Decoding bacterial dialogues in oral care: the power of quorum sensing in caries treatment and prevention

Òscar Expósito[1], Monika Buchholz[1], Alejandro Guirado[1], Ana Gallego[1], Maria Mas[1], Pau Riera[1], Sara Laplana[1], Tarik Ruiz[1], Sandra Ruiz[1], Ainhoa Bilbao[2], Felipe Goñi-de-Cerio[2]

[1]**Vytrus Biotech** S.A. C/ Sant Gaietà 121, 2nd floor. 08221 Terrassa, Barcelona (Spain); m.buchholz@vytrus.com

[2]**GAIKER**, Department of Biotechnology, Parque Tecnológico, Ed. 202. 48170 Zamudio, Spain.

Abstract (Maximum of 200 words)

Quorum sensing (QS) is crucial in the pathogenicity of dental caries and periodontitis, orchestrating bacterial communication within the oral microbiota. The complexity of the oral microbiome and the limitations of traditional treatments pose significant challenges in caries prevention. Our research investigates an innovative approach using a unique combination of Algae extract and Plant Phyto-complexes to target QS mechanisms and modulate oral microbiota dynamics. We conducted *in vitro* biofilm formation assessments with *Streptococcus mutans*, PCR assays for QS gene expression analysis, and ex vivo gingival and dental models to evaluate bacterial growth and inflammation. This biotechnological strategy demonstrated a significant inhibition of biofilm formation and QS activity, underscoring its bacteriostatic potential in caries prevention and treatment. Our findings emphasize the efficacy of natural compounds in disrupting bacterial communication pathways, presenting a novel, holistic strategy for maintaining oral health and combating antibiotic resistance in dental care. This breakthrough highlights the promise of leveraging QS modulation in transforming oral healthcare, heralding a new era in caries management and prevention.

Keywords: Algae extract Extract, Phyto-Complexes, Oral Care, Cariology, Quorum Sensing

INTRODUCTION

The human **oral cavity** hosts a highly diverse and abundant microbial community, comprising **over 700 different bacterial** species. This microbial composition and its implications for health and disease have been the subject of extensive research in recent years. Despite its significance, the oral cavity remains relatively understudied compared to the gut or skin, even though it contains one of the most unique microbial communities in the human body. **A milliliter of saliva contains approximately 10^8 microbial cells**, underscoring the importance of saliva in transporting microorganisms and signaling molecules **[8].**

In the field of cariology, **quorum sensing** has predominantly focused on the role of ***Streptococcus mutans*****, with *Candida albicans* also receiving some attention. *S. mutans* serves as a model organism for studying the interactions within quorum sensing networks**. Importantly, *S. mutans* belongs to the microbiota of the oral cavity, but when there is an increase in its population (dysbiosis), *S. mutans* turns virulent and begins forming biofilms. **These biofilms are a critical factor in the pathogenesis of dental caries, making the study of quorum sensing and bacteriostatic mechanisms within *S. mutans* particularly significant [5].**

The majority of oral diseases, including caries and periodontitis, result from **microbial dysbiosis** and biofilm formation. Consequently, recent research has emphasized the potential of quorum quenching (QQ), also known as **quorum sensing inhibition**, as a crucial bacteriostatic strategy for modulating the virulent behavior of *S. mutans*. This approach aims to **mitigate oral diseases by disrupting bacterial signaling networks**, thereby attenuating pathogenic activities. By interfering with quorum sensing mechanisms it is possible to effectively reduce biofilm formation and modulate bacterial virulence, offering a promising biological approach for the prevention and treatment of oral diseases **[5]. Dental carious lesions**, one of the most prevalent oral health issues globally, are intrinsically linked to various **salivary factors**, such as lowered pH, which causes tooth demineralization, and decreased concentrations of salivary calcium and phosphate ions, insufficient for remineralization. The acidic environment in the oral cavity is primarily driven by the presence of acidogenic bacterial species, notably *Streptococcus mutans.* ***S. mutans*** **thrives in the acidic environment of the oral cavity and contributes to the formation of dental biofilms**, commonly known as dental plaque. The bacterium metabolizes dietary sugars to produce lactic acid, which lowers the pH

in the oral cavity, leading to demineralization of the tooth enamel and subsequent cavity formation **[1].** The pathogenicity of *S. mutans* is largely attributed to its ability to produce extracellular polysaccharides from sucrose, which facilitate the adherence of bacterial cells to the tooth surface and promote the formation of robust biofilms. **These biofilms serve as a protective environment for the bacteria, making them more resistant to removal by mechanical cleaning and less susceptible to antimicrobial agents [2].**

The dominance of *S. mutans* in dental caries ethiology underscores the extensive research on **quorum sensing (QS) mechanisms [23-29]** within this species, aiming to identify QS inhibitors capable of attenuating caries development **[1,2,5,16].** Quorum sensing is a sophisticated bacterial communication system that regulates gene expression in response to population density. In *S. mutans*, QS is integral to biofilm formation and virulence, particularly in the context of dental plaque **[5].** QS mechanism consists of the release and detection of signalling molecules known as autoinducers, to communicate and orchestrate collective behaviours that enhance their survival and pathogenic potential **[2].** Autoinducer-2 (AI-2) is a signalling peptidic molecule utilized as a **universal signalling molecule by both Gram-positive and Gram-negative bacteria for interspecies communication**. In *S. mutans*, AI-2 also facilitates the quorum sensing process, enabling the bacteria to regulate gene expression in response to population density. AI-2 accumulation in the extracellular environment binds to a periplasmic receptor, initiating a signal transduction cascade that promotes gene transcription and virulence **[6, 18-20]. The process of QS occurs through AI signalling molecules**, which diffuse into the surrounding environment, making the concentration of these signaling molecules dependent on fluid flow. Research on *Pseudomonas aeruginosa* biofilms has shown that increased flow rates require higher biofilm biomass to induce QS, highlighting the importance of considering fluid dynamics in QS research **[7].** These insights are critical for developing effective QS inhibitors that can function under physiological conditions.

Saliva plays a crucial role in maintaining oral health by modulating the microbial environment, providing antimicrobial proteins, and aiding in the remineralization process. The link between saliva and QS research in biofilms is significant as fluid flow influences the distribution and concentration of QS signalling molecules, thus impacting biofilm formation and microbial communication. Understanding this relationship can lead to better strategies for disrupting harmful biofilms and promoting oral health **[8, 15].**

Also, **the oral cavity** is lined with a mucous membrane or **oral mucosa**, composed of two main tissue components: the stratified squamous epithelium, known as the oral epithelium, and the underlying connective tissue layer called the lamina propria **[3]. Collagen plays a crucial role in maintaining the structural integrity and function of the oral mucosa**, acting as a scaffold for tissue repair and regeneration. Increasing Pro-collagen levels in the oral cavity can enhance tissue resilience and accelerate healing processes, addressing the high turnover rate and mechanical stress the oral tissues endure **[4].** The **oral mucosa's superior healing capacity compared to skin** can be attributed to several factors, including its unique cellular composition and environment. Oral mucosa typically heals faster and with less scarring due to its high vascularization, rich supply of growth factors, and the presence of salivary components that facilitate rapid tissue repair. The extracellular matrix (ECM) in the oral mucosa contains a higher proportion of Type III collagen, which supports better cell migration and regeneration **[10]**. **The salivary environment provides constant moisture and a plethora of antimicrobial agents, creating optimal conditions for wound healing [11]**.

