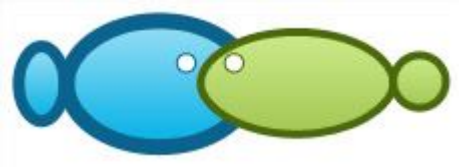

Comparison of some immune components in epidermal mucus of three species of freshwater fishes

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Abstract. The mucus secreted in the epidermis of fish skin acts as the first line of defense between fish and pathogens in their living environment. Fish epidermal mucus has been reported to prevent the colonization of pathogenic bacteria. The study aimed to evaluate the activity of several immune-related enzymes (lysozyme and alkaline phosphatase) as well as the bacterial activity of the epidermal mucus of three types of freshwater fish; tilapia (*Oreochromis niloticus*), snakehead fish (*Channa striata*), and local catfish (*Clarias batrachus*) (against shrimp pathogenic *Vibrio harveyi*, *V. parahaemolyticus,* and *V. alginolyticus*). Crude and acidic extracts of epidermal mucus of fish were prepared and their antibacterial activity was tested by disc diffusion method against three bacterial pathogens of vannamei shrimp (*Litopenaeus vannamei*); *Vibrio harveyi, V. parahaemolyticus,* and *V. alginolyticus*, which were then compared with the antibiotic Streptomycin as a positive control and acetic acid as a negative control. Of the 18 tests performed (six types of epidermal mucus extracts against three different bacterial strains), 11 tests showed antibacterial activity. Acid-extracted epidermal mucus showed the strongest inhibitory potential against pathogenic bacteria *V. harveyi, V. parahaemolyticus*, and *V. alginolyticus* compared to crude extract. The acidic and crude extracts were further tested for lysozyme activity, and the results showed that the acidic extracts of *O. niloticus* had the highest level of lysozyme activity followed by the crude extract of *C. bathracus* and *O. niloticus*. In contrast, crude extract of *C. striatus* showed the highest level of alkaline phosphatase activity, but no differences were found among the extracts examined. Thin layer chromatography of all extracts showed violet with ninhydrin test and yellowish-brown spots with sulphuric acid, that's indicating the presence of peptides and steroids respectively. Epidermal mucus contains some immune components that can help overcome the problem of antibiotic resistance of many pathogenic bacteria in shrimp culture.

Key Words: acidic, crude, epidermal mucus, *Litopenaeus vannamei*, lysozyme.

Introduction. White-leg shrimp (*Litopenaeus vannamei*) is one of the leading shrimp species cultivated for human consumption throughout the world (Manoppo et al 2011), with sales of around 6.4 million tons or the equivalent of 30.8 billion rupiahs in 2012 (FAO 2014). In 2013, the value of Indonesia's shrimp exports was 33.1% or up 3.87% from the contribution value in 2012 (KKP 2013). The characteristics possessed by this species of shrimp are the reason it can be used as one of the leading aquaculture commodities. White-leg shrimp has many advantages, including being relatively resistant to disease, growing relatively fast, being able to use space more efficiently, and being more tolerant of environmental changes (FAO 2015).

However, the development of shrimp farming cannot be separated from disease attacks. The disease is a major obstacle to increasing aquaculture production because it can cause relatively high mortality. The attack disease caused the export volume of Indonesian shrimp farming to decrease from 64.9 thousand tons in 2018 to 62.64 thousand tons in 2019 (KKP 2019). One type of disease that can cause mass mortality in cultured shrimp is vibriosis. This disease is caused by bacteria of the *Vibrio* genus such as *V. harveyi*, *V. alginolyticus*, *V. parahaemolyticus*, and *V. vulnificus* (Asplund 2013; Nor-Amalina et al 2017), and infection with these various types of vibrios occurs at every

stage of development, starting from the hatching stage, larval rearing to growing out in ponds (De-Souza Valente & Wan 2021).

Matsumoto et al (2000) reported that *V. harveyi* was one of the causative agents of mass mortality during the rearing of Penaeid larvae. Disease attacks occur at all stages of life but are more common in hatcheries. *V. parahaemolyticus* is a halophilic bacterium distributed in temperate and tropical coastal waters worldwide. Some types can cause acute gastroenteritis in humans, often after ingesting contaminated seafood. *V. alginolyticus* has been isolated from sick white-leg shrimp showing poor growth, anorexia, inactivity, redness of uropods and telsons, muscle blurring, and vaginal discharge, leading to death. *V. alginolyticus* is a Gram-negative rod-shaped bacterium in which, in histological preparations, melanized hemocytic granulomas occur in the connective tissue around the hemal sinus accompanied by hemocytic aggregation in necrotic muscle (Liu et al 2004).

Until now, antibiotics are still widely used to fight bacterial infections in the aquaculture industry. For example, florfenicol and oxolinic acid are mainly used to control vibriosis in cod fry in Norway. Quinolones and flumequine are widely used to treat cold and classic water vibriosis. However, bacterial resistance to antibiotics has been found in several types of *Vibrio* spp. bacteria due to the overuse of antibiotics in the treatment of diseases in the aquaculture industry (Cabello et al 2013).

This problem has received great attention due to the increasing resistance of pathogens *V. parahemolyticus*, *V. harveyi*, *V. alginolyticus*, and *V. vulnificus* to many antibiotics on the market and used clinically (Kang et al 2017). In addition, some antibiotic resistance causes severe economic losses for the aquaculture industry (Zhu et al 2017). Extensive use of antibiotics not only promotes the development of antibiotic resistance but also contaminates food, water, and sediment. Defoirdt et al (2011) reported that the increasing use of antibiotics such as chloramphenicol, cotrimoxazole, erythromycin, and streptomycin to control *Vibrio* infection in tiger prawns (*Penaeus monodon*), causes bacteria to become resistant and antibiotics are no longer effective.

