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ORIGINAL ARTICLE

Combinatorial effects of *trans*-cinnamaldehyde with fluoride and chlorhexidine on *Streptococcus mutans*

A.R. Balasubramanian[1], S. Vasudevan[1], K. Shanmugam[1], C.M. Lévesque[2], A.P. Solomon[1] and P. Neelakantan[3]

1 Quorum Sensing Laboratory, Centre for Research in Infectious Diseases (CRID), School of Chemical and Biotechnology, SASTRA Deemed to be University, Thanjavur, India
2 Faculty of Dentistry, University of Toronto, Toronto, ON, Canada
3 Faculty of Dentistry, The University of Hong Kong, Hong Kong, Hong Kong SAR

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biofilm, dental caries, *trans*-Cinnamaldehyde, *Streptococcus mutans*, synergy, virulence.

Correspondence
Prasanna Neelakantan, Discipline of Endodontology, Division of Restorative Dental Sciences, Faculty of Dentistry, The University of Hong Kong, Hong Kong SAR.
E-mail: prasanna@hku.hk

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Abstract

Aims: The aim of this study was to investigate the effects of *trans*-cinnamaldehyde (TC) and its synergistic activity with chlorhexidine (CHX) and fluoride against *Streptococcus mutans*.

Methods and Results: *Streptococcus mutans* UA159 was treated with TC alone and in combination with CHX or sodium fluoride. The synergy profile was analysed using the Zero Interaction Potency model. TC showed strong synergism (synergy score of 21·697) with CHX, but additive effect (synergy score of 5·298) with fluoride. TC and the combinations were tested for acid production (glycolytic pH drop) and biofilm formation by *S. mutans*, and nitric oxide production in macrophages. TC significantly inhibited sucrose-dependent biofilm formation and acid production by *S. mutans*. Mechanistic studies were carried out by qRT-PCR–based transcriptomic studies which showed that TC acts by impairing genes related to metabolism, quorum sensing, bacteriocin expression, stress tolerance and biofilm formation.

Conclusions: *trans*-Cinnamaldehyde potentiates CHX and sodium fluoride in inhibiting *S. mutans* biofilms and virulence through multiple mechanisms. This study sheds significant new light on the potential to develop TC as an anti-caries treatment.

Significance and Impact of the Study: Oral diseases were classified as a 'silent epidemic' in the US Surgeon General's Report on Oral Health. Two decades later, >4 billion people are still affected worldwide by caries, having significant effects on the quality of life. There is an urgent need to develop novel compounds and strategies to combat dental caries. Here, we prove that TC downregulates multiple pathways and potentiates the CHX and fluoride to prevent *S. mutans* biofilms and virulence. This study sheds significant new light on the potential to develop TC in combination with CHX or fluoride as novel treatments to arrest dental caries.

Introduction

Dental caries (tooth decay) is the most prevalent, noncommunicable, infectious disease, affecting more than 4 billion people worldwide. It is driven by dysbiosis of oral biofilms, characterized by the dominance of acid-producing and acid-tolerant bacteria in the multi-species dental plaque biofilm (Lamont *et al.* 2018). *Streptococcus mutans*, the primary cariogenic micro-organism, initiates dysbiosis by metabolizing dietary carbohydrates to produce acids that demineralize the enamel. *Streptococcus mutans* is equipped with an array of well-adapted, intricately woven pathways that result in polysaccharide production, DNA repair and competence (Lemos *et al.*

2019). Its ability to survive in the presence of acids (acid tolerance) and other stressful environments contributes tremendously to the sustenance of dysbiosis.

Multiple factors contribute to the increased pathogenicity and persistence of *S. mutans* (Kaur *et al.* 2017; Lemos *et al.* 2019). Several bacteria demonstrate coordinated group behaviour, by releasing chemical signalling molecules. This mechanism, termed quorum sensing, regulates biofilm formation, acid production and acid tolerance in *S. mutans* (Sztajer *et al.* 2008; Kaur *et al.* 2017). Furthermore, glucosyltransferases and glucan-binding proteins facilitate its adherence to salivary pellicles and synthesize glucans, forming the basal matrix of biofilms. Acidogenicity and mutacin production (peptide antibiotics) establish an environment that flourishes with cariogenic microbiota. Cell membrane pumps and the neutralizing systems maintain pH homeostasis (Lemos *et al.* 2019).

Most commercially available oral care products contain antimicrobials such as chlorhexidine (CHX) and fluoride. The above-mentioned rich tapestry of pathways makes it a herculean task for these antimicrobials to act effectively against *S. mutans*. Fluoride is unable to modulate biofilm composition or its virulence (Thurnheer and Belibasakis 2018). Even with regular use of fluoride, carious lesions develop when sugar exposures exceed six per day (Philip *et al.* 2018). Moreover, the prolonged usage of high concentrations of CHX and fluoride (1000–2000 μg ml^{-1}) has significant side effects. Specifically, high concentrations of fluoride have been linked to fluorosis, bone weakening and developmental neurotoxicity (Hirzy *et al.* 2016; Duangthip *et al.* 2018). The toxicity of CHX to host cells, teeth staining and allergic reactions is well established (Pemberton and Gibson 2012). More importantly, the emergence of resistance to these antimicrobials is a critical concern. Therefore, the need of the hour is a compound that can target the pathogenesis, quorum sensing and signalling pathways pertaining to acid production, biofilm formation and bacteriocin production, thereby potentiating the activity of CHX and fluoride at lower concentrations, without compromising their efficacy (Kaur *et al.* 2017; Liao *et al.* 2017; Cieplik *et al.* 2019).