Therefore, the question remains: How can we revolutionize oral care by shifting our focus from traditional methods to harnessing the complex interactions within the oral microbiome? The use of **natural compounds in oral care** is gaining traction due to their biocompatibility and potential to mitigate adverse effects associated with synthetic agents. **Antioxidants**, in particular, play a vital role in the oral cavity. **They neutralize free radicals, reducing oxidative stress and inflammation, and promote the healing and regeneration of oral tissues [13, 14].** For instance, plant-based mouthwashes, such as those containing *Morinda citrifolia* (Noni) and *Ocimum sanctum* (Holy Basil), have demonstrated significant antiplaque properties and anti-inflammatory effects, making them viable alternatives to conventional mouthwashes. These natural extracts have shown efficacy in reducing pro-inflammatory cytokines, such as TNF-α, IL-α, and IL-β, in gingival crevicular fluid, further supporting their role in maintaining oral health **[9]**.

While conventional mouthwashes primarily exhibit bactericidal activity, eliminating a broad spectrum of oral bacteria, our approach aims to promote a bacteriostatic effect through quorum sensing (QS) mechanisms in *S. mutans*. By adopting a biological approach, we aim to disrupt bacterial signalling networks, thereby attenuating pathogenic activities without indiscriminately eradicating beneficial oral microbiota. This strategy not only

targets the virulence of S. mutans but also can maintains overall oral health by favouring the balance of the microbial community.
Therefore, leveraging our extensive expertise in plant science and biotechnology and microbiotas' quorum sensing mechanisms, **we have developed a biotechnological approach focusing on the unique combination of two types of actives based on Algae extract and plant-derived phyto-complex**. This approach targets oral microbiota dynamics with strong bacteriostatic and biofilm inhibitory activity on *S. mutans*, while also aiming to positively impact the inflammatory environment.

The **first active ingredient** in our research is derived from a **novel unicellular green Algae**. By optimizing the cell culture of this Algae extract, we have harnessed its unique ability to colonize diverse habitats. This unicellular organism, whether in its free-living form or in symbiosis with other microorganisms, employs various mechanisms to interact with its microenvironment. Consequently, it is hypothesized that it possesses systems capable of **modifying and controlling competing microorganisms**, **thereby making it a powerful tool for oral health**. Additionally, we anticipated other oral care activities, such as antioxidant activity and collagen stimulation, from this active ingredient.

Furthermore, we meticulously analyzed the **array of molecules produced by plants** in response to infection or damage. Our second active ingredient focuses on the combination of two specific plant molecules that can synergistically control microbial populations and potentially play a significant role in oral care.

The results demonstrated significant benefits, leading to the development of an active based on the **combination of Algae extract and Phyto-complex (Phyto-Algae blend) for advancing oral health care.** With the aim to harness the synergistic potential of the two actives and achieve a more realistic human tissue response, *ex vivo* dental models were utilized allowing us to observe bacterial biofilm modulation of the Phyto-Algae blend.

This innovative method represents a breakthrough in preventing and treating oral diseases, particularly caries, and introduces a new paradigm in oral care. **The integration of Algae extract and Plant Phyto-complex offers a comprehensive solution that not only targets quorum sensing pathways and reinforces mucosal defenses but also promotes a**

balanced microbial ecology. Our innovative approach is setting new standards in the field, demonstrating our commitment to leveraging nature's smart strategies for better oral health.

In conclusion, **the complexity of the oral microbiome** and the limitations of traditional antimicrobial treatments necessitate innovative approaches to caries prevention and treatment. **By leveraging the QS inhibitory potential of Algae extract and plant-derived phyto-complex, we aim to disrupt bacterial communication pathways**, offering a novel strategy for managing dental caries and enhancing oral health. Notably, this approach emphasizes regulating bacterial populations rather than eliminating them, aligning with recent insights into microbial management. Regulating populations can mitigate dysbiosis and maintain a balanced microbial environment, which could be crucial for long-term oral health **[30]. This study underscores the importance of integrating natural compounds into oral care regimens, providing a holistic approach to maintaining oral hygiene and combating antibiotic resistance [12,16,17].**

MATERIALS AND METHODS

In vitro screenings: anti-biofilm and antiquorum sensing assays

Streptococcus mutans was used for *in vitro* biofilm formation assessment, utilizing fluorescence staining and confocal microscopy for detailed visualization. Polymerase chain reaction (PCR) assays were selected for their precision in analyzing quorum sensing gene expression in *S. mutans*.

Anti-biofilm activity assay

Generation of representative biofilms was carried out on borosilicate glass coupons by immersion of the coupons in a suspension of microorganism with specific culture medium of Streptococcus mutans (DSMZ 20523) in the absence or presence of the compounds. The concentration used for both the Algae extract and the phyto-complex was 5%. The capacity of the compounds to avoid biofilm formation was assessed by evaluation of the anti-biofilm properties and study of the adhesion, growth and viability of biofilms on borosilicate glass coupons by quantification of viable cells attached to coupons (biofilm population) and the planktonic cells (free swimming microbial population).

Additionally, a fluorescence staining technique was employed, and live/dead biofilms were visualized using Laser Scanning Confocal Microscopy. All experiments were performed in triplicate.

Antiquorum Sensing activity assay

Polymerase chain reaction (PCR) assays were selected for their precision in analyzing quorum sensing gene expression in *S. mutans*. The expression of quorum sensing gene Autoinducer Peptide 2 (AI-2) was carried out in *S. mutans* bacterial cultures treated with Plant Phyto-complex and Algae extract at 5%. 10^5cfu·ml^{-1} of *S. mutans* was used as an inoculum and treated during 24h under anaerobic conditions with the actives. In parallel *S. mutans* suspension in absence of compounds were also incubated as control. After 24h the RNAs were extracted and thereafter the quantification and evaluation of the RNA quality obtained was assessed using the Agilent platform, Bioanalyzer 2100 (Agilent Technologies Inc, USA). Finally, the expression of the AI-2 gene was carried out by Retro-PCR-quantitative assay (RT-qPCR).

***In vitro* screenings: oral mucosa health protective effect**

In vitro cellular models were utilized to assess the efficacy of the Algae extract and the Plant Phyto-complex regarding their protective antioxidant effects on the oral mucosa, as well as their ability to promote Pro-collagen I synthesis and enhance wound healing properties.

Cytotoxicity

Fibroblasts were cultured in 96-well plates and treated with eight different concentrations of the products under study for 24 hours at 37°C and 5% CO2. Each experimental condition was tested in sextuplicate within a single experiment. Algae extract was tested at the following concentrations: 5% v/v, 3% w/v, 2.5% v/v, 2% v/v, 1% v/v, 0.5% v/v, 0.1% v/v, and 0.01% v/v. The phyto-complex was tested at the following concentrations: 4% v/v, 2% w/v, 1% v/v, 0.05% v/v, 1% v/v, 0.01% v/v, 0.1% v/v, and 0.01% v/v.

Following the 24-hour incubation with the test compounds, the cells were washed with phosphate-buffered saline (PBS) and stained with MTT solution. The plates were then incubated at 37°C for 2 hours. After this incubation period, the medium was removed, and 100 µL of DMSO was added to each well to solubilize the colored precipitate. The plates were kept

in darkness and agitated for 15 minutes. Absorbance was measured at 540 nm using an ELISA plate reader.

The percentage of cell viability was calculated using the formula:

% Cell viability = (Abs.(t)/Abs.(c) x 100

where Abs.(t) is the absorbance of the test sample, and Abs.(c) is the absorbance of the negative (untreated) control. The results were plotted on a graph against the concentrations of the test compounds to assess cell viability.

Antioxidant activity assay

The possible antioxidant effect of the actives was performed by exposing the fibroblasts cells to H_2O_2 (1mM) in presence or absence of the products to analyse treatment at two concentrations (at 2.5% and 0.5% v/v) of Algae extract and Plant Phyto-complex. After that period, 1µL of a 4mM carboxy-H2DFFDA solution was added to 400 µL of sample. Samples were then homogenized and incubated at 37°C for 30 minutes. ROS production was then evaluated by flow cytometry means (Cytomics FC500-MCL). The values obtained are the values corresponding to the median fluorescence intensity. The mean of the median from three biological replicates was then calculated.

