

Anticandidal Activity of Lactoferrin, Apolactoferrin, and Oligosaccharides on Mueller-Hinton and Sabouraud Dextrose Agar against Fluconazole Resistant-Candida Albicans

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Abstract

Background: The demand for novel, useful, potential, and safe antifungal drugs and rapid fungal susceptibility test methods due to antifungal resistance and high prevalence of *Candida albicans* infection are continuing. Therefore, this study aimed to assess and compare the antifungal activity of lactoferrin, apolactoferrin, and oligosaccharides isolated from human, bovine, goat, and formula milk against *C. albicans* on Mueller-Hinton agar supplemented with 2% glucose and 5 µg/mL methylene blue and sabouraud dextrose agar.

Methods: Lactoferrin, apolactoferrin, and oligosaccharides were extracted from human, bovine, goat, and formula milk. Lactoferrin was identified using the Bradford test and Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis. Oligosaccharides were identified using Thin-Layer Chromatography. The antifungal activity of lactoferrin, apolactoferrin, and oligosaccharides against fluconazole-resistant *Candida albicans* ATCC 10231 was determined and compared using the disk diffusion method on Mueller-Hinton agar and sabouraud dextrose agar. The inhibition zone formed around the disk was observed after 24 hours of incubation.

Results: Lactoferrin showed an inhibition zone on sabouraud dextrose agar against *C. albicans*, but not on Mueller-Hinton agar. Meanwhile, apolactoferrin and oligosaccharides showed no antifungal activity on both agar media.

Conclusion: Different agar media in the diffusion disk test can give different results even though using the same test method and substance. These results could shed light and become the useful references on why some potential antifungals could yield a different results in *in-vitro* studies, *in-vivo* studies, or clinical trials.

Keywords: Agar media, apolactoferrin, *Candida albicans*, lactoferrin, oligosaccharides

Introduction

Candidiasis is an infectious disease that is widely recognized as a major cause of mortality and morbidity in the community.¹ The spectrum of infections caused by *Candida spp.* is very vast, ranging from an infection that is not fatal and only affects the mucocutaneous layer to invasive infections which potentially reach the bloodstream known as candidaemia.²

At present, resistance to antifungals is a challenge for health workers in the world. Azole antifungals are the drugs most frequently used

as therapy for Candida infections. However, their extensive use in various countries provokes resistance to the drug. *C. albicans* isolates from candidemic patients have the lowest incidence of azole resistance (0–5%). The incidence of fluconazole resistance in *C. albicans* isolates from oropharyngeal candidiasis (OPC) is higher and depends upon previous fluconazole treatment and prior OPC infections. *C. glabrata* has the highest incidence of azole resistance among Candida clinical isolates and exhibits intrinsic decreased susceptibility to the azole antifungals. In the

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Asia-Pacific region, fluconazole resistance in *C. tropicalis* ranges from 0 to 83%. The worldwide incidence of fluconazole resistance in *C. parapsilosis* disseminated infections ranges between 2 and 5%. As *C. krusei* exhibits intrinsic resistance to fluconazole, there is some controversy about whether its increased infection rate is related to fluconazole prophylaxis or previous treatment. Due to this issue, health workers need proper methods for susceptibility testing that are cheap and rapid to get results regarding the latest antifungal as a potential candidiasis therapy.³

Milk contains various bioactive agents that act as antifungals, including lactoferrin (Lf), apolactoferrin (ApoLf), and oligosaccharides.⁴ Oligosaccharides are reported to inhibit the adhesion of *C. albicans* on intestinal epithelial cells. Several other studies have also shown that Lf, ApoLf, and oligosaccharides isolated from milk have an antifungal effect, especially against *C. albicans* and *C. krusei*.^{5,6} The problem of resistance has prompted researchers to seek a quick and accurate antifungal susceptibility test. Disk diffusion is a method of susceptibility testing that can provide qualitative results in zones of inhibition. This method is simple, flexible, and cost-effective. Additionally, it also can be used as an alternative to the broth dilution method. Several previous studies have shown that testing the same antifungal agent on different agar media can give different results.^{5,7,8}

In this study, the antifungal effects of Lf, ApoLf, and oligosaccharides contained in human milk, bovine milk, goat milk, and formula milk were examined on two different agar media (sabouraud dextrose agar (SDA) and Mueller-Hinton agar supplemented with 2% glucose and 5 µg/mL methylene blue (MH-GMB)) against *C. albicans* by disk diffusion method. This study aimed to assess and compare the antifungal activity of lactoferrin, apolactoferrin, and oligosaccharides isolated from human, bovine, goat, and formula milk against *C. albicans* on Mueller-Hinton agar supplemented with 2% glucose and 5 µg/mL methylene blue and sabouraud dextrose agar and it is hoped that it could be used as a reference for further research to find suitable media to test the antifungal activity of lactoferrin, apolactoferrin, and oligosaccharides against *C. albicans*.

