Dysbiosis by neutralizing commensal mediated inhibition of pathobionts

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Dysbiosis in the periodontal microbiota is associated with the development of periodontal diseases. Little is known about the initiation of dysbiosis. It was hypothesized that some commensal bacteria suppress the outgrowth of pathobionts by H₂O₂ production. However, serum and blood components released due to inflammation can neutralize this suppressive effect, leading to the initiation of dysbiosis. Agar plate, dual-species and multi-species ecology experiments showed that H₂O₂ production by commensal bacteria decreases pathobiont growth and colonization. Peroxidase and blood components neutralize this inhibitory effect primarily by an exogenous peroxidase activity without stimulating growth and biofilm formation of pathobionts directly. In multi-species environments, neutralization of H₂O₂ resulted in 2 to 3 log increases in pathobionts, a hallmark for dysbiosis. Our data show that in oral biofilms, commensal species suppress the amounts of pathobionts by H₂O₂ production. Inflammation can neutralize this effect and thereby initiates dysbiosis by allowing the outgrowth of pathobionts.

Although it is well known that complex poly-microbial oral biofilms are a necessity to develop tooth decay or periodontal diseases, they are often in balance with the host and consequently do not result in pathology 5,6. Not their presence as such but changes in their composition and/or metabolic activity drive pathology. The current etiological model of periodontal disease, termed ‘polymicrobial synergy and dysbiosis’, proposes that changes in the periodontal microbiota or dysbiosis deregulate the host immune response, leading to chronic inflammation 7,8. Although dysbiosis is characterized by a proportional increase of pathogenic species and a decrease of commensal species, little is known about the initiation of dysbiosis 9.

Most likely, dysbiosis is initiated by a complex interplay between the bacterial community and the host. Commensal bacteria act with the host to prevent colonization or outgrowth of pathobionts that induce inflammation and disrupt the microbial ecology 10. Pathobionts are natural members of the human microbiota that under certain perturbations to the host and/or microbiota can cause pathology 11,12. Subgingival inter-bacterial correlations and competition between commensal bacteria and pathobionts in relation to disease severity have been shown 10. As part of the oral commensal microbiota, highly abundant streptococci produce antimicrobial substances that limit the growth of pathobionts 13. It has been shown clinically that in patients with periodontal disease, there is a decline in commensal streptococci, highly abundant streptococci produce antimicrobial substances that limit the growth of pathobionts 13. It has been shown clinically that in patients with periodontal disease, there is a decline in commensal streptococci such as Streptococcus sanguinis 14,15. It was suggested that H₂O₂ is one of the most important antimicrobials produced by certain streptococci 16. For instance, S. sanguinis and Streptococcus gordonii can inhibit the growth of Streptococcus mutans and Aggregatibacter actinomycetemcomitans by H₂O₂ production 17,18. Recently, Rodriguez Herrero and coworkers showed that S. oralis, S. gordonii, S. cristatus, S. parasanguinis, S. mitis and S. sanguinis are good H₂O₂ producers, even under anaerobic conditions 19. Although, it has been suggested that H₂O₂ has an important role in the formation and the composition of the oral biofilms 20, the effect of H₂O₂ production on oral multi-species biofilm composition has not been investigated yet.

Gingival crevicular fluid (GCF) is an inflammatory exudate of serum that that covers oral biofilms 21. It can modulate the composition of oral biofilms by inhibiting or enhancing the growth of certain oral species or by...

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promoting biofilm formation and inhibiting the adhesion of certain species\textsuperscript{16}. Additionally, serum and blood components can have peroxidase activity\textsuperscript{17}. This is of significance since these could interact with the H\textsubscript{2}O\textsubscript{2} produced by commensal bacteria to suppress the outgrowth of pathobionts. Clinically it is known that absence of bleeding on probing is a good marker for periodontal stability\textsuperscript{18}. Recently, it is shown that crevicular myeloperoxidase concentrations are highly correlated with periodontal disease severity\textsuperscript{19}.

Therefore, it can be hypothesized that commensal bacteria suppress the overgrowth of pathobionts by H\textsubscript{2}O\textsubscript{2} but some serum and blood components released during inflammation can neutralize this suppressive effect, leading to the initiation of dysbiosis.

The objective of this study is to determine the neutralizing effect of serum, hemoglobin and hemin on the inhibitory effect of the commensal bacteria towards pathobionts.

