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SHORT COMMUNICATION

Characterization of lactic acid bacteria as a fish potential immunostimulant

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ABSTRACT

Aims: This study aims to characterize the intestinal carp (*Cyprinus carpio* L.) bacteria, especially lactic acid bacteria (LAB) and its potential as immune-stimulant to be applied in the prevention of diseases in fish.
Methodology and result: The bacteria were isolated from carp intestine and cultured in de Mann Rogosa Sharpe (MRS) and glucose yeast peptone agar + calcium carbonate (GYPA+$CaCO_3$) media. The obtained LABs were identified and characterized by 16S rRNA gene primers. The phylogenetic analysis on DNA sequence was performed by using BioEdit and MEGA 7.0 software. The potential immunostimulant were derived from its ability to resist the growth of *Aeromonas* sp. and *Vibrio* sp. as pathogenic bacteria by the paper disc agar diffusion method. The clear zone diameter around the paper disc were measured by using calipers. Forty bacteria that isolated from the carp were selected for lactic acid production and clear zone around the colonies were formed from the GYPA+$CaCO_3$. For further analysis, a total of ten LABs were selected based on different colony forms and the largest clear zone around the colonies. Based on phylogenetic analysis, *Enterococcus*, *Lactobacillus*, *Streptococcus* and *Lactococcus* were found as the genera of lactic acid bacteria.
Conclusion, significance, and impact of study: We discovered that there is a wide diversity among the 40 isolated bacteria. This result indicates that *Enterococcus*, *Lactobacillus*, *Streptococcus* and *Lactococcus* were the common genera. The most potential LAB as an immuno-stimulant was *Lactobacillus* sp.

Keywords: Lactic acid bacteria (LAB); *Cyprinus carpio*; immunostimulant

INTRODUCTION

The lactic acid bacteria (LAB) are aerotolerant anaerobic bacteria, they do not use oxygen in their energy production and grow under anaerobic conditions. However, they can also grow in the presence of oxygen. LAB is non-spore-forming, usually non-motile, rods or cocci, and produce lactic acid as their major end product (Stieglmeier *et al*., 2009; Khalid, 2011). There is a core group consisting of four genera; *Lactobacillus*, *Leuconostoc*, *Pediococcus* and *Streptococcus*, and recent taxonomic reviews have several new genera, and the remaining group now comprises the following: *Aerococcus*, *Alloiococcus*, *Carnobacterium*, *Dolosigranulum*, *Enterococcus*, *Globicatella*, *Lactococcus*, *Oenococcus*, *Tetragenococcus*, *Vagococcus*, and *Weissella*. *Lactobacillus*, *Carnobacterium* and some *Weissella* are rods while the remaining genera are cocci (Jin *et al.*, 1996).

The fish intestine is a composite ecosystem that contains a complex and dynamic group of microorganisms, which plays as a key role in the nutrition and health of the host. The current study of the diversity in the bacterial composition of fish intestine microbiota is largely based on the classical culture-dependent techniques, and the contribution of this approach has been reviewed (Navarrete *et al.*, 2009). However, the bacterial characterization of the carp intestine, especially LAB potentially as immune-stimulants has not been studied. LAB genera have been suggested to be used as probiotics and immune-stimulants as they have shown potential to inhibit pathogen colonization in the intestine through a number of ways such as competitive exclusion (Ringø *et al.*, 2005; Balcázar *et al.*, 2006; Ghosh *et al.*, 2014). This study aims to characterize the intestinal carp (*Cyprinus carpio* L.) bacteria, especially lactic acid bacteria (LAB) and its potential as immune-stimulant to be

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applied in the prevention of diseases in fish that are cultivated both on a small scale and large scale.

MATERIALS AND METHODS

Fish sampling

Common carp was collected in living conditions from floating net cage in Cirata reservoir, Cianjur, West Java, Indonesia. There were 15 fish samples taken. Intake of fish is done using a net, then taken to the laboratory for further analysis.

Isolation of intestinal fish bacteria

Fish samples obtained from Cirata were kept in an aquarium equipped with an aerator. Fish intestine were then surgically removed. Intake of intestinal fish was carried out aseptically in laminar air flow. First step, the fish was surface sterilized with ethanol (70%), and the gut was removed by dissection. The intestine was immersed in sterile saline. The second step, the inside of the intestine is removed by scraping. The scraping results are suspended in a sterile saline. The presence and type of bacteria were checked by performing a wet mount of the saline suspension. The bacterial suspension was streaked with ose wire on deMann Rogosa Sharpe (MRS) agar media (Oxoid) in Petri dish. The plates were incubated at 37 °C for 24 h. The colony was observed visually, and each different colony was taken to be purified. Once a single colony is obtained, then screened to identify lactic acid bacteria.

