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Potential of Lactobacillus casei shirota's strain against the biofilm-forming of Salmonella Spp: an in vitro study

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A B S T R A C T

Biofilm of *Salmonella* spp. is formed through the expression of biofilm genes associated with proteins (bapA) regulated by curli synthesis genes (csg) which carry out adhesion, colonization, maturation, and dispersion on the surface of the intestinal epithelium. This study aimed to determine the antibiofilm activity of *Lactobacillus casei* Shirota'S strain (LcS) as an inhibitor of *Salmonella spp.* biofilm formation in vitro. This research was a true experimental study using Microtiter Plate 96 wells Biofilm Assay method. The sample used was the suspension of *Salmonella* spp. The treatment was in the form of adding a LcS suspension with a concentration series of 10^{-1};10^{-2}; 10^{-3};10^{-4}; and 10^{-5}. Biofilm measurements were carried out using a microplate reader and obtained quantitative data in the form of Optical Density at a wavelength of 595nm. The results of this study showed that LcS suspension has antibiofilm activity ranging from 10^{-5} concentrations with a percentage of 36.58% ($p<0.05$). The results of exometabolism LcS can reduce *Salmonella* growth. Exopolysaccharide (EPS) and sortase-dependent proteins (SrtA) of LcS form barriers as competitive adhesion in inhibiting pathogenic biofilm formation.

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1. INTRODUCTION

Food contamination by pathogenic bacteria is an important public health problem that causes world mortality. Salmonella is transmitted through contaminated animals to humans. The animal is then processed into food (food-borne disease), causing Salmonellosis.[1] Salmonellosis is divided into two types namely typhoid which consists of typhoid fever and paratyphoid fever, the second notified is usually caused by Salmonella serovars that do not have specific hosts. The Salmonella enterica serotype group Enteritidis and Salmonella enterica serotype Typhimurium are the two Salmonella serotypes that are most commonly transmitted from animals to humans in most parts of the world.[1]

Every year one in 10 people get sick, and 33 million people lose their health in the world due to foodborne disease. Salmonella is one of the four main causes of global diarrhoea disease caused by contaminated food.[1] Centers for Disease Control and Prevention (2019) estimates Salmonella is a bacterium that causes about 1.2 million diseases, 23 thousand hospitalizations, and 450 deaths in the United States each year. Indonesia is one of the developing countries that has health problems such as typhoid fever.[2] Based on the results of "Riset Kesehatan Dasar" (Riskesdas) in 2007, the prevalence of typhoid fever reached 1.7% with typhoid morbidity reported at 81.7 per 100,000 population in Indonesia.[3]

Biofilm formation is a natural phenomenon for most bacteria through cell-to-cell communication called Quorum Sensing (QS). The process forms biofilms on the biotic surface, namely intestinal epithelium or abiotic, which are cutlery.[4] Salmonella biofilms that contaminate food can enter the body and can reach the intestinal epithelium if the amount exceeds the average infective dose can cause clinical or subclinical infections in humans is 10^5-10^8 Salmonella, but for Typhi, serotypes can reach 10^3. Biofilm Associated Proteins (Biofilm Associated Proteins (bapA) can increase the attachment of Salmonella to the intestinal epithelium and issue QS signals for colonization and form biofilms as a form of Salmonella's defence against adverse conditions such as pH, unstable temperatures and reduced nutrition.[4]

Biofilm control can be done in three ways, namely in physics, chemistry, and biology. Biofilm control in biology can use microbiological interactions, one of them by using bacteria that act as antibiofilm.[5] Biofilm control with Lactic Acid Bacteria (LAB) biosurfactants or probiotic bacteria can reduce the expression level of biofilm-related genes and block the release of signalling molecules in the QS system as a therapeutic agent to prevent infecting biofilms.[6]

