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RESEARCH ARTICLE
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New phenomena for clinicians, model of *Candida albicans* mobilization before and after biofilm formation in the intestinal mucosa of Wistar rats (*Rattus norvegicus*)

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Abstract

Background and Aim: The virulence and antifungal resistance of *Candida albicans* are recently known for their ability to form biofilm. This research aimed to construct an *in vivo* model of *C. albicans* biofilm in Wistar rats' intestinal mucosa and study their mobilization while in a planktonic and biofilm formation. In this study, there was one treatment group that was treated with three antibiotics, immunosuppressants, and *C. albicans*.

Materials and Methods: This study was divided into control and treatment groups. The data sampling was conducted after *C. albicans* inoculation. The *C. albicans* biofilm formation stage was monitored with colony-forming units method calculation every week post-inoculation and then observed by the confocal laser scanning microscope.

Results: The planktonic *C. albicans* overgrowth occurred up to 14 days after inoculation. The formation and maturation of *C. albicans* biofilm in the intestinal mucosa started in the 28th and 35th-day post-inoculation, respectively. The density of planktonic *C. albicans* in the stool was dramatically decreased on the 35th day. Before the biofilm formation, the planktonic *Candida* was carried away by food scraps to be released as a stool. However, there were minuscule or no planktonic *Candida* observed in the stool during and after biofilm formation. Instead, they were attached to the caecum's mucosa as a biofilm.

Conclusion: We have proved that the planktonic *C. albicans* with its mobile nature were carried into the stool along with the rest of the feed, as we observed a lot of *C. albicans* cells found in the stool. Meanwhile, on day 28 after administration of antibiotics and immunosuppressants, no *C. albicans* was found in the stool samples, and at the same time, we observed *C. albicans* cells and their matrix attached to the intestinal mucosa as a biofilm.

Keywords: biofilm, candidiasis, *Candida albicans*, confocal laser scanning microscope, intestinal mucosa.

Introduction

Candida albicans is a normal microflora in the gastrointestinal (GI) mucosa, upper respiratory tract, skin, mammals' genital mucosa, urethra, skin, and tissue under fingernails [1]. These microorganisms could overgrow when there is an imbalance of microbial ecosystem (dysbiosis) in the digestive tract [2]. Although *Candid*a is one of the most common genera in the human gut mycobiota [3,4], its implication in fungal dysbiosis has been reported in several GI and inflammatory conditions [5,6], as well as in Rett syndrome [7] that is associated with autism spectrum disorders (ASDs). In an appropriate environment, *Candida* will be in a free-moving planktonic form with yeast-shaped cell morphology. The lack of nutrients or the presence of harmful agents can induce *Candida* to form a biofilm [8]. A biofilm is a group of cells with a certain arrangement and enveloped by an extracellular polymer matrix [9]. Dongari-Bagtzoglou *et al*. [10] reported that the extracellular matrix (ECM) and the filament cells are the characteristics of mucosal biofilm. β-glucan on the cell wall and *C. albicans* biofilm matrix grows excessively in biofilm. The presence of β-glucan in both *C. albicans* cell wall and ECM layer contributed to the decrease of antifungal penetration into the cytoplasm of the cell, causing antifungals resistance. This study plays an important role to examine the maturation stage of *C. albicans* biofilm and its colonization on the intestinal mucosa through a confocal microscope so that the time for optimal antifungal agent therapy administration can be determined.

In the biofilm form, *Candida* cells are embedded in a matrix that is firmly attached to the intestinal membrane mucosa. Conversely, the planktonic form of *Candida* cells lives freely and detached from the mucosa, so they move down with the rest of the food

following the intestinal peristaltic movement and then come out as stool. In the case of intestinal candidiasis like ASD gut, *Candida* cells will not be detected in stool samples if they form biofilms in the GI tract. 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 [10-13], there is no information about the formation of intestinal mucosa biofilms. The model of intestinal biofilm is indispensable for examining the natural ingredients that can reduce its antifungal resistance [14,15].

