In Vitro Multi-Species Oral Biofilms Grown in Presence of H2O2 Production-Affecting Substrates Show Health-Associated Alterations in Composition, Metabolism and Virulence.

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In vitro multi-species oral biofilms grown in presence of H_2O_2 production-affecting substrates show health-associated alterations in composition, metabolism and virulence.

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ABSTRACT

Modulation of the commensal oral microbiota is a promising preventive or therapeutic strategy for oral health and can for instance be achieved by increasing the abundance and/or activity of certain species. This study evaluated whether 10 selected substrates could modulate in vitro multi-species oral biofilms towards a more health-associated state. These substrates were chosen based on the possibility that they could stimulate H_2O_2 production by certain commensal species and/or increase their abundance, as previously reported or as hypothesized based on known bacterial H_2O_2 pathways. Biofilms grown in presence of the substrates at a clinically relevant concentration of $1\%_{(w/v)}$ often showed increased abundances of commensal species and decreased abundances of periodontal pathogens. Furthermore, most biofilms also showed an altered metabolic profile. Effects on the expression of a selection of virulence genes were substrate-dependent, but often a decreased expression of certain genes could be observed. In conclusion, this study found that a selection of substrates chosen for their hypothesized beneficial effects on the commensal oral microbiota were able to modulate in vitro multi-species oral biofilms towards a more health-associated state. These modulatory effects were found to be substrate-dependent.

INTRODUCTION

The health status of the oral cavity is determined by a variety of external factors such as oral hygiene, diet and lifestyle[1,2]. Simultaneously, also the intrinsic characteristics of an individual such as age, genetic predisposition and systemic diseases are known to strongly influence one's predisposition to develop oral illnesses such as periodontal diseases[3-5]. Eventually, the oral health status will be determined by the presence or absence of a complex, calibrated interplay between the host, its environment and its commensal oral microbiota[6]. Knowledge on the latter has increased exponentially over the past decades, leading to the understanding that despite the existence of inter- and intra-individual variability, each person possesses a so-called 'core oral microbiome' that plays a crucial role in maintaining the homeostatic, symbiotic relationship between the oral microbiota and its host[7-9].

In oral health, an individual's oral microbiota mainly consists of a few hundred bacterial species that often organize into robust, highly specialized oral biofilms (dental plaque)[6,8,9]. Within these biofilms, an optimal microenvironment allows oral bacteria to flourish while they are sheltered from external aggressors and stress but where they are also in close contact with each other[10,11]. As a result, complex inter- and intra-species interactions occur that help shape the homeostatic balances. These interactions are diverse and mediated through for instance metabolic cross-feeding and the production of substances (e.g. hydrogen peroxide (H_2O_2)) that (in)directly affect the function and survival of nearby species[12-14]. In this way, the commensal oral microbiota can deal with potentially disease-provoking disruptions while maintaining its core composition and functions[15]. However, once these disruptions surpass a certain threshold and/or when the function of the commensal oral microbiota is impaired, an imbalanced relationship between host and microbiota or within the microbiota eventually leads to dysbiosis and the onset and progression of oral diseases[6,15,16].

Up until today, prevention and treatment of oral diseases are mainly accomplished by mechanical strategies (i.e. plaque removal) in combination with adjunctive antimicrobial therapy (i.e. antibiotics or antiseptics)[17-21]. However, these approaches also come with certain disadvantages, such as the often aspecific removal and killing of both pathogenic and

commensal species. Furthermore, the widespread use of antimicrobials makes that the risk for adaptation or resistance development could be lurking around the corner[19,22,23]. Therefore, the focus shifted towards the modulation of the commensal oral microbiota to prevent disruption of oral health-associated homeostatic relationships, or to restore them. Probiotics for oral health are an example of such a 'pro-microbial approach', where live microorganisms are administered that eventually provide a health benefit to the host, for instance by inhibiting pathogens through the production of H_2O_2 or acids[24,25]. Nowadays, probiotics for oral health are being successfully used in clinical practice[26,27]. Another example is the use of prebiotics, where substrates are administered that are selectively used by endogenous microorganisms resulting in a health benefit, accomplished by for instance increasing the abundance of commensal species or stimulating their metabolism or health-associated functions[28]. The potential of prebiotics for oral health has mainly been demonstrated in vitro[29-31], but also some in vivo studies were conducted, making it a rapidly evolving research field[32,33]. Lastly, several other strategies to modulate the commensal oral microbiota are under investigation, such as the use of synbiotics (combination of pro- and prebiotics) or the exploitation of specific functions like increasing the abundance of H_2O_2 producers or boosting their H_2O_2 production[14,34,35].

The current study hypothesized that certain substrates could modulate in vitro multi-species oral biofilms towards a more health-associated state. These substrates were selected based on the possibility that they could positively influence H_2O_2 production by certain oral species or increase their abundance. For some substrates, this was described in literature, whereas for others this was hypothesized based on known bacterial H_2O_2 production pathways. More specifically, arabinose, sorbitol and saccharin have been shown to stimulate H_2O_2 production by *S. oralis* and *S. sanguinis*[36], whereas pyruvate and lactate are two substrates with a central role in the two major oral streptococcal H_2O_2 production pathways[14,37,38]. Furthermore, it is known that lactic acid might serve as a substrate for certain streptococcal species resulting in the production of H_2O_2[38,39]. Potassium acetate was shown to slightly affect H_2O_2 production by *S. gordonii*[40], which led to the hypothesis this could also be the case for sodium acetate. Finally,

97 the conversion of fumarate to succinate is known to yield H_2O_2 as a by-product, and succinate
98 can in turn be re-converted to fumarate[41], which led to the inclusion of these two substrates.
99 Given the complex nature of multi-species biofilms and the fact that accurate H_2O_2
100 measurement within oral biofilms is very difficult to accomplish, this study followed a top-down
101 approach and the focus lied only on evaluating the effects the substrates have on in vitro multi-
102 species oral biofilms. Therefore, this study aimed to investigate whether the selected
103 substrates have beneficial modulatory effects on the composition, metabolic profile and
104 virulence of in vitro multi-species oral biofilms grown in presence of these substrates at a
105 clinically relevant concentration.

