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The events that may contribute to subgingival dysbiosis: a focus on the interplay between iron, sulfide and oxygen

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One-sentence summary

This review focuses on a probable series of events detrimental to oral health-associated subgingival microbiota in the presence of iron, sulfide and oxygen, and suggests strategies to overcome them.

Abstract

This minireview considers the disruption of the host-microbiota harmless symbiosis in the subgingival niche. The establishment of a chronic infection by subversion of a commensal microbiota results from a complex and multiparametric sequence of events. This review narrows down to the interplay between oxygen, iron and sulfide that can result in a vicious cycle that would favour peroxigenic and glutathione producing streptococci as well as sulfidogenic anaerobic pathogens in the subgingival niche. We propose hypothesis and discuss strategies for the therapeutic modulation of the microbiota to prevent periodontitis and promote oral health.

Introduction

Unlike the first billion years on earth, oxygen is now abundant in most environments and it shapes the ecology and microbial behaviour. Amongst several effects, oxygen oxidizes soluble ferrous iron into insoluble ferric hydroxides. Oxygen also decomposes Fe-S clusters which are the essential cofactors of redox processes and metabolisms and generates reactive oxygen species (ROS). Most microorganisms rely on Fe-S clusters inherited from ancient proteins which have evolved in anoxic conditions rich in iron and sulfide (Imlay 2006). Because of their Fe-S dependency, most microorganisms are sensitive to high levels of oxygen and iron deficiency, but also to excess in ferrous iron which generates hydroxyl radical, the most harmful ROS, in presence of the oxygen derivative H_2O_2 . The so-called anaerobes have restricted their habitat to hypoxic niches or created local hypoxic niche by producing reductive volatile compounds such as hydrogen sulfide H_2S . However, constant hypoxic conditions are rare and these communities are likely to be exposed to changing oxygen levels, which would modify their behaviour. The focus of this review is to describe the potential interplay between oxygen, iron and sulfide in the oral ecosystem, especially in the subgingival plaque, which consists of hundreds of bacterial species. This ecosystem exposed to ambient oxygen, but composed of both aerobic and anaerobic bacteria, some of them sulfidogenic, is particularly relevant for the topic.

The oral microbiota

Over 500 species have been identified in the oral ecosystem by metagenomic analyses but a large proportion remains uncharacterized (Ai *et al.* 2017). The oral bacterial composition varies from a commensal biofilm mainly composed of Gram-positive bacteria to a pathogenic biofilm dominated by Gram-negative anaerobic bacteria which can lead to periodontitis, a chronic infection and inflammation of teeth-supporting tissues. Periodontitis is predominantly caused by the pathogens of the red (*Porphyromonas gingivalis*, *Treponema denticola*, *Tannerella forsythia*) and orange complex (*Fusobacterium nucleatum*, *Prevotella intermedia*, *Peptostreptococcus micros*, *Eubacterium nodatum*) as described by Socransky *et al.* (Socransky *et al.* 1998). The green complex (*Clostridium gingivalis*, *C. sputigena*, *C. ochracea*, *Campylobacter concisus*, *Eikenella corrodens*, and *Actinobacillus actinomycetemcomitans* serotype a) is also associated with periodontal disease. The purple (*Veillonella parvula*, *Actinomyces odontolyticus*) and the yellow (*Streptococcus gordonii*,