In recent years, natural compounds have gained wide attention owing to broad spectrum therapeutic effects. *trans*-Cinnamaldehyde (TC), a bioactive compound obtained from Cinnamon trees of the genus *Cinnamomum*, is used as a spice and traditional herbal medicine (Jia *et al.* 2011; Doyle and Stephens 2019). TC has remarkable antimicrobial activity against multidrug-resistant bacteria such as methicillin-resistant *Staphylococcus aureus* and *Acinetobacter baumannii* (Jia *et al.* 2011; Karumathil *et al.* 2018). The antibacterial mechanisms of TC include targeting FtsZ (cell division protein), membrane permeabilization and ATPase inhibition, thereby revealing its potential to hit multiple targets (Doyle and Stephens 2019).

Recently, a study demonstrated the antimicrobial activity of cinnamaldehyde against *S. mutans* (He *et al.* 2019). Cinnamaldehyde appears to reduce acid production, acid tolerance and downregulate the genes essential for the nascent stage of biofilm formation. Given that cinnamaldehyde alone can downplay the virulence of *S. mutans*, exploring its capability to synergize with the gold standard antimicrobials existing in oral products would help in boosting their efficacy at very low concentration. Thus, the aim of this study was to investigate the effects of TC alone and in combination with CHX and fluoride on *S. mutans* biofilms and virulence. Specifically, we investigated the synergistic activity of TC with CHX and fluoride against *S. mutans*, the mechanisms of actions and its biocompatibility to host cells.

Materials and methods

Chemicals, bacterial strain and culture conditions

The following chemicals were purchased from HiMedia Laboratories (Mumbai, India): CHX diacetate (98% purity), sodium fluoride (99% purity) and brain heart infusion broth. TC was purchased from Sigma Aldrich (St. Louis, MO, USA) (99% purity). The stock of TC was prepared in 2% Tween20. *Streptococcus mutans* UA159 was grown in brain heart infusion (BHI) broth with 0·02 mol l^{-1} sucrose. The culture was maintained as glycerol stock at −80°C. The assays were performed by growing *S. mutans* until mid-logarithmic phase and the OD_{595} was adjusted to achieve $1{\cdot}5 \times 10^8$ CFU per ml, with the fresh broth containing 0·02 mol l^{-1} sucrose. All experiments were done in triplicates on three different occasions.

Synergy studies: checkerboard analysis

The combinatorial action of TC with CHX and fluoride was assessed using the checkerboard assay. The above-mentioned bacterial suspension was added in 96-well microtitre plates containing TC with CHX or fluoride. Varying concentrations of TC (47–500 μg ml^{-1}), CHX (250–25 μg ml^{-1}) and fluoride (1400–150 μg ml^{-1}) were taken in two-fold dilution. The plates were incubated for 24 h at 37°C in a 5% CO_2 incubator. After incubation, bacterial growth inhibition was evaluated by measuring the OD_{595}. The untreated bacterial culture was used as negative control. Tween20 (0·2%) was maintained throughout the assay. The treated and untreated cultures

were compared, and the percentage inhibition of the combination and individual treatments was calculated. Furthermore, synergism was analysed by fitting the data in Zero Interaction Potency (ZIP) model using the R package 'synergyfinder' (https://bioconductor.org/packages/release/bioc/html/synergyfinder.html).

Glycolytic pH-drop assay

The influence of TC and its combination with CHX or fluoride on acid production was evaluated by measuring the pH drop with time (Wen and Burne 2004). Mid-logarithmic cultures of *S. mutans* were treated at different conditions for 24 h. The treatment conditions considered were: TC (500 μg ml^{-1}), CHX (25 μg ml^{-1}), fluoride (150 μg ml^{-1}), TC (63 μg ml^{-1}) + fluoride (150 μg ml^{-1}) and TC (47 μg ml^{-1}) + CHX (25 μg ml^{-1}). The effect of the above treatments on the drop in pH was monitored (Digital pH meter with pH electrode, Elico) for 8 h at every 30 min.

Immunostimulatory effect and cell cytotoxicity on macrophages

The cytotoxic effect and the ability of TC in combination with CHX or fluoride to induce reactive nitrogen species in RAW 264·7 cell lines were tested (Liu *et al.* 2017). Cell cytotoxicity was evaluated using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Briefly, RAW 264·7 cells were grown in Dulbecco modified eagle medium (DMEM), HiMedia Laboratories, to a confluency of 70–80% and were treated according to the same conditions mentioned in the glycolytic pH-drop assay. Untreated cells were used as control. The cells were incubated for 6 h and MTT was added. After incubation at dark at 37°C, absorbance was measured at 570 nm.

The immunostimulatory effects of TC in combination with CHX or fluoride on RAW 264·7 cells were evaluated by quantifying the nitric oxide produced in the presence of the different treatment conditions, same as the cell cytotoxicity studies. Briefly, RAW 264·7 cells at 70–80% confluence were exposed to the treatment conditions for 6 h. After collecting the supernatants, equal volume of Griess reagent was added and incubated for 15 min at room temperature. The nitrate concentrations were then measured.

Biofilm inhibitory assays

The effect of TC on *S. mutans* biofilm inhibition was analysed using confocal microscopic imaging. Biofilms were formed on sterile glass slides for 24 h with TC (500 μg ml^{-1}). The slides were washed with sterile water to remove the planktonic cells and stained with *Bac*Light Bacterial Viability Kit (ThermoFisher Scientific, Waltham, MA, USA). Z-stacks were obtained from at least five regions of the glass slides, using a confocal laser scanning microscope (Olympus FLUOVIEW, FV1000, Tokyo, Japan) with 40X objective lens. The obtained images were further analysed using COMSTAT package in MATLAB R2017b (Heydorn *et al.* 2000).

