Original Article

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Synergistic anti-candida activities of lactoferrin and the lactoperoxidase system

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Summary ***Candida albicans*** **is a commensal fungus in human mucosal surfaces, including the oral cavity. Lactoferrin (LF) and the lactoperoxidase (LPO) system, which are host protection components in exocrine secretions, each exhibit weak anti-candida activity. We herein examined the effects of the combination of LF and the LPO system on *C. albicans*. Morphological observations indicated that the combination of LF and the LPO system reduced the mycelial volume of *C. albicans* and changed the size and shape of cells more than each agent alone. The combination of LF and the LPO system also exerted strong inhibitory effects on the cellular metabolic activity and adhesive hyphal form of *C. albicans*. A checkerboard analysis revealed that the anti-candida activity of LF and the LPO system was synergistic. These results suggest that the combination of LF and the LPO system is useful for preventing candidiasis.**

Keywords: Lactoferrin, lactoperoxidase, *Candida albicans*

1. Introduction

Candida albicans is a commensal fungus in human mucosal surfaces, including the oral cavity. Oral candidiasis is an opportunistic infection of the oral cavity caused by the overgrowth of *Candida* spp., which causes tongue pain, a burning feeling, bad breath, and decreased quality of life. Reduced saliva production, poor oral hygiene, the long-term use of antibiotics, smoking, chemotherapy, and aging have been identified as risk factors for oral candidiasis (*1*). Although antifungal agents are used to treat oral candidiasis, their repeated usage is associated with the creation of resistant microorganisms (*2,3*).

Lactoferrin (LF) and lactoperoxidase (LPO) are glycoproteins found in saliva, milk, vaginal secretions, and other exocrine secretions and play a role in host protection (*4-6*). LF exerts antimicrobial effects against fungi such as *Candida* (*7,8*). LPO catalyzes the hydrogen peroxide-dependent oxidation of thiocyanate (SCN^-), a component in saliva, to hypothiocyanite ($OSCN^-$), which is a potent antimicrobial agent against fungi, bacteria, and viruses (*5,6*). This antimicrobial system is called the LPO system. The combination of LPO, glucose oxidase (GO), glucose, and buffer salts was previously shown to exhibit *in vitro* antimicrobial activity against periodontal bacteria and *C. albicans* through the formation of $OSCN^-$ in the presence of SCN^- (*9-11*). The candidacidal activity of LF or the LPO system alone is moderate (*8,10*). Although the combined use of LF with antifungal drugs has been reported to enhance anti-*candida* effects (*12*), it cannot be taken continuously and safely on a daily basis. In the present study, we assessed the combined effects of LF and the LPO system on the cellular metabolic activity of *C. albicans* using the alamarBlue assay. We also investigated the *in vitro* antifungal effects of the combination of LF and the LPO system against *C. albicans* using a crystal violet staining method to quantify adhesive hyphal cells, which are related to pathogenicity. The aim of the present study was to develop a new method for the prevention of oral candidiasis.

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2. Materials and Methods

2.1. *Materials*

Bovine LF was produced by Morinaga Milk Industry Co., Ltd. (Tokyo, Japan). Components of the LPO system consisted of 43 mg/g of LPO purified from bovine milk (Tatua, Morrinsville, New Zealand), 430 mg/g of GO purified from *Penicillium chrysogenum* (Shin-Nihon Chemical, Aichi, Japan), 450 mg/g glucose, 24 mg/g citric acid, and 53 mg/g sodium citrate. 66 mM sodium thiocyanate solution was prepared and sterilized with filtration. LF and the LPO system were dissolved in 0.66 mM sodium thiocyanate.

Hypothiocyanite ($OSCN^-$) solution was produced enzymatically as described previously (*13*). In brief, 0.16 mg/mL LPO solution with 7.5 mM sodium thiocyanate and 3.75 mM H_2O_2 in 10 mM potassium phosphate buffer (pH 6.6) was incubated at room temperature for 15 min. After stopping the reaction by the addition of catalase, LPO and catalase were removed by centrifugation through a 10-kDa molecular mass cut-off filter (Amicon Ultra, Merck, Darmstadt, Germany) at 5000× g for 15 min. The concentration of $OSCN^-$ was measured by monitoring the reaction with 5-thio-2-nitrobezonic acid (*14*).

