

In vitro evaluation of stainless steel orthodontic wires coated with TiO_2 and $\text{TiO}_2\text{:Ag}$ for their anti-adhesive and antibacterial efficacy against *Streptococcus mutans* in a sucrose-enriched environment

Justyna Joanna Bącela^{1,*}, Beata Borak¹, Beata Sobieszczęńska^{2,3}, Urszula Walczuk³, Jerzy Detyna¹, Michał Sarul^{4,5}

¹Department of Mechanics, Materials and Biomedical Engineering, Faculty of Mechanical Engineering, Wrocław University of Science and Technology, Wrocław, Poland

²Department of Dentofacial Orthopedics and Orthodontics, Wrocław Medical University, Wrocław, Poland

³Department of Microbiology, Wrocław Medical University, Wrocław, Poland

⁴Department of Integrated Dentistry, Wrocław Medical University, Wrocław, Poland

⁵Academic Dental Clinic, Wrocław Medical University, Wrocław, Poland

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1. Introduction

Dental caries and periodontal disease, which rank among the most prevalent ailments afflicting humanity, are intricately associated with bacterial adhesion and the formation of biofilms on both natural and restored tooth substrates. Alterations in the appearance such as the manifestation of white lesions, demineralization of enamel in proximity to orthodontic braces, dental caries, or the onset of periodontal disease represent frequently encountered complications in the context of orthodontic interventions employing fixed appliances.

Dental caries, a multifaceted ailment, is subject to the influence of a multitude of factors. Its pathogenesis is intricately linked to the presence of cariogenic microorganisms such as *Streptococcus mutans* and *Lactobacilli*, in conjunction with heightened consumption of carbohydrates [1, 2]. Amongst the various carbohydrates, sucrose is widely acknowledged within the scientific community as the most cariogenic, primarily owing to its

pivotal role in promoting the synthesis of extracellular polysaccharides [3]. These polysaccharides contribute to increased porosity in the dental biofilm and maintain a low pH caused by organic acids resulting from bacterial metabolism [4]. Therefore, an elevated rate of sucrose ingestion or sucrose exposure gives rise to recurrent episodes of plaque pH reduction, consequently fostering the proliferation of acidogenic microorganisms, specifically *S. mutans* and *Lactobacilli*. Furthermore, the frequent exposure to sucrose may induce alterations in the biochemical characteristics of dental plaque, subsequently resulting in heightened levels of insoluble polysaccharides and diminished concentrations of calcium, inorganic phosphorus (Pi), and fluoride [5, 6].

The diverse elements comprising fixed orthodontic appliances provide an optimal environment for the retention of food debris and exert a substantial influence on the proliferation of bacterial plaque due to their uneven surface morphology. The accumulation of plaque in retention areas exposes teeth to an increased risk

* E-mail: justyna.bacela@pwr.edu.pl

of demineralization of the enamel and intensifies the effects of pre-existing, initial caries changes. Following standard orthodontic treatment, it has been observed that approximately 50% of patients may experience enamel demineralization and periodontal disease [7–9]. A research investigation carried out by Marcusson et al. revealed that the incidence of white spot lesions exhibited a rise, ascending from 7.2% prior to the intervention to a spectrum spanning from 24% to 40.5% following the intervention, contingent upon the specific bonding agent utilized [10]. Certain teeth, such as the lateral upper incisors, lower canines, and first molars, tend to be more susceptible to caries. Notably, demineralization during orthodontic treatment can be detected as early as four weeks into the procedure [8, 10]. To address this issue, an encouraging approach involves the utilization of coatings that possess bacteriostatic/bactericidal properties, thereby reducing bacterial adhesion to biomaterials. With the emergence of antibiotic-resistant strains of bacteria, certain metals, particularly in the form of nanoparticles, have gained attention. Nanoparticles can be used both in combination with dental materials and by coating their surfaces to reduce microbial adhesion and prevent caries.

Coatings are one of the most common types of antimicrobial medical device technologies seen in the research literature. Antiadhesive coatings are designed to prevent the formation of the first stage of biofilm colonization and eliminate the threat from the very beginning. Bacteria can adhere and grow on natural and synthetic surfaces in an aqueous environment [11]. Fixed orthodontic wires are affixed to the dentition via brackets, thereby impeding accessibility to the dental surfaces necessary for adequate oral hygiene maintenance. This phenomenon facilitates the adherence of bacterial colonies and the ensuing development of dental plaque, consequently elevating the susceptibility to demineralization, dental caries, and the initiation of periodontal pathology [9]. In order to protect against the cariogenic effect of bacteria, a wide range of agents is used, from natural polymers to coatings made of metal nanoparticles [12–14].

According to the ISO (International Organization for Standardization), nanoparticles are materials with remarkable properties that range in size from 1 to 100 nm and have been widely used as disinfectants in water, hospital environments, as food preservatives, and to coat medical devices [15, 16]. Upon achieving nanometer-scale dimensions, materials undergo modifications in their inherent characteristics, encompassing alterations in hardness, chemical reactivity, biological activity, and active surface area. This phenomenon is particularly pronounced in the case of metallic nanoparticles, wherein antimicrobial efficacy experiences augmentation attributable to the combined effects of diminished size and an elevated surface-to-volume ratio [17]. This affords enhanced interaction with the microbial membrane, thereby augmenting the biocompatibility of the substrate material. Certain metallic nanoparticles, namely zinc oxide (ZnO), titanium dioxide (TiO₂), and silver (Ag), find utility within the realm of healthcare by virtue of their antimicrobial properties [18].

TiO₂ is renowned for its exceptional biocompatibility and has found extensive application in the manufacturing of medical apparatus. Research investigations have substantiated that TiO₂, when in the anatase crystalline phase, exhibits commendable proficiency in the oxidation and disintegration of diverse biological entities, encompassing bacteria, viruses, fungi, algae, and cancer cells [19]. Laboratory experiments investigating the antibacterial and antiadherent properties of TiO₂ have shown reduced bacterial adhesion on TiO₂-coated surfaces [20], as reported by Fatani et al. in 2017. Consequently, utilizing the photocatalytic and antiadhesive characteristics of TiO₂ in clinical settings can be beneficial in preventing bacterial adhesion and the growth of bacterial colonies around orthodontic instruments. To enhance the bactericidal properties, TiO₂:Ag coatings have been developed that incorporate silver to further augment the disinfection capabilities of TiO₂ anatase [19, 21].

Silver nanoparticles are known for their antibacterial activity, relying on producing reactive oxygen species in bacterial cells, damage to the bacterial cell walls and cytoplasmic membranes, and interruption of nucleic acid replication [22].

Additionally, antibacterial silver nanoparticles' efficacy is ensured by their nanoscale size and large surface area ratio to volume [23]. The in vitro antimicrobial, antiviral, and antifungal efficacy of silver nanoparticles (AgNPs) has been substantiated through multiple investigations. AgNPs have exhibited notable effectiveness against a spectrum of microorganisms, including *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Bacillus cereus*, *Salmonella enterica* serotype *Choleraesuis*, and *Listeria innocua*, as well as the yeast *Candida albicans* [24, 25].

The utilization of thin film coatings represents a highly efficacious strategy within the realm of surface modification techniques aimed at enhancing the performance of metallic orthodontic archwires. Within the array of techniques employed for the fabrication of thin films, the sol-gel deposition method is distinguished by numerous advantages, including its notable simplicity and uniformity, facilitating comprehensive coverage of intricate structures [26, 27].

