

# The “Vitaminic-strategy” Against the Oral Bacteria *S. Mutans* and *F. Nucleatum*, Agents of Caries and Halitosis

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## Research Article

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# Abstract

## Background

The oral cavity is one of the most complex human body environments. Indeed, the continuous variation of this habitat conditions reflects the high dynamism of the resident microbial community. Two key actors in the oral diseases are the bacteria *Streptococcus mutans* and *Fusobacterium nucleatum*, both implicated in the formation of oral biofilms and consequently in the generation of common pathologies such as caries and various gingival and soft tissue inflammation diseases. In addition, *F. nucleatum* is also implicated in the halitosis phenomenon, thanks to its demonstrated ability to produce as second metabolite the hydrogen sulphide (H<sub>2</sub>S), one of the volatile sulphur compounds (VSCs) that, with methyl mercaptan (CH<sub>3</sub>SH) and the dimethyl sulphide (CH<sub>3</sub>SCH<sub>3</sub>)<sub>24</sub>, is produced by periodontopathic anaerobic bacteria and causes the awkward bad breath in halitosis patients.

## Methods

In this study, the oral preparation Veal<sup>®</sup> Oris constituted only by vitamin E and capric/caprylic acid was evaluated as a potential treatment of caries and periodontal diseases; the effect of the product at different concentrations on the growth and the ability of both strains to form biofilm was investigated. Regarding to *F. nucleatum* also the influence of Veal<sup>®</sup> Oris on the production of H<sub>2</sub>S was evaluated.

## Results

Our *in vitro* results suggested that the Veal<sup>®</sup> Oris treatment could considerably reduce the growth and biofilm formation of both *S. mutans* and *F. nucleatum*. For *F. nucleatum* an appreciable reduction of the H<sub>2</sub>S production can be also obtained.

## Conclusions

Overall, this study highlighted the potential of Veal<sup>®</sup> Oris as a more “natural” adjuvant to prevent the biofilm and plaque formation and to reduce the smelly odour of halitosis.

## Background

The oral cavity is one of the most complex environments in the human body due to the continuous and simultaneously variation of homeostasis conditions. Over 700 diverse bacterial species have been identified as potential/usual colonizers of the many niches of the mouth, i.e., the surface of teeth and all the soft tissues of the oral mucosa such as the gingiva, the cheek, the tongue, the palate and the lips (1–3). The bacterial composition of the oral microbiota is responsive to different host intrinsic and host extrinsic factors. The host intrinsic factors are those factors not modulable by the host belonging to individually genetic factors (e.g., ethnicity, gender, host immune response, medical conditions, etc.), characteristics of the oral cavity such as the temperature, the saliva composition (pH, buffer capacity,

hormones, and secreted substances) and characteristics of the attachment surfaces (e.g., roughness of tooth surface). The extrinsic factors come from the external environment, habits and lifestyle, are modulable or relatively modulable by the host and regard the hygiene, the diet, the use of drugs, smoking, medications, sex, access to dental care etc. (4–7). All these factors contribute together and along the host ageing to establish in the oral cavity the conditions suitable for the colonization and the growth of different pools of microorganisms. As a result of this complex interaction, the balance between beneficial and pathogen species is continuously altered, passing from a state of symbiosis and health to a dysbiotic state with the upcoming of oral pathologies such as caries, gingivitis and periodontitis (2,4).

Nevertheless, the new metagenomic approaches represented a vital advantage to unravel the correlations between different types of *stimuli* in the oral cavity and the enrichment of specific bacterial species, with particular interest for those species that increase their growth as cause/effect of oral diseases (3,8). Also, through sequencing methods, the relationship between beneficial and pathogenic species of the oral cavity has been extensively studied as well as the composition of the polymicrobial biofilm assemblies detected in the oral cavity on the tooth, on the gingiva or on the tongue surface, and in minor part, on other soft tissue (7). The biofilm structure constitutes a protect sub-environment in which various bacterial species interact to protect themselves from the possible surrounding perturbations and thus represents a survival strategy of cells, whose formation is often difficult to inhibit or to remove (9–11). In the oral cavity, the biofilm causes the onset of the most prevalent infectious diseases, such as the plaque and dental caries and inflammation phenomena regarding the gingiva, which cause gingivitis, periodontitis, etc. (7,12). The formation of oral biofilm is arranged on the following subsequent three stages of attachment, colonization and biofilm development (13). The involved species are identified as “early” and “late” colonizers. The early colonizers belong to the streptococci genera; among them, *S. mutans* is particularly enriched in supragingival plaque, in childhood caries and dentinal and root caries lesions. Indeed, thanks to its rapid metabolism and strong acid tolerance it is recognized as the most cariogenic bacteria of the oral cavity (6,12,13). The early colonizer species are involved in the first two stages of biofilm formation. Since their ability to bind salivary proteins, they adhere to nascent hard or soft tissues, colonize the oral niches and provide a substratum for subsequent colonizers (6,13,14). Between the first and late colonizers, the Gram-negative obligate anaerobe *F. nucleatum* is considered a key actor for the stage of maturation of the oral biofilm. Thanks to its coating adhesion molecules and polysaccharide receptors, it is able to co-aggregate and bind both with the streptococcal early colonizers and the late colonizers, mainly Gram-negative anaerobes belonging to the genera of *Bacteroidetes* and *Spirochaetes* (13–15). Apart from the bridge role in the bacterial network of biofilm assemblages, *F. nucleatum* plays an active role as a periodontal pathogen, as demonstrated by its enhanced prevalence within the deep periodontal pockets. Moreover, in these niches, *F. nucleatum* triggers the production of matrix metalloproteinases by the host contributing initially to the periodontal inflammation and the onset of irreversible periodontal diseases (15). This pathogen is also characterized by a significant haemolytic activity and the ability to produce hydrogen sulphide (H<sub>2</sub>S). In particular, the H<sub>2</sub>S, similarly to the methyl mercaptan (CH<sub>3</sub>SH) and the dimethyl sulphide (CH<sub>3</sub>SCH<sub>3</sub>)<sub>24</sub>, is one of the volatile sulphur compounds (VSCs) produced by periodontopathic anaerobic bacteria which cause the typical malodour of the

halitosis (16–20). Consistently, the uncomfortable phenomenon of halitosis is often associated with the increase of VSCs producing species, in primis, *F. nucleatum* but also *Porphyromonas gingivalis*, *Treponema denticola*, *Prevotella intermedia* and *Eubacterium* (16,17,19,20). Actions aimed to reduce the presence of *F. nucleatum* in the oral cavity could represent an excellent double-sides strategy to reduce both the formation of the oral biofilm causative of plaque and other frequent oral diseases and the uneasiness associated with the halitosis. An even more efficient approach to massive prevent/reduce the development of oral pathologies could be represented by the simultaneous inhibition of *S. mutans* as the principal first colonizer of the oral biofilm. However, at the current point of knowledge, lifestyles modulation together with the use of innovative cosmetics or medicals are the only strategies to maintain or restore a balanced microbiome and the health of the oral cavity (2,4).

Indeed, mouthwashes and many dentistry products with antimicrobial compounds such as chlorhexidine, triclosan, cetylpyridinium chloride and chlorine dioxide are often used to treat the bad breath and to inhibit/disrupt the oral biofilm (17,18). However, the use of such products is always more often associated with the increase of the bacterial resistance as well as can cause the simultaneous killing of non-pathogenic commensal species with the consequent onset of undesirable dysbiosis states. The use of natural products is a more appreciated strategy to prevent and contrast the oral pathologies, even more if those substances impact more selectively on the pathogen species of the buccal microbiota. Moreover, the major acceptance of a “natural therapy” could modify the applicability of the therapy to a wider range of patients for ages and medical conditions, not only as curative but also as preventing therapy, both in paediatric and advanced ages. Products such as propolis, cranberry, tea, *Galla chinensis*, grapes, coffee, and cacao containing polyphenols have already demonstrated their activity against the oral biofilm, as well as hinokitiol, green tea powder, and eucalyptus extract made against the oral malodour (17,18).

Further, since it was found that oral disorders and pathologies are also associated with the oxidative stress that alters the microbial balancing, the activity of antioxidant substances against the oral bacteria has been studied as well. Well known non-enzymatic antioxidants are able to neutralize the Reactive Oxygen Species (ROS) causative of many oral pathologies. They are fat-soluble vitamins (vitamin A, vitamin E-tocopherol and b-carotene), water-soluble vitamins (vitamin C and vitamin B complex), trace elements (zinc, magnesium), and bioflavonoids (plant-derived) (21). The antioxidant compounds are generally assumed through a balanced diet with consumption of fruits and vegetables that contain considerable levels of those; interestingly, their presence is sensibly minor in individuals with oral diseases (4,21,22).

