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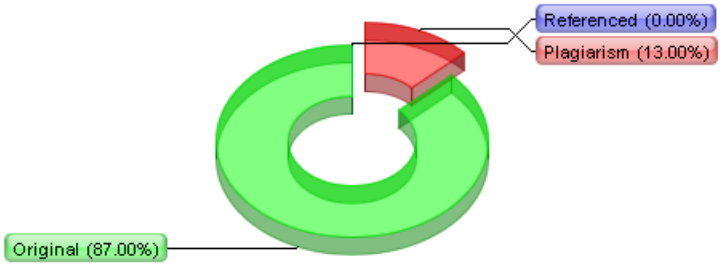

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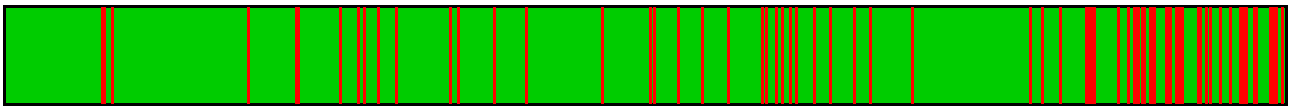

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1, Loo Loo Hariyanto Raharjo1, Harsono1, Putu Oky Ari Tania2 ,Ni'matuzahroh3 and Afaf Baktir4*1
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Email: afafi2001@yahoo.comAbstract
Background
: The incidence of candidiasis caused by Candida albicans is very high in the world. Resistance to antifungal is very common. Virulence and antifungal resistance of C. albicans are recently known by its ability to form biofilm.Objective
: to characterize the

Candida albicans biofilm formation in the intestinal mucosa of wistar rats.Methods
: This study used 32 wistar rats

and was divided into two groups, i.e., control and treatment groups. Sampling data was conducted

on days 7, 14, 21, 28, and 35 after

the inoculation of C. albicans. The biofilm formation stage of C. albicans was monitored through calculations of C. albicans cells in feces and intestinal mucosa of the rats with CFU methods (colony forming units) every week post-inoculation of C. albicans. The formation of biofilm was observed by the immunofluorescence method, using CLSM (confocal laser scanning microscope).Results
: The formation and maturation of C. albicans biofilm in the intestinal mucosa occurred on the 28th and 35th-day post-inoculation of C. albicans, respectively. Through the immunofluorescence method, the matrix of extracellular biofilm showed green color with high intensity.Conclusion
: The biofilm formation of C. albicans was successfully induced by antibiotics and immunosuppressant agent. The Candida biofilm model in vivo is useful for examining the natural ingredients in reducing biofilm in the next research.Keywords:
candidiasis, biofilm, Candida albicans, CLSM.Introduction
Candida albicans is a normal microflora in mucosa of gastrointestinal, upper respiratory tract, skin, the genital mucosa of mammals, urethra, skin, and tissue under fingernails (1). These microorganisms could be overgrowth when there is imbalance of microbial ecosystem (dysbiosis) in the digestive tract. These were caused by using therapeutic antibiotics, immunosuppressants, steroids, and drugs excessively, diabetes, increased estrogen during pregnancy, consumption of contraception pills, and obesity (2).In an appropriate environment for growth, Candida is in the planktonic form which frees with yeast-shaped cell morphology. Lack of nutrients or the presence of agents that are harmful to cells induces Candida to form biofilm (3). A biofilm is a group of cells arranged in such way that enveloped by extracellular polymer matrix (4). The virulence and resistance of many pathogenic microorganisms, including Candida, are determined by its ability to form biofilms.In recent years, it has been a lot of research on biofilm of C. albicans, whether formed on the surface of the oral mucosa (5), vagina (6), denture (7) as well as on the surface of the abiotic components (8). Dongari-Bagtzoglou et al. (2009) have reported that the extracellular matrix and filament cells are the characteristics of mucosal biofilm (5). The biofilm of C. albicans on the surface of oral mucosa is composed of yeast, commensal bacteria, hypha, and the extracellular matrix. β-glucan on the cell wall and matrix of C. albicans biofilm grow excessively. The content of β-glucan in the form of mature biofilm is twice compared to planktonic (9).

The presence of β-glucan in

both cell wall of C. albicans and extracellular matrix layer of C. albicans contributed to the lowered antifungal penetration into the cytoplasm of the cell, causing resistance to antifungals. This study plays an important role to examine the stage of maturation of C. albicans biofilm and its colonization on the intestinal mucosa through confocal microscope so that it can be determined when giving optimal antifungal agent therapy for eradication.Although the ability of C. albicans in forming biofilms on the biotic mucosa or abiotic surfaces has been widely reported in the last few years (5) (6) (7) (8), there is no information about the formation of intestinal mucosa biofilms. The model of intestinal biofilm is indispensable for examining natural ingredients that can control biofilms to reduce the antifungal resistance.Method
2.1 Research designCandida albicans
isolate

was acquired from the Laboratory of Microbiology, Faculty of Dentistry, Airlangga University.