The results of other studies previously stated that Conjugated Linoleic Acids (CLAs) results from critical metabolism as an anti-pathogenic substance from Lactobacillus secreted at 24, 48, and 72 hours of incubation significantly ($p < 0.05$) capable of suppressing the formation of Salmonella typhimurium biofilms by damaging cells membrane of pathogenic bacteria.[7] *Lactobacillus* is also able to form a biofilm protector as a barrier to prevent the attachment of pathogenic bacteria and as a competitor in the attachment of intestinal epithelium.[8]

Probiotic bacteria are bacteria that are beneficial to the body. Probiotics can produce antimicrobial substances that function as agents that can suppress the growth of enteric pathogenic bacteria. The genus Lactobacillus has the potential as a probiotic agent, which maintains the health of intestinal epithelium including being able to produce antimicrobial substances, antagonistic power against enteric pathogens that can survive at low pH, and resistant to bile salts.[9]

Lactobacillus casei Shirota strain (LcS) is a type of probiotic bacteria that have been scientifically proven to have health benefits. LcS can produce antimicrobial substances called bacteriocin consisting of acidoline, acidophylline, and lactosidin which have the ability as a broad spectrum against positive Gram and negative Gram bacteria.[10]

There are many mechanisms performed by LcS probiotics in the intestine, namely immune modulation, lactic acid production (resulting in a decrease in local pH) and competitive adhesion or transfer of pathogenic bacteria.[11] LcS is used as a probiotic bacterium in fermented milk that is circulating in the community and is widely consumed by various groups, and LcS in these research has not been tested biologically, so that researchers are interested in examining the potential of *Lactobacillus casei* Shirota strain as an inhibitor of biofilm formation *Salmonella* spp.

2. MATERIALS AND METHOD

This type of research is a pure experimental study (True Experimental) with posttest only control group design and uses a 96 wells microplate assay biofilm method to determine the activity of Lactobacillus casei strains of Shirota strain.

The tools used are Microplate reader, Incubator, Autoclave, anaerobic jar, glass object, Wells microplate 96 flat bottoms, Eppendorf micropipette and 200 µl tip size, Yellow Tip box. The materials used were isolates of Lactobacillus casei strain Shirota (Department of Microbiology FKUI), Salmonella spp. Isolate, Trypthon Soya Broth (TSB), Nutrient Agar (NA), Methanol 96%, Crystal violet 1%, Aquadest, NaCl 0.9% sterile, 0.5 McFarland, Acetic Acid 33%.

The sample of Lactobacillus casei Bacteria Shirota strain test was a bacterial suspension in fermented milk products in the Pondok Labu area, South Jakarta. Bacteria derived from bacterial suspensions were etched on NA media, then incubated for 24 hours at 37ºC in anaerobic atmosphere in anaerobic jar and stored in the refrigerator.

Salmonella bacterial test samples came from a pure culture of Salmonella Shigella Agar (SSA) from the microbiology laboratory, Faculty of Medicine, UPN "Veteran" Jakarta. Salmonella growth on SSA media is characterized by a black colony.

Lactobacillus suspension is taken 2-3 bacterial colonies from bacterial stock then input into the 40 mL TSB media, then incubated for 24 hours at 37°C, standardized turbidity using standardization <300x10^6 CFU / mL McFarland using the turbidimetry method, then take 1 mL, entered with standardization of <300x10^6 CFU / mL McFarland using the turbidimetry method into the tube and added 9 mL NaCl to the tube, then shake until homogeneous, the first tube diluting 10^{-1}. The 1 mL pipette from 10^{-1} is then put into a test tube containing 9 mL of diluent solution and is called the 10^{-2} second dilution. Do the same thing until the last dilution is 10^{-5}.

Salmonella suspension is taken 2-3 bacterial colonies from the bacterial stock and then input into a 40 mL TSB medium, then incubated for 24 hours at 37° C. Then take 100 µl, each inserted into a tube containing 10 mL TSB, then incubated for 3-5 hours at 37° C. Dilute with 0.9% NaCl until the bacterial density is equal to 0.5 McFarland standard (<300x10^6 CFU / mL).

