Faculty of Resource Science and Technology

Antibacterial Screening of Epidermal Mucus Extracts of Two Bornean *Barbodes* Fishes

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Antibacterial Screening of Epidermal Mucus Extracts of Two Bornean *Barbodes* Fishes

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DECLARATION

I declare that the work in this thesis was carried out in accordance with the regulations of Universiti Malaysia Sarawak. Except where due acknowledgements have been made, the work is that of the author alone. The thesis has not been accepted for any degree and is not concurrently submitted in candidature of any other degree.

……

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ABSTRACT

Fish are in constant interaction with their microbe-rich habitat. Fish epidermal mucus constitutes the first line of defence against its aquatic environment as it contains a wide range of innate immune components, including antimicrobial proteins. Currently, freshwater fish receives less attention from researchers compared to their marine counterparts. Furthermore, knowledge about native fish from Borneo remains scarce. Therefore, the study was aimed to establish better understanding on the epidermal mucus of two Bornean freshwater fish species *Barbodes sealei* and *Barbodes everetti*. In the study, protein recovery of five different mucus extracts with different solvents and concentrating methods namely Freeze-dried Crude (distilled water) extract (FDC), Freeze-dried Aqueous (saline) extract (FDS), Freeze-dried Acidic (3 % acetic acid) extract (FDA), Ammonium-sulphate-precipitated Aqueous (saline) extract (APS), and Ammonium-sulphate-precipitated Acidic (0.8 % acetic acid) extract (APA), was compared. Subsequently, antibacterial properties of the epidermal mucus extracts against selected bacterial strains were determined. Aiding by Liquid Chromatography with Tandem Mass Spectrometry (LC-MS/MS), major proteins from the active extracts were identified and the antibacterial proteins were also determined. For both species, the results revealed higher protein contents in AP mucus but better recovery in FD mucus. Higher protein contents could be associated with the higher level of stress experienced by fish specimens while better protein recovery might be due to minimal number of sample transfer during extract preparation. Next, four bacterial strains namely *Listeria monocytogenes* ATCC 7644, *Pseudomonas aeruginosa* ATCC 27853, *Salmonella braenderup* ATCC BAA 664, and *Vibrio cholera* were sensitive towards four out of five extracts from both species as well. Interestingly, FD acidic extract of *B. sealei* exhibited antibacterial activity at low protein concentrations (3.57 ± 0.5 µg/ml). The findings of

present study set a foundation for future antibacterial studies of fish epidermal mucus and provide interesting new avenues of research in exploring the antimicrobial potential of fish epidermal mucus.

Keywords: Antibacterial properties, Borneo, fish epidermal mucus, freshwater, protein recovery

Saringan Antibakterial Ekstrak Mukus Epidermis daripada Dua Ikan Borneo Barbodes

ABSTRAK

Ikan sentiasa berinteraksi dengan habitat yang kaya dengan mikrob. Mukus epidermis yang mengandungi pelbagai komponen keimunan semula jadi, termasuklah protein antimikrob, membentuk pertahanan barisan pertama terhadap persekitaran akuatiknya. Pada masa ini, ikan air tawar kurang diberi perhatian daripada penyelidik berbanding dengan ikan marin. Tambahan pula, pengetahuan tentang ikan endemik Borneo masih berkurangan. Justeru, kajian ini bertujuan untuk mewujudkan pemahaman yang lebih mendalami mengenai mucus epidermis daripada dua spesies ikan air tawar Borneo bernama Barbodes sealei dan Barbodes everetti. Dalam kajian ini, perbandingan pemulihan protein bagi lima ekstrak mucus dengan pelbagai pelarut dan kaedah penumpuan telah dilakukan termasuklah tiga ekstrak menggunakan kaedah pengeringan beku (FD), iaitu Crude (air suling) dengan kaedah, pengeringan beku (FDC), Aqueous (salinus) dengan kaedah pengeringan beku (FDS), Acidic (acid asetik 3 %) dengan kaedah pengeringan beku (FDA), Aqueous (salinus) dengan kaedah pemendakan ammonium sulfat (APS), dan Acidic (asid asetik 0.8 %) dengan kaedah pemendakan ammonium sulfat (APA). Selepas itu, sifat antibakteria ekstrak mukus epidermis terhadap strain bakteria terpilih telah ditentukan. Dengan teknologi keupayaan celusan tinggi, iaitu kromatografi cecair dengan spektrometri jisim beriringan (LC-MS/MS), protein daripada ekstrak aktif telah dikenal pasti dan protein antibakteria juga ditentukan. Bagi kedua-dua spesies, kandungan protein adalah lebih tinggi dalam mukus AP tetapi pemulihan adalah lebih baik dalam mukus FD. Kandungan protein yang lebih tinggi boleh disebabkan oleh spesimen ikan mengalami tahap tekanan yang lebih tinggi manakala pemulihan protein yang lebih bagus mungkin disebabkan oleh bilangan pemindahan sampel yang minimum dalam penyediaan ekstrak. Seterusnya, empat strain bakteria iaitu Listeria

monocytogenes ATCC 7644, Pseudomonas aeruginosa ATCC 27853, Salmonella braenderup ATCC BAA 664, and Vibrio cholera adalah sensitive terhadap empat daripada lima ekstrak daripada kedua-dua spesies juga. Menarik juga, ekstrak FD Acidic daripada B. sealei mempamerkan aktiviti antibakteria di tahap kepekatan protein yang rendah (3.57 ± 0.5 µg/ml). Penemuan kajian ini menetapkan asas untuk kajian antibakteria tentang mukus epidermis ikan pada masa hadapan dan menyumbang kepada penyelidikan semasa dengan pendekatan baru yang menarik untuk menerokai potensi antimikrobial mukus epidermis ikan.

Kata kunci: *Air tawar, Borneo, mukus epidermis, pemulihan protein, sifat antibakteria*

TABLE OF CONTENTS

	Page
DECLARATION	i
ACKNOWLEDGEMENT	ii
ABSTRACT	iii
ABSTRAK	v
TABLE OF CONTENTS	vii
LIST OF TABLES	xi
LIST OF FIGURES	xiii
LIST OF ABBREVIATIONS	xv
CHAPTER 1 INTRODUCTION	1
1.1 Background	1
1.2 Problem Statement	3
1.3 Objectives	6
CHAPTER 2 LITERATURE REVIEW	7
2.1 Fish of Interest	7
2.1.1 Barbodes sealei	7
2.1.2 Barbodes everetti	8
2.2 Fish Skin Mucus Collection	10
2.3 Fish Mucus Extraction	13

2.3.1 Aqueous Extract 16

2.3.2 Organic Extract 16

2.3.3 Acidic Extract 17

2.3.4 Crude Extract 17

2.4 Antibacterial Assay 17

2.4.1 Agar Disk Diffusion 18

2.4.2 Agar Well Diffusion 18

2.4.3 Broth Microdilution 18

2.4.4 Bacterial Growth Curve 19

2.5 Antibacterial Properties of Fish Skin Mucus 19

2.5.1 Class Actinopterygii 25

2.5.2 Class Elasmobranchii 36

2.5.3 Class Myxini 37

2.6 Studies of Proteomics on Fish Skin Mucus 37

2.7 Chapter Summary 39

CHAPTER 3 MATERIALS AND METHODS 40

3.1 Mucus Preparation 40

3.1.1 Fish Sampling 40

3.1.2 Epidermal Mucus Collection 42

3.1.3 Concentrating Methods of Mucus Samples 43

3.1.4 Preparation of Epidermal Mucus Extract 46

3.1.5 Bradford Protein Assay 49

3.2 Antibacterial Assay 51

3.2.1 Preparation of Growth Media 51

3.2.2 Bacterial Strain 52

3.2.3 Disk Diffusion Test 53

3.2.4 Broth Microdilution Susceptibility Test 55

3.3 Protein Characterisation of Active Mucus Extracts 57

3.3.1 SDS-PAGE 57

3.3.2 Protein Identification with LC-MS/MS 59

CHAPTER 4 RESULTS 60

4.1 Mucus Collection and Protein Recovery 60

4.1.1 Bradford Protein Assay 60

4.1.2 Protein Recovery 64

4.1.3 Comparison on the Protein Recovery between two *Barbodes* species 67

4.2 Antibacterial Assay 68

4.2.1 Preliminary Screening of Antibacterial Activity of Mucus Extracts Using Disk Diffusion Tests 68

4.2.2 Ruling out the Solvent Effect on the Antibacterial Activity of Mucus Extracts 72

4.2.3 Antibacterial Activity of Mucus Extracts 79

4.2.4 Comparison on the antibacterial activity between two *Barbodes* species 82

4.2.5 Determination of Minimal Inhibitory Concentrations Using Broth Microdilution Susceptibility Test 83

4.3 Characterisation of Protein Content in Active Mucus Extracts 85

4.3.1 SDS-PAGE 85

4.3.2 LC-MS/MS 86

CHAPTER 5 DISCUSSION 94

5.1 Mucus Protein Concentration and Protein Recovery 94

5.2 Antibacterial Activities of Different Epidermal Mucus Extracts of *B. sealei* and *B. everetti* 97

5.3 Antimicrobial Proteins (AMP) in APA of two *Barbodes* species 106

5.3.1 Histone Proteins 106

5.3.2 Heat Shock Proteins 109

5.3.3 Review on Present Study 110

CHAPTER 6 CONCLUSION AND RECOMMENDATIONS 112

6.1 Conclusion 112

6.2 Limitations 114

6.3 Future Recommendations 115

REFERENCES 117

APPENDICES 143

LIST OF TABLES

		Page
Table 2.1	Destructive and non-destructive methods for fish mucus collection	11
Table 2.2	Fish mucus extraction methods and solvents used	14
Table 2.3	Fish mucus extracts with antibacterial activity against Gram-positive bacteria	20
Table 2.4	Fish mucus extracts with antibacterial activity against Gram-negative bacteria	22
Table 3.1	BSA standard assay data for (a) dH_2O mucus samples and (b) saline mucus samples	50
Table 3.2	Gel formulations for SDS-PAGE	57
Table 4.1	Protein concentrations of epidermal mucus upon collection (Initial) vs after concentrating and extraction steps (Final) of (a) *Barbodes sealei*, and (b) *Barbodes everetti*	63
Table 4.2	Protein recovery of various epidermal mucus extracts for (a) *Barbodes sealei*, and (b) *Barbodes everetti*	65
Table 4.3	Disk diffusion test results of five epidermal mucus extracts against selected bacterial strains for (a) *Barbodes sealei* and (b) *Barbodes everetti* and (c) their corresponding negative control	69
Table 4.4	Summary of disk diffusion test results for negative controls of different solvents with respect to their epidermal mucus extracts	72
Table 4.5	Results of significance test between IZD produced by antibacterial activity of FDA and its negative control against selected bacterial strains for (a) *Barbodes sealei* and (b) *Barbodes everetti*	73
Table 4.6	Results of significance test between IZD produced by antibacterial activity of APA and its negative control against selected bacterial strains for (a) *Barbodes sealei* and (b) *Barbodes everetti*	75
Table 4.7	Antibacterial test results of five epidermal mucus extracts of (a) *Barbodes sealei* and (b) *Barbodes everetti*	80
Table 4.8	Summary of antibacterial test results of *Barbodes sealei* and *Barbodes everetti*	83
Table 4.9:	Minimal Inhibitory Concentration (MIC) Dilution Table for APA Mucus Extract of (a) *Barbodes sealei* and (b) *Barbodes everetti*	84

Table 4.10 MICs of APA mucus extracts against selected bacterial strains 84

Table 4.11 Proteins identified by LC-MS/MS from the active mucus extract APA of *Barbodes sealei* 88

Table 4.12 Proteins identified by LC-MS/MS from the active mucus extract APA of *Barbodes everetti* 91

Table 4.13 Antibacterial proteins identified from the active epidermal mucus extract (APA only) 93

LIST OF FIGURES

		Page
Figure 2.1	Bornean Spotted Barb (*Barbodes sealei*) (Photo by Mr. Badiozaman Sulaiman)	8
Figure 2.2	Clown barb (*Barbodes everetti*) (Photo by Mr. Badiozaman Sulaiman)	9
Figure 3.1	Map of Sarawak, Borneo with two different scales. Blue circle represents UNIMAS East Campus in Kota Samarahan District while the red circle represents Kubah National Park in Kuching District	41
Figure 3.2	Homemade minnow trap	41
Figure 3.3	Flow diagram of fish epidermal mucus extraction method development	44
Figure 3.4	The illustration of dialysis tubing set-up in order to remove ammonium sulphate salt from the samples	46
Figure 3.5	Four-disc arrangement and six-disc arrangement for disk diffusion test	54
Figure 3.6	Two-fold serial dilutions on 96-well microplates	56
Figure 4.1	Standard curves of absorbance at 595 nm against protein concentration for (a) Freeze-dried (FD) mucus extracts and (b) Ammonium-sulphate precipitated (AP) mucus extracts	62
Figure 4.2	Protein recovery of five epidermal mucus extracts for *Barbodes sealei* and *Barbodes everetti*	68
Figure 4.3	Presence of antibacterial activity by mucus extract where the mucus extracts exhibited significantly higher IZD than the negative control (p-value < 0.05). Extracts in Red belong to *Barbodes sealei*; Extracts in Blue belong to *Barbodes everetti*	78
Figure 4.4	Absence of antibacterial activity by mucus extracts where the negative control exhibited significantly higher IZD than the mucus extracts (p-value < 0.05). Extracts in Red belong to *Barbodes sealei*; Extracts in Blue belong to *Barbodes everetti*	78
Figure 4.5	Absence of antibacterial activity by mucus extracts where there is no significant difference (p-value > 0.05) between the mucus extracts and their negative control. Extracts in Red belong to *Barbodes sealei*; Extracts in Blue belong to *Barbodes everetti*	79

Figure 4.6 SDS-PAGE presenting protein profile of AP mucus extracts of (a) *Barbodes sealei* (From Left: Standard Protein Ladder, APS, APA) and *Barbodes everetti* (From Left: Standard Protein Ladder, APA, APS) in SDS-PAGE. Samples were loaded onto a 4% stacking and 12% resolving acrylamide gel. The staining reagent used was Coomassie Brilliant Blue R-250 (BioRad) 86

Figure 5.1 Number of transfers of dH_2O FD mucus and saline AP mucus during sample preparation 98

LIST OF ABBREVIATIONS

0.85 % NaCl	Saline
AMP	Antimicrobial Protein
ANOVA	One-way Analysis of Variance
AP	Ammonium-sulphate-precipitated
APA	Ammonium-sulphate-precipitated Acidic Extract
APS	Ammonium-sulphate-precipitated Aqueous Extract
BSA	Bovine Serum Albumin
CGS	Centre for Graduate Studies
CLSI	Clinical and Laboratory Standards Institute
FD	Freeze-dried
FDA	Freeze-dried Acidic Extract
FDC	Freeze-dried Crude Extract
FDS	Freeze-dried Aqueous Extract
FRST	Faculty of Resource Science and Technology
IZD	Inhibition Zone Diameter
LB	Miller's Luria-Bertani
LC-MS/MS	Liquid Chromatography with Tandem Mass Spectrometry
MBC	Minimum Bactericidal Concentration
MH	Mueller Hinton
MHA	Mueller Hinton Agar
MHB	Mueller Hinton Broth
MIC	Minimum Inhibitory Concentration
OD	Optical Density

Pen-Strep	Penicillin-Streptomycin
SDS-PAGE	Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis
SFC	Sarawak Forestry Corporation
UNIMAS	Universiti Malaysia Sarawak
v	Volume
w	Weight

CHAPTER 1

INTRODUCTION

1.1 Background

Fish provides ample ecosystem services for countless human and aquatic communities and have been playing a key role to maintain the balance of the ecosystem. Fossil records dating back more than 500 million years showed that the first fish appeared on earth during the Cambrian period (Helfman et al., 2009). Fish has been regarded as one of the most important resources throughout human history, from serving as a nutritious diet rich in protein and lipid, to other trade commodities including ornamental and medicinal purposes (Tilami & Sampels, 2017). Today, fish have remained the most ecologically dominant group of living vertebrates which can be found in nearly all major aquatic environments, ranging from lake to deep oceans, with a wide range of abiotic variables such as intertidal zones, temperature, salinity, and oxygen content (Helfman et al., 2009; Videler, 2012).

To date, over 33000 species were described and reported (Froese & Pauly, 2022) with more than 40 % thriving in only freshwater habitats (Lundberg et al., 2000; Tedesco et al., 2017) in spite of the fact that freshwater ecosystems cover comparatively small distribution over the surface of earth which is only around 0.8 % and constitute less than 0.02 % of the global water (Dudgeon et al., 2006). The freshwater fish classification by Myers (1938, 1949, 1951) was developed solely based on their tolerance to salt water, i.e., their patterns of distribution are often majorly bordered by a saltwater barrier. There are 23 families of freshwater fishes confined to freshwater systems in Borneo which are characterised by little tolerance to saltwater (Berra, 2007). Although the system is more of

an ecological division rather than taxonomic level, it marks the significance of freshwater fish as one of the most zoo-geographically important vertebrates (Berra, 2007). As the most diverse group of the human vertebrate kins, these freshwater fishes could evolve to be more diverse in order to adapt to the highly variable aquatic environments in terms of size, space, water chemistry, microhabitat, food resources, and competition owing to the zoo-geographical barriers. Myriads of novel defence strategies could have been developed to combat various form of invasion and infection that is yet to be explored and exploited.

Unlike terrestrial animals, fish live their entire life in an aquatic environment. Most aquatic habitats are rich in saprophytic, pathogenic, and non-pathogenic microbes such as bacteria, virus and fungi (Magnadottir, 2010). Due to the continuous contact with their living aquatic environments, these gill-breathing creatures are more susceptible to a wide variety of diseases. Generally, the vertebrate immune system is divided into innate (non-specific) and acquired (specific) immunity. Being a poikilotherm, fish metabolism is directly dependent on the surrounding temperature which has limited the efficiency of their specific immune mechanisms (Esteban, 2012). Naturally, fish rely highly on their complex system of fast-acting innate immune mechanisms to fight the constant threats to their health (Ellis, 2001; Arellano et al., 2004). In general, fish innate immune system comprises numerous organs such as scales, gills, gut, and epidermis along with the mucus secreted by the epithelial cells (Esteban, 2012). Other than that, humoral components such as various enzymes, immunoglobins, and antimicrobial peptides are also involved in fish defence mechanism, facilitated by phagocytic cells and non-specific cytotoxic cells which destroy pathogens and destroy cells infected by virus, respectively (Magnadóttir, 2006; Subramanian et al., 2007; Helfman et al., 2009).

One of the most important components of innate response in fish is the mucous layer over their body surface. Mucus is a viscous colloid gel that forms an adherent layer covering their entire body. Fish skin mucus represents a primary interface between the environment and the interior milieu. It is secreted and sloughed off continuously as they encounter, monitor, regulate myriads of microflora that are always present in the aquatic environment and prevent the pathogen adherence to the underlying tissues (Esteban & Cerezuela, 2015). On top of functioning as a physical barricade in the innate defence system, fish skin mucus also actively stops microbial infection and is regarded as one of the crucial immunological factors. It contains a wide range of innate immune components such as lysozymes, calmodulin, complement, proteolytic enzymes, lectins, C-reactive proteins, immunoglobulins, antimicrobial peptides, and proteins (Shephard, 1994; Magnadóttir, 2006; Alvarez-Pellitero, 2008; Esteban, 2012). In addition, fish skin mucus is known to have multifunctional roles in osmoregulation, protection against toxic or heavy metal substances, lubrication against abrasion, parental feeding, chemical communication, and disease resistance (Alexander & Ingram, 1992; Shephard, 1994; Mokhtar, 2017). Apart from that, skin mucus of fish has significant effects on their social behaviours between conspecifics such as shoaling or schooling, habitat searching, synchronised spawning, or release of alarm signals and the interspecific interactions such as prey-predator relationships and host-parasite interactions (Reverter et al., 2018). The versatility of skin mucus is not only vital for fish survival but also signifies great potential to be utilised by human scientific research in a non-invasive, environmental-friendly manner.

1.2 Problem Statement

The indiscriminate use of antimicrobial drugs in the treatment of infectious diseases has caused microorganisms to develop resistance to many of the commonly used antibiotics.

Action must be taken to reduce this problem such as controlling the use of antibiotics, carrying out investigations on drugs from natural sources and searching for new antimicrobial agents. The antimicrobial agents could either inhibit the growth of pathogens or kill them without or with the least toxicity to the host cell and if possible, resolve the problems of antimicrobial resistance and other side effects of the currently available antimicrobial agents. One approach is to screen for naturally occurring antimicrobial agents from native animals. These animals which represent a rich source of novel antimicrobial agents can in turn be used in the bio-pharmaceutical field in the production of a more ecologically friendly antibiotic for use in aquaculture and human healthcare. The remarkable ability of fish to survive in such a microbe-rich environment provides an impetus for further research on its skin mucus.

Presently, there is a growing body of research on the antimicrobial function of fish skin mucus strongly suggesting that it plays a role in prevention of parasitic, bacterial, and fungal invasion (Hellio et al., 2002; Subramanian et al., 2008a; Lee et al., 2020; Tiralongo et al., 2020). In addition, Chong et al. (2005) and Manivasagan et al. (2009) had reported the predominantly proteinaceous properties of epidermal mucus in various fish species and most biological active components identified were proteins or peptides. This indicates the bacterial defence mechanisms could be closely associated with their protein contents.

Although antibacterial activity in fish skin mucus has been demonstrated in many studies (Hellio et al., 2002; Dhanaraj et al., 2009; Balasubramanian et al., 2012; Rao et al., 2015; Kumari et al., 2019), the results suggest that the effects vary among different fish species and reveal specific sensitivity against certain bacteria. In addition, four main types of extraction methods namely Aqueous, Acidic, Organic and Crude which involved over 10

types of solvents had been reported to extract fish skin mucus in past studies (Lee et al., 2020). The variation in their antibacterial properties could be attributed to different methodology and strategies, coupled with various modifications used. Therefore, it is of utmost importance to note the differences of the approaches and the solvents used and their implications in order to investigate the antimicrobial potential of the fish skin mucus.

On the other hand, fish skin mucus has been characterised over the years from targeting a specific protein of interest to several groups of proteins. The protein profile of fish skin mucus from several species were characterised effectively by utilising mass-spectrometry–based proteomics technology such as Liquid Chromatography-Mass Spectrometry (LC-MS) (Ramos et al., 2012; Provan et al., 2013; Cordero et al., 2015, 2016; Magnadóttir et al., 2018) and Matrix-assisted Laser Desorption/ Ionisation Time-of-flight Mass Spectrometry (MALDI-TOF-MS) (Chong et al., 2006). In certain studies, mucus secretions were induced under different conditions such as parental care (Chong et al., 2006), bacterial infection (Rajan et al., 2013), or sea lice infection (Easy & Ross, 2009). Nevertheless, the antimicrobial properties of fish skin mucus were not always the focus of most characterisation studies while in most antimicrobial studies, the composition of fish skin mucus was not clearly addressed or elucidated.

Furthermore, the information available on fish skin mucus study is restricted to commercially important farm fish or marine species such as Atlantic cod (*Gadus morhua*) (Magnadóttir et al., 2018), Atlantic salmon (*Salmo salar*) (Provan et al., 2013), discus fish (*Symphysodon aequifasciata*) (Chong et al., 2006), European seabass (*Dicentrarchus labrax*) (Cordero et al., 2015) and gilthead seabream (*Sparus aurata*) (Cordero et al., 2016). Thus, the antimicrobial potential of mucus from their freshwater counterpart, particularly

from the native species in Borneo, is left unexplored. Therefore, increased knowledge of fish skin mucus taking part in innate defences can be of great importance owing to the challenge of combating multi-drug resistant pathogens. It is essential to carry out the respective research to redefine the bio-pharmaceutical value of the Bornean freshwater fishes.

1.3 Objectives

Two of the Bornean endemic freshwater species namely *Barbodes sealei* Herre, 1933, the Bornean spotted barb, and *Barbodes everetti* Boulenger, 1894, the clown barb, were selected for this study for their abundance in the region. The study was aimed to explore more information on the antibacterial potential of fish epidermal mucus. This was achieved through the following set of objectives:

i. To compare the protein recovery of the fish epidermal mucus from different extraction and concentrating methods

ii. To determine the antibacterial properties of different fish epidermal mucus extracts against selected bacterial strain

iii. To identify the antibacterial proteins from the active fish epidermal mucus extract

CHAPTER 2

LITERATURE REVIEW

This chapter is a slightly modified version of a review article (Lee et al., 2020) and has been reproduced here with the permission of the copyright holder.

Lee, Y., Bilung, L. M., Sulaiman, B., & Chong, Y. L. (2020). The antibacterial activity of fish skin mucus with various extraction solvents and their in-vitro evaluation methods. *International Aquatic Research*, *12*, 1–21. https://doi.org/10.22034/IAR(20).2020.670998 [Q3 74/116 in Marine and Freshwater Biology]

2.1 Fish of Interest

Barbs, carps, and minnows belong to the family Cyprinidae. In Borneo, this family is the most dominant freshwater group as they make up more than two-thirds from the total freshwater fauna (Sulaiman & Mayden, 2012).

2.1.1 Barbodes sealei

Barbodes sealei, the Bornean spotted barb (Figure 2.1) or locally known as "Turungau", is found endemic to the freshwater habitats in Borneo (Inger & Chin, 1962; Froese & Pauly, 2022).

Figure 2.1: Bornean Spotted Barb (*Barbodes sealei*) (Photo by Mr. Badiozaman Sulaiman)

Normally, this species is found in the clear or faintly murky, unpolluted forest streams with sandy or gravelly riverbed (Inger & Chin, 1962). It is distinguished by the presence of a row of equally spaced dark blotches along the flank. Though, additional dark spots may occur at the base of the dorsal and anal fins in younger individuals. This patterns typically are present in smaller specimens and full-grown adults (Kottelat et al., 1993). *B. sealei* can grow up to 14 cm of standard length. Below is its taxonomic hierarchy:

Kingdom: Animalia
Phylum: Chordata
Class: Actinopterygii
Order: Cypriniformes
Family: Cyprinidae
Species name: *Barbodes sealei* (Herre. 1933)

2.1.2 Barbodes everetti

Barbodes everetti, the clown barb (Figure 2.2), is found endemic to Borneo and Sumatra (Mills & Vevers, 1989).

Figure 2.2: Clown barb (*Barbodes everetti*) (Photo by Mr. Badiozaman Sulaiman)

This species can be found either in wild or local aquarium trade. Normally in the wild, this species inhabits the clear, fast flowing forest streams in foothill areas or quieter marginal and shallow waters such as puddles in the forest (Kottelat & Widjanarti, 2005). It is distinguished by the small dark body markings and a distinctive dark blotch at the posterior base of the dorsal fin (Pethiyagoda et al., 2012). It also exhibits sexual dimorphism where females show duller colour patterns and males develop prominent tubercles on the head during spawning conditions. *B. everetti* can grow up to 12 cm of standard length. Below is its taxonomic hierarchy:

Kingdom: Animalia
Phylum: Chordata
Class: Actinopterygii
Order: Cypriniformes
Family: Cyprinidae
Species name: *Barbodes everetti* (Boulenger. 1894)

2.2 Fish Skin Mucus Collection

Table 2.1 shows the methods of collecting fish mucus varies from one study to the other. In general, they are divided into destructive and non-destructive methods. Majority of studies for either destructive or non-destructive methods involving the fish being starved for 24 hours prior to mucus collection (Subramanian et al., 2008a; Elavarasi et al., 2013; Kumari et al., 2019).

