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Microbiology insights into boosting salivary defences through the use of enzymes and proteins

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ABSTRACT

Objectives: To assess the effects of enzymes and proteins in toothpastes to boost salivary defences and reduce oral bacteria growth and viability.
Methods: An *in vitro* study to measure levels of hypothiocyanite in saliva after treatment with a toothpaste containing enzymes and proteins (colorimetric assay). A randomised, crossover *in vivo* study measuring, with biochemical assays, the effect of a toothpaste containing enzymes and proteins on the levels of hydrogen peroxide and lysozyme in saliva. *In vitro* studies to measure, using fluorescent dyes, the effects of enzymes and proteins on bacterial membrane integrity. *In vitro* microbiology studies measuring the effects of enzymes and proteins on planktonic bacterial growth, *in vitro* studies in single and multispecies biofilms measuring the effect of toothpaste with enzymes and proteins compared to control toothpaste.
Results: Levels of hypothiocyanite, hydrogen peroxide and lysozyme are boosted after application of a toothpaste containing enzymes and proteins. The enzymes and proteins adversely affect the bacterial membrane integrity in *Streptococcus mutans* and growth of planktonic *S. mutans* and *Fusobacterium nucleatum*. In a single species biofilm model the viability of *S. mutans* was significantly reduced and in a 7 species biofilm model bacterial viability was reduced for a biofilm grown on a pellicle pre-treated with toothpaste containing enzymes and proteins.
Conclusions: The use of a toothpaste containing enzymes and proteins can boost the natural salivary defences by increasing the levels of lysozyme and hydrogen peroxide *in vivo* and hypothiocyanite *in vitro* and reduce the growth and viability of oral bacteria in microbiological models.
Clinical significance: The subtle effects reported here of enzymes and proteins in toothpastes on oral bacteria are consistent with both the gum health benefits reported for such toothpastes containing enzymes and proteins by Daly [1] and Pedersen [2] and the rebalancing of the oral microbiome reported by Adams [3].

1. Introduction

The mouth harbours one of the most diverse microbiomes in the human body with over 700 bacterial species identified to date. Maintenance of a balanced oral microbiome in symbiosis with the host is important for the promotion of health. Saliva plays an important role in maintaining this microbial balance and preventing dysbiosis [4].

A key salivary defence mechanism is the lactoperoxidase system which is activated by hydrogen peroxide in the presence of thiocyanate [5]. The presence of lactoperoxidase in both saliva and milk and the role of the enzyme in combination with hydrogen peroxide and thiocyanate in inhibiting bacterial growth in these media was first reported in the 1950s and 60 s [6–8].

Other salivary components such as lysozyme and lactoferrin play important roles in saliva's natural defences. Lysozyme has anti-bacterial properties and can break glycosidic linkage in peptidoglycans [9]. This effect is most relevant in Gram positive bacteria where a thick peptidoglycan layer is found in the cell wall. In Gram negative bacteria, the peptidoglycan layer is protected by an outer membrane, however lactoferrin has been shown to increase the permeability of this outer membrane, making it more susceptible to lysozyme penetration [10].

Midda and Cooksey demonstrated that the use of a toothpaste containing amyloglucosidase, glucose oxidase and thiocyanate provides benefits in gingival health indices after 3 months of use [11]. Other workers such as Lumikari et al. have investigated the effect of amyloglucosidase and glucose oxidase on oral bacteria [12]. More recently Shin has investigated the role of salivary enzymes and proteins (lactoperoxidase and lactoferrin) on oral malodour and salivary bacteria

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[13].

Zendium™ is a toothpaste that has been designed to boost the mouth's natural defences. It contains a three enzyme system (amyloglucosidase, glucose oxidase and lactoperoxidase) designed to enhance the production of hydrogen peroxide and hypothiocyanite in the mouth. In addition, it contains three further proteins (lysozyme, lactoferrin and bovine colostrum containing immunoglobulin IgG [14]).

Adams et al. [3] have recently reported that the use of a toothpaste containing enzymes and proteins can lead to a positive shift in the oral microbiome with an increase in the relative abundance of bacterial species associated with gum health. Further Daly et al. [1] and Pedersen et al. [2] reported improved gum health indices (gum inflammation, plaque and bleeding) over the control group when using a toothpaste containing enzymes and proteins for 3 month or at least 12 months, respectively.