Prajitno (2007) states alternative treatment is needed using natural ingredients derived from plants and aquatic biota to overcome disease in shrimp. One that can be used is mucus on fish skin. Fish skin mucus is a stable physical or chemical barrier against pathogens. In fish, the surface of the epithelial tissue is covered by a smooth mucoid layer. The mucus is a viscous colloidal substance that contains antibacterial enzymes, protein and water, and several other substances known as mucus. The mucus is an important component of the innate immune mechanism, which is regularly secreted or exfoliated to prevent the attachment of pathogens and colonization of disease-causing microbes and parasitic invasion (Arasu et al 2013). Arockiaraj et al (2013) reported that fish mucus contains several innate immunity factors such as proteins and enzymes such as lysozyme, immunoglobulins, complement proteins, lectins, C-reactive protein (CRP), proteolytic enzymes, transfer, alkaline phosphatase (ALP) and various antimicrobial peptides.

The antimicrobial potential of fish slime is highly dependent on the species and habitat of the fish (Subramanian et al 2008). Mucus in fish skin also varies greatly in thickness, viscosity, and content of glycoproteins (mucus) which are also the main component of fish mucus and all depend on the fish species (Dash et al., 2018). In addition, the extraction method also affects fish slime's antimicrobial potential. Tiralongo et al (2020) stated that organic slime extraction showed greater inhibitory activity against pathogenic bacteria in humans than in water extraction. This study was conducted to evaluate the activity of several immune-related enzymes (lysozyme and alkaline phosphatase) as well as the bacterial activity of the epidermal mucus of three types of freshwater fish; tilapia (*Oreochromis niloticus*), snakehead fish (*Channa striata*), and local catfish (*Clarias batrachus*) against white-leg shrimp pathogenic bacteria; *Vibrio harveyi, V. parahaemolyticus*, and *V. alginolyticus.*

Material and Method

Extraction of fish epidermal mucus. This research was conducted from June to September 2022, at the Fish Health Laboratory, Department of Aquaculture Technology, Pangkep State Polytechnic of Agriculture. A total of 50 fish for each species; tilapia, snakehead fish, and local catfish aged between 1.5 and 2 months with approximately 150-160 g of weight were obtained from freshwater fish farming ponds in Pinrang and Pangkep Regency, South Sulawesi Province. The fish obtained were then acclimatized separately for a week using three aquaria. During this period, each fish species was separately fed commercial feed once a day on an ad libitum basis. Every day, 20-30% of water changes were carried out in each aquarium. After one week of acclimatization, the fish from each fish species were ready to be used for taking epidermal mucus. Only healthy fish were selected for epidermal mucus collection. Dead fish and fish with damage or injuries were excluded from each rearing aquarium. The fish-rearing aquaria were cleaned daily to maintain optimal water quality and avoid microbial infection.

Before taking the mucus, the fish fasted for 24 hours. Twenty (20) fish from each of the three species were put separately one by one into a plastic container with a volume of 40 liters followed by the addition of 50 mL of 50 mM NaCl into the container. The plastic container was gently shaken for 5 minutes to trigger stress and release mucus on the fish skin's surface. Secreted mucus was collected separately from each type of fish using a 15 mL centrifuge tube. The collected mucus was immediately centrifuged for 10 minutes at 1500 rpm at 4°C. The supernatant contained at the top of the centrifuge tube was transferred to a new centrifuge and then stored in a freezer at -20°C until needed (extract stock). When mucus samples were collected, anesthetic chemicals are not used. The supernatant was further divided into two parts and extracted separately by crude and acidic methods.

Crude extraction. Crude extraction was carried out according to the method of Subramanian et al (2008) with some modifications. Around 250 mg of mucus extract in freeze-dried form was resuspended in water at a concentration of 10 mg mL^{-1} and centrifuged at 9500 × g (Beckman coulter, Avanti J-26 XPI) for 10 min at 4°C. The supernatant was collected and stored at -4°C until further antimicrobial testing.

Acidic extraction. Acidic extraction was carried out according to the method of Hellio et al (2002) and Conlon (2007) with some modifications. Around 250 mg of the lyophilized epidermal mucus was added to a moderately 1% acetic acid (HAc) (1:4) and placed in a boiling water bath for 3 min to inhibit the activity of the proteolytic enzymes. The heated mixture was homogenized using a vortex in the ice bucket for 5 minutes. The resulting homogenate was then centrifuged in a refrigerated centrifuge at 25,000 g, for 35 minutes, at 4°C and 600 mL of the homogenate supernatant was filtered through Whatman No. 1 filter paper and followed by a 0.45 micron Whatman nylon filter. The filtrate extract was stored at 4°C until further antimicrobial testing.

Enzymatic activities in epidermal mucus

Lysozyme activity. Lysozyme activity was measured by using a lysozyme detection kit (Sigma-Aldrich, Cat. no. LY0100) according to the manufacturer's instructions. The results of lysozyme activity were defined by the lysis of the *Micrococcus lysodeikticus* cells. The reactions were conducted at 25°C and absorbance at 450 nm was measured on the ultraviolet/visible spectrophotometer (Perkin Elmer, Lambda XLS, USA).

$$\text{Lysozyme activity (Unit mL}^{-1}) = \frac{A_{450}/\text{min test} - A_{450}/\text{min blank (df)}}{(0.001)(0.03)}$$

where: df = dilution factor, 0.001 = A_{450} as per the unit definition, and 0.03 = volume (in mL) of enzyme solution.

