

Candida albicans susceptibility to lactoperoxidase-generated hypoiodite

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Abstract: *In vivo*, lactoperoxidase produces hypothiocyanite (OSCN⁻) from thiocyanate (SCN⁻) in the presence of hydrogen peroxide (H₂O₂); *in vitro*, iodide (I⁻) can be oxidized into hypoiodite (OI⁻) by this enzyme. The aim of this study was to compare *in vitro* the anti-*Candida* effect of iodide versus thiocyanate used as lactoperoxidase substrate to prevent *Candida* biofilms development. *Candida albicans* ATCC 10231 susceptibility upon both peroxidase systems was tested in three different experimental designs: (i) in a liquid culture medium, (ii) in an interface model between solid culture medium and gel containing the enzymic systems, (iii) in a biofilm model onto titanium and acrylic resin. Yeast growth in liquid medium was monitored by turbidimetry at 600 nm. Material-adherent yeast biomass was evaluated by the tetrazolium salt MTT method. The iodide-peroxidase system has been shown to inhibit *Candida* biofilm formation at lower substrate concentrations (~200 fold less H₂O₂ donor) and for longer incubation periods than the thiocyanate-peroxidase system. In conclusion, efficiency of lactoperoxidase-generated OI⁻ to prevent *C. albicans* biofilm development allows refining iodine antifungal use in *ex vivo* conditions.

Keywords: denture, iodide, oral, peroxidase, saliva, titanium

Introduction

Yeasts (especially from *Candida* genus) are usually isolated from oral mucosa in healthy humans. However, saliva protects oral mucosa against candidosis; in turn, dry mouth is associated with high yeast counts and candidosis risk.¹ *Candida* incorporation into biofilms covering denture materials forms another risk factor for invasive candidosis.^{1,2} Beside oral colonization in xerostomic patients and denture wearers, yeasts have now gained more importance in oral medicine due to the increased incidence of medically/immunologically compromised patients.¹ Antifungals (azoles, polyenes) have to be reserved for curative treatments of infected patients: they are less active against *Candida* biofilms on dentures and could lead to emergent resistance if applied daily to dentures to prevent yeast colonization.^{2,3} Daily denture brushing is the main advice to give all wearers. In case of candidosis in denture wearers, decontamination of dentures is mandatory.³ Some antiphlogistic compounds, among which iodide derivatives, have been reported antifungal as well. Nevertheless, scientific literature about these does not integrate all aspects of denture care with knowledge of *Candida* biofilm development. Several iodine compounds such as povidone-iodine and hypoiodite/hypoiodous acid are already known for their anti-*Candida* properties. Povidone-iodine which has been proposed for a long time as a topical agent for skin and mucosa disinfection is still chosen as

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a control in comparative studies concerning new antifungal topical formulations.⁴⁻⁸ Iodine-povidone exhibited not only antifungal properties but also anti-adherence capacity.⁹ However, *in vitro*, its anti-*Candida* effect is reported to be rather low.⁷ Moreover, daily povidone-iodine cleaning of the mouth has been shown poorly effective in comparison with professional oral care performed weekly.⁶ Iodine-povidone is also used prophylactically in medically compromised subjects such as bone marrow transplant patients or elderly persons at risk of aspiration pneumonia.^{10,11} Iodine-releasing mucoadhesive tablets have been also proposed for treating oral infections.¹² Some antifungal effect of potassium iodide has been reported in the treatment of sporotrichosis, providing a first attempt to treat systemic mycosis; however, its activity spectrum could not be demonstrated for other systemic mycoses neither for candidosis.¹³ In the presence of hydrogen peroxide, some peroxidases can catalyze the oxidation of potassium iodide into hypoiodite (OI⁻)/hypoiodous acid (HOI) which has been shown effective against several yeasts such as *Candida*, *Saccharomyces*, *Geotrichum*, *Rhodotorula* and against some species of *Aspergillus* such as *A. fumigatus* or *A. niger*.¹⁴ The use of a lactoperoxidase system has been suggested as preservative in foods and pharmaceuticals.¹⁵ *In vitro*, peroxidase-generated hypoiodite could exert a strong inhibiting effect on *Candida albicans* at physiological concentrations but its activity in saliva was found to be under the control of thiocyanate concentration.¹⁶ The aim of this *in vitro* study was to compare the anti-*Candida* effect of iodide *versus* thiocyanate used as lactoperoxidase substrate with a view to preventing *Candida* biofilms development on materials, in the field of oral medicine in particular.

Material and methods

Materials

Candida adherence was evaluated onto titanium (powder or foil) and acrylic resins (hard or soft) which are biomaterials commonly used in the oral environment. Aliquots (0.5 g) of titanium powder (Alfa Aesar™; Karlsruhe, Germany; 99.5% purity, ~325 mesh) were transferred into sterile polystyrene cell culture tubes (BD Falcon™, USA; size: 125 × 16 mm, round bottom). Titanium pieces were cut from native foil (Advent™; UK; 99.6% purity; thickness 25 μm, size 25 mm × 5 mm); each piece was weighed and the total surface was calculated using the following algorithm: $S = 2 \{ (m/\rho) + h(L+l) \}$ where S: total surface (mm²), m: foil mass (mg), ρ: volumic mass (4.544 mg/mm³), h: foil thickness (0.025 mm), L: foil length (mm), l: foil width (mm). Total

surface averaged 246 ± 4 mm² (mean ± SEM, N = 16) with a coefficient of variation of 6.5%. Two different resins for denture (Vertex soft; and Vertex rapid simplified; Vertex Dental, Zeist, Netherlands) were investigated with and without varnishing (Palaseal; Heraeus Kulzer, Wehrheim, Germany). Acrylic resin pieces (thickness ~2 mm, size 25 mm × 5 mm) were processed according to the manufacturer's instructions. The mass of each resin piece averaged 222 ± 2 mg (mean ± SEM, N = 96) with a coefficient of variation of 8.6%.

