OLIGOSACCHARIDES IN MILK: EXPLORING NOVEL ANTIFUNGAL PROPERTIES AGAINST *CANDIDA ALBICANS* AND *CANDIDA KRUSEI*

Stella MM[1], Surja SS[2], and Arieselia Z[3].
[1]School of Medicine and Health Sciences, Atma Jaya Catholic University of Indonesia, Jakarta, Indonesia
[2]Department of Parasitology, School of Medicine and Health Sciences, Atma Jaya Catholic University of Indonesia, Jakarta, Indonesia
[3]Department of Pharmacology and Pharmacy, School of Medicine and Health Sciences, Atma Jaya Catholic University of Indonesia, Jakarta, Indonesia

Correspondence:
Zita Arieselia,
Department of Pharmacology and Pharmacy,
School of Medicine and Health Sciences,
Atma Jaya Catholic University of Indonesia,
Jakarta, Indonesia.
Email: zita.arieselia@atmajaya.ac.id

Abstract

With the increasing antifungal resistance and prevalence of candidiasis, the aim of this research is to evaluate the potential inhibitory effects and synergistic activities of oligosaccharides present in milk with azoles in inhibiting the growth of *Candida albicans* and *Candida krusei*. The oligosaccharides were derived from breast milk (OH), bovine (OB), goat (OG), and formula milk (OF), then confirmed by thin-layer chromatography (TLC). Disk diffusion and microdilution were used to evaluate the antifungal potency of oligosaccharides using disk diffusion and microdilution. The oligosaccharides only showed antifungal activity in microdilution. The statistical analysis showed moderate variability in minimum inhibitory concentration (MIC) values and higher fractional inhibitory concentration (FIC) values. A significantly wide range of MIC in the microdilution assay indicated the effectiveness of this antifungal agent varied across different samples. In the combination test, antagonistic activity was observed between fluconazole and oligosaccharides against *C. albicans* ATCC 90028. Further research is needed to study the mechanisms of antifungal activity and synergism between oligosaccharides and azoles against *Candida* species.

Keywords: Oligosaccharides, Synergistic, Azole, *Candida albicans, Candida krusei*

Introduction

Candidiasis is one of the leading etiology of morbidity and mortality in healthcare services. This infectious disease can be caused by *Candida* fungi such as *Candida albicans* and other species within the *Candida* genus, including *C. krusei*, *C. glabrata, C. parapsilosis, and C. tropicalis* (1). Its range of infections spans from non-lethal mucocutaneous layer infections to invasive infections infiltrating internal organs. Mucocutaneous candidiasis is often found in the oral cavity of infants, the vagina, moist body folds, traumatized skin, and in patients with diabetes mellitus (2). In tropical regions like Indonesia, most cases of candidiasis are superficial, affecting the skin, oral cavity, and vagina. Systemic candidiasis or candidemia occurs when *Candida* fungi enter the bloodstream and form microabscesses in the body's internal organs (3).

A fungal genus of *Candida* is among the top 10 leading causes of bloodstream infections. Approximately 33-55% of all candidemia cases occur in intensive care units (ICUs). The mortality rate ranges from 5% to 71% (4). Overall, *C. albicans* is the most common cause of candidiasis, accounting for 50-70% of cases (5). Factors such as malignancy, immunosuppressive diseases, organ transplantation, use of broad-spectrum antibiotics or corticosteroids, invasive interventions, chemotherapy, parenteral nutrition, and prosthetic device placement can increase the risk of candidiasis (6). Currently, antifungal resistance poses a significant challenge for healthcare professionals worldwide. Antifungal resistance describes the failure of therapeutic responses to fungal infections (7).

