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The intestinal model of monomicrobial and polymicrobial biofilm of *Candida albicans* and the effects of hydrolases and Bgl2 ligand

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Abstract

Background

Candida albicans is the most prevalent human fungal pathogen. In the biofilm, *C. albicans* is more resistant against antifungal due to the extracellular matrix, protecting the yeast cells. The aim of the study were (1) to prove the ability of *C. albicans* to form mix or polymicrobial intestinal biofilm with *Escherichia coli*; (2) to evaluate the interactions between *C. albicans* and *E. coli* in biofilms of the rat models; (3) to investigate the performance of glucosamine (as the Bgl2 ligand) and *A. fulica* hydrolases combination against single and polymicrobial biofilms.

Methods

The rat models were administered orally with *C. albicans* in single microbial model, and plus *E. coli* in polymicrobial model. After the biofilms appeared, these groups were treated with glucosamine and *A. fulica* hydrolases.

Results

The confocal laser scanning microscopy (CLSM) images revealed the strong association between *C. albicans* and *E. coli* in polymicrobial biofilm and the combination treatment of glucosamine and *A. fulica* hydrolases reduced the single microbial biofilm extracellular matrix by 53.58%. But in polymicrobial model, the treatment can only decreased the matrix intensity by 19.17 %.

Conclusions

Asociation between *C. albicans* and *E. coli* in polymicrobial biofilm is strong. Combination of glucosamine and *A. fulica* enzyme has ability to reduce the single microbial biofilm extracellular matrix, but not strong enough to conquer polymicrobial model.

Keywords

C. albicans, *E. coli*, intestinal polymicrobial biofilm, *A. fulica* hydrolases, Bgl2 ligand

Background

Human body is a host for a billion microorganisms. *Candida albicans* is usually found in the human normal microecosystem. *Candida* inhabits several body parts of human, such as oral cavity, gastrointestinal tract, skin and vaginal. It is a dimorphic fungi that can be commensal in a healthy host, yet it can also be an opportunistic pathogen under a certain conditions. This refers to several risk factors such as the use of antibiotics and drugs that suppress the other normal flora, and also the use of medical devices, like catheters or medical heart valve. Under this conditions, *C. albicans* might cause the clinical infections, or candidiasis [1] [2].

The virulence of *C. albicans* is most commonly related to the biofilm formation. Biofilm is a structured microbial communities covered in a matrix extracellular and attached to the surface. Biofilm shows a different phenotypic features from the planktonic

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or motile cells [3]. In nature, biofilm often found in polymicrobial model. This form offers protection to the polymicrobial cells residing under the extracellular matrix [1] [4, 5]. Although it also possible the biofilm model being monomicrobial. Polymicrobial-biofilm associated infections are responsible to significant morbidity and mortality, especially when its related to the medical devices [6, 7] [8] [9]. *C. albicans* has been reported to form a polymicrobial interactions with several bacteria species. The *C. albicans-E. coli* polymicrobial biofilm have been found in endotracheal tubes and urinary catheters [10] [2]. In this study, we observed the *C. albicans* and *E. coli* interactions in rat intestinal model using *Confocal Laser Scanning Microscope* (CLSM).

In the biofilm form, they are significantly more resistance to antimicrobial agents [11–15]. *C. albicans* biofilm is less susceptible to any antifungal or antimicrobial treatment due to several mechanisms. (1) altered growth/metabolic rate of the biofilm cells; (2) The action of resistance genes; (3) the existence of persister cells; (4) the existence of extracellular matrix [16, 17]. The extracellular matrix is secreted by the sessile communities and protect them. It mainly consists of polysaccharides, such as glucans, mannan and chitin, and it could act as a barrier and hamper the antifungal molecules to reach the cells. Although it is not the only cause of the biofilm resistance [12, 17, 18]

Since the presence of the extracellular matrix might hamper the antifungal action, the disruption of the matrix material using enzymatic treatment might be a good strategy to enhanced the antifungal performance. In our previous work, we successfully inhibited the biofilm formation using the Bgl2p ligand, glucosamine, as inhibitor molecule. The Bgl2p refers to glucosyltransferase encoded by BGL2 gene. This glucosyltransferase is responsible to the formation of covalent linkages between β -1,3-glucans and the other components of cell wall and matrix extracellular [19]. In the present work we combine the action of glucosamine ligand and the hydrolases mixture produced by *A. fulica* to support the performance of antimicrobial, hydrolyzing the extracellular matrix of monomicrobial and polymicrobial model of *C. albicans*.

