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Erythritol/chlorhexidine combination reduces microbial biofilm and prevents its formation on titanium surfaces *in vitro*

Lorenzo Drago[1,2], Monica Bortolin[1], Silvio Taschieri[3,4], Elena De Vecchi[1], Serse Agrappi[1], Massimo Del Fabbro[3,4], Luca Francetti[3,4], Roberto Mattina[5]

[1]*Laboratory of Clinical Chemistry and Microbiology, I.R.C.C.S. Galeazzi Orthopedic Institute, Milan, Italy;* [2]*Laboratory of Clinical Microbiology, Department of Biomedical Sciences for Health, University of Milan, Milan, Italy;* [3]*Department of Biomedical, Surgical and Dental Sciences, University of Milan, Milan, Italy;* [4]*Dental Clinic, I.R.C.C.S. Galeazzi Orthopedic Institute, Milan, Italy;* [5]*Department of Public Health, Microbiology and Virology, University of Milan, Milan, Italy*

BACKGROUND: The purpose of this *in vitro* study was to evaluate the antibiofilm activity of a novel air-polishing powder consisting of erythritol and chlorhexidine, assessing its ability to reduce previously grown microbial biofilm and to prevent biofilm formation on titanium surfaces.

METHODS: Clinical strains of *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Bacteroides fragilis* and *Candida albicans* isolated from peri-implantitis lesions were used. Biofilm was grown on sandblasted titanium discs and treated with erythritol/chlorhexidine. The antimicrobial activity was evaluated by determining the minimum inhibitory concentration and the minimum microbicidal concentration. The antibiofilm activity was assessed by semiquantitative spectrophotometric assay and by confocal laser scanning microscopy.

RESULTS: Erythritol/chlorhexidine displayed an inhibitory and a microbicidal activity against all the tested strains. The spectrophotometric analysis showed that the treatment was effective in both reducing the previously developed biofilm and decreasing biofilm formation on titanium surfaces. Confocal laser scanning microscopy analysis showed a significant reduction of the total biofilm volume, with an increase of the percentage of dead cells of all the microorganisms tested.

CONCLUSIONS: Erythritol/chlorhexidine displayed significant antimicrobial and antibiofilm activity against microorganisms isolated from peri-implantitis lesions. Due to its properties, it might represent a promising approach for the prevention and treatment of peri-implant diseases associated to microbial biofilm infections.

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Correspondence: Lorenzo Drago, Laboratory of Clinical Chemistry and Microbiology, I.R.C.C.S. Galeazzi Orthopedic Institute, Via R. Galeazzi 4, 20161 Milan, Italy. Tel: 00390266214839, Fax: 00390266214774, E-mail: lorenzo.drago@unimi.it
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Introduction

Peri-implant diseases, such as peri-implant mucositis and peri-implantitis, are bacterial-driven infections (1, 2). Peri-implant mucositis are reversible inflammatory lesions located in the mucosa, while peri-implantitis also affect the supporting bone and can lead to implant loss (3). The treatment of peri-implant diseases requires thorough removal of the bacterial biofilm developed around the dental implant. Non-surgical treatments consisting of mechanical and ultrasonic debridement, the use of chemical agents (e.g. disinfectants, local or systemic antibiotics) or laser application may improve clinical parameters without arresting the progression of peri-implantitis (4). Such results may be explained by the fact that these methods are not effective in eliminating bacterial biofilms from implant surfaces (5). The use of injection of abrasive water jets, also known as air-polishing devices, was evaluated in a number of recent studies, which showed that the use of glycine powder could be effective in removing bacterial biofilm without altering the morphological characteristics of the implant surface (6, 7).

