Ex vivo Yeast-Decontamination of Denture by H_2O_2/Iodide/Lactoperoxidase System: Need to Overpass the Microbial H_2O_2 Catabolism

Sebaa S[1,2], Lybaert P[1], Boucherit-Otmani Z[2], Courtois Ph[1,3], Ahariz M[1]

[1]Laboratory of Physiology and Pharmacology, Université Libre de Bruxelles, Brussels, Belgium. [2]University of Aboubekr Belkaïd-Tlemcen, Tlemcen, Algeria. [3]Haute Ecole F. Ferrer, Brussels, Belgium

Abstract

Aim: To evaluate the H_2O_2 consumption by *Candida albicans* cells as a limitation for using H_2O_2 peroxidase systems in denture decontamination from yeasts.

Material and methods: Investigations were conducted on *C. albicans* ATCC 10231 and on clinical samples isolated from 7 dentures. Strains were grown aerobically at 37°C on Sabouraud-chloramphenicol agar. H_2O_2 degradation by yeast suspensions was spectrophotometrically evaluated at 230 nm and H_2O_2 production by glucose-oxidase was quantified by the lucigenin method. *Candida* susceptibility upon H_2O_2-peroxidase systems was tested on Sabouraud solid medium for different H_2O_2 supplies in the presence of iodide and lactoperoxidase.

Results: The rate of H_2O_2 consumption by *C. albicans* ATCC 10231 was 12.1 ± 2.7 nanomoles x min^{-1} per 10^6 blastoconidia (mean ± SD, N=8). Data from 7 wild strains (*C. albicans*) ranged from 5.5 to 22.3 nanomoles x min^{-1} per 10^6 cells (mean ± SD: 13.1 ± 5.1; median: 12.2). The survival rate of *C. albicans* ATCC 10231 in the presence of H_2O_2-KI-peroxidase over a period of 30 minutes was effectively shown to be dependent on blastoconidia count. Indeed, suspensions with 10^8 blastoconidia per ml require a higher H_2O_2 intake for killing than suspensions with 10^7 blastoconidia. *Ex vivo* denture decontamination confirmed the data obtained *in vitro*.

Conclusions: The presence of H_2O_2 as a substrate is critical for a peroxidase antifungal effect but could be removed by enzymes such as catalase from yeast cells themselves. H_2O_2 supply in peroxidase systems has to overpass its consumption by *Candida* cells themselves.

Key Words: Candida, Catalase, Dentures, Hydrogen peroxide, Peroxidase, Yeast

Introduction

Oral peroxidases have been known for several decades as innate non-immune factors, which are secreted by salivary glands (sialoperoxidase) or liberated from neutrophils (myeloperoxidase), and are able to control biofilm growth on oral surfaces [1,2]. The peroxidase substrates in the oral cavity are thiocyanate (SCN^-), secreted in saliva, and hydrogen peroxide (H_2O_2), and foremost produced by some microorganisms such as streptococci. Peroxidase activity in the oral cavity produces the powerful oxidant hypothiocyanite ($OSCN^-$), which is known to present antibacterial and antifungal effects *in vitro* [3-6]. Iodide is another possible substrate, *in vitro* as well, for oral peroxidases producing hypoiodite (OI^-), which is characterized by an antimicrobial action at a lower concentration than $OSCN^-$; nevertheless, iodide secretion in saliva is found to be marginal when compared to thiocyanate secretion [7]. Different authors have proposed the incorporation of peroxidase systems into oral care products to prevent oral and dental consequences of cariogenic, periodontopathic bacteria and yeast proliferation in oral biofilms, while others have proposed using peroxidase systems for patients with halitosis or dry mouth [8-14]. Nevertheless, few (if any) studies have demonstrated their efficiency by clinical trials, *a fortiori,* on a large scale [13]. Such investigations meet biological and clinical difficulties, which implies sophisticated designs and the cautious interpretation of data. Investigators need to test several control groups to evaluate 1°) the placebo effect due to the mechanical impact of brushing or rinsing, 2°) the presence of compounds as excipients and 3°) the stimulation of hygiene by the investigator's intervention [9,10,15]. Moreover, the choice of biological markers can reveal or mask a real effect. For instance, peroxidase use may affect metabolic parameters such as ATP content without decreasing the microbial count [16]: taking into account one rather than the other can lead to reverse conclusions. Finally, transposition of *in vitro* data to *in vivo* situations must take into account the complexity of the oral environment with numerous microorganisms organized in biofilms, and not in suspension, and with numerous bacterial or salivary molecules [17,18]. Thereby, in the case of peroxidase, the presence of H_2O_2 is critical for the antimicrobial effects: this could be absent or removed by enzymes such as catalase from bacteria themselves [6].

The present study aims to investigate the role of *Candida* H_2O_2 catabolism as a limitation for using the H_2O_2-iodide-lactoperoxidase system in denture decontamination from yeasts and to highlight so the need of a physiological approach for developing new oral care products which copy saliva.

Material and Methods

Microorganisms

Yeasts were grown aerobically at 37°C on Sabouraud-agar with chloramphenicol and gentamycin (BD Diagnostics™, Erembodegem, Belgium). All *in vitro* investigations were conducted on a third subculture of *C. albicans* ATCC 10231 (Culti-Loops™, Oxoid™, Basingstoke, UK), suspended in Sabouraud broth (OXOID™ CM147, Basingstoke, UK), in phosphate buffer (0.1 M, pH 7 with 0.1 g/l glucose) or in distilled water. The suspension was approximately adjusted with a 5-McFarland standard and then to an absorbance of

Corresponding author: Dr. Courtois Ph, Laboratory of Physiology and Pharmacology (CP 604), Université Libre de Bruxelles (ULB), Route de Lennik 808, B-1070 Brussels, Belgium; e-mail: philippe.courtois@ulb.ac.be

0.800 at 600 nm before dilution for blastoconidia count or CFU count. For the *ex vivo* investigations on dentures, yeasts were isolated and identified on the basis of their colony aspect on CHROMagar™ medium [19], by chlamydoconidia formation on PCB agar and by API yeast identification system.