Minimum Inhibitory Concentration (MIC) is done by giving 1 mL of Salmonella spp suspension into 9 mL of LcS suspension with each tube containing a different concentration of LcS. The concentration was carried out by the dilution method five times the dilution series, namely 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, and 10^{-5}, then incubated for 24 hours at 37ºC, then the suspension mixture was planted and spread on selective media Salmonella Shigella Agar (SSA) and incubated 24 hours again, after incubation, the number of colonies was measured with a plate counter.

Evaluation of Salmonella biofilm formation. This evaluation is to see and prove the biofilm formation of Salmonella formed in microplates wells, put in 75 µL TSB and 25 µL bacterial suspension (<300x10^6 CFU / mL) and then incubated for 24 hours at 37° C. After incubation, the microplates were then rinsed using sterile water, and the wells on the microplate were stained with 1% violet crystal for 15 minutes, then rinse.[12]

Evaluation of Lactobacillus casei Biofilm Formation Shirota's Strain (LcS). Evaluation to see the formation of biofilms from LcS put 100 µL TSB and 100 µL bacterial suspension (<300x10^6 CFU / mL) into the microplate wells and then incubated for 24 hours at 37° C. After incubation, the microplate was then rinsed three times using NaCl and dried, gave 200 µL 96% methanol and incubated at room temperature for 10 minutes, then discarded the liquid methanol from the well and dried, and the smear on the microplate was stained with 0.1% crystal violet during 10 minutes, then rinsed three times with NaCl, dissolve biofilm with 33% acetic acid, then place it on the microplate reader to find out the optical density results at a wavelength of 595 nm.[12]

A total of 100 µL of liquid TSB media was added to the microplate wells, then 50 µL of Lactobacillus bacterial suspension was added first and continued with 50 µL of Salmonella, covered and incubated for 24 hours at 37° C. After incubation, the mixture is removed, then the microplate is washed with NaCl and dried. Microplate give 200 µL of 99% methanol and incubate for 10 minutes at room temperature, then remove the methanol liquid from the well and dry, and the microplate stained with 0.1% crystal violet for 10 minutes, then rinse three times with NaCl, dissolve it with NaCl, dissolve it biofilm with 33% acetic acid. The negative control was only given 100 µL TSB liquid media and 100 µL Salmonella bacterial suspensions in microplate wells. Positive controls were given 100 mL of Salmonella bacterial suspension and 100 mL of ceftriaxone. Solvent control was given 200 µL NaCl, and media control was given 200 µL TSB liquid media, then place it on the microplate reader to find out the optical density results at a wavelength of 595 nm.[12]

The test is carried out with three comparisons, and in the test group, the percentage of biofilm inhibition is calculated from the OD value which is processed using the following formula (*Clinical and Laboratory Standards Institute*, 2012):

$$\text{percentage of biofilm inhibition} = \frac{(C\text{-}B)\text{-}(T\text{-}B)}{(C\text{-}B)} X\ 100\% \quad (1)$$[13]

Note:
C = *Optical Density* (OD) 595 nm control media,
B = OD_{595} *negative controls*,
T = OD_{595} *treated wells*.

The results are then analyzed with the Anova oneway hypothesis test.

3. RESULTS AND DISCUSSION

Identification of *Lactobacillus casei* Shirota strain bacteria by the Gram staining method was seen in a microscope with the results of identification of the form of bacilli, single arrangement, purple colour, and Gram-positive properties. Identification of Salmonella bacteria was seen in a microscope with the results of the identification of the form of bacilli, single arrangement, red colour, and negative Gram.