The aim of this paper is to provide new insights into the effect of enzymes and proteins designed to boost salivary defences on oral bacteria *in vitro* and *in vivo* and to test the hypothesis that antimicrobial activity *vs* relevant oral microorganisms forms part of the mode of action of this technology. We report the effect of the enzymes and proteins on bacterial cell membranes, and planktonic bacteria and further investigate the effect of toothpaste containing enzymes and proteins on both single species biofilms and controlled multi-species biofilms. In all of these models we see the benefits of the enzymes and proteins in controlling bacterial growth rather than eliminating bacteria, thus accepting the hypothesis and rejecting the null hypothesis. This is consistent with the a reduction in plaque after report by Daly et al. [1] and the maintenance of a healthy balanced microbiome as described by Kilian et al. [4].

2. Materials and methods

2.1. Measurement of hypothiocyanite in irradiated saliva in vitro

The generation of hypothiocyanite from toothpaste was measured using a modification of the 5-thio-2-nitrobenzoic acid assay reported by Aune and Thomas [15] and further developed by later authors [16]. Slurries of a test toothpaste containing enzymes and proteins (as described in introduction) or control toothpaste lacking enzymes and proteins (1:5, toothpaste to saliva ratio) were prepared in irradiated saliva. Saliva was collected and pooled from appropriately informed human volunteers. The slurries were left to stand for 2 min then centrifuged, filtered (0.45μM syringe filter, Minisart) and divided into 0.5 ml aliquots in semi-micro disposable cuvettes. A 100μl aliquot of catalase (bovine liver, 10000–40000 units/mg, 4.5 mg/ml protein solution, Sigma Aldrich) was added to each cuvette, to ensure removal of residual hydrogen peroxide, followed by the addition of a 0.5 ml aliquot of thionitrobenzoic acid (Sigma Aldrich) in aqueous solution (225–250μM). The absorbance of thionitrobenzoic acid was measured at 412 nm on an Agilent 8453 UV–vis Spectrometer.

2.2. Measurement of hydrogen peroxide and lysozyme in saliva in vivo

The study used a randomised, double blind, crossover design and was conducted on site at Unilever Research and Development Port Sunlight by an independent CRO (Intertek CRS) with sample analysis conducted by Sponsor staff. The study was reviewed and approved by an independent research ethics committee and all subjects gave their written, informed consent for participation. Subjects were recruited from the Unilever R&D consumer study database *via* word of mouth, letter, text message or poster on the notice board. To be recruited onto the study, volunteers had to be over 18 years of age and have a minimum of 5 teeth per quadrant excluding third molars. Exclusion criteria included (a) anyone with a medical condition, medical history, or taking a medication which would affect the scientific validity of the study, the study staff or if the subject was to participate in the study would affect their wellbeing (b) anyone with sensitivity to or intolerance of dental/oral hygiene products or components of the study products (c) employees of the Unilever or CRO (d) diabetics and (e) pregnant or breastfeeding women. Sample size was based on a balance of logistics and obtaining sufficient data to get reasonable estimate of product difference as there was no previous data on which to base a formal calculation. Fifty healthy volunteers (45 female, 5 male, average age 39, age range 19–63) were enrolled in the study with 46 being randomised and completing (42 female, 4 male, average age 39, age range 19–63). Four subjects withdrew as they were unable to make appointments. Randomisation used a balanced AB/BA crossover design. CRO staff enrolled subjects and allocated then to random sequence based on order of attendance at study site at first test visit. Subjects used a toothpaste without enzymes or proteins after enrolment and between test visits. On each test day, subjects brushed with 1.5 g (+/− 0.05 g) of the allocated toothpaste (test toothpaste containing enzymes and proteins or control toothpaste without enzymes and proteins), for one minute and swished with the resulting slurry for 30 s and spat out, then rinsed with 5 ml water for 5 s. Approximately 2mls of unstimulated saliva was collected pre brushing and after brushing, *ca.* 3 min after rinsing. After collection the saliva samples were placed on ice and analysed for hydrogen peroxide (within 30 min) and lysozyme (within 24 h).

Levels of hydrogen peroxide and lysozyme in saliva were measured using commercial biochemical assays. A commercial Pierce peroxide assay kit was used for hydrogen peroxide quantification. This assay is based on the oxidation of ferrous to ferric ion in the presence of xylenol orange which forms a characteristic chromophore with ferric ions (λ_{max} 560 nm). Lysozyme activity was measured using the EnzChek® Lysozyme Assay Kit (Molecular Probes). In this assay the hydrolytic action of lysozyme on β-1-4 glycosidic linkages in *Micrococcus lysodeikticus* cell wall releases bound fluorescein. Fluorescein is self-quenching when bound to the cell wall and once released has a characteristic absorbance (485 nm) and emission (530 nm). For both assays, suitable calibration curves were generated using known concentration of hydrogen peroxide and lysozyme. Experiments were run in 96 well plates and absorbance and emission measured with a plate reader (Spectramax 384 (Molecular Devices) and Varioskan Flash (Thermo Scientific) respectively).