The destructive methods, which involve killing the fish directly and collecting the fish skin mucus, were adopted in a few studies like those by electrocuting (Anbuchezhian et al., 2011), killing with a sharp blow to the head (Hiwarale et al., 2016), freezing to death (Bragadeeswaran et al., 2011) or by euthanizing with a lethal dose of anaesthesia such as tricaine methanesulphate (Caruso et al., 2014).

On the other hand, non-destructive methods were reported in more studies, which aimed at introducing stress to the fish to induce excessive mucus secretion prior to mucus collection. These were further categorised into hypothermic stress, alkali stress, salt stress, and non-lethal anaesthetic stress. In hypothermic stress, the fish was kept in an enclosed container with enough water to cover the whole body and later transferred to a freezer for an hour at -20 °C without monitoring the condition of the fish (Kumari et al., 2011; Hisar et al., 2014; Al-Rasheed et al., 2018). However, in a study by Nigam et al. (2015), the water was added with crushed ice gradually, in which mucus would be ready for collection once the fish became immobile and insensitive to human touch. For alkali stress, the fish was placed in the water treated with 2 M of Sodium hydroxide, NaOH (pH 11.5) solution for 25 min after placing the fish in 3-amino-benzoic acid ethyl ester (0.6 g/L) for 5 min (Al-Arifa et al., 2011). Since the alkali-treated mucus collections have chemical residues that might affect

Table 2.1: Destructive and non-destructive methods for fish mucus collection

Type	Method	Condition	Reference
Destructive	Electrocution	Fish kept for a week in laboratory running water before electrocuted.	Anbuchezhian et al. (2011)
	Sharp blow to the head	Fish killed by sharp blows to the head until death.	Hiwarale et al. (2016)
	Freeze to death	Fish transported to the laboratory and kept at -20 °C until death.	Bragadeeswaran et al. (2011)
	Chemical euthanisation	Fish anesthetised and euthanised at a lethal dose of Tricaine methanesulphate (MS-222) at 0.1 g/L.	Caruso et al. (2014)
Non-destructive	Hypothermic stress	Fish kept in an enclosed container with water placed in the freezer for one hour at -20 °C or add ice gradually until insensitive to human touch.	Kumari et al. (2011); Nigam et al. (2015); Katra et al. (2016); Al-Rasheed et al. (2018)
	Alkali stress	Fish kept in water treated with 3-aminobenzoic acid ethyl ester (0.6 g/L) for 5 min, followed by 2M NaOH (pH 11.5) for 25 min. Neutralisation of mucus by 2 N Tris Hydrochloride buffer (optional).	Al-Arifa et al. (2011)
	Salt stress	Fish kept in highly saline water containing NaCl salt.	Wibowo et al. (2015)
	Anaesthetic stress	Fish kept in an anaesthetic bath for 4 hour or injected with a sub-lethal dose of MS-222 (100 mg/L) or clove oil (40 ppm).	Subramanian et al. (2008a); Al-Arifa et al. (2011); Guardiola et al. (2014a, 2014b, 2017); Rao et al. (2015)
No Treatment	Mucus was collected directly without any prior treatment.		Magariños et al. (1995); Kuppulakshmi et al. (2008); Wei et al. (2010); Loganathan et al. (2011, 2013); Balasubramanian et al. (2012); Elavarasi et al. (2013); Ramesh (2013); Subhashini et al. (2013); Haniffa et al. (2014); Islam et al. (2014); Nwabueze (2014); Patil et al. (2015); Manikantan et al. (2016); Tyor & Kumari (2016)

its antibacterial activities, the mucus samples collected would be neutralised to normal pH (7.5) by adding 2 N Tris Hydrochloride buffer (Al-Arifa et al., 2011). For salt stress, the fish was kept in water with high salinity by adding sodium chloride, NaCl (Wibowo et al., 2015). In anaesthetic stress, anaesthetic solutions such as tricaine methanesulphate (Subramanian et al., 2008a; Al-Arifa et al., 2011; Guardiola et al., 2014a, 2014b; Rao et al., 2015) or clove oil (Guardiola et al., 2017) were introduced into the fish in a sub-lethal dose.

There are also studies that did not induce any non-lethal stress prior to mucus collection (Magariños et al., 1995; Hellio et al., 2002; Balasubramanian et al., 2012; Patil et al., 2015). Although the effects of stress induction on the quality of the extracted fish mucus content is unclear, a study by Al-Arifa et al. (2011) illustrated the anaesthesia-treated mucus samples showing higher protein concentrations and exhibiting significantly more antibacterial activity than the alkali-treated mucus samples from major carps, *Labeo rohita*. After stress treatment or immediately without any treatment, fish skin mucus was scraped through the body dorso-laterally with a sterile plastic spatula (Hellio et al., 2002; Kuppulakshmi et al., 2008; Balasubramanian et al., 2012; Patil et al., 2015; Al-Rasheed et al., 2018). It was reported that the physicochemical property of fish skin mucus is side dependent, and there could be variation in their composition (Fernández-Alacid et al., 2019). However, mucus from the ventral side was not collected, to avoid possible intestinal and sperm contamination. Some mucus-scraping alternative tools were also used in other studies such as sterile blade (Manivasagan et al., 2009), glass slide (Magariños et al., 1995; Fernández-Alacid et al., 2018), and cell scraper (Hiwarale et al., 2016). Besides, skin mucus was also collected by sloughing off the body surface of the fish in several studies. This was done by first washing the fish to remove any apparent dirt that might be the source of contamination, then transferring and leaving it inside a sterile polyethylene bag for

approximately 20 min, and finally collecting the mucus by scrubbing or moving back and forth to slough off the mucus (Subramanian et al., 2008a; Rao et al., 2015; Wibowo et al., 2015). The fish were then returned to recovery tanks. The scraping or scrubbing of the fish body surface should not be done excessively, as it might cause epidermal lesions that could contaminate the mucus samples (Fernández-Alacid et al., 2018). The mucus samples collected were usually stored at 4 °C or below to prevent protein degradation.

It is recommended to conduct a non-destructive method of mucus collection by scraping off the skin mucus from the body surface of anaesthetised fish, as this approach not only minimises the stress of manipulation, but also allows researchers to collect a large quantity of mucus samples from the same fish. The composition of skin mucus produced by fish varies, when subjected to a stressful condition (Cerezuela et al., 2016). Therefore, applying a certain stressor might aid in demonstrating the antibacterial properties of fish mucus more effectively. Hypothermic treatment through a chronic cold condition, instead of chemically induced stress, is recommended to preserve certain antimicrobial peptides which might be present in the skin mucus produced by the fish (Sanahuja et al., 2019).

2.3 Fish Mucus Extraction

Various solvents were used in the extraction of the fish mucus samples. Table 2.2 shows the extraction methods that are categorised into three major types of solvents, i.e. aqueous, acidic and organic extracts.

Table 2.2: Fish mucus extraction methods and solvents used

Type	Extraction Solvent	Condition/Key Steps	Reference
Aqueous	Physiological Saline (0.85 % NaCl) or simply known as Saline	Mucus to solvent ratio = 1:1. Centrifugation (15 min, 5000 rpm, 25 °C). Sterile filtration with 0.45 µm syringe filter (optional).	Magariños et al. (1995); Kuppulakshmi et al. (2008); Dhanaraj et al. (2009); Manivasagan et al. (2009); Bragadeeswaran et al. (2011); Loganathan et al. (2011); Balasubramanian et al. (2012); Caruso et al. (2014); Haniffa et al. (2014); Islam et al. (2014); Nwabueze (2014); Tyor & Kumari (2016); Kumari et al. (2019)
	Phosphate-buffered Saline (PBS)	Mucus to solvent ratio = 1:1. No centrifugation.	Vennila et al. (2011)
	100 mM Ammonium Bicarbonate	Mucus to solvent ratio = 1:1. Centrifugation (30 min, 30000 x g, 4 °C).	Anbuchezhian et al. (2011); Elavarasi et al. (2013); Al-Rasheed et al. (2018)
	Tris-buffered Saline (TBS)	Mucus to solvent ratio = 1:1. Centrifugation (10 min, 500 x g, 4 °C).	Guardiola et al. (2014a, 2014b, 2017)
	Distilled water	Mucus to solvent ratio = 1:1. Centrifugation (30 min, 30000 x g, 4 °C). Filtration of suspended solid with Whatman filter paper.	Hellio et al. (2002); Wei et al. (2010); Kumari et al. (2011); Ramesh (2013); Subhashini et al. (2013); Nigam et al. (2015); Rao et al. (2015); Katra et al. (2016)
Organic	Ethanol and Dichloromethane	Mucus to solvent ratio = 1:1. Centrifugation (30 min, 30000 x g, 4 °C).	Hellio et al. (2002); Subramanian et al. (2008a); Vennila et al. (2011); Subhashini et al. (2013); Rao et al. (2015); Wibowo et al. (2015); Katra et al. (2016); Manikantan et al. (2016)
	Acetone and Methanol	Mucus to solvent ratio = 1:1. Centrifugation (15 min, 5000 rpm, 25 °C).	Varghese & Arathy (2011)

Table 2.2: continued

Acidic	1 % Acetic Acid	Mucus to solvent ratio = 1:4. Pre-centrifuged boiling water bath (3 min). Centrifugation (35 min, 25000 x g, 4 °C). Filtration with filter paper and 0. 45 µm syringe filter.	Al-Rasheed et al. (2018)
	3 % Acetic Acid	Mucus to solvent ratio = 1:1. Centrifugation (15 min, 10000 x g, 4 °C).	Kumari et al. (2011); Nigam et al. (2015)
	10 % Acetic Acid	Mucus to solvent ratio = 1:1. Pre-centrifuged boiling water bath (5 min). Centrifugation (35 min, 18000 x g, 4 °C). Sterile filtration with 0.22 µm syringe filter (optional).	Subramanian et al. (2008a); Vennila et al. (2011); Rao et al. (2015)
	0.1 % Trifluoroacetic Acid	Mucus to solvent ratio = 1:1. Centrifugation (15 min, 10000 x g, 4 °C).	Kumari et al. (2011); Nigam et al. (2015)
Crude	Mucus (Without Solvent)	Used directly without any pre-treatment. Centrifugation (5000 rpm, 15 min, 25 °C) (optional).	Wei et al. (2010); Bragadeeswaran & Thangaraj (2011); Loganathan et al. (2013); Patil et al. (2015); Tyor & Kumari (2016); Kumari et al. (2019)

2.3.1 Aqueous Extract

For aqueous extracts, the most widely used solvent was physiological saline (0.85 % NaCl) or simply known as saline. The mucus samples were mixed thoroughly with an equal amount of sterilised saline (Magariños et al., 1995; Balasubramanian et al., 2012; Tyor & Kumari, 2016; Kumari et al., 2019) or phosphate-buffered saline (Vennila et al., 2011). The mixtures were either directly used for antibacterial screening or pre-centrifuged at room temperature at various centrifugation speeds, to obtain the supernatant for antimicrobial studies. Four other aqueous solvents were also used, such as 100 mM ammonium bicarbonate (Anbuchezhian et al., 2011; Elavarasi et al., 2013; Al-Rasheed et al., 2018), Tris-buffered Saline (TBS) (Guardiola et al., 2014a, 2014b, 2017), normal distilled water (Hellio et al., 2002; Wei et al., 2010; Katra et al., 2016) and triple distilled water (Kumari et al., 2011; Nigam et al., 2015). Unlike saline, the samples using these aqueous solvents were homogenised or stirred for two to three hours at 4 °C before centrifugation (30 min, 30,000 × g, 4 °C). Further, in some of the studies, the collected supernatants were filtered with Whatman filter paper (Hellio et al., 2002; Katra et al., 2016) or 0.45 µm syringe filter (Al-Rasheed et al., 2018).

2.3.2 Organic Extract

For organic extracts, ethanol and dichloromethane were among the most widely chosen solvents for fish skin mucus extraction (Hellio et al., 2002; Subramanian et al., 2008a; Manikantan et al., 2016). In general, the collected fish mucus was first suspended in 95 % ethanol (1 mg/ml) and centrifuged at high speed (30 min, 30,000 × g, 4 °C). The pellet was evaporated under vacuum. Besides, other organic compounds such as acetone and methanol (Varghese & Arathy, 2011) were also used in the extraction of mucus with low-

speed centrifugation (15 min, 5,000 × g, 25 °C) while Wibowo et al. (2015) mixed one part of fish skin mucus with three parts of cooled ethanol before subjecting to centrifugation.

2.3.3 Acidic Extract

For acidic extracts, the most widely used solvent was acetic acid, for its capability of inhibiting proteolytic enzyme activities (Al-Rasheed et al., 2018). Different concentrations of acetic acid were used in different studies, though the effect of their concentrations on the fish mucus antibacterial activities remained largely unknown. In general, lyophilised fish skin mucus (10 mg/ml) was mixed with low concentration acetic acid (1-10%) and then placed in a boiling water bath within minutes before subjecting to homogenisation and centrifugation (35 min, 18,000 × g, 4 °C).

2.3.4 Crude Extract

Finally, there were also studies that used crude mucus with minimum processing prior to antibacterial assays. The mucus was directly centrifuged to remove insoluble particles and the clear supernatant was collected and stored at 4 °C. Various centrifugation speeds and time were applied in different studies (Bragadeeswaran & Thangaraj, 2011; Tyor & Kumari, 2016; Kumari et al., 2019). In one study, the collected fish mucus was used directly for antibacterial screening without any extraction process (Patil et al., 2015).

2.4 Antibacterial Assay

There were different methodologies used to screen the antibacterial activity of fish skin mucus, including agar disk diffusion, agar well diffusion, broth micro-dilution and evaluation of the inhibition curves of bacterial growth by cell counting or Optical Density (OD) measurement.

2.4.1 Agar Disk Diffusion

Agar disk diffusion was the most commonly used method for antimicrobial susceptibility testing in fish skin mucus studies (Magariños et al., 1995; Kuppulakshmi et al., 2008; Hiwarale et al., 2016). This method was adopted following the standardised protocols, i.e., Clinical and Laboratory Standards Institute (CLSI) guidelines and European Committee on Antimicrobial Susceptibility Testing (EUCAST). The standards are principally similar to one another, yet different bacterial strains might require different culture media with various incubation conditions. In general, agar plates are inoculated with a standardised inoculum (0.5 McFarland standard or 10^8 CFU/ml) of the tested bacterial strains. Then, a desired amount of fish skin mucus extract is added onto filter paper discs (6 mm diameter) and placed on the agar surface. After incubation under suitable conditions (at 37 °C for 16-20 h), the diameters of clear inhibition zones exhibited by positive extract would be measured, which is also known as Inhibition zone diameter (IZD).

2.4.2 Agar Well Diffusion

Agar well diffusion was also widely used to evaluate the antimicrobial activity of fish skin mucus (Hellio et al., 2002; Haniffa et al., 2014; Al-Rasheed et al., 2018). This method is quite similar to the procedure used in the disk diffusion method. Instead of placing filter paper discs, a hole (6mm diameter) is punched aseptically with a sterile cork borer and a desired volume of the fish skin mucus extract is added into the well.

2.4.3 Broth Microdilution

Broth microdilution was also used to access the antibacterial activity of fish skin mucus (Subramanian et al., 2008a; Rao et al., 2015). Briefly, the procedure involves preparing two-fold dilutions of the mucus extracts of desired volumes, with an equal amount

of MH broth in a 96-well microtitration plate. The bactericidal activity was determined by visual inspection (clear well contents) and then confirmed by streaking or spreading an aliquot of the well contents on MHA plates. The minimum inhibitory concentrations (MICs) were defined as the lowest concentration of mucus needed to completely inhibit the bacterial growth, while minimal bactericidal concentrations (MBCs) of mucus extracts were defined as the lowest concentrations at which 99.9% of the final inoculum is killed.

2.4.4 Bacterial Growth Curve

Finally, the antibacterial properties of certain fish species were also assayed by evaluating the growth inhibition curves of bacterial strains. For instance, the antibacterial effect of the skin mucus of the common stingray, *Dasyatis pastinaca* by was assessed using the growth inhibition curves by cell counting (Fuochi et al., 2017). Though the cell counting method requires long hours of monitoring as well as manpower, it provides additional information about the dynamic interaction between the mucus extracts and the bacterial strains.

2.5 Antibacterial Properties of Fish Skin Mucus

This section reviewed the antibacterial activities of the epidermal mucus of 47 fish species from three classes, namely Actinopterygii (ray-finned fishes), Elasmobranchii (cartilaginous fish), and Myxini (hag fishes) which exhibited a broad spectrum of antibacterial activity against a total of 46 bacterial species, including 13 Gram-positive bacteria (refer to Table 2.3), one acid-fast bacteria, and 32 Gram-negative bacteria (refer to Table 2.4).

Table 2.3: Fish mucus extracts with antibacterial activity against Gram-positive bacteria

Class Order *Species*	Common Name	Habitat	Gram-positive bacteria														References
			1	2	3	4	5	6	7	8	9	10	11	12	13	14*	
Actinopterygii																	
Anguilliformes																	
Anguilla Anguilla	European Eel	F					+ b,c,d				+ b,c,d						Bragadeeswaran & Thangaraj (2011); Caruso et al. (2014)
Cypriniformes																	
Barbonymus schwanenfeldii	Tinfoil barb	F	+ d								+ d						Subhashini et al. (2013)
Catla catla	Catla	F															Balasubramanian et al. (2012); Islam et al. (2014)
Cirrhinus mrigala	Mrigal	F					+ b				+ bc						Kuppulakshmi et al. (2008); Nigam et al. (2015)
Ctenopharyngodon idella	Grass carp	F	+ b,c								+ b,c						Balasubramanian et al. (2012); Islam et al., (2014); Kumari et al. (2019)
Cyprinus carpio	European carp	F	+ b,c								+ b,c						Kumari et al. (2019)
Hypophthalmichthys molitrix	Silver carp	F															Balasubramanian et al. (2012); Islam et al. (2014)
Hypophthalmichthys nobilis	Bighead carp	F	+ b,c								+ b,c	+ b,c					Tyor & Kumari (2016); Kumari et al. (2019)
Labeo rohita	Rohu	B,F			+ c			+ c			+ c						Al-Arifa et al. (2011); Balasubramanian et al. (2012); Islam et al. (2014)
Gadiformes																	
Melanogrammus aeglefinus	Haddock	M										+ a					Subramanian et al. (2008a)
Pollachius virens	Saithe, Pollock	M	+ d	+ d	+ d						+ d		+ d				Hellio et al. (2002)
Perciformes																	
Anabas testudineus	Climbing Perch	B,F			+ a					+ a	+ a						Al-Rasheed et al. (2018)
Channa gachua	Dward snakehead	F															Dhanaraj et al. (2009)
Channa marulius	Great snakehead	F															Dhanaraj et al. (2009)
Channa micropeltes	Giant snakehead	F															Dhanaraj et al. (2009)
Channa punctatus	Spotted snakehead	B,F					+ b	+ a,b			+ a,b						Kuppulakshmi et al. (2008); Dhanaraj et al. (2009); Kumari et al. (2011)
Channa striatus	Striped snakehead	B,F			+ a,b		+ b	+ b			+ b,c			+ b		+ b	Dhanaraj et al. (2009); Wei et al. (2010); Loganathan et al. (2013); Haniffa et al. (2014); Ramesh (2013)
Oreochromis niloticus	Nile tilapia	B,F	+ a		+ a			+ a		+ a							Rao et al. (2015); Wibowo et al. (2015)
Oreochromis mossambicus	Mozambique tilapia	B,F									+ b						Elavarasi et al. (2013)
Labrus bergylta	Ballan wrasse	M															Hellio et al. (2002)
Dicentrarchus labrax	European seabass	M			+ b						+ b						Magariños et al. (1995); Caruso et al. (2014); Guardiola et al. (2014a)
Umbrina cirrosa	Shi drum	M			+ b												Guardiola et al. (2014a)
Epinephelus marginatus	Dusky grouper	M															Guardiola et al. (2014a)
Epinephelus tauvina	Greasy grouper	M									+ a						Manikantan et al. (2016)
Dentex dentex	Common dentex	M			+ b												Guardiola et al. (2014a)
Pagellus bogaraveo	Blackspot seabream	M															Caruso et al. (2014)
Sparus aurata	Gilthead seabream	M									+ b						Magariños et al. (1995); Guardiola et al. (2014a, 2014b)

Table 2.3: continued

Pleuronectiformes																
Cynoglossus arel	Largescale tonguesole	M									+ b					Bragadeeswaran et al. (2011)
Platichthys flesus	European flounder	M	+ d	+ d	+ d						+ d		+ d			Hellio et al. (2002)
Scophthalmus maximus	Turbot	M	+ d	+ d	+ d						+ d		+ d			Magariños et al. (1995)
Scophthalmus rhombus	Brill	M									+ b					Hellio et al. (2002)
Solea senegalensis	Senegalese sole	M														Guardiola et al. (2017)
Solea solea	Common sole	M	+ d	+ d	+ d						+ d		+ d			Hellio et al. (2002)
Salmoniformes																
Oncorhynchus mykiss	Rainbow trout	ALL					+ b				+ b					Ramesh (2013)
Salvelinus fontinalis	Brook trout	F										+ a				Subramanian et al. (2008a)
Siluriformes																
Arius caelatus	Engraved catfish	M									+ b					Bragadeeswaran et al. (2011)
Arius maculatus	Spotted catfish	M												+ b		Manivasagan et al. (2009); Anbuchezhian et al. (2011)
Mystus gulio	Long whiskers catfish	B,F														Anbuchezhian et al. (2011)
Mystus nemurus	Asian redtail catfish	B,F	+ a		+ a			+ a		+ a						Rao et al. (2015)
Rita rita	Rita	B,F						+ a			+ a,b					Kumari et al. (2011)
Clarias batrachus	Walking catfish	B,F			+ c						+ b,c,d					Varghese & Arathy (2011); Elavarasi et al. (2013); Loganathan et al. (2013); Patil et al. (2015)
Clarias gariepinus	African sharptooth catfish	F				+ b							+ b	+ b		Nwabueze (2014)
Heteropneustes fossilis	Asian Stinging catfish	B,F			+ b			+ b			+ b				+ b	+ b	Haniffa et al. (2014)
Elasmobranchii																
Myliobatiformes																
Dasyatis pastinaca	Common stingray	M														Fuochi et al. (2017)
Dasyatis sephen	Cowtail stingray	M									+ a					Vennila et al. (2011)
Himantura gerrardi	Whitespotted whipray	B,M									+ a					Vennila et al. (2011)
Pteraspidomorphi																
Myxiniformes																
Myxine glutinosa	Atlantic hagfish	M										+ a				Subramanian et al. (2008a)

*Acid-fast bacteria;
B = Brackish water, F = Freshwater, M = Marine;
+ Sensitive to fish skin mucus, Solvent used for extraction = [a]Acidic, [b]Aqueous, [c]Crude, [d]Organic;
Bacteria designation: 1 - *Bacillus cereus*, 2 - *Bacillus megaterium*, 3 - *Bacillus subtilis*, 4 - *Bacillus* spp., 5 - *Lactobacillus vulgaris*, 6 - *Micrococcus luteus*, 7 - *Sarcina lutea*, 8 - Methicillin-resistant *Staphylococcus aureus* (MRSA), 9 - *Staphylococcus aureus*, 10 - *Staphylococcus epidermidis*, 11 - *Staphylococcus* spp., 12 - Streptococcus *pyogenes*, 13 - *Streptococcus* spp., 14 - *Mycobacterium smegmatis*

Table 2.4: Fish mucus extracts with antibacterial activity against Gram-negative bacteria

Class Order *Species*	Common Name	Habitat	Gram-negative bacteria																																References
			1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	
Actinopterygii Anguilliformes																																			
Anguilla Anguilla	European Eel	F			+ b,c, d		+ b	+ b,d			+ c		+ c				+ b,c, d	+ b,c							+ b				+ b		+ b	+ b,c, d			Bragadeeswaran & Thangaraj (2011); Caruso et al. (2014)
Cypriniformes *Barbonymus schwanenfeldii*	Tinfoil barb	F			+ d																		+ d												Subhashini et al. (2013)
Catla catla	Catla	F	+ b	+ b	+ b			+ b					+ b	+ b				+ b								+ b	+ b								Balasubramanian et al. (2012); Islam et al. (2014)
Cirrhinus mrigala	Mrigal	F			+ b		+ b	+ b			+ b		+ a,b				+ a,b	+ a,b									+ a,b								Kuppulakshmi et al. (2008); Nigam et al. (2015)
Ctenopharyngodon idella	Grass carp	F	+ b, c	+ b	+ b,c			+ b,c					+ b,c	+ b		+ b,c		+ b								+ b	+ b								Balasubramanian et al. (2012); Islam et al., (2014); Kumari et al. (2019)
Cyprinus carpio	European carp	F	+ b, c		+ b,c			+ b,c					+ b,c			+ b,c																			Kumari et al. (2019)
Hypophthalmichthys molitrix	Silver carp	F	+ b	+ b	+ b			+ b					+ b	+ b				+ b								+ b	+ b								Balasubramanian et al. (2012); Islam et al. (2014)
Hypophthalmichthys nobilis	Bighead carp	F	+ b, c		+ b,c			+ b,c					+ b,c			+ b,c																			Tyor & Kumari (2016); Kumari et al. (2019)
Labeo rohita	Rohu	B,F	+ b	+ b	+ b,c			+ b					+ b	+ b				+ b								+ b	+ b								Al-Arifa et al. (2011); Balasubramanian et al. (2012); Islam et al. (2014)
Gadiformes *Melanogrammus aeglefinus*	Haddock	M		+ a	+ a				+ a				+ a						+ a														+ a		Subramanian et al. (2008a)
Pollachius virens	Saithe, Pollock	M			+ d			+ d				+ d	+ d								+ d														Hellio et al. (2002)
Perciformes *Anabas testudineus*	Climbing Perch	B,F	+ a		+ a								+ a		+ a					+ a	+ a														Al-Rasheed et al. (2018)
Channa gachua	Dward snakehead	F	+ b		+ b								+ b													+ b		+ b							Dhanaraj et al. (2009)
Channa marulius	Great snakehead	F	+ b		+ b								+ b													+ b		+ b							Dhanaraj et al. (2009)
Channa micropeltes	Giant snakehead	F	+ b		+ b								+ b													+ b		+ b							Dhanaraj et al. (2009)
Channa punctatus	Spotted snakehead	B,F	+ b		+ b		+ b	+ b			+ b		+ b				+ b	+ b								+ b	+ b	+ b							Kuppulakshmi et al. (2008); Dhanaraj et al. (2009); Kumari et al. (2011)
Channa striatus	Striped snakehead	B,F	+ b, c	+ c	+ b,c		+ b	+ a,b	+ c		+ b	+ b	+ a,b				+ b	+ b	+ b							+ b	+ b,c	+ b							Dhanaraj et al. (2009); Wei et al. (2010); Loganathan et al. (2013); Haniffa et al. (2014); Ramesh (2013)

