Effect of lactoperoxidase on the antimicrobial effectiveness of the thiocyanate hydrogen peroxide combination in a quantitative suspension test

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Abstract

Background: The positive antimicrobial effects of increasing concentrations of thiocyanate (SCN-) and H2O2 on the human peroxidase defence system are well known. However, little is known about the quantitative efficacy of the human peroxidase thiocyanate H2O2 system regarding Streptococcus mutans and sanguinis, as well as Candida albicans. The aim of this study was to evaluate the effect of the enzyme lactoperoxidase on the bactericidal and fungicidal effectiveness of a thiocyanate-H2O2 combination above the physiological saliva level. To evaluate the optimal effectiveness curve, the exposure times were restricted to 1, 3, 5, and 15 min.

Results: The bactericidal and fungicidal effects of lactoperoxidase on Streptococcus mutans and sanguinis and Candida albicans were evaluated by using two test mixtures of a 2.0% (w/v; 0.34 M) thiocyanate and 0.4% (w/v; 0.12 M) hydrogen peroxide solution, one without and one with lactoperoxidase. Following the quantitative suspension tests (EN 1040 and EN 1275), the growth of surviving bacteria and fungi in a nutrient broth was measured. The reduction factor in the suspension test without lactoperoxidase enzyme was < 1 for all three tested organisms. Thus, the mixtures of 2.0% (w/v; 0.34 M) thiocyanate and 0.4% (w/v; 0.12 M) hydrogen peroxide had no in vitro antimicrobial effect on Streptococcus mutans and sanguinis or Candida albicans. However, the suspension test with lactoperoxidase showed a high bactericidal and fungicidal effectiveness in vitro.

Conclusion: The tested thiocyanate and H2O2 mixtures showed no relevant antimicrobial effect. However, by adding lactoperoxidase enzyme, the mixtures became not only an effective bactericidal (Streptococcus mutans and sanguinis) but also a fungicidal (Candida albicans) agent.

Background

Maintaining daily oral hygiene is essential to prevent caries, gingivitis, and periodontitis [1-3]. To support mechanical plaque control, which is mostly insufficient [4-6], antiseptics are used in toothpastes and mouth rinses [7-10].
However, the concentrations and frequency of use of anti-
septics are limited to avoid side effects, such as discolora-
tion of teeth and tongue, taste alterations, mutations
[11,12], and, for microbiostatic active agents, the risk of
developing resistance or cross-resistance against antibiot-
ics [13]. Therefore, it would seem better to stimulate or
support the innate host defence system, such as the oral
peroxidase-thiocyanate-hydrogen peroxide system.

Human saliva contains peroxidase enzymes and lys-
ozyme, among other innate host defence systems. The
complete peroxidase system in saliva comprises three
components: the peroxidase enzymes (glycoprotein
enzyme), salivary peroxidase (SPO) from major salivary
glands and myeloperoxidase (MPO) from polymorpho-
nuclear leucocytes filtering into saliva from gingival crev-
icular fluid; hydrogen peroxide (H₂O₂), and an oxidizable
substrate such as the pseudohalide thiocyanate (SCN⁻)
from physiological sources [14,15]. SPO is almost identi-
cal to the milk enzyme lactoperoxidase (LPO) [16,17]. All
these peroxidase enzymes catalyze the oxidation of the
salivary thiocyanate ion (SCN⁻) by hydrogen peroxide
(H₂O₂) to OSCN⁻ and the corresponding acid hypothiocy-
anous acid (HOSCN), O₂SCN⁻, and possibly O₃SCN⁻ [18],
which have been shown to inhibit bacterial [19-23], fun-
gal [24], and viral viability [25]. However, the system is
effective only if its components are sufficiently available
in saliva. Salivary concentration of SCN⁻ varies consider-
ably and depends, for instance, on diet and smoking hab-
bits. The normal range of salivary SCN⁻ for nonsmokers is
from 0.5 to 2 mM (29–116 mg/l), but in smokers [26,27],
the level can be as high as 6 mM (348 mg/l). Pruitt et al.
[28], for example, see the main limiting component for
the production of the oxidation products of SCN⁻ in whole
saliva to be the hydrogen peroxide (H₂O₂) concentration.
Thomas et al. [29] showed that the combination of LPO,
SCN⁻, and 0.3 mM (10.2 mg/l) H₂O₂ caused complete
inhibition that lasted for nearly 16 h, whereas 0.3 mM
(10.2 mg/l) H₂O₂ alone had no effect. However, if no
more H₂O₂ was added, the concentration of the inhibitor
OSCN⁻ fell because of slow decomposition of OSCN⁻; and,
when OSCN⁻ fell below 0.01 mM (0.74 mg/l), the bacteria
resumed metabolism and growth. The loss of OSCN⁻ over
time is based on decomposition, not on the reaction with
bacteria [29].

