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# Clinical concentrations of peroxidases cause dysbiosis in in vitro oral biofilms

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**Background and Objective**: Little is known about the initiation of dysbiosis in oral biofilms, a topic of prime importance for understanding the etiology of, and preventing, periodontitis. The aim of this study was to evaluate the effect of different concentrations of crevicular and salivary peroxidase and catalase on dysbiosis in multispecies biofilms in vitro.

**Material and Methods**: The spotting technique was used to identify the effect of different concentrations of myeloperoxidase, lactoperoxidase, erythrocyte catalase, and horseradish peroxidase in salivary and crevicular fluid on the inhibitory effect of commensals on pathobiont growth. Vitality–quantitative real-time PCR was performed to quantify the dysbiotic effect of the peroxidases (adjusted to concentrations found in periodontal health, gingivitis, and periodontitis) on multispecies microbial communities.

**Results**: Agar plate and multispecies ecology experiments showed that production of hydrogen peroxide ($H_2O_2$) by commensal bacteria decreases pathobiont growth and colonization. Peroxidases at concentrations found in crevicular fluid and saliva neutralized this inhibitory effect. In multispecies communities, myeloperoxidase, at the crevicular fluid concentrations found in periodontitis, resulted in a 1-3 Log increase in pathobionts when compared with the crevicular fluid concentrations found in periodontal health. The effect of salivary lactoperoxidase and salivary myeloperoxidase concentrations was, in general, similar to the effect of crevicular myeloperoxidase concentrations.

**Conclusions**: Commensal species suppress pathobionts by producing $H_2O_2$. Catalase and peroxidases, at clinically relevant concentrations, can neutralize this effect and thereby can contribute to dysbiosis by allowing the outgrowth of pathobionts.

KEYWORDS
gingival crevicular fluid, in vitro model, inflammation, periodontal disease, periodontal pathogens, saliva

## 1 | INTRODUCTION

It is well known that during the progression from periodontal health to disease, the oral microbiota changes.[1,2] These changes, called dysbiosis, are reflected by increased numbers of pathobionts, natural members of the human microbiota that can cause pathology and reduce the proportion of commensal bacteria.[3-5] Pathobionts appear to dysregulate the host inflammatory response, which can lead to chronic inflammation.[3,5] Recently, it was shown that certain species of commensal bacteria suppress

the outgrowth of periodontal pathobionts by producing hydrogen peroxide ($H_2O_2$).[6,7] However, human serum, horseradish peroxidase, and blood components can neutralize this suppressive effect, leading to dysbiosis.[7] The underlying mechanism may be mediated by catalase and peroxidases, enzymes that are generally considered to be protective to the host because they can protect cells from reactive oxygen species (catalase) or produce antibacterial products such as hypothiocyanite (lactoperoxidase) or hypochlorous acid (myeloperoxidase).[8-10] Other studies indicate that myeloperoxidase may be the best biomarker for site-specific diagnosis of periodontitis[11] and that its levels decrease in gingival crevicular fluid after periodontal therapy.[12] Myeloperoxidase is present in saliva and gingival crevicular fluid, but its relationship to the development of dysbiosis has not been examined. Unlike myeloperoxidase, lactoperoxidase is mostly present in saliva, whereas myeloperoxidase is found in polymorphonuclear leukocytes, which migrate into the oral cavity at gingival crevices.[13] Lactoperoxidase should therefore enhance the presence of pathobionts in healthy saliva so that they are available to promote dysbiosis in the sulcus. Catalase is a third peroxidase and it converts $H_2O_2$ to water. It appears in the gingival sulcus of pockets whenever bleeding occurs. Bleeding of the gingiva is provoked by brushing or probing and it indicates gingival inflammation and potential periodontal instability.[14] Erythrocytes present in a sulcus or pocket at sites exhibiting periodontitis are exposed to bacterial hemolysins[15] and such erythrocytes release catalases[16], which are reported to initiate dysbiosis in oral biofilms.[7] In summary, it has been shown that commensal bacteria can suppress the overgrowth of pathobionts by producing $H_2O_2$.[6] High concentrations of catalytic molecules, such as horseradish peroxidase, remove $H_2O_2$ and cause dysbiosis.[7] Current evidence indicates that the concentrations of myeloperoxidase in the gingival sulcus in health, gingivitis, and periodontitis are related to periodontal disease severity.[11,17,18]

The aim of this study was to determine the concentrations of peroxidase in saliva or gingival crevicular fluid, when oral hygiene is insufficient (as in gingivitis or periodontitis), that are able to remove $H_2O_2$ and thus contribute to dysbiosis in multispecies biofilms in vitro.

As Leppilahti and coworkers only stated the range of myeloperoxidase concentrations used in their study,[11] the authors were contacted and asked to provide the average concentrations of myeloperoxidase and catalase used. Lactoperoxidase and myeloperoxidase were also used at concentrations found in saliva.[13] The bacteria selected for use in this study were chosen by screening the scientific literature for oral commensal bacteria with a high prevalence and association with periodontal health, a low prevalence in periodontal disease, and/or a known inhibitory activity against oral pathogens (*Streptococcus sanguinis*, *Streptococcus cristatus*, *Streptococcus oralis*, *Streptococcus parasanguinis*, *Streptococcus gordonii*, *Streptococcus mitis*, *Streptococcus salivarius*, *Actinomyces viscosus*, *Actinomyces naeslundii*, and *Veillonella parvula*).[1,2,6] By contrast, *Fusobacterium nucleatum*, *Porphyromonas gingivalis*, *Aggregatibacter actinomycetemcomitans*, and *Prevotella intermedia* are well known for their involvement and prevalence in periodontal diseases,[1,2,6] and *Streptococcus mutans* and *Streptococcus sobrinus* are known for their involvement in tooth decay.[19,20]