Culture of *Candida*

Candida albicans ATCC 10231 (Culti-Loops; Oxoid, Basingstoke, UK) was grown at 37°C on Sabouraud-gentamicin-chloramphenicol agar (Becton-Dickinson, Belgium). Yeast was harvested from the third subculture to a flask containing a Sabouraud broth (CM147; oxoid). The suspension was then adjusted to an absorbance of 0.800 at 600 nm (corresponding to $1.55 \pm 0.32 \cdot 10^7$ CFU/mL, N = 6) and always 10-fold diluted in the appropriate reactant medium. Wild strains of *C. albicans* (N = 5) and *C. glabrata* (N = 4) were isolated from dentures and identified on the basis of their colony aspect on CHROMagar medium (BD Diagnostics, Erembodegem, Belgium) and their carbohydrate assimilation pattern using the ID32C API system (bioMérieux, Marcy-l'Etoile, France). Colony diameter was measured on Petri dish photography by using the Image-Pro software version 6.0 (Media Cybernetics, Bethesda, MD, USA).

Production of *Candida* biofilms

Titanium powder (0.5 g) was suspended in 3.6 mL Sabouraud broth and *C. albicans* suspension (0.4 mL) adjusted in Sabouraud broth to an absorbance of 0.800 at 600 nm, as indicated above. After continuous stirring (Stuart SB3 rotator; Barloworld Scientific, Stone, UK) during 2 days at room temperature, titanium powder was separated by sedimentation (10 min) from liquid medium and washed three times with 4 mL sterile Sabouraud broth. Titanium powder from some sterile controls was contaminated by 4 mL of the first supernatant from the contaminated assays in order to evaluate the efficiency of the washing process. All the first supernatants were monitored by turbidimetry at 600 nm to evaluate the planktonic yeast phase. Titanium and resin foils were similarly treated. Material-adherent yeast biomass (anchored yeast phase) was evaluated by the tetrazolium salt MTT (final concentration: 1.7 mg/mL in 2:1 Sabouraud – pH 7.40 PBS) which is reduced by viable

fungi with resultant formation of MTT-formazan absorbing on a spectrophotometer at 570 nm after extraction in isopropanol.^{17,18} Main characteristics of the method: molar absorption coefficient ϵ_{570} of $17,000 \text{ M}^{-1} \times \text{cm}^{-1}$, sensitivity of 0.5×10^6 blastoconidia/mL, coefficient of variation $\leq 12.0\%$, analytical range from 0.5 to 10×10^6 blastoconidia/mL corresponding to an absorbance at 570 nm comprised between 0.206 ± 0.010 (N = 6, CV: 12.0%) and 1.792 ± 0.058 (N = 6, CV: 7.9%) respectively. Microscopic examination confirmed the presence of yeast on the surface of titanium powder as assessed by the chemical data: titanium grains were surrounded by several blastoconidia layers after a 2-day incubation, and filamentous structures (hyphae and pseudohyphae) have been observed after 3 weeks.

Lactoperoxidase adsorption on titanium

Lactoperoxidase (Biopole, Belgium) was obtained from bovine milk. Its specific activity was 12 ABTS units/protein mg at 37°C at pH 5.0 (one ABTS unit corresponded to the amount of enzyme catalyzing the oxidation of 1 mM of the ABTS substrate in one minute under the assay conditions described by Mansson-Rahemtulla et al¹⁹). Lactoperoxidase was dissolved in demineralized water or in another solution when indicated. As previously described, titanium foils were immersed during 30 minutes at 37°C in 5 mL of a 10 ABTS units/mL lactoperoxidase solution or in demineralized water alone (control).²⁰ After incubation, the liquid phase was removed and titanium foils were washed 3 times with 5 mL of demineralized water and then transferred into another tube with the last washing medium. This procedure was repeated 5 times and the liquid phase was removed; titanium pieces were then dried at room temperature.

Lactoperoxidase systems

Two different peroxidase systems were tested on biofilm formation: hydrogen peroxide (H_2O_2)/lactoperoxidase (LPO, 1 U/mL)/iodide (I^- from KI, various concentrations up to 1.2 mM) system generating hypoiodite (OI^-) and H_2O_2 /LPO (1 U/mL)/thiocyanate (SCN^- from KSCN, 1.2 mM) producing hypothiocyanite (OSCN^-). H_2O_2 was enzymatically produced by glucose-oxidase (GOD, 1.3 U/mL or 0.2 U/mL) and glucose (G) present in the culture medium (115 mM). In some experiments, peroxidase was pre-adsorbed onto titanium foils as schematized in Figure 1. The effect of the peroxidase systems on *C. albicans* growth was also evaluated in Sabouraud liquid medium by turbidimetry (absorbance at 600 nm) after a 16-hour incubation at 37°C or by yeast count on Sabouraud solid

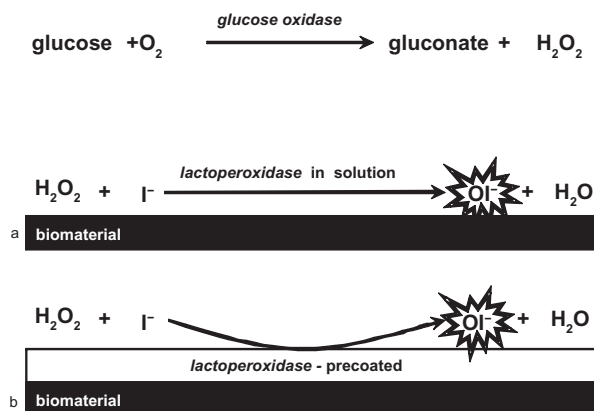


Figure 1 Peroxidase systems producing hypoiodite (OI^-) from iodide (I^-) in the presence of hydrogen peroxide (H_2O_2). The present figure schematizes both lactoperoxidase uses: **a**) dissolving in the liquid culture medium or **b**) pre-coating onto materials (titanium or resin foils).