**Results**

*Decrease of the inhibitory effect of commensals on agar plates.* In order to verify the inhibitory effect of some commensal species on pathobionts and the influence of hemin, serum, hemoglobin and peroxidase, a qualitative agar-plate method was used. On agar plates, hemin, serum, hemoglobin and peroxidase significantly lowered the inhibitory effect of commensal bacteria on *P*. intermedia, *P*. gingivalis and *A*. actinomycetemcomitans (Fig. 1). This effect was observed for all commensal species, all substrates on all pathobionts with exception for the effect of hemoglobin on the inhibition of *P*. intermedia and *P*. gingivalis by *S*. mitis (Table 1). Only peroxidase was able to completely neutralize the inhibitory effect of all the commensals. In most cases, hemin lowered the inhibition of the commensals more than serum and hemoglobin.

*Decrease of the inhibitory effect of commensals in dual-species planktonic cultures and biofilms.* Since bacteria within the oral cavity primarily live as biofilms, which can change their behavior, the decreased inhibitory effect induced by hemin, serum, hemoglobin and peroxidase in agar-plate experiments was verified in dual-species planktonic cultures and biofilms, the inhibitory effect of *S*. oralis on *P*. intermedia, *P*. gingivalis and *A*. actinomycetemcomitans was decreased (p < 0.05) by serum, hemin, hemoglobin and peroxidase (Fig. 2A and B). The effect was not significant for serum and hemoglobin on planktonic *A*. actinomycetemcomitans. For most of the dual-species experiments, adding serum, hemin, hemoglobin and peroxidase completely abolished the inhibitory effect of *S*. oralis resulting in numbers of pathobionts similar to the negative control in which *S*. oralis was not present.

*S. intermedius*, a non-H\textsubscript{2}O\textsubscript{2} producing species, did not show an inhibitory effect on the pathobionts (Fig. 2C and D). Consequently serum, hemin, hemoglobin and peroxidase could not decrease an inhibitory effect on the pathobionts but they also did not increase planktonic growth and biofilm formation in dual-species experiments. However, in these experiments, serum decreased the planktonic growth of *P*. intermedia and dual species biofilm formation of *P*. intermedia and *P*. gingivalis (p < 0.05).

*Effect of serum, hemoglobin, hemin and peroxidase on single species cultures.* In order to verify if the outgrowth of the pathobionts in the dual-species experiments was due to a decreased inhibitory effect of the commensal species and not due to an increased growth or biofilm formation of the pathobionts by the presence of hemin, serum, hemoglobin or peroxidase. The effect of these substrates on pathobiont growth and biofilm formation was evaluated. Serum, hemin, hemoglobin and peroxidase did not increase growth or biofilm formation of *P*. intermedia, *P*. gingivalis and *A*. actinomycetemcomitans (Fig. 3). Moreover, serum and hemin induced a small, but significant reduction on *P*. gingivalis biofilm formation (p < 0.05). Additionally, *A*. actinomycetemcomitans growth was decreased by serum and its biofilm formation by hemin and peroxidase (p < 0.05).

![Figure 1. Neutralization effect of serum, hemin, hemoglobin and peroxidase on the inhibitory effect of commensal species towards A. actinomycetemcomitans, P. intermedia and P. gingivalis. The commensals were spotted 24 hours before the pathogens in the center of the pictures. The pathogens were spotted at both sides of the commensals, at the left side plus the blood compound (serum (+Se), hemin (+He), hemoglobin (+Hb) and peroxidase (+Pe)) and at the right side without any blood compound.](image-url)
S. intermedius hemin and peroxidase in planktonic and biofilm conditions. (A. actinomycetemcomitans in mm (mean ± standard deviation, n = 3) against A. actinomycetemcomitans (Aa), P. intermedia (Pi) and P. gingivalis (Pg). *Statistically significant decrease of the inhibitory effect from commensal bacteria by serum, hemoglobin, hemin and peroxidase (p < 0.05). TI = Total Inhibition. − = without addition; + = with addition, 0.00 = no inhibition.

Table 1. Effect of the addition of serum, hemoglobin, hemin and peroxidase to BHI-2 agar on the antagonistic activity of commensal species. Data represent the magnitude of the zone of inhibition expressed in mm (mean ± standard deviation, n = 3) against A. actinomycetemcomitans (Aa), P. intermedia (Pi) and P. gingivalis (Pg). *Statistically significant decrease of the inhibitory effect from commensal bacteria by serum, hemoglobin, hemin and peroxidase (p < 0.05). TI = Total Inhibition. − = without addition; + = with addition, 0.00 = no inhibition.