Antifungals from the azole class are the most used drugs for the therapy of *Candida* infections. One of them, fluconazole, is often chosen as a preferred therapy due to its affordability, infrequent side effects, and widespread availability (8). However, its extensive use in various countries has led to resistance against fluconazole. Fluconazole resistance is observed in over 15% of *non-*

albicans Candida, notably in strains such as *C. glabrata* and *C. krusei*. This highlights the challenge of antifungal resistance and the need for alternative therapeutic approaches in the face of growing resistance rates (9).

Breast milk functions as an exceptional source of nutrition and bioactive factors, playing a pivotal role in promoting the optimal growth and development of newborns. (10). Breast milk can boost the performance of the immune system and protect neonates from infections. The antifungal effects of breast milk are also supported by research conducted by Mete et al. in 2006 (11). The incidence of gastrointestinal and respiratory tract infections decreased in neonates consuming breast milk compared to those not receiving breast milk (11). Oligosaccharides, found in breast milk, contributed to the development of microbiota in the intestinal tract, offering protective benefits. Oligosaccharides achieved this by preventing the adhesion and invasive interaction between pathogens and the mucosal epithelial layer. This underscored the multifaceted benefits of breast milk in supporting the health and immune defense of neonates (12).

To date, there has been no research investigating the antifungal effects of oligosaccharides found in breast milk against *C. albicans* and *C. krusei*, utilizing disk diffusion and microdilution techniques in vitro. Furthermore, there is a scarcity of studies comparing the antifungal effects of breast milk oligosaccharides with fluconazole against *C. albicans* and itraconazole against *C. krusei*. The aim of this research is to evaluate the potential inhibitory effects and synergistic activities of oligosaccharides present in milk with azoles in inhibiting the growth of *Candida albicans* and *Candida krusei*. The outcomes of this research are anticipated to serve as a foundation for future studies aiming to explore natural therapies for individuals with candidiasis, particularly neonates requiring prolonged treatment, potentially circumventing the side effects associated with antifungal medications.

Materials and Methods

This experimental study was carried out at the Microbiology and Parasitology Laboratory, School of Medicine and Health Sciences, Atma Jaya Catholic University of Indonesia, spanning from May 2019 to July 2022. Ethical clearance was obtained and approved by the Ethical Committee at the School of Medicine and Health Sciences, Atma Jaya Catholic University of Indonesia, with the registered number 17/02/KEP-FKUAJ/2019.

Isolation and identification of oligosaccharides

In this study, four types of milk were used: breast milk, bovine milk, goat milk, and formula milk. These types of milk were to be purified and isolated into oligosaccharides [breast milk (OH), bovine (OB), goat (OG), and formula milk (OF)]. Breast milk was received from a donor (1 female, 38 years old), who was still breastfeeding, having given birth within the last 1 year, and not smoking or consuming alcohol. Informed consent was obtained from the donor prior to collection. A total of 50 ml of breast milk was collected from the donor, stored in a breast milk container, and frozen for 3 months before use (maximum) at -20°C (13).

Bovine (Friesien Holstein breed) and goat (Etawa cross breed) milk were sourced from an East Jakarta cattle farm (location coordinate: -6.35512595674536, 106.90912297385142) and stored in a freezer at -20°C for up to 3 months prior to utilization. Formula milk used in the study was specifically designed for infants aged 0 to 6 months (Morinaga BMT Platinum, PT Sanghiang Perkasa, Kalbe Nutritionals). Breast milk, bovine milk, and goat milk, which had been previously stored in the freezer, underwent thawing by immersing their respective containers in warm water (36°C, 15-20 minutes) prior to the oligosaccharide separation process. In contrast, the formula milk was prepared by dissolving it in 50 mL of hot water (70°C).

Oligosaccharides were prepared according to Balogh et al. (14), briefly, by combining a 40 mL solution of methanol and chloroform 1:2 ratio with a 10 mL milk. Following this, the sample underwent vortexing for 5 to 10 minutes, creating an emulsion. Subsequently, the emulsion underwent centrifugation for 30 minutes at 6,000 rpm. The upper layer was taken for further analysis, and the lower layer (containing denatured proteins and chloroform) was discarded. This procedure was repeated, utilizing the upper layer as a sample to ensure effective separation of fats and proteins. The obtained oligosaccharides were then transferred to Eppendorf tubes and stored in the freezer for subsequent testing.