The microbiota composition in stool has been widely studied to see its effect on the development of GI disorders. For example, Tomova *et al*. [16] examined the role of the digestive microbiota on the behavior of autistic persons. The microbiota composition was determined based on their quantity in stool by various methods, from conventional methods of cell culture with total plate count to reverse transcription-polymerase chain reaction. The effect of herbal, probiotic, and medicinal ingredients on the growth of pathogenic microbiota in the digestive tract was also reported [17]. Unfortunately, the quantity of microbiota in those studies did not involve the measurement of biofilm markers [16-19], yet it is suspected that a biofilm will be formed during the herbal, probiotic, or medicinal ingredients treatment [17].

Therefore, the purposes of this study were (1) to study the mobilization of the intestinal microbiota, represented by *C. albicans*, when it is in both biofilm and planktonic form and (2) to construct an *in vivo* biofilm model of *C. albicans* in the intestinal mucosa of Wistar rats.

Materials and Methods

Ethical approval

The study has been approved by The Animal Care and Use Committee, Faculty of Veterinary Medicine, Airlangga University, Surabaya, Indonesia (number 457-KE).

Study period and location

This study was conducted from March to September 2020. The experiment with animals was managed in Animal Laboratory, Faculty of Veterinary Medicine, Airlangga University. Meanwhile, the observation of confocal laser scanning microscope (CLSM) was carried out in Central Laboratory of Biological Sciences, Brawijaya University.

Study design

The animal model used in this study was male Wistar rats (*Rattus norvegicus*). Male rats were used because they are not affected by sex hormone, like estrogen, that regulates several pro-inflammatory pathways [20]. Besides, female rats have an estrus cycle that may bias and interrupt the inflammatory process, especially in a long experiment, that is, 35 days. A total of 32 rats, 2-3-month-old, 160–170 g, were divided into two groups, that is, the control and treatment groups. The sample size was based on Federer's formula to calculate sample size (eq. 1):

$$(t–1)(n–1)\geq 15 \quad (1)$$

Where t is the number of groups (2; control group and treatment group) and n is the number of rats in each group. The sample size in each group should be 16 rats and 32 rats in total [21].

Induction of biofilm formation in intestinal mucosa

The formation of *C. albicans* biofilm in the samples' intestinal mucosa through *in vivo* induction was induced by three broad-spectrum antibiotics (tetracycline, streptomycin, and gentamicin) [22] and immunosuppressant [10]. The use of broad-spectrum antibiotics (tetracycline, streptomycin, and gentamicin) aims to disturb the homeostasis of normal microflora, so it would lead *C. albicans* cells to colonize excessively in the intestinal mucosal compared with single antibiotic usage. Gentamicin and streptomycin are aminoglycosides that inhibit the synthesis of protein of Gram-negative aerobic bacteria [23]. While, tetracycline is a broad-spectrum antibiotic that inhibits many bacteria, both Gram-positive and Gram-negative, and anaerobic bacteria [24].

The animal samples were acclimatized for 1 week in the cages and then those rats were divided into two groups based on the mentioned calculation above. The treatment group was administered with streptomycin (20 mg/kg), tetracycline (25 mg/kg), and gentamicin (7.5 mg/kg) orally every day for 5 days. On day 5, the rats were injected subcutaneously with cortisone acetate (225 mg/kg) as the immunosuppressant. On day 6, the rats were administered with *C. albicans* through orogastric gavage. During treatment, the rats were fed with standard feed from the American Institute of Nutrition (AIN-93) and spider medium (peptone, yeast, beef extract, NaCl, mannitol, and K_2HPO_4). The animals were monitored daily for factors that affect the immune system.

On 3 days before inoculation and days 7, 14, 28, and 35 after inoculation of *C. albicans*, three rats from both groups were sacrificed by ether inhalation. After that, the intestinal mucosa was isolated. The intestine was cleaved and cleaned with phosphate-buffered saline (PBS) pH 7.4. The area of the mucous membrane was measured and scraped using a spatula, which was then suspended in sterile water. The mucosa suspension was then spread to the Sabouraud Dextrose Agar media to calculate the number of colonies. The colony number of *C. albicans* in stool and intestinal mucosa was determined by colony-forming units (CFU) methods in triplicate. The formation of biofilm was observed with the immunofluorescence method, using CLSM (Olympus, FV1000 type, Japan) with 400× magnification.

