JOURNAL OF PURE AND APPLIED MICROBIOLOGY, June 2018. Vol. **12**(2), p. 601-610

Intestinal Bacteria of Common Carp (*Cyprinus carpio* L.) as a Biological Control Agent for *Aeromonas*

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http://dx.doi.org/10.22207/JPAM.12.2.18

(Received: 01 March 2018; accepted: 18 April 2018)

The microbiota of fish intestine is the most complex of all organs that interact closely with the immune system. This can be used as biological control agents that can control the disease caused by pathogenic bacteria. Thirty isolates were identified based on 16s rRNA gene sequencing. These bacteria were then tested for their pathogenicity and antagonistic activity. The bioassay was conducted by administering selected bacteria into the fish and inoculating with *Aeromonas.* Total 30 bacterial isolates were successfully isolated from the carp intestine. Morphology and DNA marker analysis shows wide diversity of bacterial consortium within the fish intestine. These bacterial community are dominated by Proteobacteria and Firmicutes. The pathogenicity test showed that 20 isolates were non-pathogenic at a density of 10^6 cfu/mL, while the rest were pathogenic to the fish. The antagonistic test showed that some isolates strongly or mildly inhibit Aeromonas and Vibrio and the rest weakly or do not inhibit the assayed bacteria. Two isolates (CgM8=*Bacillus sp.* and CgM37=*Bacillus subtilis*) are significantly better than control to protect the fish from Aeromonas infection. Two species of commensal bacteria originated from fish intestine are potential to be used as biological control agents against Aeromonas for common carp.

Keywords: *Aeromonas*; biological control; *Cyprinus carpio;* intestinal bacteria, *Bacillus subtilis.*

Common carp (*Cyprinus carpio* L.) is a freshwater fish that is widely cultivated in Indonesia. This fish has a rapid growth and high fecundity, so it has a high potential to become a source of protein. Cultivation of carp has quite a rapid development, but there are limitations in the cultivation of this fish. Like some other fish species, in the aquaculture sector, one of the limiting factors for its development is disease control[1]. *Aeromonas* is the causative agent of MAS (Motile Aeromonas Septicemia). It is either acute or subacute or even a chronic infectious disease of all freshwater fishes, characterized by rapidly fatal septicemia with a few gross signs, exophthalmia, ascitis and ulcer formation. The bacteria *Aeromonas* is a widely distributed pathogenic bacteria, and killed the fish up to 80-100% within 1-2 weeks[2]. Most cultured and wild fish are susceptible to infection with *Aeromonas* such as carp, channel catfish, eel, goldfish, snakehead fish, rainbow trout, brown trout and tilapia.

Control of bacterial diseases that have been done is to use antibiotics. But the use of this material creates a dependence on the provision of antibiotics, as well as increase production costs because the price is relatively high. The presence of antibiotics as one of the efforts to overcome diseases has many negative impacts, such as causing microbial resistance and producing

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residues that are harmful to humans who consume them. Therefore, it is important to search for other alternatives in controlling fish diseases more safely and effectively. The use of biological control agents, such as bacteria can be one of the alternatives. Bacteria that have been widely used as biological control agents in the field of aquaculture usually come from Lactic Acid Bacteria (LAB) or from the genus *Bacillus*, but these bacteria are mostly isolated from mammals or from terrestrial areas, not aquatic environments, therefore it renders these probiotics less effective when used for aquaculture[2-4].

A fish's intestine plays an important role on its health status, because the organ provides a micro-environment where there are numerous microbiota that interact with each other, and also interact with the host. The biological control agents which originate from the commensal bacteria within the fish itself are more advantageous than the bacteria from the outside because the bacteria have been proven to colonize and adapt to their host environment. Therefore, microbial communities of each aquatic species need to be identified to provide a more effective chance of developing new probiotics or new immunostimulants[5]

For different experimental purposes, the intestinal microbial flora of fish has been studied by several workers. These include microbial flora as food of fish[6-7], microbial flora's role in production of enzymes[8] and antibiotic resistance profile of indigenous flora[9-10]. The micro flora of reared fish has also been studied as a source of protection against diseases[11-13] For all these reasons, study of intestinal bacterial flora is important.