Pro-collagen I production determination

Fibroblasts cells cultured in 96-well plates were treated with 2 different concentrations for Algae extract (at 5% and 0.5%) and Plant Phyto-complex (at 2.5% and 0.5%) for 72 hours (37°C, 5% CO2). After the incubation with the test products, cell supernatants were collected and frozen. Quantification of pro-collagen I was carried out by ELISA means following manufacturer's instructions. At least, three technical replicates of each independent experiment were analysed in the ELISA.

Wound healing activity assay

For this study, fibroblasts were cultured to confluence in 24-well plates using DMEM (Dulbecco's Modified Eagle's Medium, 1 g/L glucose; Lonza Biowhittaker, Lonza Ltd., Basel, Switzerland) supplemented with 10% (v/v) fetal calf serum (FCS; Labclinics, Barcelona, Spain), 1% (v/v) penicillin/streptomycin (Lonza Biowhittaker), and 2 mM glutamine from a 200 mM

stock solution (Lonza Biowhittaker) at 37°C in a humidified incubator with 5% CO2 and 95% humidity.
Upon reaching confluence, the cell monolayer was scratched with a sterile pipette tip to generate a wound approximately 2 mm in width. The remaining cells were washed twice with warm culture medium to remove cell debris, and then the product was applied to evaluate its healing activity. Wound closure was monitored by phase contrast microscopy using a TE200 phase contrast microscope (Nikon, Tokyo, Japan). Three replicates were measured for each treatment, and 10 images of each replicate were recorded using a DS-Fi1 digital camera with the control and monitoring unit DS-L2 (Nikon).

Photographs of the cultures were taken immediately after making the wound (T0 h) and at various subsequent times (from 12 h to 72 h). The healing process was evaluated by quantifying the reduction in wound area at different times using ImageJ software (open-source image processing program). The migration rate was calculated and expressed as fold change compared to the control growth, providing a quantitative measure of the wound healing efficacy of the tested compounds.

Ex vivo

For a more realistic human tissue response, *ex vivo* gingival epithelium and dental models were utilized to observe bacterial growth modulation and pro-inflammatory markers. These methods were chosen for their specificity and relevance in assessing the compounds' impact on oral health.

Model of *S. mutans* biofilm generated and inflammatory response on gingival epithelium
In an *ex vivo* model of gingival epithelium, a total of 20 gingival epithelia were used in this trial with 4 replicates per experimental group: 3 replicates for quantification of *S. mutans* and cytokine production (IL-6 and IL-8) and 1 replicate for Scanning Electron Microscopy (SEM) analysis. Experimental groups are described in Table 1. *S. mutans* was quantify by counting Colony Forming Units (CFU) after serial dilution and plating on agar medium. Quantification of IL6 and IL8 were carried out by ELISA means following manufacturer’s instructions. And Scanning electron microscopy (SEM) was done to visualize biofilm formation in the epithelia.

Experimental Groups	Day 1	Day 2
Gingivial Epithelium +No test product +No *S. mutans*	No Product + No *S. mutans*	Quantification *S. mutans* (UFCs) IL-6, IL-8 SEM Analysis
Gingivial Epithelium +No test product + *S. mutans*	Inoculation + *S. mutans* + No product	
Gingival Epithelium + *S. mutans* + Algae extract 5%	Inoculation + *S. mutans* + Algae extract 5%	
Gingival Epithelium + *S. mutans* + Plant phyto-complex 5%	Inoculation + *S. mutans* + Plant phyto-complex 5%	

Table 1. Experimental groups for the evaluation of compounds in an *ex vivo* model of *S. mutans* biofilm generated on a gingival epithelium. IL-6 and IL-8 production was also measured.

Model of *S. mutans* biofilm generated on hydroxyapatite discs in a simulated saliva environment: evaluation of anti-biofilm properties

A stock strain of Streptococcus *mutans* (DSMZ 20523), isolated from human carious dentine, was cultured on agar medium and incubated under specified conditions. Colonies from the culture were transferred to Anaerobic Basal Broth mixed with artificial saliva (1:1) and adjusted to a concentration between $1.5x10^8$ CFU/ml and $5x10^8$ CFU/ml.

To generate biofilms, the bacterial suspension was added to 12-well plates containing Hydroxyapatite (HA) discs and incubated in Brain Heart Infusion (BHI) broth with artificial saliva (1:1) under anaerobic conditions at 37°C for 48 hours. The anti-biofilm properties of the combination of the Algae extract and phyto-complex (Phyto-Algae blend) were evaluated in an *ex vivo* model of *S. mutans* generated on HA discs in a simulated saliva environment and added to the culture medium at time 0h. Quantification of biofilm on HA discs were performed using fluorescence imaging and direct viable cell counts.

SYTO® 9 and propidium iodide from the LIVE (green)/DEAD (red) Biofilm Viability kit, followed by visualization with Confocal Laser Scanning Microscopy (CLSM) was used for the biofilms imaging. A Zeiss LSM 700 with Axio observer Z1 inverted laser scanning confocal microscope (Zeiss, Jena, Germany), 10x Plan-Neofluar and 63x Plan-Apochromat (Oil immersion)

objective lens and appropriate diode lasers (488nm and 639nm) was used to visualize both live and dead cells. Finally, the density of the biofilm was determined by counting Colony Forming Units (CFU) after serial dilution and plating on agar medium.

Anti-biofilm activity was calculated as the percent reduction of the microbial cells when measuring CFU/ml resulting from contact with tested compounds using the following formulas:

reduction % (CFU /ml) = ((B-A) / B) x 100

Log10 reduction (R)= log10 (B) - log10 (A)

where,

A: mean of viable cells after contact time with tested compounds

B: mean of viable cells in Biofilm CONTROL after contact time

RESULTS

In vitro screenings: anti-biofilm and anti-quorum sensing assays

Anti-biofilm activity assay

Streptococcus mutans is a Gram-positive, facultative anaerobic bacterium commonly found in the human oral cavity, where it significantly contributes to the formation of dental plaque. While *S. mutans* is not typically found colonizing the gums, its elevated presence in saliva has been correlated with increased severity of periodontitis. To assess the anti-biofilm effect of the Algae extract and Plant Phyto-complex on *S. mutans*, biofilms were generated in borosilicate crystal coupons by immersing them in a suspension of *S. mutans*. Subsequently, the inhibition capabilities of the Algae extract and the Plant Phyto-complex were analyzed (Figure 1A).

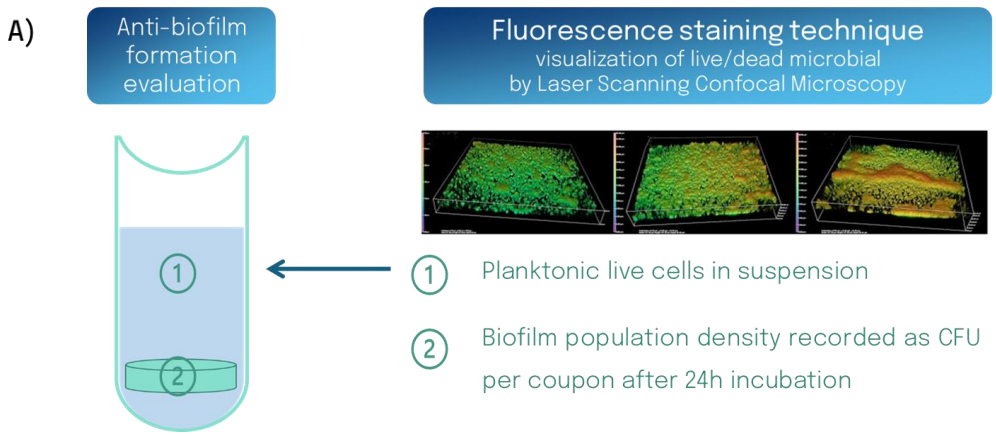

Figure 1 (A). Evaluation of the anti-biofilm formation and growth control of *S. mutans* on borosilicate glass coupons by fluorescence staining technique and visualization of live/dead microbial by Laser Scanning Confocal Microscopy with a quantification of planktonic cells in suspension and biofilm population Biofilm population density recorded as CFU after 24h incubation.

