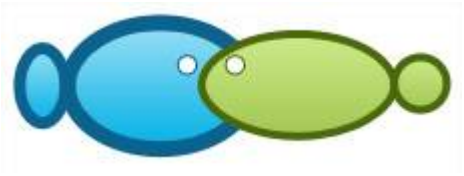

Bioactivity of carp intestinal-inhabited *Bacillus* in improving fish immunity and detection of their antimicrobial peptides (AMPs) encoding genes

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Abstract. *Bacillus* are well-known cosmopolite bacteria. Some members of this bacteria genus can cause diseases, however many of them have been proved as immunostimulant agents. Antimicrobial peptides (AMPs) are present in this genus. This study aims to evaluate the bioactivity of carp intestinal-inhabited *Bacillus* on increasing fish immunity and to detect their antimicrobial peptides (AMPs) encoding genes. The method used is an exploratory method. *Bacillus* CgM22 was isolated using classical culture method. Their ability to improve the fish's immune system was observed through blood physiology (the number of red blood cells and white blood cells) of fish induced with *Bacillus* CgM22. The physiological character of bacteria was carried out by observing their growth curve. The presence of AMPs gene was identified by PCR method using iturin, surfactin, subtilin, bacillomycin, subtilosin and fengycin primers. The results showed that the growth curve shows a stationary phase that occurs at 40-72 hours of incubation. On immunostimulation test, the number of red blood cells and white blood cells also increased compared to controls. In fish induced with *Bacillus* CgM22 10^8 cfu, the red blood cell count was 1.41 x 10^6 cells mm^{-3} and the white blood cell count was 131 x 10^3 cells mm^{-3}. In fish without induction, the number of red blood cells was 1.00 x 10^6 cells mm^{-3} and the number of white blood cells was 101 x 10^3 cells mm^{-3}. Three antimicrobial peptide genes were detected in *Bacillus* CgM22, namely subtilin (535 bp and 707 bp), surfactin (325 bp) and bacillomycin (289 bp). To conclude, *Bacillus* derived from carp intestinal have the potential to increase the immunity of carp regarding the existence of AMPs genes and can increase the number of white blood cells, red blood cells and haematocrit. The AMPs genes found in *Bacillus* CgM22 are subtilin (spa), surfactin (srf) and bacillomycin (bam).

Key Words: AMPs gene, *Bacillus*, carp, immune system, immunostimulant, PCR.

Introduction. Carp (*Cyprinus carpio* L.) is one type of freshwater fish that has important economic value, a fairly high protein content, and a fairly affordable price. Because of those reasons, carp is one of widely cultivated freshwater fish (Dianti et al 2013). Fish cultivation in the era of industrialization is increasing rapidly the demand for carp aquaculture production, in order to meet human food needs. The carp production in West Java has increased from 2017 to 2019. In 2017, carp aquaculture production reached 96,015.38 ton tons. In 2019 carp production reached 204,467.38 tons (Statistic Center 2021). Not only in West Java, the carp production in Indonesia has also increased from 2017 to 2019. In 2017, total carp aquaculture production reached 320,940.89 tons. In 2019 carp production reached 535,932.92 tons (Statistic Center 2020).

Intensive fish farming cannot be separated from the problem of disease attacks. Fish diseases can be caused by fungi, parasites, bacteria, and even viruses. One of pathogens which cause problem in common carp aquaculture is Koi herpesvirus (KHV) (Ariyanto et al 2018). This virus can cause mass mortality (80-100%) in common carps within 2 or 3 weeks (Amin et al 2018). In 2000 in Blitar, Indonesia, the first cases were reported. The estimation of economic losses due to KHV outbreak is more than IDR 5 billion within the first 3 months after the initial infection (Amin et al 2018).

The other carp's diseases can be caused by bacteria like *Vibrio* sp. and *Aeromonas* sp. Diseases caused by bacteria are also obstacle to carp cultivation, because they can also cause death resulting in significant economic losses (Lukistyowati & Kurniasih 2011). The bacteria *Aeromonas* sp. are widely distributed pathogenic bacteria, which could kill the fish up to 80-100% within 1-2 weeks (Mulyani et al 2018). The bacteria *Vibrio* sp. can also cause mass mortality in fish. Signs of disease caused by *Vibrio* include lethargy, tissue and appendage necrosis, slow growth, slow metamorphosis, body malformation, bioluminescence, muscle opacity, skin discoloration, the presence of red necrotic lesion in the abdominal muscle, and erythema (bloody blotches) at the base of the fins, around the vent, and inside the mouth (Novriadi 2016).