| Microorganism | Serum | Hemoglobin | Hemin | Peroxidase |
|---------------|-------|------------|-------|------------|
|               | −     | +          | −     | +          |
| S. oralis     | Aa    | 2.04 ± 0.07| 0.62 ± 0.17*| 2.08 ± 0.27| 1.33 ± 0.21*| 2.35 ± 0.23| 0.67 ± 0.03*| 2.31 ± 0.38| 0.00 ± 0.00*|
|               | Pi    | TI         | 1.43 ± 0.08*| TI     | 2.71 ± 0.25*| 3.63 ± 0.81| 0.46 ± 0.20*| TI         | 0.00 ± 0.00*|
|               | Pg    | TI         | 1.37 ± 0.11*| TI     | 4.36 ± 0.37| 2.58 ± 0.24*| 2.23 ± 0.02| 0.00 ± 0.00*| 2.67 ± 0.16| 0.00 ± 0.00*|
| S. sanguinis  | Aa    | 3.04 ± 0.04| 1.45 ± 0.17*| 2.67 ± 0.42| 1.94 ± 0.21*| 2.70 ± 0.25| 1.14 ± 0.03*| 3.08 ± 0.57| 0.00 ± 0.00*|
|               | Pi    | TI         | 1.29 ± 0.07*| TI     | 3.37 ± 0.14| 1.41 ± 0.28*| 2.75 ± 0.32| 0.25 ± 0.19*| TI         | 0.00 ± 0.00*|
|               | Pg    | 4.58 ± 0.11| 0.78 ± 0.15*| 3.04 ± 0.12| 2.17 ± 0.05*| 2.63 ± 0.39| 0.00 ± 0.00*| 3.65 ± 0.35| 0.00 ± 0.00*|
| S. parasanginis| Aa | 1.99 ± 0.04| 0.75 ± 0.03*| 1.41 ± 0.27| 0.47 ± 0.11*| 2.52 ± 0.14| 0.80 ± 0.02*| 1.80 ± 0.24| 0.00 ± 0.00*|
|               | Pi    | 0.00 ± 0.00*| TI     | 3.05 ± 0.05*| TI     | 3.64 ± 0.10*| TI         | 0.00 ± 0.00*| 0.00 ± 0.00*|
|               | Pg    | 2.63 ± 0.24| 0.00 ± 0.00*| 1.14 ± 0.07| 0.71 ± 0.10| 1.62 ± 0.12| 0.00 ± 0.00*| 1.87 ± 0.27| 0.00 ± 0.00*|
| S. mitis      | Aa    | 2.83 ± 0.09*| 5.26 ± 0.85| 2.56 ± 0.23*| TI     | 2.37 ± 0.05*| TI         | 0.00 ± 0.00*| 0.00 ± 0.00*|
|               | Pi    | 1.76 ± 0.05*| TI     | TI     | TI     | 1.36 ± 0.29*| TI         | 0.00 ± 0.00*| 0.00 ± 0.00*|
|               | Pg    | 2.58 ± 0.16*| TI     | TI     | TI     | 1.29 ± 0.13*| TI         | 0.00 ± 0.00*| 0.00 ± 0.00*|
| S. gordonii   | Aa    | 2.68 ± 0.11| 1.05 ± 0.11*| 3.07 ± 0.21| 2.41 ± 0.26*| 3.75 ± 0.07| 1.25 ± 0.02*| 2.11 ± 0.29| 0.00 ± 0.00*|
|               | Pi    | 1.20 ± 0.13*| TI     | 2.78 ± 0.08*| 4.42 ± 0.52| 0.78 ± 0.31*| TI         | 0.00 ± 0.00*| 0.00 ± 0.00*|
|               | Pg    | 0.24 ± 0.17*| TI     | 3.40 ± 0.15*| 3.55 ± 1.02| 0.31 ± 0.08*| 3.21 ± 0.44| 0.00 ± 0.00*| 0.00 ± 0.00*|
| S. cristatus  | Aa    | 2.25 ± 0.15| 0.94 ± 0.09*| 2.00 ± 0.32| 1.16 ± 0.18*| 1.57 ± 0.06| 0.45 ± 0.08*| 2.02 ± 0.33| 0.00 ± 0.00*|
|               | Pi    | 0.82 ± 0.15*| TI     | 2.49 ± 0.44*| TI     | 1.33 ± 0.21*| TI         | 0.00 ± 0.00*| 0.00 ± 0.00*|
|               | Pg    | 2.96 ± 0.26| 0.00 ± 0.00*| 3.05 ± 0.22| 1.98 ± 0.24*| 1.61 ± 0.15| 0.00 ± 0.00*| 2.98 ± 0.41| 0.00 ± 0.00*|

Figure 2. Neutralizing effect of serum and blood compounds in dual species interactions analyzed by PMA-qPCR (mean ± standard deviation, n = 3). (A,B) Represent the competition between S. oralis and A. actinomycetemcomitans (Aa), P. intermedia (Pi) and P. gingivalis (Pg). *Designates a statistically significant increase of the bacterial concentration in respect to BHI (p < 0.05).
Since it was technically impossible to directly measure the H$_2$O$_2$ concentration in cultures in the presence of hemin, hemoglobin or serum with the Amplex® Red Hydrogen Peroxide kit, the peroxidase activity of hemin, serum, hemoglobin and peroxidase was determined. Although all compounds showed peroxidase activity, Peroxidase and hemin showed a higher peroxidase activity when compared to hemoglobin and serum (Fig. 4). The peroxidase activity was only observed in the highest concentrations of serum and hemoglobin whereas a peroxidase activity was also detected in diluted concentrations of hemin and peroxidase.