Complete sample sets were available from 41 subjects. Missing data points arose from contamination with blood or particulate matter. Where data for hydrogen peroxide were below the lower detection limit (12 samples) they were set at the detection limit (0.033 μM). Where data for hydrogen peroxide (2 samples) were above the higher detection limit, they were set at the detection limit (50 μM). The difference between measurements post brushing was analysed using a mixed model ANCOVA with visit, product and product*visit as fixed factors, subject as random factor and pre brushing measurement as covariate to normalize for individual baseline levels. Statistical significance was set at the 95% confidence level. No transformation of the data was required since the residuals were normally distributed.

2.3. Measuring bacterial membrane integrity

Bacterial membrane integrity, measured as membrane polarity and membrane permeability, is an indicator of cell viability. Loss of membrane integrity can be used as a marker for loss of membrane transport function, loss of turgor pressure, cessation of growth and rapid cell death [17,18].

The effect of enzymes and proteins, used in toothpastes, on bacterial membrane polarity was measured using *Streptococcus mutans* ATCC 700610 as the test organism according to the method previously described by Clementi [19] using the membrane potential-sensitive probe, $DiBAC_4$ (Bis-(1,3-Dibutylbarbituric Acid) Trimethine Oxonol). The effect on bacterial membrane permeability was similarly measured using *Streptococcus mutans* ATCC 700610 or *Fusobacterium nucleatum* ATCC

25586 as the test organism using the fluorescent nucleic acid binding dye, SYTOX green.

Combinations of amyloglucosidase, glucose oxidase, lactoperoxidase, lactoferrin, lysozyme and bovine colostrum (IgG) were prepared in sterile, de-ionised water (in the ratio present in commercial toothpaste). Total protein concentrations ranged between 0.7 and 120 μg/ml. Log phase cells of *S. mutans* or *F. nucleatum* were washed re-suspended and adjusted to a standard optical density of OD_{660nm} 0.2 in Phosphate Buffered Saline (PBS) to which was added either 250 nM $DiBAC_4$ or 1000 nM SYTOX green. Fluorescence was monitored before and after the addition of the enzyme and protein mix using a fluorescence detection plate reader (490 nm excitation, 516 nm emission for $DiBAC_4$ and 504 nm excitation, 540 nm emission for SYTOX green, Varioskan Flash (Thermo Scientific)). Changes in fluorescence were determined as the fraction of the mean of the fluorescence readings before and after the addition of the enzyme and protein mix to a culture of *S. mutans* or *F. nucleatum* and expressed as a percentage. For control experiments, PBS was added to *S. mutans* or *F. nucleatum* test cultures at the same time point as the test samples.

2.4. Inhibition of bacterial growth (planktonic monoculture)

Planktonic mono-species assays were used to measure the effects of enzymes and proteins, used in toothpastes, on bacterial growth and physiological activity. Combinations of amyloglucosidase, glucose oxidase, lactoperoxidase, lactoferrin, lysozyme and bovine colostrum (IgG) were prepared in sterile, de-ionised water (in the ratio present in commercial toothpaste) and dispensed into microdilution plates (96 wells) in BHI broth buffered to either pH 5.3 or pH 7. Total protein concentrations ranged between 1.4 and 38 mg/ml. Wells were inoculated with *S. mutans* ATCC 700610 (pH 5.3 and 7) or *Fusobacterium nucleatum* ATCC 25586 (pH 7) (1.0×10^8 CFU/ml). Cultures were prepared in 15% CO_2 or anaerobic conditions (10% hydrogen, 10% carbon dioxide, 80% nitrogen) respectively and incubated in a sealed microdilution plate at 37 °C for 18 h. Optical density (OD 610 nm) was measured every 30 min (Varioskan Flash (Thermo Scientific)) and growth rates were compared by approximation of total growth from the area under the curve (AUC) using the method of Hasenbrink [20] (using Microsoft Excel).

