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THE BIOLOGICAL FUNCTIONS OF IMMUNOGLOBULIN Y (IgY) MOLECULES IN AGAINST INFECTION OF *Enterococcus faecalis* ORIGIN OF RED TILAPIA

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ABSTRACT

Red tilapia (*Oreochromis* hybrid) is Indonesia's leading freshwater fishery commodity susceptible to streptococcal bacterial infection. Many studies have been conducted on various efforts to prevent and treat this disease, one of which uses the immunoglobulin Y (IgY) molecule from chicken egg yolk. This study aimed to observe the biological function of IgY against *Enterococcus faecalis* as a cause of streptococcal-like infection. The agglutinin function was conducted by observing the growth of *Enterococcus faecalis* in brain heart infusion (BHI) broth media which was added with IgY suspension. The function of inhibin was performed using a spectrophotometric method to measure the level of turbidity of the bacterial suspension inoculated with IgY suspension. The bactericidal potential through the complementary activation pathway for red tilapia serum was carried out using a scanning electron microscope (SEM) method to evaluate the topography of the bacterial cell wall. The results of the study can be concluded that IgY anti-*Enterococcus faecalis* has the potential as an agglutinin, inhibin, and bactericidal agent through its putative potential in complement activation in streptococcal bacterial infections in red tilapia commodities.

Key words: biological functions, *Enterococcus faecalis,* immunoglobulin Y (IgY), streptococcal-like infection

ABSTRAK

Ikan nila merah (Oreochromis hybrid) merupakan komoditas perikanan air tawar unggulan Indonesia yang rentan terserang infeksi bakteri streptokokal. Studi terhadap berbagai upaya untuk mencegah dan mengobati penyakit tersebut telah banyak dilakukan, salah satunya menggunakan molekul imunoglobulin Y (IgY) asal kuning telur ayam. Tujuan studi dilakukan untuk mengobservasi fungsi biologis IgY dalam melawan Enterococcus faecalis sebagai salah satu penyebab infeksi streptokokal. Fungsi aglutinin dilakukan dengan mengobservasi pertumbuhan Enterococcus faecalis pada media kaldu brain heart infusion (BHI) yang ditambahkan dengan suspensi IgY. Fungsi inhibin dilakukan dengan metode spektrofotometri untuk mengukur tingkat kekeruhan suspensi bakteri yang diinokulasi dengan penambahan suspensi IgY. Potensi bakterisidal melalui jalur aktivasi komplemen terhadap serum ikan nila merah dilakukan dengan metode scanning electron microscope (SEM) untuk mengevaluasi morfologi dinding sel bakteri. Hasil studi dapat disimpulkan bahwa IgY anti-Enterococcus faecalis berpotensi sebagai substansi aglutinin, inhibin, dan sebagai bakterisidal melalui dugaan potensinya terhadap aktivasi komplemen dalam infeksi bakteri streptokokal pada komoditas ikan nila merah.

Kata kunci: fungsi biologis, Enterococcus faecalis, imunoglobulin Y (IgY), infeksi streptokokal

INTRODUCTION

Red tilapia (*Oreochromis* hybrid) is an essential and superior fishery commodity for Indonesia (DJPB KKP 2018). There are many challenges for increasing production, one of which is the decline in population due to infectious diseases. Streptococcal bacterial infections such as *Streptococcus agalactiae*, *Streptococcus iniae* (Intervet/Schering-Plough Animal Health 2009; Taukhid and Purwaningsih 2011; Anshary *et al*. 2014), *Lactococcus garvieae* (Anshary *et al*. 2014), and *Enterococcus faecalis* (Rahman *et al*. 2017; Rizkiantino *et al*. 2020a) are several types of bacteria that can cause disease in tilapia. The use of antibiotics in aquaculture activities to treat bacterial diseases must be avoided to prevent antibiotic resistance or decrease the quality of fishery products due to antibiotic residues. One alternative effort to prevent or treat bacterial diseases is to use biological substances such as antibody molecules that have biological functions against infection with microorganisms in the individual's body. Immunoglobulin Y (IgY) derived from chicken egg yolk has been widely studied as immunoprophylaxis and immunotherapy for various diseases in fish, including vibriosis in Japanese pufferfish (*Takifugu rubripes*) (Zhang *et al*. 2021) and aeromoniasis in *Carassius auratus Gibelio* (Li *et al*. 2006).