RESULTS

Compositional modulation of multi-species biofilms

The effects of each substrate on the absolute and relative abundances of the different species were evaluated to determine their impact on biofilm composition. Most substrates were found to have a significant influence on absolute bacterial numbers in terms of increases/decreases in comparison with the control condition (**Fig. 1**, **Supplementary Table S1**). Biofilm formation in presence of sodium L-lactate resulted in a 1.5 log(Geq/mL) increase in *S. oralis* numbers and a 0.6 log(Geq/mL) decrease in *P. gingivalis* numbers. For sodium pyruvate, also an increase in *S. oralis* abundance was observed (+1.5 log(Geq/mL)), whereas *F. nucleatum* and *P. intermedia* abundance decreased (-1.5 and -0.3 log(Geq/mL)). Sodium acetate and D-(-)-arabinose also yielded an increase in *S. oralis* numbers (+1.4 and +1.5 log(Geq/mL)), with D-(-)-arabinose also decreasing *A. naeslundii* numbers (-0.3 log(Geq/mL)). Potassium acetate resulted for *F. nucleatum* in a decrease (-1.2 log(Geq/mL)) and in an increase for *S. gordonii* (+0.4 log(Geq/mL)) and *S. oralis* (+1.2 log(Geq/mL)). Biofilms formed in presence of lactic acid and saccharin showed decreased *A. actinomycetemcomitans* (-0.4 and -0.9 log(Geq/mL)) and *F. nucleatum* numbers (-0.8 and -1.9 log(Geq/mL)), with saccharin also resulting in decreased *P. intermedia* and *S. gordonii* numbers (-2.6 and -0.2 log(Geq/mL)). D-sorbitol led to decreased numbers of *A. actinomycetemcomitans* and *P. gingivalis* (-0.6 and -0.5 log(Geq/mL)) and increased numbers of *S. mutans*, *S. mitis* and *S. sanguinis* (+1.4, +0.2 and +1.4 log(Geq/mL)). Biofilms grown in presence of sodium fumarate showed decreased *F. nucleatum* and *S. oralis* numbers (-1.6 and -0.4 log(Geq/mL)). Sodium succinate did not result in significant changes in absolute bacterial abundances.

Most substrates also significantly affected the relative abundances of commensal species, periodontal and cariogenic pathogens (**Table 1**). For one set of substrates, control biofilms harboured 21.8±3.0% commensal species, 77.8±3.0% periopathogens and 0.4±0.1% cariogenic pathogens. (**Table 1**). D-sorbitol, saccharin, lactic acid and sodium fumarate altered the proportion of commensals to 55.2±14.8, 82.6±8.6, 65.9±7.4 and 74.5±7.5%, respectively, while decreasing the abundance of periopathogens to 43.6±15.0, 13.2±6.3, 33.3±7.2 and

24.2±7.2%, respectively. Saccharin also increased cariogenic pathogens abundance (4.2±2.3%). For the other set of substrates, the control biofilms consisted of 31.7±9.3% commensal species, 68.3±9.3% periopathogens and 0.1±0.0 cariogenic pathogens (**Table 1**). Significant shifts in biofilm composition were observed for biofilms grown in presence of sodium L-lactate (82.9±7.0% commensals, 17.0±7.0% periopathogens), potassium acetate (69.7±8.6% commensals, 30.2±8.6 periopathogens) and sodium pyruvate (0.5±0.2% cariogenic pathogens).

Metabolic modulation of multi-species biofilms

To investigate the effects of each substrate on the metabolic profiles of the biofilms, levels of organic acids in the biofilm supernatants were determined (**Table 2**). For one set of substrates, control biofilms consumed 170±2 mg/L lactate and produced 94±103 mg/L formate, 4312±119 mg/L acetate, 2722±23 mg/L propionate and 2498±121 mg/L butyrate (**Table 2**). Biofilm growth in presence of sodium fumarate resulted in significant lactate production (1490±717 mg/L), whereas saccharin and sodium succinate increased acetate production (1549±90 and 3121±449 mg/L). D-sorbitol, saccharin, lactic acid, sodium succinate and sodium fumarate increased propionate production (6726±96, 1735±37, 6166±93, 5345±54 and 4557±177 mg/L) whereas butyrate production was decreased (1397±166, 280±25, 1312±358, 1308±510 and 409±87 mg/L). For the other set of substrates, control biofilms consumed 194±0 mg/L lactate, 226±7 mg/L formate, 5104±105 mg/L acetate, 3135±182 mg/L propionate and 2449±224 mg/L butyrate (**Table 2**). Biofilms grown in presence of sodium L-lactate showed decreased lactate consumption (104±2 mg/L), increased acetate and propionate production (5907±66 and 7117±206 mg/L), and decreased butyrate production (1868±117 mg/L). Sodium pyruvate yielded elevated levels of formate, acetate, propionate and butyrate (921±74, 7817±41, 3819±40 and 2086±38 mg/L), whereas potassium acetate and sodium acetate increased acetate production (10624±14 and 8524±264 mg/L).

Virulence modulation of multi-species biofilms

A selection of virulence genes from three periodontal pathogens (*A. actinomycetemcomitans*, *F. nucleatum* and *P. gingivalis*) was made of which the expression profiles were analysed to determine the effects of each substrate on multi-species biofilm virulence (**Table 3**). Noteworthy is that significant changes in virulence gene expression relative to the control condition were only considered to be biologically relevant when there was >1.5-fold upregulation or >2-fold downregulation and that only such changes were considered.

Altogether, as can be seen based on the color scale used in **Table 3**, more substrate-gene combinations showed at least a tendency towards decreased virulence gene expression than combinations showing at least a tendency towards increased virulence gene expression. For D-sorbitol, lactic acid, sodium fumarate, D-(-)-arabinose, 6/10 genes showed a tendency towards downregulated expression, for sodium succinate and sodium acetate this were 5/10 genes, for saccharin, sodium L-lactate and sodium pyruvate 4/10 genes and for potassium acetate 3/10 genes.

For five substrates, *A. actinomycetemcomitans* virulence gene expression was found to be downregulated 3.3- to 100-fold (**Table 3**). D-(+)-sorbitol downregulated *apaH*, *cagE* and *orf859* expression (3.6-, 100- and 5.9-fold). Lactic acid, sodium fumarate, D-(-)-arabinose and sodium acetate downregulated *orf859* expression with 3.3-, 4.0-, 5.9- and 3.4-fold. On the other hand, *pgA* expression was upregulated for D-sorbitol, saccharin and sodium pyruvate (3.6-, 3.8- and 3.2-fold). For *F. nucleatum*, hemin receptor gene expression was downregulated 2.3- to 9.1-fold for D-sorbitol, lactic acid, potassium acetate and sodium acetate (**Table 3**). Hemolysin gene expression was upregulated 3.6-fold for saccharin. ABC transporter permease gene expression was downregulated 2.3-fold for D-sorbitol, whereas for saccharin, sodium succinate, sodium fumarate, sodium L-lactate, sodium pyruvate and sodium acetate, 2.3- to 18-fold upregulation was observed. Finally, *P. gingivalis fimA* expression was downregulated for sodium succinate and sodium lactate (3.3- and 3.6-fold) and upregulated for saccharin and potassium acetate (3.6- and 2.7-fold) (**Table 3**). The expression of *kgp* was 25.0-fold downregulated for saccharin and 18.8-fold upregulated for sodium pyruvate, whereas *rgpA*

expression was 3.6- to 4.8-fold downregulated for sodium succinate, sodium L-lactate and sodium acetate.

