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Efficacy Comparison of Oral and Intravaginal *Lactobacillus plantarum* Administration on Secreted Aspartyl Protease-5 (SAP5) Levels in Vulvovaginal Candidiasis

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Abstract

Vulvovaginal candidiasis (VVC) is the most common fungal infection in the vaginal mucosa caused by an overgrowth of *Candida albicans*. The pathogenesis of *C. albicans* is determined by the presence of virulence secretory factors. Secreted Aspartyl Protease 5 (SAP5) is the dominant virulence factor that plays a critical role in the morphology switching of yeast cells into hyphae which causes tissue invasion and induces an immunopathological response. Levels of SAP5 are often associated with the severity various types of infection. As a potential supportive therapy to treat VVC, identification of the probiotic's administration route determines the level of efficacy of probiotics. This study aims to compare the decrease in SAP5 levels between oral and intravaginal administration of *L. plantarum*. The study was true experimental using a completely randomized pretest-posttest group design. There were 32 female rats were divided into 8 groups i.e negative control, positive control, 3 groups of oral and 3 of intravaginal administration. Positive control and 6 treatment group were injected with estradiol valerate 0.5 mg and then inoculated with suspension of *C. albicans* 2×10^7 CFU to create VVC model. *L. plantarum* was administered orally and intravaginally with 3 concentration variants i.e. 2.25×10^{10}, 4.5×10^{10} and 9×10^{10} CFU. Both of oral and intravaginal group were given with probiotics for 14 days. SAP5 levels were measured by ELISA from vaginal lavage sample. The statistical analysis used was the Paired T-Test to measure SAP5 levels pre-post oral and intravaginal administration of *L. plantarum*. The results showed a significant decrease in SAP5 levels in oral administration ($p = 0.001 < 0.05$) and intravaginal administration ($p = 0.000 < 0.05$). From the results of the statistical analysis, it can be concluded that both oral and intravaginal administration of the probiotic *L. plantarum* has the same efficacy in reducing the virulence factor of *C. albicans* in cases of VVC.

Keywords: Vulvovaginal candidiasis, *Lactobacillus plantarum,* SAP5, *Candida albicans*

Introduction

Vulvovaginal candidiasis (VVC) is the most common gynecological infection experienced by women of childbearing age after Bacterial Vaginosis (BV) [1]. In a global epidemiological study conducted from 1985 to 2016, it was stated that every year there are an estimated 103 - 172 million cases of VVC with an annually prevalence of 3.871 cases in 100.000 female population. The finding of this prevalence is expected to be increased progressively. Based on the identification of demographic factors, the age group of 25 - 34 years is the age group that is most susceptible to VVC [2]. Increased susceptibility to VVC in women of childbearing age is often associated with high levels of estrogen such as in the luteal phase of the menstrual cycle. During the luteal phase, levels of the hormones estrogen and progesterone reach relatively high levels, like taking oral contraceptives [3]. High levels of estrogen cause a decrease in the ability of vaginal epithelial cells to inhibit the attachment and invasion of pathogenic microorganisms. Meanwhile, progesterone triggers a decrease in the inhibitory effect of neutrophils against pathogenic microorganisms [4]. The presence of other risk factors such as pregnancy, use of hormonal contraceptives and Intrauterine Devices (IUD), degenerative diseases such as uncontrolled diabetes, long-term consumption of broad-spectrum antibiotics and use of corticosteroid derivatives in immunocompromised individuals will further increase the vulnerability of women of childbearing age to VCC [5].

Vulvovaginal candidiasis (VVC) is caused by several species of fungi in the genus Candida. Epidemiological studies state that more than 90 % of VVC cases are caused by *C. albicans* and only 10 % of VVC cases are caused by species other than *C. albicans* such as *C. glabrata, C. tropicalis and C. krusei* [6]. *C. albicans* is reported to be the most common opportunistic organism found in the body which causes 60 % of mucosal infections and 40 % of candidemia infections [7,8]. In the lower genital tract, colonies of *C. albicans* generally can be found in the vaginal lumen without causing symptoms (asymptomatic). Symptomatic symptoms such as itching, burning sensation, pain and abnormal secretions will occur when *C. albicans* colonies grow excessively, invade the epithelium and secrete virulence factors that further support colony growth [9].