medium (implying thus a subsequent 48-hour cultivation on solid medium from the liquid medium). Finally, peroxidase systems were tested after incorporation in a gel consisting of a polyglycerol methacrylate polymer, starch hydrogenated hydrolysate, hydroxyethylcellulose and water. Antifungal activity of gels were evaluated by a diffusion test. For this purpose, *Candida* suspension (25 μl , adjusted to an absorbance of 0.800 at 600 nm in Sabouraud broth) was swabbed onto the surface of a Sabouraud plate centrally gel-punched (O : 15 mm). Then 1 g of tested gel was deposited in the central well. Microcolonies were evident after 24 h at 37°C but the lawn of growth was confluent or nearly confluent after 48 h. After a two-day incubation at 37°C, the radius of the inhibition zone at the agar surface was measured around the central well in four orthogonal directions. Negative control has been performed using inactivated gel by heating at 85°C during 6 hours.

Finally, H_2O_2 was detected by chemiluminescence (lucigenin method). Briefly, lucigenin (20 μM) was oxidized by H_2O_2 in alkaline solution (Na_2CO_3 buffer) with photon emission, which was measured on a bioluminometer LKB 1250 (LKB-Wallac, Turku, Finland).

Statistics

Data were analyzed using the GraphPad Prism version 5.00 (GraphPad Software, San Diego, CA USA). ANOVA analysis completed by a Dunnett's Multiple Comparison test was performed for *Candida* count and spectrophotometric data. An unpaired *t*-test of Student compared colony diameter on Sabouraud solid medium in the presence of G/GOD/ SCN^- /LPO gel versus control. Mean values were expressed with their related standard error of the mean.

Results

Effect of I⁻/LPO system in *Candida* cultures

Figure 2 illustrates the *Candida* counts after a 16-hour exposure to different lactoperoxidase system formulations incorporated in Sabouraud liquid medium. In these experiments, *Candida* counts were provided by cultures on Sabouraud solid medium after appropriate serial dilutions (from 10 to 10⁴ fold) of the incubation liquid medium; the data were expressed as log(10) CFU/mL. In the presence of 115 mM D-glucose and GOD 0.2 U/mL, the *Candida* count expressed as log(10) (5.975 ± 0.114, N = 6) was not statistically different from various control formulations: LPO/KSCN/G (5.468 ± 0.137, N = 6), LPO/KI/G (5.640 ± 0.227, N = 6), LPO/NaCl/G (5.381 ± 0.055, N = 6) and LPO/NaCl/G/GOD (5.741 ± 0.181, N = 6). In the presence of the same GOD and LPO activities (0.2 U/mL and 1 U/mL respectively), the iodide substrate (1.2 mM) inhibited yeast growth (N = 6; *P* < 0.001) while the thiocyanate substrate (1.2 mM) lightly decreased the log(10) CFU/mL count to 4.625 ± 0.527 (N = 6; *P* < 0.01); in turn, chloride (1.2 mM) – which is not a substrate for lactoperoxidase – did not affect yeast survival (5.741 ± 0.181, N = 6, *P* > 0.05).

Table 1 shows data obtained in liquid cultures (6 independent cultures for each condition in one pilot experiment) by measuring the absorbance at 600 nm after a 16-hour

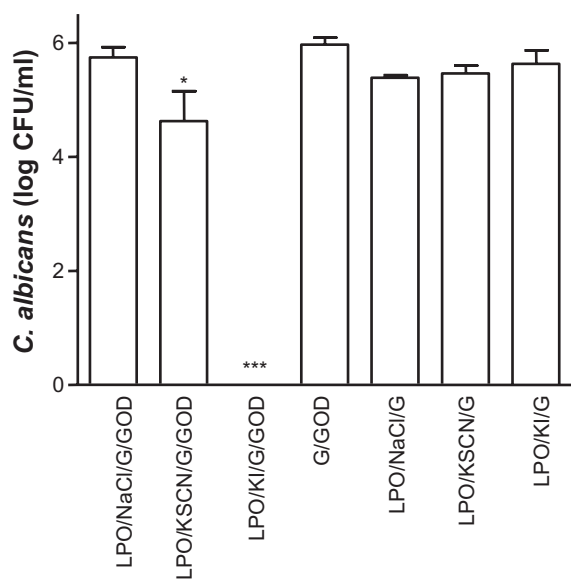


Figure 2 Anti-*Candida* effect of a 16-hour exposure to various combinations of lactoperoxidase systems. *Candida albicans* (ATCC 10231) growth in Sabouraud broth was evaluated by counting colony forming units (CFU expressed as log(10)). **Abbreviations:** G, glucose; GOD, glucose oxidase; LPO, lactoperoxidase.