Methods

Bacterial strains, media and growth conditions

The strains used in this study were *C. albicans*-ATCC 10231 and *E. coli*-ATCC 25992 purchased from Balai Besar Laboratorium Kesehatan (BBLK). Overnight cultures of *C. albicans* were grown in yeast extract-peptone-dextrose [YPD; 1% yeast extract (BD Biosciences), 2% peptone (Oxoid Ltd.), and 2% dextrose (Conda Pronadisa)] at 30°C. Overnight cultures of *E. coli* were grown in Luria Bertani [LB; 1% tryptone (Himedia); 0.5% yeast extract; and 1% NaCl (Merck)] medium at 37°C.

Animals

The animals used for the in vivo experiments were male wistar rats weighed about 200 g. All animals are acclimatized for a week and given standard ad libitum diet. The rats were divided into 5 groups, each consists of 5 individuals. Group 1 was normal group without the biofilm induction, group 2 and 4 were the biofilm-induced control group without enzymatic and antifungal treatment for mono and polymicrobial model, respectively. Group 3 and 5 were the monomicrobial biofilm-induced group with enzymatic and antifungal treatment for mono and polymicrobial model, respectively. Each of them were immunosuppressed with 225 mg/ kg b.w. cortison acetate injected subcutaneously and administered with antibiotics mixture of Tetracycline (25 mg/kg b.w.), Streptomycin (20mg/kg b.w.), and Sagestam (7.5mg/kg b.w.) orally to minimize the microbial ecosystem in the rat models. The cortison acetate was purchased from Organon, the Tetracycline was purchased from Sanbe-Indonesia, the Streptomycin was purchased from

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PT Meiji-Indonesia, and the Sagestam was purchased from Sanbe-Indonesia. All animal experiments were carried out in Laboratory Animals, Faculty of Veterinary, Airlangga University, Surabaya, Indonesia. Before the animal experiments were conducted, the ethical clearance (Reg. 271-KE) was obtained from the Animal Care and Use Committee (ACUC), Faculty of Veterinary Medicine Airlangga University.

In-vivo monomicrobial *C. albicans* biofilm induction

The biofilm induction in rat models were carried out according to Baktir (2015a) methods with several modifications. The stock culture of *C. albicans* was growth on sabouraud dextrose agar (SDA, 1% peptone, 4% dextrose, 1.5% agar) at room temperature. The single colony was inoculated on YPD broth with shaking at 120 rpm (37°C) overnight. The inoculum is then administered to the rats in both groups orally. During the biofilm induction periods, the rats diet is given at minimum portion and the rats also given 2.5 ml of the spider medium [1% nutrient broth, 1% D-mannitol (Himedia), 0.2% monopotassium phosphate (Merck)] twice a day to further induce biofilm formation. The period of this phase is 3 weeks long at minimum. The rat models from Group 2 and 4 were sacrificed in order to observe *C. albicans* biofilm macroscopically on the mucous membrane of the cecum after biofilm induction.

In-vivo polymicrobial *C. albicans*-*E. coli* biofilm induction

The polymicrobial biofilm induction was carried out using the similar methods as previous paragraph, with the addition of oral administered *E. coli* inoculum in LB medium along with *C. albicans*. The preparation of *E. coli* inoculum is described as follows. The stock culture of *E. coli* was growth on Nutrient agar (NA, 0.5% peptone, 0.3% beef extract, 1.5% agar, 0.5% NaCl) at room temperature. The single colony was inoculated on LB broth with shaking at 120 rpm (37°C) overnight.

Enzymatic, Bgl2 ligand and antimicrobial combination treatments

After the biofilm induction phase, group 2 was given the combination of enzymatic Bgl2p ligand and antimicrobial treatment. This treatment was carried out for a week. The combination treatment methods was done as follows. The enzyme consortium from *A. fulica* was harvested according to Baktir (2015b). Then the mixture of the harvested enzyme consortium, glucosamine, fluconazole (with the addition of tetracycline for polymicrobial model) were administered to the model rats from group 3 and 5, orally. The rats from group 3 and 5 were then sacrificed after the treatment period, to observe *C. albicans* biofilm macroscopically on the mucous membrane of the cecum after biofilm induction.