Besides playing a role in the specific immune system as humoral defense, antibodies or immunoglobulins also play a significant role in helping to optimize the nonspecific immune system in an individual. Antibodies can act as agglutinins, precipitins, inhibins and help activate the complement system. Agglutinins occur when antibodies can agglomerate antigens in their intact form not to become soluble, and form agglutinates. Likewise, the function of antibodies as precipitins will form precipitates if the antibodies react with their homologous antigen components in a soluble condition. The phenomenon of agglutinates and precipitates can be analogous to sugar which is an insoluble state, and the sugar will lose its ability to cause a sweet taste. The insoluble antigen will lose its ability to infect its host. The growth rate of bacteria can be inhibited due to the presence of antibodies attach in surrounding the bacteria so that the bacteria are unable to perform binary fission, and the antibody acts as inhibin (Paraf and Peltre 1991).

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Another role of antibodies is to activate complements in the individual's body as part of the nonspecific immune system (Pastoret *et al*. 1998). Complement is a heat-labile plasma protein and plays a role in killing several pathogens. These plasma proteins form a system that plays an essential role in the nonspecific immune system. This system is composed of many plasma proteins that react to opsonize pathogens and induce a series of inflammatory processes to be against the infection. Complement proteins are a class of proteases activated by their proteolysis gap. This protein has nine types, namely C1, C2, C3, C4, C5, C6, C7, C8, and C9. Zymogen, precursors for activating complement, circulates in body fluids and tissues without causing side effects to these tissues. At the site of infection, these proteins are locally activated and triggered the inflammation. Complement activation is a cascade in which cleavage and activated by the zymogen to the substrate, in this case, the complement proteins, will repeat until the activated complement protein will turn into a zymogen precursor and activate other complement proteins. There is a physiological setting to prevent excessive complement activation (Janeway *et al*. 2001).

The fish also have a complement system in common with other species. Complement proteins in serum have been reported in eel (*Anguilla anguilla*), tench fish (*Tinca tinca*), several types of freshwater teleost fish, carp (*Cyprinus carpio*), American paddlefish (*Polyodon spatula*), and salmon (Nardi 1938; Legler *et al*. 1967; Chiller *et al*. 1969; Day *et al*. 1970). A study on the complement fixation test on rainbow trout (*Salmo gairdneril*) was conducted by Dorson *et al*. (1979). The presence of these complement proteins in fish can undoubtedly increase the role of the nonspecific immune system against pathogens. The potential of the IgY molecule as a basis for prophylaxis or therapy through pellets is expected to trigger complement activation in fish and to induce the immune system work more optimally. However, further studies regarding the potential of whether IgY can activate the complement system in fish have not been carried out. To complete this research gap, this study was conducted to explore the biological function of antistreptococcal IgY molecules as agglutinin, inhibin, and biological analysis for bactericidal potential through complement activation in red tilapia.

MATERIALS AND METHODS

Agglutinin and Inhibin Potential of Immunoglobulin Y (IgY) Anti-streptococcal Infection

A total of 3 mL of brain-heart infusion (BHI) broth medium was prepared in four tubes. The first tube was added with 3 mL of microencapsulated anti-streptococcal pure IgY suspension (IgY protein concentration of 1.63 mg/mL) from previous research archives (Rizkiantino *et al*. 2020b). One ose of *Enterococcus faecalis* was added to the suspension. The second tube was added as much as 1.5 mL of the microencapsulated antistreptococcal IgY suspension (IgY protein concentration of 1.63 mg/mL) then *Enterococcus faecalis* bacteria was added one ose into the suspension. The third tube only added *Enterococcus faecalis* bacteria; as much as one öse was inoculated into the suspension without adding the anti-streptococcal IgY suspension. The fourth tube was prepared to control without culturing bacteria or adding antistreptococcal IgY suspension. The four tubes were then incubated for two days at 35° C. The suspension in the tube was observed for the first 18 hours whether there was a white cloth at the bottom of the tube to evaluate the potential of IgY as agglutinin. The suspension was then homogenized with a vortex to measure optical density (OD). The OD was measured at a wavelength of 630 nm using a microplate reader (BioTek 800TS, BioSPX, The Netherlands) at 0, 18, 24, 42, and 48 h to determine the potential of IgY as inhibin.