Peroxidase systems

Two different peroxidase systems were tested on biofilm formation: a hydrogen peroxide (H_2O_2)/lactoperoxidase (LPO, 1 U_{ABTS}/ml)/iodide (KI, 1.2 mM) system, generating hypoiodite (OI^-), and H_2O_2 / LPO (1 U_{ABTS}/ml) / thiocyanate (KSCN, 1.2 mM), producing hypothiocyanite ($OSCN^-$). H_2O_2 was concomitantly produced by glucose-oxidase (GOD, from 12 mU/ml to 12 U/ml) and Glucose (G) at a concentration of 44.4 mM.

H_2O_2 assays

H_2O_2 degradation by yeast and H_2O_2 production by glucose-oxidase suspensions were evaluated by spectrophotometry at 230 nm and by chemiluminescence (lucigenin method), respectively. Both methods present limitations such as the interference of proteins and turbidity of *Candida* suspensions in the spectrophotometric readings at 230 nm, or the inhibition of chemiluminescence by ions, *inter alia* phosphate and salt ions. The spectrophotometric method [20] is based on H_2O_2 absorbance at 230 nm (extinction coefficient [21] in 0.1 M phosphate buffer pH 7: 0.071 $mM^{-1} \times cm^{-1}$); characteristics of the method are as follows: analytical range from 5 to 30 mM, coefficient of accuracy and of variation inferior to 1.5 and 5% respectively, initial H_2O_2 concentration of 10.2 mM. The chemiluminescence method [22] is based on the oxidation of lucigenin (20 µM) by H_2O_2 in an alkaline solution (8.3 mM Na_2CO_3 adjusted at pH 10.4) producing photon emission which was then measured on a biolumineter LKB 1250 (LKB-Wallac™, Turku, Finland); characteristics of the method are as follows: analytical range from 0.1 to 4 mM, coefficient of accuracy and of variation inferior to 1.5 and 5%, respectively.

Mycological investigations

Microscopically, *C. albicans* in suspension was in the form of budding cells called blastoconidia (round 5 µm in diameter). *C. albicans* ATCC 10231 was adjusted up to 10^8 blastoconidia / ml. All swabs from resin pieces or dentures were inoculated on Sabouraud agar with chloramphenicol and gentamycin (BD Diagnostics™, Erembodegem, Belgium), which were incubated for 2 days at 37°C before Colony Forming Unit (CFU) count was performed.

Biofilms on resin pieces

Acrylic resin pieces (thickness ~2 mm, size 25 mm x 5 mm) were processed according to the manufacturer's instructions and stored at 4°C in sodium azide 0,1 % (w/v) to test the effect of peroxidase systems *in vitro*. For biofilm production, each resin piece was washed 7 times in 4 ml sterile distilled water and immersed in 4 ml Sabouraud liquid medium with 50 µl of *C. albicans* ATCC 10231 suspension adjusted at 0.800 optical density on a Beckman™ DU®-65 spectrophotometer at 600 nm. After 3-days incubation at room temperature with continuous rotary agitation (Stuart® rotator/SB3, Staffordshire, UK) at 6 rpm, the liquid phase was aspirated and each resin piece transferred into a 15 ml Falcon® polypropylene conical tube (Becton Dickinson™, Franklin Lakes, NJ, USA). Resin pieces were then incubated at 37°C during 30 min in the presence of peroxidase systems (total volume: 4 ml). At the end of incubation time, peroxidase solution was aspirated and resin pieces plated onto Petri dishes.

Dentures

Denture wearers were free of denture stomatitis or any other oral disease and were not exposed to antifungal or antibacterial medication. *Ex vivo* yeast decontamination by peroxidase systems was performed on a total of 23 maxillary complete dentures which were previously shown to be *Candida sp.* positive by swabbing the fitting surface of the denture. *Ex vivo* decontamination procedure consisted in diving denture in peroxidase solution during 30 minutes before a second swabbing.

Statistics

Data were analyzed through unpaired t-test of Student, ANOVA, Chi-square and Fischer's exact tests using the GraphPad Prism version 5.00 (GraphPad Software™, San Diego, California, USA). Mean values were expressed with their related standard error of the mean, unless indicated otherwise.

Results

***Candida* count**

Candida cell number in suspension was evaluated by blastoconidia count in a Thoma cell, by Colony Forming Unit (CFU) count on Sabouraud solid medium and by turbidimetry on a spectrophotometer at 600 nm. *Table 1* reports the data obtained by both counting procedures simultaneously performed on *C. albicans* suspensions from ATCC 10231 and wild strains after adjustment by turbidimetry at a 600 nm absorbance of 0.800. In ATCC 10231 suspensions, direct microscopic count of blastoconidia (bl) in a Thoma cell and CFU count after culture on Sabouraud solid medium were 21.42 ± 0.92 x 10^6 bl/ml (N=12) and 13.00 ± 0.77 x 10^6 CFU/ml (N=12) respectively: both procedures provided data which were highly significantly different (unpaired t-test, $p<0.0001$). In *C. albicans* isolated from dentures, microscopic and CFU counts were closer: 22.93 ± 1.14 x 10^6 bl/ml (N=7) and 19.54 ± 1.37 x 10^6 CFU/ml (N=7), respectively (unpaired t-test: NS, p=0.0814). Direct counting by microscopy in reference strain suspensions (adjusted at a 600 nm absorbance of 0.800) thus provided data which approximated those found in wild strain suspensions prepared in the same manner (unpaired t-test: NS, p=0.3243). However, CFU count in 0.800 adjusted suspensions is highly significantly lower in reference strain suspensions than in wild suspensions (unpaired t-test, p=0.0003). Consequently, the ratio of blastoconidia to CFU count was 1.72 ± 0.12 (N=12) for the reference strain and 1.21 ± 0.12 (N=7) for the wild strains: this difference is significant (unpaired t-test, p=0.0125).