Streptococcus intermedius, *Streptococcus mitis*, *Streptococcus oralis*, *Streptococcus sanguis*) complexes mainly consist of facultative anaerobic and Gram-positive bacteria associated with oral health. They are also involved in cooperative metabolisms in the periodontal biofilm. In the subgingival plaque, facultative anaerobic or microaerophilic microorganisms predominate. *P. gingivalis* is a keystone pathogen because of its ability to subvert the immune response (Maekawa *et al.* 2014) and to activate the virulence of pathobionts (Hajishengallis, Darveau and Curtis 2012). The optimal growth rate of *P. gingivalis* takes place in anaerobic condition, but it possesses an O₂ consuming cytochrome *bd* oxidase that contributes to its aerotolerant phenotype (Leclerc *et al.* 2015) as well as an O₂-generating SOD (superoxide dismutase) instead of a non-O₂-generating SOR (superoxide reductase) (Lynch and Kuramitsu 1999). Therefore, like the genotypically-related *Bacteroides fragilis*, *P. gingivalis* may be considered as a nanaerobe which means an organism that can grow with nanomolar concentrations of oxygen and benefit from it although it does not require oxygen for growth (Morris and Schmidt 2013). A species-specific co-aggregation of *P. gingivalis* with oral streptococci such as *S. gordonii* promotes initial colonization (Lamont, Hersey and Rosan 1992). *P. gingivalis* and *T. denticola* exhibit a symbiotic relationship in nutrient utilization, growth promotion, and synergetic biofilm formation (Ng *et al.* 2016, Meuric *et al.* 2013). It was also suggested that *P. gingivalis* may benefit from a mutual colonization with *Candida albicans*, a fungal species commonly encountered in the oral ecosystem. The growth of the filamentous form of the fungi under oxic conditions may offer a protective environment for the growth of *P. gingivalis* by depleting oxygen (Bartnicka *et al.* 2019). As described for other nanaerobes such as *B. fragilis* the genomes of the pathogens *P. gingivalis*, *Prevotella intermedia* and *Tannerella forsythia*, all from the family of the bacteroidetes, possess a typical cytochrome *bd* oxidase with a long Q-loop, an hydrophilic region facing the outside of the cells and connecting transmembrane helices of subunit I (Borisov *et al.* 2011). Some microaerophilic organisms are also part of the oral microbiota, such as *Campylobacter concisus* (Tanner *et al.* 1981). Several studies showed *C. concisus* to be associated with bleeding in young adults with rapidly progressive periodontitis (Kamma, Nakou and Manti 1994, 1995). The presence of O₂-dependent or O₂-tolerant microorganisms in the oral biofilm of periodontal patients suggests that the anaerobic-strict picture of the pathogenic subgingival biofilm is controversial and that changing pO₂ (partial pressure of oxygen) may modulate the behaviour of the community.

[1] Oxygen in the oral ecosystem

Oxygen is abundant in the oral cavity. The primary source of oxygen is the free entry of atmospheric oxygen through the mouth. Another oxygen supplier is the gingival crevicular fluid (GCF) from the tissues surrounding the gingival crevice or periodontal pocket where the subgingival biofilm generally develops (Loesche *et al.* 1983). GCF is formed by the passage of fluid from capillaries into the tissues and then in the gingival sulcus. The pO_2 in the periodontal pockets, which are the pathologically deepened gingival sulci, is low but not negligible. During the development of oral biofilms, the redox potential decreases: electrodes in place for up to 7 days in patients showed a fall in oxidation-reduction potential as plaque bacteria accumulated (Kenney and Ash 1969). For reference, under an atmospheric pressure of 760 mm Hg (sea level), the partial pressure of oxygen is 160 mm Hg. Deep periodontal pockets (7-10 mm) have a pO_2 of 11.6 mmHg and the moderately deep periodontal pockets (5-6 mm) have a pO_2 of 15 mmHg. The pO_2 of healthy sites is unavailable for comparison due to the limitation posed by length of the sensor used. The mean pO_2 of untreated periodontal pockets (13.2 mmHg) marks the upper limit of hypoxia (Mettraux, Gusberti and Graf 1984). Moreover, under inflammatory condition, oxygen levels rise due to elevated blood flow and haemoglobin. The pocket pO_2 showed correlation with probing depth and with oxygen saturation of haemoglobin (Tanaka *et al.* 1998).

[2] Production of H_2O_2

If not the starting event, oxidative stress contributes to the snowball effect due to the interdependence between oxidative stress and inflammation. Streptococci from the *mitis* and *sanguinis* group dominate the initial colonization of the tooth surface (Rosan and Lamont 2000; Mark Welch *et al.* 2016; Wake *et al.* 2016). They are facultative anaerobes and the main producers of H_2O_2 in response to oxygen. This metabolite was first considered beneficial for oral health by maintaining a symbiotic microbiota and preventing pathogens (Kreth, Zhang and Herzberg 2008; Redanz *et al.* 2018).

The sources of microbial H_2O_2 in the oral biofilm have been reviewed by Redanz *et al.* (Redanz *et al.* 2018). Briefly, the main enzyme involved in hydrogen peroxide production is the streptococcal protein SpxB, a pyruvate oxidase that converts pyruvate to acetyl phosphate and H_2O_2 and consumes oxygen (Fig. 1). Minor synthesis of H_2O_2 may arise from detoxifying

enzymes from various species such as SOD (superoxide dismutase) and SOR (superoxide reductase) that reduce superoxide anions into less reactive H₂O₂. Another streptococcal enzyme capable of generating H₂O₂ is the FMN-dependent lactate oxidase (LctO) which performs the aerobic oxidation of lactate into pyruvate (Fig.1). Finally, under conditions where peptone and amino acid are the only available nutrients, such as in saliva, H₂O₂ together with ammonia and α -ketoacid may be produced by the oxidation of amino acid by the L-amino acid oxidase (LAAO) (Fig.1) (Tong *et al.* 2008). The generation of H₂O₂ is also a constant process in host eukaryotic cells, which increases during inflammation in response to pathogens. When stimulated by pathogens, host cells release cytokines, recruit polymorphonuclear leukocytes which synthesize proteolytic enzymes and ROS. The primary sources of ROS are the NADPH oxidase (Nox), the mitochondrial respiratory chain and SOD (Sculley and Langley-Evans 2002; Sies 2017).