A novel minimally abrasive powder (AIR-FLOW® PLUS, EMS Electro Medical Systems, Nyon, Switzerland) containing erythritol and chlorhexidine has been developed for use in commercially available air-polishing devices. Such powder has a finer granulometry than glycine powder (14 vs. 25 μm) which limits the damage to hard and soft tissues. Its efficacy has been investigated in few clinical trials, showing promising results for subgingival air-polishing during periodontal maintenance (8–10). Comparative studies regarding its application in the treatment of peri-implantitis are lacking. Only a case report of guided

bone regeneration in sites with implants affected by peri-implantitis has been published to date (11).

The purpose of this *in vitro* study was to evaluate the antibiofilm activity of this new low-abrasive powder, assessing its ability to reduce previously developed microbial biofilm and to prevent its formation on titanium surfaces.

Materials and methods

Study design

The study was approved by IRCCS Galeazzi Orthopedic Institute. It was an *in vitro* study, as so it did not require the approbation of an ethics committee.

Erythritol/chlorhexidine solution

An air-polishing powder (AIR-FLOW® PLUS, EMS Electro Medical Systems, Nyon, Switzerland) consisting of 99.7% erythritol and 0.3% chlorhexidine (mass % of active ingredient in the powder) was used in this study. The powder was dissolved in sterile saline to obtain a solution at a concentration of 100 mg/ml.

Microbial strains

The microbial strains used in this study were isolated from clinical samples collected from patients with peri-implantitis at the Laboratory of Clinical Chemistry and Microbiology of IRCCS Galeazzi Orthopedic Institute. In particular, *Pseudomonas aeruginosa*, *Staphylococcus aureus, Candida albicans* and *Bacteroides fragilis* strains were used. These microorganisms were previously identified by biochemical assays (Vitek® 2 Compact, bioMérieux, Marcy l'Etoile, France) and stored at −80°C before analysis. Before use, strains were thawed and reconstituted in appropriate medium for 24 h at 37°C. All strains had been screened before the beginning of the study in order to assess their ability to produce biofilm according to the spectrophotometric assay described by Christensen et al. (12).

Evaluation of the antimicrobial activity

The antimicrobial activity was evaluated by determining the minimum inhibitory concentration (MIC) and the minimum microbicidal concentration (MMC). The MIC, defined as the lowest concentration of an antimicrobial substance that inhibits the visible growth of a microorganism, was determined by broth microdilution method, in accordance with the European Committee on Antimicrobial Susceptibility Testing guidelines (13). Briefly, a microbial suspension at an optical density equal to 0.5 McFarland standard in Brain Heart Infusion broth (BHI; bioMérieux, Marcy l'Etoile, France) was prepared for each strain. After obtaining a microbial load of 1×10^5 CFU/ml using appropriate dilutions, 20 µl of each suspension was inoculated in a 96-well microplate containing 180 µl of a serial BHI twofold dilution of a starting erythritol/chlorhexidine solution at a concentration of 100 mg/ml. Growth controls were performed inoculating the microbial suspension in BHI alone. MIC values were read after 24 h of incubation at 37°C. The MMC, defined as the lowest concentration of an antimicrobial substance able to kill 99.9% of the initial inoculum, was determined by subculturing 10 µl of microbial suspension from wells showing no visible growth in the MIC tests. MMC values were read after 24 h of incubation at 37°C. The assays were conducted in duplicate for each microbial strain and repeated two times.

Biofilm development

Biofilm was developed on sterile discs made of titanium alloy Ti6Al4V, of 25 mm diameter × 5 mm thickness (Adler Ortho, Cormano, Italy). Discs' surface was treated by automated sandblasting (corundum granulometry of grain size 250 µm), producing a roughness of 1.5–4.5 µm. Discs were placed in six-well polystyrene plates containing BHI for 24 h at 37°C. All microorganisms, except *B. fragilis,* were grown in BHI in aerobic atmosphere. *B. fragilis* was grown in anaerobiosis in BHI supplemented with 5% defibrinated sheep blood (Liofilchem, Roseto degli Abruzzi, Italy). Then, broth was removed, together with not adherent microorganisms, and new broth was added. Plates were incubated at 37°C in proper conditions for additional 48 h, until a mature biofilm was obtained.