DISCUSSION

Research on novel preventive and therapeutic interventions for oral health is rapidly evolving, with one of the focuses lying on the modulation of the commensal oral microbiota as a 'pro-microbial' approach. Such modulation can for instance be achieved by increasing the abundance and/or activity of certain species, eventually resulting in a more balanced oral microbiota. This study evaluated whether 10 selected substrates could modulate in vitro multi-species oral biofilms towards a more health-associated microbiological composition, an altered metabolic activity and a decreased virulence gene expression profile. The selection of the evaluated substrates was based on the possibility that they could stimulate H_2O_2 production by certain commensal species and/or increase their abundance, which has been described in literature or was hypothesized based on known bacterial H_2O_2 pathways. Biofilm growth in presence of the substrates at a clinically relevant concentration of $1\%_{(w/v)}$ often resulted in a microbiological composition with increased abundances of commensal species and decreased abundances of periodontal pathogens. Furthermore, most substrate conditions also altered the metabolic profiles of these biofilms. The effects on virulence gene expression, based on a selection of 10 important virulence genes of 3 periodontal pathogens, were highly substrate-dependent, but for several substrates a decreased expression of certain genes could be observed. Altogether, this study provides novel findings on oral biofilm modulation by 10 substrates selected for their possible effects on the activity and/or abundance of certain commensal oral bacteria. To our knowledge, this work is the first one to simultaneously investigate the modulatory effects of these specific substrates on the microbiological composition, metabolic and virulence profiles of complex, in vitro multi-species oral biofilms.

The substrates included in this study were selected based on previous findings in literature and/or their involvement in bacterial H_2O_2 production pathways. Arabinose, sorbitol and saccharin have been shown to stimulate H_2O_2 production by *S. oralis* and *S. sanguinis*[36].

Pyruvate and lactate are two substrates with a central role in the two major oral streptococcal H_2O_2 production pathways[14,37,38]. Furthermore, it is known that lactic acid might serve as a substrate for certain streptococcal species resulting in the production of H_2O_2[38,39]. Potassium acetate was shown to slightly affect H_2O_2 production by *S. gordonii*[40], which led to the hypothesis this could also be the case for sodium acetate. Finally, the conversion of fumarate to succinate is known to yield H_2O_2 as a by-product, and succinate can in turn be re-converted to fumarate[41]. The selected substrates thus have a clear link with H_2O_2-producing commensal oral species, but the current study merely focused on the effects of these substrates on multi-species oral biofilms rather than on the potential underlying mechanisms of these effects. The rationale for this is that accurate determination of H_2O_2 production within complex oral biofilms has not been achieved yet. Due to diffusion restrictions, the effects of H_2O_2 can be very localized and take mainly place within the biofilm[42,43]. Furthermore, determining the effects of the substrates on certain aspects of a complex multi-species community could be considered to be more relevant than merely investigating their mode of action in a simpler setting. Since H_2O_2 plays an important role in shaping oral bacterial communities during biofilm development[14,43], this study evaluated the effect of the presence of the substrates during oral biofilm formation.

Dysbiosis is one of the main hallmarks of oral disease development and is characterized by a decreased prevalence and/or function of commensal species, whereas the opposite is true for (potentially) pathogenic species[15,44,45]. Consequently, modulation of the oral microbiota envisions the achievement of increased abundances of commensals and/or decreased abundances of pathogens. The majority of the substrates tested in this study achieved at least one, and often both, of these goals. This shows potential for these substrates as modulators of oral biofilms, since previous studies on potential prebiotic substrates for oral health reported similar effects on multi-species biofilm composition[30,31,33,46]. The role of species like *A. actinomycetemcomitans*, *F. nucleatum*, *P. gingivalis* and *P. intermedia* in the initiation and progression of periodontal diseases has been well-characterized[11,44,47,48]. However, some streptococci like *S. oralis*, *S. mitis*, *S. sanguinis* and *S. gordonii* are well-known H_2O_2-producers

246 that play a role in shaping oral communities during biofilm development, whereas pathobionts
247 like *P. gingivalis* and *P. intermedia* are susceptible to H_2O_2-mediated toxicity[49]. For substrates
248 with a compositional effect, changes in abundances were generally observed for one or more
249 of the above-mentioned species. For instance, sodium lactate, sodium pyruvate and potassium
250 acetate all resulted in increased *S. oralis* numbers while simultaneously also a decrease in one
251 or two periodontal pathogens like *P. gingivalis*, *P. intermedia* and *F. nucleatum* was observed.
252 On the other hand, sorbitol was found to increase *S. mitis* and *S. sanguinis* numbers while also
253 decreasing *A. actinomycetemcomitans* and *P. gingivalis* numbers. The effects of other
254 substrates like saccharin or lactic acid were generally limited to decreases in periopathogens.
255 However, this does not automatically imply that such substrates have no effects on the activity
256 of commensal species, as saccharin and lactic acid have been shown to increase H_2O_2
257 production by certain oral streptococci[36,38,39]. Altogether, the majority of the substrates tested
258 in this study shifted the biofilm composition towards a more health-associated one. It can be
259 hypothesized that, besides increasing the abundance of certain commensals, this could also
260 be mediated by stimulating the activity of these species.

261 Insights into the metabolic profile of oral communities can provide valuable information
262 on the role they play in oral health or disease. In periodontal disease, inflammophilic species
263 characterized by asaccharolytic and proteolytic metabolisms are enriched in abundance and
264 show increased activity[15,50,51]. This eventually provides for a reciprocally reinforced feedback
265 loop between inflammation and dysbiosis, allowing such species to thrive and which acts as
266 an important disease driver[15]. Species like *Porphyromonas*, *Prevotella* and *Fusobacterium* are
267 characterized by such metabolic profiles through which peptides and amino acids are
268 converted into organic acids like formate, acetate, propionate and butyrate[50,52]. In this study, it
269 was remarkable that most of the substrate conditions showed decreases in butyrate
270 production. Although butyrate is known to play a protective role in the gut, butyrate production
271 in the oral cavity is known to be associated with periodontal inflammation[53-55]. Therefore, the
272 observed decreased butyrate levels can be considered as a favourable metabolic change.
273 Similar findings on decreased butyrate levels were previously reported in an in vitro study