Macroscopic identification of biofilm

Identification of intestinal model biofilm was done by macroscopic observations of mucous membrane of the cecum after splitting it and photograph it using camera (Canon eos m3).

Confocal laser scanning microscopy (CSLM)

The samples preparation were carried out as following method. The cleaned and sliced cecum were fixated in 10% formalin buffer, drown in paraffin, and cut at 5µm thickness. Then the cecum tissues were deparaffinized using xylol (2 times) for 10 min each, hydrated using ethanol (absolute ethanol, 90% and 70% consecutively), washed using PBS for 5 min and blocked using 2% BSA in PBS at room temperature for 1 h, and re-washed using PBS for 8 min. A 100 µg/ml of concavaline A (con A; bioWORLD) was dropped to the sample and then incubated at room temperature for 1h. At last, the samples were re-washed using PBS, reblocked using 2% BSA in PBS and the samples were observed using a confocal microscope (Olympus FV1000).

Statistical analysis

All of the data were subjected to independent *T-Test* to test the significant differences

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between two groups. The data normality was calculated using Shapiro Wilk assay (for the number of sample <50). The statistical analysis was conducted using SPSS/16.0 software. P values of <0.05 were considered as indicating a significant difference between the control and treatment groups. All values are expressed as mean ± standard deviation.

Results

Macroscopic observation of monomicrobial and polymicrobial *C. albicans* biofilm formation *in vivo* in rat model

The macroscopic image of monomicrobial *C. albicans* biofilm formation *in vivo* in rat model from group 2 appeared as white lesions located in mucous membrane of the cecum as presented in Fig. 1a. The normal rat model (Group 1) displayed a normal mucous membrane of the cecum without white lesions as presented in Fig. 1b.

The *in vivo* polymicrobial biofilm model from group 4 appeared as white lesions located in the outer mucous membrane of the cecum as presented in Fig. 2a. The normal rat model displayed a normal outer mucous membrane of the cecum without white lesions as presented in Fig. 2b. There was also a huge difference in cecum size between the normal group and group 4 model.

The CLSM analysis of monomicrobial and polymicrobial *C. albicans* biofilm formation *in vivo* in rat model before and after treatments

The Confocal imaging was employed to assess the association between *C. albicans* and *E. coli* from the same polymicrobial model sample with a different colouring. The confocal image of *C. albicans* and *E. coli* showed there was association between these 2 species, since *C. albicans* appeared mostly in the same location as and *E. coli*. It could be seen from the Yellow-orange coloured superimposed image (Fig. 3a). The treatment resulted in the decreased intensity of fluorescence. It could be seen from the dimmer-looks in the confocal image of the treatment group or group 2 (Fig. 3b), and also the quantitative data of the mean intensity that were also decreased in the treatment group (Fig. 4). The lowered intensity was proportional to the decreased cell amount, since the fluorescence appeared in a consequence of the specific bind from the dye with the cells part.

The effect of treatment *C. albicans* cells and matrix in polymicrobial model can also be observed with the confocal imaging. The treatment resulted in the decreased of *C. albicans* cells and matrix, demonstrated by the lower intensities of the confocal image fluorescence (Fig. 5b), compared to the no treatment group (Fig. 5a). The lowered intensity was proportional to the decreased matrix extracellular (Fig. 4).

For the monomicrobial model, the results were also in agreement with the polymicrobial biofilm model. The intensities of the *C. albicans* cells and the matrix reduced in the treatment group, demonstrated by the dimmer confocal image (Fig. 6). Quantitatively, the monomicrobial demonstrated lower amount of cells and matrix (by the lower intensities) compared to the polymicrobial in the treatment group (Fig. 7).

Discussion

Biofilm lifestyle provides protection for the cells resided within the thick extracellular matrix layer. Biofilm allows the cells to develop resistance against the antimicrobial up to 1000-fold greater than its planktonic cells form, leave biofilm eradication as a very difficult task to do [20, 21]. For *C. albicans*, the biofilm grown on surface shown increase in Minimum Inhibitory Concentrations (MIC) of antifungals, including fluconazole, significantly compared to the planktonic cells [21, 22].