Virulence factors have an important role in the mechanism of candidiasis. There are several virulence factors of *C. albicans,* including phenotypic switching, morphological dimorphism, thigmotropism, adhesion proteins, secretion of hydrolase enzymes and biofilm formation [10]. The 3 main hydrolase enzymes secreted by *C. albicans* include Secreted Aspartyl Protease (SAP), phospholipase and hemolysin [11,12]. Secreted Aspartyl Protease (SAP) produced by *C. albicans* is classified into 10 types according to the coding gene. Each SAP has a specific role in the pathogenesis of the infection. SAP1-3 is related to morphological changes, SAP4-6 is related to the transition from yeast to hyphae and SAP7-10 has not identified its specific role. SAP5 is the most frequently associated virulence factor with the onset of mucosal candidiasis immunopathology. Because the morphological changes from yeast cells to hyphae form cause damage to host tissue, immunopathological responses including migration of neutrophils and production of pro-inflammatory cytokines as a defense mechanism in the host are induced. The transition from yeast cell form to hyphae is not only in the form of morphological changes but there is a systematic process involving genetic processes in *C. albicans* cells. Identification of SAP5 levels can be an important finding that reflects the level of tissue damage due to *C. albicans* invasion [13].

The imbalance between the number of *C. albicans* as an opportunistic fungus and *Lactobacillus* spp. as the main normal flora in the vaginal mucosa is the one of important mechanisms for the occurrence of VVC. *Lactobacillus* spp. physiologically produce lactic acid which plays a role in creating a vaginal environment with an acidic pH < 4.5. *Lactobacillus* spp. also produces bacteriocins and biosurfactants which function to protect the vaginal mucosa from various types of pathogenic microorganisms [14]. In VVC conditions where the number of *Lactobacillus* spp. has decreased, it causes a decrease in lactic acid production so women with VVC often experience an increase in vaginal pH. An alkaline vaginal pH facilitates more progressive growth of pathogens characterized by increased levels of the proteolytic carboxylase enzyme which triggers the sloughing of squamous cells, inflammation of the vaginal mucosa and production of abnormal secretions in the vagina [15].

In the last few decades, *Lactobacillus* probiotics have become an alternative strategy to treat various types of infections. Giving probiotics is predicted to be able to overcome VVC through several mechanisms including increasing the number of *Lactobacillus* spp. in the vagina, increasing lactic acid production which directly impacts on decreasing vaginal pH, increasing cervicovaginal mucus production, repairing the mucosal barrier which prevents adhesion and invasion of *C. albicans* colonies, increasing defense components in the mucosa that have an impact on improving the clinical manifestations of VVC [16,17]. Currently, *Lactobacillus* probiotics are available in various forms, but oral forms are the most widely known [18]. The effectiveness of using oral probiotics in cases of infection in the reproductive organs is influenced by a homing mechanism that can modulate innate and adaptive immune responses in the vaginal mucosa [19]. However, Dibo *et al.* [20] stated that for women with reproductive disorders, intravaginal administration of probiotics is considered more appropriate because probiotic immunomodulation of the immune system in the vaginal mucosa occurs directly. The efficacy of the probiotic route has an important urgency to study because it is related to the effectivity rate of using *Lactobacillus* probiotics as a supportive therapy for VVC. So, this study aims to compare SAP5 levels before and after administration of probiotic *L. plantarum* through the oral and intravaginal in VVC with *in vivo* model study.

Materials and methods

Study design

This research is a true experiment model with a pretest-posttest randomized experimental group design conducted in the laboratory *in vivo*. The experiment was divided in 2 phases. The 1st phase is the vaginal colonization of *C. albicans*. The 2nd phase is *L. plantarum* administration to the subjects which confirmed the presence of *C. albicans* colonization.

Animal housing and grouping

Thirty-two female rats of Wistar strain (*Rattus norvegicus*) (aging 8 - 10 weeks and average weighing 90 - 110 g) were purchased from the breeder of State Islamic University of Maulana Malik Ibrahim Malang, Indonesia and have been confirmed healthy by the Veterinary Laboratory of the Faculty of Medicine, Universitas Brawijaya. They were acclimatized for 7 days at the Experimental Animal Research Laboratory, Faculty of Medicine, Universitas Brawijaya. Acclimatization aims to allow rats to adapt to the laboratory environment. After acclimatization, the rats were divided equally into 8 groups are negative control group (NC), positive control group (PC), 3 oral treatment groups (P1, P2, P3) and 3 intravaginal treatment groups (P4, P5, P6). In each group, there are 4 rats placed in 2 standard-size cages. Food and drink are provided *ad libitum*. Light and darkroom settings are 12-h cycles. While the temperature and humidity of the room are set at 27 - 28 °C.

