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Characterization of *Bacillus subtilis* and *B. licheniformis* potentials as probiotic bacteria in Vanamei shrimp feed (*Litopenaeus vannamei* Boone, 1931)

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Abstract. *Andriani Y, Safitri R, Rochima E, Fakhrudin SD. 2017. Characterization of* Bacillus subtilis *and* B. licheniformis *potentials as probiotic bacteria in Vanamei shrimp feed (*Litopenaeus Vannamei *Boone, 1931). Nusantara Bioscience 9: 188-193.* This study aimed to characterize the *Bacillus subtilis* and *Bacillus licheniformis* potentials as probiotic bacteria in Vanamei shrimp feed (*Litopenaeus vannamei* Boone, 1931). A two-phase experimental descriptive method in a laboratory scale was applied in this study. The first phase was the bacterial growth curve observation of *B. subtilis* and *B. licheniformis*. The second phase was the characterization of the two bacteria in terms of their tolerance to an acidic condition, temperature, and bile salt concentration. The measured parameter was the number of colonies in predetermined acidic conditions, temperatures, and bile salt concentrations. The number of growing bacterial colonies was calculated using the total plate count method. Results show that *B. subtilis* had an optimal growth point at the eighth hours, achieving a total population of 6.138×10^{13} CFU/mL, while the optimal growth point for *B. licheniformis* was reached at the tenth hour with 4.299×10^{13} CFU/mL. *Bacillus subtilis* and *B. licheniformis* were also tolerant at 40, 50, and 60°C. The acid tolerance testing was conducted at pH of 2, 4, and 6. It was revealed that *Bacillus subtilis* were intolerant to acidic condition at pH = 2, in which the number of colonies decreased drastically. On the contrary, *B. licheniformis* was tolerant to pH = 2, which was evident from the absence of a drastic decrease in the number of colonies. Both *B. subtilis* and *B. licheniformis* were tolerant to pH 4 and 6. High tolerance to 0.3% and 0.5% bile salt concentration was observed in both *Bacillus subtilis* and *B. Licheniformis* with a high number of colonies of 10^{10} CFU/mL. Therefore, it is concluded that both *B. subtilis* and *B. licheniformis* can be developed into probiotics.

Keywords: *Bacillus licheniformis*, *Bacillus subtilis*, feed, probiotic, Vanamei shrimp

INTRODUCTION

Vanamei Shrimp (*Litopenaeus vannamei* Boone, 1931) is a type of shrimps commonly cultivated in Indonesia. One of the challenges faced when cultivating Vanamei shrimp is the high mortality rate due to disease attacks, such as *white spot disease* (WSD), which leads to reduced shrimp production and creates a loss for the farmer. One of the approaches that can be applied to cope with this condition is through the use of probiotics (Gatesoupe 1999). Probiotics are commonly applied through feed used in shrimp cultivation. The addition of probiotic bacteria in the feed will be useful to regulate the existing microbe environment in the intestine, prevent the growth of an intestinal pathogenic microorganism, and improve feed efficiency by releasing enzymes that are able to improve feed digestion process (Anwar et al. 2016). A good digestion process will optimize the use of feed consumed, leading to increased feed efficiency and reduced the amount of feed organic waste in the form of feces and other metabolism waste, e.g. urine and ammonia. This will maintain the quality of water.

The types of bacteria that are potentially used as probiotic bacteria include, among others, *Bacillus subtilis* and *B. licheniformis*. *Bacillus* is ubiquitous and widely distributed in most places, including in shrimp digestive tract. These bacteria are very suitable to use because they do not produce toxin, easy to grow, do not need expensive substrates, able to strive in high temperature, and do not produce any metabolic side product. The development of *Bacillus*-based probiotics in Vanamei shrimp cultivation has a good prospect and potential (Linggarjati et al. 2013). The stages that have to be passed before using microbes as probiotics are, among others, viability testing, temperature tolerance testing, acid tolerance testing, bile salt tolerance testing, antimicrobial testing, autoaggregation testing, and co-aggregation testing. This study, however, only focused on testing the microbial tolerance to acid, temperature, and bile salt concentration. These tests are expected to be able to convince us that the microbes can survive and thrive in shrimp digestive tract by the ability to tolerate the temperature, acidity, and bile salt concentration in the tract. The aim was to characterize the potential use of *Bacillus subtilis* and *Bacillus licheniformis* as probiotic bacteria in Vanamei (*Litopenaeus vannamei* Boone, 1931) shrimp feed through observation of the probiotic bacterial growth curve, followed by acid, temperature, and bile salt tolerance testing.