C. albicans biofilm consists of blastospores-type cells as basal layer, and superficial

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layer of extracellular matrix and hyphal-type cells. The extracellular matrix composed of carbohydrates (monomers: glucose, N-acetylglucosamine, mannose, and rhamnose), proteins, phosphorus, uronic acid and hexosamine [17, 23]. One of the carbohydrate component is β -1,3-glucans, a glucose polymer. This component are thought to be the main component of the extracellular matrix in biofilms. β -1,3-glucans are synthesized by glucan synthase, a membrane-bounded protein, using UDP-glucose as a substrate and secretes it to the extracellular matrix. This component in *C. albicans* biofilm cells may contribute to antifungal drug resistance. Some studies reported that β -1,3-glucans can be found in the supernatant around biofilm and in the matrix, and the level of this substance increased in a high concentration during biofilm formation phase. The other evidence is that enzymatic treatment of the β -1,3-glucans using β -1,3-glucanase at quite high concentration eradicates biofilms but has no significant effect to planktonic cells. The enzymatic treatment using β -1,3-glucanase at low concentration could not really disrupt biofilms, yet, in combination with fluconazole, enhance this antifungal performance [24–26]. The previous study by Nett (2007) also suggested that β -1,3-glucans bind fluconazole in biofilm, so this binding decreased this antifungal performance to control biofilm-associated cells. Therefore, disrupt β -1,3-glucans component using β -1,3-glucanase is a good strategy to support antifungal performance.

The role of several different genes in the biofilm formation process has been investigated. The glucan transferases, Phr1p and Bgl2p, encoded by *PHR1*, *BGL2*, and exoglucanase, encoded by *XOG1* were predicted to be exist in extracellular matrix, and have roles to deliver and accumulate β -1,3-glucans in the extracellular matrix. The mutant strains lack of these genes show more susceptibility to fluconazole [27]. The competitive inhibition of the enzymes responsible for the construction of biofilm, like *Bgl2p* can be an effective way to promote antimicrobial potential in killing cells directly.

A. fulica is a natural source of several hydrolase enzymes. They produces the enzyme mixture through the digestive gland to help the digest the food. However, several undigestible materials were digested by the enzymes produced by microbial in their gastrointestinal tract. The enzyme mixtures from the digestive tract of this species were carbohydrases, such as mannosidase, glucosidase, chitinase, and β -glucanases, proteinase and lipase. [28–31]. *A. fulica* utilization might reduce the enzyme production cost, since this species is quite abundant species, especially in a tropical and humid area like Indonesia. The enzyme consortium from *A. fulica* has been successfully lysed fungal cell wall of *Candida* sp. [32]. The *Candida* sp. cell wall have similar components as the matrix extracellular with β -1,3-glucans and β -1,6-glucan being a major carbohydrate component (50-60%). So the enzyme mixtures from *A. fulica* is very potential as an affordable and effective antibiofilm treatment. This is also supported by Nett (2007) report, that the treatment of β -1,3-glucanase is very effective to eradicate *C. albicans* biofilm.

So, we hypothesized the combination of treatment using *A. fulica* mixture hydrolases and the *Bgl2* ligand, the glucose substrate look-a-like, glucosamine (Fig. 8), might work synergistically to enhance extracellular matrix eradication and increase the killing of pathogenic cells resides within extracellular matrix. The hydrolase decrease matrix by hydrolyzing its complex polymer components, mainly β -1,3-glucan, to be its oligomers or monomers. The ligand glucosamine, bind to the specific binding site of the glucan transferase, replacing its original substrate that is glucose and inhibit the β -1,3-glucan delivery and accumulation in matrix, thus further disrupt the biofilm formation. However, in this study, we did not compare the performance of this potential antibiofilm as combination and separate components. We compared the action of this combined treatment when applied to the monomicrobial and polymicrobial biofilm model *in vivo*.