Table 2.4: continued

Oreochromis niloticus	Nile tilapia	B,F	+ c			+ c			+ c								+ c			+ c											+ d				Rao et al. (2015); Wibowo et al. (2015)
Oreochromis mossambicus	Mozambique tilapia	B,F	+ b			+ b							+ b	+ b																					Elavarasi et al. (2013)
Labrus bergylta	Ballan wrasse	M				+ d			+ d				+ d	+ d								+ d													Hellio et al. (2002)
Dicentrarchus labrax	European seabass	M				+ b					+ b			+ b									+ b			+ b	+ b			+ b	+ b	+ b			Magariños et al. (1995); Caruso et al. (2014); Guardiola et al. (2014a)
Umbrina cirrosa	Shi drum	M				+ b					+ b												+ b				+ b				+ b				Guardiola et al. (2014a)
Epinephelus marginatus	Dusky grouper	M				+ b					+ b												+ b				+ b				+ b				Guardiola et al. (2014a)
Epinephelus tauvina	Greasy grouper	M	+ a			+ a			+ a			+ a			+ a				+ a							+ a					+ a	+ a			Manikantan et al. (2016)
Dentex dentex	Common dentex	M				+ b					+ b												+ b				+ b				+ b				Guardiola et al. (2014a)
Pagellus bogaraveo	Blackspot seabream	M																														+ b			Caruso et al. (2014)
Sparus aurata	Gilthead seabream	M				+ b					+ b												+ b				+ b				+ b				Magariños et al. (1995); Guardiola et al. (2014a, 2014b)
Pleuronectiformes																																			
Cynoglossus arel	Largescale tonguesole	M																	+ b									+ b				+ b			Bragadeeswaran et al. (2011)
Platichthys flesus	European flounder	M				+ d			+ d				+ d	+ d								+ d													Hellio et al. (2002)
Scophthalmus maximus	Turbot	M				+ d			+ d				+ d	+ d								+ d													Magariños et al. (1995)
Scophthalmus rhombus	Brill	M									+ b																+ b								Hellio et al. (2002)
Solea senegalensis	Senegalese sole	M									+ b																+ b				+ b				Guardiola et al. (2017)
Solea solea	Common sole	M				+ d			+ d				+ d	+ d								+ d													Hellio et al. (2002)
Salmoniformes																																			
Oncorhynchus mykiss	Rainbow trout	ALL				+ b		+ b	+ b			+ b		+ b				+ b	+ b									+ b							Ramesh (2013)
Salvelinus fontinalis	Brook trout	F		+ a		+ a				+ a				+ a						+ a														+ a	Subramanian et al. (2008a)
Siluriformes																																			
Arius caelatus	Engraved catfish	M																	+ b									+ b				+ b			Bragadeeswaran et al. (2011)
Arius maculatus	Spotted catfish	M				+ b			+ b					+ b							+ b				+ b			+ b							Manivasagan et al. (2009); Anbuchezhian et al. (2011)
Mystus gulio	Long whiskers catfish	B,F				+ b		+ b	+ b			+ b		+ b																					Anbuchezhian et al. (2011)
Mystus nemurus	Asian redtail catfish	B,F	+ a			+ a			+ a					+ a			+ a																		Rao et al. (2015)
Rita rita	Rita	B,F																		+ a															Kumari et al. (2011)
Clarias batrachus	Walking catfish	B,F	+ b			+ b,d			+ b,c,d				+ b,c,d	+ b,c,d				+ c									+ b		+ b						Varghese & Arathy (2011); Elavarasi et al. (2013); Loganathan et al. (2013); Patil et al. (2015)
Clarias gariepinus	African sharptooth catfish	F					+ b																												Nwabueze (2014)

Table 2.4: continued

Heteropneustes fossilis	Asian Stinging catfish	B,F				+ b							+ b	+ b						+ b								+ b							Haniffa et al. (2014)
Elasmobranchii Myliobatiformes																																			
Dasyatis pastinaca	Common stingray	M				+ na			+ na					+ na																					Fuochi et al. (2017)
Dasyatis sephen	Cowtail stingray	M				+ a			+ a										+ a									+ a							Vennila et al. (2011)
Himantura gerrardi	Whitespotted whipray	B,M				+ a			+ a										+ a									+ a							Vennila et al. (2011)
Pteraspidomorphi Myxiniformes																																			
Myxine glutinosa	Atlantic hagfish	M		+ a		+ a				+ a				+ a						+ a														+ a	Subramanian et al. (2008a)

F = Freshwater, B = Brackish water, M = Marine;

+ Sensitive to fish epidermal mucus, Solvent used for extraction = [a]Acidic, [b]Aqueous, [c]Crude, [d]Organic;

Bacteria designation: 1 - *Aeromonas hydrophila*, 2 - *Aeromonas salmonicida* subsp. *salmonicida*, 3 - *Aeromonas veronii* bv. *Sobria*, 4 - *Escherichia coli*, 5 - *Escherichia* spp., 6 - *Klebsiella oxytoca*, 7 - *Klebsiella pneumoniae*, 8 - *Listonella anguillarum*, 9 - *Photobacterium damselae* subsp. *piscicida*, 10 - *Proteus mirabilis*, 11 - *Proteus vulgaris*, 12 - *Pseudomonas aeruginosa*, 13 - *Pseudomonas fluorescens*, 14 - *Salmonella choleraesuis*, 15 - *Salmonella enterica* serovar *Enteritidis*, 16 - *Salmonella enterica* serovar *Paratyphi*, 17 - *Salmonella enterica* serovar *Typhi*, 18 - *Salmonella enterica* serovar *Typhimurium*, 19 - *Salmonella* spp., 20 - *Serratia marcescens*, 21 - Shewanella *putrefaciens*, 22 - *Shigella boydii*, 23 - *Shigella* spp., 24 - *Vibrio alginolyticus*, 25 - *Vibrio anguillarum*, 26 - *Vibrio cholerae*, 27 - *Vibrio fischeri*, 28 - *Vibrio fluvialis*, 29 - *Vibrio harveyi*, 30 - *Vibrio parahaemolyticus*, 31 - *Vibrio* spp., 32 - *Yersinia ruckeri*

2.5.1 Class Actinopterygii

Ray-finned fishes are the most diverse class of vertebrates, comprising about 99% of freshwater and marine species in the world (Pandey & Shukla, 2007). The majority of the fish species (43 out of 47) reviewed are grouped under Actinopterygii, which consists of seven orders (Anguilliformes, Cypriniformes, Gadiformes, Perciformes, Pleuronectiformes, Salmoniformes, and Siluriformes).

2.5.1.1 Order Anguilliformes

Anguilla anguilla, the European eel (Family: Anguillidae) is the most abundant species in its genus. Among all the strains tested, *S. paratyphi*, which is one of the common shrimp culture pond pathogens, showed the greatest sensitivity towards the crude mucus extract with IZD = 10 mm. Broad-spectrum antibacterial properties were also presented by their aqueous, crude and organic mucus extracts (Bragadeeswaran & Thangaraj, 2011; Caruso et al., 2014) against various pathogens, including two Gram-positive and nine other Gram-negative bacteria which are likely to be spread in their habitat. Among them, three *Vibrio* species and *S. aureus* were more resistant against the mucus extracts with the relatively low IZD values (<1 mm).

2.5.1.2 Order Cypriniformes

Cyprinidae is the largest and most diverse fish family in Cypriniformes. There was a total of eight species screened for their mucosal antibacterial properties, including one barb species and seven carp species. As the only barb species studied, the organic mucus extract of *Barbonymus schwanenfeldii*, the tinfoil barb (Subhashini et al., 2013) exhibited similar antibacterial effect (IZD ranged from 7 to 9 mm) against *B. cerues*, *S. aureus*, *E. coli* and *S. boydii*. However, no activity was observed from the aqueous mucus extract of the same species. The aqueous mucus extracts from four of the carp species (Balasubramanian et al.,

2012; Islam et al., 2014; Kumari et al., 2019) namely *Catla catla*, the catla, *Ctenopharyngodon idella*, the grass carp, *Hypophthalmichthys molitrix*, the silver carp, and *Labeo rohita*, the rohu show varying activities against eight Gram-negative bacteria. *P. aeruginosa* was reported to be the most sensitive strain (IZD = 29 mm) towards the skin mucus of catla, while *K. pneumonia* and *V. cholera* were more resistant against the skin mucus of grass carp with IZD of only 7 mm.

As opposed to Al-Arifa et al. (2011), three Gram-positive bacteria, namely *S. aureus*, *S. lutea* and *B. subtilis*, were reported to show increasing order of susceptibility towards the epidermal mucus extract of rohu produced by inducing anaesthesia and alkali stress. This demonstrates that the effect of stress induced during skin mucus collection would affect the content of the extracts and consequently the spectrum of antibacterial activity. In addition, another species from *Hypophthalmichthys* genus revealed different results in which the aqueous extract (Tyor & Kumari, 2016; Kumari et al., 2019) of *H. nobilis*, the bighead carp, also inhibited the growth of three additional Gram-positive bacteria, namely *S. aureus*, *B. cerues* and *S. epidermidis*, with increasing order of sensitivity.

The acidic and aqueous mucus extracts (Kuppulakshmi et al., 2008; Nigam et al., 2015) of *Cirrhinus mrigala*, the mrigal, also revealed broad-spectrum antibacterial activity against two Gram-positive and eight Gram-negative bacteria, in which *S. paratyphi* was the most resistant strain against both mucus extracts with relatively lower IZD values (3–4 mm). However, it is noteworthy that the aqueous mucus extract of the mrigal was a more effective antibacterial agent than the antibiotic chloramphenicol (10 µg/ml), against *K. oxytoca* and *V. cholerae*. Lastly, the crude and aqueous extracts of *Cyprinus carpio*, the European carp

(Kumari et al., 2019) had shown broad spectrum of antibacterial activity against seven human and fish pathogenic bacteria with MIC values ranged from 25 to 50 µg/ml.

2.5.1.3 Order Gadiformes

Skin mucus of two cod species (Family: Gadidae) namely *Melanogrammus aeglefinus*, known commonly as the haddock, and *Pollachius virens*, known commonly as the Pollock, were reported as having antibacterial activity against both Gram-positive and Gram-negative bacteria. The acidic mucus extracts of haddock (Subramanian et al., 2008a) revealed varying bactericidal activities against various pathogens, including one Gram-positive (*S. epidermis* C621) and six Gram-negative bacteria (*A. salmonicida* A449, *E. coli* D31, *L. anguillarum* 02-11, *P. aeruginosa* Z61 and K799, *S. typhimurium* C610, *Y. ruckeri* 96-4). The lowest MBC value (14 µg/ml) was observed against human pathogens such as *E. coli* D31 and *S. typhimurium* C610 and fish pathogen *Y. ruckeri* 96-4, while *S. epidermis* C621 and *P. aeruginosa* K799 were among the more resistant strains against haddock skin mucus, with MBC = 192 µg/ml. The organic mucus extracts of Pollock (Hellio et al., 2002) exhibited effective inhibition against ten different strains containing five Gram-positive and five Gram-negative bacteria, in which the lowest MIC value (12 µg/ml) was shown against *B. megaterium* CIP 6620T and the more resistant strains with MIC value = 96 µg/ml in the study were *S. aureus* ATCC25923 and *Serratia marcescens* CIP67.55.

2.5.1.4 Order Perciformes

Perciformes (Perch-like fish) is one of the most studied fish which can be subdivided into seven families, namely Anabantidae, Channidae (Snakeheads), Cichlidae (Cichlids), Labridae (Wrasses), Moronidae (Temperate basses), Scianidae (Drums), Serranidae (Groupers), and Sparidae (Porgies).

The only species from Anabantidae, *Anabas testudines* was reported to exhibit broad-spectrum of antibacterial activities against both Gram-positive and Gram-negative bacteria (Al-Rasheed et al., 2018). The acidic mucus extracts of these climbing perches showed strong antibacterial activity against *P. aeruginosa* which have the highest value (12.65 ± 0.47 mm) of IZD (not including the diameter of the disc), followed by *A. hydrophilia* (10.5 ± 1.73 mm) and *E. coli* (9.5 ± 0.58 mm). Interestingly, it also exhibited an observable inhibition activity (IZD = 0.87 ± 0.25 mm) on MRSA ATCC 43300. This suggests that a potentially effective antimicrobial activity was exhibited by this climbing perch species, which could be exploited to overcome the bacteria with growing resistance towards commonly-used antibiotics. Besides, it also showed varying level of activities against three other Gram-negative bacteria and two Gram-positive bacteria. No activity was detected in the aqueous mucus extract of the species.

The wide spectrum of antibacterial properties of five snakehead species (Family: Channidae) namely *Channa gachua* (Dwarf snakehead), *C. marulius* (Great snakehead), *C. micropeltes* (Giant snakehead), *C. punctatus* (Spotted snakehead), *C. striatus* (Striped snakehead) were also reported. The aqueous mucus extracts of all *Channa* species (Dhanaraj et al., 2009) exhibited significant inhibitory activities against five Gram-negative bacteria, with *V. fischeri* being the most sensitive strain with IZD = 30 mm towards the skin mucus of spotted snakehead while *A. hydrophila* was the most resistant strain against the skin mucus of dwarf snakehead. The results were slightly contradicted by the study of (Rao et al., 2015), where *E. coli* ATCC 25922 was resistant to the aqueous extracts of giant snakehead and striped snakehead, which could be related to different types of solvents used for mucus extraction. The acidic, aqueous and crude mucus extracts of *C. striatus*, the striped

snakehead, exhibited broad-spectrum antibacterial activity in other studies as well (Wei et al., 2010; Loganathan et al., 2013; Ramesh, 2013; Haniffa et al., 2014).

Other than the bacterial species mentioned above, a great array of inhibitory activity was observed against 16 other bacteria, including five Gram-positive, ten Gram-negative and one acid-fast bacteria. Gram-negative bacteria such as *A. salmonicida* and *E. coli* were among the most sensitive strains reported with higher IZD values (15 mm and 17 mm), while Gram-positive bacteria such as *S. aureus* was the most resistant strain with only 6.5 mm of IZD value. Besides, the antibacterial properties of *C. punctatus*, the spotted snakeheads were also reported elsewhere (Kuppulakshmi et al., 2008; Kumari et al., 2011), where its acidic and aqueous extracts exhibited strong inhibition against nine other Gram-positive and Gram-negative bacteria. The interesting findings were highlighted as the aqueous mucus extract of spotted snakehead exhibited a far better antimicrobial activity than chloramphenicol (10 μg/ml) against *V. cholerae* and *S. aureus*. The rest of the bacteria showed varying resistance against the mucus extracts, in which the most resistant strains reported were *S. paratyphi* and *S. typhi*.

Tilapias are among the most important commercial cichlids (Family: Cichlidae) found in the world. The skin mucus of two tilapia species namely *Oreochromis niloticus*, Nile tilapia and *Oreochromis mossambicus*, Mozambique tilapia were reported to be bactericidal against a broad range of Gram-positive and Gram-negative bacteria. The acidic mucus extract of Nile tilapia (Rao et al., 2015) showed strong bactericidal effect against nine bacterial strains, consisting of four Gram-positive microbes including *B. cereus* HQ 1852830, *B. subtilis* ATCC 11774, *M. luteus* ATCC 4698 and MRSA ATCC 33591 and five Gram-negative pathogens, viz. *A. hydrophila* ATCC 49140, *E. coli* ATCC 25922, *K.*

pneumoniae ATCC 700603, *S. typhimurium* IMR S391 and *S. enteritidis* IMR S966. *B. subtilis*, *M. luteus* and *E. coli* were among the more sensitive strains with MBC value of 15.96 µg/ml, which the others exhibited greater resistance against the tilapia mucus with MBC value of 31.91 µg/ml. In another study by Wibowo et al. (2015), ethanol mucus extract of the same species demonstrated moderate antibacterial activity against *V. harveyi* with MIC value of 4.5 ug/ml and MBC value of 17.99 µg/ml. Other than that, the aqueous mucus extracts of Mozambique tilapia (Elavarasi et al., 2013) also exhibited broad-spectrum inhibitory activities against one Gram-positive and four Gram-negative bacteria with the most sensitive strain – *A. hydrophila* with highest IZD = 12.76 ± 1.68 mm and the most resistant strain - *K. pneumonia* with lowest IZD = 8.08 ± 0.36 mm.

Besides, other fish species that are highly exploited in aquarium trade and commercial fisheries were also found in different antibacterial studies. Families of Labridae, Moronidae and Scianidae, with each represented by only one species, namely *Labrus bergylta*, the Ballan wrasse, *Dicentrarchus labrax*, the European seabass and *Umbrina cirrosa*, the Shi drum, respectively, were screened for their antibacterial properties while two species from family Serranidae namely *Epinephelus marginatus*, the Dusky grouper and *E. tauvina*, the Greasy grouper were reported in antibacterial studies. The organic mucus extract (30 µl, 1 mg/ml) of Ballan wrasse (Hellio et al., 2002) was reported to show effective inhibition against five terrestrial pathogens consisting of Gram-negative bacteria only. *Bacillus* species was more sensitive towards the mucus extract with greater IZD value, while *Streptococcus* species was more resistant against the skin mucus with the lower IZD value, compared to the other strains tested. However, Katra et al. (2016) reported that none of the bacterial strains tested were sensitive towards the organic mucus extract (10 µl, 1 mg/ml) of

the species, although the same extraction protocol was applied. The negative results in the latter could be due to the failure of reaching the MIC value against the strains tested.

Next, the aqueous mucus extracts of European bass and Shi drum showed antibacterial effect against several Gram-negative and Gram-positive bacteria with varying sensitivity. Different aqueous solvents such as saline (Caruso et al., 2014), sterile seawater (Magariños et al., 1995), and Tris-buffered saline (Guardiola et al., 2014a) were used, yielding similar results. Besides, the aqueous mucus extract of Dusky grouper (Guardiola et al., 2014a) and the acidic mucus extract of greasy grouper (Manikantan et al., 2016) demonstrated intense antibacterial activity against various human and fish pathogens. The greatest inhibitory activities shown against human and fish pathogens were *P. mirabilis* (IZD = 26.0 ± 0.3mm) and *V. parahaemolyticus* (IZD = 25.0 ± 0.1 mm) respectively, while the most resistant human and fish pathogens were *E. coli* (IZD = 14.0 ± 0.3 mm) and *V. alginolyticus* (IZD = 15.0 ± 0.1 mm). The acidic mucus extract of greasy grouper should be highlighted, as it showed higher antibacterial activity against all pathogens tested, other than the antibiotic ampicillin.

Breams are one of the widely exploited seafood sources for humans. The aqueous mucus extracts of two seabream species (Family: Sparidae) namely *Pagellus bogaraveo*, the blackspot seabream and *Sparus aurata*, the gilthead seabream, revealed contradicting antibacterial activities. The blackspot seabream (Caruso et al., 2014) showed activity only against one Gram-negative bacteria – *V. parahaemolyticus*, while the gilthead seabream (Magariños et al., 1995; Guardiola et al., 2014a, 2014b) exhibited inhibition upon a wide array of Gram-positive and Gram-negative bacterial strains namely *S. aureus*, *E. coli*, *P. damselae*, *S. putrefaciens*, *V. anguillarum* and *V. harveryii*. One of the tested strains, *B.*

subtilis, on the other hand, has exhibited a better growth when incubated in the mucus extract of Gilthead seabream. Another member from the same family, *Dentex dentex* which is also known as the Common dentex, exhibited effective antibacterial activity against various Gram-positive and Gram-negative bacteria as well (Guardiola et al., 2014a).

2.5.1.5 Order Pleuronectiformes

Flatfishes are one of the popular bottom-feeding fishes in commercial fisheries. One of the many families is Cynoglossidae, represented by one species of tonguefish, *Cynoglossus arel*, also known as largescale tonguesole. The aqueous mucus extracts of *C. arel* (Bragadeeswaran et al., 2011) had shown good activity against four human pathogens including *S. typhi*, *V. parahaemolyticus*, *S. aureus* and *V. cholerae* with increasing order of sensitivity (increasing IZD values). Further, many other important food fish under this order, including flounders (Family: Pleuronectidae), turbots (Family: Scophthalmidae) and soles (Family: Soleidae) were reported to be effective in inhibiting certain bacterial growth as well. The organic mucus extracts of *Platichthys flesus*, the European flounder, *Scophthalmus rhombus*, the brill and *Solea solea*, the common sole (Hellio et al., 2002) exhibited varying antibacterial activities against ten Gram-positive and Gram-negative bacteria, in which the lowest MIC value (12 µg/ml) was observed against *E. coli* K12 ATCC 23176 and *P. aeruginosa* ATCC 27853 while *B. megaterium* CIP 6620T was more resistant with MIC value of 96 µg/ml. Further, two studies (Magariños et al., 1995; Guardiola et al., 2017) demonstrated interesting results from two other flatfish species, *S. maximus and S. sonegalensis*, from genus *Scophthalmus* and *Solea*, respectively. Both species showed strong antibacterial activities against two common fish pathogens, namely *Photobacterium damselae* subsp. *piscicida* and *V. anguillarum*, whose natural hosts are a wide variety of marine fish (Romalde & Magariños, 1997) and may greatly impact commercial fisheries.

2.5.1.6 Order Salmoniformes

Two salmonid species (Family: Salmonidae) namely *Oncorhynchus mykiss*, the Rainbow trout and *Salvelinus fontinalis*, the brook trout were reported to present broad-spectrum antibacterial activities. The aqueous extract of rainbow trout (Ramesh, 2013) revealed antibacterial activities against a broad range of pathogens, including two Gram-positive and eight Gram-negative bacteria with varying susceptibility (IZD ranged from 7 to 12 mm) while acidic mucus extracts of brook trout (Subramanian et al., 2008a) exhibited bactericidal activity against various pathogens consisting of one Gram-positive (*S. epidermis* C621) and six Gram-negative bacteria (*A. salmonicida* A449, *E. coli* D31, *L. anguillarum* 02-11, *P. aeruginosa* Z61 and K799, *S. typhimurium* C610, *Y. ruckeri* 96-4). The result from brook trout mucus extract against *S. typhimurium* C610 was highlighted with MBC value as low as 10 µg/ml, while *P. aeruginosa* K799 was the most resistant strain against brook trout skin mucus with MBC = 273 µg/ml. However, the aqueous mucus extract of rainbow trout in another study (Hisar et al., 2014) showed no activity against any of the bacterial strains tested. This could be due to the contamination of mucus extracts upon collection and lack of treatment to eliminate the contaminants such as the absence of bacterial filtration step in the study.

2.5.1.7 Order Siluriformes

Siluriformes (catfishes) was among the most studied order for antibacterial research on its epidermal mucus. A total of eight species of catfishes from four families, including marine catfish - Ariidae (sea catfish), and freshwater catfish - Bagridae (bagrid catfish), Clariidae (airbreathing catfish) and Heteropneustidae (airsac catfish) were screened for their antibacterial activity.

Two marine catfish species, namely *Arius caelatus*, engraved catfish and *A. maculatus*, spotted catfish were reported to show antibacterial activities against different bacteria tested. The aqueous mucus extract of engraved catfish (Bragadeeswaran et al., 2011) showed activity against four terrestrial pathogens, *S. typhi*, *V. cholerae*, *S. aureus*, and *V. parahaemolyticus* with increasing order of susceptibility (increasing IZD values), while the aqueous mucus extract of spotted catfish (Manivasagan et al., 2009; Anbuchezhian et al., 2011) showed a wider spectrum of inhibitory activity against seven strains comprising one Gram-positive and six Gram-negative bacteria with varying sensitivity (IZD ranged from 7 to 10 mm).

Compared to marine catfishes, the antibacterial properties of freshwater catfish were reported more extensively which consists of three bagrid catfish species - *Mystus gulio* (Long whiskers catfish), *Mystus nemurus* (Asian redtail catfish), *Rita rita*, two air-breathing catfish species - *Clarias batrachus* (Walking catfish), *Clarias gariepinus* (African sharp-tooth catfish) and one air-sac catfish species - *Heteropneustes fossilis* (Asian Stinging catfish).

The aqueous mucus extracts of *M. gullio*, the long whiskers catfish (Anbuchezhian et al., 2011) showed bacteriostatic activity against Gram-negative bacteria only, including five common human pathogens, in which *P. aeruginosa* was the most sensitive strain (IZD = 14 mm), while *K. oxytoca* was the most resistant strain against the catfish skin mucus (IZD = 10 mm). However, the acidic mucus extract of Asian redtail catfish, which is from the same genus *Mystus*, exhibited a bactericidal effect on nine pathogens, including both Gram-positive and Gram-negative bacteria. In Rao et al. (2015), its extracts had shown twice stronger antimicrobial activity against Gram-positive microbes including *B. cereus* HQ 1852830, *B. subtilis* ATCC 11774, *M. luteus* ATCC 4698 and MRSA ATCC 33591 with

MBC = 11.96 µg/ml, than Gram-negative pathogens including *A. hydrophila* ATCC 49140, *E. coli* ATCC 25922, *K. pneumoniae* ATCC 700603, *P. aeruginosa* ATCC 27583 and *S. enteritidis* IMR S966 with MBC = 23.91 µg/ml). This could be due to the largely impermeable cell wall within Gram-negative bacteria that prevent the active compound in the extracts from penetrating the cell, making it more resistant to the mucus extracts than Gram-positive bacteria.

Other than that, the acidic mucus extract of *R. rita* (Kumari et al., 2011) was found to inhibit two Gram-positive and one Gram-negative bacteria namely *S. typhi*, *M. lutues*, and *S. aureus* with increasing order of sensitivity (increasing IZD from 8 to 17 mm). Notably, different strengths of inhibition were observed between the acidic (IZD = 17.0 ± 2.58 mm) and aqueous (IZD = 9.75 ± 1.70 mm) mucus extracts of this species against *S. aureus*. These bagrid catfish had demonstrated the effect of different solvents used in extraction that may change the antibacterial activity (i.e. increasing IZD or from bacteriostatic to bactericidal) of the fish mucus as well as the spectrum of its activity.

Clarias batrachus, the walking catfish, exhibited broad-spectrum antibacterial activities in many studies. The aqueous and organic mucus extracts of the walking catfish (Loganathan et al., 2011; Varghese & Arathy, 2011; Elavarasi et al., 2013) revealed a great spectrum of activity against various human and fish pathogens. Different strains tested had shown varying sensitivity towards different mucus extracts, where *E. coli*, *K. pneumonia* and *S. aureus* were more sensitive towards organic extracts while *P. aeruginosa* and *P. vulgaris* were more sensitive towards aqueous extracts. The aqueous mucus extract of the other *Clarias* species, *C. gariepinus* (Nwabueze, 2014) had demonstrated significantly greater inhibition against four common pathogens, when the experimental fish was treated with

ginger diet rather than conventional fish feed. This further suggested that the fish diet is crucial in determining the antibacterial strength of fish mucus.

The only species of air-sac catfish species, *H. fossilis* (Haniffa et al., 2014) also presented a broad-spectrum antibacterial activity. Its aqueous mucus extracts revealed inhibitory activities against ten different strains, where Gram-positive bacteria were more sensitive (IZD = 9 to 11 mm) than gram-negative bacteria (IZD = 4 to 6 mm).

In conclusion, freshwater catfish exhibited a broader spectrum of antibacterial properties than marine catfish. More studies should be done on both marine and freshwater catfish species to understand the potential of their antibacterial properties.

2.5.2 Class Elasmobranchii

Three stingray species (Order: Myliobatiformes; Family: Dasyatidae), namely *Dasyatis pastinaca* (common stingray), *D. sephen* (cowtail stingray) and *Himantura gerrardi* (whitespotted whipray) were also screened for their mucosal antibacterial properties. The crude mucus extract (16.50 µg/µl) of common stingray (Fuochi et al., 2017) was reported to strongly inhibit the growth of gram-negative bacteria such *E. coli*, *K. pneumoniae*, and *P. aeruginosa*, while the acidic mucus extracts of cowtail stingray and whitespotted whipray (Vennila et al., 2011) revealed varying MIC values against various pathogens. In this study, the extracts of both stingray species had shown lower MIC value against gram-negative bacteria (*E. coli*, *S. iyphi* and *V. cholerae*) than gram-positive bacteria (*S. aureus*). Nevertheless, no activity was shown by the aqueous and organic mucus extracts for the two species.

