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**ISOLATION, SCREENING, AND CHARACTERIZATION OF PROBIOTICS (LACTIC ACID BACTERIA) ANTAGONISTIC AGAINST *Candida albicans***

**ISOLASI, SKRINING, DAN KARAKTERISASI PROBIOTIK (BAKTERI ASAM LAKTAT) YANG BERSIFAT ANTAGONIS TERHADAP *Candida albicans***

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## ABSTRACT

Candidiasis in female reproductive tract are mainly caused by *Candida albicans*. This infection often causes serious problems, particularly on their reproductive tract (genital part). Until recently, control of this infection has relied on the use of antibiotics. However due to numerous bad side effects of antibiotics, lactic acid bacteria have been proposed as an alternative method to control the growth of *Candida albicans*. Therefore, this research was aimed to isolate, screen, and characterize lactic acid bacterial isolates (LAB) antagonistic against *Candida albicans* (the causative agent of candidiasis infection in reproductive tract of human). LABs were isolated from various fermented foods, such as *tape ketan* and *kimchi*. Isolation of LABs was conducted by applying dilution and spread plate method on MRS agar medium supplemented with BCP indicator to distinguish LABs from non acid-producing bacteria. Colonies with indication to produce acid were screened for antagonistic activity against *C. albicans* on MRS agar and followed by characterization of those isolates (Gram stain, catalase production test, oxydase production, gas production test, resistance test to low pH conditions and to high level of NaDC (sodium deoxicollic), and test for ability to convert colic acid (CA) into deoxicollic acid (DCA)). The results showed that 46 LAB isolates were successfully isolated from samples of *tape ketan* and *kimchi*. Among those, 7 isolates showed antagonistic activity against *C. albicans* in *in vitro* tests. All these 7 candidates were also found to be resistance to low pH conditions (up to pH 2) and to high level of NaDC (up to 0.6 mM). Four most potential isolates were further testes for ability to convert colic acid into deoxycolic acid and none showed positive result, indicating that they all showed initial potential and safe for future human probiotic development (especially to be used to treat patients infected by *C. albicans*).

*Keywords:* *C. albicans*, lactic acid bacteria, *tape ketan*, *kimchi*.

## INTISARI

Kandidiasis pada saluran reproduksi wanita umumnya disebabkan oleh *Candida albicans*. Infeksi ini sering menimbulkan masalah serius, khususnya pada saluran reproduksi. Sampai saat ini, pengendalian infeksi candida sangat tergantung pada penggunaan antibiotik. Namun, banyaknya efek samping antibiotika pada pasien yang mulai diketahui, maka bakteri asam laktat mulai banyak diusulkan sebagai salah satu metoda alternatif untuk mengontrol pertumbuhan *Candida albicans*. Oleh karena itu, penelitian

ini ditujukan untuk mengisolasi, menskrining, dan mengkarakterisasi bakteri asam laktat (BAL) yang bersifat antagonis terhadap *Candida albicans* (penyebab penyakit keputihan pada saluran reproduksi wanita). BAL diisolasi dari berbagai makanan terfermentasi, seperti tape ketan dan kimchi. Isolasi BAL dilakukan dengan menerapkan metoda pengenceran dan sebar pada permukaan medium MRS agar yang ditambahkan dengan indikator BCP untuk membedakan isolat BAL dengan bakteri lain yang tidak menghasilkan asam. Koloni yang tumbuh dan menunjukkan indikasi sebagai penghasil asam diisolasi dan diskriming aktivitas antagonisnya terhadap biakan *C. albicans*, yang dilanjutkan dengan karakterisasi (pewarnaan Gram, uji katalase, oksidase, uji produksi gas, uji ketahanan terhadap pH rendah, dan uji kemampuan mengubah asam kolat menjadi asam deksikolat) setiap isolat yang menunjukkan zona hambatan pada biakan *C. albicans*. Hasil penelitian menunjukkan bahwa sebanyak 46 isolat BAL berhasil diisolasi pada penelitian ini, dan sebanyak 7 isolat menunjukkan aktivitas antagonism terhadap *C. albicans*. Semua isolat antagonis tersebut juga menunjukkan sifat resisten terhadap lingkungan pH rendah (sampai pH 2). Empat isolat yang paling potensial berdasarkan uji-uji tersebut selanjutnya diuji kemampuannya dalam mengubah asam kolat menjadi asam deoksikolat, dan semua isolat menunjukkan hasil negatif dalam uji ini. Hasil ini mengindikasikan bahwa keempat isolat tersebut menunjukkan indikasi awal untuk dikembangkan menjadi probiotik potensial, khususnya untuk menanggulangi penyakit keputihan yang disebabkan oleh *C. albicans*.