Confirmation of the presence of oligosaccharides in a sample was achieved, according to Dreisewerd et al., through thin-layer chromatography (TLC) (14). A 4 µL sample was applied to a TLC Silica gel 60F254 plate, measuring 20-20 cm (EMD/Merck, Darmstadt, Germany), then subjected to chromatography using an eluent mixture of water/ acetic acid/ n-butanol (1:1.1:2, v/v/v). Following the run, TLC bands/spots were visualized through staining with DAP dye, comprising a solution of aniline, diphenylamine, phosphoric acid, and acetone (Merck KGaA, Darmstadt, Germany). Subsequently, the TLC plate was placed in an oven (Camag, Switzerland) at 120°C for about 10-15 minutes until the bands became visible. The standards applied consisted of xylooligosaccharides standards obtained from xylose, xylobiose, xylotriose, xylotetraose, xylopentaose, and xylohexaose in addition to glucose and mannose (15)

Antifungal assay

The potency of antifungal activity and synergistic effects of oligosaccharides against *C. albicans* ATCC 10231 (resistant to fluconazole), *C. albican*s ATCC 90028 (susceptible to fluconazole), and *C. krusei* (wild type) were tested using disk diffusion and microdilution methods. Microdilution tests were conducted on all the mentioned species and strains to determine the inhibitory concentration of the test substance compared to existing standard antifungals. The

evaluation of synergistic activity between oligosaccharides and antifungals was evaluated using the disk diffusion method to determine the size of inhibition zones after combination and microdilution to ascertain the fractional inhibitory concentration (FIC).

Each species was subcultured on CHROMAgar (Oxoid, United Kingdom) to confirm the species. About 33 grams of CHROMAgar was dispersed slowly in 1 liter of aquadest, then boiled at 100°C while stirring regularly. CHROMAgar then plated in agar plates. *C. albicans* exhibited green colonies, while *C. krusei* colonies appeared pink. The disk diffusion method followed the Clinical Laboratory Standard Institute (CLSI) M44 with modifications. Sabouraud dextrose agar (SDA, Oxoid, United Kingdom) was used as the test medium, deviating from the recommended Mueller-Hinton agar. Blank disks (Oxoid, United Kingdom) were used for disk diffusion (16). The microdilution method followed CLSI M27 with modifications. Sabouraud dextrose broth (SDB, Oxoid, United Kingdom) was used instead of Mueller-Hinton broth as the test medium. A 96-well microplate (Biologix, USA) served as the microdilution plate (17).

Fluconazole (Hangzhou Hyper Chemicals) was served as a positive control for *C. albicans*, meanwhile itraconazole (Murli Krishna Pharma Pvt. Ltd.) was used as a positive control for *C. krusei* due to its intrinsic resistance to fluconazole (18–20). Distilled water was used as a negative control for all three fungal species. After 24 hours incubation at 35°C, the results of the disk diffusion, represented by inhibition zones, and the microdilution, represented by minimum inhibitory concentration (MIC), were compared. The potential synergistic activity between oligosaccharides and azole-class antifungal drugs was tested using both the disk diffusion and microdilution methods. In both methods, the combination of fluconazole and oligosaccharides was tested against *C. albicans*, and the combination of itraconazole and oligosaccharides was tested against *C. krusei*.

In the disk diffusion test, this combination involved dipping antifungal disks into oligosaccharides and placing them on agar media inoculated with *Candida spp.* The addition of inhibition zones was documented and categorized as follows: i) antagonistic if there was a decrease in the inhibition zone, ii) additive if there was an increase in the inhibition zone but did not exceed the combined inhibition zones of the antifungal and oligosaccharide alone, iii) synergistic if there was a greater increase in the inhibition zone than the combined inhibition zones of the antifungal and oligosaccharide (21).