Screening for LAB

The LAB screening step is carried out by growing the purified colony with characteristics grow apart, white and 0.1-3 mm in size on MRS were reinoculated on glucose yeast peptone agar + calcium carbonate (GYPA+$CaCO_3$) medium, then incubated at 37 °C for 48 h. Furthermore, the colonies that grow, white and there are clear zones around them were isolated and stored on the MRS medium to be molecularly identified.

Molecular identification

Molecular identification begins with the isolation of bacterial DNA using the boiling method, after which isolate DNA is used as a template for amplification of the 16S rRNA genes was undertaken using the forward primer F, 5'-GAGTTTGATCCTGGCTCAG-3' and the reverse primer R, 5'-GGCTACCTTGTTACGACTT-3' (Mulyani, 2018). Intestinal bacteria were identified by 16S rRNA gene sequencing (Macrogen) as described by Ringø *et al.* (2006). All sequences were corrected in Bioedit and blasted in Eztaxon, and further analyzed with MEGA7 software.

Test of lactic acid bacteria (LAB) as immune-stimulant

The potential of LAB as immune-stimulant was derived from its ability to resist the growth of *Aeromonas* sp. and *Vibrio* sp. as pathogenic bacteria in aquaculture by the paper disc agar diffusion method. Pathogenic bacteria (*Aeromonas* sp. and *Vibrio* sp.) were spread on nutrient agar medium using L glass. The paper disc containing LAB were placed on the medium with pathogenic bacteria. Furthermore, the plates were incubated for 30 °C at 24 h until a clear zone is formed. Diameter of clear zone around the paper disc was measured by using callipers.

RESULTS AND DISCUSSION

Isolation of LAB

A total of 40 bacterial strains were isolated from the intestine of common carp and culture on MRS agar. The colony characteristic of the isolates were varied characters. The bacteria's ability to produce lactic acid in bacterial isolates was obtained by adding $CaCO_3$ to GYPA medium. This is to see the ability of bacterial isolates in forming a clear zone in the medium, which means to produce lactic acid. Ten LABs were selected for further analysis based on different colony forms and the largest clear zone around the colonies.

Molecular characteristic and phylogenetic analyses

The LAB obtained is identified and characterized by 16S rRNA gene primers. Phylogenetic analyses on the sequencing result were performed using BioEdit and Mega 7 software. The community of LAB from intestine carp is characterized by 16S rRNA gene fragment analysis. The genus of LAB obtained has similarities to the genus of LAB from the GenBank. Based on the phylogenetic analysis of the identified bacteria, the genera of LAB obtained was *Enterococcus*, *Lactobacillus*, *Streptococcus* and *Lactococcus*.

Phylogenetic relationship of the 16S rRNA gene fragment sequences obtained from intestine carp showed that the majority of the sequences recovered from intestine were *Lactococcus*, and only one sequence was closed to *Lactobacillus* (Figure 1). Research of Balcázar *et al.* (2008) showed that the LAB found in fish intestine include *Lactococcus lactis*, *Lactobacillus plantarum*, and *Lactobacillus fermentum*. All those three bacteria can inhibit adhesion of several fish pathogens.

Potential immunostimulant of LAB

The potential immune-stimulant of LAB is derived from its ability to inhibit the growth of Aeromonas sp. and Vibrio sp. as pathogenic bacteria by the paper disc agar diffusion method. The ten LABs were tested for their

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antagonistic ability to exhibited different clear zones. This indicates that the LAB has different capabilities to inhibit the growth of pathogenic bacteria (Figure 2 and Figure 3).

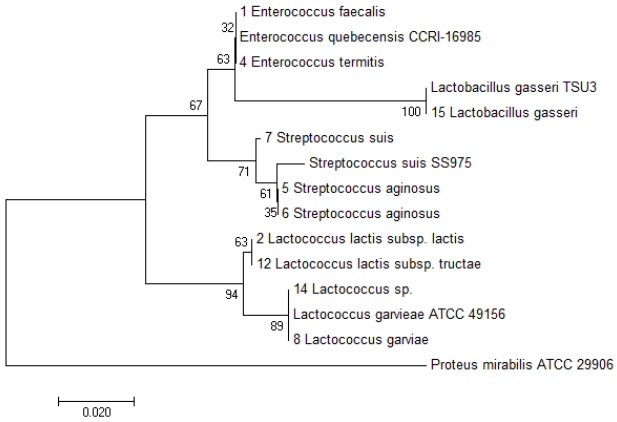

Figure 1: Phylogenetic relationships of lactic acid bacteria from intestine carp.

Figure 2: The clear zone of bacterial inhibition around paper disc on *Aeromonas* sp.