Figure 1 (B-C) showed that tested compounds, Algae extract and the Plant Phyto-complex, have a notable efficacy in both coupon-based and suspension-based assays. The obtained results for the coupon-based assay demonstrated that the Algae extract and the Plant Phyto-complex reduce the levels of Colony Forming Units (CFU) by 59.54% and 99.65%, respectively. For the suspension-based assay, the Algae extract and the Plant Phyto-complex reduced the levels of Colony Forming Units (CFU) by 96.92% and 97.37%, respectively, demonstrating that both compounds not only prevent biofilm formation but also effectively control the growth of *S. mutans*. The control group exhibited sustained growth from the initial inoculum to the day of measurement. In contrast, the treatment groups, which were exposed to the Algae extract and Plant Phyto-complex, showed lower growth rates compared to the control. Notably, there was no observed bacterial death, and a slight growth was still present in the treatment groups. This indicates that our active ingredients do not exhibit bactericidal properties, but rather exert a bacteriostatic effect by limiting bacterial growth. This is significant as it suggests that these compounds can control bacterial populations without completely eradicating them. By leveraging the QS inhibitory potential of Algae extract and Plant Phyto-complex, we aim to disrupt bacterial communication pathways, offering a novel strategy for managing dental caries and enhancing oral health.

The study underscores the importance of integrating natural compounds into oral care regimens. Such an approach provides a holistic method for maintaining oral hygiene and combating antibiotic resistance, thereby promoting a balanced microbial environment crucial for long-term oral health. This strategy aligns with the current understanding of microbial ecology and Quorum sensing strategies, where the regulation of populations rather than their elimination is favored to maintain a healthy microbiome **[30].**

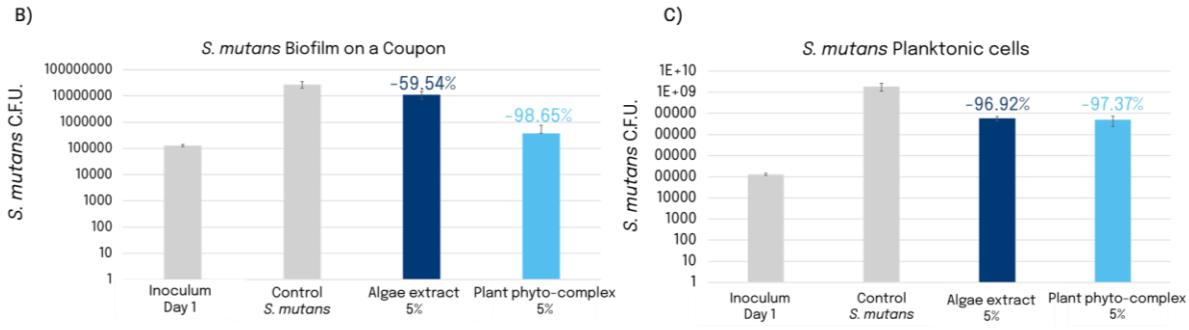

Figure 1 (B-C). Effect of the tested compounds on biofilm formation modulating capacity. Anti-biofilm evaluation of Algae extract at 5% and Plant Phyto-complex at 5% against *Streptococcus mutans*. Graph showing the behaviour of *S. mutans* planktonic cell population and biofilm cell population (CFU) after 5 days in presence of the test compound was conducted .

Overall, the findings suggest that the Algae extract and Plant Phyto-complex, not only inhibit the formation of dental biofilms but also regulates growth in planktonic cells, highlighting their promising application in oral care products aimed at preventing plaque formation and mitigating caries development.

Determination of the production of AI-2 by *S. mutans*

In this study, tested compounds, Algae extract and Plant Phyto-complex, were found to significantly reduce AI-2 expression in *Streptococcus mutans* after 24 hours of incubation. This reduction in AI-2, a key signal molecule produced as a signal indicative of Quorum Sensing for both Gram-positive and Gram-negative bacteria, highlights the efficacy of these compounds in disrupting bacterial communication **(Figure 2).** It is observed that the control has an increase in the in the AI-2 expression, indicating that QS is highly active during growth and virulence. When the compounds were added, it can be observed a reduction of 5.83-folds for the Algae extract and 5.75 folds for de Plant Phyto-complex compared to the control conditions. These exceptionally results indicate a substantial reduction in AI-2 production, demonstrating the significant impact of the treatment on quorum sensing inhibition.

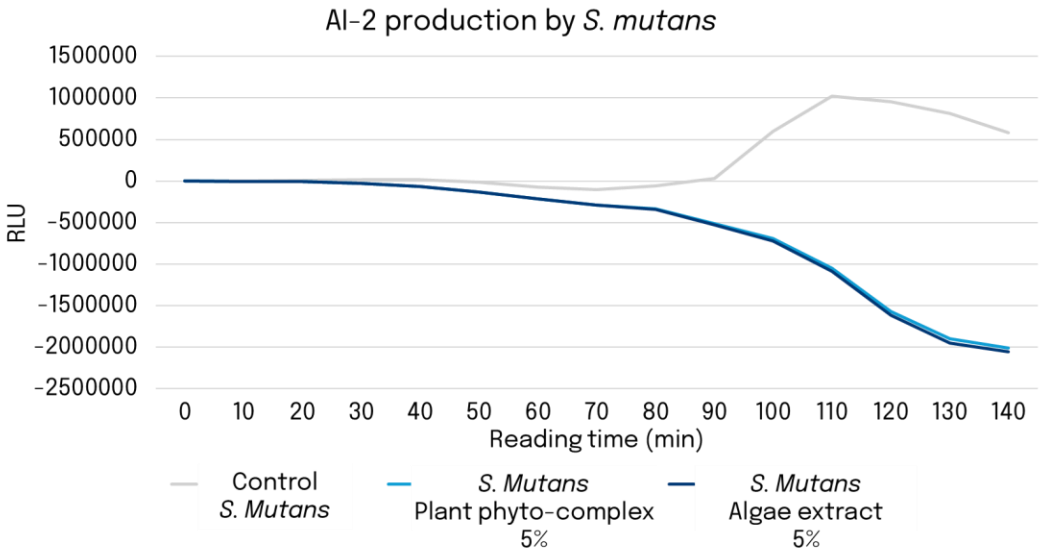

Figure 2. Production of AI-2 by *S. mutans* after 24 hours incubation with tested compounds.

Notably, Plant Phyto-complex and Algae extract respectively exhibited a high level of inhibition, indicating their superior potential in interfering with QS pathways in *S. mutans*. These findings suggest that these compounds are particularly effective in targeting and reducing QS activity, thereby potentially preventing biofilm formation and reducing bacterial virulence.

In vitro screenings: protective effect of oral mucosa health

Cytotoxicity assay

The cytotoxicity of the compounds was evaluated in fibroblasts. None of the compounds exhibited high cytotoxicity, indicating a favourable safety profile for these compounds. However, the Plant Phyto-complex at 4% concentration showed slight cytotoxicity.