MATERIALS AND METHODS

Materials and instruments

The instruments used in this study were autoclave, Bunsen, stirring rod, Petri dish, funnel, Beaker glass, incubator, Erlenmeyer flask, laminar air flow, refrigerator, micropipette, analytical scale, inoculating loop, oven, heater, pH meter, tube rack, low-temperature centrifuge (refrigerated), spray dryer, stirrer, tube cap, water bath, and vortex. Meanwhile, the materials used were 70% alcohol, aluminum foil, aquadest, 0.3% and 0.5% bile salt, physiological NaCl solution, 1 N NaOH, Nutrient Agar (NA) medium, Nutrient Broth (NB) medium, deMann Rogosa Sharpe Agar (MRSA) medium, Phosphate Buffer Saline (PBS), and plastic wrap.

Methods

This was a descriptive experimental study in laboratory scale. Data analysis was performed descriptively.

Probiotic bacterial growth curve

The plotting of probiotic bacterial growth curve for *B. subtilis* and *B. licheniformis* was performed based on Total Plate Count (TPC) and pour plate methods. First, 10% of bacterial suspension was cultured in 100 mL MRSB (10 g/L bacteriological peptone; 8 g/L meat extract; 4 g/L yeast extract; 20 g/L dextrose; 1 g/L Tween 80; 2 g/L dipotassium phosphate; 5 g/L sodium acetate; 2 g/L ammonium citrate; 0.2 g/L magnesium sulfate; 0.05 g/L manganese sulfate) and NB (1 g/L lab lemco powder; 2 g/L yeast extract; 5 g/L peptone; 5 g/L sodium chloride) media, which were then incubated for 72 hours. Every 2 hours, 1 mL of bacterial suspension was cultured into the MRSA (10 g/L bacteriological peptone; 8 g/L meat extract; 4 g/L yeast extract; 20 g/L dextrose; 1 g/L Tween 80; 2 g/L dipotassium phosphate; 5 g/L sodium acetate; 2 g/L ammonium citrate; 0.2 g/L magnesium sulfate; 0.05 g/L manganese sulfate; 10 g/L bacteriological agar) and NA (5 g/L peptone; 3 g/L meat extract; 12 g/L bacteriological agar) media. Every 2 hours for 24 hours, 1 mL of bacterial suspension was retrieved. The parameter observed was the bacterial colony growth every 2 hours. A growth curve was then plotted, and analysis was performed descriptively (Saropah 2012).

Acid tolerance test

The acid tolerance testing of *B. subtilis* and *B. licheniformis* probiotic bacteria was performed using modified Hardianingsih et al. (2006) method. A starter of *Bacillus* bacteria was made with a turbidity of McFarland 3 or $9x10^8$ CFU/mL bacterial cells. The pH of the NA liquid medium was set to 2, 4, and 6 using 1% acetic acid and 1% NaOH as the buffer solution. Each treatment was inoculated with 10% of bacterial starter and then incubated for 6 hours. After that, 1 mL of bacterial suspension from each pH treatment was cultured in NA medium and the bacteria were counted using the *Total Plate Count* (TPC) method. The parameter measured was the number of colonies grown in each bacterial isolate on NA medium with different pH. If the number of bacterial colonies was more than 10^8, bacteria were considered to have a high tolerance to pH.

