

Research Article

The Impact of Triclosan-Containing Toothpaste on the Overall and Cariogenic Salivary Microbiota Among Primary School Children

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Abstract

Objectives: Triclosan-diphenyl ether derivate is an agent, which belongs to the Non-Ionic antimicrobial agents group. This study aims to estimate the salivary levels of *Streptococcus mutans* and *Lactobacillus species* in the saliva before and after teeth brushing with a dentifrice containing triclosan/copolymer and to compare the number of whole salivary flora by saliva analysis before and after teeth brushing.

Methods: To accomplish our objective, we used a Colgate Total Plus Whitening®, Colgate-Palmolive dentifrice. The group consisted of 24 healthy schoolchildren aged 9 - 13 of both sexes. The counts of *Streptococcus mutans* and *Lactobacillus species* were determined with commercial CRT bacteria strips produced by Ivoclar-Vivadent, Liechtenstein. The total count of the saliva microbial was determined by standard microbiological methods.

Results: A significant reduction in salivary MS and LB levels was observed in all samples as well as a decrease in the total count of aerobe and anaerobe bacteria and yeast.

Conclusion: The effectiveness of Colgate Total Plus Whitening® toothpaste in reducing salivary aerobes is statistically significant, but its impact on reducing salivary anaerobic microbes is statistically negligible. The bacteria *Streptococcus mutans* experiences a decrease that is greater than tenfold, while the bacterium *Lactobacillus species* experiences a reduction of around tenfold.

Keywords: Children; School; Salivary Microbiota; Teeth Brushing

Clinical Relevance

Scientific Rationale for Study

Multiple studies highlight the essential role of cariogenic bacteria in developing tooth caries. Triclosan is an antibacterial chemical that has received limited research attention. This work enhances our understanding of the effects of triclosan in predominantly lowering the cariogenic salivary bacteria.

Principal Findings

The data obtained demonstrate the antibacterial efficacy of triclosan in reducing both aerobic and anaerobic salivary flora, yeasts and the two most significant cariogenic microorganisms.

Practical Implications

This study presents the significance of maintaining regular oral hygiene using toothpastes including triclosan. Additionally, it recommends that makers of oral hygiene products consider using triclosan in their products.

Introduction

Dental caries cannot occur in the absence of germs. This perspective is widely acknowledged and supported by a substantial body of scientifically validated evidence. Specific microorganisms found in the mouth can induce dental caries in laboratory settings. Animals raised in sterile conditions, even when fed a cariogenic diet, do not develop caries. Bacteria can be identified in the decayed area through histological and microbiological analysis of the affected enamel, dentin and dentinal tubules [1].

From a preventative perspective, there is a vast array of items, both commercial and professional, that have integrated one or more distinct antimicrobial components into their makeup. In this context, we will mostly discuss fluorine, chlorhexidine, cetylpyridinium chloride, benzalkonium chloride, triclosan, Listerine and xylitol. Contemporary mechanical and chemotherapeutic methods for oral hygiene seek to alter the oral microbiota and promote the health of dental and periodontal tissue. Triclosan and Listerine are classified as nonionic antibacterial agents and are derived from phenol. They have both hydrophilic and hydrophobic properties, displaying a broad range of activity against Gram-positive and Gram-negative bacteria [2]. Both compounds have traditionally been employed in the antiseptic treatment of the skin. Since the late 1980s, they have been utilized as oral antiseptics [2].

Triclosan is a chemical that has a broad-spectrum antibacterial and antimicrobial activity. The American company Ciba Specialty Chemicals produces it under the name Irgasan DP 300, but other manufacturers outside the United States also make it. Triclosan is widely used in personal care products like toothpastes, deodorants, soaps, antiperspirants, body washes, detergents, dishwashing detergents, cosmetic creams and lotions due to its ability to inhibit the growth of a wide range of bacteria, including both Gram-positive and Gram-negative bacteria. Additionally, it is employed as an addition in the manufacturing of plastics, polymers and textiles to confer upon them antibacterial characteristics. Triclosan is a compound derived from diphenyl ether, specifically known as 2,4,4'-trichloro-2'-hydroxydiphenyl ether or 5-chloro-2 (2,4-dichlorophenoxy) phenol. To optimize the efficacy of triclosan, a copolymer surface protection layer is included [3,4].