2.5. Effect on viability in single species biofilms

Simple biofilm models provide insights into the effects of oral health care technology on the specific species of interest within a biofilm. Daytime, stimulated saliva was collected from an appropriately consented panel of human volunteers and pooled (typically from a minimum of 15 subjects). The collected saliva samples were pooled and filter-sterilised. The wells of a 96 well microtitre plate were coated with sterile saliva for 2 h to form a salivary pellicle then treated with test or control toothpaste (diluted 1:2 in sterile, de-ionised water) and rinsed with sterile, de-ionised water. The saliva coating and treatment steps were immediately repeated. 50 μl aliquots of bacterial cultures of *S. mutans* ATCC 700610 (1.0×10^8 CFU/ml in BHI) or *F. nucleatum* ATCC 10953 (2.7×10^8 CFU/ml in BHI) were added and incubated overnight at 37 °C in 15% CO_2 or anaerobic conditions (10% hydrogen, 10% carbon dioxide, 80% nitrogen) respectively. PrestoBlue, a resazurin-based cell viability reagent (Invitrogen), was used to detect metabolically active cells within the biofilms according to the method of Mariscal [21]. Fluorescence was measured using a fluorimeter (Varioskan Flash (Thermo Scientific) excitation 540 nm, emission 590 nm).

2.6. Effect on viability in a seven species biofilm

In vitro biofilm models using a defined inoculum of mixed bacterial species allow for limited species-species interactions and community effects. Such mixed species models enable insight into the effects of oral health care technologies on species of interest within a pre-defined biofilm consortium. Stock suspensions of bacteria (Gram positive - *Streptococcus mitis* NCTC 12261, *Streptococcus intermedius* DSM 20573, *Streptococcus oralis* NCTC 11427, *Actinomyces naeslundii* DSM 17233 and Gram negative - *Veillonella dispar* NCTC 11831, *Fusobacterium nucleatum* ATCC 10953 and *Prevotella intermedia* DSM 20706) were grown and standardised to 1×10^7 cfu/ml in artificial saliva. Thermanox plastic coverslips were cultured overnight in 500 μl artificial saliva [22] to form a salivary pellicle. Spent saliva was then removed and coverslips were treated with a slurry of toothpaste (1:2 dilution in sterile, de-ionised water), water or 0.2% chlorhexidine (positive control) for 2 min before washing with PBS to remove product from the surface. Bacterial suspensions were collectively added to the pre-treated Thermanox coverslips and incubated anaerobically (85% N_2, 10% CO_2, 5% H_2) at 37 °C. Replicate biofilms were analysed (two-way ANOVA) after 2, 4 and 8 h to measure viability of bacteria within the biofilm using a resazurin based viability indicator (Alamar Blue, Thermofisher) according to manufacturer's instructions.

3. Results

3.1. Measurement of hypothiocyanite in irradiated saliva in vitro

Two toothpastes (a test toothpaste containing enzymes and proteins and a vehicle control toothpaste containing no enzymes or proteins) were tested. The test toothpaste generated significantly higher levels of hypothiocyanite (109.3μM ± 3.1μM) than the control toothpaste, consistent with a triple enzyme cascade of amyloglucosidase, glucose oxidase and lactoperoxidase generating glucose, hydrogen peroxide and then hypothiocyanite respectively. The vehicle control toothpaste gave a measurement of 1.3 μM ± 2.1, indicative of no hypothiocyanite generation *in vitro*.

3.2. Measurement of hydrogen peroxide and lysozyme in saliva

3.2.1. Hydrogen peroxide results

The concentration of hydrogen peroxide in saliva was 64% greater after brushing with the toothpaste containing enzymes and proteins (4.2 μM) than after brushing with the control toothpaste without enzymes and proteins (2.7 μM). The difference was statistically significant ($p < 0.05$) (ANCOVA).

3.2.2. Lysozyme results

The lysozyme activity in saliva was 92% greater after brushing with the toothpaste containing enzymes and proteins (197 U/ml) than after brushing with the control toothpaste without enzymes and proteins (98 U/ml). The difference was statistically significant ($p < 0.05$) (ANCOVA).

3.3. Bacterial membrane integrity

A combination of amyloglucosidase, glucose oxidase, lactoperoxidase, lactoferrin, lysozyme and bovine colostrum (IgG) at a total protein concentration of 5.2 μg / ml or 7.8 μg / ml was added to *S. mutans* and *F. nucleatum* test cultures respectively. This resulted in a significant increase in fluorescence of $DiBAC_4$ and (separately) of SYTOX green compared to the control (PBS) ($p < 0.01$, Student's t-test). For membrane polarity using $DiBAC_4$ an increase of 33.3% ± 11.7% in fluorescence was observed for treatment of *S. mutans* with the enzymes and proteins compared to 0.75% ± 4.4% for control (Table 1), while for membrane integrity using SYTOX green increases in fluorescence of 44.4% ± 9.3% and 57.6% ± 22.8% were observed for treatment of *S. mutans* and *F. nucleatum* respectively with enzymes and proteins compared to 0.1% ± 0.8% and 12.5 ± 4.7% for the respective controls (Table 1). The increase in fluorescence indicated an increase in membrane depolarization and membrane permeabilisation respectively.

