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Inhibition of biofilm formation and virulence factors of cariogenic oral pathogen *Streptococcus mutans* by natural flavonoid phloretin

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ABSTRACT

Objectives: To evaluate the effect and mechanism of action of the flavonoid phloretin on the growth and sucrose-dependent biofilm formation of *Streptococcus mutans*.

Methods: Minimum inhibitory concentration, viability, and biofilm susceptibility assays were conducted to assess antimicrobial and antibiofilm effect of phloretin. Biofilm composition and structure were analysed with scanning electron microscopy (SEM) and confocal laser scanning microscopy (CLSM). Water-soluble (WSG) and water-insoluble glucan (WIG) were determined using anthrone method. Lactic acid measurements and acid tolerance assay were performed to assess acidogenicity and aciduricity. Reverse transcription quantitative PCR (RT-qPCR) was used to measure the expression of virulence genes essential for surface attachment, biofilm formation, and quorum sensing.

Results: Phloretin inhibited *S. mutans* growth and viability in a dose-dependent manner. Furthermore, it reduced *gtfB* and *gtfC* gene expression, correlating with the reduction of extracellular polysaccharides (EPS)/bacteria and WIG/WSG ratio. Inhibition of *comED* and *luxS* gene expression, involved in stress tolerance, was associated with compromised acidogenicity and aciduricity of *S. mutans*.

Conclusions: Phloretin exhibits antibacterial properties against *S. mutans*, modulates acid production and tolerance, and reduces biofilm formation.

Clinical significance: Phloretin is a promising natural compound with pronounced inhibitory effect on key virulence factors of the cariogenic pathogen, *S. mutans*.

Introduction

Dental caries is one of the most prevalent biofilm-mediated diseases worldwide modulated by multiple risk factors including consumption of dietary sugars, inadequate fluoride exposure, reduced saliva flow, poor oral hygiene and deleterious shift in oral microbiota [1,2]. Prevalence of cariogenic bacteria such as *Streptococcus mutans* in dental biofilm plays a discriminatory role in disease establishment and progression [3,4]. *S. mutans* gained a significant evolutionary advantage for persistence and surface colonisation via its ability to metabolize a large variety of carbohydrates present in human diet. Among them, sucrose has proven to be the most cariogenic [5]. *S. mutans* has evolved several pathogenic traits essential for dental biofilm development and caries progression. First, it produces virulence enzymes such as fructosyltransferase (Ftf) and glucosyltransferase (Gtfs) to synthesise extracellular polysaccharides from dietary sugars. Thus,

Gtfs convert sucrose into water-soluble glucans (WSG) and water-insoluble glucans (WIG) essential for structured biofilm matrix formation [4,6,7]. Second, it uses several cell wall-associated proteins (e.g. SpaP, WapA and GbpC) to promote initial surface colonization and interbacterial interaction [8,9]. Third, *S. mutans* can metabolize dietary sugars for acid production (acidogenicity), which promotes further enamel demineralization and tooth decay [5,10]. Finally, *S. mutans* can tolerate various environmental stress conditions, such as an acidic environment in dental biofilm [11,12], particularly by exploiting two types of quorum sensing systems, ComCDE and LuxS-AI-2 [13–15]. Therefore, therapeutic strategies, which target virulence factors of *S. mutans*, are of great interest for caries prevention and maintenance of oral health.

Currently available antimicrobial compounds used in oral healthcare products (e.g. chlorhexidine [16], metal nanoparticles [17] or anti-microbial peptides

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[18,19]) are broad-spectrum antibacterial agents that are often associated with side effects and limitations, such as risk of resistance development [20], altered sensation, cellular toxicity or compound stability [21]. In recent years, plant polyphenols and flavonoids have gained increased attention as alternative or adjunctive anti-caries compounds based on their broad availability, low toxicity and prominent antimicrobial and antibiofilm capacity including direct inhibition of key virulence factors of cariogenic bacteria [22,23].

The flavonoid family includes more than 6000 compounds, and based on their chemical structure, they are divided into eight main subgroups, such as flavones, flavonols, flavonones, flavononols, flavan-3-ols, anthocyanins, isoflavones and chalcones [24]. Thus, extracts from *Camelia sinensis* enriched in flavan-3-ols (used for black, green and oolong tea production) exhibited antibacterial and antibiofilm activities against *S. mutans* and *Streptococcus sobrinus* [25–27]. Flavone apigenin, an abundant component found in propolis, is an effective inhibitor of *S. mutans* virulence gene expression and one of the most potent inhibitors of Gtfs enzymes [28–32]. Flavonols quercetin and kaempferol destroy *S. mutans* biofilm via multiple mechanisms including cytotoxic effect and reduction of insoluble and soluble glucan formation [33]. Naringenin, a flavanone from citrus plants, exhibits antibacterial effects against *S. mutans* and suppresses the adhesion and maturation stages of *S. mutans* biofilm formation [34]. Non-toxic cranberry flavonoids, such as A-type proanthocyanidins (PACs) and myricetin, have been shown to inhibit the activity of *S. mutans* Gtfs [35,36], sortase A (SrtA) [37] and EPS-mediated bacterial adhesion in multispecies dental biofilm [38]. Moreover, a few recent studies have indicated that flavonoids could potentially exhibit selectivity against oral pathogens. Thus, plant extract enriched with apigenin, quercetin and their derivatives selectively reduced *S. mutans* population in a dual-species biofilm with *S. sanguinis* in the presence of glucose [39]. Flavonol morin leads to a decreased viability of aciduric bacteria in polymicrobial biofilms obtained from saliva [40]. Additionally, disk diffusion assay showed that morin, luteolin and naringin effectively inhibited the growth of pathogens found in dental plaque including *Aggregatibacter actinomycetemcomitans*, *Enterococcus faecalis*, *Escherichia coli*, *Staphylococcus aureus* and *Candida albicans*, while remained inactive against commensal bacteria such as *Streptococcus sanguinis* and *Streptococcus oralis* [41].