Orthodontic wires covered with a functional coating are used in the oral cavity environment. Hence, their use is to reduce the risk of caries via evaluation of their antibacterial properties in a carbohydrate-rich environment. This study aimed to assess the antibacterial and antiadherent characteristics of stainless steel orthodontic archwires with surface modifications of TiO_2 and $\text{TiO}_2\text{:Ag}$ in the presence of sucrose. The research focused on the evaluation of these modified wires' effects on *Streptococcus mutans*, a bacterial species known for its involvement in the development of dental caries.

2. Materials and methods

2.1. Materials

2.1.1. Stainless steel orthodontic archwires

The 304V stainless steel wires (70% Fe, 19% Cr, 9% Ni, 1.5% Mn, 0.5% Si) were provided by the manufacturer of orthodontic wires (Adenta GmbH). Initially, the wires had a rectangular cross-section with dimensions of 0.016×0.022 inches. To prepare the wire samples, they were subjected to

a cleaning process involving immersion in acetone and distilled water for a duration of 15 min, while employing an ultrasonic bath [28]. The surfaces of the stainless steel orthodontic wires underwent a modification process using a sol-gel thin film dip-coating technique, which involved coating them with TiO_2 and $\text{TiO}_2\text{:Ag}$. For the purpose of the microbiological investigation, the wire samples underwent autoclaving at 121°C under steam pressure of approximately 15 pounds per square inch for a duration of 15 minutes.

2.1.2. Sol-Gel synthesis

The sol-gel synthesis was divided into two parts: the preparation of TiO_2 sol and nano- $\text{TiO}_2\text{:Ag}$ sol. In the formulation of a titanium dioxide (TiO_2) solution, 6 ml of titanium (IV) isopropoxide (97%, Aldrich) were conjoined with 85 ml of isopropanol (Eurochem BDG, Poland), and 0.5 ml of acetic acid (99%, Aldrich). To ameliorate surface topography, 1 wt% of polypropylene glycol (PPG, molar mass = 1000, Alfa Aesar) was incorporated into the TiO_2 solution. The resultant amalgamation was subjected to agitation using a magnetic stirrer at ambient temperature for a duration of 3 hours. Thereafter, the agitated solution was permitted to undergo maturation for a period of 24 hours at 4°C [29].

In order to formulate the nano- $\text{TiO}_2\text{:Ag}$ sol, 1 gm of silver nitrate (AgNO_3 ; Sigma-Aldrich) was dissolved in a solution comprising 2.4 ml of deionized water, 10 ml of acetic acid, and 12 ml of isopropanol. The resultant solution was then amalgamated with the TiO_2 precursor solution at ambient conditions for a duration of 3 hours, employing a magnetic stirrer [30].

2.1.3. Coatings procedure

The dip-coating technique was used to apply a protective layer onto the stainless steel wires. Each individual orthodontic wire was submerged within the sol solution for a duration of 1 minute and subsequently withdrawn at a consistent velocity of 65.8 millimeters per minute, thereby ensuring uniform coverage. The coated wires were subsequently subjected to a drying phase lasting 60 minutes at a temperature of 120°C , with a controlled

Table 1. Various categories of orthodontic wires that have undergone surface modifications are utilized in microbiological examinations

Group A	The experimental group included stainless steel orthodontic archwires coated with a functional thin film of TiO ₂ :Ag.
Group B	The experimental group consisted of stainless steel orthodontic archwires with a base coating of a thin TiO ₂ film.
Group C	The control group comprised stainless steel orthodontic archwires without any coating.

thermal ramp of 0.5°C per minute. This procedure was iterated twice to achieve a thicker thin film. Ultimately, the coated wires were subjected to an annealing process for a duration of 120 minutes at 500°C, employing a thermal transition rate of 1°C per minute, all conducted within the laboratory's ambient environmental conditions. After annealing, the linear segments of orthodontic wires were severed into 1–0 cm-length specimens. The details of the final subgroup of the studied material can be found in Table 1.

2.2. Methods

2.2.1. Description of the tested bacterial strain and conditions for bacterial culture

The coatings were subjected to testing using a specific strain of *Streptococcus mutans* (*S. mutans*), known as ATCC 25175. To prepare the bacterial culture, *S. mutans* was grown in brain heart infusion (BHI) broth at a temperature of 37°C for a duration of 12 hours. After incubation, 1 ml of the 18-hour culture was centrifuged at 16,000 rpm for 10 minutes at a temperature of 4°C. The bacterial pellet obtained was subsequently resuspended in a solution of phosphate buffer (PBS) with a pH of 7.4. The bacterial suspension's density was quantified via spectrophotometry and found to be 6×10^8 colonyforming units (CFU) per milliliter. Subsequently, the suspension of *S. mutans* was diluted at a 1:100 ratio employing an artificial saliva solution comprising Lab-lemco (0.05 g), yeast extract (0.1 g), protease peptone (0.25 g), mucin (0.125 g), NaCl (0.01 g), KCl (0.01 g), and CaCO₃ (0.015 g)

per liter, following the methodology outlined by Pratten and Barnett in their 1998 publication [31].

2.2.2. The evaluation of *S. mutans* attachment onto the examined coatings applied to the orthodontic archwire

The researchers conducted an experiment to assess the ability of *S. mutans* to adhere to and colonize various coatings on orthodontic archwires. The archwires were either uncoated or coated with a TiO₂ coating (B) or a TiO₂:Ag coating (A). In order to prepare the archwires for experimentation, they were submerged in artificial saliva, either with or without 3% sucrose, and subsequently exposed to mucins from the saliva by incubating them at room temperature with gentle agitation. Next, the saliva-dipped archwires were infected with a suspension of *S. mutans* and kept in a microaerophilic environment at 37°C for 4 hours. Following the incubation period, the archwires were subjected to a saline rinse procedure in order to eliminate bacterial entities loosely adhered to the surfaces. Then, the archwires were placed in a broth and subjected to sonication to detach the bacteria from the wire surfaces. The bacterial suspensions were appropriately diluted and applied onto agar plates. These plates were then placed in an incubator to facilitate growth, allowing the colonies of *S. mutans* to develop. The quantity of colonies present on the coated archwires was subsequently assessed to gauge the extent of colonization relative to the uncoated archwires, which were designated as the control group at 100% baseline colonization. This experimental protocol was iterated three times with triplicate samples, and the reported outcomes signify the mean value derived from a minimum of three measurements.

2.2.3. Evaluation of *S. mutans* biofilm formation employing a quantitative culturing technique

The experiment was designed with the primary objective of evaluating the capacity of *Streptococcus mutans*, a bacterium of interest, to initiate and develop a biofilm on various surface coatings employed on orthodontic archwires. This assessment was carried out under controlled conditions

using an artificial saliva medium, with two distinct experimental conditions: one with the presence of a 3% sucrose solution and the other in the absence of sucrose. Biofilm formation was assessed following incubation periods of 24, 48, and 96 hours at a temperature of 37°C, under microaerophilic conditions, consistent with the protocol employed for the adherence assay. Stainless steel archwires, both uncoated and bearing surface treatments of TiO₂ coating and TiO₂:Ag coating, were subjected to prior incubation in artificial saliva. Subsequently, these archwires were exposed to *S. mutans* infection and maintained under the prescribed environmental conditions. To mitigate potential acidification during biofilm development, the culture medium was replenished daily, with pH measurements conducted using a dedicated pH meter on a daily basis. Following a four-day incubation period, the archwires that had been exposed to infection were subjected to thorough washing and then subjected to sonication in TSB broth to facilitate the detachment of adherent bacterial populations. The resulting bacterial suspensions were adequately diluted before being plated onto TSA agar plates to facilitate the quantification of bacterial colonies. The colony counts arising from the coated archwires were subsequently compared to those from the uncoated counterparts, which served as the baseline (considered 100%). This experimental procedure was replicated three times in triplicate, with the average outcomes obtained from three independently conducted experiments for each type of archwire sample being reported.