Regarding vitamin E, it is the major fat-soluble antioxidant in all cell membranes, exerts some anti-inflammatory properties and enhances the humoral immune response. Its positive effect on the oral health has been already documented with improvements of all periodontal parameters, principally the decreasing of plaque index and biofilm formation, of probing depth, clinical attachment level, and bleeding on probing (21–23).

Therefore, in this study, we *in vitro* tested the innovative product Veal<sup>®</sup> Oris against *S. mutans* and *F. nucleatum*, the two bacteria causatives of the most recurrent oral diseases. This product is composed of only two components, vitamin E as alpha-tocopherol and Caprylic/Capric Triglyceride obtained from coconut oil and glycerine. The effect of Veal<sup>®</sup> Oris at different concentrations was evaluated both on the growth and biofilm formation of *S. mutans* and *F. nucleatum*, and for *F. nucleatum* also on the production of the volatile compound H<sub>2</sub>S.

## Methods

The strains tested in the present study were *S. mutans* DSM 20523 and *F. nucleatum subsp. vincentii* DSM 19507. The product tested was the Veal<sup>®</sup> Oris (by Hulka s.r.l.).

The strains were cultured in Columbia Broth at the temperature of 37°C for 48 hours. The Optical Density at 600 nm (600 nm O.D.) of grown cultures was measured and dilutions were prepared to obtain 4 ml inocula with a starting O.D. of 0,1. For each strain, aliquots of 150 ul were distributed into the wells of a 96 well plate both without and with Veal<sup>®</sup> Oris at concentrations of 5, 10 and 20% v/v (50, 100 and 187 ul/ml, respectively) (Fig. 1, yellow and green wells). Also, experimental controls were produced with only Columbia Broth medium not added and added with the same concentrations of Veal<sup>®</sup> Oris (Fig. 1, grey wells). Each treatment was reproduced in triplicates and triplicates of 96 well plates were assessed. Liquid sterile paraffin was used to cover the surface of each inoculated well and to provide the suitable condition of oxygenation, also to standardize the growth conditions.

The plates were sealed, and the O.D. at 600 nm was measured at starting point (T0) using the VICTOR X5 multilabel plate reader (Perkin-Elmer). The O.D. measurement was performed in triplicates, and the O.D. values were calculated as mean values. After the first measuring, the plates were incubated at 37°C. The O.D. measuring was performed again after 24 and 48 hours (T24 and T48) to estimate the growth variation. The values obtained for strains without and with Veal<sup>®</sup> Oris were normalized for values measured for the respective controls, i.e., broth inoculated without or with the different concentrations of the tested product. The growth of strains inoculated with 5, 10 and 20% v/v of Veal<sup>®</sup> Oris was then compared with the growth obtained without the adding of Veal<sup>®</sup> Oris, and the concentration-effect of the product was evaluated (ANOVA, Tukey HSD with  $p < 0.05$ ).

After the last O.D. measuring, at 48 hours of incubation, to determine the rate of biofilm produced under Veal<sup>®</sup> Oris treatments respect to the cultures without the product, the inoculated wells were subjected to the staining procedure suggested by Stepanovic et al. (2000), with few experimental modifications (24). The wells were emptied and washed three times using 250 ul of 0.9% NaCl. A volume of 200 ul of methanol solution was added to each well and maintained in incubation for 15 min to fix at the plastic surface of wells the adhering cells. The methanol solution was discarded, and the plates were dried under the biological laminar flow in an upset-down position. Subsequently, 200 ul of crystal violet solution (Gram staining kit, Biolife Italiana srl) were added and maintained in incubation for 5 min. The staining solution was removed firstly by aspiration and then washing the plates under a moderate tap water flow.

The dye entrapped into the adherent cells was resolubilized, adding 160 ul of 33% glacial acetic acid. Finally, the Optical Density (O.D.) at 570 nm of each well was measured in triplicates using the Perkin-Elmer VICTOR X5 multilabel plate reader (24–27). The O.D. value for each treatment was calculated as the average of measures.

The calculated values are to be considered as proportional to the amount of the staining resuspended, thus proportional to the number of cells fixed at the well surface as a biofilm.

Therefore, the biofilm formed by strains grown with 5, 10 and 20% v/v of Veal<sup>®</sup> Oris was compared to the biofilm formed without the product and also the effect of the increasing concentrations of Veal<sup>®</sup> Oris on the biofilm formation was evaluated. More precisely, the biofilm formed by the strains in the control conditions (without Veal<sup>®</sup> Oris) was considered as the 100% of biofilm formation; then, the reduction of biofilm due to the Veal<sup>®</sup> Oris treatments was estimated as the percentage difference between the biofilm formed without (100% of biofilm) and with 5, 10 and 20% of Veal<sup>®</sup> Oris (ANOVA, Tukey HSD with  $p < 0.05$ ).

Since the genus *Fusobacterium* has been largely demonstrated as associated with the halitosis (16,19,28,29), the strain *F. nucleatum subsp. vincentii* DSM 19507 was also investigated regarding its ability to form the malodourous product H<sub>2</sub>S under Veal<sup>®</sup> Oris treatments.

The *F. nucleatum* was cultivated in Columbia Broth at 37°C for 48 hours. After growth, the culture was centrifuged at 5000 rcf for 3 minutes to collect the cells at the bottom of the culturing tube. The bacterial pellet was resuspended into 40 ml of Sulphide Indole Motility (SIM) medium. The formulation of the SIM medium allows the detection of H<sub>2</sub>S produced by the strain, since it contains ferrous ammonium sulphate, which reacts with this volatile compound and forms ferrous sulphide, a black precipitate, that finally acts as the indicator of H<sub>2</sub>S presence (30,31). Aliquots of the *F. nucleatum* culture in SIM medium were treated with the test concentrations of Veal<sup>®</sup> Oris, i.e., 5, 10 and 20% v/v, respectively. In detail, 3 ml aliquots of the culture were distributed in 15 ml falcon tubes that contain 150 (5% v/v), 300 (10% v/v) e 600 (20% v/v) ul of Veal<sup>®</sup> Oris, respectively. Each Veal<sup>®</sup> Oris treatment was assessed in triplicates. Also, 3 ml triplicates of blank and control tubes were produced respectively with SIM medium alone and with *F. nucleatum* culture without Veal<sup>®</sup> Oris adding. Finally, 1 ml of liquid sterile paraffin was added at the top of the tubes to provide the anaerobic growth condition. The tubes were incubated at 37°C for 48 hours in static condition. After incubation, the formation of black precipitated was observed and spectrophotometrically estimated. The absorbance of all inocula was measured within the wide range 400–700 nm at 1 nm steps, with the Lambda 25 spectrophotometer (Perkin-Elmer). The final values of absorbance for different treatments were calculated as the average of values measured for each set of triplicates. The absorbance spectrum of each treatment with Veal<sup>®</sup> Oris was compared with the control one (no Veal<sup>®</sup> Oris adding) (ANOVA, Tukey HSD with  $p < 0.05$ ).

## Results

Following are described the significant variations (ANOVA, Tukey HSD with  $p < 0.05$ ) induced by Veal® Oris treatments on the growth and biofilm formation of *S. mutans* and *F. nucleatum* and only for *F. nucleatum* on the strain ability to produce H<sub>2</sub>S.

As showed in Fig. 2, when compared with the untreated control, the *in vitro* growth of *S. mutans* was only slightly increased by Veal® Oris at 5 or 10% (v/v), with percentage increments respectively of 17 and 14%. Surprising, Veal® Oris at a concentration of 20% did not exert a significant increment of the bacterial growth.

Conversely, the same Veal® Oris doses were all significantly active in reducing the biofilm formation capability of the cariogenic species *S. mutans*. A biofilm reduction of 26% was obtained with Veal® Oris at 5% (v/v) and of 32% with Veal® Oris at 10 and 20% (v/v).

