Egyptian Journal of Aquatic Biology & Fisheries
Zoology Department, Faculty of Science,
Ain Shams University, Cairo, Egypt.
ISSN 1110 – 6131
Vol. 29(1): 2409 – 2430 (2025)
www.ejabf.journals.ekb.eg

Characterization of the Antimicrobial Peptides Gene of *Bacillus subtilis* in the Intestine of Common Carp and Its Relationship with Toll-Like Receptor Expression in the Kidney and Liver

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ARTICLE INFO

Article History:
Received: Oct. 10, 2024
Accepted: Feb. 13, 2025
Online: Feb. 22, 2025

Keywords:
Aquatic,
Immunogenic,
Immunostimulants,
Microbiota,
Probiotics

ABSTRACT

Bacillus subtilis is recognized as a probiotic bacterium with the potential to enhance fish immunity through the production of antimicrobial peptides (AMPs). This study evaluated a strain of *B. subtilis* isolated from fish intestines in previous research, designated as *Bacillus* CgM22, which has the potential to act as a probiotic and immunostimulant. Therefore, further research is needed to analyze the AMPs gene in *Bacillus* CgM22 at the molecular level and to assess the expression of the toll-like receptor (TLR2) gene in the kidneys and livers of carp following induction. The study involved molecular analysis of the AMPs gene, followed by induction in carp. The experiment included four treatments: A (control), B (bacterial culture at 10^8 CFU/mLat 15 mL/kg feed), C (5 mL/kg supernatant), and D (10 mL/kg supernatant), each with three replicates, where the treatments were mixed into the feed. Afterward, liver and kidney samples were analyzed for TLR2 gene expression. The results indicated the presence of surfactin and subtilin markers, with the detection of several AMPs genes, including SrfAA (surfactin) and SpaS (subtilin). The highest TLR2 gene expression in kidney and liver samples was observed in treatment B (addition of *Bacillus* CgM22 culture at 10^8 CFU/mL at 15mL/ kg feed). These findings suggest that *Bacillus* CgM22 plays a role in modulating fish immunity by enhancing the body's defense mechanisms against pathogens. Overall, this study indicates that *B. subtilis* CgM22 has potential as a probiotic alternative to improve carp health through immune stimulation.

INTRODUCTION

Global aquaculture production reached 130.9 million tons in 2022, worth USD 312.8 billion, which is 59 percent of global fisheries and aquaculture production. Inland aquaculture accounts for 62.6 percent of cultivated aquatic animals, marine and coastal aquaculture 37.4 percent. For the first time, aquaculture surpassed capture fisheries in terms of aquatic animal production with a total of 94.4 million tons, representing 51 percent of the world's total and reaching a record of 57 percent of production intended for human consumption **(FAO, 2024)**.

Freshwater fish production in Indonesia is currently dominated by carp. According to **DJPB KKP (2021)**, it was found that the volume of carp production was 514,643 tons in 2020. This is supported by the fact that carp (*Cyprinus carpio*) is a commodity that has a fairly preferred preference with a high selling price compared to other freshwater fish such as the catfish and tilapia **(Mudlofar *et al.*, 2013)**. Freshwater fish commodities, especially carp, have good potential and will continue to grow every year.

According to the data **DJPB KKP (2021)**, carp production showed an increase during 2017 – 2021 by 24.95%, with the highest increase of 215,419 tons in the period 2017 – 2018. However, fish production has not reached the target to date. The target in 2020 is 182,741 tons less than the target of 697,384 tons. The fish production target has not been achieved mainly due to environmental factors, the presence of pathogenic microorganisms and host conditions **(Afifah *et al.*, 2014)**. Environmental factors are caused by chemicals such as pesticides that are not biodegradable that caused increase of pathogen organisms in population **(Yaseen *et al.*, 2024)**. Pathogenic bacteria are the primary threat to carp farming, causing significant productivity declines. Some types of bacteria that are known to be detrimental to carp farming activities are *Edwardsiella tarda*, *Yersenia* sp. *Pseudomonas* sp., and *Aeromonas hydrophila* **(Pardamean *et al.*, 2021)**. *Aeromonas hydrophila* are found on almost all parts of the body of freshwater fish. These organisms can adhere by adhesion and feed on cell fluid on the mucus or in the epidermis **(Pangathousands *et al.*, 2018; Azhar *et al.*, 2022)**.