Various attempts have been made to overcome pathogenic infections in fish culture ponds. One of them is using antibiotics. However, long-term use of antibiotics can result in the emergence of bacterial strains that are resistant to these drugs. Resistance can occur due to the accumulation of antibiotics in the body of the cultured fish. The use of antibiotics will produce residues that can pollute the water. In addition, antibiotics can produce toxins and are residual for consumers so that they can cause food allergies and even poisoning (Dewi et al 2014).

One of the actions that can be taken to reduce the use of antibiotics in fish farming is to use natural ingredients. They are expected to be friendly for the surrounding environment (Romero et al 2012). Natural ingredients can be used as a substitute for synthetic antibiotics if they contain secondary metabolite compounds that function as antioxidants, and have antibacterial, anticancer, and anti-inflammatory properties (Naqvi et al 2019). The use of probiotic bacteria is also an alternative to replace the use of antibiotics in fish farming (Romero et al 2012).

Bacillus is one of the bacteria genera found in the intestinal tract of fish. These bacteria are commensal because they are known to produce antimicrobial substances in the form of bacteriocins (Suriani & Muis 2016) and antimicrobial peptides (AMPs) (Fitri 2012). AMPs can neutralize endotoxins in Gram-negative bacteria, have broad-spectrum antibacterial properties, and can destroy microbial cells. In addition to their antimicrobial effect, AMPs also have several important roles as inflammatory mediators (Luo & Song 2021). In addition, several studies have also shown that the bacteria *Bacillus* sp. are able to improve the immune system of carp (Septiarini et al 2012), increase feed efficiency in striped catfish (*Pangasianodon hypophthalmus)* (Setiawati et al 2013), increase catfish (*Clarias* sp.) survival (Sukenda et al 2016), and increase non-specific immune responses in vannamei shrimp (*Litopenaeus vannamei*) (Wananda et al 2022).

The way to see the effect of supplementation of *Bacillus* on the immune response of fish is by observing its haematological parameters. Leukocytes (white blood cells) and erythrocytes (red blood cells) are part of the immune system. Leukocytes can act to protect against infections, meanwhile erythrocytes play a direct and significant role in innate immunity and inflammation (Anderson & Siwicki 1995). Therefore, based on the above explanation, this study was conducted to evaluate the bioactivity of carp intestinal-inhabited *Bacillus* supplementation on increasing fish immunity and to detect their antimicrobial peptides (AMPs) encoding genes.

Material and Method

Supplementation of Bacillus CgM22 into the feed of carp. The isolates used were the results of research by Mulyani et al (2018). The culture density of *Bacillus* CgM22 was measured according to the treatment, namely: (A) without the addition of *Bacillus* CgM22; (B) 10^6 CFU mL^{-1}; (C) 10^8 CFU mL^{-1}; and (D) 10^{10} CFU mL^{-1}, using a Cary 60 UV-Vis spectrophotometer from Agilent. If a density of 10^{10} CFU mL^{-1} is obtained, then a dilution method is used to obtain the densities of 10^6 CFU mL^{-1} and 10^8 CFU mL^{-1}. The dilution procedure was carried out by taking 1 mL of the bacterial suspension (10^{10} CFU mL^{-1}) and then put it in the distilled water diluent media containing 9 mL and homogenized with a vortex (density produced at this stage is 10^9 CFU mL^{-1}). Then we did the same thing to get a density of 10^8 CFU mL^{-1} and 10^6 CFU mL^{-1}. The amount of the solution of *Bacillus* sp. mixed into feed was as much as 10 mL kg^{-1} (Setiawati et al 2013).

First, the feed is mixed with a binder, as much as 2% of the solution to be made. Then the solution of *Bacillus* CgM22 was sprayed onto the feed. After that, it was air-dried and stored in a refrigerator at 4°C. The purpose of storing feed in the refrigerator is to maintain the quality of the bacteria so as not to be contaminated and to make the bacteria in a dormant state so as not to change its density.

Feeding of carp with Bacillus CgM22. Carp jveniles were fed with feed supplemented with *Bacillus* CgM22 with the density according to the treatment, namely:

- treatment A: control (without *Bacillus* CgM22);
- treatment B: the addition of *Bacillus* CgM22 density 10^6 cfu mL^{-1} in feed;
- treatment C: addition of *Bacillus* CgM22 density 10^8 cfu mL^{-1} in feed;
- treatment D: addition of *Bacillus* CgM22 density 10^{10} cfu mL^{-1} in feed.

