

Study of Lactic Acid Bacteria Activities from Human Oral Mucosa for *Candida albicans* Inhibition

Evi Lauw¹, Khusnul Hatimah Ilham¹, Febliani Taruk Palinggi¹, Desy Setiady² and Sartini¹

¹ Faculty of Pharmacy, Hasanuddin University, Makassar, Indonesia

² Faculty of Dental Medicine, Hasanuddin University, Makassar, Indonesia

Abstract

Lactic acid bacteria are known to have potential in producing antimicrobial compounds, including antifungal agents. This study aims to determine whether the lactic acid bacteria from human oral mucosa have the potential to produce the secondary metabolite to inhibit the candidiasis, which caused by pathogen fungi, *Candida albicans*. Lactic acid bacteria were isolated from three healthy respondents based on age difference that is an infant, child, and adult. Lactic acid bacteria isolation using the deMann Rogosa Sharpe Broth (MRSB)-liquid medium, followed by purification stage with the quadrant scratch method using the deMann Rogosa Sharpe Agar (MRSB)-solid medium. The purified isolates obtained were characterized and identified by Gram staining, biochemical tests, and inhibition zone diameter measurement toward pathogenic fungi *Candida albicans*. The results were obtained from each of the two lactic acid bacteria isolates from each respondent with characteristics including gram-positive bacteria with short stem cell shape, possibly as *Lactobacillus sp.* It is conclude that the lactic acid bacteria of oral mucosa from adult had greater anticandidal activity than infant and child.

Article History

Received 23 May 2017

Accepted 10 November 2017

Keyword

Lactic Acid Bacteria
Human Oral Mucosa
Antifungal Activity
Candida albicans

Introduction

During the last one decade, the prevalence of infectious diseases caused by pathogenic fungi, which is candidiasis, has increased sharply (Fortún *et al.*, 2012). Candidiasis is a mucosal fungal infection on humans of all ages that affect the skin and mucous membranes of oropharynx, oesophagus, and vagina (Fortún *et al.*, 2012; Gajer *et al.*, 2012). There are at least 15 distinct *Candida* species that cause human disease, but more than 90% of invasive disease is caused by the five most common pathogens, *C. albicans*, *C. glabrata*, *C. tropicalis*, *C. parapsilosis*, and *C. krusei* (Silva-Dias *et al.*, 2014). *Candida albicans* continues to be the most prevalent and problematic of all *Candida* species (Perera *et al.*, 2015; Paul *et al.*, 2014). The pathogenicity of *Candida* species is attributed to certain virulence factors, such as the ability to evade host defences, adhesion and biofilm formation (on host tissues and or on medical devices), and the production of tissue-damaging hydrolytic enzymes, such as proteases, phospholipases and haemolysins (Paul *et al.*, 2014). Moreover, the morbidity and

mortality associated with candidiasis are still very high, even using the actual antifungal drugs. Annually, 50% of adults and up to 30% of children die of candidiasis (Paul *et al.*, 2016; Martinez *et al.*, 2015).

The major classes of antifungal drugs used for treatment of *Candida* species infections are azoles, polyenes, and echinocandins (Martinez *et al.*, 2015). According to previous experimental results, irrational use may lead to colonization of *Candida sp.* that are resistant to these drugs. It has been reported that conventional treatment for treating candidiasis with fluconazole has a case of resistance (Martinez *et al.*, 2015; Costa *et al.*, 2015). The occurrence of *Candida* species infections becoming more difficult to treat due to the growth of immunogenic diseases, endocrine disorders, malnutrition, the disproportionate use of immunosuppressive drugs, and broad spectrum antibiotics (Costa *et al.*, 2015). It is believed that the increase of *C. albicans* infections is due to its intrinsically low susceptibility to azoles, including the imidazole and the oral-parenteral triazoles (e.g., fluconazole, voriconazole) (Gayford JJ & Harskell R, 2010). Additionally, it is known that the acquired resistance is resulted of rare mutations that are selected by drug pressure (Samaranayake, 2012).