Alkaline phosphatase (ALP) activity. ALP activity of crude and acidic extracts from epidermal mucus of three types of freshwater fish was measured (Glory diagnostic, GD ALP100) using 2 types of reagents. Eight (8) mL of reagent 1 was mixed with 2 mL of reagent 2, stored at 2-8^{o}C, then 20 L of each extracted sample was added to 1 mL of the mixture reagent. The mixture was allowed to be homogeneous and then incubated at room temperature for 1 minute. The measurement of activity was done using a spectrophotometer with a wavelength of 405 nm. The measurement was repeated in the 2nd, 3rd, and 4th min. Then the average absorbance was calculated.

Minimum inhibitory concentration (MIC) test. The purpose of the MIC test was to determine the lowest concentration of epidermal mucus capable of inhibiting the growth of *V. harveyi*, *V. parahaemolyticus*, and *V. alginolyticus* according to the method recommended by Dahak & Taourirte (2013). MIC tests were carried out by serial dilutions of the two extracts in six concentrations of 10, 20, 30, 40, 50, and 60 µL mL^{-1}. Bacteria with a density of 2 × 10^{6} were grown in Mueller Hinton broth and incubated at 37°C. MIC was determined as the lowest concentration required to inhibit microbial growth. All tests were performed in triplicate, using 2% dimethylsulfoxide (DMSO) as a negative control.

Antibacterial activity test. The antibacterial activity of the epidermal mucus of each fish species was tested separately using the agar diffusion method (Loh et al 2014). The inhibition test of epidermal mucus extracts was carried out on the pathogenic bacteria *V. harveyi, V. parahaemolyticus*, and *V. alginolyticus*. A volume of 0.5 µL of isolates of pathogenic bacteria with a density of 10^{6} CFU mL^{-1} was cultured on Tryptic Soy Agar media with the addition of 2% NaCl (w/v). After the media containing the pathogenic bacteria culture solidified, then paper discs (2 mm, Toyo Roshi Kaisha Ltd, Japan) were dripped with each epidermal mucus extract and 0.5 L of Streptomycin ® 100 mg antibiotic as a positive control, while acetic acid as a negative control was performed in 3 replications and then incubated at 30°C for 24-h. To determine the amount of antibacterial activity, observations and measurements of the diameter of the clear zone formed around the paper disc were carried out. The filtrate containing antibacterial substances will inhibit pathogenic bacteria as evidenced by the presence of a clear zone around the paper disc.

Thin layer chromatography (TLC). Crude extracts and acidic extracts were applied to a thin-layer chromatographic plate (Merck) with a capillary tube and placed in a chamber containing chloroform:methanol (80:20) as a developing solvent. After development, compounds were visualized as purplish pink spots on spraying with 10% ethanolic sulphuric acid as a detecting agent followed by heating at 100^{o}C till the spots were visible.

Data analysis. Data obtained for TLC and MIC were analyzed descriptively while others were analyzed using one-way analysis of variance (ANOVA) followed by Duncan's multiple range test which was used to determine the significant variation between the epidermal mucus of the three test fish species and the antibiotics used. The t-test was used to determine the significant difference between the zone of inhibition (ZOI) obtained from the two types of extracts; crude and acidic obtained from three species of fish tested against three types of shrimp pathogenic bacteria; *V. harveyi*, *V. parahaemolyticus*, and *V. alginolyticus*. The significance of the data was analyzed at the probability value of $p < 0.05$. All data were analyzed using SPSS Version 22.

Results

Enzymatic activities in epidermal mucus. Crude and acidic epidermal mucus extracts from the three fish species showed differences in lysozyme and ALP activities as shown in Table 1. Lysozyme activity was significantly higher ($p < 0.05$) in the acidic extract of *O. niloticus* epidermal mucus (181.80±30.77 U mg^{-1}). But lysozyme activity was relatively low in the crude extract of *C. striata* epidermal mucus (32.40±7.16 U mg^{-1}) and acidic extract of *C. striata* epidermal mucus (30.11±6.36 U mg^{-1}).

Table 1

Lysozyme activity from crude and acidic extracts of fish epidermal mucus

Epidermal mucus extracts	*Lysozyme activity (U mg^{-1})*	*Alkaline phosphatase activity (U mg^{-1})*
Acidic *O. niloticus*	181.80±30.77^{c}	0.287±0.159^{ab}
Acidic *C. striata*	30.11±6.36^{a}	0.241±0.026^{a}
Acidic *C. bathracus*	100.40±10.73^{b}	0.275±0.050^{ab}
Crude *O. niloticus*	136.60±0.91^{bc}	0.275±0.003^{ab}
Crude *C. striata*	32.40±7.16^{a}	0.303±0.005^{b}
Crude *C. bathracus*	142.50±53.30^{bc}	0.263±0.002^{ab}

Note: Values in the same column with the same letter are not significantly different ($p > 0.05$).

Data on the measurement of ALP activity showed that crude extract of *C. striata* epidermal mucus had the highest activity (0.303±0.005 U mg^{-1} protein), followed by acidic-extracted *O. niloticus* (0.287±0.159 U mg^{-1} protein). However, no significant differences were found among *O. niloticus* acidic extract, *C. bathracus* acidic extract, *O. niloticus* crude extract, and *C. batrachus* crude extract. On the other hand, a significant difference appears in the different extraction methods for *C. striata*. The acidic extract of *C. striata* had the lowest ALP activity ($p < 0.05$) (0.241±0.026 U mg^{-1} protein).

Thin layer chromatography. Epidermal mucus from the three types of freshwater fish both extracted by crude and acidic methods contained peptide compounds with a purple color change after staining with ninhydrin, and a yellowish-brown color using sulfuric acid (Table 2).