identifying potential prebiotic substrates for oral health[46]. However, commensal species like *Actinomyces* and *Streptococcus* have a saccharolytic metabolism, leading to the production of lactate, acetate and formate[52]. Given that several substrate conditions showed increases in one or two streptococcal species, one would expect to observe an increase in lactate levels, although this was not the case. This can be explained by the complexity of multi-species biofilms, which are characterized by a wide variety of interspecies interactions and metabolic cross-feeding[11,52,56-58]. Lactate produced by streptococci forms a nutritional source for *Actinomyces* and *Veillonella* species, which results in the production of formate, acetate and propionate (*Veillonella* spp.) or acetate (*Actinomyces* spp.)[52,56,57]. Formate has been shown to have an inverse relationship with the severity of periodontal disease[53], and in the current study, it was increased in the sodium pyruvate condition. On the other hand, in some studies, it has also been associated with undesired effects on oral epithelial cells in vitro, which is also the case for acetate and propionate[59]. However, given the entanglement of metabolic pathways within complex multi-species oral biofilms, it is difficult to fully interpret the impact of all metabolic shifts observed in this study.

Pathogenic bacteria in dysbiotic oral communities are often characterized by a pronounced virulence that allows them to persist, thrive and contribute to disease progression[60,61]. The virulence genes evaluated in this study were selected based on their well-known involvement in periodontal disease onset and progression[62-67]. Effects on virulence gene expression were highly dependent on the substrate and pathogenic species under consideration. For instance, downregulated *apaH*, *cagE* and *orf859* expression in *A. actinomycetemcomitans* was observed for the sorbitol condition. These genes encode virulence factors involved in the invasion of non-phagocytic cells (*apaH*)[62], conjugation, DNA transport and virulence factor secretion (*cagE*)[66] and intracellular survival (*orf859*)[62]. *Orf859* expression was also downregulated in several other conditions. Remarkable was the increased *pgA* expression in the sorbitol, saccharin and sodium pyruvate conditions. *PgA* encodes a protein involved in the synthesis of a polysaccharide with an important role in aggregation and biofilm formation[63]. Similar observations for *pgA* expression in modulated oral

biofilms were also previously observed[46], and this could be explained as a response to external stress, something reported for other *Aggregatibacter* species[68]. For *F. nucleatum*, downregulation was often observed for the gene encoding a hemin receptor, which is highly immunogenic and plays an important role in hemin uptake[65]. Apart from sorbitol and lactic acid, most conditions showed upregulated ABC transporter permease gene expression. As its gene product is involved in membrane transport[65], this could also be a response to the induced environmental changes. For *P. gingivalis* the effects were also diverse. Most substrates led to decreased *rgpA* expression, a gingipain gene encoding an arginine-specific cysteine protease involved in several processes such as disturbance of host defense systems and tissue degradation[67]. *FimA* and *kgp* expression, encoding a fimbrilin involved in attachment to oral surfaces and a gingipain gene encoding a lysine-specific cysteine protease, respectively[67], were sometimes downregulated and sometimes upregulated, depending on the substrate.

Altogether, the effects of the substrates on the virulence profiles of the biofilms were found to be highly diverse. Nevertheless, it is important to look at the overall effect of the substrates, since oral diseases are caused by the concerted virulence, (metabolic) function and composition of synergistic polymicrobial biofilms[6,16]. From that point of view, most substrates had beneficial modulatory effects on at least one, and often two or all three of these aspects. To conclude, future research should look into some of the limitations and aspects that were not addressed in the current study. For instance, a broader selection of virulence genes could provide further insight into changes in virulence, and also evaluating the effects on the inflammatory potential of the biofilms towards oral cells could be of interest. Furthermore, now the effects of the substrates on a complex multi-species biofilm have been established, the underlying mechanisms of these effects should be investigated. Given the rationale for the selection of the substrates, this should first focus on the influence they might have on streptococcal H_2O_2 production. In conclusion, this study found that a selection of substrates chosen for their hypothesized beneficial effects on the abundance and/or activity of commensal oral bacteria were able to modulate in vitro multi-species oral biofilms towards a more health-associated state. More specifically, biofilms grown in presence of the substrates at a clinically

relevant concentration often showed a beneficial shift in microbiological composition, an altered metabolic profile and sometimes a decreased virulence, the latter of which was highly dependent on the substrate under consideration.

MATERIALS AND METHODS

Bacterial strains, growth media and culture conditions

Aggregatibacter actinomycetemcomitans ATCC 43718, *Fusobacterium nucleatum* ATCC 10953, *Porphyromonas gingivalis* ATCC 33277 and *Prevotella intermedia* ATCC 25611 were used as representative periodontal pathogens, *Streptococcus mutans* ATCC 25175 and *Streptococcus sobrinus* ATCC 33478 as representative cariogenic pathogens and *Actinomyces naeslundii* ATCC 51655, *Actinomyces viscosus* ATCC 15987, *Streptococcus gordonii* ATCC 49818, S*treptococcus mitis* ATCC 49456, *Streptococcus oralis* DSM 20627*, Streptococcus sanguinis* LMG 14657 and *Veillonella parvula* DSM 2008 as representative commensal species. Bacteria were grown on blood agar (Oxoid, Ltd, Basingstoke, UK) supplemented with 5 µg/mL hemin, 1 µg/mL menadione (both Sigma-Aldrich Co, St.-Louis, USA) and 5% sterile horse blood (E&O Laboratories Ltd, Bonnybridge, Scotland). *A. actinomycetemcomitans*, *S. gordonii*, *S. mitis*, *S. mutans*, *S. oralis*, *S. sanguinis* and *S. sobrinus* were grown aerobically (37°C, 5% CO_2) whereas *A. naeslundii*, *A. viscosus*, *F. nucleatum*, *P. gingivalis*, *P. intermedia* and *V. parvula* were grown anaerobically (37°C, 80% N_2, 10% H_2, and 10% CO_2). Single species planktonic cultures were grown in brain hearth infusion (BHI) broth (Difco Laboratories, Detroit, USA) as described previously[30]. Multi-species biofilms were grown in modified BHI broth (BHI-2)[30].

Bioreactor-derived multi-species community

A 13-species community was established in a bioreactor (Biostat B Twin 1L bioreactor, Sartorius Stedim Biotech GmbH, Goettingen, Germany) under controlled environmental conditions, as described in detail elsewhere[30].