Microbiota in fish intestines have been extensively studied, such as *Oncorhynchus mykiss*[14], *Garra mullya*[15], *Ictalurus punctatus*, *Micropterus salmoides*, and *Lepomis macrochirus*[5]. Bacterial communities of several carp species have also been studied, namely grass carp (*Ctenopharyngodon idellus*), crucian carp (*Carassius cuvieri*), and bighead carp (*Hypophthalmichthys nobilis*)[16]. While the potentials of bacteria as probiotics, immunostimulants and / or biological control agents have been investigated, such as *Bacillus subtilis* on Koi and Nila[17-18], Lactobacillus on Flounder and Grouper[19], *Enterococcus faecalis* for Snakehead fish[20]. In this study we have analyzed the intestinal bacterial of a Common carp. There are few studies that discuss intestinal bacteria, but studies exploring Common carp intestinal bacteria as a biological control agent for *Aeromonas* are even rarer.

The aim of this study was to isolate the Common carp's intestinal bacteria, to study the morphological characterization and molecular identification of the isolated bacteria, to analyze the diversity of the bacteria based on the 16s rRNA gene sequence, and to explore its potential as a biological control agent for *Aeromonas*.

MATERIALS AND METHODS

Collection of fish and Isolation of intestinal bacteria

Common carp was collected from Floating nets cage in Cirata reservoir, West Java, Indonesia. The fresh fish was rinsed with sterile aquadest and surface sterilized with ethanol (70%). The intestine were removed by dissection in sterile condition, then scraped the inner part and suspended in sterile saline and serially diluted in test tube. The presence of bacteria were checked by performing wet mount of the saline suspension. Bacterial suspension was then streaked on NA (Nutrient Agar) and MRS (de Man Rogosa Sharpe) agar media. The plates were incubated at 37^{o}C for 24 h.

Molecular identification

Bacterial isolates were identified by 16s rRNA gene sequencing (Macrogen, Korea). All sequences were edited in BIOEDIT. Sequencing data were compared with DNA databases using the BLAST in EzTaxon (www.EzBioCloud.net). Neighbor-joining analysis was performed using MEGA7 software package. The confidence limits for the tree topology were estimated using 1000 x bootstrap analysis.

Pathogenicity and Antagonistic Test

Thirty bacterial isolates were tested to their pathogenicity test to fish survival. Antagonistic activity of the isolates were tested *in-vitro* against three bacterial pathogens (*Aeromonas hydrophila*, *Staphylococcus epidermidis* and *Vibrio* sp.)

Testing bacteria in fish (Induction and Challenge experiment)

A direct immersion method was done to deliver the bacteria as a biological control agent into the fish. The selected 3 isolates (8, 36 and 37)

were administered by soaking the fish into the bacterial suspension over 30 minutes separately. Bacterial density was set at up at 10^6 cfu/ml before admistering into the fish and then maintaining the fish for 12 days to induce the immune system. During maintanance, the fish were feed twice daily with a standard fish pellet. Challenge test to see the effects of selected bacteria on the fish survival, a pathogenic strain of Aeromonas was inoculated into the fish tank at a density of 10^6 cfu/ml. The challenge test was performed over 4 days before counting the fish survival for each treatment.

Data Analysis

The challenge experiment data were analyzed using variance analysis (ANOVA) with Complete Randomized Design (Four treatments with three replicates). If the result of ANOVA showed a significant effect ($p < 0.05$), further test was carried out using Duncan's Multiple Range Test (DMRT) with a confidence significance level of 5%.