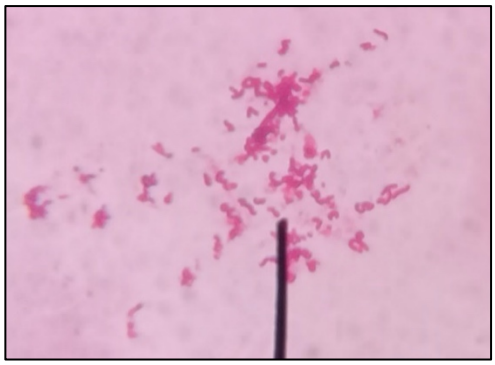

Figure 1. Lactobacillus Microscopic Identification Results (Gram Staining)

Table 1 shows that group 10^{-1} had the most significant growth inhibition than the other groups. In contrast, group 10^{-5} showed the lowest number in inhibiting the growth of Salmonella spp. in each group with different concentrations. The higher the concentration of LcS suspension, the less the amount of Salmonella colonies that grow in SSA.

Table 1. Results of Measurement of Total Growth of Salmonella Spp. on the MIC

Group	Number of Salmonella sp. colonies Within 0.1 millilitres of bacterial suspension
10^{-1}	43.6
10^{-2}	46
10^{-3}	80.4
10^{-4}	125
10^{-5}	161

Salmonella spp is a bacterium that has a flagella that functions to move and attach to the surface of an object, when the process of biofilm formation flagella will tend to stick to the surface, thus forming a biofilm ring that will be seen on the surface of the object.[4] Figure 1 shows a purplish-blue biofilm ring on the surface of the microplate wells. The picture shows the top view seen throughout the microplate wells are blue, to see the shape of the ring can only be seen in the side view picture. Evaluation to see the formation of biofilms from Salmonella in each microplate wells, if the microplate remains purplish-blue, then a biofilm has been formed in the microplate wells.[12]

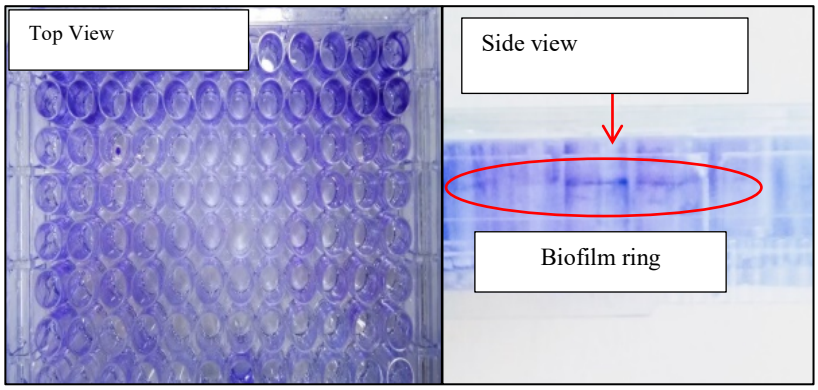

Figure 2. Evaluation of biofilm formation

Evaluation of the formation of LcS biofilms was carried out to monitor the formation of biofilms and LcS growth without the suspension of Salmonella spp. through optical density values. The results obtained were OD values in the form of planktonic and biofilm in the dilution method.