Substrates

The substrates used in this study were selected based on the following two criteria: (1) shown in literature to (possibly) stimulate H_2O_2 production by a limited number of oral bacterial species; and/or (2) (in)direct involvement in known pathways of oral bacterial H_2O_2 production. All substrates were dissolved in BHI-2 without mucin at a concentration of 2%$_{(w/v)}$, followed by pH adjustment to 7.4 and filter sterilization. For the biofilm experiments, one volume of this was supplemented with one volume of sterile BHI-2 with double-concentrated mucin (2 x), yielding sterile BHI-2 solutions (with 1 x mucin) with a final substrate concentration of 1%$_{(w/v)}$. Following substrates were selected for this study: D-(-)-arabinose, lactic acid, potassium acetate, saccharin, sodium fumarate, sodium L-lactate, sodium pyruvate, sodium succinate (all Sigma-Aldrich Co, St. Louis, USA), sodium acetate and D-sorbitol (both VWR, Radnor, USA).

Multi-species biofilm formation assays, DNA extraction and quantification

Biofilms were grown horizontally on Calcium Deficient Hydroxyapatite (CAD-HA) disks (Hitemco Medical, Old Bethpage, USA) on the bottom of a 24-well plate in presence of a substrate. Samples from the bioreactor-derived multi-species community were diluted 1:5 in fresh BHI-2 with 2 x mucin, after which 1 mL was added to each well containing a HA disk. Equal volumes (1 mL) of 2%$_{(w/v)}$ substrate solutions in BHI-2 without mucin were added to the bacterial suspensions (final multi-species community dilution of 1:10, final substrate concentration of 1%$_{(w/v)}$ in BHI-2). As a negative control, BHI-2 without substrate supplementation was used. Biofilms were allowed to grow for 48 h under micro-aerophilic (6% O_2, 7% CO_2, 7% H_2, 80% N_2) conditions (170 rpm, 37°C). All experiments were repeated on three different days. After 48 h, biofilms were gently washed with phosphate buffered saline (PBS, pH 7.4) to detach non-adherent cells, after which remaining biofilms were disrupted by trypsinization and bacterial cells were harvested as described before[30]. DNA from only living bacteria was extracted using a previously described propidium monoazide (PMA) treatment[30]. Bacterial numbers were determined using a quantitative polymerase chain reaction (qPCR) assay as described by Slomka et al.[30], whereas species-specific primers and probes were listed by Herrero et al.[69].

Organic acid analysis of multi-species biofilm supernatants

Concentrations of lactate, acetate, formate, propionate and butyrate in the filter sterilized supernatant of the multi-species biofilm assays were determined with a 761 Compact Ion Chromatograph (Metrohm, Switzerland) with a Metrosep Organic acids 250/7.8 column and a Metrosep Organic acids Guard/4.6 guard column, with the eluent consisting of 1 mM H_2SO_4 at a flow rate of 0.8 mL min^{-1}. Organic acid production/consumption was calculated as the organic acid concentrations detected in the filter sterilized supernatants, minus the concentrations of those organic acids detected in sterile BHI-2 with or without supplemented substrate.

RNA extraction and virulence gene expression analysis

Biofilm-coated disks were dip-rinsed in PBS (pH 7.4) to remove unattached cells, followed by bacterial RNA extraction as described previously[60]. Briefly, RNA was obtained through a mechanical disruption and acid phenol-chloroform extraction as described by Vandecasteele et al.[34] in combination with the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. After quality and integrity assessment, a concentration-dependent normalization of all RNA samples was performed, followed by conversion of RNA to complementary DNA (cDNA), all as described previously[60]. Expression of bacterial virulence genes was analysed through SYBR RT-qPCR and normalized for bacterial housekeeping gene (species-specific 16S rRNA or other genes) expression. Reaction mixtures were prepared and assay conditions were performed as described by Herrero et al.[60]. Specific sequences of each primer pair can be found elsewhere[60]. Data were determined as a function of the threshold cycle (CT) values and relative virulence gene expression was calculated according to the ΔΔCT method ($2^{-(\Delta CTexp - \Delta CTcontrol)}$).

Statistical analysis

Statistical analysis was done using GraphPad Prism v.7.04 for Windows (GraphPad Software, La Jolla, USA). Normality of the residuals was assessed through a Shapiro-Wilk test and a normal quantile plot. For most experiments, comparisons with the control were made and statistically significant differences ($P < 0.05$) were determined through a one-way ANOVA (confidence level of 95%) followed by Dunnett's correction for simultaneous hypothesis testing. Changes in absolute bacterial abundances expressed as the difference between the value of

the control condition and the value of the substrate condition were analysed through a two-tailed, one sample t test to detect differences significantly different from 0 (no difference between control condition and substrate condition).

DATA AVAILABILITY

The authors declare that all data supporting the findings of this study are available within the paper and its supplementary information files.

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AUTHOR CONTRIBUTIONS

T.V. contributed to conception, design, data acquisition and analysis, data interpretation, drafted and critically revised the manuscript; D.V. contributed to data acquisition and analysis, data interpretation and critically revised the manuscript; W.V.H. and N.Z. contributed to data interpretation and critically revised the manuscript; K.B. and N.B. contributed to design, data interpretation and critically revised the manuscript; W.T. contributed to conception, design, data analysis and interpretation, and critically revised the manuscript.

ADDITIONAL INFORMATION

Competing interests statement

All authors report no conflicts of interest related to this study.

Supplementary information

Supplementary information accompanies the manuscript on the *Scientific Reports* website https://www.nature.com/srep.