2.2.4. Microscopic observation of biofilm formed by *S. mutans* on orthodontic archwires

To facilitate the visualization of biofilm formation, orthodontic archwires were subjected to exposure with *S. mutans* for distinct time intervals of 24, 48, and 96 hours. Subsequent to the specified incubation periods, the archwires were immobilized through immersion in a 4% buffered formalin solution for a duration of 10 minutes, succeeded by a series of three sequential rinses employing phosphate buffer. To augment the perceptibility of the samples, they were subjected to staining with acridine orange at a concentration of 1 mg/ml

dissolved in phosphate buffer for a time span of 10 minutes. Following this staining process, two additional rinses were conducted, thereby ensuring the removal of excess stain. Subsequently, the treated archwires were scrutinized under a fluorescence microscope (Olympus BX51 model).

2.3. Statistical analyses

Statistical analyses were undertaken employing the TIBCO Statistica package to scrutinize the hypotheses postulated within the confines of this research. The package was used to examine basic descriptive statistics of the quantitative variables under investigation, as well as to assess the normality of their distribution using the Shapiro-Wilk test. This test is widely recognized as the most reliable method for checking the normality assumption of a random variable. It is advantageous because of its high statistical power, lending it a greater probability of detecting departures from the null hypothesis compared to other similar tests.

A series of one-way analysis of variance (ANOVA) tests were performed in a between-group design. In cases where the assumption of equal variances across groups was violated, Welch's ANOVA was employed as an alternative.

To assess the homogeneity of variances across the study groups, Levene's test was used. Furthermore, after the primary analysis, post hoc examinations were performed employing the Newman-Keuls procedure to meticulously assess and juxtapose distinctions among the cohorts under investigation. The statistical analysis is included in Section 7, supplementary materials.

3. Results

3.1. The fundamental statistical characteristics of the measured quantitative variables and the evaluation of their distribution's normality

Firstly, the quantitative variables under analysis were subjected to basic descriptive statistics. The normality of the distribution of these variables was assessed through the application of the Shapiro-Wilk test.

Table 2. Fundamental descriptive statistics for the examined quantitative variables (experiment with sucrose)

	N	M	Me	SD	Sk.	Kurt	Min.	Maks.	W	p
pH										
t = 24 h										
A+	10	4.31	4.27	0.30	−0.38	0.78	3.72	4.75	0.95	0.652
B+	9	4.32	4.31	0.27	−0.72	1.40	3.77	4.71	0.92	0.429
C+	9	3.90	3.75	0.24	0.83	−1.72	3.72	4.22	0.68	0.001
t = 48 h										
A+	9	4.10	4.03	0.16	2.34	5.88	3.98	4.50	0.68	0.001
B+	10	3.92	3.93	0.10	−1.36	2.57	3.69	4.03	0.89	0.156
C+	9	3.88	3.96	0.18	−0.60	−1.67	3.64	4.08	0.81	0.029
t = 96 h										
A+	10	4.22	4.27	0.22	−0.55	−0.37	3.82	4.55	0.94	0.568
B+	9	3.80	3.78	0.12	1.41	1.88	3.69	4.06	0.86	0.092
C+	8	3.82	3.87	0.15	−0.26	−1.92	3.64	4.00	0.86	0.111
Cultures [CFU/ml]										
t = 24 h										
A+	5	3.16×10^6	1.70×10^6	3.83×10^6	2.20	4.87	1.1×10^6	10×10^6	0.63	0.001
B+	5	5.33×10^6	2.30×10^6	5.05×10^6	1.12	−0.38	1.6×10^6	13×10^6	0.81	0.105
C+	4	1.40×10^7	1.45×10^7	5.14×10^6	−0.54	1.55	7.3×10^6	19.8×10^6	0.96	0.774
t = 48 h										
A+	4	3.47×10^7	2.41×10^7	3.85×10^7	0.97	−0.66	4.50×10^6	8.60×10^7	0.87	0.291
B+	5	1.27×10^8	1.49×10^8	6.30×10^7	−1.01	1.39	2.81×10^7	1.96×10^8	0.93	0.597
C+	4	2.19×10^8	2.15×10^8	4.91×10^7	0.48	1.52	1.63×10^8	2.83×10^8	0.96	0.786
t = 96 h										
A+	4	2.53×10^6	2.50×10^6	1.77×10^6	0.01	−5.96	1.00×10^6	4.15×10^6	0.75	0.038
B+	4	6.00×10^6	5.50×10^6	2.45×10^6	0.54	−2.94	4.00×10^6	9×10^6	0.86	0.262
C+	4	4.89×10^6	5.28×10^6	1.43×10^6	−0.91	−1.00	3.00×10^6	6×10^6	0.86	0.268
Adhesion [CFU/ml]										
t = 4h										
A+	4	7.25×10^4	6.50×10^4	4.65×10^4	0.56	−2.48	3.00×10^4	1.30×10^5	0.92	0.519
B+	4	2.50×10^5	2.35×10^5	1.68×10^5	0.25	−4.06	9.00×10^4	4.40×10^5	0.90	0.437
C+	4	3.48×10^5	2.75×10^5	1.63×10^5	1.94	3.77	2.50×10^5	5.90×10^5	0.72	0.019

Abbreviations: N: the number of measurements; M: mean; Me: median; SD: standard deviation; Sk: skewness, Kurt.: kurtosis; Min and Max: the lowest and highest value of the distribution; W: Shapiro-Wilk test result; p: significance level; A: functional coating (TiO₂:Ag); B: base coating (TiO₂); C: original state (uncoated wires); p<0.05 is marked in bold.

Table 2 provides a comprehensive overview of the fundamental statistical characteristics for the examined quantitative variables in our study, particularly focusing on experiments involving the presence of sucrose for three different groups of orthodontic archwires: coated with TiO₂:Ag (A), coated with TiO₂ (B) and uncoated (C) wires. The symbol ‘+’ denotes the addition of sucrose to the environment.

The variables under investigation include the pH level, the formation of *S. mutans* biofilms, and adhesion tests. Based on the data presented in Table 2 (studies with sucrose), it can be observed

that the conducted tests did not provide sufficient evidence to reject the null hypothesis of normality for the empirical distribution being analyzed. The table includes the test results for pH level, the formation of *S. mutans* biofilms and adhesion test.

4. The results of the post hoc analysis for each experimental group are detailed below

Within the 24-hour timeframe, notable disparities were observed between the adapted archwires

(with functional and base coatings) and the control sample (lacking any coating) in both experiments (with and without sucrose). Because the testing duration was extended to 48 hours and 96 hours, the archwire endowed with coating A consistently yielded markedly elevated pH levels within the salivary milieu, when contrasted with the archwire treated with coating B and the uncoated wire. It is pertinent to observe that the significance threshold necessary for the rejection of the null hypothesis exhibited a remarkably elevated magnitude across all the aforementioned comparisons.