The feed was given for 14 days. According to Irianto et al (2003) probiotic supplementation in feed for 14 days was able to increase the number of kidney macrophages and fish phagocytic activity. The frequency of feed given was twice a day as much as 3% of the fish weight at satiation.

Fish haematological profile

White blood cell count. Blood of carp that has been prepared is sucked using a leukocyte toma pipette containing white stirring grains to a scale of 0.5. Then the Turk's solution was added by sucking it up to a scale of 11. Then stir the pipette for 3-5 minutes until well mixed. Then the first two drops of the blood solution in the pipette were discarded, while the next drops were added to the haemocytometer and covered with a cover glass. Furthermore, the white blood cell count was carried out under a 400x magnification microscope (Zubaidah et al 2018).

Red blood cell count. The blood of carp that has been prepared is sucked using an erythrocyte toma pipette containing red stirring grains to a scale of 1. Then Hayem's solution is added by sucking it up to a scale of 11. Then stir the pipette for 3-5 minutes until well mixed. Then the first two drops of the blood solution in the pipette were discarded, while the next drops were added to the haemocytometer and covered with a cover glass. Subsequently, the number of red blood cells was counted under a 400x magnification microscope (Yanuhar et al 2020).

Hematocrit. Fish were taken and dissected at the base of the tail. Fish blood is sucked with a capillary tube until it reaches 3/4 of the capillary tube. The end of the capillary tube is closed with crytoceal by plugging it in the tip in crytoceal to a depth of 1 cm. The capillary tube was centrifuged at a speed of 3000 rpm for 5 minutes with the volume cylinder position opposite the centrifuge rotation. Measurement of the value of the hematocrit level was done by comparing the volume of solid red blood cells with the total volume of blood on the hematocrit scale (Zubaidah et al 2018).

Bacterial growth curve. Before making a growth curve, bacterial colonies from nutrient agar were taken using a needle loop and put into an Erlenmeyer containing Nutrien broth and cultured at 30°C in an incubator shaker. Bacterial growth curve was carried out every 2 hours by observing the absorbance value of bacterial suspension using a spectrophotometer.

Detection of antimicrobial peptides (AMPs) encoding genes of Bacillus CgM22

Bacterial DNA isolation. Isolation of genomic DNA of *Bacillus* CgM22 was conducted using Wizard DNA Purification (Promega), with protocol according to the manufacturer.

Bacterial DNA amplification by PCR. Amplification of the gene encoding AMPs was carried out by polymerase chain reaction (PCR) to determine the presence of AMPs using six primer pairs, which can be seen in Table 1. The components of the PCR reaction and the

PCR cycle can be seen in Table 2. The PCR results were seen through electrophoresis in 1% agarose gel at 80 volts for 30 minutes. DNA amplification products were then visualized in UV light and photographed using a digital camera.

Amplification was carried out using a PCR thermal cycler programmed for pre-denaturation, namely 1 cycle at 95°C for 1 minute, denaturation of 35 cycles at 95°C for 1 minute, annealing of 35 cycles at 58°C for 30 seconds, and extension of 35 cycles at 72°C for 1 minute (ituC, srfA, and fenB1), for sboA the annealing process was set at 35 cycles at 54°C for 30 seconds, for bamC the annealing process was set at 35 cycles at 50°C for 30 seconds, while for spaS the denaturation process was set at 30 cycles at 95°C for 30 seconds, and annealing of 30 cycles at 50°C for 30 seconds (Mora et al 2015).

Table 1

AMPs primer

Primer	*Gene*	*Nucleotide sequence (5'→3')*
ITUCF	Iturin (itu)	GGCTGCTGCAGATGCTTTAT
ITUCR		TCGCAGATAATCGCAGTGAG
SRFAF	Surfactin (srf)	TCGGGACAGGAAGACATCAT
SRFAR		CCACTCAAACGGATAATCCTGA
SPASF	Subtilin (spa)	GGTTTGTTGGATGGAGCTGTA
SPASR		GCAAGGAGTCAGAGCAAGGT
FenB1F	Fengycin (fen)	TACCAATCGCAATGTCGTGT
FenB1R		CTTCGATTTCTAACAGCCGC
BamC2F	Bacillomycin (bam)	CTGGAAGAGATGCCGCTTAC
BamC2R		AAGAGTGCGTTTTCTTCGGA
Sbo1F	Subtilosin (sbo)	TCGGTTTGTAAACTTCAACTGC
Sbo1R		GTCCACTAGACAAGCGGCTC

Table 2

Component reaction of PCR

Component	*Volume (µL)*
Green Taq Master Mix 2x	12.5
Nuclease free water	8
Primer forward	1.25
Primer reverse	1.5
DNA template	2

Statistical analysis. Effectiveness of *Bacillus* supplementation on fish hematology which includes the changes of leukocytes and erythrocytes number can be analyzed by using the Analysis of Variance (ANOVA) F test; if there is a significant difference between treatments, then it is tested further by using Duncan's Multiple Distance test with an error rate of 5% to determine the best treatment.