Biological Analysis of Bactericidal Potential Through Activation of the Complement System in Red Tilapia Serum

Biological analysis using scanning electron microscope (SEM) imaging method. Preparation of red tilapia serum as complement protein source from six healthy red tilapia measuring ±250 g. Fish sedation was conducted using the low-temperature method by immersing the fish in ice water for ±3 minutes. As much as 0.25 mL of blood was drawn through the caudal vein for each fish. The blood was then incubated at 4° C for serum collection. Serum was used as a source of fish complement serum. Serum was also examined using the AGPT method to evaluate whether the serum contained antibodies to the bacterium *Enterococcus faecalis* before being used in the test. If the agar gel precipitation test (AGPT) result is negative, the serum can be used and stored at -20° C. The use of test fish for blood sampling has obtained ethical approval from the Animal Ethics Commission of the Faculty of Veterinary Medicine, Bogor Agricultural University (IPB University) with certificate number: 010/KEH/SKE/V/2021.

The analysis was performed by preparing four 1.5 mL microtubes. Tube 1 contained 1 mL of pure *Enterococcus faecalis* bacterial suspension (bacteria concentration 2.1×10^8 CFU/mL) in 0.9% NaCl solution to control normal and live bacterial cells. Tube 2 contained 100 µL of anti-*Enterococcus faecalis* IgY suspension (IgY concentration 1.63 mg/mL), 100 µL of non-inactivated tilapia serum, 100 µL of *Enterococcus faecalis* bacterial suspension (concentration 2.1×10^8 CFU/mL), and a solution PBS pH 7.4 ad. 1 mL as a test tube. Tube 3 contained 100 µL of anti-*Enterococcus faecalis* IgY suspension (IgY concentration 1.63 mg/mL), 100 µL of inactivated tilapia serum at 56° C for 30 minutes, 100 µL of *Enterococcus faecalis* bacterial suspension (concentration 2.1×10^8 CFU/mL), and PBS solution pH 7.4 ad. 1 mL as a negative control. Tube 4 contained 100 µL of *Enterococcus faecalis* bacterial suspension (concentration 2.1×10^8 CFU/mL), 100 µL of the fosfomycin 50 µg/mL, and

PBS solution pH 7.4 ad. 1 mL as a positive control against antibiotics that are bactericidal by inhibiting the formation of cell walls. The four tubes were then incubated for 60 minutes at room temperature. The suspension was then prepared for observation under a SEM JSM IT200 at 10 000× magnification to see the topography of the bacterial cell wall in three dimensions (3D) for treatment at the Zoology Characterization Laboratories, National Research, and Innovation Agency, Bogor, West Java, Indonesia. Data analysis of the potential test results of anti-streptococcal immunoglobulin Y (IgY) as agglutinins, inhibins, and their potential in activating complement in red tilapia serum were analyzed qualitative-descriptive analysis.

RESULTS AND DISCUSSION

Agglutinin and Inhibin Potential of Immunoglobulin Y (IgY) Antistreptococcal Infection

Based on observations of the potential agglutinin test, it was found that the incubation results in the first 18 hours showed that in the tube containing 1.5 mL and 3 mL of antistreptococcal IgY suspension (IgY concentration 1.63 mg/mL) with *Enterococcus faecalis* bacteria, there was a white lump precipitate at the bottom of the tube. The brain heart infusion (BHI) broth media became clearer when compared to tubes containing only bacterial suspension without the addition of IgY suspension (Figure 1).

The optical density (OD) measurement results on the inhibin test are presented in Figure 2. The treatment tube containing 1.5 mL and 3 mL of antistreptococcal IgY suspension (IgY concentration 1.63 mg/mL) with *Enterococcus faecalis* bacteria showed a line curve under the treatment tube containing only bacterial suspension without the addition of IgY suspension. When compared between the treatment with the addition of 1.5 mL and 3 mL of the antistreptococcal IgY suspension, it was seen that there was no significant difference in the measured OD reduction between the addition of IgY suspension to the two-fold volumes.