Biofilms consisting of single microbial species have been extensively studied in the

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past, however, more recent investigations have found that polymicrobial biofilms are the dominant form in nature. In this study, we investigated the interaction between bacteria and fungi in a polymicrobial biofilm model composed of *E. coli* and *C. albicans*. Both of these species are also predominant pathogens that can grow as biofilms on medical devices [2]. The result of this study demonstrated that the extracellular matrix disruption using ligand-enzyme combination treatment in monomicrobial biofilm model was more effective compared to the treatment in polymicrobial biofilm model. The extracellular matrix intensity significantly decreased by 53.58% in monomicrobial. But in polymicrobial model, the treatment can only decrease the matrix intensity by 19.17 %, despite the fact that it was also considered significantly decreased (Fig. 4 and Fig. 7). The gap in the ligand-enzyme performance to decrease the matrix material, might be due to the strong and synergistic association between *E. coli* and *C. albicans*. The previous study stated that *E. coli* might facilitate the *C. albicans* attachment to the host surface and resulted in the strengthen binding between the cells and the host[33, 34] .

Conclusions

The treatment method of matrix hydrolysis and BglI inhibition in combination with antimicrobial successfully reduced biofilm matrix, *E. coli* and *C. albicans*. The extracellular matrix intensity significantly decreased by 53.58% in monomicrobial model. But in polymicrobial model, the treatment can only decrease the matrix intensity by 19.17 %. It might be due to the strong and synergistic association between *C. albicans* and *E. coli*, resulted in the less susceptible treatment in polybial model.

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Declarations

Authors' contributions

AB conceived the study and designed it together with, NAA, MSY, HLG, M and SS. AB conducted the experiments with contribution from NAA, MSY, HLG, and M. HLG collected microorganism isolates. M performed the statistical study with contributions from MSY. NAA drafted the manuscript with contribution from AB, M, MSY, HLG and SS. All author have read and approved the final manuscript.

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Ethics approval and consent to participate

We would like to thank DIPA BOPTN-Directorate General of Higher Education of Indonesia (DIKTI) for the research funding. We also indebted to the Animal Care and Use Committee (ACUC) Faculty of Veterinary Medicine, Airlangga University who has tested the feasibility of the material and provide recommendation pre-clinical studies using animal test (*Rattus novergicus*).

Consent for publication

Not applicable

Availability of data and materials

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Competing interests

The author(s) declared that they have no competing interests.

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References

1. Harriott MM, Noverr MC. **Importance of Candida-bacterial polymicrobial biofilms in disease.** Trends Microbiol. 2011;**19**:557–63.
2. Samaranyake YH, Bandara HMHN, Cheung BPK, Yau JYY, Yeung SKW, Samaranyake LP. **Enteric gram-negative bacilli suppress Candida biofilms on Foley urinary catheters.** Acta Pathol Microbiol Immunol Scand. 2014;**122**:47–58.
3. Gulati M, Nobile CJ. **Candida albicans biofilms : development , regulation , and molecular mechanisms.** Microbes Infect. 2016;**18**:310–21.
4. Davey ME, O'toole GA. 2000 Davey **Microbial Biofilms: from Ecology to Molecular**

Genetics. *Microbiol Mol Biol Rev.* 2000.

5. Morales DK, Hogan DA. **Candida albicans Interactions with Bacteria in the Context of Human Health and Disease.** 2010;**6**:6–9.

6. Røder HL, Sørensen SJ, Burmølle M. **Studying Bacterial Multispecies Biofilms: Where to Start?** *Trends Microbiol.* 2016;**24**:503–13.

7. Burmølle M, Ren D, Bjarnsholt T, Sørensen SJ. **Interactions in multispecies biofilms: do they actually matter?** *Trends Microbiol.* 2014;**22**:84–91.

8. Peters BM, Jabra-Rizk MA, O'May GA, William Costerton J, Shirtliff ME. **Polymicrobial interactions: Impact on pathogenesis and human disease.** *Clin Microbiol Rev.* 2012;**25**:193–213.

9. Tsui C, Kong EF, Jabra-rizk MA. **Pathogenesis of Candida albicans Biofilm.** *Pathog Dis Adv Access.* 2016.

10. Vandecandelaere I, Matthijs N, Nelis HJ, Depuydt P, Coenye T. **The presence of antibiotic-resistant nosocomial pathogens in endotracheal tube biofilms and corresponding surveillance cultures.** 2013;**1**:142–8.

