T.C
YEDİTEPE UNIVERSITY
INSTITUTE OF HEALTH SCIENCES
DEPARTMENT OF PEDIATRIC DENTISTRY

EVALUATION OF THE ANTIMICROBIAL EFFECTS AND CYTOTOXICITY OF VARIOUS MOUTHWASHES APPROPRIATE FOR THE USE OF CHILDREN, *IN VITRO*

DOCTOR OF PHILOSOPHY THESIS

DENTIST
PIRIL BAŞARAN

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DENTIST
PIRIL BAŞARAN

ADVISOR
PROF. DR. ELİF SUNGURTEKİN EKÇİ

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	Title, Name-Surname (Institution)
Chair of the Jury:	Prof. Dr. Senem SELVİ KUVVETLİ Yeditepe University
Member/Examiner (Advisor):	Prof. Dr. Elif SUNGURTEKİN EKÇİ Yeditepe University
Member/Examiner:	Prof. Dr. Didem ÖZDEMİR ÖZENEN Yeditepe University
Member/Examiner:	Prof. Dr. Emine Nursen TOPÇUOĞLU İstanbul University
Member/Examiner:	Prof. Dr. Mehmet Sertaç PEKER Marmara University

APPROVAL

This thesis has been deemed by the jury in accordance with the relevant articles of Yeditepe University Graduate Education and Examinations Regulation and has been approved by the Administrative Board of the Institute with a decision dated …… and numbered ……...

Prof. Dr. Bayram YILMAZ

Director of the Institute of Health Sciences

DECLARATION

I hereby declare that this thesis is my own work and that, to the best of my knowledge and belief, it contains no material previously published or written by another person nor material which has been accepted for the award of any other degree except where due acknowledgment has been made in the text.

DT. PIRIL BAŞARAN

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ABBREVIATIONS and SYMBOLS

XTT:2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2Htetrazoliumhydroxide

MTS:3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2Htetrazolium

MTT: 3-(4, 5-dimethyl thiazolyl-2)-2, 5-diphenyltetrazolium bromide

A. viscosus: *Actinomyces viscosus*

ASTM: American Society for Testing and Materials

AFM: Atomic Force Microscopy

BHI: Brain-Heart Infusion

Ca: Calcium

CPC: Cetylpyridinium Chloride

CHX: Chlorhexidine

CLSI: Clinical and Laboratory Standards Institute

CLSM: Confocal Laser Scanning Microscopy

DMEM: Dulbecco's Modified Eagle Medium

EPS: Extracellular Polymeric Substances

ELISA: Enzyme-Linked Immunosorbent Assay

FBS: Fetal Bovine Serum

FDA: Food and Drug Administration

FTIR: Fourier Transform Infra-Red

HDF: Human Dermal Fibroblasts

H_2O_2: Hydrogen Peroxide

IPS: Intracellular Polysaccharide

ISO: International Organization for Standardization

L. acidophilus: *Lactobacillus acidophilus*

L. casei: *Lactobacillus casei*

L. fermentum: *Lactobacillus fermentum*

L. paracasei: *Lactobacillus paracasei*

L. plantarum: *Lactobacillus plantarum*

LF: Lactoferrin

LYZ: Lysozyme

MDP: Microbial Dental Plaque

MJI: Microjet Impingement

MIC: Minimum Inhibitory Concentration

MW: Mouthwash

MS: Mutans Streptococci

PSA: Penicillin-Streptomycin-Amphotericin B Solution

PBS: Phosphate-Buffered Saline

PCR: Polymerase Chain Reaction

K: Potassium

s-HA: Salivary Pellicle-Coated Hydroxyapatite

SEM: Scanning Electron Microscopy

Na: Sodium

NaF: Sodium Fluoride

SnF: Stannous Fluoride

S. cricetus: *Streptococcus cricetus*

S. downei: *Streptococcus downei*

S. ferus: *Streptococcus ferus*

S. mitis: *Streptococcus mitis*

S. mutans: *Streptococcus mutans*

S. macacae: *Streptococcus macacae*

S. rattus: *Streptococcus rattus*

S. sobrinus: *Streptococcus sobrinus*

SLS: Sodium Lauryl Sulfate

TLC: Thin-Layer Chromatography

Q-PCR: Quantitative Real-Time Polymerase Chain Reaction

QCM: Quartz Crystal Microbalance

Xan: Xanthorrhiza Oil

VSC: Volatile Sulfur Compounds

ABSTRACT

BASARAN P. 2023. Evaluation of the Antimicrobial Effects and Cytotoxicity of Various Mouthwashes Appropriate for the Use of Children, *In Vitro.* Yeditepe University Institute of Health Sciences Doctorate Thesis, Istanbul.

The study aimed to compare the antimicrobial effects and cytotoxicity of five mouthwashes containing chlorhexidine (CHX), fluoride, probiotics, and their combinations (Klorhex: 0.2 % CHX, Humble Co.: CHX+NaF, Listerine: NaF, Luvbiotics: probiotic+NaF, Henry Blooms: probiotic). In this *in vitro* study, the antimicrobial activity was screened against *Streptococcus mutans (S. mutans)* using the disc diffusion assay. To evaluate the antibiofilm efficacies, an *in vitro* biofilm model was used. The *S. mutans* biofilm was formed on the hydroxyapatite discs coated with the artificial saliva. The resulting biofilms were rinsed with mouthwashes, and the discs were examined with a confocal laser scanning microscope (CLSM). In the final part of the study, cytotoxicity of the mouthwashes was determined by 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4 sulfophenyl)-2H-tetrazolium (MTS) assay on human dermal fibroblast (HDF) cell lines. The cell cultures were exposed to three different concentrations (5%, 10%, and 20%) of the mouthwashes for 30 seconds, and the cell viability of HDFs was calculated. According to the disc diffusion assay, the highest inhibition zone values among the groups were obtained in the Klorhex, Luvbiotics, and Listerine groups, respectively, and no inhibition zones were formed in Henry Blooms and Humble Co. groups. In the CLSM analysis, the most effective antibiofilm activity was seen in Klorhex, Luvbiotics, Listerine, Henry Blooms, and Humble Co. groups, respectively. According to the MTS assay, the cell viability decreased at certain levels in Klorhex, Luvbiotics, and Listerine groups. It was observed that almost all cells died in these groups at high concentrations (10% and 20%). Also, in the Henry Blooms group, the cell viability reduced highly at 20% concentration; only the Humble Co. group showed high cell viability at all concentrations. It was found that the cell viability decreased in all groups as the concentration increased. As a result of this study, it was observed that mouthwashes with higher antimicrobial activity also had higher cytotoxicity; only the Humble Co. group had lower antimicrobial and cytotoxic effects. However, more *in vitro* and *in vivo* studies are needed for more valid results.

Keywords: *Mouthwash, Probiotics, Lactobacillus paracasei, Streptococcus mutans, Chlorhexidine, Fluoride, Biofilm, Antibacterial Effect, Cytotoxicity.*

ABSTRACT (TURKISH)

BASARAN P. 2023. Çocuklarda Kullanıma Uygun Farklı İçerikli Gargaraların Antimikrobiyal Etkilerinin ve Sitotoksisitelerinin *in vitro* Olarak İncelenmesi. Yeditepe Üniversitesi Sağlık Bilimleri Enstitüsü, Doktora Tezi, İstanbul.

Bu çalışmanın amacı, klorheksidin (CHX), fluorid, probiyotik ve bunların kombinasyonlarını içeren beş gargaranın antimikrobiyal etkilerini ve sitotoksisitesini karşılaştırmaktır (Klorhex: 0.2 % CHX, Humble Co.: CHX+NaF, Listerine: NaF, Luvbiotics: probiyotik+NaF, Henry blooms: probiyotik). Bu *in vitro* çalışmada, disk difüzyon yöntemi kullanılarak gargaraların *Streptococcus mutans (S. mutans)* üzerine antimikrobiyal etkileri incelenmiştir. Gargaraların biyofilm üzerine etkinliklerini değerlendirmek için ise bir *in vitro* biyofilm modeli kullanılmıştır. *S. mutans* biyofilmi, yapay tükürük ile kaplanmış hidroksiapatit diskler üzerinde oluşturulmuştur. Ortaya çıkan biyofilm üzerine gargaralar uygulanmış ve diskler konfokal lazer taramalı mikroskopta incelenmiştir. Çalışmanın son bölümünde gargaraların insan dermal fibroblast hücre hattı (HDF) üzerine sitotoksisitesi 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4 sulfophenyl)-2H-tetrazolium (MTS) testi ile belirlenmiştir. Hücre kültürleri 30 saniye boyunca üç farklı konsantrasyonda (%5, %10 ve %20) gargaralara maruz bırakılmıştır ve HDF'lerin hücre canlılığı hesaplanmıştır. Disk difüzyon testi sonuçlarına göre gruplar arasında en yüksek inhibisyon zonu değerleri sırasıyla Klorhex, Luvbiotics ve Listerine gruplarında elde edilirken, Henry Blooms ve Humble Co. gruplarında inhibisyon zonu oluşmamıştır. Konfokal mikroskop ile analizin sonuçlarına göre en etkili antibiyofilm aktivitesi sırasıyla Klorhex, Luvbiotics, Listerine, Henry Blooms ve Humble Co. gruplarında görülmüştür. MTS testine göre Klorhex, Luvbiotics ve Listerine gruplarında hücre canlılığı önemli seviyede azalmıştır ve yüksek konsantrasyonlarda (%10 ve %20) neredeyse tüm hücrelerin öldüğü gözlenmiştir. Ayrıca Henry Blooms grubunda da, hücre canlılığı %20 konsantrasyonda oldukça azalmış, sadece Humble Co. grubu, tüm konsantrasyonlarda yüksek hücre canlılığı göstermiştir. Tüm gruplarda konsantrasyon arttıkça hücre canlılığının azaldığı görülmüştür. Bu çalışma sonucunda antimikrobiyal aktivitesi yüksek olan gargaraların sitotoksisitesinin de daha yüksek olduğu, sadece Humble Co. grubunun düşük düzeyde antimikrobiyal ve sitotoksik etkiye sahip olduğu görülmüştür. Ancak, daha geçerli sonuçlar elde etmek için daha fazla *in vitro* ve i*n vivo* çalışma yapılmasına ihtiyaç vardır.

Anahtar Kelimeler: *Gargara, Probiyotik, Lactobacillus paracasei, Streptococcus mutans, Klorheksidin, Fluorid, Biyofilm, Antibakteriyel Etki, Sitotoksisite.*

1. INTRODUCTION and PURPOSE

Dental caries is one of the most prevalent chronic infectious diseases impacting people globally (1,2). It occurs over time due to a complicated interaction between acid-producing bacteria, fermentable carbohydrates, and various host components such as teeth and saliva (2,3).

Dental biofilm is a bacterial layer accumulating on the tooth's surface and producing acids due to its metabolism (4,5). This bacterial activity creates an acidic environment to solve dental minerals causing dental caries (6).

Carious lesions are caused by poor oral hygiene, which leads to biofilm formation on tooth surfaces. The mechanical removal of dental biofilm by frequent brushing is the most efficient method for plaque prevention, although its effectiveness heavily depends on the individual's competency (7,8).

Based on our current understanding of caries development, thoroughly examining dental caries involves more than just looking for demineralization sites and considering treatment methods. A complete dental caries therapy should involve diagnosing cariogenic bacteria and plaque acidogenicity, removing cariogenic bacteria, decreasing plaque acidogenicity, and increasing tooth remineralization (9).

The primary goal of preventive dental care is to eliminate biofilm formation. Methods that selectively restrict oral pathogens or modify the microbial ecology of biofilm are gaining attention to manage the community-based microbial disease. The probiotic technique is one of the most widely utilized strategies for modifying microorganism populations (10). Probiotic technology promises a revolutionary way to preserve oral health by using the naturally present helpful bacteria exist in healthy oarl flora as a natural protection to the microorganisms considered harmful to teeth and gums (11,12).

Antimicrobials such as toothpaste and mouthwash are used for chemical plaque control products because of their role as delivery systems (7). Among these, mouthwash is a secure and stable way to eliminate bacterial development and decrease persistent colonization by delivering antimicrobial chemicals (12).

These chemicals can inhibit the adhesion, colonization, and metabolism of bacteria. Unfortunately, the regular use of these agents has several adverse consequences, including tooth

discoloration and medication resistance. The development of bacterial resistance to such drugs has become widespread, which presents a significant challenge. This issue has prompted the development of alternate drug resistance tactics. The probiotic technique is an alternative to mouthwashes to prevent these drawbacks (11,13).

Chlorhexidine (CHX), sodium fluoride, essential oils, hyaluronic acid, cetylpyridinium chloride, triclosan, octenidine, delmopinol, polyvinylpyrrolidone, and other natural compounds are commonly utilized in mouthwash formulations, and their complex composition often makes cytotoxicity assessments difficult (14).

Oral rinsing brings the oral mucosa into contact with the mouthwash. Although adverse reactions to oral rinse are rare, there have been reports of allergic reactions to certain components (14). These uncommon events have encouraged research on mouthwashes, specifically *in vitro* studies, to determine whether they have an antibacterial function and are ultimately cytotoxic under *in vitro* conditions (14).

Previous studies have reported that the agents mentioned above (fluoride, chlorhexidine, probiotics) could prevent dental caries; however, limited studies are comparing the antimicrobial efficacy and cytotoxicity of mouthwashes containing these agents mainly used in pediatric patient population (1,7,11,13). Therefore, this study aimed to evaluate the antimicrobial and cytotoxicity effects of mouthwashes containing fluoride, chlorhexidine, probiotics, and their combinations suitable for children's use.

In this study, the H_1 hypothesis was that there would be a significant difference among the groups in antimicrobial efficacy and cytotoxicity. Also, the null hypothesis (H_0) was that there would be no significant difference among the groups in antimicrobial efficacy and cytotoxicity.

2. GENERAL INFORMATION

2.1. Dental Caries

The metabolic action of the dental biofilm, which covers the surface of the tooth, causes the chemical and localized loss of tooth structure that can be defined as dental caries (3). It is a bacterial infection that progresses with prolonged exposure and is transmissible. In its early stages, caries progression is reversible (15). Multiple variables, including saliva, exposure to fluoride, and sugar in the diet, impact the dynamic balance between demineralization and remineralization, ultimately determining the progression of the disease (15,16). Dental caries may form on every tooth surface, including enamel, dentine, and primary and permanent teeth cementum. But it is more prevalent in areas where the dental biofilm is more protected, allowing it to grow, such as grooves, pits, and fissure surfaces (3,16).

There are several hypotheses on how dental caries develop. Attempts to explain the origin of dental caries date back to 5000 BC, when tooth worms were assumed to be the cause of dental caries. However, the father of modern dentistry, Pierre Fauchard, disproved this hypothesis of the tooth worm. In the 1680s, scientist Antonie van Leeuwenhoek found bacteria in the tartar from his teeth. He sketched what are now called cocci, fusiform, and spirochete bacteria in his notes (10).

Miller was a pioneer in the field of oral microbiology. He presented the “chemoparasitic theory,” claiming that oral bacteria would transform fermentable carbohydrates into acid, causing demineralization of teeth in vulnerable hosts who ate these carbohydrates often. However, Miller's idea could not explain that microbial dental plaque is the source of bacteria and the acids created by these bacteria (17).

G. V. Black described dental plaque in 1898 as "gelatinous microbic plaques," now known as dental plaque, a gelatin-like substance that carried microorganisms. Black hypothesized that the acid attacks from bacteria within these plaques caused caries formation based on his observations (18).

Black's study and Miller's chemoparasitic hypothesis formed the basis for our knowledge of the causes of dental caries and established dental plaque's importance in caries formation. Studies of dental plaque have also become an important marker of oral microbiology's overall growth (10).

In the past, many theories have been made about how caries start. Firstly, it was believed that just a few bacterial species, especially the gram-positives, such as *Streptococcus mutans (S. mutans)*, *Streptococcus sobrinus (S. sobrinus)*, and lactobacillus were responsible for the caries formation. This definition is called the “specific plaque hypothesis” (19). In contrast, according to the “nonspecific plaque hypothesis,” dental caries is caused by the activity of plaque microflora consisting of more than one bacterial species. In this hypothesis, many different microorganisms could cause disease (19).

More recently, Phil Marsh's "Ecological plaque hypothesis" postulates that the interactions between the microorganisms inside a biofilm are more important than the existence of any one microorganism within the biofilm itself in determining the biofilm's features (Figure 1) (20,21).

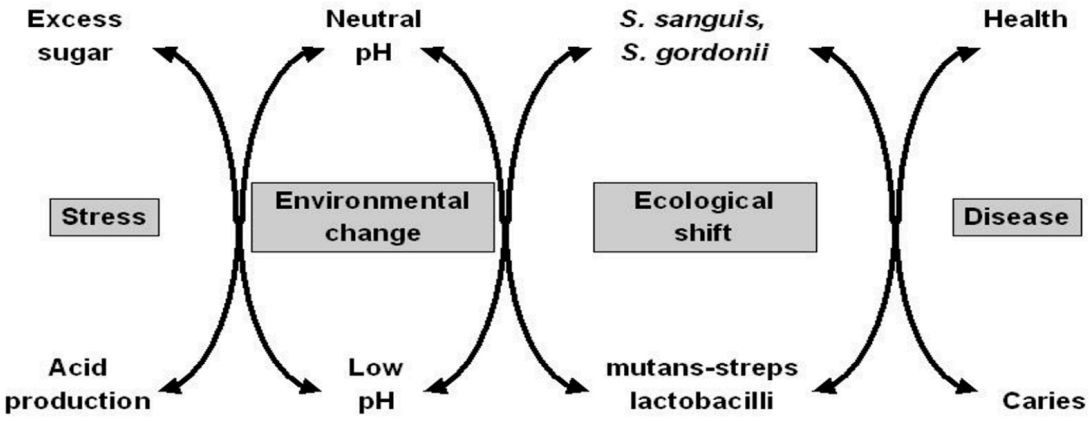


Figure 1: The ecological plaque hypothesis (21).

This hypothesis proposes that dysbiosis, a variation in the equilibrium of local bacterial species due to environmental changes, is the disease's primary cause. The interaction among the bacteria in the biofilm which affects the properties of the biofilm, stimulating the growth of acid-producing and acid-tolerant bacteria, causes an alteration in the bacterial community, with acidogenic gram-positive bacteria predominating at the tooth surface (20).

2.2. Biofilm Formation and Dental Caries

Before the 1960s, it was widely thought that growth in the overall number of bacteria, rather than qualitative changes in the content of the microbiota, was the cause of dental and periodontal disease. The term "biofilm," originally coined by Costerton in 1978, describes a bacterial colony encapsulated within a matrix. Bacteria inside the biofilm have been demonstrated to have distinct morphologies from their non-biofilm ones. They are stronger and more resilient to external traumas. Within the biofilm, there are many metabolite exchanges and different levels of interactions between other species (21).

The dental biofilm is a very advanced microbiological framework that consists of bacterial species that attach to the tooth surface (16). The oral biofilm occurs in a state of balance with host defenses and is essential for preserving the integrity of oral tissues. Dental caries result from an ecological imbalance in this physiological equilibrium between tooth minerals and the oral biofilm (15,22,23).

Dental caries develops when microbial dental plaque (MDP) is permitted to accumulate and is not cleared from the environment for a certain amount of time. MDP is defined as a sticky, amorphous, gelatinous, organic accumulation containing different types of microorganisms, leukocytes, macrophages, dead epithelial cells, salivary glycoproteins, and some food residues on the tooth that cannot be mechanically removed with saliva (24,25).

MDP is a community of microorganisms living on the surface of the tooth as a biofilm and is held together by polymers from both the host and the microorganisms. The development of dental plaque follows a specified series of events, which leads to forming a microbial community that is physically and functionally structured and abundant in species. The bacterial community that forms MDP is highly organized and genetically diverse. This microbial ecology-based pathogenic hypothesis helps explain the interaction between dental plaque and suggests new disease treatment and preventive methods (20,26).

Bacteria grow on teeth in microcolonies and are covered in an organic matrix of polysaccharides, proteins, and DNA secreted by the cells. This matrix offers protection against desiccation and host defense and greater resistance to antimicrobial agents and antibiotics than other planktonic members of the same species (23).

Plaque development is a dynamic process. Bacteria undergo the same process of adhesion, growth, organization, separation, and re-adhesion (27). Within a few seconds of the teeth being exposed to the oral environment, an outer layer known as the acquired pellicle has formed on the tooth surfaces. This layer includes glycoproteins and proteins derived from gingival crevicular fluid and saliva. Following the formation of the acquired pellicle, a few bacteria begin to bind to this layer (16,28). Bacteria–bacteria interactions stimulate further colonization and result in the formation of complex microbial communities. Quorum sensing is crucial for the continuous presence of numerous species in oral biofilms (28,29). The bacteria stick to the pellicle because of hydrophobic ion, electrostatic, and Van der Waals forces. Over time, as bacteria get more organized, biofilm formation starts to increase. As the biofilm's thickness increases, less oxygen can enter the matrix through diffusion (27).

The extracellular matrix is a critical part of biofilm, and this matrix is composed of extracellular polymeric substances (EPS). It is important as a biologically active scaffold for determining the form of the biofilm and serving as a physical support for vital nutrients, water, and enzymes (Figure 2) (16).

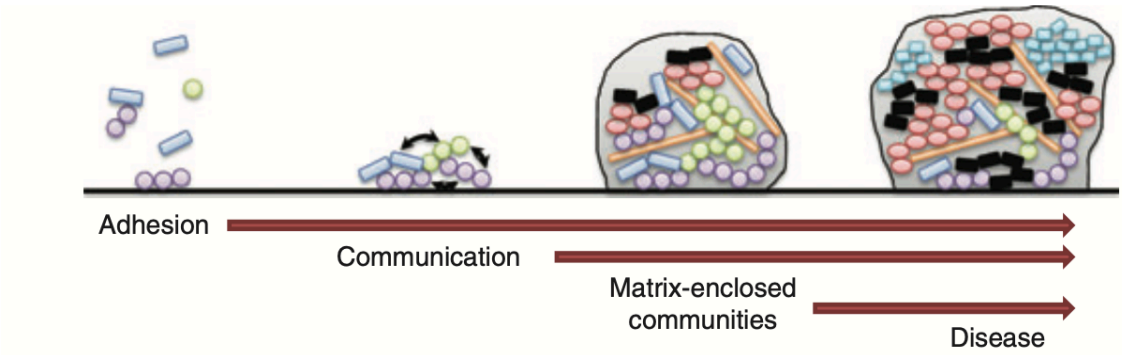


Figure 2. Phases of dental plaque development (28).

2.3. Caries-Associated Microorganisms

The oral microflora is one of the most colonized parts of our body, containing hundreds of different bacterial, viral, and fungal species (30). There are almost 700 other bacterial species that have colonized various parts of the oral microflora, including the teeth, tongue, oral mucosa, palate, and periodontal pockets (20).

Dental caries result from the decalcification of tooth hard tissue due to bacterially produced organic acid (31,32). This process results from interactions over time between bacteria that create organic acid, a substrate that the bacteria may metabolize, and other host components, including teeth and saliva (15,22). According to current understanding, gram-positive bacteria predominate in supragingival plaque, while gram-negative anaerobic bacteria predominate in subgingival plaque (10).

The two most significant cariogenic dental pathogens are mutans streptococci (MS) and lactobacilli (33). *Streptococcus mutans (S. mutans), Streptococcus sobrinus (S. sobrinus), Lactobacillus acidophilus (L. acidophilus), Lactobacillus casei (L. casei), Lactobacillus fermentum (L. fermentum), Lactobacillus plantarum (L. plantarum), Actinomyces viscosus (A. viscosus)* are the microorganisms responsible for caries formation (16,33,34).

The presence of Streptococcus mutans has been found to have a significant correlation with the development of dental caries. However, caries can also ocur in the absence of this particular group of bacteria. Especially, early childhood caries have been linked to a newly discovered species called *Scardovia wiggsiae*, which implies that this might be a novel caries pathogen. In some studies, although there are no *S. mutans* in the flora, it has been determined that this bacterial group can cause caries formation (35).

Acid production (acidogenic) and tolerance, the ability to survive and multiply in a low pH environment (aciduric), and extracellular and intracellular polysaccharide synthesis are the main features of microorganisms that cause dental caries (32). Cariogenic bacteria can produce weak organic acids (lactic, acetic, formic, propionic acid) by fermenting carbohydrates, and these acids can easily dissolve enamel and dentin minerals. This makes dental caries bacteria acid-tolerant, carrying out the dental caries process in an acidic environment (36).

Lactic acid has been known as the primary reason to declining pH in dental plaque (36). The acid accumulation results in a pH decline to the critical pH, which contributes to tooth hard tissue demineralization (21). The major acidogenic bacteria are *Streptococcus mutans*, *Lactobacilli* spp., and *Actinomyces* spp. (16).

Some oral bacteria are early colonizers that express biochemical components that enable them to attach the tissues they are attempting to colonize (37). The early colonizers, primarily oral streptococci, attach loosely and irreversibly to the acquired pellicle. By coaggregation,

secondary colonizers attach to the early colonizers, promoting the changes in the environment caused by the pioneer bacteria during the oral biofilm proliferation (16,28).

The later colonizers frequently have features that enable them to attach to the earlier colonizers, either giving competitive advantages or metabolic benefits. When dental plaque is present different types of bacteria are often next to each other or mixed to make unique structures that help the bacteria adhere or grow (19).

Bacteria may be passed on to infants by their parents and other caregivers during the early years of life, and they have the potential to multiply in soft tissues such as the tongue, palate, and mucous membranes even before the appearance of the first teeth (15).