In the microdilution test, the results of the potential synergistic activity are expressed as the FIC (fractional inhibitory concentration) index. FIC can be determined with the following formula:

FIC Index = FIC (A) + FIC (B)

where:

$$\text{FIC(A)} = \frac{MIC\ (A)\ in\ combination}{MIC\ (A)\ alone} \text{ and FIC(B)} = \frac{MIC\ (B)\ in\ combination}{MIC\ (B)\ alone}$$

A synergistic activity is indicated when the FIC value is ≤ 0.5, antagonistic activity is suggested when FIC > 4, additive activity falls within the range of FIC between 0.5 and 1, and indifference is noted for FIC values between 1 and 4 (22).

In the microdilution test to determine FIC, each well was filled with 50 µL of SDB. In the first column, 50 µL of oligosaccharides and 50 µL of fluconazole antifungal solution (Hangzhou Hyper Chemicals Limited) against *C. albicans*, and itraconazole (Murli Krishna Pharma Private Ltd.) against *C. krusei* were added. Subsequently, two-fold dilutions were performed from the first column to the 10th column. Afterward, *Candida spp.* was added in an amount of 50 µL to the wells in columns 1 through 11. Column 11, without oligosaccharides and antifungal, served as the negative control, and column 12 served as a parameter in case the microdilution process encountered contamination. Meanwhile, for the positive control, a separate microdilution test was conducted to determine the MIC of the tested antifungal. The MIC result of the oligosaccharides was presented as a percentage concentration relative to the total solution in those wells. MIC of the antifungal is expressed in µg, representing the minimal concentration of antifungal present in the entire solution of the well.

The results of the TLC analysis are shown in Figure 1. The formed bands on the TLC plate suggested the presence of oligosaccharides in the sample at a relatively high concentration, indicated by the appearance of thick bands. The sample seemed to encompass sugars from the third and fifth positions, implying that the isolated oligosaccharides were likely xylotriose, xylotetraose, and xylopentaose. To determine the specific types of oligosaccharides, it is necessary to use the standards that match the sample source for confirmation.

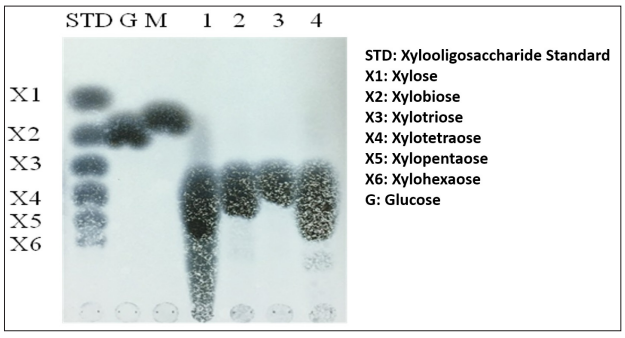

Figure 1: Oligosaccharides on thin-layer chromatography

STD: Xylooligosaccharide Standard (X1-X6); X1=Xylose; X2=Xylobiose; X3=Xylotriose; X4=Xylotetraose; X5=Xylopentaose; X6=Xylohexaose; G=Glucose
Standard; M=Mannose Standard; 1=Formula milk oligosaccharide; 2=Goat milk oligosaccharide; 3=Cow milk oligosaccharide; 4=Breast milk oligosaccharide

All types of oligosaccharides in the disk diffusion test did not show any antifungal activity in the form of inhibition zones against all *Candida* species in this study. While the azole group showed an inhibition zone in *Candida spp.* [*C. albicans* ATCC 90028 (28 mm); *C. albicans* ATCC 10231 (23 mm); *C. krusei* (31 mm)].