S. sanguinis and *S. gordonii* are often called "oral health compatible organisms", due to their H₂O₂-related antimicrobial properties against anaerobic pathogens. However, the effect of H₂O₂ on the microbiota cannot be limited to the inhibition of pathogen viability or a global beneficial input on oral health. H₂O₂ is a non-radical product with low reactivity with organic molecules. Its longer life span (1ms) compared to free radicals such as O₂^{•-} (1 μ s) and OH⁻ (1 ns) makes it a good signalling molecule (Dahiya *et al.* 2013).

[3] The Fenton chemistry and the sources of Iron

The toxicity of H₂O₂ mainly depends on transition metal concentration in the surrounding, especially ferrous iron or copper, because of the Fenton reaction [H₂O₂ + Fe²⁺ → ·OH + Fe³⁺ + OH⁻] using both substrates to generate the highly reactive hydroxyl radical (OH·). H₂O₂, via the production of OH·, may be toxic not only for other bacteria including potential pathogens but also for host cells. The effect of H₂O₂ on eukaryotic oral host cells is either beneficial or deleterious depending on its concentration. At low levels (28.9 μ M maximum concentration for 4 h and reduced up to 13.5 μ M for 24h), it can stimulate the proliferation of human periodontal ligament fibroblasts *in vitro*, but it also displays cytotoxic effect at higher concentrations (1mM), including on endothelial cells (Choe *et al.* 2012). Some results suggest that streptococcal H₂O₂ is not only cytotoxic to epithelial cells, but also promotes bacterial evasion of the host defence systems in the oral cavity (Okahashi *et al.* 2014; Liu *et al.* 2017; Erttmann and Gekara 2019). Moreover, H₂O₂ produced by *S. gordonii* can serve as an electron acceptor to increase the persistence of *Aggregatibacter actinomycetemcomitans* by a

shift from an anaerobic to an aerobic mode of growth, an interaction termed "cross-respiration". *A. actinomycetemcomitans* is associated with aggressive periodontitis, presumably due to the production of the leukotoxin A (Stacy *et al.* 2014, 2016). Therefore, the balance between good and bad for oral health may be, amongst several factors, a matter of concentration (entry, release and turnover) of free ferrous iron and H₂O₂ inside target cells. Oxygen-derived free radical resulting from Fenton reaction serves to amplify the inflammatory response, by favouring the expression of cytokines in endothelial cells, promoting the recruitment of neutrophils and inflammatory cells. As a result of tissue damage, mitochondria, xanthine oxidase and neutrophils produce more reactive oxygen species (Closa and Folch-Puy 2004; Mittal *et al.* 2014).

In the oral environment, iron may come from the gingival crevicular fluid (GCF), saliva and dietary sources. GCF is an inflammatory exudate derived from the periodontal tissues and is composed of serum, tissue breakdown products, inflammatory mediators, and sometimes antibodies. During the development of gingivitis, the flow rate of GCF increases (Goodson 2003). In the host, iron is bound in complexes such as lactoferrin, haemoglobin and transferrin. Lactoferrin is a glycoprotein with iron-binding properties, secreted by exocrine glands and neutrophils. It binds to two atoms of iron and retains iron at a much lower pH (up to pH 3.0) than the main systemic transporter transferrin (up to pH 5.5) (Baker and Baker 2005). Lactoferrin is an important defence factor against *Streptococcus mutans* and periodontopathic bacteria. It has the ability not only to inhibit bacterial growth and biofilm development, but also to limit free-iron overload, and therefore reduce reactive oxygen formation and inflammatory processes (Berlutti *et al.* 2011). A correlation between lactoferrin concentration in the GCF and the number of PMNs in GCF was reported (Adonogianaki, Moughal and Kinane 1993). Although lactoferrin can be used as an iron source for some pathogens such as *Neisseria gonorrhoeae* (Blanton *et al.* 1990) or *Campylobacter jejuni* (Miller *et al.* 2008), its role as an iron source for periodontal bacteria is not yet proven.

Hemic iron is mainly provided by haemoglobin (Hb). Haemoglobin derived from microbleeding in gingival sulci was detected in GCF samples from periodontal sites (Ito *et al.* 2016). Iron acquisition from Hb by oral/periodontal bacteria may be facilitated by the H₂O₂ produced by the streptococci: the initial oxidation of oxyHb into metHb, a form of haemoglobin that contains the ferric [Fe³⁺] form of iron is carried out by H₂O₂. This further promoted the activity of the gingipains, proteases of *P. gingivalis*, which cleave such iron

proteins as the first step of heme uptake. Also, the dissociation of the ferric iron from heme protoporphyrin is easier than in the case of ferrous iron (Brown *et al.* 2018).