FIGURE LEGENDS

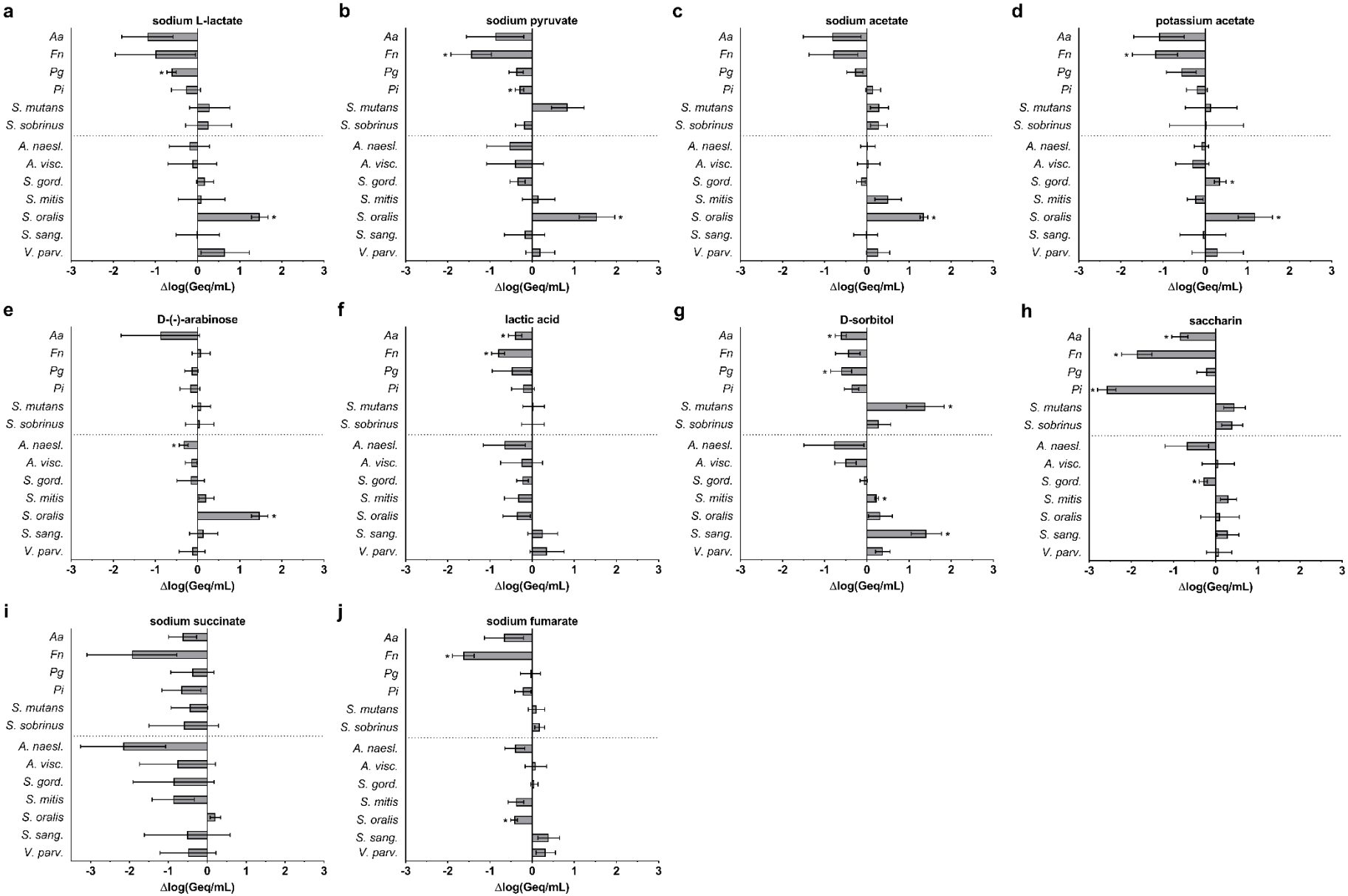

Figure 1 Changes in absolute composition of multi-species biofilms grown in presence of H_2O_2 production-affecting substrates. Multi-species biofilms were grown during two series of experiments in the absence (=control; BHI-2 medium) or presence of the different substrates (**a**: sodium L-lactate; **b**: sodium

pyruvate; **c**: sodium acetate; **d**: potassium acetate; **e**: D-(-)-arabinose; **f**: lactic acid; **g**: D-sorbitol; **h**: saccharin; **i**: sodium succinate; **j**: sodium fumarate) dissolved in BHI-2 medium at a concentration of $1\%_{(w/v)}$. Changes in absolute abundances of each species in comparison with the control are shown as mean ± SD (n = 3) and expressed in Δlogarithmic value of genome equivalents per millilitre (Δlog(Geq/mL)). Δlog(Geq/mL) was calculated by subtracting the log(Geq/mL) values of the control condition for each species from the log(Geq/mL) values of the substrate condition for each species. Δlog(Geq/mL) values that are statistically significantly different from 0 (corresponding to no difference between control condition and substrate condition) are shown in bold and are marked with '*****' ($P < 0.05$, two-tailed one sample t test).

Aa: *A. actinomycetemcomitans*; *Fn*: *F. nucleatum*; *Pg*: *P. gingivalis*; *Pi*: *P. intermedia*; *A. naesl.*: *A. naeslundii*; *A. visc.*: *A. viscosus*; *S. gord.*: *S. gordonii*; *S. sang.*: *S. sanguinis*; *V. parv*: *V. parvula*.

Table 1 Changes in relative composition of multi-species biofilms grown in presence of H_2O_2 production-affecting substrates

	relative abundance (%Geq/mL)		
	commensals	periopathogens	cariogenic pathogens
control	21.8 ± 3.0	77.8 ± 3.0	0.4 ± 0.1
D-sorbitol	**55.2 ± 14.8 ***	**43.6 ± 15.0 ***	1.2 ± 0.4
saccharin	**82.6 ± 8.6 ***	**13.2 ± 6.3 ***	**4.2 ± 2.3 ***
lactic acid	**65.9 ± 7.4 ***	**33.3 ± 7.2 ***	0.8 ± 0.3
sodium succinate	47.5 ± 16.6	51.6 ± 16.8	0.8 ± 0.3
sodium fumarate	**74.5 ± 7.5 ***	**24.2 ± 7.2 ***	1.3 ± 0.3

	relative abundance (%Geq/mL)		
	commensals	periopathogens	cariogenic pathogens
control	31.7 ± 9.3	68.3 ± 9.3	0.1 ± 0.0
sodium L-lactate	**82.9 ± 7.0**	**17.0 ± 7.0 ***	0.1 ± 0.0
sodium pyruvate	62.4 ± 13.6	37.1 ± 13.6	**0.5 ± 0.2 ***
D-(-)-arabinose	33.5 ± 19.6	66.4 ± 19.6	0.1 ± 0.0
potassium acetate	**69.7 ± 8.6**	**30.2 ± 8.6 ***	0.1 ± 0.1
sodium acetate	49.6 ± 8.1	50.3 ± 8.1	0.1 ± 0.0

Multi-species biofilms were grown during two series of experiments (upper part and lower part) in the absence (=control; BHI-2 medium) or presence of the different substrates dissolved in BHI-2 medium at a concentration of $1\%_{(w/v)}$. Relative abundances of commensals, periopathogens and cariogenic pathogens are shown as mean ± SD (n = 3) and expressed in %genome equivalents per millilitre (%Geq/mL). Statistically significant changes in comparison with the control condition are shown in bold and are marked with '*' ($P < 0.05$, ANOVA + Dunnett's correction for simultaneous hypothesis testing).