Instead, the results obtained for *F. nucleatum*, showed in Fig. 3, revealed that Veal® Oris does not influence the growth of the strain at any tested concentrations. On the contrary, regardless to applied concentration, it significantly reduces the bacterium ability to form biofilm. Indeed, interestingly the treatments with Veal® Oris at 5, 10 and 20 % (v/v) caused similar biofilm reductions of respectively 50, 52 e 40 %.

Moreover, regarding the effect of Veal® Oris on the ability of *F. nucleatum* to produce the “smelly” volatile compound H<sub>2</sub>S of halitosis, after the incubation time (see the methods section) the darkening of SIM medium was observed for all inocula, without or with Veal® Oris adding, as an expected consequence of H<sub>2</sub>S production by the tested strain. However, as showed in Fig. 4, differences of the medium darkening were detected between the untreated and Veal® Oris treated inocula (Fig. 4). With respect to the control, a progressive reduction of the dark precipitate was observed with the incremental concentrations of the Veal® Oris treatments.

These findings were confirmed through the spectrophotometric analysis. The absorbance spectra obtained for the untreated samples clearly differed from the spectra obtained for the samples treated with Veal® Oris (Fig. 5).

The range of maximal absorbance for all samples was comprised between the wavelengths of 400–420 nm; among this range, the curves of Veal® Oris treatments showed respect to the control, a progressive reducing of absorbance along the increments of Veal® Oris concentration (Fig. 6). This result suggested that the production of H<sub>2</sub>S by *F. nucleatum* was inversely influenced by the adding of Veal® Oris in a concentration-dependent manner.

To get inside the quantitative differences in H<sub>2</sub>S producing in absence/presence of Veal® Oris, the wavelength of maximum absorbance for the control strain was identified as the wavelength at which the dark precipitate is detected. The absorbance value is to be intended as proportional to the dark precipitate produced and finally to the H<sub>2</sub>S produced by the strain. Therefore, at the identified wavelength, the

absorbance measured for the control was compared with the ones measured under the Veal<sup>®</sup> Oris treatments.

In detail, the heatmap in Fig. 7A shows that the major absorbance values for the control were measured within the sub-range of the wavelength 400–409 nm with the maximum value registered at 407 nm. Comparing the absorbance measured for the treated samples within this range with the ones of the control, the progressive reduction of absorbance (H<sub>2</sub>S production) along the increments of Veal<sup>®</sup> Oris is clearly highlighted (Fig. 7B). Finally, comparing the absorbance measured for the treated samples with the control at the single wavelength of maximal absorbance i.e., 407 nm, a reduction of 6, 9 and 13% was calculated for the treatment with 5, 10 e 20% (v/v), respectively (Fig. 7C).

## Discussion

In this study, the action of the oral preparation Veal<sup>®</sup> Oris was investigated against *S. mutans* and *F. nucleatum*, two oral bacteria that can be considered as the key actors of the most common oral diseases.

*S. mutans* is one of the most cariogenic species of the mouth environment (6,18,32); since its ability to colonize the oral surfaces, its rapid metabolism and the strong acid tolerance, it manages the first stage of the biofilm formation as the principal early colonizer (12,15). *F. nucleatum* intervenes in the later stage of the oral biofilm maturation, when it acts as bridge-species between the early colonizers and the late colonizers, the latter ones needed to assemble the complex structure of a mature and resistant oral biofilm (6,13,15). Moreover, *F. nucleatum* is also implicated in the development of gingivitis and periodontal diseases as well as, thanks to its ability to produce the malodorous sulphur compound H<sub>2</sub>S, its enrichment in the oral cavity is associated to the development of halitosis (16,17,20,28).

The product Veal<sup>®</sup> Oris tested in this study is made by only two components from natural sources, the vitamin E (α-tocopherol acetate) and the caprylic/capric triglyceride. This formulation was tested at the concentration of 5, 10 and 20% (v/v) on cultures of *S. mutans* and *F. nucleatum*. The effect of the Veal<sup>®</sup> Oris treatments on the growth and the biofilm formation of both strains was evaluated. In addition for *F. nucleatum* the influence on the H<sub>2</sub>S production was also estimated. Regarding to the effect of Veal<sup>®</sup> Oris on the *in vitro* growth of the two oral strains, the growth of *F. nucleatum* was not promoted by the product. A weak stimulation was obtained for *S. mutans* with the lower concentrations of 5% and 10% (v/v), whereas at the higher tested concentration of 20% (v/v), no significant variation was registered. More interestingly, regarding the biofilm formation, the results suggested that the combination of vitamin E with capric/caprylic acid clearly decrease the *in vitro* ability of both *S. mutans* and *F. nucleatum* to assemble the biofilm structure. Indeed, depending on Veal<sup>®</sup> Oris concentration, a percentage reduction of biofilm ranging from 26–32% for *S. mutans* and 40–52% for *F. nucleatum* was estimated.

Although no studies have been so far conducted with a similar mixture of vitamin E (alpha-tocopherol) and capric/caprylic acid, our results are consistent with the hypothesis of a synergistic antimicrobial/antibiofilm action of the two components. Undoubtedly, the vitamin E as alpha-tocopherol

acetate has already confirmed its antibiofilm action against bacteria associated with Urinary tract infections (UTIs), mainly belonging to the genera *Staphylococcus* and *Proteus* (27,33). Regardless of the methods used for the biofilm estimation and depending on the strain tested, the application of alpha-tocopherol acetate induced a reduction of biofilm around 50% or more (27,33). Unfortunately, very few investigated is the potential action of tocopherols on the growth of the buccal bacteria and on their ability to form the oral biofilm. However, Smolarek et al. showed that toothpaste added with tocopherols produced an *in vitro* antimicrobial activity against *S. mutans* and *E. faecalis* (34); although in this study, no characterization as alpha, beta, gamma, and delta-tocopherol has been performed, the results clearly suggested the potential beneficial use of tocopherols for the maintenance of the oral health.

Nevertheless, some important experimental findings suggested that, among the others, the tocopherol acetate is the vitamin E ester more suitable and potentially more active for the treatment of the oral tissues. Indeed, the acetate tocopherol could be significantly delivered to the surface of the gingival tissues, it could be metabolized by the buccal and gingival epithelium to the free form of vitamin E, and finally, it is able to deeply penetrate to the metabolically active gingival tissue (35–37). At the same time, our hypothesis that the effect of Veal<sup>®</sup> Oris is the result of a synergistic action of the two components is consistent with some previous observations about the bactericidal effects of medium-chain fatty acids (MCFAs), whom the capric/caprylic acid of Veal<sup>®</sup> Oris belong to. Among the lacking literature describing the activity of MCFAs against oral bacteria, the study conducted by Huang et al. confirmed that the short, medium and long-chain fatty acids exhibit patterns of inhibition against a panel of oral bacteria comprising *S. mutans*, *Streptococcus gordonii*, *Streptococcus sanguis*, *Candida albicans*, *Aggregatibacter actinomycetemcomitans*, *F. nucleatum*, and *Porphyromonas gingivalis* (38). The inhibitory action of fatty acids appeared clearly species-specific, since it is related to the profile of fatty acids produced by each species itself; it could be thought as a “system of cross-balancing” of the oral microbiota ecology constituted by a pool of molecules active against competitors of the same environment. For instance, it was demonstrated that caprylic acid (25 ug/ml) completely inhibits the growth of *F. nucleatum* and reduces by 50% the growth of *S. mutans*. Interestingly, these results are consistent with the evidence that *F. nucleatum* produces butyric, isovaleric, and propionic acids but does not produce capric/caprylic acid to whom, therefore, it is sensitive. Similarly, since *S. mutans* is a major acidogenic and aciduric microorganism in the oral cavity, it showed greater resistance to the effects of the major part of the fatty acids, comprising the capric/caprylic acid (38).

Instead, a potential antibiofilm action of capric/caprylic acid has not been highlighted. Rather, the 1-monoglyceride of capric acid, the monocaprin, tested against *S. aureus*, *C. albicans*, and *S. mutans* showed inhibitory action against the strains in the planktonic phase, but no effect when the same strains were tested in the biofilm growing state (39).