Table 2

Rapid detection of the presence of bioactive compounds from fish epidermal mucus thin layer chromatography

Epidermal mucus extracts	*Color changes in TLC*				*Estimated compounds*
	UV 254	*UV 366*	*Ninhydrin*	*H_2SO_4*	
Acid *O. niloticus*	+	+	Purple	Yellowish brown	Peptide and steroid
Acid *C. striata*	+	+	Purple	Yellowish brown	Peptide and steroid
Acid *C. bathracus*	+	+	Purple	Yellowish brown	Peptide and steroid
Crude *O. niloticus*	+	+	Purple	Yellowish brown	Peptide and steroid
Crude *C. striata*	+	+	Purple	Yellowish brown	Peptide and steroid
Crude *C. bathracus*	+	+	Purple	Yellowish brown	Peptide and steroid

Minimum inhibitory concentration (MIC) test. Mucus extract from the three different fish showed different MIC against the three microbial strains tested. Crude and acidic extraction from all fish samples showed a minimum inhibitory effect at a concentration of 20 µL mL^{-1} against *V. harveyi*, *V. alginolyticus*, and *V. parahaemolyticus* bacteria (Table 3).

Table 3

The minimum inhibitory concentration of epidermal mucus of the selected freshwater fish against the three selected pathogenic bacteria

Pathogenic bacteria	*MIC (µL mL^{-1})*	*Mucus extracts*	*Diamater of inhibition (mm)*		
			O. niloticus	*C. batrachus*	*C. striata*
V. harveyi	20	Crude	1.05	0.90	0.85
	20	Acidic	0.95	0.65	0.80
V. parahaemolyticus	20	Crude	1.16	1.10	0.98
	20	Acidic	1.15	1.15	0.97
V. alginolyticus	20	Crude	0.95	0.85	1.06
	20	Acidic	1.04	0.96	1.12

Antibacterial test. The antimicrobial test results showed that no detectable level of antimicrobial activity was recorded in crude extracts of both *O. niloticus* and *C. striata* against the pathogen *V. harveyi*. Likewise, no antimicrobial activity was observed in the crude extract of *C. batrachus* and *C. striata* against *V. parahaemolyticus* (Table 4).

Table 4

Inhibitory zone (diameter) of crude and acidic extracts from epidermal mucus of three types of fish against three types of pathogenic bacteria

Mucus extracts	*Diameter of inhibition (mm)*		
	V. harveyi	*V. parahaemolyticus*	*V. alginolyticus*
1. Crude *O. niloticus*	-	1.07 ± 0.08^{a}	1.22 ± 0.02^{b}
2. Crude *C. batrachus*	2.06 ± 0.02^{b}	-	0.87 ± 0.02^{a}
3. Crude *C. striata*	-	-	1.10 ± 0.18^{ab}
4. Streptomycin	-	-	-
5. Acidic acid	-	-	-
1. Acid *O. niloticus*	2.30 ± 0.01^{c}	1.06 ± 0.05^{a}	1.28 ± 0.19^{c}
2. Acid *C. batrachus*	-	-	1.05 ± 0.10^{a}
3. Acid *C. striata*	0.83 ± 0.03^{a}	-	1.01 ± 0.05^{a}
4. Streptomycin	-	-	-
5. Acidic acid	-	-	-

The inhibitory effect of *O. niloticus* epidermal mucus which was extracted by acidic method showed the maximum inhibitory effect (2.30±0.01) against *V. harveyi* compared to *O. niloticus* epidermal mucus extracted by crude method (no inhibition zone). The same thing was observed in the inhibitory effect of *O. niloticus* epidermal mucus against *V. alginolyticus* bacterium. The inhibitory effect of *O. niloticus* epidermal mucus extracted by the acidic method was more significant than that of *C. striata* and *C. batrachus* extracted by the acidic method. The acidic extract of the epidermal mucus of *O. niloticus* could inhibit three types of pathogenic bacteria in white-leg shrimp, *V. harveyi*, *V. parahaemolyticus,* and *V. alginolyticus* while the acidic extract of *C. striata* could only inhibit *V. harveyi* and *V. alginolyticus*. The acidic extract of *C. batrachus* could only inhibit *V. alginolycticus*. *O. niloticus* epidermal mucus extracted by the crude or acidic method was found to inhibit the growth of *V. parahaemolyticus* and the same was not found in the epidermal mucus of *C. batrachus* and *C. striata.* Bacterial resistances of *V. harveyi*, *V. parahaemolyticus*, and *V. algnolyticus* to the antibiotic streptomycin were indicated by the absence of a clear zone around the disc paper that was first dropped with streptomycin.

Discussion. Fish epidermal mucus and its components provide the first line of defense against pathogenic organisms. Esteban (2012), Guardiola et al (2014), and Dash et al (2018) explained that the components of fish skin mucus that are often found are antimicrobial peptides, acids, alkaline phosphatase, and lysozyme, where these components act as inhibitory agents against various infectious diseases. In fish skin mucus of our selected fish species we have found lysozyme, alkaline phosphatase, and bactericidal activity. In addition, this study also estimated the content of peptides and steroids in the epidermal mucus. The components evaluated in this study showed variations between the fish species.

Measurement of lysozyme activity is essential considering the role of this enzyme as the first line of inhibition of the innate immune system of fish and shrimp. Magnadottir (2006) and Baba (2021) stated that this enzyme is bacteriolytic in fish epidermal mucus which helps protect against bacterial infection. Furthermore, Guardiola et al (2014), Ghafoori et al (2014), and Baba (2021) added that the activity of lysozyme in mucus showed differences between the tested fish species reflecting differences in the level of resistance of fish to pathogenic bacteria. Lysozyme plays a role in the body's defense of fish by breaking the β-1,4-glycoside bond between N-acetyl glucosamine acid and N-acetyl muramic acid on peptidoglycan so that it can damage bacterial cell walls (Jana et

al 2017). As a result, water can then enter the cell and cause the cell to swell and eventually burst, a process called lysis. Due to its capacity to disrupt the bacterial cell wall, lysozyme has been considered an endogenous antibiotic (Ferraboschi et al 2021).