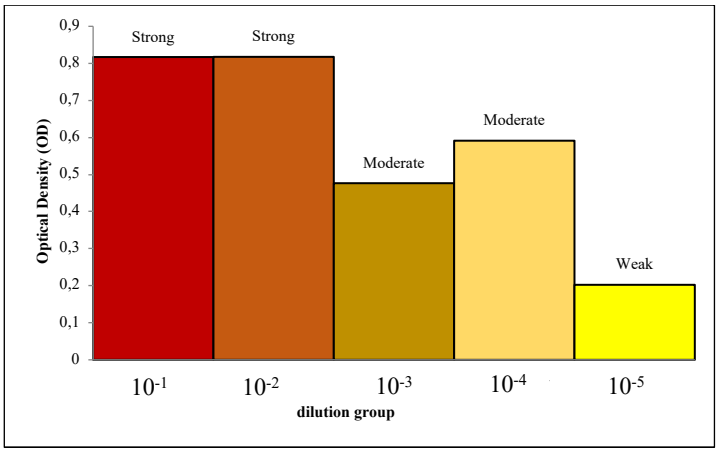

Figure 3. Results of Optical Density for LcS Biofilm Formation

The results of the formation of LcS biofilms can be classified according to the magnitude of OD values compared to OD values of media control (ODC), i.e. wells that only contain liquid media. According to Borges et al. (2012), the classification of biofilms according to OD values is as follows, if (OD≤ODC) then categorized as non-biofilm producers, if (ODC <OD≤2 × ODC) then classified as weak biofilm producer, if (2 × ODC <OD ×4 × ODC) then categorized as moderate biofilm producer, and if (4 × ODC <OD) then classified as keen biofilm producers.[14] Figure 2 shows the LcS OD values at 10-1 and 10-2 dilutions forming a strong biofilm, the OD LcS values at 10-3 and 10-4 dilutions form a moderate biofilm, and the LcS OD values at 10-5 dilutions form a weak biofilm. This shows that the higher the concentration of LcS bacterial suspension, the greater the OD value produced.

Lactobacillus antibiofilm activity can be seen from the value of optical density with different bacterial conditions namely planktonic and biofilm contained in the microplate wells of each test group and control group.

Table 2. Results of Optical Density (OD) of All Groups on Microplate 96 Wells with Two Repetitions

Group	Optical Density Results for All Groups			
	Planktonic		Biofilm	
	1	2	1	2
Positive Control	0.19433	0.18544	0.20809	0.24558
Negative Control	1.47607	1.47002	0.50370	0.51246
Test group				
10-1	1.22957	1.25635	0.48704	0.46604
10-2	1.26903	1.30692	0.44372	0.43266
10-3	1.29641	1.31218	0.38462	0.39403
10-4	1.31553	1.34341	0.33899	0.33365
10-5	1.33861	1.35872	0.28161	0.29158
Media Group	0.07075	0.07078	0.15655	0.16113
Solvent Group	0.04016	0.04012	0.10534	0.16334

Table 2 shows that positive control containing Salmonella bacterial suspension with Ceftriaxone antibiotics showed lower planktonic and biofilm OD values compared to the test group, indicating that Salmonella was still sensitive to the antibacterial effect of Ceftriaxone. The negative group only contained Salmonella bacteria with the media showing the highest planktonic and biofilm OD values of the other groups, and this indicates that Salmonella can form biofilms totally without the intervention of the antibacterial effects of ceftriaxone or LcS suspension. Media groups and solvent groups showed the lowest OD values of all groups because they did not contain bacteria in the wells and showed no biofilm formation.

The planktonic test group at the highest concentration ie group 10^{-1} showed the lowest OD value compared to other planktonic test groups. The lowest concentration in group 10^{-5} shows the highest OD value compared to other planktonic test groups, and the conclusion is that the growth of Salmonella in planktonic form decreases with increasing concentration of LcS suspension illustrated as shown in Figure 4.

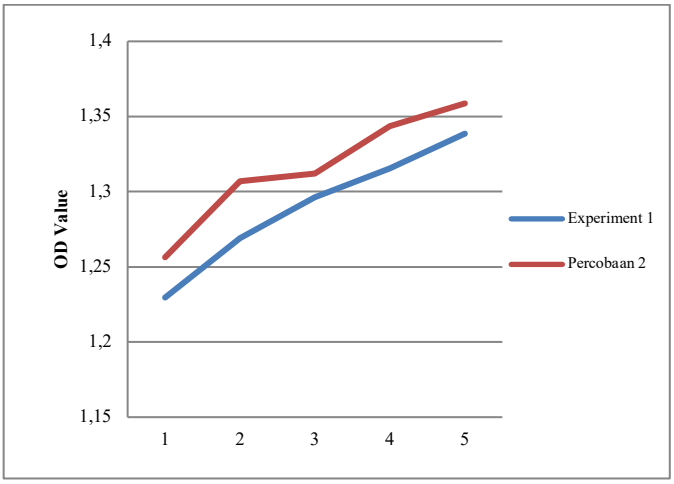

Figure 4. Results of Optical Density Inhibition of Formation of Salmonella Biofilm spp.