Gene expression analysis: qRT-PCR studies

The overall effect of TC on *S. mutans* metabolism, biofilm inhibition and virulence (Table S1) was elucidated using the gene expression analysis. The total RNA at three different time points (early, mid and late log phase) from TC-treated *S. mutans* cells (47 μg ml^{-1}; 37°C; 5% CO_2) was extracted following the manufacturer's guidelines (HiMedia RNA Extraction Kit MB613). The quality and quantity of the extracted RNA were evaluated using standard agarose gel electrophoresis and NanoDrop (Thermo Scientific) respectively. Total RNA was converted to cDNA using the iScript™ cDNA Synthesis Kit using the manufacturer-recommended protocol. The gene expression levels of the considered genes were analysed using qRT-PCR. 16s rRNA was used as the reference gene and the gene expression was calculated by $2^{-\Delta\Delta CT}$ method. The PCR conditions were followed as described previously (Kaur *et al.* 2017).

Statistical analysis

The results were expressed as mean ± SD. GraphPad Prism software version 6·05 (GraphPad Software Inc., San Diego, CA) was used for performing the statistical analysis. Significance was checked with Dunnett *t*-test for multiple comparisons and paired Student *t*-test ($P \leq 0{\cdot}05$).

Results

TC potentiates the antimicrobial activity of chlorhexidine and fluoride

When compared with the individual treatment, combination with TC resulted in an 80-fold reduction in the concentration of CHX to achieve ~80% growth inhibition (Fig. 1). At a concentration of 25 μg ml^{-1}, CHX was able to inhibit only 42·55%. Similarly, there was a 13-fold reduction in the concentration of fluoride required to achieve ~50% growth inhibition. At a concentration of 150 μg ml^{-1}, fluoride could inhibit only 24·76% growth. TC inhibited 15·68 and 21·80% growth at 47 μg ml^{-1} and 63 μg ml^{-1} respectively. Thus, the combinations

resulted in an increased antimicrobial activity with reduced concentrations of the three compounds.

The obtained data were further analysed by fitting into the ZIP model (Yadav *et al.* 2015) (Fig. 1a,c) wherein the combined response does not affect the individual potency of the drugs. From the response plot, it is apparent that CHX showed a strong synergism (synergy score of 21·697), whereas fluoride showed an additive effect (synergy score of 5·298) with TC.

TC reduces *S. mutans* acidogenicity

Acid production (acidogenicity) was investigated by monitoring the pH drop for the different treatments upto 8 h (Fig. 2). Treatment with TC alone (500 μg ml^{-1}) maintained an average pH of 6·7 until 8 h. Notably, the combinatorial treatments maintained a pH of 6·5–6·8 for 8 h, and this was comparable to the respective individual treatments alone. The trend in our results is in agreement with a recent report on the anti-acidogenic effect of cinnamaldehyde up to 120 min (He *et al.* 2019). Thus, TC successfully reduced acid production by *S. mutans*.

TC reduces *S. mutans* biofilm biomass, alters biofilm distribution and architecture

Our results are in accordance with a recent study that showed the antibiofilm activity of TC against (He *et al.* 2019). Biofilm analysis revealed a significant difference in the architecture of untreated and treated biofilms (Fig. 3). Biofilm biomass, average thickness, dimensionless roughness coefficient, surface area to volume ratio and diffusion distances were analysed. It is evident that the untreated *S. mutans* biofilms were thicker with higher biomass (32 μm^3 μm^{-2}) than the treated biofilm (1·9 μm^3 μm^{-2}) (Fig. 3b). The maximum biofilm thickness of the untreated sample was 84 μm whereas the maximum biofilm thickness was only 27·8 μm in the presence of TC. Our results showed that the roughness was significantly more in TC-treated biofilms than in the untreated ones. The surface-to-volume ratio was also increased in the treatment groups than in the untreated samples. The diffusion distance was also observed to be reduced from 11·69 (control) to 0·68 after TC treatment.

TC in combination with CHX and fluoride is not toxic to host cells and does not contribute to nitric oxide production

The effect of TC on cell viability and nitric oxide has been shown previously (Kim *et al.* 2018). We questioned if the combinatorial treatments demonstrated similar effects. Figure 4 depicts the nontoxic property of the combinations investigated in this study. It is apparent that TC treatment alone resulted in increased cell viability as compared to control. Furthermore, at the combinatorial concentrations of CHX (25 μg ml^{-1}) and fluoride (150 μg ml^{-1}), no toxicity was observed. The viability of cells in the combination treatment was comparable to the individual treatment of CHX and fluoride. With respect to nitric oxide production, there was no significant difference in the nitric oxide production in the macrophages with different treatments. Our results show that the different treatments maintain the nitric oxide equivalent to control, indicating that they do not have immunostimulatory effect at the concentrations considered.

TC downregulates genes related to metabolism, biofilm and virulence

In order to decode the mechanism of action of TC in the potentiating activity, gene expression studies were conducted at the synergy concentration of TC (47 μg ml^{-1}). The studies were conducted in a time-dependent manner (Fig. 5), focusing on the exponential phase (early, mid and late). The genes required for biofilm formation, virulence and metabolism of *S. mutans* were chosen for analysis in this work.

The timescale mapping of the gene expression profile showed that during the early log phase, *atpD*, *bsmH* and *altA* were markedly downregulated. There was a 3 $\log_{10}$ fold reduction in the *atpD* expression, indicating a disturbance in stress tolerance. The other genes which had a notable reduction of 1·5 $\log_{10}$ fold were *idh* and *covR*.