Reduction of the inhibitory effect of commensals in multi-species ecologies. Bacteria within the oral cavity are part of complex microbial ecologies. Since the observed effects in dual-species experiments might be different in more complex ecologies, the effect of hemin, hemoglobin, serum and peroxidase on simplified and complex multi-species ecologies was examined. In both biofilm models, commensal biofilms containing S. oralis, S. gordonii, S. cristatus, S. parasanguinis, S. mitis and S. sanguinis were challenged with either only 3 pathobionts (simplified ecology) or with a complex 14 species ecology (complex ecology). The commensal biofilm significantly inhibited the planktonic and biofilm concentrations of P. gingivalis and P. intermedia both in simplified (Fig. 5A and B) and complex multi-species ecologies (Fig. 5C and D). Its effect on A. actinomycetemcomitans concentrations was limited in simplified multi-species ecologies (Fig. 5A and B). Although it was more pronounced in complex multi-species ecologies, it did not reach statistical significance (Fig. 5C and D). In general, serum, hemin, hemoglobin and peroxidase decreased the inhibitory effect of the commensal biofilm on planktonic and biofilm concentrations of the pathobionts. This inhibition resulted in an outgrowth of A. actinomycetemcomitans, P. gingivalis and P. intermedia respectively of up to 2.37 (±0.98), 4.48 (±0.62) and 2.94 (±0.38) log$_{10}$ CFU/ml in complex planktonic multi-species ecologies and of up to 2.21 (±0.46), 8.17 (±0.14) and 3.12 (±0.18) log$_{10}$ CFU/ml in complex multi-species biofilms. The neutralizing effect of serum and hemoglobin was less than that of hemin and peroxidase.

The presence of the commensal biofilm did not only result in decreased planktonic concentrations of P. gingivalis and P. intermedia (Table 2). In the planktonic ecology, also a decreased concentration of S. salivarius and increased concentrations of S. sanguinis and S. oralis were observed when the commensal biofilm was present (p < 0.05). On the other hand, the biofilm concentrations of S. mutans, S. sobrinus and S. salivarius were decreased and the biofilm concentrations of F. nucleatum and S. sanguinis were increased (p < 0.05).

Moreover, in the complex multi-species ecologies (Table 2), the presence of serum increased the concentration of planktonic P. gingivalis (p < 0.05) and the concentrations of P. intermedia, P. gingivalis,
A. actinomycetemcomitans and S. oralis in the biofilm (p < 0.05). Additionally, it decreased the planktonic concentrations of F. nucleatum, A. viscosus, S. oralis, S. sobrinus and S. gordonii (p < 0.05) and the concentrations of F. nucleatum, A. viscosus and A. naeslundii in the biofilm (p < 0.05).

In contrast, the planktonic concentrations of A. actinomycetemcomitans and P. intermedia as well as the biofilm concentrations of A. actinomycetemcomitans, P. gingivalis, P. intermedia, A. viscosus and S. sanguinis were increased in the presence of hemoglobin (p < 0.05).

The addition of hemin increased the planktonic concentrations of P. gingivalis and P. intermedia but decreased the concentrations of A. viscosus, S. mutans, S. gordonii and S. mitis (p < 0.05). In the biofilms, hemin increased the concentrations of P. gingivalis and P. intermedia and decreased the concentrations of A. viscosus, A. naeslundii, S. sanguinis and S. gordonii (p < 0.05).

Peroxidase increased the planktonic concentrations of P. gingivalis and P. intermedia (p < 0.05) and the biofilm concentrations of P. intermedia, P. gingivalis, A. actinomycetemcomitans (p < 0.05).