RESULTS

Bacterial Characterization and Identification

Thirty bacterial strains were isolated from the intestine of Common Carp. Bacteria that grew on NA medium were dominated by Gram negative bacilli, while bacteria that grew on MRS medium were mostly Gram positive bacilli or cocci. Molecular identification of the intestinal bacteria based on 16s rRNA gene was performed. Phylogenetic tree was constructed from the 30 isolates. The phylogenetic analysis suggested that CgM36 isolate was closely related to *Enterococcus faecalis* (Fig. 1). The same analysis was also used to identify the other isolates (Table 1)

Table 1 indicates that Firmicutes and Proteobacteria are dominantly found in the fish intestine. In this study, the NA culture medium (CgN) was dominated by *Proteus*. Molecular data showed, their proximity is close to *Proteus mirabilis* species. In addition, other genus were also found such as *Enterobacter* , *Bacillus*, as well as *Niveispirillum* and *Blautia*. In the MRS agar medium, cultured bacteria (CgM) which belong to the Lactic Acid Bacteria group were also obtained from *Lactococcus* and *Enterococcus* genera. Other generas were also recovered from MRS medium including *Staphylococcus* and *Rumeliibacillus*.

Pathogenicity Test

Before choosing as a biological control agent, the bacterial isolates were confirmed first for their pathogenic effects to the host. The fish model should be challenged with the bacterial isolates, under normal or stress conditions[3]. In this research, the fish was immersed in a suspension of the candidate bacteria. From the pathogenicity test, thirty isolates , show the varied survival rate as shown in figure 2.

There were 20 non-pathogenic isolates at density of 10^6 cfu/mL, while the other 10 were pathogenic. Eight pathogenic isolates were obtained from NA medium (2, 4, 9,10, 15, 16,17, 18) and 2 isolates were obtained from MRS agar medium (1 and 19a).

Antagonistic Test

Bacterial antagonism occure in nature, therefore microbial interactions play a major role in the equilibrium between beneficial and potentially pathogenic microorganisms. However, the composition of microbial communities can be altered by any human activities including animal husbandry practices and environmental conditions that stimulate the proliferation of certain bacterial species. The microbiota in the gastrointestinal tract of aquatic animals are also influenced by aquatic microbes. Intestinal microbial manipulation constitutes a viable tool to reduce or eliminate the incidence of opportunist pathogens[21].

In this study, thirty bacterial isolates were tested for their antagonisity against three pathogenic bacteria, *Aeromonas* and *Vibrio* (representative from pathogenic Gram negative bacteria group), and *Staphylococcus* (representative from pathoegenic Gram positive bacterial group). The results of the antagonistic test are shown in Figure 3.

Figure. 3 shows that there are 12 isolates of intestinal bacteria can inhibit Aeromonas and 7 bacterial isolates can inhibit *Aeromonas* and *Vibrio.* There is no isolate that only inhibits Vibrio without inhibiting *Aeromonas.* The data also show that 8 isolates can inhibit *Staphylococcus* and 4 isolates can inhibit all three bacteria.

Bioassay

From the results of bacterial identification, pathogenic and antagonistic tests, three of bacterial species were selected for further testing. The selected bacterial isolates were obtained from

isolate CgM8 (*Bacillus sp.*), CgM36 (*Enterococcus faecalis*) and CgM37 (*Bacillus subtilis*). All three bacterial species were introduced to the fish, and incubated first for 12 days for the induction of the immune system. Afterwards, they were then challenged with *Aeromonas*. The highest percentage of survival rate of the fish was obtained from the treatment with isolate CgM37 (Figure. 4).

DISCUSSION

Media for the cultivation of microorganisms contain the substances necessary to support the growth of microorganisms. Due to the diversity of microorganisms and their diverse metabolic pathways, there are numerous media[22]. Nutrient agar (NA) is a medium for the isolation, cultivation and maintenance of a wide variety of microorganisms. NA is one of the most commonly used media in bacteriological procedures such as water testing, procedures run on food products or culture stock, for growth of samples in bacterial tests, and for isolating organisms in pure cultures. In this study, the NA culture (CgN) obtained was dominated by *Proteus*, although morphologically seen differently, but after molecular analysis, its proximity was similar to *Proteus mirabilis* species. *Proteus* is widely distributed in animals, polluted water, and is also found in the intestines of humans. In this research, this genus dominated intestinal microbial communities cultured with NA medium. The dominance of this species is in accordance with the opinion of Schaffer and Pearson[23] which states that *Proteus mirabili*s is well-known in clinical