The genes, *ftsZ, gyrA, gbpB, relA, bsmI, dnaK, comDE, comA, covR,* were downregulated in the mid-log phase. *covR* was the most downregulated gene with a 6 $\log_{10}$ fold reduction, indicating the anti-virulence effect of TC, followed by *gbpB* (2 $\log_{10}$ fold reduction). Another striking downregulation was observed for the ComDE quorum sensing system. The histidine kinase, response regulator and the ABC transporter of ComDE quorum system were downregulated in the mid-log phase. This has implications on competence development and biofilm formation. *relA, dnaK* and *bsmI* were downregulated approximately 1·5 $\log_{10}$ fold. A notable downregulation of *ftsZ* and *gyrA* was also observed.

In the late log phase, there was a time and density-dependent downregulation of a majority of the following genes: *gtfC, spaP, immA, immB, nlmC, idh, recA, brpA, comB, vicR, comX, luxS*. The consistent downregulation in all the three time points establishes the effect of TC on major genes pertaining to biofilm formation, immunity and global virulence regulators. *luxS* was downregulated 3 $\log_{10}$ fold, followed by *brpA* (2·5 $\log_{10}$ fold). Genes required for biofilm formation, namely *gtfC* for glucan

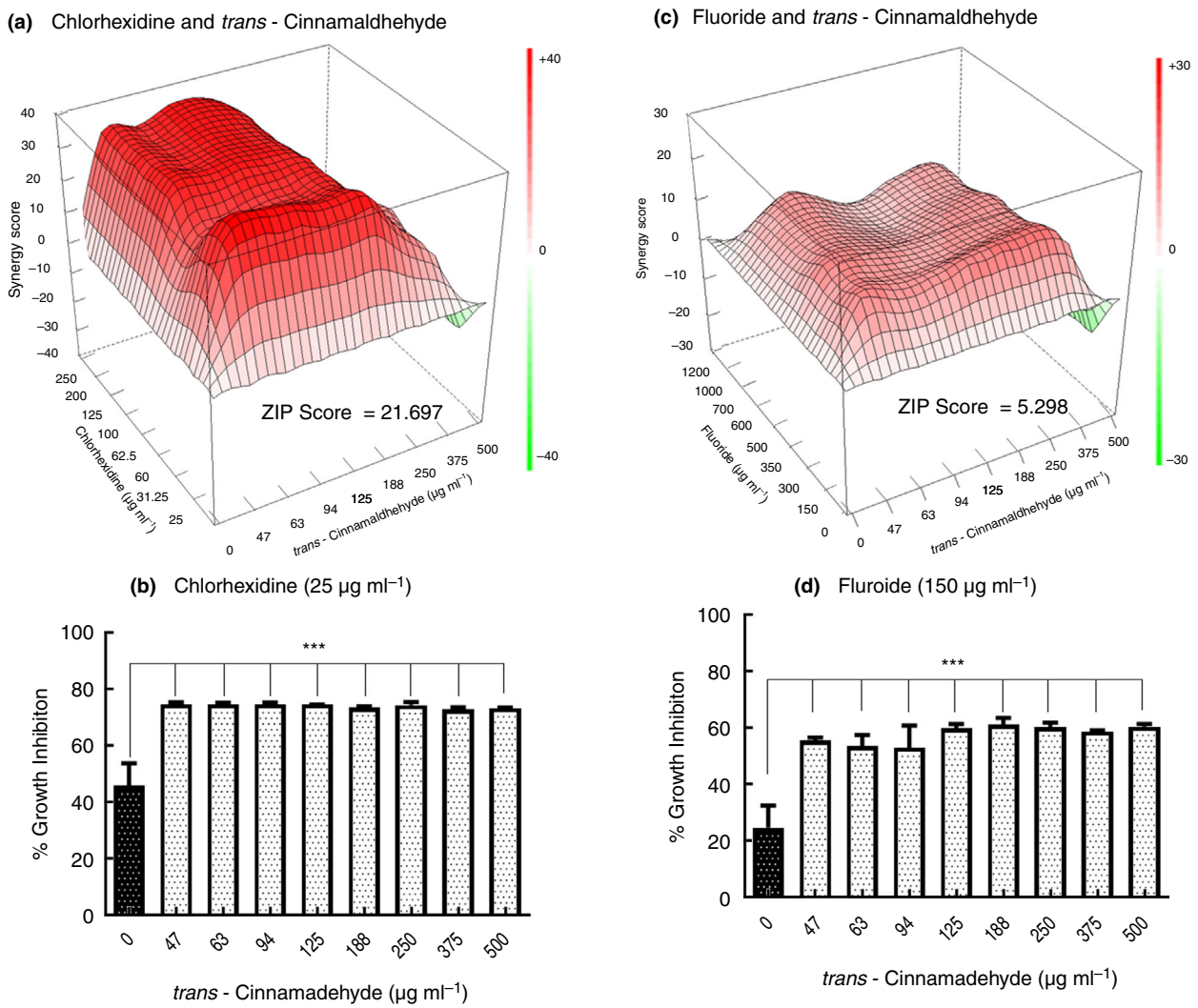

Figure 1 Synergistic action of *trans*-cinnamaldehyde (TC) with fluoride and chlorhexidine. (a) The Zero Interaction Potency (ZIP) plot of chlorhexidine and TC, (b) the antimicrobial potentiation of chlorhexidine (25 µg ml^{-1}) at different concentrations of TC, (c) the ZIP plot of fluoride and TC, (d) the antimicrobial potentiation of fluoride (150 µg ml^{-1}) at different concentrations of TC. Dunnett *t*-test was performed to compare the inhibitory activity of chlorhexidine with and without the presence of TC and fluoride with and without the presence of TC. $P < 0{\cdot}05$ was considered statistically significant.