Evaluation of antibiofilm activity

Evaluation of the activity against preformed biofilm

In order to evaluate the activity of erythritol/chlorhexidine to reduce microbial biofilm previously formed on sandblasted titanium discs, biofilm was allowed to grow for 72 h, as described above. Then, erythritol/chlorhexidine was added to the growth medium at different concentrations (corresponding to 2 × MIC, 1 × MIC and ½ × MIC) and the plates were further incubated for 0, 6, 12, 24, 48 and 72 h. Untreated biofilm incubated in the same conditions was used as positive control. The test was conducted in duplicate for each strain and repeated two times.

Prevention of biofilm formation

In order to evaluate the ability to prevent biofilm formation, microbial biofilm was allowed to grow on titanium discs placed in six-well plates containing different concentrations of erythritol/chlorhexidine (corresponding to 2 × MIC, 1 × MIC and ½ × MIC) for 24 h at 37°C. Then, growth medium was removed, and new broth was added. The plates were further incubated for 6, 12, 24, 48 and 72 h. Untreated biofilm incubated in the same conditions was used as positive control. The test was conducted in duplicate for each strain and repeated two times.

Semiquantitative analysis of biofilm by spectrophotometric assay

The amount of biofilm was determined by a spectrophotometric assay, adopting the method developed by Christensen et al. (12). At the end of each incubation time, discs were washed two times with sterile saline and air-dried. Dried discs were stained by immersion in a solution of 5% crystal violet for 10 m. After staining, they were washed with sterile saline to remove the dye in excess, left to completely air dry and finally placed in six-well plates containing 3 ml of 96% ethanol in order to re-solubilize the dye included into the biofilm. Crystal violet absorbance was measured at 595 nm using a microplate reader. Measurements were carried out in triplicate for each disc.

Quantitative confocal laser scanning microscopy (CLSM) analysis

Confocal laser scanning microscopy analysis was carried out following a method previously developed for quantitative analysis of biofilm (14, 15). The microbial biofilm was grown on titanium discs and treated with erythritol/chlorhexidine, as described above. The chosen concentration was 2 × MIC, and the incubation time was 72 h. The biofilm was analysed before and after treatment. Staining was performed with a fluorescent stain (Filmtracer™ LIVE/DEAD® Biofilm Viability Kit; Life Technologies, Carlsbad, CA, USA) containing a mixture of two dyes: SYTO 9 (green stain) and propidium iodide (red stain), 3 μl of each component in 1 ml of saline solution. In the case of *P. aeruginosa*, only SYTO 9 was used. Discs were incubated with fluorescent stains at room temperature for 15 m in the dark. To minimize the air contact and maintain constant sample moisture condition, a coverslip was placed on the specimen. Stained biofilm was examined with a confocal laser microscope (Leica model TCS SP5; Leica Microsystems CMS GmbH, Mannheim, Germany) using a 20× dry objective (HC PL FLUOTAR 20.0 × 0.50 DRY) plus a 2× electronic zoom. A 488-nm laser line was used to excite SYTO 9, while the fluorescent emission was detected at 500–540 nm. Propidium iodide was excited with a 561-nm laser line, while its fluorescent emission was detected at 600–695 nm. In order to avoid false co-localization between fluorescent stains due to microbial movement, a simultaneous acquisition mode of the two channels was adopted, in which the laser beam scanned the visual field at a frequency of 700 Hz. Using a third laser line (633 nm) in a reflection mode, it was possible to determine both titanium disc (starting acquisition point) and coverslip (ending acquisition point) reflecting surfaces. Images from five randomly selected fields (area 2×10^5 μm^2) were acquired for each sample. Sequential optical sections of 2 μm were collected along the *z*-axis over the complete thickness of the biofilm. The resulting stacks of images were quantified using an image processing package (Fiji, ImageJ 1.47 q, National Institute of Health, USA) and subsequently rendered into three-dimensional mode using an image analysis software (Imaris 7.2.3; Bitplane AG, Zurich, Switzerland).