## 2 | MATERIAL AND METHODS

### 2.1 | Bacteria and media

The following bacteria were used as commensal species in this study: *S. sanguinis* LM14657, *S. cristatus* ATCC 49999, *S. gordonii* ATCC 49818, *S. parasanguinis* DSM 6778, *S. mitis* DSM 12643, *S. oralis* DSM 20627, *S. salivarius* TOVE-R, *A. viscosus* DSM 43327, *A. naeslundii* ATCC 51655, and *V. parvula* DSM 2008. *Streptococcus mutans* ATCC 20523, *S. sobrinus* ATCC 20742, *P. intermedia* ATCC 25611, *P. gingivalis* ATCC 33277, *F. nucleatum* ATCC 20482, and *A. actinomycetemcomitans* ATCC 43718 were used as pathobionts. All bacteria were maintained on blood agar (Oxoid, Basingstoke, UK) supplemented with 5 mg/mL of hemin (Sigma, St Louis, MO, USA), 1 mg/mL of menadione (Calbiochem-Novabiochem, La Jolla, CA, USA) and 5% sterile horse blood (E&O Laboratories, Bonnybridge, UK). Overnight liquid cultures were prepared in Brain-Heart Infusion (BHI) broth (Difco, Detroit, MI, USA). Competitive inhibition experiments were performed in Brain Heart Infusion-2 (BHI-2) broth or agar containing Brain Heart Infusion medium (Difco) supplemented with 2.5 g/L of mucin (Sigma-Aldrich, St Louis, MO, USA), 1.0 g/L of yeast extract (Oxoid), 0.1 g/L of cysteine (Calbiochem, San Diego, CA, USA), 2.0 g/L of sodium bicarbonate and 0.25% (v/v) glutamic acid (Sigma-Aldrich). The bacteria were cultured under aerobic (5% $CO_2$) or anaerobic (80% $N_2$, 10% $H_2$, and 10% $CO_2$) conditions. Optical densities (ODs) were measured and adjusted using spectrophotometry at an OD of 600nm (GeneQuant 100 Spectrophotometer; GE Healthcare, Bucks, UK). To verify the effect of myeloperoxidase, lactoperoxidase, catalase, and horseradish peroxidase on the antimicrobial effect of commensal bacteria, the effect of these enzymes on the growth of the pathobionts on agar plates and in complex 14-species communities was evaluated.

### 2.2 | Peroxidases

Myeloperoxidase from human leukocytes (Sigma-Aldrich) was dissolved in BHI-2 at concentrations of 8.91 ng/mL, 53.12 ng/mL, and 1.18 μg/mL, corresponding to the clinical concentrations of myeloperoxidase found in human gingival crevicular fluid from periodontally healthy patients, patients with gingivitis, and patients with periodontitis, respectively.[11] Catalase (from human erythrocytes) and horseradish peroxidase (Sigma-Aldrich) were also dissolved in BHI-2 at the same concentrations as myeloperoxidase.[11] Lactoperoxidase (Sigma-Aldrich) from bovine milk (equal in activity to salivary peroxidase) and myeloperoxidase were adjusted to the salivary concentrations of 1.90 μg/mL and 3.60 μg/mL, respectively, in BHI-2.[13]

### 2.3 | Inhibitory experiments on agar plates

In brief, 7 μL of an overnight culture of a commensal species was spotted onto an agar plate and incubated for 24 hours in aerobic conditions, as described by Herrero et al.[6,7] Afterwards, 7 μL of the

peroxidase solution was spotted next to spots of the commensal species and left to dry for 5 minutes at 37°C under aerobic conditions. Afterwards, 7 μL of an overnight pathobiont culture was spotted on top of the peroxidase spots and at the opposite side of the commensal spot, as a control. Afterwards, the agar plates were incubated under anaerobic conditions (Figure S1). After 48 hours of anaerobic incubation, a calibrated photograph was taken of the agar plate. The magnitude of inhibition was measured from the edge of the commensal colony to the border of the inhibited pathogen colony using ImageJ (http://rsb.info.nih.gov/ij/download.html) (Figure S1).[6,7]

## 2.4 | Biofilm experiments

Overnight cultures of 6 $H_2O_2$-producing species of commensal bacteria (*S. oralis*, *S. gordonii*, *S. cristatus*, *S. parasanguinis*, *S. mitis*, and *S. sanguinis*),[6,7] were centrifuged and each pellet was resuspended in BHI-2 broth (adjusted to an $OD_{600}$ 0.5 representing $1 \times 10^8$ colony-forming units [CFUs]/mL). Equal volumes of these solutions were mixed together and 1 mL of the mix was inoculated into each of 6 wells of a 24-well plate which was then incubated under aerobic conditions. After 24 hours, 500 μL of BHI-2 was added to 1 well, myeloperoxidase, catalase, and horseradish peroxidase, each adjusted to a crevicular fluid concentration of 8.91 ng/mL (periodontally healthy), 53.12 ng/mL (gingivitis), and 1.18 μg/mL (periodontitis), were added to a further 3 wells, myeloperoxidase adjusted to a salivary concentration of 3.60 μg/mL was added to 1 well, and lactoperoxidase adjusted to a salivary concentration of 1.90 μg/mL, was added to 1 well. Additionally, 1 mL of a bioreactor ($OD_{600}$ 0.5~ $1 \times 10^8$ CFU/mL)-derived complex multispecies co-culture of 14 species (containing, mean[SD], 7.40[0.61] $Log_{10}$ genome equivalents [Geq]/mL of *A. actinomycetemcomitans*, 8.09[1.47] $Log_{10}$ Geq/mL of *F. nucleatum*, 8.08[0.26] $Log_{10}$ Geq/mL of *P. gingivalis*, 7.01[0.23] $Log_{10}$ Geq/mL of *P. intermedia*, 6.62[0.92] $Log_{10}$ Geq/mL of *S. mutans*, 7.21[0.52] $Log_{10}$ Geq/mL of *S. sobrinus*, 6.54[0.31] $Log_{10}$ Geq/mL of *A. naeslundii*, 7.45[0.23] $Log_{10}$ Geq/mL of *S. gordonii*, 6.43[0.20] $Log_{10}$ Geq/mL of *A. viscosus*, 5.72[1.05] $Log_{10}$ Geq/mL of *S. salivarius*, 5.16[0.97] $Log_{10}$ Geq/mL of *S. mitis*, 6.80[0.40] $Log_{10}$ Geq/mL of *S. sanguinis*, 5.75[0.45] $Log_{10}$ Geq/mL of *S. oralis*, and 8.74[0.49] $Log_{10}$ Geq/mL of *V. parvula*) was centrifuged (1438 *g*, 10 minutes), resuspended in BHI-2 (adjusted to an $OD_{600}$ 0.5 representing $1 \times 10^8$ CFU/mL), and added to each well. After 24 hours of anaerobic incubation, 1 mL was removed from each well and analyzed using vitality qPCR. Afterwards, the remaining supernatant was removed and the biofilms at the bottom of the wells were washed with phosphate-buffered saline. The biofilms were detached by incubation with 500 μL of 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 resuspended in 1 mL of phosphate-buffered saline and analyzed using vitality qPCR.