The typical concentration of peroxidases in whole saliva is
roughly 5 μg/ml, whereas the MPO concentration (3.6 μg/
ml) is approximately twice the amount of SPO (1.9 μg/
ml) [30]. Therefore, even if SPO is deficient, MPO activity
would probably be adequate for SCN⁻ oxidation in mixed
saliva [30]. The study by Adolphe et al. [31] showed that
the lactoperoxidase system's antimicrobial efficiency can
be enhanced by better concentration ratios of the LPO sys-
tem components. However, this finding was postulated
for only near physiological conditions and did not con-
sider a concentration of thiocyanate and H₂O₂ higher
than the physiological one.

Rosin et al. [32] showed that, in the saliva peroxidase sys-
tem, increasing SCN⁻/H₂O₂ above its physiologic saliva
level reduced plaque and gingivitis significantly compared
to baseline values and a placebo. A new dentifrice formu-
lated on these results showed the same effects regarding
plaque and gingivitis prevention in comparison to a
benchmark product containing triclosan [33]. However,
the effects were not sufficient to recommend using the
SPO system to effectively prevent oral diseases in the long
run.

Thus, the question arose, Is it possible to increase antimi-
icrobial effectiveness by adding not just thiocyanate and
hydrogen peroxide but also LPO to oxidize as much the
SCN⁻ anions as possible to become an effective antimicro-
bial agent? Therefore, we conducted a standardized quan-
titative suspension test at a fixed concentration level of all
three components above the physiological one to evaluate
the influence of LPO on the lactoperoxidase-thiocyanate-
hydrogen peroxide system relative to its bactericidal and
fungicidal effectiveness against Streptococcus mutans and sanguinis and Candida albicans.

Results
The reduction factors (RF) of the test suspensions without
and with LPO on the viability of Streptococcus mutans,
Streptococcus sanguinis, and Candida albicans at differ-
ent time points (1, 3, 5, and 15 min) are shown in tables
1, 2 &3. The accompanying suspension tests with single compo-
nents (SCN⁻, LPO) and combinations of two components
(LPO+SCN⁻, LPO+H₂O₂) showed no clinically relevant
effects (RF ≤ 0.3) at all time points. Only the single com-
ponent H₂O₂ showed a reduction factor of 1.5 after 15
min.

Streptococcus mutans
The antibacterial reductions of the thiocyanate-hydrogen
peroxide system without LPO increased with time and
were statistically significantly different between 5 and 15
min. However, they remained at a very low level (RF < 1).
Thus, the suspension without LPO had practically no bac-
tericidal effectiveness. The suspension with LPO showed a
distinct antibacterial reduction (RF 7.49) after 5 min,
which means the complete killing of all cells. Thus, a fur-
ther increase of the reduction factor was not possible. The
comparison between groups A (without LPO) and B (with
LPO) showed a statistically significant difference in favour
of group B after 5 and 15 min (Table 1).
Streptococcus sanguinis

The antibacterial reductions of the thiocyanate-hydrogen peroxide system without LPO increased with time but only to a very low level (RF ≤ 1) with practically no bactericidal effectiveness. The suspension with LPO showed an effective antibacterial reduction after 5 min (RF 4.01 ± 3.88) and after 15 min (RF 8.12 ± 0.22). The RFs between 3 and 5 min were statistically significantly different. The comparison between groups A and B showed a statistically significant difference in favour of B (with LPO) after 15 min (Table 2).

Candida albicans

The antifungal reduction of the thiocyanate-hydrogen peroxide system without LPO (Group A) increased with time but only to a very low level (RF < 1) with practically no fungicidal effectiveness. The suspension with LPO (Group B) showed an effective fungicidal reduction after 3 min (RF 6.78 ± 0.25), which means the complete killing of all microbes. Thus, a further increase of the reduction factor was not possible.