Table 1
Percentage change in fluorescence values (of $DiBAC_4$ or SYTOX green) after the addition of amyloglucosidase, glucose oxidase, lactoperoxidase, lactoferrin, lysozyme and bovine colostrum (IgG) to *S. mutans* or *F. nucleatum* test cultures compared to no active control.

Fluorescent Dye	Bacterium	No. of replicate experiments	Change in fluorescence (enzymes and proteins) / %	Standard deviation	Change in fluorescence (control) / %	Standard deviation
$DiBAC_4$	*S. mutans*	5	33.3	11.7	0.75	4.4
SYTOX green	*S. mutans*	3	44.4	9.3	0.1	0.8
SYTOX green	*F. nucleatum*	3	57.6	22.8	12.5	4.7

Lysozyme was also tested alone showing a significant difference for both membrane depolarisation and membrane permeabilisation over a no active control for 120μg/ml of lysozyme (but not at lower levels). Similarly, lactoferrin showed a significant difference in membrane depolarisation over a no active control for 120μg/ml of lactoferrin. These results are consistent with a synergistic effect between lysozyme and other components such as lactoferrin.

3.4. Inhibition of bacterial growth (planktonic monoculture)

A combination of amyloglucosidase, glucose oxidase, lactoperoxidase, lactoferrin, lysozyme and bovine colostrum (IgG) at a protein concentration of 1.4 mg/ml in buffered BHI broth gave significantly reduced growth of *S. mutans* (at pH 5.3 and 7) and *F. nucleatum* (at pH 7) when compared to a no active control tested under the same conditions ($p < 0.001$, Student's *t*-test). See Fig. 1.

3.5. Effect on viability in single species biofilms

Treatment of a salivary pellicle with a toothpaste containing enzymes and proteins followed by exposure to representative oral bacteria (*S. mutans* or *F. nucleatum)* was associated with a significant reduction in viability of the resultant biofilm, as measured by a fluorescent, resazurin based viability indicator (PrestoBlue) with treatment being associated (Fig. 2) with a significant ($p < 0.005$, Student's *t*-test), 40% reduction in fluorescence of *S. mutans* treated biofilm (from 330 to 199 RFU (relative fluorescence units) and a directional, non-significant ($p = 0.074$, Student's *t*-test) reduction in viability of 23% (from 400 to 308 RFU) for *F. nucleatum* (Fig. 3) relative to the control toothpaste.

3.6. Effect on viability in a seven-species biofilm

Treatment of salivary pellicle with a toothpaste containing enzymes and proteins before introduction to a 7-species biofilm model (Gram positive – *S. mitis, S. intermedius, S. oralis, A. naeslundii* and Gram negative – *V. dispar, F. nucleatum and P. intermedia*) was associated with a significant reduction in viability of the biofilm compared to treatment with a toothpaste without enzymes and proteins as measured by a resazurin based viability indicator (Alamar Blue). This can be seen in Fig. 4, with a significant reduction ($p < 0.001$, using ANOVA) of treatment relative to the control at each of the time points, 2, 4 and 8 h after treatment and inoculation. Treatment was associated with a 30 percent reduction in viability at 2 h (56%–26%), a 27 percent reduction at 4 h (69%–42%) and a 47 percent reduction after 8 h (78%–31%) relative to the control toothpaste.

4. Discussion

Advances in oral microbiology have repeatedly changed our understanding of oral health and disease. The key feature of the non-specific plaque hypothesis of the 19th century was that plaque quantity alone correlated with disease. This was displaced as advances in selective culture of oral microorganisms in the 1970's led to the identification of disease associated species and the development of the so-called Specific Plaque Hypothesis linking disease to the growth of specific pathogenic species [23].

The idea that a small number of specific pathogens define oral health has in turn been displaced by the development of the Ecological Plaque Hypothesis [24] focusing on the importance of maintaining an appropriate symbiotic balance within the total ecology of the mouth and avoiding the dysbiosis associated with poor oral health [25].

The ecological plaque hypothesis and the application of advanced next generation sequencing techniques [26,27] has led to recognition that maintenance of a balanced oral microbiome in symbiosis with the host is important in promoting health [4]. The application of a selective pressure such as the boosting of naturally occurring salivary defences [28] through the use of enzymes and proteins from a toothpaste is now recognised as an effective and clinically proven strategy for shifting the balance of the oral microbiome towards health [3].