Immunoglobulins as proteins that play a role in the immune system can function in the body's antigens clearance and make antigens uninfected to their hosts. The presence of antigen attachment fragments on immunoglobulins (paratopes) makes antigens capable of attaching and interacting with antigens (epitopes) so that they are bound by immunoglobulins (Schroeder and Cavacini 2010). Studies conducted by Williams and Gibbons (1972); Wold and Adlerberth (2000) stated that the potential for agglutinins in immunoglobulins (secretory IgA) which can bind to antigens in the form of enteric bacteria, can play a role in the process of preventing bacteria from penetrating the epithelial barrier. Roche *et al*. (2015) also proved that antibodies could block bacteria from colonizing. Sunwoo *et al.* (2010) reported that a specific IgY concentration of 0.54 mg/mLcould inhibit the growth of *Escherichia coli* 987P bacteria in broth media. In the current study, the IgY potential for agglutinin was qualitatively proven that IgY could agglutinate *Enterococcus faecalis* bacteria at the bottom of the tube to make the broth culture medium clearer. The potency of inhibin also proves a decrease in turbidity in the spectrophotometric results of the bacterial culture suspension added with pure anti-*Enterococcus faecalis* IgY with a concentration of 1.63 mg/mL. These two findings have confirmed that IgY can act as a substance that can agglomerate or inhibit the growth of *Enterococcus faecalis* bacteria.

Biological Analysis of Bactericidal Potential Through Activation of the Complement System in Red Tilapia Serum

A photomicrograph was obtained based on imaging results using a SEM, presented in Figure 3. The results

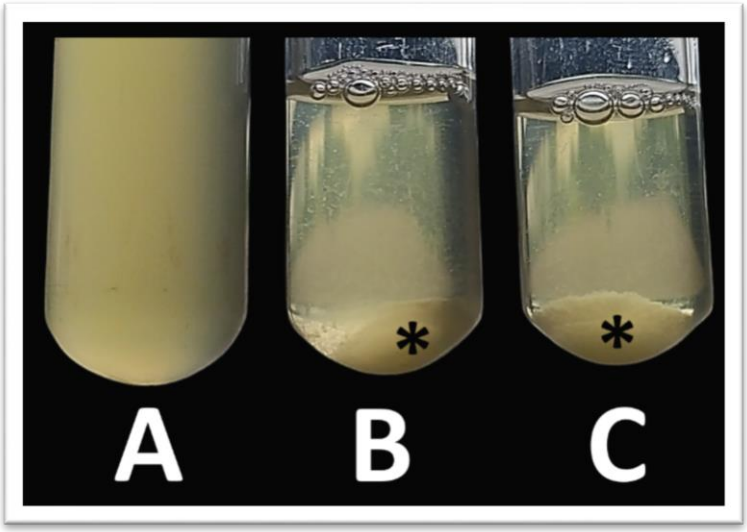

Figure 1. The agglutinin potential of antistreptococcal IgY against *Enterococcus faecalis* bacteria. A= Bacterial culture of *Enterococcus faecalis* without the addition of IgY antistreptococcal infection, B= Bacterial culture of *Enterococcus faecalis* with the addition of 1.5 mL of IgY antistreptococcal infection (IgY concentration 1.63 mg/mL), C= Bacterial culture of *Enterococcus faecalis* with the addition of 3 mL of IgY antistreptococcal infection (IgY concentration 1.63 mg/mL), (Asterisk) White lumpy precipitate at the bottom of the tube (agglutinate)

showed that the cell shape of the *Enterococcus faecalis* bacteria underwent cell wall topography changes when incubated in a suspension containing IgY anti-*Enterococcus faecalis* and the addition of red tilapia serum without inactivation. This treatment showed the holes at the cell poles, deep craters, indentations, and the cell were broken. Moreover, the *Enterococcus faecalis* cells which were incubated in a 50 µg/mL concentration of fosfomycin antibiotic suspension showed morphological changes in the missing of septum cleavage.

Complement activation is the oldest nonspecific immune system and can be found in low to high animals. Members of the phylum Echinoderms are one example of lower animals with a complement system as self-defense against infection (Zhu *et al*. 2005). Protein components that initiate activation of the complement system by alternative pathways and lectins can also be found in ancient jawless fish such as lampreys (Nonaka *et al*. 1984). More modern fish from the classes Chondrichthyes (cartilaginous fish) and Osteichthyes (bony fish) have developed a specific immune system in the form of antibodies or immunoglobulins, causing these fish species to be able to develop a third complement system associated with antigen-antibody binding that forms aggregate, namely the classical pathways (Smith 1998; Nonaka and Smith 2000; Boehm *et al*. 2012; Nonaka 2014).