Several researchers have conducted experiments to improve the immune system. The benefits of immunostimulants are that they can increase the non-specific immune response of *Clarias* sp. **(Isnansetyo *et al.*, 2014)**, *Sparus aurata* and *Dicentrarchus labrax* **(Carbone & Faggio 2016)**, the European seabass (*Dicentrarchus labrax*) **(Guardiola *et al.*, 2016)**, *Oreochromis niloticus* **(Isnansetyo *et al.*, 2016)**, and shrimp **(Yudiati *et al.*, 2016)**. Immunity can be increased by administering probiotics. Probiotics are living microorganisms that provide health benefits to their hosts **(Plaza-Diaz *et al.*, 2019)**. Probiotics have a function that can increase the host's response to disease and can be used as a biocontrol agent to reduce disease attacks. One of the probiotics that can be used is *Bacillus subtilis*. This bacteria species can stimulate immunity *in vivo* and *in vitro* **(Mohapatra *et al.*, 2013)**. Induction of *B. subtilis* which is added to feed can express genes from the immune system and further physiologically increase the body's resistance to disease attacks.

Based on the results of the study of **Feliatra *et al.* (2012)**, *Bacillus* sp. has the ability to control pathogenic bacteria, inhibit the growth of other bacteria and can produce antibiotics that are toxic to other microbes **(Sukenda *et al.*, 2016)**. *Bacillus* also has metabolites capable of acting as protease inhibitors including chondrillasterol, cholestane, trifluoroacetic acid, octadesenoic acid, stigmasterol, 9-octadesenoic acid, hexadecanoic acid, macrolactin A, Subtilosin A, Leodoglucomide, Gramicidine S, and Tyrocidine A **(Mulyani *et al.*, 2024)**. *Bacillus subtilis* is a probiotic bacterium widely recognized for its

beneficial role in enhancing fish immunity, primarily through the production of antimicrobial peptides (AMPs). These AMPs act as natural defense molecules, inhibiting the growth of pathogenic bacteria and reducing the risk of infections in aquaculture systems **(Valero *et al.*, 2020)**.

The mechanism of bacterial immunity to defend itself against pathogens includes physical defenses such as skin and mucosal epithelium and other factors such as Antimicrobial Peptides (AMPs). Antimicrobial peptides (AMPs) function as the body's innate defense system in many organisms. Characterization of AMPs in *Bacillus subtilis* is to identify the presence of the desired type of AMPs and to ensure that they conform to the appropriate characteristics. These AMPs play a role in the immune system's response by activating and mobilizing immune cells, resulting in the destruction of microbes and controlling inflammation **(Argentina *et al.*, 2019)**. These AMPs can identify the presence of the expected type of AMPs and ensure that they conform to the appropriate characteristics. One of the ingredients contained in supernatants *B. subtilis* namely polymixin, colistin, circulin and peptide antibiotics such as subtilin, subtilosin A, functional amyloid TasA and sublancin **(Willenbacher *et al.*, 2016)**.

Currently, there has been a lot of research on the potential *Bacillus* sp. Based on the results of the research of **Mulyani *et al.* (2018)**, several types of bacteria *Bacillus* which is isolated from the intestines of carp are obtaind. Furthermore, bacteria *Bacillus* is also identified to select candidates for alternative sources of immunostimulants, and one of them is found in *Bacillus* subtilis (*Bacillus* CgM22) **(Sofandi *et al.*, 2021)**. In immunostimulation tests, 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 number of red blood cells was 1.41 x 10^6 mm^{-3} cells while in control it was 1 x 10^6 mm^{-3}. Moreover, the number of white blood cells was 131 x 10^3 mm^{-3} cells while in control it was 101.07 x 10^3 mm^{-3} **(Mulyani *et al.*, 2023)**.

The immune response of carp to microorganisms attacking them is also observed when they are given Bacillus. **Mufidah *et al.* (2015)** found that the TLR (Toll-like receptor) of carp increases when exposed to antigens from bacteria. Toll like receptor or TLR is an important receptor because it is able to detect the presence of antigen infections and increase innate immune response or inflammatory response to pathogens **(Primary *et al.*, 2021)**. However, there is still limited molecular data on the antimicrobial peptides (AMPs) gene produced by *B. subtilis* and its potential link to Toll-like receptor 2 (TLR2) gene expression. Based on this description, it is necessary to conduct research on the molecular analysis of AMPs genes *Bacillus* CgM22 which comes from the intestines of fish. Then *Bacillus* CgM22 was induced in carp and subsequently analyzed for expression of the TLR 2 gene from the kidney and liver.

MATERIALS AND METHODS

Sample collection, bacterial isolation and identification of *Bacillus subtillis*

The *Bacillus subtilis* used in this study was identified in previous research by **Mulyani *et al.* (2018)**. Their study analyzed the microbiota of the fish intestine, identifying 30 bacterial isolates through 16S rRNA gene sequencing. Among these, *Bacillus subtilis* (CgM22) was found with pathogenicity and antagonistic tests revealed that *Bacillus subtilis* was non-pathogenic at a density of 10^6 CFU/mL and demonstrated significant protective effects against *Aeromonas* infection. These findings suggest that *Bacillus subtilis* from the fish intestine has potential as a biological control agent.