Figure 1 shows the relationship between the absorbance at 600 nm and the number of blastoconidia present in the suspension as part of a rectangular hyperbola with the

Table 1. *Evaluation of 2 different procedures for Candida count in H_2O suspension adjusted at a 600 nm absorbance of 0.800, from ATCC 10231 and wild strains. Count data are expressed as millions of blastoconidia cells or CFU per ml (mean ± SEM).*

	Microscopic count (10^6 cells/ml)	CFU count (10^6 CFUs/ml)
ATCC 10231	21.42 ± 0.92 (N=12)	13.00 ± 0.77 (N=12)
Wild strains	22.93 ± 1.14 (N=7)	19.54 ± 1.37 (N=7)

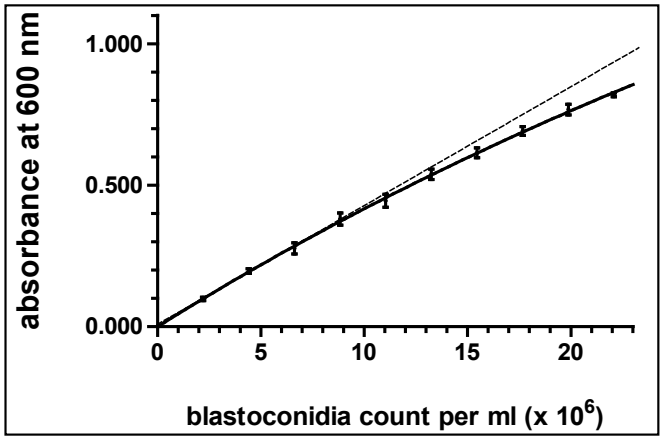

Figure 1. *Relation between absorbance at 600 nm and Candida blastoconidia count (blastoconidia cell / ml x 10⁶). Error bars in this graph indicate the standard deviation (N = 3 from one C. albicans ATCC 10231 suspension).*

equation y=4.5x / 98.7 + x (r=0.9985). In these experiments (N=3), a 20-fold dilution of a suspension containing 20 × 10^6 blastoconidia per ml decreased the 600 nm absorbance from 0.813 ± 0.007 to 0.049 ± 0.002, that to say by a ratio of 16.7. A supplementary set of experiments (N=6) confirmed this ratio: a 20-fold dilution of *Candida* suspension decreased the suspension absorbance from 0.794 ± 0.007 to 0.048 ± 0.002 (ratio: 16.5).

Figure 2 shows the variation in blastoconidia number (expressed as the percentage of the initial value) after 30-180 minutes incubation in water *versus* Sabouraud broth. In water, blastoconidia number at 30 min fluctuated from 95.2 ± 6.4% of the initial amount (N=6) and 113.9 ± 8.1% (N=6) at 150 min. In broth, blastoconidia count varied between 107.0 ± 5.2% (N=6) at 60 min and 131.3 ± 10.4% (N=6) at 180 min. Data observed in water remained within the range of the mean ± 3 SEM for 180 min. Nevertheless, in broth, the count data remained within the same range for only 90 min.

Measuring H_2O_2 consumption by blastoconidia

Blastoconidia of *C. albicans* ATCC 10231 decreased the absorbance at 230 nm of different H_2O_2 solutions. *Figure 3a* (upper graph) shows that the H_2O_2 consumption by *C. albicans* ATCC 10231 blastoconidia is directly proportional to blastoconidia count. The equation of the fitted line is y=11,3 x and the regression coefficient r is 0.9575. These results are representative of six independent experiments. *Figure 3b* (lower graph) illustrates the relation between the H_2O_2 consumption rate by yeast cells and its initial concentration, which can be assimilated to a rectangular hyperbola: y=138.6 x/(123.7 + x), with a coefficient of regression r equaling 0.9940. The maximum rate (V_{max}) corresponded to a H_2O_2 concentration outside the superior analytical range of the spectrophotometric method (30 mM). The Lineweaver-Burk double-inverse plot linearized the rectangular hyperbola (fitting equation: y=0.78 × - 0.01; regression coefficient: r=0.9872), where x-intercept corresponding to $-1/K_m$ and the y-intercept to $1/V_{max}$ allowed the calculation of an apparent K_m of 55.1 mM and a theoretical V_{max} of 70.6 nanomoles/min. The consumption rate of H_2O_2 increased from 5.7 nmol for 0.5 × 10^6 blastoconidia to 28.3 nmol for 2.5 x 10^6 blastoconidia.