The study has been approved by The Animal Care and Use Committee, Faculty of Veterinary Medicine,

Airlangga University no 457-KE. Animals were monitored daily for distress to environmental factors that affect the immune system.The animal model used in this study was male wistar rats (
Rattus novergicus). The use of male rats as experimental animals was due to the fact that males are not affected by sex hormones estrogen that regulate several pro-inflammatory pathways (10). A total of 32 rats, 2-3-month-old, weight of 160-170 g, were divided into two groups, i.e., the control and treatment groups. Based on Federer's formula to calculate sample size: (t−1)(n−1) ≥ 15; t: number of group were 2 groups, control group, and treatment group,

rats in each group. The sample size in each group was 16 rats, and total animals were 32 rats (11).2.2 Induction of Biofilm Formation in Intestinal Mucosa of Wistar ratsMale wistar rats (32 rats) were acclimatized for 1 week with each group had 16 rats which each cage filled with 4 rats. The treatment group administered with

gentamycin (7.5 mg/kg) every day for 5 days per oral. On day 5, rats

) via subcutan as immunosuppressant. On day 6, rats were administered with C. albicans through orogastric gavage. During treatment, rats were fed with standard feed from American Institute of Nutrition (AIN-93)

On day 3 pre- and day 3, 7, 14, 28, and 35 post-inoculation of C. albicans, three rats from the control and treatment groups were terminated. The termination was done by giving ether inhalation. The intestinal mucosa was isolated. The intestine was cleaved and cleaned

extent of the mucous membrane was measured in area and scraped using a spatula, and then suspended in sterile water. The mucose suspension was then spread to the SDA media to calculate the number of colonies.The colony number of C. albicans in feces and intestinal mucosa was determined by using CFU methods (colony forming units) in SDA medium. The formation of biofilm was observed with the immunofluorescence method, using CLSM (Confocal Laser Scanning Microscope (Olympus, FV1000 type, 400×)). This data collection was conducted in triple2.3 The Quantitative Analysis of C. albicans Cells (CFUs)CFU analysis in the feces of wistar ratsFresh fecal' samples were collected 10 min after the first feces were produced. The fecal samples were then placed in aseptic pots. The fecal samples (1 g) w
ere transferred into falcon tubes containing 9 mL PBS and then the solution was homogenized. Serial dilution was performed from 10−1 to 10−6, in which 1 mL of suspension from 10−4 dilution was spread onto

r containing 50 ug/mL of ampicillin and 100 ug/mL of streptomycin. Those steps were repeated for 10−5 and 10−6 of dilution. The inoculums

days. Colony numbers were counted with the following formula:CFU analysis in the intestinal mucosa of wistar rats
Cecums
were collected and cleaned with PBS in order to remove the feces and contaminants attached to the mucosal surface. Then, each cecum was cut about 1 cm, and the inner part (mucosa) was scraped by using spatula then diluted with 10−1-10−3 dilution, to obtain intestinal mucosa suspension. A total amount of 0.2 mL of intestinal mucosa suspension was spread to YPD agar containing 50 ug/mL of ampicillin and 100 ug/mL streptomycin, incubated at room temperature for 2-3 days and then colony formed was counted with the following formula:2.4
Observation

Intestinal Mucosa of Wistar Rats with CLSMThe part of intestines w

as split up, cleaned, and then fixed with formalin buffer solution 10%, embedded in paraffin and cut off with a thickness of 5 um. The first stage was deparaffinization with xylol (twice) for 10 min, then terraced with ethanol (absolute ethanol, 90%, 70% for 5 min). Then the preparates were soaked in PBST (Potassium Buffer Saline Tween) for 5 min three times, and then in citrate buffer 10 mm pH 6 for 15 min at 120'C. Further preparations were washed with PBST (thrice) for 5 min, blocking with BSA 2%