Identification and functional characterization of flavonoids with inhibitory properties against cariogenic pathogen *S. mutans* are essential to select potential candidates for an effective anti-caries therapy. Phloretin is a dihydrochalcone (Figure 1a, upper panel) found mainly in apple, pear and strawberry fruit and has been shown to possess a strong antimicrobial and antibiofilm activity against various bacteria including *Bacillus subtilis* [42], *Pseudomonas aeruginosa* [43] enterohemorrhagic *E. coli* O157:H7 strain [44] and *Streptococcus pyogenes* [45]. However, its effect on *S. mutans* remains unknown. In this study, using a broad spectrum of assays, we demonstrate that phloretin exhibits strong inhibitory effect on *S. mutans* growth and biofilm formation by targeting its key virulence factors such as acid production and tolerance. These results warrant further exploration of phloretin as an antimicrobial and antibiofilm compound relevant for dental medicine.

Materials and methods

Bacterial strains and growth conditions

S. mutans strain ATCC 25,175 was used in this study (Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures, DSM 20,523, serotype c, isolated from carious dentine). The bacteria were routinely cultured in Brain Heart Infusion Broth (BHI, Oxoid, Thermo Scientific) aerobically at 37°C in 5% CO_2. For the biofilm assay, BHI was additionally supplemented with 1% (wt/vol) sucrose (BHIS). Phloretin was purchased from Sigma Aldrich, dissolved in 100% dimethyl sulfoxide (DMSO) to a stock concentration of 100 mg/ml, aliquoted and stored at −20°C.

Bacterial susceptibility assay

The minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) of phloretin were determined using a broth microdilution assay in 96-well polystyrene plates. Fresh *S. mutans* culture (OD_{600} = 0.5–0.6) was diluted 1:100 into 96-well plate using BHI supplemented with serial dilutions of phloretin and DMSO. The final concentration of DMSO in the assay did not exceed 5%. BHI supplemented with 5% DMSO was used as a negative control. The microplate was incubated aerobically at 37°C for 24 h. Optical density was measured at 600 nm using a microplate reader (Synergy HTX, Biotek, Agilent Technologies, Basel, Switzerland). MIC was determined by the lowest concentration of phloretin required to inhibit detectable bacterial growth. To assess the phloretin effect on *S. mutans* viability and determine MBC, 10-fold serial dilutions were made for each condition from the MIC microplate and 5 μl aliquots were spotted on BHI agar plate. MBC was determined by the lowest concentration of phloretin required to completely prevent bacterial growth on
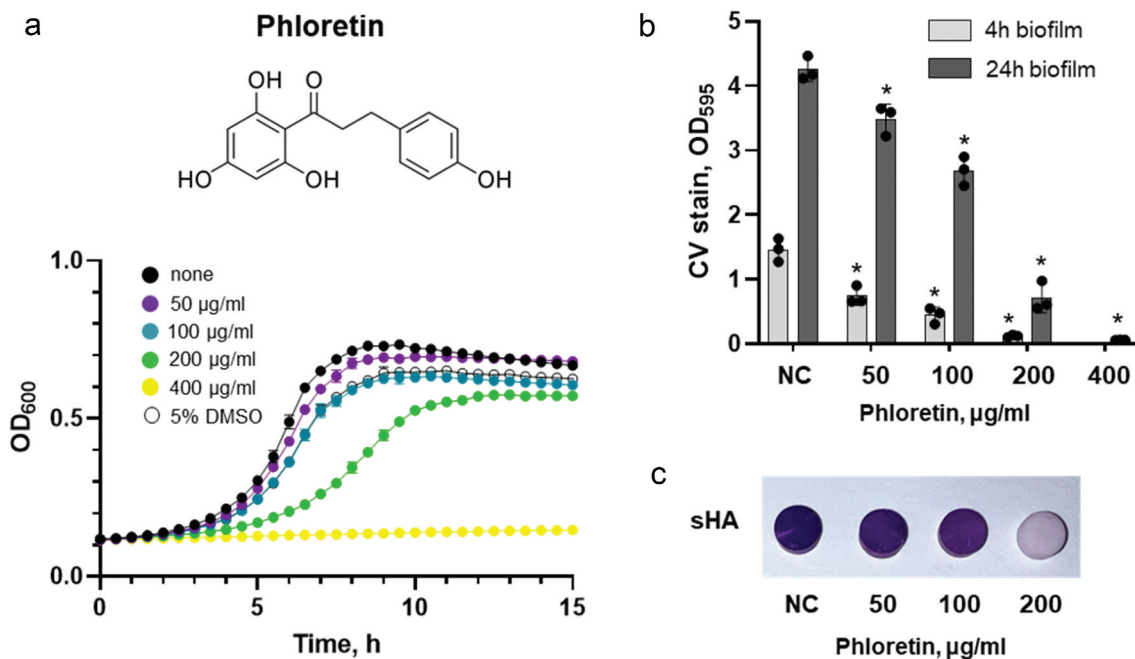
Figure 1. Antibacterial and antibiofilm effect of phloretin on *S. mutans*. (a) growth curves of *S. mutans* in the presence of phloretin (coloured curves) in comparison to non-treated control (black curve) and solvent control (5% DMSO, open symbols). (b) the effect of phloretin on early (4 h, light grey) and late (24 h, dark grey) biofilm of *S. mutans*. Biofilm mass was quantified by CV staining and measuring absorbance at 595 nm. Bars represent the mean of three biological replicates. Error bars show standard deviation. (c) the effect of phloretin on 24 h biofilms of *S. mutans* formed on sHA. Representative sHA disks with *S. mutans* biofilm treated with 50 µg/ml, 100 µg/ml and 200 µg/ml of phloretin for 24 h and stained with crystal violet. NC, negative control (bacteria treated with 5% DMSO).

BHI agar plate. MIC and MBC assays were performed independently for three bacterial cultures.

Biofilm inhibition assay

Crystal violet staining assay was used to evaluate the inhibitory effect of flavonoids on biofilm formed on polystyrene microplate or on saliva-coated hydroxyapatite disks. For biofilm assay in microplate, a fresh *S. mutans* culture (OD$_{600}$ = 0.5–0.6) was diluted 1:100 using BHIS supplemented with DMSO and, when indicated, two-fold serial dilutions of phloretin in DMSO. Final concentration of DMSO did not exceed 5%. Bacterial suspension was incubated aerobically at 37°C. After 4 h or 24 h of incubation, excess medium including the planktonic cells were removed, the plate was washed twice with sterile water and air-dried for 20 min. An equal volume of 0.1% (wt/vol) crystal violet solution was added to each well and incubated for 10 min at room temperature. The crystal violet solution was then removed and the plate was washed with water. To dissolve the dye, an equal volume of 25% acetic acid was added to each well, and absorbance at 595 nm was measured using a microplate reader (Synergy HTX, Biotek, Agilent Technologies, Basel, Switzerland).