## 2.5 | Bioreactor-derived multispecies community

A multispecies community was established in a BIOSTAT B TWIN (Sartorius, Göttingen, Germany) bioreactor. In brief, 750 mL of BHI-2 broth was added to the vessel together with 5.0 mg/mL of hemin, 1.0 mg/mL of menadione, and 200 μL/L of Antifoam Y-30 (Sigma-Aldrich). The medium was pre-reduced over 24 hours at 37°C by bubbling 100% $N_2$ and 5% $CO_2$ through the medium under continuous stirring at 300 rpm. The pH was set to pH 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*, *F. nucleatum*, *A. actinomycetemcomitans*, and *V. parvula* were adjusted to an OD of 1.4 and 750μl of each culture was added to the bioreactor. During the first 48 hours, the medium was not replaced. Thereafter, the medium was replaced at a rate of 200 mL/24 hours.

## 2.6 | Vitality qPCR

DNA extraction and vitality qPCR with propidium monoazide (Biotium Inc., Hayward, CA, USA) was performed.[21] Propidium monoazide was dissolved in 20% dimethylsulfoxide to produce stock concentrations of 1 mg/mL. These were stored at −20°C in the dark. Ten microlitres of propidium monoazide stock solution was added to 90-μL culture aliquots to achieve a final concentration of propidium monoazide of 100 μg/mL. Following a 5-min incubation in the dark, samples were exposed for 10 minutes to a 650 W halogen light source placed 20 cm above the samples. The samples were kept on ice during this period. DNA was extracted from bacterial samples using a QIAamp DNA Mini kit (Qiagen Ltd., Hilden, Germany) in accordance with the manufacturer's instructions. A qPCR assay was performed using a CFX96 Real-Time System (BioRad, Hercules, CA, USA). The TaqMan 5′ nuclease assay PCR method was used for detection and quantification of bacterial DNA. Primer and probe sequences are as described in Table S1.[7] TaqMan reactions contained 12.5 μL of Mastermix (Eurogentec, Seraing, Belgium), 4.5 μL of sterile $H_2O$, 1 μL of each primer and probe, and 5 μL of template DNA. Primers and probes were used at different concentrations depending on the organism. Assay conditions for all primer/probe sets consisted of an initial 2 minutes at 50°C, denaturation for 10 minutes at 95°C, followed by 45 cycles of 95°C for 15 seconds and 60°C for 60 seconds. The bacterial counts are expressed as Geq/mL because the concentration was calculated based on plasmid standard curves.[21]

## 2.7 | Statistical analysis

The data from agar-plate inhibition experiments were compared for each combination of compound, species, and pathogen. For each combination of compound, species, and pathogen, a comparison was made between inhibition in the presence of the compound and inhibition in the absence of the compound. As such, the analysis existed, each time, of a comparison between two groups (presence and absence of the compound). Five different types of inhibition data existed:

I one group showed no growth and the other group showed no inhibition. No statistical analysis was performed.
II one group showed no growth and the other group showed some growth. A one-sided *t*-test was applied to test whether the group

with some growth was significantly different from the group with no growth.

III one group showed no inhibition at all and the other group showed some growth. A one-sided t-test was applied to test whether the group with some growth was significantly lower than the distance for no inhibition at all.

IV both groups showed some inhibition. A paired *t*-test was applied to compare the groups.

V both groups showed some inhibition and at least one observation showed no inhibition at all.

A frailty model, with run as random factor, was fit to compare the groups and to take into account the censored character of the data that reflected no inhibition at all. *P*-values were corrected for simultaneous hypothesis testing according to Šidák in order to obtain a global significance level of 95% for all the comparisons made for a specific compound.

For every combination of enzyme, sample type, and species from planktonic and biofilm data, a linear mixed model was fit with compound as fixed factor and the log-transformed CFU count as dependent variable. The run was modeled as a random factor. A weighing inversely proportional to the observed variance of each compound was applied. A normal quantile plot showed that the data were normally distributed. Compounds were compared with the control and a correction for simultaneous hypothesis testing was applied according to Dunnett when one control was used and according to Šidák when two controls were used.

## 3 | RESULTS

### 3.1 | Decrease of the inhibitory effect of commensals on agar plates

All three peroxidases lowered the inhibitory effects of most of the commensal bacteria on most of the pathobionts but this was more

**TABLE 1** Effect of the addition of myeloperoxidase (MPO), lactoperoxidase (LPO), catalase, and horseradish peroxidase (HRP) to BHI-2 agar on the antagonistic activity of commensal species of bacteria

| | MPO | | LPO | | Catalase | | HRP | |
|---|---|---|---|---|---|---|---|---|
| **Bacterial species** | **−** | **+** | **−** | **+** | **−** | **+** | **−** | **+** |
| *S. oralis* | | | | | | | | |
| Aa | 1.85 ± 0.03 | 1.64 ± 0.02 | 1.52 ± 0.01 | 0.00 ± 0.00* | 1.56 ± 0.02 | 0.00 ± 0.00* | 3.12 ± 0.03 | 0.53 ± 0.07* |
| Pi | TI | 1.19 ± 0.02* | TI | 0.00 ± 0.00* | TI | 0.00 ± 0.00* | TI | 0.00 ± 0.00* |
| Pg | 4.54 ± 0.34 | 2.14 ± 0.18* | TI | 0.00 ± 0.00* | 2.36 ± 0.06 | 0.00 ± 0.00* | 2.39 ± 0.10 | 0.00 ± 0.00* |
| *S. sanguinis* | | | | | | | | |
| Aa | 2.79 ± 0.04 | 2.57 ± 0.04 | 2.05 ± 0.14 | 0.53 ± 0.04 | 1.51 ± 0.07 | 0.00 ± 0.00* | 2.74 ± 0.10 | 0.97 ± 0.12* |
| Pi | TI | 2.10 ± 0.12* | TI | 0.00 ± 0.00* | TI | 0.00 ± 0.00* | TI | 0.00 ± 0.00* |
| Pg | 2.69 ± 0.07 | 1.30 ± 0.03* | 2.44 ± 0.21 | 0.00 ± 0.00* | 3.27 ± 0.06 | 0.00 ± 0.00* | 2.99 ± 0.08 | 0.00 ± 0.00* |
| *S. parasanguinis* | | | | | | | | |
| Aa | 2.11 ± 0.04 | 1.36 ± 0.08 | 1.53 ± 0.06 | 0.64 ± 0.05* | 1.10 ± 0.03 | 0.00 ± 0.00* | 2.06 ± 0.20 | 0.55 ± 0.18* |
| Pi | TI | 0.52 ± 0.05* | TI | 0.00 ± 0.00* | TI | 0.00 ± 0.00* | TI | 0.00 ± 0.00* |
| Pg | 1.92 ± 0.05 | 1.52 ± 0.01 | 1.81 ± 0.20 | 0.00 ± 0.00* | 1.94 ± 0.09 | 0.00 ± 0.00* | 1.76 ± 0.05 | 0.00 ± 0.00* |
| *S. mitis* | | | | | | | | |
| Aa | TI | 5.00 ± 0.07* | 4.35 ± 0.28 | 1.08 ± 0.13* | TI | 0.19 ± 0.04* | TI | 1.82 ± 0.03* |
| Pi | TI | TI | TI | 0.95 ± 0.08* | TI | 0.00 ± 0.00* | TI | 0.78 ± 0.10* |
| Pg | TI | 4.23 ± 0.08 | TI | 0.00 ± 0.00* | TI | 0.00 ± 0.00* | TI | 0.00 ± 0.00* |
| *S. gordonii* | | | | | | | | |
| Aa | TI | 4.36 ± 0.05* | 5.30 ± 0.15 | 1.63 ± 0.04* | 1.78 ± 0.06 | 0.00 ± 0.00* | 5.32 ± 0.38 | 1.69 ± 0.05* |
| Pi | TI | 3.81 ± 0.07* | TI | 0.38 ± 0.07* | TI | 0.00 ± 0.00* | TI | 0.00 ± 0.00* |
| Pg | TI | 3.88 ± 0.08* | 3.31 ± 0.22 | 0.00 ± 0.00* | 3.90 ± 0.12 | 0.00 ± 0.00* | 4.22 ± 0.23 | 0.00 ± 0.00* |
| *S. cristatus* | | | | | | | | |
| Aa | 3.16 ± 0.05 | 2.82 ± 0.02 | 2.53 ± 0.05 | 0.72 ± 0.01* | 1.32 ± 0.03 | 0.00 ± 0.00* | 2.65 ± 0.11 | 0.63 ± 0.07* |
| Pi | TI | 2.67 ± 0.07* | TI | 0.00 ± 0.00* | TI | 0.00 ± 0.00* | TI | 0.00 ± 0.00* |
| Pg | 3.44 ± 0.24 | 1.99 ± 0.52 | 3.42 ± 0.11 | 2.66 ± 0.06 | 2.20 ± 0.02 | 0.00 ± 0.00* | 2.23 ± 0.26 | 0.00 ± 0.00* |