Characterization of the AMPs gene in *Bacillus subtilis*

The DNA isolation process of *Bacillus* CgM22 uses the Wizard Genomic DNA Purification Kit (Promega). The final result is a DNA solution that is ready to be used as a template for the next process, namely amplification and electrophoresis. Bacterial DNA isolates are used as prints. Amplification of AMPs-coding genes is done by Polymerase Chain Reaction (PCR) to determine the presence of AMPs. The primers used for gene amplification are surfactin (srfAA) srfAA-F : 5' TCGGGACAGGAAGACATCAT 3' and srfAA-R : 5' CCACTCAAACGGATAATCCTGA 3'. Meanwhile, for the amplification of subtilin genes (spaS), spaS-F 5' GGTTTGTTGGATGGAGCTGTA 3', spaS-R 5' GCAAGGAGTCAGAGCAAGGT 3' is used **(Mora *et al.*, 2011)**.

The components used in the PCR reaction include 12.5µl of Green Taq Master Mix 2x, 8µl of Nuclease Free Water, 1.25µl of Forward Primer, 1.25µl of Reverse Primer, and 2µl of DNA template. PCR cycles performed for *Bacillus* CgM22 with SpaS primer is with the first stage of 1 cycle of predenaturation at 95°C for 1 minute, 35 cycles of denaturation at 95°C for 1 minute, and annealing 35 cycles at 51°C for 30 seconds. Meanwhile, in the SrfAA primer, the annealing is 35 cycles with a temperature of 49°C for 30 seconds and Extension 35 cycles at 72° for 10 seconds **(Amatulloh *et al.*, 2021)**.

This analysis was performed using BLAST (Basic Local Alignment Search Tool) compared to the nucleotide reference database derived from the GenBank (https://www.ncbi.nlm.nih.gov/nucleotide/). Sequence alignment is performed using the BioEdit program to remove nucleotides **(Hall *et al.*, 2011)**. This alignment was further constructed with a phylogenetic tree using the ClustalW program integrated into the MEGA 11 software (Molecular Evalutionary Genetic Analysis) **(Tamura *et al.*, 2021)**. Genetic distance was calculated using the pairwise distance method and phylogenetic tree design (1,000 replicates) and the neighbor-joining tree bootstrap model Kimura Two parameters **(Sahaba *et al.*, 2021)**.

Preparation of *Bacillus subtilis* culture and supernatant

The *Bacillus* CgM22 isolate was re-isolated to obtain a single colony. Bacterial cultivation was carried out on nutrient sgar (NA) medium using the streak plate method.

The sterilized NA medium was aseptically poured into a petri dish and left to cool. *Bacillus* CgM22 was then inoculated into nutrient broth (NB) and incubated at 30°C for 72 hours. The resulting bacterial broth culture was transferred into a cuvette, and its density was measured using a spectrophotometer at a wavelength of 620nm. A serial dilution was then performed to achieve a density of 10^8 CFU/mL.

Experimental design and fish treatment

The method at the fish rearing stage is an experimental method with a Complete Random Design model that uses four treatments and three replicates. The treatment given was different at each concentration of *Bacillus CgM22* supernatant mixed into artificial feed. Treatment A is a control with no addition in the feed. Treatment B forms the addition of 10^8 CFU *Bacillus* CgM22 bacterial cultures with a dose of 15ml/ kg of feed. Treatment C is the addition of supernatant from *Bacillus CgM22* with 5ml/ kg of feed. Treatment D is the addition of supernatant from *Bacillus CgM22* at a dose of 10ml/ kg of feed. Feed per day is only 2 times a day using the adlibitum feeding method by calculating feed needs based on their body weight **(Narejo *et al.*, 2023)**. Before feeding the fish in each aquarium, their weight is measured to determine the appropriate amount of feed to provide daily. Probiotic feeding is carried out over a 2-month period during the maintenance phase **(Septiarini *et al.*, 2012)**.

RNA extraction and cDNA synthesis

The steps in gene expression analysis using RT-qPCR (Real Time PCR) begin with RNA isolation followed by PCR using an optimized primer. RNA isolation is carried out by the Phenol-Chloroform method. The RNA concentration was then measured again using a microplate reader. RNA isolates are amplified using RT-qPCR (Real time PCR) machines AriaMx Real-Time PCR System Agilent and sample count according to the Sensoquist kit. The primers used are β-actin and TLR 2.The primer used in RT-qPCR (Real Time PCR) consists of several primer pairs in Table (1).