in PBST at room temperature for 1

h and washed PBST (thrice) for 8 min. Dropped prepared with Con-A in PBST (one time) and incubation for 1 h then washed with PBST (thrice) for 8 min. Blocking again with BSA 2% in PBST at room temperature 1 h and washed PBST (thrice) for 8 min. The last stage was the incubation at 28'C with antibody-anti Candida in the BSA 2% for 1 h and washed PBST (thrice) for 8 min and the slide was observed by using CLSM. Biofilm formation was observed descriptively. Observation consists of fluorescent intensity. Green fluorescent showed thickness of biofilm matrix and red fluorescent showed Candida albicans.3 Results and Discussion3.1 Induction of Biofilm Formation in Intestinal Mucosa of wistar ratsThe formation of C. albicans biofilm in the intestinal mucosa of Rattus norvegicus through induction in vivo was induced by using three broad-spectrum antibiotics (tetracycline, streptomycin, and gentamycin) (12) and immunosuppressant (5).The use of broad -spectrum antibiotics (tetracycline, streptomycin, and gentamycin) aims to disturb homeostasis of normal microflora, in order for C. albicans cells to colonize more excessively in the intestinal mucosal of wistar rats compared with single antibiotic usage. Gentamycin and streptomycin are aminoglycosides which inhibits synthesis of protein of Gram-negative aerobic bacteria, while tetracycline is broad-spectrum antibiotic that inhibits many bacteria

both Gram-positive and Gram-negative

also anaerobic.The formation of biofilm was determined through cell number of C. albicans in the feces and intestinal mucosal of wistar rats at certain periods expressed in CFU/mL.3.2 Monitoring of C. albicans Cell Number in Feces of wistar ratsThe monitoring of C. albicans overgrowth in the intestinal mucosa needed fresh feces which were collected in less than 10 min. The increased number of C. albicans cells at certain period as parameter of overgrowth of C. albicans.Sampling and platting feces before antibiotics and immunosuppressed (pre-test) administrations aimed to find out the initial amount of C. albicans cells as normal microflora in intestinal tract of wistar rats. The number of C. albicans cells of both control and treatment group was initially almost the same. In the next period, the number of C. albicans cells in control group tends to constant, while treatment groups were increased.

The overgrowth of C. albicans occurred 14 days after inoculation. On day

21 until 35, the number

of C. albicans cells in the

feces decreased (Figure 1).Figure 1
. The density of C. albicans in feces of wistar rats.B
efore administered with C. albicans, the number of C. albicans cells of both control and treatment groups was almost the same. In the next period, the number of C. albicans cells in control group tended to constant, while treatment groups changed. On the third until seventh days after administering, the number

of C. albicans cells in the

feces of treatment groups slightly increased. On the fourteenth day, the number of C. albicans cells in feces of treatment groups drastically increased. This indicated that antibiotics and immunosuppressant lead to disturbance of the balance of microflora in the intestinal mucosal of wistar rats. Conversely, C. albicans was able to survive then overgrowth, colonize, and attach on the feces of wistar rats.On the twenty-first days, the number

of C. albicans cells in the

feces was decreased. This condition was directly related to limitation of nutrition supply during this time. At the same time, C. albicans started to form biofilm. This situation started in the fourteenth. In this period, biofilm formation was initiated. Then followed by biofilm maturation, which occurs in thirty-fifth days.The number of C. albicans cells expressed in CFU/mL as performed by Rosenbach (2010) and White (2007) which were analyzing the colonization

of C. albicans in the intestinal

mucosa of rats with the aim of studying the genes regulating the colonization. Rosenbach (2010) reported that C. albicans WT (wild-type) has the capability of colonization in intestinal mucosa until the twenty-first days after inoculation. The cell number of C. albicans in the feces increased on the seventh day and decreased on the fourteenth days and the twenty-first days. The differences were caused by the use of antibiotics without immunosuppressant in our research.3.3
Monitoring of C. albicans Number Cells in the Intestinal Mucosa of wistar ratsThe biofilm formation can be indicated by the increasing cells number

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of C. albicans in the intestinal

mucosa of the treatment group. On the fourteenth days, overgrowth

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of C. albicans in the intestinal

mucosa occurred; therefore, the cell count of C. albicans was started on the fourteenth days after inoculation (Figure 2).Figure 2.
The density

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of C. albicans cells in the

intestinal mucosa of wistar ratsT
he lowest number

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of C. albicans cells in the

intestinal mucosa was observed on the fourteenth day. At this stage, C. albicans was overgrowth and nutrition intake from the intestinal mucosa of wistar rats.