Table 1. Molecular identify of intestinal bacteria of Common Carp based on 16s rRNA gene

No.	Isolate	Top-hit taxon	Top-hit strain	Similarity (%)
1	CgN1	*Enterobacter cloacae*	LMG 2683(T)	92.48
2	CgN2	*Bacillus flexus*	NBRC 15715(T)	95.80
3	CgN3	*Bacillus flexus*	NBRC 15715(T)	95.50
4	CgN4	*Bacillus cereus*	ATCC 14579(T)	95.59
5	CgN5	*Proteus mirabilis*	ATCC 29906(T)	88.50
6	CgN6	*Proteus mirabilis*	ATCC 29906(T)	80.87
7	CgN9	*Bacillus carboniphilus*	JCM 9731(T)	68.48
8	CgN10	AF426002_s *Niveispirillum sp.*	UNSW7	59.79
9	CgN12	FJ369991_s *Blautia sp.*	TS55_a03b11	55.44
10	CgN13	*Proteus mirabilis*	ATCC 29906(T)	85.36
11	CgN14	*Proteus mirabilis*	ATCC 29906(T)	85.13
12	CgN15	*Proteus mirabilis*	ATCC 29906(T)	88.44
13	CgN16	*Proteus mirabilis*	ATCC 29906(T)	80.82
14	CgN17	*Proteus mirabilis*	ATCC 29906(T)	87.14
15	CgN18	*Proteus mirabilis*	ATCC 29906(T)	77.03
16	CgM1	*Bacillus haynesii*	NRRL B-41327(T)	99.79
17	CgM5	*Bacillus licheniformis*	ATCC 14580(T)	98.30
18	CgM6	*Staphylococcus gallinarum*	ATCC 35539(T)	99.41
19	CgM8	CP013984_s *Bacillus sp.*	DE111	99.54
20	CgM15	*Staphylococcus gallinarum*	ATCC 35539(T)	99.55
21	CgM16	*Lactococcus garvieae*	ATCC 49156(T)	99.12
22	CgM18	*Bacillus zhangzhouensis*	DW5-4(T)	100.00
23	CgM19	*Rummeliibacillus stabekisii*	KSC-SF6g(T)	99.87
24	CgM22	CP013984_s *Bacillus sp.*	DE111	98.57
25	CgM34	*Bacillus tequilensis*	KCTC 13622(T)	98.29
26	CgM36	*Enterococcus faecalis*	ATCC 19433(T)	98.22
27	CgM37	*Bacillus subtilis*	KCTC 13429(T)	99.34
28	CgM38	CP013984_s *Bacillus sp.*	DE111	99.56
29	CgM19a	*Bacillus albus*	N35-10-2(T)	96.70
30	CgM20a	*Bacillus megaterium*	NBRC 15308(T)	95.21

laboratories and microbiology survey as the species that swarms across agar surfaces, overtaking any other species present in the process. *P. mirabilis* is often isolated from the gastrointestinal tract, although whether it is a commensal, a pathogen, or a transient organism, is somewhat controversial.

MRS agar is used for the isolation, cultivation, and maintenance of *Lactobacillus* species from clinical specimens, foods, and dairy products. It is a good medium for the cultivation of lactic acid bacteria. MRS agar contains polysorbate, acetate, magnesium, and manganese that act as growth factors for Lactobacillus. In order to act as medium, MRS is enriched by various nutrients to support the growth of lactic acid bacteria, but not very selectively, so it still allows other types of bacteria to grow.