For biofilm assay on hydroxyapatite, hydroxyapatite disks (5 mm diameter, HiMed Inc., USA) were coated with pooled filter-sterilized clarified human saliva at room temperature for 30 min to simulate the formation of an acquired pellicle-like layer (sHA). sHA disks were placed into the wells in a 96-well culture plate containing bacteria in BHIS with flavonoid dilutions. The culture plate was incubated for 24 h as described above. After incubation, sHA disks were washed with water to remove loosely attached cells and stained with crystal violet. The crystal violet solution was then removed, and sHA disks were thoroughly washed with water. An equal volume of 25% acetic acid was used to dissolve the dye for further quantification.

Saliva preparation

Whole saliva stimulated by chewing paraffin tablets was collected on ice from healthy donors free from systemic or oral topical medications, filtered through a 70-µm filter and clarified by centrifugation (21,000 g, 4°C, 40 min). The supernatant was further filtered through a double filter system of 0.45µm and 0.22 µm (Millex Millipore, Switzerland), aliquoted and stored at −20°C. Pooled saliva was prepared by mixing saliva from seven volunteers. All samples collected for this study involving human volunteers were stored anonymously and in accordance with the ethical standards of the institutional and national research committee and with the 1964 Helsinki declaration. All volunteers

were informed about the study and upon agreeing to participate, their consent was registered.

Biofilm analysis and structural imaging

For the biofilm analysis by confocal laser scanning microscopy, the culture of *S. mutans* treated either with 5% DMSO (negative control) or phloretin was prepared as described above and 0.5 ml was added to the eight-well chambered borosilicate coverglass plate. Plates were incubated aerobically for 24 h. For quantification of bacteria and EPS composition in biofilm, *S. mutans* was cultured with 1 μM Alexa Fluor 647-labeled dextran conjugate (Invitrogen) for 24 h, while bacterial cells were labelled with SYTO 9 (Invitrogen) after the incubation, according to the manufacturer's instruction. For viability assay in biofilm, bacteria were stained with SYTO 9 and Propidium iodide (PI) after incubation according to the manufacturer's instructions (LIVE/DEAD BacLight bacterial viability staining kit, Invitrogen). Biofilm composition analysis and live/dead staining were performed separately on independent biofilms. The biofilms were visualized on Leica SP8 confocal laser scanning microscope (Leica Microsystem, Switzerland) using x63 oil immersion objective. The images were collected using 500/618 nm, 575/656 nm and 650/778 nm spectra for SYTO9, PI and Alexa Fluor 647, respectively. Z-stacks of biofilm images were acquired with 0.46 μm intervals at five arbitrary positions per sample. Biofilm scanning from the bottom to the top resulted in 75–78 slices for control biofilms and decreased to 35–40 slices for biofilm treated with 200 μg/ml of phloretin. Three-dimensional reconstruction and analysis of the biofilms were performed with Imaris 9.0.0 (Bitplane, Switzerland).

Scanning electron microscopy was used to analyse the architecture of *S. mutans* formed on sHA. The biofilms were prepared as described above. After 24-h incubation, the biofilms were washed three times with sterile PBS to remove planktonic and loosely adherent cells, and then fixed with 4% glutaraldehyde aqueous solution at 4°C overnight. The disks were washed once with PBS, and the samples were dehydrated in increasing concentrations of ethanol (30%, 50%, 70%, 90% and 100%), 10 min each. Samples were additionally dried using critical point drying (CPD) with liquid carbon dioxide and finally coated with a 20 nm gold layer. Disks were observed by SEM imaging (ESEM XL30, Philips, Eindhoven, The Netherlands) at magnifications of 200×, 2,000× and 8,000×. Each sample was scanned at three arbitrary selected locations. Image analysis was performed using ImageJ software (National Institutes of Health, USA). To analyse the cell morphology, at least three SEM images scanned with a magnification of 8,000× and 20,000× were used.

Water-soluble and water-insoluble EPS measurement

The effect of 100 μg/ml and 200 μg/ml of phloretin on water-soluble glucan (WSG) and water-insoluble glucan (WIG) production was estimated by the anthrone-sulphuric methods as described previously [46] with modifications. Briefly, a fresh *S. mutans* culture (OD_{600} = 0.5–0.6) was diluted in 24-well plates in BHIS with phloretin. Biofilm was formed for 24 h at 37°C. After incubation, the culture medium was removed, and the biofilm was carefully rinsed with sterile PBS. One millilitre of sterile water was added to each well, and the biofilm was scraped from the bottom of the well with a sterile spatula. Water-soluble glucan from the suspension was dissolved by vigorous vortexing. The suspension was centrifuged at 6,000 g for 10 min, and the supernatant was collected in a separate tube for WSG measurement. WIGs was extracted from the cell sediment in 2 ml of 0.4 M NaOH with constant agitation for 2 h at 37°C. After incubation, cells were collected by centrifugation (6,000 g, 10 min). Supernatants from aqueous and alkaline extraction were mixed with 3 volumes of anthrone-sulfuric acid reagent and heated at 95°C for 6 min. The absorbance at 625 nm was measured in a 96-well plate using a microplate reader (Synergy HTX, Biotek, Agilent Technologies, Basel, Switzerland). The concentrations of WSG and WIG were calculated according to the standard curve prepared with the glucose standards (**Figure S4**). The experiment was performed in triplicates (independent bacterial cultures). Biofilm formed in BHIS with 5% DMSO was used as a negative control.

Lactic acid measurement

The effect of phloretin on *S. mutans* lactic acid production was evaluated for 50 μg/ml, 100 μg/ml, 200 μg/ml, and 400 μg/ml. A fresh *S. mutans* culture (OD_{600} = 0.5–0.6) was harvested and washed twice with PBS. Cells were resuspended 1:10 in buffered peptone water (BPW) supplemented with 0.2% sucrose in a 24-well plate. Two-fold serial dilutions of phloretin in DMSO were added to the wells. Bacterial suspension supplemented with DMSO was used as a negative control. Bacteria were incubated aerobically at 37°C for 2 h. *S. mutans* cells were then removed by centrifugation (8,000 g, 5 min, 4°C), and the supernatant was used to measure lactic acid concentration with a lactate assay kit (MAK064, Sigma-

Aldrich, Switzerland), according to the manufacturer's instruction. The measurements were performed with three biological replicates.