The mechanism of action of triclosan involves the breakdown of the bacterial cell wall, inhibition of bacterial enzymes and extraction of bacterial lipopolysaccharides [5]. This action results in a decrease or suppression of glucose absorption, which in turn leads to a decrease or absence of acid formation. An increased concentration results in membrane damage and leads to bacteriolysis [2]. The objectives of this examination were to determine the presence of *Streptococcus mutans* and *Lactobacillus* species in saliva before and after using Colgate Total Plus Whitening® toothpaste and to compare the quantitative representation of the complete microbial flora in the patient's mouth, by analyzing the saliva before and after brushing the teeth with Colgate Total Plus Whitening® toothpaste.

Study Population and Methodology

The study comprised 24 youngsters, both male and female, between the ages of 8 and 13. To enhance precision and accuracy in the study, each of the groups under investigation was utilized as a control group as well. This entailed collecting the material for analysis (saliva) before and after cleaning the teeth. The participants exhibited good overall and dental health, followed similar hygiene practices and diets and had the same DMFT index. The responders were selected from the Clinic for Pediatric and Preventive Dentistry at the Dental Clinical Center in Skopje, Republic of North Macedonia. Before the start of the tests, each participant's parent was questioned and the collected data was recorded in custom survey forms.

Material

Colgate Total Plus Whitening® toothpaste, manufactured by Colgate-Palmolive, United States, includes the active antibacterial ingredient Triclosan. The composition of the product includes water, sorbitol and PVM/MA. The ingredients of this product are copolymer, sodium lauryl sulfate, sodium hydroxide, sodium fluoride, sodium saccharin and fragrance.

Plan and Methods for Conducting an Examination

- Participants and their parents were instructed to abstain from eating for a minimum of 12 hours before the assessment
- The responders neglected dental hygiene till our technique was implemented
- Participants had not received any antibiotics for a minimum of 30 days before to the commencement of the trial and were also not taking antibiotics during the treatment period

Methodology for Assessing the Antimicrobial Efficacy

1. Collecting the initial saliva sample without prior stimulation of production
2. Applying toothpaste to the toothbrush and brushing the teeth for roughly 3 minutes
3. Following brushing, the oral cavity was flushed with a volume of 200 milliliters of water, which took roughly 15 seconds
4. Following a 10-minute interval, a second saliva sample was collected without external stimulation

Techniques for Quantifying the Presence of *Streptococcus mutans* and *Lactobacillus* species, as well as Measuring the Overall Microbial Count in Saliva

To ascertain the overall quantity of germs, a sample of saliva was collected using specialized sterile vials by expelling 3-5 ml of saliva. Quantitative representation of *S. mutans* and *Lactobacillus* was determined using CRT bacteria®, which are commercially available strips manufactured by Vivadent, Schaan, Liechtenstein. These strips contain microbial media that selectively isolate *Streptococcus mutans* on one side and *Lactobacillus* species on the other side. The process begins by extracting the agar carrier from the test bottle. Next, a NaHCO_3 tablet is positioned at the base of the bottle and the protective coatings on both sides of the agar surfaces are eliminated. Using the pipette provided in the package, thoroughly moisten both agar surfaces with saliva. By tilting the agar carrier slightly, the surplus saliva is expelled. Afterward, the carrier is placed back into the vial, sealed and dispatched for microbiological investigation. The whole procedure for quantifying the abundance of *Streptococcus mutans* and *Lactobacillus* species is shown in Fig. 1.