*Kata-kata kunci: C. albicans, lactic acid bacteria, tape ketan, kimchi*

## INTRODUCTION

Candidacies infection in reproductive tract of human is mainly caused by *Candida albicans* (Kundu and Garg, 2012). This infection has frequently been found among females and often causes serious problems, particularly on their reproductive tract (genital part). This type of infection has been reported to increase as a function of time, particularly among women with immune-compromise conditions (Zarrin and Mahmoudabadi, 2009).

Various types of Candidacies infections have been reported worldwide. Inter-digital candidacies infection and onicomycosis candida, for examples, are more frequently found in tropical areas and in cold climate areas, respectively (Ramali, 2013; Kullberg and Arendrup, 2015). Vaginal candidacies infection and vulvo-vaginal infections are considered as the second most frequent cases of vaginal infection among females. According to Suyoso (2013) 75% females may experience an episode of vaginal candidacies infection during their life, caused either by *C. albicans* or *C. glabrata*. Among those reported cases, infection caused by *C. albicans* may reach 80%-90% of those cases (Pappas *et al.*, 2003; Suyoso, 2013; Kullberg and Arendrup, 2015). Candida infection is mainly

due to the disturbance of vaginal normal flora, especially on the groups of lactic acid bacteria that play important roles to inhibit the growth of pathogenic Candida in the vagina.

Until recently, treatment of patients with candidacies infection has relied on antibiotics with topical properties. Itrakonazol, flukonazol, and nystatin, for examples, are dominant types of antibiotics used in the therapies (Hoan and Rahardja, 2007 ; Salehei *et al.*, 2012; Suyoso, 2013). To reduce bad side effects of antibiotics, alternative approaches to control candidacies infection, such as application of probiotics has been intensively studied in the last two decades (Hilton *et al.*, 1992; Taylor, 2004; Manzoni *et al.*, 2006; Martinez *et al.*, 2009). Probiotics reduce candidacies infections by inhibiting the growth of the causative agents, and therefore they may be applied synergically with antibiotic therapies (Wagner *et al.*, 1997; Manzoni *et al.*, 2006; Martinez *et al.*, 2009; Senok, *et al.*, 2009).

Based on the above background, potential probiotic candidates antagonistic against *C. albicans* were isolated, screened, and characterized in this research. All probiotic candidates were isolated from fermented foods, such as *tape ketan* and *kimchi* purchased from supermarkets around Denpasar city.

## MATERIALS AND METHOD

### Isolation of Probiotic Candidates

Probiotic candidates were isolated from samples of *tape ketan* or *kimchi*, purchased from supermarkets in Denpasar city, by applying dilution and spread method as specified in Ramona *et al.* (2015). Some 10 g samples were added to 90 ml of saline solutions to obtain dilution rate of  $10^{-1}$ . This sample was further diluted in saline solution to obtain dilution rates of  $10^{-2}$  to  $10^{-6}$ . Some 0.1 ml samples from dilution rates of  $10^{-3}$  to  $10^{-6}$  were next spread on MRS agar medium supplemented with BCP indicator, incubated at 37 °C for 24 – 48 hours, and observed for acid-producing bacterial colonies. Colonies with indication to produce acidic compounds were purified and stored at 4 °C until needed for further experiments.