H_2O_2 consumption rate by *C. albicans* ATCC 10231 was 12.1 ± 2.7 nanomoles x min^{-1} per 10^6 blastoconidia (mean ± SD, N=8). Data from 7 *C. albicans* wild strains ranged from 5.5 to 22.3 nanomoles x min^{-1} per 10^6 cells (mean ± SD: 12.1 ± 2.7; median: 12.2). This H_2O_2 consumption did not vary from one subculture to another. *Figure 4* compares the results obtained in three successive subcultures for 7 clinical strains of *C. albicans*: H_2O_2 consumption did not differ in 3 serial

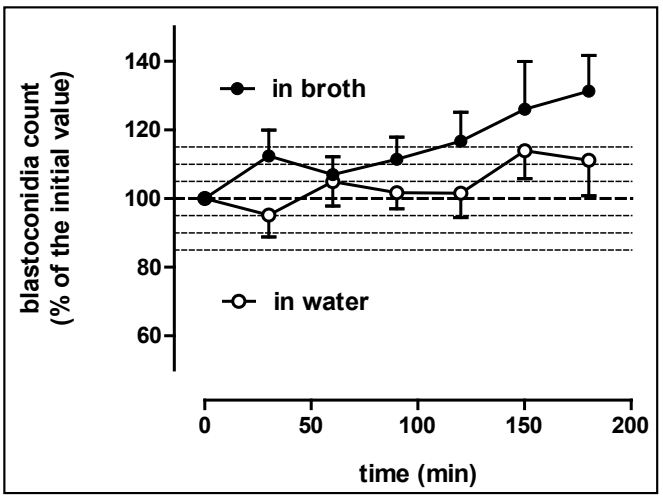

Figure 2. *Blastoconidia survival rate in water versus blastoconidia growth in broth related to time (min). The data are expressed as the percentage of the initial value. Error bars in this graph indicate the standard error of the mean (N = 6).*

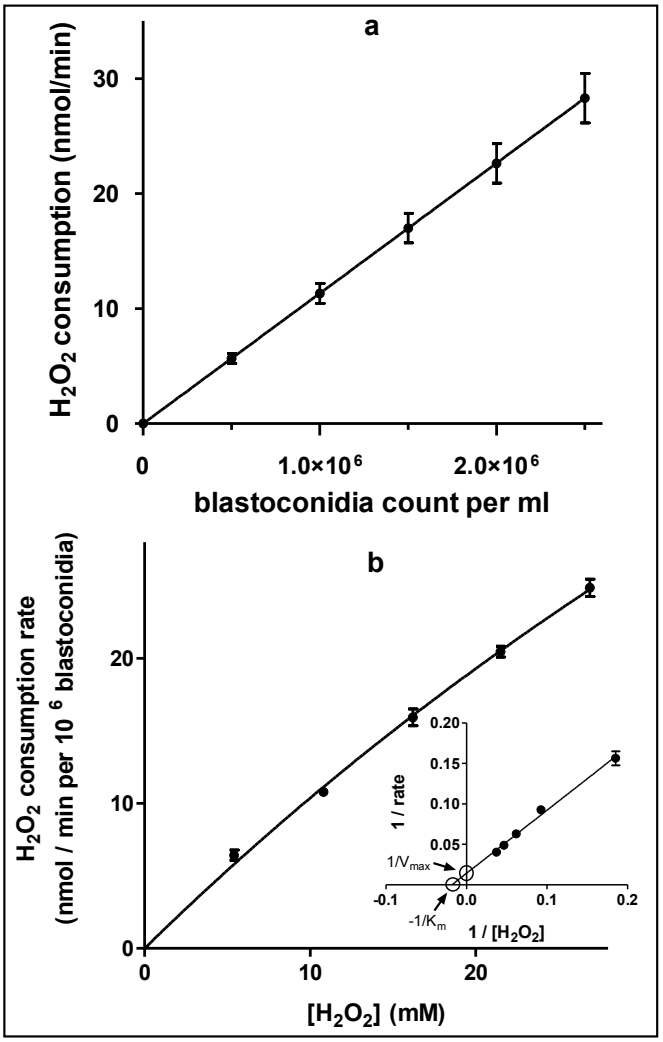

Figure 3. *H_2O_2 consumption by yeast cells. Upper graph **a**: relationship between H_2O_2 disappearance rate and blastoconidia count.*
***b**: Lower graph: kinetic aspects of H_2O_2 consumption by yeast cells, with Lineweaver-Burk double inverse plot as insert.*

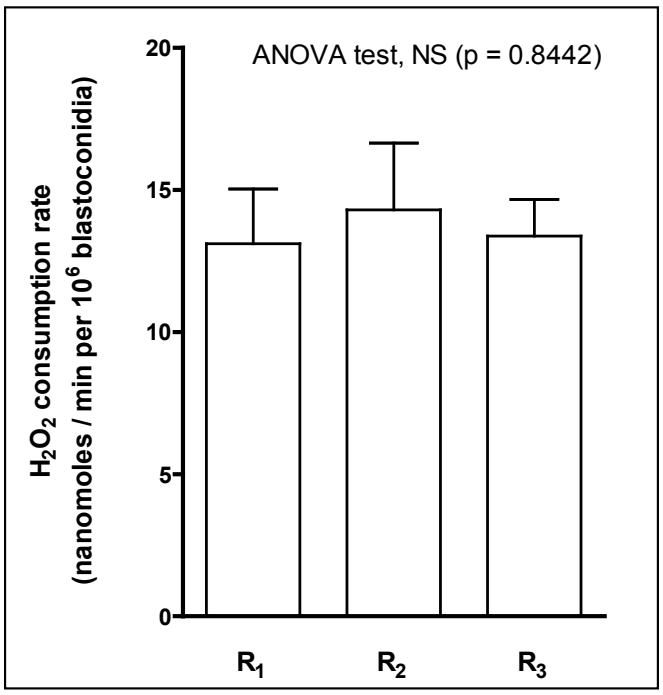

Figure 4. *H_2O_2 consumption in 3 successive subcultures (R_{1-3}) of Candida albicans clinical isolates (N=7).*