Acid tolerance assay

The effect of phloretin on the acid tolerance of *S. mutans* was determined by measuring its viability after 2 h of exposure to a pH of 5.0. The *S. mutans* cells were grown in BHI until the mid-log phase, harvested, washed with sterile PBS and then resuspended at a final concentration of 1.0×10^7 CFU/ml in buffered BHI, pH 5.0. Phloretin was added at concentrations 50 μg/ml, 100 μg/ml, 200 μg/ml and 400 μg/ml. Untreated bacterial suspension supplemented with 5% DMSO was used as the negative control. *S. mutans* cells grown in BHI pH 7.2 treated with the same phloretin concentration were used as a viability control. After incubation at 37°C for 2 h, aliquots from each condition were taken and serially diluted 10-fold in sterile PBS for further CFU counts. Aliquots of 100 μl were spread on BHI agar plate and incubated aerobically at 37°C for 2 days (**Figure S5**). The assays were independently performed with three biological replicates.

Transcriptional analysis of virulence genes

To determine the effect of phloretin on the gene expression levels in biofilm, *S. mutans* cells were cultured in 12-well plate for 24 h with 200 μg/ml phloretin as described above for the biofilm assay. Culture without treatment served as a control. After 24-h incubation, supernatants were carefully removed from the wells and biofilms were scraped with sterile spatula and resuspended in 1 ml RNAprotect bacterial reagent (Qiagen). After an incubation at RT for 5 min, pellets were collected by centrifugation for 10 min at 10,000 × g. Stabilized pellet was resuspended in 5 ml cold PBS and homogenized by sonication on ice to remove soluble polysaccharides. Three cycles of ultrasonic washing were performed with 30 s pulse. Next, the cells were resuspended in a lysis buffer with 15 mg/ml lysozyme and incubated for 15 min at RT. One millilitre TRIzol reagent (Invitrogen) was added to the suspension, and then transferred to the 2ml screw-cap tubes with 0.5 mm acid-washed glass beads. The cells were lysed by vertexing for 10 min at the highest speed. Total RNA was extracted using RNeasy kit (Qiagen) with an on-column DNase digestion step. The cDNA was synthesized using GoScript™ Reverse Transcriptase system with random primers (Promega, Switzerland). To quantify the mRNA expression, qRT – PCR was performed using FastStart Universal SYBR Green Master (ROX)

(Roche Diagnostics, Mannheim, Germany). The PCR was performed in CFX96 Real-Time System (BioRad) in accordance with the manufacturer's recommendations for determining the threshold cycle values (Ct). $2^{-\Delta\Delta Ct}$ method was used to analyse the data. The sequences of the primers used in this study are listed in Supplementary Table 1. 16S rRNA was used as an internal control.

Statistical analysis

All assays were performed in biological replicates (independent bacterial cultures). The results are presented as mean ± standard deviation (SD). Statistically significant differences were determined with a one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparisons test: *$P <$ 0.05. The results were graphed using GraphPad Prism version 9.0.2 (GraphPad Software, LLC, USA).

Results

Phloretin inhibited S. mutans *viability and biofilm formation*

The MIC and MBC of phloretin tested on planktonic *S. mutans* culture was 400 μg/ml. At sub-inhibitory concentration, gradual growth attenuation and reduction of cell viability were observed indicating a dose-dependent inhibitory effect of phloretin (Figure 1a, Figure S1). Dose-dependent biofilm inhibition was also observed in 4 h and 24 h biofilm assay with MBIC of 200 μg/ml and 400 μg/ml, respectively (Figure 1b). Similarly, on saliva-coated hydroxyapatite (sHA) surfaces, a condition mimicking a human oral environment, in 24 h, 200 ug/ml of phloretin drastically reduced biofilm formation (Figure 1c, Figure S2) resulting in a scarce number of surface-associated cells detected by SEM (Figure 2). These cells also underwent a notable change in shape and structure (Figure 2, Figure S3). In comparison to non-treated cells with symmetric ellipsoidal shape, in the presence of 200 μg/mL phloretin, bacteria appeared elongated, with multiple dysmorphic or swollen cells and asymmetric division sign (Figure S3A, red arrows). On average, non-treated control cells were 0.72 ± 0.11 μm long, while the length of the cells treated with 200 μg/ml phloretin was 1.1 ± 0.2 μm (Figure S3B). These observations might potentially indicate a perturbation in the coordination of cell elongation and cell division which is also reflected in compromised growth kinetics (Figure 1a). As assessed by the live/dead staining technique combined with CLSM, phloretin significantly reduced the proportion of live cells in 24 h biofilm, while the proportion of cells with compromised membrane, stained with PI, increased with increasing phloretin concentration (Figure 3).
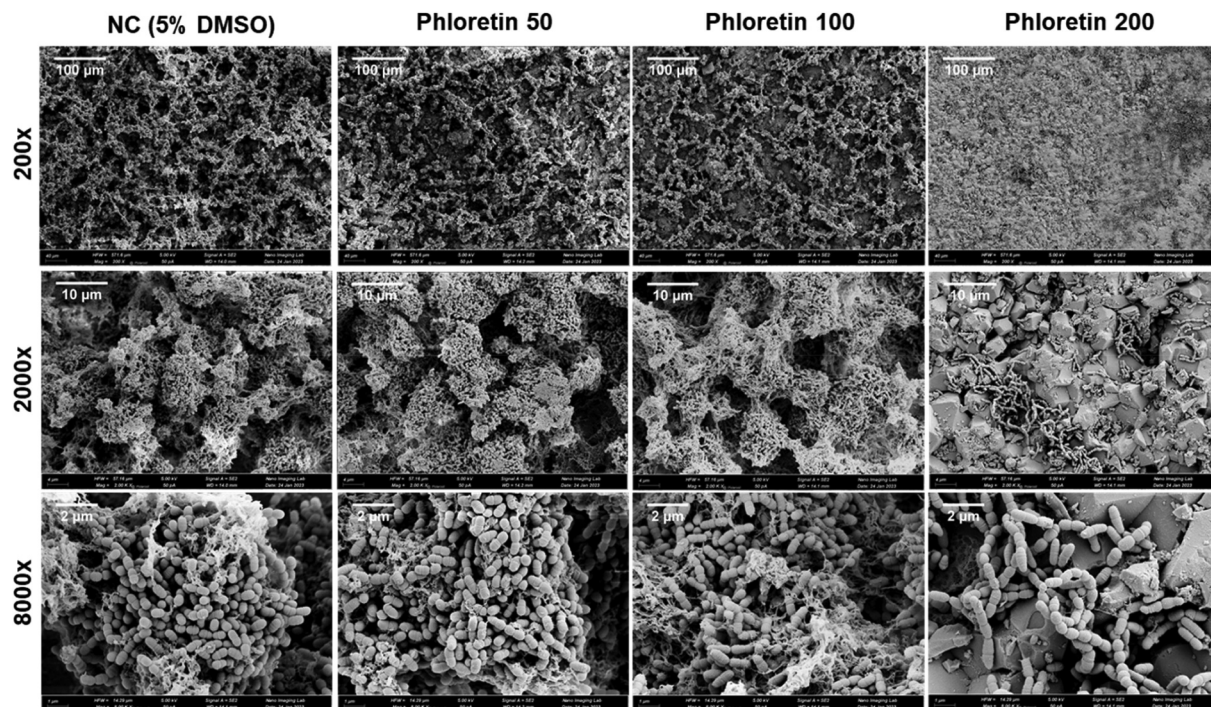
Figure 2. Phloretin effect on *S. mutans* adherence to HA. Representative scanning electron microscopy images (SEM) of *S. mutans* biofilms treated with 50 µg/ml, 100 µg/ml and 200 µg/ml of phloretin compared to negative control containing 5% DMSO. Images were taken at ×200, ×2 000 and ×8000 magnifications.