The recent efforts of various excellent researches tremendously broadened on the complex mechanisms of normal flora (Scatassa *et al.*, 2015). They were shown that living creatures without normal flora are more susceptible to pathogenic microbes (Kanmani *et al.*, 2013; Amadou *et al.*, 2013; Amenu, 2013). In this sense, the authors highlighted the importance of normal flora and that the addition of secondary metabolite to improve the efficacy of candidiasis. The result of a study showed that there was an increase in oral and dental health, as well as a decrease in the number of *Candida albicans* fungi in school children who consumed milk or cheese containing *Lactobacillus rhamnosus strain GG.3* which belongs to the lactic acid bacteria group (Canabarro *et al.*, 2012). According to the authors, some strains of lactic acid bacteria have a positive activity in reducing the amount of *Candida albicans* in the saliva of the human oral cavity (Amenu, 2013; Canabarro *et al.*, 2012).

Recent studies revealed the involvement lactic acid bacteria, especially the genus *Lactobacillus*, can be isolated from animal and human digestive tracts, including upper gastrointestinal organs such as the oral cavity (Corona *et al.*, 2016). It has obtained *Lactobacillus gasseri* isolates identified using 16S-rRNA sequencing from oral mucosa of a 4-month-old infant (Settanni *et al.*, 2013). However, this article showed anti-fungal activity of lactic acid bacteria isolates from three most potent sources as producers of anti candidiasis metabolite compounds that are an infant, child, and adult. The significance of this study is due to its contribution to knowledge on the determination of the ability of lactic acid bacteria isolates from the oral cavity of humans as anti candidiasis agents.

Materials and Methods

Preparation of Tools and Materials

The tools used in this research were Autoclave (All American), Incubator (Memmert), Laminary Air Flow, Microscope (Olympus), Oven, Caliper, Analytical Scales (Ohaus), Sterile Swab (OneMed), petri disc, Chemical Glass (Pyrex), Bunsen, Cotton, Measuring cylinder (Pyrex), handscoon, electric stove, cloth mask, round loop, straight loop, spoon, test tube, vial tube, etc.

The materials used in this research were 70% alcohol, aqua distillate, pure *Candida albicans* culture (obtained from Microbiology Laboratory, Faculty of Pharmacy, Hasanuddin

University), ketoconazole, deMann Rogosa Sharpe Agar and Broth (MRSA and MRSB) media, and Potato Dextrose Agar (PDA) medium.

Experimental Methods

Isolation of Lactic Acid Bacteria

Oral mucous samples were taken from each of the three samples individually, infant, child, and adult. Sampling is done by rubbing into the inside cheeks in samples using a sterile swab sticker. The swab result is then inserted into a vial containing the MRSB medium as a transport medium, then immediately taken to the laboratory.

Samples of the oral mucosa were diluted by 1 mL pelleted and fed into 9 mL of sterile distilled water, then diluting the dilutions from 10^{-1} to 10^{-5} . A total of 1 mL of liquid from each dilution was introduced into Petri dishes and 10 mL of MRSA and CaCO_3 1% were added and homogenized, then incubated at 37 °C for 1x24 hours. When a clear zone is formed around the colony, then the colony is suspected as lactic acid bacteria.

Purification of Lactic Acid Bacteria Isolates

Lactate Acid Bacteria isolates were then inoculated by scraping them onto the medium in a petri dish then incubated for 1x24 hours at 37 °C at the aerobic incubator. Then purified by inoculating the MRSA medium inclined in the test tube, after which incubated for 1 × 24 hours at 37 °C in the aerobic incubator.

Characterizations and Identifications of Lactic Acid Bacteria

Macroscopic Test

Pure lactic acid bacteria isolates were inoculated on MRSA medium in petri dishes, after which incubated for 1x24 hours at 37 °C in aerobic incubators. Then, observed colonies.

Microscopic Test

Gram staining method was used by isolate growing on MRSB medium placed on the preparation. Then, fixed and added 2-3 drops of gram A (crystal violet), left for 1 minute. Wash with running water, dried with filter paper. Add gram B (mordant). Left 1 minute. Spilled with gram C (95% ethanol) for 10-20 seconds. Wash with running water, dried with filter paper. Add gram D (safranin) for 1 minute. Wash with running water, dried with filter paper. It was observed under a microscope with 100x widening by using immersion oil. Gram positive bacteria will be purple and gram negative will be red (Ventimiglia *et al.*, 2015).

Biochemical Test

Biochemical tests in this study consist of the several tests as following;

Carbohydrate Fermentation Test

Cultures of lactic acid bacteria inoculated into Lactose Broth, Sucrose Broth, Glucose Broth, and Mannitol Broth media were incubated for 1 x 24 hours at 37 °C. Thus, observed changes in medium color and gas formation.