2.3.1. Mutans Streptococci (MS)

Mutans streptococci are a gram-positive, spherical, non-motile, facultative anaerobic bacterial group. Hydrogen peroxide, a by-product of them, is used by them to hinder the growth of other bacteria (38) The optimal temperature for their reproduction is 37°C. In addition, these bacteria are acidogenic and aciduric and associated with carious lesions. The frequency and rate of MS are positively correlated with caries activity (16,34,38).

The neonatal oral cavity, which is sterile after birth, is rapidly colonized by the mother's microflora. Intimate contacts, such as sharing meals or spoons, lead to vertical transmission of *Streptococcus mutans* from mother to child in terms of the transmission mechanism. Vertical transmission refers to intra-familial transfer, especially in mothers, while horizontal transmission refers to transmission from the environment and other people (39,40).

MS can be colonized in the mouth, especially in the soft tissues, upper respiratory tract, esophagus, genitourinary region, and skin (33). Species with different serotypes of MS include *S. mutans, S. sobrinus, S. rattus, S. ferus, S. macacae, S. cricetus, and S. downei* (34).

2.3.1.1. *Streptococcus Mutans (S. mutans)*

The bacteria most closely related to dental caries is *S. mutans*, recognized as the primary pathogen of dental caries (33). J. Kilian Clarke identified *S. mutans* from the location of human dental caries in 1924; it is a gram-positive facultative coccus. They are stained dark blue or purple on the gram stain (Figure 3) (33). Clarke demonstrated that this type of bacteria could

ferment several sugars and produce a pH of 4.2 in glucose broth. However, he could not show that these bacteria contributed to caries (41). In 1960, Fitzgerald and Keyes demonstrated that caries could be caused by a single strain of streptococcus (41).

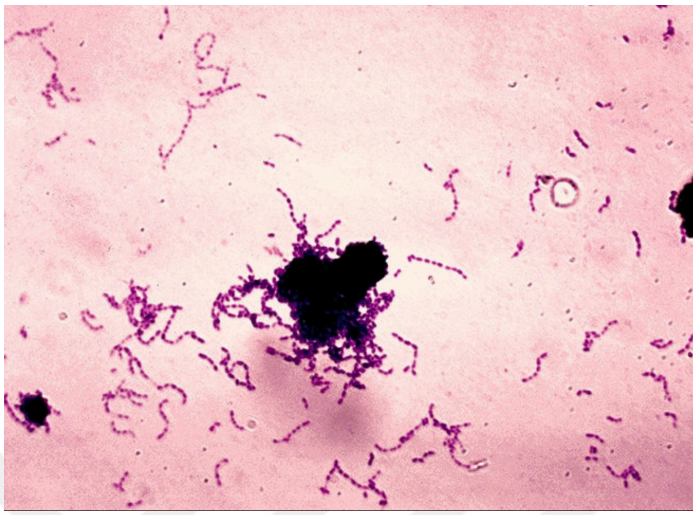


Figure 3. *S. mutans* gram staining (42).

Due to its acidogenic, aciduric, and adhesion abilities, *S. mutans* has been linked to the pathogenicity of dental caries. It is known that extracellular polysaccharide production by *S. mutans* is closely related to dental biofilm formation and cariogenicity (16,34).

Cariogenic virulence of *S. mutans* involves many features of adhesion for primary attachment to the surface of the saliva-coated tooth, such as producing extracellular polysaccharides such as fructans and glucans to increase stability on the tooth surface and enable dental plaque accumulation, producing intracellular polysaccharides, and producing an acidic environment and supporting aciduric microflora (16,34).

The biosynthesis of glucans, which contribute to biofilm formation by producing a glue-like polysaccharide matrix, plays an important role in pathogenicity. By forming the dental plaque matrix, glucans allow the bacteria to adhere to the tooth more tightly and allow bacterial adhesion after adhesion, while fructans; it is metabolized in a non-carbohydrate environment (43). These fructans serve as temporary storage reservoirs in the plaque and may also help the bacteria adhere to the tooth's surface. Short-term extracellular carbohydrate needs are met by fructans hydrolyzed by the fructanase enzyme FruA to produce fructose and then internalized or used as an energy source (43).

On the other hand, the intracellular polysaccharide (IPS) is a polysaccharide with a structure similar to glycogen. It can be used for energy production in the presence or absence of sugar in the environment and can be converted to acid. The IPS can be used as a source of carbohydrates for fermentation upon nutrient depletion (44).

2.4. Factors in the Formation of Dental Caries

Dental caries is a chronic, multifactorial disease that clinical, microbiological, behavioral, social, and environmental variables are all involved in the process, and caries can be caused by any one of them (45). Dental caries is a lifestyle disease, and therefore, behavioral variables that a person can control are involved. These variables involve improper baby feeding, poor oral hygiene practices, and poor dietary habits, such as frequent use of refined carbohydrates and oral medications containing sugar. Environmental factors include nutrition, time, socioeconomic status, and education level (3,15,46).

Early childhood colonization by mutans streptococci and other cariogenic bacteria is a significant risk factor for the development of caries. Children with a history of caries or whose mother, caregiver, or siblings have caries should be considered a higher risk for the condition (3,15).

Physical and biological risk factors for dental caries include insufficient salivary flow and composition, a large number of cariogenic bacteria, insufficient exposure to fluoride, gingival recession, immunological components, the requirement for specialized medical treatment, and hereditary variables (Figure 4) (15,22,46).

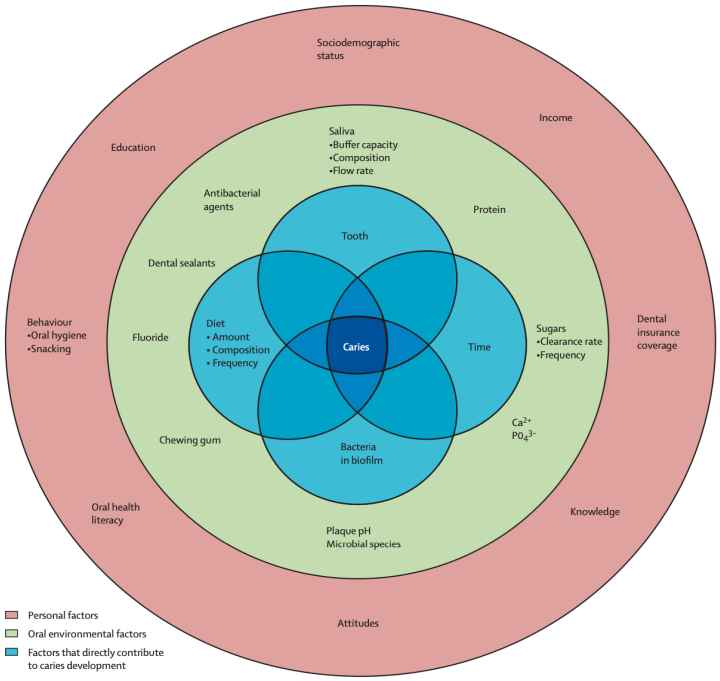


Figure 4. Illustration of the factors involved in caries development (47).

Key determinants in the development and progression of dental caries include the location of the teeth in the dental arch, the time since the eruption, and the morphology and structure of the tooth surfaces. The plaque buildup and susceptibility of teeth to acid assaults are directly related to how accessible the teeth are in the arch and how easy they are to clean (3). The occlusal surfaces of posterior teeth are more vulnerable because of the increased plaque buildup and difficulties in cleaning these areas. The lower incidence of caries can be attributed to reduced plaque deposition on the lingual surfaces of the lower anterior teeth, simpler cleaning, and more benefit from the washing impact of saliva (3). Also, plaque builds up in the mouth due to inadequate restorations, crowding, orthodontic treatment, and tooth alignment, which opens the way to developing dental caries (27).

Another element that influences a tooth's resistance to caries is the structure of the enamel itself. The solubility of dental enamel depends on the type, size, and distance of hydroxyapatite crystals. Teeth are more prone to caries because tooth enamel has not fully developed and is difficult to clean in the mouth after the eruption (27).

In the process of developing dental caries, the function that diet plays is critically significant. As a byproduct of the digestion of fermentable carbohydrates, cariogenic bacteria produce organic acids. The plaque's pH quickly drops due to these released acids (in 1-3 minutes). Plague acidity may rise to a normal value (pH=7) that can be reached in 30-60 minutes. Dr. Robert Stephan was the first to describe this pH increase and decrease in 1943. The "Stephan Curve" describes these pH variations after food consumption (Figure 5) (48,49).

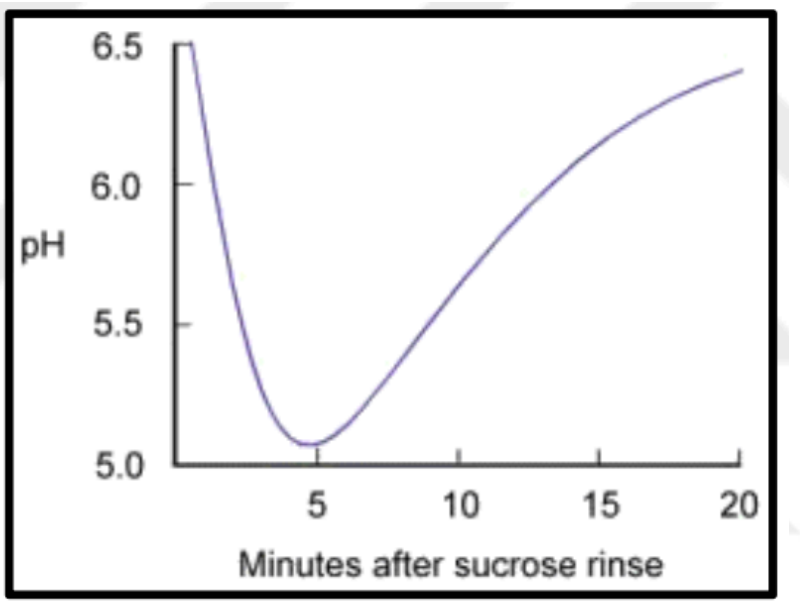


Figure 5. Stephan Curve (49)

According to a study, how frequently sugary foods are consumed is more important than how much they are consumed. Dental caries result from the persistently low plaque pH carried on by regularly eating foods with a high cariogenicity (50).

The time that sweet meals remain in the mouth also influences how cariogenic they are. Sticky meals can stay on teeth for an extended period, whereas quickly dissolving ones can be cleansed (50,51). The meal at which cariogenic meals are ingested is another critical consideration. The production of saliva is greater after a meal. As a result, eating cariogenic foods with meals decreases their carious action (51).

Saliva is critical in controlling the balance between demineralization and remineralization in a cariogenic environment. The major (submandibular, sublingual, and

parotid) and minor salivary glands of the mouth secrete saliva. Saliva is composed of 99% of water and 1% of organic and inorganic substances (52). The organic components of saliva include amino acids, urea, uric acid, mucin, immunoglobulins, ammonia, glucose, lipid, cholesterol, and proteins. In contrast, the inorganic components are calcium, sodium, potassium (K), sulfate, chlorine, and bicarbonates. Mucin, albumin, immunoglobulins, enzymes, hormones, DNA, polypeptides, oligopeptides, antibacterial proteins, and viruses are common components of saliva's organic composition (53).

Saliva has many protective functions, such as lubricating the oral tissues, sugar clearance, protecting the soft tissues of the mouth from abrasion, facilitating the digestion of carbohydrates, flushing the mouth to remove food particles, antimicrobial activity against microorganisms, keeping the environment rich in calcium, phosphate, and acid-buffering agents (53,54). Saliva includes several natural or acquired (antibody) defense systems and inhibits oral bacteria. Salivary antimicrobial proteins are non-specific immune agents. These are lysozyme, lactoferrin, lactoperoxidase, immunoglobulins, mucin, and agglutinin. The mucous proteins in saliva, known as mucins, serve many purposes, including protecting against pathogens and avoiding dehydration (53).

IgA suppresses bacterial aggregation and neutralizes byproducts from bacteria, viruses, and their antigen-antibody process. Lysozyme disintegrates a microorganism's cell wall. Lactoferrin exhibits bacteriostatic qualities, inhibits the colonization of bacteria, and binds the iron that is required for the growth of organisms. It is also bactericidal against MS (54).

It is known that the pH value of normal saliva is between 6.5-7.5. When the ph level drops below the critical pH, the tooth enamel starts to break down. One of the most vital duties of saliva is to neutralize and buffer the organic acids formed in the mouth. Saliva has three buffering systems: carbonic acid-bicarbonate, phosphate, and protein buffering systems (53,54).

When saliva is stimulated, the carbonic acid-bicarbonate system works, but when saliva is unstimulated, the phosphate buffering system becomes active. However, the proteins in saliva start to contribute to buffering if pH drops below 4-4.5. The most crucial buffering mechanism is the carbonic acid-bicarbonate system. As it passes into the microbiological dental plaque, it neutralizes the acids and serves as a buffer, inhibiting the development of caries (54).

The inorganic component of saliva contains sodium (Na) and calcium (Ca). The management of the acid-base balance is carried out by sodium, while calcium contributes to the rise in bicarbonate and pH. It is also known that fluoride in the saliva inhibits demineralization and accelerates remineralization (53). The remineralization of teeth, which helps prevent caries, is assisted by the saturation of saliva with minerals. The maturation and remineralization of enamel can be facilitated by saliva containing high amounts of calcium and phosphate (52,53).

2.5. Dental Plaque Control Methods

Microbial dental plaque management involves the daily removal of oral biofilm and dental plaque and the protection of their formation on the hard and soft tissue surfaces of the oral cavity. It can be accomplished mechanically, chemically, or by combining the two methods (55,56). Supragingival plaque is the type most often seen on the smooth surfaces of the teeth along the gingival lines. Plaque can also be found in the gingival sulcus, called subgingival plaque (56).

Most preventative procedures focus on removing plaque and reducing its effects to maintain a healthy oral environment. In addition, proper plaque removal requires frequent and effective oral hygiene practices (55).

2.5.1. Mechanical Plaque Control

Mechanical plaque control, which includes cleaning dental, interdental, and oral surfaces with toothbrushes and manual interproximal aids such as dental floss, interdental brushes, and irrigation devices, is an effective method of removing plaque biofilm and preserving oral and periodontal health (55). Mechanical plaque treatment can be successful, but it requires great attention to detail, motivated patients, and a proper lifestyle (such as a healthy diet, no smoking, etc.) (57).

2.5.1.1. Toothbrushes

Since they were first invented, toothbrushes have undergone a series of progressive improvements that have helped make them more effective at cleaning teeth. Traditional tools like fingers, sticks, and twigs were not as good at cleaning as today's toothbrushes (55).

Electric rotation-oscillation toothbrushes, sonic toothbrushes, and even solar-powered toothbrushes have lately been created thanks to ongoing research and development. These modern toothbrushes do more than simply scrape teeth with a plastic handle; they also encourage improved oral health with less effort and extra cleaning features (55).

2.5.1.2. Dental Floss and Interdental Brushes

The most often suggested instrument for eliminating plaque from proximal surfaces is dental floss. Using dental floss in combination with brushing significantly reduces plaque formation and gingival problems (55).

Interdental brushes are another tool that can be used to clean proximal surfaces. In the 1960s, these interdental brushes were made as an alternative to wood sticks. They consist of soft nylon filaments twisted into a wire. There are many different sizes and shapes of interdental brushes in the market (55).

2.5.1.3. Irrigation Devices

A dental water jet, water flosser, or oral irrigator is an electric device that pumps out a pulsed stream of water under controlled pressure. Its purpose is to remove interdental and subgingival plaque biofilm from the surfaces of teeth to reduce inflammation (55).

They cannot remove adherent plaque but clean non-adhesive plaque and food residues. These devices provide effective cleaning in fixed appliances or prostheses, but they are not suitable for use in the presence of untreated deep periodontal pockets (55).

2.5.2. Chemical Plaque Control

Mechanical oral hygiene is effective in preventing dental disease. Although effective plaque management is essential, most people struggle to keep it under control for extended periods (58). Antimicrobial chemotherapeutics and mechanical plaque control in individuals' daily oral care routines increase the effectiveness in controlling plaque and preventing periodontal disease (59).

Chemical plaque control is used in addition to mechanical methods, not instead of them. Chemical methods are only used as an adjuvant to control plaque. Mechanical methods continued to be the primary way to control plaque (59). Chemical plaque control includes the suppression of plaque deposition, the inhibition of microbial colonization, the removal and disintegration of existing plaque, the prevention of plaque production, and the transformation of pathogenic plaque into a less pathogenic form (60).

The use of chemical agents can be carried out in different ways. Toothpaste, mouthwashes, sprays, chewing gums, and varnishes are some of the main carriers. Mouthwashes and toothpaste are the most commonly used (59).

2.5.2.1. Classification of Chemical Plaque Control Agents

Chemical plaque control agents were classified as follows according to their effects by the European Federation of Periodontology at the 1996 European Workshop on Periodontology (61):

a. **Antimicrobial agents:** Chemical agents with a bactericidal or bacteriostatic effect *in vitro* that cannot be extended to a proven impact against plaque *in vivo*.

b. **Plaque-reducing/inhibitory agents**: Chemical agents that can change the quantity or quality of plaque may or may not be effective in preventing gingivitis and/or cavities.

c. **Antiplaque agents:** Chemical agents that produce prolonged and profound plaque reduction are strong enough to assist with gingivitis and/or tooth decay.

d. **Antigingivitis agents:** Chemical agents like anti-inflammatory drugs that can reduce gingival inflammation without affecting the dental plaque.

Antiplaque agents were subdivided by Kornman in 1986 (62), according to the antimicrobial efficiency and their substantivity, into three distinct generations;

a. **First-generation agents:** Antibacterial agents that are not very effective and only work for a short amount of time but can reduce plaque scores by 20–50%, such as antibiotics, fluorides, plant extracts, phenolic derivatives, oxidizing agents, and quaternary ammonium compounds.

b. Second-generation agents: Antibacterial agents that exhibit good substantivity over a long period and lower the plaque score by 70–90%, such as bisbiguanides; chlorhexidine is the best example, also triclosan with either copolymer or zinc citrate.

c. Third-generation agents: Agents that impair or cease bacterial adhesion without having any impact on bacteria, e.g., amine alcohols such as delmopinol. This group can also include products containing oxygenating agents, hexetidine, sanguinarine, and saturated pyrimidine.

According to Mandel in 1988 (62), antimicrobial agents were classified into five categories;

a. Antiseptic agents that can kill or stop the proliferation of all plaque organisms and have a wide range of antibacterial activity.

b. Antibiotics that can kill or inhibit a particular type of bacteria.

c. Enzymes that can separate or disperse the matrix that holds plaque together or change the plaque activity.

d. Modifying agents that are non-enzymatic, dispersing, denaturing, or modifying agents can change the composition of the plaque or the metabolic activity of the plaque bacteria.

e. Antiadhesives which prevent all or some of the oral bacteria from adhesion to the pellicle.

Chemotherapeutic agents can be divided according to their individual properties into three categories by Eley in 1999 (63);

a. Group A agents (antiplaque): They include chemicals with good substantivity, antibacterial spectrum, and sound antiplaque effects. They have such a strong inhibitory effect on plaque formation. So, they can be used instead of mechanical cleaning methods for a short time when the person can't use them well. Bisguanides are the only agents with these characteristics. Staining is the main problem of the bisguanides, which is closely related to their substantivity. Therefore, their extended usage is not recommended. This group includes chlorhexidine, salifluor, and delmopinol.

b. Group B agents (plaque-inhibitory): This category includes drugs with weak or no substantivity yet a broad range of antibacterial activity. Therefore, they prevent plaque

formation but do not actively remove it. As a result, they are not a suitable replacement for brushing, although they can be used as adjuncts to mechanical plaque control. These chemicals include cetylpyridinium chloride, essential oil, and triclosan rinses.

c. Group C agents (low to moderate activity): These are antiseptic mouthwashes that have been demonstrated to have antibacterial activities *in vitro,* but in clinical investigations, they have been shown to have varied plaque inhibitory effects from moderate to low. These agents would have minimal or no adjuvant impact when used with mechanical cleaning, so they should not be advised for this purpose. They would be expected to have a largely cosmetic role, such as breath-freshening. These include sanguinarine, oxygenating agents, hexetidine, povidone-iodine, and saturated pyrimidine rinses.

2.5.2.2. Mechanism of Action of the Chemical Plaque Control Agents

The clean surfaces of the teeth are prone to diseases because of plaque accumulation. Patient motivation is essential for mechanical plaque control. In addition to mechanical plaque management, chemical plaque control agents affect the metabolism and composition of biofilms. Antiplaque chemicals act by eliminating or disrupting present biofilms and inhibiting the growth of new biofilms. There are bactericidal or bacteriostatic agents available (58,60).

Depending on the targeted microorganisms, these agents may have a limited or broad spectrum. The effectiveness of antimicrobial agents is related to their contact time and concentration. Over time, the bacteria in the oral biofilm develop resistance to the antimicrobial agents used (60).

1- Different mechanisms of action may achieve chemical plaque control with a quantitative (reduction of the number of microorganisms) and/or qualitative (altering the vitality of the biofilm) effect (74). Agents that could inhibit the maturation or development of Anti-adhesive: Initial bacterial adherence to tooth surfaces is prevented because the active ingredient creates a pellicle on the tooth surface, inhibiting bacterial adhesion and preventing bacterial colonization. They impact the main microorganisms that cause plaque formation: E.g., amine alcohol and delmopinol, which interfere with bacterial matrix formation.

2- Antimicrobial: A combination of the two processes or one of them alone might be used by antimicrobial agents to prevent plaque development. The first type is bacteriostatic and

involves bacterial proliferation reduction. The antimicrobial agent's second function is bactericidal, which kills any microorganisms adhering or already adhered to the tooth surface: E.g., chlorhexidine or antibiotics.

3- Plaque-removal: Using a chemical agent in a mouthwash is anticipated to efficiently clean all tooth surfaces, similar to how a toothbrush eliminates microorganisms from a tooth surface. Because of this, chemical plaque removal products are often known as "chemical toothbrushes." E.g., hypochlorite, enzymes, and amine alcohol.

4- Anti-pathogenic: Changing pathogenicity of biofilms without destroying the microorganisms or boosting the hosts' immune systems.

2.5.2.3. Delivery Formats for Chemical Plaque Control Agent

Various products have been used to deliver chemical agents into the mouth to control plaque formation, such as toothpaste, mouthwashes, sprays, irrigators, varnishes, and chewing gums (59). The most commonly used products are toothpaste and mouthwash (64).

2.5.2.3.A. Toothpastes

Toothpaste is a term for all kinds of products used with a toothbrush to clean and/or polish teeth. In daily life, adding more toothpaste has been shown to be helpful because it gives people a feeling of freshness in their mouths, which they like (65,66). Toothpastes have changed a lot since they were first made thousands of years ago. They used to be made of crushed eggshells or ashes, but now they are often made with more than 20 different ingredients (66).

Several products on the market contain different chemicals. Compounds that fight caries, periodontal disease, bad breath, erosion, and hypersensitivity can be among these. Also, toothpastes have abrasives to clean and whiten teeth, flavors to freshen breath, and dyes to make the toothpaste look better (66).

The toothpastes use enzymes, amine alcohols, herbal or natural products, triclosan, bisbiguanides (chlorhexidine), quaternary ammonium compounds (cetylpyridinium chloride), and different metal salts (zinc salts, stannous fluoride (SnF), SnF with amine fluoride) as active ingredients (65,66).

2.5.2.2.B. Mouthwashes

Mouthwashes are now the most common and least complicated delivery method for antiplaque agents (59). Mouthwashes or mouth rinses are medicated, unsterile aqueous solutions used for rinsing the mouth to reduce oral bacteria, remove food debris, and reduce oral malodor while providing a pleasant taste. They are often categorized as cosmetic, therapeutic, or a combination (67).

It is well-documented that products similar to mouthwash have been used for oral hygiene for a very long time in ancient history. Evidence shows that recipes and formulations beneficial to oral health have existed for at least 6000 years (64).

According to writings from 2700 BC, ancient Egyptian and Chinese societies employed substances including beer, wine, honey, alum, vinegar, and even infant urine as a mouthwash for thousands of years. Fauchard (1690–1761) recommended rinsing with fresh urine in France. Alcohol-based mouth rinses, including white wine and beer, were also popular with the Romans (64).

Cosmetic rinses are over-the-counter or commercial solutions that help to eliminate oral debris before or after brushing, reduce bacteria in the mouth, prevent bad breath, and give the mouth a refreshing flavor. Therapeutic rinses often have the same benefits as their cosmetic counterparts, but they also have an active ingredient, like fluoride or CHX, that helps protect against oral diseases (67,68).

A mouthwash's ability to reach hard-to-reach places, ease of administration, and positive reception by patients are just a few of its many benefits. Lately, it has been acknowledged that, despite advanced technologies, the amount of mechanical oral hygiene practice is insufficient. It is advised to use antimicrobial mouthwashes to reduce plaque and gingivitis. Mouthwashes comprise a combination of the active ingredient (typically an antibacterial ingredient), surfactants, humectants, flavors, sweeteners, coloring, and preservatives (67,68). Mouthwashes have one or more therapeutic agents such as antibacterial, antiplaque, anti-inflammatory, anti-calculus, anti-caries, healing promoting, anti-hypersensitivity, and anti-halitosis agents as the active ingredient(s) (67,68)

The ingredients in mouthwashes can vary, although they are often less complex than those in toothpaste. They are basic aqueous solutions with coloring, flavoring, and preservatives (64). Ethanol is frequently used in manufacturing to stabilize active chemicals and lengthen the product's shelf life. Alcohol-based mouthwashes have been the center of many concerns (63). Most mouthwashes include less than 10% alcohol, but some have more than 20% alcohol. Alcohol-free mouthwashes are also being developed by several companies (64).