However, contradictory results were obtained in the microdilution test. In this test, oligosaccharides showed antifungal activity (Table 1). The comparison of MIC results for *C. krusei* wild-type and *C. albicans* ATCC 90028 produced by OH, OB, OG, and OF were equivalent. Meanwhile, the worst MIC result for *C. albicans* ATCC 10231 was shown by OB. Statistical analysis of MIC was shown in Table 2. The wide range of MIC in Table 2 indicated that there was variability in the effectiveness of oligosaccharides across different samples.

Table 1: Minimum inhibitory concentration of oligosaccharides

Test drug/ Sample	Minimum Inhibitory Concentration (MIC)		
	***Candida albicans* ATCC 90028**	***Candida albicans* ATCC 10231**	***Candida krusei* (wild-type)**
Fluconazole	1 µg/mL	32 µg/mL	-
Itraconazole	-	-	0.125 µg/mL
OH	12.5%	1.5625%	1.5625%
OB	12.5%	3.125%	1.5625%
OG	12.5%	1.5625%	1.5625%
OF	12.5%	1.5625%	1.5625%

OH: Human oligosaccharides; OB: Bovine oligosaccharides; OG: Goat oligosaccharides; OF: Formula milk oligosaccharides

Table 2: Statistical analysis of minimum inhibitory concentration (MIC)

MIC	N	Minimum	Maximum	Mean	Std. Deviation
MIC	15	0.1250	32.0000	6.479167	8.6486047
Valid N	15				

In the combination test (Table 3), there was antagonistic activity between fluconazole and oligosaccharides against *C. albicans* ATCC 90028. The high standard deviation (SD) in Table 4 further confirmed that the MIC values were widely dispersed from the mean. The standard deviation of FIC (6.38) suggests that there was higher variability around the mean value (Table 4). The disk diffusion test for oligosaccharides revealed the absence of an inhibition zone around the disk, suggesting that the oligosaccharides extracted from breast milk did not impede the growth of *C. albicans* and *C. krusei* in this particular method.

Nevertheless, the microdilution test indicated that oligosaccharides exhibit inhibitory effects on the growth of fluconazole-resistant *C. albicans* and *C. krusei* wild-type at specific doses, demonstrating a dose-dependent manner. The MIC results showed that 12.5% oligosaccharides were equivalent to 1 µg/mL fluconazole in fluconazole-susceptible *C. albicans*. Meanwhile, in fluconazole-resistant *C. albicans*, the MIC results showed that 1.5625% (OH, OG, OF) and 3.125% (OB) are equivalent to 32 µg/mL fluconazole. The MIC results against *C. krusei* in the microdilution test indicated that 1.5625% oligosaccharides are equivalent to 0.125 µg/mL itraconazole. These results demonstrate the antifungal activity of oligosaccharides present in breast milk in inhibiting *C. albicans* and *C. krusei* in the microdilution method.

Table 3: Fractional inhibitory concentration (FIC) on microdilution

Oligosaccharides		MIC with Azole Combination on *Candida albicans* ATCC 90028	FIC	Note
Human	Fluconazole OH	16 µg/mL 12.5%	17	Antagonistic
Bovine	Fluconazole OB	16 µg/mL 12.5%	17	Antagonistic
Goat	Fluconazole OG	8 µg/mL 6.25%	8.5	Antagonistic
Formula	Fluconazole OF	4 µg/mL 3.125%	4.25	Antagonistic

MIC: minimum inhibitory concentration; FIC: fractional inhibitory concentration; OH: human oligosaccharides; OB: bovine oligosaccharides; OG: goat oligosaccharides; OF: formula milk oligosaccharides

Table 4: Statistical analysis of FIC

	N	Minimum	Maximum	Mean	Std. Deviation
MIC	8	3.1250	16.0000	9.796875	5.1477880
FIC	4	4.25	17.00	11.6875	6.37500
Valid N	4				

Discussion

This was the first study reported on the antifungal activity comparison of milk oligosaccharides from various sources such as human breast milk, bovine, goat, and formula milk. In this study, the inhibitory efficacy of antifungal agents was altered by the assay technique, growth medium, and resistance profile of the *Candida* species. The statistical analysis showed moderate variability in MIC values and higher in FIC values. The significantly wide range of MIC values indicated that the effectiveness of oligosaccharides

varied across different samples and required different concentrations to achieve inhibition.