Table 2 Organic acid production/consumption by multi-species biofilms grown in presence of H_2O_2 production-affecting substrates

	OA production/consumption (mg/L)				
	lactate	formate	acetate	propionate	butyrate
control	-170 ± 2	94 ± 103	4312 ± 119	2722 ± 23	2498 ± 121
D-sorbitol	-13 ± 222	383 ± 100	3889 ± 52	**6726 ± 96 ***	**1397 ± 166 ***
saccharin	-194 ± 0	-78 ± 0	**1549 ± 90 ***	**1735 ± 37 ***	**280 ± 25 ***
lactic acid	85 ± 42	13 ± 59	4791 ± 320	**6166 ± 93 ***	**1312 ± 358 ***
sodium succinate	-136 ± 8	28 ± 52	**3121 ± 449 ***	**5345 ± 54 ***	**1308 ± 510 ***
sodium fumarate	**1490 ± 717 ***	114 ± 41	3864 ± 98	**4557 ± 177 ***	**409 ± 87 ***

	OA production/consumption (mg/L)				
	lactate	formate	acetate	propionate	butyrate
control	-194 ± 0	226 ± 7	5104 ± 105	3135 ± 182	2449 ± 224
sodium L-lactate	**-104 ± 2 ***	203 ± 6	**5907 ± 66 ***	**7117 ± 206 ***	**1868 ± 117 ***
sodium pyruvate	-146 ± 34	**921 ± 74 ***	**7817 ± 41 ***	**3819 ± 40 ***	**2086 ± 38 ***
D-(-)-arabinose	-134 ± 10	236 ± 6	4965 ± 167	2985 ± 60	2274 ± 91
potassium acetate	-167 ± 38	201 ± 4	**10624 ± 14 ***	3150 ± 64	2547 ± 39
sodium acetate	-145 ± 34	177 ± 13	**8524 ± 264 ***	3078 ± 158	2145 ± 26

Multi-species biofilms were grown during two series of experiments (upper part and lower part) in the absence (=control; BHI-2 medium) or presence of the different substrates dissolved in BHI-2 medium at a concentration of $1\%_{(w/v)}$. Organic acid production/consumption (shown as mean ± SD (n = 3) and expressed in mg/L) was calculated as the organic acid concentrations detected in the filter sterilized supernatants, minus the concentrations of those organic acids detected in sterile BHI-2 with or without supplemented substrate. Values preceded by a negative sign ('-') indicate organic acid consumption (net decrease), whereas all other values indicate organic acid production (net increase). Statistically significant changes in comparison with the control condition are shown in bold and are marked with '*' ($P < 0.05$, ANOVA + Dunnett's correction for simultaneous hypothesis testing). OA: organic acid.

Table 3 Changes in virulence gene expression of multi-species biofilms grown in presence of H_2O_2 production-affecting substrates

	relative fold change in virulence gene expression									
	SORBI	**SACCH**	**LA**	**SS**	**SF**	**SL**	**SP**	**ARA**	**PA**	**SA**
Genes	***A. actinomycetemcomitans***									
apaH	**0.28 (0.04-1.84)**	0.61 (0.14-2.63)	1.39 (0.47-4.17)	1.70 (0.15-18.94)	2.56 (0.33-9.85)	1.81 (0.96-3.42)	0.73 (0.7-0.76)	0.61 (0.39-0.94)	0.84 (0.36-1.97)	2.67 (0.37-19.52)
cagE	**0.01 (0.01-0.01)**	0.48 (0.15-1.48)	2.85 (0.7-11.61)	3.11 (0.61-15.91)	0.92 (0.03-28.02)	3.49 (1.41-8.61)	1.07 (0.59-1.94)	0.81 (0.21-3.11)	2.00 (1.31-3.05)	3.29 (1.45-7.45)
orf859	**0.17 (0.05-0.53)**	1.13 (0.30-4.24)	**0.30 (0.11-0.78)**	0.55 (0.04-7.20)	**0.25 (0.07-0.86)**	0.34 (0.07-1.56)	0.66 (0.42-1.03)	**0.17 (0.03-0.96)**	0.69 (0.51-0.93)	**0.29 (0.07-1.18)**
pgA	**3.57 (1.44-8.84)**	**3.80 (1.34-10.75)**	0.96 (0.72-1.27)	0.49 (0.2-1.22)	1.84 (0.44-7.66)	0.68 (0.17-2.68)	**3.19 (0.62-16.54)**	1.57 (0.34-7.4)	1.62 (1.1-2.38)	1.53 (1.12-2.09)
Genes	***F. nucleatum***									
ABC tr. p.	**0.44 (0.20-0.95)**	**17.98 (13.4-24.2)**	0.45 (0.16-1.26)	**3.96 (1.15-13.67)**	**4.98 (3.51-7.06)**	**3.71 (2.66-5.19)**	**2.29 (0.85-6.16)**	1.16 (0.80-1.70)	3.49 (1.60-7.59)	**3.96 (1.59-9.91)**
hemin rec.	**0.11 (0.02-0.53)**	3.05 (0.67-13.99)	**0.15 (0.03-0.75)**	0.42 (0.11-1.62)	0.51 (0.17-1.50)	1.15 (0.35-3.82)	0.33 (0.02-4.28)	0.39 (0.07-2.30)	**0.43 (0.09-1.96)**	**0.29 (0.15-0.58)**
hemolysin	1.08 (0.48-2.44)	**3.60 (2.02-6.41)**	0.72 (0.31-1.66)	1.63 (0.38-7.04)	0.66 (0.24-1.85)	1.27 (0.53-3.03)	1.15 (0.17-7.79)	0.99 (0.51-1.92)	1.58 (1.31-1.92)	1.03 (0.36-2.96)
Genes	***P. gingivalis***									
fimA	1.56 (0.18-13.18)	**3.58 (2.07-6.18)**	0.61 (0.23-1.60)	**0.30 (0.13-0.71)**	0.42 (0.13-1.39)	**0.28 (0.13-0.60)**	1.46 (0.26-8.20)	0.60 (0.17-2.14)	**2.71 (1.35-5.45)**	0.46 (0.13-1.67)
kgp	0.45 (0.07-2.89)	**0.04 (0.01-0.18)**	2.33 (1.76-3.08)	1.43 (0.23-8.90)	0.41 (0.36-0.46)	1.31 (0.3-5.74)	**18.79 (7.13-49.53)**	2.20 (0.24-19.88)	3.68 (1.75-7.76)	1.23 (0.3-5.08)
rgpA	1.01 (0.6-1.72)	0.40 (0.12-1.41)	0.58 (0.13-2.58)	**0.28 (0.04-2.10)**	0.32 (0.29-0.35)	**0.21 (0.06-0.72)**	0.73 (0.22-2.41)	0.61 (0.14-2.58)	1.80 (0.49-6.63)	**0.25 (0.02-3.20)**

relative fold change values										
<0.1	**0.1-0.3**	**0.3-0.5**	**0.5-0.7**	**0.7-0.9**	**0.9-1.1**	**1.1-1.4**	**1.4-2.0**	**2.0-3.3**	**3.3-10.0**	**>10.0**