Depending on the components, mouthwashes are commonly used to inhibit bacterial plaque formation, minimize the oral cavity's microbial load, and reduce halitosis. Before oral surgeries, the antibacterial activity of mouthwashes is used to decrease dental plaque and gingivitis. Cosmetic mouthwashes may not kill microorganisms but have a pleasant flavor and can reduce bad breath (14,64).

Standard oral rinse formulations often include additives such as essential oils, chlorhexidine digluconate, detergents, and ethanol. Anti-inflammatory glucocorticoids, morphine, and lidocaine as anesthetics, nystatin as antifungal, antimicrobials, and antihistamine are all ingredients that may be found in oral mouthwashes. Chemicals, including glycerin, sodium benzoate, flavorings, colorings, and stabilizers, are also present in mouthwashes (14,58,62).

2.6. Characteristics of the Ideal Mouthwash

The following characteristics have been suggested for the ideal mouthwash:

An ideal mouthwash must be effective against disease and have an antimicrobial capacity against pathogenic flora. However, the only target of these agents should be the pathogenic bacteria. The control of pathogens should not lead detrimental changes in the remaining ecosystem.

i. It must reduce plaque and gingivitis and have possible anti-inflammatory effects.
ii. It must have high substantivity to the oral surface.
iii. The mouthwash should have the ability of fresh mouth sensation.
iv. It can be compatible with oral tissues and must be safe with minimal side effects.
v. It should not cause staining to the oral tissues.
vi. An ideal mouthwash should not cause toxic effects or allergies to the oral tissues.

vii. It must be stable at room temperature for an extended period.

viii. It must have acceptable taste and cost (64).

2.7. Chemical Anti-plaque Agents Formulated in Mouthwashes

2.7.1. Antibiotics

Antibiotics are antimicrobial substances that can kill certain types of bacteria by affecting microbial metabolism. Different groups of antibiotics, such as penicillin, tetracyclines, metronidazole, vancomycin, kanamycin, and spiramycin, have been shown to work on dental biofilm. Even though there is evidence that local administration of certain antibiotics is effective for gingivitis, antibiotics are not routinely used as supragingival agents because of the risk of developing resistant organisms, patient hypersensitivity, and fungal superinfections (62).

2.7.2. Enzymes

Enzymes are separated into two categories based on how they control plaque accumulation. The first group comprises dextranase, mutanase, protease, and lipase enzymes. They break up dental biofilm by making it hard for bacteria to adhere to teeth or by disintegrating plaque. The enzymes glucose oxidase and amyloglucosidase are in the second group. They work by making the immune system of the host. There is limited scientific evidence about how they affect gingivitis in real life, and there aren't any long-term studies either (69).

2.7.3. Amine Alcohols

Amine alcohols, octapinol, and delmopinol are surface-active substances having antibacterial action. They work by preventing the development of the biofilm matrix, which results in less bacterial adhesion. Delmopinol also prevents S. mutans from producing glucan and lessens bacterial acid production. Due to their side effects, such as burning sensation, tooth discoloration, and mucosal numbness, their usage is restricted (69).

2.7.4. Detergents

Toothpaste and mouthwash generally contain detergents due to their foaming and surfactant activity. Sodium lauryl sulfate (SLS) is the most common detergent with little antibacterial and antiplaque function. SLS adsorbs and penetrates the bacterial cell membrane, leaking internal components and causing cell lysis. Because it removes the mucosa's protective mucin layer and causes negative symptoms such as stomatitis, desquamation, cheilitis, and burning sensation, its application is restricted to dentifrices (70,71).

2.7.5. Metal Salts

Metal salts have been acknowledged to have antimicrobial effects, such as preventing plaque build-up, with most research focusing on zinc, tin, and copper. Polyvalent metal salts work well to stop plaque formation, but there are problems with their taste and toxicity.

As in zinc chloride, zinc citrate, zinc sulfate, zinc acetate, etc., zinc is an antibacterial ingredient added to mouthwashes and toothpaste. Zinc ions can suppress bacterial growth, plaque development, the glycolytic sequence in oral anaerobic bacteria, and the conversion of urea to ammonia. Since zinc ions have an affinity for sulfur, and create inert zinc sulfides, which are a little soluble and suppress the expression of volatile sulfur compounds (VSCs) and reduce halitosis (62,72).

2.7.6. Oxygenating Agents

Hydrogen peroxide (H_2O_2), peroxycarbonate, and sodium peroxyborate are oxygenating compounds with an antibacterial action by releasing oxygen. Oxygenating agents have been utilized as disinfectants in several dental specialties. Hydrogen peroxide has been used for dental plaque management and has lately gained popularity as a bleaching agent. Likewise, peroxyborate can be used to treat acute ulcerative gingivitis (69).

2.7.7. Quarternary ammonium compounds

Monocationic surface-active quaternary ammonium compounds include benzoyl chloride and cetylpyridinium chloride (CPC). CPC's wide range of antibacterial activity allows it to eliminate gram-positive pathogens quickly. CPC is equally effective against germs as

CHX. However, it has less of an impact on plaque and gingivitis. CPC's side effects are less common than CHX's (69,73).

2.7.8. Phenols and Essential Oils (EO)

Essential oils, which are made of phenolic chemicals, have been used for a long time as antiseptics and disinfectants. They combine eucalyptol, benzoic acid, thymol, methyl salicylate, menthol, and boric acid in an alcohol solvent. They damage bacterial cells and inhibit enzymes, which makes them suitable antimicrobials. These have the effect of reducing bacterial proliferation, aggregation, and pathogenicity. They also function as antioxidants, anti-inflammatories, and prostaglandin inhibitors.

Listerine combines thymol, methylsalicylate, eucalyptol, and menthol on a hydroalcoholic basis. The agent is used in the form of a mouthwash. This mouthwash can be combined with mechanical plaque removal in patients with gingivitis (62,74).

Charles et al. (2004) conducted a clinical study to evaluate the effectiveness and the potential adverse effects of a CHX (0.12%) and EO containing (Listerine) mouthwashes. They concluded that after 6- months of use, these mouthwashes had similar antiginigivitis and antiplaque efficacy and the adverse effects of CHX mouthwash may affect regular use (75).

Marchetti et al. (2017) evaluated the antiplaque effects of an alcohol-based essential oils mouth rinse (EO+) and an alcohol-free mouth rinse containing essential oils (Listerine Zero) compared with positive control of 0.20% chlorhexidine mouth rinse. Results showed that Listerine Zero has the same antiplaque effect on dental plaque regrowth as EO+ and a less antiplaque effect than the control (CHX) and the placebo (saline) groups (76).

In a systematical review, the effects of the two mouthwashes (EO anda CHX containing) on dental plaque and periodontal tissues were evaluated. Results of the study showed that EO mouthwash appeared to be a strong alternative to CHX mouthwash for the periodontal problems (77).

2.7.9. Triclosan

Triclosan, a non-ionic, bisphenolic antimicrobial, has been extensively used in antiperspirants, detergents, and other medicinal products for many years. It has recently been incorporated into toothpaste and oral rinses (64). It's antibacterial activity is broad-spectrum and it has plaque-inhibitory effect. It impairs plaque metabolism and bacterial cell walls. Inhibiting cyclooxygenase and lipoxygenase pathways decrease prostaglandin and leukotriene production, which may lessen inflammation. When added to toothpaste, triclosan with copolymer, zinc citrate, or pyrophosphate improved antibacterial action and lasted 8 hours (69).

2.7.10. Bisbiguanides

As a result of attaching to cell membranes, bisbiguanides, including CHX, alexidine, and octenidine, can demonstrate antiplaque action and kill numerous bacteria by disrupting their cell wall. CHX is widely regarded as the gold standard anti-gingivitis and anti-plaque agent because of its ability to increase cell membrane permeability and subsequently cause cellular macromolecules to coagulate. It is also efficient against gram-negative and gram-positive bacteria, anaerobes, aerobes, fungi, yeasts, and lipid-encapsulated viruses (63).

2.7.11. Hexetidine

A pyrimidine derivative known as hexetidine has some antibacterial properties *in vitro* against gram-positive and gram-negative bacteria and yeast. However, due to limited oral retention and antimicrobial action that could not continue for more than 90 minutes, *in vivo* data did not reveal plaque-inhibitory or antiplaque efficacy (69).

2.7.12. Povidone Iodine

Povidone-iodine exhibits antibacterial activity against various microorganisms, including bacteria, viruses, fungi, and protozoa. Povidone is an iodophor because it transports free iodine to the membranes of bacteria. It decreases gingivitis and radiation mucositis symptoms and helps prevent plaque buildup. In those with iodine sensitivities or preexisting thyroid issues, it should be avoided (69)

2.7.13. Natural Products

Natural products, extracts from plants, fungi, microorganisms, animals, and the sea are used in oral hygiene products for many years, and both the market and the professional community have seen a significant rise in demand. They have anti-inflammatory and antioxidant properties that are good for oral health. They work by preventing bacteria from adhering to tooth and surfaces of the dental restorations. Peppermint oil, aloe vera, tea tree oil, sanguinarine, echinacea, chamomile, myrrh, propolis, sage, clove, rhatany, meswak, turmeric, neem, green tea, xylitol, and hyaluronan have been in high demand on the market and among professionals (62,74,78).

2.7.13.1. Xylitol

Xylitol is a five-carbon natural alcohol that is nonfermentable and originates from vegetables, fruits, and berries. It is manufactured by artificially isolating xylan-rich plant components, and US Food and Drug Administration (FDA) has authorized it as a natural sweetener. It is formulated in mouthwashes, toothpaste, syrups, gums, and lozenges (79).

Xylitol reduces the number of MS in saliva and dental plaque by interfering with their energy production mechanisms, resulting in an ineffective energy cycle and cell mortality. It prevents these microorganisms from adhering to the tooth surface and causing acid production. Oral microorganisms cannot practically ferment xylitol, which is xylitol's distinctive quality. As a sweetener, xylitol is well tolerated by the human body, but its absorption in the small intestine is prolonged. Consuming 5–6 grams of xylitol three or more times per day is optimal for inhibiting the growth of *S. mutans* (79).

Lebel et al. (2020) evaluated the cetylpyridinium chloride (CPC)-based mouthwashes containing sodium fluoride and xylitol (X-PUR Opti-Rinse 0.05% NaF and X-PUR Opti-Rinse 0.2% NaF) for their antimicrobial activity against oral pathogens associated with dental caries, periodontal disease, and candidiasis. They found that the CPC-based mouthwashes supplemented with sodium fluoride (0.05% or 0.2%) and xylitol (10%) were highly active against oral pathogens (80).

2.8. Fluoride Compounds

Fluoride has played an important role in reducing dental caries prevalence and severity. Several caries-preventive mechanisms of fluoride are present. Low fluoride levels in plaque and saliva are responsible for the inhibition of the demineralization of the enamel and the promotion of the remineralization of demineralized enamel when applied topically. Additionally, fluoride inhibits dental caries by suppressing the metabolic activity of cariogenic bacteria (81). Fluoride aids in preventing dental caries by facilitating remineralization with fluorapatite and fluoro-hydroxyapatite, thereby enhancing enamel resistance to acid attack (82,83).

Different methods, such as water fluoridation, fluoride toothpaste, mouth rinses, varnishes, and gels, have contributed significantly to the decline of dental caries. Although numerous alternatives have been proposed, fluoride mouthwash is an efficient supplement to mechanical cleansing. Fluoridated mouthwashes have been developed to make the application easier and to save time (83,84). The purpose of fluoridated mouthwashes is to increase the free fluoride level in the saliva and to keep it at a certain level. Fluoridated mouthwash has been reported to increase salivary fluoride concentration for several hours (85).

Fluoride is generally available in various concentrations as acidulated phosphate fluoride or sodium fluoride (NaF). Besides, stannous fluoride (SnF_2), amine fluoride, and ammonium fluoride compounds can also be preferred in mouthwashes. Two primary rinsing methods are low-potency sodium fluoride at 0.5% and high-potency sodium fluoride at 0.2%. Low-potency ones are for daily applications, and high-potency ones are for weekly applications (82–84).

Patients at high risk for dental caries, such as those with xerostomia following irradiation and chemotherapy, those with difficulty conducting oral hygiene procedures, and those undergoing fixed orthodontic treatment, are advised to use fluoride mouthwashes. As with any fluoride delivery system, children younger than six must be treated cautiously (82,83).

Pizzo et al. (2008) investigated the plaque-inhibiting effects of two mouth rinses which contain amine fluoride/stannous fluoride (ASF) and EO. Results of the study demonstrated that ASF- and EO-containing mouth rinses had significantly greater antiplaque activity than

negative control (saline solution). However, there were no significant differences between the ASF and EO groups. They concluded that EO and ASF rinses may be an effective alternative to CHX rinses to maintain oral hygiene due to the minimal numerical difference in the plaque index between the rinses (86).

Dehghani et al. (2015) evaluated the efficacy of a mouthwash that includes a combination of 0.05% NaF and 0.06% CHX. They found that the combination significantly increased the oral hygiene both microbiologically and clinically. The combination was tested and compared with 0.05% NaF and 0.06% CHX mouthwashes separately. It was observed that there was a significant improvement in the plaque index (PI), modified gingival index (MGI), and bleeding index (BI) with the use of CHX + NaF mouth rinses. However, there was no statistically significant difference when comparing the use of CHX alone. Additionally, the effect of NaF alone was found with a significant decrease in *S. mutans* and lactobacilli. Despite not having additional effects on S. mutans, the combined CHX + NaF group still revealed a synergistic effect (87).

2.9. Chlorhexidine

Chlorhexidine was developed in the UK by Imperial Chemical Industries in 1953. Since its introduction in clinical and veterinary medicine, the synthetic antimicrobial agent CHX has been used extensively as a broad-spectrum antiseptic. In 1954, it was initially used on humans as an antimicrobial cream for wounds (63,88).

CHX is often used as water-soluble salts like digluconate, acetate, and hydrochloride (89). It is a broad-spectrum antimicrobial that belongs to the bisbiguanide family; in low concentrations, CHX has bacteriostatic properties. However, at higher concentrations, it exhibits bactericidal properties. The antibacterial effect of this compound is caused by an increase in the cellular membrane permeability of the bacteria, which is then followed by the precipitation of the cytoplasm and the coagulation of the macromolecules in the cytoplasm (63,90).

Chlorhexidine is effective against both gram-negative and gram-positive bacteria, including anaerobes and aerobes, as well as fungi, yeasts, and viruses, especially the human immunodeficiency virus and the hepatitis B virus (63).

The symmetrical chlorhexidine molecule comprises two biguanide groups, four chlorophenyl rings, and a central hexamethylene bridge. At pH levels greater than 3.5, CHX is di-cationic and has two positive charges on either side of the hexamethylene bridge (Figure 6) (91).

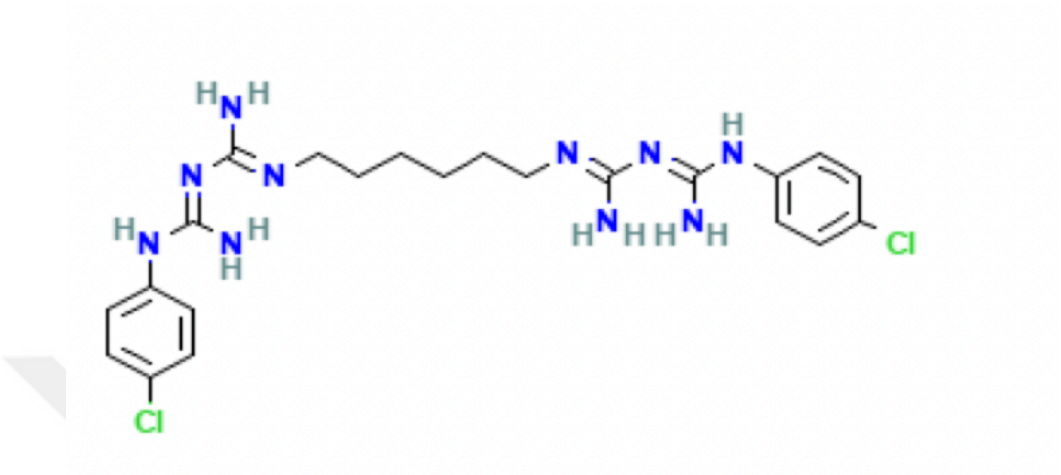


Figure 6. Structure of the CHX molecule (91).

As a microorganism reaches the tooth surface, one charged end of the CHX molecule bonds to the tooth surface while the remaining two connect with the bacterial membrane, a process known as the pincushion effect. It reversibly and strongly adheres to the hard and soft tissues in the oral cavity and is slowly released, providing 8-12 hours of persistent antibacterial action (92).

2.9.1. Mechanism of Action of Chlorhexidine

Mechanism of antibacterial action of chlorhexidine is believed to be as follows. Typically, the bacterial cell is negatively charged. The cationic chlorhexidine molecule is immediately attracted to the negatively charged bacterial cell surface, with a considerable affinity for phosphate-containing substances. This compromises the integrity of the bacterial cell membrane, and chlorhexidine is attracted to the inner cell membrane. Chlorhexidine binds to phospholipids in the inner membrane, causing the inner membrane to become more permeable and potassium ions to flow out (90).

In this bacteriostatic stage, chlorhexidine's effects are reversible; neutralizers remove excess CHX, which results in the recovery of the bacterial cell. This suggests that the structural

alterations to the cytoplasmic membrane induced by low chlorhexidine concentrations are negligible compared to those induced by higher drug concentrations (bactericidal levels) (90).

Elevating the quantity of chlorhexidine damages the membrane increasingly more, as shown by the size of the permeable species lost by the cell. When the concentration of chlorhexidine rises, the leakage of low-molecular-weight cytoplasmic components decreases, indicating the coagulation and precipitation of the cytoplasm caused by the creation of phosphate complexes, such as adenosine triphosphate and nucleic acids (90).

2.9.2. Usage of Chlorhexidine in the Field of Dentistry

Several chlorhexidine formulations include oral rinses, gels, chips, sprays, toothpaste, varnishes, and chewing gum. The most frequent form of CHX is a mouthwash with a concentration between 0.1 and 0.2% (89,93).

Rinses between these concentrations are equally effective when administered in equivalent dosages. Rinsing for 30 or 60 seconds depends on the adsorption rate of antiseptics to the oral surfaces (50% of chlorhexidine binds to receptors after 15 seconds), which differs between individuals. The optimal dosage schedule is twice daily for 12 hours (89).

It is used in addition to oral hygiene and professional prophylaxis. It is used after oral surgery, like root planning or periodontal surgery. CHX is used as a preoperative rinse or irrigation to reduce bacteremia, the total count of bacteria in the mouth, and aerosol contamination from sonic and ultrasonic devices (69,89).

It is administered to individuals who are medically compromised and susceptible to oral candidiasis. As an adjuvant to antibiotic prophylaxis, chlorhexidine prevents oral bacteria from causing bacteremia and operatory contamination. Additional applications for chlorhexidine include subgingival irrigation, the treatment of denture stomatitis, hypersensitivity, and oral malodor (69,94).

Chlorhexidine is usually used short-term for biofilm prevention or treatment. CHX may be used as an adjunct to control the mechanical plaque and prevent the formation of biofilm after periodontal treatment and in patients with intermaxillary fixation, acute mucosal, and gingival infections or to control pathogenic microorganisms in necrotizing gingivitis,

candidiasis, peri-implant mucositis, peri-implantitis, and periodontitis. CHX alone can prevent postoperative infections. When chlorhexidine rinses are applied before and after extractions, scaling, root planning, or periodontal surgery, wound healing is accelerated (69,89,95).

In individuals with periodontitis, permanent or removable orthodontic equipment, gingival hypertrophy or overgrowth, dental implants, or disabilities, chlorhexidine can be used long-term to avoid biofilm development. CHX may be used long-term to prevent oral and systemic problems in patients with immunosuppression at high risk of oral infections. CHX also treats and prevents oral problems, including chemo/radiation-induced oral mucositis in head and neck cancer patients, caries, candidiasis, recurring aphthous ulcers, and halitosis (69,94).

In comparison with different concentrations of CHX mouthwashes, after the usage of 21 days, 0.2% CHX resulted in lower dental plaque scores than 0.06% and 0.12% CHX (96).

Jose et al. (2015) designed a study to compare clinical parameters following use of 0.2% CHX-alcohol and 0.2% CHX-alcohol-free, and brushing with a fluoride toothpaste was compared to brushing alone for six weeks. According to the study, CHX mouth rinse with or without alcohol combined with brushing significantly reduces gingival inflammation, plaque, and bleeding scores compared to brushing alone (97).

Haerian-Ardakani et al. (2015) investigated the antimicrobial effects of 0.2% CHX, Persica mouthwash (PM), and Listerine on bacteria. They found that a 0.2% CHX mouthwash has more effect than Listerine on facultative and aerobic bacteria gathered from plaque samples of the individuals with gingivitis, and the antibacterial activity of CHX persists longer (98).

2.9.3. Toxicity and Adverse Effects of Chlorhexidine

Chlorhexidine has limited gastrointestinal absorption and toxicity. It's non-teratogenic. It has an unpleasant taste, affects taste sensation, creates oral mucosal erosion, and leaves brown discoloration on teeth and restorations that is difficult to eliminate. Staining of the tongue and mucous membranes may be caused by chromogenic food substances precipitating on them. The breakdown of chlorhexidine results in the release of parachloroaniline. Catalysis of Maillard

browning events occurs, which is non-enzymatic. Protein denaturation and metal sulfide production develop, and anionic dietary chromogens precipitate (89,94).

There should be a 30-minute break between toothpaste and mouthwash with chlorhexidine. It is recommended since most toothpastes include detergents that are mostly anionic. Being dicationic, the chlorhexidine molecule prefers to interact with anionic agents, decreasing the substantivity of chlorhexidine mouth rinse (89,94).

2.10. Probiotics

The World Health Organization has accepted the following definition of "probiotics" as "living microorganisms that confer a health advantage on the host when administered in adequate amounts" (99). The beneficial effects of probiotics on human health were first suggested by Elie Metchnikoff in 1908, and she stated that regular consumption of live microorganisms in fermented milk products prolongs life span by positively affecting microflora (100).

Although probiotic microorganisms are generally known as bacterial strains, such as Lactobacillus, Lactococcus, Bifidobacterium, Streptococcus, Enterococcus, and Bacillus, some yeast strains belonging to the Saccharomyces genus are also used in probiotic products (100).

2.10.1. Effects of Usage of Probiotics on Oral Flora

Probiotic technology is a novel method to maintain oral health, as it employs the naturally occurring helpful bacteria found in healthy mouths to provide a natural defense against the microorganisms believed to be harmful to teeth and gums (11). The oral microbiome is a significant component of the human microbiome (101).

The oral microbiome is a significant component of the general microbiome of a human (101).

Probiotics are thought to work through similar mechanisms in the mouth as they do in other body areas. They are believed to primarily act via the following mechanisms:

- compete for adhesion sites or nutrients with possible pathogens,

- producing bacteriocins or other compounds that inhibit the development of pathogens,
- activation and modification of the mucosal immune system; increase of mucin synthesis,
- regulation of cell proliferation and apoptosis
- strengthening the barrier of the intestine (99,102).

Pathogen colonization may be blocked if adhesion receptors are associated with microorganisms with minimal or low virulence potential and the ability to create beneficial compounds. In this way, probiotics have been used to prevent the colonization of microorganisms and to modify immunological and inflammatory responses (99,102).

Probiotics in the oral cavity have unique features. Oral probiotics must adhere to and accumulate in oral tissue, particularly non-shedding, hard surfaces, and grow in the biofilm. They should not ferment sugar or carbohydrates, either. Otherwise, the pH will begin to decrease, which may lead to dental cavities. (99,102).

Probiotic mouthwashes contain living organisms such as lactobacilli or Bifidobacterium, which are considered to be part of oral microflora and could reduce the level of *S. mutans* in saliva through multiple mechanisms, including the production of antimicrobial agents (hydrogen peroxide, lactic acid, and bacteriocins), modulating the inflammatory response, and competing with pathogens for adhesion to surfaces (103). Probiotic mouthwash decreases the pathogenic oral flora and stabilizes the beneficial flora in the oral cavity (104). A clinical study revealed that probiotic mouth rinse has a potential therapeutic value and is an effective and safe alternative to chlorhexidine mouthwash, appearing to be a boon for treating dental caries and periodontitis (105).

Jothika et al. (2015) analyzed the short-term effectiveness of CHX, probiotics, and fluoride mouthwashes on *S. mutans* levels. According to the results of this *in vivo* study, CHX, NaF, and probiotic mouthwashes decrease the number of plaque *S. mutans*. Probiotic mouthwash found effective and equivalent to CHX and NaF mouthwashes. Thus, probiotic mouthwash can also be an alternative oral hygiene regimen (1)

Sajedinejad et al. (2018) assessed the antibacterial effects of Nanosil, CHX, and probiotic mouthwash on some main oral perio-pathogens. They concluded that probiotic mouthwash had more significant antibacterial effects than others (104).

Bande et al. (2021) investigated the efficacy of a probiotic and chlorhexidine mouthwash in reducing plaque accumulation and gingival inflammation among schoolchildren aged 6–8. In this 14-day randomized controlled trial, they found that probiotic mouthwash effectively reduced plaque accumulation and gingival inflammation in 6- to 8-year-old children (105).