Then, we are confident to suppose that the highlighted effect of Veal<sup>®</sup> Oris on the growth and biofilm formation of *S. mutans* and *F. nucleatum* is the result of combined action of the two-component, more focused on the antimicrobial action for the caprylic/capric acid, whereas to the antibiofilm effect for the alpha-tocopherol acetate. The synergistic nature of the interaction seems to be further confirmed by previous studies, at least regarding the antimicrobial activity of caprylic/capric acid. Indeed, medium-

chain fatty acids such as caprylic and lauric acids demonstrated an enhanced bactericidal effect when used in association with essential oils (i.e. carvacrol, eugenol, b-resorcylic acid, trans-cinnamaldehyde, thymol, and vanillin) that originates by extraction from plant and seeds, similarly to the alpha-tocopherol (40,41). Consistently, we are confident to suppose a similar relationship of synergism between caprylic/capric acid and the alpha-tocopherol acetate of Vea® Oris as well. The enhancing activity may be due to the same mechanism of synergism identified by Chapple et al. (2013) for ascorbate and  $\alpha$ -tocopherol. These two compounds, recycling each other, produce together increased antioxidant effect respect to the ascorbate alone (42).

In addition, Vea® Oris demonstrated an interesting inhibitory effect on the ability of *F. nucleatum* to produce H<sub>2</sub>S, responsible with other volatile compounds, of the smelly odour in halitosis. The *in vitro* decrement of H<sub>2</sub>S induced by 5, 10, and 20% (v/v) of Vea® Oris ranged between only 5–12%, and interestingly the variation occurred in a product concentration-dependent manner. Although further investigations are needed to go insight the H<sub>2</sub>S inhibitory action of both components, we are confident to speculate that the observed effect is principally attributable to the vitamin E. Indeed, Ben Lagha et al. (2017) demonstrated that the green tea extract and the principal tea catechin, the epigallocatechin-3-gallate (EGCG), at a high concentration of 2000 ug/ml markedly reduced the H<sub>2</sub>S production by *F. nucleatum* (about 80% of reduction); a weaker but significant reduction as well was verified at concentrations  $\leq$  500 ug/ml for both substances and also for the theaflavins (43). A similar inhibitory action was verified for the thymoquinone, the aromatic compound obtained as the most prominent constituent of *N. sativa* seeds essential oil (44). Used at the concentration of 100 ug/ml it significantly decreases H<sub>2</sub>S level by 40% in *F. nucleatum*, and at an even lower concentration of 6.25  $\mu$ g/ml it reduced of 45% the H<sub>2</sub>S production in *Porphyromonas gingivalis* as well. These data lead us to suppose that the aromatic molecular structure of alpha-tocopherol as for the aforementioned natural compounds makes them able to compete with some intermediate of the H<sub>2</sub>S production pathway. Going deeply inside the molecular mechanism of inhibition, we could speculate that the inhibitory action acts at genetic level as it is for the cetylpyridinium chloride (CPC), a quaternary ammonium compound often used in oral hygiene products (45). Daily use of mouth rinses containing CPC was found to reduce VSCs and oral malodor through both a direct antimicrobial activity against the oral halitosis bacteria such as *F. nucleatum* and *Phorphiromonas gingivalis*, and through the capacity of the molecule to suppress the expression of the bacterial genes *mgI* and *cdI* that lead the VSC production (45). Nevertheless, deeper investigations are needed to confirm the same genetic inhibition mechanism for the tested product.

## Conclusions

The present study highlighted the potential of using Vea® Oris both to counteract the formation of biofilm and plaque and as adjuvant in the halitosis treatment. Not to be overlooked is that, since its natural derivation its application in the oral cavity prospects important advantages respect to a chemical derived product firstly in term of safety and approval by patients. Add to this, the antioxidant and anti-inflammatory properties of vitamin E could contribute to the maintainance of the health state of the oral

mucosa, as found for other mucosal tissues (46–51). These encouraging results certainly set the stage for further *in vitro* and desirable also *in vivo* studies.

## Abbreviations

VSCs: volatile sulfur compounds; ROS: reactive Oxygen Species; O.D.: optical density; SIM medium: sulphide indole motility medium; UTIs: urinary tract infections; MCFAs: medium-chain fatty acids; EGCG: epigallocatechin-3-gallate; CPC: cetylpyridinium chloride.

## Declarations

## Ethics approval and consent to participate

Not applicable

## Consent for publication

Not applicable

## Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

## Competing interests

All the authors firmly declare they avoided circumstances that might affect their judgement or impartiality when performing their jobs, thus they declare that there are no conflicts of interest.

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## Authors' contributions

L.P. conducted the investigations, formal analysis, conceptualization and writing. I.M. and G.P.P. contributed to the investigations, the writing and reviewing. M.A.C., N.V. and A.P. aided in procurement of

resources and reviewing. D.N. contributed in data validation, and study supervision. G.G. was responsible of funding acquisition and supervision. R.D.M. was responsible for the funding acquisition, planned the study and managed the conceptualization, validation and supervision. All authors read and approved the final manuscript.

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## References

1. Lu M, Xuan S, Wang Z. Oral microbiota: A new view of body health. *Food Sci Hum Wellness*. 2019;8(1):8–15.
2. Kilian M, Chapple ILC, Hannig M, Marsh PD, Meuric V, Pedersen AML, et al. The oral microbiome - An update for oral healthcare professionals. *Br Dent J*. 2016;221(10):657–66.
3. Palmer RJ. Composition and development of oral bacterial communities. *Periodontol* 2000. 2014;64(1):20–39.
4. Cornejo Ulloa P, van der Veen MH, Krom BP. Review: modulation of the oral microbiome by the host to promote ecological balance. *Odontology*. 2019;107(4):437–48.
5. Willis JR, Gabaldón T. The human oral microbiome in health and disease: From sequences to ecosystems. *Microorganisms*. 2020;8(2):1–28.
6. Guo L, Shokeen B, He X, Shi W, Lux R. Streptococcus mutans SpaP binds to RadD of Fusobacterium nucleatum ssp. polymorphum. *Mol Oral Microbiol*. 2017 Oct;32(5):355–64.
7. Xu P, Gunsolley J. Application of metagenomics in understanding oral health and disease. *Virulence*. 2014;5(3):424–32.
8. Belda-Ferre P, Alcaraz LD, Cabrera-Rubio R, Romero H, Simón-Soro A, Pignatelli M, et al. The oral metagenome in health and disease. *ISME J*. 2012;6(1):46–56.
9. Singh S, Singh SK, Chowdhury I, Singh R. Understanding the Mechanism of Bacterial Biofilms Resistance to Antimicrobial Agents. *Open Microbiol J*. 2017;11(1):53–62.
10. Yin W, Wang Y, Liu L, He J. Biofilms: The microbial “protective clothing” in extreme environments. *Int J Mol Sci*. 2019;20(14).
11. Harper RA, Carpenter GH, Proctor GB, Harvey RD, Gambogi RJ, Geonnotti AR, et al. Diminishing biofilm resistance to antimicrobial nanomaterials through electrolyte screening of electrostatic interactions. *Colloids Surfaces B Biointerfaces*. 2019;173(August 2018):392–9.
12. Jwa SK. Efficacy of moringa oleifera leaf extracts against cariogenic biofilm. *Prev Nutr Food Sci*. 2019;24(3):308–12.
13. Benítez-Páez A, Belda-Ferre P, Simón-Soro A, Mira A. Microbiota diversity and gene expression dynamics in human oral biofilms. *BMC Genomics*. 2014;15(1):1–13.