The quantitative analysis of *C. albicans* cells (CFUs)

CFU analysis in the stool

Fresh fecal samples were collected 10 min after the first stool was produced. The fecal samples were then placed in aseptic pots. One gram of the sample

was transferred into falcon tubes containing 9 mL PBS pH 7.4 and then homogenized. A serial dilution was performed from 10^{-1} to 10^{-6}, in which 1 mL of suspension from 10^{-4} dilution was spread onto Yeast Extract-Peptone-Dextrose (YPD) agar containing 50 µg/mL of ampicillin and 100 µg/mL of streptomycin. Those steps were repeated for the 10^{-5} and 10^{-6} of dilution. The inoculums were incubated at 37°C for 3-4 days and the colony numbers were counted with the formula presented in Eq. (2) after the incubation period.

$$\text{CFU / ml / gram} = \frac{\text{number of colony} \times \text{dilution}}{\text{volume of inoculum} \times \text{weight of feces}} \quad (2)$$

CFU analysis in the intestinal mucosa

The rat model's cecum was collected and cleaned with PBS pH 7.4 to remove the stool and contaminants attached to the mucosal surface. Then, each cecum was cut about 1 cm, the inner part (mucosa) was scraped using a spatula, and then diluted with 10^{-1}–10^{-3} dilution to obtain cecum mucosa suspension. A total of 0.2 mL of cecum mucosa suspension was spread to YPD agar containing 50 µg/mL of ampicillin and 100 µg/mL streptomycin, incubated at room temperature for 2-3 days, and then the formed colony was counted with the formula shown on Eq. (3).

$$\text{CFU / mL / cm}^2 = \frac{\text{number of colony} \times \text{dilution}}{\text{volume of inoculum} \times \text{wide area}} \quad (3)$$

C. albicans biofilm observation

The intestines part was cut, cleaned, and then fixed with 10% formalin buffer solution. After that, it was embedded in paraffin and cut to 5 µm thickness. The first stage was deparaffinization with xylol for 10 min twice and then terraced with ethanol (absolute ethanol, 90%, and 70% for 5 min). After that, the prepared tissue was soaked in PBST (Potassium Buffer Saline Tween) for 5 min 3 times and in 10 mM pH 6 citrate buffer at 120°C for 15 min. Then, the prepared tissue was washed with PBST 3 times for 5 min, blocked with 2% bovine serum albumin (BSA) in PBST at room temperature for 1 h, and then washed 3 times with PBST for 8 min. After that, the prepared tissue was incubated in Concavalin-A (Con-A) in PBST for 1 h and then washed with PBST 3 times for 8 min. The blocking process with BSA 2% in PBST at 25ºC 1 h was done once more and then washed with PBST 3 times for 8 min. The last stage was the incubation of antibody-anti *Candida* in the BSA 2% for 1 h at 28°C and then finally washed with PBST 3 times for 8 min to remove the excess antibody. The final slide was observed using CLSM. The observation consists of fluorescent intensity; green fluorescent showed the thickness of biofilm matrix; and red fluorescent showed *C. albicans* cells. The biofilm formation was observed descriptively.

Results and Discussion

C. albicans cell count in the stool

The monitoring of *C. albicans* overgrowth in the intestinal mucosa needed fresh stool that was collected in less than 10 min. The increased number of *C. albicans* cells at a certain period is a parameter of *C. albicans* overgrowth. Sampling and platting stool before antibiotics and immunosuppressed (pre-test) administrations aimed to find out the baseline amount of *C. albicans* cells in the intestinal tract. The baseline number of *C. albicans cells* of both the control and treatment groups was similar. In the next period, the number in the control group tended to constant, while it was increased in the treatment group. The overgrow of *C. albicans* occurred 14 days after inoculation, and on day 21 until 35, the number of *C. albicans* cells in the stool was decreased (Figure-1).

From the 3rd until the 7th day after administration, the number of *C. albicans* cells in the stool of the treatment group was slightly increased and became drastically increased on the 14th day. This indicated that the antibiotics and immunosuppressants disturbed the balance of microflora in the intestinal mucosal. Conversely, *C. albicans* were able to survive, overgrow, colonize, and attach to the stool. On the 21st day, the number of *C. albicans* cells in the stool was decreased. This condition was directly related to the limited nutrition condition that started on the 14th day. In this period, biofilm formation was initiated and then followed by biofilm maturation on the 35th day.