Alhallah et al. (2022) evaluated the effectiveness of nonchemical (probiotics) and chemical (fluoride) mouthwashes on plaque accumulation in children with different time periods of use. They found that both nonchemical and chemical mouthwashes are effective for maintaining oral health. However, probiotics are considered more effective than fluoride in reducing plaque accumulation after a month of use (106).

2.10.1.1. *Lactobacillus paracasei (L. paracasei)*

L. paracasei gathered from healthy humans showed anticandidal and antibacterial activities against *S. mutans, S aureus, S sanguis, S. salivarius, P. gingivalis, A viscosus, Candida grabata, Candida tropicalis,* and *Candida albicans* (107).

Chuang et al. (2011) evaluated the efficacy of the probiotic *L. paracasei* GMNL-33 tablets for decreasing the number of caries-associated microbials in healthy adults. They found that at least a 2-week period of orally administered usage may be needed for *L. paracasei* GMNL-33 to become effective in its probiotic action (108).

Rossoni et al. (2018) evaluated the antibiofilm effects of cell-free supernatants of Lactobacillus strains from the oral cavity on *S. mutans*. This study showed that most Lactobacillus strains isolated from the oral cavities of caries-free subjects could release bioactive substances that inhibit the growth of *S. mutans* in planktonic cultures. The strains *L. paracasei* 11.6, *L. paracasei* 25.4, *L. fermentum* 20.4, and *L. paracasei* 20.3 presented antibiofilm activity against *S. mutans* in different methods of biofilm quantification (109).

2.11. Antimicrobial Activity Evaluation Methods

Antimicrobial susceptibility tests are applied to determine an antimicrobial agent's *in vitro* activity against a particular bacterial strain. Many different methods are used in antimicrobial susceptibility tests (110).

2.11.1. Diffusion Methods

Tests that assess or screen an extract's or a pure compound's antibacterial activity in vitro. Methods such as the agar disk-diffusion test, antimicrobial gradient method (E test), and other diffusion methods (agar well diffusion method, agar plug diffusion method, cross streak method, poisoned food method) are included in this group (110).

2.11.1.1. Agar Disk-Diffusion Method

The official technique is the Agar disk-diffusion test, established in 1940 and used in many clinical microbiology laboratories for routine antibiotic susceptibility testing. The Clinical and Laboratory Standards Institute (CLSI) has published many authorized and accepted standards for testing bacteria and yeasts (110,111).

In this process, agar plates are inoculated with a standardized inoculum of the test microorganism. The test compound is applied in the required concentration on filter paper discs (approximately 6 mm in diameter) and then placed on the agar surface. The Petri dishes are incubated in the proper environments. The test microorganism's germination and growth are typically inhibited by an antimicrobial agent that diffuses into the agar, and the diameters of the inhibitory growth zones are then determined (110).

The disk-diffusion assay has many advantages over other methods, such as its simplicity, low cost, ability to test many microorganisms and antimicrobial agents, and ease of interpreting results. But the minimum inhibitory concentration (MIC) cannot be calculated using the agar disk-diffusion method because it is impossible to determine how much antimicrobial agent has diffused into the agar medium (110).

2.11.2. Thin-layer chromatography (TLC)– Bioautography

The TLC-bioautography approach is beneficial for assessing organic plant extracts' antibacterial and antifungal activities. Three bioautographic procedures, including agar diffusion, direct bioautography, and agar-overlay assay, have been outlined for evaluating anti-microbial drugs using this method (110).

2.11.3. Dilution Methods

Dilution procedures are the most suitable for the determination of MIC values since they enable the estimation of the antimicrobial agent's concentration in the agar (agar dilution) or broth medium (macro-dilution or micro-dilution) (110).

2.11.4. Time-kill Test

The time-kill test is ideal for determining a substance's bactericidal or fungicidal activity. The time-kill test demonstrated a concentration- or time-dependent antibacterial action (110).

2.11.5. ATP bioluminescence Assay

The ATP bioluminescence test measures the adenosine triphosphate (ATP) bacteria or fungi create. Since ATP is the chemical energy form of all living cells, each cell contains a constant amount. Consequently, it estimates the microbial population in samples (110).

2.11.6. Flow Cytofluorometric Method

The ability to quickly identify injured cells depends on using the right dyes for staining. Three subpopulations (dead, living, and wounded cells) can be separated using this approach in addition to lysed cells. Flow cell counting is a technique for detecting antimicrobial resistance based on evaluating the probable effects on the viability and cell damage of the tested molecule and organism (110).

2.12. Biofilm Assessment Methods

Biofilm assays describe biofilm features such as the quantity and kind of microorganisms, the population's vitality (dead/living cells), age, thickness, structure, and

surface topography. These assays involve several techniques, such as microbiological culture, colorimetric, microscopic, physical, biochemical, and molecular methods (112).

2.12.1. Microbiological Culture Techniques

Colony Forming Units (CFU) are defined as the number of bacteria that attach to a surface, which can be used to directly count the biofilm that forms on a substrate. But the CFU may only be able to find bacteria that can divide their cells quickly enough to form colonies (112).

Many methods suggest that sonication or centrifugation should be used to separation of biofilm bacteria from the adhesion surface. Supernatant collected after sonication and centrifugation is typically used to calculate CFU in such situations (112).

2.12.2. Colorimetric Techniques

A semi-quantitative technique that is based on dye absorption by bacteria. This test involves staining the bacterial cells of the biofilm with a dye (like crystal violet), disrupting it with alcohol or a surfactant, and measuring the dye's intensity with a spectrophotometer. This basic technique enables the quick quantification of bacteria found in biofilms (113).

2.12.3. Microscopic techniques

One of the direct ways to study bacterial adherence is using a microscope. In bacterial adhesion tests, the primary criteria measured by microscopes are the number of adhering cells, the amount of the material's surface area covered by bacteria, the kind, vitality, and dispersion of bacteria, and the number of adherent cells (112).

Microscopy techniques such as light microscopy, fluorescence microscopy, scanning electron microscopy, transmission electron microscopy, confocal laser scanning microscopy, atomic force microscopy, and epifluorescent microscopy can be utilized to assess bacterial adherence (112).

In the microscopical technique, a suitable fluorescent (such as propidium iodide) or non-fluorescent dye is used to stain the bacterial biofilm (e.g., safranin). The sample preparation

stages, such as fixation and dehydration, are a drawback of this approach. These procedures may alter the structure of the initial plaque (112).

2.12.3.1. Scanning Electron Microscopy (SEM)

Scanning Electron Microscopy (SEM) has been a reliable tool for analyzing biofilms for a long time. The shape and structure of microbial biofilms have been studied with the help of high-resolution electron microscopy (112).

Manfred von Ardenne invented scanning electron microscopy in 1938, and the Cambridge Instrument Company released it publicly in 1965. High-voltage electrons are sent to the tooth surface, where they come into contact with it while scanning the surface and capture the resulting signals, which are then reflected on a screen after being amplified. It enables in-depth analysis of alterations to the enamel surface's topographic structure (114).

Due to its combination of focus depth, discriminating power, and image analysis, it is frequently used in research. SEM-produced images have excellent depth of field. Surface characteristics, morphology, and atomic or elemental sequences can all be seen during SEM studies. SEM provides a 300 times greater focal depth and 20–100,000 times clearer vision than light microscopy. It may give incredibly detailed pictures at different magnifications (x20-x10,000 or higher) (112,115).

The samples need to be coated in a substance that reflects electrons for the surface to be visible. A thin layer of platinum (Pt), gold (Au), gold-palladium (Au/Pd) alloy, or carbon (C) can be applied to samples. This process is known as "sputtering" or "sputter coating" (115).

The main problem with these methods is that they require a lot of steps to prepare the sample, such as dehydration, fixation, freeze-drying or drying to the critical point, and sputtering. These treatments may have a significant impact on the original shape of the biofilm (112).

2.12.3.2. Confocal Laser Scanning Microscopy (CLSM)

CLSM is a non-invasive, three-dimensional (3D) microscopic imaging technology that is widely used. The CLSM method is used to evaluate materials utilizing a variety of fluorescent dyes. A focused laser source provides light throughout the application of the procedure, and

scans are created as lines on the sample. Scanned samples are layered in the x, y, and z planes to create high-quality three-dimensional images (112).

CLSM is an essential method for analyzing and quantifying biofilm structures, but it can only be used on 50–200 mm thick structures (112). The thickness and dispersion of the cells in a biofilm structure can be determined using CLSM imaging. Additionally, CLSM can be used to analyze pH gradients in biofilms. Biofilms' internal pH is determined using a fluorescent lifetime imaging approach and fluorescein as a pH indicator. For *in vitro* biofilm analysis, a fluorescent dye combination (LIVE/DEAD BAC light) with CLSM has been established as standard procedure. Separate vials of the two-component dyes are included in the LIVE/DEAD Bacterial Viability Kit from Molecular Probes in Eugene, Oregon (SYTO 9 and propidium iodide). Following the manufacturer's recommendations, the dyes employed to color the biofilm bacteria are prepared in a 1:1 combination. Under CLSM examination, the live cells reflect green light, whereas the dead cells reflect red light (112).

In contrast to SEM imaging, CLSM allows working with teeth that are still moistened naturally. This approach also has the benefit of enabling defects on sample surfaces to be neglected. Problems are prevented because processes like cutting in thin parts and drying samples using precise equipment are not used. There are also disadvantages, such as the sample preparation takes a long time, costs a lot, and requires much knowledge (112,116).

2.12.3.2.A. COMSTAT Software and Biofilm Quantification

COMSTAT, written as a script in MATLAB (MathWorks- R2018a), is a computer software with ten different features and is used to measure the quantitative characterization of three-dimensional biofilm photographs (117,118). The CLSM image stacks are used as the raw data by COMSTAT (118).

Image stack thresholding in COMSTAT is done using a fixed threshold value chosen by the user. COMSTAT loads a black-and-white series of images and sets a threshold value of 10-50. The threshold value formed a binary image using the original images' pixel intensity. This image was then compared to the initial group of greyscale images taken. After thresholding the photos, the data volume is evaluated. The data to be studied depends on the variables chosen and the number of stacks captured using CLSM (117,119).

For measuring cell viability, the biomass image analysis function is utilized. Biomass is calculated by dividing the volume of all voxels containing biomass pixels in all stack pictures by the substratum area of the image stack. Biomass is measured in $\mu m^3/\mu m^2$. Each biofilm sample's live and dead cell biomass is determined individually from their image stacks (117,119).

COMSTAT is a ten features algorithm for quantifying three-dimensional biofilm image stacks that can calculate average thickness, area in a specific layer, total biomass, and volumes of microcolonies identified at the substratum. It also gets multivariate data, such as the thickness distribution that can be used to measure the 3-D structures in the biofilm (118,120).

2.12.4. Physical Methods

Biofilm growth is measured using fundamental physical variables such as weight (wet and dry), thickness, area, and density values. Thickness measurement by light microscopy is often accurate in thin biofilms but cannot be in thick biofilms. (KAYNAK)

2.12.5. Biochemical Methods: Biomass and Extracellular Matrix

The total number of microorganisms in a particular region is called microbial biomass. The wet or dry weight of the complete biofilm, measurements of the contents of the cells, and measurements of the cellular activities or viable cells are all included in the measurement of microbial biomass, which is thought to be a quick procedure. (KAYNAK)

2.12.6. Molecular Biological Methods

Molecular biology methods have given us a lot of information about the genes of biofilm bacteria. Most of these techniques are used to make standard tests to study the factors that affect how bacteria stay together and form biofilms. Using defined regulatory mutants and microarray analysis have been important ways to study how biofilms form (112).

The enzyme-linked immunosorbent assay (ELISA) is a sensitive way to determine if a sample has antigens or antibodies of interest. When standard curves are used with ELISA, they can measure quantities. Using new high-resolution and specific assays to determine how different genes are expressed in biofilm communities may be easier if we can find out how

different genes are expressed. But the tests we have now may only show the average signal or response from all the cells in the biofilm (50).

The polymerase chain reaction (PCR) is a technique for replicating small DNA sequences rapidly. The technique utilizes thermal cycling and enzymatic DNA replication. As the PCR process moves forward, the DNA produced is employed as a template for replication, starting a chain reaction that exponentially amplifies the DNA template. This analysis technique is mainly used as a qualitative tool for determining if a particular bacterial DNA is present or absent. A real-time polymerase chain reaction, also known as a quantitative real-time polymerase chain reaction (Q-PCR), is a technique used to simultaneously amplify and measure a specific DNA molecule (50).

2.12.7. Miscellaneous Advanced Techniques

Recently, the forces of contact between bacteria cells and between bacteria cells and substrates have been studied using atomic force microscopy (AFM). The bacterial cell or substrate particle is linked to an AFM tip to utilize it to identify the forces that interact with other bacterial cells and between the bacterial cell and substrate (50,51).

The chemical composition of mature biofilm formations has been studied using Fourier Transform Infra-Red (FTIR) spectroscopy. A test sample interacts with infrared light during an FTIR spectroscopic investigation. In this interaction, some infrared radiation passes through the sample, and the sample absorbs a part of it. The resultant spectrum provides a molecular fingerprint of the material, showing molecule-level absorption and transmission. Chemical components on a biofilm structure may be qualitatively and quantitatively analyzed using FTIR spectroscopy (50).

2.13. Biocompatibility and Cytotoxicity

Biocompatibility is the ability of a substance to interact with soft or hard tissue without causing local or systemic toxicity or allergic, thrombogenic, mutagenic, or carcinogenic tissue reactions. Toxicity refers to the tissue reaction to non-biocompatible compounds (52,53).

Cytotoxicity, the determining factor of biocompatibility, can be defined as damage to cell function and structure due to disrupting the synthesis chain of macromolecules (54,55).

Although biocompatibility is not a fundamental characteristic of the material, it is a dynamic state that can change over time and under different circumstances. Because the material can change over time, and the body's response can vary due to things like getting older or chronic disease (52,53).

The kind of material, the host area, and the function of the substance are the factors that affect a material's biocompatibility. Components of dental materials can produce local toxicity in tissues in touch with the application region and systemic toxicity by mixing with saliva or the bloodstream. The increased inflammation based on these released components can also harm the surrounding tissues (52,53).

Today, in addition to mechanical and aesthetic considerations, a material's medical effectiveness should be assessed for its biological safety and compatibility with surrounding tissues. In other words, biocompatibility is crucial when choosing the dental materials utilized in the clinic (56).

When determining the biocompatibility of the material used in dentistry, it is advisable to follow a sequencing approach that begins with the *in vitro* tests phase and then moves on to the *in vivo* tests step and, finally, the clinical usage tests stage. This procedure aims to identify high-risk groups through in vitro testing, which is the initial stage of the process, and then to disable those groups so that they cannot be utilized in clinical applications or investigations involving animals (53,57). The three types of in vitro testing include cytotoxicity, genotoxicity (mutagenicity), and estrogen tests (53,58).

2.13.1. *In vitro* Cytotoxicity Tests

The most used test technique for *in vitro* biocompatibility research is cytotoxicity testing. Studies to evaluate the cytotoxicity of dental materials often utilize cell cultures (58). At present, the International Organization for Standardization (ISO), a worldwide federation of national standards bodies (ISO member bodies), is presenting national standards for in vitro biocompatibility testing methods in ISO 10993-5 (54,59).

For *in vitro* cytotoxicity screening, the recommended testing methods include (54);

1. Cell culture method:
   a. Direct cell culture method: - Direct cell culture test
      - Culture extract test
   b. Barrier test method

2. Agar diffusion test
3. Filter diffusion test
4. Dentin barrier test

2.13.1.1. Cell Culture Method

Cell culture tests generally investigate the cytotoxicity of dental materials. This technique allows for the quick determination of the physical and chemical effects, mutagenic factors, and structural and chromosomal disorders caused by a material that cannot be examined *in vivo* quickly. Cell culture tests are based on the principle that the components that make up the dental materials are placed directly on the cells in the culture. The dose-response curve determines the potential cytotoxicity (122,125,130).

It has the advantages of directly observing the effects on cells, examining short-term interactions, and repeating the applications. With this, it has disadvantages such as being affected by hygienic conditions, being expensive, and differentiation or death of cells in successive passages after primary culture.

The cell culture method can be applied in 2 different ways: direct cell culture and barrier test method: In the direct cell culture method, the dental material or components are used directly to the cells in culture for short periods. In this method, the material is in physical contact with the cells or culture medium. In the barrier test method, artificial barrier systems allow the diffusion of components and mimic dentin permeability (54,55,60).

The most used cell lines in cytotoxicity tests are primary cells or continuous cell lines (L-929 mouse fibroblast cells, BALB/3T3 mouse embryo fibroblasts, MDPC-23 mouse odontoblast cell, WI-38 human embryonic lung cells, HeLa human epithelial cell, ECV-304 human endothelial cell) (54,61,62).

Primary cells are isolated from a particular tissue or organ and placed in culture for more than 24 hours, providing an experimental environment similar to in vivo conditions (54,61).

Continuous cell lines are primary cells that have been transformed and can proliferate indefinitely (54,61).

While creating cell cultures, settings with a temperature of 37°C, 5% CO2, and 95% humidity are ideal. Antioxidants and antibiotics are also given to the culture. Physical and pathological changes in cultivated cells are analyzed using light or electron microscopy. Even if the acquired results indicate possible material-related cytotoxicity, these findings should not directly correlate with clinical conditions. They cannot fully provide in vivo conditions (54,61,62).

2.13.1.2. Agar Diffusion Test

In this method, the material whose cytotoxicity is examined has to diffuse through 1.5% agar. After 24 hours of diffusion of the material whose toxicity will be examined with agar, the membrane permeability and cell death amount that change after the toxic effect are determined by measuring the trypan blue and neutral red staining or decolorization area. If the material does not dissolve or diffuse on agar, this method cannot evaluate the amount of cytotoxicity (54,62).

2.13.1.3. Filter Diffusion Test

Since many materials used in the restorative treatment are not in direct contact with cells, it is thought that it would be more objective to put a filter between the material and the cell culture (54,62).

For the material examined in this method to have a cytotoxic effect, it must be able to diffuse through a 0.45 μm cellulose filter. As the primary cell line, epithelial cells and

fibroblasts are generally preferred; these cells are placed on one side of the filter and the examined material on the other. Cell damage and cell death caused by the cytotoxic effect of materials diffused through the filter are determined by neutral red staining (54,62).

2.13.1.4. Dentin Barrier Test

While dental materials are applied to the tooth tissue, the dentin tissue acts as a protective barrier to the pulp. In this method, which was developed to imitate this feature of dentin tissue, the diffusion ability of the tested material is measured. Unlike other tests, in this method, which mimics the oral environment more, human dentin tissue or bovine dentin discs are used as a barrier between the monomers released from the dental material and the target cell (130,133).

2.13.2. Cytotoxicity Assessment Methods

Materials have the potential to alter the place where they are applied. These alterations include increased cell mortality, membrane integrity degradation, cell adhesion reduction, cell shape change, cell proliferation decrease, biosynthesis, and enzymatic activity decline (55,63). Four different evaluation methods are used to assess these cytotoxic effects (55).

2.13.2.1. Viability Assessment Methods

These tests allow colorimetric or fluorescence measurements to determine the percentage of viable cells in culture due to toxic effects (55,62). They are performed with dyes such as trypan blue, erythrosine, or naphthalene black taken into cells with impaired membrane integrity or by using dyes such as diacetyl fluorescent or neutral red taken into living cells with intact membrane integrity (55,61).

2.13.2.2. Survival Evaluation Methods

In these tests, which evaluate only dead cells, the toxic effects of the cells can be seen in the first few days or later. In determining cell survival, the ability to form colonies at low cell density is evaluated (61,62).

With this method, the amount of cytotoxicity produced by the cells that encounter the biomaterial in the short term can be assessed (61,62).

2.13.2.3. Proliferation Evaluation Methods

Counting cells in culture after a few days is used to determine the effect of various material components on cell proliferation. H-Thymidine and Bromodeoxyuridine tests are among the proliferation evaluation methods (55).

2.13.2.4. Metabolic Cytotoxicity Assessment Methods

In this method, which generally requires more sensitive work, metabolic or proliferative capacities of cells and enzyme activity degradation are measured and evaluated with the help of a microplate reader spectrophotometer (61,62). With these tests, direct cell survival evaluation cannot be made. However, these tests determine the net increase in the number of cells, total protein amount, or DNA increase (125,132).

The 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4 sulfophenyl)-2H-tetrazolium (MTS) test is frequently used in the cytotoxicity evaluation of dental materials. It is a tetrazolium salt. Proliferated cells transform MTS into dark pink-red formazan crystals with increased mitochondrial dehydrogenase enzyme activity, and the absorbance of the formed formazan is measured at 492 nm (64). In cases where the dehydrogenase activity in the cell is affected due to the cytotoxic effect of the applied material, blue-colored formazan does not occur; formazan formation is only seen in living cells with active mitochondria. Formazan formation is determined by measuring the optical density with a spectrophotometer or by detecting the formazan light around the test sample with an electron microscope (60,61). The MTS technique has the benefits of being simple to use, inexpensive, and providing precise, reliable outcomes (64).

3. MATERIALS and METHODS

All experimental materials were prepared and sterilized at Yeditepe University, Faculty of Dentistry, Department of Pediatric Dentistry Clinic. The disc diffusion and antibiofilm assays were performed at Istanbul University, Faculty of Dentistry, Department of Oral Microbiology. Biofilm-covered discs were examined by confocal laser scanning microscopy (CLSM). The biofilm viability on the hydroxyapatite discs was calculated using the COMSTAT software. CLSM imaging was performed at Istanbul University Aziz Sancar Experimental Medicine Research Institute. SEM imaging of the biofilm was done in Yeditepe University Biocidal and Research and Development Laboratory.

After the antimicrobial activity part, the MTS method was used on human dermal fibroblasts in the cytotoxicity assay. The cytotoxicity study was performed at Yeditepe University, Faculty of Genetics and Bioengineering.

A power analysis was performed using the G*power 3.1 program to determine the sample size for each group. The number of discs for each group was determined using the study of *Golestannejad et al.* (65), and the effect size was calculated as 0.49. Therefore, it was determined that at least ten discs should be included in each group for biofilm assay, and disc diffusion and MTS tests need to be performed at least four times to achieve 80% power at the α=0.05 level.

3.1. Materials

There were six study groups in the study. Five commercially available mouthwashes containing probiotics, fluoride, chlorhexidine, and their combinations and distilled water (negative control) were used. The mouthwashes used in the study are shown in Figure 7, and the content, active ingredients, and the manufacturers' information are shown in Table 1.

The study groups were as follows;

Group 1: Henry Blooms Probiotic Mouthwash
Group 2: Luvbiotics Mouthwash
Group 3: Listerine Smart Rinse
Group 4: The Humble Co. Natural Mouthwash

Group 5: Klorhex (Positive control)

Group 6: Distilled water (Negative control)

The hydroxyapatite discs used in the experiment for antimicrobial activity testing were 100% synthetic hydroxyapatite (HAD40, Himed, USA) (Figure 8). A total of 72 discs were used, 12 discs in each group. Ten discs from each group were used to measure remaining bacterial viability and biofilm thickness with a CLSM, and two discs from each group were used to obtain images with SEM.

In the second part of the study, a cytotoxicity test was performed. The human dermal fibroblast cell line was used to evaluate the cytotoxic effects of the mouthwashes used in the study. The human dermal fibroblasts PCS-201-012™ (ATCC, USA) were obtained from Yeditepe University Regenerative Biology Laboratory (passage number: 20).



Figure 7. Mouthwashes used in the study.

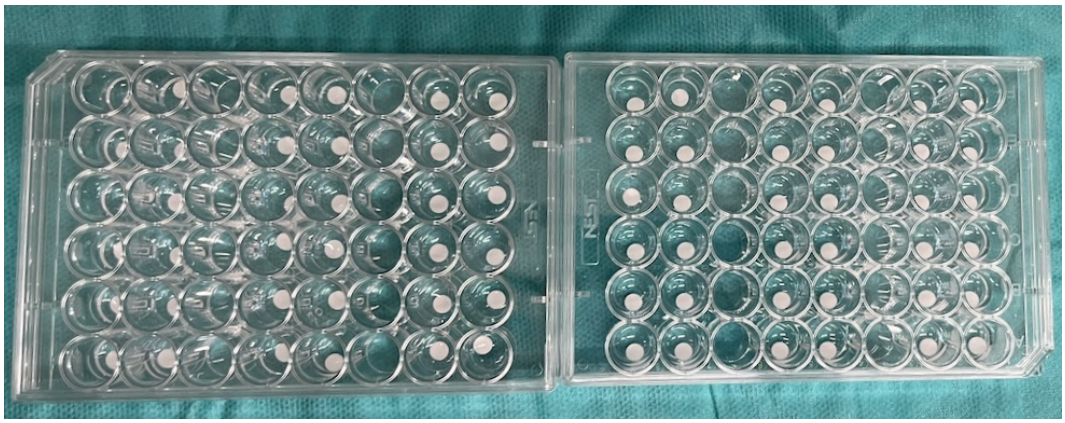


Figure 8. Hydroxyapatite discs used in the study.

Table 1. Content, active ingredients, and manufacturer information of the mouthwashes used in the study.