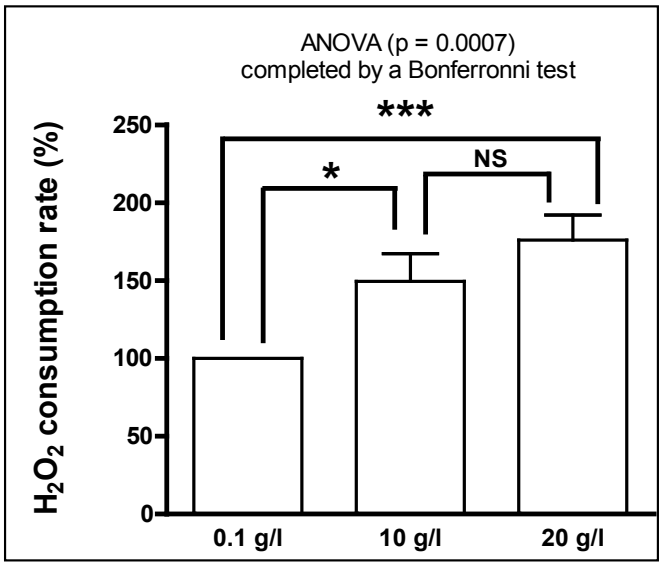

Figure 5. *H_2O_2 consumption per minute by clinical Candida albicans isolates at 3 different concentrations of glucose. The data (mean ± SEM) are expressed as the percentage of the value found at the lowest glucose concentration.*

subcultures (ANOVA: p=0.8442, NS) averaging 13.1, 14.3 and 13.4 nmol/min per 10^6 blastoconidia. *Figure 5* shows the effect of glucose concentration in the reaction medium on H_2O_2 consumption by blastoconidia. Increasing the amount of glucose in the reaction mixture significantly multiplied the H_2O_2 consumption by blastoconidia. Indeed, the increase of glucose concentration from 0.1 g/l to 10 or 20 g/l multiplied H_2O_2 consumption by 1.5 and 1.8, respectively, in a significant manner (ANOVA complemented by a Bonferroni test: $p < 0.01$).

Measurement of H_2O_2 production by G/GOD

Table 2 reports data concerning the H_2O_2 production by GOD (1.2 U/ml and 12 U/ml) after 30-min incubation in H_2O at 37°C. As expected, at 37°C, 12 U/ml GOD activity induced a stronger production of H_2O_2 (1.04 ± 0.04 µmol/ml, N=7) than 1.2 U/ml solution (0.45 ± 0.02 µmol/ml, N=7): the impaired

Table 2. *H_2O_2 concentration reached after a 30 min-incubation of glucose (44.4 mM/glucose oxidase (1.2 versus 12.0 U/ml) in distilled water at 37°C. H_2O_2 was assayed by the lucigenin method.*

	GOD (1.2 U/ml)	GOD (12.0 U/ml)
mean value ± SEM (µmol/ml) CV (%)	0.45 ± 0.02 14.2	1.04 ± 0.04 10.5

t-test showed a highly significant difference ($p < 0.0001$). *Figure 6a-b* examines the production of H_2O_2 in an aqueous medium by GOD in the absence of peroxidase LPO as a function of GOD activity (upper graph 6a) and as a function of time (lower graph 6b). In the presence of 44.4 mM glucose, increasing GOD activity in a manner of geometric progression from 12 mU/ml to 12 U/ml multiplied H_2O_2 production by a factor of nearly 15 (after 30 min at 37°C).

Detoxification of H_2O_2 by catalase

Figure 7 illustrates the inhibition of *C. albicans* ATCC 10231 growth in liquid medium (monitored by turbidimetry at 600 nm) in the presence of the glucose (155 mM)/glucose oxidase (12 U / ml) system after incubation for 5 hours. The absorbance at 600 nm was 0.170 ± 0.004 (N=6) in the control suspensions but absorbance was only 0.033 ± 0.002 (N=6) in the presence of G/GOD (12 U/ml). The addition of catalase (5000 U/ml) to G/GOD solution removed this inhibitory effect: the absorbance is then equal to 0.185 ± 0.007 (N=6) after 5-hours of incubation. In the presence of G/GOD without catalase, growth inhibition was statistically significant (ANOVA completed by Bonferroni test $p<0.001$) from the control and G/GOD/catalase conditions. *Figure 8* reports the survival rate of planktonic yeast cells in the presence of the G/GOD/KI/LPO system as a function of the amount of GOD-produced H_2O_2 during 30-min of incubation. The system was then evaluated using 2 different *Candida* suspensions: one containing 10^7 blastoconidia per ml, and the other containing 10^8 blastoconidia per ml.

The first suspension was very sensitive to the peroxidase system, while the second was more resistant. *Figure 8* presents the mean values of 7 independent experiments.

Effect of G/GOD/KI/ peroxidase system on *in vitro* preformed *Candida*-biofilms

Figure 9 compares the candidacidal effect of hydrogen peroxide, hypothiocyanite and hypoiodite enzymatically produced at 37°C or room temperature. Two different amounts of GOD (1.2 and 12 U/ml) were evaluated. Only hypoiodite produced by a peroxidase system at 37°C containing 12 U of GOD per ml reduced the *Candida* number harvested from biofilms on resin foils down to at least 2 log units (Kruskal-Wallis test complemented by a post-test of Dunns: $p<0.01$). *Figure 10* shows *Candida* blastoconidia adherent on resin foils after 3 washings.