Transferrin is a glycoprotein with 2 iron-binding sites (composed of 39 cysteines), found in the periodontal pocket together with haemoglobin and lactoferrin. Transferrin and its iron-saturated form, holotransferrin support the growth of several oral bacteria such as *Campylobacter rectus* (Grenier and Tanabe 2011) and *P. gingivalis* (Brochu *et al.* 2001; Goulet *et al.* 2004). Increased transferrin saturation is generally due to genetic haemochromatosis that provokes iron egress from macrophages and enterocytes in relation with hepcidin deficiency. Increased transferrin saturation is not only associated with increased mortality in patients with sepsis (Lan *et al.* 2018) but also with dysbiosis and severe periodontitis (Boyer *et al.* 2018). Moreover, as previously stated, iron saturation and transferrin concentration increase in the GCF during periodontitis (Adonogianaki, Moughal and Kinane 1993).

Along with the external sources of iron in the GCF, oxygen radicals, especially superoxide anions, destroy the ubiquitous 4Fe-4S clusters of the host and proteins of the microbiota, releasing free ferrous iron in the surrounding. The fate of the dissolved ferrous iron depends on the properties of the environment such as oxidative status and pH. As the pH increases, the speed of the oxidation of ferrous iron increases and produces ferric oxides and hydroxides. Altogether free iron overload enhanced by oxygen and combined with oxygen derivatives may be one of the triggers that favour the dysbiosis and inflammatory processes, provided some bacteria (pathogens and H₂O₂ producers) can protect themselves against O₂/H₂O₂ toxicity in the presence of ferrous iron.

[4] The role of reductive compounds, H₂S and glutathione

Peroxygenic streptococci limit their reliance to Fe-S cluster protein and use manganese in place of Fe to avoid H₂O₂ self-toxicity (Jakubovics, Smith and Jenkinson 2002; Tseng *et al.* 2002). However, the sensitivity of anaerobes to oxidative stress mainly relies on the sensitivity of the Fe-S cluster of their pyruvate-ferredoxin oxidoreductase (PFOR). PFOR is a major metabolic enzyme involved in energy generation through oxidative decarboxylation of pyruvate, as demonstrated with the model *Bacteroides thetaiotaomicron* (Pan and Imlay 2001; Lu, Sethu and Imlay 2018). The periodontal pathogens *P. gingivalis*, *P. intermedia* and *T. forsythia* use PFOR which allowed the development of a new antimicrobial compound, amoxicile, specifically targeting the anaerobic pathogen via the inhibition of PFOR

(Hutcherson *et al.* 2017). Anaerobes need reduced environment rather than low oxygen concentration for growth. PFOR help bacteria to thrive in a reduced environment because of low-potential electron transfer pathway, in which PFOR passes low-potential electrons toward hydrogen formation and/or NAD reduction. The redox potential measured in saliva was +158 to 542 mV while it was lower in periodontal pockets (-300 mV) and gingival crevices (-200 mV). Indeed, the redox potential is reduced by microbial metabolism during the development of oral biofilm (Kenney and Ash 1969). Hydrogen sulfide is a metabolite which is produced in high amount by microorganisms in the periodontal environment (Horowitz and Folke 1973). H₂S production add to oral malodour: halitosis is caused by various volatile sulfur compounds (VSCs), including H₂S but also ethanethiol, S-ethyl thioacetate, diethyl disulfide, dimethyl sulfide and methyl mercaptan. The amount of VSCs has been shown to significantly increase in patients with periodontal disease. H₂S produced in subgingival pocket probably contributes to halitosis even though the predominance of methyl mercaptan produced by the microbiota of the tongue has been shown (Yaegaki and Sanada 1992). Such metabolites with low redox potential (E₀ +0.17 V), potentially contribute to the reductive and protective environment for anaerobes and nanaerobes in subgingival pockets. The effect of H₂S greatly varies with environmental conditions. H₂S molecules dissolve in water and dissociate into H⁺, HS⁻ and S²⁻ ions. HS⁻ and to a lower extent, H₂S are chemical reductants capable of scavenging ROS and inducing defence response in eukaryotic cells (Xie *et al.* 2014). H₂S can be as toxic as cyanide due to its ability to bind with and precipitate metal cations. Likewise, for some bacterial species, exogenous H₂S can suppress H₂O₂-mediated oxidative stress. It was shown that H₂S suppressed the oxidative stress in *E. coli* by sequestering free iron that reacted with H₂O₂ in Fenton chemistry (Mironov *et al.* 2017). Another study on Streptococci showed that H₂S reduced intracellular bound ferric iron to form unbound ferrous iron that can act as a substrate for Fenton chemistry (Dunning, Ma and Marquis 1998). Therefore, H₂S may be beneficial to oral anaerobes (Lai and Chu 2008) but exerts a toxic effect on other microorganisms, by inhibition of dismutase, catalase, and sequestration of trace metals in the surrounding or increase of free cytosolic ferrous iron. The toxic effect of H₂S when studied on streptococci, was iron-dependent. The addition of the iron chelator deferoxamine obliterated the bactericidal effect of H₂S (Ooi and Tan 2016). In *Shewanella oneidensis*, the coordinated effect of H₂S and H₂O₂ was described as being time-dependent. On simultaneous addition of H₂O₂ and H₂S to cultures, H₂S promoted H₂O₂ toxicity via catalase inactivation. However, on later supplementation of H₂O₂, H₂S promoted H₂O₂ resistance, probably by lowering external iron concentrations, activating the influx of iron and OxyR-dependent stress defences (Wu *et*