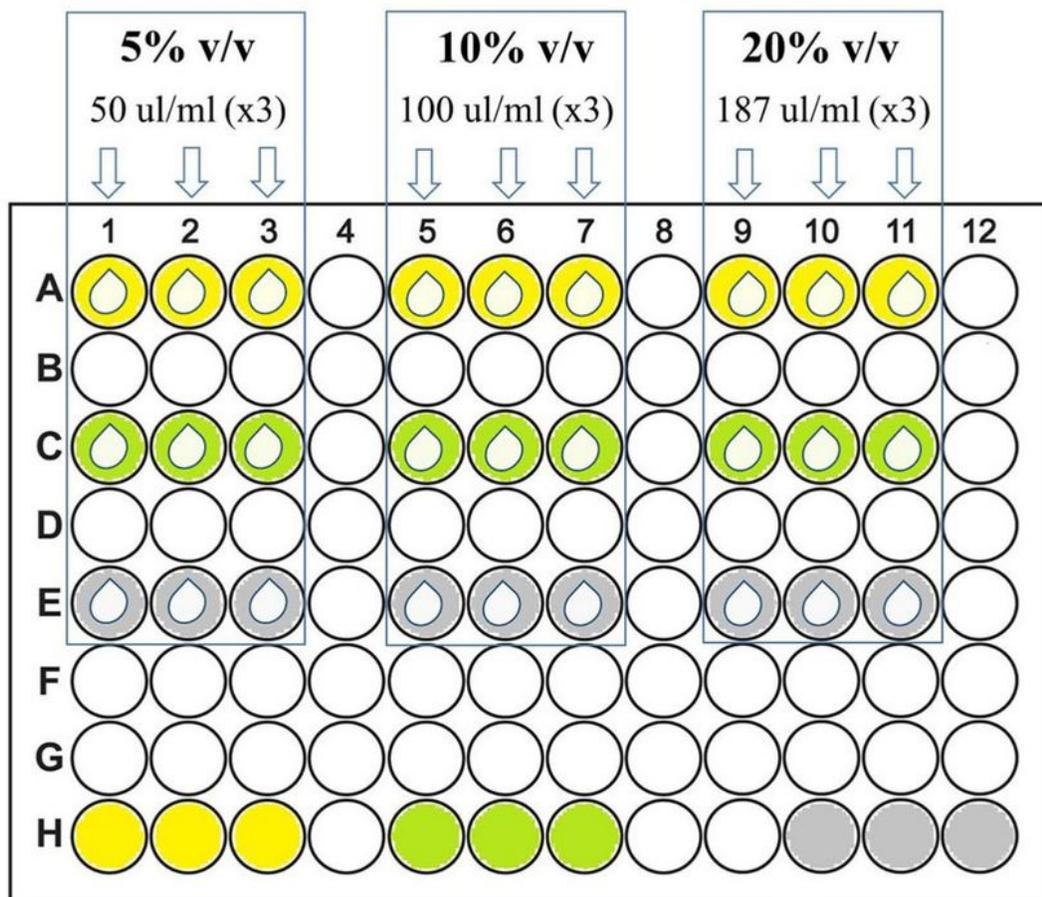
14. Karched M, Bhardwaj RG, Asikainen SE. Coaggregation and biofilm growth of *Granulicatella* spp. with *Fusobacterium nucleatum* and *Aggregatibacter actinomycetemcomitans*. *BMC Microbiol.* 2015;15(1):1–10.
15. Thurnheer T, Karygianni L, Flury M, Belibasakis GN. *Fusobacterium* Species and Subspecies Differentially Affect the Composition and Architecture of Supra- and Subgingival Biofilms Models. *Front Microbiol.* 2019;10(July).
16. Washio J, Sato T, Koseki T, Takahashi N. Hydrogen sulphide-producing bacteria in tongue biofilm and their relationship with oral malodour. *J Med Microbiol.* 2005;54(9):889–95.
17. Suzuki N, Nakano Y, Watanabe T, Yoneda M, Hirofuji T, Hanioka T. Two mechanisms of oral malodor inhibition by zinc ions. *J Appl Oral Sci.* 2018;26:1–7.
18. Kuang X, Chen V, Xu X. Novel Approaches to the Control of Oral Microbial Biofilms. *Biomed Res Int.* 2018;2018.
19. Kato H, Yoshida A, Awano S, Ansai T, Takehara T. Quantitative detection of volatile sulfur compound-producing microorganisms in oral specimens using real-time PCR. *Oral Dis.* 2005;11(SUPPL. 1):67–71.
20. Yoshida A, Yoshimura M, Ohara N, Yoshimura S, Nagashima S, Takehara T, et al. Hydrogen Sulphide Production From Cysteine and Homocysteine by Periodontal and Oral Bacteria. *J Periodontol.* 2009;80(11):1845–51.
21. Gomes Muniz FWM, Nogueira SB, Vasconcelos Mendes FL, Rösing CK, Mendes Moreira MMS, De Andrade GM, et al. The impact of antioxidant agents complimentary to periodontal therapy on oxidative stress and periodontal outcomes: A systematic review. *Arch Oral Biol.* 2015;60(9):1203–14.
22. Hernández-Ríos P, Pussinen PJ, Vernal R, Hernández M. Oxidative stress in the local and systemic events of apical periodontitis. *Front Physiol.* 2017;8(NOV):1–8.
23. Kensche A, Reich M, Kümmerer K, Hannig M, Hannig C. Lipids in preventive dentistry. *Clin Oral Investig.* 2013;17(3):669–85.
24. Stepanović S, Vuković D, Dakić I, Savić B, Švabić-Vlahović M. A modified microtiter-plate test for quantification of staphylococcal biofilm formation. *J Microbiol Methods.* 2000;40(2):175–9.
25. Pietrangelo L, Bucci A, Maiuro L, Bulgarelli D, Naclerio G. Unraveling the composition of the root-associated bacterial microbiota of *Phragmites australis* and *Typha latifolia*. *Front Microbiol.* 2018;9(AUG).
26. S. S, Cirkovic I., Ranin L., Švabić-Vlahović M. Biofilm formation by *Salmonella* spp. and *Listeria monocytogenes* on plastic surface. *Lett Appl Microbiol.* 2004;38:428–32.
27. Vergalito F, Pietrangelo L, Petronio G, Colitto F, Alfio Cutuli M, Magnifico I, et al. Vitamin E for prevention of biofilm-caused Healthcare-associated infections. *Open Med.* 2020;15(1):14–21.
28. Basic A, Blomqvist M, Dahlén G, Svensäter G. The proteins of *Fusobacterium* spp. involved in hydrogen sulphide production from L-cysteine. 2017;1–10.

29. Greabu M, Totan A, Miricescu D, Radulescu R, Virilan J. Hydrogen Sulphide, Oxidative Stress and Periodontal Diseases : A Concise Review. 2016;(Fig. 1):1–13.
30. Mahon CR, Lehman DC, Manuselis G. Textbook of Diagnostic Microbiology - E-Book. Elsevier Health Sciences; 2014.
31. Pommerville JC. Alcamo's Fundamentals of Microbiology. Jones and Bartlett Publishers; 2004.
32. Anderson AC, Rothballer M, Altenburger MJ, Woelber JP, Karygianni L, Lagkouvardos I, et al. In-vivo shift of the microbiota in oral biofilm in response to frequent sucrose consumption. *Sci Rep*. 2018;8(1):1–13.
33. Bidossi A, Bortolin M, Toscano M, De Vecchi E, Romanò CL, Mattina R, et al. In vitro comparison between  $\alpha$ -tocopheryl acetate and  $\alpha$ -tocopheryl phosphate against bacteria responsible of prosthetic and joint infections. *PLoS One*. 2017;12(7):1–12.
34. De Camargo Smolarek P, Esmerino LA, Chibinski AC, Bortoluzzi MC, Santos EB dos, Kozlowski VA. In vitro antimicrobial evaluation of toothpastes with natural compounds. *Eur J Dent*. 2015;9(4):580–6.
35. Green AK, Alcock J, Cox TF, Abraham PJ, Savage D, McGrady M. Delivery of vitamin E acetate and sunflower oil to gums from fluoride toothpaste containing 0.1% vitamin E acetate and 0.5% sunflower oil. *Int Dent J*. 2007;57(S2):124–8.
36. Scott AE, Alcock J, Carlile MJ, Griffiths HR. Metabolism of vitamin E acetate by reconstituted human gingival and buccal epithelium. *Int Dent J*. 2007;57(S2):135–9.
37. Philpotts CJ, Harding CR, Carlile MJ, Sadler CJ, Wright J. Ex vivo delivery and penetration of  $\alpha$ -tocopherol acetate and linoleic acid to gingival tissue from a toothpaste formulation. *Int Dent J*. 2007;57(S2):129–34.
38. Huang CB, Alimova Y, Myers TM, Ebersole JL. Short- and medium-chain fatty acids exhibit antimicrobial activity for oral microorganisms. *Arch Oral Biol*. 2011;56(7):650–4.
39. Thorgeirsdóttir TÓ, Kristmundsdóttir T, Thormar H, Axelsdóttir Í, Holbrook WP. Antimicrobial activity of monocaprin: A monoglyceride with potential use as a denture disinfectant. *Acta Odontol Scand*. 2006;64(1):21–6.
40. Kim SA, Rhee MS. Highly enhanced bactericidal effects of medium chain fatty acids (caprylic, capric, and lauric acid) combined with edible plant essential oils (carvacrol, eugenol,  $\beta$ -resorcylic acid, trans-cinnamaldehyde, thymol, and vanillin) against *Escherichia coli* O15. *Food Control*. 2016;60:447–54.
41. Cho TJ, Park SM, Yu H, Seo GH, Kim HW, Kim SA, et al. Recent advances in the application of antibacterial complexes using essential oils. *Molecules*. 2020;25(7):1–22.
42. Chapple IL, Matthews JB, Wright HJ, Scott AE, Griffiths HR, Grant MM. Ascorbate and  $\alpha$ -tocopherol differentially modulate reactive oxygen species generation by neutrophils in response to Fc $\gamma$ R and TLR agonists. *Innate Immun*. 2013;19(2):152–9.
43. Ben Lagha A, Haas B, Grenier D. Tea polyphenols inhibit the growth and virulence properties of *Fusobacterium nucleatum*. *Sci Rep*. 2017;7(March):1–10.