(soluble and insoluble) production and *spaP*, showed reduced expression. The DNA repair systems, *idh* and *recA* were also consistently downregulated. The response regulator of a two-component system that is important to *S. mutans* virulence, *vicR* was downregulated 1·5 $\log_{10}$ fold. In addition to the above-mentioned genes, *atlA* was also downregulated ~2 $\log_{10}$ fold.

Discussion

Streptococcus mutans orchestrates a well-established pathogenesis, using intricate cross-regulatory mechanisms. These mechanisms provide a unique edge for *S. mutans* to survive and establish its predominance amongst the multitude of oral microbiota. Thus, it is paramount to develop a combinatorial therapeutic approach which works through multiple mechanisms. Phytomolecules possess ethnopharmacological properties and have shown to have broad spectrum antimicrobial activities. In this regard, the current report demonstrates the proof-of-concept that TC, a known natural compound from cinnamon, can be used in combination with CHX or fluoride to combat *S. mutans* biofilms and virulence.

The checkerboard assay for synergy studies proved the potentiation activity of TC. This may be attributed to various factors such as biofilm inhibition, suppression of

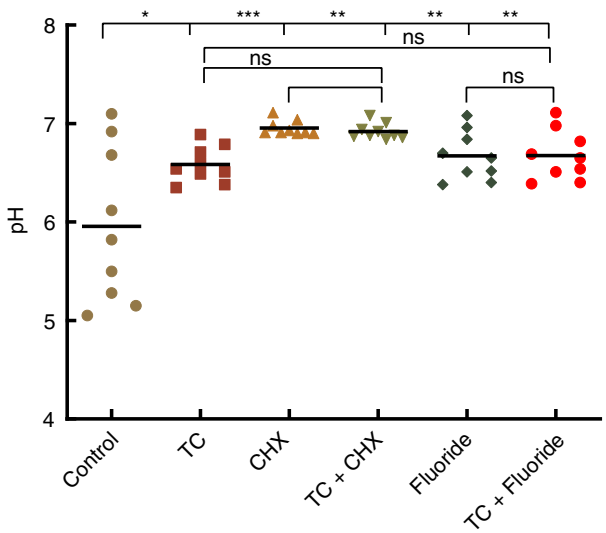

Figure 2 Effect of TC on acid production: The effect of different treatments on glycolytic pH drop of *Streptococcus mutans*. Dunnett *t*-test was performed for multiple comparison analysis. * denotes $P < 0{\cdot}05$, ** denotes $P < 0{\cdot}01$ and *** denotes $P< 0{\cdot}001$.

virulent phenotypes and quorum sensing inhibition (Vasudevan *et al.* 2018). Previous studies corroborate that TC inhibits biofilm formation during initial stage (4 h) as well as at the maturation stage, 24 h (He *et al.* 2019). It was shown previously that there is a close relationship between bacteriocin immunity proteins and modulation of antimicrobial sensitivity in *S. mutans*. Lack of bacteriocin immunity proteins production was shown to increase the susceptibility of *S. mutans* to antimicrobials, especially CHX and fluoride (Wang *et al.* 2013). Hence, it is likely that TC suppressed the immunity proteins, thereby providing a way for CHX and fluoride to act. Considering all these factors, it can be regarded that TC may act in multiple pathways to establish its synergy activity.

The results of this study prove that TC can potentiate the activity of CHX and fluoride at reduced concentrations. Thus, the mechanism of potentiation by TC was mapped through different phenotypic assays. Acid production (acidogenicity) and acid tolerance (aciduricity) are key virulence mechanisms of *S. mutans*, relevant to dental caries (Matsui and Cvitkovitch 2010). Through these two mechanisms, *S. mutans* and the other acid-tolerant cariogenic pathogens survive in the biofilms, while the health-associated mutualistic species do not survive. This results in a vicious cycle of more acid production, which is closely associated with enamel demineralization and cavity formation (Takahashi and Nyvad 2008). The impairment of acid production by TC obtained in this study is comparable to previous studies (He *et al.* 2019). The current study proves the efficacy of TC to reduce acid production up to 8 h, and it should be noted that in combination with CHX or fluoride, there was no significant change observed in the pH drop. This shows that the reduction in acid production by CHX and fluoride is unperturbed by TC. The direct effect of TC alone and the combinations on enamel demineralization requires further validation, yet the implications of the reduced acidogenicity can be extrapolated to the reduced enamel dissolution.

Biofilm is considered to be the strong defence mechanism of *S. mutans*. With its three-dimensional matrix, biofilm encompasses exopolysaccharide, eDNA and several other proteins which acts a diffusion barrier to antimicrobials. Physical parameters such as biofilm thickness, biofilm mass, diffusion distance and roughness coefficient define the biofilm architecture. Roughness coefficient indicates biofilm heterogeneity (Heydorn *et al.* 2000). Our results signify an uneven distribution of biofilm with less biomass in the treated samples, which can be easily eliminated by host clearance mechanisms. The importance of diffusion distances is well documented and diffusion distances are more in biofilms (Stewart 2003). When a biofilm is perturbed, there is an easy solute movement, leading to the reduction in the diffusion distance (Stewart, 2003). The architectural analysis support the anti-biofilm property of TC.