Disk diffusion was used to provide qualitative visualization of the *Candida spp.* susceptibility towards oligosaccharides, fluconazole, and itraconazole. Meanwhile, the microdilution could be used to show the minimum concentration required to inhibit the growth of *Candida spp.* The use of different assay media, even with the same methods and test substances, can yield different results. This could also provide insight into the reasons behind variations in the outcomes of certain antifungals that appear promising in experiments (in vitro) compared to their performance in both in vivo studies and clinical trials. (24, 25).

The absence of an inhibition zone from oligosaccharides is suspected to be caused by the inhibitory mechanism of oligosaccharides against *t*hese *Candida* species. In a study conducted by Nilakanta et al. (12), oligosaccharides have been found to exert inhibitory effects on *C. albicans* growth. This inhibition was attributed to the blocking of surface structures essential for the binding of *C. albicans* to epithelial cells. Additionally, oligosaccharides demonstrate the ability to impede the pathogenicity of *C. albicans* by hindering the hyphae growth (12, 25–27).

Furthermore, oligosaccharides have been observed to decrease the morphogenesis ability of *C. albicans*, particularly during the hyphae initiation. Within the body, these oligosaccharides play a dual role as prebiotics, suppressing the *C. albicans* growth. This effect is notably enhanced when oligosaccharides are combined with normal microbiota such as *Bifidobacterium infantis, Bacteroides spp.*, *Lactobacillus spp.*, and the probiotic in the intestines (28,29).

In the study by Seyfarth et al. (30), oligosaccharides demonstrated better antifungal effects against C. krusei compared to *C. albicans*, which are susceptible to fluconazole. The *C. krusei* growth could be slowed down at concentrations above 0.025%. After 24 hours, the increase in dosage beyond 0.1% could lead to additional inhibition of *C. krusei* growth. Based on Mann-Whitney statistical analysis, the expansion of *C. krusei* was significantly suppressed at a concentration of 0.25%. Conversely, the growth of *C. albicans* was not suppressed by oligosaccharides at concentrations above 1% (30).

In addition, this study suggests the utilization of the disk diffusion method as a screening test for evaluating the potential of antifungal agents. To ensure comprehensive assessment, it is advisable to employ various agar media for comparison, especially in cases where differences in inhibitory test results may arise, as observed in this study. Furthermore, isolates intended for vulnerability testing should undergo retesting, incorporating confirmation tests like macrodilution, microdilution, or E-test for a more thorough validation of results.

Conclusion

The milk oligosaccharides exhibited a significantly wide range of MIC in the microdilution assay, indicating the effectiveness of this antifungal agent varied across different samples. However, the oligosaccharides did not show any inhibitory zones in the disk diffusion test. Oligosaccharides also demonstrated antagonistic activity when combined with fluconazole against fluconazole-sensitive *C. albicans*. Further studies are needed to unravel the mechanisms underlying the antifungal activity and synergistic effects between different types of oligosaccharides and azoles against *Candida* species. This experiment could pave the way for the potential use of these compounds as adjuvant therapy or primary treatment for candidiasis.

Acknowledgement

We would like to show our gratitude to the Faculty of Medicine and Health Sciences, Atma Jaya University, for facilitating this research. Special thanks to dr. Irene, SpA, for her valuable input and guidance, which contributed to the success of the study. We also extend our appreciation to the Indonesian Institute of Sciences (LIPI) and the laboratory team (Linda Widyawati A.Md. Keb., Ariska Verri Marantika, Ida Afiyah, Sri Handayani, M. Yashir A.Md. Kes., Agus Siswanto), as well as other research colleagues who have contributed to the project.