al. 2015). For anaerobic bacteria, H₂S production helps to maintain a reduced environment and to decrease dissolved O₂. A study of Lai and Chu has revealed that H₂S production by *T. denticola* resulted in a dose-dependent removal of dissolved O₂ in the growth cultures (Lai and Chu 2008).

H₂S also increases intracellular glutathione (GSH) synthesis in streptococci. GSH protects streptococci against both their H₂O₂ (Zheng *et al.* 2013) and H₂S produced by neighbouring bacteria (Ooi and Tan 2016). GSH (γ -L-glutamyl-L-cysteinyl-glycine) is an abundant non-protein thiol present in many bacteria and all eukaryotes (Masip, Veeravalli and Georgiou 2006). Glutathione biosynthesis generally occurs via a conserved two-step mechanism in which a γ -glutamylcysteine ligase (GshA) uses l-glutamate and l-cysteine to form γ -glutamylcysteine and then, a glutathione synthetase (GshB) condenses γ -glutamylcysteine with glycine to give glutathione (Fig.2). H₂S reduces cystine into cysteine in extracellular space and increases the expression of glutamate-cysteine ligase that converts cysteine into γ -glutamyl cysteine, the direct precursor of GSH. The susceptibility of oral streptococci to H₂S is dependent on intracellular glutathione levels, which increase in the presence of the H₂S donor NaHS *in vitro* (Ooi and Tan 2016).

GSH synthesis by eukaryotic and prokaryotic cells may promote H₂S production by sulfidogenic bacteria if released in the surrounding. H₂S is produced by several anaerobic bacteria of the oral microbiota mainly from cysteine, which originated from the catabolism of cysteine-containing proteins/peptides and glutathione, via cysteine desulfhydration. The possible pathway for production of H₂S from the degradation of GSH is depicted in Figure.2. As the initial step, the γ -glutamyltransferase GGT breaks down glutathione into cysteinyl glycine (Cys-Gly) and glutamate. Next, the cysteinyl glycinase CGase degrades Cys-Gly into glycine and l-cysteine. Finally, the β C-S lyases, pyridoxal-5'-phosphate (PLP)-dependent enzyme (L-cysteine desulfhydrase LCD for cysteine) catalyse the α , β -elimination of sulfur amino acids (l-cysteine, l-cystathionine, l-cystine, S-(2-aminoethyl)-l-cysteine, and S-methyl-l-cysteine) to the corresponding sulfur-containing molecules (H₂S in case of cysteine), pyruvate and ammonia. The NH₃ produced by this reaction may contribute to the alkaline pH encountered in periodontal pockets. This pathway is certainly relevant in the oral biofilm *in vivo* since glutathione was identified as a suitable substrate for H₂S production by the major pathogen *T. denticola* (Chu *et al.* 2002, 2003, 2008). Recently a *T. denticola* mutant lacking the gamma-glutamyltransferase enzyme GGT was constructed (Chu *et al.* 2020). The mutant cannot produce H₂S from glutathione. Interestingly, the mutant has also lost the ability to