C. albicans and suspension preparation

Isolate of *C. albicans* obtained from clinical specimen of a patient with vaginal candidiasis were sent to the Department of Microbiology, Faculty of Medicine, Universitas Brawijaya. The isolate was identified with some microbiological procedures such as culture in *Saboraud Dextrose Agar* (SDA), Gram staining, Potassium Hydroxide test and Germinating Tube test to analyze the suitability of *C. albicans* characteristics. For use in the experiment, *C. albicans* cells were cultured in SDA medium overnight before use and then were diluted NaCl. Density was measured using a spectrophotometer with a wavelength of 520 λm. Pure *C. albicans* colony was isolated using sterile inoculating loop and combined with 5 mL of 0.9 % NaCl. The absorbance of the suspension was determined using spectrophotometry with a wavelength of 520 nm. Mixing 4.1 mL of *C. albicans* solution with 5.9 mL of sterile NaCl a suspension containing 2×10^7 CFU/mL.

Establishment of pseudo estrus and VVC model in rat

The procedure of the VVC model in this study refers to the "Animal Models for Candidiasis" procedure [21]. After 7 days of acclimatization, on the 8th day, the positive control group and the 6 treatment groups were injected with estradiol valerate 0.5 mg diluted in 100 µL sesame oil subcutaneously in the lower abdomen to induce pseudo estrus. Injection of estradiol valerate to induce pseudo estrus in rats was repeated on day 15th and 21th. On the 12th day, the positive control group and the 6 treatment groups were intravaginally inoculated with 2×10^7 CFU/mL cell suspension of *C. albicans* dissolved in 20 µL mL of NaCl using a micropipette. Meanwhile, the negative control group was only inoculated with 20 µL mL of PBS. Before *C. albicans* inoculation intravaginally, rats were anesthetized with 100 mg/kg BW of ketamine hydrochloride.

Identification of pseudo estrus in rats

On the 13th day, vaginal smear was performed using a sterile cotton swab. After 3 rounds, the samples were placed on glass slides and allowed to dry. Then the sample was fixed using a bunsen burner followed by Methylene Blue staining and observed under a microscope.

Identification of VVC model in rats

On the 14th day, *C. albicans* inoculation was confirmed by performing a vaginal lavage of each rat with 100 µL PBS to collect their vaginal fluid and stored it in sterilized 1.5 mL Eppendorf Tubes (ET). Vaginal cultured in SDA medium and Gram stained to ensure the infection was consistently distributed among rats. After the results of culture in SDA media and Gram staining showed the characteristics of *C. albicans isolate*, then the next intervention could be given. All subjects were confirmed to have the same degree of VVC because the suspension were inoculated intravaginally was uncontaminated material. The suspension volume is precision ensured using a micropipette and spectrophotometry to count of OD. So, all subject inoculated with the same volume of suspension. In addition, to ensure that all cases of VVC were homogeneously experienced by all subjects, we performed pH assessment by digital meter. From the results of pH measurements, it was found that the pH value in all subject was in the range 7.3 to 7.9.

Exposure to *L. plantarum*

Isolate of *L. plantarum* ATCC 8014 obtained from Inc. Agavi Indonesia, a consulting firm engaged in various food sectors, such as industrial processing, distribution and consumption. The isolate was identified with some microbiological procedures such as culture in *de Mann Rogosa Sharpe* (MRS) Agar, Gram staining, catalase test and glucose test to analyze the suitability of *L. plantarum* characteristics. After ensuring the successful establishment of rat model candidiasis, suspension *L. plantarum* was prepared with 3 concentration variations that are 2.25×10^{10}, 4.5×10^{10} and 9×10^{10} CFU/mL. The density of *L. plantarum* suspension was measured using a spectrophotometer with wavelength 625 λm. Each of these concentration variations was administered through oral and intravaginal. In both oral and intravaginal treatment group, each of the subject was given *L. plantarum* suspension once a day for 14 days. In the oral group, *L. plantarum* suspension was administered using a gastric tube. Whereas in the intravaginal group, using an intravaginal micropipette.

Table 1 Scheme of intervention among all of treatment group in the study.

Group intervention	NC	PC	P1	P2	P3	P4	P5	P6
Estradiol valerate injection	-	+	+	+	+	+	+	+
C. albicans inoculation	-	+	+	+	+	+	+	+
L. plantarum oral 2.25×10^{10} CFU/mL	-	-	+	-	-	-	-	-
L. plantarum oral 4.5×10^{10} CFU/mL	-	-	-	+	-	-	-	-
L. plantarum oral 9×10^{10} CFU/mL	-	-	-	-	+	-	-	-
L. plantarum intravaginal 2.25×10^{10} CFU/mL	-	-	-	-	-	+	-	-
L. plantarum intravaginal 4.5×10^{10} CFU/mL	-	-	-	-	-	-	+	-
L. plantarum intravaginal 9×10^{10} CFU/mL	-	-	-	-	-	-	-	+

Study sample and result measurement

On the 29th day, rats were anesthetized with 100 mg/kg BW of ketamine hydrochloride before performing vaginal lavage to take vaginal fluid as a sample. Secreted Aspartyl Protease 5 (SAP5) was measured using an Enzyme-linked immunosorbent assay (ELISA) kit manufactured by BT Lab China.