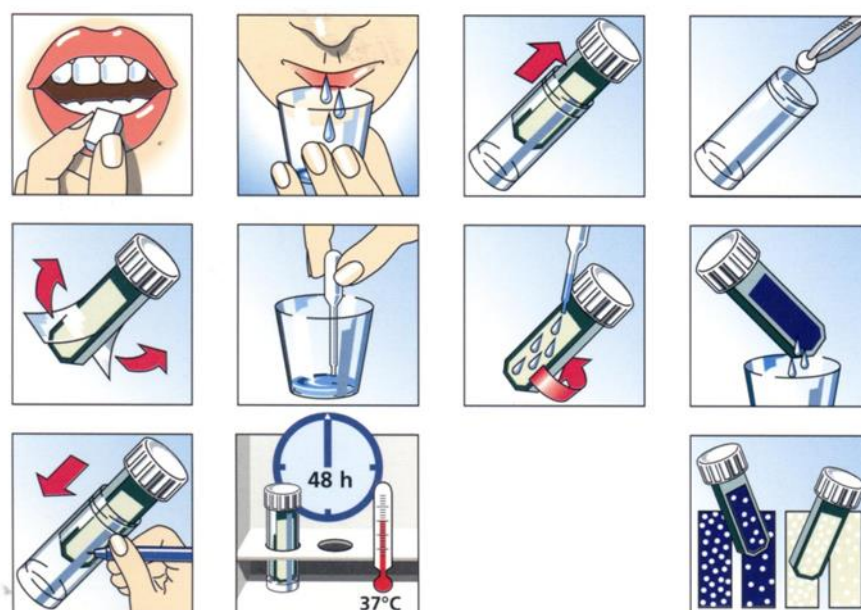


Figure 1: Protocol for quantifying the abundance of *Streptococcus mutans* and *Lactobacillus* species.

Microbiological Processing of Samples

The microbiological analyses were conducted at the Institute of Microbiology and Parasitology at the Faculty of Medicine in Skopje, Republic of North Macedonia. The results of the microbiological analyses for each individual were recorded in specifically designed spreadsheets.

Quantitative Analysis of *Streptococcus mutans* and *Lactobacillus* species in Saliva

The act of planting is accomplished by directly collecting the sample. Following a 48-hour incubation period at a temperature range of 35-37°C, the colonies that have developed (referred to as Colony Forming Units - CFU) are enumerated. If the number of colonies is low, they are directly counted. If the number of colonies is high, they are compared to a reference standard provided in the manufacturer's instructions. The interpretation of the colony count is categorized into four ranges: 10,000, 10,000-100,000, 100,000-1,000,000 and >1,000,000 CFU. *Streptococcus mutans* colonies appear transparent on a blue substrate, while *Lactobacillus* species colonies appear grayish-white on a green substrate (Fig. 2.) The number of bacterium cells is estimated by counting the colonies, as it is assumed that each colony originates from a single cell and is hence referred to as a "colony forming unit (CFU)".

Once dried, the strips are stored in a refrigerator at a temperature range of 2-8°C. They should be kept away from light and can survive for several years. This allows for the convenience of being able to read them at any given moment.

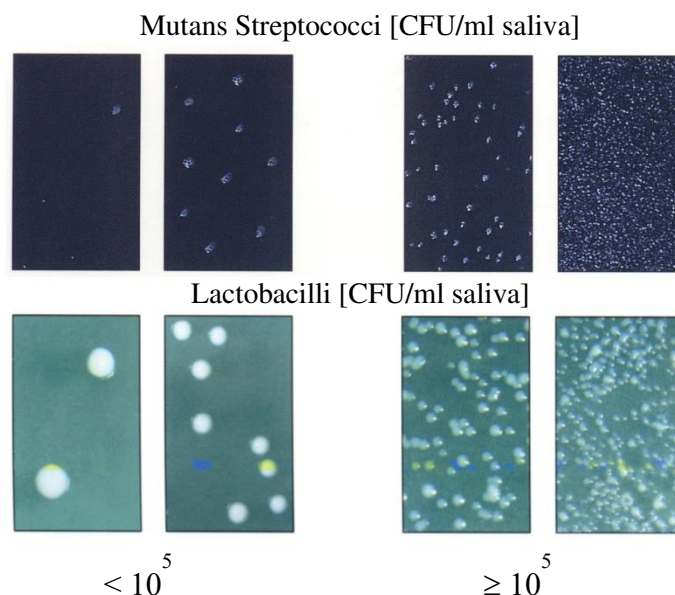


Figure 2: Colony-forming units for mutans streptococci and lactobacilli (standard from manufacturer's instructions).

The samples for the microbiological evaluation were appropriately treated, namely by being placed on nutrient media to isolate and identify aerobic and anaerobic bacteria as well as fungi.

Procedure for the Identification of Aerobic Microorganisms

To isolate aerobic bacteria, a sample was obtained from each saliva sample and placed on a nutrient-rich blood agar medium. The culture was then incubated in a thermostat at a temperature of 37°C for 24 hours to allow the aerobic bacteria to grow and multiply.