Data are given as mean ± SD (n = 3) and represent the zone of inhibition, in mm, against *A. actinomycetemcomitans* (Aa), *P. intermedia* (Pi), and *P. gingivalis* (Pg).

*Statistically significant decrease of the inhibitory effect of the commensal bacteria by MPO, LPO, catalase, and HRP ($P < .05$).

−, without addition; +, with addition; 0.00, no inhibition; TI, total inhibition.

pronounced for *P. intermedia* and *P. gingivalis* than for *A. actinomycemcomitans* (Table 1 and Figure S1).

### 3.2 | Outgrowth of pathobionts in multispecies ecologies exposed to the different concentrations of peroxidase found in crevicular fluid

The reduction, observed above, of the inhibitory effect of commensal bacteria by myeloperoxidase, catalase, and horseradish peroxidase at concentrations found in the crevicular fluid of patients with periodontitis was verified in complex multispecies ecologies. The concentrations of myeloperoxidase, catalase, and horseradish peroxidase used in these experiments were 8.91 ng/mL, 53.12 ng/mL, and 1.18 μg/mL, representing gingival health, gingivitis, and periodontitis, respectively.[11]

The commensal biofilm significantly inhibited the planktonic and biofilm concentrations of *P. gingivalis* and *P. intermedia* in a complex multispecies ecology model composed of 14 oral species (Figure 1). The inhibitory effect on *A. actinomycetemcomitans* and *F. nucleatum* concentrations was limited, without reaching statistical significance (Table 2, Table S2 and S3). In general, myeloperoxidase, catalase, and horseradish peroxidase lowered the inhibitory effect of the commensal biofilm on planktonic and biofilm concentrations of the periopathobionts. A positive, dose-dependent neutralization effect of myeloperoxidase, catalase, and horseradish peroxidase on the inhibitory effect of commensal bacteria was observed (Figure 1). The concentrations of myeloperoxidase, catalase, and horseradish peroxidase found in crevicular fluid of patients with periodontitis showed the strongest neutralizing effect on the inhibitory effect of commensal bacteria. At concentrations found in gingivitis or in periodontal health, these enzymes had weaker neutralizing effects (Figure 1, Table 2, Table S2 and S3).

The crevicular fluid concentrations of myeloperoxidase, catalase, and horseradish peroxidase found in patients with periodontitis resulted, respectively, in an outgrowth of *P. gingivalis* of, on average, 1.31(0.28), 3.25(0.07), and 2.89(0.43) $Log_{10}$ Geq/mL in complex planktonic multispecies ecologies (Figure 1A) and of 1.44(0.02), 2.89(0.27), and 3.76(0.01) $Log_{10}$ Geq/mL in complex multispecies biofilms (Figure 1B), all values given as mean(SD). For *P. intermedia*, the outgrowth was, on average, 2.84(0.24), 2.97(0.04), and 2.92(0.01) $Log_{10}$ Geq/mL in planktonic multispecies ecologies (Figure 1C) and 3.16(0.19), 3.23(0.05), and 3.14(0.18) $Log_{10}$ Geq/mL in complex multispecies biofilms (Figure 1D). The outgrowth was pronounced most when the enzymes were used at concentrations found in periodontitis. However, there was a clear concentration-dependent effect observed when using enzyme concentrations found in periodontal health vs enzyme concentrations found in gingivitis or vs enzyme concentrations found in periodontitis (Figure 1).

The outgrowth of *P. gingivalis* and *P. intermedia* was also significantly higher when enzyme concentrations found in periodontitis were compared with enzyme concentrations found in periodontal health (Figure 1). The difference in myeloperoxidase concentration between periodontal health and periodontitis resulted in an