Temperature tolerance test

The temperature tolerance test was performed using the following steps. Culture tubes were filled with 9 mL of sterile NaCl solution, added by 1 mL of probiotic bacterial suspension. Samples were then heated in a water bath at 40, 50, and 60°C for 15 minutes. After the heating time was achieved, the tubes were removed immediately and cooled down under running water until a temperature of 30°C was achieved. Pure culture or plating was then performed by inoculating the isolate on NA media with 10^{-1}-10^{-6} serial dilutions and a ratio of sample and diluent of 1:9. One milliliter of the sample was then placed in a Petri dish and NA medium was poured until it was frozen. Incubation was performed at 37°C and colony count was done. Temperature tolerance was assessed by counting the number of colonies that grew from each isolate on solid medium that was incubated at 40, 50, and 60ºC. If the number of bacteria grown was 10^8 CFU/mL, the bacteria were stated as having a high tolerance to temperature (Sukasih et al. 2005).

Bile salt tolerance test

Testing was performed by placing 1 mL of probiotic bacteria into 9 mL of control NB and bile-salt-containing NB. The concentrations of bile salt used were 0.3% and 0.5%. Mixing was then performed using vortex and the samples were incubated at 37°C for 6 hours. After incubation, the bacteria were harvested using 5000g centrifugation at 4°C and rinsed twice using phosphate buffer solution. Pellet was diluted using 10 mL of aquadest, which was followed by a serial dilution, dish plating, and incubation at 37°C for 48 hours. The number of colonies grown was counted, both in control and treatment (Lian et al. 2003).

Antimicrobial activity against pathogen test

The method used for antimicrobial activity test was the agar diffusion well method. The antimicrobial activity testing was performed by taking 1 gram microcapsule to be placed in 9 mL of NB followed by vortexing. Samples were then incubated for 24 hours at 37°C. Free probiotic cell supernatant was gained through centrifugation for 20 minutes followed by sterilization. As much as 20 µl free cell supernatant and pellet from each sample were placed into the wells of NA solid media that had been inoculated with the test bacteria. The media were incubated for 24 hours at 37°C. The clear zone formed showed inhibition of test bacteria growth from the supernatant. The inhibition zone (clear zone, in mm) around the well was measured using a caliper (Sari 2011).

RESULTS AND DISCUSSION

Probiotic bacterial growth curve

One of the requirements that should be met by a bacterium to become the probiotic bacterial candidate is the

ability to thrive in its environment. The ability of bacteria to grow in an inoculum-substrate and exponential phase (log phase) can be observed by counting the number of microbial cells in each time unit. Of all bacterial growth phase, the log phase is a very important phase to know because of this period comprises a very rapid microbial growth (Pelczar et al. 1986).

A growth curve is plotted to understand the life phase of each probiotic microbe. During the incubation process, the NB (Nutrient Broth) medium was used for *B. subtilis* and *B. licheniformis* culture media. The growth curve was observed for 24 hours and the bacterial density was measured every 2 hours. From the results of study, it was revealed that the optimum growth of *B. subtilis* was seen in the 8th hour (point 4) while *B. licheniformis* reached its optimum growth in the 10th hour (point 5) (Figure 1).

From Figure 1, it can be understood that the adaptation phase (lag phase) of *B. subtilis* was very short, which was between the 0th and 4th hours. The same was true for *B. licheniformis*. In short, this adaptation phase was due to the fact that the same media was used for the bacterial regeneration, leading to a short adaptation period to the new environment. The duration of the adaptation phase is very much determined by the number of cells inoculated, physiological condition, and suitable morphology as well as the culture media needed (Fardiaz 1992). The logarithmic phase (exponential phase) of the two probiotics happened from the 4th to the 8th hours. The logarithmic phase represents a constant cell division, which doubles the number of cells with the same rate, constant metabolism activities, and balanced growth condition.

A study from Vijayalakshmi et al. (2013) suggested that the lag phase of *B. subtilis* is very short (4 hours), followed by the exponential phase at the 32nd hour and the stationary phase up to the 48th hour where the bacterial growth will decrease afterward. Meanwhile, *B. licheniformis*'s lag phase was seen at the 8th hour, followed by the exponential phase at the 32nd hour, and the stationary phase that ends at the 48th hour.