***Ex vivo* investigations**

Table 3a illustrates the effect of G (8 mg/ml)/GOD (1.2 U/ml) *versus* the G (8 mg/ml) / GOD (1.2 U/ml) / KI (0.2 mg/ml)/LPO (1 ABTS U/ml) system in *ex vivo* denture decontamination at room temperature. The colony count after swabbing showed a decrease of at least 1 logarithmic unit in 1 out of 5 cases after denture immersion in G/GOD solution and in 2 out of 5 cases after immersion in the G/GOD/KI/LPO mixture, when compared with the control swabbing before decontamination. A Chi-square test (p=0.4762) failed to show any significant difference between the groups. *Table*

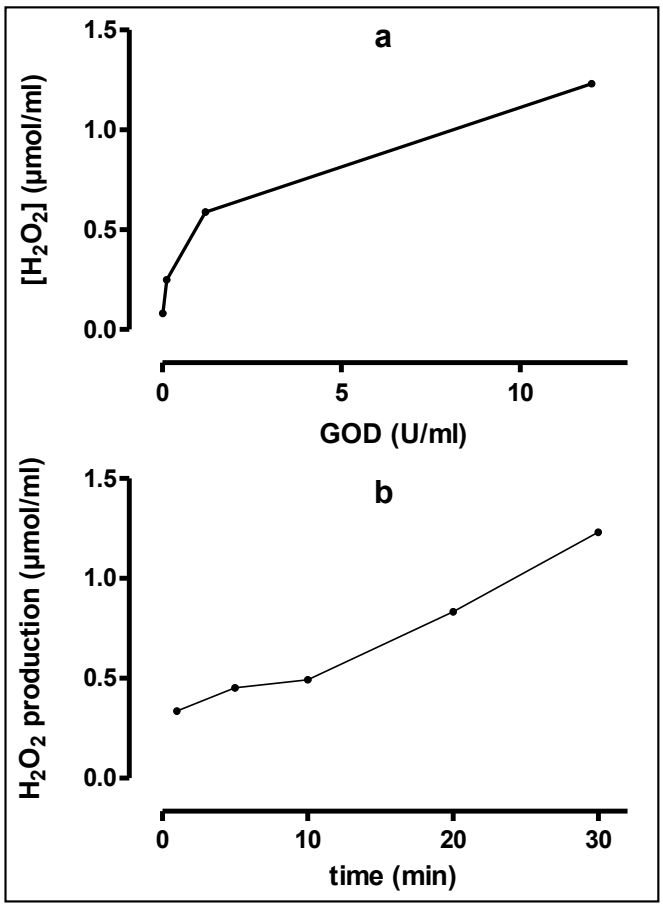

Figure 6. *Upper graph* ***a)*** *H_2O_2 (µmol/ml) produced by G/GOD at different GOD activities after a 30-min incubation.* ***b).*** *Lower graph: time course of H_2O_2 production (µmol/ml) in the presence of 12 U/ml GOD activity.*

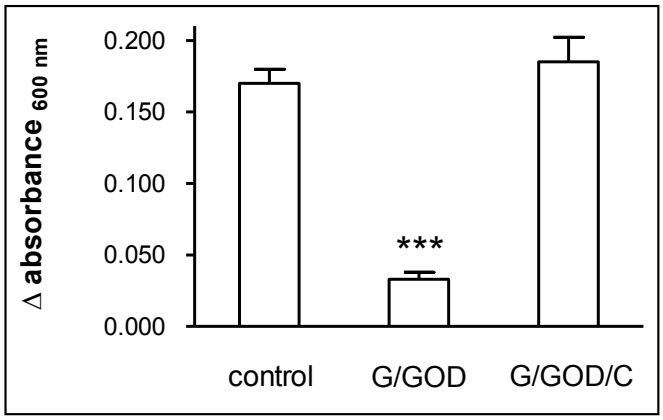

Figure 7. *Effect of catalase (C) on the inhibition of yeast growth by the G/GOD system at 12 U/ml GOD after a 5-hours incubation.*

3b illustrates the effect of 30-min of incubation at 37°C in the presence of a lactoperoxidase system similar to the first one except for a higher GOD activity (12 U/ml). After swabbing, the CFU count showed a decrease of at least 1 logarithmic unit in 6 out of 6 cases when the dentures were immersed in the solution, while incubation in water caused no significant change. A Chi-square test (p=0.0005) confirmed a highly significant difference.

Discussion

For several decades, peroxidase systems have been incorporated in oral hygiene products on the basis of *in vitro* testing against strains isolated from oral microflora, while *in vivo* data and clinical trials are still lacking to prove their