2.2. *Preparation of medium for C. albicans hyphal formation*

One milliliter of fetal calf serum (FCS, Thermo Fisher Scientific, MA, USA) was aseptically added to 39 mL of RPMI 1640 medium (Sigma Chemical Co., Mo., US) with L-glutamine and sodium bicarbonate (Sigma-Aldrich, MO, USA), aseptically supplied with 0.03 g of penicillin (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan), 0.03 g of kanamycin (FUJIFILM Wako Pure Chemical Corporation) and 2.39 g of HEPES (4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid, Dojindo, Kumamoto, Japan) in 500 mL of the same medium. This 2.5% FCS containing RPMI 1640 medium was three-fold diluted by adding sterile distilled water. The diluted FCS-RPMI medium was used in this study.

2.3. *Assay for the inhibition of the cellular metabolic activity of C. albicans*

C. albicans TIMM1768, a clinically isolated serotype A strain obtained from the Teikyo University Institute of Medical Mycology, was used in experiments. Cells were cultured on Sabouraud dextrose agar, which contained 1% Bactopeptone (Becton Dickinson, MD, US), 2% glucose, and 1.5% agar, and suspended the diluted FCS-RPMI medium. One hundred microliters of the *C. albicans* suspension (5×10^3 cells/mL) in a 96-well microtiter plate was prepared. After the addition of 100-μL serial dilutions of LF and the LPO system, the microtiter plate was incubated at 37°C for 16 h in 5% CO_2 in air. After the incubation, 20 μL of alamarBlue (Thermo Fisher Scientific) was added to each of the wells. Samples were incubated at 37°C for 20 h in 5% CO_2 in air. Absorbance at 570 and 594 nm was measured spectrophotometrically. The percent inhibition of cellular metabolic activity was calculated as follows. 100 − (absorbance (samples)/absorbance (control)) × 100 (%).

2.4. *Assay for the growth inhibition of C. albicans in the hyphal form*

The assay for the hyphal growth of *C. albicans* was performed using the method previously reported (*15,16*). The assay was performed under the same culture conditions as described above. One hundred microliters of the *C. albicans* suspension (5×10^3 cells/mL) in a 96-well microtiter plate (MS-8096-F, Sumitomo Bakelite, Tokyo, Japan) was prepared. After the addition of 50-μL each of serial dilutions of LF and the LPO system to well of plate, the microtiter plate was incubated at 37°C for 16 h in 5% CO_2 in air. The hyphal growth content of *C. albicans* was assessed by the crystal violet staining assay. The medium in the wells was discarded by inverting the microplate. Adhesive *Candida* mycelia were sterilized by immersion in 70% ethanol. The plate was washed twice in distilled water. Mycelia were stained with 0.01% crystal violet (Merck, Darmstadt, Germany) for 20 min and washed 3 times with water. After drying the microplate, 150 μL of isopropanol containing 0.04 N HCl and 50 μL of 0.25% sodium dodecyl sulfate were added to the wells. Samples were mixed by a plate mixer for 2 min to extract crystal violet from mycelia. The absorbance at 620 nm of triplicate samples was measured spectrophotometrically. The percent inhibition of *Candida* was calculated as follows: 100 − (absorbance (samples)/absorbance (control)) × 100 (%).

A checkerboard analysis was used to assess anti-*candida* combinations, and the results obtained were evaluated based on standard criteria (*17*). Concentrations of the combination of LF and the LPO system showing 50% inhibitory concentration (IC_{50}) against *C. albicans* were assessed by the crystal violet staining method and plotted in closed circles on arithmetic scales. The fractional inhibitory concentration (FIC) index was calculated as follows: (lowest inhibitory concentration of LF in combination/IC_{50} of LF alone + lowest inhibitory concentration of the LPO system in combination/IC_{50} of the LPO system alone). FIC index values of ≤ 0.5, 1.0, and > 4.0 represented synergism, additivity, and antagonism, respectively.