Table 1 *Candida albicans* (ATCC 10231) growth in Sabouraud broth evaluated by turbidimetry (absorbance at 600 nm) after a 16-hour incubation in the presence of the iodide/lactoperoxidase system at different iodide concentrations

Culture conditions	Δ Absorbance at 600 nm mean ± SEM (N)	%
Positive control	0.894 ± 0.022 (6)	100.0 ± 3.5 (6)
G/GOD	0.917 ± 0.020 (6)	102.6 ± 3.4 (6)
G/GOD/LPO	0.935 ± 0.021 (6)	104.6 ± 3.5 (6)
G/GOD/1.20 mM KI	0.929 ± 0.011 (6)	103.4 ± 2.8 (6)
G/GOD/LPO/1.20 mM KI	0.026 ± 0.011 (6)***	2.9 ± 1.2 (6)***
G/GOD/LPO/0.60 mM KI	0.029 ± 0.013 (6)***	3.2 ± 1.5 (6)***
G/GOD/LPO/0.30 mM KI	0.659 ± 0.033 (6)***	73.7 ± 4.1 (6)***
G/GOD/LPO/0.15 mM KI	0.891 ± 0.010 (6)	99.7 ± 2.7 (6)
G/GOD/LPO/0.07 mM KI	0.884 ± 0.026 (6)	98.9 ± 3.8 (6)
Negative control	0.000	0.0

Notes: Data were analysed by ANOVA completed by a Dunnett's Multiple Comparison test (positive control condition considered as control group). **Abbreviations:** G, glucose; GOD, glucose oxidase; LPO, lactoperoxidase; SEM, standard error of the mean; N, number of determinations.

exposure to the lactoperoxidase system in the presence of different iodide substrate concentrations comprised between 0.07 and 1.20 mM. As already demonstrated by culture on solid medium, GOD at 0.2 U/mL in the absence of lactoperoxidase (thus producing H₂O₂) did not affect the *Candida* growth as observed by spectrophotometry: the absorbance at 600 nm yielded then 102.6 ± 3.4% (N = 6) of that found after culture in control broth (100.0 ± 3.5%, N = 6). Both 0.6 and 1.20 mM KI concentrations in the presence of G/GOD/LPO provided absorbance values (0.029 ± 0.013 and 0.026 ± 0.011, respectively) that were not statistically different from the paired negative controls. The other KI concentrations (0.07, 0.15 and 0.30 mM) tested with lactoperoxidase were insufficient to abolish *Candida* growth: absorbance at 600 nm respectively yielded 98.9 ± 3.8, 99.7 ± 2.7 and 73.7 ± 4.1% of the paired positive control. In 6 independent experiments, the lowest active iodide concentration estimated to 0.6 mM in the pilot experiment has been successfully corroborated by subcultures of the infected liquid media on solid Sabouraud agar: in the presence of 0.6 and 1.2 mM KI, no colony-forming unit (CFU) could be observed.

In another set of 6 independent experiments, iodide versus thiocyanate substrates (1.2 mM both) were incorporated in gel containing glucose, lactoperoxidase and GOD. The anti-*Candida* effect of these gels was simultaneously evaluated by counting after gel-covering yeast-napped agar and by measuring inhibition radius in a radial diffusion test: Table 2 summarizes the data. The data were compared

Table 2 *Candida albicans* (ATCC 10231) growth on Sabouraud solid medium covered by a gel containing an oxidative system (G/GOD producing H₂O₂, G/GOD/KSCN/LPO producing hypothiocyanite and G/GOD/KI/LPO producing hypoiodite)

Oxidative system	GOD activity (U/gel mL)	Survival rate (%)	Inhibition radius (mm)
G/GOD	0.000	100.0 ± 9.8 (6)	0.0 ± 0.0 (3)
	0.150	105.9 ± 8.7 (6)	0.0 ± 0.0 (3)
	1.500	4.9 ± 2.4 (6)***	0.0 ± 0.0 (3)
	3.000	0.0 ± 0.0 (6)***	0.0 ± 0.0 (3)
G/GOD/KSCN/LPO	0.000	107.4 ± 9.3 (6)	0.0 ± 0.0 (3)
	0.050	88.5 ± 4.6 (6)*	0.0 ± 0.0 (3)
	0.250	66.9 ± 4.2 (6)***	0.0 ± 0.0 (3)
	0.500	16.9 ± 1.8 (6)***	0.0 ± 0.0 (3)
	1.000	13.6 ± 4.1 (6)***	0.0 ± 0.0 (3)
	1.500	3.0 ± 1.3 (6)***	0.3 ± 0.2 (3)
	3.000	0.0 ± 0.0 (6)***	0.0 ± 0.0 (3)
	6.000	0.0 ± 0.0 (6)***	0.7 ± 0.2 (3)*
G/GOD/KI/LPO	0.000	113.9 ± 1.9 (6)	0.0 ± 0.0 (3)
	0.005	43.0 ± 10.9 (6)***	0.3 ± 0.1 (3)
	0.010	0.0 ± 0.0 (6)***	1.0 ± 0.3 (3)
	0.020	0.0 ± 0.0 (6)***	1.8 ± 0.3 (3)**
	0.040	0.0 ± 0.0 (6)***	4.9 ± 0.1 (3)***
	0.400	0.0 ± 0.0 (6)***	5.2 ± 0.3 (3)***
	1.000	0.0 ± 0.0 (6)***	6.9 ± 0.7 (3)***

Notes: Minimal inhibitory activity of GOD was evaluated by colony forming unit count and by a radial diffusion test after a 2-day incubation at 37°C. Survival rate was expressed as percentage (%) of the paired control without GOD, and the radius around a gel-filled wall was expressed in millimeters (mm). Data for each tested gel were analysed by ANOVA completed by a Dunnett's Multiple Comparison test (no GOD activity condition for each oxidative system was considered as a control group).