Phloretin inhibited EPS production in biofilm

To evaluate the effect of phloretin at concentrations of 50 µg/ml, 100 µg/ml and 200 µg/ml on biofilm structure and the biomass of bacteria and EPS, CLSM was used combined with a double-staining, where bacteria were labelled with green fluorescent SYTO 9 and EPS were stained with red-labelled fluorescent dextran. For the efficient image analysis, biofilms were cultured in an eight-well chambered borosilicate coverglass system for 24 h. Three-dimensional biofilm images indicated that, with increasing concentration, phloretin gradually reduced bacterial and EPS biomass. Treatment with 50 µg/ml and 100 µg/ml resulted in thinner and less compact biofilms, while 200 µg/ml prevented surface attachment of the majority of cells in comparison to thick biofilms grown under non-treated conditions (Figure 4a). Notably, at concentration 50 µg/ml and 100 µg/ml of phloretin, there was no substantial change in EPS/bacteria or WIG/WSG ratio between treated and non-treated conditions. However, at concentration 200 µg/ml, phloretin reduced EPS/bacteria ratio and slightly increased the proportion of WSG against WIG (Figure 4b-d).

### Phloretin modulated *S. mutans* lactic acid production and acid tolerance

To assess the effect of phloretin on *S. mutans* acidogenicity, lactic acid production in bacterial cultures that were treated with 50 µg/ml, 100 µg/ml, 200 µg/ml and 400 µg/ml of phloretin for 2 h was monitored. As shown in Figure 5a, phloretin reduced lactic acid production in a dose-dependent manner. Thus, 100 µg/ml of phloretin reduced lactic acid production by 50%, while 200 µg/ml and 400 µg/ml, concentrations used to prevent early and late biofilm formation, reduced up to 80% of lactic acid produced by *S. mutans*. Similarly, 200 µg/ml and 400 µg/ml of phloretin strongly inhibited *S. mutans* aciduricity (Figure 5b). The survival of bacterial cells in acidic environment was markedly reduced in the presence of phloretin compared to the control bacteria treated at a pH 7.2.