Table 1 indicates that Proteobacteria and Firmicutes are dominantly found as the common phyla. The results of this study are similar to those found by other studies. Proteobacteria, Firmicutes and Actinobacteria were the dominant allochthonous microbiota in the gut content of grass carp cultured in pond[24] while Luo *et al*. [25] identicated Proteobacteria, Firmicutes, Bacteroides and Actinobacteria as the dominant allochthonous bacteria in the intestine of transgenic carp. Al Harbi and Udin[26] studied that many of the bacteria found in Tilapia intestine were predominantly from proteobacteria and firmicutes. In the rainbow trout intestine, Kim and Austin[14] found that Aeromonadaceae, Enterobacteriaceae and Pseudomonadaceae representatives were the dominant cultured bacteria.

In zebrafish, the morphologically different bacteria found were *Aeromonas*, *Vibrio*, *Photobacterium*, *Pseudomonas*, *Comamonas*, *Ochrobactrum*, and *Staphylococcus*[27]. One prominent difference found in the present study compared to the study of Roeselers *et al*.[28] was that Firmicutes represented the second most abundant phylum. Firmicutes represents one of the most abundant phyla in the mammalian intestine, and members of Clostridia within Firmicutes are obligate anaerobes[29].

Perez *et al*.[30] stated that the intestinal microbiota of freshwater species tend to be dominated by members of the genera *Acinetobacter*,

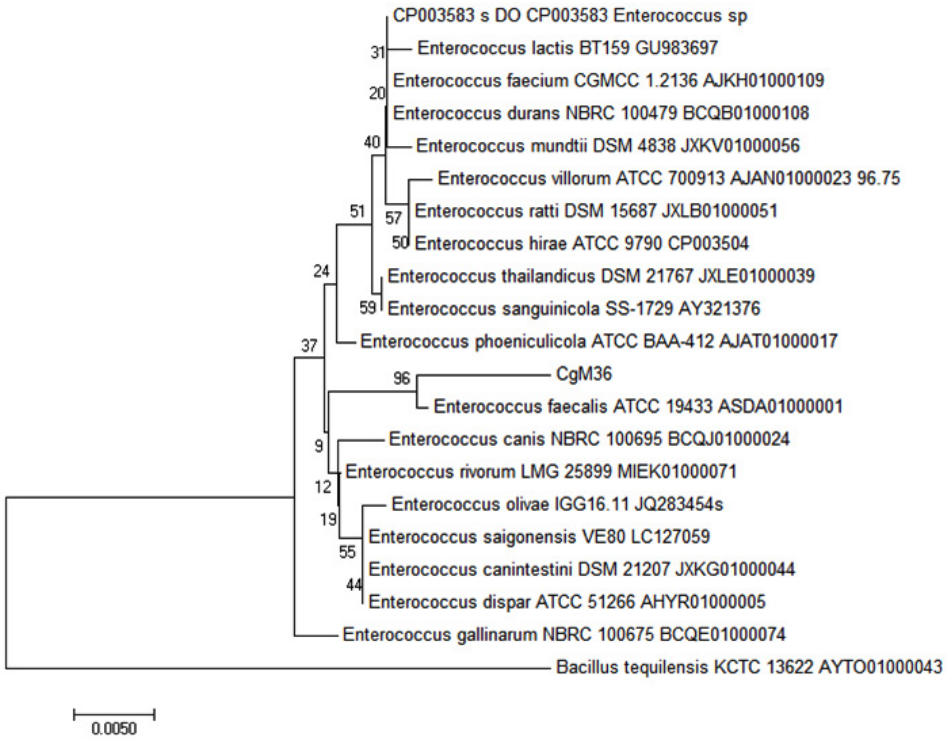

Fig. 1. Phylogenetic analysis of CgM36 isolate based on 16s rRNA gene sequence with MEGA 7

Aeromonas, *Flavobacterium*, *Lactococcus* and *Pseudomonas*, representatives of the family Enterobacteriaceae, and obligate anaerobic bacteria of the genera *Bacteroides, Clostridium*, and *Fusobacterium*. The intestinal content in grass carp, crucian carp, and bighead carp were dominated by four major phyla, including Fusobacteria, Firmicutes, Proteobacteria and Bacteroidetes[16].