Results and Discussion

Carp heematology. Haematological observations of fish were carried out to determine the health status of fish before and after treatment with *Bacillus* CgM22. Haematological parameters include the number of red blood cells (erythrocytes), the number of white blood cells (leukocytes), and hematocrit.

Carp white blood cell count. Observation of the number of white blood cells (leukocytes) was carried out to determine any changes in the number of leukocytes in carp before and after treated with probiotic *Bacillus* CgM22 (Figure 1).

Based on Figure 1 and Table 3, the average number of white blood cells (leukocytes) in fish before treatment was 104,800 cells mm^{-3}. The highest mean number of leukocytes after *Bacillus* CgM22 supplementation was in treatment C of 131,467 cells

mm^{-3}, followed by treatment B with an average of 115,467 cells mm^{-3}, treatment D with an average of 108,533 cells mm^{-3}, and treatment A with an average of 101,067 cells mm^{-3}. From these data, it could be concluded that the number of leukocytes in carp during observation is still in the normal range (from 20000 to 150000 cells mm^{-3}) (Moyle & Chech 2004).

The number of leukocytes in the fish body is used as a determinant of the health status of the fish. Leukocytes are one of the blood components that function as non-specific defenses that will localize and inhibit pathogens through phagocytosis (Anderson & Siwicki 1995). Kresno (2001) said that the increase in leukocyte cells is a reflection of the success of the fish immune system in developing a cellular (non-specific) immune response.

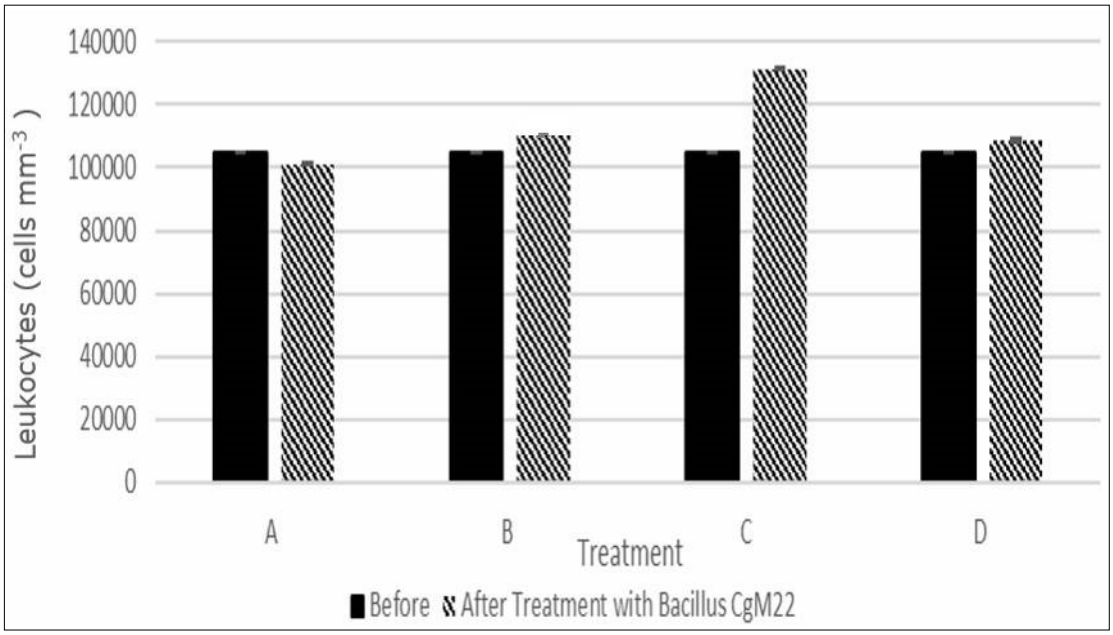

Figure 1. Average white blood cell count.

Table 3

The average number of white blood cells after treatment with *Bacillus* CgM22

Treatment	*Average number of leukocyte (x 10^3 cells mm^{-3})*		*Enhancement of leukocytes (%)*
	Before	*After*	
A (control)	104.8	101.07 ± 0.46^{a}	-3.7^{a}
B (10^6 cfu mL^{-1})	104.8	115.47 ± 0.402^{bc}	9.3^{bc}
C (10^8 cfu mL^{-1})	104.8	131.47 ± 0.01^{c}	20.3^{c}
D (10^{10} cfu mL^{-1})	104.8	108.53 ± 0.646^{b}	3.4^{b}

Note: Different superscript letters within the same column show a significant difference ($p < 0.05$).