2.5.3 Class Myxini

The only hagfish species studied, *Myxine glutinosa*, Atlantic hagfish (Order: Myxiniformes; Family: Myxinidae) was reported to have a good spectrum of antibacterial activity as well. Its acidic mucus extracts (Subramanian et al., 2008a) were reported to be bactericidal against both Gram-positive and Gram-negative bacteria, which included one Gram-positive (*S. epidermis* C621) and six Gram-negative bacteria (*A. salmonicida* A449, *E. coli* D31, *L. anguillarum* 02-11, *P. aeruginosa* Z61 and K799, *S. typhimurium* C610*, Y. ruckeri* 96-4). Notably, the screening revealed the lowest MBC value reported (6.1 µg/ml) against Gram-negative strains namely *E. coli* D31 and *Y. ruckeri* 96-4, while *S. epidermis* C621 was more resistant to hagfish skin mucus with MBC = 82.5 µg/ml. However, no activity was observed for the aqueous and organic mucus extract of Atlantic hagfish.

2.6 Studies of Proteomics on Fish Skin Mucus

For the past decade, studies on the composition of fish skin mucus, particularly their protein contents, had been conducted extensively as more high throughput methods were developed and enhanced for deep proteome analysis. Conventional 1D or 2D polyacrylamide gel electrophoresis was among the most widely used techniques to purify protein in fish skin mucus for its simplicity and accuracy. Following that, various approaches, which are primarily utilised in current proteomics, namely mass spectrometry with LC/MS-MS and MALDI-TOF/TOF would be applied to further identify and quantify the protein contents (Aslam et al., 2017).

For general characterisation, the fish skin mucus reported as such in several studies (Patel & Brinchmann, 2017; Coelho et al., 2019; Shahrudin et al., 2019) were collected

without any biological, behavioural, chemical, and physical influence prior to proteome analysis.

However, in order to achieve a more informative and specific result, there were also studies that collected the fish skin mucus samples under various influencing factors. One of the many factors involved bacterial infections. For instance, in the study of Rajan et al. (2013), the skin mucus of the fish individuals diagnosed with vibriosis which was caused by one member of gram-negative bacteria namely *Vibrio angullarum* was collected while in Xiong et al. (2020), the fish groups were infected with another gram-negative bacteria, *Edwardsiella ictalurid* which is a type of fish bacterial pathogen found in the aquaculture industry. Other than that, some studies also introduced parasitic infection to the fish prior to skin mucus collection such as *Lepeophtheirus salmonis*, the sea lice (Easy & Ross, 2009; Provan et al., 2013) and *Ichthyophthirius multifiliis* (Saleh et al., 2019) which both were ectoparasites commonly found on the body surface of farmed fish.

Fish was also subjected to physical stress in previous study in order to examine the protein contents of their skin mucus under extreme conditions such as chronic cold stress (Sanahuja et al., 2019), healing of chronic wound (Cordero et al., 2017; Kwan & Ismail, 2018) or overcrowding stress (Cordero et al., 2015). Elsewhere, Chong et al. (2005) reported the protein profiling of fish skin mucus during parental-care phase while Cordero et al. (2016) and Micallef et al. (2017) introduced probiotic intake or made alterations on the fish diet and reported the effects on their skin mucus proteome. Interestingly, there was also study such as Fæste et al. (2020) which compared the proteomic profiling of fish skin mucus with different collection methods namely absorption, wiping and scraping. By comparing the

proteomic data, a better understanding could be established with regards to the fish host responses against various conditions.

2.7 Chapter Summary

Although multiple fish mucus extraction approaches were available, their efficiencies were incomparable because different solvents might target different antimicrobial compounds and the fish species tested were also different, i.e. they were not biologically identical, with distinct ecological niches and defence mechanisms. Among these extraction methods, acidic solvents are recommended and most widely used as they can inhibit proteolytic enzyme activities which might cause major protein degradation prior to antibacterial screening (Al-Rasheed et al. 2018).

Agar disk diffusion assay was commonly used in antibacterial tests of fish skin mucus because of its simplicity, low-cost performance, robustness to screen enormous numbers of bacterial strains, and the ease of interpreting outcomes. However, a clear zone of bacterial growth inhibition does not necessarily signify the death of the tested strains. Therefore, this method is unable to differentiate between bactericidal and bacteriostatic effects. While the broth micro-dilution methods require more complexity in preparing different concentrations of the extracts prior to antibacterial activity screening, quantitative results can be obtained as in MIC and MBC values. Further, the miniaturisation of these tests has made this method a more viable, reproducible, and cheaper approach for antibacterial susceptibility testing. In addition, if one is interested in evaluating the on-going interaction between sample extracts and the bacterial strains, the cell counting method could provide a more accurate result with detailed information.

CHAPTER 3

MATERIALS AND METHODS

3.1 Mucus Preparation

3.1.1 Fish Sampling

Several site surveys were carried out across numerous forest streams and drainages from different villages and national parks in Sarawak prior to fish sampling (i.e. Kubah National Park, Gunung Gading National Park, Santubong National Park and UNIMAS East Campus). Based on the pilot survey experiences, two species of interest were observed in abundance year-round at two different sites respectively (Figure 3.1): *Barbodes sealei* were seen congregating in one of the storm drains with clear water located at UNIMAS East Campus in Kota Samarahan District (1.4649 °N, 110.4269 °E) while populations of *Barbodes everetti* was observed in one of the clear shallow stream pools located at the foothill areas of Kubah National Park in Kuching District (1.6128 °N, 110.1969 °E). There were also other fish species captured such as *Barbodes kuchingensis*, *Channa lucius* and *Clarias leiacanthus* but excluded from the study due to various reasons such as failed acclimatisation to lab conditions (pre-experimental death), the miniature size of the species or low number of healthy live specimens of the same species (relatively low amount of mucus that can be obtained).

Passive sampling techniques were used in this study. The capture of fish was done by entrapment tools which are different sizes of homemade minnow traps made of 9.5 L plastic water bottles (Figure 3.2). Commercial fish feed pellets (5 g) that are bigger than the mesh size of trapping minnows was used as attractants. The sampling was conducted during the rainy seasons in inland areas of Sarawak from mid-October to December 2017.

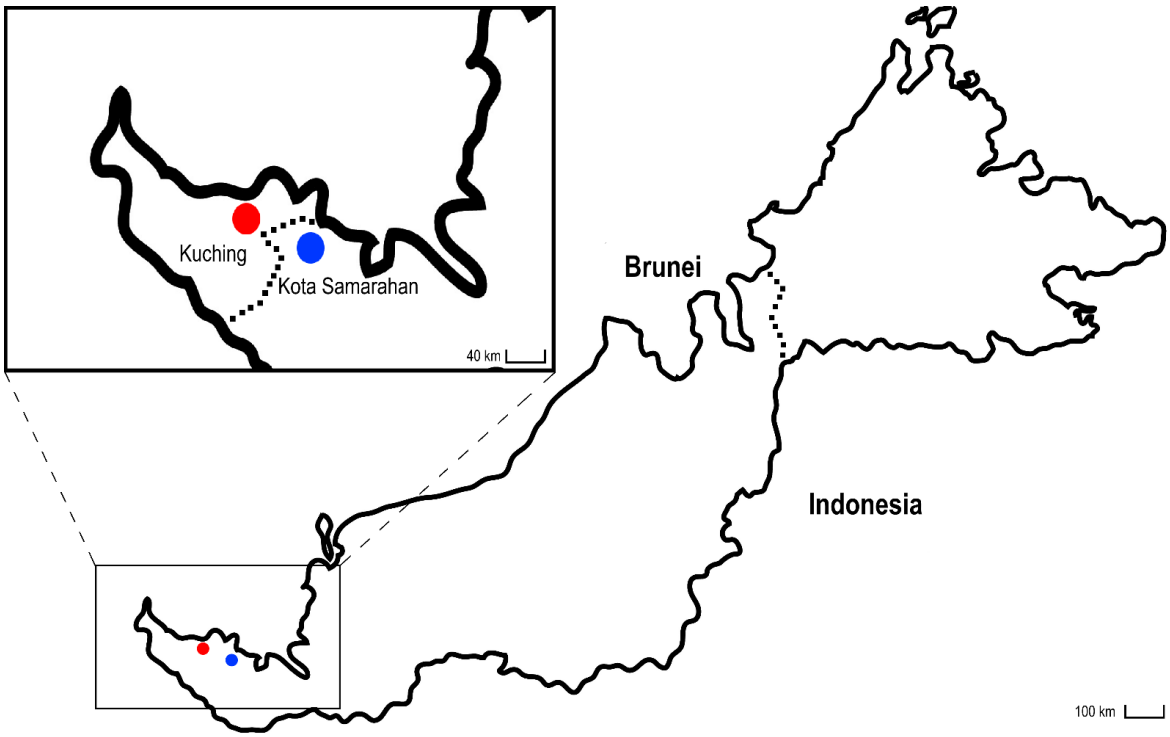


Figure 3.1: Map of Sarawak, Borneo with two different scales. Blue circle represents UNIMAS East Campus in Kota Samarahan District while the red circle represents Kubah National Park in Kuching District

Figure 3.2: Homemade minnow trap

Normally, the trappings were more fruitful after heavy rainfalls. The trappings were done only after the rain stopped as the high velocity water might damage or carry along the traps too far from their original placements. Five traps were deployed at each site randomly and anchored to big heavy rocks. The traps were checked again after two to three hours. All the specimens caught were transferred to a plastic aquarium tank (45 × 30 × 30 cm^3) with two portable oxygen air pumps and brought back to the laboratory. Prior to further experiments, all the specimens of the same species would undergo one week of acclimatisation period to laboratory condition within the same tank. Throughout the period, dissolved oxygen was maintained at an optimal level with the help of oxygen air pumps with sponge filter and the fish was fed to satiation daily with commercial feed. In addition, half of the water in the tank was changed with dechlorinated water every two to three days. Trappings were done several times to replenish fish specimens. Further processing was summarised in a flow diagram on fish epidermal mucus extraction method development (Figure 3.3).

3.1.2 Epidermal Mucus Collection

This study was conducted under the approval of the UNIMAS Animal Ethics Committee, Universiti Malaysia Sarawak (UNIMAS/AEC/R/F07/020). The epidermal or skin mucus of the fish was collected by modified sloughing off methods from Wei et al. (2010). Two types of solvents were used which were sterile distilled water (dH_2O) and sterile physiological saline - 0.85 % (w/v) NaCl or simply known as saline.

At first, the fish were starved for 24 h. Twenty-five specimens of healthy fish of the same species (standard length ranged from 8 to 14 cm) were chosen where fish with signs of disease or lesions were excluded from the study. Each chosen specimen was rinsed with

water to remove any apparent dirt and transferred into an enclosed zip-locked polyethylene bag with 50 ml of dH_2O. No direct physical contact was made by the usage of latex hand gloves. The fishes were gently massaged in the bag for 10 to 15 min for better mucus secretion and then returned to a recovery tank. Skin mucus that was sloughed off in the bag then poured and pipetted into a sterile 50 ml falcon graduated tube which was labelled and stored at -20 °C. For saline, 30 specimens were selected to slough off mucus from their body surface in 30 ml of the solvent instead. The collected samples were labelled and stored at 4 °C which would be used directly for extraction. Each session of skin mucus collection was conducted at least two days apart to minimise stress on the fish and keep them healthy and alive for a sustainable source of fresh mucus samples.

3.1.3 Concentrating Methods of Mucus Samples

Depending on these two initial solvents, the raw mucus collected will undergo either a freeze-drying process to pre-concentrate the samples before subsequent extraction (dH_2O) or direct extraction where the concentration process via ammonium sulphate precipitation occurs in a later stage (saline).

3.1.3.1 Freeze-drying

In order to carry out the freeze-drying, fifty ml of dH_2O sample was pre-frozen in an 80 ml fast-freeze flask (Labconco; Cat no. 7542200) that was compatible with the freeze-drying machine (Labconco; FreeZone -105 °C 4.5 Litre Cascade Benchtop Freeze Dry System; Cat no. 7382033). The freeze-dried samples were carefully weighed and divided into three parts for extraction purpose.

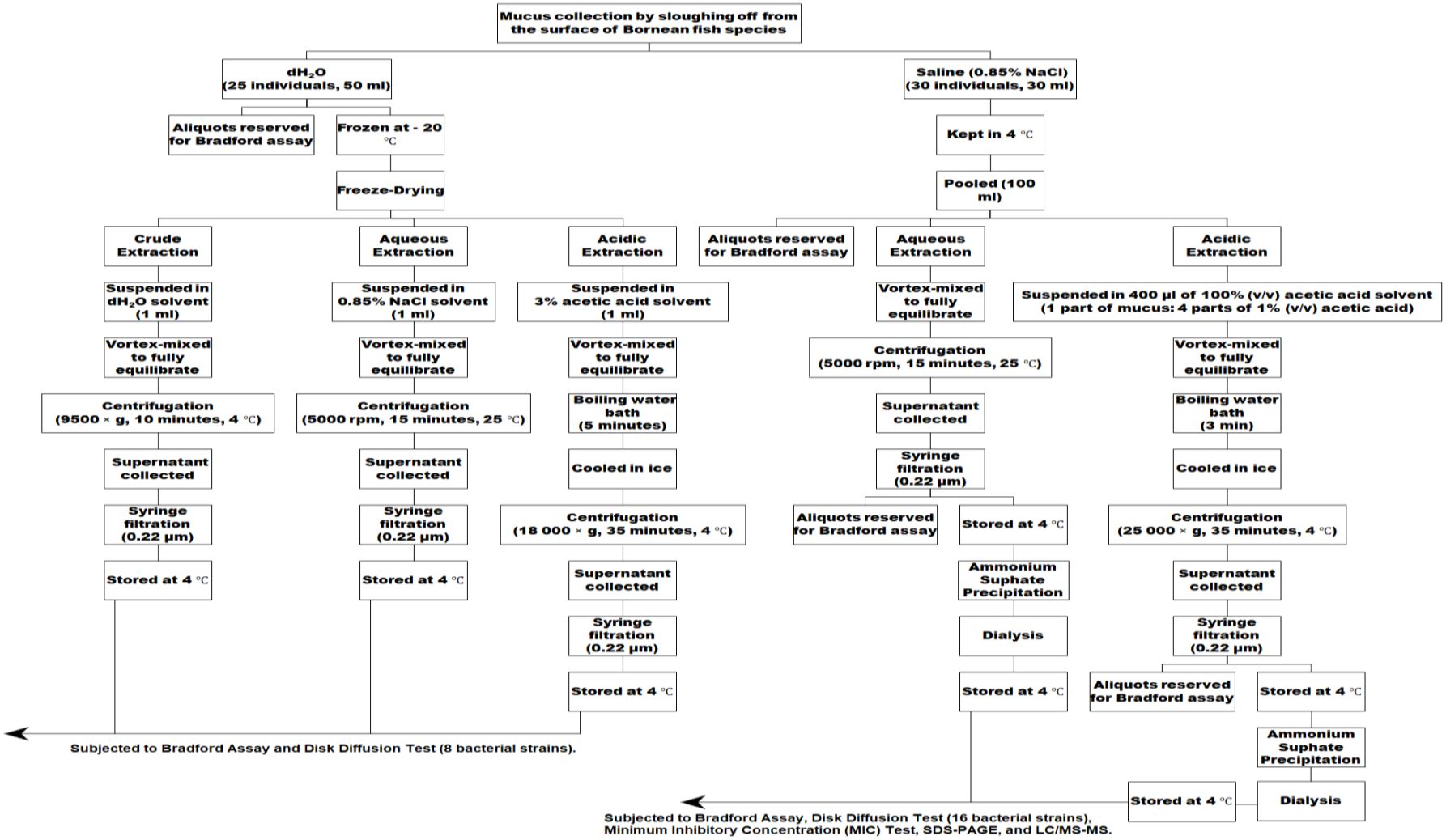


Figure 3.3: Flow diagram of fish epidermal mucus extraction method development

3.1.3.2 Salting-out of proteins by ammonium sulphate precipitation and dialysis

Fifty ml of refrigerated saline mucus extract samples was poured into a 100 ml beaker which was placed in an ice bath (500 ml beaker full of ice). By using online program from EnCor Biotechnology (2022), the amount of solid ammonium sulphate needed to prepare a solution of saturation level up to 90 % to precipitate the whole proteins at 4 °C was calculated as 27.96 g in which the final volume of the solution was 59.85 ml. Any lump of ammonium sulphate solid was first broken up using a mortar and pestle. Solid ammonium sulphate was gradually added into the samples and stirred using a magnetic stirrer (Labtech; Model LMS-1003) for one hour to fully equilibrate or until all the solutes completely dissolved. The solution was aliquoted into sterile microcentrifuge tubes at 2.0 ml/tube and subjected to centrifugation (10000 × g, 4 °C, 15 min) in a high-speed refrigerated centrifuge (Hitachi, Japan; Model CF15RX) to pellet out protein. The supernatants were discarded, and the leftover from the solution was aliquoted into the same tubes at 2.0 ml/tube and subjected to centrifugation again until all was used up. The pellets from each tube were pooled and resuspended by pipette-mixing in 500 µl of the negative control solvent (saline or moderately 0.8 % (w/v) acetic acid) using a 1000 ml pipette in which it resembles the nature of the solvent used during mucus extraction until completely dissolved. The pipette content was then dispensed into a sterile 2 ml microcentrifuge tube and labelled.

The resuspended samples were then subjected to dialysis to remove the ammonium sulphate salts (Figure 3.4). First, the modified dialysis tubing cellulose membrane (Sigma-Aldrich; Flat width 33 mm; MWCO 14 kDa) was hydrated by soaking in the appropriate buffer (negative control solvent) for 5 min. Then, a single knot was tied firmly at one end to prevent leakage. Once secured, the sample from each tube was pipetted into the tubing. The air above the sample was carefully pressed out of the tubing in which a second knot was tied

on the other end. The sample in tubing was immersed completely at the bottom of a pre-refrigerated 500 ml beaker containing the respective solvents. To prevent the tubing from floating to the surface, a small heavy object was tied on one end. The beaker containing the samples was incubated overnight at 4 °C to dialyse out the ammonium sulphate. The dialysed samples were pipetted into a sterile 2 ml microcentrifuge tube and stored at 4 °C until further use.

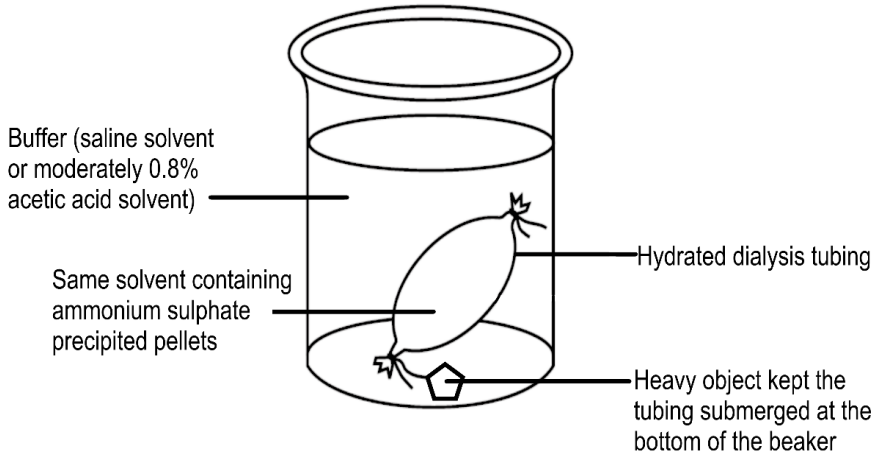


Figure 3.4: The illustration of dialysis tubing set-up in order to remove ammonium sulphate salt from the samples

3.1.4 Preparation of Epidermal Mucus Extract

The samples for both species were grouped in such a way: i) freeze-dried dH_2O mucus separated into three parts for crude (FDC), aqueous (FDS) and acidic (FDA) extractions and ii) saline mucus divided into two parts for aqueous (APS) and acidic (APA) extractions followed by ammonium sulphate precipitation and dialysis. Thus, a total of five epidermal mucus extracts for each species would be subjected to further experiments.

3.1.4.1 Crude Extraction

A modified method adapted from Subramanian et al. (2008a) was used for preparing crude extracts. Lyophilised dH_2O mucus (1 mg/ml) was first suspended in a sterile 1.5 ml microcentrifuge tube containing sterile dH_2O solvent and vortex-mixed to dissolve completely. The tube was spun in a high-speed refrigerated centrifuge (Hitachi, Japan; Model CF15RX) at 9500 × g for 10 min at 4 °C to separate insoluble particles. The clear supernatant (FDC) was loaded into a 5 ml Terumo Luer-lock syringe, filtered (Minisart® NML SFCA syringe filter, 0.22 µm pore size, 28 mm diameter), labelled and stored in a sterile 1.5 ml microcentrifuge tube at 4 °C until further use. Negative control (dH_2O) was prepared according to the same extraction protocols without adding lyophilised dH_2O mucus.

3.1.4.2 Aqueous Extraction

The extraction of dH_2O and saline mucus was performed following the aqueous extraction protocols in Loganathan et al. (2011) with slight modification. The preparation of aqueous extracts was principally similar to that of crude extracts. Lyophilised dH_2O mucus (1 mg/ml) was first suspended in a sterile 1.5 ml microcentrifuge tube containing sterile 0.85% NaCl (saline solvent) and vortex-mixed to dissolve completely. The tube was then centrifuged (LaboGene; Scan Speed Mini Microcentrifuge) at 5000 rpm for 15 min at 25 °C to separate precipitates in suspension. The clear supernatant (FDS) was loaded into a 5 ml Terumo Luer-lock syringe and filtered (Minisart® NML SFCA syringe filter, 0.22 µm pore size, 28 mm diameter), labelled and stored in a sterile 1.5 ml microcentrifuge tube at 4 °C until further use. Negative control (0.85 % NaCl) was prepared according to the same extraction protocols without adding lyophilised dH_2O mucus.

For saline mucus, 50 ml of the pooled samples was vortex-mixed to fully equilibrate and aliquoted into 2 ml sterile microcentrifuge tubes at 2.0 ml/tube. After subjected to centrifugation (same condition), the clear supernatants (APS) were pooled into a 50 ml Terumo Luer-lock syringe and filtered into a 50 ml falcon graduated tube which would be labelled and stored at 4 °C until further use. Negative control (0.85% NaCl) was prepared according to the same extraction protocols by replacing the mucus with saline solvent.

3.1.4.3 Acidic Extraction

The methods modified from Al-Rasheed et al. (2018) and Wei et al. (2010) were used to prepare acidic extracts. Lyophilised dH_2O mucus (1 mg/ml) was suspended in a sterile 1.5 ml microcentrifuge tube containing 3 % (v/v) acetic acid solvent and vortex-mixed to dissolve completely. Following that, the tube was placed in a boiling water bath for 5 min to inhibit proteolytic enzyme activity (Conlon, 2007) and immediately cooled in ice bath (1000 ml beaker full of ice cubes). Next, the tube was spun in a high-speed refrigerated centrifuge (Hitachi, Japan; Model CF15RX) at 18000 × g for 35 min at 4 °C. The clear supernatant (FDA) was loaded into a 5 ml Terumo Luer-lock syringe, filtered (Minisart® NML SFCA syringe filter, 0.22 µm pore size, 28 mm diameter), labelled and stored in a sterile 1.5 ml microcentrifuge tube at 4 °C until further use. Negative control (3 % (v/v) acetic acid solvent) was prepared according to the same extraction protocols without adding lyophilised dH_2O mucus.

For saline mucus, 400 µl of 100 % (v/v) glacial acetic acid solvent (Brand: Merck, Germany; Cat no. 1000632511) was added to vortex-mix with 50 ml of pooled saline mucus to fully equilibrate in order to produce a solution containing one part of mucus and four parts of moderately 1 % (v/v) acetic acid solvent, followed by a three-minute boiling water bath and cooling in ice bath. The contents were aliquoted into sterile microcentrifuge tubes at 2.0

ml/tube and the tubes were subjected to centrifugation 25000 × g for 35 min at 4 °C. The clear supernatants (APA) were pooled into a 50 ml Terumo Luer-lock syringe and filtered into a 50 ml falcon graduated tube which would be labelled and stored at 4 °C until further use. Negative control (moderately 0.8 % (v/v) acetic acid solvent) was prepared according to the same extraction protocols by replacing the mucus with saline solvent.

3.1.5 Bradford Protein Assay

Bradford reagent contains Coomassie Brilliant Blue G-250 dye (brown colour) which can form blue-coloured complexes, shifting the maximum absorbance from 465 to 595 nm when binding with protein samples. Normally, the complexes complete the binding process within 5 min which will be stable for approximately one hour. In this study, protein contents of the fish epidermal mucus were quantified by Bradford assay method (Bradford, 1976) according to the instructions of manufacturer (HiMedia, India) with slight modifications.

Protein concentrations of skin mucus were assayed upon collection, after extraction and after concentration to compare protein recovery. The assays utilised two different volumes of test samples which were 200 µl for lyophilised dH_2O mucus extract samples and 100 µl for saline mucus extract samples, thus two standard curves were generated. The standard protein used in the study was bovine serum albumin (BSA). For dH_2O samples (200 µl), increasing volumes of BSA (1 mg/ml) viz. 0, 4, 8, 12, 16, and 20 µl were first pipetted into six 1.5 ml microcentrifuge tubes and adjusted to a total volume of 200 µl in each tube by adding dH_2O [Table 3.1(a)]. Next, the same volume of skin mucus extract samples with unknown concentrations were also pipetted into microcentrifuge tubes, followed by the addition of 1 ml of Bradford reagent. The contents in the tubes were vortex-mixed and incubated at room temperature for 10 min. After that, the mixtures were transferred into

cuvettes and the absorbances were measured at 595 nm using a spectrophotometer (Metertech, Taiwan; Model SP 830 Plus). For saline samples (100 µl), the series of BSA volumes used was 0, 2, 4, 6, 8, 10, and 12 µl and the total volume was adjusted to 100 µl by adding saline instead [Table 3.1(b)].

Table 3.1: BSA standard assay data for (a) dH_2O mucus samples and (b) saline mucus samples

Tube No.	Blank	1	2	3	4	5	6
BSA Protein Standard (µg / 200 µl)	0	4	8	12	16	20	dH_2O mucus samples
Stock (µl)	0	4	8	12	16	20	200 µl
Diluent (µl) – dH_2O	200	196	192	188	184	180	
Bradford Reagent (ml)	1	1	1	1	1	1	1
Absorbance at 595nm	Refer to Appendix A						

(a)

Tube No.	Blank	1	2	3	4	5	6	7
BSA Protein Standard (µg / 100 µl)	0	2	4	6	8	10	12	Saline mucus samples
Stock (µl)	0	2	4	6	8	10	12	100 µl
Diluent (µl) – saline	100	98	96	94	92	90	88	
Bradford Reagent (ml)	1	1	1	1	1	1	1	1
Absorbance at 595nm	Refer to Appendix A							

(b)

The procedures were done in triplicate and the absorbance obtained were tabulated (Refer to Appendix A). A standard curve of absorbance against BSA protein concentration was plotted in Microsoft Excel with linear regression equation ($R^2 > 0.98$) in $y = a + bx$ where x = protein concentration (µg /200 µl or µg /100 µl), y = absorbance value at 595 nm, a = y-intercept of regression line and b = slope of regression line. From the equation, protein concentrations of mucus extracts (µg /200 µl or µg /100 µl) could be solved with respect to their absorbance value at 595 nm which were converted into µg/ml. All the samples were assayed in triplicate, and all the data were presented as means ± standard deviation (Refer to Appendix B).

Independent Student's t-test was used to determine significant differences among the protein concentrations of various dH_2O mucus extracts and saline mucus extracts from both fish species. Statistical significance was considered at $p \leq 0.05$. All statistical analyses were conducted using IBM SPSS Statistic 27 (64 bit).