Table 1: Reduction factors of the test thiocyanate hydrogen peroxide microbial suspension without and with LPO to Streptococcus mutans at different time points.

| Time [min] | Group A Without LPO | Group B With LPO | A vs. B² |
|-----------|---------------------|-----------------|---------|
| 1         | 0.23 ± 0.26         | 0.03 ± 0.17     | 0.128   |
| 3         | 0.21 ± 0.36         | 0.53 ± 0.22     | 0.026   |
| 5         | 0.25 ± 0.12         | 7.49 ± 0.64³    | < 0.001 |
| 15        | 0.69 ± 0.43         | 7.41 ± 0.69³    | < 0.001 |

1) Wilcoxon test with a significant level of < 0.05
2) Mann-Whitney U test with a significance level of < 0.001
3) Complete killing of all cells in test suspension

Table 2: Reduction factors of the test thiocyanate hydrogen peroxide microbial suspension without and with LPO to Streptococcus sanguinis at different time points.

| Time [min] | Group A Without LPO | Group B With LPO | A vs. B² |
|-----------|---------------------|-----------------|---------|
| 1         | 0.10 ± 0.90         | 0.13 ± 0.12     | 0.710   |
| 3         | 0.16 ± 0.15         | 0.78 ± 0.67     | 0.073   |
| 5         | 0.27 ± 0.17         | 4.01 ± 3.88³    | 0.073   |
| 15        | 1.03 ± 0.60         | 8.12 ± 0.22³    | < 0.001 |

1) Wilcoxon test with a significant level of < 0.05
2) Mann-Whitney U test with a significance level of < 0.001
3) Complete killing of all cells in test suspension
The RFs between 3 and 5 min were statistically significantly different. The comparison between groups A and B showed a statistically significant difference in favour of B (with LPO) after 3 min (Table 3).

### Discussion

The applied quantitative suspension tests are recognized European norm tests for evaluating bactericidal (EN 1040) and fungicidal efficacy (EN 1275) of a newly developed antiseptic [34,35]. In contrast to common antimicrobial tests (inhibition tests), these quantitative suspension tests facilitate, for example, the strict distinctions between bacteriostatic/fungistatic and bacteriocidal/fungicidal effects by neutralizing the active agent. The tests are also useful for determining a quantitative curve for concentration and time of an antiseptic. Thus, the tests are suitable for evaluating the effect of LPO on the lactoperoxidase-thiocyanate-hydrogen peroxide system's antimicrobial effects. However, the results must be interpreted within the limitations of an in vitro test.

The industrially produced LPO enzyme such as that used in toothpaste [36] was used because of its reproducible quality. Human SPO is slightly different from industrially produced LPO. However, the main characteristics of the industrially produced LPO are identical to saliva peroxidase [16,17]. Based on this similarity, industrially produced LPO is used instead of SPO in studies and is often referred to as LPO in the literature [37].

The efficiency of the LPO system depends – besides the concentration of its components – on exposure time and pH value [29,31]. Therefore, to determine when the LPO system or the oxidation products reached their initial optimal bactericidal and fungicidal effectiveness, tests were conducted at the exposure times of 1, 3, 5, and 15 min.

All tests were conducted at the pKₐ (pH 5.3) of HOSCN/OSCN⁻ [38], because pretests showed that the lactoperoxidase-thiocyanate-hydrogen peroxide system was effective at 5.3 pH. Lumikari et al. [23] found the optimum pH to be about 5.0. Increasing the HOSCN/OSCN⁻ concentration by adding H₂O₂ could raise the inhibition of Streptococcus mutans in human saliva [21,36] but only at a pH around 5 and not at neutral pH because of the shift of OSCN⁻ to HOSCN by a low pH value in favour of HOSCN. Unlike OSCN⁻, HOSCN has no charge, which facilitates penetration through the lipophilic bacterial cell membrane and raises the antimicrobial effectiveness of the saliva antiperoxidase system [18]. Thus, the most effective product of the LPO system works around the pH, where the biofilm/saliva pH level is pathologically effective.

To completely ensure that the tested effect of the lactoperoxidase enzyme on the thiocyanate-hydrogen peroxide system above the physiological concentration level was not based primarily on single components (H₂O₂, SCN⁻, LPO) or on combination of two components (LPO+SCN⁻, LPO+H₂O₂), accompanying suspension tests were conducted.