11. Taylor P, Mayer FL, Wilson D, Hube B, Mayer FL, Wilson D, et al. **Candida albicans pathogenicity mechanisms.** 2013; December 2014:**37**–41.

12. Nobile CJ, Johnson AD. **Candida albicans Biofilms and Human Disease.** *Annu Rev Microbiol.* 2015;**69**:71–92.

13. Fu J, Ding Y, Wei B, Wang L, Xu S, Qin P, et al. **Epidemiology of Candida albicans and non-C.albicans of neonatal candidemia at a tertiary care hospital in Western China.** *BMC Infect Dis.* 2017;**17**:1–6.

14. Bitew A, Abebaw Y. **Vulvovaginal candidiasis: Species distribution of Candida and their antifungal susceptibility pattern.** *BMC Womens Health.* 2018;**18**:1–10.

15. Ou HT, Lee TY, Chen YC, Charbonneau C. **Pharmacoeconomic analysis of antifungal therapy for primary treatment of invasive candidiasis caused by Candida albicans and non-albicans Candida species.** *BMC Infect Dis.* 2017;**17**:1–9.

16. Seneviratne CJ, Jin L, Samaranyake LP. **Biofilm lifestyle of Candida: A mini review.** *Oral Diseases.* 2008;**14**:582–90.

17. Pierce C, Vila T, Romo J, Montelongo-Jauregui D, Wall G, Ramasubramanian A, et al. **The Candida albicans Biofilm Matrix: Composition, Structure and Function.** *J Fungi.* 2017;**3**:14.

18. Zarnowski R, Westler WM, Lacmouh GA de, Marita JM, Bothe JR, Bernhardt J, et al. **Novel entries in a fungal biofilm matrix encyclopedia.** *MBio.* 2014;**5**:e01333–e01314.

19. Hartland RP, Emerson GW, Sullivan PA. **A Secreted Formula-glucan-branching Enzyme from Candida albicans.** *Proc R Soc B Biol Sci.* 1991;**246**:155–60.

20. Sherry L, Rajendran R, Lappin DF, Borghi E, Perdoni F, Falleni M, et al. **Biofilms formed by Candida albicans bloodstream isolates display phenotypic and transcriptional heterogeneity that are associated with resistance and pathogenicity.** 2014;**1**:1–14.

21. Lara HH, Urbina DGR, Pierce C, Ribot JLL, Jiménez MJA, Yacamán MJ. **Effect of silver nanoparticles on Candida albicans biofilms: an ultrastructural study.** *J Nanobiotechnology.* 2015;**1**:1–12.

22. Chandra J, Kuhn DM, Mukherjee PK, Hoyer LL, McCormick T, Ghannoum MA. **Biofilm formation by the fungal pathogen Candida albicans: Development, architecture, and drug resistance.** *J Bacteriol.* 2001;**183**:5385–94.

23. Lal P, Sharma D, Pruthi P, Pruthi V. **Exopolysaccharide analysis of biofilm-forming Candida albicans.** *J Appl Microbiol.* 2010;**109**:128–36.

24. Al-Fattani MA, Douglas LJ. **Biofilm matrix of Candida albicans and Candida tropicalis: chemical composition and role in drug resistance.** *J Med Microbiol.*

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2006;**55**:999–1008.

25. Andes D, Nett J, Oschel P. **Development and characterization of an in vivo central venous catheter *Candida albicans* biofilm model.** *Infect Immun.* 2004;**72**:6023–31.

26. Nett J, Lincoln L, Marchillo K, Massey R, Holoyda K, Hoff B, et al. **Putative role of beta-1,3 glucans in *Candida albicans* biofilm resistance.** *Antimicrob Agents Chemother.* 2007;**51**:510–20.

27. Taff HT, Nett JE, Zarnowski R, Ross KM, Sanchez H, Cain MT, et al. **A *Candida* Biofilm-Induced Pathway for Matrix Glucan Delivery: Implications for Drug Resistance.** *PLoS Pathog.* 2012;8.

28. The Comparative Physiology of Digestion. 1961;252:245–52.

29. Leparoux S, Colas B. **Digestive juice of *Achatina achatina* as a potential source of transglycosylation enzymes.** *Int J Biochem.* 1994;**26**:247–54.