The complement works through three stages. First, when complement is activated in large quantities, it can assist the phagocytosis process by binding to pathogens and opsonizing them. Second, small fragments of dissociated complement can act as chemoattractants to trigger phagocytic cells towards complement-activated sites. Third, the final process of cascade activation can make a hole in the bacterial cell membrane or the infected host cell so that the cell undergoes lysis. Three pathways can activate a cascade of complement proteins: the classical, the mannan-binding lectin (MB-lectin), and the alternative pathways. The role of antibodies in complement activation is in the classical pathways, where the complement can be activated due to the complex binding between antigen and antibody. The antigen-antibody complex binds to the complement component complex C1 on the single-molecule C1q. The C1q molecule is a protein that contains lectins and has six globular heads surrounded by other zymogens, namely C1r and C1s, forming a complex $(C1r:C1s)_2$ and having a tail conformation formed from collagen. The C1q molecule can also directly bind to the surface of specific pathogens and then trigger complement activation in the absence of antibodies. More than one bond between globular C1q and the pathogen's surface causes a conformational change in the complex

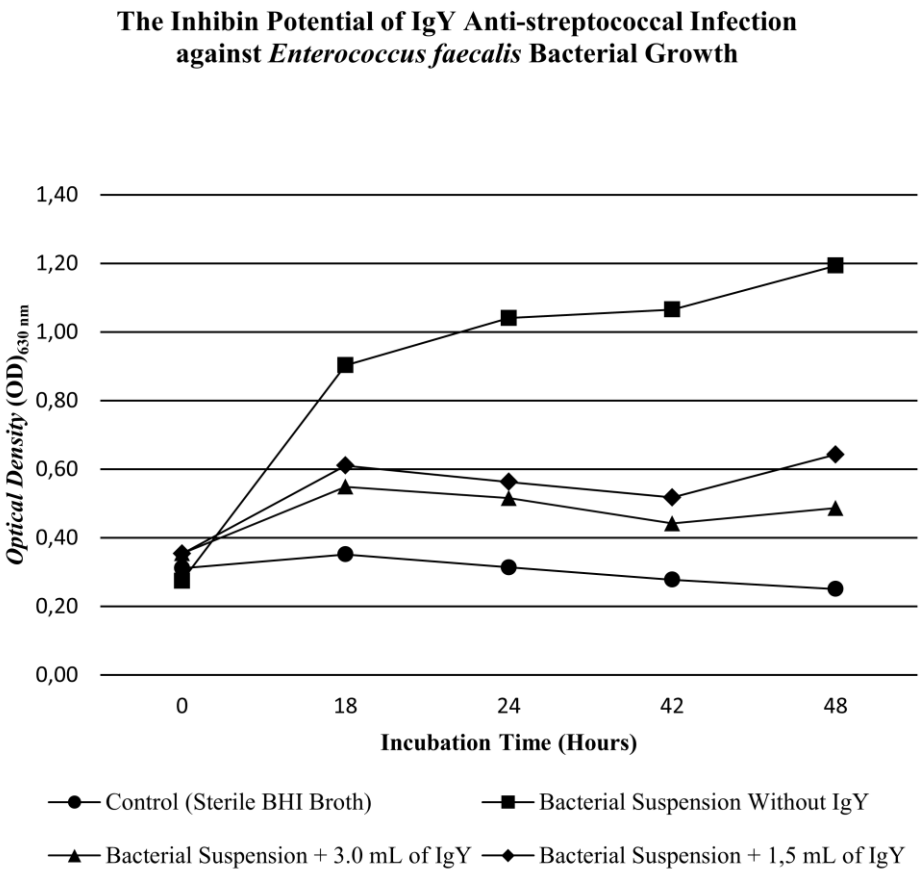

Figure 2. Graph of growth of *Enterococcus faecalis* bacteria inhibited by IgY antistreptococcal-like infection. There was no significant difference in the measured decrease in OD between the two volumes of 1.5 mL and 3 mL of IgY antistreptococcal infection suspension (concentration 1.63 mg/mL), However, the difference was noticeable compared to the tubes containing only bacterial suspension without IgY suspension

$(C1r:C1s)_2$. The active form of C1r can trigger autocatalytic enzymatic activity on C1r so that C1r becomes the active form and associates with C1s to produce an active serine protease. In the presence of an antigen-antibody complex, the fragment of complement (Fc) of the antibody or immunoglobulin can bind to the C1q molecule and change the conformation of the complex $(C1r:C1s)_2$ in the same way that the antibody does not form a complex with the antigen (Janeway *et al*. 2001).