44. Khader M, Eckl PM. Thymoquinone: An emerging natural drug with a wide range of medical applications. *Iran J Basic Med Sci.* 2014;17(12):950–7.
45. Liu J, Ling J, Wu CD. ScienceDirect Cetylpyridinium chloride suppresses gene expression associated with halitosis. 2013;8:2–7.
46. Kamisah Y, Qodriyah HMS, Chua KH, Nur Azlina MF. Vitamin E: A potential therapy for gastric mucosal injury. *Pharm Biol.* 2014;52(12):1591–7.
47. Asad Iqbal MD, Khan M, Kumar P, Kumar A, Ajai K. Role of vitamin e in prevention of oral cancer:-A review. *J Clin Diagnostic Res.* 2014;8(10):ZE05–7.
48. Nallapu V, Balasankulu B, Vuppalapati H, Sambhana S, Mala D, Koppula S. Efficacy of vitamin E in oral submucous fibrosis: A clinical and histopathologic study. *J Indian Acad Oral Med Radiol.* 2015;27(3):387.
49. El-Housseiny AA, Saleh SM, El-Masry AA, Allam AA. The effectiveness of vitamin “E” in the treatment of oral mucositis in children receiving chemotherapy. *J Clin Pediatr Dent.* 2007;31(3):167–70.
50. Ohta Y, Kobayashi T, Imai Y, Inui K, Yoshino J, Nakazawa S. Effect of oral vitamin E administration on acute gastric mucosal lesion progression in rats treated with compound 48/80, a mast cell degranulator. *Biol Pharm Bull.* 2006;29(4):675–83.
51. Kamsiah J, Muhaizan W, Gapor M., Roslin O. Mucosal Protective Effects of Vitamin E on Aspirin-Induced Gastric Lesions in Rats. *International Journal of Pharmacology.* 2005.

## Figures

## Vea® Oris adding

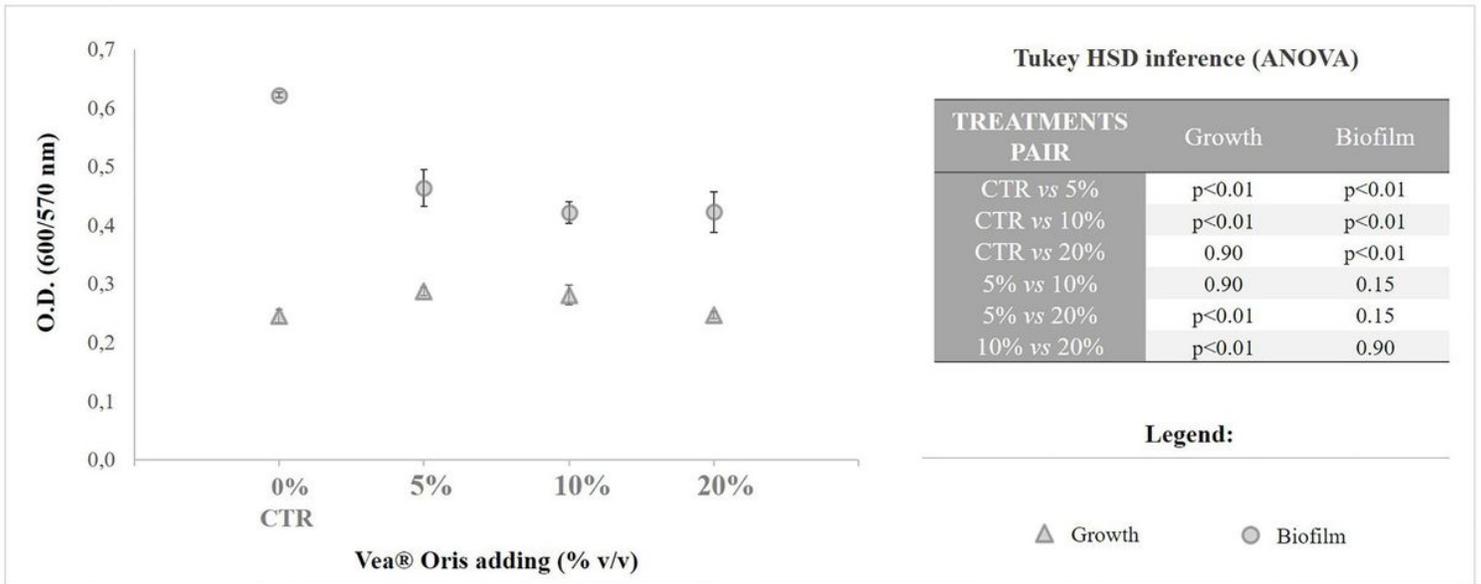


### Legend:

	Added with Vea® Oris	NOT added with Vea® oris (CTRs)
<i>S. mutans</i>		
<i>F. nucleatum</i>		
medium		

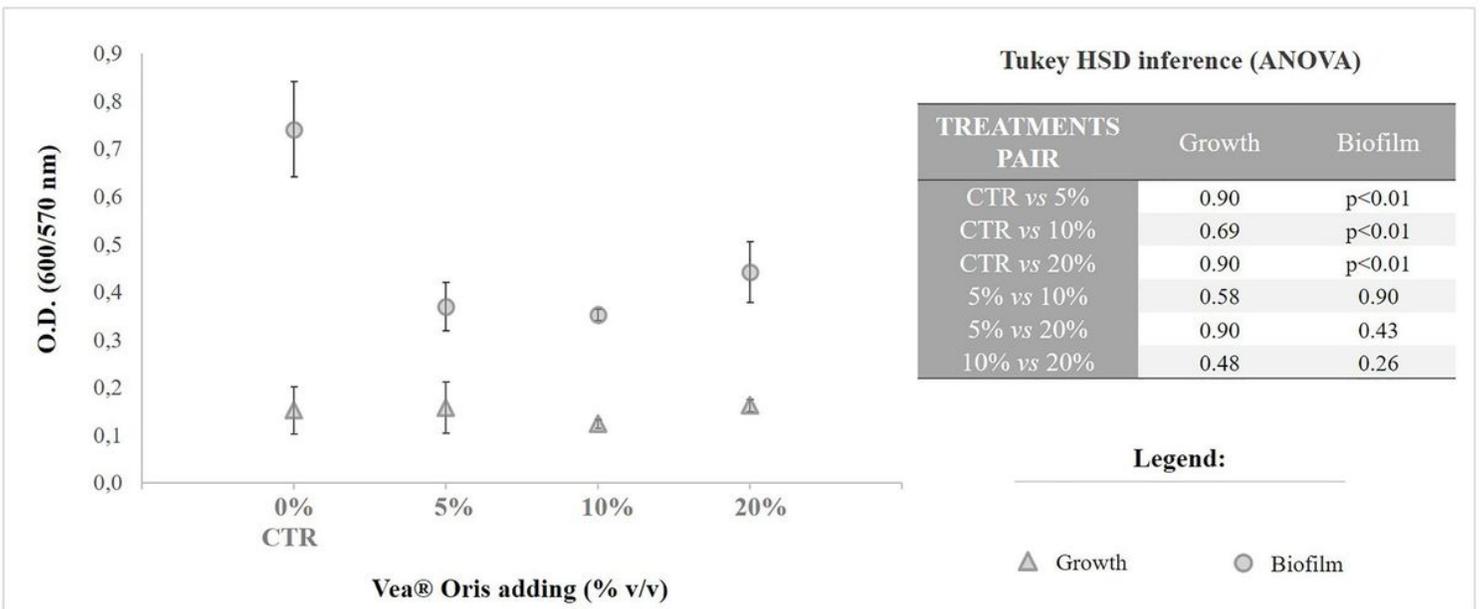
**Figure 1**

Schematic of the experimental setup. *S. mutans* without Vea® Oris (yellow wells) and with 5, 10 and 20% of Vea® Oris (yellow wells with drop); *F. nucleatum* without Vea® Oris (green wells) and with 5, 10 and 20 (green wells with drop). Controls of growing medium not added with Vea® Oris (grey wells) and added with the tested Vea® Oris concentrations (grey wells with drop).