Based on these observations, the concentrations for subsequent studies were selected to balance efficacy and safety. The low cytotoxicity profiles of these compounds underscore their potential as safe and effective agents for further investigation in anti-plaque and anti-caries applications **(Figure 3, A-B)**.

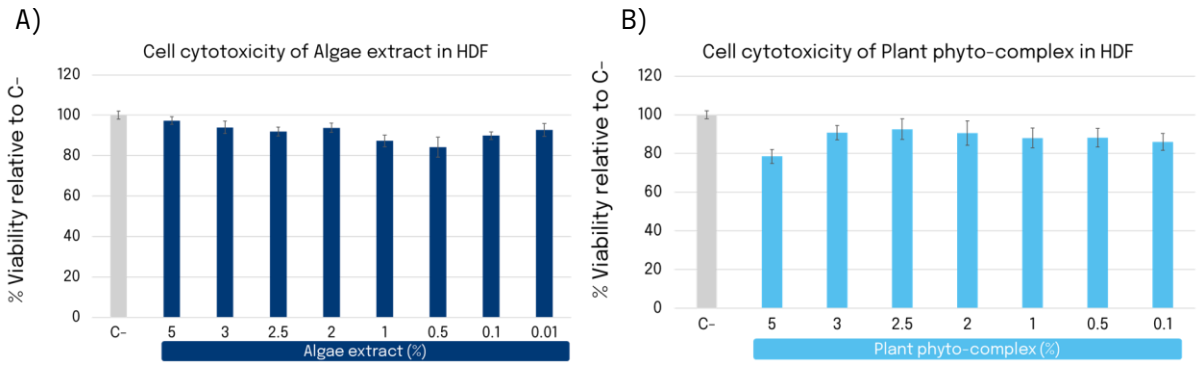

Figure 3 (A-B). Effect of the tested compounds on fibroblasts cell viability. Cells exposed to different concentration of the compounds under study (Plant Phyto-complex or Algae extract) for a period of 24 hours. Cell viability (metabolic activity) was measured by MTT means. Bars represent the average of the 6 technical replicates. Error bars represent the standard deviation. C- represents cells without any treatment.

Antioxidant activity assay

The tested compounds demonstrated significant antioxidant activity at a concentration of 2.5%, providing a clear protective effect against reactive oxygen species (ROS) production induced by H_2O_2 in fibroblasts. This protective effect highlights the potential of these compounds to mitigate oxidative stress under the conditions of the study **(Figure 4, A-B).**

Among the compounds tested, the Algae extract exhibited the most pronounced dose-response at the concentrations 2.5% and 0.5%. Specifically, the Algae extract showed an increase in antioxidant activity by 80.97% compared to the negative control. The efficacy at 2 different concentrations was comparable to that of the positive control, where cells treated with a known potent antioxidant, ascorbic acid (100 µM).

This significant increase highlights the potent antioxidant capabilities of the Algae extract in enhancing cellular defense mechanisms against oxidative stress.

On the other hand, Plant Phyto-complex was tested at 2 different concentrations and showed demonstrated an increase of the antioxidant activity by 30.3%, compared to the negative control. The efficacy at 2.5% was higher to that of the positive control.

In contrast, the negative control, where cells were not treated with any product, did not exhibit any protective effects against ROS production. These results underscore the potential of the

Algae extract, in particular, as effective antioxidants in protecting fibroblasts from oxidative damage.

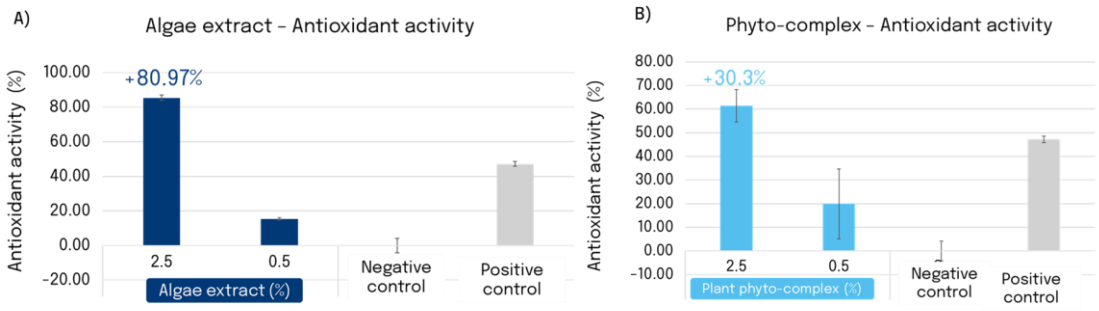

Figure 4 (A-B). Effect of the tested compounds on fibroblasts ROS production. Cells exposed to different concentration of the compounds under study (Plant Phyto-complex (B) and Algae extract (A)) for a period of 30 minutes. After that period of time the ROS production (Reductive Oxygen Species activity) was measured by Flow Cytometry Bars represent the average of the 4 technical replicates. Error bars represent the standard deviation. Control negative represents healthy cells without any treatment. C+ represents cells treated with antioxidant Ascorbic Acid 100 µM.

Pro-collagen I production determination

The efficacy of Algae extract and Plant Phyto-complex in promoting pro-collagen I production was evaluated. The 5% Algae extract enhanced a 59.84% pro-collagen I production compared to the negative control (C-). This was the most pronounced effect observed, highlighting the Algae extract as a potent stimulator of pro-collagen I synthesis.

In contrast, the combination of Plant Phyto-complex did not show a significant effect on Pro-collagen synthesis. However, at lower concentrations, the Plant Phyto-complex also exhibited a tendency to enhance Pro-collagen synthesis, with an increase of 29.93% compared to the negative control.

These findings underscore the potential of the Algae extract in significantly boosting collagen production, while the Plant Phyto-complex also shows some promise, particularly at lower concentrations.

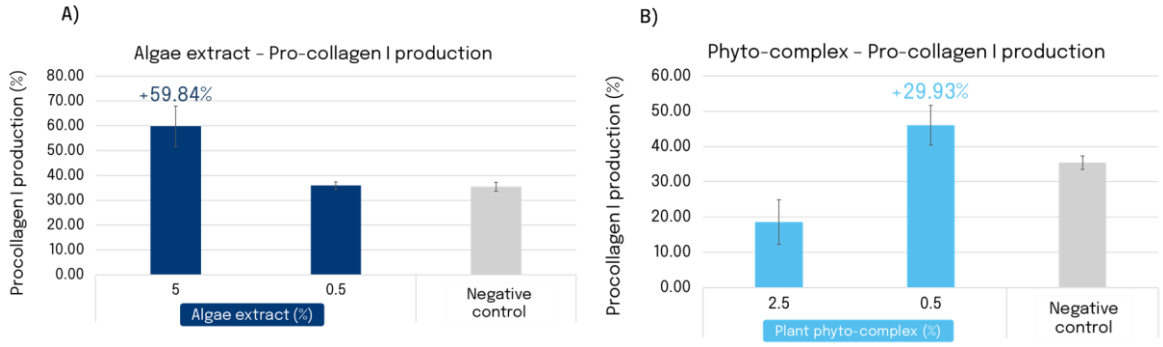

Figure 5. Effect of the tested compounds on the synthesis of Pro-collagen I by fibroblasts. Cells were exposed to different concentrations of the tested compounds for a period of 72 hours. After that period, Pro-collagen I secretion was measured by ELISA means. Bars represent the average of three technical replicates. Error bars represent the standard deviation. Negative control represents cells without treatment.

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Wound healing activity assay

The wound healing study was conducted using confluent fibroblast monolayers that were mechanically disrupted to create a cell-free area (scratch), following the described procedure. The wound healing process was evaluated by measuring the healed area (or wound area covered by cells) using Image J software. The mean healed area and standard deviation from ten images analyzed for each experimental condition are presented in **Figure 6**.