grow in the presence of oxygen when incubated with glutathione and showed a reduced virulence *in vitro* against human gingival fibroblasts. The data also pointed out that the levels of cell death correlated with the amounts of H₂S produced. *P. gingivalis* does not possess GGT, but it is capable of producing H₂S from cysteinyl glycine, therefore, possessing CGase activity (Tang-Larsen *et al.* 1995). Neither of the two pathogens possesses the *gshA* and *gshB* genes for GSH synthesis. On the contrary, *in silico* genome analysis (unpublished analysis) indicates that the primary coloniser *S. gordonii* possesses a GshF-homologue gene. The product of this gene is a bifunctional enzyme that may carry out glutathione biosynthesis by catalysing both reactions on its own. Several studies pointed out that other pathogenic and free-living bacteria possess a single enzyme to catalyse the γ -glutamylcysteine ligase reaction and the condensation of γ -glutamylcysteine with glycine to give glutathione (Gopal *et al.* 2005; Janowiak and Griffith 2005; Vergauwen, De Vos and Van Beeumen 2006; Stout *et al.* 2012). Indeed, the SGO-1990 gene product of *S. gordonii* showed 100% identity with the bifunctional GshF enzyme of *Streptococcus sanguinis* (Cui *et al.* 2019). What remains to be investigated is whether reactive oxygen species enhance GSH synthesis in streptococci. Such activation was described in different bacteria and is as per the role of GSH in oxidative stress responses. For example, in *Pseudomonas aeruginosa*, hydrogen peroxide, cumene hydroperoxide, and *t*-butyl hydroperoxide increase the expression of *gshA* and *gshB* involved in glutathione synthesis (Wongsaroj *et al.* 2018). If ROS promotes GSH synthesis in the oral microbiota, oxidative stress conditions from the presence of H₂O₂ and ferrous iron could indirectly trigger H₂S synthesis by pathogenic oral bacteria via GSH cross-feeding and initiate a vicious circle leading to an H₂S-mediated reductive environment where anaerobic bacteria would flourish. The use of GSH by H₂S-producing bacteria may also explain why in GCF, the amount of GSH is lower in cases of chronic periodontal disease compared to healthier sites (Chapple 2002; Grant *et al.* 2010; Savita *et al.* 2015).

The cysteine-rich transferrin may also play a role in the oral dysbiosis not only by providing iron but also by favouring the synthesis of volatile sulfur compounds. The transferrin concentration increases in the gingival crevicular fluid during periodontitis (Adonogianaki, Moughal and Kinane 1993). As previously said, some bacteria can use it as both iron and nutrient source in the host (Goulet *et al.* 2004). *In vitro*, iron-saturated transferrin supported the growth of *P. gingivalis* via degradation of the protein (Brochu *et al.* 2001). Boyer *et al.* (2018) observed a significant link between the periodontitis severity and increased transferrin saturation in patients with iron-overload disease (haemochromatosis). However, the levels of

transferrin in the GCF of haemochromatosis patients compared to healthy patients are not known. The use of transferrin as a suitable substrate for H₂S synthesis by oral bacteria remains to be investigated as well. Finally, following an initial observation that mutants of *S. sanguinis* and *S. gordonii* depleted of the pleiotropic carbon catabolite regulator CcpA lost their ability to inhibit H₂O₂-sensitive strains of *S. mutans*, the authors identified pyruvate as the compound, overproduced by these mutants and responsible for the protective effect. This study highlighted a new mechanism for regulating H₂O₂ homeostasis within microbiota, which would depend on pyruvate secretion and H₂O₂ scavenging by some members of the community and which will enrich studies on interactions and dysbioses related to oxidative stress (Redanz *et al.* 2020).

Conclusion

This review combines separate data, mainly obtained *in vitro*, to propose hypotheses that will need to be tested *in vivo* or in a relevant context such as multispecies biofilms. Oxygen always being present in oral sites, even at low levels, allows H₂O₂ production by peroxygenic bacteria, mainly streptococci. Combined with H₂O₂, the levels of free ferrous iron may be one of the signals that will promote dysbiosis. Free iron along with the H₂O₂ in the oral sites can give rise to the Fenton reaction and thus produce ROS, which in turn generates more free iron via Fe-S degradation. The generation of toxic hydroxyl radicals may induce the production of GSH by some bacteria, and then indirectly of H₂S since GSH can be used as a substrate. H₂S protects anaerobes and may trigger more GSH production by H₂O₂ producing organisms. H₂S can also reduce ferric iron to promote the Fenton reaction or chelate ferrous iron and decrease the Fenton chemistry *in vitro*. The major points raised by this speculation is whether oxidative stress in a first step and then H₂S can significantly increase the production of glutathione by oral bacteria so that it can serve as a substrate for the production of H₂S. The whole picture is depicted in Figure 3. Ultimately, a treatment containing both an oxygen donor and a non-metabolizable iron chelator could be of interest to treat and prevent periodontal diseases as it may favour the development of commensal aerobic organisms without inducing deleterious oxidative stress. Besides, inhibitors of enzymes involved in the production of volatile sulfur compounds may be worth investigating especially on periodontal diseases in relation with increased levels of cysteine-containing proteins such as transferrin (hemochromatosis-related periodontitis). An *in vitro* study has identified propargylglycine as a potent inhibitor of volatile sulfur compound synthesis in *P. gingivalis*. The inhibitor hampers the activity of methionine gamma-lyase deaminase (Kandalam *et al.* 2018). Further detailed and focused