Statistical analysis

All variations concentrations of probiotic *L. plantarum* that were given orally and intravaginally and SAP5 levels were analyzed using SPSS 21 statistical analysis (SPSS, Chicago, IL, USA). Data normality was analyzed using the Shapiro-Wilk test followed by the Paired t-test. The paired t-test aimed to determine whether there were significant differences in SAP5 levels before and after oral and intravaginal administration of probiotics *L. plantarum* in VVC rat models.

Ethical statement

All procedures in this study have received ethical approval from the Ethical Committee Faculty of Medicine, Universitas Brawijaya with an Ethical Approval Letter number 34 / EC / KEPK - S2 / 02 / 2023.

Results and discussion

Establishment of pseudo estrus in rat

Based on microscopic observations of vaginal smear samples stained with Methylene Blue, pseudo estrus is dominated by keratinized epithelial cells that are mostly anucleate, devoid of neutrophils characterized by blue cytoplasm that overlaps with jagged edges (**Figure 1**).

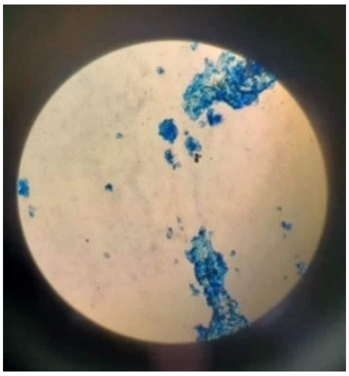
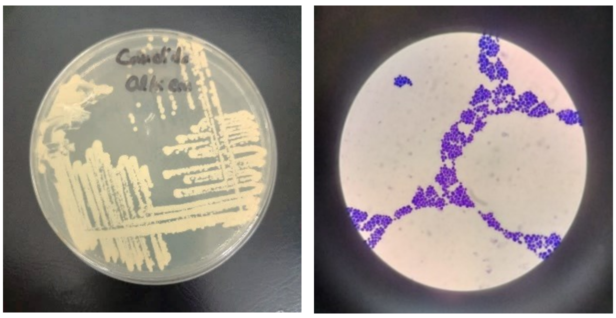


Figure 1 Microscopic view of the vaginal smear of rat was injected with estradiol valerate. Vaginal smears stained by Methylene Blue.

Assessment of the estrus cycle refers to changes in the physiology and anatomy of the animal. Commonly used methods include visual assessment, vaginal cytology, histological examination of the reproductive organs and interpretation of the biochemical properties of urine. Cytological examination of vaginal smear samples is the most widely used method in identifying the reproductive cycle of female animals because requiring a short time, is non-invasive and easy to observe microscopically [22]. In various studies of animal models of candidiasis, one of the most widely used approaches is to induce the estrous cycle by administering estrogen derivate like estradiol valerate, estradiol benzoate, ethinyl estradiol, etc. Histological and cytological changes in the vaginal mucosa depend on fluctuations of the steroid hormone, especially estrogen. When rats experience pseudo estrus as a result of being induced with estradiol, vaginal mucosal epithelial cells become cornified. The formation of cornified cells due to high concentrations of estrogen in the body causes thickening of the vaginal wall which then undergoes freezing and finally releases. The absence of a nucleus is caused by a process of keratinization followed by cell desquamation because a large amount of keratin blocks the diffusion of nutrients from the capillaries to the vaginal tissue [23].

Establishment model VVC

Establishment VVC *in vivo* models can be identified by several methods i.e. macroscopic observation by culturing vaginal fluid in SDA media and microscopically by Gram staining. Vaginal fluid cultured in SDA media showed round-shaped colonies, convex, smooth surfaces, yellowish-beige in color with an odor like yeast. Whereas in Gram staining, the results observed using a microscope with an objective lens obtained round-shaped colonies (cocci) and purple in color (**Figure 2**).



A **B**

Figure 2 Macroscopic view of *C. albicans* were cultured in SDA media (A); microscopic view of *C. albicans* by Gram staining (B).

The primary identification medium used to identify *C. albicans* is SDA. However, SDA cannot be used as a differential medium because other species of *Candida* spp. cannot be distinguished clearly [24]. Culturing *C. albicans* in SDA will show the growth of round colonies with convex and smooth surfaces, yellowish cream in color with a characteristic yeast-like odor after incubation for 24 - 48 h at 37 °C [25].