In this present study, the results determined for lysozyme activity in *O. niloticus*, *C. striata*, and *C. batrachus* showed significant differences between the sample fish species. The lysozyme activity in the skin mucus of tilapia (*O. niloticus*) extracted by acidic method showed higher activity than others. It has been documented that significantly higher lysozyme activity in the skin mucus of seawater fish species is higher than that of freshwater fish (Subramanian et al 2007). However, the opposite has also been reported (Fast et al 2002). The level and activity of lysozyme in skin mucus depends on environmental conditions and fish species (Jung et al 2012; Nigam et al 2012).

Alkaline phosphatase is part of the essential lysosomal enzymes, is associated with the immune system, and has also been identified in fish mucus (Nigam et al 2012). Cheng et al (2018) claimed ALP protects against pathogenic bacteria by changing the surface structure of the pathogen. ALP enzyme plays an important role in the absorption of calcium in the water, the formation of calcium phosphate, and the secretion and formation of chitin. This role may be possible because of the hydrolytic activity of this enzyme (Sheikhzadeh et al 2012). The results found in the present study are in line with the results obtained by Nigam et al (2012) which showed a significant difference in certain alkaline phosphatase activities in the epidermal mucus of *C. striata* extracted by crude method and low in *C. striata* extracted by the acidic method. Subramanian et al (2007) documented a higher concentration of alkaline phosphatase in epidermal mucus of fish species such as hagfish (*Myxine glutinosa*) and koi fish (*Cyprinus rubrofuscus*) compared to rivers trout (*Salmo trutta*), haddock (*Melanogrammus aeglefinus*), arctic char (*Salvelinus alpinus*), and Atlantic cod (*Gadus morhua*). Further, Guardiola et al (2014) reported that alkaline phosphatase was produced in seabream (*Sparus aurata*), sea bass (*Dicentrarchus labrax*), shi drum (*Umbrina cirrosa*), and common dentex (*Dentex dentex*), while in black grouper (*Mycteroperca bonaci*) the amount of alkaline phosphatase was found high or low, respectively. This study found no significant association between alkaline phosphatase activity and the other non-specific immune-related parameters in the fish mucus. In the present study, the lysozyme enzyme activity of the acidic extract of *O. niloticus* showed the strongest activity, and the crude extract of local catfish showed the weakest activity, the opposite was indicated by alkaline phosphatase enzyme activity. In line with this, Nigam et al (2012) stated that the role of alkaline phosphatase activity has not been determined in the innate immunity of fish mucus (Nigam et al 2012). However, alkaline phosphatase activity is considered to indicate physical or chemical stress, immunostimulation, and microbial and parasitic infections (Subramanian et al 2008; Baba 2021).

Lysozyme and ALP found in epidermal mucus may play an important role in the immune function of fish; of these two enzymes, lysozyme has been identified in several fish species. The enzymatic activity has been compared between fish species or characterized after fish were exposed to pathogens, stress, or environmental factors such as temperature or salinity (Caruso et al 2011; Loganathan et al 2013). For example, in the present study, the lysozyme activity was at different levels in all extracted skin mucus and it appears to have a different effect on susceptibility to bacterial attacks. Another important factor is the presence of proteases that may play a protective role against pathogens by; i) directly degrading pathogens (Subramanian et al 2007), ii) inhibiting their colonization and invasion due to modification in the consistency of the mucus surface and/or increasing the sloughing of this mucus layer (Aranishi et al 1998), and iii) activate and enhance the production of other innate immunity components present in fish mucus such as the complement system including lysis of infectious organisms, activation of inflammation, opsonization and immune clearance, immunoglobulin, or antibacterial peptides (Fernandez et al 2002). However, protease activity has not been observed in the present study.

Epidermal mucus can be a source of antimicrobial peptides (AMPs). The role of AMPs is increasingly being recognized as an important factor in host defense mechanisms

and is found in organisms ranging from microbial, and plant to animal species (Fernandes et al 2002; Kennedy et al 2009). AMPs also play an important role in fish compared to mammals because fish rely more on their innate immune system (Hancock & Scott 2000).

Fish epidermal mucus may have been used for disease control purposes because of its antimicrobial compounds. The granular glands in fish produce certain secretions that may be effective against microbes. These antimicrobial compounds are associated with and dispersed from the epidermal mucus-producing epidermal cells of fish. Fish by-products are rich in peptides, steroids, and enzymes as obtained in this study. Several antimicrobial agents are present in the mucus of bony fish that bind to microbes and destroy them (Tiralongo et al 2020).

This study shows that all fish slime contained peptides and steroids. Tyor & Kumari (2016) reported that fish mucus is secreted by epidermal goblet cells and consists of mucin and other substances such as inorganic salts, immunoglobulins, proteins, and lipids suspended in the water giving it characteristic lubricating properties. Furthermore, fish mucus is a slimy secretion, consisting of mucins and a combination of other substances such as inorganic salts, immunoglobulin, proteins, and lipids suspended in the water giving it characteristic lubricating properties which help in choking pathogens (Kumari et al 2019). Salerno et al (2007) concluded that the peptides found in fish mucus include alpha-helical peptide piscidins or moronecidins, pleurocidins, dicentracins, and chrysopsins. Peptides are known for their broad-spectrum activity against parasitic microorganisms at very low minimum inhibitory concentrations (MIC) (Deslouches & Di 2017). The concentration of all epidermal mucus extracts that was able to inhibit the growth of the three pathogenic bacteria tested was found at the lowest concentration of 20 µL mL^{-1}. In addition, steroids found in the epidermal mucus, such as glucocorticoids, diffuse from the blood to the epidermal mucus because of their lipophilic nature (Bertotto et al 2010). The glucocorticoid response to environmental disturbances is of increasing interest because it can be used to measure the impact of stressors on individuals (Wikelski & Cooke 2006).

