

Minimum Inhibitory Concentration of Probiotic Soy-Milk Yoghurt (SOYGHURT) Towards Porphyromonas gingivalis [IN VITRO]

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Abstract—The main cause of chronic periodontitis is *Porphyromonas gingivalis* that can be found in subgingival plaque. *Porphyromonas gingivalis* can be inhibited by probiotic product such as yoghurt. Yoghurt is a beverage from milk that proceed by fermentation with adding lactic acid bacteria. Yoghurt is usually made of from cow's milk, but in this study are been made by soymilk. The aim of this study is to know the inhibition effect of yoghurt probiotic to *Porphyromonas gingivalis* growth. Soyghurt is made with adding lactic acid bacteria with 2%, 3%, 4%, and 5% concentration. The inhibition test of this probiotic is done with an agar-well diffusion assay. The result was analyzed with one way ANOVA and LSD test. The result of this study shows that there's difference of inhibition zone of *Porphyromonas gingivalis* among 2%, 3%, 4%, and 5% ($p < 0,05$). The result of this study shows that the widest inhibition zone are in 4% concentrate (14,63 mm) and 5% concentrate one (17,53 mm) which are in weak group based on Ahn classification. The conclusion of this study is the is soyghurt has an inhibition effect of the growth of *Porphyromonas gingivalis* in 4% and 5% concentrate.

Keywords: *probiotic, soyghurt, porphyromonas gingivalis, chronic periodontitis*

I. INTRODUCTION

Probiotics are not only good for gastrointestinal, but also for oral health [1]. Currently probiotics have been developed in various products such as cheese, tablets, gels, pastes, liquids or powders contained in various

fermented foods or beverages such as beverages yogurt [2,3]. Yoghurt is processed by adding an organism culture such as lactic acid bacteria (BAL). The most commonly used lactic acid bacteria are *Lactobacillus bulgaricus* and *Streptococcus thermophilus* [4]. *Porphyromonas gingivalis* is the most common bacteria in chronic periodontitis which present in subgingival plaques and are anaerobic Gram-negative bacteria [5,6,7].

Yogurt is generally made from cow's milk. Cow's milk can cause allergic reactions in some individuals because it contains lactose, so it takes other milk alternatives that do not cause allergic reactions such as soy milk [8].

Based on the background, the authors are interested to conduct research on the inhibitory power probiotic yogurt soy milk (soyghurt) to *Porphyromonas gingivalis* in vitro.

II. MATERIALS AND METHODS

This is a laboratory experimental research with post test only control group design. The research was conducted at the Laboratory of Science and Technology of Milk Processing Department of Animal Husbandry Faculty of Agriculture and Microbiology Laboratory of Veterinary Faculty of Syiah Kuala University of Banda Aceh.

Samples in this study were isolates of *Porphyromonas gingivalis* ATCC (American Type

Culture Cell) 33277 and probiotic drink of soymilk yogurt (soyghurt). Soy milk is divided into 4 parts and then added yogurt starter with *Streptococcus thermophilus* and *Lactobacillus bulgaricus* of 2%, 3%, 4%, and 5% [9].

The total bacterial population (in CFU-colony forming unit) was calculated on MRSmedia [10].

Total Lactic Acid Bacteria = number of colonies counted x 1/dilution factor.

Porphyromonas gingivalis are cultured and confirmation was done by Gram staining. Colonies of *Porphyromonas gingivalis* were inserted into a reaction tube containing 0.9% NaCl of 5 ml and homogenized using a vortex [10,11]. The *Porphyromonas gingivalis* suspension was then diluted from 10^{-1} to 10^{-6} and then colonies of *Porphyromonas gingivalis* were calculated [10]. Inhibitory measurements were performed by well diffusion method. The results of the study were interpreted by classification by Ahn [11].

TABLE I. CLASSIFICATION OF INHIBITORY ZONE DIAMETER OF PROBIOTIC SOY-MILK YOGHURT (SOYGHURT) ACCORDING TO AHN

Concentration	Inhibition Zone (mm)	(p)
Klorheksidin 0,2%	19,50	0,000*
Aquades	0,00	
2%	5,57	
3%	9,60	
4%	14,63	
5%	17,53	

The results of this research data were analyzed using One-Way Anova.