Procedure for the Identification of Anaerobic Microorganisms

To isolate anaerobic bacteria, the sample material was spread over Schaedler's blood agar, which was supplemented with vitamin K3. The plates were then placed in McIntosh pots and incubated under anaerobic conditions for 48 hours at a temperature of 37°C.

Procedure for the Identification of Fungi

To isolate fungus, the sample was placed on Sabouraud nutritional medium and cultured at a temperature of 37°C for 72 hours.

Semi-Quantitative Assessment of the Overall Microbial Count in Saliva

To semi-quantitatively determine the total number of microorganisms, 50 microliters of saliva were placed on each of the three substrates indicated before using a calibrated sieve with a diameter of 4 mm, following the typical procedure for processing. To achieve isolated colonies by dilution, the material was planted in three sectors of the Petri dish, covering half of the dish in Sector 1. Subsequently, the plate underwent sterilization with the application of heat. Following this, the material from the final two lines of Sector 1 was introduced into the lower quarter of the Petri dish, designated as Sector 2. Ultimately, the dish underwent sterilization once again and material from the final two lines of Sector 2 was introduced into the last quarter of the Petri dish (Sector 3).

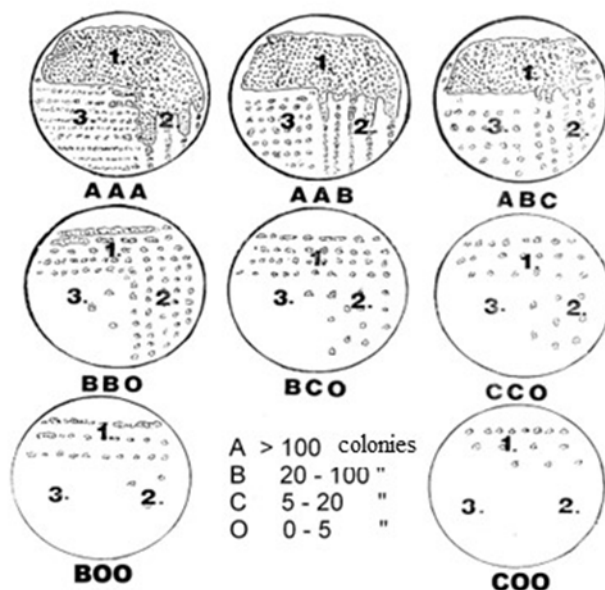


Figure 3: Sectors with growth density.

The results were assessed using a semi-quantitative approach, where the growth density was represented by capital letters: A, B and C (Fig. 3). The initial letter denotes the level of growth density in the first sector, the subsequent letter denotes the level in the second sector and the final letter denotes the level in the third sector. The sector labeled with the letter A indicates a high density of colony growth that is too many to count (A > 100 colonies in the sector). The sector labeled B represents a medium density of colony development, ranging from 20 to 100 colonies. The sector labeled C indicates a low density of colony growth, ranging from 5 to 20 colonies. Finally, the sector labeled O represents the lowest density of colony growth, ranging from 0 to 5 colonies [6,7].

Colony Identification

The growing colonies were identified using the conventional bacteriological approach for isolating aerobic and anaerobic bacteria. The grown bacteria were definitively identified using an automated technique called the VITEK system®, developed by Bio Merilux in France (Fig. 4). This system is capable of rapidly determining the presence of over 500 different types of microorganisms, including 7 types of Actinomycetes, 13 types of Bacterioides, 5 types of Corynebacterium, 7 types of Lactobacillus, 8 species of Neisseria, 9 species of Prevotella, 22 species of Streptococcus, 16 species of Candida and other microorganisms commonly found in the oral cavity. The gadget provides real-time feedback on the accuracy % of the identification.

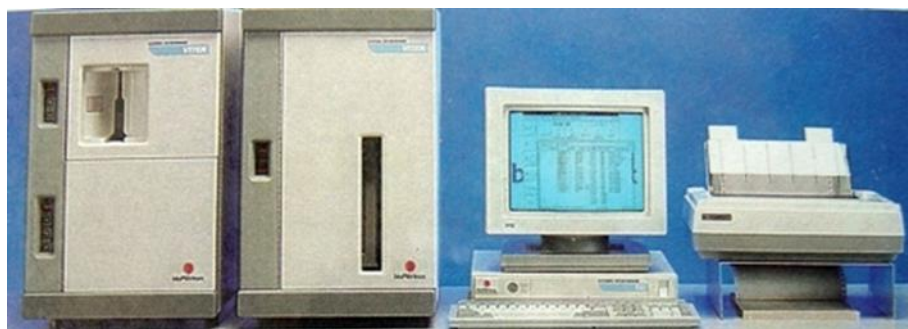


Figure 4: VITEK system®.