30. Maeda I, Shimohigashi Y, Kihara H, Ohno M. **Purification and Characterization of a Cellulase from the Giant Snail *Achatina fulica*.** *Biosci Biotechnol Biochem.* 1996;**60**:122–4.

31. Agogbua SIO, Anosike EO, Ugochukwu EN. **Partial Purification And Some Properties Of Arylsulphatases From The Gut Of The Giant African Snail , ACHATINA ACHATIN A.** 1978;**5915**:169–73.

32. Ezeronye OU, Okerentugba PO. **Optimum conditions for yeast protoplast release and regeneration in *Saccharomyces cerevisiae* and *Candida tropicalis* using gut enzymes of the giant African snail *Achatina achatina*.** *Lett Appl Microbiol.* 2001;**32**:190–3.

33. Makrides HC, MacFarlane TW. **An investigation of the factors involved in increased adherence of *C. albicans* to epithelial cells mediated by *E. coli*.** *Microbios.* 1983;**38**:177–85.

34. Brucker K De, Tan Y, Vints K, Cremer K De, Braem A, Verstraeten N, et al. in a **Polymicrobial *E. coli* / *Candida albicans* Biofilm.** 2015;**59**:3052–8.

Figures

Figure 1 - Macroscopic image of monomicrobial *C. albicans* biofilm.

Macroscopic image of monomicrobial *C. albicans* biofilm in cecum mucous membrane of the group 2 rat model (a) and the normal rat model from group 1 (b). Biofilm shown in the red circle, appeared as white lesions.

Figure 2 - Macroscopic image of polymicrobial *C. albicans* biofilm in cecum mucous outer membrane.

Macroscopic image of polymicrobial *C. albicans* biofilm in cecum mucous outer membrane of the normal rat model from group 1 (a) and the group 4 rat model (b). Biofilm shown in the red circle, appeared as white lesions.

Figure 3 - CLSM image of *C. albicans* and *E. coli* cells

Confocal image of *C. albicans*-*E. coli* polymicrobial biofilm model. (a) non treatment polymicrobial biofilm group. (b) treatment polymicrobial biofilm group. The green colored image displayed *C. albicans* cells, colored using polyclonal-Antibody Anti-Candida-FITC. The red colored image displayed *Escherichia coli* cells, colored using SYTO-59. Yellow-orange colored displayed the superimposed image of *C. albicans* and *E. coli* confocal image.

Figure 4 - Quantitative data from CLSM assessment in polymicrobial biofilm model.

The mean intensities of *E. coli*, *C. albicans* and matrix were shown in the data below the chart. All of the *E. coli*, *C. albicans* and matrix consecutively shown the decreased intensities in the treatment group by 31.51%, 39.03%, 19.17%. ** indicates the significant differences ($p < 0.05$)

Figure 5 - CLSM image of *C. albicans* cells and matrix

Confocal image of *C. albicans* and matrix of the polymicrobial biofilm model. (a) non treatment polymicrobial biofilm group. (b) treatment polymicrobial biofilm group. The green colored image displayed matrix, colored using Concavaline A. The red colored image displayed *C. albicans* cells, colored using Polyclonal-Antibody Anti-Candida conjugated to TRITC. Yellow-orange colored displayed the superimposed image of *C. albicans* and matrix confocal image.

Figure 6 - CLSM image of *C. albicans* cells and matrix

Confocal image of *C. albicans* and matrix of the monomicrobial biofilm model. (a) non treatment monomicrobial biofilm group. (b) treatment monomicrobial biofilm group. The green colored image displayed matrix, colored using Concavaline A. The red colored image displayed *C. albicans* cells, colored using Polyclonal-Antibody Anti-Candida conjugated to TRITC. Yellow-orange colored displayed the superimposed image of *C. albicans* and matrix confocal image.

Figure 7 - Quantitative data from CLSM assessment in polymicrobial biofilm model

Quantitative data from CLSM assessment in monomicrobial biofilm model. The mean intensities of *C. albicans* and matrix were shown in the data below the chart. Both of the *C. albicans* and matrix consecutively shown the decreased intensities in the treatment group by 49.83% and 53.58%. ** indicates the significant differences ($p < 0.05$).

Figure 8 – The structure of glucosamine

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