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Effect of storage temperature on *Streptococcus mutans* viability

Influência da temperatura de armazenamento na viabilidade de Streptococcus mutans

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Resumo

Introdução: O correto armazenamento e manutenção adequada de material biológico viável representam importante papel nas pesquisas microbiológicas em virtude da oportunidade de desenvolvimento de pesquisas futuras. **Objetivo:** Avaliar a viabilidade de *Streptococcus mutans* (*S. mutans*) previamente cultivados e armazenados sob diferentes temperaturas há aproximadamente oito anos. **Material e método:** Foram avaliados 393 isolados bacterianos armazenados em freezer a -80°C (G1) e 200 em freezer a -20°C (G2). Alíquotas de cada amostra foram semeadas em meios de cultura ágar sangue e ágar Mitis Salivarius Bacitracina Sacarose. Após incubação sob condições de microaerofilia em estufa a 37°C, durante 72 horas, observou-se: presença, morfologia e pureza do crescimento bacteriano. Os dados obtidos foram analisados por meio de estatística descritiva. **Resultado:** A viabilidade microbiana foi observada em praticamente todas as amostras (99,7%) em G1. Por outro lado, todas aquelas estocadas a -20°C foram consideradas inviáveis. **Conclusão:** A viabilidade de *S. mutans* é influenciada pela temperatura de armazenamento das amostras, sendo que as cepas permanecem viáveis quando estocadas em condições ideais de temperatura (-80°C), mesmo quando armazenadas por longo período de tempo.

Descritores: *Streptococcus mutans*; cárie dentária; viabilidade microbiana.

Abstract

Introduction: Proper storage conditions and maintenance of viable biological material plays an important role in microbiological research, allowing for the opportunity to conduct future studies. **Objective:** To evaluate the viability of *Streptococcus mutans* strains that were previously grown and stored under different temperatures for approximately eight years. **Material and method:** In this study, we evaluated 393 bacterial isolates that were stored in a freezer at -80°C (G1) and 200 isolates stored in a freezer at -20°C (G2). Aliquots of each sample were plated on blood agar and mitis-salivarius bacitracin sucrose agar-solidified medium. After incubating under microaerophilic conditions in an incubator at 37°C for 72 hours, the presence, morphology and purity of bacterial growth was observed. The data were analyzed by means of descriptive statistics. **Result:** Microbial viability was observed in almost all samples (99.7%) in G1, whereas all isolates stored at -20°C were considered inviable. **Conclusion:** The viability of *S. mutans* is influenced by the storage temperature of the samples, and the strains remain viable when stored under ideal temperature conditions (-80°C), even when stored for a long period of time.

Descriptors: *Streptococcus mutans*; dental cavity; microbial viability.

INTRODUCTION

Due to its potent virulence factors, *Streptococcus mutans* is considered the microorganism of greatest cariogenicity among microbial species that colonize the human oral cavity[1,2]. Consequently, the scientific community has investigated this bacterium for decades using various biochemical, serological and genetic techniques[3].

To this end, microbiology laboratories strive to preserve bacterial cultures while considering both microorganism survival and viability. In particular, methods are used that do not promote the production of mutations or variabilities in the species[4].

Most of the knowledge of microbial physiology comes from studies of isolated cell lines grown in an environment that provides adequate conditions for their propagation[5]. Therefore, when the primary objective is the detection of bacterial colonies or individual cells, a selective medium should be used that contains components that almost exclusively allow for the development of the target group or species[6].

For microorganisms to grow, they require the elements that make up their chemical composition, as well as metabolic energy to synthesize macromolecules and maintain essential chemical

gradients. Analysis of the progression of this process should thus use carefully controlled nutrients and growth factors, such as pH, temperature, aeration, salt concentration and ionic strength[5].

In view of the biotechnological and scientific development of microbiology, ensuring the survival of cultures and preserving their morphological, physiological and genetic characteristics is essential for the preservation of microbial growth, since different storage conditions affect cellular survival[7].

Water is the primary component required for the maintenance of cellular functions and must be present in the cell for biochemical processes to occur. When water is converted to ice, cellular metabolism is stopped[8]. In this sense, the term viability refers to the ability of a microorganism to grow and multiply after being thawed and inoculated in the appropriate culture medium[9].