The difference in timing until the peak of the growth curve was reached in this study was more influenced by the agitation factor during the bacterial culture. *Bacillus subtilis* grows and produces a lot of enzymes with a low agitation speed (50 rpm) while *B. licheniformis* needs a high agitation speed (150 rpm) to grow and produce enzymes optimally. Genckal and Tari (2006) stated that *Bacillus* sp. that shows a higher agitation speed might reduce the production of an enzyme that may reduce the need for oxygen.

Temperature tolerance test

The temperature tolerance test for probiotics is performed to understand the ability of the probiotics to maintain the number of microbial colonies (CFU/g) during higher temperatures (40°C, 50°C, and 60°C). High tolerance to temperature is one of the requirements that have to be met by probiotic bacteria. This study used the TPC method and the results (Figure 2) showed that the density of the two bacterial type at 40°C, 50°C, and 60°C was not reduced significantly when compared to the initial density of $1.5x10^8$ CFU/mL (Table 1).

Results in Table 1 show that *B. subtilis* and *B. licheniformis* were able to thrive in a temperature of up to 60°C. This is because the *B. subtilis* and *B. licheniformis* probiotics are Gram-positive bacteria. Gram positive bacteria have a very thick cell wall where 90% of the cell wall is a peptidoglycan layer. This leads to better resistance against physical disturbance (heat) when compared to Gram-negative bacteria. According to Wibisono et al. (2016), each microbe has a different resistance to temperature. Furthermore, some microbes will die when they are heated to a certain constant temperature (T), leading to a logarithmic reduction in the number of bacteria that survive in that temperature.

Bacillus has the ability to grow at high temperature with the endospore it produced; hence, the enzymes produced by *Bacillus* will be more stable at high temperature. This is because the enzymes used as bio-catalysator in the digestive system of a living being have a weakness of denaturating in high temperature (CUCALS 2016). It is further stated that *B. subtilis* is a thermophilic bacterium that can grow in a temperature range of 45-55°C and is able to grow at an optimum temperature of 60-80°C.

Acid tolerance test

The acid tolerance test for probiotics is performed to understand the ability of the bacteria to survive in an acid environment with a pH of 2, 4, and 6. The selection of these three PH value is based on the acid environment in the gut. Probiotic bacteria have to have a high tolerance to the acidity level of the environment because the bacteria have to survive and function when they are in the shrimp digestive tract. This study shows that *B. licheniformis* can survive at H 2-6, while *B. subtilis* experience a reduction in the number of colonies in acid condition pH = 2 (Table 2). Compared to the initial number of *B. subtilis* and *B. licheniformis* of $1.5x10^8$ CFU/mL at the time the test was started, the bacterial density was still dense up to pH = 2. On the contrary, *B. subtilis* had the lowest density at that level of acidity (Figure 3).

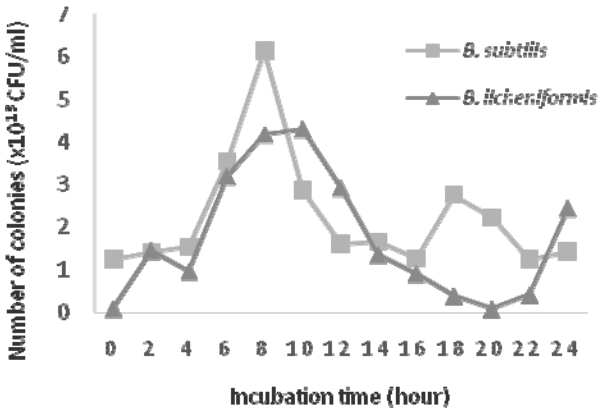


Figure 1. *B. subtilis* and *B. Licheniformis* growth curve

The ability of bacteria to survive in acid and base conditions is one of the bacterial isolate criteria that have to be considered when considering any bacteria as probiotic bacteria (Doeschate and Coyne 2008). Based on their tolerance to an acidic condition, it is assumed that B. *subtilis* and *B. licheniformis* are able to survive in shrimp digestive tract. The pH range of shrimp gut during non-digestion condition is between 1.5-2.0 and between 4.5-5.0 after eating while the pH in the intestine is between 6-7.5 (Jacobsen 1999). UK Standards for Microbiology Investigations (2015) stated that *B. subtilis* is generally a mesophilic bacterium in terms of temperature and neutrophilic bacterium in terms of pH for growth but also tolerance to higher pH levels.