### Screening of Probiotic Candidates Antagonistic against *C. albicans*

Antagonistic activity of probiotic candidates was assessed by applying the dual culture assay as specified by Ramona (2003). Some 20 µl suspensions of *C. albicans* were spread evenly on MRS agar medium to produce lawn of *C. albicans* and let it dry for 5 minutes at ambient temperature. Cells of probiotic candidates were next picked from single colonies (with indication to produce acid) on MRS agar using a tooth pick and stab inoculated on the lawn of *C. albicans*, incubated at 37 °C for 24 hours, and observed for inhibition zone around the probiotic candidates. Probiotic isolates with inhibition zone were purified and stored at -80 °C in MRS broth medium with 30% glycerol until required in subsequent experiments.

### Gram Stain

Gram staining of probiotic candidates followed the method as specified by Ramona (2003). Cells of purified probiotic candidates (24 hours of age) were fixed on an object glass, exposed with crystal violet solution for 1 minute, washed with distilled water, exposed with several drops of lugol solution for 1 minute, washed again with distilled water, washed for 5 seconds with 96% ethanol, and washed again

with distilled water before being counter stained with safranin for 1 minutes. After 1 minute elapsed, the slide was washed with distilled water, dried, and observed under a microscope.

### Catalase Test

This catalase test followed the method specified by Ramona (2003). Fixed cells of probiotic candidates on an object glass were added with several drops of 10% H<sub>2</sub>O<sub>2</sub> solution and observed for air bubble production. Positive test was indicated by bubble production. Lactic acid bacteria showed negative result for this test.

### Gas Production Test from Glucose Metabolism

This test followed the method specified by Sujaya *et al.* (2008). A hot loop was stabbed into suspension of probiotic candidates in MRS broth supplemented with glucose and observed for bubble formation. Positive test was indicated by bubble formation. Homo-fermentative lactic acid bacteria showed negative result in this test, and selected as potential probiotic candidates.

### Test for Resistance to Acidic Conditions

These tests adopted the method applied by Sujaya *et al.* (2008). Isolate suspensions that showed optical density of more than 0.1 following exposure to low pH conditions indicated that they were resistance to acidic conditions.

### Test for Conversion of Colic Acid (CA) into Deoxycolic Acid (DCA)

Some 50 µL of LAB suspension was inoculated into 5 ml MRS *broth* pH 8.0 supplemented with 20 µl *colic acid* and incubated at 37°C for 24 hours (Kurdi *et al.*, 2000; Yoshida, 2004). Some 1 mL of this suspension was next centrifuged for 5 minutes at 5000 rpm, its supernatant (100 µL) was dispensed in an eppendorf tube, added with 500 µL ethyl acetate and 20 µl HCl, centrifuged for 5 minutes at 5000 rpm., its supernatant was transferred into a new eppendorf tube, evaporated for 48 hours at ambient temperature, and added with 15 µL methanol. Subsequently, 1 µL DCA, CA, and extracts of each LAB isolates were

spotted on aluminum silica gel, dried with hair dryer, placed in a chamber containing eluent solution (10 ml Cyclohexane, 15 mL ethyl acetate and 4 mL acetic acid), air dried, sprayed with *molibddoposporic acid* and placed in an oven until spots of each isolate appeared on the aluminum silica gel (Sujaya *et al.*, 2008). The Rf values were calculated by dividing the distance traveled by the spots with the distance traveled by the eluent solution (Lipsy, 2010).

**RESULTS**

Some 46 lactic acid bacteria (LAB) were successfully isolated from samples of fermented food (*tape ketan* and *kimchi*). Seven isolates showed antagonistic activity against *C. albicans* with various degree of inhibition zones *in vitro* (Figure 1). The characteristics of these 7 isolates are shown in Table 1.