Based on Table 3, the addition of *Bacillus* Cgm22 into the feed affects the number of leukocytes. This can be seen in the Duncan test that all treatments with *Bacillus* supplementation (B, C, and D) were able to increase the number of leukocytes significantly compared to treatment without *Bacillus* supplementation (A/control). Furthermore, it was also seen that the supplementation of *Bacillus* at 10^8 cfu mL^{-1} (treatment C) in this research has given the best result in the increase of leukocytes.

Carp red blood cell count. Observation of the number of red blood cells (erythrocytes) of carp was carried out to determine the changes in the number of erythrocytes of carp before and after being supplemented with *Bacillus* CgM22 bacteria into the feed. The number of erythrocytes in fish is used to determine the physiological condition and indicate the health status of the fish (Lukistyowati & Kurniasih 2011). Figure 2 and Table

4 display the changes of the number of erythrocytes before and after being supplemented with *Bacillus*.

The average number of red blood cells of carp before being given treatment is 1,000,000 cells mm^{-3}. The highest value of the average number of red blood cells after being treated with *Bacillus* CgM22 in treatment C was 1,413,333 cells mm^{-3}. The number of erythrocytes in treatment B was 1,330,000 cells mm^{-3}, then followed by treatment D as many as 1,240,000 cells mm^{-3} and the lowest number in treatment A was 1,000,000 erythrocytes (cells mm^{-3}). The number of carp red blood cells during observation was still in the normal range. Irianto (2005) said that the number of fish erythrocytes in general ranges from 1,050,000 to 3,000,000 cells mm^{-3}. Overall, the number of erythrocytes of fish treated with *Bacillus* supplementation (B, C, D) are higher than in fish without supplementation (A/control).

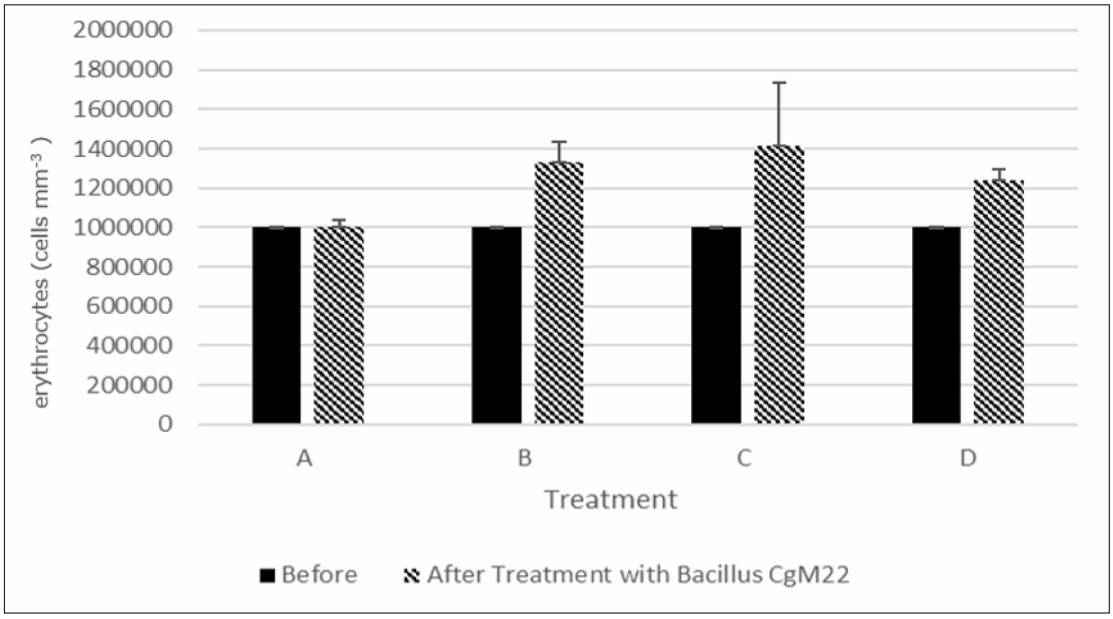

Figure 2. Average red blood cell count.