Several previous studies have revealed that the epidermal mucus of some fish species has strong anti-bacterial properties and activity against various microbial pathogens (Subramanian et al 2008; Balasubramanian et al 2012). Our data also confirm this and evidence that epidermal mucus from three freshwater fish species showed bactericidal activity against bacterial pathogens with substantial differences between fish species and bacterial strains.

Regarding the antimicrobial activity of the acidic extract of epidermal mucus, Hancock & Sahl (2006) reported that extracting fish mucus under acidic conditions causes the higher ability of the protein to become more soluble. Although the data obtained showed varied results, a tendency seemed to indicate that the acidic extract of mucus contained the greatest bactericidal activity (Wei et al 2010; Venilla et al 2011). It is further explained that the bactericidal activity might be due to the activity of the lysozyme enzyme and the data of this study support this hypothesis. But, the data found on alkaline phosphatase show different things. Thus, based on epidermal mucus data, other compounds with antimicrobial implications, or a combination of many factors, play a role in bactericidal activity. In this sense, several antimicrobial peptides have been identified in epidermal mucus capable of exerting bactericidal activity (Valero et al 2013).

Several studies have shown that mucus-producing cells in the epidermal layer differ between fish species and, therefore, may influence the composition of secreted epidermal mucus (Subramanian et al 2008). Furthermore, the biochemical properties of epidermal mucus may appear different depending on the ecological and physiological conditions, stress management, growth, and maturity stages. In this study, the results of the antibacterial activity of the three tested freshwater fish also further confirmed that epidermal mucus can be a source of antimicrobial products. Previous study conducted by Nurtamin et al (2016) also showed strong antibacterial activity in the mucus of *Anguilla* sp.. Ebran et al (2000) stated that only the hydrophobic component of epidermal mucus extracted by the crude method from freshwater and marine fish showed strong pore-forming properties, which correlated well with antibacterial activity.

Contrary to the findings of this study, Ebran et al (2000) observed that three proteins (45 kDa, 49 kDa, and 65 kDa) of the epidermal mucus supernatant of eel (*Anguilla anguilla*), tench (*Tinca tinca*), and rainbow trout (*Oncorhynchus mykiss*) exhibited different MIC values, ranging from 1 to 5 µg mL^{-1}. In other words, the same fish species or different fish species show different MICs against different or the same bacterial strains. The existence of different ages, habits, and habitats of different fish can be the reason behind this variation. Further studies on the extract of epidermal mucus could be developed to identify antimicrobial peptides in fish and their precise role in immunity.

Conclusions. In this study, crude and acidic extraction of epidermal mucus from three test fish species that were challenged with pathogenic bacteria showed a broad spectrum of bactericidal activity against the tested shrimp pathogenic bacteria. The antibacterial activity of epidermal mucus may be due to the fish mucus's peptides, steroids, and enzyme contents. Therefore, fish epidermal mucus contains antibacterial compounds that can be used as alternative antibiotics that can be used in aquaculture. Epidermal mucus can be a natural product, which can help reduce the problem of antibiotic resistance in the shrimp farming industry.

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

References

Aranishi F., Mano N., Hirose H., 1998 Fluorescence localization of epidermal cathepsins L and B in the Japanese eel. Fish Physiology and Biochemistry 19:205-209.

Arasu A., Kumaresan V., Sathyamoorthi A., Palanisamy R., Prabha N., Bhatt P., Roy A., Thirumalai M. K., Gnanam A. J., Pasupuleti M., Marimuthu K., Arockiaraj J., 2013 Fish lily type lectin-1 contains β-prism 2 architecture: immunological characterization. Molecular Immunology 56(4):497-506.

Arockiaraj J., Gnanam A. J., Muthukrishnan D., Gudimella R., Milton J., Singh A., Muthupandian S., Kasi M., Bhassu S., 2013 Crustin, a WAP domain containing antimicrobial peptide from freshwater prawn *Macrobrachium rosenbergii*: immune characterization. Fish and Shellfish Immunology 34(1):109-118.

Asplund M. E., 2013 Ecological aspects of marine *Vibrio* bacteria - exploring relationships to other organisms and a changing environment. PhD thesis, University of Gothenburg, Kristineberg, 48 pp.

Baba E., 2021 Analysis of some immune parameters in the skin mucus of four cultured fish species. The Israeli Journal of Aquaculture 73 doi:10.46989/001C.29916

Balasubramanian S., Baby-Rani P., Arul-Prakash A., Prakash M., Senthilraja P., Gunasekaran G., 2012 Antimicrobial properties of skin mucus from four freshwater cultivable fishes (*Catla catla*, *Hypophthalmichthys molitrix*, *Labeo rohita* and *Ctenopharyngodon idella*). African Journal of Microbiology Research 6(24):5110-5120.

Bertotto D., Poltronieri C., Negrato E., Majolini D., Radaelli G., Simontacchi C., 2010 Alternative matrices for cortisol measurement in fish. Aquaculture Research 41(8): 1261-1267.

Cabello F. C., Godfrey H. P., Tomova A., Ivanova L., Dölz H., Millanao A., Buschmann A. H., 2013 Antimicrobial use in aquaculture re-examined: its relevance to antimicrobial resistance and to animal and human health. Environmental Microbiology 15(7):1917-1942.