Table 1: Characteristics of the 7 LABs isolates antagonistic against *C. albicans*

Isolates Codes	Characteristics				
	Gram Stain	Catalase	Gas Production	Oxidase	Cell Morphology
Kim 26	Positive	Negative	Negative	Negative	Rod
Kim 45	Positive	Negative	Negative	Negative	Spherical
Tape 3	Positive	Negative	Negative	Negative	Rod
Tape 5	Positive	Negative	Negative	Negative	Spherical
BD01	Positive	Negative	Negative	Negative	Rod
BD02	Positive	Negative	Negative	Negative	Rod
BD04	Positive	Negative	Negative	Negative	Rod



Figure 1: *In vitro* inhibition zones produced by LAB isolates on *C. albicans*

Table 2: Resistance of 7 LAB isolates against acidic conditions (low pH)

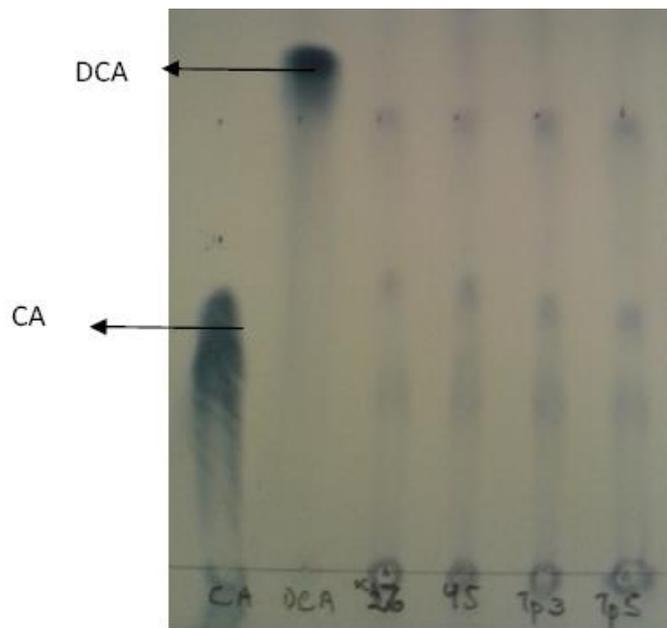
Isolate codes	Growth indication (OD reading at λ 660 nm)*			
	Control (pH 6.5)	pH 2	pH 3	pH 4
Kim 26	+++ (2.44±0.36)	+++ (2.23±0.20)	+++ (2.59±0.13)	+++ (2.61±0.13)
Kim 45	+++ (2.66±0.01)	+(0.45±0.35)	+++ (2.61±0.07)	+++ (2.69±0.06)
Tape 3	+++ (2.43±0.06)	+++ (1.36±0.12)	+++ (2.43±0.01)	+++ (2.45±0.01)
Tape 5	+++ (2.34±0.04)	++ (0.51±0.45)	+++ (2.38±0.02)	+++ (2.39±0.01)
BD01	+++ (2.34±0.22)	+++ (1.98±0.30)	+++ (2.28±0.15)	+++ (2.40±0.19)
BD02	+++ (2.50±0.03)	+++ (2.32±0.27)	+++ (2.49±0.08)	+++ (2.53±0.05)
BD04	+++ (2.26±0.14)	+++ (2.15±0.07)	+++ (2.20±0.12)	+++ (2.26±0.19)

\*Each absorbance value in Table 2 ±standard deviation is an average of triplicates  
 - = not resistance against acidic conditions (OD reading < 0.1)  
 +=slightly resistance against acidic conditions (OD reading 0.1 – 0.5)  
 +++= resistance against acidic conditions (OD reading 0.51 – 1.0)  
 ++++= highly resistance against acidic conditions (OD reading > 1.0)

In the resistance tests against acidic conditions (low pH), all 7 isolates shown in Table 1 were able to grow well in such extreme conditions, indicating that they showed initial potential for probiotic development, especially to be used to treat patients infected by *C. albicans*. The results of this test are shown in Table 2. LAB isolates to be developed as human probiotics must not have the ability to convert

colic acid into deoxicolic acid.

In this test, all tested isolates (4 isolates from Table 2) did not convert colic acid into deoxicolic acid. This indicated that all of them fulfilled one of the most important properties needed for candidate of human probiotic development, although further tests need to be conducted. The results of this test (on 4 most potential isolates) are shown in Figure 2.