2.5. *Statistical analysis*

Statistical analyses were performed between two groups using the two-tailed Student's *t*-test. *P* values of < 0.05

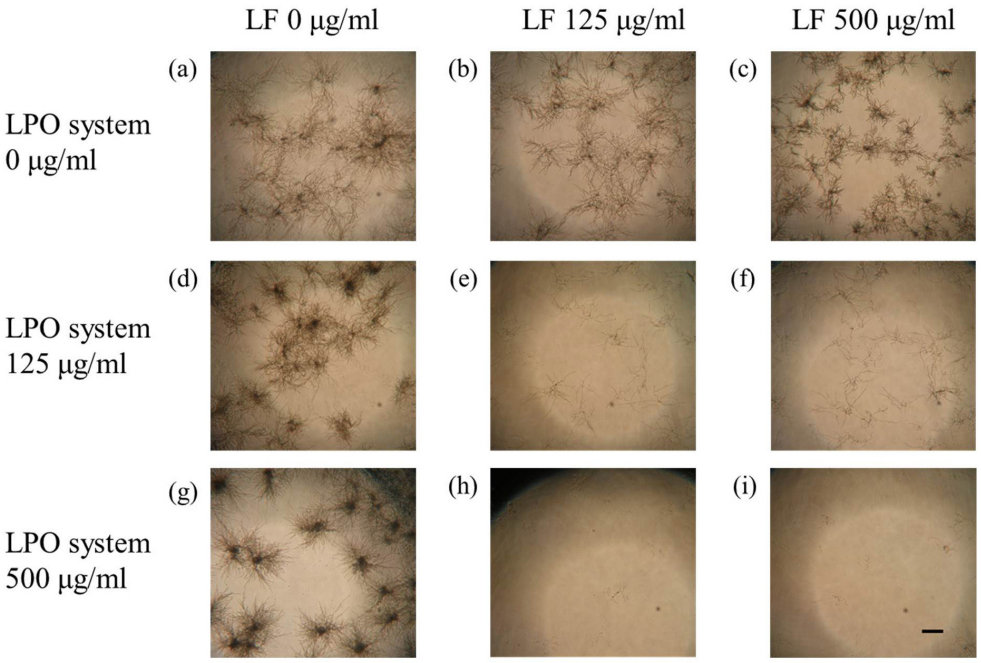

Figure 1. Phase-contrast micrographs of the hyphal growth of *C. albicans*. Approximately 5×10^3 cells/mL of *C. albicans* TIMM1768 were incubated with different doses of LF and the LPO system at 37°C for 16 h in 5% CO_2 in air. **(a)** Control culture showing the prominent development of hyphae; **(b)** culture with LF (125 μg/mL) alone showing the unchanged development of hyphae; **(c)** culture with LF (500 μg/mL) alone showing fewer hyphae; **(d)** culture with the LPO system (125 μg/mL) alone showing a slightly changed hyphal shape; **(e, f)** culture with LF (125 or 500 μg/mL) and the LPO system (125 μg/mL) showing a marked reduction in the mycelial volume of *C. albicans* and changes in the size and shape of cells; **(g)** culture with the LPO system (500 μg/mL) alone showing the changed hyphal shape of *C. albicans* to a slightly more isolated and smaller colony-like appearance; **(h, i)** culture with LF (125 or 500 μg/mL) and the LPO system (500 μg/mL) showing almost no hyphae. Bar, 100 μm.

were considered to indicate a significant difference.

3. Results

3.1. *Effects of LF and the LPO system on the growth morphology of C. albicans*

The effects of LF and the LPO system on the hyphal form of *C. albicans* were investigated morphologically. Figure 1 shows the hyphal growth of *C. albicans* after an incubation at 37°C for 16 h. LF alone at 500 μg/mL did not reduce the mycelial volume of *C. albicans*; however, morphologically, the hyphal length of *C. albicans* under these conditions was shorter than those of the control (Figure 1c). The LPO system alone at 500 μg/mL morphologically altered the hyphal shape of *C. albicans* to a slightly more isolated and smaller colony-like appearance (Figure 1g). The combination of 125 μg/mL of LF and 125 μg/mL of the LPO system markedly reduced the mycelial volume of *C. albicans* and altered the size and shape of cells (Figure 1e).

3.2. *Effects of LF and the LPO system on the cellular metabolic activity of C. albicans*

AlamarBlue is a redox indicator that changes its color and fluoresces in response to metabolic activity (*18*). This method is commonly used to quantitatively assess the viability and proliferation of microorganisms. Table 1 shows the effects of the combination of LF and the LPO system on cellular metabolic activity. No marked inhibition was observed in the presence of 125 μg/mL of LF or the LPO system alone (Table 1). LF alone did not exert inhibitory effects, even at 2,000 μg/mL (data not shown). The LPO system alone at 500 μg/mL only exerted weak inhibitory effects (30.7% inhibition, data not shown). In contrast, the combination of more than 7.8 μg/mL of LF and more than 31 μg/mL of the LPO system completely inhibited metabolic activity.