The wound healing activity of the tested compounds was assessed by determining the mean percentage increase in the healed area (or wound closure) compared to the non-treated control cells. The wound healing activity of the tested compounds was assessed by determining the mean percentage increase in the healed area (or wound closure) compared to the non-treated control cells. As illustrated in **Figure 6**, the Algae extract at a concentration of 0.1% significantly promoted wound healing, resulting in a 57.81% increase in the regenerated area compared to the negative control. Interestingly, lower concentrations of the Algae extract also demonstrated an improvement in wound healing, highlighting its efficacy at various dosages.

Conversely, although some trends were observed, the Plant Phyto-complex did not show a significant effect on wound healing (data not shown).

None of the tested compounds matched the efficacy of the positive control (C+), where cells were treated with a known wound healing agent, Epidermal Growth Factor (EGF) at 20 ng/ml. Nonetheless, the Algae extract exhibited a notable promotion effect in wound healing, underscoring the distinct effects of both ingredients analyzed.

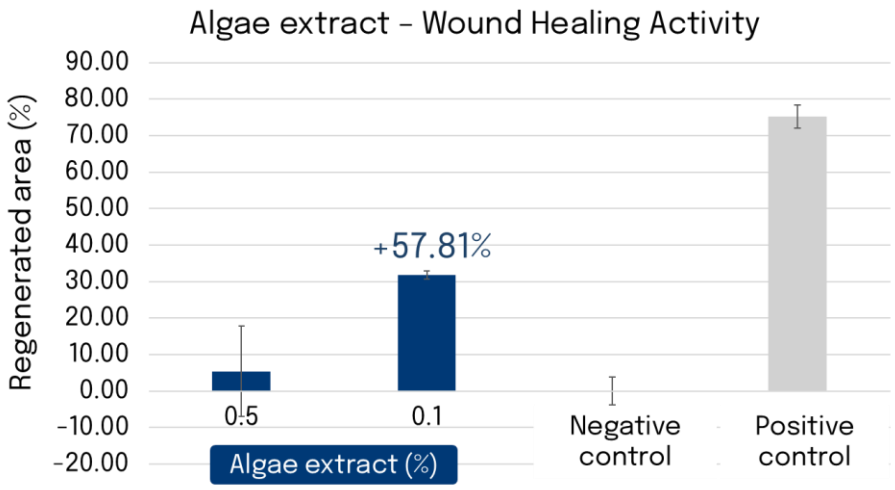

Figure 6. Evaluation of the wound healing activity of the Algae extract (0.5-0.1%) by means of scratch test on fibroblasts. Percentage of increase in healed area (%) after 24h vs negative control.

Ex vivo assays

Evaluation of compounds against *S. mutans* biofilm generated on gingival epithelium

Quantification of *S. mutans* in gingival epithelium

The study evaluated the inhibition of *Streptococcus mutans* colonization on gingival epithelium with and without the application of active compounds. The results in **Figure 7** indicate that Algae extract does not significantly affect the colonization of *S. mutans* on the gingival epithelium.

In contrast, the combination of Plant Phyto-complex demonstrated a notable reduction in the colonization of *S. mutans*. As observed in the SEM analysis, inhibition of colonization on the gingival epithelium is evident **(Figure 8).** These findings highlight the efficacy of the

combination of Plant Phyto-complex in reducing bacterial colonization. This suggests their potential as beneficial agents in preventing *Streptococcus mutans* colonization and, consequently, mitigating the risk of associated oral health issues.

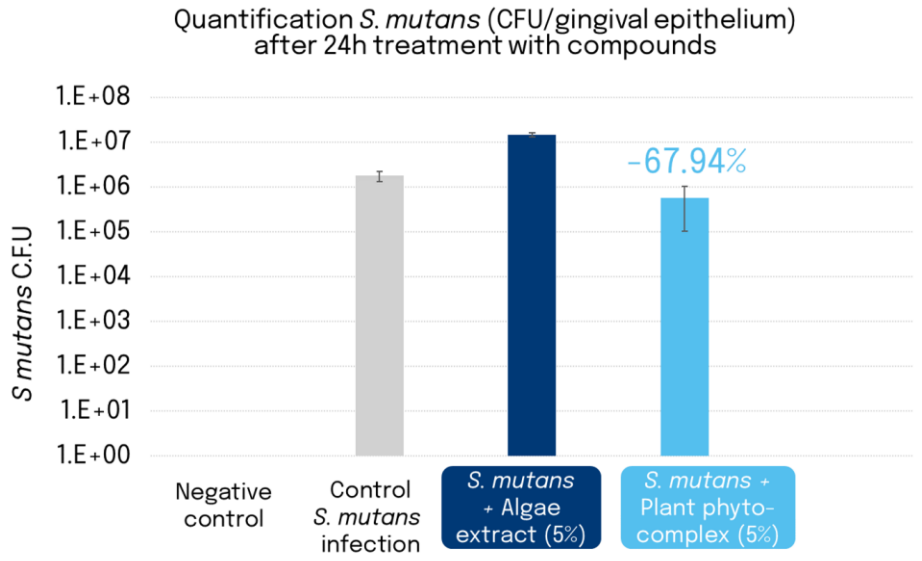

Figure 7. Quantitative measurement of microbial cells attached (biofilm) to gingival epithelium.

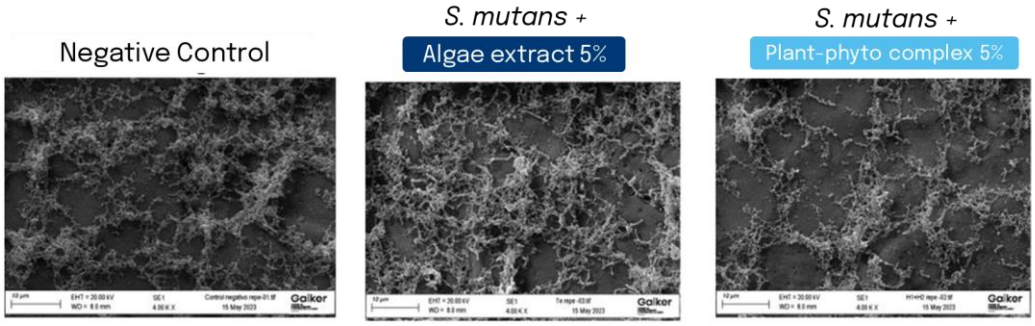

Figure 8. Images obtained by SEM. Coupon 1 with *S. mutans* Biofilm on gingival epithelium (63X objective) treated with Algae extract and Plant Phyto complex at 5%.

Production of IL-8 and IL-6

In our study, we aimed to observe a reduction in pro-inflammatory cytokines within the gingival epithelium, which is critical for mitigating inflammation and promoting oral health.

The Plant Phyto-complex was the only tested compound to demonstrate a notable reduction in the levels of pro-inflammatory cytokines, specifically IL-6 and IL-8. This reduction was

observed to be 60.02% for IL-6 and 62.61% for IL-8 when compared to the negative control. This indicates the robust anti-inflammatory properties of the Plant Phyto-complex, suggesting that not only seem to inhibit pathogenic mechanisms but also enhance the body's natural defense systems in the oral cavity.