Phloretin reduced virulence gene expression of *S. mutans*

To further investigate the effect of phloretin on *S. mutans*, gene expression levels of three glycosyltransferases, *gtfBCD*, cell wall-anchored adhesins *spaP*, *gbpC* and their transpeptidases *srtA* as well as the main quorum sensing components, *comED* and *luxS*, were analysed. To this end, biofilms were treated with 200 µg/ml phloretin, the concentration at which phloretin had a pronounced effect on *S. mutans* viability, biofilm formation, and acid production and tolerance. The results (Figure 6) revealed a reduction in transcriptional levels of *gtfB* and *gtfC*, responsible for WIG production, but not *gtfD* responsible for WSG synthesis. The results also revealed that 200 µg/ml phloretin significantly reduced gene expression of tested cell wall-associated adhesins and their modifying enzyme SrtA, with the strongest effect on *gbpC* gene expression. Both *comED* and *luxS* quorum
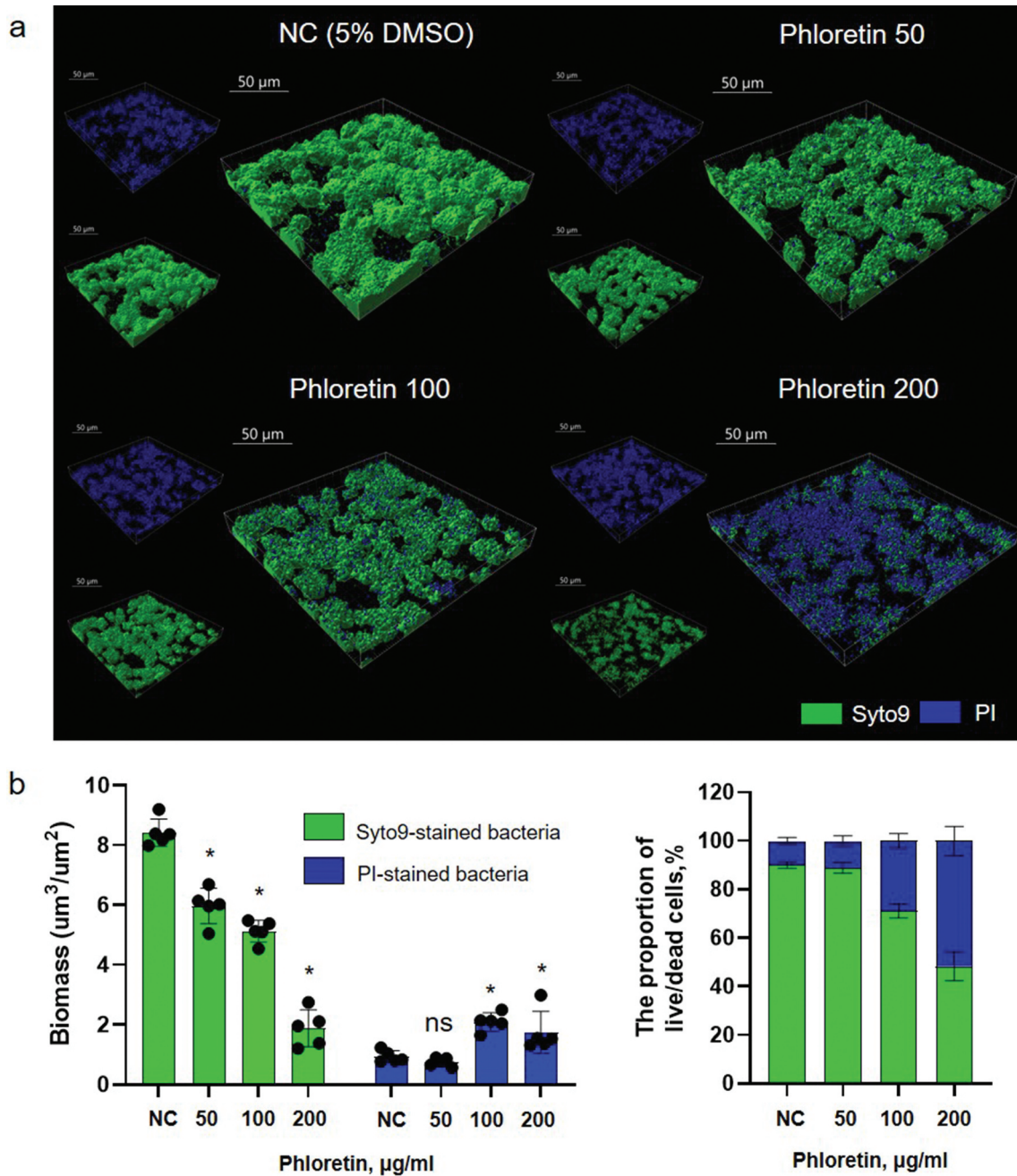
Figure 3. Antimicrobial effect of phloretin on *S. mutans* biofilm based on confocal laser scanning microscopy. (a) representative images of live/dead-stained 24-h biofilms. Live bacteria are shown in green and dead (PI-stained) in blue. Three-dimensional reconstructions were performed with Imaris 9.0.0. (b) right panel: bacterial biomasses as calculated by Imaris. Left panel: the proportion of live/dead cells. In all panels, bars represent the mean of five values calculated for arbitrary selected positions per sample. Error bars show standard deviation. NC, negative control (bacteria treated with 5% DMSO).

sensing systems were also significantly downregulated upon phloretin treatment.

Discussion

Natural flavonoids represent an attractive selection of structurally and functionally diverse molecules with potent antibacterial and antibiofilm properties. Given their abundancy, low toxicity in human cells and pleiotropic effect on bacterial physiology, flavonoids seem to be prominent compounds for antibacterial and antibiofilm therapies in dental research. Thus, plant extracts and essential oils enriched in flavonoids as well as single purified compounds have demonstrated high efficacy against *S. mutans* biofilm formation and its virulence factors in multiple *in vitro* studies [47–50]. Several flavonoids, e.g. apigenin, quercetin, naringenin and proanthocyanins, have been successfully used to reduce caries in animal studies [30,51,52]. Finally, despite very limited and heterogeneous data from clinical investigations [53], some studies have demonstrated an inhibitory effect
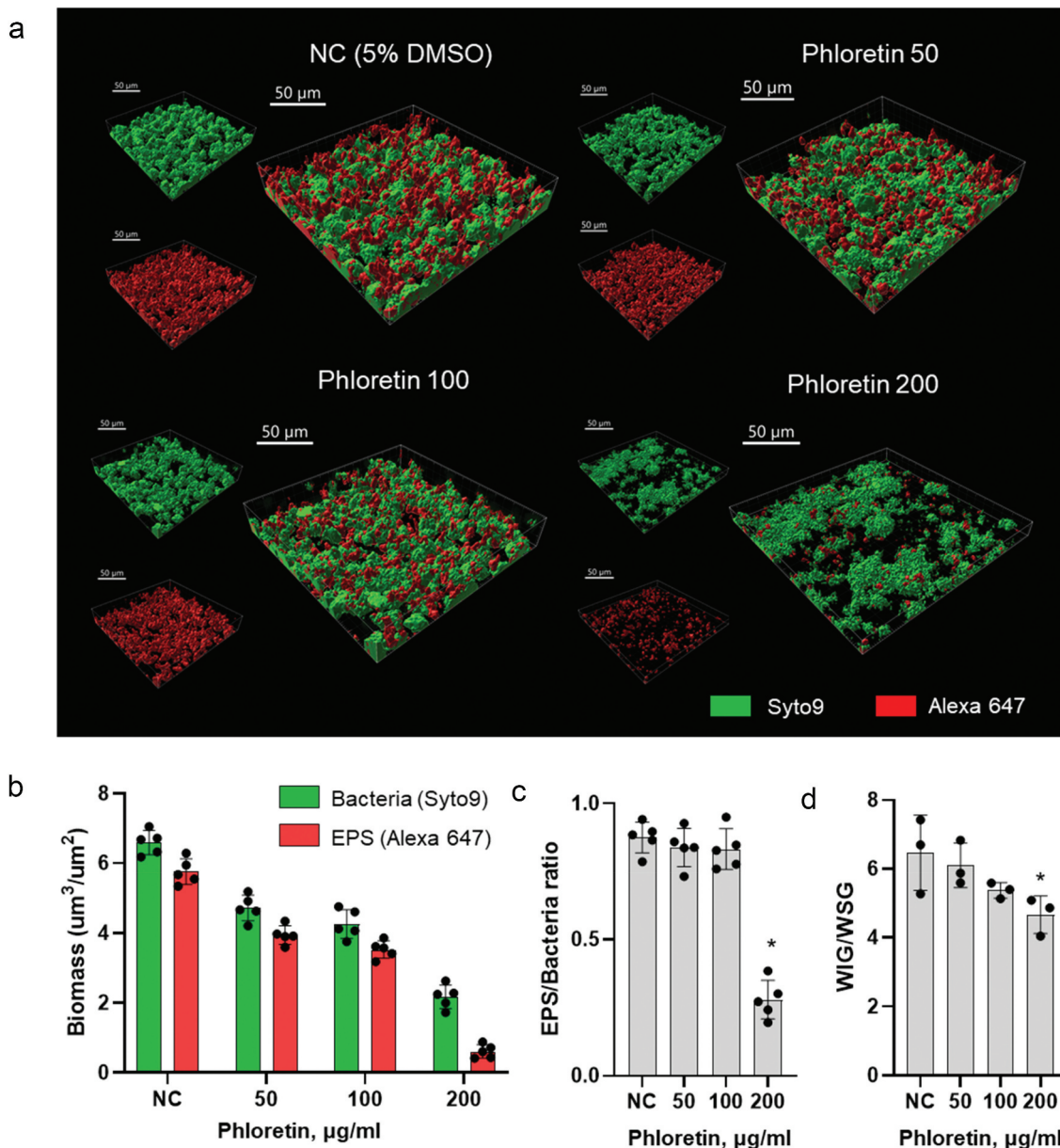
Figure 4. Biofilm structure and composition affected by phloretin. (a) representative images of the double-labelled 24-h biofilms. Bacterial cells are shown in green and EPS in red. Three-dimensional reconstructions were performed with Imaris 9.0.0. (b) bacterial and EPS biomasses as calculated by Imaris. (c) EPS/bacteria biomass ratio as calculated by Imaris. (d) phloretin effect on biofilm-associated water-soluble and water insoluble glucan ratio, as measured by anthrone method. In all panels, bars represent the mean of five values calculated for arbitrary selected positions per sample. Error bars show standard deviation. NC, negative control (bacteria treated with 5% DMSO).