Table 1. Primer used in RT-qPCR

Primer	Primer Sequence	PCR Product Size (bp)	Reference
β-actin -Forward	CCCTGGCCCAGCAGCAATG	300	**Fink *et al.*, 2016**
β-actin -Reverse	TCTGCGCAGTTGAGTCGGCG		
qTLR 2 - Forward	TCAACACTCTTAATGTGAGCCA	160	
qTLR 2 -Reverse	TGTGCTGGAAAGGTTCAGAAA		

The components used in the RT-qPCR reaction consisted of 5μL of 2x SensiFAST SYBR No-ROX One Step Mix, 0.4μL of 10μM forward primer, 0.4μL of 10μM reverse

primer, 0.1µL of reverse transcriptase, 0.2µL of RiboSafe RNAse inhibitor, 1.9µL of nuclease-free water, and 2µL of RNA template with a concentration of 250ng **(SensiFast, 2019)**.

TLR2 gene expression analysis by qRT-PCR

The RT-qPCR (Real Time PCR) program consists of several stages with specific cycle and temperature settings. The first stage was a reverse transcription which is carried out at 45°C for 10 minutes with one cycle. Next, the polymerase activation stage was carried out at a temperature of 95°C for 2 minutes with one cycle. The amplification process consisted of three main steps, namely denaturation at 95°C for 5 seconds, annealing at 55°C for 20 seconds, and extension at 72°C for 5 seconds, all of which were carried out over 40 cycles. After amplification, the melt stage was carried out in three steps, namely at 95°C for 30 seconds, 65°C for 30 seconds, and 95°C for 30 seconds, each with one cycle. This setting was adapted as outlined by **SensiFast (2019)**.

Next, gene expression analysis was carried out with RT-qPCR (Real time PCR). The results of the relative ratio of gene expression were analyzed by comparing between the samples. The determination of the relative ratio of genes to real-time PCR quantification was determined by the crossingpoint (cp) deviation of the target gene and the β-actin gene following the 2-ddCT method.

Data analysis

PCR visualization data were analyzed using Corel Draw X and Microsoft Excel to ensure accurate representation and interpretation. Additionally, electrophoresis visualization data of antimicrobial peptides (AMPs) found in carp gut bacteria were examined to identify the presence and expression patterns of these peptides. Furthermore, RT-qPCR (Real-Time PCR) results for kidney and liver characterization were analyzed descriptively to assess gene expression levels and potential immune responses. This comprehensive approach provides valuable insights into the molecular characteristics of AMPs and their role in fish health. The RT-qPCR quantification results were then analyzed using the following equation:

$$ddC_T = (C_{T\,Target} - C_{T\,Actin})_{Time\,x} - (C_{T\,Target} - C_{T\,Actin})_{Time\,0}$$

Keterangan:

CT: Fluorescence detected every cycle. The principle is that when the fluorescence line crosses the RT PCR device, the device will recode the cycle until it is complete.

ddCT : Δ ΔCT;

Time x : Expression of the target gene at the target time point;

Time 1 : Expression of the target gene is normalized to β-actin. In principle, the CT value of the housekeeping gene and the sample gene shows their relative relationship.

RESULTS AND DISCUSSION

Molecular analysis of *Bacillus* CgM;22 AMPs gene

Bacillus CgM22 from the gut is a suitable candidate to be a probiotic. Therefore, molecular analysis is needed to identify antibacterial products produced by bacteria *Bacillus*. Antimicrobial peptides (AMPs) are one type of peptide that can inhibit bacteria or as natural antibiotic genes and are a promising alternative to new generation antibiotics **(Cotter *et al.*, 2013)**. Strains of the genus *Bacillus* produces a wide range of antimicrobial peptides with several different basic chemical structures **(Gebhardt *et al.*, 2002; Stein 2005)**. The AMPs observed include surfactants, subtylosin and subtilin **(Corvey *et al.*, 2003; Abriouel *et al.*, 2011)**. This can be identified by conducting molecular analysis of the AMP gene which is then sequenced **(Sumi *et al.*, 2015)**. Based on the AMP, PCR was carried out with the primer used, and the results are shown in Fig. (1).