Methods

This in vitro experimental study was conducted in January–February 2020 in Parasitology Laboratory, School of Medicine and Health

Sciences, Atma Jaya Catholic University of Indonesia, Jakarta. This study has been approved by Ethical Committee with ethical clearance number: 17/02/KEP-FKUAJ/2019.

Four types of milk were used in this study, human, bovine, goat, and formula milk. Human milk was obtained from a breastfeeding donor with the criteria that the donor had just given birth 1 year earlier, was still breastfeeding her baby, did not smoke, or consumed alcohol. For donors, informed consent was requested first. Human milk was put in specialized human milk containers. Bovine and goat milk is obtained from cattle farms in East Jakarta. The milk was stored in a freezer with a temperature of -20°C for a maximum of 3 months before use and thawed by immersing the milk container in lukewarm water before usage.⁹ Formula milk used for babies aged 0 to 6 months contains 50 mg lactoferrin/100 grams of milk. Ten grams of formula milk was dissolved in 50 mL of water.

Milk that was used in this study was purified and isolated into human milk lactoferrin (hLf), bovine milk lactoferrin (bLf), goat milk lactoferrin (gLf), and formula milk lactoferrin (fLf). Lactoferrin was isolated and purified using the modified Hassan Abdalla method.¹⁰ The procedures involved include alkalizing the milk, making the solution in contact with open air, and adding organic solvents. Milk alkalization was carried out by mixing 50 mL of milk with 3 mL of 40% NaOH so that the NaOH concentration becomes 2.4 g/L. Let the alkalized milk come into contact with the open air for one night. After being left for one night, the solution will form 2 layers where the lower layer is red, and the upper layer is white milk fat, which is formed due to the saponification reaction. The red layer was taken for the next procedure, and the fat layer was removed. The red solution was then filtered using Whatman paper number 1. The filtration results were mixed using acetone as much as two times the filtration volume to precipitate lactoferrin. The residue was washed with acetone several times until the lactoferrin is entirely precipitated. The evaporation of acetone from Lf is modified by inserting the residue into a vacuum jar desiccator for one night, while the Hassan Abdalla method uses low-temperature centrifugation. When the residues appeared dry, lactoferrin was stored in a cuvette tube in the -20°C freezer until they are used for the test.¹⁰

Lactoferrin was identified using the Bradford test to determine the presence or absence of protein content and sodium dodecyl

sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) to determine the molecular weight. A positive result is indicated by a blue color in the solution, while a negative result is indicated by the absence of a blue color change. The SDS-PAGE procedure was carried out in several steps, including SDS-PAGE gel preparation, running buffer preparation, reducing sample buffer preparation, sample preparation, and SDS-PAGE running. The molecular weight of lactoferrin can be read by observing the bands formed, compared with available markers.

Lactoferrin was inserted into the dialysis membrane in 100 mL of 0.1 M citric acid solution and left for one night at room temperature. A dialysis membrane containing Lf was then transferred to a beaker containing 100 mL of aquadest and leave it for one night at -4°C. After that, the apolactoferrin formed in the dialysis membrane was taken and stored in the Eppendorf tube for further testing.¹¹

Milk samples were isolated into human milk oligosaccharides (OH), bovine milk oligosaccharides (OB), goat milk oligosaccharides (OG), and formula milk oligosaccharides (OF). Oligosaccharides were made by mixing 10 mL of milk sample and 40 mL of chloroform and methanol in a ratio of 2: 1. Then, the samples were inserted into the vortex for 5 to 10 minutes. The resulting emulsion was centrifuged for 30 minutes at 6000 rpm. The layer formed at the top of the solution was taken. Meanwhile, the layer formed at the bottom of the solution (chloroform and denatured protein) was removed. This entire procedure was repeated using the layer formed on top of the solution to ensure fat and protein separation from the sample. The oligosaccharides were then moved into the Eppendorf tube and stored in the freezer for further testing.¹²