Discussion

Dysbiosis in oral bacterial communities is characterized by a microbial shift, which is translated in an increase of pathobionts and a decrease of commensal species. It has been shown that some commensal species can suppress the growth of pathobionts by H2O2 under specific environmental conditions. More specifically, the sequence of colonization and the presence of oxygen are major influencing factors. Additionally, dysbiotic biofilms are enriched in virulence factors that stimulate the host inflammatory response. Although it can be deduced that both absence of commensal species and the presence inflammation are key factors, the factors driving dysbiosis are unclear. Moreover, as far as the authors know, dysbiosis has never been induced in in vitro multi-species biofilms. In this study, it was hypothesized that commensal bacteria suppress the overgrowth of pathobionts by H2O2 but some serum and blood components released during inflammation can neutralize this suppressive effect, leading dysbiosis. It was shown on agar plates and in dual-species biofilms that H2O2 production by commensal species and presence inflammation are key factors, the factors driving dysbiosis are unclear. Moreover, as far as the authors know, dysbiosis has never been induced in in vitro multi-species biofilms. In this study, it was hypothesized that commensal bacteria suppress the overgrowth of pathobionts by H2O2 but some serum and blood components released during inflammation can neutralize this suppressive effect, leading dysbiosis. It was shown on agar plates and in dual-species biofilms that H2O2 production by commensal bacteria decreases pathobiont growth and colonization. Although it was not directly tested in the current study, Herrero et al. showed that the amount of inhibition on pathobiont growth is determined by oxygen availability. Commensal bacteria could produce H2O2 and inhibit pathobiont growth under anaerobic condition. However, H2O2 production and pathobiont inhibition was significantly higher under aerobic conditions. Therefore, oxygen availability must play an important role into the transition from homeostasis to dysbiosis. If a non-H2O2 producing species was used in the current study, no inhibition was observed. Peroxidase and blood components neutralize the inhibitory effect of H2O2 primarily by a peroxidase activity since they did not stimulate the...
growth and biofilm formation of the pathobionts directly. In multi-species environments, neutralization of H$_2$O$_2$ by peroxidase or blood components resulted in 2 to 3 log increases in pathobionts which can be considered as a hallmark for dysbiosis.

The agar plate experiments showed an inhibition of the pathobionts when grown in the vicinity of the commensals. This inhibition was completely neutralized in the presence of peroxidase. These results were in concordance with previous studies$^{11,12,22}$ that also identified H$_2$O$_2$ as the main inhibitory substance. It was observed that the magnitudes of inhibition described by Van Essche et al. were markedly smaller than the ones reported in the current study although the same bacterial strains were used$^{23}$. This was attributed to the blood agar medium which was used in the Van Essche study and pointed towards an interference of certain blood compounds on the inhibition effect of these streptococcus species.

Gingival inflammation is characterized by an increase in GCF production, which is similar to serum, and bleeding tendency. Among many other components, blood contains serum, hemin and hemoglobin$^{24}$ but the hemolytic capacity of pathobionts can also increase their concentration$^{25}$. It is already described that hemoglobin$^{26}$ also generate layers of haems with catalytic activity to degrade the H$_2$O$_2$ using hemin and hemoglobin$^{27}$. Additionally, serum and GCF contains catalytic molecules such as myeloperoxidase that neutralizes the antimicrobial effect of H$_2$O$_2$ thus preventing the outgrowth of pathobionts. Furthermore, changes in the oxygen availability within the biofilm might also lower the H$_2$O$_2$ production and subsequently contribute to the outgrowth of pathobionts.

### Methods

**Bacterial strains and media.** All used bacterial species (Streptococcus sanguinis LM14657, Streptococcus cristatus ATCC 49999, Streptococcus gordonii ATCC 49818, Streptococcus parasanguinis DSM 6778, Streptococcus
mitis DSM 12643, Streptococcus oralis DSM 20627, Streptococcus salivarius TOVE-R, Streptococcus intermedius DSM 20573, Streptococcus mutans ATCC 20523, Streptococcus sobrinus ATCC 20742, Actinomyces viscosus DSM 43327, Actinomyces naeslundii ATCC 51655, Prevotella intermedia ATCC 25611, Porphyromonas gingivalis ATCC 33277, Fusobacterium nucleatum ATCC 20482, Aggregatibacter actinomycetemcomitans ATCC 43718 and Veillonella parvula DSM 2008) were maintained on blood agar (Oxoid, Basingstoke, UK) supplemented with 5 mg/mL hemin (Sigma, St. Louis, USA), 1 mg/mL menadione (Calbiochem-Novabiochem, La Jolla, USA) and 5% sterile horse blood (E&O Laboratories, Bonnybridge, Scotland). Overnight liquid cultures were prepared in Brain Heart Infusion (BHI) broth (Difco, Detroit, USA). Competitive inhibition experiments were performed in Brain Heart Infusion 2 (BHI-2) broth or agar containing Brain Heart infusion (Difco, Detroit, USA) supplemented with 2.5 g/L mucin (Sigma-Aldrich, St. Louis, USA), 1.0 g/L yeast extract (Oxoid, Basingstoke, UK), 0.1 g/L cysteine (Calbiochem, San Diego, USA), 2.0 g/L sodium bicarbonate and 0.25% (v/v) glutamic acid (Sigma-Aldrich, St. Louis, USA). The bacteria were cultured under aerobic (5% CO2) or anaerobic (80% N2, 10% H2 and 10% CO2) conditions. Optical densities were measured and adjusted using spectrophotometry (OD600, GeneQuant Spectrophotometer, Buckinghamshire, UK).