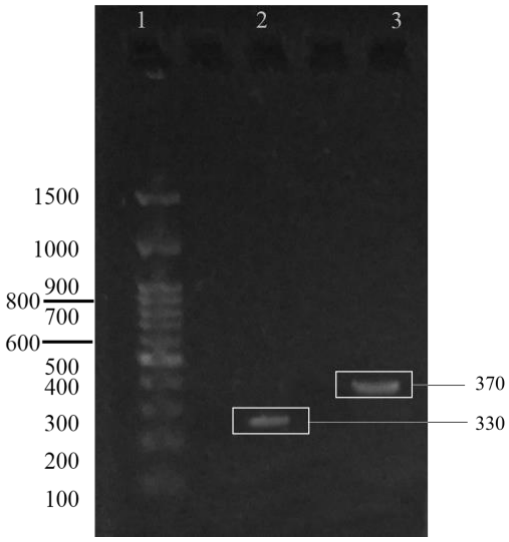

Fig. 1. Electrophoresis results of AMPs PCR samples from *Bacillus* CgM22. Lane (1): 100 bp DNA ladder, Lane (2): Surfactin (SrfAA), Lane (3): Subtilin (SpaS)

Based on these results, it was found that the sample appeared on *Bacillus* CgM22 with surfactin and subtyline markers at 330 and 370bp. Surfactin is one of the cyclic lipopeptide biosurfactants with the characteristics of an 8-member compound consisting of 7 amino acids and a hydroxycanoic acid part. This surfactin is produced by several strains of *Bacillus* which is able to induce the induction response of the resistance system in various species **(Ongena *et al.*, 2007; Anonymous *et al.*, 2013; Cawoy *et al.*, 2014; Rodríguez *et al.*, 2018)**. Treatment with surfactin contained in *Bacillus* from plants can reduce disease showing that treatment with biomolecules is as effective as inoculation with bacteria. This can increase the defense capacity of plants and can produce a faster and stronger defense reaction when affected by disease **(Pieterse *et al.*, 2014)**.

While, subtilin is a peptide antibiotic (lantibiotic) that contains lantionin which is a group of linear antibiotics. The spaS gene encodes the subtyline prepeptide, in which the

amino acid is enzymatically modified by the products of the spaB and spaC genes **(Velho *et al.*, 2013)**. The spaS gene is essential for the production of the antimicrobial peptide subtilin **(Stein, 2005)**. Subtilin derived from *Bacillus* also produces bacteriocin-coding genes that can increase resistance to *Staphylococcus aureus* in food **(Chhetri *et al.*, 2019)**.

Surfactin and subtilin detected in strains of *Bacillus* CgM22 from the intestines of this carp are produced by the strain *Bacillus* which associates with plants. The AMPs produced include surfactin and subtilin. Surfactin and subtylin can have the potential for the biocontrol of plant pathogens and support plant growth **(Chen *et al.*, 2009; Mora *et al.*, 2011)**. Some antimicrobial peptides are genes that are encoded and synthesized in ribosomes, while others are produced non-ribosomes by multienzyme mechanisms **(Cotter *et al.*, 2005)**.

The PCR sample results were then analyzed for sequence to investigate kinship with Genbank data **(Nelson-Sathi *et al.*, 2013)**. The data contained in the genbank show that the sequences of PCR products include AMPs sequences **(Velho *et al.*, 2013; Chen *et al.*, 2022)**. The results obtained describe the identity and molecular diversity of the PCR products. The next sample is then sequenced using the Sanger method.

The Sanger or dideoxy method has become the most widely practiced method for DNA sequencing **(Furutani *et al.*, 2022)**. A variety of automated fluorescence-based methods can sequence DNA fragments with thousands of nucleotides in just a few hours **(Durmaz *et al.*, 2015)**. The results of sequencing by the sanger method are with nucleotide lengths of 474 on the suractin marker and 269 on subtyline. This amount is in accordance with the results of the PCR products obtained, as the results of AMPs sequencing with the bacterial Sanger method on shellfish samples show similarities with the results of PCR products **(Gerdol *et al.*, 2012)**.

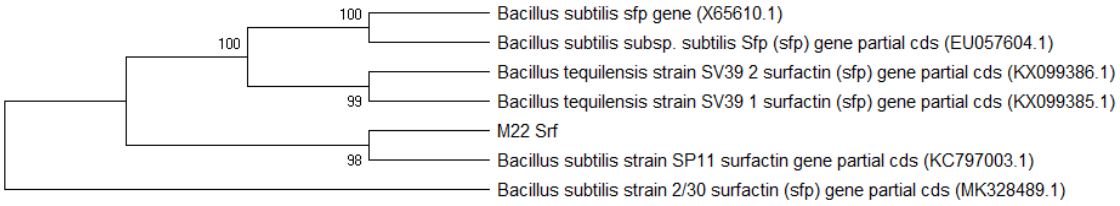

Fig. 1. Phylogeny tree of surfactin gene (SrfAA) from *Bacillus* CgM22

Sequencing results *Bacillus* CgM22 with surfactin primer can be seen in phylogeny trees (Fig. 2). Homological results of SrfAA sequence from isolate *Bacillus* CgM22 has a homological similarity to *B. subtilis* strain SP11 surfactin gene partial cds (KC797003.1) with a bootstrap value of 98. The value of this bootstrap is relatively high and trustworthy, as the categories of bootstrap values include high (>85%), moderate (70–85%), weak (50–69%), or very weak (<50%) **(Kress *et al.*, 2002)**.