S. mutans has a predominantly fermentative metabolism through which it obtains energy primarily through the use of sugars[6]. Thus, its growth tends to be efficient using enriched solid or liquid media[5]. In this context, although the literature indicates different culture media for *S. mutans* isolation[10,11], the selective agar medium mitis-salivarius containing 0.2 U/mL bacitracin and 20% sucrose (MSBS) has been the most used medium for this purpose[12].

Considering that scientific studies often require stocks of microorganism strains that can be used at different times for experiments, maintaining their viability for varying periods of time should be ensured. Based on the hypothesis of permanence of microbial viability in previously cultured samples, the aim of this study was to assess the viability of *S. mutans* stored under different temperatures for a long period of time.

MATERIAL AND METHOD

The biological material used in this study was collected in a previous study conducted between 2002 and 2005, developed through an agreement between the Bauru School of Dentistry/University of São Paulo (FOB/USP) and the University of Pittsburgh/USA. The project was submitted for evaluation and approved by the Ethics Committee on Human Research under opinion no. 073/2011.

In the present study, 593 *S. mutans* samples were isolated from the saliva of 20 caries-active adults (age: 32.9 ± 6.9 years and number of decayed, missing, and filled tooth surfaces (DMFS): 43.7 ± 12.8). The participants were members of Brazilian families with low socioeconomic status living in areas with sub-optimal concentrations of fluoride (0.60 to 0.79 mg F/L). All mothers were primiparous and had salivary levels of streptococci from the *mutans* group ≥ 10^6 CFU/mL.

The biological material was stored for approximately eight years in brain and heart infusion (BHI) broth containing 20% glycerol. A portion of the samples (393) were stored in a freezer at -80°C (G1), and the remainder (200) were stored in a freezer at -20°C (G2).

For the microbial viability analysis, a 10 µL aliquot of each thawed and homogenized sample was streaked with a loop onto Petri dishes containing agar-solidified blood agar medium (BIOCEN, Campinas, São Paulo, Brazil) or the selective medium MSBS agar[12] (DIFCO Laboratories, Detroit, Michigan, USA).

The dishes were placed in anaerobic jars and incubated under microaerophilic conditions in an incubator at 37°C for 72 hours. After this period, and with the aid of a stereoscopic microscope (OLYMPUS SZ 40, Shinjuku-ku, Tokyo, Japan), the presence, morphology and purity of bacterial growth were assessed to determine the viability of the sample.

Samples considered viable were plated and frozen. A representative *S. mutans* colony from each MSBS agar plate was selected and aseptically transferred to a tube containing 5 mL of BHI broth (DIFCO Laboratories, Detroit, Michigan, USA). The tubes were incubated in an incubator at 37°C for 48 hours under microaerophilic conditions. Once bacterial growth was observed, 500 L of the plated material were suspended in BHI broth with 20% glycerol and stored in a freezer at -80°C.

The data were summarized and analyzed using descriptive statistics to determine the absolute and relative frequencies of the samples considered viable.

RESULT

Of the 393 samples in G1, 392 (99.7%) were considered viable, whereas none of the samples in G2 were viable (Figure 1 - Sample viability distribution according to storage temperature).

Figure 2 (macroscopic appearance of colony morphology after plating on MSBS agar) shows the growth pattern of *S. mutans* in the analyzed samples. The presence of small black colonies with rough and convex morphology, similar to a blackberry, was observed. The colonies strongly adhered to the substrate and were free of bacterial or fungal contamination, indicating the purity of the culture.

DISCUSSION

Quality control, from the time of collection to the use of the sample, is extremely important to obtain reliable results when the samples are reused in prospective studies[13]. Therefore, prior evaluation of cell viability is essential for conducting experiments using microorganisms frozen for various periods of time.