The presence or absence of oligosaccharides content in a sample was ascertained by performing Thin-Layer Chromatography (TLC). Samples (4 μ L) were spotted on TLC Silica gel 60F254, 20–20 cm (EMD/Merck, Darmstadt, Germany) and run on eluent consisting of a mixture of solutions: n-Butanol/Acetic Acid/water (2:1.1:1, v/v/v). The TLC tape/spot was colored using DAP dye consisting of a solution of diphenylamine, aniline, acetone, and phosphoric acid (Merck KGaA, Darmstadt, Germany), and incubated in an oven at 120°C for approximately 10–15 min until the yield band was visible. The standards used in this TLC consist of xylooligosaccharide standards from xylose (X1), xylobiose (X2), xylotriose

(X3), xylohexaose (X4), xylopentaose (X5), and xylohexaose (X6), glucose (G), and mannose (M). The oligosaccharides tested were OH (1), OB (2), OG (3), and OF (4).

The potential antifungal activity of Lf, ApoLf, and oligosaccharides against *C. albicans* ATCC 10231 (resistant to fluconazole) was tested using the Kirby-Bauer disk diffusion method. This species was subcultured in CHROM Agar (Oxoid Brilliance™ Candida agar, England) for species confirmation. *C. albicans* appears as a green colony on the agar. The disk diffusion method was carried out according to the clinical laboratory standard institute (CLSI) M44. This study used sabouraud dextrose agar (SDA, Oxoid, United Kingdom) and Mueller-Hinton agar supplemented with 2% glucose and 5 μ g/mL methylene blue (MH-GMB, Oxoid, United Kingdom). Blank disk (Oxoid, United Kingdom) was dipped into Lf, ApoLf, and oligosaccharides. Fluconazole (Hangzhou Hyper Chemicals Limited, China) was used as a positive control, and the negative control was aquadest. The disk diffusion results in inhibition zones were compared after incubation for 24 hours at 35°C.

Results

Lactoferrin was formed as a red paste (Figure 1). The Bradford test gave positive results indicated by a blue color change in all samples (Figure 2). These results indicate protein in all milk. Based on SDS-PAGE analysis, there was a faint band between the 72 and 95 kDa marker bands (Figure 3), which indicated the molecular weight of proteins in all samples. The above results strongly suggest the presence of Lf in the isolates. ApoLf appeared as a yellowish-white paste with difficulty dissolving in water (Figure 1).

The results of the TLC analysis are shown in Figure 4. The formed band revealed that the sample contained oligosaccharides with a high concentration by a thick band's appearance. The samples extended between the third and fifth sugars indicating that the oligosaccharides isolated from human milk were most likely xylotriose, xylohexaose, and xylopentaose.

Lactoferrin from various types of milk against *C. albicans* ATCC 10231 showed inhibition zones in SDA. The largest zone of inhibition was shown by bLf and gLf of 23 mm. Meanwhile, the smallest zone of inhibition was produced by fLf of 16.67 mm. However, in MH-GMB, there was no inhibition zone against *C. albicans* ATCC 10231 (Table). The inhibition



Figure 1 Lactoferrin (above); apolactoferrin (below)

test of apolactoferrin and oligosaccharides against *C. albicans* did not show any inhibition zone on either SDA or MH-GMB.

Discussion

This in vitro experimental study shows that

using different test media can give different results even though using the same test method and substance. These results indicate that the susceptibility test results of the substances that have the potential to be antifungal are influenced by the type of assay, the composition of the assay media, and the tested isolates'