Table 4

The average number of red blood cells after treatment with *Bacillus* CgM22

Treatment	*Average number of red blood cells (x 10^6 cells mm^{-3})*		*Changes in erythrocyte levels (%)*
	Before	*After*	
A (control)	1.00	1 ± 0.036^{a}	0^{a}
B (10^6 cfu mL^{-1})	1.00	1.33 ± 0.104^{bc}	25^{bc}
C (10^8 cfu mL^{-1})	1.00	1.413 ± 0.32^{c}	29^{c}
D (10^{10} cfu mL^{-1})	1.00	1.24 ± 0.055^{b}	19^{b}

Note: Different superscript letters within the same column show a significant difference ($p < 0.05$).

The data in Table 4 showed that the supplementation of *Bacillus* has a significant effect on increasing the number of red blood cells in carp. The Duncan test showed that in the treatment A (without *Bacillus* supplementation), the number of red blood cells was significantly lower than all treatments with *Bacillus* supplementation (B, C, and D). The highest number of erythrocytes was obtained from the supplementation of *Bacillus* at 10^8 cfu mL^{-1} (treatment C).

Hematocrit. Hematocrit is the ratio between the volume of blood cells and blood plasma and affects the regulation of red blood cells. Observations of carp hematocrit levels were carried out to determine changes in carp hematocrit levels before and after the addition of *Bacillus* CgM22 into feed. Hematocrit levels can be seen in Figure 3.

Based on the graph in Figure 3, the average number of hematocrit level of the carp before treatment is 33%. The average number of hematocrit levels has increased after the addition of *Bacillus* CgM22 into the feed. In treatment C it had the highest value of 39%, followed by treatment B with 37%, treatment D with 34%, and treatment A with 33%.

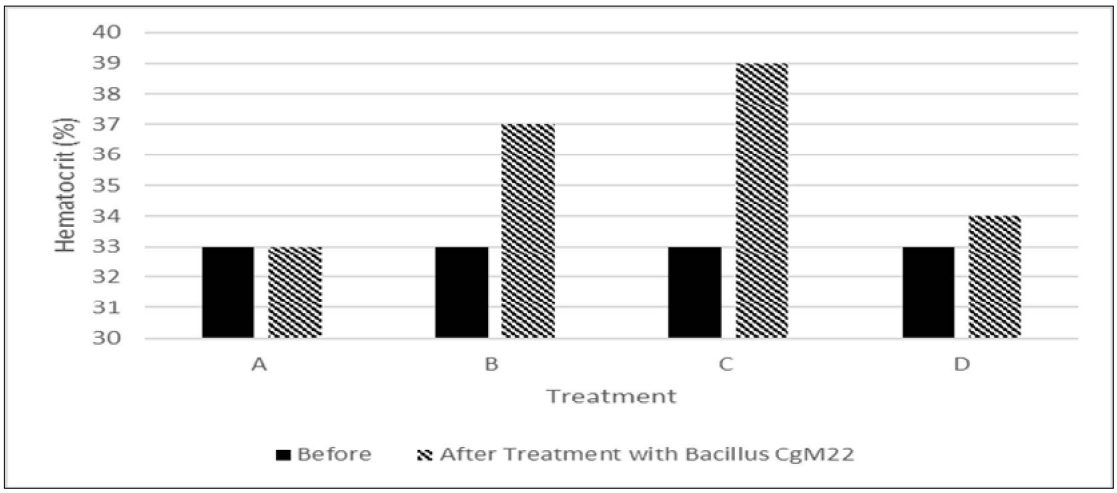

Figure 3. Hematocrit level of carp.

Bacterial growth curve. Bacterial growth curve is a curve that shows the growth phases of bacteria. According to Maier et al (2009), the growth phases of bacteria are the lag phase, the exponential phase, the stationary phase, and the death phase. These phases are indicated by the difference in the growth period which is indicated by the difference in growth speed on the bacterial growth curve. The lag phase is the initial phase of bacterial growth where the growth rate of bacteria is still zero because the new bacteria adapt to the new media environment so that the bacteria are more focused in defending themselves to grow on the new media. The exponential phase is a phase where the growth of bacteria has increased rapidly. The third phase is the stationary phase where the amount of cell growth is accompanied by cell death so that the growth curve becomes flat again. The last phase is the death phase where the number of bacterial cells begins to decrease.

Based on Figure 4, it can be seen that *Bacillus* CgM22 experienced a lag phase at 0 to 18 hours, an exponential phase at 20 hours to 40 hours, then experienced a stationary phase at 42 hours to 72 hours. There is no death phase because the bacterial growth process is still ongoing. While the results of research conducted by Harris (2016) showed that *B. subtilis* has started to enter the exponential phase or log phase from the first day or 24 hours to 72 hours, then a stationary phase on the fourth day and experienced a death phase as indicated by the presence of decrease in absorbance value which decreased on the fifth and sixth day.