Lane 1 is deposited with control colic acid (CA)

Lane 2 is deposited with control deoxicolic acid (DCA)

Lanes 3 -6 are deposited with supernatants of isolates kim 26, kim 45, tape 3, and tape 5.

Figure 2: Chromatogram of test for ability of LAB isolates to convert CA into DCA

## DISCUSSION

Probiotic candidates (especially LAB) can easily be isolated from any sources, such as fermented food (Parvez *et al.*, 2006), milk (Sintyadewi *et al.*, 2015), or feces of healthy infants (Ramona *et al.*, 2015). In our research, some 46 LAB isolates were successfully isolated from *tape ketan* and *kimchi* or in line with that reported by Sujaya *et al.* (2008). In an *in vitro* isolation, most of microbes cannot be cultivated in synthetic medium. This is due to most of their growth requirements cannot be fulfilled by the medium composition (Ramona, 2003). This was

probably the main cause of the limited number of probiotic isolates obtained in our research.

Among those 46 isolates, 7 isolates were found to inhibit the growth of *C. albicans* (the causative agent of candidiasis in human) in dual culture assay (Figure 1). Dual culture assay is a very convenient method used to initially recognize antagonistic activity of an organism against others. This method has been used widely either to screen potential biocontrol agents (Ramona, 2003) or probiotic candidates to be used to control human pathogenic agents (Scapin *et al.*, 2013). Inhibition zone around an isolate can be used as a consideration to select this isolate for further experiments.

Isolates that showed antagonistic activity in the previous study were tested for resistance in low pH conditions. All those 7 isolates performed well in this test where all of them reached high level of cell densities following exposure to pH 2 – 4 (Table 2). Generally all isolates were suppressed when exposed to pH 2 and better growth response was observed in higher pH conditions. This indicated that all isolates tolerated low pH conditions very well and met one of most important requirements for probiotic development, especially to counter the growth of *C. albicans* in acidic condition within vagina. Although it was not elucidated in this research, resistance properties of LAB isolates in low pH conditions have been reported to be due to their ability to maintain their cytoplasmic pH condition higher than that of their surroundings (Sieggumfeldt *et al.*, 2000; Cotter and Hill, 2003; Slonczewski *et al.*, 2009). This is done by actively pumping H<sup>+</sup> ions out of the cells (Sieggumfeldt *et al.*, 2000; Cotter and Hill, 2003) to increase the internal pH of the cell so that they can well survive in acidic conditions. Our results are in line with those found by Uni *et al.* (2013), Ramona *et al.* (2015), and Sintyadewi *et al.* (2015).

In addition to resistance test against low pH conditions, an isolate to be developed for human for probiotic need to be tested for ability to convert colic acid (CA) into deoxicolic acid (DCA) (Sujaya *et al.* 2008; Pato, 2003 and Liong, 2008). Isolates with this ability will increase the DCA concentration when exposed to CA. High concentration of DCA in human body has been suspected to induce cancer in human colon (Kawano *et al.*, 2010; Wells *et al.*, 2003). Therefore for safety reasons, isolates that show ability to do so must be rejected for human probiotic development, although they show high potential based on other tests (Pato, 2003 and Liong, 2008). In our research, none of tested isolates were able to convert CA into DCA, indicating that they have potential to be developed as human probiotics, although many important properties of these isolates, such as resistance against antibiotic used medically to treat *C. albican* and ability to adhere on vaginal

lining, need to be elucidated, so that they are effective to control this pathogen.

## CONCLUSION

Some 46 LAB isolates were isolated from samples of *tape ketan* and *kimchi* and 7 of those isolates inhibited the growth of *C. albicans* *in vitro* on MRS agar. All of these isolates were found to be resistance against low pH conditions (up to pH 2). In the test of conversion of CA into DCA on 4 potential isolates, none of them showed this ability, indicating that they all have good prospect to be developed for future human probiotics, particularly to be used to control *C. albicans*, although further tests need to be conducted.

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