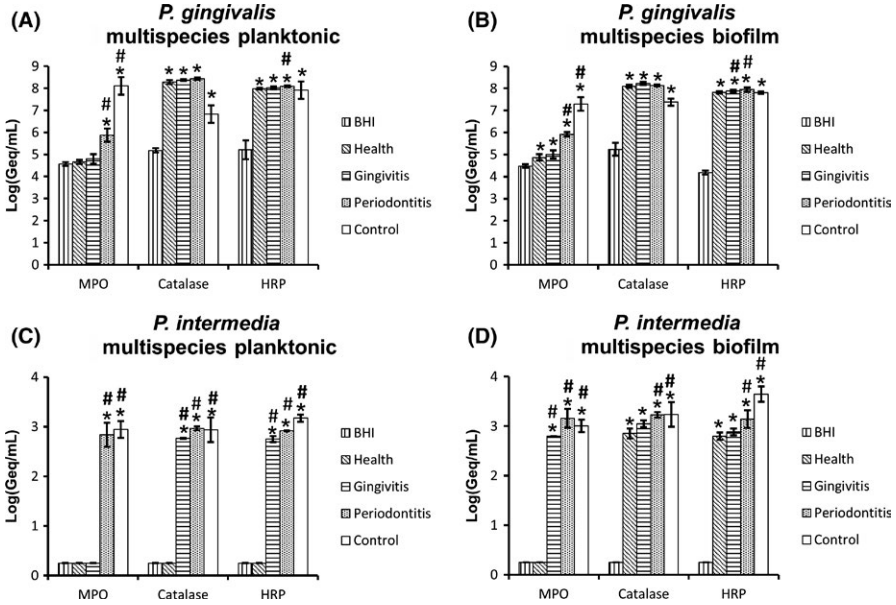


**FIGURE 1** Neutralization effect of myeloperoxidase (MPO), catalase (CT), and horseradish peroxidase (HRP) on complex multispecies biofilms. Control refers to a 14-species community without addition of the 6 species of commensal bacteria and BHI refers to an experimental condition without peroxidases. (A, B) Concentration of *Porphyromonas gingivalis* (Pg) after exposure to MPO, CT, and HRP, in planktonic and biofilm conditions. (C, D) Concentration of *Prevotella intermedia* (Pi) after exposure to MPO, CT, and HRP, in planktonic and biofilm conditions. Data are expressed as mean ± SD (n = 3) $Log_{10}$ genome equivalents (Geq)/mL. *Statistically significant difference of the bacterial concentration in comparison with BHI ($P < .05$). #Statistically significant difference of the bacterial concentration in comparison with that in periodontal health

outgrowth of *P. gingivalis* of 1.21(0.33) $Log_{10}$ Geq/mL in planktonic conditions and of 1.05(0.09) $Log_{10}$ Geq/mL in biofilms (Figure 1A,B). The outgrowth of *P. intermedia* was 2.84(0.24) $Log_{10}$ Geq/mL in planktonic cultures and 3.16(0.19) $Log_{10}$ Geq/mL in biofilms (Figure 1C,D) when comparing enzyme concentrations found in periodontal health with enzyme concentrations found in periodontitis. For catalase and horseradish peroxidase, no concentration-dependent effect was observed. Catalase and horseradish peroxidase resulted in a *P. gingivalis* outgrowth of 2-3 $Log_{10}$ Geq/mL in multispecies biofilms (Figure 1).

Myeloperoxidase, catalase, and horseradish peroxidase also affected the growth of the other species in the community (Table 2, Tables S2 and S3). The presence of myeloperoxidase at a concentration found in gingivitis or periodontitis resulted in a significant increase in the planktonic growth of *A. actinomycetemcomitans*, *A. viscosus*, *S. sobrinus*, *S. gordonii*, *S. oralis*, *S. salivarius*, or *V. parvula* when compared with the levels of these species found after exposure to myeloperoxidase levels found in periodontal health. In biofilms, the presence of myeloperoxidase at concentrations found in gingivitis or periodontitis significantly increased the growth of *V. parvula*. Conversely, the presence of catalase and horseradish peroxidase at concentrations found in periodontal health significantly reduced the growth of *S. mutans*, *S. oralis*, and *S. gordonii* compared with the BHI control in planktonic ecologies. At concentrations found in gingivitis and periodontitis, catalase significantly decreased the growth of *S. mutans*, *S. sobrinus*, and *A. viscosus* compared with the BHI control in biofilm ecologies. Similarly, the presence of catalase at crevicular fluid concentrations found in health, gingivitis, and periodontitis reduced the numbers of *S. sobrinus* in the biofilms. Additionally, the growth of *S. mutans* was reduced when catalase concentrations found in gingivitis and periodontitis were used, and the growth of *A. viscosus* was reduced when catalase concentrations found in gingivitis were used. The presence of horseradish peroxidase did not decrease the abundance of any of the bacterial species present in the biofilm. Rather, horseradish peroxidase stimulated an increase in the numbers of *S. gordonii* when used at concentrations found in periodontal health and gingivitis and an increase in the numbers of *A. naeslundii* and *A. viscosus* at when used at concentrations found in periodontitis, compared with the BHI control.

### 3.3 | Outgrowth of pathobionts in multispecies ecologies following exposure to the concentrations of lactoperoxidase and myeloperoxidase found in saliva

The effect of salivary concentrations of lactoperoxidase and myeloperoxidase on periopathobionts was investigated in multispecies ecologies (Table 3). Salivary concentrations of lactoperoxidase and myeloperoxidase resulted in outgrowth of *P. gingivalis* of, on average, 1.00(0.23) and 1.14(0.18) $Log_{10}$ Geq/mL, respectively, in planktonic (Figure 2A) and of, on average, 1.29(0.15) and 1.50(0.11) $Log_{10}$ Geq/mL, respectively, in biofilms (Figure 2B). In addition, salivary concentrations of lactoperoxidase and myeloperoxidase also resulted in an outgrowth of *P. intermedia* of 0.95(0.23) and 1.06(0.26) $Log_{10}$ Geq/mL, respectively, in planktonic biofilms (Figure 2A) and of 1.23(0.23) and 1.28(0.11) $Log_{10}$ Geq/mL, respectively, in multispecies biofilms (Figure 2B). In general, the effect of myeloperoxidase was slightly stronger than the effect of lactoperoxidase.

The presence of salivary concentrations of lactoperoxidase and myeloperoxidase resulted also in an effect on the growth of different species of the community (Table 3). The presence of lactoperoxidase reduced significantly the planktonic growth of *A. naeslundii* and *A. viscosus* and the growth of *S. sobrinus* and *S. oralis* in biofilms. The presence of myeloperoxidase stimulated increased growth of *A. actinomycetemcomitans* and *S. mutans* in biofilms.