Statistical Analysis of the Results

In our analysis, we employed many statistical tests including the Chi-square test (χ^2), the Fisher Exact test, the arithmetic mean, the standard deviation (δ) and the Wilcoxon matched-pairs signed-ranks test.

Results

A Comparison of the Quantitative Representation of the Whole Microbial Population in Saliva Before and After Using Colgate Total Plus Whitening® Toothpaste

To facilitate the presentation of the results, we categorized the sectors based on the number of colonies into sectors with high growth density (AAA, AAB and AAC), sectors with moderate growth density (ABB, ABC, ABO and ACO) and sectors with low or no colony growth (BBO, BCO and OOO). We designated the first item by using a pair of plus signs (+ +), the second item with a single plus sign (+) and the third item with a combination of plus and minus signs (+ -). Table 1 displays the outcomes of the semi-quantitative assessment of aerobic and anaerobic bacteria in the saliva of the participants prior to and during the use of Colgate Total Plus Whitening toothpaste. The product has a statistically significant effect on reducing aerobic bacteria ($p < 0.05$), but it does not have a statistically significant effect on reducing anaerobic bacteria ($p > 0.05$) (Table 1.)

Growth Density		Aerobic		Anaerobic	
		before	after	before	after
+ +	AAA	/	/	/	/
	AAB	4	/	3	/
	AAC	3	/	1	/
+	ABB	1	/	2	/
	ABC	13	4	13	4
	ABO	3	6	3	6
	ACO	/	13	2	13
+ -	BBO	/	1	/	1
	BCO	/	/	/	/
	OOO	/	/	/	/
		$\chi^2 - p < 0,05$ ($p = 0,012$)		$\chi^2 - p > 0,05$ ($p = 0,07$)	

Table 1: Quantification of the growth rate of aerobic and anaerobic bacteria in saliva before and after the use of Colgate Total Plus Whitening® toothpaste.

Fig. 5 and 6 illustrate the semi-quantitative method used for determining the overall count of aerobic and anaerobic bacteria in separate Petri dishes.



Figure 5: Growth density sectors of aerobic bacteria colonies.



Figure 6: Growth density sectors of anaerobic bacteria colonies.

Quantification of the abundance of *Streptococcus mutans*, *Lactobacillus species* and *Candida albicans* before and after the use of Colgate Total Plus Whitening® toothpaste. Table 2 displays the CFU/ml count of cariogenic bacteria before and after cleaning the teeth with Colgate Total Plus Whitening® toothpaste. Participants in whom no microorganisms were isolated before brushing their teeth are disregarded in the data obtained after toothpaste use. The number of these participants is shown in brackets.

	<i>Streptococcus mutans</i> CFU/ml		<i>Lactobacillus species</i> CFU/ml	
	Before	After	Before	After
Without Growth	(1)	/	(3)	/
10²⁻³	1	2	/	1
10³⁻⁴	2	4	1	3
10⁴⁻⁵	1	8	4	6
10⁵⁻⁶	5	9	3	6
10⁶⁻⁷	14	/	13	5
N	23	23	21	21

Table 2: Number of subjects with CFU of *Streptococcus mutans* and *Lactobacillus species* before and after using Colgate Total Plus Whitening® toothpaste.

Given the significant variations seen in the quantitative measurement of cariogenic bacteria before and after teeth brushing, which can vary from 0 to over 1,000,000, the subsequent tabular analysis will display the data using a logarithmic scale. Table 3 presents a summary of the number of subjects in whom colonies of cariogenic microorganisms were isolated. The reduction of these microorganisms is expressed using a logarithmic Reduction Factor (log RF) ranging from 0 to ≥ 4 . This factor specifically applies to *Streptococcus mutans* and *Lactobacillus species*. It is calculated by subtracting the logarithm of the Colony-Forming Units (CFU) before using the product from the logarithm of the CFU after using the product. The participants without isolated colonies were excluded from consideration and the total Number (N) represents the actual number of subjects included in the studies.