Motility Test

The culture of lactic acid bacteria was inoculated puncture on an erect SIM medium and incubated for 1 x 24 hours. If isolate growth spreads far from puncture indicates motile isolate, whereas if growth only occurs puncture discharge, it indicates no motile isolate.

Catalase Test

Suspension isolates drops as much as two drops on glass object, and then spilled 3% H₂S₂ solution. The positive catalase reaction is shown by the presence of foam or foam that occurs after the addition of the solution for 1 min.

Test of Optimum Growth Temperature

A total of 1 mL of pure culture suspension were each inoculated into 4 tubes containing 9 mL MRSB media on which the purple brom-kresol indicator was split. The medium is incubated at a temperature of 10 °C, 15 °C, 37 °C and 45 °C for 1-5 x 24 hours under suitable conditions. The media color change from purple to yellow signifies the growth of culture.

Test of Optimum Growth pH

A total of 1 loop of pure isolates was each inoculated into 4 tubes containing MRSB media on which the purple brom-kresol indicator was split. The medium was incubated at pH 2, 4, 6 and 8 for 1- 5 x 24 hours at 37 °C. The media color change from purple to yellow signifies the growth of culture.

Test of Isolate Activity against *Candida albicans*

The isolated lactic acid bacteria were grown in MRS Broth media for 24-48 hours. The result of the fermentation will be precipitated its cell mass. Then the supernatant fluid containing the bioactive component was tested for its anticandidal activity of *Candida albicans* by the paper disc-diffusion method. The isolates were prepared for the test with two replications of each sample.

Results and discussion

Lactic Acid Bacteria Isolation

This research began with sterilization as the most important stage in the implementation of research related to microorganisms. Furthermore, the manufacture of a special medium for the growth of lactic acid bacteria is MRSB (deMann Rogosa Sharpe Broth) as the transport medium. The next stage is the oral mucosal sampling of three people samples; infant, child, and adult from three volunteers with healthy dental and mouth conditions. Sampling is done by using sterile swab swabs and immediately inserted into the transport medium immediately after taking to minimize the occurrence of contamination. The purpose of this early stage is to obtain lactic acid bacteria from the oral mucosa and increase the number of cells (cultivation) before the next stage.

This process used MRSA (deMann Rogosa Sharpe Agar) medium in a petri dish and calcium carbonate (CaCO₃ 1%) added to the medium in order to identify differences of lactic acid bacteria compared to the other types of bacteria. The validation of the lactic acid bacteria was performed by the muddiness of medium colour (Vestman *et al.*, 2013). If it contained lactic acid bacteria, the calcium from CaCO₃ in MRSA can react with lactic acid produced by lactic acid bacteria to form soluble calcium lactate, which makes the colour of the medium becomes transparent (Vestman *et al.*, 2013).

Lactic Acid Bacteria Purification

The next stage was the purification of isolates. This isolation was carried out each into 2 petri dishes for each sample. The observation of colony growth will be done on days 1, 3, 5, and 7. On the 5 the day, a transparent zone has been formed on the MRSA medium that is thought to be a colony of lactic acid bacteria. The purification results are repeated twice with the aim of obtaining more pure isolates and increasingly free from mixtures of other types of bacterial colonies.

Subsequently, the isolates were moved into the test tube containing the inclined MRSA in the test tube for the further stages, i.e. identification of isolates. Isolates were named by code, i.e. infant's mouth isolates (MB, MB2), child's mouth isolates (MA, MA), and adult's mouth isolates (MD, MD2).

Lactic Acid Bacteria Characterizations and Identifications

In this study, the identification of lactic acid bacteria microscopically was done by the gram staining method. The staining results for the three types of isolates (MB, MA, MD) showed purple bacterial cells, so the isolates in this study included gram-positive bacteria. This supports the theory that lactic acid bacteria, one of which is from the genus *Lactobacillus*, is a type of gram-positive bacteria that in gram staining will have a purple appearance under an optical microscope (Ventimiglia *et al.*, 2015; Norder *et al.*, 2013).