Probiotic bacteria are able to maintain the intracellular pH to be more alkaline than the extracellular pH; however, the reduction of intracellular pH happens along with the reduction of the extracellular pH, which supports the bacteria's tolerance to acid. Bacteria can reduce the intracellular pH to be around neutral when the extracellular pH is decreasing; however, the energy used is high due to the big gap in proton gradient which leads to organic acid anion accumulation in cytosol which is toxic to cells (Magfirah et al. 2015).

Bile-salt tolerance test

The bile salt concentration tolerance testing for probiotics is performed to understand the probiotic's ability to survive in high bile salt concentration, specifically in the digestive tract that has 0.3% and 0.5% bile salt concentration. Good probiotic bacteria should also resistant to high bile salt concentration in the digestive tract (Table 3).

Resistance to acid and bile salt is a prerequisite for a bacterium to become a probiotic bacterium, because when the bacterium enters the host, it will pass the gut with the acidic environment and the bile salt with alkaline pH in the intestine (Widanarni et al. 2012). The results of this study show that *B. subtilis* and *B. licheniformis* bacteria has a high resistance to 0.3% and 0.5%, bile salt concentration, which is proven by the high number of probiotic colonies, i.e. 10^{10} CFU/mL (Figure 4). Tannock (1999) stated that the number of probiotic bacteria in the digestive tract should be 10^6-10^8 CFU/mL to be able to influence health.

The tolerance of both *Bacillus* species in this study to bile salt concentration is originated from the neutral bile salt pH level, i.e. 7-8, which is suitable with the pH for *Bacillus* growth of 5-9 (Hatmanti 2000). In addition, bile salt tolerance may also be gained from the Gram-positive bacterial cell wall components. Cell membrane fatty acid can reduce cell leak caused by bile salt through increased lipid stability. Gram-positive bacterial cell wall predominantly consists of lipid, which is important to maintain the membrane structure by reducing the attachment ability of bile salt on the bacterial cell wall (Kimoto et al. 2002).

To be able to thrive and grow in the digestive tract, the bacterial lactic acid as the probiotic culture has to be able to go through various environmental threats. One of the threats is faced when the bacteria enter the upper intestinal tract where the bile is secreted into the intestine. The bile secretion is a mixture of bile acid, cholesterol, fatty acid, phospholipids, bile pigment, and some detoxified xenobiotics. This combination is bactericidal for the commensal microorganism in the body, except for several intestinal resident bacteria that are resistant to bile salt (Nuraida et al. 2011). The higher the bile salt concentration is, the higher the cell mortality. The ability of bacteria to be resistant to bile salt is caused by, among others, thicker peptidoglycan layer and cell wall, which are the properties of Gram-positive bacteria. This prevents lysis of Gram-positive bacteria when they are exposed to bile salt.

The bile salt influences the bacterial permeability. Bacteria that are vulnerable to bile salt are assumed to have high permeability, allowing a big amount of intracellular material to leak and lysis of the cell. Bile salt has a property of a surface active compound that it can go through and react with the cytoplasm membrane side, leading to changes and damages in membrane structures. The various fatty acid structures in bacterial cell membrane create differences in permeability and are assumed to influence the bacteria's resistance to bile salt (Magfirah et al. 2015).

Antimicrobial activity test

Probiotic antimicrobial activity testing in various carrier materials to digestive pathogenic bacteria is performed to understand the ability of probiotics to inhibit digestive pathogenic bacteria such as *Escherichia coli* that produces enterotoxin, i.e. Labile Toxin (LT), Stable Toxin (ST) and cytotoxin, and *Salmonella typhimurium* that produces enterotoxin and cytotoxin. The results of the clear zone diameter measurement on probiotics in various carriers against pathogenic bacteria in antimicrobial activity testing are listed in Table 4.