The result on the pathogenicity test shows that there are 20 non-pathogenic isolates at the density of 10^6 cfu/mL, while the other isolates are pathogenic. In this study, there are several

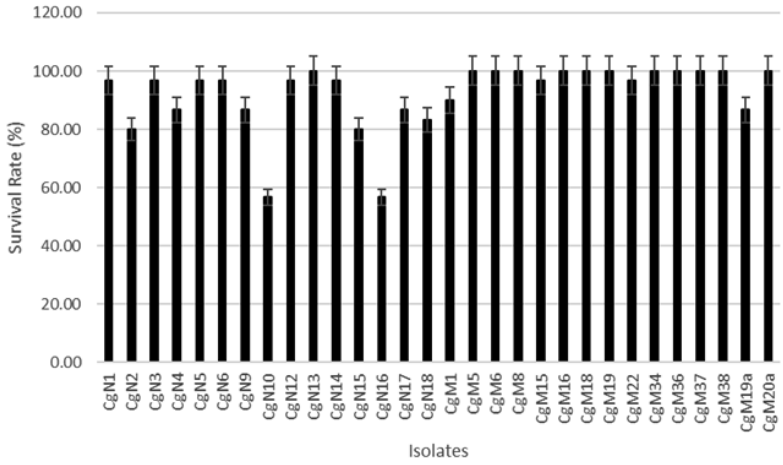

Fig. 2. The Survival Rate of Common carp after infected with bacterial isolates at a density of 10^6 cfu/mL

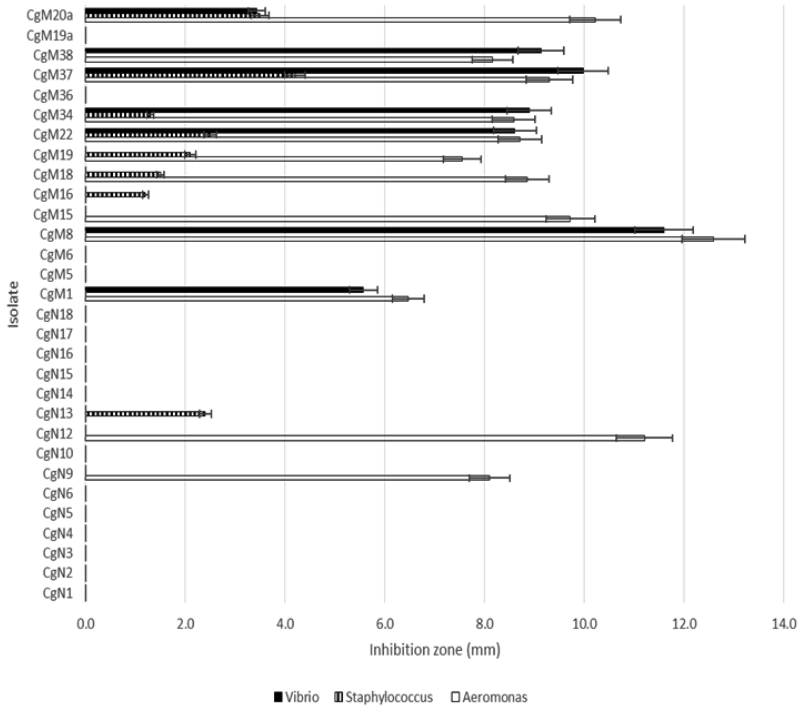

Fig. 3. The result of *in vitro* antagonism test of intestinal bacterial isolates against *Aeromonas*, *Vibrio* and *Staphylococcus*