of flavonoid-rich mouth rinse on dental plaque accumulation [54]. However, more multidisciplinary research and clinically relevant studies are needed to explore the full potential of flavonoids in anticaries strategies.

In order to gain more knowledge on antibacterial mechanisms of flavonoids and to explore their structure-activity relationship against one of the main cariogenic bacterium, *S. mutans*, a flavonoid with a strong antimicrobial and antibiofilm properties against several pathogenic microbes, phloretin [55], has been tested in this study. Phloretin is a dihydrochalcone, a natural plant-defence antioxidant compound found mainly in apples, pears, and strawberry fruit. Dihydrochalcones, similar to other flavonoids with antibacterial properties, act mainly by damaging cell membranes via direct interaction of their free hydroxyl groups with phospholipids and other biological molecules in bacterial membranes and cytoplasm [56]. This correlates well with the documented antibacterial activity of phloretin against multiple Gram-positive bacteria including *S. aureus*, *L. monocytogenes* and *S. typhimurium* [57]. In this study, phloretin showed a prominent concentration-dependent antibacterial, and, therefore, antibiofilm effect against *S. mutans*. At concentrations above 400 μg/ml, it completely diminished bacterial growth and viability in planktonic culture
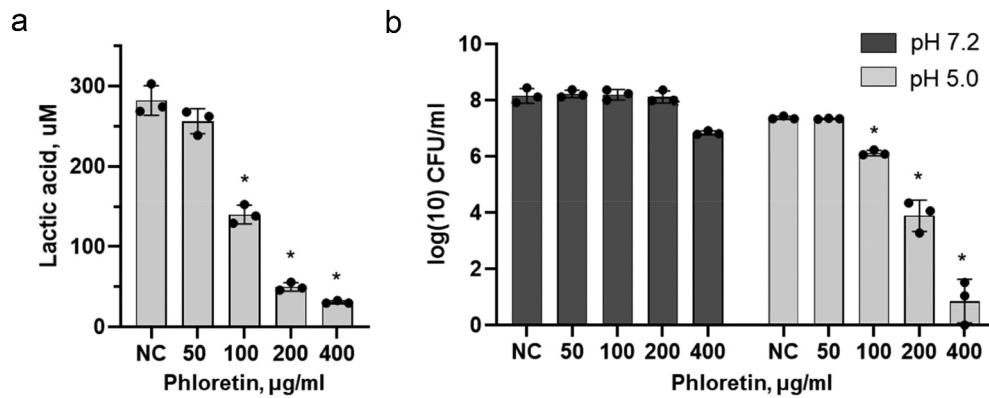
Figure 5. Effect of phloretin on lactic acid production and acid tolerance by *S. mutans*. (a) production of lactic acid by *S. mutans* treated with 50 µg/ml, 100 µg/ml, 200 µg/ml and 400 µg/ml of phloretin. (b) survival of *S. mutans* cells after 2 h of treatment with phloretin at pH 7.2 and pH 5.0 as measured by colony forming units (CFU/ml). Bars represent the mean of three biological replicates. Error bars show standard deviation. NC, negative control (bacteria treated with 5% DMSO).
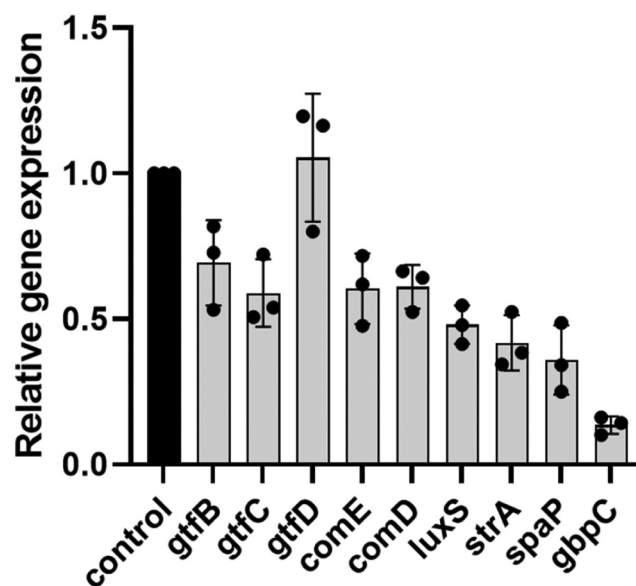
Figure 6. Inhibitory effect of phloretin on the virulence gene expression levels in 24-hour biofilm. The relative expression levels were quantified by real-time PCR with 16S rRNA as an internal control. Black bar represents non-treated control, grey bars stand for biofilm treated with 200 µg/ml of phloretin. Bars represent the mean of three biological replicate. Error bars show standard deviation. NC, negative control (bacteria treated with 5% DMSO).

upon 24 h of incubation (Figure 1, S1). CLSM also revealed the impact of phloretin on *S. mutans* viability in biofilm (Figure 3). Staining of surface-associated *S. mutans* cells with two fluorescent nucleic acid stains, PI and SYTO 9 for dead and live cells, respectively, revealed that, in concentration-dependent manner, phloretin substantially reduced the proportion of live cells and increased the proportion of cells with damaged cellular membrane.