III. RESULT

The result of total calculation of BAL at 2% concentration was $2,42 \times 10^7$ CFU/ml, 3% concentration $3,7 \times 10^7$ CFU/ml, concentration 4% $6,3 \times 10^7$ CFU/ml, and at concentration 5% as much as 8.2×10^7 CFU/ml. The results of the calculation of BAL colonies at concentrations of 2%, 3%, 4%, and 5% in probiotics soyghurt showed that the number of BAL colonies exceeded the minimal amount of BAL in yoghurt is 10^7 CFU / ml.

Porphyromonas gingivalis that will be cultured first taken on the Blood Nutrient media, subsequently, *Porphyromonas gingivalis* cultured using nutrient agar media and incubated for 72 hours. Results of culture on nutrient media shows the colonies of *Porphyromonas gingivalis* in the form of small round and yellowish-colored.

Gram staining was performed for *Porphyromonas gingivalis* confirmation test. Gram staining results seen under a light microscope with 10 x 100 magnification appears *Porphyromonas gingivalis* pink/ reddish and short rod-shaped with rounded edges

TABLE II. INHIBITORY ZONE DIAMETER OF PROBIOTIC SOY-MILK YOGHURT (SOYGHURT) ACCORDING TO AHN

Bright Zone Diameter	Growth Inhibitory Respond
>20 mm	Strong
16-19 mm	Immediate
10-15 mm	Weak
<10 mm	None

The results of probiotics inhibitory of soy-yoghurt (soyghurt) of various concentrations with the method of wells on Mueller Hilton Agar (MHA) media which has been poured 1 ml suspension of *Porphyromonas gingivalis*.

The results of the inhibitory on growth test of *Porphyromonas gingivalis* using probiotic drink soyghurt showed that at concentration 2% (5,57 mm), concentration 3% (9,60 mm) and on negative control (KN) using aquadest showed no presence of inhibitory zone response. In probiotic drink of soymilk yogurt (soyghurt) with concentration 4% (14.63 mm) there is zone of inhibition to *Porphyromonas gingivalis* with weak response. At concentrations of 5% (17.53 mm) of probiotic soyghurt and positive control group (KP) using 0.2% chlorhexidine showed a zone of inhibition against *Porphyromonas gingivalis* with immediate response.

ANOVA Test Result Effect of Probiotics Soy-milk Yoghurt (Soyghurt) Against *Porphyromonas gingivalis*. In this research, data was analyzed using one way ANOVA. In the normality test shows that the data diameter

TABLE III. MEASUREMENT OF DIAMETER ZONE PROBIOTIC YOGHURT SOY-MILK (SOYGHURT) AGAINST PORPHYROMONAS GINGIVALIS

Treatment	Average	Inhibitory Zone Diameter According to Ahn
Positive control	19.50 mm	Immediate
Negative control	0.00 mm	None
(2%)	5.57 mm	None
(3%)	9.60 mm	None
(4%)	14.63 mm	Weak
(5%)	17.53 mm	Immediate

* There was a significant difference after testing with ANOVA (p <0.05)

In this research, data was analyzed using one way ANOVA. In the normality test showed that the inhibitory zone diameter data probiotic yoghurt of soy milk (soyghurt) normal distribution with $p > 0.05$. Furthermore, the homogeneity tests to see the similarity of data. The result of homogeneity test of data shows the value of $p = 0.285$ which means $p > 0.05$ so that it can continue the use of Anova test. ANOVA test performed shows the value $p = 0.00$ which means $p < 0.05$ so that H_0 is rejected and H_a accepted. Furthermore, the Least Significance Difference (LSD) test was performed to see each treatment group, negative control group, and positive control.