Serum and blood components. Hemin, human hemoglobin and horseradish peroxidase (Sigma-Aldrich, St. Louis, USA) were dissolved in BHI-2 at concentrations of 5 mg/mL hemin, 0.44 mg/mL hemoglobin33 and 16 μg/mL peroxidase. Human serum was obtained by venipuncture of a single, systemically healthy, male volunteer with no oral disease and who had not taken any antibiotics for 1 year. Peripheral venous blood was immediately centrifuged at 264 × g for 30 min at room temperature. The serum was removed and frozen at −20 °C after aliquotation.

Ethics Statement. The use of human serum was approved by the ethical committee of the KU Leuven and registered with identifier B322201628215. The procedures were executed according to the Helsinki Declaration and the regulations of the University Hospital, which are approved by the ethical committee. The adult subject provided a written and oral consent after having explained to him the purpose of the study. The subject is aware that the results will be used in a scientific study. An informed consent was obtained from all subjects.

Antagonistic experiments on agar plates. The spotting technique was used to quantify the inhibitory effect of 6 commensal species on 3 pathobionts and to identify neutralization effects by serum and blood components11. An overnight culture of a commensal species was adjusted to a concentration of 10^9 CFU/mL. This solution was spotted on an agar plate and incubated under aerobic conditions. After 24 hours, an overnight culture of the pathobiont (10^9 CFU/mL) was spotted next to the commensal spot. After 48 hours of anaerobic incubation, a calibrated photograph was taken from the agar plate and the magnitude of inhibition was measured from the edge of the commensal colony to the border of the inhibited pathobiont colony using ImageJ (http://rsb.info.nih.gov/ij/download.html).
Dual-species planktonic and biofilm experiments. *S. oralis* was selected as the model commensal species with a strong inhibitory effect on pathobionts on *H*₂*O₂* production. *S. internedius* was used as a not-inhibiting commensal species (negative control)⁹. 10 mL of an overnight culture of these commensals was centrifuged (1438 × g, 10 minutes). The supernatant was discarded and the pellet was re-suspended in 10 mL BHI-2 broth. The density was adjusted to 1 × 10⁸ CFU/ml. 6 ml of this solution was transferred to 6 wells (1 mL/well) of a 24 well-plate (Greiner, Frickenhausen, Germany) and incubated under aerobic conditions. After 24 hours, 500 μL of BHI-2 broth, serum, hemoglobin, hemin or peroxidase was added to the cultures. Additionally, an overnight culture of a pathobiont (*A. actinomycetemcomitans, P. gingivalis* or *P. intermedia*) was centrifuged (1438 × g, 10 minutes) and re-suspended in BHI-2 (1 × 10⁸ CFU/ml). 1 mL of this bacterial solution was inoculated in each well with *S. oralis* or *S. internedius* and in an additional well containing 1.5 mL BHI-2 (negative control). After 24 hours of anaerobic incubation, 1 mL was taken from each well, centrifuged (1438 × g, 10 min), re-suspended in PBS and analyzed via vitality q-PCR. Afterwards, the remaining supernatant was removed and the biofilms at the bottom of the wells were washed with phosphate buffered saline (PBS). The biofilms were detached with 500 μL 0.05% Trypsin-EDTA (Gibco, Paisley, UK) for 15 minutes at 37 °C, transferred to Eppendorf tubes, centrifuged (6010 × g, 10 minutes) and after discarding the trypsin, the biofilm pellets were re-suspended in 1 mL of PBS and analyzed by vitality q-PCR.

Effects on the growth and biofilm formation of pathobionts. An overnight culture of a pathobiont (*A. actinomycetemcomitans, P. gingivalis* or *P. intermedia*) was centrifuged (1438 × g, 10 minutes) and re-suspended in BHI-2 (1 × 10⁸ CFU/ml). 1 mL of this bacterial solution was inoculated in each well plus 500 μL of BHI-2 broth, serum, hemoglobin, hemin or peroxidase. The wells were incubated for 24 hours under anaerobic conditions where after planktonic bacteria and the biofilms analyzed as described above.