Table 4 presents that the probiotic clear zone diameters for *B. licheniformis* supernatant against *E. coli* pathogenic bacteria were 14.3 mm. Fitri and Betty (2010) stated that the potential antimicrobial ability could be seen from the size of the inhibition zone, which is classified as follows: (i) a clear zone diameter of ≤5 mm shows that the bacteria in this test are resistant; (ii) a clear zone diameter of between 5 and 10 mm represents that the bacteria in this test are less sensitive; (iii) a clear zone diameter of between 10 and 20 mm shows that the bacteria in this test are sensitive; and (iv) a clear zone diameter of ≥20 mm presents that the bacteria in this test are very sensitive. From the results of this study, it is revealed that all clear zone diameters of *B. subtilis* and *B. licheniformis* probiotics against the *E. coli* and *S. typhimurium* pathogens present evidence that these probiotics are sensitive to the pathogens (Figure 5). From Table 4, it is also apparent that the supernatant has a higher ability to inhibit the growth of the two pathogenic bacteria than the pellets. The antimicrobial substance is secreted from the cytoplasm membrane into the extracellular part. The supernatant is an extracellular component while pellet is an intracellular component (Ananthanarayanan and Dubhashi 2015).

The probiotic antimicrobial activity is specific for pathogenic bacteria. Ouoba et al. (2006) stated that the *B. subtilis* antimicrobial activities tend to actively inhibit Gram-negative bacteria. This antimicrobial activity is

Table 1. Number of *B. subtilis* and *B. licheniformis* microbial colonies in various temperatures

Type of bacteria	Number of colonies in various temperatures (CFU/mL)		
	40°C	50°C	60°C
B. subtilis	$2.855x10^{10}$	$8.114x10^{10}$	$1.338x10^{10}$
B. licheniformis	$2.924x10^{10}$	$1.475x10^{10}$	$1.418x10^{10}$

Table 2. Number of *B. subtilis* and *B. licheniformis* colonies in various pH

Probiotic bacteria	Number of colonies in various pH (CFU/mL)		
	2	4	6
B. subtilis	$3.333x10^{4}$	$4.407x10^{6}$	$1.711x10^{7}$
B. licheniformis	$2.035x10^{7}$	$2.345x10^{7}$	$9.367x10^{6}$

Table 3. Number of colonies *B. subtilis* and *B. licheniformis* probiotics in various bile salt concentrations

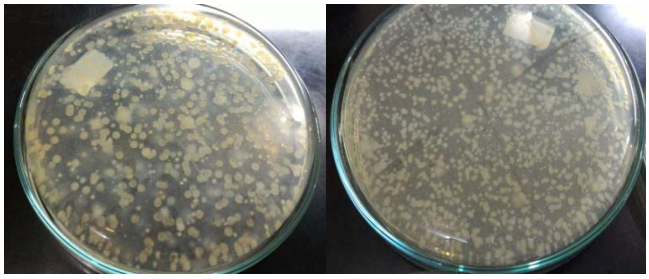
Probiotic bacteria	Number of colonies in various colonies in various concentration (CFU/mL)	
	0.3%	0.5%
B. subtilis	$5.605x10^{10}$	$4.243x10^{10}$
B. licheniformis	$6.668x10^{10}$	$4.913x10^{10}$

Table 4. Results of *B. subtilis* and *B. licheniformis* probiotic clear zone measurement against *E. coli* and *S. typhimurium* pathogens

Probiotic bacteria		Clear zone diameter (mm)	
		E. coli	*S. typhimurium*
B. subtilis	Supernatant	13.0	13.3
	Pellet	12.3	12.0
B. licheniformis	Supernatant	14.3	13.2
	Pellet	13.3	12.7