3.2 Antibacterial Assay

All the experiments involving bacteria were conducted in adherence to recommended guidelines by Clinical and Laboratory Standards Institute (CLSI), notably with the preparation of media, incubation conditions and practices of common aseptic techniques. On another note, the experiments were conducted in a laminar flow cabinet (Airstream® Class II Type A2 Biological Safety Cabinets) to minimise sources of contamination.

3.2.1 Preparation of Growth Media

The preparation of three types of growth media were subjected to manufacturer's instructions namely Miller's Luria-Bertani (LB) broth (Brand: Merck; Cat no. 110285), Mueller Hinton agar (MHA) (Brand: Oxoid; 500g; Product code: CM0337B) and Mueller Hinton broth (MHB) (Brand Oxoid; 500g; Product code: CM0405B) which would be used throughout the antibacterial assays.

For LB broth, 2.5 g of the broth powder was dissolved completely in 100 ml of distilled water in a screw-capped glass bottle which was sterilised by autoclaving at 121 °C for 15 min. Cooled broth was stored at 4 °C for future use.

For MHA, 38 g of the agar powder was added into 1000 ml of distilled water in a screw-capped glass bottle. The solution was stirred and brought to boil until the solutes completely dissolved using a hotplate magnetic stirrer (Labtech; Model LMS-1003) and

sterilised by autoclaving at 121 °C for 15 min. The freshly autoclaved medium in liquid form was immediately poured into sterile petri dishes or circular plates (Brand: FAVORIT; 90 x 15 mm; cat no. P9222-0001) or sterile 5 ml glass vials which were tilted at an angle where the medium inside was at a slanted position relative to the vial which increases the surface area of the agar. Once the agar had solidified, the plates or glass vials were sealed with parafilm and stored at 4 °C for future use.

For MHB, 10.5 g of the broth powder was dissolved completely in 500 ml of distilled water in a screw-capped glass bottle which was sterilised by autoclaving at 121 °C for 15 min. After cooled, the broth was stored at 4 °C for future use.

3.2.2 Bacterial Strain

The bacterial strains used in this study were selected from available LB-glycerol bacterial isolates collection provided by Microbiology Laboratory at Faculty of Resource Science and Technology, which included Gram-positive *Bacillus cereus* ATCC 33019, *Listeria monocytogenes* ATCC 7644, *Staphylococcus aureus* ATCC 25923, and Gram-negative *Aeromonas hydrophila*, *Escherichia coli* O157:H7, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* ATCC 27853, *Salmonella braenderup* ATCC BAA 664, *Salmonella enteritidis* ATCC 13036, *Salmonella typhi* ATCC 14028, *Salmonella typhimurium*, *Shigella boydii* ATCC 9207, *Shigella flexneri* ATCC 12022, *Shigella sonnei* ATCC 25931, *Vibrio cholerae*, and *Yersinia enterocolitica*.

In order to revive the bacterial isolates from the stock cultures, the frozen cultures were first thawed until all ice crystals had melted. Next, 100 µl from the culture was inoculated into 5 ml LB broth in a graduated falcon tube which was then incubated at 37 °C for 16 h. To prepare the stock culture for the study, 300 µl of the fresh culture of each

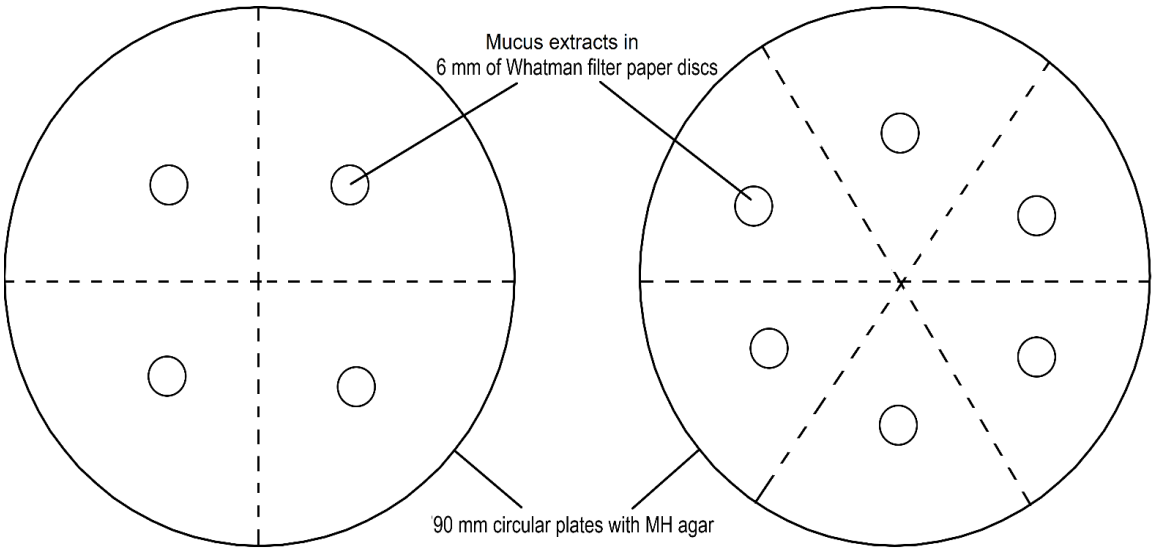
bacterial strain was pipette-mixed using a 1000 ml pipette with 1.2 ml sterile LB medium with 37.5 % (v/v) glycerol which made up 1.5 ml of 30 % glycerol stock solution. The stock culture was then stored at -20 °C while the rest would be subjected to preparation of short-term working cultures on MHA slants. By using a flamed inoculation loop, the fresh cultures were gently streaked onto the surface of pre-prepared MHA slants in a glass vial which was incubated at 37 °C for 16 h. Subsequently, a single colony from each bacterial culture was picked with a flamed loop from the agar slant culture and inoculated into 5 ml of MHB in a graduated falcon tube which was then incubated at 37 °C for 16 h. OD value of each bacterial culture was measured using a spectrophotometer (Metertech, Taiwan; Model SP 830 Plus) with sterile saline as blank and adjusted with sterile saline to achieve a standardised inoculum of OD_{600} = 0.1 which would be used within 30 min for antibacterial assays.

3.2.3 Disk Diffusion Test

Preliminary antibacterial screening of mucus extracts was performed using standard Kirby-Bauer disc diffusion method (Bauer et al., 1966) as per CLSI guidelines. Lyophilised dH_2O mucus extracts (FDC, FDS, and FDA) were tested against eight out of sixteen bacterial strains due to limited samples, while saline mucus extracts (APS and APA) were tested against all bacterial strains. Prior to bacterial inoculation, refrigerated pre-prepared MHA plates (25 ml, 4 mm in depth) were brought back to room temperature. A hole puncher was also used to create 6 mm discs from filter paper (Brand: Whatman; Grade 1, 11 µm pore size) which was placed in a falcon graduated tube and sterilised by autoclaving at 121 °C for 15 min. The discs in the sealed tube were dried overnight in the oven at 60 °C.

First, 100 µl of the standardised inoculum (OD_{600} = 0.1) was transferred into MHA plate and distributed evenly using a bent glass rod (4 mm diameter) as a cell spreader. Next,

6 mm filter paper discs were placed on the agar surface in even contact by a pair of flamed pointed tip forceps which would be impregnated with 20 µl of test samples. Each plate was confined to either four-disc arrangement or six-disc arrangement (Figure 3.5).



Note: Dotted line equally divides the designated region for each disc to avoid possible overlapping of the inhibition zone. Disc content could be either mucus extract, negative control or positive control (Pen-Strep solution or ciprofloxacin disc)

Figure 3.5: Four-disc arrangement and six-disc arrangement for disk diffusion test

The agar plates were then incubated at 4 °C to allow pre-diffusion of the mucus extracts for one hour, followed by another incubation at 37 °C for 16-20 h. Twenty µl of 1X Penicillin-Streptomycin (Pen-Strep) (Stock concentration: 100X; Cat no. 15070063) or Ciprofloxacin antimicrobial disc (Brand: Oxoid; 5µg; Cat no. CT0425B) was used as positive control.

The plates were observed from the back against a dark background with reflected light for a clear inhibition zone around the disc. The inhibition zone diameters (IZD) were measured up to two decimal places with a digital calliper. All the experiments were done in

triplicate for all types of mucus extracts of both species against each bacterial strain. To rule out the possible influence of solvent used in mucus extracts, the experiments were repeated with negative controls and results were compared. Data were presented as means ± standard deviation.

Independent Student t-test and One-way analysis of variance (ANOVA) followed by Duncan's multiple range test was used to determine significant variation among the antibacterial strength of mucus extracts or positive control against different bacterial strains. Independent Student t-test was also used to determine significant difference between IZD means of mucus extracts with their respective negative control, in the case that both exhibited bacterial inhibition against the same strain. Statistical significance was considered at $p \leq 0.05$. Mucus extracts which showed significantly higher IZD than their negative controls were proceeded to minimal inhibitory concentration (MIC) test. All statistical analyses were conducted using IBM SPSS Statistic 27 version.

3.2.4 Broth Microdilution Susceptibility Test

Minimal inhibitory concentrations (MICs) of the mucus extracts that showed significantly higher antibacterial activity than their negative controls were determined using broth microdilution susceptibility test in accordance with CLSI guidelines. The tests were performed on 96-well (12 × 9) microtiter plates (microplates). Prior to experiments, protein concentration of the mucus extract across the wells was calculated in µg/ml and tabulated. Briefly, 50 µl of MHB was first dispensed into all wells except for the first well for each row on the microplates. Then, 100 µl of stock mucus extracts was aliquoted into the first well. Next, 50 µl was transferred into the next MHB-contained well and the mixture was homogenised by repeatedly pipetting up and down with a 200 µl pipette. As a result, the

concentration of the subsequent well was diluted by a factor of two. Two-fold serial dilutions were performed until 11th well where 50 µl of the last diluted mucus extracts were discarded. No extract was added to the last well and it was assigned as the negative growth control (Figure 3.6).

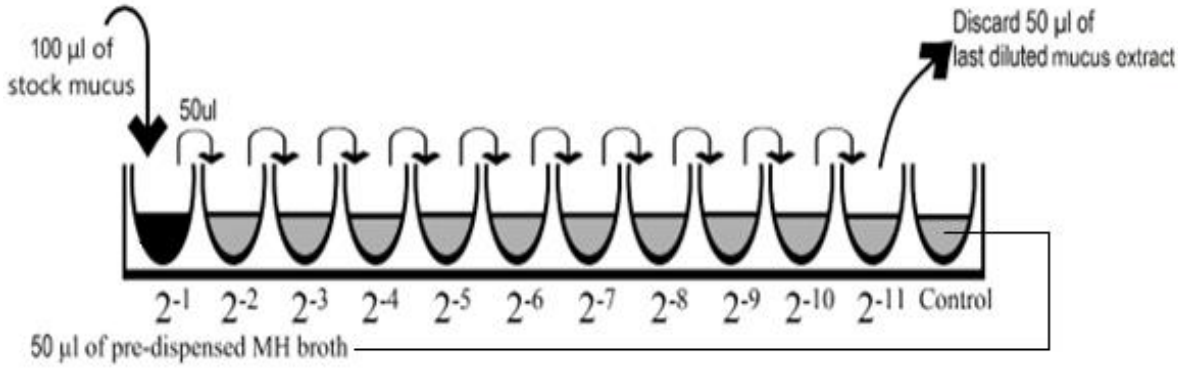


Figure 3.6: Two-fold serial dilutions on 96-well microplates

All the wells across the rows were then inoculated with 50 µl of the standardised inoculum (OD_{600} = 0.1) of the designated bacterial strains and mixed thoroughly by pipetting up and down. Finally, the microplates were incubated at 37 °C for 16-20 h. The bacteriostatic activity was examined by visual inspection with the aid of a mirror reflecting the bottom of the microplates. Clear well contents indicated the absence of bacterial growth and MIC values were determined by the clear well with the lowest mucus extract concentrations. The experiments were performed in triplicate.

Independent Student's t-test was used to evaluate if there are significant differences between the antibacterial strength of active mucus extracts from different fish species against the same bacterial strain or the same mucus extract against different bacterial strains.

Statistical significance was considered at $p \leq 0.05$ and all statistical analyses were conducted using IBM SPSS Statistic 27 version.

3.3 Protein Characterisation of Active Mucus Extracts

3.3.1 SDS-PAGE

SDS-PAGE (Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis) buffer system by (Laemmli, 1970) is a discontinuous buffer system for the separation of proteins. The system involves heating of proteins in SDS-2-mercaptoethanol buffer which results in irreversible denaturation. This allows the unfolded polypeptide molecules to become completely linearised with a constant charge-to-mass ratio proportional to their molecular weights. Proteins are then separated according to their molecular weights. In the study, the protein profiles of concentrated mucus extracts (APS and APA) were determined by SDS-PAGE buffer system according to manufacturer's instructions (BioRad Mini-PROTEAN® Tetra Cell; Cat no. 165-8000) with slight modifications and the gel was prepared according the formulation table provided in the user manual (Table 3.2).

Table 3.2: Gel formulations for SDS-PAGE

Gel (Percentage)	dH2O (ml)	Acrylamide/Bis (ml)	Gel buffer (ml)	10% (w/v) SDS (ml)
4%	6.1	1.3	2.5	0.1
5%	5.7	1.7	2.5	0.1
6%	5.4	2.0	2.5	0.1
7%	5.1	2.3	2.5	0.1
8%	4.7	2.7	2.5	0.1
9%	4.4	3.0	2.5	0.1
10%	4.1	3.3	2.5	0.1
11%	3.7	3.7	2.5	0.1
12%	3.4	4.0	2.5	0.1

The gel cassette (0.75 mm gel thickness) was first assembled and made sure there was no leakage. Next, the 12% resolving and 4% stacking gel monomer solutions were prepared by combining all reagents following the gel formulation table in Table 3.3. The resolving solution was first mixed with APS and TEMED and immediately pipetted into the gel cassette, leaving a 1.5 cm gap which was then overlaid with dH_2O. The solution was polymerised and solidified after approximately 30 min. The overlay was then discarded, and a stacking solution mixed with APS and TEMED was added until the cassette was full and covered with a ten-well comb teeth to make the wells for sample loading. The stacking gel would take another 30 min to solidify. While waiting, 950 µl of SDS reducing buffer was mixed with 50 µl of 2-mercaptoethanol to make a sample buffer. The mucus extract (10 µl) was diluted with a sample buffer in the ratio of 1:1 and incubated at 95 °C for 4 min. The whole assembly was placed in the running buffer tank (10x Tris/glycine/SDS, pH 8.3) and subjected to electrophoresis after all the samples, along with 4 µl of Chromatein pre-stained protein ladder (Vivantis Technology, Malaysia; Cat no. PR0602) as standard, were loaded into the wells. After electrophoresis, the gel was removed carefully from the cassette and stained with Coomassie Brilliant Blue R-250 (BioRad) solution on a 100-rpm shaker overnight at room temperature. After that the gel was destained in a plastic container with a destaining solution which consisted of 5 %(v/v) methanol, 7 %(v/v) acetic acid and 88% dH_2O on a 100-rpm shaker at room temperature. The destaining solution was changed multiple times when it became opaque with blue stains and the step was repeated until the bands in the gel become visible. The distinct protein bands were viewed under naked eye and compared with the protein ladder to determine their molecular weight.

3.3.2 Protein Identification with LC-MS/MS

LC-MS/MS (Liquid chromatography with tandem mass spectrometry) is a powerful analysis tool that incorporates both separating power of liquid chromatography and the highly sensitive and selective mass analysis capability of triple quadrupole mass spectrometry. The most predominant five bands from SDS-PAGE of the active mucus extracts were chosen, excised and sent for protein sequencing by LC-MS/MS (Proteomic International).

Briefly, protein samples were first trypsin digested and fragmented peptides were analysed by electrospray ionisation mass spectrometry using the Shimadzu Prominence nano HPLC system coupled to a Sciex 5600 TripleTOF mass spectrometer. Tryptic peptides were loaded onto an Agilent Zorbax 300SB-C18, 3.5 µm and separated with a linear gradient of water/acetonitrile/0.1 % formic acid (v/v). Spectra were analysed to identify proteins of interest using Mascot sequence matching software (Matrix Science) with UniProt database (Taxonomy: Actinopterygii; 2 775 399 sequences; August 2019). The results with list of identified proteins were received in HTML file and further analysed.

CHAPTER 4

RESULTS

Mucus was collected using two types of media namely dH_2O and 0.85 % NaCl (saline). For dH_2O mucus, the concentrating method was freeze-drying (FD) and three types of extractions with their respective solvents were applied namely Crude Extraction (dH_2O), Aqueous Extraction (0.85 % NaCl) and Acidic Extraction (3 % acetic acid) while for saline mucus, two types of extractions with their respective solvents were applied, namely Aqueous Extraction (0.85 % NaCl) and Acidic Extraction (0.8 % acetic acid) and the concentrating method used was ammonium sulphate [$(NH_4)_2SO_4$] precipitation (AP). Thus, a total of five mucus extracts for both fish species (*Barbodes sealei* and *Barbodes everetti*) namely Freeze-dried Crude extract (FDC), Freeze-dried Aqueous extract (FDS), Freeze-dried Acidic extract (FDA), Ammonium-sulphate-precipitated Aqueous extract (APS), and Ammonium-sulphate-precipitated Acidic extract (APA) would proceed with further assays.

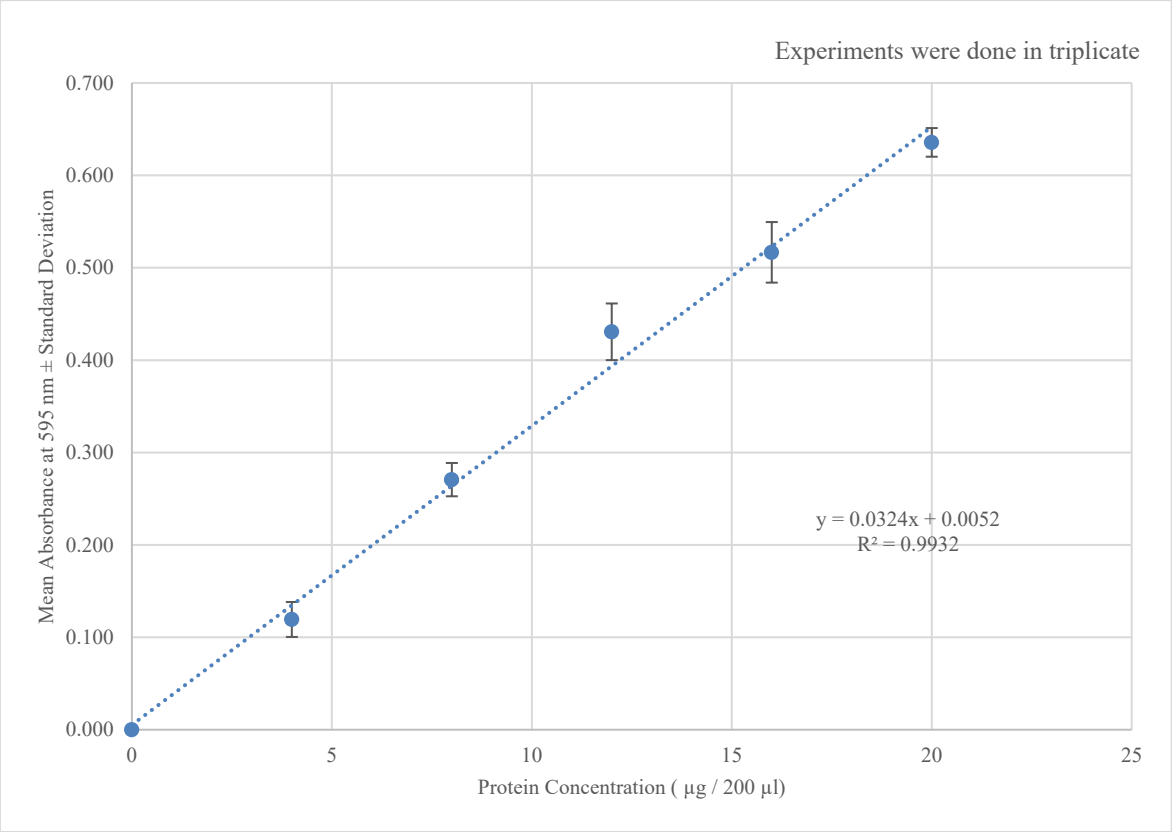
4.1 Mucus Collection and Protein Recovery

4.1.1 Bradford Protein Assay

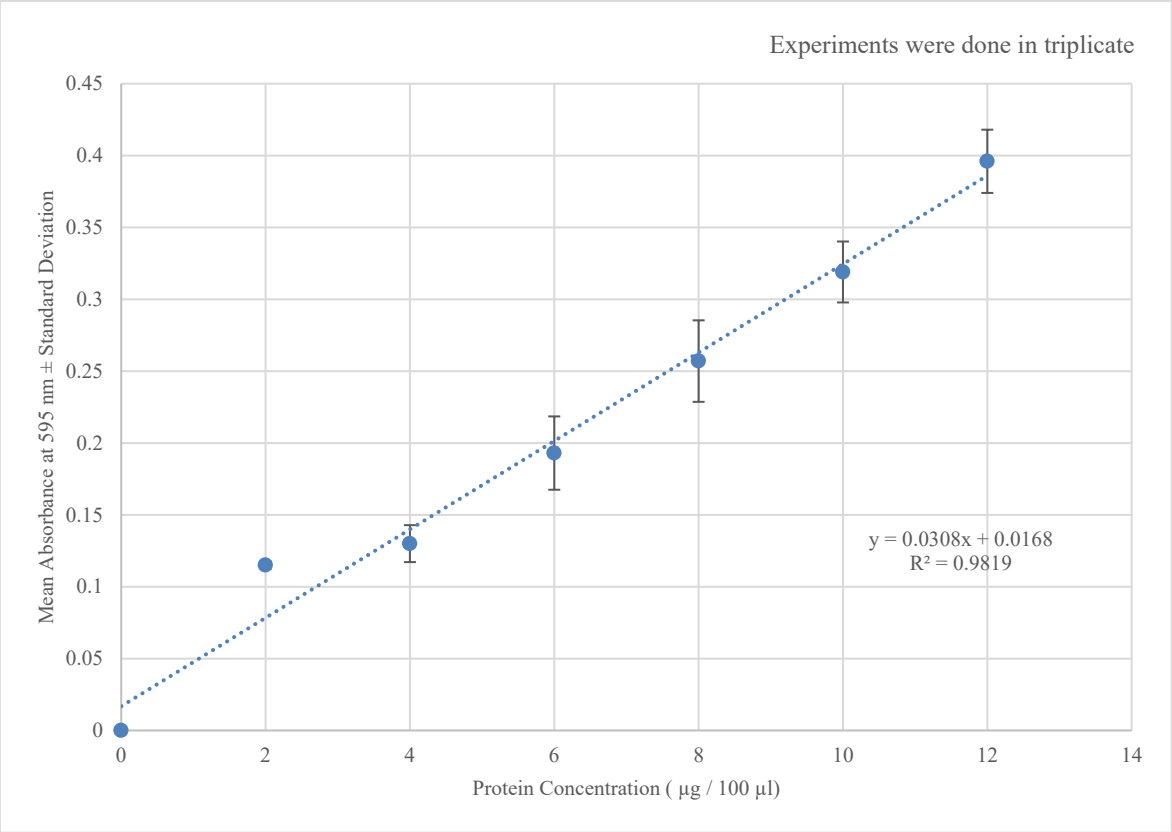
The A_{595} values of BSA standards were prepared in triplicate and tabulated (Appendix A). The standard protein standard curves were plotted with linear regression line equation in the form of $y = ax + b$ where $y = A_{595}$ and x = Protein concentration. Both FD (µg/200 µl) and AP (µg/100 µl) mucus concentrations obtained were converted into standard units (µg/µl) (Appendix B) for further interpretation.

For each FD mucus extract, the protein concentration was calculated using linear regression line as in Figure 4.1(a) with equation $y = 0.0324x + 0.0052$ ($R^2 = 0.9932$) whereas

the protein concentration of each AP mucus extract was calculated using linear regression line as in Figure 4.1(b) with equation y = 0.0308x + 00168 (R^2 = 0.98189).



(a)



(b)

Figure 4.1: Standard curves of absorbance at 595 nm against protein concentration for (a) Freeze-dried (FD) mucus extracts and (b) Ammonium-sulphate precipitated (AP) mucus extracts

Two groups of epidermal mucus samples, viz. dH_2O mucus and saline mucus were collected from each species and further processed into five types of extracts (FDC, FDS, FDA, APS, and APA), adding up to a total of ten epidermal mucus extracts from two fish species. The initial protein concentration upon collection of the mucus samples and the final concentration of mucus extracts were compared and the number of folds were computed. Generally, both initial and final protein concentrations (Table 4.1) between FD mucus and AP mucus showed, though not statistically, obvious numerical differences.

Table 4.1: Protein concentrations of epidermal mucus upon collection (Initial) vs after concentrating and extraction steps (Final) of (a) *Barbodes sealei*, and (b) *Barbodes everetti*

Epidermal Mucus Extract	Protein Concentration (µg / ml)		Number of Fold(s)
	Initial	Final	
FDC	6.66 ± 0.85	77.23 ± 10.07[a]	12-fold
FDS	6.66 ± 0.85	46.37 ± 2.76[b]	7-fold
FDA	6.66 ± 0.85	3.57 ± 0.50[c]	<1-fold
APS	212.19 ± 15.94	2473.25 ± 301.30[A]	12-fold
APA	212.19 ± 15.94	2414.37 ± 299.79[A]	11-fold

(a)

Epidermal Mucus Extract	Protein Concentration (µg / ml)		Number of Fold(s)
	Initial	Final	
FDC	6.45 ± 1.01	41.12 ± 2.85[b]	6-fold
FDS	6.45 ± 1.01	55.78 ± 7.91[a]	9-fold
FDA	6.45 ± 1.01	60.46 ± 7.90[a]	9-fold
APS	217.81 ± 17.77	2031.69 ± 332.47[A]	9-fold
APA	217.81 ± 17.77	2354.63 ± 417.55[A]	11-fold

(b)

FDC: Freeze-dried Crude Extract, FDS: Freeze-dried Aqueous Extract (FDS), FDA: Freeze-dried Acidic Extract, APS: Ammonium-sulphate-precipitated Aqueous Extract, APA: Ammonium-sulphate-precipitated Acidic Extract; All experiments were performed in triplicate; All values were in Mean ± Standard Deviation. Different letters in superscripted lower case of the final concentration of FD mucus extracts from the same column represent statistically significant difference (p-value ≤ 0.05); Different letters in superscripted upper case of the final concentration of AP mucus from the same column represent statistically significant difference (p-value ≤ 0.05); Comparison with < three groups are analysed by Independent Student's t-test; Comparison with ≥ three groups are analysed by One-way ANOVA followed by Duncan's multiple range test

For both species, the initial protein concentration of AP mucus (212.19–217.81 µg/ml) was approximately 30 times more concentrated than that of FD mucus (6.45–6.66 µg/ml). Consequently, the final concentration of AP mucus extracts and FD mucus extracts exhibited a similar trend. For *Barbodes sealei*, AP mucus extracts (2414.37–2473.25 µg/ml) were 600 times more concentrated than FD mucus extracts (3.57–77.23 µg/ml) whereas the concentration of AP mucus extracts (2031.69–2354.63 µg/ml) of *Barbodes everetti* were 50 times more concentrated that FD mucus extracts (41.12–60.46 µg/ml).