Surfactin exhibits a wide range of interactions with target cell membranes and has potential for a wide range of applications related to the immune system **(Seydlová & Svobodová, 2008)**. Surfactin is a lipopeptide-type biosurfactant produced primarily by species *Bacillus*, consisting of a peptide loop of seven amino acids and a chain of hydrophobic fatty acids (C12-C16) **(Bartal *et al.*, 2018)**. These molecules have been shown to exhibit a variety of biological activities including anti-mycoplasm **(Vollenbroich *et al.*, 1997)**, anti-tumor **(Duarte *et al.*, 2014)**, anti-inflammatory activity **(Zhang *et al.*, 2015)** and anti virus **(Money *et al.*, 2017)**. Other research results proved that surfactin is produced by species *Bacillus* positive grams including *B. subtilis, B. amyloliquefaciens, B. licheniformis,* and *B. pumilus* **(Alvarez *et al.*, 2012; Chen *et al.*, 2015a; Jiao *et al.*, 2017)**. This is in accordance with the *B. amyloliquefaciens* FZB42 and strains *Bacillus* & others are AMPs, which are lipopeptide genes (surfactants, bacillomycin D, and fengycin) that play a role in the production of bioactive metabolites with antimicrobial activity **(Dabiré *et al.*, 2021; Haddoudi *et al.*, 2021)**. Moreover, **Mora *et al.* (2011)** reported that the srfAA gene and other genes such as bmyB, bacA, and fenD strengthen the competitive role of survaccin, bacyllomicin, fengycin, and bacilysin in boosting immune activity. The study of **High *et al.* (2018)** indicated that in the isolate *B. velezensis* Y6 derived from plants produces AMPs iturin, fengycin and surfactants.

Surfactin carries out its antibacterial activity process by acting on the plasma membrane through the pore formation mechanism. In addition, suractin can break down bacterial biofilms by lowering the percentage of alkali-soluble polysaccharides and decreasing the regulation of gene expression involved in the formation of biofilms such as icaA and icaD **(Liu *et al.*, 2019)**. In addition, suractin can also induce the grapevine immune system in response to infection **(Farace *et al.*, 2015)**.

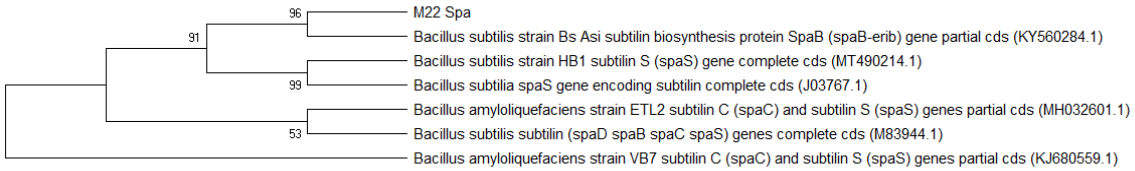

Fig. 2. Phylogeny tree gene subtilin (SpaS) from *Bacillus* CgM22

Sequencing results *Bacillus* CgM22 with a subtilin primer can be seen in the phylogeny tree of Fig. (3). Homological results of SpaS sequence from isolate *Bacillus* CgM22 has a homological similarity to *B. subtilis* strain Bs Asi subtilin (spaB-erib) (KY580284.1) with a bootstrap value of 96. This value is high and trustworthy. As it is known, antimicrobial peptides (AMPs) in *B. subtillis* are dominated by biosynthetic genes, namely survacitin, bacylisin, fengycin, bacyllomicin, subtilin and iturin **(Amruta *et al.*, 2016; Kumar *et al.*, 2017)**. This gene can result in the inhibition of the pathogen's radial growth **(Mora *et al.*, 2011)**.

AMPs produced by group species *B. subtilis* among them are subtilin, ericin, sublancin, and subtilosin A. Subtilin shows antibacterial activity against pathogenic bacteria such as *B. cereus, E. coli, S. aureus* and *L. monocytogenes* **(Sutyak *et al.*, 2008; Compaore *et al.*, 2013; Kumariya *et al.*, 2019)**. Strains *Bacillus* and others also produce identical antibiotics including lanthipeptide subtilin **(Helfrich & Stein 2022)**. Strains *B. subtilis* W168 also produces subtilin and produces other antibiotics **(Zhang *et al.*, 2022)**.