While the Gram staining aims to identify groups of gram-positive or gram-negative organisms. In addition, Gram staining can identify fungi based on their morphology. Based on the gram staining method, *C. albicans* are round in shape and gram-positive because they bind the purple color of the crystal violet solution. Organisms in the gram-positive group have thick cell walls containing 90 % peptidoglycan layer, causing purple pigments from crystal violet solutions to be absorbed on the cell walls and will survive the washing process using laxative agents such as Lugol and alcohol [26].

Identification of *Lactobacillus plantarum*

Based on the results of culture on MRSA media, it was found that there were round-shaped colonies with a smooth non-fibrous surface, the average colony size was between 0.5 to 2 mm, and shiny milky white in color with a sour aroma typical of fermented products. The results of gram staining method were observed using a microscope with an objective lens magnification of 100 times, obtained purple rod-shaped colonies (bacilli). In the catalase test, the catalase test result was negative where there were no air bubbles in the *L. plantarum* isolate tube which was dripped with 3 drops of 3 % H_2O_2 solution. The sugar fermentation test was carried out using a mannitol medium in which 4 drops of *L. plantarum* isolate were dropped and then incubated for 48 to 72 h at 37 °C, indicating a color change from red to yellow (**Figure 3**).

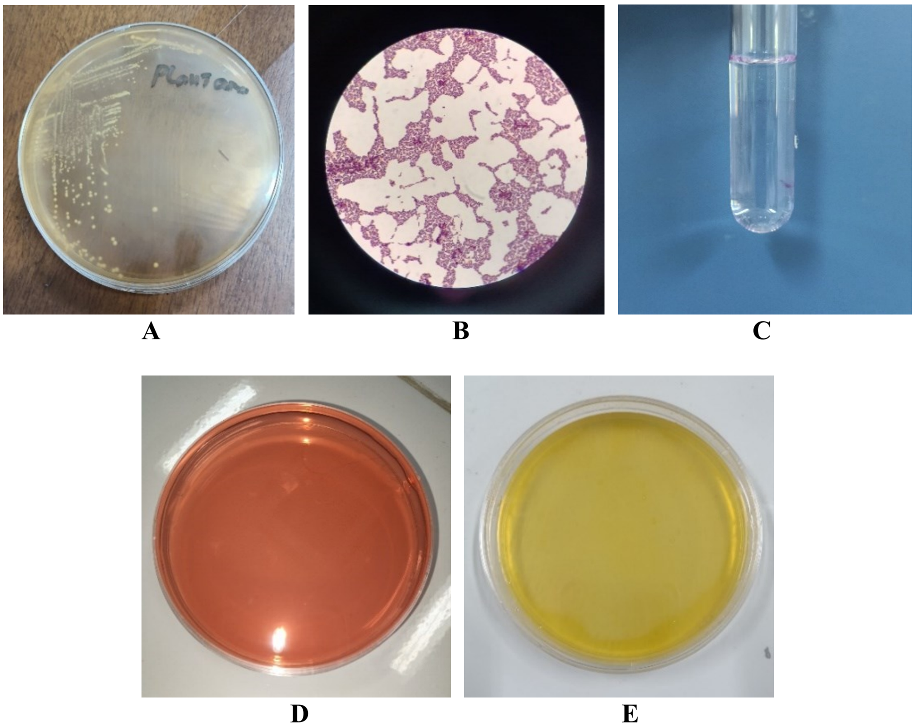


Figure 3 Macroscopic view of *L. plantarum* was cultured in MRS media (A); microscopic view of *L. plantarum* by Gram staining (B); there's no air bubbles were found in the tube indicating a negative result of catalase test (C); there's a change in the color of mannitol in the plate from red into yellow (D, E).