Freezing involves complex phenomena in which the cells lose water. This procedure is slow and causes the formation of ice crystals outside the cell faster than inside the cell. Because the water in the

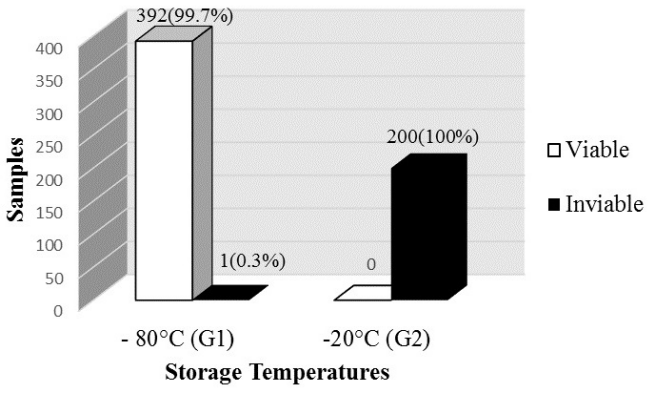

Figure 1. Sample viability distribution according to storage temperature.

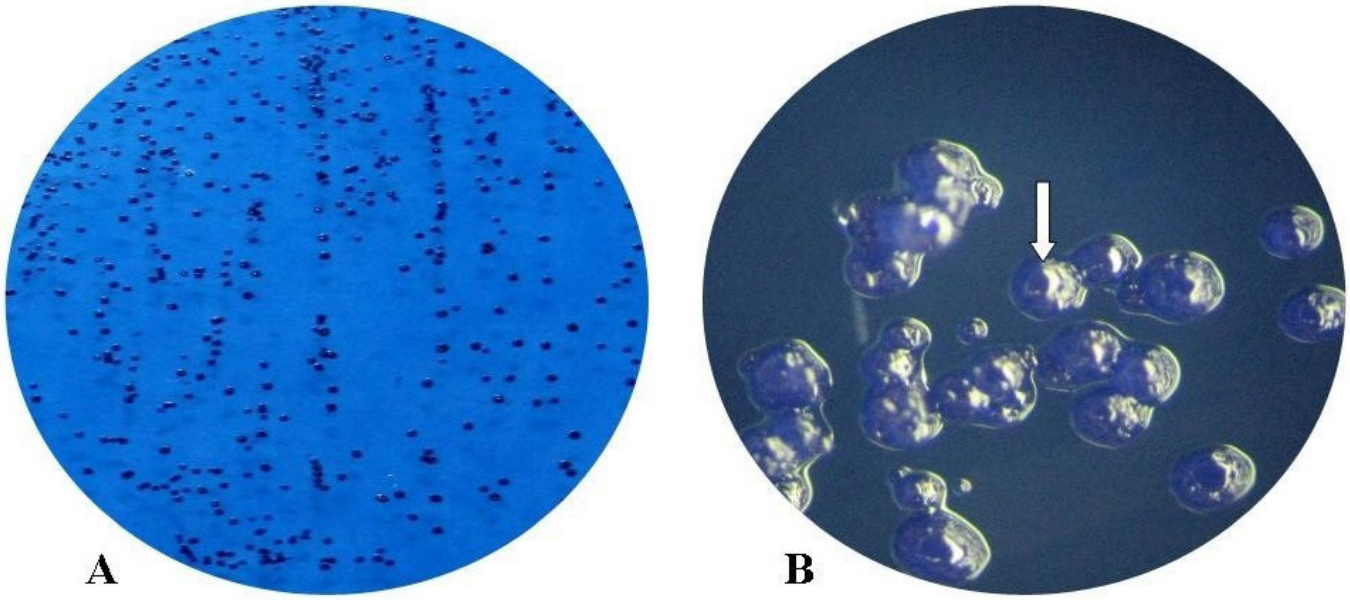

Figure 2. Macroscopic appearance of colony morphology after plating on MSBS agar (A: 30 magnification, B: 50× magnification; the arrow indicates a droplet of extracellular polysaccharides).

extracellular medium is consumed, there is an osmotic imbalance across the cell membrane, leading to migration of water out of the cell. An increase in the acid-base ratio of intracellular material compromises cell survival. In addition, ice crystal expansion can destroy the cell walls[8,14].

To prevent cell dehydration, protective agents should be used during the freezing process. These agents are more effective when they penetrate the cell, as they delay intracellular freezing, reducing harmful osmotic effects and preventing the formation of ice crystals and the consequent rupture of the cell wall. Thus, the addition of protective agents to the cells during freezing allows them to be preserved for an indefinite length of time and resume their normal function when thawed. Glycerol is often used as a preservative in the freezing of bacterial cultures because it is biochemically compatible with cellular structures[8,14].

