Williams E, et al. Human saliva modifies growth, biofilm architecture, and competitive behaviors of oral streptococci. *mSphere* . 2024;9(2). doi:10.1128/msphere.00771-23
9. Westergren G, Krasse B. Evaluation of a micromethod for determination of *Streptococcus mutans* and *Lactobacillus* infection. *Journal of Clinical*

- Microbiology . 1978;7(1):82-83.
doi:10.1128/jcm.7.1.82-83.1978
10. Muñoz LF, Uribe AM, Reyes MC, Castro LA, Rodríguez MJ. Salivary Streptococcus mutans colony-forming unit count in patients with and without orthodontic appliances. Acta odontológica latinoamericana/Acta odontológica latinoamericana . 2022;35(3):171-177. doi:10.54589/aol.35/3/171
 11. Houchmandzadeh B, Ballet P. A novel procedure for CFU plating and counting. HAL (Le Centre pour la Communication Scientifique Directe) . Published online December 8, 2022. Accessed August 2025. <https://hal.science/hal-03890465>
 12. Souror YR, Waly A, khelifa YHA, Abuzenada BM. Mutans streptococci growth on glass ionomer incorporated with chlorhexidine: in-vivo study. Brazilian Dental Science . 2020;23(2). doi:10.14295/bds.2020.v23i2.1909
 13. Bansal K, Shamoo A, Atif M, Batra P, Chaudhry R. Isolation and detection of bacterial species on mitis salivarius bacitracin agar from the plaque samples of caries active children. Journal of Indian Society of Pedodontics and Preventive Dentistry . 2024;42(1):15-21. doi:10.4103/jisppd.jisppd_17_24
 14. Tusi SK, Jafari A, Marashi SMA, Niknam SF, Farid M, Ansari M. The effect of antimicrobial activity of Teucrium Polium on Oral Streptococcus Mutans: a randomized cross-over clinical trial study. BMC Oral Health . 2020;20(1):130-130. doi:10.1186/s12903-020-01116-4