3.3. *Inhibitory effects of LF and the LPO system on the adhesive hyphal form of C. albicans*

The *in vitro* antifungal effects of LF and the LPO system on *C. albicans* were also investigated using a crystal violet staining method to quantify adhesive hyphal cells. The IC_{50} of LF alone and the LPO system alone were 1,000 and 400 μg/mL, respectively (Table 2). In contrast, the combination of 7.8 μg/mL of LF and 50 μg/mL of the LPO system exerted inhibitory effects (82.6%). This combined effects of LF and the LPO system were characterized by the checkerboard analysis (Figure 2). The points indicate the concentration of each compound achieving more than 50% inhibition against *C. albicans* by the crystal violet staining method. The FIC index of this combination was 0.134, and this value

Table 1. Effects of the combination of LF and the LPO system on the cellular metabolic activity of *C. albicans* TIMM1768 using the alamarBlue assay

LPO system (μg/mL)	% inhibition, LF (μg/mL)				
	0	2.0	7.8	31	125
125	3.2 ± 3.2	2.2 ± 4.3	101.4 ± 1.1*	101.2 ± 1.1*	102.0 ± 0.3*
31	0.0 ± 3.2	0.0 ± 5.4	98.9 ± 1.1*	97.8 ± 1.1*	98.9 ± 1.1*
7.8	-3.2 ± 3.2	1.1 ± 12.9	-2.2 ± 5.4	9.7 ± 3.2	78.5 ± 7.5*
2.0	-1.1 ± 5.4	-2.2 ± 5.4	-1.1 ± 8.6	1.1 ± 1.1	1.1 ± 3.2
0	0.0 ± 3.2	-3.2 ± 4.3	-4.3 ± 6.5	4.3 ± 3.2	2.2 ± 3.2

Data represent the mean ± SE of three to six experiments. *$p < 0.01$ significantly different from the control (no added agents).

Table 2. Effects of the combination of LF and the LPO system on the adhesion of the hyphal form of *C. albicans* TIMM1768 using the crystal violet staining assay

LPO system (μg/mL)	% inhibition, LF (μg/mL)						
	0	2.0	7.8	31	125	500	1000
600	60.7	68.5	73.4	88.5	89.4	90.6	90.8
400	51.1	64.2	69.1	89.6	89.0	89.8	90.2
300	48.5	45.6	52.3	87.7	88.8	89.2	90.2
200	21.5	38.4	86.5	87.5	88.6	89.6	90.0
100	21.5	11.2	86.9	87.5	88.5	88.5	89.8
50	-19.0	81.6	82.6	85.9	86.5	89.2	89.4
0	0.0	6.8	5.7	-0.8	9.7	44.5	56.3

A checkerboard analysis showed percent inhibition on the adhesion of the hyphal form. The underlined combination was used to calculate the FIC index for the combination of LF and the LPO system.

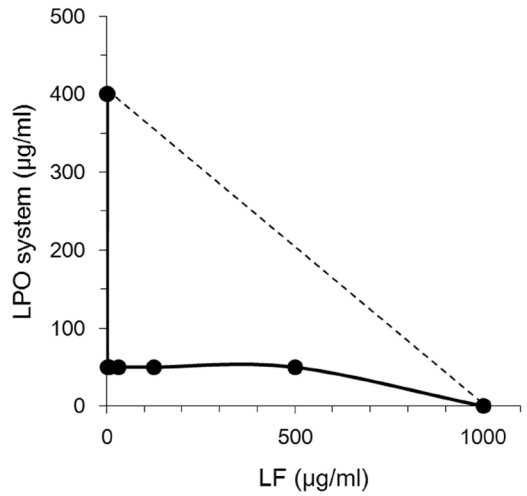

Figure 2. The anti-candida activity of the combination of LF and the LPO system was examined using a checkerboard analysis. The IC_{50} of LF and the LPO system against the adhesive hyphal growth of *C. albicans* TIMM1768 was assessed by the crystal violet staining method and plotted in closed circles on arithmetic scales. A putative additive effect is represented by the dashed line.

indicated synergy (≤ 0.5). These results suggest that the combination of LF and the LPO system exerts strong cooperative effects against the adhesive hyphal form of *C. albicans*.