Inactivated fish's complement system can act as a bactericidal agent through the classical and alternative pathways. The pathway that plays a role in killing bacteria through activation of the complement system is strongly influenced by the bacteria's virulence, strain, and capsule components (Boshra *et al*. 2006). Several studies have been conducted on bacteria that cause diseases in fish, such as *Lactococcus garvieae*, *Streptococcus iniae*, *Vibrio anguillarum*, and *Flavobacterium psychrophilum*. A study conducted by Barnes and Ellis (2004) showed that *Lactococcus garvieae* with capsules could be highly susceptible to lysis through classical complement activation in the presence of antibodies, whereas *Lactococcus garvieae* without capsules could be killed by alternative pathways without requiring the presence of specific

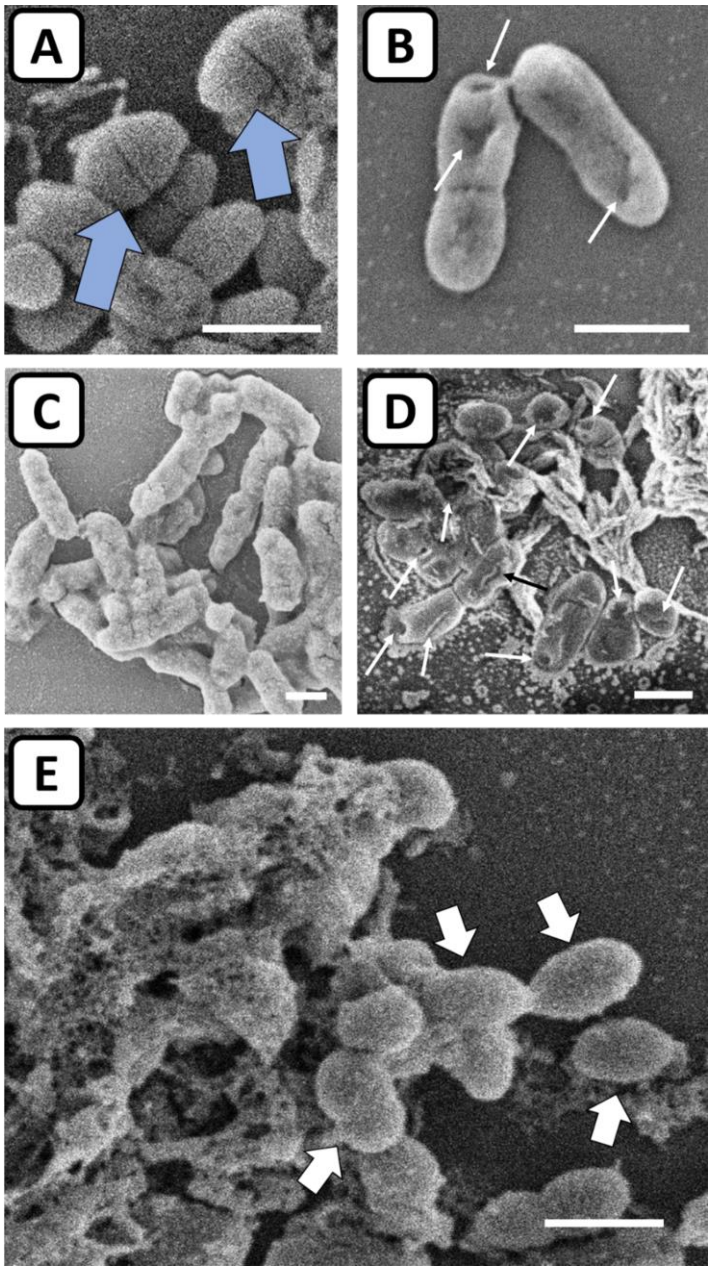

Figure 3. Scanning electron microscope (SEM) imaging of the topographic observations of *Enterococcus faecalis* bacterial cells. A= Normal *Enterococcus faecalis* bacterial cells (blue arrows), B= Bacterial cells of *Enterococcus faecalis* with damaged cell wall topography showing holes at the cell poles, indentations, and deep craters (white arrows), C= *Enterococcus faecalis* bacterial cells incubated in IgY anti-*Enterococcus faecalis* and the addition of inactivated red tilapia serum at 56° C for 30 minutes, D= *Enterococcus faecalis* bacterial cells incubated in the IgY suspension of anti-*Enterococcus faecalis* and the addition of tilapia serum without inactivation. There were holes at the cell poles, deep craters, indentations, and lysed cells (white and black arrows), E= *Enterococcus faecalis* bacterial cells incubated in the antibiotic suspension fosfomycin with a concentration of 50 µg mL^{-1}. Cells showed morphological changes in the missing septum cleavage (white arrows). Bar scale= 1 µm

antibodies. Barnes *et al*. (2003) reported the opposite and found in *Streptococcus iniae*, which in encapsulated *Streptococcus iniae* was resistant to the classical pathways, but could be killed by alternative pathways. Differences in strains of bacteria can also influence whether the bacteria are resistant or sensitive to the lytic effects caused by the activation of complement through classical or alternative pathways. The study conducted by Boesen *et al*. (1999a) and Boesen *et al*. (1999b) showed that *Vibrio anguillarum* strain O1 could be lysed through classical pathways because very susceptible to the presence of antibodies from vaccinated fish serum, whereas *Vibrio anguillarum* strain O2a cannot be killed through the classical pathways of complement activation due to the O-antigen side chain in this strain able to protect bacteria from complement-mediated bactericidal effects.