In addition, besides the microscopic tests and gene identification above, the isolates were tested for their antimicrobial activity by the diffusion method in order to use paper discs. It was measured from horizontal, vertical, and diagonal, then the calculated for the average diameter. Based on antimicrobial activity test from oral mucosa isolate to *Candida albicans* fungi causing infectious disease of candidiasis, and obtained the result of the zone diameter as follows.

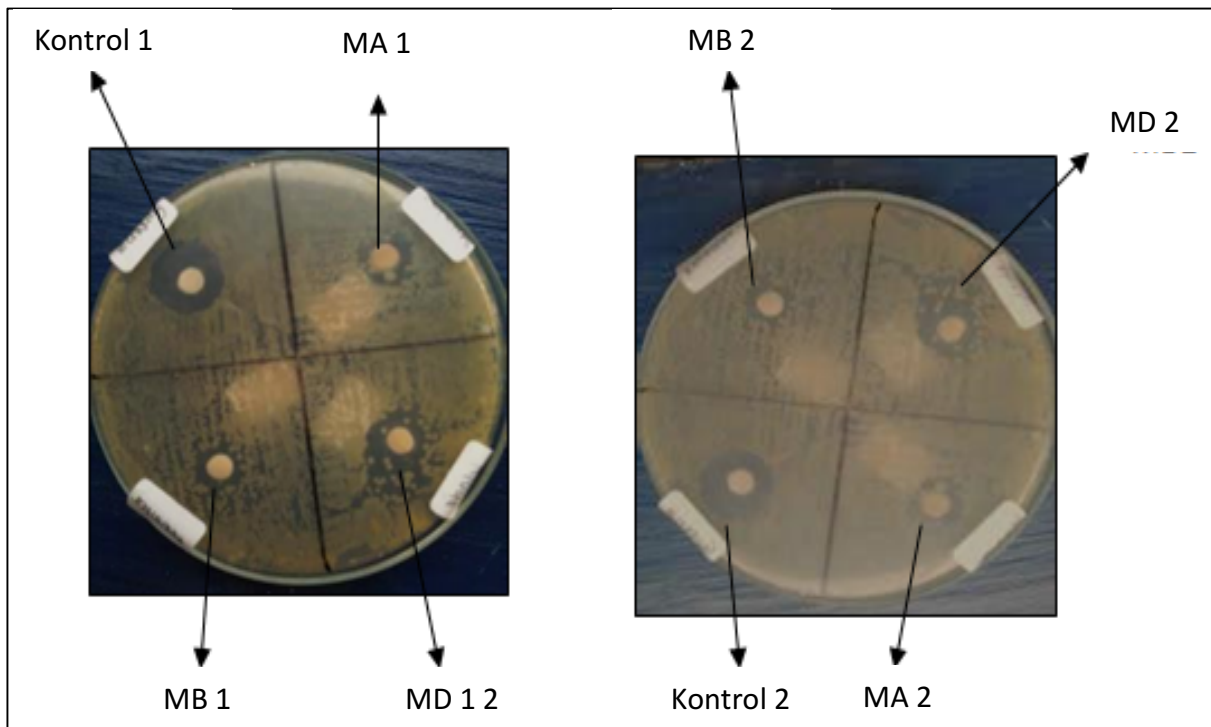


Figure 1. Inhibition zone diameter of lactic acid bacteria isolates from human oral mucosa

Table 1. Inhibition zone diameter measurement of lactic acid bacteria isolates from human oral mucosa

Types of Isolate	Replication		Diameter ± Standard Deviation (mm)
	I	II	
Ketoconazole (Control +)	19.1	20.4	19.19 ± 0.16
	18.7	19.3	
	19.4	18.3	
<i>Average</i>	19.07	19.30	
Infant (MB)	8.1	7.3	7.22 ± 0.02
	6.5	7.5	
	7.1	6.8	
<i>Average</i>	7.23	7.20	
Child (MA)	9.3	10.3	9.72 ± 0.58
	9.5	9.9	
	9.1	10.2	
<i>Average</i>	9.30	10.13	
Adult (MD)	16.4	15.8	16.10 ± 0.09
	16.2	16.1	
	15.9	16.2	
<i>Average</i>	16.17	16.03	

From the table above, it can be seen that the average inhibitory zone diameter of the oral mucosa of infants (MB and MB2) is 7.23 mm and 7.20 mm (average 7.22 mm ± 0.02 mm), lactic acid bacteria isolate from the oral mucosa of children (MA) is 9.30 mm and 10.13 mm (average 9.72 mm ± 0.58 mm), and lactic acid bacteria isolate from adult mouth mucosa (MD) were 16.17 mm and 16.03 mm (average 16.10 mm ± 0.09 mm). While the results of inhibitory power using ketoconazole antifungal drugs as a comparison were 19.07 mm and 19.30 mm (average 19.19 mm ± 0.16 mm). It is showed that lactic acid bacteria isolates from adult mouth mucosa (MD) had the best inhibitory power seen from their larger inhibitory diameter zones compared to both infant isolate (MB) and child isolate (MA).