TABLE IV. RESULT OF LSD TEST OF MINIMUM INHIBITORY CONCENTRATION (MIC) PROBIOTIC SOY-MILK YOGHURT (SOYGHURT) TOWARDS PORPHYROMONAS GINGIVALIS

LSD test	Control Positive	Control Negative	2%	3%	4%	5%
Control Positive	-	.000*	.000*	.000*	.000*	.000*
Control Negative	.000*	-	.000*	.000*	.000*	.000*
2%	.000*	.000*	-	.000*	.000*	.000*
3%	.000*	.000*	.000*	-	.000*	.000*
4%	.000*	.000*	.000*	.000*	-	.000*
5%	.000*	.000*	.000*	.000*	.000*	-

*p<0,05 there was significant difference

Based on the table, it can be seen that LSD further test in probiotic drinking group of soy-milk yoghurt with concentration 2%, 3%, 4%, and 5% have significant difference with all control group negative (aquades) and positive control group (chlorhexidine 0.2%) with a test value of $p < 0.05$. Based on statistical test result in this research, it can be concluded that soyghurt with concentration of 2% and 3% have no antibacterial potency to growth of *Porphyromonas gingivalis* while concentration 4% and 5% have antibacterial potency growth of *Porphyromonas gingivalis*.

IV. DISCUSSION

This study used soyghurt probiotics as main in growth addition of yoghurt starter *Lactobacillus bulgaricus* and *Streptococcus thermophilus* with concentration of 2%, 3%, 4% and 5% then total colonies of lactic acid bacteria (BAL) were calculated. Total BAL in probiotic soyghurt with 2%, 3%, 4%, and 5% yoghurt starter concentration increased total colony at each concentration. Increased BAL colonies due to different amounts of BAL colonies in each yoghurt starter, so the more starter used, the more colonies of BAL. This is in accordance with Nizori (2008) which states that the more yoghurt starter added, the more total BAL colonies in yogurt [12].

The bacteria tested in this study were *Porphyromonas gingivalis* which was first taken on agar medium agar and cultured on nutrient agar medium by zigzag stroke. Nutrient media show colonies of *Porphyromonas gingivalis* yellowish white color, smooth, convex, and 1-2 mm in size. This is in accordance with research Kusumawardani (2010) which states that the bacteria *Porphyromonas gingivalis* that grows to form a convoluted colony, smooth shiny, and 1-2 mm in diameter [13].

In this study, bacterial confirmation test using Gram staining was done to find out that the cultured bacteria were *Porphyromonas gingivalis* and not contaminated with other bacteria. The results of the confirmation test after Gram staining and seen under light microscope shows that *Porphyromonas gingivalis* look like short stems with rounded tips (coco-bacilli) and are pink.

This is according to research Kusumawardani (2010) which stated that the bacteria *Porphyromonas gingivalis* is a coco-bacilli [13]. Pink color on *Porphyromonas gingivalis* fathomed that *Porphyromonas gingivalis* binding the last given color that is color of safranin. The safranin color binding occurs because the fat that present in the outer membrane of the *Porphyromonas gingivalis* dissolves when it is given alcohol and releases the color of the violet crystals previously given. Violet crystals released from the surface of *Porphyromonas gingivalis* cause red-colored safranins attached to the surface of Gram-negative bacteria [14].

In this study the results of the test concentration of soy yoghurt probiotics (soyghurt) showed the inhibition of *Porphyromonas gingivalis*. Inhibition of probiotic soy yoghurt (soyghurt) against *Porphyromonas gingivalis* in this study to be seen at the formation of a clear zone around the wells that contain soy milk yogurt (soyghurt) with a concentration of 2%, 3%, 4% and 5%. The formation of a clear zone around the wells allegedly due to antibacterial compounds contained in soy milk yogurt probiotic drinks (soyghurt) such as lactic acid, bacteriocins, diacetyl, and hydrogen peroxide that can inhibit the growth of *Porphyromonas gingivalis*. In this study, the probiotic soy yoghurt milk (soyghurt) can inhibit the growth of *Porphyromonas gingivalis* is also suspected to be caused by a sour taste in the probiotic yoghurt drink soy milk (soyghurt) that can affect the growth of *Porphyromonas gingivalis*. Ray's research (2008) showed that soyghurt probiotic drink has a pH of 4.0 that has a bactericidal effect [15]. The decrease in environmental pH in the oral cavity is caused by bacteriocin produced by BAL which can inhibit the growth of pathogenic bacteria [16,17].