Besides their antimicrobial activity, many flavonoids specifically influence bacterial biofilm formation mechanisms. Thus, morin inhibits adhesion potential of *S. mutans* and *S. aureus* via direct inhibition of SrtA enzyme, a transpeptidase responsible for anchoring surface adhesins and virulence factors to the cell wall [58,59]. Many flavonoids

such as apigenin, quercetin or their derivatives disturb EPS production and quorum sensing in many bacteria including *S. aureus* and *E. coli* [60], and *P. aeruginosa* [43] via direct interaction with effector proteins. In *S. mutans*, apigenin remains one of the most potent inhibitors of Gtfs enzymes [29]. In addition to this, flavonoids could suppress gene expression of biofilm-associated virulence factors [28,61,62]. The molecular mechanism of this regulation remains largely unknown; however, a recent study indicated that flavonoids can directly modulate the activity of transcriptional regulator from a two-component signal transduction system [63]. This study revealed that in a dose-dependent mode, phloretin reduced *S. mutans* biofilm formation on various surfaces including sHA representing conditions

similar to teeth in a natural oral environment (Figures 1b-c, 2, Figure S2). The antibiofilm activity of phloretin, most probably, worked via the inhibition of bacterial growth and viability since a reduced number of planktonic cells limits biofilm formation. However, at a concentration of 200 μg/ml, it significantly reduced EPS-to-bacteria and WIG-to-WSG proportion, which indicates that phloretin could specifically target EPS synthesis. This was further corroborated with gene expression analysis where phloretin inhibited mRNA levels of *gtfB* and *gtfC*, glycosyltransferases responsible for WIG production, but not *gtfD*, responsible for WSG synthesis (Figure 6). Additionally, its inhibitory effect on *srtA*, *spaP* and *gbpC* gene expression could contribute to the reduction in surface adhesion and interbacterial communication in biofilms.

Acidogenic and aciduric properties are among the most critical virulence factors of *S. mutans*. Its ability to metabolise dietary sugars to lactic acid and survive in low-pH environment promotes pathological changes in dental biofilms with dominance of acid-tolerant species and subsequent tooth enamel demineralization. Inhibition of *S. mutans* acidogenicity and aciduricity is one of the key aspects to reduce its virulence and to promote non-cariogenic oral biofilm. Phloretin tested in this study exhibited dose-dependent reduction of *S. mutans* acidogenicity and survival in acidic environment with the most prominent effect at 200 μg/ml and 400 μg/ml (Figure 5). Essentially, its inhibitory effect was detected within a few hours of *S. mutans* exposure to phloretin and is not attributed to its bactericidal activity. Presumably, phloretin directly targeted lactate dehydrogenase (LDH) and other metabolic enzymes involved in stress tolerance, similarly to its effect on LDH, catalase and isocitrate dehydrogenase (IDH) of *S. aureus* [57].

In a complex multispecies cariogenic biofilm, *S. mutans* employs its quorum sensing systems to modulate inter- and intraspecies communication and stress response. Thus, histidine kinase sensor protein ComD and cognate response regulator ComE, are critical for the density-dependent quorum sensing [64], while LuxS-mediated signalling is essential for acid and oxidative stress tolerance in *S. mutans* biofilm [15,65]. The *comD*, *comE* and *luxS* genes were downregulated in biofilms treated with 200 μg/ml phloretin suggesting its interference with *S. mutans* quorum sensing (Figure 6).

Although the exact mechanism of the antibacterial and antibiofilm effects of phloretin needs further investigation, the observation from the current study makes phloretin a promising candidate to be tested in conditions relevant to clinical practice. This study only explores the antibacterial and antibiofilm effects of phloretin on 24 h monospecies biofilm of *S. mutans, in vitro*. However, *in vivo*, cariogenic biofilm development depends on multiple components of oral microbiome. Hence, further studies of phloretin effect on multispecies dental biofilms and, in particular, on commensal oral bacteria are required to elucidate the pharmacological potential of phloretin for oral health maintenance. It is worth noting that flavonoid effects could be species-specific [42]. Thus, the reciprocal impact of phloretin on the biofilm formation of pathogenic and commensal *E. coli* strains has been previously described [44]. Furthermore, to be a successful adjunct to oral hygiene, phloretin can be tested in a dynamic environment with shorter exposure time (a few minutes) as well as in combination with other agents, e.g. fluoride, other flavonoids or probiotics, to enhance their cariostatic properties without increasing exposure parameter.

Despite a long history of flavonoid investigation in dental research, their introduction into the therapeutic products is subject to several limitations and challenges such as low compound solubility in aqueous medium or reduced substantivity due to a very dynamic environment in oral cavity [66]. However, recent progress in molecular chemistry and material science has provided attractive solutions to increase flavonoid solubility and to improve controlled drug delivery systems. Thus, microparticles, films and oral tablets with natural flavonol morin have been recently tested to increase its water solubility and improve drug release kinetics [67,68]. Different types of nanoparticles are currently being tested to improve the bioavailability of quercetin against oral diseases [69]. Finally, flavonoid incorporation, e.g. apigenin, into dental composites, is tested to modulate cariogenic bacteria virulence [70].

Conclusions

In conclusion, natural flavonoids retain a large potential as effective antimicrobial and antibiofilm agents for oral science. Phloretin, tested in this study, showed a prominent inhibitory effect against *S. mutans* virulence, mainly by compromising its viability, acidogenicity and acid tolerance. These observations make phloretin a promising candidate to test in more clinically relevant systems including specific oral healthcare products ranging from mouth rinses to toothpastes for caries preventive therapies.

Author contributions

Conception and design, V.S.; data curation, analysis and interpretation, LR and VS; original draft preparation, review and editing, V.S., L.R. and M.M.B., project administration and supervision, V.S. and M.M.B. All authors have read and agreed to the published version of the manuscript.

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