Caruso G., Denaro M. G., Caruso R., Mancari F., Genovese L., Maricchiolo G., 2011 Response to short term starvation of growth, haematological, biochemical and non-specific immune parameters in European sea bass (*Dicentrarchus labrax*) and blackspot sea bream *(Pagellus bogaraveo)*. Marine Environmental Research 72(1-2):46-52.

Cheng C. H., Guo Z. X., Ye C. X., Wang A. L., 2018 Effect of dietary astaxanthin on the growth performance, non-specific immunity, and antioxidant capacity of pufferfish (*Takifugu obscurus*) under high-temperature stress. Fish Physiology and Biochemistry 44:209-218.

Conlon J. M., 2007 Purification of naturally occurring peptides by reversed-phase HPLC. Nature Protocols 2(1):191-197.
Dahak K., Taourirte M., 2013 Comparative study of *in vitro* antimicrobial activities of *Foeniculum vulgare* Mill. (Umbelliferae) extract. OnLine Journal of Biological Sciences 13(4):115-120.
Dash S., Das S. K.., Samal J., Thatoi H. N., 2018 Epidermal mucus, a major determinant in fish health: a review. Iranian Journal of Veterinary Research 19(2):72-81.
De-Souza Valente C., Wan A. H. L., 2021 *Vibrio* and major commercially important vibriosis diseases in decapod crustaceans. Journal of Invertebrate Pathology 181: 107527.
Defoirdt T., Sorgeloos P., Bossier P., 2011 Alternatives to antibiotics for the control of bacterial disease in aquaculture. Current Opinion in Microbiology 14(3):251-258.
Deslouches B., Di Y. P., 2017 Antimicrobial peptides with selective antitumor mechanisms: prospect for anticancer applications. Oncotarget 8(28):46635-46651.
Ebran N., Julien S., Orange N., Molle G., 2000 Isolation and characterization of novels glycoproteins from fish epidermal mucus: correlation between their pore-forming properties and their antibacterial activities. Biochemica et Biophysica Acta 1467(2): 271-280.
Esteban M. A., 2012 An overview of the immunological defenses in fish skin. Hindawi International Scholarly Research Notices 2012:853470.
FAO, 2014 A quarterly update on world seafood markets. FAO, Rome, Globefish Highlights 3, 62 pp.
FAO, 2015 Climate change and food security: risks and responses. FAO, Rome, 98 pp.
Fast M. D., Sims D. E., Burka J. F., Mustafa A., Ross N. W., 2002 Skin morphology and humoral non-specific defence parameters of mucus and plasma in rainbow trout, coho and Atlantic salmon. Comparative Biochemistry and Physiology Part A 132(3): 645-657.
Fernandes J. M. O., Smith V. J., 2002 A novel antimicrobial function for a ribosomal peptide from rainbow trout skin. Biochemical and Biophysical Research Communications 296(1):167-171.
Ferraboschi P., Ciceri S., Grisenti P., 2021 Applications of lysozyme, an innate immune defense factor, as an alternative antibiotic. Antibiotics (Basel) 10(12):1534.
Ghafoori Z., Heidari B., Farzadfar F., Aghamaali M., 2014 Variations of serum and mucus lysozyme activity and total protein content in the male and female Caspian kutum (*Rutilus frisii kutum*, Kamensky 1901) during reproductive period. Fish and Shellfish Immunology 37(1):139-146.
Guardiola F. A., Cuesta A., Abellan E., Meseguer J., Esteban M. A., 2014 Comparative analysis of the humoral immunity of skin mucus from several marine teleost fish. Fish and Shellfish Immunology 40(1):24-31.
Hancock R. E. W., Scott M. G., 2000 The role of antimicrobial peptides in animal defenses. Proceedings of the National Academy of Sciences of the USA 97(16): 8856-8861.
Hancock R. E. W., Sahl H. G., 2006 Antimicrobial and host-defense peptides as new anti-infective therapeutic strategies. Nature Biotechnology 24(12):1551-1557.
Hellio C., Pons A. M., Beaupoil C., Bourgougnon N., Le Gal Y., 2002 Antibacterial, antifungal and cytotoxic activities of extracts from fish epidermis and epidermal mucus. International Journal of Antimicrobial Agents 20(3):214-219.
Jana M., Ghosh A., Santra A., Kar R. K., Misra A. K., Bhunia A., 2017 Synthesis of novel muramic acid derivatives and their interaction with lysozyme: action of lysozyme revisited. Journal of Colloid and Interface Science 498:395-404.
Jung T. S., Del Castillo C. S., Javaregowda P. K., Dalvi R. S., Nho S. W., Park S. B., Jang H. B., Cha I. S., Sung H. W., Hikima J. I., Aoki T., 2012 Seasonal variation and comparative analysis of non-specific humoral immune substances in the skin mucus of olive flounder (*Paralichthys olivaceus*). Developmental and Comparative Immunology 38(2):295-301.

Kang C. H., Shin Y. J., Jang S. C., Yu H. S., Kim S. K., An S., Park K., So J. S., 2017 Characterization of *Vibrio parahaemolyticus* isolated from oysters in Korea: resistance to various antibiotics and prevalence of virulence genes. Marine Pollution Bulletin 118(1-2):261-266.

Kementerian Kelautan dan Perikanan, 2019 Trend ekspor impor periode Januari – April Tahun 2015-2019. Available at: https://kkp.go.id/djpdspkp/artikel/12107-trend-ekspor-impor-periode-januari-april-tahun-2015-2019. Accessed: September, 2019. [in Indonesian]

Kennedy J., Baker P., Piper C., et al., 2009 Isolation and analysis of bacteria with antimicrobial activities from the marine sponge *Haliclona simulans* collected from Irish waters. Marine Biotechnology11(3):384-396.

Kumari S., Tyor A. K., Bhatnagar A., 2019 Evaluation of the antibacterial activity of skin mucus of three carp species. International Aquatic Research 11:225-239.