Abbreviations: G, glucose; GOD, glucose oxidase; LPO, lactoperoxidase.

with those observed with gel alone. The lowest active GOD activity is quite similar in the presence of both oxidative G/GOD and G/GOD/KSCN/LPO systems: 3 U of GOD per gel mL provided a total inhibition of *Candida* growth. In turn, GOD incorporated in G/GOD/KI/LPO gels already suppressed the *Candida* survival at an activity of 10 mU per gel mL. These data obtained at the interface agar/gels were confirmed by a radial diffusion test. This second procedure resulted in an inhibition area round a gel-filled central wall only in the presence of G/GOD/KI/LPO oxidative system: at an activity of 10 mU per gel mL, inhibition radius was 1.0 ± 0.3 mm (N = 3). Highest GOD amounts (20, 40, 400 and 1000 mU/gel mL) provided an increase of this inhibition radius to 1.8 ± 0.3 mm (N = 3), 4.9 ± 0.1 mm (N = 3), 5.2 ± 0.3 mm (N = 3) and 6.9 ± 0.7 mm (N = 3) respectively. In turn, no inhibition area could be observed around the central wall filled with G/GOD or G/GOD/KSCN/LPO gels.

Efficiency of iodide-lactoperoxidase system (GOD 0.5 U/gel mL) has been confirmed on five *C. albicans* and four *C. glabrata* wild strains isolated from yeast-colonized dentures. Then, survival rates in the presence of iodide-peroxidase system were 1.8 ± 0.6% (N = 5) and 2.7 ± 1.3% (N = 4) of the paired control for *C. albicans* and *C. glabrata* wild strains respectively versus 95.3 ± 5.1% (N = 5) and

88.3 ± 12.5% (N = 4) in the presence of thiocyanate-peroxidase system.

Effect of lactoperoxidase systems on *Candida* biofilm formation

Both lactoperoxidase systems, one with thiocyanate and the other with iodide as peroxidase substrate, were tested in a model of yeast biofilm on titanium powder. Yeast growth in the supernatant (planktonic phase) was monitored by turbidimetry at 600 nm and titanium-adherent yeast biomass (anchored phase) was evaluated by spectrophotometry at 570 nm in a tetrazolium salt MTT assay. Table 3 reports the absorbances obtained in different conditions for both assays simultaneously performed. In the presence of 1.3 U/mL GOD, both G/GOD/SCN⁻/LPO and G/GOD/I⁻/LPO systems prevented *Candida* growth in planktonic and attached phases during at least 4 days. Indeed, the absorbances at 600 nm (testing the planktonic phase) were 0.017 ± 0.008 (N = 9) and 0.059 ± 0.023 (N = 9) respectively versus 0.658 ± 0.056 (N = 12) for the control without oxidative system: this reduction of supernatant turbidity is statistically significant (ANOVA test: $P < 0.0001$, Dunnett: $P < 0.001$). The absorbances at 570 nm (reflecting the anchored phase) were 0.065 ± 0.021 (N = 9) and 0.040 ± 0.018 (N = 9) respectively versus 0.788 ± 0.061 (N = 13) for the control

Table 3 *Candida albicans* biofilm formation after 2 versus 4 days incubation with OSCN⁻ and OI⁻ produced in the presence of 0.2 versus 1.3 U/mL GOD

		Control	G/GOD/KSCN/LPO	G/GOD/KI/LPO
GOD:	planktonic phase	0.452 ± 0.075 (3)	0.453 ± 0.005 (3) NS	0.030 ± 0.001 (3)***
0.2 U/mL	(absorbance at 600 nm)			
incubation:	anchored phase	1.153 ± 0.073 (3)	0.366 ± 0.019 (3)***	0.112 ± 0.059 (3)***
2 days	(absorbance at 570 nm)			
GOD:	planktonic phase	0.757 ± 0.100 (3)	0.927 ± 0.042 (3)	0.029 ± 0.001 (3)***
0.2 U/mL	(absorbance at 600 nm)			
incubation:	anchored phase	0.820 ± 0.027 (3)	1.101 ± 0.030 (3)***	0.125 ± 0.007 (3)***
4 days	(absorbance at 570 nm)			
GOD:	planktonic phase	0.658 ± 0.056 (12)	0.017 ± 0.008 (9)***	0.059 ± 0.023 (9)***
1.3 U/mL	(absorbance at 600 nm)			
incubation:	anchored phase	0.788 ± 0.061 (13)	0.065 ± 0.021 (9)***	0.040 ± 0.018 (9)***
4 days	(absorbance at 570 nm)			

Notes: The planktonic phase was monitored by turbidimetry (absorbance at 600 nm) and the anchored phase by the MTT method (absorbance at 570 nm). Data from both peroxidase system conditions were compared to the control condition by ANOVA completed by a Dunnett's Multiple Comparison test.

Abbreviations: G, glucose; GOD, glucose oxidase; LPO, lactoperoxidase.