The results of OD values for Salmonella biofilm inhibition are expressed as percentages, to find out how much the percentage of LcS inhibition. The percentage of LcS inhibition against Salmonella was obtained based on the calculation of the formula between two bacterial suspensions with different concentrations because this research method used two concentrations with two types of bacteria that have different characteristics and characteristics.

Table 3. Percentage Results of Lactobacillus casei Inhibition of Shirota Strain Against Formation of Salmonella spp.

Group	Percentage of LcS Inhibition Against Salmonella Biofilm	
	Test 1	Test 2
10^{-1}	95.20%	86.79%
10^{-2}	82.72%	77.29%
10^{-3}	65.70%	66.29%
10^{-4}	52.55%	49.10%
10^{-5}	36.02%	37.13%

Table 3 shows the group with 10^{-1} concentration had the highest percentage of inhibition compared to other groups. The group with a concentration of 10^{-5} had the lowest percentage of inhibition compared to other groups. These results can be concluded that the higher the concentration, the higher the percentage of inhibition of LcS bacteria against Salmonella.

The value of optical density biofilm has a variety of results at each concentration. Concentration 10^{-1} produces an average value of optical density 0.47654 with an average value of the percentage of inhibition of 91.00%, at a concentration of 10^{-2} produces an average value of optical density of 0.43819 with an average value of the percentage of inhibition of 80.01%, at concentration of 10^{-3} produces an average value of optical density 0.389325 with an average value of the percentage of inhibition of 66.00%, at a concentration of 10^{-4} produces an average value of optical density of 0.33632 with an average value of the percentage of inhibition of 50.83%, at concentration of 10^{-5} produces an average value of optical density 0.286595 with an average value of the percentage of inhibition of 36.58%.

Lactobacillus suspension in concentrations of 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, and 10^{-5} can inhibit the formation of Salmonella spp biofilms. With the average percentage of the minimum inhibitory power found at a concentration of 10^{-5} that is equal to 36.58% and the average percentage of the maximum inhibitory power is found at a concentration of 10^{-1} that is equal to 91.00%. Increasing the concentration of Lactobacillus suspension can increase the activity of spending antibiofilm such as biosurfactants and exopolysaccharides so that the percentage of inhibition against pathogenic bacteria increases. The results of the study were also supported by the results of the Minimum Concentration Concentration (MIC) test. The results of this test indicate that there is a decrease in the number of colonies in the SSA agar media with higher LcS concentrations.

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Salmonella biofilm spp. able to form with antigen virulence activity Biofilm associated proteins (bapA) are large cell-surface proteins needed for biofilm formation. Biofilms are encoded by the bapA gene and secreted through the type-I protein secretion system (bapBCD operon) which is located at

the end of the bapA gene. The expression of BAPA is coordinated with the genes encoding curli fimbriae and cellulose, through the action of curli synthesis genes (csgD). Both Long polar fimbriae (Lpf) and plasmid-encoded fimbriae (Pef) contribute to the initial steps of biofilm formation. The bapA gene is also a gene that lives in Salmonella. Salmonella produces O-antigen capsules that are regulated with fimbria and extracellular matrix cellulose.[4]

Biofilm biological control with these bacteria is different from other biofilm control. In this study, the value of optical density is influenced by two types of bacteria with different characters. The mechanism of LcS in inhibiting Salmonella as explained in the previous literature review, namely the exopolysaccharide and various other proteins released by LcS, so that it adheres to the surface of the wall as a protective barrier the same as when it is in the intestinal epithelium. This can affect the value of optical density biofilm in each well, and the optical density value is greater when LcS concentration is greater, inversely proportional to the shape of bacteria in a planktonic state that the optical planktonic value is getting smaller when LcS concentration is greater, this is because LcS produces exometabolite which acts as an antibacterial so that the growth of pathogenic bacteria is inhibited.[15]