Within the oral cavity, salivary enzymes bind to the acquired pellicle, changing the surface properties and influencing bacterial adhesion and immobilizing the enzymes themselves. Indeed, lysozyme and peroxidases are both found on the tooth surfaces and levels can be up to 40% higher than in saliva [29,30]. Both enzymes show similar enzyme activity when free in saliva or immobilized on the tooth surface [29,31]. Hannig has additionally demonstrated that glucose oxidase and lactoperoxidase accumulate in the pellicle after brushing with toothpastes containing enzymes [32].

The *in vitro* data reported here demonstrate that a toothpaste containing enzymes and proteins can generate significant levels of hypothiocyanite (greater than 100 μM) when mixed with irradiated saliva. This is consistent with glucose oxidase generating hydrogen peroxide from glucose in saliva [33] and in turn lactoperoxidase generating hypothiocyanite from the hydrogen peroxide and thiocyanate in the saliva [5]. Hypothiocyanite levels reported in fresh saliva are variable, perhaps driven in part by the highly reactive nature of the chemical species but also variability in measurement techniques. The average levels reported are between 15 and 50μM with large standard deviations [34].

Hydrogen peroxide levels measured in saliva were 64% higher after brushing with a toothpaste containing enzymes and proteins compared to the control toothpaste. This is consistent with the proposed role of exogenous amyloglucosidase and especially glucose oxidase in generating glucose and hydrogen peroxide respectively [11,35]. Hydrogen peroxide levels in the oral biofilm are expected to be many times higher than those found in the saliva as elegantly demonstrated by Liu et al. [36]. The lysozyme activity measured in saliva after use of a toothpaste containing enzymes and proteins compared to a control increased by 92%.

The data for hypothiocyanite generation, and elevated salivary levels of hydrogen peroxide and lysozyme taken together are strong evidence of boosted salivary defences after use of a toothpaste containing enzymes and proteins.

Having established elevated levels of key natural antimicrobial salivary defence factors after use of a toothpaste containing enzymes and proteins, we investigated the effect of those same enzymes and proteins on key microbial species in an array of microbiology models ranging in complexity from single species suspension assays to single and multispecies oral biofilm assays.