Competing interests

The authors declare that they have no competing interests.

Ethical Clearance

Our study received approval from the Ethical Committee, School of Medicine and Health Sciences, Atma Jaya Catholic University of Indonesia, registered under 17/02/KEP-FKUAJ/2019.

Financial support

We would like to show our gratitude to the Faculty of Medicine and Health Sciences, Atma Jaya University, for providing support and funding (grant number: 44/PHK-PKPD/SEKR/06/2011) for the progress and completion of this research.

References

1. Pappas PG, Kauffman CA, Andes DR, Clancy CJ, Marr KA, Ostrosky-zeichner L, et al. Clinical Practice Guideline for the Management of Candidiasis: 2016 Update by the Infectious Diseases Society of America. Clin Infect Dis. 2016; 1–50.
2. Sudoyo AW, Setiyohadi B, Alwi I, Simadibrata M, Setiadi S. Buku Ajar Ilmu Penyakit Dalam Jilid 1. Interna Publishing. 2014. p: 1973–1983.
3. Brooks GeoF, Carroll KC, Butel JS, Morse SA, Mietzner TA. Jawetz, Melnick & Adelberg's Medical

Microbiology, 26th Edition. Journal of Chemical Information and Modeling. 2013. p: 1689–1699.
4. Bouza E, Muñoz P. Epidemiology of candidemia in intensive care units. Int J Antimicrob Agents. 2008; 32(suppl. 2):87–91.
5. Arendrup MC. Epidemiology of invasive candidiasis. Curr Opin Crit Care. 2010; 16(5):445–52.
6. Yapar N. Epidemiology and risk factors for invasive candidiasis. Ther Clin Risk Manag. 2014; 10(1):95–105.
7. Kontoyiannis DP, Lewis RE. Antifungal drug resistance of pathogenic fungi. Lancet. 2002; 359(9312):1135–44.
8. Whaley SG, Berkow EL, Rybak JM, Nishimoto AT, Barker KS, Rogers PD. Azole antifungal resistance in *Candida albicans* and emerging non-albicans *Candida* Species. Front Microbiol. 2017; 7(Jan):1–12.
9. Cuenca-Estrella M, Rodriguez-Tudela JL. The current role of the reference procedures by CLSI and EUCAST in the detection of resistance to antifungal agents in vitro. Expert Review of Anti-infective. Therapy. 2010 Mar; 8(3):267-76.
10. Cacho NT, Lawrence RM. Innate immunity and breast milk. Front Immunol. 2017; 8 (May):584.
11. Mete E, Bavbek N, Dayi S, Erkmen M, Andiran F. In vitro antifungal effect of human milk. Allergy and asthma proceedings: the official journal of regional and state allergy societies. 2006; 27(5):412–4.
12. Nilakanta H, Drews KL, Firrell S, Foulkes MA, Jablonski KA, Gonia S, et al. Human Milk Oligosaccharides Inhibit *Candida albicans* Invasion of Human Premature Intestinal Epithelial Cells. J Nutr. 2014; 7(1):830.
13. Rollo DE, Radmacher PG, Turcu RM, Myers SR, Adamkin DH. Stability of lactoferrin in stored human milk. Journal of Perinatology. 2014 Apr; 34(4):284-286.
14. Dreisewerd K, Kölbl S, Peter-Katalinić J, Berkenkamp S, Pohlentz G. Analysis of native milk oligosaccharides directly from thin-layer chromatography plates by matrix-assisted laser desorption/ionization orthogonal-time-of-flight mass spectrometry with a glycerol matrix. J Am Soc Mass Spectrom. 2006; 17(2):139–50.
15. Rahmani N, Kashiwagi N, Lee JM, Niimi-Nakamura S, Matsumoto H, Kahar P, et al. Mannan endo-1,4-β-mannosidase from *Kitasatospora sp.* isolated in Indonesia and its potential for production of mannooligosaccharides from mannan polymers. AMB Express. 2017; 7(1):1–11.