Mouthwash	Content Information	Active Ingredients	Manufacturer	Lot Number
Henry Blooms Probiotic Mouthwash	Aqua, Glycerin, Polysorbate 20, Xylitol, *Lactobacillus paracasei* GMNL-33, Sodium benzoate, Stevia, Peppermint Oil, Potassium sorbate, Spearmint Oil, natural preservative Salinatural® MIN (Peppermint Oil, Spearmint Oil, Clove Oil, Neem Leaf extract, Rosemary Oil)	Xylitol, *Lactobacillus paracasei* GMNL-33	**Henry Blooms, Australia**	**B18838**
Luvbiotics Mouthwash	Aqua, Glycerin, Xylitol, Disodium Phosphate, Sodium Phosphate, PEG-40 Hydrogenated Castor Oil, Polysorbate 20,	Xylitol, *Lactobacillus paracasei* GMNL-33, Cetylpryridinium	**Luvbiotics, England**	**89454**

	Aroma (Menthol, Mentha Arvensis Leaf Oil, Mentha Piperita Oil, Mentha Spicata Herb Oil, Anethole, Eucalyptus Globulus Leaf Oil, Menthyl Salicylate, Menthyl Lactate), Cetylpryridinium Chloride, Sodium Fluoride, Lactobacillus (*L.paracasei* GMNL-33), Limonene.	Chloride, Sodium Fluoride		
Listerine Smart Rinse	Water, Sorbitol, Flavor, Phosphoric Acid, Sucralose, Sodium fluoride 0.02% (0.01% w/v fluoride ion), Cetylpyridinium Chloride, Disodium Phosphate, Sodium Saccharin, Menthol, Blue 1, Green 3.	Sodium fluoride 0.02%, Cetylpyridinium Chloride	**Johnson & Johnson, USA**	**0212LZ**
The Humble Co. Natural Mouthwash	Aqua, Glycerin, PEG-40 Hydrogenated Castor Oil, Betaine, Aroma, Sodium Saccharin, Mentha Piperita Oil, Chlorhexidine Digluconate (0.02%), Aloe Barbadensis Leaf Juice, Sodium Fluoride, Citric Acid, Sodium Benzoate, Potassium Sorbate.	Chlorhexidine Digluconate, Sodium Fluoride	**The Humble Co., Sweden**	**30133619**
Klorhex (0,2 %) (positive control)	Aqua, Glycerin, Chlorhexidine Digluconate (%0.2), Aroma, Mentha arvensis herb oil.	Chlorhexidine Digluconate (%0.2)	**Drogsan, Turkey**	**22G014601**

3.2. Methods

3.2.1. Disc Diffusion Assay

3.2.1.1. Preparation of the Bacteria Suspension

A 24-hour culture of *Streptococcus mutans* ATCC 25175 was prepared in 10 ml of Brain-Heart Infusion (BHI) broth according to a 0.5 MF turbidity tube (10^8 CFU/ml) and diluted 100-fold (Final concentration; 10^6 CFU/ml). The suspension was diluted 10-fold for control, and 0.1 ml was cultivated on a solid medium.

3.2.1.2. Determination of the Antimicrobial Efficacy of Mouthwashes

The antimicrobial activity of mouthwashes against *S. mutans* was investigated by disc diffusion assay. 100 µl of the prepared bacterial suspension was taken and applied to Müller-Hinton agar media with sterile swabs. After *S. mutans* inoculation, blank 6 mm paper discs (Bioanalyse, Turkey) impregnated with 10 µl mouthwash were placed on the medium (Figure 9). Petri dishes were incubated at 37°C for 24 hours in a 5% CO_2 environment. This experiment was repeated on seven petri dishes. The inhibition zones formed around the discs due to the incubation of the petri dishes were measured in millimeters with a ruler (Figures 10 and 11). The fact that bacteria did not grow in a particular area around the mouthwash indicated that the mouthwash had antibacterial activity.



Figure 9. Paper discs used for mouthwash impregnation.

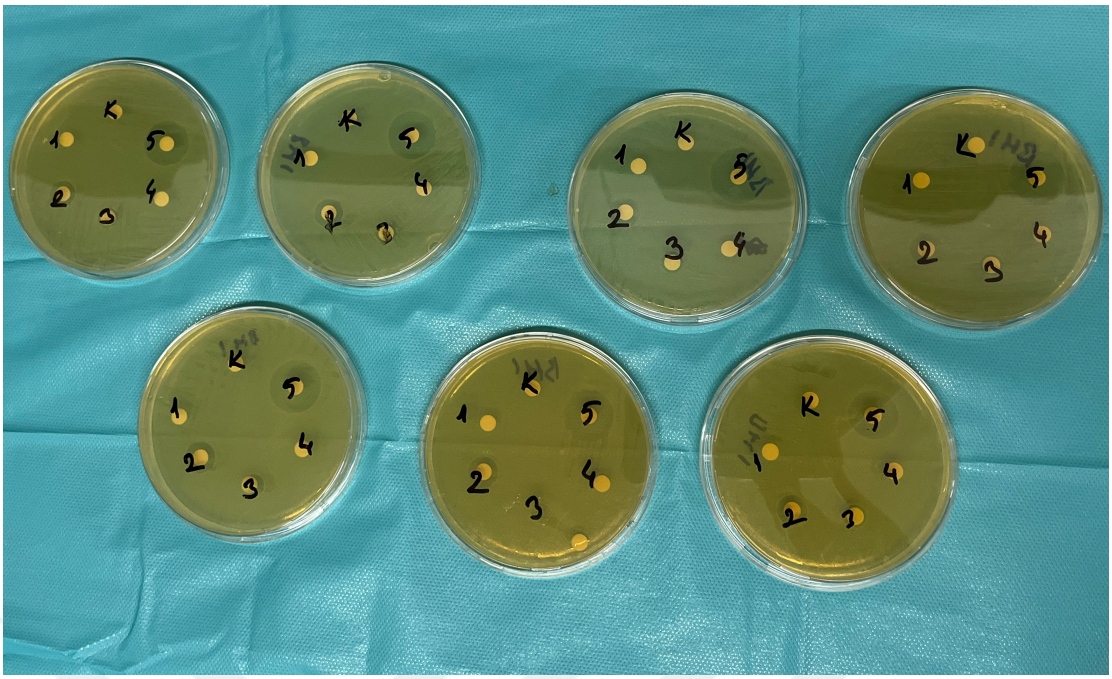


Figure 10. Image of inhibition zones formed around the mouthwash-impregnated discs on the medium.

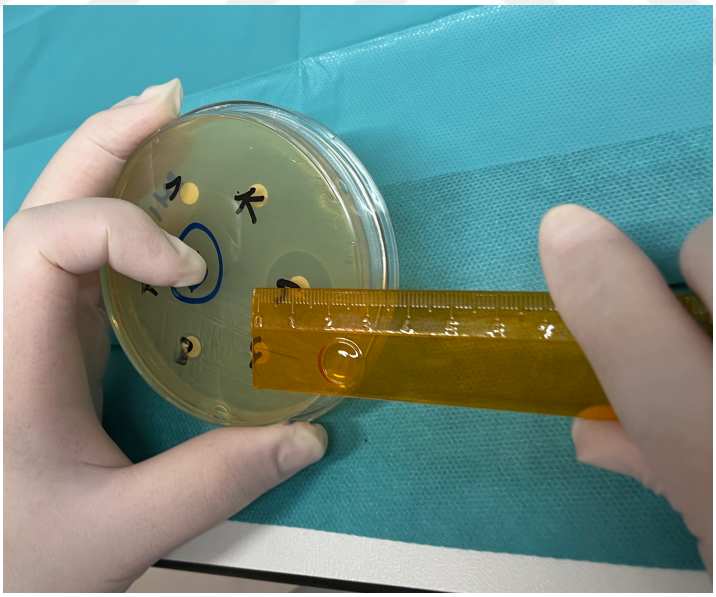


Figure 11. Inhibition zone measurement.

3.2.2. Evaluation of the Effects of Mouthwashes on *S. Mutans* Biofilm

After determining their antimicrobial efficacy with disc diffusion assay, the effect of mouthwashes on *S. mutans* biofilm was evaluated. For this purpose, mouthwashes were applied on the *S. mutans* biofilm layer formed on hydroxyapatite discs *in vitro*.

3.2.2.1. Artificial Saliva Preparation

The artificial saliva was prepared in the laboratory according to the Pratten et al. formula (66). This formula included 1 g/L Lab-lemco powder, 2 g/L yeast extract, 5 g/L proteose peptone, 2.5 g/L hog gastric mucin, 0.35 g/L sodium chloride, 0.2 g/L calcium chloride, and 0.2 g/L potassium chloride in distilled water. After autoclaving, 1.25 mL of a 40% urea solution was added. The pH of the saliva substitute was adjusted to 7. The solution was then filtered (0.22 µm) into a sterile container (Figure 12).



Figure 12. Filtration of artificial saliva.

3.2.2.2. Biofilm Formation

Sterile 48-well plates were used for the *in vitro* biofilm experiment. Prepared sterile hydroxyapatite discs (HAD40, Himed, USA) were placed in each well. 500 µl of artificial saliva

was added to the test discs for salivary pellicle coating and incubated for one hour at 37°C in a shaker (Figure 13). After incubation, the discs in saliva were rinsed with phosphate-buffered saline (PBS) solution. 1.6 ml of sucrose-containing BHI Broth (MilliporeSigma 290, MA, USA) with sucrose and 200 µl of bacterial suspension were added to the discs placed in the new well (Figure 14). Tissue culture petri dishes with 48-wells were placed in a 5% CO_2 environment and incubated at 37°C for 24 hours. After incubation, the discs were rinsed with 2 ml of PBS and put in new wells.



Figure 13. Saliva added onto discs placed in a 48-well plate.

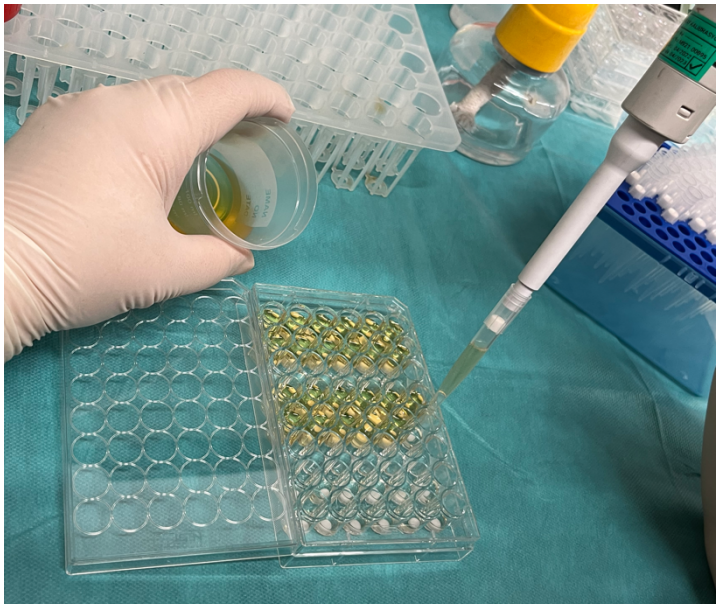


Figure 14. Addition of sucrose-containing BHI and bacterial suspension onto discs.

3.2.2.3. Application of Mouthwash on Biofilm-Formed Discs

Mouthwashes were added enough to cover the biofilm-formed discs in the wells and kept in an incubator at 37°C with shaking for 30 seconds at 90 rpm (Figures 15 and 16). After the application, the discs were washed with sterile saline for 10 seconds.



Figure 15. Adding mouthwash to biofilm-formed discs.

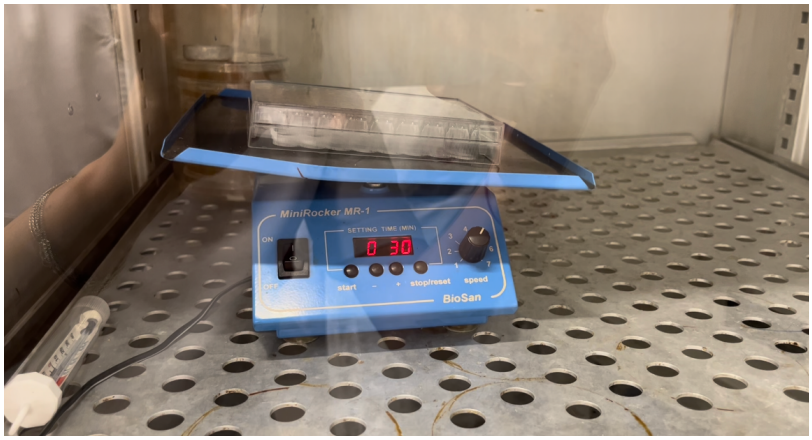


Figure 16. Shaking the discs for 30 seconds.

3.2.2.4. Evaluation with Confocal Laser Scanning Microscope (CLSM)

Sixty discs from six groups, ten samples from each group, were separated for evaluation under confocal microscopy. The discs were taken into 48-well plates, and 20 µl of Live/dead Backlight Kit (Invitrogen, Thermo Fisher Scientific, USA) dye solution was added. The separated samples were painted with dyes, covered with aluminum foil in the dark, and stored in the refrigerator at $4^{o}C$ until sent for examination.

In the lightless laboratory, images were obtained from three different points of each hydroxyapatite disc with the help of optical lenses using a confocal laser scanning microscope (Leica CLSM, Heidelberg, Germany) and a wavelength of 488 and 532 nm (Figure 17). Three different spots of each sample were imaged separately, and a series of photographs of each spot showed the bacterial viability status in red and green.

To measure the remaining bacterial viability with a confocal laser scanning microscope, the COMSTAT software was used. COMSTAT is a program that analyzes image stacks of biofilms recorded by confocal microscopes. With this software, the biomass of remaining the bacteria was analyzed separately as dead and live. In this study, the percentage of the dead cell amounts were also calculated in order to compare the effectiveness of mouthwashes on biofilm.

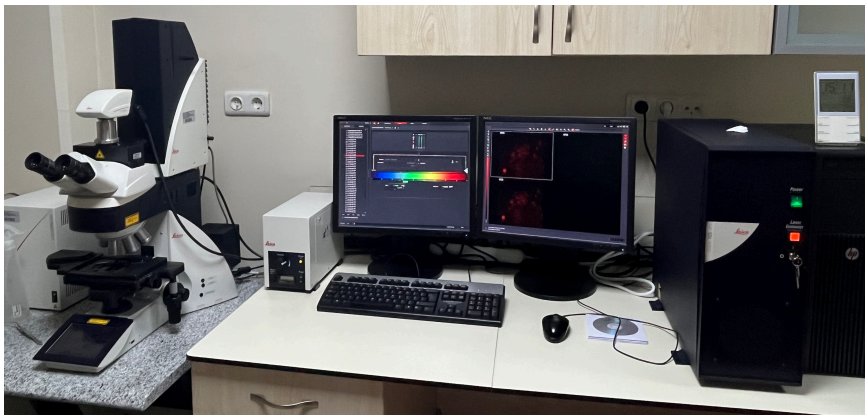


Figure 17. Confocal Laser Scanning Microscope (Leica, Heidelberg, Germany).

3.2.2.5. Scanning Electron Microscope (SEM) Imaging and Evaluation

Two hydroxyapatite discs from each of the six groups were placed in 48-well plates after the mouthwash application. 1 ml of 4% formaldehyde was added, covered with parafilm, and prepared for imaging with SEM (Figure 18).

The samples were placed in separate plates and kept in each solution for 30 minutes at 100%, 96%, 70%, 50%, and 25% ethyl alcohol concentrations and then left to dry in the open air for 24 hours. Dried samples were coated with gold and palladium for 45 seconds with the sputtering device (Leica EM ACE200, Germany), and images were examined on SEM (Carl Zeiss, EVO 40, Germany) (Figure 18) at x1000, x2500, x7500, and x15000 magnifications, respectively.

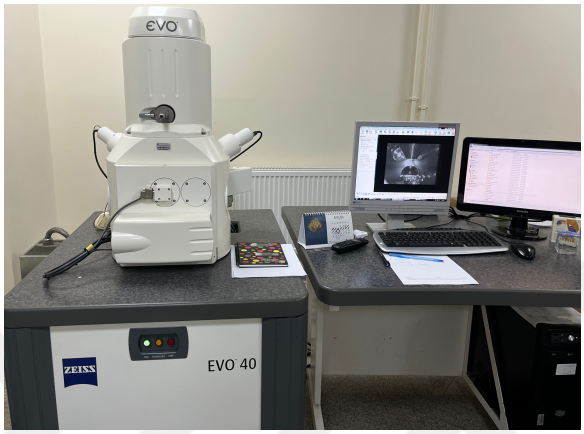


Figure 18. Scanning Electron Microscope (Carl Zeiss, EVO 40, Germany)

3.2.3. Evaluation of Cytotoxicity of Mouthwashes Used in the Study

In the cytotoxicity assay of the study, the MTS (3-(4,5 dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) method was used on the human dermal fibroblast cell line (Figure 19a, 19b).

3.2.3.1. Preparation of Cell Culture

The human dermal fibroblast (HDF) cell line was used to determine the cytotoxic effects of mouthwashes. HDF was taken from liquid nitrogen and grown in high glucose Dulbecco's Modified Eagle Medium (DMEM) (Gibco 11965084) containing a nutrient mixture of 10% Fetal Bovine Serum (FBS) and 1% Penicillin-Streptomycin-Amphotericin B Solution (PSA). Five thousand cells/200 µl were seeded into 96-well plates, and they waited 24 hours for their attachment.

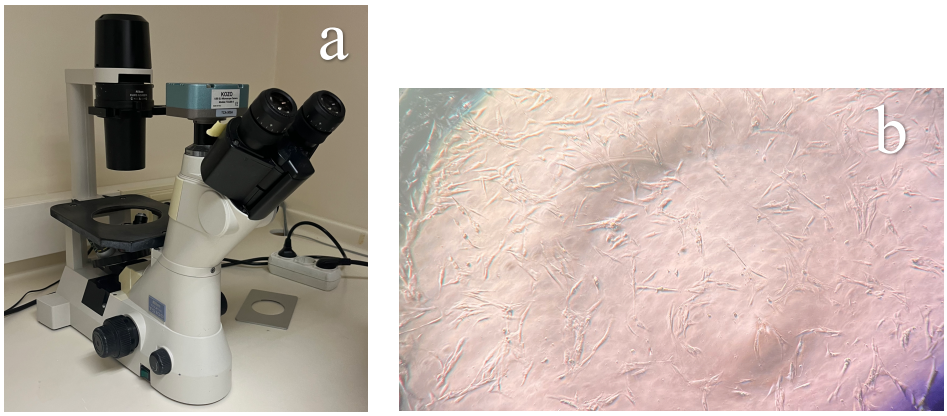


Figure 19. Light microscope (Eclipse TS100 Nikon, Japan) (a) and control of the HDF used in the study (b).

3.2.3.2. Application of MTS Test

For the MTS test, 5000 cells were seeded at each well of the 96-well plate and incubated at 37°C for 24 hours. Five different mouthwash materials tested in our study were dissolved in High Glucose DMEM (Gibco, Thermo Fisher Scientific, USA) and prepared at three different concentrations of (5%, 10%, and 20%) and the normal medium used for the negative control (untreated) group.

During the experiment, the cells' medium was first drawn, then the cell cultures were exposed to mouthwashes for 30 seconds (Figure 20). After this process, 20 µl of MTS solution was added to the cells and incubated for 2 hours under standard culture conditions.

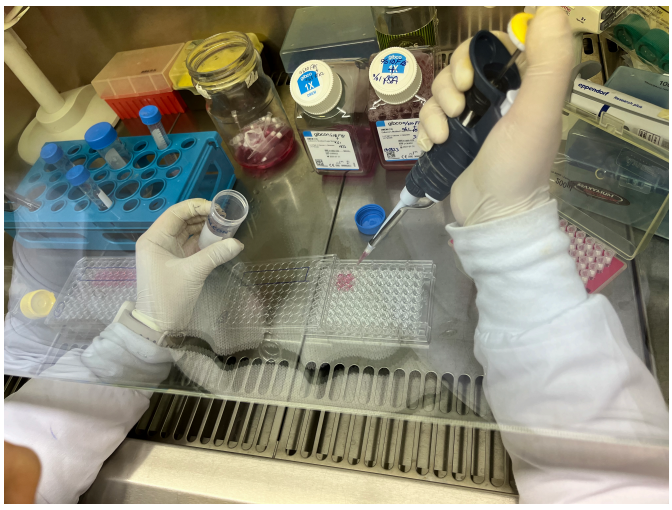


Figure 20. Mouthwash application onto cells.

Measurements were made with a spectrophotometer device (Multimode Reader Varioskan Lux, Thermofisher, USA) at a wavelength of 490 nm (Figure 21). Data were obtained by evaluating each plate separately. The absorbance of the blank group was subtracted from the absorbance of the samples. The experiment was repeated four times.

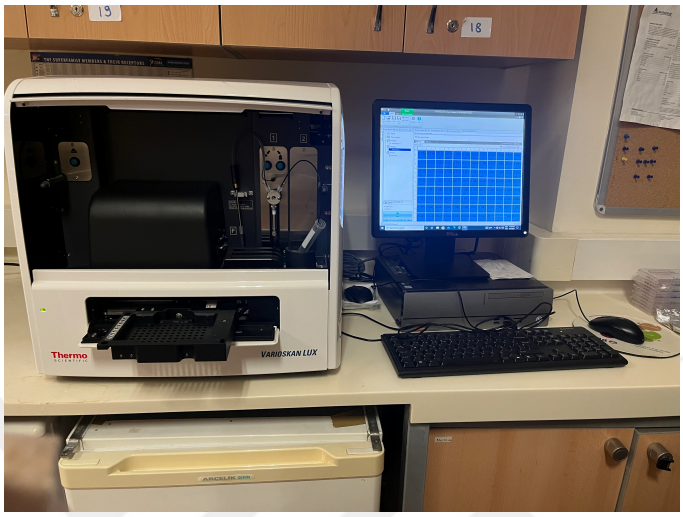


Figure 21. Spectrophotometer device (Multimode Reader Varioskan Lux, Thermofisher, USA).

3.3. Statistical Analysis

In this study, NCSS (Number Cruncher Statistical System) 2007 statistical software (Utah, USA) was used for the data analysis. In addition to descriptive statistics (mean, standard deviation, median, interquartile range), the Shapiro-Wilk normality test was used to confirm the distribution of the variables. The paired t-test was used for evaluating repetitive measures of normally distributed variables, one-way analysis of variance for comparisons between multiple groups, Tukey multiple comparison test for subgroup comparisons, Wilcoxon test for evaluating repeated measures of non-normally distributed variables, Kruskal Wallis Test for comparing multiple groups, and Dunn's multiple comparison test for subgroup comparisons. The results were evaluated at the significance level of $p<0.05$.

4. RESULTS

4.1. Disc diffusion assay

Inhibition zone values determined with the agar disc diffusion part of this *in vitro* study are shown in Table 2. The mean values of inhibition zones were Klorhex > Luvbiotics > Listerine > Henry Blooms = Humble Co. = Control, respectively. A statistically significant difference was observed between all study and control groups' mean *S. Mutans* inhibition zones (p=0.0001). Henry Blooms and Humble Co. groups showed no antibacterial effect on *S. mutans* biofilm similar to the negative control group.

Table 2: Inhibition zone values after the mouthwash application.

S. Mutans **Inhibition zones (mm)**	**Mean±SD**	**Median (IQR)**
Henry Blooms	0±0	0 (0-0)
Luvbiotics	12.29±0.76	12 (12-12)
Listerine	8.14±0.38	8 (8-8)
Humble Co.	0±0	0 (0-0)
Klorhex	19±1	19 (18-19)
Control	0±0	0 (0-0)
p‡	**0.0001***	

‡Kruskal Wallis test, *p<0.05.

IQR: Inter Quartile Range

The mean inhibition zone of Klorhex was statistically significantly higher than that of the other groups (p=0.001). The mean inhibition zone of Luvbiotics was statistically significantly higher than the mean inhibition zones of Henry Blooms, Listerine, Humble Co., and the control groups (p=0.001, p=0.0001). The mean inhibition zone of Listerine was found to be statistically significantly higher than the mean of the inhibition zone of Henry Blooms, Humble Co., and the control groups (p=0,0001). No statistically significant difference was

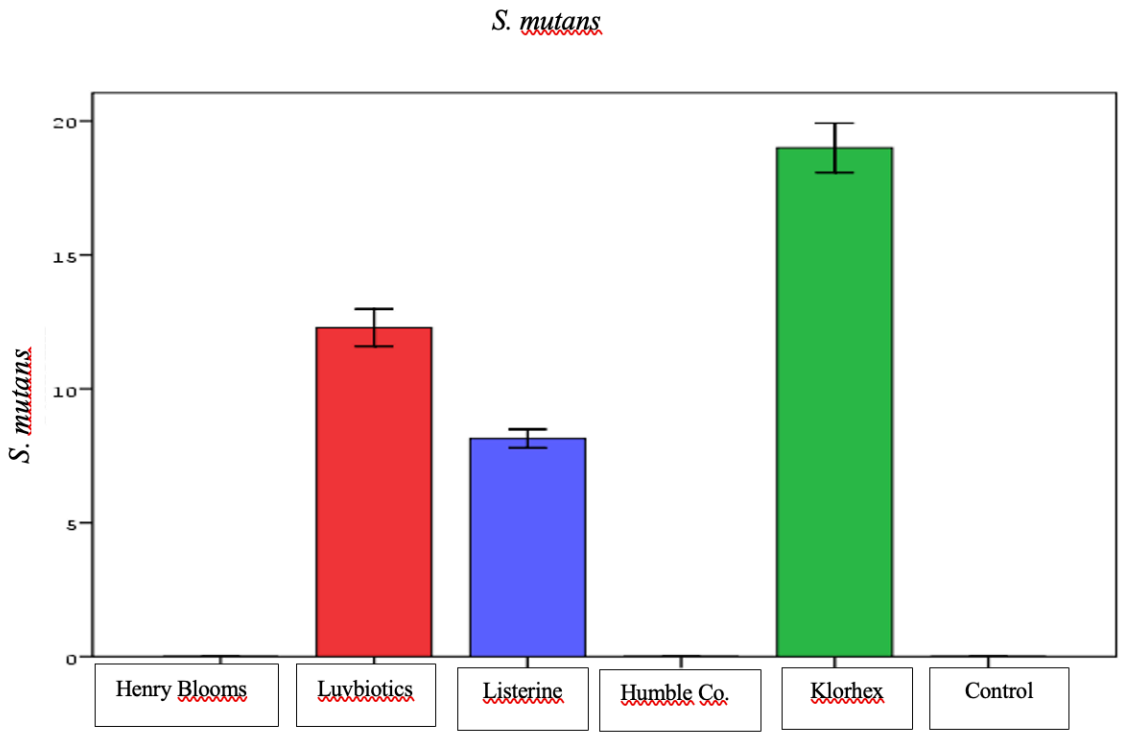
observed between the other groups (p>0.05) (Table 3). The distribution of the mean inhibition zones formed on the discs is given in Graphic 1.