Statistical analysis

Results were expressed as mean ± standard deviation. Comparisons between two groups were performed using unpaired Student's *t*-test. Comparisons between more than two groups were performed using two-way ANOVA followed by Bonferroni *post hoc* tests. A value of $P \leq 0.05$ was used as the significance level.

Results

Evaluation of the antimicrobial activity

Erythritol/chlorhexidine showed an inhibitory and a microbicidal activity against all the tested strains, with MIC values varying from 0.78 to 25 mg/ml and MMC values ranging from 1.56 to 50 mg/ml, depending on the tested microorganism (Table 1).

Table 1 Antimicrobial activity of erythritol/chlorhexidine

Microbial strain	*MIC (mg/ml)*	*MMC (mg/ml)*
Pseudomonas aeruginosa	25	50
Staphylococcus aureus	0.78	1.56
Candida albicans	6.25	12.5
Bacteroides fragilis	12.5	25

Evaluation of the activity against preformed biofilm

Results were expressed as percentage of biofilm compared to the control (Fig. 1, panel A). For all the microorganisms tested, erythritol/chlorhexidine at the concentration of 2 × MIC significantly reduced the amount of biofilm starting from 12 h of incubation. *S. aureus* showed a significant reduction also after 6 h. The concentration of 1 × MIC produced a significant reduction after 48 h on the biofilm of *S. aureus, P. aeruginosa* and *C. albicans*, while it seemed to have no significant effect on *B. fragilis.* The concentration of ½ × MIC seemed to display no activity against the biofilm produced by the tested strains.

For each microorganism and each treatment concentration, the reduction of biofilm from 0 to 72 h was also calculated. A decrease of the previously formed biofilm could only be observed at the concentration of 2 × MIC, with a percentage of 77% ± 5% for *S. aureus*, 70% ± 4% for *P. aeruginosa,* 45% ± 6% for *C. albicans* and 45% ± 8% for *B. fragilis*.

Prevention of biofilm formation

Results were expressed as percentage of biofilm compared to the control (Fig. 1, panel B). For all the microorganisms tested, the concentration of 2 × MIC significantly reduced the biofilm formation over time, starting from 6 h of incubation. The concentration of 1 × MIC produced a slower, but still significant, reduction in the biofilm of *S. aureus* (after 12 h)*, P. aeruginosa, C. albicans* (after 24 h) and *B. fragilis* (after 72). The concentration of ½ × MIC failed to reduce biofilm development.

CLSM analysis

Figure 2 displays the three-dimensional rendering of the microbial biofilms before and after treatment. The corresponding quantifications obtained through the image analysis software are shown in Fig. 3 and Table 2.

A significant lower total biomass volume was observed for all strains after a 72-h treatment with erythritol/chlorhexidine at the concentration of 2 × MIC (Fig. 3). In particular, reductions of 45% ± 8%, 35% ± 4%, 32% ± 13% and 29% ± 6% were observed for *P. aeruginosa*, *S. aureus*, *C. albicans* and *B. fragilis*, respectively.

The composition of *S. aureus*, *C. albicans* and *B. fragilis* biofilm in terms of live and dead cells was also evaluated (Table 2). A significantly higher percentage of dead cells was observed in treated samples than in controls for all microorganisms ($P < 0.0001$).