## 4 | DISCUSSION

One should consider that little is known about the initiation of dysbiosis in periodontal diseases,[3,5] which is obviously of prime importance for understanding its etiology and preventing periodontitis. Recently it has been shown that some commensal bacteria inhibit the outgrowth of pathobionts by $H_2O_2$ production.[6,7] The production of $H_2O_2$ is, in essence, not restricted to commensal, albeit beneficial, bacteria. For instance, *Streptococcus pneumoniae*, a common pathogen of the upper respiratory tract, can inhibit the growth of *Haemophilus influenza* by producing $H_2O_2$.[22] However, $H_2O_2$ production has never been described in periodontopathogens. None of the species used in this study produced $H_2O_2$ except for *S. oralis*, *S. gordonii*, *S. cristatus*, *S. parasanguinis*, *S. mitis*, and *S. sanguinis*.[6,7] Serum, high concentrations of horseradish peroxidase, and certain blood compounds can neutralize the inhibitory effect of $H_2O_2$ in multispecies biofilms, which results in dysbiosis.[7] The data from the current study show that, in vitro, clinically relevant peroxidases and catalase at clinically relevant peroxidases (myeloperoxidase, lactoperoxidase) and catalase, when used at clinically relevant concentrations (i.e. as found in gingivitis and periodontitis) can neutralize the inhibitory effect of commensal bacteria on pathobiont growth on agar plates, in planktonic cultures, and in multispecies biofilms. Moreover, increasing the concentration of myeloperoxidase from that found in gingival crevicular fluid of periodontally healthy patients to that found in patients with periodontitis resulted in a 1-3 $Log_{10}$ Geq/mL increase in pathobionts. These in vitro data might explain the correlation between periodontitis and myeloperoxidase concentrations and suggest that the concentration of myeloperoxidase found in gingival crevicular fluid can contribute to the dysbiosis seen in subgingival plaque of patients with periodontal disease. In concordance with these data, previous studies have shown that the addition of peroxidases could neutralize the inhibitory effect of commensal bacteria on agar plates and multispecies communities.[6,7,23,24] However, the concentrations of peroxidases used in these studies were not clinically relevant. The results indicate that an increase in clinically relevant peroxidase concentrations causes the outgrowth of pathobionts, such as *P. gingivalis*, *A. actinomycetemcomitans*, and *P. intermedia*. The factors that influence the concentrations and activity of myeloperoxidase or lactoperoxidase are not well known. However, *A. actinomycetemcomitans* can increase

**TABLE 2** Concentrations of the different species of bacteria in the 14-species biofilms after exposure to different crevicular fluid concentrations of myeloperoxidase (MPO)

| | Planktonic | | | | | Biofilm | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Bacterial species | Without MPO | MPO Health | MPO Gingivitis | MPO Periodontitis | Control | Without MPO | MPO Health | MPO Gingivitis | MPO Periodontitis | Control |
| *Aa* | 5.85 ± 0.03 | 5.88 ± 0.02 | 5.88 ± 0.01 | 5.94 ± 0.02$^{*\Delta}$ | 5.98 ± 0.02$^{*\Delta}$ | 5.45 ± 0.42 | 5.75 ± 0.07 | 5.88 ± 0.11 | 5.98 ± 0.05$^{\Delta}$ | 6.04 ± 0.08$^{\Delta}$ |
| *Pi* | 0.00 ± 0.00 | 0.00 ± 0.00 | 0.00 ± 0.00 | 2.84 ± 0.24$^{*\Delta}$ | 2.94 ± 0.17$^{*\Delta}$ | 0.00 ± 0.00 | 0.00 ± 0.00 | 2.79 ± 0.01$^{*\Delta}$ | 3.16 ± 0.19$^{*\Delta}$ | 3.01 ± 0.13$^{*\Delta}$ |
| *Pg* | 4.57 ± 0.09 | 4.66 ± 0.09 | 4.81 ± 0.21 | 5.88 ± 0.30$^{*\Delta}$ | 8.11 ± 0.36$^{*\Delta}$ | 4.48 ± 0.08 | 4.87 ± 0.15$^{*}$ | 5.01 ± 0.19$^{*}$ | 5.92 ± 0.10$^{*\Delta}$ | 7.29 ± 0.31$^{*\Delta}$ |
| *Fn* | 8.81 ± 0.09 | 8.79 ± 0.12 | 9.23 ± 0.31 | 9.05 ± 0.15 | 8.99 ± 0.10 | 9.10 ± 0.06 | 9.27 ± 0.09 | 9.31 ± 0.12 | 9.34 ± 0.10$^{*}$ | 9.22 ± 0.13 |
| *A. naeslundii* | 4.92 ± 0.16 | 4.81 ± 0.32 | 4.65 ± 0.15 | 4.74 ± 0.08 | 4.85 ± 0.12 | 7.62 ± 0.13 | 7.46 ± 0.05 | 7.44 ± 0.08 | 7.32 ± 0.09 | 7.41 ± 0.03 |
| *A. viscosus* | 7.71 ± 0.07 | 7.63 ± 0.05$^{\#}$ | 7.75 ± 0.04$^{\Delta}$ | 7.67 ± 0.01 | 7.70 ± 0.15 | 8.23 ± 0.22 | 8.34 ± 0.09 | 8.25 ± 0.19 | 8.22 ± 0.09 | 8.02 ± 0.09 |
| *S. mutans* | 6.77 ± 0.13 | 6.70 ± 0.05 | 6.72 ± 0.01 | 6.79 ± 0.05 | 6.90 ± 0.06$^{\Delta}$ | 8.05 ± 0.10 | 8.06 ± 0.02 | 7.97 ± 0.16 | 7.84 ± 0.05$^{\Omega}$ | 7.77 ± 0.10$^{\Omega}$ |
| *S.sobrinus* | 5.25 ± 0.05 | 5.23 ± 0.04 | 5.33 ± 0.01$^{\Delta}$ | 5.35 ± 0.01$^{*\Delta}$ | 5.64 ± 0.14$^{*\Delta}$ | 6.53 ± 0.19 | 6.56 ± 0.04 | 6.54 ± 0.13 | 6.39 ± 0.08 | 6.53 ± 0.03 |
| *S. sanguinis* | 6.73 ± 0.03 | 6.72 ± 0.05 | 6.78 ± 0.02 | 6.76 ± 0.02 | 6.03 ± 0.15$^{\#\Omega}$ | 8.61 ± 0.14 | 8.73 ± 0.04 | 8.62 ± 0.10 | 8.66 ± 0.03 $^{\Omega}$ | 7.47 ± 0.16$^{\#\Omega}$ |
| *S. gordonii* | 9.50 ± 0.06 | 9.43 ± 0.05 | 9.56 ± 0.03$^{\Delta}$ | 9.59 ± 0.11 | 9.72 ± 0.04$^{*\Delta}$ | 9.96 ± 0.10 | 9.93 ± 0.03 | 9.89 ± 0.06 | 9.88 ± 0.01 | 9.99 ± 0.06 |
| *S. oralis* | 7.62 ± 0.08 | 7.62 ± 0.04 | 7.74 ± 0.02$^{\Delta}$ | 7.66 ± 0.08 | 6.46 ± 0.12$^{\#\Omega}$ | 8.63 ± 0.12 | 8.61 ± 0.01 | 8.51 ± 0.09 | 8.47 ± 0.06 $^{\Omega}$ | 7.59 ± 0.07$^{\#\Omega}$ |
| *S.salivarius* | 4.56 ± 0.36 | 4.22 ± 0.39 | 4.97 ± 0.06$^{\Delta}$ | 4.71 ± 0.005 | 4.40 ± 0.21 | 5.08 ± 0.17 | 5.22 ± 0.18 | 5.16 ± 0.14 | 5.12 ± 0.07 | 4.79 ± 0.08 $^{\Omega}$ |
| *V. parvula* | 7.11 ± 0.12 | 7.19 ± 0.09 | 7.47 ± 0.13$^{*\Delta}$ | 7.74 ± 0.04$^{*\Delta}$ | 7.78 ± 0.13$^{*\Delta}$ | 9.61 ± 0.13 | 9.79 ± 0.13 | 9.84 ± 0.04$^{*}$ | 10.08 ± 0.03$^{*\Delta}$ | 10.17 ± 0.13$^{*\Delta}$ |
| *S. mitis* | 4.65 ± 0.12 | 4.49 ± 0.16 | 4.70 ± 0.04 | 4.55 ± 0.06 | 3.99 ± 0.15$^{\#\Omega}$ | 5.42 ± 0.12 | 5.44 ± 0.05 | 5.38 ± 0.08 | 5.36 ± 0.09 | 3.74 ± 0.17$^{\#\Omega}$ |