**Figure 2**

Influence of Veal® Oris at 5, 10 and 20% (v/v) on the growth and biofilm formation for *S. mutans* (left). The ANOVA analysis, ANOVA, Tukey HSD test, p<0.05 (right).



**Figure 3**

Influence of Veal® Oris at 5, 10 and 20% (v/v) on the growth and biofilm formation for *F. nucleatum* (left). The ANOVA analysis, ANOVA, Tukey HSD test, p<0.05 (right).

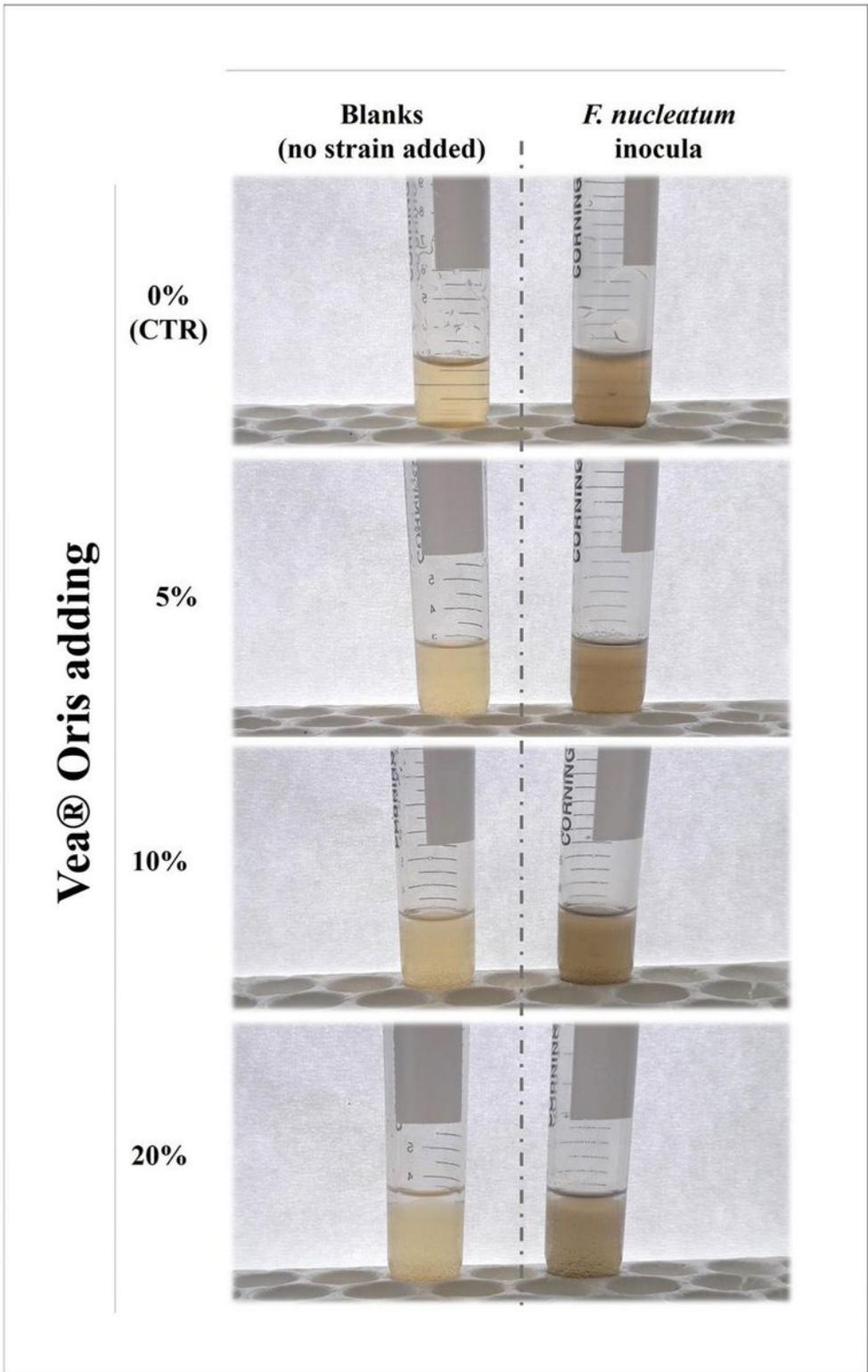


Figure 4

Effect of Vea® Oris on H<sub>2</sub>S production. Darkening of *F. nucleatum* cultures in SIM medium not added (0% v/v) and added with 5, 10 and 20% (v/v) of Vea® Oris.

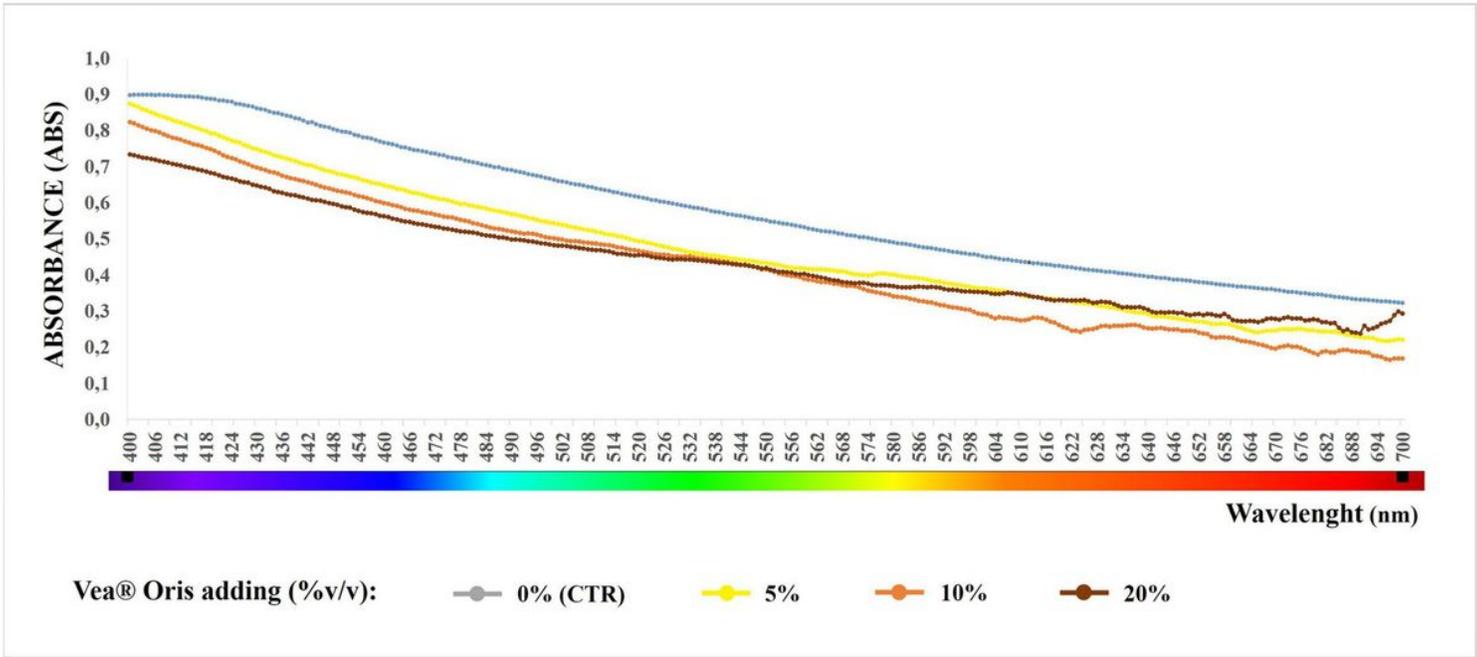
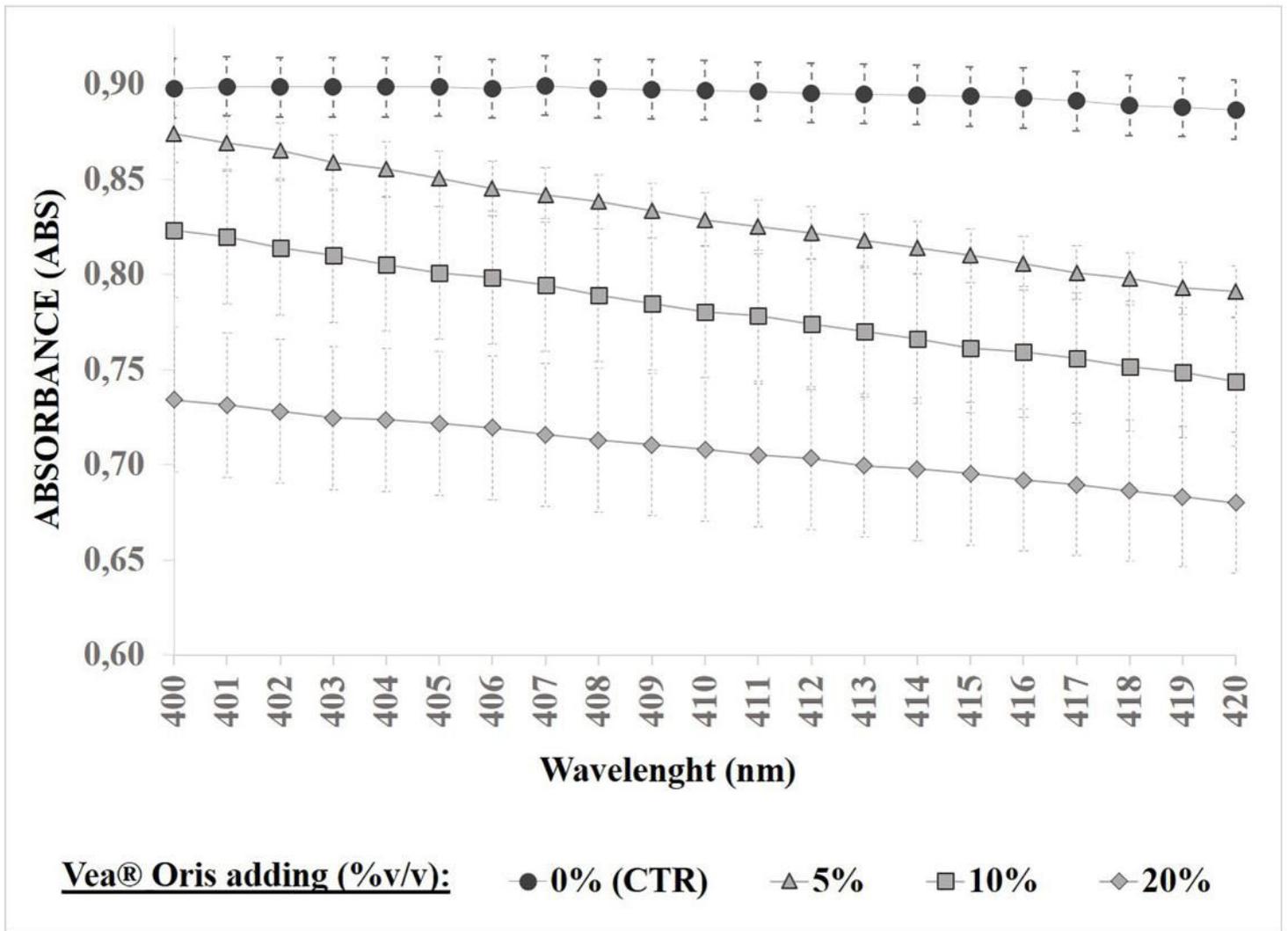