Table 3: Binary comparison of the inhibition zone values among the groups.

	Inhibition zone (p*)
Henry Blooms / Luvbiotics	**0.0001**
Henry Blooms / Listerine	**0.0001**
Henry Blooms / Humble Co.	0.999
Henry Blooms / Klorhex	**0.001**
Henry Blooms / Control	0.999
Luvbiotics / Listerine	**0.001**
Luvbiotics / Humble Co.	**0.0001**
Luvbiotics / Klorhex	**0.001**
Luvbiotics / Control	**0.0001**
Listerine / Humble Co.	**0.0001**
Listerine / Klorhex	**0.001**
Listerine / Control	**0.0001**
Humble Co./ Klorhex	**0.001**
Humble Co./ Control	0.999
Klorhex / Control	**0.001**

***Dunn's Multiple Comparison Test**

p<0.05



Graphic 1. Diagram representing the size of the inhibition zones after the mouthwash application.

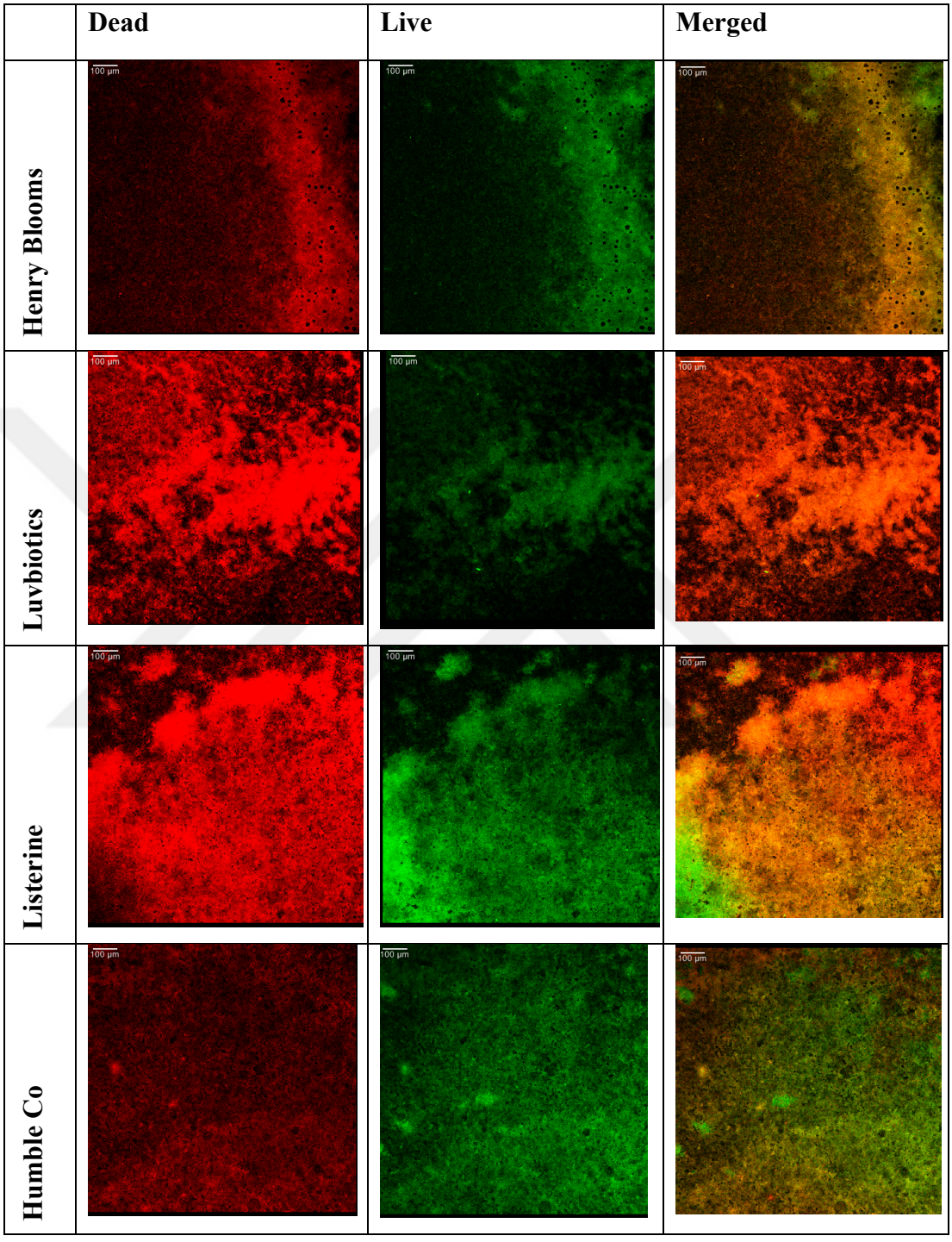
4.2. Evaluation of the Effectiveness of Mouthwashes on *S. mutans* Biofilm

The number of viable bacteria on the biofilm and the ratio of dead-live bacteria were examined with a confocal laser scanning microscope (CLSM, Leica, Heidelberg, Germany).

In live/dead staining, green dots show live cells, red dots show dead cells, and yellow dots show live and dead cells combined.

In the images taken with CLSM after mouthwash application, the highest density of dead cells was found in the Klorhex, Luvbiotics and Listerine groups, respectively; while in the Henry Blooms and Humble Co. groups, higher live cell density was observed. In the negative control group, there was almost no dead cells.

Representative samples of the images obtained with CLSM over discs are shown in Figure 22.



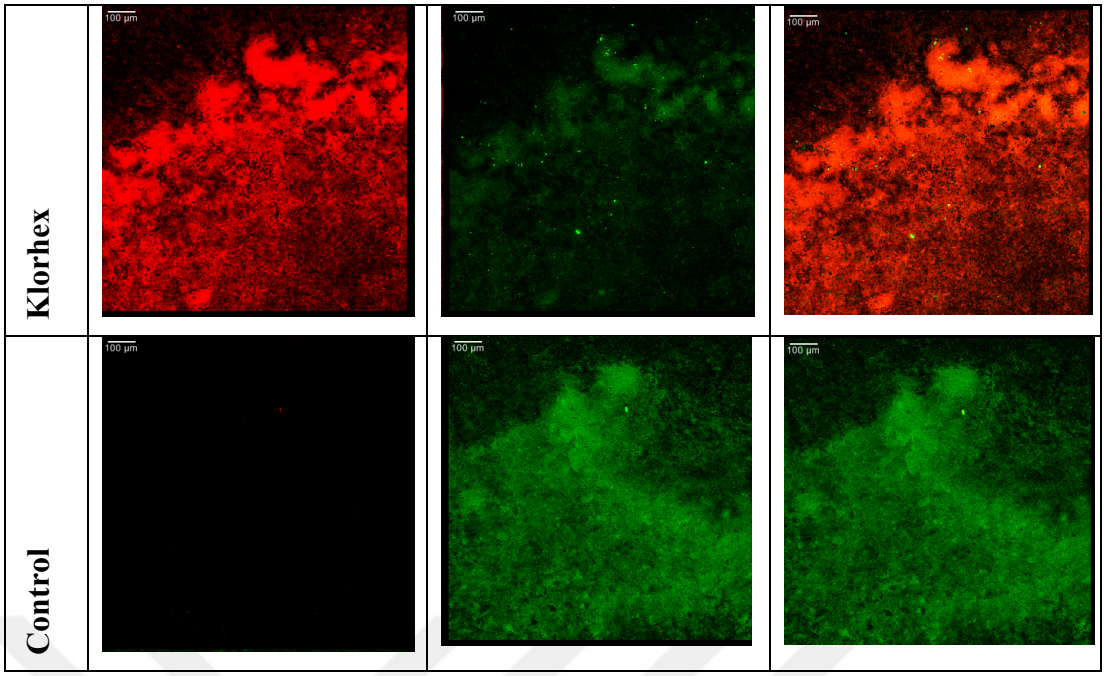


Figure 22. Sample images of the all biofilms on HA discs after exposure to the mouthwashes, by CLSM (Bar denotes: 100 µm).

4.3. Evaluation of the Biomass of the Remaining Bacteria

Dead and alive biomass of the remaining bacteria after the mouthwash application are given in Table 4. The dead bacteria biomass of the groups was Luvbiotics > Listerine > Klorhex > Humble Co. > Henry Blooms > control, respectively. The alive bacteria biomass of the groups was control > Humble Co. > Listerine > Luvbiotics > Henry Blooms > Klorhex, respectively. According to the results, a statistically significant difference was observed within the mean of dead and live bacteria biomass ($\mu m^3/\mu m^2$) amounts of all groups ($p=0.0001$). However, the intragroup comparison revealed that no statistically significant difference was observed between the biomass of the dead and live bacteria of the Henry Blooms group ($p=0.128$). The dead bacteria biomass of Luvbiotics, Listerine, and Klorhex groups was statistically significantly higher than the live biomass ($p=0.001$, $p=0.011$, and $p=0.0001$, respectively). The dead bacteria biomass of Humble Co. and control groups was statistically significantly lower than the live biomass of these groups ($p=0.002$ and $p=0.0001$) (Table 4).

Table 4: Dead and live biomass of the remaining bacteria after mouthwash application

Biomass ($\mu m^3/\mu m^2$)	Dead		Live		p*
	Mean±SD	Median (IQR)	Mean±SD	Median (IQR)	
Henry Blooms	6.73±6.61	4.29 (0.32-12.69)	8.95±5.98	7.93 (5.48-13.34)	0.128
Luvbiotics	19.74±9.64	19.91 (12.67-25.57)	11.48±6.91	11.89 (6.07-17.15)	**0.001**
Listerine	18.81±7.99	16,06 (12,19-27,2)	13.64±6.92	12.55 (8.82-18.47)	**0.011**
Humble Co	9.22±9.93	5,73 (0,03-17,35)	17.24±8.23	17.61 (11.49-22.17)	**0.002**
Klorhex	18.23±10.15	18,29 (10,45-24,4)	6.34±8.59	0.8 (0.02-12.56)	**0.0001**
Control	0.86±2.78	0,01 (0-0,01)	19.11±8.17	17.6 (13.82-25.73)	**0.0001**
p‡	**0.0001**		**0.0001**		

‡Kruskal Wallis test *Mann Whitney U test

IQR: Inter Quartile Range

p<0.05

The intergroup comparison of the mean biomass of the dead and live bacteria is given in Table 5. Accordingly, the mean dead biomass of the control group was statistically significantly lower than that of the other groups (p=0.0001). The Henry Blooms group's dead bacteria biomass was statistically significantly lower than that of the Luvbiotics, Listerine, and Klorhex groups (p=0.0001). In contrast, no significant difference was found between Henry Blooms and Humble Co. (p=0.767). The Humble Co. group's dead bacteria biomass was statistically significantly lower than that of the Luvbiotics, Listerine, and Klorhex groups

(p<0.05). No statistically significant difference was observed between the other groups (p>0.05).

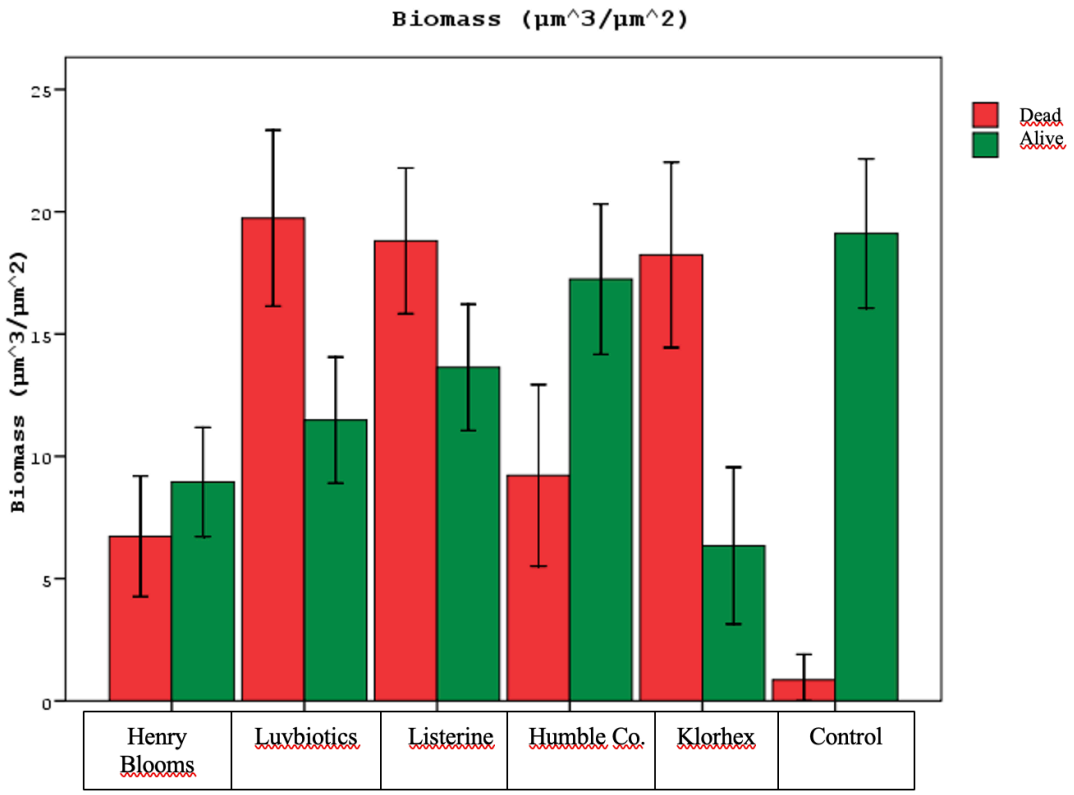
The mean of live bacteria biomass of the Klorhex group was found to be statistically significantly lower than that of the Henry Blooms, Luvbiotics, Listerine, Humble Co., and control groups (p=0.033, p=0.008 and p =0.0001, respectively). The live bacteria biomass of the Henry Blooms group was statistically significantly lower than that of the Listerine, Humble Co., Klorhex, and control groups (p=0.014, p=0.0001, p=0.033, and p=0.0001, respectively). The live bacteria biomass of the Luvbiotics group was statistically significantly lower than that of the Humble Co. and control groups (p=0.006, p=0.001). The Listerine group's live biomass was statistically significantly lower than that of the Humble Co. and control group (p=0.004, p=0.001). However, the other groups had no statistically significant difference (p>0.05). The distribution of the mean dead and live bacteria biomass according to the study groups is given in Graphic 2.

Table 5: Binary comparison of live and dead Biomass ratios

	Biomass ($\mu m^3/\mu m^2$) (p*)	
	Dead	**Live**
Henry Blooms / Luvbiotics	**0.0001**	0.156
Henry Blooms / Listerine	**0.0001**	**0.014**
Henry Blooms / Humble Co.	0.767	**0.0001**
Henry Blooms / Klorhex	**0.0001**	**0.033**
Henry Blooms / Control	**0.0001**	**0.0001**
Luvbiotics / Listerine	0.824	0.375
Luvbiotics / Humble Co.	**0.0001**	**0.006**
Luvbiotics / Klorhex	0.574	**0.008**
Luvbiotics / Control	**0.0001**	**0.001**
Listerine / Humble Co.	**0.001**	0.06
Listerine / Klorhex	0.848	**0.0001**
Listerine / Control	**0.0001**	**0.004**
Humble Co./ Klorhex	**0.001**	**0.0001**
Humble Co./ Control	**0.0001**	0.337
Klorhex / Control	**0.0001**	**0.0001**

***Dunn's Multiple Comparison Test**

p<0.05



Graphic 2. Diagram representing the biomass of dead and alive bacteria after the mouthwash application**.**

4.4. Evaluation of the Percentages of the Dead Cell Biomass

The percentage of dead bacteria biomass after mouthwash application according to groups is given in Table 6. The dead bacteria percentage was found as Klorhex > Luvbiotics > Listerine > Henry Blooms > Humble Co. > control, respectively. According to the results, a statistically significant difference was observed between the percentage of dead bacteria biomass ($\mu m^3/\mu m^2$) of all study and control groups (p=0.0001).

Table 6: Comparison of the percentages of the ratio of the dead cell biomass to the total biomass after the mouthwash application of each group

Biomass ($\mu m^3/\mu m^2$)	Dead (%)	
	Mean±SD	**Median (IQR)**
Henry Blooms	40.49±25.9	39.36 (23.06-54.58)
Luvbiotics	64.16±19.14	62.9 (55.52-72.75)
Listerine	57.92±13.13	56.8 (50.88-65.2)
Humble Co	27.41±26.76	29.8 (0.35-48.97)
Klorhex	79.84±20.54	87.95 (59.55-99.77)
Control	3.5±8.99	0.03 (0-0.1)
p‡	**0.0001**	

‡Kruskal Wallis test

IQR: Inter Quartile Range

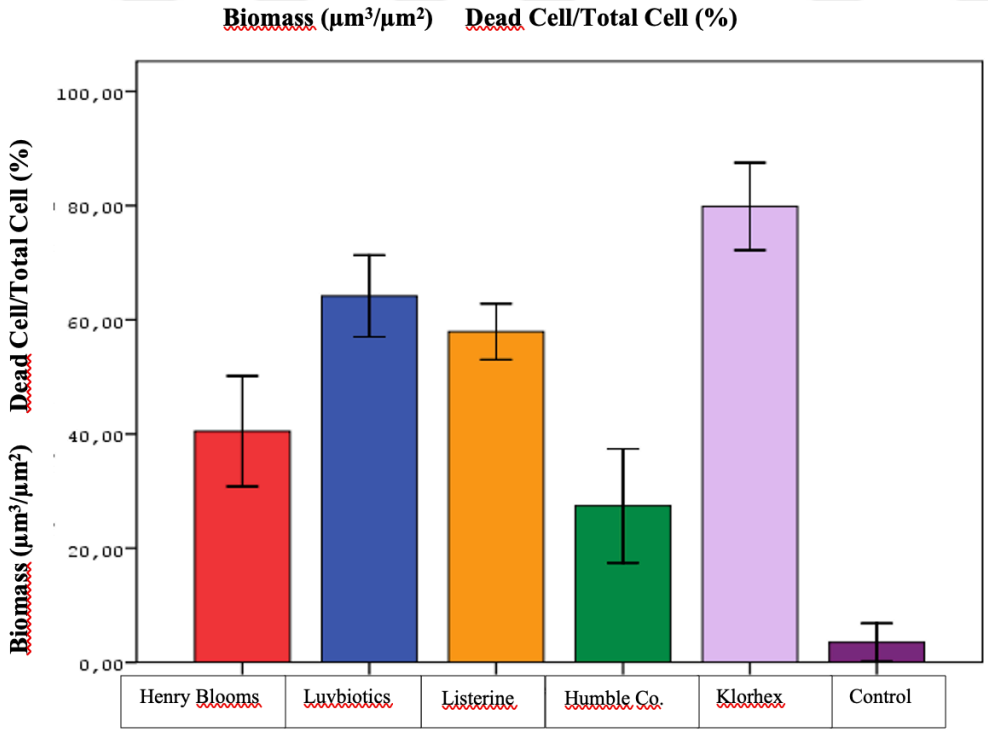
p<0.05

The highest percentage of the dead bacteria biomass was found in the Klorhex group, whereas the lowest was found in the control group. The difference between the groups having the highest and lowest percentages and the other groups was statistically significant ($p<0.05$). All the groups' percentages differed statistically significantly except for Henry Blooms and Humble Co. and Luvbiotics and Listerine (Table 7). The distribution of the percentage of dead bacteria biomass among the study and control groups is shown in Graphic 3.

Table 7: Binary comparison of the percentages of the dead cell to the total cell in terms of biomass

	Dead/Total (p*)
Henry Blooms / Luvbiotics	**0.0001**
Henry Blooms / Listerine	**0.001**
Henry Blooms / Humble Co.	0.069
Henry Blooms / Klorhex	**0.0001**
Henry Blooms / Control	**0.0001**
Luvbiotics / Listerine	0.143
Luvbiotics / Humble Co.	**0.0001**
Luvbiotics / Klorhex	**0.023**
Luvbiotics / Control	**0.0001**
Listerine / Humble Co.	**0.0001**
Listerine / Klorhex	**0.0001**
Listerine / Control	**0.0001**
Humble Co. / Klorhex	**0.0001**
Humble Co. / Control	**0.0001**
Klorhex / Control	**0.0001**

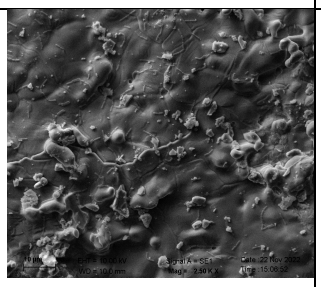
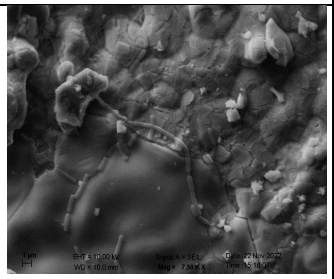
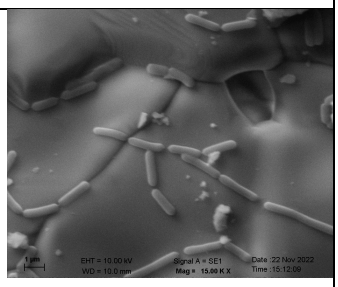
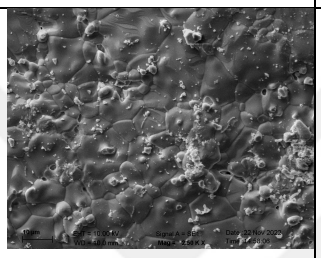
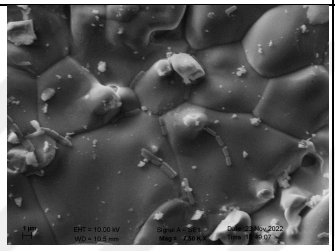
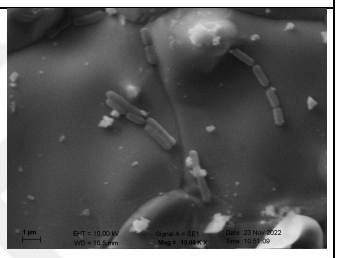
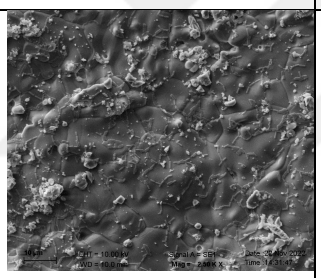
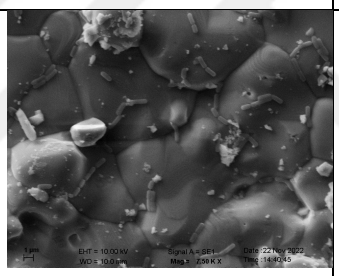
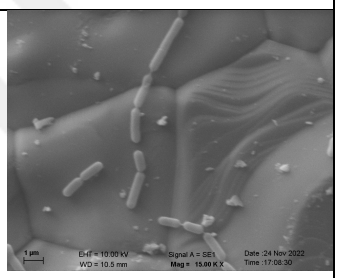
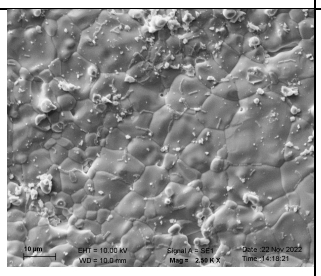
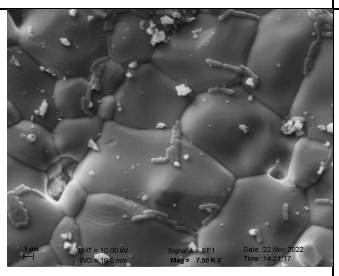
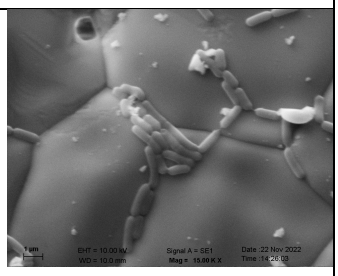
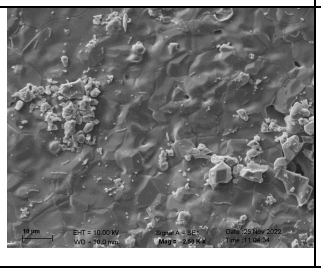
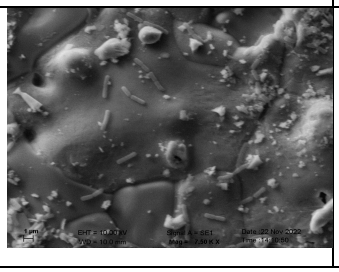
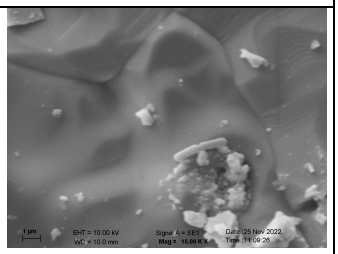
***Dunn's Multiple Comparison Test** $p<0.05$



Graphic 3. Diagram showing the dead cell percentages after mouthwash applications.