Results of TLR 2 gene expression analysis from fish kidney and liver

Based on the molecular analysis of AMPs in *Bacillus* CgM22 isolated from the fish intestine, the immune system in carp is induced through the feed. **Wang *et al.* (2021)** have investigated TLR2 gene expression levels in the kidney and liver across different treatment groups. The results of RNA concentration and purity measurements showed that the three samples had sufficient quality and quantity to proceed to the stage of RNA gene expression analysis with RT-qPCR (Real time PCR). Housekeeping gene used is β-actin **(Dheda *et al.*, 2004)**. The use of β-actin is used because it is abundant in eckaryotic cells and is expressed continuously at each stage of development and in all tissues of eukaryotic organisms **(Dheda *et al.*, 2004)**. Furthermore, for the normalization of RNA input, the gene expression level was analyzed with $2^{-\,ddCt}$ **(Livak & Schmittgen, 2001)**.

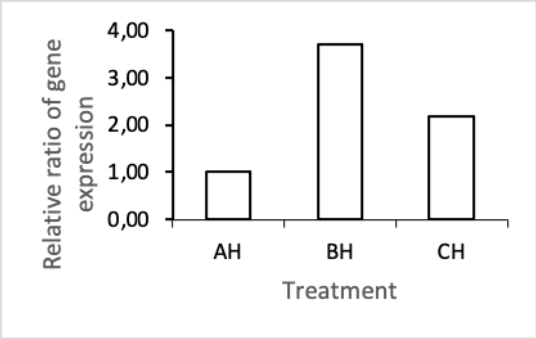

Fig. 3. Relative expression of RNA in the Liver (TLR2 normalized with β-actin) (AH) Control, (BH) bacterial culture at 10^8 CFU/mLat 15 mL/kg feed (CH) Addition of Supernatant from *Bacillus* CgM22 as much as 5 ml/kg of feed

Based on the results obtained regarding the relative ratio of TLR gene expression in the liver organs treated A, B, and C (Fig. 4), there are differences in each treatment. During the sample processing, sample D was not in good condition and unsuccessful, so only three samples were continued. The results of the calculation by the ddCT analysis method according to **Zhang *et al.* (2013)**. AH treatment is very different from BH and CH treatment which is given Bacillus CgM22. Relative ratio of TLR 2 gene expression was highest in BH treatment to culture administration treatment Bacillus CgM22 108 CFU/mL at a dose of 15 ml/kg feed and the lowest in the AH treatment with control treatment. This is suspected because the culture content is higher than the content

contained in the supernatant. It is worthy to mention that the culture content in *Bacillus subtilis* can be widely identified regarding its antimicrobial activity **(Biver *et al.*, 2018)**.

Based on the results of the gene expression research in shrimp, an increase in TLR2 in the liver was produced in *Oreochromis niloticus* and increase immunity against *Streptococcus agalactiae* **(Kuebutornye *et al.*, 2020)**. In addition, gene expression in fish liver was also analyzed in the rainbow trout fish (*Oncorhynchus mykiss*) to find out the immune genes. In the study, the genes observed were genes related to immunity such as SAA, IL-8, IL-1β, TGF-β, TNF-α, IFN-γ and IgM against immunity and resistance to disease caused by *Y. ruckery* **(Yilmaz, 2019)**. The results showed that gene expression in the liver showed increased immunity and disease resistance. This is also in line with research by **Adeshina *et al.* (2020)** that the administration of probiotics to carp (*Cyprinus carpio*) showed results in increased gene expression compared to control treatment.

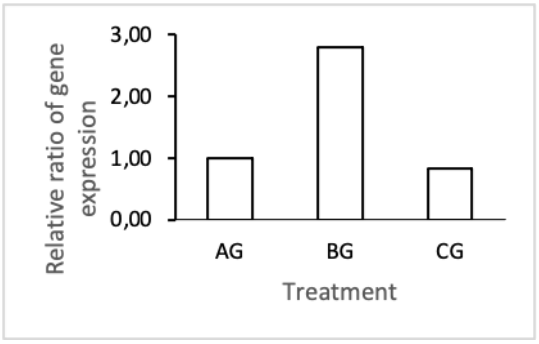

Fig. 4. Relative expression of RNA in the kidney (TLR2 normalized with β-actin) (AG) Control, (BG) bacterial culture at 10^8 CFU/mLat 15 mL/kg feed (CG) Addition of Supernatant from *Bacillus* CgM22 as much as 5 ml/kg of feed