However, the number of folds did not show extreme variation (6–12-fold) across all the mucus extracts for both fish species with the exception of FDA of *Barbodes sealei* ending up with a more diluted protein concentration (<1-fold, 3.57 µg/ml).

However, among the mucus extracts of the same concentrating method, significant differences were observed (p-value ≤ 0.05) in FD mucus extracts. For *Barbodes sealei*, the protein concentration of FDC was the highest as compared to two other FD mucus extracts but protein concentration of FDC of *Barbodes everetti* was the lowest among all three FD extracts. For AP mucus extracts, both fish species did not show statistically significant difference in terms of protein concentrations between APS and APA.

4.1.2 Protein Recovery

To further analyse the Bradford assay data, the total amount of protein recovered after concentrating steps [Freeze-drying or $(NH_4)_2SO_4$ precipitation] and after extraction (Crude, Aqueous or Acidic) were calculated and summarised in Table 4.2(a-b) for *Barbodes sealei* and *Barbodes everetti*, respectively. FD mucus was extracted after freeze-drying while AP mucus was extracted before $(NH_4)_2SO_4$ precipitation. In the experiment, FD mucus after freeze-drying was not assayed because the sample obtained was in solid state and would be directly subjected to extraction.

In addition to the obvious differences between protein concentrations of FD and AP mucus, a similar trend occurred for the total amount of protein recovered from one specimen. AP mucus extracts of *Barbodes sealei* (48.29–48.46 µg) was up to 100 times greater than that of FD mucus extracts (0.43–9.27 µg) while the amount of protein recovered from each specimen for FD (4.93–7.26 µg) and AP (40.63–47.09 µg) mucus extracts of *Barbodes everetti* differed up to 10-fold.

Table 4.2: Protein recovery of various epidermal mucus extracts for (a) *Barbodes sealei*, and (b) *Barbodes everetti*

Types of Mucus Mucus extracts	Volume (ml)	Concentration (µg/ml)	Total Protein (µg)	No. of Specimen	Protein (µg)/Specimen	*Recovery %
FD Mucus (Initial)	*50*	*6.66 ± 0.85*	*332.82 ± 42.50*	*25*	*13.31 ± 1.70*	*100 ± 0*
FDC after Freeze-drying			Solid samples could not be assayed (NA)			
FDC after Crude Extraction (Final)	**1**	**77.23 ± 10.07**	**77.23 ± 10.07**	**~8.33**	**9.27 ± 1.21**	**69.71 ± 4.39[a]**
FDS after Freeze-drying			Solid samples could not be assayed (NA)			
FDS after Aqueous Extraction (Final)	**1**	**46.37 ± 2.76**	**46.37 ± 2.76**	**~8.33**	**5.56 ± 0.33**	**42.11 ± 4.01[b]**
FDA after Freeze-drying			Solid samples could not be assayed (NA)			
FDA after Acidic Extraction (Final)	**1**	**3.57 ± 0.50**	**3.57 ± 0.50**	**~8.33**	**0.43 ± 0.06**	**3.22 ± 0.14[d]**
AP Mucus (Initial)	*100*	*212.19 ± 15.94*	*21218.61 ± 1593.67*	*100*	*212.19 ± 15.94*	*100 ± 0*
APS after Aqueous Extraction	50	207.75 ± 19.67	10387.45 ± 983.36	50	207.75 ± 19.67	97.82 ± 2.09
APS after $(NH_4)_2SO_4$ precipitation (Final)	**1**	**2473.25 ± 301.30**	**2473.25 ± 301.30**	**50**	**48.46 ± 6.03**	**23.28 ± 1.60[c]**
APA after Acidic Extraction	50	89.89 ± 13.12	4494.59 ± 656.08	50	89.89 ± 13.12	42.82 ± 9.01
APA after $(NH_4)_2SO_4$ precipitation (Final)	**1**	**2414.37 ± 299.79**	**2414.37 ± 299.79**	**50**	**48.29 ± 6.00**	**22.85 ± 3.52[c]**

(a)

FDC: Freeze-dried Crude Extract, FDS: Freeze-dried Aqueous Extract (FDS), FDA: Freeze-dried Acidic Extract, APS: Ammonium-sulphate-precipitated Aqueous Extract, APA: Ammonium-sulphate-precipitated Acidic Extract; *Recovery = Protein per specimen (µg) of Extracts/Protein per specimen (µg) of Mucus x 100 %; All experiments were performed in triplicate; All values were in Mean ± Standard Deviation; Italicised data represents the initial state of the mucus samples; Data in Bold represents the final state of the mucus samples; Different letters in superscripted lower case of the means from the same column represent statistically significant difference (p-value ≤ 0.05); Comparison with ≥ three groups are analysed by One-way ANOVA followed by Duncan's multiple range test

Types of Mucus Mucus extracts	Volume (ml)	Concentration (µg/ml)	Total Protein (µg)	No. of Specimen	Protein (µg)/Specimen	*Recovery %
FD Mucus (Initial)	*50*	*6.45 ± 1.01*	*322.51 ± 50.60*	*25*	*12.90 ± 2.02*	*100 ± 0*
FDC after Freeze-drying			Solid samples could not be assayed (NA)			
FDC after Crude Extraction (Final)	**1**	**41.12 ± 2.85**	**41.12 ± 2.85**	**~8.33**	**4.93 ± 0.34**	**38.77 ± 5.34[b]**
FDS after Freeze-drying			Solid samples could not be assayed (NA)			
FDS after Aqueous Extraction (Final)	**1**	**55.78 ± 7.91**	**55.78 ± 7.91**	**~8.33**	**6.69 ± 0.95**	**52.68 ± 10.71[a]**
FDA after Freeze-drying			Solid samples could not be assayed (NA)			
FDA after Acidic Extraction (Final)	**1**	**60.46 ± 7.90**	**60.46 ± 7.90**	**~8.33**	**7.26 ± 0.95**	**56.58 ± 5.29[a]**
AP Mucus (Initial)	*100*	*217.81 ± 17.77*	*21781.39 ± 1776.64*	*100*	*217.81 ± 17.77*	*100 ± 0*
APS after Aqueous Extraction	50	213.05 ± 22.87	10652.60 ± 213.05	50	213.05 ± 22.87	97.67 ± 2.68
APS after $(NH_4)_2SO_4$ precipitation (Final)	**1**	**2031.69 ± 33.47**	**2031.69 ± 33.47**	**50**	**40.63 ± 6.65**	**18.90 ± 4.66[c]**
APA after Acidic Extraction	50	151.58 ± 17.38	7579.00 ± 868.82	50	151.58 ± 17.38	70.07 ± 11.50
APA after $(NH_4)_2SO_4$ precipitation (Final)	**1**	**2354.63 ± 417.55**	**2354.63 ± 417.55**	**50**	**47.09 ± 8.35**	**21.82 ± 4.98[c]**

(b)

FDC: Freeze-dried Crude Extract, FDS: Freeze-dried Aqueous Extract (FDS), FDA: Freeze-dried Acidic Extract, APS: Ammonium-sulphate-precipitated Aqueous Extract, APA: Ammonium-sulphate-precipitated Acidic Extract; *Recovery = Protein per specimen (µg) of Extracts/Protein per specimen (µg) of Mucus x 100 %; All experiments were performed in triplicate; All values were in Mean ± Standard Deviation; Italicised data represents the initial state of the mucus samples; Data in Bold represents the final state of the mucus samples; Different letters in superscripted lower case of the means from the same column represent statistically significant difference (p-value ≤ 0.05); Comparison with ≥ three groups are analysed by One-way ANOVA followed by Duncan's multiple range test

For AP mucus extracts, both species exhibited almost 100 % protein recovered for APS but slightly lower recovery for APA mucus extracts (42.82–70.07 %) after the extraction. The result is not surprising as during the centrifugation step of extraction, obvious pellets (protein loss) was observed in APA but none in APS. However, both AP extracts only managed to recover less than half of the amount of protein after $(NH_4)_2SO_4$ precipitation, resulting in only total recovery of approximately 20 %.

Total protein recovery was compared across all five epidermal mucus extracts for each species. For *Barbodes sealei*, FDC exhibited the highest protein recovered (69.71 %), followed by FDS (42.11 %) in which both showed significantly (p-value ≤ 0.05) higher recovery than AP mucus extracts. However, FDA of *Barbodes sealei* showed the lowest recovery of 3.22 % among all five extracts. On the other hand, all FD mucus extracts of *Barbodes everetti* remained consistent which exhibited significantly higher (p-value ≤ 0.05) protein recovery than that of AP mucus extracts.

4.1.3 Comparison on the Protein Recovery between two *Barbodes* species

Comparison was done on protein recovery between mucus extracts *of Barbodes sealei* and *Barbodes everetti* (Figure 4.2). FDC and FDA showed significant difference (p-value ≤ 0.05) between both fish species. However, there was no significant difference (p-value > 0.05) for one other FD mucus (FDS) as well as two AP mucus (APS and APA).

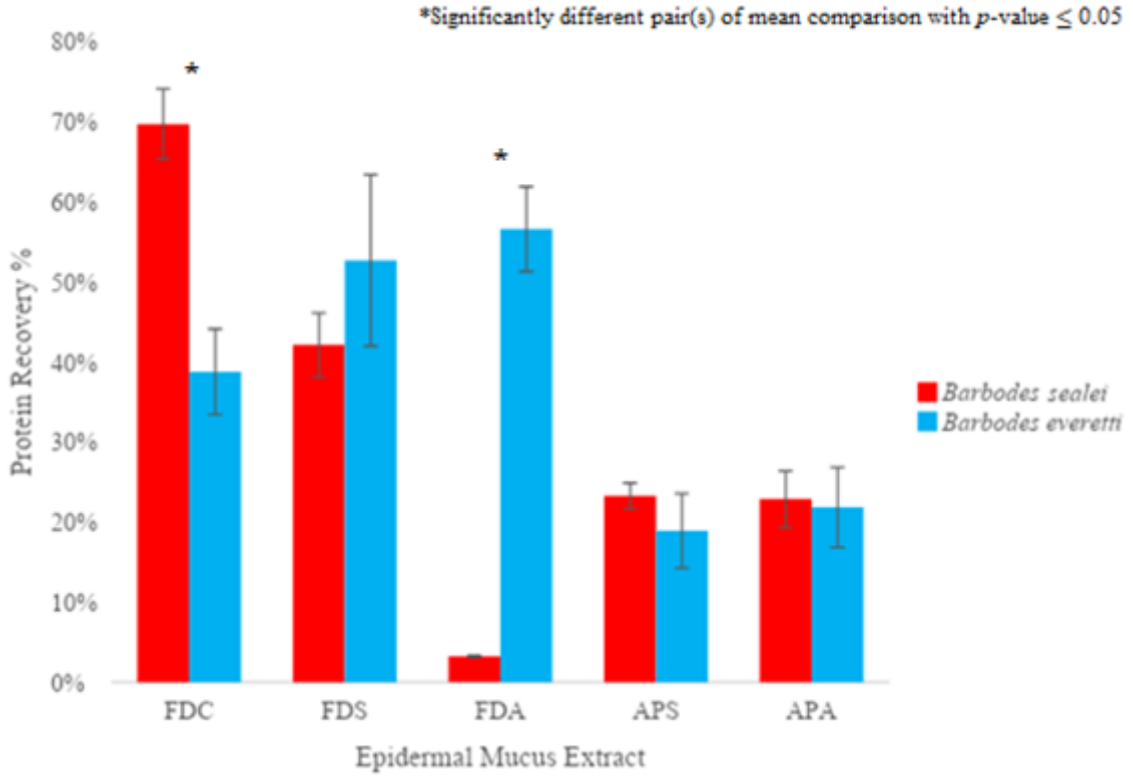


Figure 4.2: Protein recovery of five epidermal mucus extracts for *Barbodes sealei* and *Barbodes everetti*

4.2 Antibacterial Assay

4.2.1 Preliminary Screening of Antibacterial Activity of Mucus Extracts Using Disk Diffusion Tests

Ten epidermal mucus extracts (five per species) were assessed alongside negative controls (Distilled water for FDC; 0.85% NaCl for FDS and APS; 3% acetic acid for FDA; 0.8% acetic acid for APA) against three Gram-positive and five Gram-negative bacterial strains. AP mucus extracts were evaluated against eight more Gram-negative bacterial strains. 1X Pen-Strep and commercial antimicrobial discs (5 µg Ciprofloxacin) served as positive controls. Detailed antibacterial screening test results (Appendices C-E) are summarised in Table 4.3(a-c) for *B. sealei*, *B. everetti* and negative control, respectively.

Table 4.3: Disk diffusion test results of five epidermal mucus extracts against selected bacterial strains for (a) *Barbodes sealei* and (b) *Barbodes everetti* and (c) their corresponding negative control

Bacterial Strain	Inhibition Zone Diameter – IZD (mm)						
	FDC	FDS	FDA	APS	APA	[1]Positive Control	[2]Positive Control
Bacillus cereus ATCC 33019	-	-	-	-	12.16 ± 1.10	21.47 ± 0.38	24.31 ± 0.79
Listeria monocytogenes ATCC 7644	7.13 ± 0.05	7.65 ± 1.12	7.74 ± 0.45	-	-	27.12 ± 1.12	20.55 ± 0.56
Staphylococcus aureus ATCC 25923	-	-	9.54 ± 0.82	-	8.39 ± 0.42	6.96 ± 0.19	20.34 ± 0.46
Aeromonas hydrophila PRP 012	NA	NA	NA	-	9.08 ± 0.84	NA	17.37 ± 0.89
Escherichia coli O157:H7	-	-	-	-	9.15 ± 1.24	11.57 ± 3.04	21.32 ± 1.02
Klebsiella pneumoniae PRP 010	NA	NA	NA	-	8.95 ± 0.44	NA	14.28 ± 0.60
Pseudomonas aeruginosa ATCC 27853	-	-	10.99 ± 0.37	-	9.03 ± 1.41	15.47 ± 0.22	25.24 ± 0.99
Salmonella braenderup ATCC BAA 664	-	-	11.16 ±1.12	-	10.73 ± 0.16	15.20 ± 0.48	29.38 ± 2.14
Salmonella enteritidis ATCC 13036	NA	NA	NA	-	9.98 ± 1.24	NA	25.44 ± 0.66
Salmonella typhi ATCC 14028	NA	NA	NA	-	8.92 ± 1.95	NA	22.24 ± 2.11
Salmonella typhimurium	-	-	-	-	10.63 ± 0.75	18.45 ± 0.63	20.39 ± 0.87
Shigella boydii ATCC 9207	NA	NA	NA	-	8.01 ± 1.04	NA	16.60 ± 0.65
Shigella flexneri ATCC 12022	NA	NA	NA	-	9.35 ± 0.57	NA	14.07 ± 0.28
Shigella sonnei ATCC 25931	NA	NA	NA	-	9.77 ± 1.63	NA	26.32 ± 1.04
Vibrio cholerae	7.48 ± 0.09	7.38 ± 0.11	11.42 ± 2.42	-	7.50 ± 0.61	23.56 ± 4.73	25.79 ± 1.58
Yersinia enterocolitica	NA/	NA	NA	-	-	NA	29.09 ± 0.77

(a)

NA indicates no test performed against the bacterial strain; IZD includes 6mm disc diameter; All experiments are done in triplicate. All values were in Mean ± Standard Deviation; - indicates no clear zone of inhibition observed (Absence of antibacterial activity). [1]Positive Control = 1X Pen-Strep; [2]Positive Control = Ciprofloxacin (5 µg)

Bacterial Strain	Inhibition Zone Diameter – IZD (mm)						
	FDC	FDS	FDA	APS	APA	[1]Positive Control	[2]Positive Control
Bacillus cereus ATCC 33019	-	-	-	-	12.49 ± 1.39	22.95 ± 0.54	24.31 ± 0.79
Listeria monocytogenes ATCC 7644	6.93 ± 0.59	6.65 ± 0.22	7.74 ± 1.13	-	-	23.43 ± 0.91	20.55 ± 0.56
Staphylococcus aureus ATCC 25923	-	-	8.67 ± 1.51	-	8.23 ± 0.80	8.50 ± 1.67	20.34 ± 0.46
Aeromonas hydrophila PRP 012	NA	NA	NA	-	9.67 ± 0.42	NA	17.37 ± 0.89
Escherichia coli O157:H7	-	-	-	-	9.64 ± 0.54	10.09 ± 1.10	21.32 ± 1.02
Klebsiella pneumoniae PRP 010	NA	NA	NA	-	9.27 ± 1.07	NA	14.28 ± 0.60
Pseudomonas aeruginosa ATCC 27853	-	8.76 ± 0.57	11.08 ± 0.32	-	10.04 ± 0.45	17.27 ± 1.07	25.24 ± 0.99
Salmonella braenderup ATCC BAA 664	-	-	9.46 ± 1.45	-	11.58 ± 1.01	15.98 ± 1.69	29.38 ± 2.14
Salmonella enteritidis ATCC 13036	NA	NA	NA	-	9.64 ± 1.12	NA	25.44 ± 0.66
Salmonella typhi ATCC 14028	NA	NA	NA	-	10.27 ± 0.24	NA	22.24 ± 2.11
Salmonella typhimurium	-	-	8.65 ± 0.66	-	8.37 ± 2.09	14.32 ± 1.58	20.39 ± 0.87
Shigella boydii ATCC 9207	NA	NA	NA	-	8.96 ± 1.03	NA	16.60 ± 0.65
Shigella flexneri ATCC 12022	NA	NA	NA	-	9.70 ± 0.54	NA	14.07 ± 0.28
Shigella sonnei ATCC 25931	NA	NA	NA	-	10.10 ± 1.88	NA	26.32 ± 1.04
Vibrio cholerae	7.38 ± 0.64	7.75 ± 0.57	10.13 ± 0.46	-	7.35 ± 1.35	20.39 ± 0.56	25.79 ± 1.58
Yersinia enterocolitica	NA	NA	NA	-	-	NA	29.09 ± 0.77

(b)

NA indicates no test performed against the bacterial strain; IZD includes 6mm disc diameter; All experiments are done in triplicate. All values were in Mean ± Standard Deviation; - indicates no clear zone of inhibition observed (Absence of antibacterial activity). [1]Positive Control = 1X Pen-Strep; [2]Positive Control = Ciprofloxacin (5 µg)

Bacterial Strain	Inhibition Zone Diameter – IZD (mm)						
	FDC	FDS	FDA	APS	APA	[1]Positive Control	[2]Positive Control
Bacillus cereus ATCC 33019	-	-	-	-	14.91 ± 0.10	22.95 ± 0.54	24.31 ± 0.79
Listeria monocytogenes ATCC 7644	-	-	7.98 ± 0.15	-	-	23.43 ± 0.91	20.55 ± 0.56
Staphylococcus aureus ATCC 25923	-	-	8.58 ± 1.07	-	8.16 ± 0.48	8.50 ± 1.67	20.34 ± 0.46
Aeromonas hydrophila PRP 012	NA	NA	NA	-	9.83 ± 1.94	NA	17.37 ± 0.89
Escherichia coli O157:H7	-	-	-	-	10.13 ± 0.34	10.09 ± 1.10	21.32 ± 1.02
Klebsiella pneumoniae PRP 010	NA	NA	NA	-	8.52 ± 1.37	NA	14.28 ± 0.60
Pseudomonas aeruginosa ATCC 27853	-	-	9.16 ± 0.28	-	8.19 ± 0.63	17.27 ± 1.07	25.24 ± 0.99
Salmonella braenderup ATCC BAA 664	-	-	7.29 ± 0.19	-	9.03 ± 0.56	15.98 ± 1.69	29.38 ± 2.14
Salmonella enteritidis ATCC 13036	NA	NA	NA	-	9.77 ± 1.89	NA	25.44 ± 0.66
Salmonella typhi ATCC 14028	NA	NA	NA	-	8.65 ± 1.25	NA	22.24 ± 2.11
Salmonella typhimurium	-	-	7.93 ± 0.25	-	9.48 ± 0.02	14.32 ± 1.58	20.39 ± 0.87
Shigella boydii ATCC 9207	NA	NA	NA	-	9.01 ± 0.62	NA	16.60 ± 0.65
Shigella flexneri ATCC 12022	NA	NA	NA	-	11.42 ± 0.42	NA	14.07 ± 0.28
Shigella sonnei ATCC 25931	NA	NA	NA	-	10.11 ± 1.01	NA	26.32 ± 1.04
Vibrio cholerae	-	-	13.02 ± 0.88	-	8.30 ± 0.78	20.39 ± 0.56	25.79 ± 1.58
Yersinia enterocolitica	NA	NA	NA	-	-	NA	29.09 ± 0.77

(c)

NA indicates no test performed against the bacterial strain; IZD includes 6mm disc diameter; All experiments are done in triplicate. All values were in Mean ± Standard Deviation; - indicates no clear zone of inhibition observed (Absence of antibacterial activity). [1]Positive Control = 1X Pen-Strep; [2]Positive Control = Ciprofloxacin (5 µg)

4.2.2 Ruling out the Solvent Effect on the Antibacterial Activity of Mucus Extracts

As for the preliminary screening results, broad-spectrum antibacterial activity was exhibited by various extracts for both species. Notably, negative controls of two mucus extracts, viz. FDA (3 % acetic acid) and APA (0.8 % acetic acid) (Table 4.4) also exhibited clear zones of inhibition against the bacterial strains tested while three other negative controls remained inactive against the bacterial strains tested. In order to rule out the possible influence of solvent used in antibacterial activity exhibited by FDA and APA, the IZD values of the extracts and their corresponding negative controls were tested for significance using Student t-test to further verify the presence of antibacterial activity by mucus extract.

Table 4.4: Summary of disk diffusion test results for negative controls of different solvents with respect to their epidermal mucus extracts

Mucus Extract	Negative Control	Observation
FDC	Distilled water	-
FDS	0.85 % NaCl (Saline)	-
FDA	3 % acetic acid	+
APS	0.85 % NaCl (Saline)	-
APA	0.8 % acetic acid	+

+ indicates there is a clear zone of inhibition against bacterial strains tested (Presence of antibacterial activity);
- indicates no clear zone of inhibition observed (Absence of antibacterial activity)

4.2.2.1 Freeze-dried Acidic Extract (FDA)

FDA of *Barbodes sealei* were active against five out of eight bacterial strains while FDA of *Barbodes everetti* were active against six out of eight bacterial strains. IZD of active FDA for both species were compared with the negative control (3 % acetic acid) (different plate) against the same bacterial strain and the results of significance test were summarised in Table 4.5 (a-b).

For *Barbodes sealei*, FDA had shown significantly greater IZD against *Pseudomonas*

Table 4.5: Results of significance test between IZD produced by antibacterial activity of FDA and its negative control against selected bacterial strains for (a) *Barbodes sealei* and (b) *Barbodes everetti*

Bacterial Strain	Inhibition Zone Diameter – IZD (mm)			
	FDA	Negative Control (3% acetic acid)	*p*-value	Note
Listeria monocytogenes ATCC 7644	7.74 ± 0.45	7.98 ± 0.15	0.438	NS
Staphylococcus aureus ATCC 25923	9.54 ± 0.82	8.58 ± 1.07	0.287	NS
***Pseudomonas aeruginosa* ATCC 27853**	**10.99 ± 0.37**	**9.16 ± 0.28**	**0.002**	**Extract > Control**
***Salmonella braenderup* ATCC BAA 664**	**11.16 ± 1.12**	**7.29 ± 0.19**	**0.004**	**Extract > Control**
Vibrio cholerae	11.42 ± 2.42	13.02 ± 0.88	0.345	NS

(a)

Bacterial Strain	Inhibition Zone Diameter – IZD (mm)			
	FDA	Negative Control (3% acetic acid)	*p*-value	Note
Listeria monocytogenes ATCC 7644	7.74 ± 1.13	7.98 ± 0.15	0.733	NS
Staphylococcus aureus ATCC 25923	8.67 ± 1.51	8.58 ± 1.07	0.937	NS
***Pseudomonas aeruginosa* ATCC 27853**	**11.08 ± 0.32**	**9.16 ± 0.28**	**0.001**	**Extract > Control**
Salmonella braenderup ATCC BAA 664	9.46 ± 1.45	7.29 ± 0.19	0.062	NS
Salmonella typhimurium	8.65 ± 0.66	7.93 ± 0.25	0.152	NS
Vibrio cholerae	10.13 ± 0.46	13.02 ± 0.88	0.007	Control > Extract

(b)

IZD includes 6mm disc diameter; All experiments are done in triplicate; All values were in Mean ± Standard Deviation; Student's t-test was used where p-value > 0.05 indicates no significant (NS) difference between mucus extract and its negative control (Absence of antibacterial activity by mucus extract) while p-value ≤ 0.05 (Data in Bold) indicates significant difference between mucus extract and negative control (Presence of antibacterial activity by mucus extract)

aeruginosa ATCC 27853 (*p*-value = 0.002) and *Salmonella braenderup* ATCC BAA 664 (*p*-value = 0.004) than that of its negative control which suggested mucus extracts has a more significant role in the IZD demonstrated (Presence of antibacterial activity by mucus extract) while there was no significant difference (*p*-value > 0.05) between IZD of FDA and its negative control against three other bacterial strains tested which suggested mucus extracts has no significant effect in the IZD demonstrated (Absence of antibacterial activity by mucus extract).

On the other hand, FDA of *Barbodes everetti* also exhibited significantly greater zone of inhibition against *Pseudomonas aeruginosa* ATCC 27853 (*p*-value = 0.001) than that of its negative control which suggested mucus extracts has a more significant role in the IZD exhibited (Presence of antibacterial activity by mucus extract). Contrastingly, FDA showed significantly smaller zone of inhibition against *Vibrio cholerae* (*p*-value = 0.007) than that of its negative control which suggested the negative control has a more significant role in the IZD exhibited (Absence of antibacterial activity by mucus extract). Besides, there was no significant difference (*p*-value > 0.05) was observed between IZD of FDA and its negative control against four other bacterial strains tested which suggested mucus extracts has no significant effect in the IZD exhibited (Absence of antibacterial activity by mucus extract)

4.2.2.2 Ammonium-sulphate-precipitated Acidic Extract (APA)

On the other hand, APA of both *Barbodes sealei* and *Barbodes everetti* were active against fourteen bacterial strains. IZD of active APA for both species were compared with the negative control (moderately 0.8 % acetic acid) (same plate) against the same bacterial strain and the results of significance test were summarised in Table 4.6(a-b).