<i>log RF</i>	<i>Streptococcus mutans</i>	<i>Lactobacillus species</i>
0	2	4
1	7	14
2	14	3
3	/	/
≥ 4	/	/
N	23	21

Table 3: Number of participants exhibiting a logarithmic reduction factor for Colgate Total Plus Whitening® Toothpaste.

Fig. 7. displays the quantity of *Candida albicans* colonies. Prior to product usage, yeast was extracted from a total of eight individuals, with Subjects 1, 5 and 8 exhibiting a greater number of Colony-Forming Units (CFU). Upon product usage, the quantity of generated colonies is decreased by an average factor of 5.

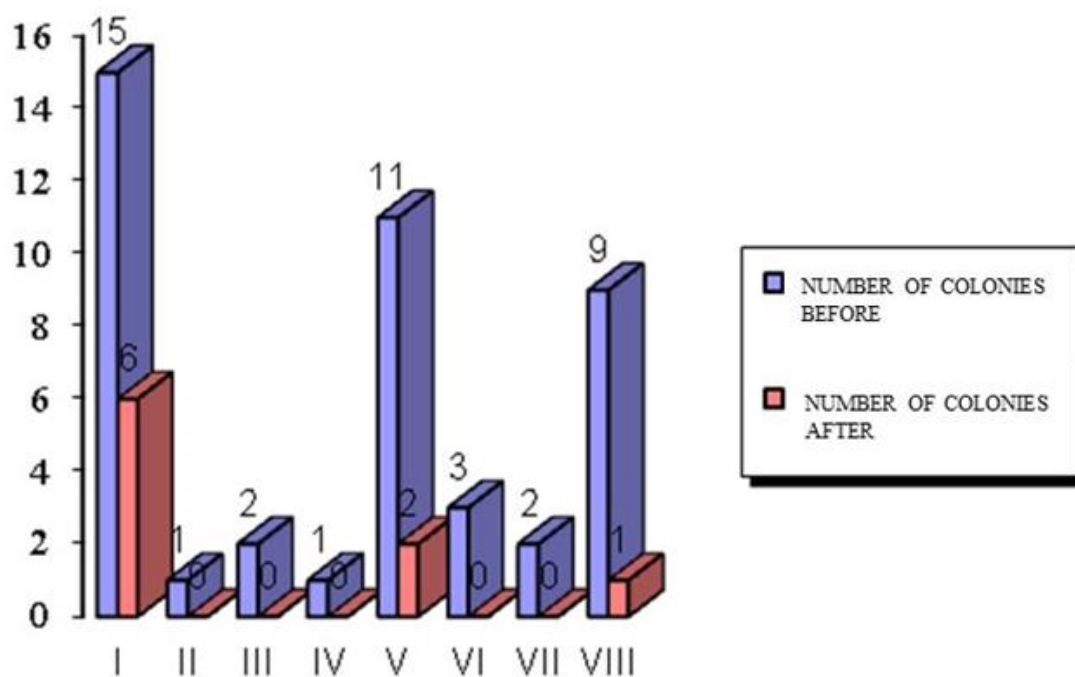


Figure 7: Colony-Forming Units (CFU) in individuals with *Candida albicans* before and after the use of Colgate Total Plus Whitening® toothpaste.

Fig. 8 illustrates the approach for calculating growth sectors for *Candida albicans*, which is similar to the semi-quantitative method used to identify aerobic and anaerobic microbes mentioned earlier.



Figure 8: Growth density sectors of *Candida Albicans*.

The impact of triclosan's action on reducing cariogenic bacteria in saliva is demonstrated in Table 4, which presents logarithmic reduction factors for each participant individually. Table 4 displays the impact of Colgate Total Plus Whitening® toothpaste on the individual decrease of *Streptococcus mutans* and *Lactobacillus species*. The arithmetic mean ($\bar{x}$) and standard deviation (σ) are provided for each logarithmic number, namely log PRE, log POST and log RF. Furthermore, while calculating these parameters, <https://doi.org/10.46889/JDHOR.2024.5308>

individuals with a log PRE value of 0, indicating no presence of germs, were excluded from the analysis. The total number (N) represents the actual number of patients included in the analysis.