Peroxidase activity of blood compounds and peroxidase on H₂O₂. 50 μL of a 100 μM *H*₂*O₂* solution was mixed with either 10 μL horseradish peroxidase (10 U/ml) (control) or 10 μL serum or 10 μL hemoglobin (0.44 mg/ml) or 10 μL hemin (5 mg/ml) or 10 μL peroxidase (16 μg/ml). Additionally, 50 μL of a 100 μM *H*₂*O₂* solution was mixed with 10 fold serial dilutions of peroxidase, serum, hemoglobin, hemin and horseradish peroxidase in PBS (up to 1 in 1000). To 50 μL of these solutions, 50 μL of Amplex Red (50 μM) was added (Amplex® Red Hydrogen Peroxide/Peroxidase Assay Kit, Life technologies). After 20 minutes, the absorbance was measured at 560 nm (Powerwave XS Microplate Spectrophotometer, BioTek Instruments, Winooski, USA) according to the manufacturer's instructions in order to determine the peroxidase activity of serum, hemin, hemoglobin and peroxidase.

Simplified multi-species planktonic and biofilm experiments. Similar to the dual-species experiments, overnight cultures of six commensal bacteria (*S. oralis, S. gordonii, S. cristatus, S. parasanguinis, S. mitis* and *S. sanguinis*), with inhibitory effects by producing *H*₂*O₂*, were centrifuged, re-suspended in BHI-2 broth (1 × 10⁸ CFU/mL). Equal volumes of these solutions were mixed and inoculated in 6 wells of a 24 well plate and incubated under aerobic conditions. After 24 hours, BHI-2, serum, hemoglobin, hemin and peroxidase were added to the wells as described above. Additionally, 1 mL of an overnight co-culture of 3 pathobionts (*A. actinomycetemcomitans, P. gingivalis* and *P. intermedia*) was centrifuged (1438 × g, 10 minutes), re-suspended in BHI-2 (1 × 10⁸ CFU/ml) and added to the wells. The latter co-culture was obtained from overnight cultures of the pathobionts (*simplified ecology*) which were centrifuged (1438 × g, 10 minutes) and re-suspended in 10 mL of BHI-2. 1 mL of each pathobiont culture was added to 7 mL of BHI-2 and incubated for 24 hours under anaerobic conditions to obtain the co-culture. The wells were incubated for 24 hours under anaerobic conditions where after planktonic bacteria and the biofilms analyzed as described above.

Complex multi-species planktonic and biofilm experiments. The experimental set-up was identical to the set-up used for the simplified multi-species experiments with the exception that instead of using an overnight co-culture of 3 pathobionts, a bioreactor derived complex multi-species co-culture of 14 species (*complex ecology*), as described below, was used. The wells were incubated for 24 hours under anaerobic conditions where after planktonic bacteria and the biofilms analyzed as described above.

Bioreactor derived multi-species community. A multi-species community was established in a BIOSTAT B TWIN (Sartorius, Germany) bioreactor. 750 mL of BHI-2 broth was added to the vessel together with 5.0 mg/mL hemin, 1.0 mg/mL menadione and 200 μL/L Antifoam Y-30 (Sigma, St. Louis, USA). The medium was pre-reduced over 24 hours at 37°C by bubbling 100% N₂ and 5% CO₂ in the medium under continuous stirring at 300 rpm. pH was set at 6.7 +/− 0.1. After 24 hours, overnight cultures of *S. sanguinis*, *S. gordonii*, *S. salivarius*, *S. mitis*, *S. oralis*, *S. mutans*, *S. sobrinus*, *A. viscosus*, *A. naeslundii*, *P. intermedia*, *P. gingivalis*, *E. nucleatum*, *A. actinomycetemcomitans* and *V. parvula* were adjusted to an OD of 1.4 and added to the bioreactor. During the first 48 hours, the medium was not replaced. After that, the medium was replaced at a rate of 200 mL/24 hours.

Vitality q-PCR. DNA extraction and vitality q-PCR using propidium monoazide was previously described⁹. Table 3 shows primer and probe sequences used in this study.

Statistical analysis. All experiments were repeated on 3 different days. To account for the censored character of the inhibition data from agar plates experiments, differences between treatments (with serum, hemoglobin, hemin or peroxidase) and control (without addition of blood compound or peroxidase) for the inhibition data were analyzed by means of a survival regression model for gaussian data. Comparisons between treatments and control were made for each combination of substance (blood compounds or peroxidase) and bacteria and corrected for simultaneous hypothesis testing according to Sidak. For planktonic and biofilm data, a linear mixed
model was fit to model the log-transformed CFU counts using substrate (blood compounds or peroxidase) and sample type (planktonic or biofilm) as fixed factors and run as random factor. Since a residual analysis showed that the model was heteroscedastic, weights, proportional to the inverse of the predicted value, were applied. Comparisons with BHI and control were made separately by calculating the appropriate contrasts and a correction for simultaneous hypothesis testing according to Dunnett was applied. Data were analyzed using S-plus 8.0 for Linux (Tibco, Palo Alto, CA, USA).