Multi-species biofilms were grown during two series of experiments in the absence (=control; BHI-2 medium) or presence of the different substrates dissolved in BHI-2 medium at a concentration of 1%$_{(w/v)}$. Changes in the expression of a selection of virulence genes from three periodontal pathogens present in the multi-species biofilms were determined. Fold changes in virulence gene expression were calculated with the $2^{\wedge-\Delta\Delta Ct}$ method and were determined relative to the control (BHI-2). Data are shown as geometric mean and C.I. (n = 3) of the $2^{\wedge-\Delta\Delta Ct}$ values. Values between 0 and 1 indicate downregulation relative to the control, values >1 indicate upregulation relative to the control. Statistically significantly different fold changes relative to the control with a value <0.5 (more than 2-fold downregulated) or >1.5 (more than 1.5-fold upregulated) are considered biologically relevant and are shown in bold ($P < 0.05$, ANOVA + Dunnett's correction for simultaneous hypothesis testing). The color

scale indicates the magnitude of the fold change in virulence gene expression relative to the control. SORBI: D-sorbitol; SACCH: saccharin; LA: lactic acid; SS: sodium succinate; SF: sodium fumarate; SL: sodium L-lactate; SP: sodium pyruvate; ARA: D-(-)-arabinose; PA: potassium acetate; SA: sodium acetate; ABC tr. p.: ABC transporter permease; hemin rec.: hemin receptor; C.I.: 95% confidence interval.

Figures

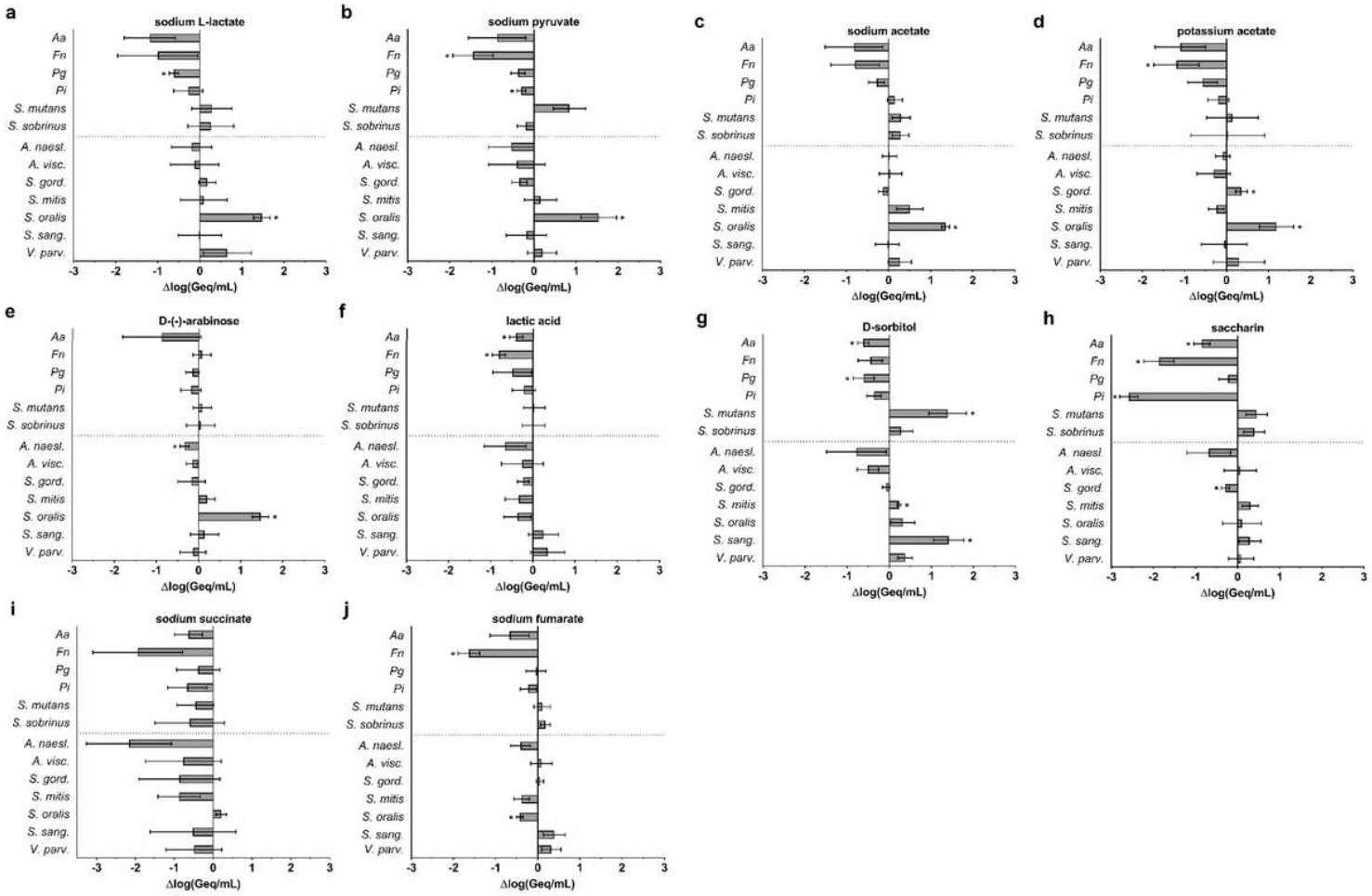

Figure 1

Changes in absolute composition of multi-species biofilms grown in presence of H2O2 production-affecting substrates. Multi-species biofilms were grown during two series of experiments in the absence (=control; BHI-2 medium) or presence of the different substrates (a: sodium L-lactate; b: sodium pyruvate; c: sodium acetate; d: potassium acetate; e: D-(-)-arabinose; f: lactic acid; g: D-sorbitol; h: saccharin; i: sodium succinate; j: sodium fumarate) dissolved in BHI-2 medium at a concentration of 1%(w/v). Changes in absolute abundances of each species in comparison with the control are shown as mean ± SD (n = 3) and expressed in Δlogarithmic value of genome equivalents per millilitre (Δlog(Geq/mL)). Δlog(Geq/mL) was calculated by subtracting the log(Geq/mL) values of the control condition for each species from the log(Geq/mL) values of the substrate condition for each species. Δlog(Geq/mL) values that are statistically significantly different from 0 (corresponding to no difference between control condition and substrate condition) are shown in bold and are marked with '*' ($P < 0.05$, two-tailed one sample t test). Aa: A. actinomycetemcomitans; Fn: F. nucleatum; Pg: P. gingivalis; Pi: P. intermedia; A. naesl.: A. naeslundii; A. visc.: A. viscosus; S. gord.: S. gordonii; S. sang.: S. sanguinis; V. parv: V. parvula.

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