Figure 5

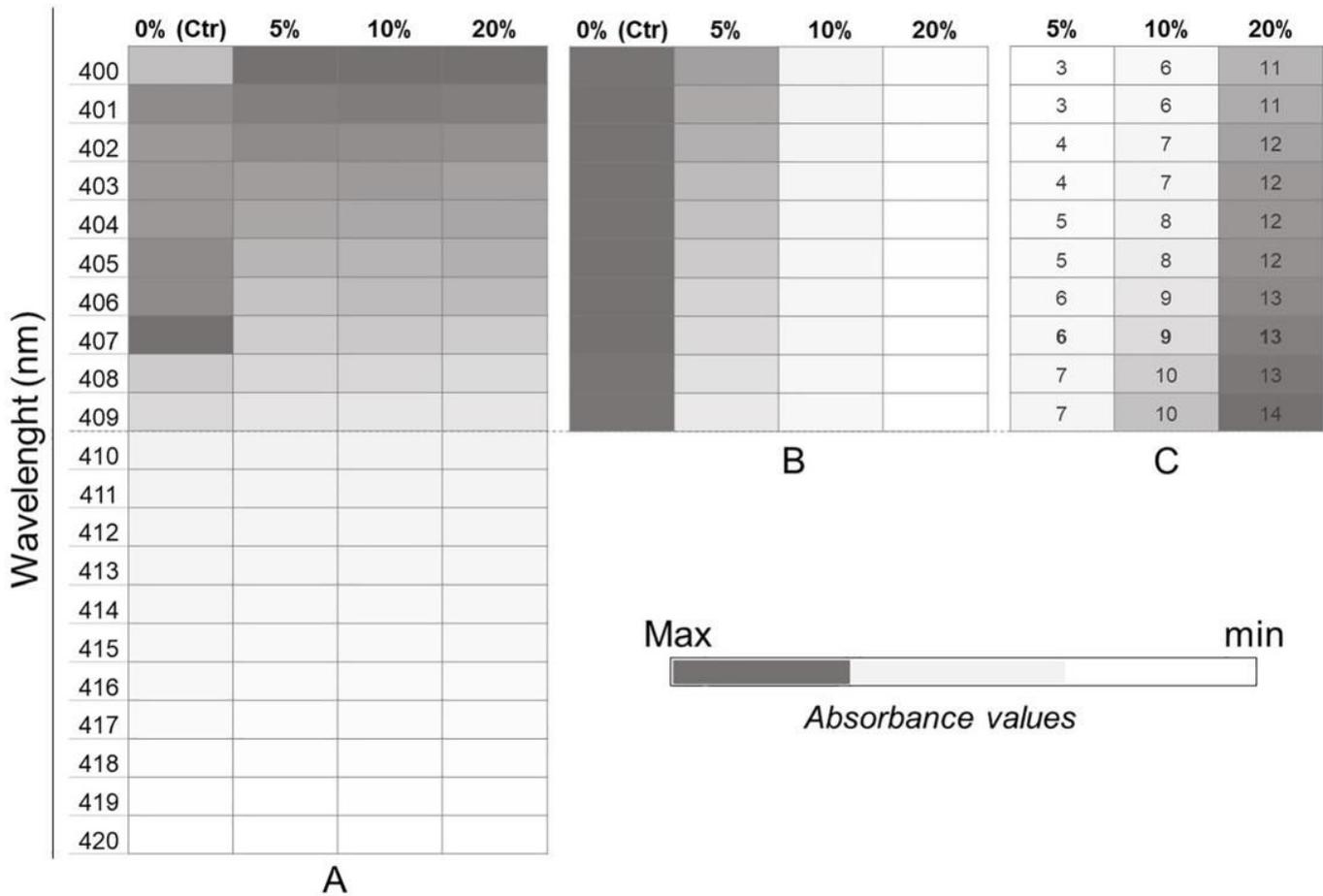
Absorbance spectra (range 400-700 nm) of *F. nucleatum* cultures not treated (grey curve, 0%, CTR) and treated with 5,10 and 20% of Veal® Oris (yellow, orange, and brown curves, respectively).



**Figure 6**

Absorbance curves of *F. nucleatum* cultures not treated (black-circle indicators, 0% (CTR)) and treated with 5,10 and 20% of Veal® Oris (triangle, square and rhombus indicators, respectively).

## Vea® Oris adding (v/v%)



**Figure 7**

Variation of H<sub>2</sub>S production shown as variation of the absorbance values. Absorbance variation depicted by grey colour gradient for *F. nucleatum* cultures along with increments of Vea® Oris with respect to untreated samples (0%, CTR). The variation (grey gradient) is shown between the range 400-420 nm as absolute values for each treatment (A) and between the range of 400-409 nm as relative values between treatments(B). Absorbance variation (colour gradient) and percentage reduction (numerical values) registered for each treatment with respect to the untreated samples (C). The absorbance variation is proportional to the variation of H<sub>2</sub>S production.