The *in vitro* phenotypic assays demonstrate that the combination has antimicrobial and anti-virulence effect. In order to develop this as a therapeutic combination, it

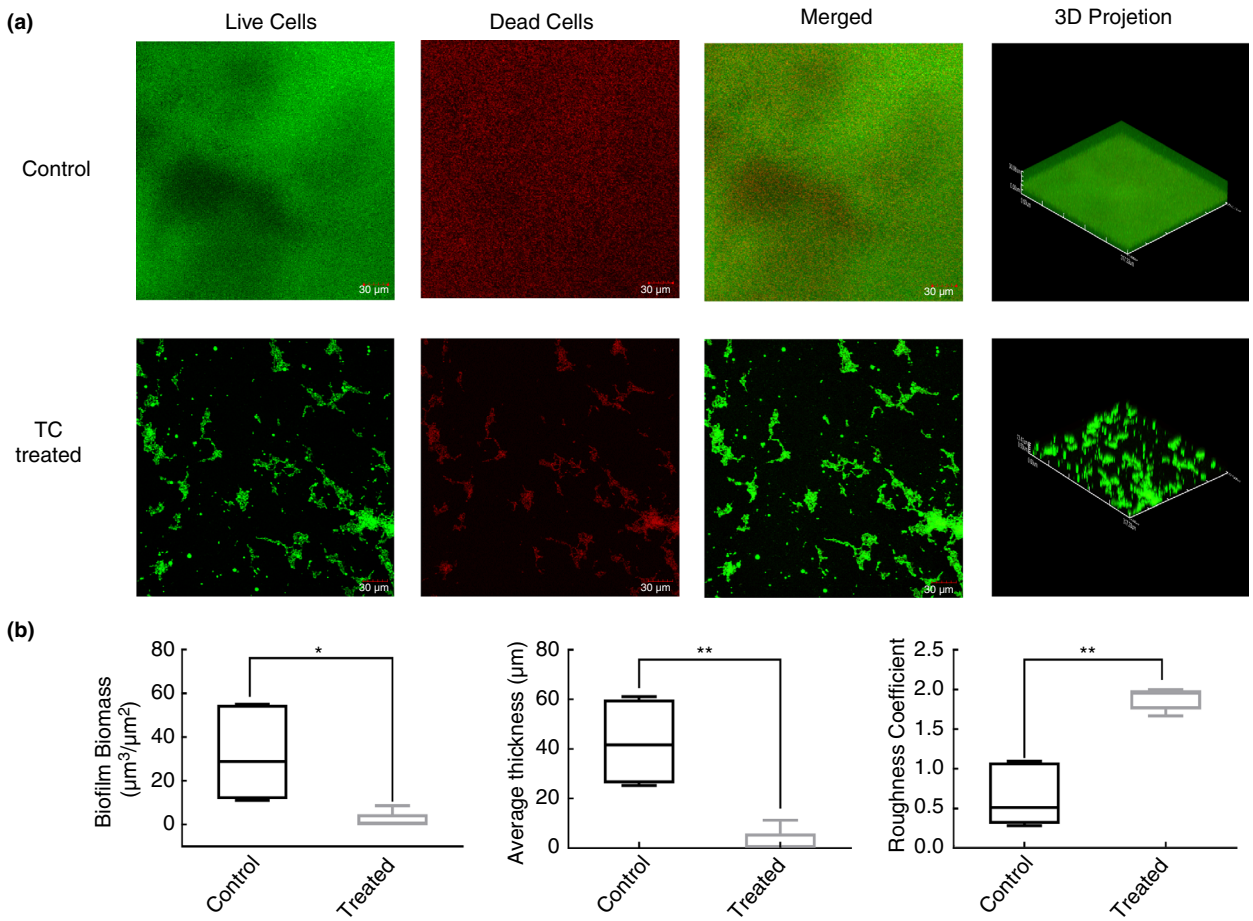

Figure 3 Qualitative analysis of biofilm inhibition of TC. (a) The panel shows the reduced biofilm biomass when treated with TC. (b) The analysis of the biofilm using COMSTAT 1. The biofilm biomass, average thickness and dimensionless roughness coefficient are represented in the figure. Student *t*-test was used for the significance analysis. $P < 0{\cdot}05$ was considered significant. * denotes $P < 0{\cdot}05$, ** denotes $P < 0{\cdot}01$ and ***denotes $P < 0{\cdot}001$.

should be proved as nontoxic and should not induce any inflammatory reaction. The nontoxic effects of TC reported previously were limited to concentrations of 100 µmol l^{-1} (Lu *et al.* 2018). Our results demonstrate that TC, in addition to being nontoxic, improves the cell viability at a concentration of 500 µg ml^{-1}. The increased cell proliferation of TC needs further analysis. Previously, it has been shown that CHX is toxic to these cells at a higher concentration (Bonacorsi *et al.* 2004). Our results show that the potentiation activity of TC contributes to the reduced concentration of CHX required, thereby reducing the toxicity. Nitric oxide is one of the key effectors of inflammatory response and is activated in the event of bacterial invasion (MacMicking *et al.* 1997). The anti-inflammatory property of TC is well established (Kim *et al.* 2018). Through this study, we demonstrate the effects of the combinatorial treatments on nitric oxide production. The production of nitric oxide has both useful and deleterious effect on the cells (Wang *et al.* 2018). The results achieved for CHX are comparable to previously published results (Bonacorsi *et al.* 2004). In the case of fluoride, it was reported that fluoride increases the nitric oxide production in serum and that is related to the damage of soft tissue (Liu *et al.* 2003). Here, the nitric oxide production was maintained *status quo*, inferring that our combinatorial treatment may not induce tissue damage.