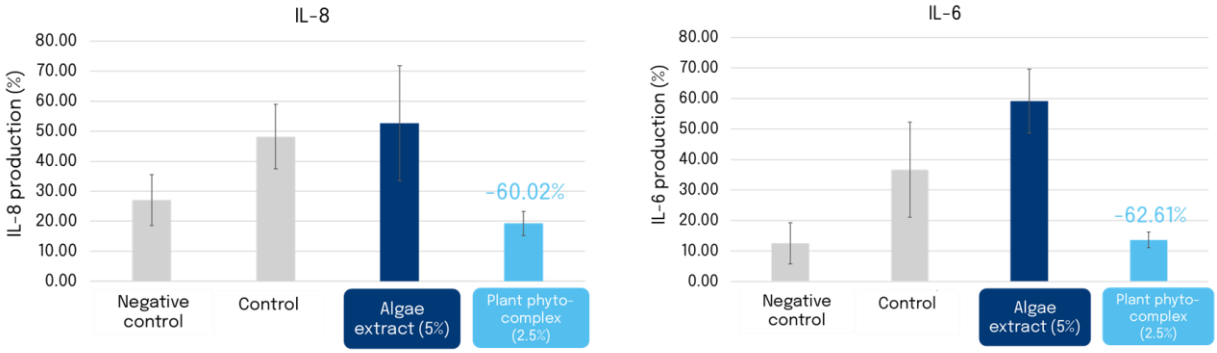

Figure 9 (A-B). IL-8 and IL-6 secretion on gingival epithelium under different concentrations for the test compounds. Negative control represents cells without stimulation. Positive control represents cells with LPS stimulation.

Evaluation of compounds against *S. mutans* biofilm generated on hydroxyapatite discs in a simulated saliva environment: Determination of biofilm adherence on the surface of HA coupons.

Based on the significant benefits demonstrated for the Plant Phyto-complex and Algae extract, we developed an innovative active ingredient for advancing oral care combining both of the compounds: the Phyto-Algae blend. The Algae extract demonstrates superior results in antioxidant activity, wound healing, and pro-collagen I production, while the Plant Phyto-complex excels in modulating *S. mutans* biofilm formation in gingival epithelium and significantly reducing inflammatory responses. Consequently, these compounds complement each other, offering a comprehensive solution that enhances oral health through different mechanisms. This synergistic approach leverages the distinct advantages of each ingredient, thereby providing a more effective and multifaceted strategy for oral health improvement.

With the aim to harness the synergistic potential of the two actives and achieve a more realistic human tissue response, *ex vivo* dental models, on a simulated saliva medium, was utilized, allowing us to observe bacterial growth modulation from the Phyto-Algae blend.

To evaluate the compounds in an *ex vivo* model of *Streptococcus mutans* biofilm generated on hydroxyapatite discs in a simulated saliva medium, the anti-biofilm properties were assessed by studying the adhesion and viability of the biofilms on HA coupons. This was done by counting Colony Forming Units (CFU). It important to emphasize that in this assay, we only aimed to assess the formation of biofilm on the tooth surface, as we had already tested the bacteriostatic effect on the planktonic cells in previous in vitro screenings, including anti-biofilm and anti-quorum sensing assays. This focused approach allowed us to specifically determine the compounds' efficacy in preventing biofilm formation on dental surfaces.

The effect of Phyto-Algae blend was particularly noteworthy. As shown in **Figure 10**, this combination demonstrated a complementary effect, significantly inhibiting biofilm density by 99.86%, as evidenced by the CFU counts. This result highlights the potential of the combined treatment to effectively reduce biofilm formation and improve oral health.

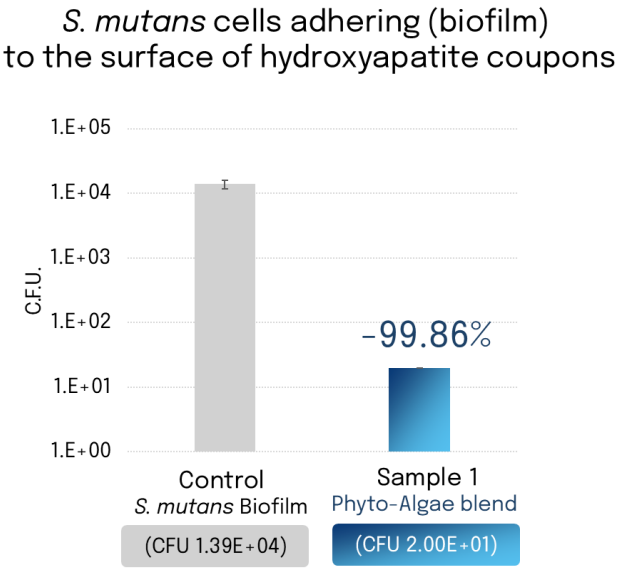

Figure 10. *S. mutans* cells adhering (biofilms) to the surface of hydroxyapatite coupons. The biofilm population density on hydroxyapatite (HA) discs was assessed by quantifying viable cells using the serial dilutions and plating method. The total number of live bacterial cells was determined by counting the Colony Forming Units (CFU) on the plates.

By means of fluorescent labelling and visualisation of the adhered cells by confocal laser scanning microscopy (Green: viable cells/Red: non-viable cells), the effect of the Phyto-Algae blend was assessed, as illustrated in **Figure 11**. It was observed that the control group

exhibited very high growth and nearly 100% viability of *S. mutans* (A). In contrast, treatment with the Phyto-Algae blend significantly reduced both the growth and viability of *S. mutans* (B).

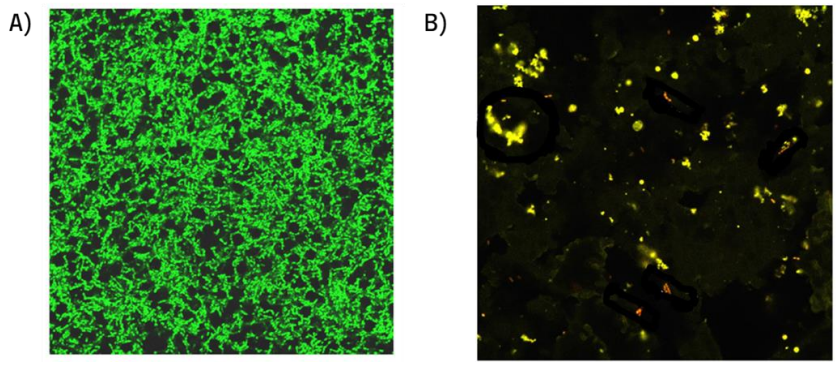

Figure 11. Effect of the combination of Phyto-Algae blend extract assessed by Confocal laser microscopy. (A) *S. mutans* biofilm control group growth exhibited nearly 100% viability. (Green: viable cells/Red: non-viable cells). (B) S*. mutans* biofilm treated with Phyto-Algae blend exhibit a reduction in the biofilm formation.

DISCUSSION

Our comprehensive study yielded several key findings, highlighting the **efficacy of the combination of Algae extract and phyto-complexes** in transforming oral care. These results underscore the potential of these natural compounds in preventing and mitigating various aspects of oral diseases, particularly those caused by *Streptococcus mutans*.