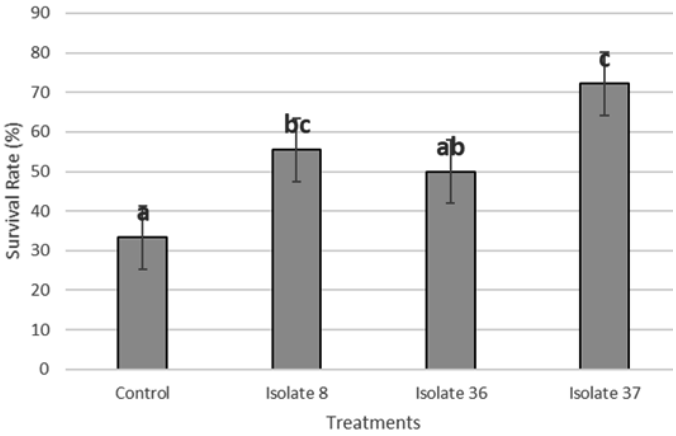

Fig. 4. The survival rate of Common carp treated with bacterial isolates CgM 8, 36, and 37

isolates that are pathogenic when tested. The type of bacteria that are pathogenic include *Bacillus* and *Proteus*. In general, bacterial pathogens are caused by their ability to produce endotoxins and exotoxins, such as LPS, and their ability to produce certain enzymes or proteins that can damage the immune system in fish. In addition, some bacteria have the ability to lyse cells or blood hosts, so that the tissue or organ hosts become damaged[31].

The antibacterial effect of bacteria is due to the production of antibiotics, bacteriocins, siderophores, enzyme and/or hydrogen peroxide and the alteration of pH values by the production of organic acid[2]. The inhibition of pathogenic bacteria occurs by destroying the cell wall causing lysis or inhibiting cell wall growth in the growing bacterial cell, altering the permeability of the cytoplasmic membrane, causing leakage and resulting in nutrients exiting the cell. In addition, antagonistic bacteria can also inhibit the synthesis of proteins and nucleic acids by denaturing proteins and destroying nucleic acids so that their function as genetic material is lost. Then it also inhibits intracellular enzyme activity that disrupts cell metabolism[32]. Antibacterial compounds can also increase lysozyme activity, which can be used as immunogenic parameters. The lysozyme enzyme works by lysis of bacterial cell walls such as hydrolyzing N-acetylglucosamine and N-acetylmuramic acids in peptidoglycan, so that with the loss of cell walls, the bacteria die[33]. Lyszozymes are more active in Gram-positive bacteria than in Gram-negative bacteria, because the peptidoglycan content in its cells more abundant[34].

Based on this study, cultured bacterial isolates have varying abilities in inhibiting pathogenic bacteria, (*Aeromonas, Vibrio and Staphylococcus*), seen from clear zones formed around disc paper (figure 3). The clear zone is caused by bacterial isolates to produce metabolites which are antibacterial compounds that can inhibit the development of pathogenic bacteria. Antibacterial compounds work by disrupting components of the cell wall, causing plasmolysis resulting in inhibition of growth or death of pathogenic bacteria[35]. The ability of bacterial isolates in inhibiting the growth of pathogenic microbes is one indicator that the isolate has potential as a biological control agent. From the results of antagonistic test of cultured bacteria showed that not all isolates have the ability to inhibit three pathogenic bacteria (*Aeromonas, Vibrio, Staphylococcus*).

The results of the antagonistic test of intestinal bacteria cultured on *Aeromonas* showed that the isolates grown on NA medium which had antagonistic activity in this study were isolates CgN 9 and 12, while isolates grown on MRS medium which had antagonistic activity, were isolates CgM 1, 8, 15, 18, 19, 22, 34, 37, 38, and 20a. The isolates produced clear zones ranging from 6.5 to 12.6 mm. Pan *et al*.[36] states that the clear zone diameter 0-3 mm has a weak inhibition, the 3-6 mm clear zone diameter has good inhibition, and the clear zone diameter of > 6 mm has strong inhibition.

Therefore, all clear zones formed in inhibition of *Aeromonas* are included in the strong inhibition.

The result of the antagonistic test of intestinal bacteria on *Vibrio* pathogen showed that the bacterial isolates had a moderate to strong inhibition. The isolates of CgM 1 and 20a resulted in moderate inhibition, with clear zones ranging from 3.4 to 5.6, whereas CgM 8, 22, 34, 37 and 38 isolates had strong inhibitory powers, resulting in clear zones ranging from 8.6 - 11.6 mm.