The potentiation effect of TC was analysed further using gene expression analysis. The synergistic effect of TC may be due to the effects on virulence or metabolism or both. Previous reports on TC against *S. mutans* (He *et al.* 2019) as well as other micro-organisms (Jia *et al.* 2011; Karumathil *et al.* 2018) provide a strong clue that TC has both antimicrobial and anti-virulence effects, with

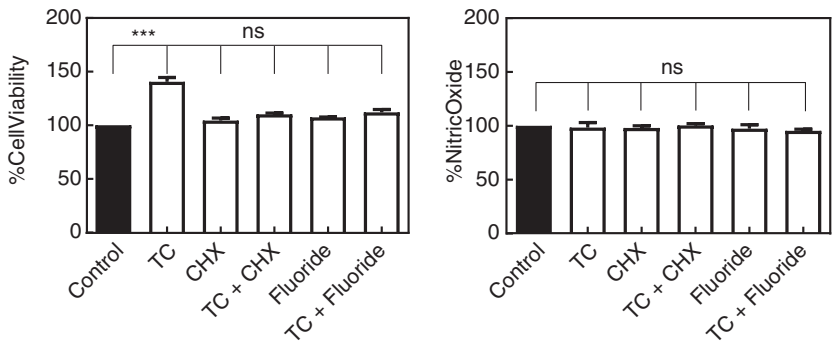

Figure 4 Effect of TC on macrophages. The cell cytotoxicity and nitric oxide production of the combination treatment. Dunnett *t*-test was performed for multiple comparison analysis. * denotes $P < 0{\cdot}05$, ** denotes $P < 0{\cdot}01$ and *** denotes $P < 0{\cdot}001$.

effects on genes related to metabolism as well as virulence (Wen and Burne 2004; Sztajer *et al*. 2008; Winkler and Hoch 2008; Senadheera *et al*. 2009). There is a close association amongst the genes considered in this work. Figure 5 shows an overall picture of the virulence processes downregulated by TC. The temporal mapping of the TC-treated *S. mutans* gene expression supports the possible mechanism of potentiation activity. The bacteriocin immunity proteins play a major role in providing immunity to the bacterial cells during antimicrobial treatments namely fluoride and CHX (Wang *et al*. 2013). Thus, to potentiate the activity of traditional antimicrobials, the compound should downregulate the pathways associated with the above processes. Genes encoding for bacteriocin immunity proteins, *immA* and *immB* (Wang *et al*. 2013) and mutacin namely *nlmC* (Senadheera *et al*. 2012; Liu *et al*. 2014) were downregulated in a time-dependent manner. The genes associated with cell wall biogenesis—*atlA* (Ahn and Burne 2006) were downregulated ~2 $\log_{10}$ fold and cell wall integrity: *brpA* (Bitoun *et al*. 2012) which was downregulated ~3 $\log_{10}$ fold, correlates with the TC potentiation activity.

In the context of virulence mechanisms, aciduricity and acidogenicity are the key players in *S. mutans*. There are multiple genetic mechanisms associated with this property. Of them, maintaining homeostasis is a forerunner. In order to maintain homeostasis, DNA repair mechanisms are upregulated in the event of acidogenicity (Senadheera *et al*. 2009). In this regard, the genes: *atpD, dnaK* (stress tolerance) and the DNA repair systems, *idh* and *recA* were consistently downregulated ~1·5 $\log_{10}$ fold preventing the stress tolerance in the presence of TC. Thus, the maintenance of pH 6·5 upto 8 h by TC, and in combination with the antimicrobials may be attributed to the downregulation of the DNA repair mechanisms. Similarly, multiple mechanisms are involved in biofilm formation (Lemos *et al*. 2019). Quorum sensing systems, notably LuxS (Wen and Burne 2004), ComDE (Kaur *et al*. 2017) and VicRK (Senadheera *et al*. 2005), have a direct regulation of biofilm formation. BrpA, biofilm regulatory protein, in addition to its role in cell envelope integrity and stress tolerance, has a major role in the biofilm formation (Wen and Burne, 2002). TC was shown to have a regulatory role in such pathways to have anti-biofilm activity either directly or indirectly. *vicR* (Senadheera *et al*. 2005, 2009, 2012) was downregulated 1·5 $\log_{10}$ fold. Com system (*comDE*, *comA* and *comB*) was downregulated ~1·5 $\log_{10}$ fold. In addition, genes required for biofilm formation namely *gtfC* (~2 $\log_{10}$ fold) for glucan (soluble and insoluble) production and *spaP* (1·5 $\log_{10}$ fold) showed reduced expression. Glucan-binding protein B has been shown to have immunodominant activity in addition to biofilm formation (extracellular role). Downregulation of this protein has severe effects on initiation of biofilm as well as the associated cell surface phenotypes (Duque *et al*. 2011), TC reduced the expressed ~2 $\log_{10}$ fold. Other virulence-associated genes, namley *relA*, which was downregulated 1·5 $\log_{10}$ fold, plays an important role in the stringent response and in the expression of virulence-related traits in *S. mutans* (Nascimento *et al*. 2008) and *bsmI* (bacteriocin production) were downregulated approximately 1·5 $\log_{10}$ fold.

A notable downregulation of *ftsZ* and *gyrA* was observed, indicating that TC influences growth and metabolism of *S. mutans*. The downregulation of the global response regulator, *covR* has a profound effect on the virulence mechanism (Dmitriev *et al*. 2011; Alves *et al*. 2016). This gene has been shown to be significantly upregulated during the exponential phase (Chong *et al*. 2008) and a 6-$\log_{10}$ fold reduction by TC indicates its anti-virulence effects.