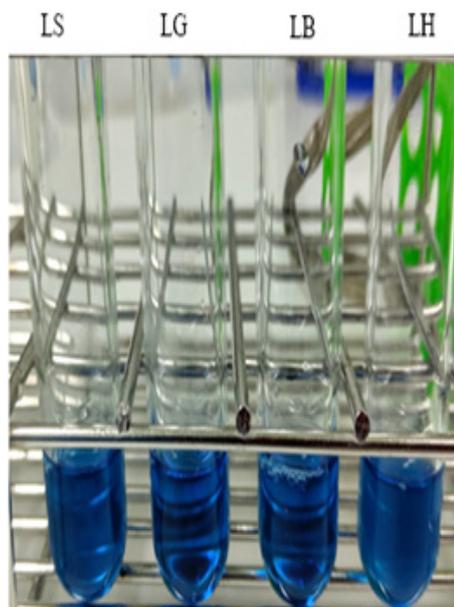


Figure 2 The Bradford Assay for Lactoferrin

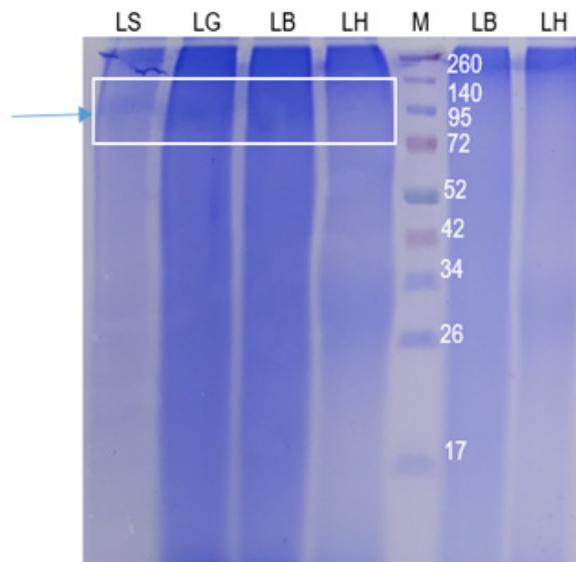


Figure 3 Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) for Lactoferrin

resistance profile. This could also shed light on why some potential antifungals could yield different in *in-vitro* studies, in-vivo studies, or clinical trials.^{7,8}

In this study, both hLf, bLf, gLf, and fLf inhibited the growth of *C. albicans* in SDA, but not in MH-GMB. A study by Andrés et al.¹³ shows that Lf's antifungal activity depends on

the cells' energy metabolism. In the absence of oxygen, starved *C. albicans* was almost resistant to different lactoferrin concentrations, unlike non-starved cells exposed to this protein under oxygenic conditions, which were susceptible in Lf concentration-dependent way. *Candida spp.* needs a sugar-rich medium to grow properly.¹³ This is supported by Miramón et al.¹⁴, the

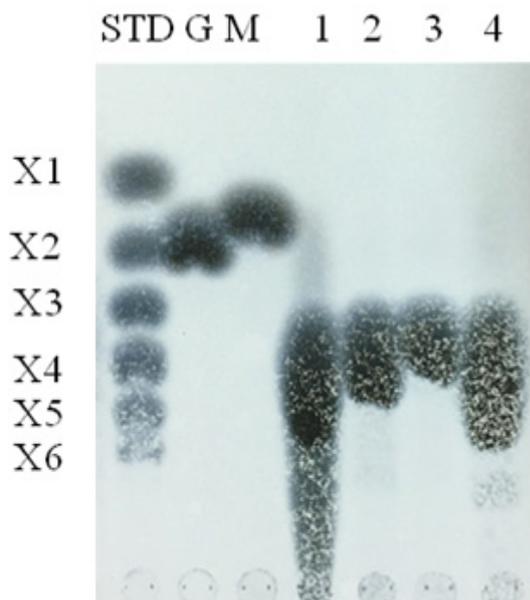


Figure 4 Thin Layer Chromatography (TLC) for Oligosaccharides

Table Inhibition Zone on Mueller-Hinton and Sabouraud Dextrose Agar Comparison

Antifungal Agents	Inhibition Zone (mm)							
	Mueller-Hinton Agar				Sabouraud Dextrose Agar			
	I	II	III	Mean	I	II	III	Mean
Fluconazole	25	23	27	25	24	23	22	23
Aquadest	0	0	0	0	0	0	0	0
hLf*	0	0	0	0	22	20	21	21
bLf**	0	0	0	0	25	23	21	23
gLf***	0	0	0	0	25	21	23	23
fLf****	0	0	0	0	17	17	16	16.67

Note: *hLf: human lactoferrin, **bLf: bovine lactoferrin, ***gLf: goat lactoferrin, ****fLf: formula lactoferrin

contributions of carbohydrate starvation, oxidative, and nitrosative stress play a crucial role in fungal resistance.¹⁰ SDA has much more glucose than Mueller-Hinton agar (MHA), making SDA widely used for *Candida spp.* culture preservation.⁷