Following a 24-hour incubation period, it was noted that the pH of the salivary milieu exhibited a 5% elevation when contrasted with the control sample in the presence of the TiO₂:Ag coating. Subsequently, at the 48-hour and 96-hour time points, a 4% increment in pH was discerned in the experimental group conducted in the absence of sucrose [32]. In the presence of sucrose, the TiO₂:Ag coating caused a 9% increase in pH after 24 hours, followed by a 5% increase at 48 hours and a further 9% increase at 96 hours, as indicated in Figure 1.

4.1. The evaluation of the adherence capability of *S. mutans* to the orthodontic archwires under examination

The antiadhesive surface of orthodontic archwires, which is an important parameter for preventing microorganism colonization in the oral cavity, was evaluated after a 4-hour experiment.

In the case of the experiment without sucrose, one can see the strong significance of the difference in the averages in groups A and C (the significance level is far from the threshold level) in favor of the group with the active coating. Also worth noting is that there are differences between the other groups in this experiment but not significant.

For the sucrose experiments, a significant difference is seen between groups A and C, although, as we recall, such differences were not observed in the ANOVA test. A discussion of the significance of the differences in this case would be too far-fetched (the Newman-Keuls test is simply more sensitive), but this figure leads us to conclude that

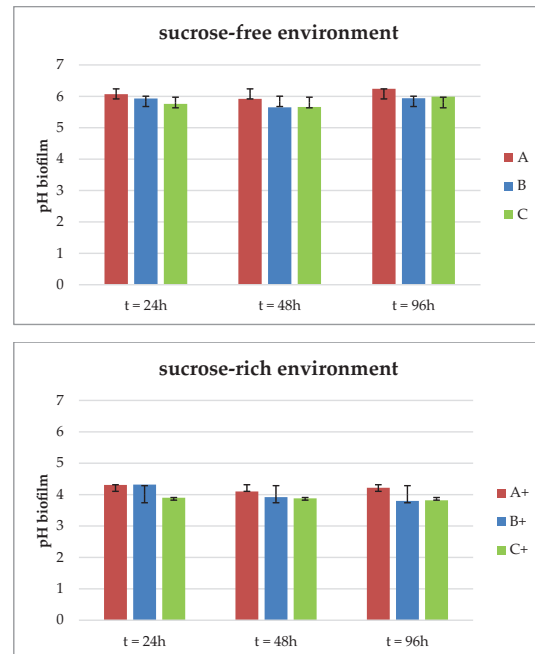


Fig. 1. pH value of saliva environment without [33] and with sucrose (+) for three types of samples: (A) orthodontic wires with TiO₂:Ag coating, (B) orthodontic wires with TiO₂ coating and (C) uncoated orthodontic wires. The measurements were taken at three different time intervals: 24 h, 48 h, and 96 h

there are significant differences in adhesion values for the groups indicated, with a lower value of this parameter observed for the samples with the active coating.

It is evident that sucrose loading has a clear effect on the reduction of adhesion. In each of the cases studied, samples with an applied active coating (A) show significantly lower adhesion than the reference samples (without any coating). In addition, samples with active coating allowed results with significantly less variation compared to the other groups.

Table 1S in the supplementary materials section) reveals a strong significance in the difference between groups A and C. The marked dissimilarity in outcomes lends a distinct advantage to the cohort endowed with the A coating. Nevertheless, it is imperative to underscore that the distinctions observed among the remaining experimental

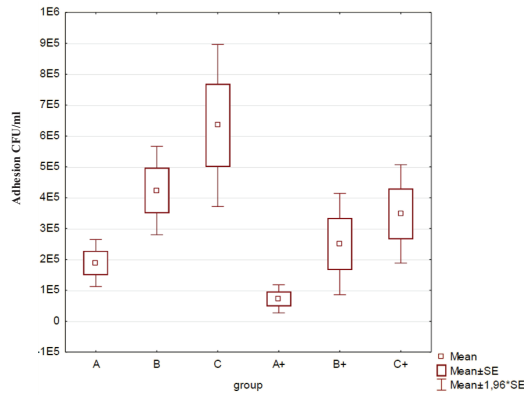


Fig. 2S. Box-plots for comparison regarding adhesion to the surface of three types of orthodontic archwires: (A) orthodontic wires with TiO₂:Ag coating, (B) orthodontic wires with TiO₂ coating, and (C) uncoated orthodontic wires under differential coating conditions for experiments without [33] and with sucrose (+)

groups, while statistically significant, do not attain a magnitude of substantial significance. The A coating elicited a noteworthy 74% reduction in *S. mutans* adhesion to the wire surface over a span of 4 hours, irrespective of the presence or absence of sucrose. When comparing the results of both experiments in a box-plot plot (Fig. 2S), it becomes evident that the presence of sucrose reduces adhesion. In all cases tested, samples with the A coating showed significantly lower adhesion compared to reference samples without any coating. Additionally, samples with the functional TiO₂ coating exhibited less variation compared to the other groups.

Based on the data provided, it is discernible that the inclusion of the TiO₂:Ag coating (A) engendered a diminution in bacterial adhesion when juxtaposed with the control specimen bereft of any coating (C) [32]. Furthermore, Figure 3S demonstrates that the biofilm formed on the TiO₂:Ag coating (A) demonstrated signs of fragmentation and heightened vulnerability to detachment. In contrast, the biofilm on the uncoated archwire (C) exhibited the formation of three-dimensional clusters, enveloped by a bacterial matrix layer (Fig. 3S A+, B+, C+). It is possible that the presence of coating A hinders the formation of a durable

and firmly attached bacterial biofilm on orthodontic wire, making it easier to remove during oral rinsing.

4.2. The impact of surface coatings on orthodontic archwires regarding the development of *S. mutans* biofilms

In the subsequent phase of analysis, an investigation was carried out to ascertain whether the presence of bacterial biofilms is affected by the coating applied on the surface of orthodontic archwires. In order to assess significant differences between pairs of data, the researchers utilized the Newman-Keuls post-hoc tests. The results of the Newman-Keuls test can be found in Table 1S (results without sucrose) and Table 2S (results with sucrose) in the supplementary materials section.

The application of a TiO₂:Ag coating on the surface of the orthodontic archwire resulted in a substantial decrease in biofilm formation. After 24 hours, there was a notable reduction of 77% compared to the reference sample. This reduction further improved to 84% after 48 hours. However, after 96 hours, the reduction decreased to 48% when compared to the reference sample. In contrast, the TiO₂ base coating exhibited a 62% decrease in biofilm formation after 24 hours. After 48 hours, the reduction decreased to 42%. Surprisingly, in the presence of sucrose, there was an unexpected increase of 23% in biofilm formation after 96 hours compared to the reference sample. These findings are illustrated in Figure 4S.