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| STRAIN                                      | Primer/Probe (5’/3’)                  | Fragment length |
|---------------------------------------------|--------------------------------------|-----------------|
| *Aggregatibacter actinomycetemcomitans*     | Forward GAA CCT CTA CTC TTG ACA TCC GAA | 80 bp           |
|                                             | Reverse AGA ACT AGA TGG GTT TGT GCC TTA CGG |                |
|                                             | Probe CAC TGA ACT CAA GCC CGG CAG TTT CAA |                |
| *Fusobacterium nucleatum*                   | Forward GGC CTC AAC GTT CAG CC        | 162 bp          |
|                                             | Reverse GGC ATT CCT ACA AAT ATC TAC GAA |                |
|                                             | Probe CTC TAC ACT TGT AGT TCC G       |                |
| *Porphyromonas gingivalis*                  | Forward CAC GAA TCC CGC CTG C         | 68 bp           |
|                                             | Reverse CAC TGA ACT CAA GCC CGG CAG TTT CAA |       |
|                                             | Probe CAC TGA ACT CAA GCC CGG CAG TTT CAA |       |
| *Prevotella intermedia*                     | Forward CAC CAT GAA TCC CGC ATG CA    | 99 bp           |
|                                             | Reverse TGG GGG ACT TGA GTG CAC GC     |                |
| *Streptococcus mutans*                      | Forward GCC ATAG TCAA CAC TCA TGA ATT GA | 114 bp         |
|                                             | Reverse TGG AAA TGA CGG TCG CCG TTA TGA A |       |
| *Streptococcus sobrinus*                    | Forward TTC AAA GGC AAG ACC AAG GTA TGT | 88 bp           |
|                                             | Reverse GCC CGT TGA TAT GCT GTG TGT C |                |
|                                             | Probe CTT GCA GCC CTT GGC TCA CAG CGG TCA CAG |       |
| *Actinomyces naeslundii*                    | Forward TGC AAA CTC AGC AAG TAG CCG   | 96 bp           |
|                                             | Reverse AGA GGGA CGG CAA AAG ACA GC  |                |
| *Streptococcus gordonii*                    | Forward TGT AAG GAG CCA CCG GTG TCG TCG TCG TCG | 177 bp       |
|                                             | Reverse GTT AGC TGT TGG ATT GTG TGC C |                |
|                                             | Probe AGA ACA GGC CGT TCA GAG CAA |                |
| *Actinomyces viscosus*                      | Forward GTG AAG GAG CCA CCG GTG TCG TCG TCG | 155 bp       |
|                                             | Reverse GCC AAC AAA CTC TCC CCA GGC |                |
|                                             | Probe ATG AGT GGC GAA GTG AGT AAG AC |                |
| *Streptococcus salivarius*                  | Forward ACC GTT GAC CTT ACG GTA GC    | 192 bp          |
|                                             | Reverse ACC GTA ACG TGG GAA AAG ATG C |                |
|                                             | Probe GTA GCG TCA GAG TCG TTG AC     |                |
| *Streptococcus oralis*                      | Forward ACC AGA AGC GAA AGA AGA AT   | 229 bp          |
|                                             | Reverse ACG TTC GGG CAA GGG ATC TTT CT |                |
|                                             | Probe AAG GCT GGT GTG GCT GAA GAA GT |                |
| *Streptococcus mitis*                       | Forward GGC TCG TAG TCT GGA GAT G    | 133 bp          |
|                                             | Reverse TAG TGC GTC GTC CCA AGG AA   |                |
|                                             | Probe CGA AGA GCA CCA ATA GCA CCT CCC |                |
| *Streptococcus sanguinis*                   | Forward CCA AAT TGT TGC AAA TCC AAA GG | 75 bp           |
|                                             | Reverse GCT ATC CCT CCC TGT CTT TGA |                |
|                                             | Probe AAA GGA AGA TGG CTG CTC GAC AGA ACC CG |       |
| *Veillonella parvula*                       | Forward GAC GAA AGT CTG ACG GAG CA   | 171 bp          |
|                                             | Reverse TGC CAC CTA GGT ATT ACC GC   |                |
|                                             | Probe AGC TCT GTT AAT CGG GAC GAA AGG C |                |

Table 3. Primers and probes used for the detection and quantification by vitality qPCR.
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Conceptualization, E.R.H., K.B., N.B. and W.T.; Methodology, E.R.H., E.H.S. N.B. and W.T.; Analysis, E.R.H. and W.T.; Investigation, E.R.H. and V.S.; Writing—Original Draft, E.R.H.; Writing—Review & Editing V.S., K.B., N.B. and W.T.; Funding Acquisition, W.T. and M.Q.; Resources, W.T., M.Q. and N.B.; Supervision, W.T., N.B. and K.B.

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