Values are given as mean ± SD (n = 3) $Log_{10}$ genome equivalents (Geq)/mL."Control" refers to a 14-species community without the addition of the 6 species of commensal bacteria. "Without MPO" refers to a condition without peroxidases. MPO was adjusted to clinical concentrations found in the crevicular fluid of periodontally healthy patients and of patients with gingivitis and periodontitis.
*and $^{\Delta}$designate a statistically significant increase of the bacterial concentration in respect to "without MPO" and to "MPO health", respectively ($P < .05$). $^{\#}$and $^{\Omega}$designate a statistically significant decrease of the bacterial concentration in respect to "without MPO" and to "MPO health", respectively ($P < .05$).

**TABLE 3** Concentrations of the different species of bacteria in the 14-species biofilms that were exposed to salivary concentrations of lactoperoxidase (LPO) and myeloperoxidase (MPO)

| | Planktonic | | | | Biofilm | | | |
|---|---|---|---|---|---|---|---|---|
| Bacterial species | Without LPO and MPO | LPO Saliva | MPO Saliva | Control | Without LPO and MPO | LPO Saliva | MPO Saliva | Control |
| *Aa* | 7.44 ± 0.11 | 7.46 ± 0.06 | 7.57 ± 0.06 | 7.76 ± 0.07* | 6.61 ± 0.16 | 6.77 ± 0.12 | 6.82 ± 0.08* | 7.61 ± 0.08* |
| *Pi* | 5.35 ± 0.37 | 6.30 ± 0.15* | 6.40 ± 0.16* | 7.19 ± 0.21* | 5.51 ± 0.25 | 6.75 ± 0.10* | 6.79 ± 0.18* | 7.84 ± 0.13* |
| *Pg* | 6.38 ± 0.18 | 7.38 ± 0.09* | 7.51 ± 0.01* | 8.32 ± 0.07* | 6.24 ± 0.10 | 7.52 ± 0.11* | 7.74 ± 0.01* | 8.22 ± 0.11* |
| *Fn* | 8.98 ± 0.10 | 8.89 ± 0.04 | 8.90 ± 0.05 | 8.93 ± 0.16 | 9.01 ± 0.04 | 9.12 ± 0.23 | 9.12 ± 0.16 | 9.12 ± 0.12 |
| *A. naeslundii* | 2.98 ± 0.14 | 2.63 ± 0.07# | 2.91 ± 0.15 | 3.28 ± 0.54 | 6.37 ± 0.15 | 6.31 ± 0.37 | 6.41 ± 0.17 | 6.57 ± 0.21 |
| *A. viscosus* | 7.23 ± 0.02 | 7.10 ± 0.04# | 7.10 ± 0.09 | 7.34 ± 0.11 | 7.98 ± 0.10 | 8.15 ± 0.29 | 8.09 ± 0.04 | 8.21 ± 0.09* |
| *S. mutans* | 6.02 ± 0.03 | 5.93 ± 0.06 | 5.99 ± 0.10 | 6.00 ± 0.18 | 6.56 ± 0.04 | 6.52 ± 0.05 | 6.66 ± 0.06* | 6.62 ± 0.10 |
| *S. sobrinus* | 6.92 ± 0.02 | 6.86 ± 0.05 | 6.82 ± 0.13 | 8.04 ± 0.10* | 7.95 ± 0.11 | 7.67 ± 0.13# | 7.69 ± 0.10 | 9.31 ± 0.11* |
| *S. sanguinis* | 7.06 ± 0.06 | 6.99 ± 0.07 | 7.09 ± 0.08 | 6.62 ± 0.08# | 8.10 ± 0.05 | 8.13 ± 0.09 | 8.20 ± 0.07 | 8.04 ± 0.02# |
| *S. gordonii* | 9.43 ± 0.07 | 9.42 ± 0.11 | 9.47 ± 0.09 | 9.95 ± 0.11* | 10.30 ± 0.09 | 10.43 ± 0.38 | 10.42 ± 0.11 | 10.55 ± 0.14 |
| *S. oralis* | 7.36 ± 0.08 | 7.29 ± 0.06 | 7.23 ± 0.19 | 6.70 ± 0.11# | 8.03 ± 0.07 | 7.87 ± 0.10# | 7.97 ± 0.03 | 7.85 ± 0.24 |
| *S. salivarius* | 3.95 ± 0.47 | 3.99 ± 0.29 | 4.21 ± 0.18 | 5.49 ± 0.16* | 4.60 ± 0.21 | 5.06 ± 0.43 | 4.73 ± 0.08 | 5.15 ± 0.46 |
| *V. parvula* | 7.97 ± 0.01 | 7.96 ± 0.05 | 7.96 ± 0.09 | 8.45 ± 0.04* | 10.06 ± 0.07 | 10.05 ± 0.16 | 10.11 ± 0.03 | 10.11 ± 0.03 |
| *S. mitis* | 4.93 ± 0.23 | 4.86 ± 0.12 | 4.86 ± 0.17 | 5.02 ± 0.13 | 5.45 ± 0.13 | 5.26 ± 0.23 | 5.58 ± 0.11 | 5.13 ± 0.25 |

Values are given as mean ± SD (n = 3) $Log_{10}$ genome equivalents (Geq)/mL. "Control" refers to a 14-species community without addition of the 6 species of commensal bacteria. "Without LPO and MPO" refers to a condition without peroxidases.
*Designates a statistically significant increase of the bacterial concentration in respect to "Without LPO and MPO" ($P < .05$). #Designates a statistically significant decrease of the bacterial concentration in respect to "Without LPO and MPO" ($P < .05$).