De Man Rogosa Sharpe (MRS) agar is a selective medium for the isolation and culture of Lactic Acid Bacteria (LAB) especially *Lactobacillus* spp., both from clinical and non-clinical specimens. Apart from being enriched by the nutrients for *Lactobacillus* spp., MRS agar contains sodium acetate and ammonium citrate which can inhibit the growth of gram-negative bacteria and fungi. So MRS agar is called the best selective medium for the growth of *Lactobacillus* spp. [27]. Based on the identification method using Gram-staining, *L. plantarum* is a bacillus Gram-positive bacterium. Although it can identify bacteria based on Gram-positive and Gram-negative groups, the Gram-staining method cannot be used as the only method of identifying an isolate of a microorganism. In addition, it also cannot be used as an indicator to establish a diagnosis. The catalase test is one of the simplest methods to identify groups of bacteria that produce the catalase enzyme. The catalase enzyme is an enzyme that catalyzes the release of oxygen from hydrogen peroxide that mediate the breakdown of hydrogen peroxide (H_2O_2) into oxygen (O_2) and water (H_2O). Based on the test results, *L. plantarum* showed a negative catalase which is indicated by the absence of air bubbles. It is shown that *L. plantarum* isolates cannot mediate the decomposition of H_2O_2 to produce O_2. The last, sugar fermentation test aims to identify the ability of bacteria to ferment various types of carbohydrates. There are 15 types of carbohydrates used to identify the fermentability of *L. plantarum* isolates such as L-arabinose, cellobiose, D-fructose, lactose, melezitose, melibiose, mannitol, D-mannose, raffinose, rhamnose, D-ribose, salicin, sorbitol, sucrose, trehalose and D-xylose. Based on the results of the sugar fermentation test using mannitol, *L. plantarum* isolates showed positive fermentation results marked by a change in the color of the solution from red to yellow. The ability to ferment glucose is because *L. plantarum* has an α-amylase enzyme which plays a role in converting glycogen produced by endocervical cells into lactic acid which maintains vaginal pH stability [28].

SAP5 levels

Table 1 is the results of measuring SAP5 levels in each subject (n = 4) in the 8 treatment groups. In the intervention group, both oral groups (P1-3) and intravaginal groups (P4-6) with each 3 variances of *L. plantarum* concentrations showed a decrease in SAP5 levels in all subjects. Whereas in the negative control group (NC) and positive control group (PC), SAP5 levels showed an increase in all subjects.

Table 1 Result of SAP5 levels measurement in each subject of treatment group pre and post administration of *Lactobacillus plantarum.*

Group	Kind of intervention	Subject	SAP5 Levels (ng/mL)	
			Pre[a]	Post[b]
Control	Negative control (NC)	1	0.145	0.501
		2	0.269	0.491
		3	0.331	0.243
		4	0.179	0.305
	Positive control (PC)	1	14.289	18.501
		2	13.668	17.888
		3	17.639	19.176
		4	16.252	17.719
Intervention	2.25×10^{10} CFU/mL oral (P1)	1	6.151	4.852
		2	9.165	8.196
		3	5.258	5.045
		4	5.578	4.881
	4.5×10^{10} CFU/mL oral (P2)	1	8.555	4.776
		2	9.652	7.094
		3	9.995	5.307
		4	9.746	6.772
	9×10^{10} CFU/mL oral (P3)	1	10.885	5.594
		2	12.965	5.974
		3	12.582	9.805
		4	11.906	3.859
	2.25×10^{10} CFU/mL intravaginal (P4)	1	10.125	7.455
		2	11.895	4.208
		3	15.895	10.492
		4	11.625	3.385
	4.5×10^{10} CFU/mL intravaginal (P5)	1	15.885	11.821
		2	12.667	6.456
		3	12.889	9.788
		4	16.497	11.523
	9×10^{10} CFU/mL intravaginal (P6)	1	12.954	8.512
		2	9.385	5.966
		3	11.885	6.125
		4	10.492	7.909

Source: Primary data

[a]SAP5 level before administration of *Lactobacillus plantarum* through the oral and intravaginal

[b]SAP5 level after administration of *Lactobacillus plantarum* through the oral and intravaginal

Analysis statistic

Data of SAP5 levels were distributed normally ($p = 0.387 > 0.05$). Findings of normal data distribution qualify for the paired t-test. In the statistical analysis using the paired t-test, the data homogeneity test was not carried out because the sample groups before and after treatment were the same subject. The results of the paired t-test for SAP5 levels before and after administering of *L. plantarum* were classified based on the route of administration, i.e. oral route with 3 variations of *L. plantarum* concentration (n = 12) and intravaginal route which also has 3 concentration variations (n = 12). The results of the paired t-test in the oral group showed *p*-value = 0.001 ($p < 0.05$). While the results of the paired t-test in the intravaginal group showed *p*-value = 0.000 ($p < 0.05$). The finding of p-value < 0.05 in the oral and intravaginal groups showed that there was a decrease in SAP5 levels after administration of *L. plantarum* both in the oral (mean = 3.358) and intravaginal route (mean = 4.872) (**Table 2**).