5. Discussion

This study aimed to assess the antibacterial and antiadherent characteristics of stainless steel orthodontic archwires with surface modifications of TiO₂ and TiO₂:Ag in the presence of sucrose. The orthodontic archwires were manufactured from 304V stainless steel. The primary distinction between 304V and 304L stainless steel lies in their carbon content and intended use. While 304L has lower carbon content, enhancing its corrosion resistance in welded applications, 304V is a free-machining variant designed for improved

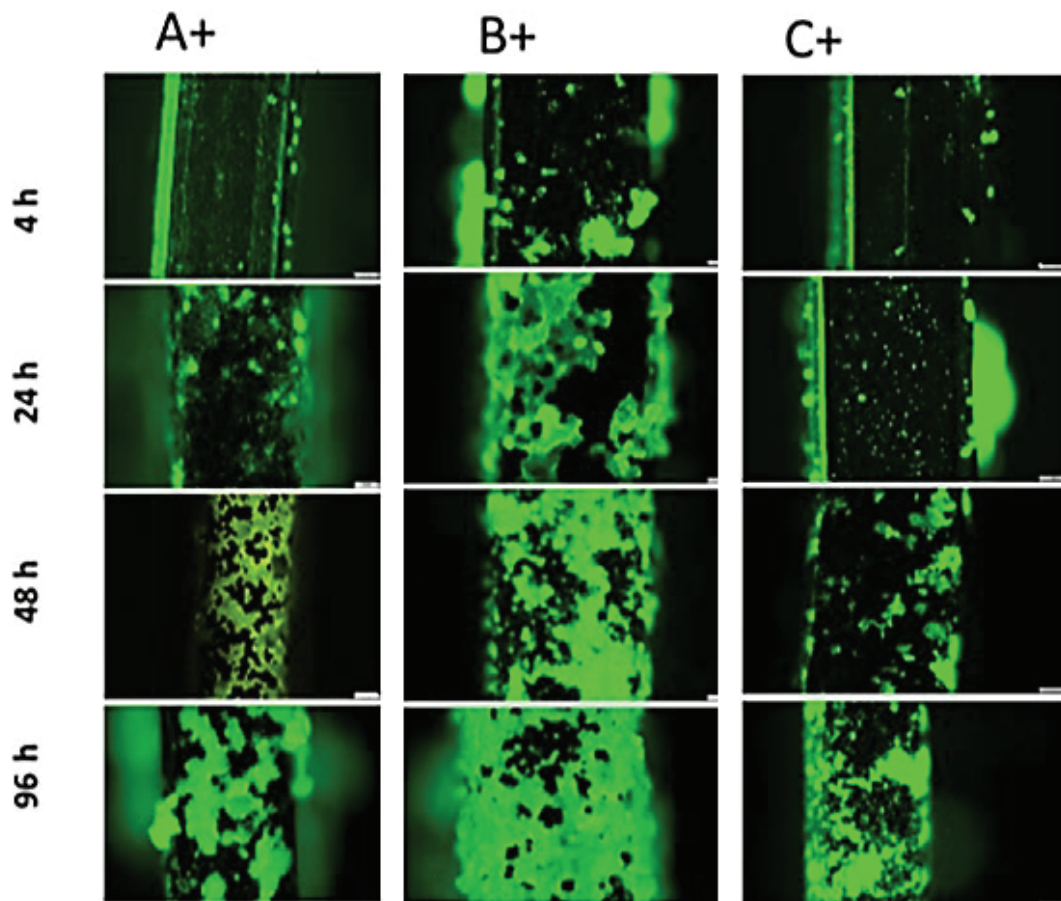


Fig. 3S. Representative images of coating surfaces with streptococcal biofilms produced in the presence (+) of sucrose. (A) orthodontic wires with $\text{TiO}_2\text{:Ag}$ coating, (B) orthodontic wires with TiO_2 coating and (C) uncoated orthodontic wires. Fluorescence microscope, magnification 400x

machinability, making it suitable for applications where ease of machining is a priority. In the context of orthodontic archwires, 304V stainless steel is specifically designed for improved machinability, making it easier to shape and manipulate during the manufacturing process.

In an environment devoid of sucrose, the $\text{TiO}_2\text{:Ag}$ coating exhibited significant effects over a span of 24, 48, and 96 hours. After a 24-hour period, a discernible 5% elevation in the pH level of the synthetic saliva was observed in comparison to the control sample. Similarly, after both 48 and 96 hours, there was a 4% increase in pH. With respect to bacterial adhesion, the $\text{TiO}_2\text{:Ag}$ coating exhibited a substantial reduction of 74% in *S. mutans* adhesion to the archwire surface after 4 hours, while the base TiO_2 coating reduced it by 33%.

Additionally, the $\text{TiO}_2\text{:Ag}$ coating demonstrated a significant decrease in *S. mutans* adherence. In terms of biofilm formation, the $\text{TiO}_2\text{:Ag}$ coating displayed a remarkable reduction of 98% after 24 hours, 73% after 48 hours, and 40% after 96 hours on the surface of the orthodontic archwire, as compared to the reference sample. Conversely, the TiO_2 base coating reduced biofilm formation by 97% after 24 hours, 39% after 48 hours, and 71% after 96 hours in comparison to the reference sample [28].

The current investigation reveals that in a sucrose-rich environment, the survival rate of *S. mutans* gradually rises over a period of 48 hours, after which it begins to decline, ultimately reaching zero after 96 hours. Such a phenomenon was already described in 1976 by Donoghue

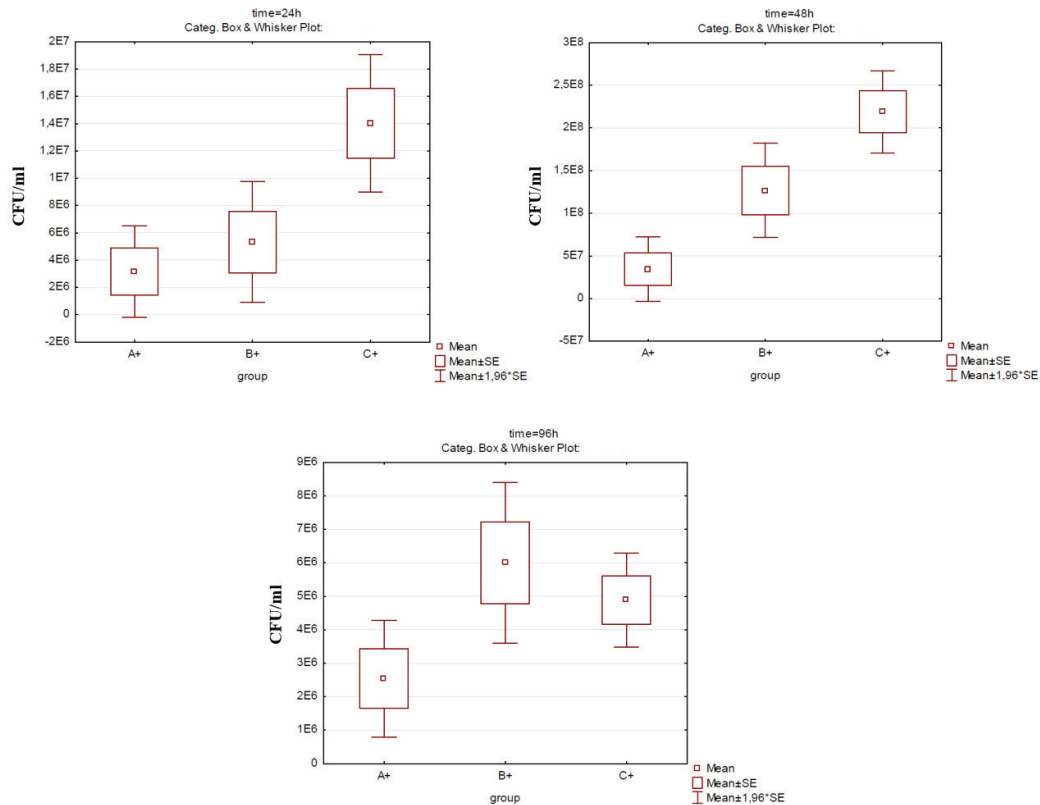


Fig. 4S. Box-plots for comparison the population of bacteria present on specimens subjected to a functional coating (A+), a basic coating (B+), and control specimens (an uncoated orthodontic wire – C+) after 24, 28, and 96 h