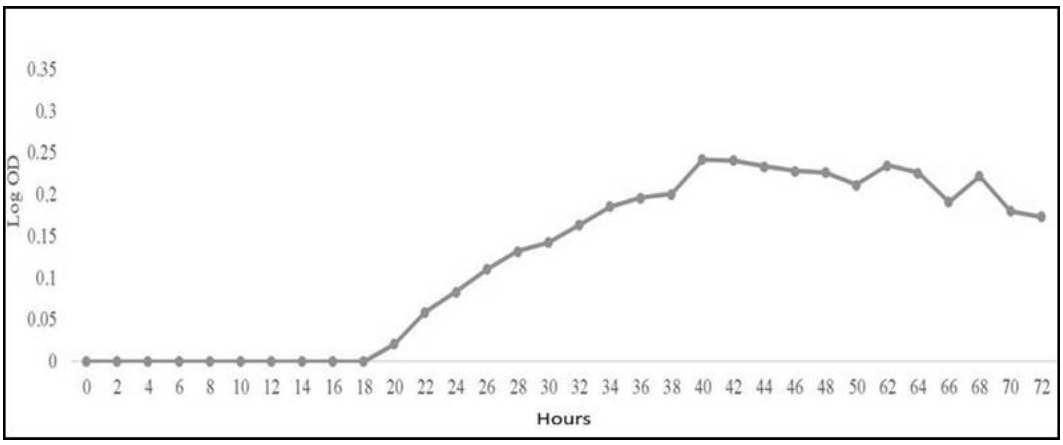

Figure 4. Growth curve of *Bacillus* CgM22.

Each phase of bacterial growth is determined by several factors. According to Pin & Baranyi (2008), the length of the lag phase is influenced by factors such as bacterial species, changes in environmental conditions and the condition of the bacteria cultured in the previous medium, besides that the nutrient content available in the medium also affects the time in the lag phase. This is in accordance with the statement of Madigan et al (2012) that if the culture is transferred from a nutrient-rich medium to a medium that has few nutrients, the culture will experience a lag phase because bacterial cells need a complete complement of enzymes to adapt to a new environment and the lag phase does not occur when bacteria are transferred to a new medium with the same environmental conditions or nutritional content as the previous medium. Brooks et al (2013) states that the length or shortness of the adaptation phase or the lag phase is determined by the number of cells inoculated, the appropriate physiological and morphological conditions and the media required. Factors that affect the log phase or exponential phase according to Setyati et al (2015) are the media where it grows such as nutrient content and pH, as well as environmental conditions including temperature and humidity. According to Brooks et al (2013) in the stationary phase, a lot of secondary metabolites were produced because bacteria defend themselves to survive by releasing their secondary metabolites and some are poisoned by changing environmental conditions due to the metabolites they produce. The factor that influences the absence of a stationary phase on the bacterial growth curve is the carbon content in the medium. Berg (2007) states that bacteria are easier to use simple carbon sources than complex carbon sources, for example Wahyuningsih & Zulaika's (2018) research shows that the stationary phase does not appear on the growth curve in carboxymethylcellulose (CMC) medium and appears on nutrient broth (NB) medium because the carbon source is in the medium. NB is simpler than cellulose. The growth rate or length of time in each growth phase can be caused by differences in bacterial species in environmental conditions, if the environmental conditions have fewer nutrients, the growth of bacteria will be slower. According to Quinn et al (2011) bacterial growth is influenced by nutrition, environmental factors, and genetic factors.

Bacterial growth occurs asexually which is known as binary fission. Binary fission occurs at regular intervals marked by exponential additions or multiples (Faizah et al 2017; Hidayatulloh et al 2019). The development of the growth curve also serves as an optimization of the production time of antimicrobial secondary metabolites by sampling the starter culture. The existence of this time optimization can help to determine the length of incubation time needed to find out the right time to harvest bacteria (Warsi & Sulistyani 2018). The bacterial suspension will become more murky with increasing incubation time, this indicating that the bacteria were still growing (Hidayatulloh et al 2019).