investigations on each of these scenarios will widen the understanding concerning the transition from oral health to disease and perhaps open up precise targets against periodontal pathogens. One should not neglect another theory that is worth considering, which is that inflammation plays a central role and modulates the polymicrobial oral biofilm, a model termed "Inflammation-Mediated Polymicrobial-Emergence and Dysbiotic-Exacerbation" (IMPEDE) (Van Dyke, Bartold and Reynolds 2020). Although our hypothesis has intentionally focused on microbial interactions, the two models are not mutually exclusive.

The authors declare that there are no conflicts of interest

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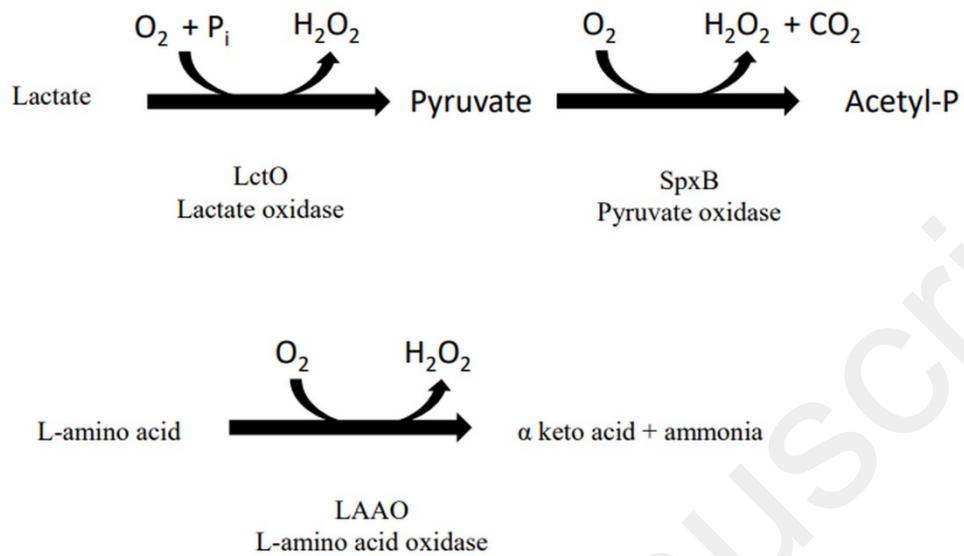


Figure 1. Possible H_2O_2 -producing enzymes in oral microbiota.

The FMN-dependent lactate oxidase (LctO) performs the aerobic oxidation of lactate into pyruvate. SpxB is a pyruvate oxidase that converts pyruvate to acetyl phosphate and H_2O_2 and consumes oxygen. When peptone and amino acid are the only available nutrients, the oxidation of amino acid by the L-amino acid oxidase (LAAO) produces H_2O_2 together with ammonia and α -ketoacid.

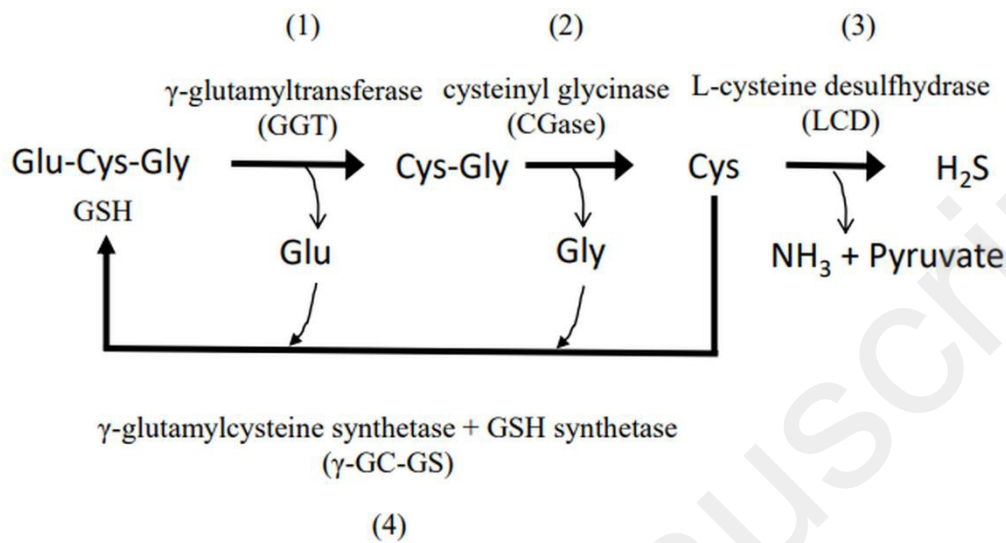


Figure 2. Common pathway of cysteine degradation to H₂S and GSH synthesis.