without oxidative system: this reduction of MTT staining was statistically significant (ANOVA: $P < 0.0001$, Dunnett: $P < 0.001$). In the presence of 0.2 U/mL GOD, G/GOD/I-/LPO kept both planktonic and attached phases sterile during 4 days while G/GOD/SCN⁻/LPO only delayed *Candida* adhesion onto titanium from day-2 to 4 without effect on its planktonic growth during the same time period. At this GOD activity (0.2 U/mL) and at day 2, the absorbance at 600 nm was 0.453 ± 0.005 (N = 3) for the thiocyanate system and 0.030 ± 0.001 (N = 3) for the iodine system versus 0.452 ± 0.075 (N = 3) for the control without oxidative system: the reduction of supernatant turbidity in the presence of G/GOD/KI/LPO was statistically significant (ANOVA: $P = 0.0007$, Dunnett: $P < 0.001$). The absorbances at 570 nm were 0.366 ± 0.019 (N = 3) and 0.112 ± 0.059 (N = 3) respectively versus 1.153 ± 0.073 (N = 3) for the control without oxidative system: this reduction of MTT staining was statistically significant (ANOVA: $P < 0.0001$, Dunnett: $P < 0.001$). A 2-day incubation in the presence of OSCN⁻ thus decreased the attached biomass to $31.7 \pm 2.6\%$ (N = 3) of the paired control without effect on the growth in the supernatant ($100.2 \pm 2.8\%$ of the paired control, N = 3). However, after a 4-day exposure to OSCN⁻, the attached biomass reached $95.5 \pm 6.6\%$ (N = 3) of the control measured at day 2. The absorbances at 600 nm were then (on day 4) 0.927 ± 0.042 (N = 3) for the thiocyanate system and 0.029 ± 0.001 (N = 3) for the iodine system versus 0.757 ± 0.100 (N = 3) for the control without oxidative system: this reduction of supernatant turbidity in the presence of G/GOD/KI/LPO was statistically significant (ANOVA: $P < 0.0001$, Dunnett: $P < 0.001$) while the increase in the presence of G/GOD/KSCN/LPO was not.

The absorbances at 570 nm were 1.101 ± 0.030 (N = 3) and 0.125 ± 0.007 (N = 3) respectively versus 0.820 ± 0.027 (N = 3) for the control without oxidative system: the increase with KSCN and decrease with KI substrate of MTT staining were statistically significant (ANOVA: $P < 0.0001$, Dunnett: $P < 0.001$).

In another set of experiments, *Candida* biofilm formation was also examined on titanium foils and on different resins for dentures. All the data for resins were pooled since no statistical difference could be observed according to resin characteristics (hard or soft, varnished or not). The G/GOD/I-/LPO peroxidase system was able to prevent *Candida* growth in liquid in which any materials were immersed (Figure 3) and to prevent *Candida* biofilm formation onto materials after 2-day incubation. Similar results were found when lactoperoxidase was precoated onto materials. In the presence of G/GOD/KI/LPO, the absorbance at 600 nm of the culture broth was 0.029 ± 0.001 (N = 24) and 0.038 ± 0.009 (N = 24) for lactoperoxidase solubilized in broth and for lactoperoxidase precoated onto resin foils respectively versus 1.327 ± 0.076 (N = 24) for the control. In the case of titanium foils, results were 0.041 ± 0.002 (N = 4) and 0.045 ± 0.002 (N = 4) versus 1.691 ± 0.038 (N = 4). In the presence of G/GOD/KI/LPO, the absorbance at 570 nm after MTT processing was 0.035 ± 0.006 (N = 24) and 0.023 ± 0.004 (N = 24) for lactoperoxidase solubilized in broth and for lactoperoxidase precoated onto resin foils respectively versus 0.161 ± 0.032 (N = 24) for the control. In the case of titanium foils, results were 0.041 ± 0.002 (N = 4) and 0.045 ± 0.002 (N = 4) versus 1.691 ± 0.038 (N = 4). Thus, a 2-day incubation in the presence of generated OI⁻ decreased the turbidity of the liquid culture medium when compared to that observed in the paired

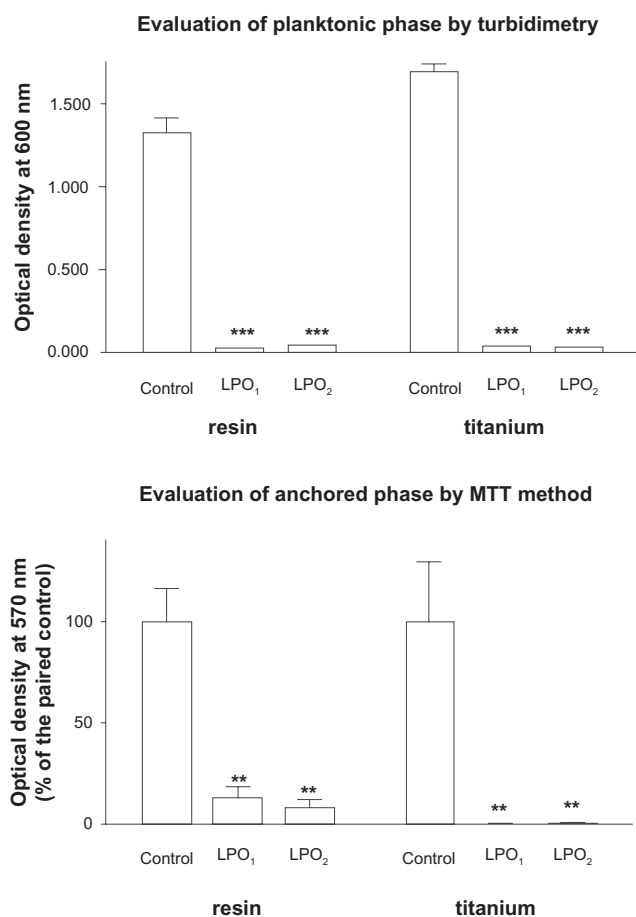


Figure 3 *Candida albicans* biomass in planktonic phase (evaluated by turbidimetry at 600 nm) and anchored phase (evaluated by spectrophotometry at 570 nm) onto materials (titanium or resin) after a 2-day incubation. Lactoperoxidase-adsorbed materials (LPO₂) were compared with the same material exposed to the enzyme in solution (LPO₁) after a 2-day incubation in the presence of OI⁻ produced by various peroxidase systems.

control without the peroxidase system (ANOVA: $P < 0.0001$, Dunnett: $P < 0.001$) as well with solubilized lactoperoxidase as with the enzyme precoated onto the material surfaces; these data were confirmed by negative cultures on Sabouraud agar. Moreover, the absorbance at 570 nm (reflecting the attached yeast phase onto resins) was reduced down to $21.1 \pm 5.6\%$ ($N = 24$) of the paired control and $14.3 \pm 3.8\%$ ($N = 24$) for solubilized and precoated lactoperoxidase respectively (ANOVA: $P < 0.0001$, Dunnett: $P < 0.001$); the decrease of anchored *Candida* biomass was still more severe onto titanium foil and dropped to $1.0 \pm 3.1\%$ of the control (ANOVA: $P < 0.0001$, Dunnett: $P < 0.0001$).