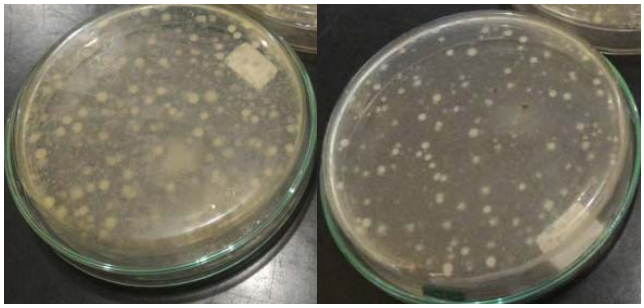
shown by the formation of the clear zone with the certain diameter that is produced by the antibiotic compound activity. Antibiotics is a secondary metabolism product produced by an organism in a small amount that can kill or inhibit other microorganisms (Pelczar and Chan 1981). More than 45 antimicrobial molecules are produced by *Bacillus*. The antibiotic agents produced by *Bacillus* are categorized into, among others, bacteriocin, amino acid, and non-amino acid (Powedchagun et al. 2011).

According to Hafsan (2014), the antimicrobial substrates are produced by probiotic bacteria since the exponential phase. However, most bacteriocin is produced in a large amount during the stationary phase. Bacteriocin can damage the permeability of the bacterial cell membrane by creating pores on the cell, leading to leakage and disturbed cell stability. Unstable cell membrane may inhibit bacterial cell growth and may eventually lead to cell death.



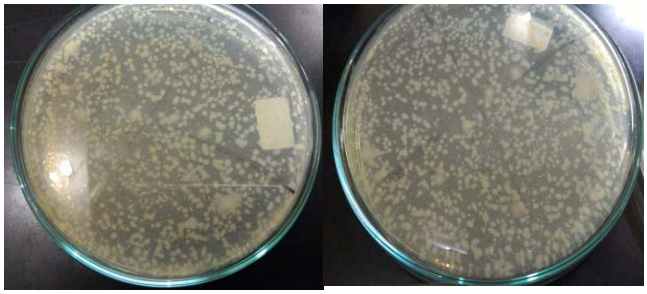
A B

Figure 2. TPC of *B. subtilis* (A) and *B. licheniformis* (B) colonies during temperature tolerance testing



A B

Figure 3. TPC for *B. subtilis* (A) and *B. licheniformis* (B) in acid tolerance testing



A B

Figure 4. TPC of B. subtilis (A) and B. licheniformis (B) in bile salt tolerance testing

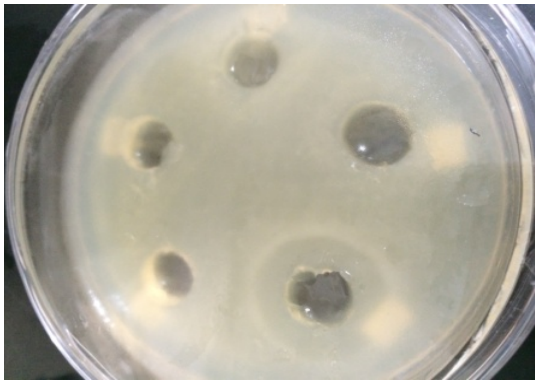


Figure 5. Clear zone formed in probiotic antimicrobial activity test against pathogenic bacteria

From the growth curve, temperature tolerance, and acid tolerance tests, it can be concluded that *B. subtilis* and *B. licheniformis* can be used as probiotics in Vanamei shrimp feed. The optimum growth of *B. subtilis* and *B. licheniformis* is reached at the 8th and 10th hours, respectively. Both bacteria are tolerance to 40, 50, and 60°C temperature but *B. subtilis* cannot tolerate an acid environment with pH = 2. *B. subtilis* and *B. licheniformis* have high tolerance to 0.3% and 0.5% bile salt concentration which is proven by a high number of probiotic colonies, i.e. 10^{10} CFU/mL. In antimicrobial activity testing, *B. subtilis* and *B. licheniformis* are highly sensitive to *E. coli* and *S. typhimurium* pathogens. The ability of *B. subtilis* and *B. licheniformis* to survive in shrimp digestive tract should be studied through a follow-up study that performs a biological testing on the use of probiotic bacteria in Vanamei shrimp.

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