Discussion

Peri-implant diseases are infections associated with a complex microbiota that induce a local and systemic

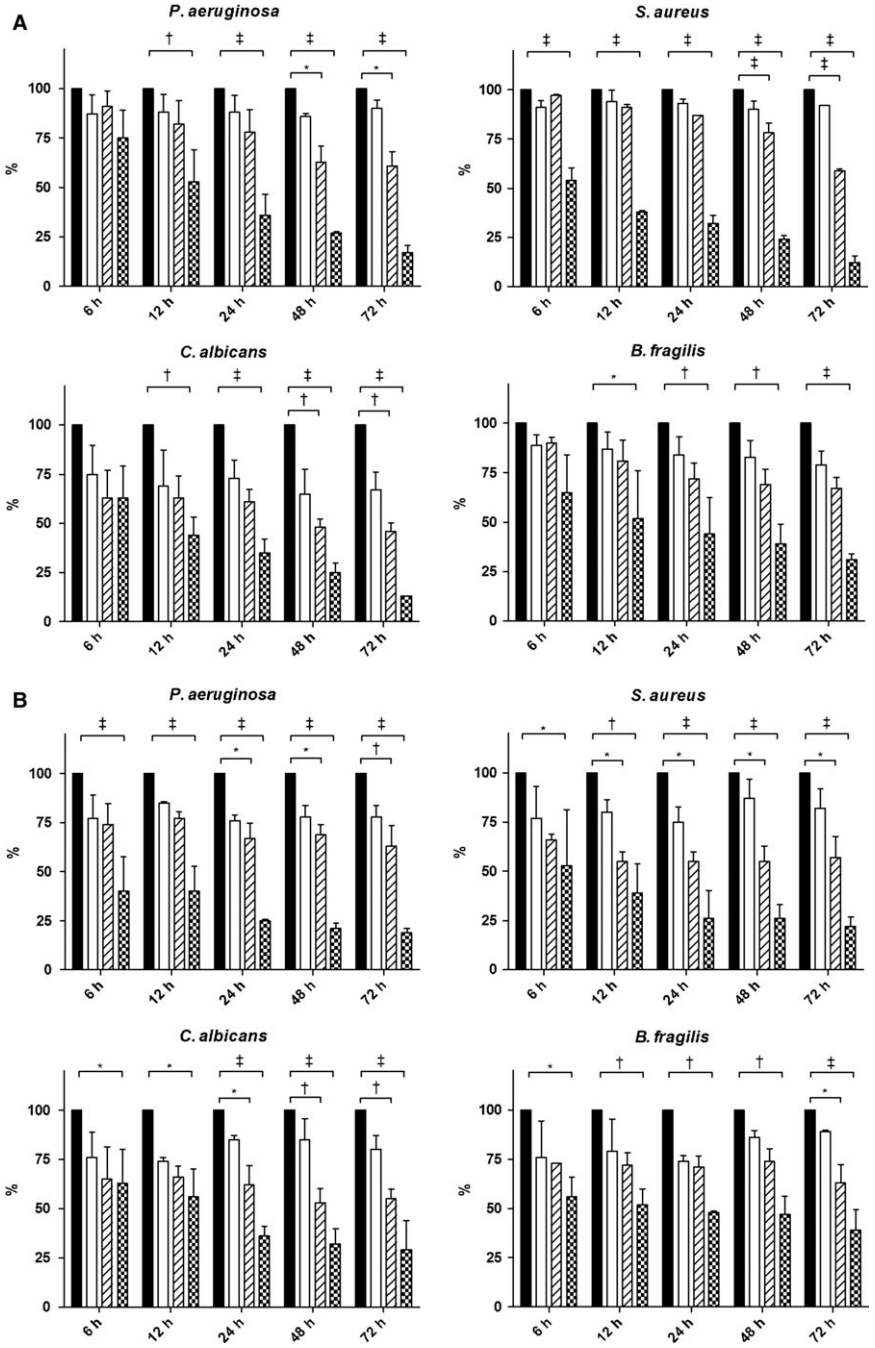

Figure 1 Semiquantitative analysis of microbial biofilm by spectrophotometric assay. Panel A shows reduction (%) of the previously formed biofilm, while panel B shows reduction (%) of biofilm development on sandblasted titanium discs at various time points by different concentrations of the erythritol/chlorhexidine powder. Data are expressed as mean ± standard deviation. Black bars = control, white bars = ½ × MIC, dashed bars = 1 × MIC, squared motif bars = 2 × MIC. *$0.01 < P < 0.05$, †$0.001 < P < 0.01$, ‡$P < 0.001$.

inflammatory response that may lead to peri-implant tissue breakdown and eventual implant loss (16). Therefore, the accurate removal of the biofilm developed on the dental implant surface is necessary to address this condition. The aim of this study was to evaluate the antibiofilm properties of a novel air-polishing powder based on erythritol and 0.3% chlorhexidine, recently introduced in the clinical setting for the treatment of periodontal and peri-implant diseases.