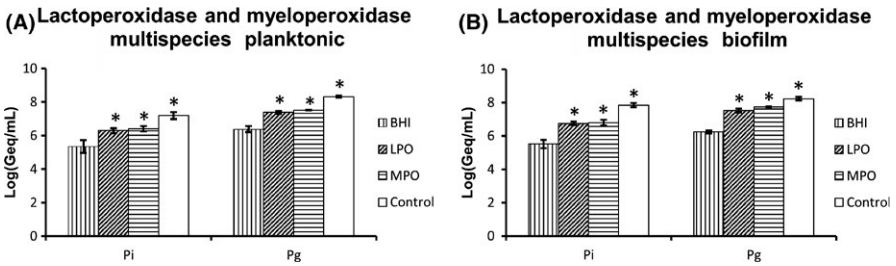


**FIGURE 2** Neutralization effect of myeloperoxidase (MPO) and lactoperoxidase (LPO) adjusted to salivary concentrations on complex multispecies biofilms. Control refers to a 14-species community without the addition of the 6 commensal bacteria, and BHI refers to an experimental condition without peroxidases. (A, B) Concentration of *Porphyromonas gingivalis* (Pg) and *Prevotella intermedia* (Pi) after exposure to lactoperoxidase (LPO) and myeloperoxidase (MPO), in planktonic and biofilm conditions. Data are expressed as mean ± SD (n = 3) $Log_{10}$ genome equivalents (Geq)/mL. *Statistically significant difference of the bacterial concentration in comparison with BHI ($P < .05$)

significantly the release of myeloperoxidase by neutrophils,[25] which may enhance the dysbiosis observed in periodontal diseases. For other pathobionts, such an effect has not yet been investigated. However, in studies of experimental gingivitis, the concentration of myeloperoxidase has been shown to increase after only 4 days.[26,27] Also, in subjects who smoke (smoking is a significant risk factor for periodontitis), elevated levels of myeloperoxidase have been found in saliva and serum, apparently as a result of stimulation of neutrophils by nicotine.[28,29] Genetic influences can also affect the activity and concentration of oral peroxidases. A clinical study showed an association between MPO-463 gene polymorphisms and aggressive periodontitis.[30] The myeloperoxidase activity of individuals with the MPO-463GG variant has been reported to be higher than that of individuals with the MPO-463AA variant. The MPO G/G genotype has been associated with coronary artery disease, leukemia, cancer, multiple sclerosis and higher risk for periodontal disease.[31,32] Interestingly, myeloperoxidase deficiency, a frequently occurring genetic disorder which is related to a reduced quantity or function of myeloperoxidase, is rarely associated with clinical symptoms unless the patient also suffers from diabetes mellitus, which leads to disseminated candidiasis and other fungal infections.[33] Until now, not even a case report has been published on whether the levels of gingivitis or periodontitis are increased in patients with myeloperoxidase deficiency. In this context, it would be interesting to investigate

if patients with myeloperoxidase deficiency are more resistant to gingivitis or periodontitis.

Bleeding periodontal pockets are an indicator for periodontal diseases.[14] Some blood compounds have been shown to induce dysbiosis in multispecies ecologies.[7] Recently, an association between bacterially mediated hemolysis and the clinical parameters of periodontitis has been described.[15] The catalase released from erythrocytes, as a result of the action of bacterial hemolysin, can contribute to the dysbiotic effect.[16,34] Our results show that catalase from erythrocytes decreased the suppressive effect of $H_2O_2$ produced by commensal bacteria, leading to the outgrowth of pathobionts with 2-3 $Log_{10}$ Geq/mL in oral multispecies biofilms. Similar effects have been reported for other blood components with peroxidase activity, such as serum, hemoglobin, and hemin.[7] The neutralization of $H_2O_2$ by serum, hemoglobin, and hemin increased the colonization of *P. gingivalis* by 3 $Log_{10}$ Geq/mL in oral multispecies biofilms but resulted in a higher outgrowth of *P. intermedia* and *A. actinomycetemcomitans* in multispecies biofilms. These differences in outgrowth can be explained by the concentrations of serum, hemoglobin, and hemin used in that study, which were higher than the concentrations used in the present study.

Saliva also harbors different peroxidases, mainly lactoperoxidase and myeloperoxidase.[13] The results of our study showed that salivary concentrations of lactoperoxidase and myeloperoxidase increased the growth of pathobionts by 1-2 $Log_{10}$ Geq/mL in multispecies biofilms. These data are in line with recent clinical studies, which indicated a positive correlation between myeloperoxidase concentrations in saliva and clinical signs of gingival or periodontal diseases.[35,36]

Overall, this study suggests that when dental plaque persists over a long period of time because of poor dental hygiene and/or when the susceptibility of the host changes, peroxidases resulting from the inflammatory response can play an important role in the development of dysbiosis of oral biofilms by disrupting the inhibition of pathobiont growth by commensal bacteria. Plaque accumulation also creates a more anaerobic environment, which limits oxygen availability at the base of the sulcus, subsequently lowering $H_2O_2$ production and thus contributing to the dysbiotic effect. Unfortunately, it is not known when dysbiosis becomes irreversible with respect to $H_2O_2$ production and no studies are available that directly correlate concentrations of oral peroxidase with microbiological data.

One should consider that the 16-species biofilm model does not take into account the host–microbiome interactions. This might be of importance because myeloperoxidase also promotes the formation of neutrophil extracellular traps, which can enhance its antimicrobial activity through different mechanisms.[37] Additionally, myeloperoxidase produced by neutrophils can utilize $H_2O_2$ to generate hypochlorous acid to kill bacteria.[33] The impact of these scenarios was not captured in the current model and therefore needs to be validated in in vivo animal models.

The chronological steps in the initiation of dysbiosis are far from being elucidated. To date, it is not clear if an increase in myeloperoxidase concentration triggers a shift toward a dysbiotic biofilm or if other factors (eg, environmental factors, such as oxygen or pH) initiate the occurrence of dysbiotic biofilms, which in turn induce the release of higher concentrations of myeloperoxidase. At this point the influence and role of these factors in the initiation of dysbiosis remains to be investigated in future studies.

In conclusion, under the given experimental conditions, clinically relevant peroxidases and catalase can contribute to dysbiosis at clinically relevant concentrations in 16-species in vitro oral biofilms.

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## CONFLICT OF INTEREST

The authors have stated explicitly that there are no conflicts of interest in connection with this article.

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## SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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