The genes considered for this study have overlapping roles. For instance, VicRK, a two-component signal

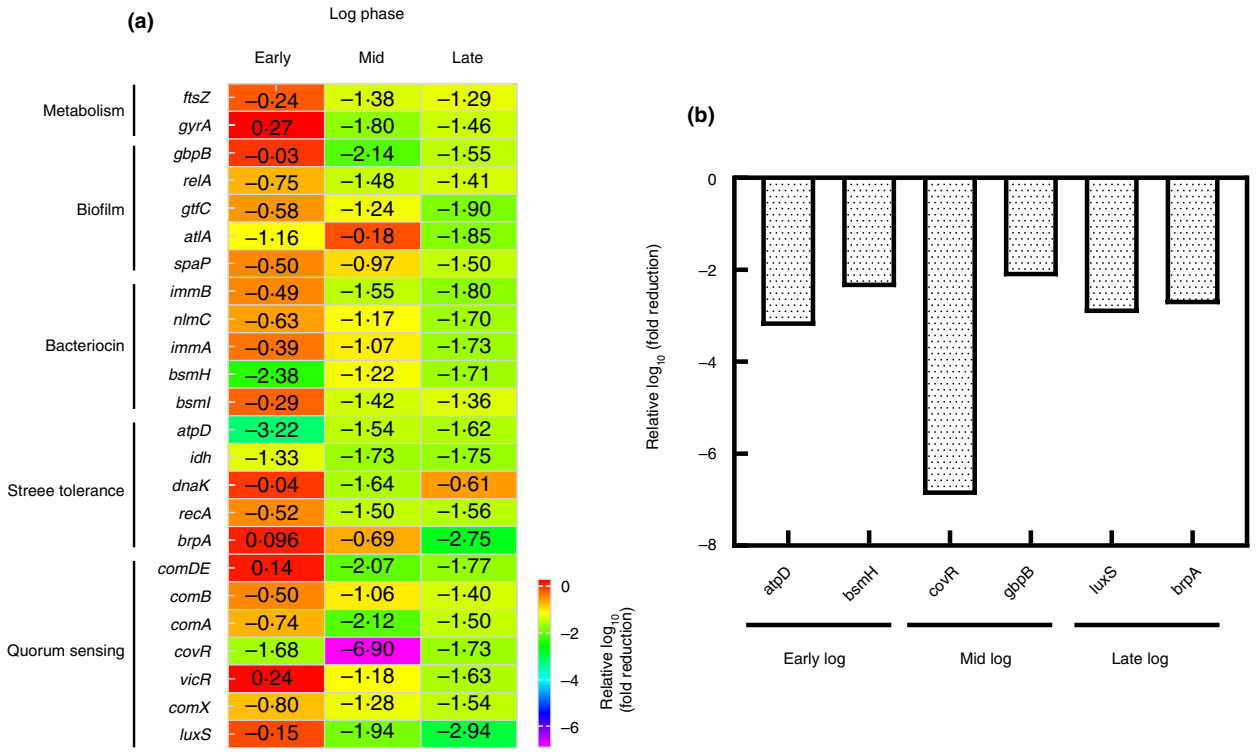

Figure 5 TC downregulates metabolism, biofilm and virulence genes. (a) Downregulation (log_{10} fold reduction) of the gene expression responsible for metabolism, biofilm formation and virulence. The normalized gene expression values are shown with red and green indicating an upregulation and downregulation respectively. (b) Representative genes downregulated at each time point are depicted.

transduction systems (TCSTS) of *S. mutans* is closely associated with acid production and acid tolerance (Senadheera *et al.* 2009), in addition to its role in biofilm formation (Senadheera *et al.* 2005), mutacin production and cell death (Senadheera *et al.* 2012). It was shown that *atlA* is responsible for cell surface biogenesis, biofilm maturation and autolysis (Shibata *et al.* 2005; Ahn and Burne 2007; Jung *et al.* 2009). LuxS is an interspecies quorum sensing system of *S. mutans* and also plays an important role in metabolism (Sztajer *et al.* 2008), while *brpA* encodes for the biofilm regulatory protein. Both these genes are known to regulate major virulence factors including biofilm formation, bacteriocin and stress tolerance (Wen and Burne 2002; Wen *et al.* 2006). LuxS, a versatile interspecies communication mechanism of *S. mutans,* regulates pathways associated with biofilm formation (Merritt *et al.* 2003), stress tolerance and mutacin production (Merritt *et al.* 2005), as well as the cellular metabolism. The downregulation of *brpA*, *vicR*, *comDE*, *spaP* and *recA* may be due to direct effects of TC or a rippling effect due to the downregulation of *luxS* (Wen *et al.* 2011). *brpA* that encodes for an important regulatory protein was downregulated upon TC treatment, is known to control *atpD*, *dnaK* and *recA* (Wen *et al.* 2006, 2018) in addition to its role in stable biofilm formation (Wen and Burne 2002). CovR and VicR act in a coordinated fashion and regulate the biofilm growth and cell envelope biogenesis (Stipp *et al.* 2013). Thus, the qRT-PCR results clearly demonstrate that TC has effects on multiple genes involved in different virulence mechanisms of *S. mutans*. The most striking feature of TC was that it exerts both antimicrobial and anti-virulent activity on *S. mutans* and curbs its pathogenesis.

Combinatorial regimens of natural compounds with the antimicrobials used as daily home-care regimens have several advantages. This study demonstrated for the first time that TC, a known natural compound from cinnamon exhibited synergistic effects with CHX against *S. mutans* biofilms and virulence, at significantly reduced concentrations of CHX. This study sheds significant new light on the potential to develop TC in combination with CHX or fluoride as novel treatments to prevent dental caries.

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Author contributions

All the authors contributed equally to the manuscript.

Transparency declaration

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

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. List of genes and RTPCR primer sequences used for the TC potentiation study.