Inhibitory effect of soyghurt probiotic drink on growth of *Porphyromonas gingivalis* obtain at concentration 4% (14.63 mm) classified as weak category according to Ahn classification. Probiotic yogurt of soymilk (soyghurt) concentration of 5% (17.53 mm) has an inhibitory effect on growth of *Porphyromonas gingivalis* and belonging to immediate category according to Ahn classification. The formation of inhibitory zone resistance probiotics drinks soyghurt at concentrations of 4% and 5% due to the presence of adequate antibacterial compounds to inhibit the growth of *Porphyromonas gingivalis* colonies. Antibacterial compounds in probiotics drink soyghurt are produced by *Lactobacillus bulgaricus* and *Streptococcus thermophilus* which can inhibit the growth of *Porphyromonas gingivalis*.

Lactobacillus bulgaricus and *Streptococcus thermophilus* are able to inhibit the attachment of pathogenic bacteria, colonization, biofilm formation and development. It can affect the host response that includes collagenase inhibition, reduction of inflammatory molecules, inhibition of cytoprotective production of proteins on the cell surface, the cytokines that trigger apoptosis prevention and response modulation host [4]. This is consistent with research by Aslam (2011) which states that *Streptococcus*

thermophilus has the ability to inhibit the activity of Gram-positive and Gram-negative [17]. The results Singh et al (2011) and Koll et al (2005) also support the results of this study which states that the yogurt contains *Lactobacillus bulgaricus* can inhibit the growth of *Porphyromonas gingivalis* [4,19].

In this study, probiotics of soyghurt concentration of 2% (5.57 mm) and 3% (9.60 mm) did not have an inhibitory response to *Porphyromonas gingivalis* according to Ahn classification in the absence of an inhibitory response to suspected *Porphyromonas gingivalis* because the antibacterial compounds produced by *Lactobacillus bulgaricus* and *Streptococcus thermophilus* are not able to fight the virulence factor of *Porphyromonas gingivalis*.

Porphyromonas gingivalis has virulence factors such as protease enzymes. The protease enzyme may damage the bacteriocins produced by the antibacterial *Lactobacillus bulgaricus* and *Streptococcus thermophilus* [20]. The absence of an inhibitory response to *Porphyromonas gingivalis* is thought to be caused by the lower probiotic pH of soy-yogurt at a concentration of 2% and 3% concentrations of 4% and 5%. Low pH yogurt cannot provide a bactericidal effect. This is in accordance with Nizori (2008) study which states that the lower the starter yoghurt is given the lower the sour taste in yoghurt [12]. The sour taste in yoghurt can affect the pH of yogurt that has a bactericidal effect [16].

A 0.2% chlorhexidine used as a positive control in this study which had an inhibitory effect on *Porphyromonas gingivalis* growth with moderate category according to Ahn classification. A 0.2% chlorhexidine has broad-spectrum antibacterial activity against Gram-positive and Gram-negative bacteria. Antibacterial activity in chlorhexidine 0.2% can damage bacterial cell walls by forming pores on the cell membrane. The results of the Najafi (2012) study suggest that 0.2% chlorhexidine may inhibit bacteria in plaque [21]. The distilled water used as a negative control in this study and did not indicate a bacterial inhibition zone response because the distilled water had no antibacterial compounds capable of inhibiting *Porphyromonas gingivalis* growth.

Based on the result of this study, it can be concluded that soy yoghurt probiotic drink (soyghurt) has inhibitory effect to *Porphyromonas gingivalis* at concentrations of yoghurt 4% and 5%. Suggestion on this research, inhibition probiotic test of soyghurt on *Porphyromonas gingivalis* using in vivo method and we can see Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) probiotics soyghurt on *Porphyromonas gingivalis*.

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