One of the most significant outcomes of this study was the observed **reduction in biofilm formation by *S. mutans in vitro* (Figure 1, B-C).** The control group exhibited sustained bacterial growth from the initial inoculum to the day of measurement. In contrast, treatment groups exposed to the Algae extract and Plant Phyto-complex showed lower growth rates compared to the control, with no observed bacterial death and slight growth still present. This indicates that the active ingredients exert a bacteriostatic effect by limiting bacterial growth without eradicating them. **Leveraging the QS inhibitory potential of Algae extract and Plant Phyto-complex**, this approach disrupts bacterial communication pathways, offering a novel strategy for managing dental caries and enhancing oral health. The study underscores the importance of integrating natural compounds into oral care regimens, providing a holistic

method for maintaining oral hygiene and combating antibiotic resistance. This strategy promotes a balanced microbial environment crucial for long-term oral health, aligning with current microbial ecology and quorum sensing strategies that favor regulating populations over their elimination **[30]**. Also, the results obtained indicate that the tested Algae extract and phyto-complex are highly effective in preventing the initial stages of caries development **(Table 2). While *S. mutans* is not typically found colonizing the gums, its elevated presence in saliva has been correlated with increased severity of periodontitis [21,22]. Overall, the findings suggest that the Algae extract and Plant Phyto-complex, not only inhibit the formation of dental biofilms but also reduce bacterial growth (it has been demonstrated *in vitro* and *ex vivo* in gingival and dental models), highlighting their promising application in oral care products aimed at preventing plaque formation and mitigating caries development.**

Additionally, there was a noticeable **decrease in quorum sensing (QS) activity, specifically in the levels of autoinducer peptides (AI-2),** which are key molecules in the QS pathways of *S. mutans*. This **disruption of bacterial communication pathways** is vital in mitigating the progression of oral diseases. The reduction in AI-2 expression by the Algae extract and the Plant Phyto-complex underscores their potential in interfering with QS and thereby preventing biofilm formation and reducing bacterial virulence. This highlights the **importance of saliva in the effectiveness of these compounds,** since the process of QS occurs through AI signaling molecules, which diffuse into the surrounding environment making the concentration of these signaling molecules dependent on saliva flow **(see Figure 2)**. These insights are critical for developing **effective QS inhibitors that can function under physiological conditions**, then saliva influences the distribution and concentration of QS signaling molecules, thereby impacting biofilm formation and microbial communication **[14,15].** Understanding this relationship is critical for developing effective strategies to disrupt harmful biofilms and promote oral health **[17].**

The results of both, the biofilm and quorum sensing (QS) assays, **collectively demonstrate that the active compounds, comprising Algae extract and Plant Phyto-complex, effectively modulate bacterial growth without exerting a biocidal effect.** These findings are significant as they highlight the ability of these compounds to regulate bacterial populations and virulence through QS mechanisms.

In vitro cellular models revealed that the application of the active ingredients led to a significant **increase in antioxidant response (Table 2)**. This is crucial for maintaining oral mucosal health, as oxidative stress is a major contributor to the degradation of oral tissues. The *in vitro* study also highlighted the efficacy of the compounds in **promoting collagen synthesis**. Specifically, the Algae extract significantly increased collagen production, which is essential for maintaining the structural integrity of the oral mucosa. The **enhanced collagen production by the Algae extract underscores its potential in promoting tissue repair and regeneration**, which is vital for oral health **[3,4].** Furthermore, the wound healing assay indicated the **Algae extract improved wound healing at low concentrations (Table 2)**. The higher potential of the Algae extract compared to the Plant Phyto-complex in these in vitro assays justifies the development of an active based on the **combination of the Algae extract and the Phyto-complex (Phyto-Algae blend) for advancing oral health care,** with the aim to harness the complementary potential of the two actives and achieve a more realistic human tissue response on *ex vivo* dental models.

Finally, *the ex vivo* gingival epithelium model was utilized to observe *S. mutans* bacterial growth modulation and pro-inflammatory response **(Table 2)**. These methods were chosen for their specificity and relevance in assessing the compounds' impact on oral health. **In gingival models**, we observed that the **Plant Phyto-complex effectively avoided biofilm formation and reduced inflammation**, whereas the Algae extract did not demonstrate significant anti-biofilm or anti-inflammatory effects. **The Plant Phyto-complex showed a pronounced reduction in pro-inflammatory cytokines, specifically IL-6 and IL-8**, underscoring its potential in mitigating inflammation and enhancing the body's natural defense mechanisms in the oral cavity. This indicates the **robust anti-inflammatory properties of the Plant Phyto-complex and its ability to inhibit pathogenic mechanisms**.

Once we had all the results, it became evident that **combining the Algae extract and Plant Phyto-complex** would provide a comprehensive solution for oral care. The **Algae extract exhibited significant antioxidant activity, enhanced collagen synthesis, and wound healing properties**. Meanwhile, the **Plant Phyto-complex demonstrated strong anti-biofilm formation and anti-inflammatory effects**. Combining these two compounds could potentially offer a **synergistic approach to controlling dental plaque**. To validate this hypothesis, we tested the combination (Algae extract plus phyto-complex) in dental epithelium models, where

the **Phyto-Algae blend effectively reduced *S. mutans* colonization on hydroxyapatite discs within a simulated saliva environment**. By inhibiting biofilm formation on hydroxyapatite discs, these compounds help prevent the establishment of *S. mutans* on tooth surfaces, which is a critical factor in caries progression **[1,2].** This significant reduction in colonization highlights the potential of the Phyto-Algae blend as a beneficial agent in preventing bacterial colonization and mitigating the risk of associated oral health issues. This innovative combination leverages the distinct benefits of both components to provide a holistic and effective approach to oral care **[7,11,12].**

In conclusion, the findings of this study underscore the transformative potential of the resulting combination of the Algae extract and Plant Phyto-complex in oral care, **exhibiting significant anti-biofilm, quorum sensing inhibitory, antioxidant, and collagen-promoting activities, with minimal cytotoxicity so minimizing the potential negative side effects.** Their integration into oral care products could lead to innovative and effective solutions for preventing dental caries, enhancing wound healing, and promoting overall oral health. Further research and development are warranted to fully harness the potential of these natural compounds in oral health applications.

Efficacy assays *in vitro & ex vivo*	Algae extract	Plant-phyto complex	Algae-Phyto blend
Anti-biofilm coupon (5%)	↓59.54%	↓98.65%	-
Anti-biofilm plaktonic (5%)	**↓96.92%**	↓97.33%	-
Anti-Quorum Sensing (5%)	↓583.88%	↓575.67%	-
Cytotoxicity	Non-cytotoxic	Non-cytotoxic	-
Antioxidant activity	2.5% (85%)	Light at 2.5%	-
Pro-collagen I	Good at 5%	Light at 0.5%	-
Wound healing activity	Light at 0.1-0.5%	-	-
Gingival Biofilm (5%)	-	↓67.94%	-
Gingival IL-8 (5%)	-	↓60%	-
Gingival IL-6 (5%)	-	↓62%	-
Hydroxyapatite Biofilm	-	-	↓99.86%

Table 2. *In vitro* and *ex vivo* assays summary.

CONCLUSION

Our findings reveal that Algae extract extracts and **plant-derived phyto-complexes offer a promising new avenue in oral care.** By targeting quorum sensing pathways and reinforcing mucosal defenses, these compounds provide a dual strategy against caries. This represents a paradigm shift from conventional antimicrobial strategies**, promoting a balanced microbial ecology rather than indiscriminate eradication.**

The innovative concept of leveraging quorum sensing disruption to condition the oral microenvironment presents an integrated approach to managing oral microbiota. The demonstrated efficacy of **Plant Phyto-complex and Algae extract in reducing biofilm formation and quorum sensing (QS) assays collectively demonstrate that the active compounds, effectively modulate bacterial growth and regulate bacterial populations and virulence through QS mechanisms without exerting a biocidal effect**. Consequently, this regulatory capability, combined with their demonstrated efficacy in enhancing collagen synthesis and mitigating oxidative stress, highlights the potential of these compounds as powerful agents in oral healthcare. This comprehensive approach not only **addresses microbial imbalances but also supports the structural and functional integrity of oral tissues, offering a multifaceted strategy for maintaining optimal oral health**.

As we look towards the future, a pivotal question remains: Could this biotechnological approach be the key to unlocking a new era in cariology and broader oral care enhancement? **The integration of these natural compounds into oral care formulations could pave the way for advanced, effective solutions that promote dental health and prevent oral diseases, marking a significant advancement in the field of dentistry**. The promising results encourage further research and development, underscoring the potential for a transformative impact on oral health practices.

CONFLICT OF INTEREST STATEMENT

NONE.

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