Examples of known coding genes for 3 representative species of the oral biofilm [*Porphyromonas gingivalis* TDC60 (PGTDC), *Treponema denticola* ATCC 35404 (TDE), and *Streptococcus gordonii* Challis NCTC7868 (SGO)] are: (1) TDE0444; (2) TDE0300; (3) TDE1669, PGTDC60-1459; (4) SGO-1990.

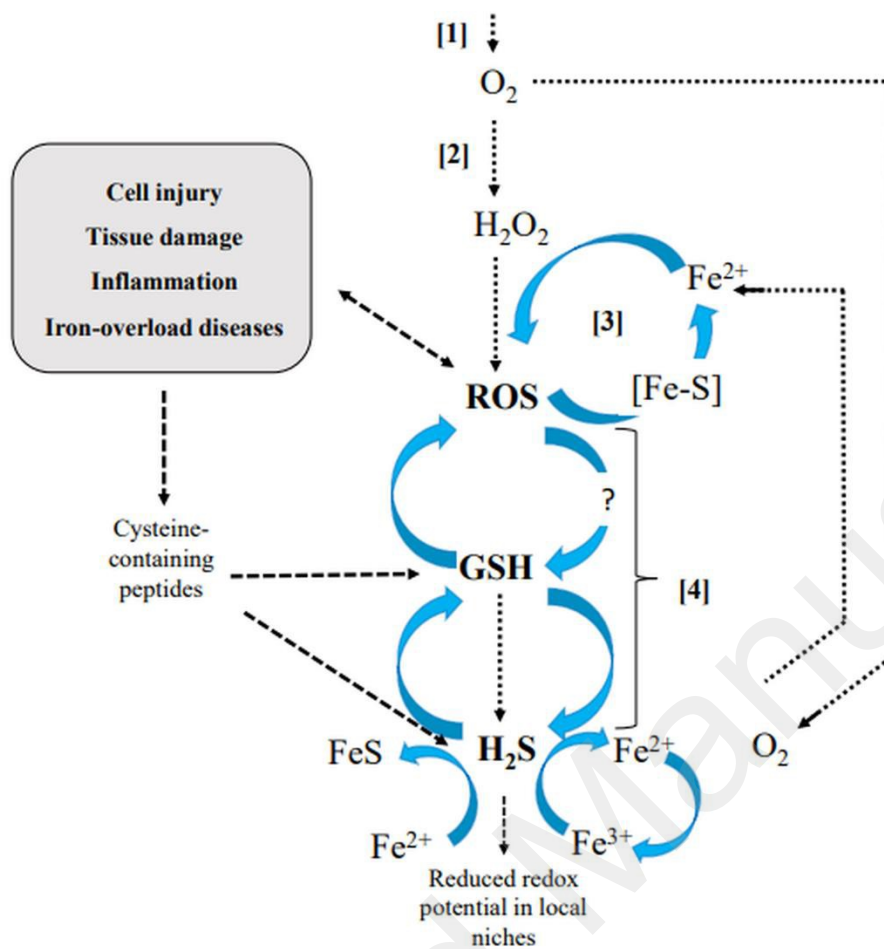


Figure 3. Hypothetical cascade of events leading to dysbiosis in periodontitis: the possible role of oxygen, iron and H_2S in the multifactorial process.

The arrows indicate metabolic, regulatory or inhibitory relationships. The numbers in brackets refer to the corresponding chapter in the text. The question mark indicates a link not yet experimentally verified in oral bacteria but identified in other bacteria. Briefly, oxygen always being present in oral sites may promote H_2O_2 production by peroxygenic bacteria, mainly streptococci. The Fenton chemistry involving ferrous iron and H_2O_2 may generate ROS (hydroxyl radical) which in turn may produce more free iron via Fe-S degradation. The generation of toxic hydroxyl radicals may induce the bacterial production of GSH as stress response, and then indirectly induce the formation of H_2S since GSH can act as a substrate *in vitro*, like other cysteine-containing peptides from the gingival crevicular fluid. H_2S protects anaerobes by lowering the redox potential in local niches. It may trigger more GSH production by H_2O_2 producing organisms (as described in streptococci), and can reduce ferric iron or can decrease the Fenton chemistry since H_2S can react with non-heme iron, free or

present in iron-sulfur cluster containing proteins, to generate insoluble precipitates (FeS). Altogether, this vicious circle starting from O₂ and ferrous iron may promote anaerobic pathogens and inflammation.

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