C. albicans cell count in the intestinal mucosa

The lowest number of *C. albicans* in the intestinal mucosa was observed on the 14th day in the treatment group. At this stage, *C. albicans* was overgrown and siphon the nutrition from the rat's intestinal mucosa. A lack of nutrients induces biofilm formation *in vitro* [25]. After that, the number of *C. albicans* cells in the intestinal mucosa tended to be constant

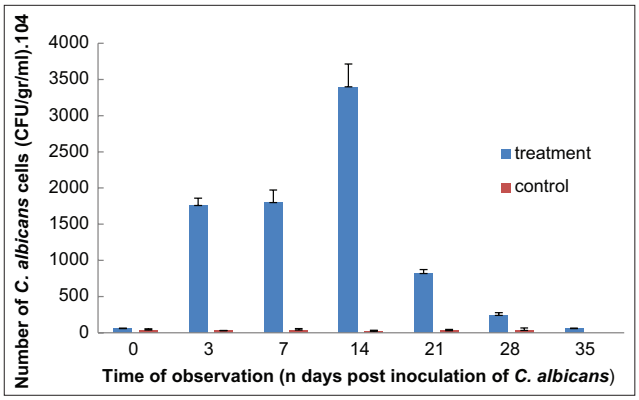

Figure-1: The density of *Candida albicans* in the stool.

from the 14th until the 21st day after inoculation. On the 21st day, the number of *C. albicans* in the intestinal mucosa of the treatment group was increased slightly, because the colonization and the first stage of biofilm formation were started at this time. On the 28th day, the intestinal mucosa *C. albicans* cells in the treatment group were increased and became sharply increase on the 35th day. At this stage, the biofilm in the intestinal mucosa was in the mature stage. The number of *C. albicans* cells in the intestinal mucosa of the control group remained similar in all observations (Figure-2).

The number of *C. albicans* cells is expressed in CFU/mL as performed by Rosenbach [22],which analyzed the colonization of *C. albicans* in the intestinal mucosa of rats to study the colonization regulating genes. Rosenbach *et al*. [22] reported that WT (wild-type) *C. albicans* can colonize the intestinal mucosa until the 21st day after inoculation. The colonization of *C. albicans* depends on the colonization regulatory genes [25]. The Cph2p gene is an important regulatory gene and is expressed during colonization [22].

Biofilm formation in the intestinal mucosa

The biofilm formation of *C. albicans* was observed using CLSM targeted to ECM produced by *C. albicans* as well as their presence. This method used the combination of fluorescent staining of Con-A and polyclonal anti-*Candida* conjugated with tetramethylrhodamine isothiocyanate (TRITC). Con-A selectively binds the mannose and glucose residues in the polysaccharide constituent of both cell and ECM of *C. albicans* biofilm, while the polyclonal anti-*Candida* selectively binds to *C. albicans* cells [10,26,27]. Thus, the existence of ECM of *C. albicans* biofilm can be observed by Con-A blue-green fluorescence signal. The signal intensity indicates the thickness of the ECM. The *C. albicans* cells can be seen from the red signal from TRITC. The darker the red signal, the higher the cell count in the biofilm.

Figure-3 showed the reconstruction of treatment groups' intestinal mucosa in 3D during the *C. albicans* biofilm formation. In the early stage of biofilm formation (the 7th, 14th, and 21st days after inoculation), the mucosal membrane exhibited low fluorescence signal for *C. albicans* cells and ECM, indicating that the ECM has not been formed and the *C. albicans* count was low at the early stage. Meanwhile, the biofilm has been formed in a mature phase on the 28th and 35th days after inoculation. At this stage, the biofilm's biomass expands and the ECM was accumulated and thickened [20-22,25]. It could be seen from Figure-3a that there is no visible color luminescence. Meanwhile, there is red fluorescence signals in Figure-3b and intense blue-green in Figure-3c. The correlation between *C. albicans* cells with their ECM can be observed through the yellow fluorescence signal in Figure-3c.