Chlorhexidine is a well-known broad-spectrum antimicrobial substance commonly used in dentistry as an irrigant and as a component of mouthwashes. The manufacturer actually adds chlorhexidine as a preservative, not with the intention to have a therapeutic effect. Actually, although chlorhexidine is effective in reducing the viability of microorganisms (17), it seems to have little or no efficacy in removing biofilms, due to its limited diffusion into the extracellular polymeric matrix (EPS) (18–20).

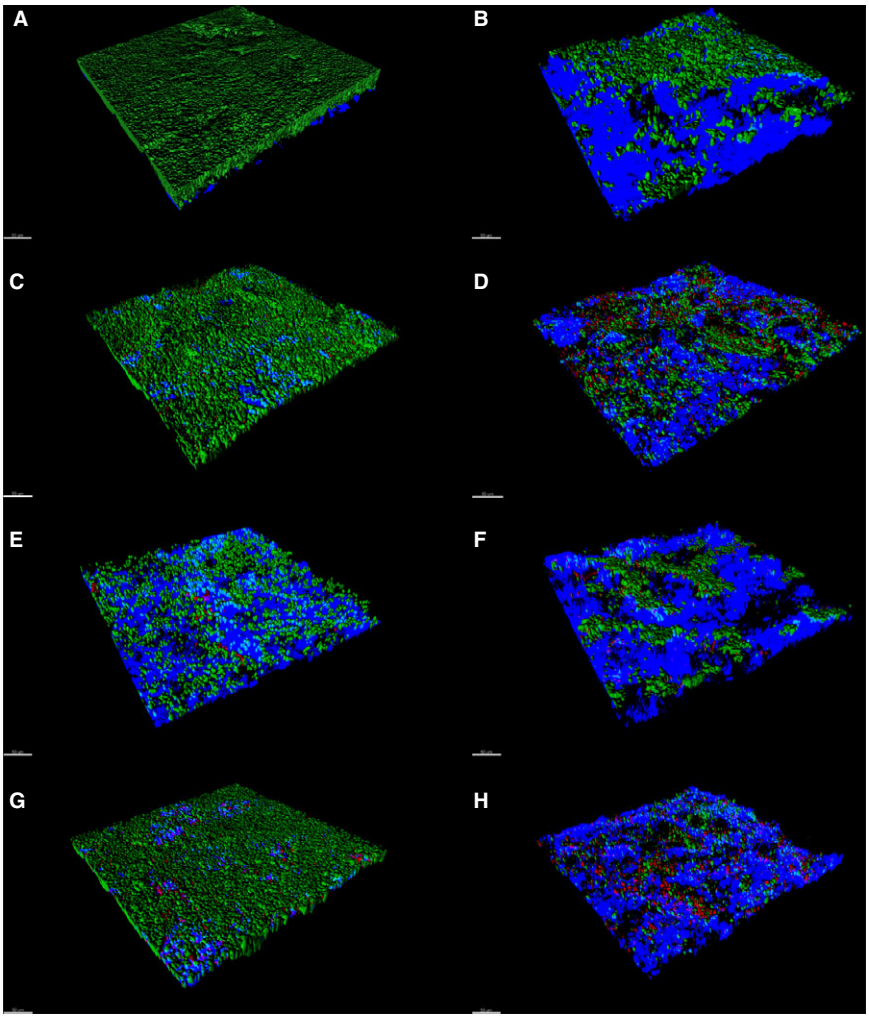

Figure 2 Three-dimensional reconstruction of microbial biofilms. The left panels (A, C, E, G) represent biofilms of *Pseudomonas aeruginosa* (A), *Staphylococcus aureus* (C), *Candida albicans* (E) and *Bacteroides fragilis* (G) before treatment, while the right panels (B, D, F, H) represent biofilms of *P. aeruginosa* (B), *S. aureus* (D), *C. albicans* (F) and *B. fragilis* (H) after treatment with 2 × MIC erythritol/chlorhexidine for 72 h. Green = viable cells, red = dead cells, blue = titanium. Scale bar = 50 μm.

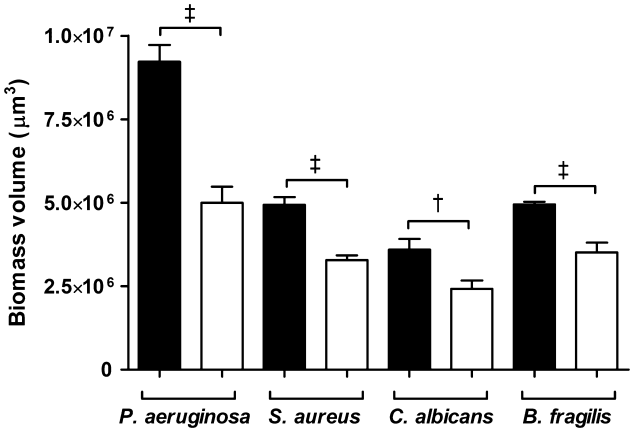

Figure 3 Total biomass volume quantified by confocal laser scanning microscope. The figure shows the total biomass volume of microbial biofilms before and after treatment with 2 × MIC erythritol/chlorhexidine for 72 h. Data are expressed as mean ± standard deviation. Black bars = controls; white bars = treated samples. †$0.001 < P < 0.01$, ‡$P < 0.001$.

Table 2 Composition of microbial biofilms in terms of live and dead cells

Microbial strain	*Before/after treatment*	*% live cells*	*% dead cells*
Staphylococcus aureus	Before	91 ± 3	9 ± 3
	After	45 ± 8	55 ± 8
Candida albicans	Before	93 ± 3	7 ± 3
	After	44 ± 18	56 ± 18