Secreted Aspartyl Protease (SAP) is a protein that has an important role to maintain the life of the fungus in the host's body. In the case of mycosis in humans, SAP production is often associated with the presence of *C. albicans, C. parapsilosis* and *C. tropicalis*. SAP secretion is related to genetic activity in fungal cells. mRNA that has been synthesized and entered the cytoplasm, then is translated to be transferred to the rough endoplasmic reticulum. Upon entering the endoplasmic reticulum, the N-terminal signal is removed by the peptidase enzyme. After that, the mRNA will be transferred to the Golgi apparatus and will be processed by the proteinase Kex2 enzyme. Before being processed by the Kex2 enzyme, the retained enzyme is inactive. It is in this inactive form that the zymogen then enters the secretory vesicles so that it can be released into extracellular [29]. There are 10 types of SAP that are currently known, among all types of SAP, SAP1-8 is associated with protein hydrolysis and tissue damage. SAP1-3 expression is associated with changes in fungal phenotypic morphology. This process occurs due to the rearrangement of fungal chromosomes and the regulation of the SIR2 (Silent Information Regulator) gene. The most common examples of colony changes are white, oval-shaped and fine-textured colonies that can change to coarse-textured and opaque gray colonies. Opaque fungal cells produce SAP1 and SAP3. While the white fungal cells will produce SAP2. Conversely, SAP4-6 will be secreted especially during the morphological transition from cells to hyphae. Among SAP4-6, only SAP5 plays a role in tissue colonization, penetration and invasion [29,30].

Secreted Aspartyl Protease 5 (SAP5) has an important role in the pathophysiology of infection in both mucosal candidiasis and candidemia because it is identified as the most dominant virulence factor expressed by hyphae. Hyphae become an important morphology in dimorphic fungal species that cause damage to mucosal tissue as an initial stage of the emergence of an immunopathological response [31,32]. SAP5 plays an important role in supporting dimorphic properties in *C. albicans* because SAP5 is involved in the mechanism of adhesion, colonization, invasion and tissue damage by *C. albicans* [33]. Moreover, SAP5 specifically facilitates the adhesion of *C. albicans* to the mucosa along with the ALS3 protein, predominantly involved in the transition mechanisms from yeast cells to hyphae and invasion of tissues [31]. Based on the results of measurements of SAP5 levels (**Table 1**), the group of rats that were inoculated with *C. albicans* (PC) showed higher levels of SAP5 than the group of rats that were not inoculated with *C. albicans* (NC). The PC and NC groups had increased levels of SAP5, but the increase in PC group showed a relatively higher increase compared to the NC group. The aspartate protease enzyme is a type of endopeptidase enzyme group that is normally found in the tissues of prokaryotic, eukaryotic, plant and animal organisms. aspartate protease plays a role in regulating the activity of various proteins, modulating protein interactions and contributing to cellular information processes [34]. So that an increase in SAP5 levels in the NC group is a physiological thing. Meanwhile, significantly increased levels of the aspartate protease enzyme in cases of candidiasis indicate that there is a process of degradation of various host proteins, both cell membrane proteins [35,36]. In candidemia patients, vulvovaginal candidiasis and dental caries levels and expression of SAP5 can be identified. So, it shows that SAP5 is one of the important virulence factors in the pathogenesis of *C. albicans*. SAP5 is an important target in modeling potential antifungal agents [9].

The results of a comparative analysis of SAP5 levels before and after *L. plantarum* administration showed that *L. plantarum* probiotics either orally or intravaginally had similar effectiveness in reducing SAP5 levels. Giving probiotics *Lactobacillus* spp. orally can increase the natural and adaptive immune response through the introduction of Pattern Recognition Receptors (PRR) in the intestinal epithelium with Microbe Associated Molecular Patterns (MAMPs). When entering the intestinal mucosa, probiotics will interact with dendritic cells. Gut Associated Lymphoid Tissue (GALT) and intestinal mucosa are reservoir tissues for dendritic cells. The presence of receptors on dendritic cells will facilitate the detection process on the surface of pathogenic cells. In addition, dendritic cells act as inductors for various cascades that modify cytokines due to the stimulation of Toll-Like Receptors (TLR) and C-Type Lectin Receptors (CLR).

After probiotics enter the intestinal epithelium, dendritic cells and macrophages will migrate to the mesenteric lymph nodes to present antigens to T cells. Probiotic Lactobacillus are also reported to be able to regulate the role of T-reg cells through TLR2 so that they can reduce inflammatory reactions [37].