and Newman [1]. The increasing concentration of organic acids in the environment, which are a product of the metabolic pathway of carbohydrates, is believed to be a factor causing a decrease in cell survival. Simultaneously, the presence of these organic acids is accountable for the cariogenic impact of *S. mutans*. The formation of a bacterial biofilm serves the purpose of safeguarding bacteria against the detrimental impact of decreased environmental pH. Thus, it is imperative to acknowledge that any intervention leading to diminished bacterial survival within the initial 48-hour period and impeding their capacity to develop a biofilm would yield an anti-cariogenic outcome. As the study showed, the tested functional coating caused a significant reduction in bacterial survival both in the first 48 hours and along the entire time interval. In the study conducted, it was observed that the presence of sucrose led to a more pronounced reduction in the pH of the surrounding environment

by *S. mutans*. Additionally, when sucrose was in contact with the $\text{TiO}_2\text{:Ag}$ functional coating, a significant drop in pH was observed compared to the same coating in an environment without sucrose. However, the $\text{TiO}_2\text{:Ag}$ functional coating raised the pH of the environment much more when sucrose was present.

S. mutans metabolizes various carbohydrates to organic acids, causing primarily cariogenic destruction of the tooth surface, but also contributing to the change in the properties of metal alloys used, for example, in orthodontic treatment [30]. The influence exerted on the properties of orthodontic wires holds significant importance for the effectiveness and safety of orthodontic treatment [12]. Various surfaces tend to attract oral microorganisms, including *S. mutans*. These microorganisms possess a propensity to form biofilms, which are intricate, multi-layered colonies that enhance their survival

in the environment. The capacity of bacteria to adhere and accumulate on the tooth surface can be heightened through the metabolic breakdown of different carbohydrates [31, 32]. The presence of carbohydrates visibly augments the ability of *S. mutans* to generate a biofilm and secrete organic acids, which are the primary metabolites responsible for the detrimental effects of these bacteria [33].

The research findings revealed an intriguing pattern. Upon analyzing the results, it was observed that in a sucrose-rich environment, bacteria exhibited a reduced inclination to adhere to metal wires. However, the presence of carbohydrates led to a noteworthy decrease in the pH level throughout all the studied time intervals. In summary, the presence of sucrose diminished the propensity of *S. mutans* to form bacterial biofilms on orthodontic wire surfaces, but it also resulted in increased secretion of organic acids, thereby augmenting the cariogenic and detrimental impact on the metal components. The efficacy of the tested functional coating varied depending on the presence of sucrose in the environment. The TiO₂:Ag coating elevated the pH level by 5%–9% in all tested time intervals in a carbohydrate-rich environment (Fig. 1).

The research results demonstrate that the presence of sucrose in the environment leads to a significant decrease of 40%–60% in bacterial attachment to the wire surface. The extent of reduction in bacterial adhesion varies depending on the type of coating applied. This observation is supported by the data depicted in Figure 2S, which compares the experimental results with and without the inclusion of sucrose.

Previous research conducted by Boyd *et al.* [34] has shown that the presence of a high concentration of sucrose leads to a reduction in the adherence of *S. mutans* biofilm to titanium surfaces. However, these findings contradict the claims made by Decker *et al.* [32]. To fully understand and provide a comprehensive explanation for the observed phenomenon of decreased biofilm formation ability of *S. mutans* on nickel-titanium alloy surfaces in sucrose-rich environments, further investigation is required. Nonetheless, one of the key mechanisms contributing to the anti-cariogenic

effect of silver nanoparticles is their ability to enhance this phenomenon of diminished biofilm formation.

Takahashi *et al.* previously proposed the hypothesis that when a high sucrose concentration is present, the reduction of the pH in the surrounding environment exerts a more pronounced influence [31]. Sucrose is commonly found in the human oral environment as part of the diet and can influence bacterial adhesion, biofilm formation, and acidification of the surroundings. Consequently, the authors suggest that studies evaluating the antibacterial properties of coatings should also consider the presence of carbohydrates. Numerous existing studies have confirmed the antibacterial properties of TiO₂ or TiO₂:Ag coatings, but they have overlooked the potential impact of additional sucrose loading in their experimental designs [9, 35–37]. This oversight is significant as the presence of sucrose could potentially contribute to the development of dental caries. The researchers in this study have provided evidence to demonstrate that in the presence of sucrose, bacteria exhibit decreased abilities to adhere and form biofilms; however, they tend to acidify the surrounding environment to a greater extent. Furthermore, the introduction of silver nanoparticles significantly reduces the bacteria's ability to adhere in the presence of sucrose, especially after 48 hours and 96 hours. This effect is more pronounced within the initial 24-hour period when sucrose is absent from the environment.

In summary, it can be deduced that the inclusion of sucrose leads to a considerable increase in acidity in the environment surrounding *S. mutans*, irrespective of whether they are influenced by silver ions or not. Nevertheless, the existence of the bioactive TiO₂:Ag coating predominantly counteracts this effect, particularly when the pH has decreased significantly due to the presence of carbohydrates.

Based on a clinical analysis of outcomes, it can be inferred that when individuals undertake measures to limit the effects of sucrose, such as modifying their diet and practicing oral hygiene, the functional coating is anticipated to offer an additional advantage. It is anticipated that the coating

will effectively impede the capacity of *S. mutans* bacteria to form a biofilm and elevate the pH levels in the surrounding milieu. When sucrose appears in the mouth, the TiO₂:Ag coating will work even more effectively. Its anti-adhesive effect will be even stronger after 48–96 h. The pH level of the surrounding environment is expected to experience a considerable decrease, although the functional coating will effectively raise it to a greater extent. It should be noted, however, that the coating's impact on pH adjustment is not considered a primary action, but rather a supplementary measure that supports other health-enhancing practices like regular brushing and a nutritious diet.

In considering avenues for future research, the intriguing observations and complexities uncovered in this study prompt the identification of several directions for further investigation. Given the pivotal role of orthodontic wires as medical devices, it is imperative to delve deeper into understanding the dynamics between coating compositions, environmental conditions, and bacterial responses. While our study provides valuable insights into the short-term effects of coatings on bacterial adhesion and pH levels, a more extensive examination of the long-term durability and stability of these coatings is essential. Longitudinal studies could elucidate the sustained impact of functional coatings over extended durations, simulating the conditions that orthodontic wires encounter during the course of treatment. Transitioning from laboratory conditions to clinical applications is a crucial step. Future research should bridge the gap by conducting in vivo studies to validate the efficacy and safety of the TiO₂:Ag coating in real-world orthodontic scenarios. Assessing its performance in the presence of diverse oral environments and patient-specific factors will contribute to the clinical translatability of the proposed intervention.