	<i>Streptococcus mutans</i>			<i>Lactobacillus Sppcies</i>		
	log PRE	log POST	log RF	log PRE	log POST	log RF
N	23	23	23	21	21	21
$\bar{X}$	5,57	4,04	1,52	5,48	4,52	0,95
δ	1,41	0,95	0,65	1,10	1,14	0,58

Table 4: Assessing the degree of variations in the reduction of cariogenic bacteria in saliva.

Table 5 displays the concise outcomes of the several categories of bacteria and yeasts found in the participants' saliva, as determined by the Vitek system®.

Microorganisms That Are Found In Isolation	Percentage
1. Facultatively aerobic cocci and bacilli-S. Viridans группа	
<i>Streptococcus mutans</i>	30 %
<i>Streptococcus sanguis</i>	19 %
<i>Streptococcus mitis</i>	17 %
<i>Streptococcus oralis intermedius</i> (viridans – others)	15 %
<i>Streptococcus pneumoniae</i>	/
<i>Neisseria</i> (nonpathogenic species)	7 %
<i>Corynebacterium</i> (nonpathogenic species)	5 %
2. Microaerophilic and anaerobic Gram-positive bacilli	
<i>Lactobacillus species</i>	11 %
<i>Lactobacillus casei</i>	7 %
<i>Lactobacillus fermenti</i>	5 %
<i>Lactobacillus brevis</i>	3 %
<i>Actinomyces</i>	23 %
<i>Bifidobacterium</i>	4 %
<i>Propionobacterium</i>	1 %
3. Anaerobic Gram-positive cocci	
<i>Veillonella</i>	4 %
<i>Peptostreptococcus</i>	7 %
<i>Peptococcus</i>	2 %
4. Anaerobic Gram-negative cocci	
<i>Fusobacterium</i>	5 %
<i>Bacteroides</i>	3 %
5. Yeasts	
<i>Candida albicans</i>	10 %

Table 5: Microorganisms identified in unstimulated saliva.

Discussion

During the analysis of the data, we considered not only the assessment of the chemical antibacterial component but also the impact of extra saliva output and mouth rinse with water on the number of germs. Triclosan is a chemical that has a broad-spectrum antibacterial and antimicrobial activity. It is classified as a non-ionic antibacterial agent and is derived from phenol [3]. It has both hydrophilic and hydrophobic qualities and may effectively target both Gram-positive and Gram-negative bacteria [2,4,8-13]. Oral products containing triclosan have undergone clinical studies to assess their effectiveness in decreasing plaque, gingivitis and calculus development [14]. Two approaches have been devised to enhance the therapeutic effectiveness of these products. The initial approach involves the combination of triclosan and zinc citrate to enhance and optimize the qualities of

being anti-plaque and anti-calculus [15]. The second approach, which we find more intriguing, involves incorporating a copolymer of polyvinylmethyl ether and maleic acid into triclosan. This is done to prolong its retention duration and improve its effectiveness [3,5,14]. Several studies conducted using a combination of triclosan/Zn-citrate and triclosan/copolymer in toothpastes have demonstrated a notable decrease in plaque, gingivitis and calculus development. The findings from these trials indicate that there were no significant disruptions to the microorganisms present in the mouth and there was no development of resistance to triclosan [16-18]. This study aimed to investigate the antibacterial properties of Colgate Total Plus Whitening® toothpaste, manufactured by the US-based company Palmolive. According to the survey data provided in Fig. 9, the majority of respondents (38%) reported using toothpastes from the diverse selection offered by Colgate. Regardless of its name, each one comprises a mixture of triclosan and copolymer in its composition. The efficacy of this product has been confirmed in multiple scholarly publications and coupled with an extensive global marketing effort, it presently holds the top position in toothpaste sales. The confirmation and recommendation of the Association of Dentists of the Republic of North Macedonia validate this practice in our nation.

The semi-quantitative analysis of microorganisms in saliva revealed a shift in the distribution of colonies of aerobic and anaerobic microorganisms. Specifically, colonies from sectors with dense and medium-density growth (AAB, AAC and ABC) were redistributed to sectors with weak growth (ABC, ABO and ACO). No isolated colonies were seen in the first sector of the very dense growth (AAA) sectors during the study of both aerobic and anaerobic bacteria, before the application of the agent. No individuals exhibiting little or absent colony development (BBO, BCO and OOO sectors) were seen following the administration of the agent. The statistical analysis conducted (χ^2) reveals a significant statistical difference in aerobic microorganisms following the use of toothpaste ($p < 0.05$, $p = 0.012$). The tests undertaken for anaerobic microbes suggest that there is no significant statistical difference after teeth brushing ($p > 0.05$, $p = 0.07$).