Liu C. H., Cheng W., Hsu J. P., Chen J. C., 2004 *Vibrio alginolyticus* infection in the white shrimp *Litopenaeus vannamei* confirmed by polymerase chain reaction and 16S rDNA sequencing. Diseases of Aquatic Organisms 61(1-2):169-174.

Loganathan K., Arulprakash A., Prakash M., Senthilraja P., 2013 Lysozyme, protease, alkaline phosphatase and esterase activity of epidermal skin mucus of freshwater snake head fish *Channa striatus*. International Journal of Research in Pharmaceutical and Biosciences 3:17-20.

Loh K. M., Ang L. T., Zhang J., et al, 2014 Efficient endoderm induction from human pluripotent stem cells by logically directing signals controlling lineage bifurcations. Cell Stem Cell 14(2):237-252.

Magnadottir B., 2006 Innate immunity of fish. Fish and Shellfish Immunology 20(2):137-151.

Manoppo H., Sukenda, Djokosetiyanto D., Sukadi M. F., Harris E., 2011 [Enhancement of non-specific immune response, resistance and growth of *Litopenaeus vannamei* by oral administration of nucleotide]. Jurnal Akuakultur Indonesia 10(1):1-7. [in Indonesian]

Matsumoto C., Okuda J., Ishibashi M., Iwanaga M., Garg P., Rammamurthy T., Wong H., Depaola A., Kim Y. B., Albert M. J., Nishibuchi M., 2000 Pandemic spread of an O3:K6 clone of *Vibrio parahaemolyticus* and emergence of related strains evidenced by arbitrarily primed PCR and toxRS sequence analyses. Journal of Clinical Microbiology 38(2):578-585.

Nigam A. K., Kumari U., Mittal S., Mittal A. K., 2012 Comparative analysis of innate immune parameters of the skin mucous secretions from certain freshwater teleosts, inhabiting different ecological niches. Fish Physiology and Biochemistry 38(5):1245-1256.

Nor-Amalina Z., Dzarifah M. Z., Mohd-Termizi Y., Amal M. N. A., Zamri-Saad M., Ina-Salwany M. Y., 2017 Phenotypic and genotypic characterization of *Vibrio* species isolates from *Epinephelus* species in Selangor, Malaysia. In: Proceedings of the International Conference on Advances in Fish Health, April 4-6, Universiti Putra Malaysia, Serdang, Selangor, Malaysia.

Nurtamin T., Nurman R. Y., Hafizah I., 2016 Antibacterial activity of eel (*Anguilla* spp.) mucus against *Salmonella typhi*. The Indonesian Biomedical Journal 8(3):179-182.

Prajitno A., 2007 Penyakit ikan-udang: bakteri. Penerbit Universitas Negeri Malang, Malang, 115 pp. [in Indonesian]

Salerno G., Parrinello N., Roch P., Cammarata M., 2007 cDNA sequence and tissue expression of an antimicrobial peptide, dicentracin; a new component of the moronecidin family isolated from head kidney leukocytes of sea bass *Dicentrarchus labrax*. Comparative Biochemistry and Physiology. Part B Biochemistry and Molecular Biology 146(4):521-529.

Sheikhzadeh N., Pashaki A. K., Nofouzi K., Heidarieh M., Tayefi-Nasrabadi H., 2012 Effects of dietary Ergosan on cutaneous mucosal immune response in rainbow trout (*Oncorhynchus mykiss*). Fish and Shellfish Immunology 32(3):407-410.

Subramanian S., MacKinnon S., Ross N. W., 2007 A comparative study on innate immune parameters in the epidermal mucus of various fish species. Comparative Biochemistry and Physiology Part B: Biochemistry & Molecular Biology 148:256-263.

Subramanian S., Ross N. W., MacKinnon S. L., 2008 Comparison of antimicrobial activity in the epidermal mucus extracts of fish. Comparative Biochemistry and Physiology. Part B Biochemistry and Molecular Biology 150(1):85-92.
Tiralongo F., Messina G., Lombardo B., Longhitano L., Li Volti G., Tibullo D., 2020 Skin mucus of marine fish as a source for the development of antimicrobial agents. Frontiers in Marine Science 7:541853.
Tyor A. K., Kumari S., 2016 Biochemical characterization and antibacterial properties of fish skin mucus of fresh water fish, *Hypophthalmichthys nobilis*. International Journal of Pharmacy and Pharmaceutical Sciences 8(6):6-10.
Valero Y., Chaves-Pozo E., Meseguer J., Esteban M., Cuesta A., 2013 Biological role of fish antimicrobial peptides. In: Antimicrobial peptides: properties, functions and role in immune response. Seong M. D., Hak Y. I. (eds), Nova Science Publishers, Inc., pp. 31-60.
Vennila R., Kumar K. R., Kanchana S., Arumugam M., Vijayalakshmi S., Balasubramaniam T., 2011 Preliminary investigation on antimicrobial and proteolytic property of the epidermal mucus secretion of marine stingrays. Asian Pacific Journal of Tropical Biomedicine 1(2):239-243.
Wei O. Y., Xavier R., Marimuthu K., 2010 Screening of antibacterial activity of mucus extract of snakehead fish, *Channa striatus* (Bloch). European Review Medical and Pharmacological Sciences 14:675-681.
Wikelski M., Cooke S. J., 2006 Conservation physiology. Trends in Ecology and Evolution 21(1):38–46.
Zhu Y. G., Zhao Y., Li B., Huang C. L., Zhang S. Y., Yu S., Chen Y. S., Zhang T., Gillings M. R., Su J. Q., 2017 Continental-scale pollution of estuaries with antibiotic resistance genes. Nature Microbiology 2:16270.

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