Based on Fig. (5), the results of the relative ratio of TLR gene expression in the renal organs treated A, B, and C were different in each treatment. Due to poor condition and unsuccessful processing, sample D was excluded, leaving only three samples for further analysis. Relative ratio of highest TLR gene expression in BH and GH treatment to culture treatment *Bacillus* CgM22 was 15 ml/kg and the lowest in AH and CG treatments. This is in accordance with the results of TLR2 gene expression in the liver that the culture treatment is the highest treatment. Gene-related expression Toll-like receptors (TLR) which belongs to the group of glycoproteins that function as transmembrane surface receptors and are involved in the immune response. TLR is a key component of the non-specific immune response that can recognize components of microorganisms. The TLR then initiates signaling pathways to activate cytokines, chemokines, and antimicrobial peptides **(Petry & Gaspari, 2009)**. TLR can improve the binding and regulation of costimulation molecules involved in specific and non-specific immune responses. Therefore, it can be said that TLR has an important role in a specific and specific immune response in providing an immune response to microorganisms or

pathogens. Lymphocyte immune cells are one of the immune cells expressing TLR in a specific immune system **(Erniati & Ezraneti, 2020)**.

Toll-like receptor is a recognition molecule for several pathogens, including bacteria, viruses, fungi, and parasites. TLR2 forms heterodimers with TLR1 and TLR6. This is the first step in a series of events leading to a significant innate immune response, the development of adaptive immunity to the pathogen and protection from immune sequelae associated with infection with this pathogen. Immune response to canonical and non-canonical responses to TLR2 ligands that affect the state of the disease or protection from disease so that it can increase immunity in the body **(Oliveira-Nascimento *et al.*, 2012)**.

This is in accordance with several studies showing probiotics from *Bacillus* strains such as *B. cereus, B. licheniformis* and *B. subtilis* can improve the immunity of aquatic fish by increasing the inflammatory response. In this study, higher levels of expression of TLR-4, MyD88 and IL-6 were observed in carp fed *B. subtilis* and there was an increase in carp immunity **(Summer *et al.*, 2013; Galagarza *et al.*, 2018; Midhun *et al.*, 2019; Xue *et al.*, 2020)**.

Other studies have also reported that gene expression in the kidney *Cyprinus carpio* induced by various strains of *Bacillus* including *B. subtilis C-3102, B. licheniformis, B. amyloliquefaciens, B. coagulans* increased expression of immune-related genes compared to control treatment **(Summer *et al.*, 2011; Chen *et al.*, 2015b; Huang *et al.*, 2015; Jiang *et al.*, 2022; Jiang *et al.*, 2022)**. The presence of antimicrobial peptide (AMP) genes in *Bacillus subtilis* can influence TLR2 gene expression and modulate the immune response in carp. *Bacillus subtilis* produces various AMPs, such as surfactins, iturins, and fengycins, which have been shown to interact with the host's immune system. In grass carp (*Ctenopharyngodon idella*), stimulation with *Bacillus subtilis* has been observed to upregulate the expression of both pro-inflammatory and anti-inflammatory cytokines in dendritic cells, indicating an immunomodulatory effect **(Zhou *et al.*, 2019)**. The kidney organ is an important organ for capturing and cleaning bacteria **(Yılmaz & Ergün, 2018)**. In addition, the kidney are organs that function as the main regulators in immune-endocrine interactions and even neuro-immuno-endocrine interactions **(Altunoglu *et al.*, 2017)**. The increased expression of the TLR2 gene in liver and kidney samples, which are important organs in the immune system, indicates that the addition of *Bacillus* CgM22 enhances immunity in carp (*Cyprinus carpio*).

CONCLUSION

Molecular analysis of antimicrobial peptide (AMP) genes revealed that the *SrfAA* sequence from *Bacillus* CgM22 shares 98% similarity with the partial *surf*actin gene of *B. subtilis* strain SP11 (KC797003.1). Similarly, the SpaS sequence shares 96% similarity with the *subtilin* (*spaB-erib*) gene of *B. subtilis* strain Bs Asi (KY580284.1). The highest *TLR2* gene expression, observed in treatment B (*Bacillus* CgM22 at 10^8 CFU/mL,

administered at 15 mL/kg of feed), demonstrated its potential to enhance carp resistance, with a 3.7-fold increase in the liver and 2.7-fold in the kidney. These results highlight the need for further research on the antimicrobial peptide activity of *Bacillus* species in various fish species.

ACKNOWLEDGMENT

Thanks to all the committee members of the International Seminar of Indonesian Seas: Catalyst for Ocean Sustainability (ISCO) 2024, initiated by Faculty of Fisheries and Marine Sciences, Universitas Padjadjaran, who have facilitated the publication process of this manuscript until it was published in the Egyptian Journal of Aquatic Biology and Fisheries.

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