In considering the implications of our research and charting directions for future investigations, it becomes apparent that further mechanical testing is warranted to comprehensively evaluate the performance of orthodontic wires under the influence of different coatings. The incorporation of mechanical assessments, such as three-point bending tests, can provide valuable insights into the

structural integrity and durability of the wires. This would contribute to a holistic understanding of the interplay between the functional coatings and the mechanical properties of orthodontic wires, ensuring their effectiveness and safety in a clinical context. Moreover, delving into the nanoscale level could unveil additional dimensions of their impact on bacterial adhesion and biofilm formation. Investigating these finer details at the nanoscale level could elucidate the intricate interactions between the coating properties and bacterial behavior. Nanostructural characteristics play a pivotal role in influencing the adhesion of microorganisms, and an in-depth exploration of these features can augment our comprehension of the underlying mechanisms governing the observed antibacterial effects.

In conclusion, these suggested directions for future research aim to propel the field of orthodontic materials towards enhanced functionality, longevity, and patient-centric outcomes. By addressing these research avenues, we can advance the understanding of orthodontic coatings and pave the way for transformative innovations in orthodontic care.

6. Conclusions

The method employed demonstrated efficacy when applied to orthodontic wires, resulting in the creation of a coating that exhibited effective antibacterial properties against *S. mutans* in vitro.

1. The introduction of sucrose resulted in an average 30% reduction in the pH of the surroundings, as *S. mutans* consume sugar and generate organic acids, causing a decline in pH. Conversely, the TiO₂:Ag coating demonstrated a pH elevation of 5%–9% throughout all observed time intervals in a carbohydrate-rich environment.
2. In the experimental setting, the addition of sucrose led to a noteworthy reduction of 40%–60% in the adherence of *S. mutans* to the wire surface as compared to the sucrose-free test. Following a duration of 4 hours, the application of the TiO₂:Ag

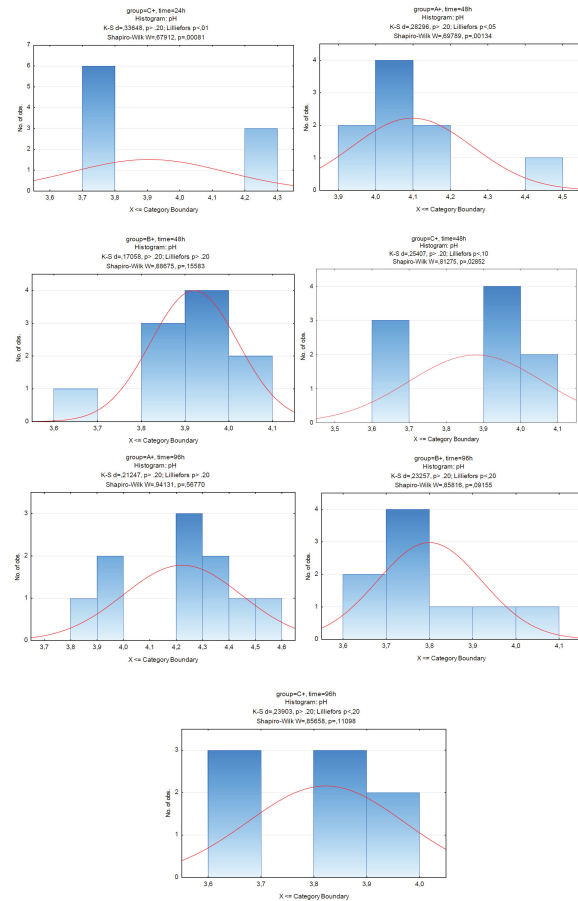
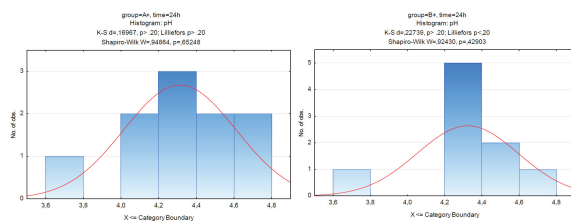
coating exhibited a remarkable reduction of 76% in the adherence of *S. mutans* to the wire surface. The application of a $\text{TiO}_2\text{:Ag}$ coating onto stainless steel wire has exhibited significant anti-adhesive characteristics when confronted with predominant oral pathogens, specifically *Streptococcus mutans*.

3. The application of a $\text{TiO}_2\text{:Ag}$ coating on the orthodontic archwire surface resulted in a noteworthy reduction in the formation of biofilm in an environment containing high levels of sucrose. After 24 hours, a decrease was observed in biofilm formation by 77% compared to the control sample. This reduction further increased to 84% after 48 hours and 48% after 96 hours, relative to the reference sample.
4. In vitro studies have revealed that archwires coated with $\text{TiO}_2\text{:Ag}$ possess antibacterial properties, which consequently contribute to the prevention of caries and the accumulation of plaque.

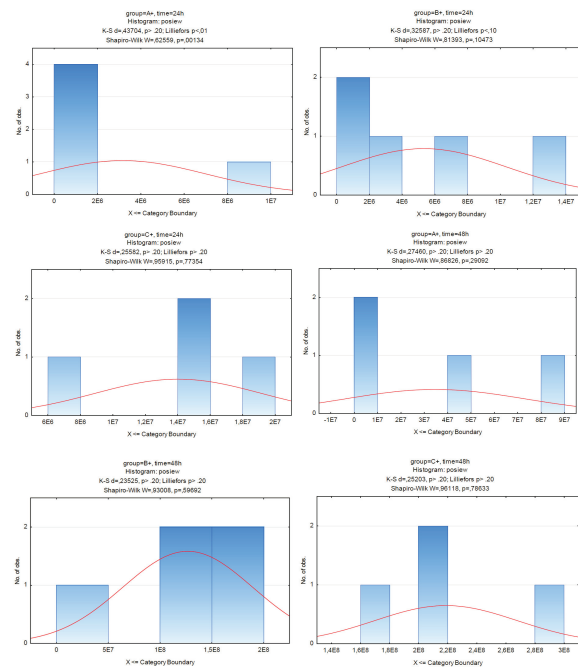
The presence of sucrose in the environment leads to a notable increase in the acidification level of *S. mutans*, irrespective of the impact of silver ions. However, the presence of the bioactive $\text{TiO}_2\text{:Ag}$ coating effectively mitigates this effect, particularly when there is a substantial reduction in pH caused by the presence of carbohydrates. Applying an antibacterial coating to orthodontic wires can assist in maintaining proper hygiene practices during fixed appliance treatments.