We also assessed the inhibitory effects of LF combined with $OSCN^-$ solution using the crystal violet staining method. We produced this $OSCN^-$ solution enzymatically, and removed LPO and GO from the solution. When an $OSCN^-$ concentration of 225 μM was used, 500 μg/mL of LF exerted inhibitory effects against the adhesive hyphal form of *C. albicans* (56.7%, data not shown). On the other hand, LF alone at 500 μg/mL did not exert any inhibitory effects against the adhesive hyphal form (0.0%, data not shown). The $OSCN^-$ solution at a concentration of 225 μM exerted weak inhibitory effects (24.0%, data not shown). These results suggest that $OSCN^-$ solution, which is a potent antimicrobial product generated by the LPO system, also functions with LF against *C. albicans*.

4. Discussion

LF and LPO are antimicrobial components found in saliva and each exert weak candidacidal effects (*8,10*). We herein demonstrated that the combination of LF and the LPO system exerted synergistic inhibitory effects on the cellular metabolic activity and adhesion of the hyphal form of *C. albicans*. The effects on the combination of LF and the LPO system (FIC index = 0.134) was more synergistic than that of the combined use of LF with antifungal drugs (FIC index = 0.187-190) (*12*). The hyphal form of *C. albicans* invades deeper host tissues and initiates clinical disease (*1*). Therefore, the inhibition of metabolism and adherence is assumed to be a more effective approach for preventing candidiasis.

LF with $OSCN^-$ solution also exerted inhibitory effects against *C. albicans*. This result suggests that $OSCN^-$, which is a product of the LPO system, is an active component. The activity of the LPO system was

more effective than $OSCN^-$ alone, and may be explained by the LPO system also producing short-lived, highly reactive intermediates, such as superoxythiocyanate (O_2SCN^-) or trioxythiocyanate (O_3SCN^-) (*19*). The reaction through GO, which is a component of the LPO system, generates hydrogen peroxide, which exhibits antifungal activity (*20*). Since previous studies reported that hydrogen peroxide was immediately reduced in the presence of peroxidase (*21*), the contribution of hydrogen peroxide to the effects of the LPO system may have been negligible. Furthermore, hydrogen peroxide exerts toxic effects against mammalian cells (*22*). The LPO system is safer than hydrogen peroxide alone because lactoperoxidase and thiocyanate protects against the toxic effects of hydrogen peroxide.

Previous studies suggested that the main antifungal mechanism of action of LF was dependent on iron and occurred through the direct interaction of LF with the fungal cell surface, leading to cell membrane damage and leakage (*8*). On the other hand, $OSCN^-$ reacts with microbial sulfhydryl groups and inhibits various functions, such as the membrane transport of sugars and amino acids, glycolysis, and respiration (*19*). Alterations in microbial membranes increased this efficacy of the LPO system, possibly by promoting the access of $OSCN^-$ to essential cell compounds (*23*). Accordingly, we speculate that cell membrane damage caused by LF may increase cell permeability to reactive $OSCN^-$ produced by the LPO system, and increase modifications to essential intracellular components. This mechanism of action may have a role in the synergistic inhibitory effects observed; however, further studies are needed to confirm this.

Reductions in salivary flow rates may increase the risk of opportunistic infections including oral candidiasis (*24*). The concentrations of LF and LPO in exocrine secretions have been reported to be 5-10 and 2 μg/mL in saliva, respectively (*25,26*). These concentrations of LF and LPO in saliva were lower than IC50 in the present study. Hence, LF and the LPO system in saliva do not exert anti-*candida* effects alone. The synergistic inhibitory effects of LF and the LPO system against saliva may be the reason for the co-localization of LF and LPO in mammalian exocrine secretions.

In conclusion, we herein demonstrated that the combination of LF and the LPO system exerted synergistic inhibitory effects against *C. albicans*. These results suggest that food or a dietary supplement containing LF and the LPO system will be helpful for patients with oral issues caused by the excessive growth of *C. albicans*. Further clinical trials are needed to assess the preventive effects of this combination of LF and the LPO system on oral candidiasis.

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