Clear zone formed due to the antagonistic character of the test bacteria against pathogenic bacteria. Effects of antagonistic microorganism produced proteases, bacteriocins, lysozymes, hydrogen peroxide, the formation of ammonia, diacetyl, and alteration of pH values by the production of organic acids. The LAB is known to produce compounds such as bacteriocins that inhibit the growth of other microorganisms (Sugita *et al.*, 2002; Gullian *et al.*, 2004; Balcázar *et al.*, 2006).

Based on Balcázar *et al.* (2008), *L. lactis* can reduce the adhesion of all pathogenic bacteria tested (*Aeromonas hydrophila*, *Aeromonas salmonicida*, *Yersinia ruckeri* and *Vibrio anguillarum*). While *L. plantarum* can only reduce adhesion of *A. hydrophila* and

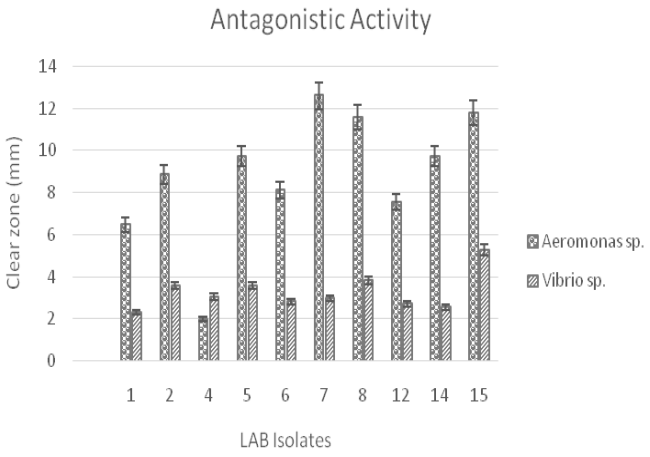

Figure 3: The result of *in vitro* antagonism test of LAB isolates.

A. salmonicida, *L. fermentum* may reduce the adhesion of pathogenic bacteria tested except *V. anguillarum*.

Based on this study, all the selected LAB can inhibit *Aeromonas* and *Vibrio* pathogen bacteria. It was indicated by the formation of clear zones around the disc paper (Figure 2). The clear zone that formed by LAB produces bactericidal metabolites or antibacterial compounds that will damage bacterial cell walls and will cause plasmolysis. In addition, cytoplasmic membranes can be easily damaged by antimicrobials and lead to the growth inhibition or the death of pathogenic bacteria (Brooks *et al.*, 2008). The ability of bacterial isolates to inhibit the growth of pathogenic microbes is the one of indicator that the isolate has the potential as immune-stimulant.

The study shows, almost all isolates bacteria can inhibit *Aeromonas* bacteria except one isolate (isolate 4) (Figure 3). It was shown by the formation of the larger clear zones in *Aeromonas* antagonist test, compared with the antagonistic test on *Vibrio* bacteria. The isolates 1, 2, 5, 6, 7, 8, 12, 14 and 15 yielded clear zones ranging from 6.5-12.6 mm, whereas isolate 4 only yields clear zone of 2.0 mm.

The results of an antagonistic test of LAB on *Vibrio* pathogen showed that bacterial isolates had moderate to poor inhibitory ability. The isolate 2, 4, 5, 8 and 15 had a moderate inhibitory ability with clear zones ranged of 3.05 mm to 5.27 mm. Besides, the isolate 1, 6, 7, 12 and 14 have the weak inhibitory ability with clear zone ranged from 2.30-2.96 mm. According to Pan *et al.* (2009), the bacteria with strong inhibitory ability would form a clear zone of more than 6 mm, the moderate inhibitory ability formed 3-6 mm clear zone, while the weak inhibitory ability formed a 0-3 mm clear zone.

The differences in lactate acid bacterial inhibition resistance to pathogenic bacteria were affected by the ability of lactic acid bacteria to produce antibacterial compound and the thickness of the cell wall composition of pathogenic bacteria.

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The ability of isolates of LABs in inhibiting pathogenic bacteria is caused by acids or other substances such as bacteriocin (Aslim *et al.*, 2005). In addition to producing bacteriocin, the role of LAB as antagonistic bacteria of immune-stimulatory bacterial candidates is to produce the organic acids, such as lactic acid and acetic acid, e.g. the activity of LAB against pathogens in Turbot fish (Vazquez *et al.*, 2005).

CONCLUSION

Based on the results of molecular characterization and phylogenetic analyses, ten isolates of lactic acid that isolated from the gut of common carp, consisted of four genera such as *Enterococcus*, *Lactobacillus*, *Streptococcus* and *Lactococcus*. Nine potential isolates as immunostimulant were found, those are the isolate 1, 2, 5, 6, 7, 8, 12, 14, and 15. These isolates have the strong ability to inhibit the growth of *Aeromonas* bacteria. As a result, clear zone diameter ranges from 6.5 mm to 12.6 mm were formed. However, further studies are needed to identify the mechanism of interaction between the intestinal bacteria and fish as their host.

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