The results of the antagonistic test of intestinal bacteria against Staphylococcus showed that the bacterial isolates had a weak to moderate inhibition. Isolate CgN 13 produced a weak inhibition, with a clear zone of 2.4 mm. Isolates CgM 16, 18, 19, 22, and 34 also had weak inhibition, ranging from 1.2 to 2.5. While the isolates CgM 37 and 20a had a moderate inhibition, which produced a clear zone of 4.2 and 3.5 mm.

Differences in intestinal bacterial inhibition to the pathogenic bacteria tested were influenced by the differences in the ability of these bacteria to produce antibacterial compounds, as well as the thickness and composition of pathogenic bacterias'cell wall.

Bioassay was performed using three selected intestinal bacteria, which were isolate CgM8 (*Bacillus sp*.), CgM36 (*Enterococcus faecalis*) and CgM37 (*Bacillus subtilis*). Isolate CgM8 (*Bacillus sp*.) produced the highest inhibition against Aeromonas and Vibrio pathogens, then CgM36 (*Enterococcus faecalis*), which gave the best inhibitory ability against three pathogenic bacteria and CgM37 (*Bacillus subtilis*) produced very weak clear zones in antagonistic tests. The statistical test of the survival of fish on bioassay proved that the two isolates gave significantly different results than controls, proving that these isolates could act as biological control agents, although further studies are still needed.

These bacteria are normal intestinal microflora in common carp. If these species are common in this fish, this findingis not in line with the study of Wu *et al*.[37] who states that lactic acid bacteria can not build large populations in the intestine of grass carp. The results of this study are in line with the statement of Ghosh *et al*.[38] that LAB isolated from the gut of *M. cephalus* provides an opportunity to develop a sustainable and organic means of combating the aquaculture pathogens. It has been demonstrated by Balcázar[39] that a mixture of bacterial strains (*Bacillus* and *Vibrio* sp.) had a beneficial effect on the growth and survival of juveniles of white shrimp besides improving their immunity against *Vibrio harveyi* and white spot syndrome virus. Most probiotics proposed as biological control agents in aquaculture belong to lactic acid bacteria and the genus *Bacillus*, although other genera can also be included[2].

Probiotics or immunostimulant bacteria as biological control agents in aquaculture have several possible modes of action. Those modes are: production of inhibitory compounds; competition for chemicals or available energy; competition for adhesion sites; enhancement of the immune response; improvement of water quality; interaction with phytoplankton; source of macro and micronutrients; and enzymatic contribution to digestion[2].

Some bacteria have been used as immunostimulants, they are susceptible to phagocytosis by macrophages and they stimulate cytokine synthesis. So their immunostimulant effect is due to the release of a mixture of cytokines[40]. Among the cytokine synthesis enhancers, the most effective is bacille Calmette-Guerin (BCG). A live and attenuated vaccine from *Mycobacterium bovis* BCG strain results in elevated B-cell and T-cell mediated response, enhancing phagocytosis and providing resistance to infection[41].

Immunostimulatory bacteria have a major role in inducing innate immune cells to stimulate and modulate the mucosal immune system by decreasing the production of proinflamatory cytokines through NFºB pathways, increasing the production of anti-inflammatory cytokines (IL-10), increasing IgA defenses and affecting the maturation of dendritic cells[42] . Some bacterial products with immunomodulatory properties such as lipopolysaccharide (LPS), peptidoglycan and lipoteichoic acid (LTA) from Bifidobacteria may activate macrophages to evoke an immune response. The bacteria can also increase the production of anti-inflammatory cytokines and reduce the production of proinflammatory cytokines thereby reinforcing the intestinal mucosal barrier[43].

ACKNOWLEDGEMENT

Acknowledgments are addressed to the Ministry of Research, Technology and Higher Education of the Republic of Indonesia through the Post-Graduate Scholarship to provide funding and support for this research.

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