4.5. Evaluation of Scanning Electron Microscope Analysis

Within the scope of evaluating the antibacterial activity, the images obtained from the hydroxyapatite discs by SEM were evaluated at x2500, x7500, and x15000 magnifications.

	x2500	x7500	x15000
Henry Blooms			
Luvbiotics			
Listerine			
Humble Co.			
Klorhex			

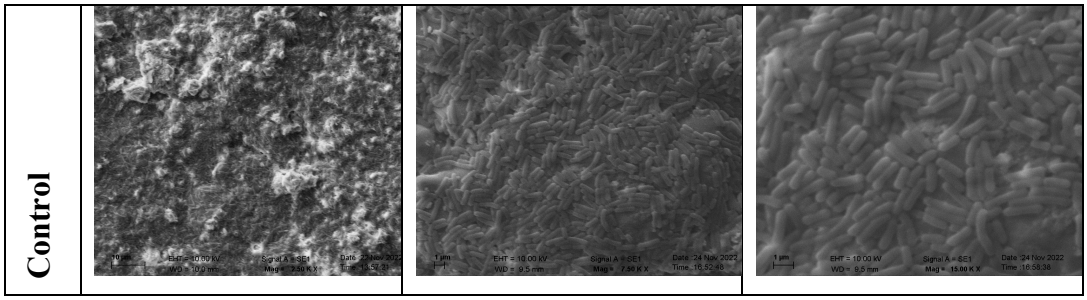


Figure 23. Scanning electron microscope (SEM, FEI Versa 3D, USA) images of the hydroxyapatite discs (x2500, x7500, x15000, respectively.)

At the x15000 magnification, *S. mutans* biofilm densities can be seen clearly. Biofilm formation was evident in the control group. A higher amount of *S. mutans* biofilm was observed in the Henry Blooms and Humble Co. groups after the mouthwash applications compared to the discs in other groups. It was determined that the amount of biofilm was lower in the discs in the Klorhex, Luvbiotics, and Listerine groups after the mouthwash application compared to the discs in the control group. Especially in the Henry Blooms group, in addition to *S. mutans,* Lactobacillus bacteria in the mouthwash are also observed at the x7500 and x15000 magnifications.

4.6. MTS Assay

MTS assay results for determining cell viability after using the mouthwashes with different concentrations for 30 sec. are shown in Table 8. All mouthwashes revealed a statistically significant difference when used in various concentrations except for the Humble Co. group ($p=0.276$). Regarding the concentrations, there was a statistically significant difference between all groups ($p<0.05$).

Table 8: Intra-group comparison of the cell viability at different concentrations of the mouthwashes

	5%		10%		20%		p‡
	Mean±SD	**Median (IQR)**	**Mean±SD**	**Median (IQR)**	**Mean±SD**	**Median (IQR)**	
Henry Blooms	36.55±9.34	38.11 (26.93-44.61)	23.06±5.58	24.71 (17.25-27.23)	1.73±2.25	1.09 (0-4.09)	**0.012**
Luvbiotics	46.26±16.3	51.43 (28.99-58.36)	4.78±2.4	5.21 (2.32-6.8)	0±0	0 (0-0)	**0.006**
Listerine	28.66±8.48	31.91 (19.87-34.2)	0.85±0.99	0.79 (0-1.77)	0±0	0 (0-0)	**0.01**
Humble Co.	73.57±34.67	71.48 (42.49-106.75)	60.4±12.87	60.13 (48.13-72.95)	47.07±13.14	51.85 (33.42-55.94)	0.276
Klorhex	10.11±3.5	9.7 (7.11-13.53)	0.63±1.26	0 (0-1.88)	0±0	0 (0-0)	**0.009**

‡Kruskal Wallis test

IQR: Inter Quartile Range

p<0.05

The results of the intragroup comparisons are given in Table 9. Accordingly, in the Henry Blooms group, the mean value of the viable cells in the 20% concentration group was statistically significantly lower than the 5% and 10% concentration groups (p=0.020). However, there was no statistically significant difference between the mean values of the viable cells in the 5% and 10% concentration groups (p=0.083).

In the Luvbiotics group, the mean value of the viable cells in the 20% concentration group was statistically significantly lower than the 5% and 10% concentration groups (p=0.014). Also, the mean value of the viable cells in the 10% concentration group was statistically significantly lower than the 5% concentration group (p=0.021).

In the Listerine group, the mean value of the viable cells in the 10% and 20% concentration groups was statistically significantly lower than that in the 5% concentration

group (p=0.020, p=0.014). However, there was no statistically significant difference between the mean values of the viable cells in the 10% and 20% concentration groups (p =0.131).

In the Humble Co. group, there was no statistically significant difference between the mean value of the viable cells in the 5%, 10%, and 20% concentration groups (p=0.276).

In the Klorhex group, the mean value of the viable cells in the 10% and 20% concentration groups was statistically significantly lower than that in the 5% concentration group (p=0.018, p=0.014). However, there was no statistically significant difference between the mean values of the viable cells in the 10% and 20% concentration groups (p=0.317).

Table 9: Intra-group comparison of the cell viability of the mouthwashes among different concentrations.

		30 Sec. (p*)
Henry Blooms	**%5 / %10**	0.083
	%5 / %20	**0.020**
	%10 / %20	**0.020**
Luvbiotics	**%5 / %10**	**0.021**
	%5 / %20	**0.014**
	%10 / %20	**0.014**
Listerine	**%5 / %10**	**0.020**
	%5 / %20	**0.014**
	%10 / %20	0.131
Humble Co.	**%5 / %10**	NA
	%5 / %20	NA
	%10 / %20	NA
Klorhex	**%5 / %10**	**0.018**
	%5 / %20	**0.014**
	%10 / %20	0.317

Dunn's Multiple Comparison Test, p<0.05

(NA: Not Applicable)

The intergroup comparison of the mouthwashes in different concentrations is shown in Table 11. When used in 5% concentration, the mean value of the viable cells of the Klorhex group was statistically significantly lower than the Henry Blooms, Luvbiotics, Listerine, and Humble Co. groups (p=0.021). After using Humble Co., the number of viable cells was statistically significantly higher than Listerine (p=0.043). However, there was no statistically significant difference between the other groups (p>0.05).

In 10% concentration, the mean value of the Klorhex group's viable cells was statistically significantly lower than the Henry Blooms, Luvbiotics, and Humble Co. groups (p=0.018, p=0.038). In contrast, no statistically significant difference was found with the Listerine group (p=0.741). The mean value of the viable cells of the Listerine group was found statistically significantly lower than that of the Henry Blooms, Luvbiotics, and Humble Co. groups (p=0.020, p=0.042). The mean value of the Humble Co. group's viable cells was statistically significantly higher than that of the Henry Blooms and Luvbiotics groups (p=0.021). The mean value of the viable cells of the Henry Blooms was statistically significantly higher than that of the Luvbiotics group (p=0.021).

In 20% concentration, the mean value of the viable cells of the Humble Co. group was statistically significantly higher than the mean values of the viable cells of the Henry Blooms, Luvbiotics, Listerine, and Klorhex groups (p=0.014). However, there was no statistically significant difference between the other groups (p>0.05). The distribution of the mean viable cell values after using mouthwashes in different concentrations is shown in Graphic 4.

Table 10: Comparison of the cell viability at different concentrations of the mouthwashes after 30 seconds of application.

	5%		10%		20%	
Groups	**Mean±SD**	**Median (IQR)**	**Mean±SD**	**Median (IQR)**	**Mean±SD**	**Median (IQR)**
Henry Blooms	36.55±9.34	38.11 (26.93-44.61)	23.06±5.58	24.71 (17.25-27.23)	1.73±2.25	1.09 (0-4.09)
Luvbiotics	46.26±16.3	51.43 (28.99-58.36)	4.78±2.4	5.21 (2.32-6.8)	0±0	0 (0-0)
Listerine	28.66±8.48	31.91 (19.87-34.2)	0.85±0.99	0.79 (0-1.77)	0±0	0 (0-0)
Humble Co.	73.57±34.67	71.48 (42.49-106.75)	60.4±12.87	60.13 (48.13-72.95)	47.07±13.14	51.85 (33.42-55.94)
Klorhex	10.11±3.5	9.7 (7.11-13.53)	0.63±1.26	0 (0-1.88)	0±0	0 (0-0)
p‡	**0.01**		**0.002**		**0.003**	

‡Kruskal Wallis test

IQR: Inter quartile range

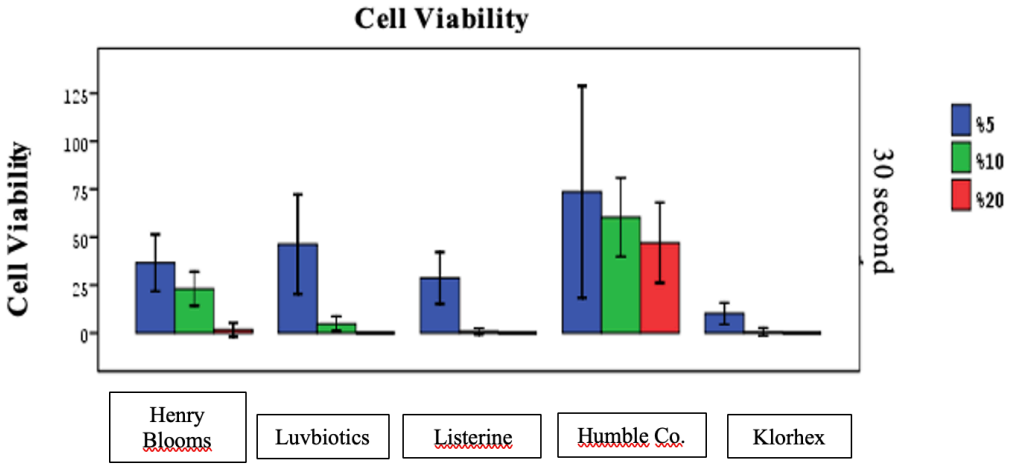
p<0.05

Table 11: Binary comparison of the cell viability at different concentrations of the mouthwashes after 30 seconds of application

	30 Sec.(p*)		
	5%	**10%**	**20%**
Henry Blooms / Luvbiotics	0.386	**0.021**	0.131
Henry Blooms / Listerine	0.248	**0.02**	0.131
Henry Blooms / Humble Co.	0.149	**0.021**	**0.02**
Henry Blooms / Klorhex	**0.021**	**0.018**	0.131
Luvbiotics / Listerine	0.149	**0.042**	1
Luvbiotics / Humble Co.	0.149	**0.021**	**0.014**
Luvbiotics / Klorhex	**0.021**	**0.038**	1
Listerine / Humble Co.	**0.043**	**0.02**	**0.014**
Listerine / Klorhex	**0.021**	0.741	1
Humble Co. / Klorhex	**0.021**	**0.018**	**0.014**

***Dunn's Multiple Comparison Test**

$p<0.05$



Graphic 4. Diagram representing the ratio of cell viability of the HDF cells after different mouthwashes applications at different concentrations.

5. DISCUSSION

The majority of oral diseases can be avoided by practicing good dental hygiene on a regular basis. Most people practice routine brushing; however, mouthwash has a synergistic effect on the oral cavity when used as a supplement (1). Mouthwash use is an easy-to-use plaque control method because it is an easily accessible material, can be implemented in the short term, and does not require patient skill or cooperation (67).

This present study evaluated whether adding probiotics to mouthwashes, which are thought to provide a significant benefit when used to support brushing, especially in children with high caries risk, will make a difference in antimicrobial efficacy and cytotoxic effects. Therefore, five commercial mouthwashes were investigated concerning their antimicrobial and cytotoxic effects. The null hypothesis of the study was that there would be no significant difference in antimicrobial efficacy and cytotoxicity between the groups. In this study, the null hypothesis is partially rejected because there is a significant difference among the groups in antimicrobial efficacy and cytotoxicity.

The infectious condition known as dental caries is caused by the interaction of the tooth surface, saliva, cariogenic bacteria, and diet. The etiology of dental caries may be linked with a wide variety of causes. The most important cariogenic bacteria in dental caries is *S. mutans*. It is present in the oral cavity of the majority of humans. *S. mutans* requires adhesion to the tooth surface and the presence of acids produced by the fermentation of sucrose and other carbohydrates in the environment to cause tooth decay (68). Mutans streptococci, including *S. mutans* and *S. sobrinus* species, are the most cariogenic microorganisms in humans due to their high adhesion and acid production properties (69). But *S. mutans* is the microorganism that is mostly responsible for acid production, and it is among the first microorganisms to colonize the caries lesion. When the balance of oral microflora is disturbed, opportunistic microorganisms come to the fore and initiate the disease process. Studies have proven that *S. mutans* plays an important role in caries formation (16,34,38).

Antimicrobial treatments against caries-causing bacteria are considered to effectively prevent or control dental caries. For this reason, research has focused on eliminating *S. mutans*

(1,69–71). Therefore, its direct relation to dental caries, *S. mutans* was chosen to serve as an early colonizer on dental biofilm in the current investigation.

It is critical to remove plaque in order to preserve periodontal tissues and maintain good oral health. Toothbrushes, interdental cleaners, and irrigators are used in mechanical dental plaque control. However, some studies state that mechanical dental plaque control is effective but insufficient for its removal. It is recommended that mechanical and chemical plaque control should be carried out together to ensure oral hygiene (71,72).

5.1. Microbiological Assay

The antimicrobial activity is preferably the first parameter investigated regarding providing oral hygiene. Antimicrobial properties are provided by various herbal and chemical agents added to mouthwash and toothpaste (73). Some tests are needed to evaluate the antimicrobial effects of these agents, which have been developed with the spread of scientific developments. Initial tests (antimicrobial, antibacterial, cytotoxic, mutagen) and secondary tests (sensitivity, mucosal irritation) should be performed before an improved material or ingredient can be put into clinical use. *In vitro* tests contain initial test models for situations where protection is desired. With appropriate tests, mimicking *in vivo* events *in vitro is possible*. These tests are simple, fast, and inexpensive, making them frequently preferred (74).

Many methods have been used to determine the antimicrobial activity of different active substances. Clinical and Laboratory Standards Institute (CLSI) standards are widely used in applying antimicrobial susceptibility tests and evaluating results. According to this standard, antimicrobial susceptibility tests are accepted as tests applied to determine the effectiveness of an antimicrobial agent against a certain microorganism species. Two methods (dilution and diffusion) are used in antimicrobial susceptibility tests (75,76). “Diffusion tests” are applied on the agar surface with different methods. In the “Agar diffusion method,” wells of certain diameters are opened on the culture-containing medium, and the petri dishes are incubated after adding a homogeneously dissolved essential oil mixture into these wells. The effectiveness of the substance used at the end of the incubation period is determined by the inhibition zones formed around the well. The antimicrobial effect of the substance used is measured, and the antimicrobial effect increases in direct proportion to the diameter of the inhibition zone formed (75,76).

Another method used within the scope of the “Disc diffusion test” is widely used and based on the principle of diffusion of the antimicrobial agent absorbed into the paper discs into the medium where the tested microorganism is inoculated. Paper discs impregnated with certain amounts of antimicrobial agents are placed in the solid medium where the test microorganism is inoculated. Inhibition zones formed around the disc as a result of the incubation of the petri dishes are measured in millimeters, and after the results are evaluated according to the standard zone tables, the sensitivity of the microorganism to the antimicrobial agents used is determined (75,76). The disc diffusion method was utilized in the current investigation to estimate the antibacterial activity of each of the mouthwashes.

Two of the mouthwashes used in the study contain probiotics (*L. paracasei* GMNL-33). One of them includes only probiotics, while the other has a combination of sodium fluoride and probiotics. The other mouthwash groups used in this study contain sodium fluoride, sodium fluoride, and chlorhexidine, and 0.2 % CHX served as a positive control.

The cationic bisbiguanide chlorhexidine gluconate has low toxicity and broad-spectrum antibacterial activity (77). Among the chemotherapeutic agents used in mouthwashes, due to their broad-spectrum antimicrobial activity, chlorhexidine formulations are regarded as the gold standard in anti-plaque and anti-gingivitis mouthwashes (78). In addition to its antibacterial properties, its primary benefit is its “substantivity.” Since CHX is cationic makes it easier for it to connect to the tooth structure and the oral mucosa. This results in less pellicle generation and more substantivity as a result of delayed and prolonged drug release. It effectively prevents gingivitis by blocking plaque regrowth (77).

CHX has been shown in many studies to inhibit plaque formation, decrease gingivitis, and reduce oral bacteria (78,79). The use of CHX mouthwash on a consistent basis, can cause staining of the tooth, irritation of the oral tissues, burning feeling in the mouth, and a noticeable alteration in taste. Due to these disadvantages of CHX, an ideal anti-plaque agent has not yet been developed (1,80). In this study, 0.2 % CHX-containing mouthwash was used as a positive control.

Chen et al. **(2011)** evaluated the antimicrobial efficacy of two commercially available mouthwashes (Listerine and Corsodyl) on a mono-species biofilm model on orthodontic brackets *in vitro*. The XTT reduction test was used to determine the S. mutans biofilm's cell

viability on the brackets after it had been exposed to both of the mouthwashes. Fluorescence microscopy and CLSM were utilized in order to observe the biofilm. Both Listerine and Corsodyl were shown to have antimicrobial properties on the biofilm, however the results revealed that Listerine had more bactericidal effect, while Corsodyl was more effective at preventing the growth of bacteria (81).

***Lakade et al.* (2014)** compared the antimicrobial effect of chlorhexidine mouthwash (0.2%) and mouthwash containing 0.05% sodium fluoride, 5% xylitol, and 0.03% triclosan in reducing the counts of plaque Mutans streptococcus in plaque. In this clinical study, the plaque samples were collected, and after incubation, Mutans streptococcus count was estimated on the strips from the Dentocult SM kit. They reached the conclusion that chlorhexidine gluconate was a more efficient antibacterial agent in lowering the number of Mutans streptococci that were present in plaque (82).

***Demirel et al.* (2015)** evaluated the antimicrobial activities of commonly used mouthwashes against different oral microorganisms by disc diffusion and MIC tests *in vitro*. They found CHX and CPC-containing mouth rinses more effective than other tested products (83).

In the present study, CHX and CPC-containing mouth rinses (Klorhex, Luvbiotics, and Listerine) were found to have more antimicrobial and antibiofilm activity than the other tested products, regardless of their fluoride or probiotics content. 0.2% CHX-containing mouthwash (Klorhex) had the highest antimicrobial and antibiofilm activity against *S. mutans*.

Fluoride is another ingredient commonly added to various oral healthcare products. Sir H. Trendley Dean introduced fluoride to dentistry in 1934 because of its capacity to reverse the process of tooth demineralization while simultaneously enhancing the remineralization process. Fluoride has well-known effects on bacterial metabolism and oral flora. There is a wide variety of fluoride mouthwashes and toothbrushes on the market today, which can help preserve dental health and decrease the incidence of cavities (1,84).

***Sajadi et al.* (2015)** compared the salivary *S. mutans* count after two weeks of consumption, and the incidence of adverse effects of chlorhexidine, fluoride and chlorhexidine-fluoride mouthwashes. In this clinical study, 120 students (12 to 14 years old) were selected.

According to the results, all mouthwash groups decreased counts of *S. mutans*. The most reduction in bacteria count were seen with fluoride-chlorhexidine mouthwash, however this group had greater adverse effects than the other two groups. (85).

Guggenheim and Meier **(2011)** compared the antimicrobial effects of chlorhexidine mouth rinses available on the Swiss market and Listerine against poly-species biofilm model and concluded that all solutions containing CHX as well as Listerine reduced the number of microorganisms in biofilms significantly (86).

Becker et al. **(2021)** evaluated effectiveness of a mouthwash containing reduced concentration of CHX and CPC, on the oral biofilms on the hydroxyapatite discs (HA) and micro-rough titanium (Ti) surface. Acrylic appliances containing Ti and HA discs were worn by four healthy volunteers for *in situ* formation of plaque. Biofilms were formed on the discs and exposed to: 0.1% CHX (positive control), 0.05% CHX + 0.05% CPC or sterile saline (negative control). According to the study, high antibacterial effects and decrease of biofilm viability were seen after the application of CHX, and its combination with CPC (87).

Low concentrations of CHX containing mouthwashes in combination with CPC could be strong antibacterial agents with reduced side effects for long-term applications. Unlike ***Becker et al.*** **(2021),** in this study, the antimicrobial efficacy of Humble mouthwash (containing CHX at a low concentration (0.02%)) was found to be very low. This result might be attributed to the absence of CPC in Humble mouthwash.

CPC is a quaternary ammonium compound that belongs to the category of cationic surface-active agents. Initially, its effectiveness was only moderate. Still, a synergistic effect is projected to increase overall antibacterial efficacy when chlorhexidine is used in conjunction with the CPC (87,88)

The primary purpose of oral rinses is to remove microorganisms from the mouth, but their efficacy depends on formulation, concentration, quantity of components, and duration of rinsing (83). In this present study, it was found that the antimicrobial activity of Humble mouthwash was very low despite the presence of CHX and fluoride in it. This difference may also be due to varieties in ingredients or insufficient concentration of CHX and fluoride in the mouthwash.

According to the results of our study, the antimicrobial activity against *S. mutans* was mostly seen in the Klorhex group, followed by the Luvbiotics and Listerine groups. The antimicrobial activity of the other groups (Henry Blooms and Humble Co.) against *S. mutans* was very low.

Probiotic bacteria are living microorganisms that, when administered in sufficient quantities, positively impact the host's health (78). Salivary S. mutans levels may be decreased by using products containing probiotics like Bifidobacterium or Lactobacillus. (1). Nowadays, many dairy products like milk, cheese, and yogurt, contain probiotics. They exert their effects through a variety of mechanisms, including the production of antimicrobial compounds, competing for nutrition, elimination of pathogenic organisms, and regulating of the body's immune response (78,89). Strain features, fermentation technology, viability or nonviability, microencapsulation and stability, all influence the efficacy of probiotics. (78).

Oral health care may also profit from using probiotic therapy as an alternate treatment option. Tablets, lozenges, mouthwashes, powder, straw, and powdered straw are some vehicles that can be used to administer probiotics (13). *In vivo* studies show that daily consumption of probiotic bacteria *Lactobacillus reuteri* ATCC 55730 by different vehicles reduces salivary MS significantly (90–92).

Nowadays, probiotic mouthwashes that contain living microorganisms are recommended (13,93,94). Because of they have no risk to the oral flora and do not promote antibiotic resistance, they were created as a natural defence mechanism against harmful bacteria (1,78).

Zahradnik et al. (2009) investigated the efficacy of a probiotic MW and its safety, they concluded that it was secure for usage on a regular basis as a supplement for the purpose of preserving the oral health (95).

Elgamily et al. **(2018)** conducted an *in vitro* study to test the antibacterial effectiveness of a probiotic based experimental mouthwash against *Streptococcus mutans.* Two additives; Zamzam and probiotic was tested against *S. mutans* with the disc diffusion method. They found the experimental mouthwash was effectively reduce *S. mutans* (78)

It should be emphasized that there were variations in the potential for dental caries among different lactobacilli. Some Lactobacillus species were inked to the development of caries, however some strains may not have a caries-inducing effect (91,96). Along with *L. paracasei*, the well-documented *Lactobacillus rhamnosus* GG are some of these bacterial strains (91,97).

L. paracasei's probiotic advantages to human and dental health have been extensively researched (98–100). In a study, *L. paracasei* obtained from healthy individuals was found to be effective against a wide variety of oral pathogens (101). According to a study ***Koˆll-Klais et al.* (2005)** was conducted, *Lactobacillus paracasei, Lactobacillus salivarius, Lactobacillus rhamnosus* and *L. plantarum* showed the strongest antimicrobial activity against *S. mutans* (102).

***Simark-Mattsson et al.* (2007)** assessed probiotic characteristics of oral lactobacilli. In that study, lactobacilli were obtained from plaque and saliva from children and adolescents. They found that *Lactobacillus paracasei*, *L. rhamnosus,* and *Lactobacillus plantarum* species were showed maximum effect against *S. mutans* (103).

***Teanpaisan and Piwat* (2014)** investigated the effect of *L. paracasei* SD1 on the growth of mutans streptococci and yeasts and the persistence of *L. paracasei* SD1 *in vivo.* Forty healthy young adults were randomly assigned to either the test (received milk powder contained *L. paracasei* SD1) or placebo (standard milk powder) groups. They concluded that short-term (4 weeks) daily consumption of milk powder containing *L. paracasei* SD1 reduced the numbers of mutans streptococci in saliva, and this bacterium represents a promising candidate for oral probiotics (99).

***Manmontri et al.* (2019)** investigated the effects of *Lactobacillus paracasei* SD1 on the *S. mutans* in children's plaque and saliva samples. A 6-month of daily or three days a week; probiotic or placebo milk usage was evaluated. The *S. mutans* and total lactobacilli counts in the plaque and saliva samples were analyzed by quantitative polymerase chain reaction (qPCR). 6-month of daily and triweekly consumption of the *L. paracasei* SD1 was found significantly effective on reducing the *S. mutans* counts, and this effect continues for at least six months after discontinuation (98).