Figure 2. The collection of potent lactic acid bacteria isolates as anti candidiasis

For additional data, biochemical tests consisting of carbohydrate fermentation test, motility test, catalase test, optimum pH test, and optimum temperature test were conducted. The aim of these five biochemical tests is to confirm that the isolates obtained from the oral mucosa of humans in this study can satisfy the characteristics of lactic acid bacteria with specific physiological and metabolic activities specifically.

The results of carbohydrate fermentation test on the three isolates (MB, MA, MD) with medium LB, GB, and SB showed the three results of each positive able to ferment carbohydrate. The positive result is marked by the colour change of the medium to yellow and the formation of gas in the *Durham* tube. This is consistent with the reference that lactic acid bacteria include heterofermentative bacteria, which are capable of fermenting sugars (monosaccharides and disaccharides) to produce acidic and CO₂ gases. The acidic conditions in the medium result in a change of medium colour and bubble formation (Minervini *et al.*, 2012; Pepe *et al.*, 2013).

The result of the motility test of all three isolates showed the growth of colonies perpendicularly in the direction of inoculation using a loop, and without spreading around the inoculants. If the bacterial cell is motile, its colonies will spread throughout the medium. Therefore, the three isolates (MB, MA, MD) are non-motile bacteria (Minervini *et al.*, 2012; Pepe *et al.*, 2013).

The result of catalase test of lactic acid bacteria isolated from oral mucosa of the three samples (MB, MA, MD) was negative catalase. This is in accordance with the reference to the fact that lactic acid bacteria do not produce catalase enzymes so that the test results are negative as a result of the absence of H₂O₂ degradation by catalase enzymes (Minervini *et al.*, 2012; Pepe *et al.*, 2013).

The optimum pH test results in this study were pH 4. At pH 4, the most turbid visible medium was thought to be the highest growth rate of lactic acid bacteria in this study. The previous study stated that lactic acid compounds capable of inhibiting the growth of pathogenic microbes by lowering the pH of the environment to 3 to 4.5 so that other microbial growth will be hampered (Minervini *et al.*, 2012; Pepe *et al.*, 2013).

The optimum temperature test results are at 45 °C, which is the highest temperature of this test. The highest growth of colonies in the form of the cloudy-looking medium occurred at 45 °C compared to 25 °C and 37 °C. Based on the reference, lactic acid bacteria can grow at a temperature of 20-50 °C (Minervini *et al.*, 2012; Pepe *et al.*, 2013).

Based on the five biochemical tests, the three types of isolates are catalase negative, nonmotile, and include Gram positive (+) bacteria with basil (stem) morphology. In this study, it can be said that the isolates obtained from the mucosa of the human mouth (MB, MA, MD) belong to the lactic acid bacteria group, were from the genus *Lactobacillus* based on the biochemical test results.

Conclusions

The anti candidiasis activity of lactic acid bacteria isolates from the adult oral mucosa (MD) most potentially inhibited the growth of *Candida albicans* that is 16.10 mm, when compared to lactic acid bacteria isolates from the infant (MB) and the child (MA)

Acknowledgment

The authors are grateful to Ministry of Research, Technology, and Higher Education Indonesia which has funded this research through Student Creativity Program-Exact Research

with contract number 0547/B3.1/KM/2017. We also grateful to the lecturer Dr. Sartini, M.Si., Apt., for her valuable help and to Hasanuddin University, which has facilitated us, so that the research could successfully conduct.

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To cite this article:

Lauw, E., Ilham, K.H., Palinggi, F.T., Setiady, D. & Sartini. 2017. Study of Lactic Acid Bacteria Activities from Human Oral Mucosa for *Candida albicans* Inhibition. *International Journal of Applied Biology*. 1(2):58-66.