Administration of probiotics through the oral route has a direct effect on lamina propria cells and Peyer's patch which induces the secretion of secretory Immunoglobulin A (sIgA) which is able to neutralize toxins produced by pathogens. This induction is also triggered by the stimulation of TLR2. Plasma cells are released into the bloodstream and eventually return through the intestinal vascular endothelium to fill the lamina propria (LP) where they are needed to release antibodies into the intestinal lumen. Intestinal endothelial cells that recognize these homing factors serve as gatekeepers to recruit immune cells to infected mucosal sites [38]. Whereas in the vaginal lumen, there are 4 main subsets of Antigen Presenting Cell (APC) in the lower reproductive tract, namely Langerhans cells, lamina propria-dendritic cells, CD14 cells (cluster of differentiation 14) and macrophages [39]. Adaptive response in the vaginal mucosa will arise when APC mobilizes to the lymph nodes. Dendritic cells and Langerhans cells activate Th2 cells while CD14 and macrophages respond to pathogens via TLRs and activate Th1 [40]. Apart from APC, the vagina and ectocervix also contain NK cells which function to limit the growth of pathogens. Antibodies directly contribute to the neutralization of pathogens and their toxins, complement-induced opsonization and lysis. Plasma cells that secrete IgG and IgA can be found in the lamina propria of the vagina [39].

In oral administration, the effect on decreasing SAP5 levels through direct and indirect mechanisms. The direct mechanism occurs because *L. plantarum* survives in the GIT tract and then attaches (adhesions) to the vaginal mucosa through cross-contamination from the rectum [41]. Through the mechanism of cross contamination, the number of Lactobacillus colonies in the vaginal mucosa will increase progressively. *L. plantarum* had competition with *C. albicans* both for attachment sites and get nutrition. The growth of *Lactobacillus* colonies was also accompanied by an increase in bioactive compounds such as bacteriocins, biosurfactants, lactic acid (types L and D), diacetyl, hydrogen peroxide which inhibited the growth, attachment and invasion of *C. albicans* and neutralized the aflatoxins produced by *C. albicans* [42]. The progressive decrease in *C. albicans* colonies following by a decrease in SAP5 levels. Whereas in the indirect mechanism, the emergence of clinical benefits of *L. plantarum* occurs through interactions with components of immune cells. Through interaction with TLR2, *Lactobacillus* is able to shift the immune response from Th1 to Th2 characterized by a decrease in pro-inflammatory cytokines [43]. In addition, L. *plantarum* increase immunoregulatory compounds such as proline and glutamine. Proline and glutamine are non-essential amino acids that function as cell protectors against oxidative stress by maintaining membrane integrity, increasing cell growth, increasing lactic acid in the mucosa [44,45]. The presence of lactic acid in the mucosa becomes a toxic condition for *C. albicans* so it will inactivates the invasion mechanism in the epithelium characterized by a decrease in SAP5 levels.

Same as oral administration, intravaginal administration also has 2 important mechanisms, direct and indirect. Based on direct mechanism, Darby and Jones [46] stated that *L. plantarum* has the ability to attach to vaginal epithelial cells and can significantly reduce *C. albicans* cell adhesion which contributes to a decrease in SAP5. When attached to vaginal epithelial cells, *L. plantarum* will also be recognized by TLR and NLR. This recognition will increase the phagocytic activity of macrophages to phagocytize *C. albicans* both in the form of single yeast and hyphae. The more *C. albicans* hyphae that are phagocytosed, the lower the SAP5 level [47].

Even though the results of measurements of SAP5 levels both showed a significant decrease in the oral group (p = 0.001) and the intravaginal group (p = 0.000) but in terms of the average results of the decrease, the decrease in SAP5 levels was higher in the intravaginal group (mean = 4.872) compared to the oral group (mean = 3.35841) (**Table 2**). Intravaginal administration of *L. plantarum* probiotics can directly reach the infected site so that the effects of immune modulation, vaginal microbiota modulation, induction of anti-inflammatory cytokine secretion and vaginal homeostasis can occur more quickly [48]. Whereas in oral consumption, although technically it feels easier and more comfortable, its effectiveness is also influenced by factors in gastrointestinal tract. In several studies that have been conducted, oral administration of *Lactobacillus* spp. probiotics can reduce the number of *Gardnerella vaginalis* bacteria that cause BV. Thus, the administration of probiotics both orally and intravaginally can have a positive impact both locally at the site of infection and systemically.

Table 2 Paired T-test SAP5 levels.

		Mean	N	α*	Sig. (2-tailed)
Pair 1	Pre-Oral Intervention	9.36733	12	0.05	0.001
	Post-Oral Intervention	6.00892	12	0.05	
Pair 2	Pre-Intravaginal Intervention	12.67533	12	0.05	0.000
	Post-Intravaginal Intervention	7.80333	12	0.05	

*Confidence Interval for Difference (CI) 95 %

Conclusions

Administration of the probiotic *Lactobacillus plantarum* either orally or intravaginally showed similar and significant effectiveness in reducing SAP5 levels. The results of this study indicate that both the oral and intravaginal routes are potential routes of administration of *Lactobacillus plantarum* probiotics as supportive therapy in treating VVC. So that the determination of the probiotic consumption route can be adjusted to the convenience and ease of application.

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