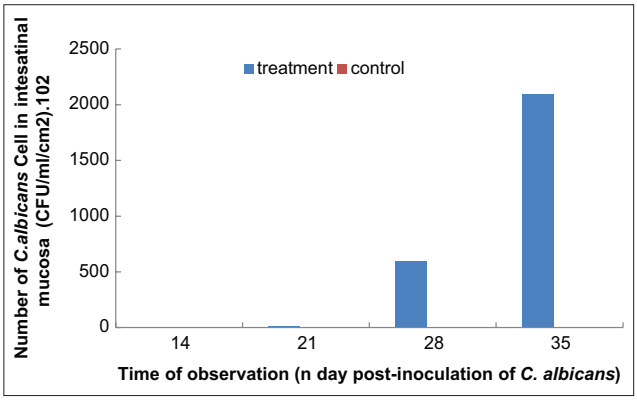

Figure-2: The density of *Candida albicans* cells in the intestinal mucosa.

Several studies used CLSM to observe the structure of biofilms formed *in vitro* and *in vivo*. Nett *et al*. [12] used two sets of fluorescent dyes, the first was FUN-1 and Con-A and the second was Calcofluor white and SYTO 9, to examine the formation of *C. albicans* biofilms on the surface of the dentures. The structure of the *C. albicans* biofilm is composed of fungal cells, ECM, hyphae, and bacteria. *C. albicans* biofilms in the vagina are composed of yeast and hyphal cells that are embedded in the ECM, depicted through Con-A staining [11]. The structure of the *C. albicans* biofilm in the rat tongue mucosa has been investigated by Dongari-Bagtzoglou *et al*. [10]. Epithelial cells, neutrophils, and commensal bacteria interaction in the mucosal biofilms were observed with CLSM using the fluorescence *in situ* hybridization method [10].

The presence of ECM on *C. albicans* biofilm is important as a shelter to evade the host's immune response and increases their resistance to antifungal drugs. In another study, the body's immune response begins in the early stages of *C. albicans* biofilm formation in the intestinal rats, so an immunomodulatory therapy can be developed against candidiasis to inhibit the formation of *C. albicans* biofilms [28].

Meanwhile, the absence of *C. albicans* colonies in the stool does not mean that the individual has no candidiasis. Our study observed that the number of *C. albicans* in the sample's stool was decreased until the 28th day. At this point, *C. albicans* was not mainly found in the stool, but it grew excessively in the intestinal mucosa. There was a thick and spreading white layer in the intestinal mucosa and the thickest layer was observed on the 28th day.

According to the results, the negative presence of *C. albicans* in the stool should be carefully interpreted, as the result cannot be concluded if the patient has candidiasis. *C. albicans* biofilm should be examined before reaching the final diagnosis of candidiasis. The results above reveal that this study successfully created and characterized the biofilm formation model of *C. albicans* in the intestinal mucosa of Wistar rats. Furthermore, the characterization of the formed biofilm is an interest for future research.