Competition between I⁻ and SCN⁻ for lactoperoxidase

Figure 4 shows the susceptibility of *Candida* growth when both KSCN and KI substrates were simultaneously tested

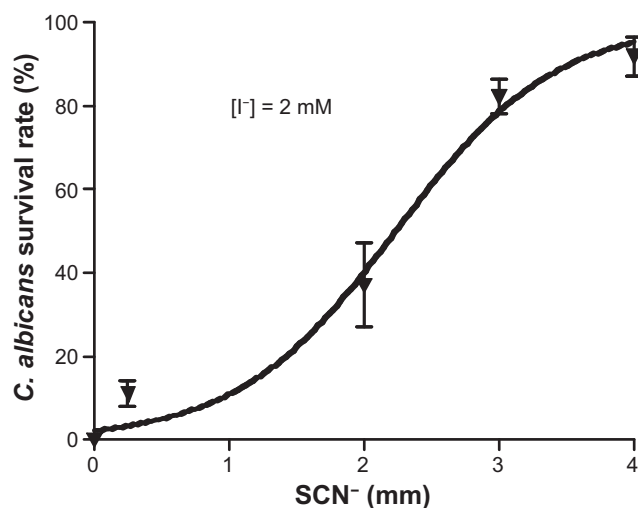


Figure 4 Effect of SCN⁻/I⁻ competition for lactoperoxidase in oral gel on *Candida* growth on Sabouraud agar. Survival rate (expressed as the percentage of the control without any peroxidase substrate) was assayed for different KSCN concentrations (0–4 mM) in the presence of 2 mM KI.

with the lactoperoxidase and the glucose/glucose oxidase sequence. The data demonstrate the competition between SCN⁻ at different concentrations and 2 mM I⁻ through *Candida* survival rate after covering napped Petri dishes with peroxidase gels. The gel alone was unable to limit *C. albicans* growth and H₂O₂ produced by glucose oxidase (GOD 0.2 U/mL) in the presence of glucose was insufficient in the experimental conditions to inhibit the yeast, which is catalase positive. G/GOD/I⁻/LPO reduced the CFU count to zero but addition of SCN⁻ (0.25, 2, 3 and 4 mM) progressively decreased this antifungal effect. Only a slight although significant (unpaired Student's *t*-test: $P < 0.0001$) anti-*Candida* effect of G/GOD/SCN⁻/LPO was observed after 48 hours incubation by measuring colony diameter: 2.23 ± 0.01 mm ($N = 102$) for SCN⁻/LPO condition versus 2.57 ± 0.01 mm ($N = 102$) for control. In 3 experiments, H₂O₂ produced by G/GOD in water reached 3.1 ± 0.3 mM after 4-hours incubation at 37°C in absence of the lactoperoxidase sequence. Addition of peroxidase and of substrates (thiocyanate or iodide) to G/GOD abolished chemiluminescence (Figure 5).

Discussion

Iodine compounds have long been used in oral health care against fungi, particularly against yeasts, with limited efficiency. Among the different forms of iodine mentioned for their antifungal properties, only hypoiodous acid/hypoiodite has been reported to be active *in vitro*.¹⁶ Production of this iodine compound simultaneously needs an iodide intake and the presence of peroxidase activity with H₂O₂. Its strong

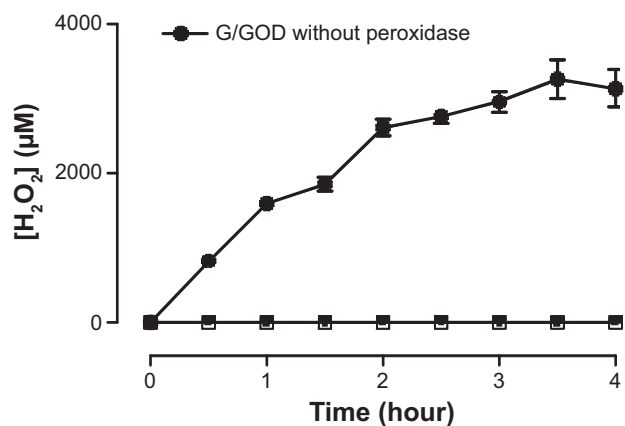


Figure 5 Iodide/peroxidase (■) and thiocyanate/peroxidase (□) effect on H_2O_2 concentration produced by glucose (G)/glucose oxidase (GOD) and measured by the lucigenin method.

oxidation capacity and the need for enzymes, which in turn can be immunogenic, have prevented its therapeutic use *in vivo*. Moreover topical use could be limited by the presence of a competitive substrate for peroxidase, as thiocyanate is present in exocrine secretions. Nevertheless, it may be envisaged for *ex vivo* use as food preservative or device cleaner where thiocyanate is absent from the support environment to be protected.