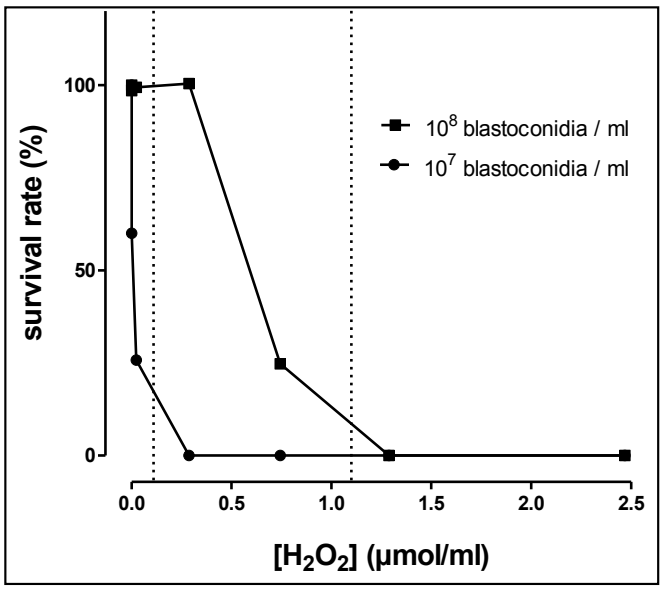

Figure 8. *C. albicans ATCC 10231 blastoconidia survival (%) as a function of H_2O_2 (mM) in 2 yeast suspensions containing respectively 10^7 (●) and 10^8 (■) blastoconidia/ml. Dotted lines indicate theoretical detoxification cut-off inferred from measuring the H_2O_2 consumption per min by blastoconidia in Figure 3 data.*

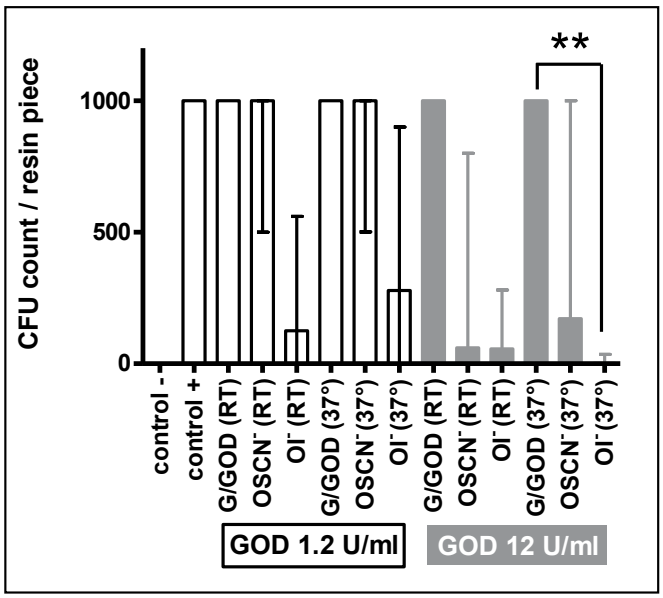

Figure 9*. Decontamination of resin foils upon preformed Candida-biofilm by peroxidase systems after a 30 minutes incubation at 37°C. The height of the columns corresponds to the median values and bars to the lower and upper limit rounded to 1000 CFU. Statistical test: Kruskal-Wallis complemented by a post-test de Dunns (**=p < 0.01).*

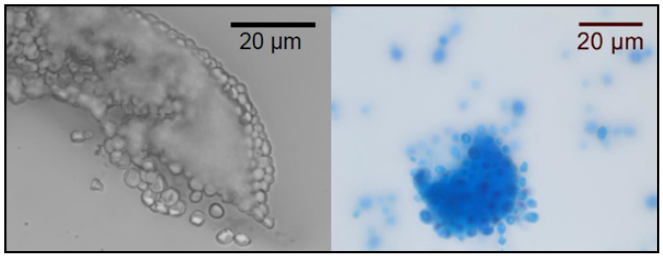

Figure 10*. Candida blastoconidia on a resin granule (magnification: 400 x). Left: direct examination; right: Blue Cotton staining.*

in vivo efficiency in the oral environment. H_2O_2 degradation by some bacteria and yeast (unless a critical cut-off level of abundance is overpassed) can explain the *in vivo* inefficiency of peroxidase systems which need H_2O_2 as a substrate.

Table 3. *Ex vivo decontamination of dentures by KI-lactoperoxidase system. 3a (upper part): G/GOD/KI/L versus G/GOD at room temperature; 3b (lower part): G/GOD/KI/LPO versus H_2O at 37°C.*

	G/GOD	G/GOD/KI/LPO
reduction	1	2
no reduction	4	3
total	5	5
Chi-square: p=0.4762 (N=10), NS Fisher's exact test: p=1.0000 (N=10), NS		
	H_2O	**G/GOD/KI/LPO**
reduction	0	6
no reduction	6	0
total	6	6
Chi-square: p=0.0005 (N=6) Fisher's exact test: p=0.0022 (N=6)		

Moreover, oral microflora biofilm mode of life is presented as an additional factor of resistance to antimicrobials.