Detection of antimicrobial peptides (AMPs) encoding genes of Bacillus CgM22. Identification of antimicrobial peptide-coding genes requires bacterial DNA as template DNA and a pair of specific primers for target genes. Advances in molecular biology have facilitated the development of specific primers and probes that can be used for PCR-based detection of specific antimicrobial-producing bacteria (de Souza & Raaijmakers 2003). The specific primers used were subtilin, surfactin, iturin, bacillomycin, subtilosin, and fengycin (Cao et al 2012). These primers meet the general criteria for a primer, which consist of 18-25 nucleotides, G-C percentage of 50% or more, Tm not exceeding 70°C (Sambrook & Russel 2001). The amplification of antimicrobial peptide coding genes can be done using specific primers characterized by the appearance of PCR product bands on 1% agarose gel. The resulting band fragments vary because there are differences in the nucleotide sequence where the primer is attached. The amplified ribbon is highly dependent on the quality, suitability of the template, and the primer used (Mulyani et al 2011).

The presence of the AMPs gene in *Bacillus* CgM22 was identified by PCR method using the primers iturin, surfactin, subtilin, bacillomycin, subtilosin and fengycin. Then the electrophoresis visualization was performed using a 100 bp DNA marker. The results of visualization of the electrophoresis process of *Bacillus* CgM22 can be seen in Figure 5 and Table 5.

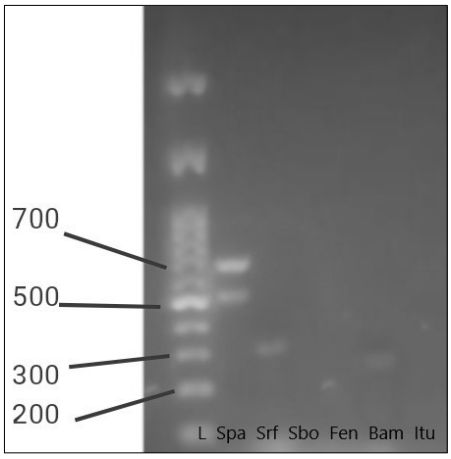

Figure 5. Visualization of PRC products o *Bacillus* CgM22: L = ladder; Spa = subtilin; Srf = surfactin; Sbo = subtilosin, Fen = fFengycin; Bam = bacillomycin; Itu = iIturin.

Table 5

AMPs genes detected in *Bacillus* CgM22

Bacteria	*AMPs gene*	*Band lenght (bp)*
B. subtilis	Bacillomycin (BamC2)	289
	Surfactin (Srf)	325
	Subtilin (Spa)	535
	Subtilin (Spa)	707

Based on Table 5, it can be seen that three antimicrobial peptide genes were detected in *Bacillus* CgM22 namely subtilin (535 bp and 707 bp), surfactin (325 bp) and bacillomycin (289 bp). Based on Mulyani et al's research (2018), *Bacillus* CgM22 is *Bacillus subtilis*. Gupta & Pandey (2019) stated that *B. subtilis* produces iturin, surfactin, and subtilin. Research results of Varma et al (2017) showed that antimicrobial peptides that have been found in *B. subtilis* were basilicin, fengycin, and bacillomycin. The research results of Cao et al (2012) showed that the only antimicrobial peptides detected in *B. subtilis* were fengycin and bacillomycin from the 6 types of antimicrobial peptides used, namely subtilisin, subtilosin, bacillomycin, iturin, fengycin, and surfactin. While Kim et al (2010) succeeded in finding the antimicrobial peptides iturin, fengycin, and surfactin in *B. subtilis*. Huang et al (2007) also succeeded in finding antimicrobial peptides surfactin and fengycin in *B. subtilis*.

The differences in peptide genes detected in these studies are thought to be caused by several factors, including the antimicrobial peptide gene that does not express the gene or the observation process that is less than optimal This is because the factors that influence the non-appearance or undetectability of antimicrobial peptide genes in bacteria are not optimal during the process and lack of expression of essential genes (Tapi et al 2010). Riley & Wertz (2002) stated that the production of antimicrobial peptides is considered a characteristic of unstable bacteria because according to bacterial growth conditions, the exchange between metabolism and gene expression affects the biosynthesis of antimicrobial peptides during bacterial growth. Westerdahl et al (1991) stated that *Bacillus* bacteria were able to produce antibacterial substances. This is because *Bacillus* is able to inhibit pathogens directly or induce systemic resistance in the host.

Conclusions. *Bacillus* derived from carp intestinal have the potential to increase the immunity of carp regarding the existence of AMPs genes and can increase the number of leukocytes (white blood cells), erythrocytes (red blood cells) and haematocrit. The AMPs genes found in *Bacillus* CgM22 are subtilin (spa), surfactin (srf) and bacillomycin (bam). The role of microorganisms in fish disease is of growing interest, and future work in this area should shed light not only on the effects of potential probiotics on host immunity but also on the host microbiome.

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Conflict of interest. The authors declare that there is no conflict of interest.

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