In the present study, peroxidase system with 1.2 mM iodide required less H_2O_2 than with thiocyanate at the same concentration to inhibit *Candida* growth. Similar observations were reported for *Curvularia* haloperoxidase systems where 0.1 mM iodide contributed to reduce *Rhodotorula sp.* and *Candida sp.* in a more efficient manner than other substrates of the enzyme.²¹ In the present investigation, *C. albicans* was shown to be more susceptible to iodide/peroxidase systems in 3 different experimental designs: (i) in a liquid culture medium, (ii) in an interface model between solid medium culture (Sabouraud agar) and gel, (iii) in a biofilm model onto titanium powder and acrylic resin. In the case of the lactoperoxidase system using 1.2 mM iodide solubilized in a liquid medium, yeast growth was completely inhibited after 16 hours incubation. By contrast, when thiocyanate was used as substrate in the same lactoperoxidase system, the number of colony forming units reached at least 4 logarithmic units (4.625 ± 0.527 , $N = 6$) and more than 5 in different control conditions (from 5.3810 ± 0.055 , $N = 6$ to 5.741 ± 0.181 , $N = 6$). This difference of 1 logarithmic unit between G/GOD/KSCN/LPO and control means that the CFU/mL of liquid culture represented about 10% of that found in the control tubes while the iodine/lactoperoxidase system totally suppressed the yeast count. Similarly, turbidimetry data (A_{600nm}) confirmed

the growth inhibition in the presence of iodide substrate in comparison with thiocyanate, the other compounds of the peroxidase systems being identical and used at the same concentrations. Incorporation of a lactoperoxidase system in gel, in order to cover yeast-napped solid medium surfaces, led to similar conclusions. The lowest active amount of GOD (providing H_2O_2 from glucose) was at least 200 fold less important in the presence of KI when compared to KSCN. Moreover, at a GOD activity of 0.2 mU/mL, the iodide/lactoperoxidase system inhibited *Candida* biofilm formation on titanium and resin materials immersed in an infected broth while thiocyanate/lactoperoxidase was only able to delay it. In all these experiments, the enzyme sequence G/GOD was used as H_2O_2 donor. In the presence of a peroxidase sequence, H_2O_2 is totally transformed into hypohalous compound. Indeed, H_2O_2 can be detected by chemiluminescence (lucigenin method) but only in the absence of peroxidase. Addition of peroxidase and of substrates (thiocyanate or iodide) abolished chemiluminescence (Figure 5) so that media with G/GOD, G/GOD/KSCN/LPO and G/GOD/KI/LPO enabled to specifically evaluate the effects of H_2O_2 , $OSCN^-$ and OI^- respectively without interference of one on the other.

The efficiency of an iodide/peroxidase system demonstrated *in vitro* through this investigation is difficult to transfer to either animal or human. Beside the toxicity of oxidant products on host cells and the immunogenicity of enzymes isolated for example from bovine milk, the oxidation of iodide is under the control of thiocyanate, which is not only present in several exocrine secretions (for example in human saliva) but is also preferentially used as substrate by lactoperoxidase. Indeed, simultaneous incorporation of both substrates in the same gel provided a decrease of the beneficial effect of 2 mM iodide in the presence of increasing concentrations of thiocyanate ranging from 0.25 to 4 mM, which correspond to the normal range of thiocyanate in saliva. The ability of OI^- to affect yeast growth and survival must be questioned in regard of the other iodine compounds, since clinical trials have shown limited or weak beneficial effects of other iodine formulations used for *in vivo* antifungal purposes. Re-examining this in the light of data obtained *in vitro* with the iodide/lactoperoxidase system, one could hypothesize that these poor results *in vivo* were due to small amounts of OI^- formed by peroxidases, present in the investigated medium, which preferentially oxidize thiocyanate. Finally, the molecular mechanisms, which would explain the specific toxicity of hypoiodite in comparison with other oxidant

products of peroxidase activities remain unknown. Previous studies have shown that *C. albicans* blastoconidia incubated with $^{125}\text{I}^-$ and lactoperoxidase could fix radioactive iodide through their own H_2O_2 metabolism and that this iodination was mostly detected in a cell wall mannoprotein.^{22,23} Further studies concerning the role of this specific iodination target could lead to a better understanding of the specific mechanisms of OI^- toxicity in *C. albicans*. Implication of these data for dentistry, especially for prosthesis care, is questionable since candidosis represents a frequent complication in edentulous patients, resulting from the high prevalence of dentures contaminated by *Candida*. Indeed, a previous screening had shown *Candida* contamination of upper prosthesis in 76% of denture wearers hospitalized in geriatric units for long term care.² The most frequent species isolated in this series were *C. albicans* (78%), *C. glabrata* (44%) and *C. tropicalis* (19%). Carriage of more than one yeast species was found in 49% of the contaminated dentures. There was a significant association between denture contamination and palatal mucosa colonization making mandatory *ex vivo* denture decontamination together with *in vivo* mucosa disinfection. Iodide/ H_2O_2 /lactoperoxidase system use in dentistry runs up not only against SCN^- presence in saliva, but also against catalase presence in some micro-organisms, among which different oral bacteria beside *Candida* itself. Catalase contributes to decrease the amount of available H_2O_2 substrate necessary for the peroxidase, and therefore the OI^- production. Overpassing both limitations of peroxidase use in oral medicine needs further research about OI^- enzymatic production in the oral environment.

Conclusion

In the absence of thiocyanate, iodide/peroxidase system has been shown active *in vitro* against *C. albicans* 1) in a liquid culture medium, 2) in an interface model (Sabouraud agar covered by a peroxidase gel), and 3) finally in a biofilm model onto titanium and acrylic resin. Efficiency of lactoperoxidase-generated OI^- to prevent *C. albicans* biofilm development allows refining iodine antifungal use in *ex vivo* conditions (for instance denture or material decontamination) when environment is free of thiocyanate.

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Disclosure

The authors report no conflicts of interest in this work.

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