Previous research stated that biofilms from *E. coli* O157: H7, *Listeria monocytogenes*, and *S. typhimurium* in Lactic Acid Bacteria (LAB) suspensions for 24, 48, and 72 hours were significantly reduced ($p < 0.05$). The use of probiotic biofilms can be an alternative approach to reduce the formation of pathogenic biofilms in the food industry, without giving risks to consumers. The application of competitive biofilms formed by LAB bacteria can produce natural antimicrobial substances and biosurfactants can provide new opportunities for controlling pathogenic bacteria and avoiding infectious diseases from food contamination. Development of protective biofilms with probiotic LAB present in food can help avoid the problem of contamination into the food chain.[8]

Lactobacllus casei Shirota's strain in fermented milk has two unique abilities in its role as inhibitors of the formation of pathogenic bacterial biofilms that infect humans. First, probiotic bacteria can adhere firmly to the surface of the intestinal epithelium and survive in the extracellular polymer matrix with exopolysaccharide (EPS) and sortase-dependent proteins (SrtA) components maintaining the barrier function as a competitive adhesion in inhibiting the formation of pathogenic biofilms. Secondly, bacteriocin is the result of Lactobacillus metabolism which can act as an antibacterial, besides bacteriocin, there are other Lomet's exometabolime results such as lactic acid, hydrogen peroxide can suppress the growth of Salmonella.[16] Bacterial biofilms can form on biotic or abiotic surfaces. *Lactobacillus casei* Shirota's strain (LcS) can attach to the surface of objects or human intestine in response to the formation of protective barriers against pathogenic bacteria. There are several genetic and environmental factors that influence the formation of these microbial structures in the digestive tract. Lucks gene and pheromone peptide plantaricin A (Plna) owned by Lactobacillus, have an important role in regulating interactions between microbes in the human intestinal system. The role of other Lactobacillus on the intestinal surface is to increase the expenditure of several proteins (Dnak, GroEL, ClpP, GroES and catalase) to stimulate immunomodulation.[17]

This study uses a microplate surface as a biofilm coating site which describes the attachment of biofilms on the surface of the intestinal epithelium. The difference is the inhibitory response of the formation of pathogen biofilms by Lactobacillus with favourable environmental conditions for the stimulation of additional inhibitory agents such as various proteins in the body and immunomodulation which can only occur in the digestive tract and can be done in vivo, whereas microplate provides an environment with laboratory standards by in vitro. The response is only obtained from the ability of Lactobacillus such as the removal of probiotic bacterial surface adhesion proteins and forming EPS as an attachment response, as well as the release of exometabolism as an antibacterial agent. This ability is carried out by Lactobacillus itself without the aid of immune system stimulation and protein from the body.[18]

4. CONCLUSION

Based on the results of the analysis and discussion of the research that has been done, it can be concluded Lactobacillus casei Shirota's strain has the potential to inhibit the formation of Salmonella spp biofilms. In vitro at the suspension concentration of Lactobacillus bacteria 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, and 10^{-5}. The quantitative biofilm detection method using violet crystal staining on

microtiter plates has the result that the higher the concentration of Lactobacillus bacterial suspension, the higher the inhibitory power for the formation of Salmonella spp biofilms.

For the next research can be done with the field emission scanning electron microscopy method to see the structural differences produced by Lactobacillus and Salmonella in biofilms attached to the microplate wells. It measured the growth curve of Lactobacillus casei Shirota strain as an inhibitor of the formation of Salmonella spp. Biofilms, to determine the number of bacteria that can inhabit. Test other types of pathogenic bacteria that produce biofilms or can also test probiotic bacteria or bacteria that have other antibacterial properties. Test the potential of Lactobacillus casei Shirota strain as an inhibitor of the formation of Salmonella spp biofilms. In vivo and carry out other biofilm control tests, such as physics and chemistry.

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CONFLICT OF INTEREST

We declared in this work, not any conflict of interest.

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