In this study, all analyzed samples were suspended in BHI broth containing 20% glycerol since being previously cultured and isolated. However, the difference in storage temperature significantly affected microbial viability, since only *S. mutans* samples stored at -80°C remained viable.

This finding corroborates the study of Costa, Ferreira[15]. In that study, the authors classified microorganism storage methods according to the maximum preservation time. For long-term storage (above two years), storage in a freezer at -80°C or in liquid nitrogen at -196°C is suggested. However, freezing at relatively low temperatures of between -4°C and -20°C is suggested for storing strains for medium lengths of time (three months to two years) due to the significant reduction in cellular metabolism.

According to Perry[16], the storage of biological material at -80°C allows for good cell recovery and can be used when no liquid nitrogen is available. Numerous recent studies[17-20] have successively stored *S. mutans* stocks maintained at -80°C.

Abreu, Tutunji[21] analyzed the viability of 1,500 samples of various bacterial and fungal species (*Escherichia coli*, *Enterococcus* sp., *Staphylococcus* sp., *Aeromonas* sp., *Xanthomonas campestris*, *Candida* sp. and *Salmonella* sp.) stored at -20°C and concluded that while this temperature is suitable for most microorganisms, some cells are sensitive and do not survive for long periods of time. Most of the samples remained viable in the first year of freezing, particularly *Escherichia coli* and *Enterococcus* sp.

In the present study, none of the *S. mutans* samples stored at -20°C (G2) were viable. In addition to the temperature not being considered ideal with respect to the time of storage of the microorganisms, inadequate management of the freezer may also have contributed to the occurrence of inviable cells. In this case, constant temperature oscillations may have caused thawing followed by recrystallization, causing irreversible damage to the bacterial cell membrane. However, it should be noted that inviable cells in media considered suitable for many purposes can be analyzed using molecular biology techniques that do not require viable cells.

According to Zhao, Zhang[22], the different cell recovery methods used to resume metabolic activity also affect viability, and appropriate culture media should be used in the cell regeneration process, allowing the resumption of metabolic processes interrupted by freezing, with the least amount of osmotic and physical disturbance.

In this study, all viable samples showed microbial growth when plated on both blood agar and MSBS agar. A closer analysis of the microbial growth on MSBS agar (Figure 2B) revealed the production of shiny droplets of insoluble extracellular polysaccharides at the top of the colonies, characteristic of *S. mutans* grown in the presence of sucrose[23]. The extracellular polysaccharides act as "biological adhesives" that allow bacterial adhesion to the dental surface and to other bacteria, contributing to the success of certain species in biofilms[24].

Only one of the samples stored at -80°C (G1) was inviable when plated on MSBS agar but was viable on blood agar, producing beta

hemolysis. However, *S. mutans* is typically alpha hemolytic. To confirm this observation, several colonies from the blood agar plate were plated onto MSBS agar, and no cell proliferation was observed. Thus, the possibility that the observed growth was *S. mutans* was discarded, as contamination of the biological material was probable.

The Brazilian National Health Council, via Resolution No. 441 of May 12, 2011, when considering the use of stored biological material, established that "stored samples may be used in new studies approved by the institutional Research Ethics Committee and, when applicable, by the National Research Ethics Committee; research projects that intend to use stored samples should include a justification for the use of the material and a copy of the informed consent form (ICF) used when the material was collected, containing authorization for storage and possible use in future research, and the ICF specific for the new study or the request for its dispensation[25]."

Lastly, it should be noted that biological materials can pose risks to health and the environment if not properly disposed of. It is recommended that in cases where sample viability has been detected, the material should be disposed of according to the biosafety standards for biological risk[13]. According to the guidance of the Brazilian National Sanitary Surveillance Agency (Resolution No. 306 of December 7, 2004), waste containing biological agents, which may present a risk of infection due to their greater virulence or concentration, cannot leave the generating unit without prior treatment. Such waste must be subjected to microbial inactivation and subsequently collected in special garbage bags for infectious waste (white bags)[25].

CONCLUSION

The viability of *S. mutans* is affected by the storage temperature of the samples, and the strains remain viable when stored under ideal temperature conditions (-80°C), even if stored for a long period of time.

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CONFLICTS OF INTERESTS

The authors declare no conflicts of interest.

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