With one exception, all accompanying single component tests showed no clinically relevant antimicrobial effectiveness (RF: ≤ 0.3). Only the single component H₂O₂ showed a moderate reduction factor of 1.5 after 15 min. This result is in line with the known bactericidal effect of H₂O₂ [29]. However, in combination with LPO, the effect of H₂O₂ was reduced compared to its single

### Table 3: Reduction factors of the test thiocyanate hydrogen peroxide suspension without and with LPO to Candida albicans at different time points.

| Group | Without LPO | With LPO | A vs. B² |
|-------|-------------|----------|----------|
| Time  | Reduction factor | Comparisons within A³ | Reduction Factor | Comparisons within B³ |
| [min] | Mean ± SD | p | p | p | Mean ± SD | p | p | p | p |
| 1     | 0.12 ± 0.19 | 0.496 | 0.077 |
| 3     | 0.26 ± 0.26 | 0.141 | 6.78 ± 0.25³ | 0.004 | 0.551 | < 0.001 |
| 5     | 0.15 ± 0.13 | 0.004 | 6.75 ± 0.22³ | 1.000 | < 0.001 |
| 15    | 0.93 ± 0.58 | 6.74 ± 0.26³ | 1.000 | < 0.001 |

1) Wilcoxon test with a significant level of < 0.05
2) Mann-Whitney U test with a significance level of < 0.001
3) Complete killing of all cells in test suspension
effect. We assume that the radicals, which are produced by the reaction of LPO with H$_2$O$_2$ [39], are short-lived intermediates that cannot react bactericidally under the test conditions.

All suspension tests without LPO at all time points showed no or no clinically relevant antimicrobial effectiveness (highest RF: Streptococcus mutans 0.6, Streptococcus sanguinis 1.0, and Candida albicans 0.9). The low reduction potential could be based on H$_2$O$_2$ itself or, to a small extent, on the oxidation without enzyme of SCN$^-$ to OSCN$^-$ by H$_2$O$_2$, especially at higher exposure times.

On the other hand, all suspensions with LPO showed remarkably high antimicrobial effectiveness. In the quantitative suspension test, the lactoperoxidase-thiocyanate-hydrogen peroxide system (group B) showed its maximal reduction (complete) of Streptococcus mutans (RF 7.49) after a 5-min incubation time. Both reduction factors (after 5 and 15 min) were statistically significantly different from group A (without LPO).

The results show the large effect of the LPO enzyme on antibacterial effectiveness of the lactoperoxidase-thiocyanate-hydrogen peroxide system, which can be a powerful bactericide, not just bacteriostatic, if all components are above their physiological levels. It is assumed that the effect is based on not just the described shift of OSCN$^-$ to HOSCN (pH 5.3) [38] but also a higher amount of the more effective LPO-caused oxidation products, O$_2$SCN$^-$ and O$_3$SCN$^-$ [21,23,28].

In the case of Streptococcus sanguinis, the reduction factor at 5 min (RF 4.01) was statistically significantly higher in comparison with the reduction factor at 3 min (RF 0.78) of Group B (with LPO). However, there was no statistically significant difference between the reduction factors at 5 min in either group (A and B), despite a great difference in their mean values. The reason was the large standard deviation of in RF (4.01 ± 3.88).

We assume that, when the 5-min measurement was taken, the bactericidal effect by HOSCN/OSCN$^-$ was already occurring in some experiments but not yet in others. One of the reasons could be the NAD(P)H-SCN$^-$ oxidoreductase system, which Streptococcus mutans and Streptococcus sanguinis and other bacteria have. This system can reduce HOSCN/OSCN$^-$ to the less effective components, SCN$^-$ and H$_2$O$_2$. Streptococcus sanguinis has more of this reducing enzyme than does Streptococcus mutans. Thus, we assume that a higher concentration of HOSCN/OSCN$^-$ is needed to achieve a similar bactericidal effect on Streptococcus sanguinis than on Streptococcus mutans [40,41], meaning more time in the experiment. After 15 min, the test suspension with LPO had a similar antibacterial effectiveness on Streptococcus sanguinis (RF 8.12 ± 0.22) as on Streptococcus mutans (RF 7.41 ± 0.69).

Rosin et al. [32] used more than the physiological level of SCN$^-$/H$_2$O$_2$ in a toothpaste to increase the human oral defence system. This toothpaste reduced gingivitis and inhibited plaque. The enhancement of these effects by an optimal combination not only of H$_2$O$_2$ and thiocyanate, but also of LPO enzyme, for mouth rinses or toothpaste formula is certainly possible and should be considered in further clinical studies.

In our study, the LPO system was bactericidal at pH 5.3 to Streptococcus mutans and sanguinis. However, experiments by Thomas et al. [29] showed that the LPO system was effectively bacteriostatic, but not bactericidal, at pH 7 during a 1-h incubation. This finding may mean that the LPO system might shift from bacteriostatic to bactericidal at a point when the Streptococcus mutans causes low pH (<5.5), leading, for example, to demineralisation of tooth hard substances. Thus, the system could be a reservoir, getting its highest antibacterial activity when it is most needed: at a point when pH falls as a result of bacterial lactic acid production.