The biological analysis study on the interaction between IgY protein and red tilapia complement protein which was carried out by observation using a SEM, showed that there were changes in the topography of *Enterococcus faecalis* bacteria cells when incubated together with pure IgY suspension that was specific for *Enterococcus faecalis* with the addition of healthy red tilapia serum of *Enterococcus faecalis*-free as a source of complement protein. These changes were observed as holes at the cell poles, deep craters, indentations, and the presence of lysed bacterial cells (Figure 3D). The bacteria *Enterococcus faecalis* were incubated with IgY anti-*Enterococcus faecalis* and inactivated serum only exhibited opsonized cell topography, which is thought to be a collection of IgY protein molecules. This suggests a suspected bactericidal potential from the activation of the classical pathways of complement-linked antigen-antibody (IgY) aggregates in red tilapia, although in mammals, IgY molecules are unable to activate the complement system and interact with Fc receptors in stimulating the inflammatory response (Campbell *et al*. 1980; Rubinstein *et al*. 1991; Abdou *et al*. 2013).

The positive control of bactericidal antibiotics by inhibiting the formation of cell walls in the form of fosfomycin showed morphological changes in bacterial cells in the form of missing septum cleavage. *Enterococcus faecalis* has peptidoglycan (PG) hydrolase enzyme, which has activity against N-acetylglucosamineidase, such as MurA and AtlA. This enzyme is responsible for septum cleavage, which allows derived cells to detach during the binary fission process (Mesnage *et al*. 2008) and is a new virulent factor possessed by *Enterococcus faecalis* and can be used as a therapeutic target in overcoming this opportunistic pathogen infection (Salamaga *et al*. 2017). Fosfomycin is a broad-spectrum antibiotic widely used in its potency to treat *Enterococcus faecalis* infection. This antibiotic has a mechanism of action by inhibiting enzymes that catalyze the first step in synthesizing peptidoglycan as one of the leading cell wall components of *Enterococcus faecalis* bacteria, such as the MurA enzyme, a type of UDP-N-acetylglucosamine-enolpyruvyl transferase (Silver 2017). This causes it to inhibit the formation of septum cleavage, which can be observed in *Enterococcus faecalis* bacterial cells incubated with the addition of fosfomycin through SEM imaging (Figure 3E).

CONCLUSION

Based on studies that have been conducted on various biological functions of anti-*Enterococcus faecalis* immunoglobulin Y (IgY) molecules, it can be concluded that these molecules have a function in helping to neutralize bacteria through the agglutination process and inhibit colony growth. The presence of bactericidal potential mediated by complement through the classical pathways due to the presence of bacterial-IgY aggregates is a potential that can be utilized to prevent and treat streptococcal-like infections caused by *Enterococcus faecalis* in red tilapia commodities. This can be used as an alternative to antibiotic preparations that are more environmental-friendly for aquaculture activities.

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