Focusing on species with a well-established association with oral

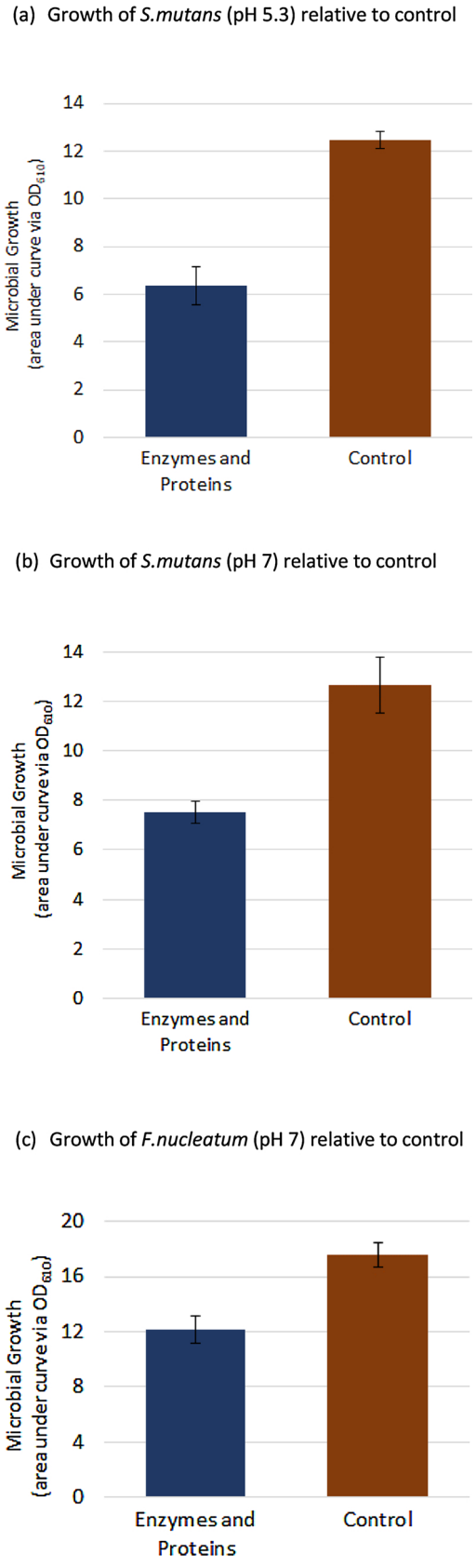

Fig. 1. Mean Area under Curve (AUC) values derived from the growth curves of *S. mutans* (pH 5.3 and 7 respectively) and *F. nucleatum* respectively at pH 7 in the presence of amyloglucosidase, glucose oxidase, lactoperoxidase, lactoferrin, lysozyme and bovine colostrum (IgG) compared to a no active control. Error bars show one standard deviation from four replicate experiments.
(a) Growth of *S.mutans* (pH 5.3) relative to control.
(b) Growth of *S.mutans* (pH 7) relative to control.
(c) Growth of *F.nucleatum* (pH 7) relative to control.

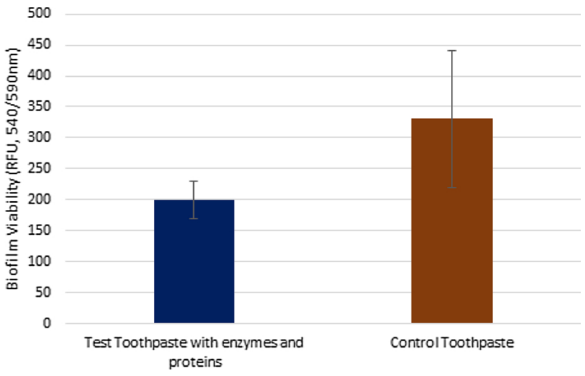

Fig. 2. Viability of *S. mutans* biofilm grown on salivary pellicle as measured by PrestoBlue. Data plotted are means for 12 replicate experiments. Difference between test and control $p < 0.005$, Student's t-test. Error bars indicate one standard deviation.

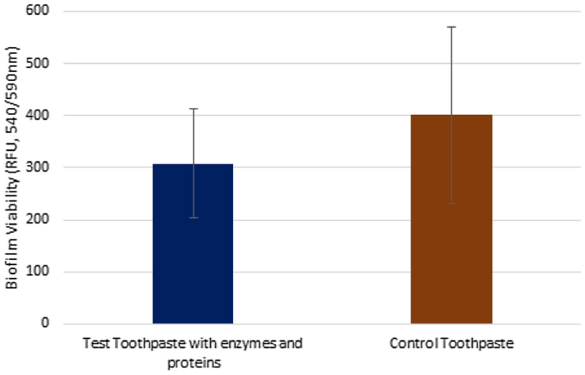

Fig. 3. Viability of *F. nucleatum* biofilm grown on salivary pellicle as measured by PrestoBlue. Data plotted are means for 12 replicate experiments. Difference between test and control $p = 0.074$, Student's t-test. Error bars indicate one standard deviation.

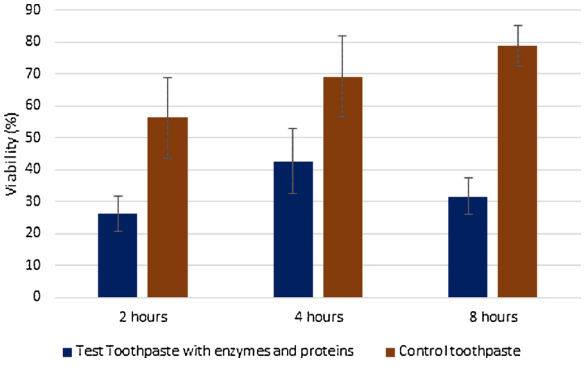

Fig. 4. Viability of seven species biofilms grown on salivary pellicle as measured by Alamar Blue. Data plotted are means for 6 replicate experiments. Error bars indicate one standard deviation (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

disease, we conducted microbial assays of cell membrane permeability and function showing that the combination of enzymes and proteins used in toothpaste (at a concentration of 5.2 μg / ml) can disrupt the cell membranes of *S. mutans* (at a concentration of 5.2 μg / ml) and *F. nucleatum* (7.8 μg / ml). The observations of membrane perturbation by enzymes and proteins are consistent with the effects of lysozyme previously seen by Bleiweis et al. [37] who noted extensive degradation of cell wall structure and loss of cell viability from lysozyme treatment of *S. mutans* and the effects of lactoferrin on *S. mutans* cell walls reviewed by Farnaud et al. [38]. Additionally the higher concentrations of lysozyme and lactoferrin alone (120 μg / ml) required for significant perturbation of membrane integrity points to a strong synergy between the enzymes and proteins. Enhanced antibacterial effects have been previously reported from the combination of salivary enzymes including lysozyme with the peroxidase system [39]. In addition to membrane perturbation, the bacterial growth inhibition assays reported here also demonstrate a reduction in growth of the representative oral pathogens *S. mutans*, and *F. nucleatum* by the combination of enzymes and proteins as low as 1.4 mg/ml.