Evaluation of procedures

This study investigated the balance between a H_2O_2 donor system and the H_2O_2-detoxification power in *C. albicans* strains submitted to the H_2O_2/SCN^-/I^-/LPO system. It aimed to validate *Candida* count procedures and to evaluate yeast survival rate under different experimental conditions dictated by chemical (pH, inhibitors...), enzymatic (optimal pH, substrates concentration, kinetic aspects...) and bacteriological (sterility, growth conditions...) requirements. The optimal pH for biofilm formation and for GOD activity covers a comparable wide acidic range of 4.0-7.0 and 5.0-6.5, respectively [23,24]. CFU count (sometimes) provides results that are slightly inferior to blastoconidia count, probably because budding cells and aggregated cells are distinctly counted under a microscope, but both initiated single colony forming units on Petri dishes. *C. albicans* isolates survived in water without growth for at least 2 hours, allowing the preparation of different reaction media for investigations, while the same strain started to grow after 90 minutes when these cells were re-suspended in fresh broth. All of the experiments were performed on a third sub-culture: H_2O_2 consumption was shown to remain stable in 3 serial subcultures (ANOVA, NS, p=0.8442). In the absence of peroxidase, G/GOD produced enough H_2O_2 to reduce *Candida* growth when GOD activity was 12 U/ml (*Figure 7*), but H_2O_2-degrading enzyme activities (foremost catalase and glutathione peroxidase) were able to reverse this anti-*Candida* effect. In the presence of a peroxidase sequence (with KI or KSCN substrate), no H_2O_2 could be measured, implying that the anti-*Candida* effect of peroxidases systems is due to the products (OI^-/$OSCN^-$) of peroxidase activity and not due to the H_2O_2 provided. At a H_2O_2 concentration that is not effective against *Candida*, the addition of peroxidase with a substrate to H_2O_2 donors makes the combination antifungal; the beneficial effect of such a mixture has already been described [1,4,6,25]. Evaluation of H_2O_2 production and consumption depended on the reaction medium composition: proteins which absorb in the UV spectrum make the chemiluminescence method more suitable for H_2O_2 production measurement, while phosphate buffer ions and salts impede chemiluminescence, making the spectrophotometric method more appropriate for H_2O_2 consumption measurements. Whole fungal cells caused a manageable turbidity that was compatible with spectrophotometry, but inconvenient for lucigenin chemiluminescence. Interestingly, both procedures for H_2O_2 measurement used in this investigation were sensitive enough to fulfill the objectives of this study.

In vitro **investigations**

The rate of H_2O_2 consumption by *C. albicans* ATCC 10231 (12.1 ± 2.7 nanomoles x min^{-1} per 10^6 blastoconidia, mean ± SD, N=8) was found to be close to that obtained from 7 clinical strains which ranged from 5.5 to 22.3 nanomoles x min^{-1} per 10^6 cells. Increasing the glucose concentration from 0.1 to 10 or 20 g/L multiplied the rate of H_2O_2 disappearance by a factor of 1.5 and 1.8, respectively: this observation supports the probability of fluctuation in the oral environment, where glucose depends on alimentary intakes. Analysis of H_2O_2 consumption by blastoconidia indicated that the rate of disappearance obeys Michaelis-Menten laws, with an apparent K_m calculated by the double-reciprocal Lineweaver-Burk plot. This investigation did not envisage further the molecular pathways leading to H_2O_2 decrease in a medium containing *C. albicans* blastoconidia which were known to be catalase-positive. The presence of catalase in *C. albicans* can be related to the high H_2O_2 concentration (from 4 to 88 mM quoted in the literature [25]) required to inhibit clinical isolates. Previous studies [26-28] have demonstrated a protective role of catalase upon H_2O_2 in *Escherichia coli* and *Pseudomonas aeruginosa* biofilms by studying the association of catalase-positive and -negative strains. These authors concluded on a group protection effect rather than an individual effect allowing catalase-negative bacteria to survive in the presence of H_2O_2-producing micro-organisms when left in aggregates such as biofilms. In this study, planktonic *Candida* inhibition by the peroxidase system has been shown to be effective when the GOD-produced H_2O_2 concentration was superior to the capacities of yeast suspensions to metabolize it. Moreover, this study reported that the KI/peroxidase system became efficient with regard to *Candida* biofilm preformed on resin foils in comparison to the mere G/GOD sequence when a higher GOD activity was supplemented (from 1.2 to 12 U/ml) and when the enzyme solution was incubated at 37°C *versus* room temperature, as this contributed to increasing the enzyme activity as well. A previous investigation had already demonstrated a higher efficacy for hypoiodite than for hypothiocyanite produced by the SCN^-/peroxidase system [29].

Ex vivo **investigations**

A previous paper [15] investigated the different species usually found on dentures. Swabbing of the palatal side of the prosthesis was yeast-positive in 66 out of 87 subjects (75.9%) while the adjacent mucosal surface was positive in 72.4% of cases; 68.2% of subjects simultaneously showed contamination of both denture and mucosa. The 3 species mainly isolated were *C. albicans* (77.9% of yeast-positive subjects), *Candida glabrata* and *Candida tropicalis* (44.1 and 19.1% of swabs respectively). The contaminated maxillary dentures were shown heavily colonized (more than 50 CFU) in 90.9% cases and more than one yeast species was found in 50.0% of contaminated dentures. Moreover, other studies [30] reported that denture wearers often develop denture stomatitis linked to *C. albicans* contamination, what could be prevented by adequate prosthesis care.

In the present investigation, the data obtained *in vitro* were used to define suitable conditions to decontaminate *Candida*-

positive dentures by immersing them in a solution containing the G/GOD/KI/LPO system at 37°C for 30 min. The dentures were swabbed before and after a 30-min immersion in the enzyme solution at 37°C. Data were compared with those obtained after immersion in G/GOD alone or in water. The G/GOD (1.2 U/ml) and KI/lactoperoxidase systems were ineffective at room temperature. In contrast, a reduction superior to one logarithmic unit was always observed when the GOD activity was 12 U/ml in a G/GOD/KI/LPO system incubated at 37°C.

Conclusion

To be efficient, H_2O_2 supply in peroxidase systems has to overpass its consumption by *Candida* cells themselves. The survival rate of *C. albicans* ATCC 10231 in the presence of H_2O_2/KI/peroxidase for 30 minutes was effectively shown to be dependent on yeast capacity to decrease H_2O_2 levels. Similarly, *ex vivo* denture decontamination by the peroxidase system is possible with higher GOD activities that are able to overpass H_2O_2 diversion by oral biofilms. These data have to be considered as a base to develop further clinical studies on a larger scale, illustrating well the need to deepen the knowledge of the physiological oral complexity before developing oral care products which copy saliva.

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Conflict of interests

The authors declare no conflict of interests.

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