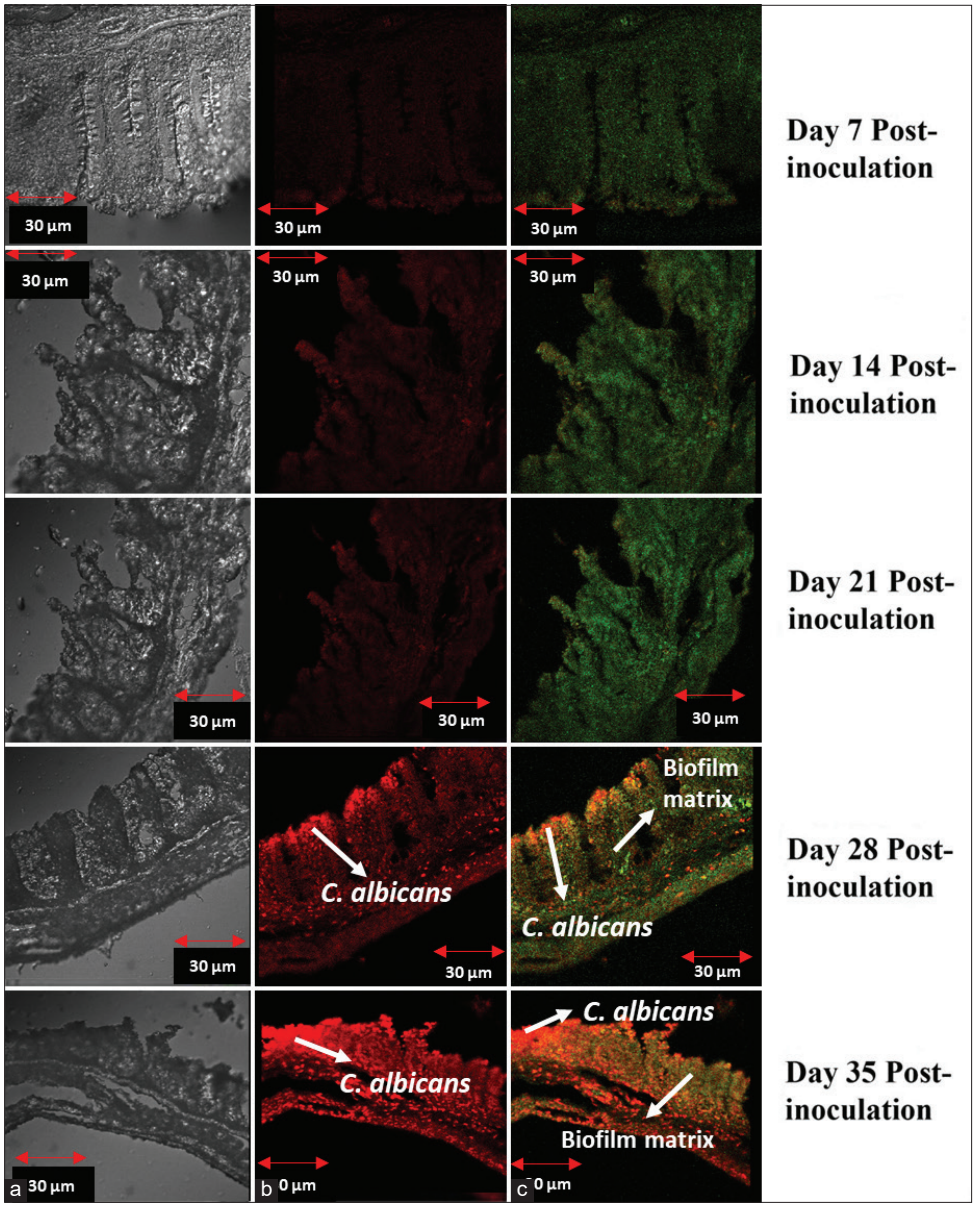

Figure-3: *Candida albicans* biofilm formed in the intestinal mucosa of Wistar rats, observed with confocal laser scanning microscope using immunofluorescence method. (a) the observasion of intestine tissue with confocal laser scanning microscope (CLSM) without fluorescence staining so there are no visible color luminescence; (b) the observasion of intestine tissue stained with polyclonal anti-*Candida* conjugated tetramethyl rhodamine isothiocyanate (TRITC) (red) for the presence of *C. albicans* cells; (c) intestinal mucosa with double staining for the presence of extracellular matrix and *C. albicans* Con-A (green) and *C. albicans* cells stained by polyclonal anti-*Candida* conjugated TRITC (red).

Conclusion

The intestinal biofilm of *C. albicans* was successfully formed through antibiotics and immunosuppressant agent induction. The formation and maturation of *C. albicans* biofilm were observed on the 28th and the 35th days after *C. albicans* administration, indicated by the high intensity of ECM signal and the increasing number of *C. albicans*. The mobilization of *C. albicans* cells from the intestinal mucosa to stool was observed when there is a shift from biofilm to planktonic *Candida*. It means the negative tests of *C. albicans* in stool samples have not shown that someone to be free of *Candida* in their GI tract, but it may be candidiasis that attaches to the surface of the intestinal mucosa as a biofilm instead.

Authors' Contributions

HW, NN, and AB: Conceived the idea. MM, LHR, and POAT: Conducted the experiment. MM and HW: Conducted data analysis and interpretation. MM, NN, and AB: Wrote the draft of the paper. NN, LHR, POAT, and HW: Revised the manuscript. All authors read and approved the final manuscript.

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Competing Interests

The authors declare that they have no competing interests.

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