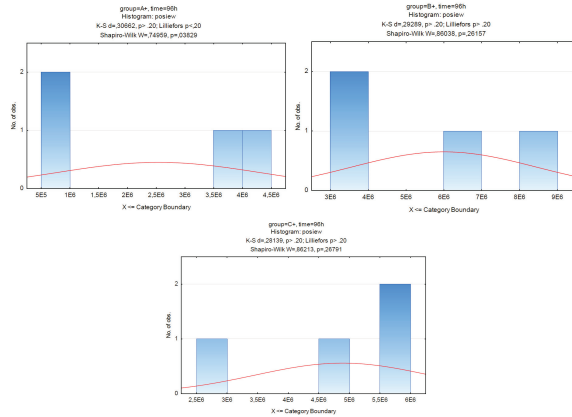
7. Supplementary material

7.1. Histograms of pH level depending on the coating used (with sucrose)

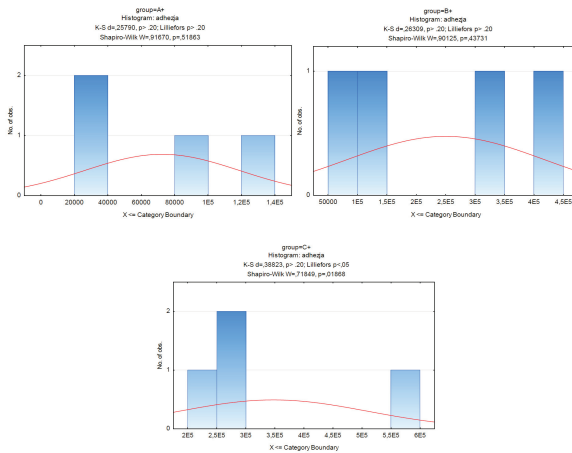


7.2. Histograms of culture results depending on the coating used (with sucrose)





7.3. Histograms of adhesion level after 4h depending on the coating used (with sucrose)



7.4. Results of the Newman-Keuls post hoc test

The results of the post-hoc tests for the different experimental groups are shown in Tables 1S and 2S. As can easily be seen, after 24 h there are significant differences in the sucrose-free and sucrose-grown groups between the modified arcs (with active and base coating) and the reference sample (without coating). After longer testing times (48 h and 96 h), the active-coated sample induces a significantly higher pH in the environment than the base-coated and uncoated arcs. The significance level for rejecting the null hypothesis is very high in each of the comparisons indicated.

In the case of the experiment without sucrose, one can see the strong significance of the difference

Table 1S. Results of the Newman-Keuls post hoc test for the dependent variable of pH levels; experiment without sucrose

t = 24 h			
Group	{1}	{2}	{3}
A {1}		0.0678	0.0007*
B {2}	0.0678		0.0236
C {3}	0.0007	0.0236	
t = 48 h			
Group	{1}	{2}	{3}
A {1}		0.0067	0.0035
B {2}	0.0067		0.8958
C {3}	0.0035	0.8958	
t = 96 h			
Group	{1}	{2}	{3}
A {1}		0.0058	0.0084
B {2}	0.0058		0.5474
C {3}	0.0084	0.5474	

*Significant differences for $p < 0.05$ are marked in bold.

Table 2S. Results of the Newman-Keuls post hoc test for the dependent variable of pH levels; experiment with sucrose

t = 24 h			
Group	{1}	{2}	{3}
A+ {1}		0.9353	0.0035*
B+ {2}	0.9353		0.0075
C+ {3}	0.0035	0.0075	
t = 48 h			
Group	{1}	{2}	{3}
A+ {1}		0.0176	0.0117
B+ {2}	0.0176		0.5602
C+ {3}	0.0117	0.5602	
t = 96 h			
Group	{1}	{2}	{3}
A+ {1}		0.0002	0.0002
B+ {2}	0.0002		0.7863
C+ {3}	0.0002	0.7863	

*Significant differences for $p < 0.05$ are marked in bold.

in the averages in groups A and C (the significance level is far from the threshold level) in favor of the group with the active coating. Also worth noting is that there are differences between the other groups in this experiment but not significant.

For the sucrose experiments, a significant difference is seen between groups A and C, although,

Table 3S. Results of the Newman-Keuls post hoc test for bacterial cultures

Without sucrose, t = 4 h			
Group	{1}	{2}	{3}
A {1}		0.0590	0.0041*
B {2}	0.0590		0.0866
C {3}	0.0041	0.0866	
With sucrose, t = 4 h			
Group	{1}	{2}	{3}
A+ {1}		0.1012	0.0472
B+ {2}	0.1012		0.3420
C+ {3}	0.0472	0.3420	

*Significant differences for $p < 0.05$ are marked in bold;

Table 4S. Results of the Newman-Keuls post hoc test for bacterial cultures, experiments without sucrose

t = 48 h			
Group	{1}	{2}	{3}
A {1}		0.0895	0.0048*
B {2}	0.0895		0.0529
C {3}	0.0048	0.0529	
t = 96 h			
Group	{1}	{2}	{3}
A {1}		0.1159	0.0488
B {2}	0.1159		0.0058
C {3}	0.0488	0.0058	

*Significant differences for $p < 0.05$ are marked in bold.

as we recall, such differences were not observed in the ANOVA test. A discussion of the significance of the differences in this case would be too far-fetched (the Newman-Keuls test is simply more sensitive), but this figure leads us to conclude that there are significant differences in adhesion values for the groups indicated, with a lower value of this parameter observed for the samples with the active coating.

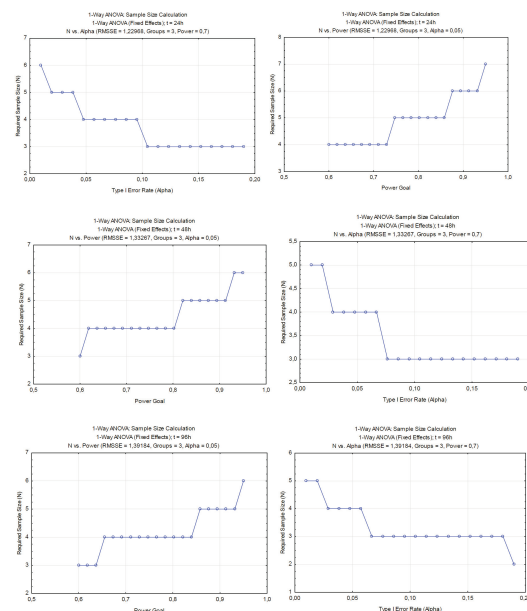
It is evident that sucrose loading has a clear effect on the reduction of adhesion. In each of the cases studied, samples with an applied active coating (A) show significantly lower adhesion than the reference samples (without any coating). In addition, samples with active coating allowed results with significantly less variation compared to the other groups.

Table 5S. Results of the Newman-Keuls post hoc test for bacterial cultures, experiments with sucrose

t = 24 h			
Group	{1}	{2}	{3}
A+ {1}		0,4960	0.0121*
B+ {2}	0.4960		0,0166
C+ {3}	0.0121	0.0166	
t = 48 h			
Group	{1}	{2}	{3}
A+ {1}		0.0282	0.0013
B+ {2}	0.0282		0,0282
C+ {3}	0.0013	0.0282	
t = 96 h			
Group	{1}	{2}	{3}
A+ {1}		0.0749	0.1198
B+ {2}	0.0749		0.4368
C+ {3}	0.1198	0.4368	

*Significant differences for $p < 0.05$ are marked in bold.

7.5. Graphs of sample sizes as a function of test power and type I error



Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Author Contributions

Conceptualization, J.D., M.S.; methodology, J.B., Z.K.-G., A.Z.; validation, J.B., B.S., U.W.; formal analysis, J.B., B.B.,

J.D.; investigation, J.B., B.S., U.W.; resources, J.B., B.B., Z.K.-G., U.W., B.S.; writing — original draft preparation, J.B.; writing — review and editing, B.B., J.D., M.S.; visualization, J.B., Z.K.-G.; supervision, M.S., J.D., B.K., B.B., B.S., U.W.; project administration, J.D., M.S.; funding acquisition, J.D., M.S. All authors have read and agreed to the published version of the manuscript.

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Wrocław University of Science and Technology, Smoluchowskiego 25, 50-372 Wrocław, Poland

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