Colgate Total Plus Whitening® toothpaste effectively decreases the presence of *Candida albicans* species [19]. In individuals with one to two colonies, the reduction is total, while in those with a higher number of colonies, it is twice. Out of the initial 44 colonies that were isolated before tooth brushing, only 9 colonies were isolated after the procedure was adopted. This indicates an average reduction of 5 times.

This toothpaste contains triclosan as an active component, along with sodium fluoride at a concentration of 0.32% and sodium lauryl sulfate. Both of these compounds are classified as anionic antibacterial agents. This toothpaste also includes fluorine and its concentration in this product should be taken into account as an additional factor that might impact the decrease of bacteria in saliva. Sodium lauryl sulfate, while being classified as an antibacterial agent, is not in an active state and is largely utilized as a surfactant to emulsify and cleanse surfaces. When examining the findings, it is important to consider the method of utilizing this tool, namely the mechanical removal of germs by brushing and the subsequent washing of the mouth. These actions can lead to a decrease in the quantity of bacteria present in saliva.

Triclosan, an antibacterial compound, has a broad range of effectiveness against bacteria, including the cariogenic bacteria *Streptococcus mutans* and *Lactobacillus species* [19-21]. The findings obtained for the quantitative representation of the cariogenic microorganisms *Streptococcus mutans* and *Lactobacillus species* indicate a notable disparity in the decrease of these bacteria's population following the application of the agent. The effect is more evident in decreasing the population of *Streptococcus mutans* bacteria compared to reducing the population of *Lactobacillus species*. This observation is derived from the investigation of the impact of Colgate toothpaste on decreasing the population of cariogenic bacteria, as shown by the number of participants exhibiting a logarithmic decrease factor. The results indicate that there were two respondents with a log RF value of 0 for the bacterium *Streptococcus mutans* and four respondents with a log RF value of 0 for the bacteria *Lactobacillus species*. The logarithmic reduction factor of 2, which represents the greatest decrease achieved by this drug, was observed in 14 participants for the bacteria *Streptococcus mutans* and only in three subjects for the bacterium *Lactobacillus species*. Analysis of the data obtained from the average values of the log reduction factors for Colgate Total Plus Whitening® toothpaste revealed a decrease of almost ten times for *Streptococcus mutans* (log RF = 1.52) and a reduction of nearly ten times for *Lactobacillus species* (log RF = 0.95).

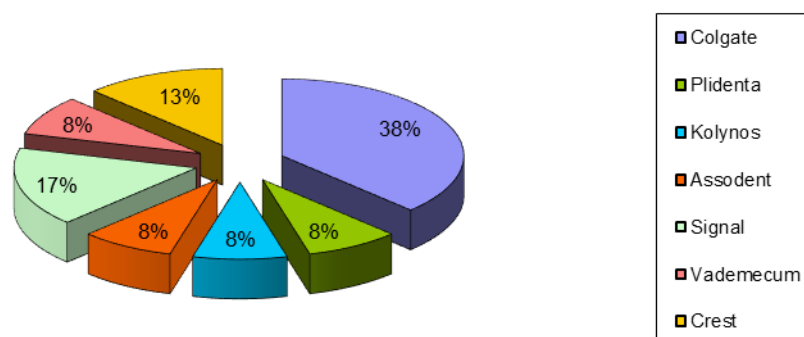


Figure 9: The use of toothpaste among the participants of the survey.

Conclusion

The effectiveness of Colgate Total Plus Whitening® toothpaste in reducing salivary aerobes is statistically significant, but its impact on reducing salivary anaerobic microbes is statistically negligible. The bacteria *Streptococcus mutans* experiences a decrease that is greater than tenfold, while the bacterium *Lactobacillus species* experiences a reduction of around tenfold.

Conflict of Interest

All authors declare that they have no conflicts of interest.

Author Contributions

All authors contributed to the conception and design of the work. All authors contributed to the acquisition of data. AD conceived the ideas, collected the data and led the writing. JS collected the data. All authors gave final approval for publication.

Acknowledgment

We thank all children and their parents for their participation in the study.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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