Different compositions and properties where SDA is more acidic (pH 5.6±0.2) while MH-GMB has a more neutral pH (pH 7.2–7.4) could also produce a different result.^{9,11} A study showed that, the susceptibility of fluconazole-susceptible *C. albicans* and fluconazole-resistant *C. albicans* to fluconazole could give different results at pH 7 and when the pH decreased.⁸ This suggests that differences in the test media's pH can affect the antifungal activity against *C. albicans*.^{8,11,12}

Mueller-Hinton agar was used as the medium in this study because it is the agar medium most often used for susceptibility testing. However, previous studies showed that *C. albicans* experienced poor growth with MHA. This makes MHA, not a suitable medium for testing antifungal susceptibility with inhibitory mechanisms such as fluconazole against *C. albicans* strains. One of the Lf antifungal mechanism targets is the cell membrane, similar to fluconazole antifungal mechanism. This study also showed no inhibition of Lf against *C. albicans*, so MHA should not be used in carrying out the Lf test against *C. albicans*.^{13,14}

The antifungal susceptibility test of apolactoferrin against *C. albicans* on both SDA and MH-GMB media did not show any inhibition zone, indicating that the isolated apolactoferrin could not inhibit the growth of *C. albicans* in this assay. The absence of a zone of inhibition of apolactoferrin against *C. Albicans* is thought to be due to technical constraints. The apolactoferrin cannot be adequately absorbed into the disk because of its insoluble substance

nature. However, these results contrast with a study which showed that apolactoferrin could directly inhibit the growth of *C. albicans*, while Lf does not show any antifungal activity.⁵ Media with iron concentrations above 0.5 mmol/L can eliminate fungal growth's inhibitory effect, even at 10% concentrations. Iron concentration in the growth medium of *C. albicans* is essential for the regulation of iron-uptake mechanisms that can affect the virulence of apolactoferrin. This study has used 24-well dishes (Nunclon) in the assay method and RPMI 1640 with L-glutamine and without antibiotics or serum as the assay medium for fungal growth.⁵ Furthermore, the lactoferrin used in this study also came from Sigma Chemical Co. (St. Louis, MO, USA). From this, it can be concluded that the test media and the isolation technique of the related agent can affect the results of the inhibition test against *C. albicans*. The antifungal activity against *C. albicans* cells is not solely due to the chelation of iron by lactoferrin but also involves another complex mechanism involving genetic resistance when lactoferrin comes into contact with *Candida* cells.^{5,15}

The oligosaccharides against *C. albicans* showed no inhibition zone around the disk in both SDA and MH-GMB. This indicates that the oligosaccharides isolated from various types of milk could not inhibit the growth of *C. albicans* ATCC 10231 in the disk diffusion method. This is thought to be related to the mechanism of inhibition of oligosaccharides against *C. albicans*. Oligosaccharides suppress the growth of *C. albicans* by blocking the surface of the *C. albicans* cell structures that are needed to bind to epithelial cells. Oligosaccharides also inhibit the pathogenicity of *C. albicans* by inhibiting the growth of its hyphae. Also, the morphogenesis of *C. albicans* is inhibited by the presence of oligosaccharides, especially during

the initiation of hyphae.^{6,16-19} Saccharides can also function as prebiotics in the body that suppress the growth of *C. albicans*, especially when combined with *Lactobacillus spp.*, *Bacteroides spp.*, and *Bifidobacterium infantis*, which are normal microbiota and probiotics in the intestine.²⁰

In addition, from the results of this study that is limited to disk diffusion test, this method should only be used as a screening test to determine the antifungal agent's potency. Various agar media should also be used to compare differences in the inhibition test results, as in this study. Susceptibility assays such as macrodilution, microdilution, or E-test should also be done after the disk diffusion test to confirm the result.

One of the limitations of this study was only the disk diffusion method was conducted. Therefore, more research is required to determine these agents' antifungal mechanisms in various growth media and susceptibility tests. Moreover, it is recommended to examine lactoferrin, apolactoferrin, and oligosaccharides activity by performing in vivo tests on animal models.

To conclude, different agar media in the disk diffusion test for the same agent and isolate can give different results. Lactoferrin has shown potential inhibition against *C. albicans* in SDA, but not in MH-GMB. Meanwhile, apolactoferrin and oligosaccharides did not show any antifungal activity in both types of agar. These results could shed light and become the useful references on why some potential antifungals could yield a different results in in-vitro studies, in-vivo studies, or clinical trials.

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