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A lack of nutrients induces biofilm formation in

vitro (13). On the twenty-first days, the number

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of C. albicans cells in the

intestinal mucosa increased slightly, because colonization and first stage of formation biofilm was started. The colonization of C. albicans depends on regulatory genes of colonization (14). The gen Cph2p is an important regulatory gene and expressed in intestinal mucosa of wistar rats during colonization (12). During this period, the number

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of C. albicans cells in the

intestinal mucosa of wistar rats tended to constant on the fourteenth until twenty-first days after inoculation.On day 14, the number of C. albicans cells in intestinal mucosa is very least. But on day 21 until 35, it is increased slightly. On the twenty-eighth days, the number

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of C. albicans cells in the

intestinal mucosa of wistar rats at treatment groups was increased and the thirty-fifth days tended to sharply increase. At this stage, biofilm in the intestinal mucosa of wistar rats was in a mature stage.3.4
Observation of Biofilm Formation in the Intestinal Mucosa of Wistar ratsThe biofilm formation of C. albicans was observed using CLSM based on immunofluorescence method targeted to extracellular matrix produced

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by C. albicans as well as the

presence of C. albicans. This method used combination of fluorescent staining of Concanavalin A (Con A) and Polyclonal anti-Candida that are conjugated TRITC (Tetramethyl Rhodamine Isothiocyanate). Con-A selectively binds mannose and glucose residues of polysaccharide constituent of either

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the cell wall as well as

extracellular matrix of C. albicans biofilm, while polyclonal anti-Candida selectively binds cells of C. albicans. Thus, the existence of matrix extracellular biofilm of C. albicans can be observed through the blue-green color of the Con-A fluorescence. The greener color fluorescence indicates the thicker extracellular matrix, while the

existence of cells of C. albicans can be seen from the red color after colorization with polyclonal anti-Candida conjugated with TRITC. The darker red color indicates the more cell number of C. albicans in the biofilm.Figure 3.

C. albicans biofilm formed in the intestinal mucosa of wistar rats and observed with CLSM using immunofluorescence method. Tissue without staining (A), with Polyclonal anti-Candida conjugated TRITC (red)

cells (B), with double staining of Con-A (green) and Polyclonal anti-Candida conjugated TRITC (red) for the presence of extracellular matrix and C. albicans (C).Figure 3 shows r

econstruction of intestinal mucosa of wistar rats in 3-D for the treatment groups during formation biofilm C. albicans. The early stage of biofilm formation (the seventh, fourteenth, and twenty-first days after inoculation), the area of mucosal membrane was less fluorescence and the intensity of extracellular matrix was low. This means that at the early stage, the extracellular matrix has not been formed and the C. albicans is still in low. Meanwhile, on the twenty-eighth and thirty-fifth days after inoculation, the biofilm has been formed in a mature phase. At this stage, the biofilm biomass expands, and extracellular matrix is getting accumulated and thickened (15) (16) (17). It could be seen from the blue-green and red fluorescence colors were very strong with a high intensity of extracellular matrix. The existence of correlation between cells of C. albicans with extracellular matrix can be observed through the yellow fluorescence colors in Figure 3C. Several other studies have used CLSM to observe the structure of biofilms formed in vitro and in vivo. Nett (2010) used two sets of fluorescent dyes (FUN-1 and Con-A) and (Calcoflour white and SYTO 9) to examine the formation of C. albicans biofilms on the surfaces of dentures (7). The structure of the C. albicans biofilm is composed of fungal cells, extracellular matrix (EPS), hyphae, and bacteria. C. albicans biofilms in the vagina are composed of yeast and hyphal cells that are embedded in the extracellular matrix, depicted through staining using Con-A (6). The structure of the C. albicans biofilm in the mucosa of the rat tongue has been investigated by Dongari-Bagtzoglou, A. (2009). Epithelial cells, neutrophils, and commercialized bacteria interacting in mucosal biofilms were observed with CLSM using the fluorescence in situ hybridization (FISH) method (5).The presence of extracellular matrix on C. albicans biofilm is important as a shelter and sustains the immune response from the host cell and could resist to antifungal drugs. In other studies, the body's

stages of formation of the mouse C. albicans intestinal biofilm, so that in the future, an immunomodulatory therapy can be developed against candidiasis so that it can inhibit the formation of C. albicans biofilms (18).The absence

does not mean that the individual has no candidiasis. This can be seen on the 28th day where C. albicans was not found in the stool at the same time grew bushy (overgrowth) in the intestinal mucosa.Thus, this research has successfully created and characterized the biofilm formation model

mucosa of wistar rats. Furthermore, the characterization of the biofilm formed is of interest for future research.Conclusion

The biofilm

formation of C. albicans was successfully induced by antibiotics and immunosuppressant agents. The formation and maturation of C. albicans biofilm have been observed on days 28 and 35 after administration of C. albicans, indicated by the high intensity of extracellular matrix and the increasing cell number of C. albicans.Acknowledgement

of the Republic of Indonesia for the research funding.References

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