Table 4.6: Results of significance test between IZD produced by antibacterial activity of APA and its negative control against selected bacterial strains for (a) *Barbodes sealei* and (b) *Barbodes everetti*

Bacterial Strain	Inhibition Zone Diameter – IZD (mm)			
	APA	Negative Control (0.8% acetic acid)	*p*-value	Note
Bacillus cereus ATCC 33019	12.16 ± 1.10	14.91 ± 0.10	0.033	Control > Extract
Staphylococcus aureus ATCC 25923	8.39 ± 0.42	8.16 ± 0.48	0.565	NS
Aeromonas hydrophila PRP 012	9.08 ± 0.84	9.83 ± 1.94	0.574	NS
Escherichia coli O157:H7	9.15 ± 1.24	10.13 ± 0.34	0.260	NS
Klebsiella pneumoniae PRP 010	8.95 ± 0.44	8.52 ± 1.37	0.626	NS
Pseudomonas aeruginosa ATCC 27853	9.03 ± 1.41	8.19 ± 0.63	0.400	NS
***Salmonella braenderup* ATCC BAA 664**	**10.73 ± 0.16**	**9.03 ± 0.56**	**0.007**	**Extract > Control**
Salmonella enteritidis ATCC 13036	9.98 ± 1.24	9.77 ± 1.89	0.878	NS
Salmonella typhi ATCC 14028	8.92 ± 1.95	8.65 ± 1.25	0.850	NS
Salmonella typhimurium	10.63 ± 0.75	9.48 ± 0.02	0.057	NS
Shigella boydii ATCC 9207	8.01 ± 1.04	9.01 ± 0.62	0.226	NS
Shigella flexneri ATCC 12022	9.35 ± 0.57	11.42 ± 0.42	0.007	Control > Extract
Shigella sonnei ATCC 25931	9.77 ± 1.63	10.11 ± 1.01	0.776	NS
Vibrio cholerae	7.50 ± 0.61	8.30 ± 0.78	0.234	NS

(a)

IZD includes 6mm disc diameter; All experiments are done in triplicate; All values were in Mean ± Standard Deviation; Student's t-test was used where p-value > 0.05 indicates no significant (NS) difference between mucus extract and its negative control (Absence of antibacterial activity by mucus extract) while p-value ≤ 0.05 (Data in Bold) indicates significant difference between mucus extract and negative control (Presence of antibacterial activity by mucus extract)

Bacterial Strain	Inhibition Zone Diameter – IZD (mm)			
	APA	Negative Control (0.8% acetic acid)	*p*-value	Note
Bacillus cereus ATCC 33019	12.49 ± 1.39	14.91 ± 0.10	0.070	NS
Staphylococcus aureus ATCC 25923	8.23 ± 0.80	8.16 ± 0.48	0.903	NS
Aeromonas hydrophila PRP 012	9.67 ± 0.42	9.83 ± 1.94	0.896	NS
Escherichia coli O157:H7	9.64 ± 0.54	10.13 ± 0.34	0.257	NS
Klebsiella pneumoniae PRP 010	9.27 ± 1.07	8.52 ± 1.37	0.496	NS
***Pseudomonas aeruginosa* ATCC 27853**	**10.04 ± 0.45**	**8.19 ± 0.63**	**0.014**	**Extract > Control**
***Salmonella braenderup* ATCC BAA 664**	**11.58 ± 1.01**	**9.03 ± 0.56**	**0.019**	**Extract > Control**
Salmonella enteritidis ATCC 13036	9.64 ± 1.12	9.77 ± 1.89	0.923	NS
Salmonella typhi ATCC 14028	10.27 ± 0.24	8.65 ± 1.25	0.093	NS
Salmonella typhimurium	8.37 ± 2.1	9.48 ± 0.02	0.408	NS
Shigella boydii ATCC 9207	8.96 ± 1.03	8.67 ± 0.71	0.708	NS
Shigella flexneri ATCC 12022	9.70 ± 0.54	11.42 ± 0.43	0.012	Control > Extract
Shigella sonnei ATCC 25931	10.10 ± 1.88	9.77 ± 0.74	0.793	NS
Vibrio cholerae	7.35 ± 1.35	8.30 ± 0.78	0.350	NS

(b)

IZD includes 6mm disc diameter; All experiments are done in triplicate; All values were in Mean ± Standard Deviation; Student's t-test was used where p-value > 0.05 indicates no significant (NS) difference between mucus extract and its negative control (Absence of antibacterial activity by mucus extract) while p-value ≤ 0.05 (Data in Bold) indicates significant difference between mucus extract and negative control (Presence of antibacterial activity by mucus extract)

APA of *Barbodes sealei* showed significantly greater IZD against *Salmonella braenderup* ATCC BAA 664 than that of its negative control (*p*-value = 0.007) which suggested mucus extracts has a more significant role in the IZD observed (Presence of antibacterial activity by mucus extract) (Figure 4.3) while significantly smaller IZD values against *Bacillus cereus* ATCC 33019 (*p*-value = 0.033) and *Shigella flexneri* ATCC 12022 (*p*-value = 0.007) by APA were observed when compared to its negative control which suggested the negative control has a more significant role in the IZD observed (Absence of antibacterial activity by mucus extract) (Figure 4.4). Furthermore, there was no significant difference (*p*-value > 0.05) between IZD of APA and its negative control against 11 other bacterial strains tested which suggested mucus extracts has no significant effect in the IZD observed (Absence of antibacterial activity by mucus extract) (Figure 4.5).

Besides that, APA of *Barbodes everetti* also exhibited significantly greater zone of inhibition against *Pseudomonas aeruginosa* ATCC 27853 (*p*-value = 0.014) and *Salmonella braenderup* ATCC BAA 664 (*p*-value = 0.019) than that of its negative control which suggested mucus extracts has a more significant role in the IZD observed (Presence of antibacterial activity by mucus extract) (Figure 4.3) while APA showed significantly smaller IZD against *Shigella flexneri* ATCC 12022 (*p*-value = 0.012) than its negative control which suggested the negative control has a more significant role in the IZD observed (Absence of antibacterial activity by mucus extract) (Figure 4.4). However, there was no significant difference (p-value > 0.05) was observed between IZD of APA and its negative control against 11 other bacterial strains tested which suggested mucus extracts has no significant effect on the IZD observed (Absence of antibacterial activity by mucus extract) (Figure 4.5).

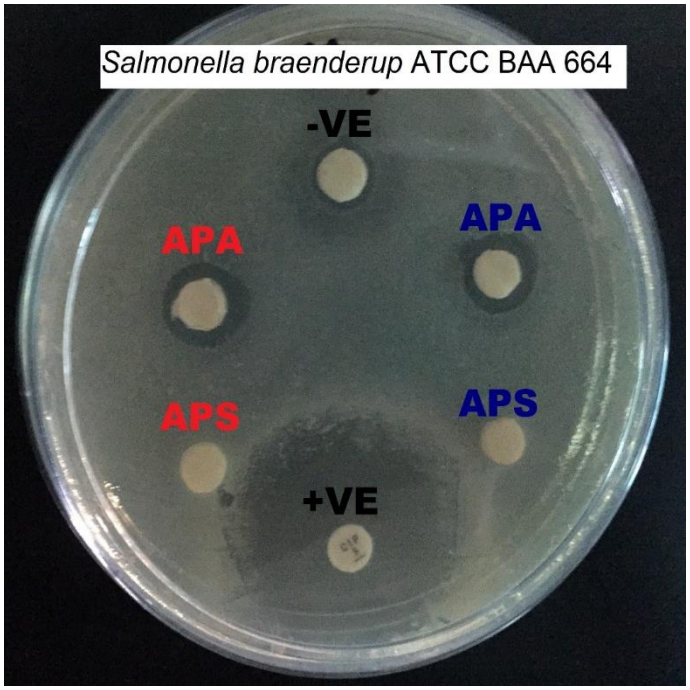


Figure 4.3: Presence of antibacterial activity by mucus extract where the mucus extracts exhibited significantly higher IZD than the negative control (p-value < 0.05). Extracts in Red belong to *Barbodes sealei*; Extracts in Blue belong to *Barbodes everetti*

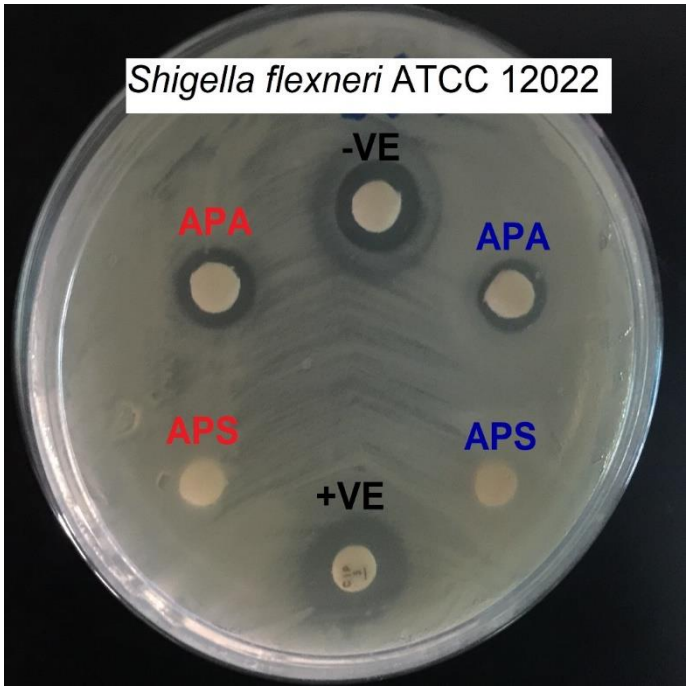


Figure 4.4: Absence of antibacterial activity by mucus extracts where the negative control exhibited significantly higher IZD than the mucus extracts (p-value < 0.05). Extracts in Red belong to *Barbodes sealei*; Extracts in Blue belong to *Barbodes everetti*

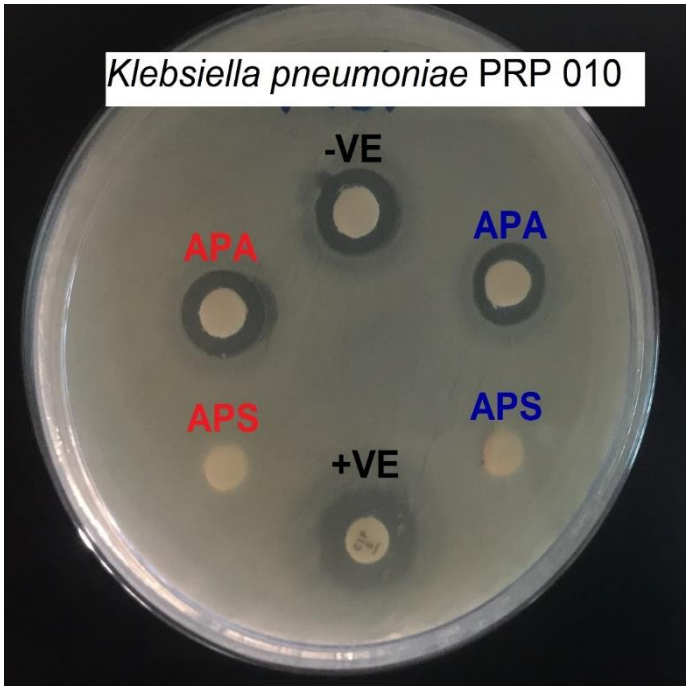


Figure 4.5: Absence of antibacterial activity by mucus extracts where there is no significant difference (p-value > 0.05) between the mucus extracts and their negative control. Extracts in Red belong to *Barbodes sealei*; Extracts in Blue belong to *Barbodes everetti*

4.2.3 Antibacterial Activity of Mucus Extracts

After ruling out the influence of the solvents on the antibacterial activity exhibited by mucus extracts, four out of five mucus extracts from both *Barbodes sealei* and *Barbodes everetti* demonstrated antibacterial activity against one Gram-positive and three Gram-negative bacterial strains in which clear zones of inhibitions were observed and if applicable, significantly higher than those of their respective negative controls. APS from both species remained inactive against all the bacterial strains tested (No clear zone of inhibition). On the other hand, two positive controls used in the study, namely 1X Pen-Strep and Ciprofloxacin exhibited broad-spectrum antibacterial activity with varying strength against all the bacterial strains tested. Final results were summarised in Table 4.7(a-b).

Table 4.7: Antibacterial test results of five epidermal mucus extracts of (a) *Barbodes sealei* and (b) *Barbodes everetti*

Bacterial Strain	Inhibition Zone Diameter – IZD (mm)						
	FDC	FDS	FDA	APS	APA	[1]Positive Control	[2]Positive Control
Gram-positive							
Bacillus cereus ATCC 33019	-	-	-	-	-	21.47 ± 0.38[bc]	24.31 ± 0.79[b]
Listeria monocytogenes ATCC 7644	7.13 ± 0.05[b]	7.65 ± 1.12[a]	-	-	-	27.12 ± 1.12[a]	20.55 ± 0.56[c]
Staphylococcus aureus ATCC 25923	-	-	-	-	-	6.96 ± 0.19[f]	20.34 ± 0.46[c]
Gram-negative							
Aeromonas hydrophila PRP 012	NA	NA	NA	-	-	NA	17.37 ± 0.89[d]
Escherichia coli O157:H7	-	-	-	-	-	11.57 ± 3.04[e]	21.32 ± 1.02[c]
Klebsiella pneumoniae PRP 010	NA	NA	NA	-	-	NA	14.28 ± 0.60[e]
Pseudomonas aeruginosa ATCC 27853	-	-	10.99 ± 0.37[a]	-	-	15.47 ± 0.22[d]	25.24 ± 0.99[b]
Salmonella braenderup ATCC BAA 664	-	-	11.16 ±1.12[a]	-	10.73 ± 0.16	15.20 ± 0.48[d]	29.38 ± 2.14[a]
Salmonella enteritidis ATCC 13036	NA	NA	NA	-	-	NA	25.44 ± 0.66[b]
Salmonella typhi ATCC 14028	NA	NA	NA	-	-	NA	22.24 ± 2.11[c]
Salmonella typhimurium	-	-	-	-	-	18.45 ± 0.63[cd]	20.39 ± 0.87[c]
Shigella boydii ATCC 9207	NA	NA	NA	-	-	NA	16.60 ± 0.65[d]
Shigella flexneri ATCC 12022	NA	NA	NA	-	-	NA	14.07 ± 0.28[e]
Shigella sonnei ATCC 25931	NA	NA	NA	-	-	NA	26.32 ± 1.04[b]
Vibrio cholerae	7.48 ± 0.09[a]	7.38 ±0.11[a]	-	-	-	23.56 ± 4.73[b]	25.79 ± 1.58[b]
Yersinia enterocolitica	NA	NA	NA	-	-	NA	29.09 ± 0.77[a]

(a)

NA indicates no test performed against the bacterial strain; IZD includes 6mm disc diameter; All experiments are done in triplicate. All values in Mean ± Standard Deviation indicates presence of antibacterial activity; - indicates absence of antibacterial activity; [1]Positive Control = 1X Pen-Strep; [2]Positive Control = Ciprofloxacin (5 µg). Different letters in superscripted lower case of the means from the same column represent statistically significant difference (p-value ≤ 0.05); Comparison with < three groups are analysed by Independent Student's t-test; Comparison with ≥ three groups are analysed by One-way ANOVA followed by Duncan's multiple range test

Bacterial Strain	Inhibition Zone Diameter – IZD (mm)						
	FDC	FDS	FDA	APS	APA	[1]Positive Control	[2]Positive Control
Gram-positive							
Bacillus cereus ATCC 33019	-	-	-	-	-	21.47 ± 0.38[bc]	24.31 ± 0.79[b]
Listeria monocytogenes ATCC 7644	6.93 ± 0.59[a]	6.65 ± 0.22[c]	-	-	-	27.12 ± 1.12[a]	20.55 ± 0.56[c]
Staphylococcus aureus ATCC 25923	-	-	-	-	-	6.96 ± 0.19[f]	20.34 ± 0.46[c]
Gram-negative							
Aeromonas hydrophila PRP 012	NA	NA	NA	-	-	NA	17.37 ± 0.89[d]
Escherichia coli O157:H7	-	-	-	-	-	11.57 ± 3.04[e]	21.32 ± 1.02[c]
Klebsiella pneumoniae PRP 010	NA	NA	NA	-	-	NA	14.28 ± 0.60[e]
Pseudomonas aeruginosa ATCC 27853	-	8.76 ± 0.57[a]	11.08 ± 0.32	-	10.04 ± 0.45[a]	15.47 ± 0.22[d]	25.24 ± 0.99[b]
Salmonella braenderup ATCC BAA 664	-	-	-	-	11.58 ± 1.01[a]	15.20 ± 0.48[d]	29.38 ± 2.14[a]
Salmonella enteritidis ATCC 13036	NA	NA	NA	-	-	NA	25.44 ± 0.66[b]
Salmonella typhi ATCC 14028	NA	NA	NA	-	-	NA	22.24 ± 2.11[c]
Salmonella typhimurium	-	-	-	-	-	18.45 ± 0.63[cd]	20.39 ± 0.87[c]
Shigella boydii ATCC 9207	NA	NA	NA	-	-	NA	16.60 ± 0.65[d]
Shigella flexneri ATCC 12022	NA	NA	NA	-	-	NA	14.07 ± 0.28[e]
Shigella sonnei ATCC 25931	NA	NA	NA	-	-	NA	26.32 ± 1.04[b]
Vibrio cholerae	7.38 ± 0.64[a]	7.75 ± 0.57[b]	-	-	-	23.56 ± 4.73[b]	25.79 ± 1.58[b]
Yersinia enterocolitica	NA	NA	NA	-	-	NA	29.09 ± 0.77[a]

(b)

NA indicates no test performed against the bacterial strain; IZD includes 6mm disc diameter; All experiments are done in triplicate. All values in Mean ± Standard Deviation indicates presence of antibacterial activity; - indicates absence of antibacterial activity; [1]Positive Control = 1X Pen-Strep; [2]Positive Control = Ciprofloxacin (5 µg). Different letters in superscripted lower case of the means from the same column represent statistically significant difference (p-value ≤ 0.05); Comparison with < three groups are analysed by Independent Student's t-test; Comparison with ≥ three groups are analysed by One-way ANOVA followed by Duncan's multiple range test

Among the active mucus extract, all FD mucus extracts demonstrated antibacterial activity while only one of the two AP mucus extracts exhibited antibacterial activity. Mucus extracts in dH_2O and saline such as FDC and FDS inhibited the growth of both Gram-positive (*Listeria monocytogenes* ATCC 7644) and Gram-negative bacteria (*Pseudomonas aeruginosa* ATCC 27853 and *Vibrio cholerae*). However, mucus extract in low concentration of acetic acid (0.8-3 %) such as FDA and APA demonstrated a selective activity in which the extracts were active towards Gram-negative bacteria only (*Pseudomonas aeruginosa* ATCC 27853 and *Salmonella braenderup* ATCC BAA 664).

Active mucus extracts that inhibited more than one bacterial strain were also compared. FDC of *Barbodes sealei* was significantly more active (p–value < 0.05) against Gram-negative *Vibrio cholerae* (IZD = 7.48 ± 0.09 mm) than Gram-positive *Listeria monocytogenes* ATCC 7644 (IZD = 7.13 ± 0.05) while FDS of *Barbodes everetti* also exhibited significantly higher activity (p–value < 0.05) against Gram-negative bacterial strains such as *Pseudomonas aeruginosa* ATCC 27853 (IZD = 8.76 ± 0.57 mm), followed by *Vibrio cholerae* (IZD = 7.75 ± 0.57 mm) as compared to that of Gram-positive *Listeria monocytogenes* ATCC 7644 (IZD = 6.65 ± 0.22 mm). For FDA of *Barbodes sealei* and APA of *Barbodes everetti*, similar activity was observed (p–value > 0.05) against both Gram-negative bacterial strains which were Pseudomonas aeruginosa ATCC 27853 and *Salmonella braenderup* ATCC BAA 664.

4.2.4 Comparison on the antibacterial activity between two *Barbodes* species

Listeria monocytogenes ATCC 7644, *Pseudomonas aeruginosa* ATCC 27853, and *Vibrio cholerae* were most often inhibited by mucus extracts, which included four out of ten epidermal mucus extracts studied while *Salmonella braenderup* ATCC BAA 664 was

inhibited by three. Interestingly, the inhibitory activities against certain bacterial strains by mucus extract from one of the fish species tested only were also observed, i.e. the growth of *Pseudomonas aeruginosa* ATCC 27853 was inhibited by FDS and APA of *Barbodes everetti* while the growth of *Salmonella braenderup* ATCC BAA 664 was inhibited by FDA of *Barbodes sealei*. The sensitivity of the bacterial strains against the mucus extracts tested in the study was summarised in Table 4.8.

Table 4.8: Summary of antibacterial test results of *Barbodes sealei* and *Barbodes everetti*

Bacterial Strain	FDC	FDS	FDA	APS	APA
Listeria monocytogenes ATCC 7644					
Pseudomonas aeruginosa ATCC 27853					
Salmonella braenderup ATCC BAA 664					
Vibrio cholerae					

Red-coloured box = Bacterial strains inhibited by *Barbodes sealei* only; Blue-coloured box = Bacterial strains inhibited by *Barbodes everetti* only; Purple-coloured box = Bacterial strains inhibited by both fish species. Black-coloured box = Bacterial strains resistant to both fish species; Other resistant bacterial strains not shown in the table = Gram-positive: *Bacillus cereus* ATCC 33019 and *Staphylococcus aureus* ATCC 25923. Gram-negative: *Aeromonas hydrophila* PRP 012, *Escherichia coli* O157:H7, *Klebsiella pneumoniae* PRP 010, *Salmonella enteritidis ATCC 13036*, *Salmonella typhi* ATCC 14028, *Salmonella typhimurium*, *Shigella boydii* ATCC 9207, *Shigella flexneri* ATCC 12022, *Shigella sonnei* ATCC 25931, and *Yersinia enterocolitica*.

4.2.5 Determination of Minimal Inhibitory Concentrations Using Broth Microdilution Susceptibility Test

Prior to the experiment, the concentrations of APA mucus extracts of both *Barbodes* species for each dilution until the 11th well was tabulated [Table 4.9(a-b)]. Additional test (Broth microdilution susceptibility test) was performed to further characterize the antibacterial properties of APA mucus extracts of both fish species by determining their MICs against the bacterial strains tested (Table 4.10). Active FD mucus extracts were not included due to insufficient concentrations (Data not shown).

Table 4.9: Minimal Inhibitory Concentration (MIC) Dilution Table for APA Mucus Extract of (a) *Barbodes sealei* and (b) *Barbodes everetti*

Well	Dilution Factor	Protein Concentration of APA in Each Well (µg/ml)		
		1	2	3
	*Stock Concentration	2384.94	2727.79	2130.39
1	2^{-1}	1192.47	1363.90	1065.19
2	2^{-2}	596.23	681.95	532.60
3	2^{-3}	298.12	340.97	266.30
4	2^{-4}	149.06	170.49	133.15
5	2^{-5}	74.53	85.24	66.57
6	2^{-6}	37.26	42.62	33.29
7	2^{-7}	18.63	21.31	16.64
8	2^{-8}	9.32	10.66	8.32
9	2^{-9}	4.66	5.33	4.16
10	2^{-10}	2.33	2.66	2.08
11	2^{-11}	1.16	1.33	1.04
12	Control	Control	Control	Control

(a)

Well	Dilution Factor	Protein Concentration of APA in Each Well (µg/ml)		
		1	2	3
	*Stock Concentration	2618.70	1873.25	2571.95
1	2^{-1}	1309.35	936.62	1285.97
2	2^{-2}	654.68	468.31	642.99
3	2^{-3}	327.34	234.16	321.49
4	2^{-4}	163.67	117.08	160.75
5	2^{-5}	81.83	58.54	80.37
6	2^{-6}	40.92	29.27	40.19
7	2^{-7}	20.46	14.63	20.09
8	2^{-8}	10.23	7.32	10.05
9	2^{-9}	5.11	3.66	5.02
10	2^{-10}	2.56	1.83	2.51
11	2^{-11}	1.28	0.91	1.26
12	Control	Control	Control	Control

(b)

*Stock concentration is based on Bradford assay (Refer to Appendix A); # represents replicates of samples

Table 4.10: MICs of APA mucus extracts against selected bacterial strains

Bacterial Strain	Minimal Inhibitory Concentration - MIC (µg/ml)					
	APA of *Barbodes sealei*			APA of *Barbodes everetti*		
	1	2	3	1	2	3
Pseudomonas aeruginosa ATCC 27853	NA	NA	NA	654.68	468.31	642.99
Salmonella braenderup ATCC BAA 664	298.12	340.97	266.30	327.34	234.16	321.49

Active FD mucus extracts were not included due to insufficient concentrations (Data not shown); NA indicates broth microdilution test not performed against the bacterial strain; # represents replicates of samples;

APA of *Barbodes sealei* were able to inhibit the growth of *Salmonella braenderup* ATCC BAA 664 with MIC values ranged from 266.30–340.97 µg/ml. As compared to APA of *Barbodes everetti*, a lower range of MIC values was obtained (234.16–327.34 µg/ml) against the same bacterial strain. Other than that, APA of *Barbodes everetti* also showed a much higher MIC range (468.31–654.68 µg/ml) to inhibit the growth of *Pseudomonas aeruginosa* ATCC 27853.

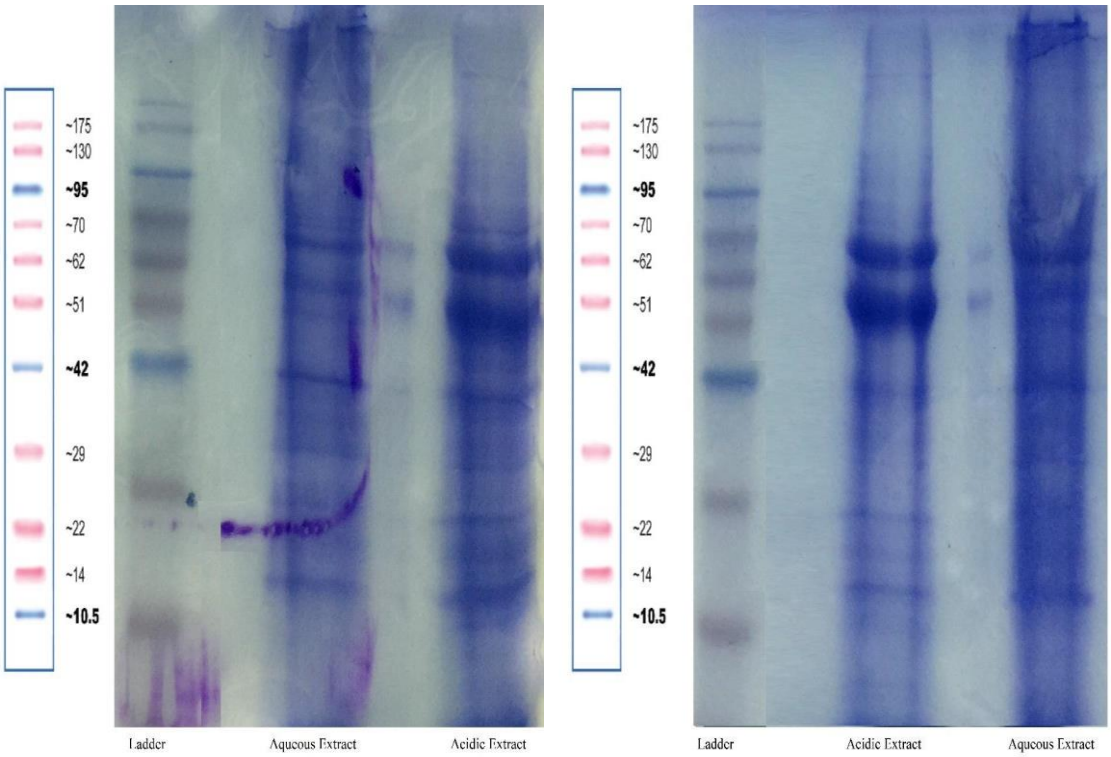
4.3 Characterisation of Protein Content in Active Mucus Extracts

The only active mucus extracts subjected to protein characterisation analysis were Ammonium-sulphate-precipitated Aqueous Extract (APS) and Ammonium-sulphate-precipitated Acidic Extract (APA). Active FD mucus extracts, i.e. Freeze-dried Crude Extract (FDC), Freeze-dried Aqueous Extract (FDS), and Freeze-dried Acidic Extract (FDA) was not included in the study due to insufficient concentrations.