Bacteroides fragilis	Before	82 ± 18	18 ± 5
	After	31 ± 5	69 ± 5

Data are expressed as mean ± standard deviation.

Erythritol is a natural sugar alcohol used as sweetener in many countries. It is efficiently excreted by the kidneys, so it does not affect blood sugar or insulin levels and is unlikely to cause gastrointestinal side effects, as opposed to other sugar alcohols (21). Like the other sugar alcohols, erythritol is a 'tooth-friendly' sweetener and does not induce dental caries, probably due to the lack of fermentation of this sugar by oral microorganisms (22).

In this study, the evaluation of the antibiofilm activity of this air-polishing formulation was carried out by means of a spectrophotometric assay followed by CLSM analysis, adopting a well-established method developed in our laboratory (14, 15). To mimic the conventional surface of dental implants, sandblasted titanium discs were used as substrates for the development of biofilm. The choice of using clinical strains of *S. aureus, P. aeruginosa, B. fragilis* and *C. albicans* was made because all these species have been identified among microorganisms associated with peri-implantitis (23, 24).

A preliminary assessment of the MIC and of the MMC was carried out in order to evaluate the optimal concentrations to be used in the spectrophotometric and CLSM assays. The formulation showed both an inhibitory and a microbicidal activity against all the tested stains. The microbicidal activity may be due to the presence of chlorhexidine, as no microbicidal activity has been observed for erythritol yet. Available data only indicate an inhibitory effect of this sugar alcohol on the growth of several strains of streptococci *in vitro* (25, 26).