In the *in vivo* context, the biofilm mode of growth is key to the development of plaque. Bacteria growing as a surface adhered biofilm are expected to show increased resistance to antimicrobial agents due to a range of factors such as reduced penetration of agents, altered metabolic activity and / or different gene expression [40]. Furthermore, in the complex, poly-microbial context of the mouth, biofilms may show emergent properties, meaning that the biofilm community may demonstrate properties hard to predict from the individual species from which the biofilm is composed [41]; for these reasons a series of single and multispecies biofilm assays were conducted to investigate the impact of enzymes and proteins on simulated oral biofilms.

Single species biofilm assays showed that the viability of *S. mutans* was affected by the application of a toothpaste containing enzymes and proteins. This effect is observed at levels 1/3 of those found in the toothpaste, a level consistent with that likely to be found in the mouth during brushing when the toothpaste is diluted by saliva [42]. This assay demonstrates that the effects of enzymes and proteins seen in planktonic cultures are also applicable to single species biofilms when the enzymes and proteins are delivered from a toothpaste and that the enzymes and proteins are effective on both planktonic and sessile bacteria.

Mono-culture biofilm population models allow for high-throughput and detailed analysis of key microbial species. However, predictions based on mono-culture assays are limited, as the oral cavity harbours a complex microbiota. Biofilm heterogeneity strongly influences the success of oral treatment strategies, as intra- and interspecies interactions play an important role in susceptibility to antimicrobial agents and in maintaining a balanced microbiota [43]. The use of closed, well defined multi-species biofilm models adds complexity and allows for additional biofilm community behaviours such as cooperation and competition [41]. The choice of well-defined communities helps to maintain biofilm reproducibility and allows for clearly defined interspecies interactions.

We used a defined 7 species biofilm model based on the work of Millhouse [22], incorporating Streptococcus species, *F. nucleatum*, *A. naeslundii* and *V. dispar*, which are associated with the creation of more complex biofilms that may support species more associated with mature oral biofilms, and *Prevotella intermedia,* which is strongly associated with periodontitis [44]. Treatment with a toothpaste containing enzymes and proteins gave a significant reduction in cell viability compared to the control, providing data consistent with that generated in simpler models. It should be noted that in this model no effect was seen for the toothpaste with enzymes and proteins on the total biomass, as measured by crystal violet, or the biofilm composition. In previous work *in vitro*, the combined action of salivary enzymes and proteins has been tested on single bacterial species, with the focus on streptococci species, especially *S. mutans*. The data reported here suggest that by enhancing the natural salivary defences with enzymes and proteins, the antimicrobial mechanisms in saliva may be increased and the growth of a range of key bacterial species on the acquired pellicle may be reduced. The combination of an upregulated lactoperoxidase system in synergy with lactoferrin and lysozyme clearly showed a reduction in cell viability in both simple and more complex microbial communities.

Reduction of the bacterial load within the oral cavity is a key treatment strategy to decrease occurrence of conditions such as gingivitis [45] and the associated gum health benefits for users of toothpaste containing enzymes and proteins have been demonstrated *in vivo* by both Daly et al. and Pedersen et al. [1,2].

5. Conclusions

In these studies we have demonstrated that the use of a toothpaste containing enzymes and proteins (Zendium™) can boost the natural salivary defences by increasing the levels of lysozyme and hydrogen peroxide *in vivo* and hypothiocyanite *in vitro*. We have further demonstrated that the enzymes and proteins have subtle effects on representative oral pathogens, reducing membrane integrity, growth and viability in a range of *in vitro* models, supporting the conclusion that the enzymes and proteins in the test toothpaste contribute to the previously reported microbiome benefits and suggest that the null hypothesis in the Introduction can be rejected.

Although no *in vitro* assay can fully capture the complexity of the *in vivo* context the subtle effects on bacteria here reported are consistent with the rebalancing of the microbiome by use of a toothpaste containing enzymes and proteins reported by Adams et al. [3] and the maintenance of a healthy balanced microbiome as described by Kilian et al. [4]. The results are further consistent with the gum health benefits reports by Daly et al. and Pedersen et al. [1,2].

Future work should address in more detail the effects of boosted salivary defences on the ecology of complex oral biofilms using tools such a transcriptomics and metabolomics and may lead to targeted and specific treatment strategies.

Conflict of interest and funding statement

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