***Pahumunto et al.* (2019)** investigated the effect of the *L. paracasei* SD1 on oral bacteria. In this study, milk powder with or without probiotic, consumed by the volunteers, once daily for six months. Saliva was collected at baseline 3-, 6-, and 12 months and total streptococci, *S. mutans,* lactobacilli, and *L. paracasei/L. casei* counts were evaluated for using real-time PCR. The ELISA method was used for salivary IgA (sIgA) examination. The results revealed that *L. paracasei* SD1 stimulate sIgA, could control *S. mutans* level and this strain may be used for the caries prevention (100). In the present study, mouthwashes containing *L. paracasei* GMNL-33 were preferred for probiotic-containing groups.

Chuang et al. (2011) evaluated the *S. mutans* and lactobacilli counts in adults, after 2-weeks intervention of *L. paracasei* GMNL-33 tablets. According to the study, there were no significant differences in the counts of the lactobacilli and *S. mutans*, and buffer capacity between the groups. However, they detected a significant reduction in the *S. mutans* count two weeks after medication. They concluded that at least 2-week period of intervention needed for *L. paracasei* GMNL-33 to become effective and this intervention showed inhibitory effect against *S. mutans* after the discontinuation (104). Unlike the above-mentioned study, the results of the present study revealed that the antimicrobial and antibiofilm activity of the group whose active ingredient was only probiotic *L. paracasei* GMNL-33 (Henry Blooms) was statistically significantly lower than most of the other groups. The group containing probiotic, fluoride, and CPC combination (Luvbiotics) was the highest after the Klorhex group regarding the antimicrobial and antibiofilm activity.

Hydroxyapatite and glass are frequently used to mimic oral hard surfaces (105). Synthetic hydroxyapatite discs are widely used in biofilm and bacterial adhesion experiments to simulate healthy enamel hydroxyapatite, which is the main component of dental enamel (106–108).

Saliva (artificial or pooled human saliva) is preferred to condition glass and hydroxyapatite disc surfaces to generate an acquired pellicle that increases bacterial adhesion for biofilm investigations (109–111). Because the bacterial composition is controlled by the selective pressure of human saliva's physical-chemical characteristics and nutrients, rather than artificial medium, the biofilm developed in artificial saliva or pooled human saliva will be a more accurate representation of *in vivo* plaque (105).

Tonguç Altin et al. (2021) investigated the antibacterial effects of different saliva-substitutes-containing-lysozyme (LYZ) or-lactoferrin (LF) on *S. mutans* in comparison with human saliva. They evaluated the adhesion of *S. mutans* on hydroxyapatite discs (108).

Wang et al. (2020) conducted a study to investigate the effect of casein phosphopeptide (CPP) on pellicle modification and initial bacterial adhesion on the saliva-coated hydroxyapatite (s-HA) discs (106).

Babu et al. **(2014**) evaluated the efficacy of three oral rinses in preventing adherence of oral bacteria to epithelial cells and pooled saliva-coated HA disks and their ability to detach adherent bacteria from the epithelial cells and HA disks (112). In this study, s-HA discs were used. An *in vitro* model was established to simulate pellicle formation, modification, and initial adhesion of *S. mutans.* Natural enamel was not used due to the different chemical compositions of the individual enamel specimens, which may impact adhesion of the bacteria to the enamel surface.

Testing the efficacy of new anti-biofilm agents via *in vitro* model systems is time- and cost-effective (105). Developing oral biofilm communities is also made simpler when *in vitro* oral biofilm models are used, which is another advantage of employing these models. *In vitro,* systems have the potential to be highly adaptable. Nutrient availability, flow, the introduction of designated species, and the passage of time may all be carefully regulated, which can assist researchers in answering particular research questions about biofilm architecture, cellular organization, and processes involved with biofilm formation (113).

Many microscope technologies are available to analyze *in vitro* oral biofilms, each with advantages and disadvantages. Using a confocal microscope, researchers can simultaneously observe oral biofilm architecture and cell viability (105).

Kreth et al. **(2004)** showed that the concentration of sucrose had a qualitative as well as quantitative impact on the total biomass of *S. mutans,* the strength of the biofilm, and the architecture of the biofilm. In this study, the biofilm cells were detached from the surface with microjet impingement (MJI) and quartz crystal microbalance (QCM) and the COMSTAT software to evaluate the total biofilm biomass, biovolume, and average film thickness. According to the results, while the biovolume of the biofilm formed without sucrose was much

reduced, the volume of the biofilm in the presence of sucrose did not decrease to the same levels (114).

Serbiak et al. **(2018)** compared the efficacy of EO mouth rinse (Listerine) versus triclosan and stannous fluoride dentifrice slurries, in terms of antimicrobial efficacy and kill penetration. In the study, saliva-coated hydroxyapatite discs were used for biofilm assay and the 3D structure of the biofilm was evaluated with CLSM using viability staining. Images were analyzed with Volocity Image Analysis Software. The results showed that the EO-containing mouth rinse had a greater antimicrobial effect at shorter treatment times than the dentifrice slurries tested and could penetrate deep into the accumulating plaque biofilm (115).

Cho et al. **(2020)** investigated the optimal nano-emulsion formulation for Curcuma xanthorrhiza oil (Xan). They compared the antimicrobial effects of the nano-emulsion, the original emulsion, Listerine, and distilled water on *S. mutans* biofilm *in vitro*. A biofilm assay with artificial saliva-coated hydroxyapatite discs was conducted, and the resulting biofilms after the treatments were evaluated with CLSM imaging. These images were assessed with COMSTAT software, and the alive and dead cell biomass were analyzed. The results revealed that the nano-emulsion's alive/dead cell ratio was less than in other groups (116) In the present study, an *in vitro* biofilm was formed on the hydroxyapatite discs coated with the artificial saliva. The resulting biofilm was rinsed with mouthwashes, and the discs were examined with a confocal microscope. Images of the 3D structure of the *in vitro* biofilms were examined with the COMSTAT software, and biomass and volumes of alive and dead bacteria were evaluated. The selected parameters were analyzed because they represent essential morphological characteristics from the formation and development of the biofilm.

Verkaik et al. **(2011)** evaluated the antimicrobial efficacies of two toothpaste formulations containing natural antimicrobials (herbal extracts and chitosan) against oral biofilms. In this study, COMSTAT enabled measurement of the biofilm volume (mm^3/mm^2) occupied by live and dead bacteria, from which the percentage of live bacteria in a biofilm volume and the percentage of removal after flowing with toothpaste supernatant or chlorhexidine (positive control) or buffer (negative control). They concluded that natural antimicrobials in herbal and chitosan-based toothpastes could be equally effective as chlorhexidine, not only concerning immediate but also delayed bacterial killing due to the

substantivity of antimicrobials in oral b (117) iofilms. The percentage of dead bacteria biomass was calculated in the present study, and antibiofilm effects were evaluated according to these results. The highest percentage of dead cell amount was seen in the Klorhex group regarding biomass. Luvbiotics and Listerine were the other most effective mouthwashes, respectively. Henry Blooms and Humble mouthwashes were found to be the groups with the lowest percentage of dead cells in terms of biomass, but no significant difference was observed between them.

Liu et al. **(2012)** examined the effect of CHX, ampicillin, and NaF on *S. mutans* UA159. Live/dead cell ratios were calculated using COMSTAT software. According to the results, untreated biofilms showed an elaborated architecture, and upon treatment with NaF, CHX and ampicillin, *S. mutans* cells were scattered. CHX and ampicillin treatment led to a decrease in live/dead cell ratios, while NaF treatment had no significant effect on this ratio (118).

In the present study, it was observed that live/dead cell ratios were lower in both CHX and NaF-CPC-containing mouthwashes (Klorhex, Luvbiotics, and Listerine groups). Still, on the other hand, live/dead cell ratios were higher in Henry Blooms and Humble Co. groups. While the number of dead cells increased, the number of dead cells remained less than the number of live cells after treatment in these groups.

Pandit et al. **(2015)** investigated the antibiofilm activity of brief CPC treatments during *S. mutans* biofilm formations. They concluded that brief CPC treatments have strong antibiofilm activity, and this activity was dependent on some features such as CPC concentration, the stage of biofilm formation, and treatment time. Results suggest that the antibiofilm activity of brief CPC treatments is time-dependent, as while the biovolume of live cells decreased, the treatment time increased. If stronger antibiofilm activity is required to control dental biofilms, it can be obtained by increasing treatment time (119).

Han Y. **(2021)** investigated the effect of the NaF on during the early and mature stages of *S. mutans* biofilm formation on s-HA disks. They concluded that inhibiting *S. mutans* biofilm growth with fluoride was simpler during the early formation stage than during the mature formation stage. In that study, the CLSM analysis and COMSTAT software was used to evaluate the biovolume and thickness of live and dead cells, and the extracellular polysaccharides (EPSs) biovolume and thickness (120).

Balasubramanian et al. **(2021)** investigated the effects of trans-cinnamaldehyde (TC) and its synergistic activity with CHX and fluoride against *S. mutans*. In this study, biofilm inhibitory assay was analyzed using confocal microscopic imaging, and the obtained images were further analyzed using COMSTAT software. Biofilm biomass, average thickness, dimensionless roughness coefficient, surface area to volume ratio, and diffusion distances were also analyzed. They found that untreated *S. mutans* biofilm was thicker with higher biomass than the treated biofilm (121). In the present study, the live amount of biomass at the substratum of the control group was significantly higher than most other groups. Only the Humble Co. group showed similar results to the control group.

Alonso-Español **et al. (2023)** conducted an *in vitro* study to compare the antimicrobial efficacy of xanthohumol and curcumin, using an *in vitro* multi-species biofilm model. CLSM, qPCR, and SEM were used for evaluation. They concluded that both agents showed antimicrobial efficacy on the biofilm. In this study, the biomass of live and dead cells was calculated using the COMSTAT software on the previously collected image films (122).

Biofilm is considered an effective bacterial defense mechanism. Biofilm's three-dimensional matrix contains exopolysaccharides and several other proteins that function as a barrier to the diffusion of antimicrobials. Biofilm architecture is defined by physical parameters such as biofilm thickness, biofilm mass, diffusion distance, and roughness coefficient (123). Similar to other studies, biomass ($\mu m^3/\mu m^2$) was used for biofilm analysis in the present study.

5.4. SEM Imaging

Jones et al. (2018) investigated the antibacterial effect of a newly formulated foam mouthwash against the biofilm grown on tooth enamel. SEM images were obtained from the enamel surface to determine the biofilm retention following mouthwash exposure (124). Similarly, the surface properties of the biofilms on the discs after mouthwash application were also examined under SEM imaging at the end of the present study.

5.5. MTS Assay

In addition to having broad-spectrum antimicrobial activity, the ideal mouthwash should not be toxic to oral cells (70). Biocompatibility is the ability of materials to coexist with living cells without harming them. It is essential to evaluate the biocompatibility of mouthwashes

because they are in constant contact with periodontal tissues and alveolar bone during their use. Substances released from mouthwashes can cause a reaction (inflammation or necrosis) in adjacent tissues such as the oral mucosa, gingiva, or alveolar bone (58). Therefore, in the last part of the present study, the possible cytotoxic effects of tested mouthwashes on human dermal fibroblasts were analyzed.

Before being used on patients, dental materials must undergo biocompatibility testing, as required by national and international standards. Cell culture tests and animal studies are used for this purpose. Dental material cytotoxicity tests using animals, is time-consuming, expensive, and morally contentious. However, *in vitro* cytotoxicity testing can easily control experimental parameters that typically create problems during in vivo studies. These tests are suitable for assessing dental material biological features, inexpensive, and repeatable (58)

The MTS test can detect the ratio of live/dead cells with the colorimetric method. Mitochondrial enzymes of living cells can cleave the tetrazolium ring of the MTS dye. As a result of this fragmentation, living cells are stained dark pink, while dead cells are not. The MTS test consists of incubating cells with MTS dye, solubilizing the reaction product, and colorimetric measurement. While detecting formazan formation by measuring the optical density, light or electron microscopy can be used as an alternative. The MTS test is a simple, reliable, and easy-to-use method that enables cytotoxicity evaluation in small culture volumes (64).

It is necessary to standardize the test methods to be applied to investigate biomaterials' properties and compare the test results (53,58). Organizations such as the American Society for Testing and Materials (ASTM), International Organization for Standardization (ISO), Council on Dental Materials, and Instruments and Equipment of the American Dental Association have standardized the application of test methods to be applied to materials (125,126). In the present study, the cell cytotoxicity test was done *in vitro* following ISO 10993-5 proceedings (126).

Cell culture tests use primary cells or cell lines from oral tissue. Cell lines can be cultured over a long period and have homogeneous morphological and physiological characteristics. In contrast, primary cells are derived directly from target tissues and have a limited lifespan and heterogeneous properties. Because they age after being cultured several times in vitro, aging means that the reproduction rate of the cells decreases, the number of

cytoplasm increases, and eventually, this situation ends with cell death (56,58). According to the data obtained from the studies, tests using primary cells and continuous cell lines give similar results. It has been observed that continuous cell lines produce much more sensitive responses than primary cells (53).

The HDF cell lines are one of the recommended cells for *in vitro* testing with cell types such as L929, Balb/3T3, and WI38. Such continuous cell lines are frequently used for testing the cytotoxic properties of dental materials, as they are easy to manufacture, elicit appropriate biological responses, and facilitate cell culture control (125,126).

Gingival fibroblasts are the target of chemical substances that are releasing by dental products. In the present study, HDF cell lines were used because of their availability, frequent use in *in vitro* tests, and rapid proliferation. In conclusion, HDFs make a useful screening model for *in vitro* toxicity testing dental materials (127–132).

Antimicrobial mouthwashes help support oral biofilm management. Many chemical agents have been evaluated, but the success of most has been highly variable. Antimicrobial agents are expected to provide more protective efficacy than therapeutic effects (133). Active chemical agents used in mouthwashes are mixed in different ways and have some important properties; they are active against disease-causing microorganisms, and they impair the effects of MDP without disturbing the normal healthy oral flora; their use is safe for humans and the environment (134).

Mouthwashes containing chlorhexidine may cause harmful effects on gingival fibroblasts, which affect gingival morphology, vitality, and function (135). Chlorhexidine, when used in low concentrations, binds strongly to the bacterial cell membrane, causing increased permeability by leaking intracellular components; when used in high concentrations, it causes direct death of the cell by precipitation of the bacterial cytoplasm. Today, 0.2% chlorhexidine is still considered the gold standard mouthwash in oral antiseptic treatment (135). Therefore, 0.2% chlorhexidine mouthwash was also used as a positive control in the present study.

When mouthwashes are taken into the mouth, they increase the secretion of saliva by stimulating the chemoreceptors, so their concentration in contact with the oral tissues decreases.

This should be considered when deciding on the concentration to test clinical conditions in cytotoxicity tests. *In vitro* cytotoxicity studies conducted to date have evaluated the effects of very different mouthwash concentrations on different cells (135–138).

***Balloni et al.* (2016)** evaluated the effects of different mouthwashes on the adhesion and proliferation mechanisms of keratinocyte cells and gingival fibroblasts. They determined that the highest dose that could be used without cell separation was with the 30%, 10%, and 30% concentrations of mouthwashes. They found that cell death was higher at higher concentrations (139). On the other hand, ***Tsourounakis et al.* (2013)** found that all cells were killed when the concentrations of the tested mouthwash were above 25% (exposure time 60 seconds). They also caused morphological changes, decreased viability, and loss of cell function at lower concentrations (140). Similar to the studies above, in the present study, HDF cells were exposed to 5%, 10%, and 20% concentrations of mouthwashes for 30 seconds.

Maintaining good oral health is critical to overall well-being. The basis of modern dentistry involves the continuity of oral tissues (141). While mouthwashes reduce the number of harmful microorganisms in the oral region, they should be able to maintain their effectiveness for a long time (142). They should be able to stay in the mouth long enough to provide antimicrobial activity, and this time should not be long enough to reveal toxic effects simultaneously (133).

Following the instructions, mouthwashes containing chlorhexidine are recommended to use two times a day by keeping them in the mouth for at least 30 seconds without diluting on a 10-15 ml scale. In addition to the use of oral hygiene applications, holding chlorhexidine in the mouth for 30 seconds can help minimize the production of MDP and inflammation. Although it has some side effects, it is easy to use, and individuals adapt immediately (133).

In clinical practice, the contact time with tissues in the oral cavity and existing microbial pathogens is often short, and patients use mouthwash in their daily routine between 30 seconds and 1 minute. When mouthwash is used for 30 seconds to 1 minute, the oral mucosal cells are exposed to it for a long exposure time (143–145).

Studies have evaluated whether chlorhexidine mouthwashes cause cell apoptosis or necrosis at different exposure times. Because of the findings of these investigations, it was

suggested that even longer exposure durations should be applied because the length of exposure in the mouth was significantly greater than the application time (143–145).

***Park et al.* (2014)** examined the effects of CHX and Listerine on the stem cells. Titanium discs were immersed in CHX or Listerine for 30 sec, 1.5 min, or 4.5 min. CLSM analysis was performed for evaluating cell morphology, and the cell counting kit-8 assay was used for analyzing the cell viability. Results showed that the application of CHX and Listerine on titanium discs had residual effects on the viability of the stem cells and that it may be suggested that the application of both mouthwashes directly into the peri-implant pocket produces toxic effects (144).

***Oliviera et al.* (2018)** evaluated cell viability effects of 5 different mouthwashes on the gingival fibroblasts, 24 hours after the application with the MTT test. Cells were exposed to mouthwashes two different times, as T1 (30 or 60) and twice as T2 (60 or 120), following the manufacturer's instructions. All evaluated mouthwashes showed effective antibiofilm activity and were highly cytotoxic to gingival fibroblasts at two different application times (146). Therefore, in the cytotoxicity assay of this study, the short application time of mouthwashes was preferred as 30 seconds, in line with the manufacturers' recommendation.

***Müller et al.* (2017)** examined cytotoxic and antimicrobial activity of twelve mouthwashes. Their antimicrobial against five bacterial strains was screened using disc diffusion assay, and mitochondrial reductase activity were tested with primary gingival L929 cells, HSC -2 epithelial cells and fibroblasts, to determine their cytotoxic effects. Data showed that moderate antimicrobial, but strong cytotoxic activity was observed in CPC containing mouthwashes, but cocamidopropyl betaine and 0.2% chlorhexidine containing mouthwashes were exhibited strong antimicrobial and cytotoxic activity (14).

***Bayraktar et al.* (2023)** evaluated the effects of a 0.2 % chlorhexidine (CHX) solution (at 30-second and 2-minute time intervals) on human gingival fibroblasts' cell viability and cytotoxicity with MTT. According to the results, 0.2 % CHX solution reduced HGF cell viability and showed cytotoxic activity on HGF during short exposure times of 30 sec and 2 min (147).

The results of the present study are consistent with the other researchers. Cell viability, as expected, was higher in the untreated group than in the mouthwash groups. With the data obtained within the limits of this study, it was determined that Klorhex, Luvbiotics, and Listerine mouthwashes significantly reduced HDF cell viability and showed strong cytotoxic activity at the high concentrations (10% and 20%) compared to other mouthwashes in this exposure time. Also, in the Henry Blooms group, the cell viability was highly reduced at 20% concentration; however, in the Humble Co. group, the highest cell viability was observed in the HDF cells, almost close to the values of the control group.

Limitations of this study

- Regarding methodological aspects, using a single species for antimicrobial efficacy and antibiofilm assay could be considered a limitation, considering the original structure of the biofilm.
- Also, the study was limited by its dependence on monolayer cultures for the cytotoxicity analysis. Because of the oral cavity is a host to many different types of cells, including epithelial cells, immune system cells and fibroblasts.
- Because this study was conducted only *in vitro*, the effects of many variable factors in the clinical setting could not be evaluated.

6. CONCLUSION

In this study, the antimicrobial efficacy and cytotoxic effects of mouthwashes suitable for use in children were investigated. According to the present study, the following results were obtained:

1. When the inhibition zone values were compared in the disc diffusion assay, the mean values of inhibition zones were Klorhex > Luvbiotics > Listerine > Henry Blooms = Humble Co. = Control, respectively. A statistically significant difference was observed between all study and control groups' mean *S. Mutans* inhibition zones ($p<0.05$).

2. Henry Blooms and Humble Co. groups showed no antibacterial effect on *S. mutans* biofilm similar to the negative control group in the disc diffusion assay ($p>0.05$).

3. The dead bacteria biomass of Luvbiotics, Listerine, and Klorhex groups was statistically significantly higher than the live biomass ($p<0.05$). The dead bacteria biomass of Humble Co. and control groups was statistically significantly lower than the live biomass of these groups ($p<0.05$).

4. Regarding the percentage of the dead bacteria biomass, the highest percentage was found in the Klorhex group, whereas the lowest was found in the control group. The difference between the groups having the highest and lowest percentages and the other groups was statistically significant ($p<0.05$).

5. MTS assay results for determining cell viability, all mouthwashes revealed a statistically significant difference when used in various concentrations except for the Humble Co. group ($p>0.05$).

6. Regarding the concentrations, there was a statistically significant difference between all groups, in terms of cell viability ($p<0.05$).

7. In the Henry Blooms group, the mean value of the viable cells in the 20% concentration group was statistically significantly lower than the 5% and 10%

concentration groups ($p<0.05$). However, there was no statistically significant difference between the mean values of the viable cells in the 5% and 10% concentration groups ($p>0.05$).

8. In the Luvbiotics group, the mean value of the viable cells in the 20% concentration group was statistically significantly lower than the 5% and 10% concentration groups ($p<0.05$). Also, the mean value of the viable cells in the 10% concentration group was statistically significantly lower than the 5% concentration group ($p<0.05$).

9. In the Listerine group, the mean value of the viable cells in the 10% and 20% concentration groups was statistically significantly lower than that in the 5% concentration group ($p<0.05$). However, there was no statistically significant difference between the mean values of the viable cells in the 10% and 20% concentration groups ($p>0.05$).

10. In the Humble Co. group, there was no statistically significant difference between the mean value of the viable cells in the 5%, 10%, and 20% concentration groups ($p>0.05$).

11. In the Klorhex group, the mean value of the viable cells in the 10% and 20% concentration groups was statistically significantly lower than that in the 5% concentration group ($p<0.05$). However, there was no statistically significant difference between the mean values of the viable cells in the 10% and 20% concentration groups ($p>0.05$).

12. When used in 5% concentration, the mean value of the viable cells of the Klorhex group was statistically significantly lower than the Henry Blooms, Luvbiotics, Listerine, and Humble Co. groups ($p<0.05$). After using Humble Co., the number of viable cells was statistically significantly higher than Listerine ($p<0.05$). However, there was no statistically significant difference between the other groups ($p>0.05$).

13. In 10% concentration, the mean value of the Klorhex group's viable cells was statistically significantly lower than the Henry Blooms, Luvbiotics, and Humble

Co. groups ($p<0.05$). In contrast, no statistically significant difference was found with the Listerine group ($p>0.05$). The mean value of the Humble Co. group's viable cells was statistically significantly higher than that of the Henry Blooms and Luvbiotics groups ($p<0.05$). The mean value of the viable cells of the Henry Blooms was statistically significantly higher than that of the Luvbiotics group ($p<0.05$).

14. In 20% concentration, the mean value of the viable cells of the Humble Co. group was statistically significantly higher than the mean values of the viable cells of the Henry Blooms, Luvbiotics, Listerine, and Klorhex groups ($p<0.05$). However, there was no statistically significant difference between the other groups ($p>0.05$).

The H_0 of the study was that there would be no significant difference in antimicrobial efficacy and cytotoxicity between the groups. In this study, the null hypothesis is partially rejected because there is a significant difference between some groups in the antimicrobial efficacy and cytotoxicity.

Mouthwashes may not always have the same antimicrobial and cytotoxic efficacy due to their complex content. Among the mouthwashes in our study, those with higher antimicrobial activity were also found to have higher cytotoxic activity. On the other hand, Henry Blooms mouthwash had low antimicrobial effect, but it had cytotoxic effects in higher concentrations. It is important to note that this situation may not be relevant to the oral cavity due to the complexity of the oral environment, which includes variables such as host immunity, saliva, pH, and enzymes.

When all the results are evaluated, although the antimicrobial activity was found low in this study, it is thought that mouthwash containing probiotics can be used as an alternative to mouthwashes with more chemical content in suitable patient groups due to its moderate antibiofilm effect and lower cytotoxicity. However, more *in vitro* and *in vivo* studies are required to evaluate mouthwashes' antimicrobial activity and cytotoxicity in the oral cavity microenvironment, and obtain more valid results.

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APPENDIX 1: Resume

Personal Information

Name	PIRIL	**Surname**	BAŞARAN
Place of Birth		**Date of Birth**	
Nationality		**ID Number**	12422986992
E-mail		**Phone**	

Education Status

Degree	**Faculty**	**Institute**	**Graduation Year**
Licence/Degree	Dentistry	Yeditepe University	2018
High School	-	TED Karabük High School	2012

Foreign Languages	**Exam Note**
English	YÖKDİL: 92.500

Computer Skills

Program	**Skill**
Microsoft Office	Good

Papers Presented at International Scientific Meetings and Published in the Proceedings Book

Management of a Dentigerous Cyst in a 7-Year-old Patient: A Case Report, IAPD Congress, Online Seminar, 2021, Poster Presentation Kubra TONGUC-ALTIN, Begüm VAROL, Piril BASARAN, Nurhan GULER
Kök Ucu Kapalı Sürekli Kesici Dişlerin Avülsiyon Sonrası Gecikmiş Replantasyonu: İki Olgu Raporu, 27. Uluslararası TPD Kongresi, Antalya, 2022, Poster Sunumu Pırıl Başaran, Gökçen Deniz BAYRAK, Elif YAMAN-DOSDOĞRU, Senem SELVİ-KUVVETLİ