After 3 min, the reduction of Candida albicans in the test suspension with LPO was already complete. Thus, of the three tested microorganisms, Candida albicans was most sensitive to the lactoperoxidase-thiocyanate-hydrogen peroxide system, even if it was buffered by phosphate. Majerus and Courtois [42], as well as Samant et al. [43], could not find a sufficient antifungal effect of the SCN$^-$/H$_2$O$_2$-LPO system. Lenander-Lumikari [22] found that C. albicans is sensitive to HOSCN/OSCN$^-$, but saliva and salivary concentrations of phosphate blocked the antifungal effect of the peroxidase systems. However, they used all components of this system at the physiological human saliva level.

Thus, the lactoperoxidase-thiocyanate-hydrogen peroxide system can be not only fungistatic [44] but also fungicidal for Candida albicans; independently, it is phosphate-buffered at salivary concentrations or higher.

C. albicans can be isolated from the mouth of most individuals, but the fungus causes oral disease such as oral mucositis in primarily immunocompromised individuals [45-47]. Further, Candida albicans is seen as a reservoir for pneumonia [48] and intestinal related diseases [49].

Theraud et al. [50] showed that chlorhexidine was fungicidal on pure cultures, yeast mixtures, and biofilms above a concentration level of 0.5% (w/w). However, Pitten et al. [51] showed that treatment with a 0.3% (w/w) chlorhexidine-based product did not provide a clinical benefit.
for cancer patients with chemotherapy-induced leukopenia. In their study, the risk of mucositis and clinical sequelae (e.g., C-reactive protein) seemed to be enhanced by chlorhexidine mouth rinse, although the counts of microorganisms on the oral mucous membranes were significantly reduced. They assumed that the reason was the reduced tissue tolerance to chlorhexidine. This assumption is supported by a study that showed a discrepancy between antiseptic activity and clinical effect on radiation-induced [52] or chemo-induced mucositis [53] by chlorhexidine mouth rinse compared with placebo. In a peritoneal explant test for evaluating tissue tolerance, chlorhexidine showed the highest cytotoxicity in comparison to an essential oil and an amine/stannous fluoride mouth rinse [54]. Thus, it could be interesting to increase host innate defence systems, such as the lactoperoxidase-thiocyanate-hydrogen peroxide system, which have no or low effectiveness at the physiological level, by increasing their level of concentration instead of using common anti-septics.

**Conclusion**

In summary, in the quantitative suspension test, the SCN- and H2O2 mixture above normal physiological saliva levels showed little or no antimicrobial effect within 15 min. However, by adding lactoperoxidase enzyme, the tested mixtures became not only an effective bactericidal (Streptococcus mutans and sanguinis) but also a fungicidal (Candida albicans) agent. Thus, all three components of the LPO-system are needed for its microbicidal effect. Subsequent studies should consider loading tests with human saliva and different concentrations of all three components.

**Methods**

The study was performed based on the European norms (EN) 1040 and EN 1275. A 9.9-ml test solution (with and without LPO) was mixed with a 0.1-ml bacteria or fungus suspension (overnight culture) and stored at 37°C.

After 1, 3, 5, and 15 min contact time, the test mixture was again well mixed (vortexed), and 1 ml was transferred into 9 ml of neutralizer (polysorbate 80 30 g/L, lecithin 3 g/L, L-histidine 1 g/L, sodium thiosulfate 5 g/L, aqua bidestilata ad 1000 mL). The neutralizer was tested in a prestudy according to the recommended neutralization test of the German Society for Hygiene and Microbiology (DHGM). After 5 min of neutralization time, 1.0 ml of the neutralized test suspension was mixed with 0.1 ml bacteria or fungus suspension (overnight culture) and stored at 37°C.

The microbial counts were expressed as their decimal logarithms. The reduction factor (RF) was calculated as follows:

$$\text{lg RF} = \text{lg (cfu c)} - \text{lg (cfu t}_{A/B})$$

where cfu c = number of cfu per ml control medium (water with standardized hardness), and cfu t_{A/B} = number of cfu per ml test group A or B.
The comparisons at the time points between groups A and B (without and with LPO, respectively) were performed with the Mann-Whitney U test and within groups with the Wilcoxon test. All statistical analyses were carried out with SPSS 11.5.

Authors' contributions
AW, HB, and AK participated in the design and coordination of the study, supervised the study, and analyzed the data. RS performed most of the laboratory work with the assistance of CHM and HB. CHS carried out the statistical analysis. AW wrote the manuscript. All authors read and approved the final version of the manuscript.

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