16. Clinical and Laboratory Standards Institute (CLSI). Method for antifungal disk diffusion susceptibility testing of yeasts; approved guideline—second edition. CLSI document M44-A2. Wayne, PA: CLSI; 2009.
17. Clinical and Laboratory Standards Institute (CLSI). Reference method for broth dilution antifungal susceptibility testing of yeasts; approved standard—third edition. CLSI document M27-A3. Wayne, PA: CLSI; 2008.
18. Marichal P, Gorrens J, Coene MC, Le Jeune L, Vanden Bossche H. Origin of differences in susceptibility of *Candida krusei* to azole antifungal agents. Mycoses. 1995; 38(3–4):111–7.
19. Katiyar SK, Edlind TD. Identification and expression of multidrug resistance-related ABC transporter genes in *Candida krusei.* Med Mycol. 2001; 39(1):109–16.
20. Lamping E, Ranchod A, Nakamura K, Tyndall JDA, Niimi K, Holmes AR, et al. Abc1p is a multidrug efflux transporter that tips the balance in favor of innate azole resistance in *Candida krusei.* Antimicrob Agents Chemother. 2009; 53(2):354–69.
21. Boonyanugomol W, Kraisriwattana K, Rukseree K. In vitro synergistic antibacterial activity of the essential oil from *Zingiber cassumunar* Roxb against extensively drug-resistant *Acinetobacter baumannii* strains. J Infect Public Health. 2017; 10(5):586-592.
22. Balouiri M, Sadiki M, Ibnsouda SK. Methods for in vitro evaluating antimicrobial activity: A review. J Pharm Anal. 2016; 6(2):71–9.
23. Danby CS, Boikov D, Rautemaa-Richardson R, Sobel JD. Effect of pH on in vitro susceptibility of *Candida glabrata* and *Candida albicans* to 11 antifungal agents and implications for clinical use. Antimicrob Agents Chemother. 2012; 56(3):1403–6.
24. 24. Rubio MC, Gil J, de Ocáriz IR, Benito R, Rezusta A. Comparison of results obtained by testing with three different agar media and by the NCCLS M27-A method for in vitro testing of fluconazole against *Candida spp*. J Clin Microbiol. 2003; 41(6):2665–8.
25. Burford-Mason AP, Weber JCP, Willoughby JMT. Oral carriage of *Candida albicans*, ABO blood group and secretor status in healthy subjects. Med Mycol. 1988; 26(1):49–56.
26. Hurd EA, Domino SE. Increased susceptibility of secretor factor gene Fut2-null mice to experimental vaginal candidiasis. Infect Immun. 2004; 72(7):4279–81.
27. Hundshammer C, Minge O. In love with shaping you—influential factors on the breast milk content of human milk oligosaccharides and their decisive roles for neonatal development. Nutrients. 2020; 12(11):1–31.
28. Marcobal A, Barboza M, Sonnenburg ED, Pudlo N, Martens EC, Desai P, et al. Bacteroides in the infant gut consume milk oligosaccharides via mucus-utilization pathways. Cell Host Microbe. 2011; 10(5):507–14.
29. Kojima Y, Ohshima T, Seneviratne CJ, Maeda N. Combining prebiotics and probiotics to develop novel synbiotics that suppress oral pathogens. J Oral Biosci. 2016; 58(1):27–32.
30. Seyfarth F, Schliemann S, Elsner P, Hipler U. Antifungal effect of high- and low-molecular-weight chitosan hydrochloride, carboxymethyl chitosan, chitosan oligosaccharide and N-acetyl-d-glucosamine against *Candida albicans, Candida krusei* and *Candida glabrata.* Int J Pharm. 2008; 353:139–148.