The results of the spectrophotometric assay showed that the treatment was able both to reduce the biofilm previously developed and to reduce biofilm formation over time on titanium surfaces *in vitro*. This formulation, especially at the 2 × MIC concentration, seems to be effective already after 6–12 h of incubation.

As observed from CLSM analysis, erythritol/chlorhexidine significantly reduced the total biofilm volume of all the microorganisms tested, with reductions ranging from 29% to 45%, depending on the strain under evaluation. These percentages are slightly lower than those obtained using the crystal violet assay. This difference may be due to the lower specificity of the spectrophotometric method, which only allows the measurement of the air-dried biomass, while CLSM analysis supplies more precise information, as it permits the three-dimensional visualization of living biofilms.

We also tested the antibiofilm activity of this air-polishing formulation in a previous study, observing a reduction of about 65% of the biofilm of *S. aureus*, *C. albicans* and *B. fragilis* at the spectrophotometric analysis (27). The experimental setting of our previous study was different from the present one, as the treatment was performed by means of an air-polishing device, so that the biofilm reduction observed could be partly due to the mechanical action of the water jet emerging from the tip of the handpiece. This bias was removed in the present study by directly dissolving the formulation into the culture medium.

The double with SYTO 9 and propidium iodide allowed the evaluation of the number of dead and live cells embedded into the biofilm of *S. aureus*, *B. fragilis* and *C. albicans.* SYTO 9 labels all bacteria in a population, while propidium iodide penetrates only bacteria with damaged membranes. Thus, bacteria with intact cell membranes (i.e. live) stain fluorescent green, whereas bacteria with damaged membranes (i.e. dead) stain fluorescent red. The percentage of dead cells was significantly higher after treatment, suggesting that the interference with cell viability is one possible mechanism of action by which this formulation is able to reduce the biofilm. In the case of *P. aeruginosa*, only SYTO 9 was used for staining of biofilm for CLSM analysis, so that a distinction could not be made between live and dead cells, limiting the biofilm evaluation to the determination of the total biomass volume. This choice was based on the previous observation that, in the case of *P. aeruginosa,* the quantification of the volume occupied by dead bacterial cells is hampered by the emission of non-specific signal due to EPS characteristics (15).

The mechanism underlying the antibiofilm of erythritol is poorly understood to date. Soderling and Hietala-Lenkkeri demonstrated that erythritol could decrease polysaccharide-mediated streptococci adherence contributing to plaque accumulation through a mechanism not dependent on growth inhibition (25). Hashino et al. (28) suggested that erythritol has inhibitory effects on *Streptococcus gordonii* and *Porphyromonas gingivalis* biofilm development through several pathways, including suppression of growth resulting from DNA and RNA depletion, attenuated extracellular matrix production and alterations of dipeptide acquisition and amino acid metabolism. Ichikawa et al. (29) showed that the addition of erythritol drastically enhanced the effect of an antifungal agent against *C. albicans* biofilm. They assumed that erythritol may play a role in dispersing biofilm and thus favouring the penetration of the fungicidal agents and weakening microbial attachment to surfaces.

It is unclear to what extent the antibiofilm activity of this air-polishing formulation may be attributed to the addition of 0.3% chlorhexidine. It is possible that the contribution of chlorhexidine to the antibiofilm activity of the formulation is due to its antimicrobial activity, which might explain the high percentage of dead cells observed with CLSM analysis. Further studies evaluating the effect of erythritol and chlorhexidine alone are required to get further insights into the mechanisms underlying the antibiofilm activity of this air-polishing formulation.

Possible limitations of the present study include the use of single-species biofilm grown on plain titanium surfaces, which might be more easily decontaminated than multi-species biofilm on screw-shaped dental implants (30). Further studies adopting multispecies models mimicking more closely the complex biofilm of dental plaque would be of great interest.

In conclusion, due to its antimicrobial and antibiofilm properties, the air-polishing powder consisting of erythritol and chlorhexidine might represent a promising approach for prevention and treatment of peri-implant diseases.

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Conflict of interest

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