4.3.1 SDS-PAGE

The SDS-PAGE analysis of mucus extracts (30-55 µg) revealed protein bands of various molecular weights ranging from 22 kDa to 95 kDa for both fish species [Figure 4.6(a-b)] which were estimated using Chromatein Pre-stained Standard Protein Ladder. Certain bands were not clearly visible. For *Barbodes sealei*, the protein profile of APS and APA exhibited protein bands with similar weights but different intensity. Both mucus extracts demonstrated several distinct bands at 24 kDa, 40 kDa, 50 kDa, 56 kDa and 66 kDa. However, protein bands between 50 kDa and 70 kDa were more prominent in APA whereas protein bands at 40 kDa were more intense in APS. As for *Barbodes everetti*, the protein profile of APS and APA also presented a similar pattern where protein bands of similar weights were observed with varying intensity. Major distinct bands were observed at 23 kDa,

29 kDa, 42 kDa, 55 kDa and 68 kDa where protein bands of APA between 50 kDa and 70 kDa were more prominent but protein bands below 42 kDa were more prominent in APS. Despite being one of the active mucus extracts in the study, FD mucus extracts for both fish species were too diluted to be visualised (data not shown).



(a) (b)

Figure 4.6: SDS-PAGE presenting protein profile of AP mucus extracts of (a) *Barbodes sealei* (From Left: Standard Protein Ladder, APS, APA) and *Barbodes everetti* (From Left: Standard Protein Ladder, APA, APS) in SDS-PAGE. Samples were loaded onto a 4% stacking and 12% resolving acrylamide gel. The staining reagent used was Coomassie Brilliant Blue R-250 (BioRad)

4.3.2 LC-MS/MS

The most predominant five bands from SDS-PAGE of the active mucus extract (APA) from each species were chosen, excised, and sent for protein sequencing by LC-

MS/MS (Proteomic International). The data obtained from LC-MS/MS analysis (Appendix F) were compared with UniProt database corresponding to the fish class Actinopterygii which resulted in 158 protein hits and 56 unique protein hits after removal of redundant proteins for *Barbodes sealei* and 61 protein hits and 31 unique protein hits for *Barbodes everetti*. Table 4.11 and Table 4.12 showed literature-based comparison about presence of these proteins in skin mucus of other fish species with *B. sealei* and *B. everetti*, respectively.

Out of 56 proteins identified from APA of *Barbodes sealei*, 19 were also reported in epidermal mucus of other fish species while 14 out of 31 proteins identified from APA of *Barbodes everetti* were reported in other fish epidermal mucus studies. Collectively, 48 proteins identified from the extracts of both fish species were never reported in epidermal mucus of other fish species. To the best of our knowledge, this is the first report of their presence in the fish epidermal mucus extracts.

Based on current literature, four of the protein identified including heat shock protein 70, histone H2A, histone H2B, and histone H4 were reported for their antibacterial activity in the past studies (Table 4.13). Interestingly, histone H2B is only present in mucus extract of *B. everetti* but absent in that of *B. sealei*. On the other hand, the three antibacterial histone proteins were also reported in skin mucus of other fish species except for heat shock protein 70 to the best of our knowledge.

Table 4.11: Proteins identified by LC-MS/MS from the active mucus extract APA of *Barbodes sealei*

Band ID	Protein Name	Reported In Fish Epidermal Mucus
3	78 kDa glucose-regulated protein	*Sparus aurata* (Sanahuja & Ibarz, 2015; Pérez-Sánchez et al., 2017) *Salmo salar* (Jensen et al., 2014)
4	Abelson helper integration site 1	
1-4	Actin, cytoplasmic 1	*Gadus morhua* (Rajan et al., 2011) *Cyclopterus lumpus* (Patel & Brinchmann, 2017; Patel et al., 2019) *Dicentrarchus labrax* (Cordero et al., 2015) *Salmo salar* (Fæste et al., 2020) *Sparus aurata* (Cordero et al., 2017; Pérez-Sánchez et al., 2017)
1-4	Actin, cytoplasmic 2-like	
1-4	Actin-depolymerizing factor	
3	AdoHcyase_NAD domain-containing protein	
3	Alanine--tRNA ligase	
4	Alpha-1-antitrypsin	*Acipenser oxyrhynchus oxyrhynchus* (Murphy et al., 2020)
2	alpha-2-macroglobulin-like	*Pelteobagrus fulvidraco* (Xiong et al., 2020)
1,3-5	Anaphase-promoting complex subunit 5	
3	ATPase family AAA domain-containing protein 5-like	
3	CAP-Gly domain-containing protein	
3	Complement C3-like protein	*Pelteobagrus fulvidraco* (Xiong et al., 2020)
3	Echinoderm microtubule-associated-like 2 isoform X1	
3	EMAP like 2	
1-3,5	GLOBIN domain-containing protein	
1	Glutathione-dependent dehydroascorbate reductase	
1	Guanine nucleotide binding protein (G protein), alpha 15 (Gq class), tandem duplicate 4	
3	Heat shock 70 kDa protein-like	
3	Heat shock cognate 70	
3	Heat shock cognate 70 kDa protein	*Cathorops spixii* (Ramos et al., 2012) *Gadus morhua* (Magnadóttir et al., 2018) *Larimichthys crocea* (Ao et al., 2015) *Sparus aurata* (Jurado et al., 2015) *Salmo salar* (Provan et al., 2013; Jensen et al., 2014)

Table 4.11: continued

3	Heat shock cognate 71 kDa protein	*Cathorops spixii* (Ramos et al., 2012) *Larimichthys crocea* (Ao et al., 2015) *Sparus aurata* (Cordero et al., 2017; Pérez-Sánchez et al., 2017)
3	Heat shock cognate 71 kDa protein-like	*Boleophthalmus pectinirostris* (Liu et al., 2019)
4	Heat shock cognate protein 70	
3	Heat shock protein 70 (Fragment)	
3	Heat shock protein family A (Hsp70) member 2	
3	Heat shock protein family A (Hsp70) member 8	
3	Heat shock protein Hsc70	
4	Hemoglobin subunit alpha-1	*Larimichthys crocea* (Ao et al., 2015)
4	Hemopexin	*Sparus aurata* (Pérez-Sánchez et al., 2017)
1-5	Histone H2A	*Channa striata* (Kwan & Ismail, 2018) *Cyclopterus lumpus* (Patel et al., 2019) *Salmo salar* (Fæste et al., 2020) *Sparus aurata* (Cordero et al., 2017) *Oncorhynchus mykiss* (Fernandes et al., 2002)
2,4,5	Histone H2A type 2-A (Fragment)	
3-5	Histone H3	*Cirrhinus mrigala* (Nigam et al., 2015) *Gadus morhua* (Magnadóttir et al., 2018) *Myxine glutinosa* (Subramanian et al., 2008b)
1-5	histone H3-like	*Pelteobagrus fulvidraco* (Xiong et al., 2020)
1, 4-5	Histone H4	*Channa striata* (Kwan & Ismail, 2018) *Cyclopterus lumpus* (Patel et al., 2019) *Dicentrarchus labrax* (Cordero et al., 2015) *Sparus aurata* (Cordero et al., 2017)
3	Hsc70	
1,3	IF rod domain-containing protein	*Sparus aurata* (Pérez-Sánchez et al., 2017)
1, 3-5	Ig-like domain-containing protein	
3	Inducible heat shock protein 70	
1	Keratin, type II cytoskeletal 8-like	*Boleophthalmus pectinirostris* (Liu et al., 2019) *Dicentrarchus labrax* (Cordero et al., 2015) *Pelteobagrus fulvidraco* (Xiong et al., 2020) *Sparus aurata* (Sanahuja et al., 2019)

Table 4.11: continued

1	Keratin 4	
3,4	leucine-rich repeat and IQ domain-containing protein 1	
5	L-lactate dehydrogenase	*Carassius auratus gibelio* (Jiang et al., 2019) *Channa striata* (Kwan & Ismail, 2018)
4	Major vault protein	*Salmo salar* (Valdenegro-Vega et al., 2014)
1	Pol-like protein	
1-5	Putative histone H2B type 2-E-like	
1	Putative threonine-rich GPI-anchored glyco isoform X2	
1	Sarcosine dehydrogenase	
3	Serotransferrin	*Channa striata* (Kwan & Ismail, 2018) *Gadus morhua* (Magnadóttir et al., 2018)
1	Si:dkey-65b12.6 (Fragment)	
2	Threonyl-tRNA synthetase	
1	Transmembrane protein 132D	
1	UmuC domain-containing protein	
4	Warm-temperature-acclimation-associated 65-kDa protein	
4	WD repeat domain 1	
4	WD_REPEATS_REGION domain-containing protein	

Table 4.12: Proteins identified by LC-MS/MS from the active mucus extract APA of *Barbodes everetti*

Band ID	Protein Name	Reported In Fish Epidermal Mucus
2	Actin-depolymerizing factor	
2	Alpha-2-macroglobulin-like protein	
2	ATPase family AAA domain-containing protein 5-like	
1	Collagen alpha-1(I) chain	
1	Collagen alpha-1(I) chain-like	*Cyprinus Carpio* (Saleh et al., 2018)
1	Collagen, type I, alpha 1b (Fragment)	
4	Heat shock protein 70 (Fragment)	
1,2,4	Hemopexin	*Sparus aurata* (Pérez-Sánchez et al., 2017)
1,3,4	Histone H2A	*Channa striata* (Kwan & Ismail, 2018)
		Cyclopterus lumpus (Patel et al., 2019)
		Salmo salar (Fæste et al., 2020)
		Sparus aurata (Cordero et al., 2017)
		Oncorhynchus mykiss (Fernandes et al., 2002)
1,3,5	Histone H2A type 2-A (Fragment)	
1	Histone H2B	*Cyclopterus lumpus* (Patel et al., 2019)
		Gadus morhua (Bergsson et al., 2005)
		Salmo salar (Fæste et al., 2020)
		Sparus aurata (Cordero et al., 2017)
1-4	Histone H3	*Cirrhinus mrigala* (Nigam et al., 2015)
		Gadus morhua (Magnadóttir et al., 2018)
		Myxine glutinosa (Subramanian et al., 2008b)
1,4	histone H3-like	*Pelteobagrus fulvidraco* (Xiong et al., 2020)
1,4	Histone H4	*Channa striata* (Kwan & Ismail, 2018)
		Cyclopterus lumpus (Patel et al., 2019)
		Dicentrarchus labrax (Cordero et al., 2015)
		Sparus aurata (Cordero et al., 2017)
1	Inter-alpha-trypsin inhibitor heavy chain 3	
1	Inter-alpha-trypsin inhibitor heavy chain H3-like	
2	Keratin, type I cytoskeletal 13-like	*Boleophthalmus pectinirostris* (Liu et al., 2019)
		Dicentrarchus labrax (Cordero et al., 2015)
		Pelteobagrus fulvidraco (Xiong et al., 2020)
		Sparus aurata (Jurado et al., 2015)

Table 4.12: continued

2	Keratin, type I cytoskeletal 17	*Sparus aurata* (Sanahuja & Ibarz, 2015)
2	Keratin, type I cytoskeletal 17-like	*Dicentrarchus labrax* (Cordero et al., 2015)
2	Keratin, type I cytoskeletal 18-like	*Cyprinus carpio* (Saleh et al., 2018) *Pelteobagrus fulvidraco* (Xiong et al., 2020)
2	Keratin, type II cytoskeletal 8-like (Fragment)	*Boleophthalmus pectinirostris* (Liu et al., 2019) *Dicentrarchus labrax* (Cordero et al., 2015) *Sparus aurata* (Sanahuja et al., 2019)
4	Protein BANP	
1-5	Putative histone H2B type 2-E-like	
4	Serine protease inhibitor	
3	Serotransferrin	*Channa striata* (Kwan & Ismail, 2018) *Gadus morhua* (Magnadóttir et al., 2018)
2,4	Si:ch211-243g18.2	
2	Thread biopolymer filament subunit alpha-like (Fragment)	
3	Transferrin variant B (Fragment)	
4	Warm temperature acclimation-related 65 kDa protein 2	
4	Warm temperature acclimation-related 65kDa protein (Fragment)	*Cyclopterus lumpus* (Patel & Brinchmann, 2017) *Sparus aurata* (Jurado et al., 2015; Sanahuja & Ibarz, 2015)
4	Warm-temperature-acclimation-associated 65-kDa protein	

Table 4.13: Antibacterial proteins identified from the active epidermal mucus extract (APA only)

Antibacterial Protein	Fish Species			References
	Barbodes sealei	*Barbodes everetti*	Other fish species	
Heat shock protein 70 (Fragment)	Present	Present	Absent	Taniguchi et al. (2013, 2015)
Histone H2A	Present	Present	Present	Chen et al. (2015) Dawson et al. (2010) Fernandes et al. (2002) Ma et al. (2017) Muñoz-Camargo et al. (2018) Park et al. (2000) Sruthy et al. (2019)
Histone H2B	Absent	Present	Present	Bergsson et al. (2005) Robinette et al. (1998) Noga et al. (2011)
Histone H4	Present	Present	Present	Knappe et al. (2009)

CHAPTER 5

DISCUSSION

5.1 Mucus Protein Concentration and Protein Recovery

Aquatic vertebrates live in intimate contact with pathogen-rich aquatic environments. The high level of exposure to potential pathogens may pose additional health hazards compared to their terrestrial counterparts. A successful infection relies greatly on the ability of bacterial adhesion on their mucosal surface (Magariños et al., 1995; Benhamed et al., 2014). Fortunately, fish are gifted with the ability to combat the infection. Fish skin plays a vital role in the prevention of colonisation of these infectious pathogens by secreting mucus layers which can trap, neutralise, and immobilise the pathogens (Reverter et al., 2018). This indicates fish epidermal mucus is a key component that might contain antibacterial substances which contribute to the mucosal innate immunity. In addition, Chong et al. (2005) and Manivasagan et al. (2009) had reported the predominantly proteinaceous properties of epidermal mucus in various fish species and most antibacterial components identified were proteins or peptides. This suggests that the bacterial defence mechanisms could be associated with their protein contents. Therefore, it is crucial to obtain high protein contents in fish mucus studies. The present study managed to determine the protein concentrations of various epidermal mucus extracts using different concentrating techniques from two fish species namely *Barbodes sealei* and *Barbodes everetti*. The total protein contents of these mucus extracts were also successfully recovered and compared.

For both fish species, the protein concentrations of FD and AP mucus and their extracts were inconsistent due to the different number of fish specimens and different volumes of solvent used during mucus collection, resulting in different ratios of volume to

specimen. Standardisation of the initial number of fish specimens used between FD and AP mucus is dependent on the number of healthy individuals available for mucus collection and therefore it is difficult to maintain constant rate of epidermal mucus secretion. Furthermore, stress conditions such as handling stress, starvation or confinement can greatly influence the epidermal mucus production as well as its composition (Helfman et al., 2009). On the other hand, mucus secretion could reduce friction in order to help the fish to escape in adverse situations (Vatsos et al., 2010). The collection of FD mucus is believed to exert a less stressful condition to the fish specimens during mucus collection as the ratio of extraction solvent volume to number of samples (2:1) is higher that of AP mucus (1:1). Thus, the inconsistency of protein concentrations in FD and AP mucus could be associated with different levels of stress experienced by each fish specimen.

Other than that, different results were yielded when comparing the concentrating ability of each epidermal mucus extract from both species; the highest number of fold (11 - 12-fold) was observed in FDC, APS, and APA of *B. sealei* but only in APA of *B. everetti*. In addition, significant variations were also observed among FD mucus extracts. Protein concentration of crude extract (FDC) was the highest, followed by aqueous (FDS) and acidic (FDA) extracts for *B. sealei*. However, a different trend was observed in *B. everetti* where lowest protein concentration was observed in FDC.

Fish epidermal mucus is primarily composed of approximately 95 % of water and glycoproteins as well as many other substances (Bansil & Turner, 2006) and the mucus composition usually varies among different fish species (Sanahuja & Ibarz, 2015). It is well established that the composition of fish mucus could be influenced by endogenous (sex and developmental stage) and exogenous factors (stress, temperature, pH or infections (Esteban,

2012; Reverter et al., 2018). The biochemical substances found in fish mucus might differ depending on the ecological and physiological factors (Loganathan et al., 2011). Thus, the different results could be due to differences in the mucus composition that reacted differently to the type of extraction and concentrating methods. Besides, these differences might also affect their solubility in different solvents and result in varying protein concentrations among the extracts between the two fish species tested. However, no obvious difference was observed between AP mucus extracts for both species. It could be due to high protein contents obtained from AP mucus that make the differences to be negligible.

Bradford assays quantify the protein depending on the molar absorbance of the dye-protein complex that is bound in Bradford's reagent-protein mixture (Zaia et al., 2005; Okutucu et al., 2007). According to Stoscheck (1990), Noble and Bailey (2009), the Coomassie Brilliant Blue (dye of Bradford's reagent) reacts fundamentally with arginine residues while react less vigorous with histidine, lysine, tyrosine, tryptophan, and phenylalanine residues in an acidic solution which causes wide variation of response to different proteins. Furthermore, bovine serum albumin (BSA), being one of the most inexpensive and easily obtained protein standards, however, exhibits a strong dye response and might lead to underestimation on protein concentrations of average samples. The protein concentrations of the products from different mucus extraction were more likely to be unstable and inconsistent. As a result, the low concentration observed in FDA might be due to inefficiency of Bradford's assay in detecting different proteins with unfitting choice of standards.

Despite the shortcomings, Bradford's method with BSA as protein standard remains a popular technique used by numerous literatures to estimate protein concentration for its

simplicity and rapidity (Noble & Bailey, 2009; Campion et al., 2017). In addition, Kruger (2009) stated that Bradford assay, when compared with another commonly used method named Lowry assay, is a more sensitive approach and prone to less interference by commonly used reagents of biological samples.

In the present study, all five epidermal mucus extracts also showed different rates of protein recovery where most FD mucus extracts yielded higher recovery compared to AP mucus extracts for both species despite having lower protein concentration. Doonan and Cutler (2003) stated that the number of purification steps should be reduced to a minimum as losses on any step is deemed inevitable. Apart from the different nature of mucus composition which would in turn alter the extraction products, preparation involving the transfer of samples might greatly impact the loss of protein as well. Figure 5.1 showed that the number of sample transfers for AP mucus extracts almost doubled that of FD mucus extracts. This explains the low recovery of protein contents in AP mucus extracts as each transfer indicates a certain degree of protein loss.

5.2 Antibacterial Activities of Different Epidermal Mucus Extracts of *B. sealei* and *B. everetti*

Present study screened all five epidermal mucus extracts of both *B. sealei* and *B. everetti* for antibacterial activities. In this preliminary screening, crude, and aqueous extracts such as FDC, and FDS showed inhibition against two (*Listeria monocytogenes* and *Vibrio cholerae*) out of 16 bacterial strains tested while acidic extracts such as FDA and APA appeared to be exhibiting wide spectrum of antibacterial activities against 15 out of 16 gram-positive and gram-negative bacterial strains tested. However, absence of antibacterial activity was observed in another aqueous extract, APS, against all the bacterial strains tested. The overall results demonstrated more promising antibacterial activity from acidic extracts

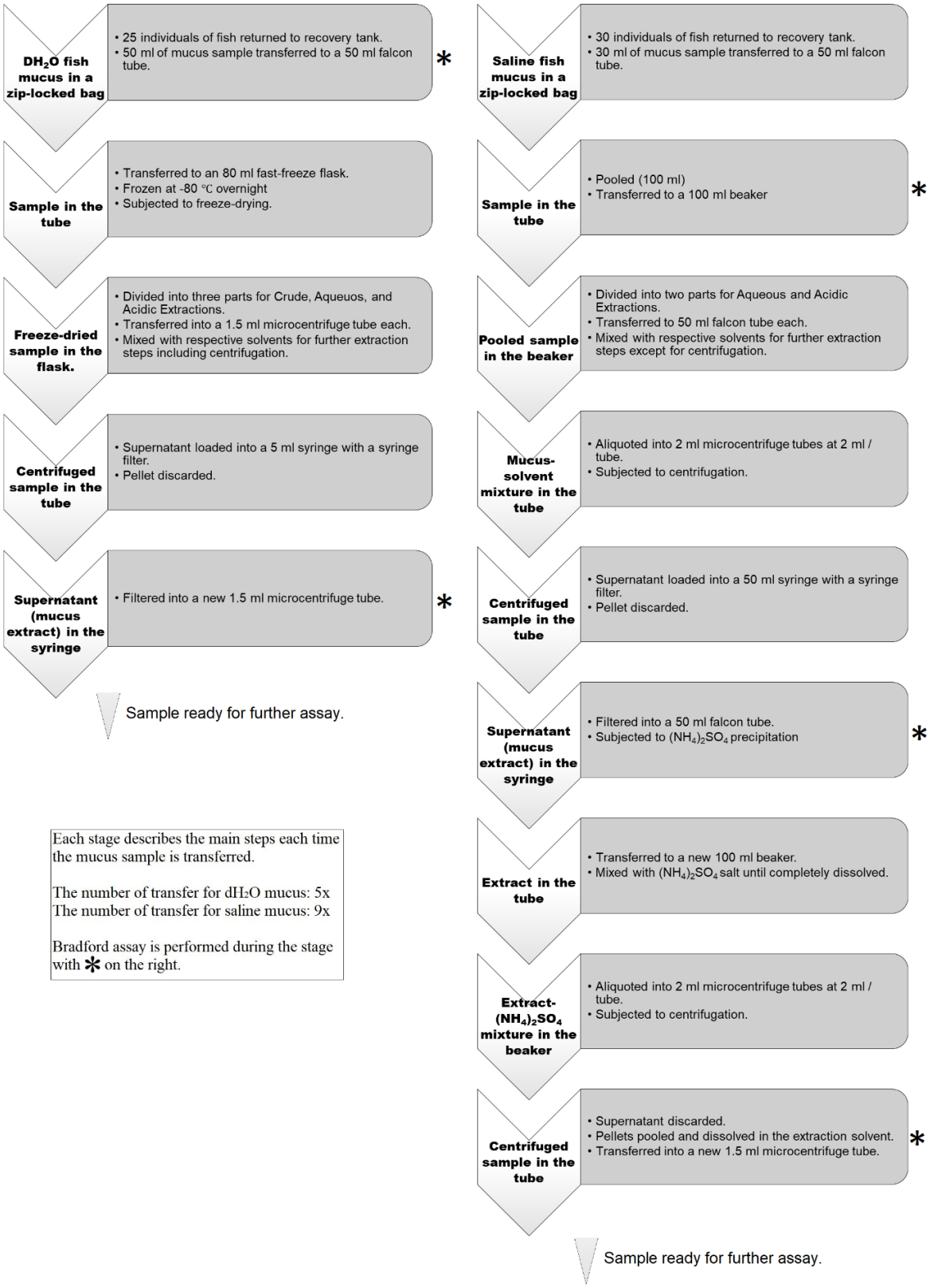


Figure 5.1: Number of transfers of dH_2O FD mucus and saline AP mucus during sample preparation

than other types of extracts, which agreed to the studies in the past decade (Wei et al., 2010; Vennila et al., 2011; Rao et al., 2015; Manikantan et al., 2016; Al-Rasheed et al., 2018). However, these authors did not take into account the influence of extraction solvents on the activity (negative control was not mentioned). In present study, the acidic negative control (acetic acid solvent) has shown to be exhibiting a broad spectrum of antibacterial activities. In fact, the role of acetic acid alone as disinfectant had been well established due to its antibacterial activity against myriads of bacterial pathogens including those were tested in present study such as *Staphylococcus aureus*, *Escherichia coli*, and *Pseudomonas aeruginosa* (Ryssel et al., 2009; Cortesia et al., 2014; Halstead et al., 2015).

In order to further verify the antibacterial properties exhibited by acidic mucus extracts, Student's t-test was performed to analyse the difference between the inhibition zone diameter of the extract and negative control. From 15 bacterial strains that were tested sensitive to FDA and APA, only two namely *Pseudomonas aeruginosa* and *Salmonella branderup* were proved to be responding to mucus extract as the IZD exhibited by acidic extract were significantly higher than that of its negative control. It was verified that the mucus extract is responsible for the inhibition zone observed on these two bacterial strains rather than the effect of an acidic solvent alone. Naturally, the other 13 bacterial strains were considered to be sensitive towards the solvents and not solely due to the mucus content. The results were not surprising as acidic mucus extracts in the study ranged from 0.8 % to 3 % but studies have shown that acetic acid was capable of exhibiting antibacterial activity even at concentrations as low as 0.166 % (Fraise et al., 2013; Wali & Abed, 2019). Strikingly, *Bacillus cereus*, *Shigella flexneri*, and *Vibrio cholerae* showed better growths in the presence of mucus extracts where the IZD exhibited by FDA and APA were significantly lower than their respective negative controls. Similar result was reported in mucus extract of Gilthead

seabream that caused the overgrowth of *B. subtilis* (Guardiola et al., 2014b). Furthermore, the findings of Minniti and coworkers (2019) suggested *Vibrio* sp. and *Pseudoalteromonas* sp. can thrive in the presence of salmon mucus by degrading and consuming it as a source of nutrient. Even though fish epidermal mucus was widely reported to be antimicrobial, its protein content might also be a potential source of nutrient for enhanced bacterial growth (Smith & Fernandes, 2009). However, it should be noted that present results only suggest the nutritious potential of skin mucus in vitro. It is believed that the defence mechanism of skin mucus on healthy living fish might differ when it comes to the bacterial invasion and is yet to be explored.

The potential of fish mucus being a nutrient source for bacterial growth can also be related with absence of antibacterial activity exhibited by the mucus extracts in present study, especially aqueous extract (APS) from both targeted fish species. As reported by Minniti et al. (2019) on salmon mucus, high protein contents in mucus extract might instead signify the presence of concentrated amount of favourable substrate for the growth of the bacterial strains tested. Contrastingly, another aqueous extract (FDS) demonstrated antibacterial activity against both gram-positive and gram-negative bacterial strains. In fact, an extensive review was conducted previously (Lee et al., 2020) and the review was consistent with the results in which many other fish species exhibited pronounced antibacterial properties on their aqueous mucus extracts (Magariños et al., 1995; Kuppulakshmi et al., 2008; Elavarasi et al., 2013; Nigam et al., 2015; Kumari et al., 2019) but other studies reported absence of antibacterial activity in aqueous mucus extracts from more than 20 fish species (Hellio et al., 2002; Subramanian et al., 2008a; Subhashini et al., 2013; Katra et al., 2016; Al-Rasheed et al., 2018). Subramanian et al. (2007) successfully characterised the aqueous mucus extracts of seven distinct marine fish species namely Arctic

char, brook trout, koi carp, striped bass, haddock, Atlantic cod, and hagfish which confirmed the presence of various hydrolytic enzymes such lysozyme and proteases. These enzymes were reported to be antimicrobial in the fish mucus (Aranishi, 2000; Smith et al., 2000). Furthermore, certain hydrolytic enzymes in fish mucus respond to pathogen invasion by activating gene expression that encodes complement protein or antimicrobial peptides. For instance, Cho and colleagues (2002a) reported that cathepsin D and matrix metalloprotease present in catfish mucus was involved in synthesizing antimicrobial peptides, named parasin I. Thus, the bactericidal properties of aqueous mucus extracts in present study could be associated with the enzymatic activities, resulting in either direct bacterial killing mechanism or indirect pathway that involve the production of bactericidal components. The absence of antibacterial activity, however, could be ascribed to unfavourable incubation conditions (temperature or pH) that led to inactivation of these enzymes or insufficient enzyme concentrations that failed to produce observable activity.

Conclusively, except for APS, all the other four mucus extracts (FDC, FDS, FDA, and APA) for both fish species had exhibited varying antibacterial activities against four bacterial strains which included one Gram-positive bacterial strain, i.e., *Listeria monocytogenes* and three Gram-negative bacterial strains, i.e., *Pseudomonas aeruginosa*, *Salmonella braenderup*, and *Vibrio cholerae*. Present results once again confirmed that fish epidermal mucus is a potential source of antimicrobial products under the right circumstances (i.e. using the right extraction and for the right strains).

Nevertheless, the positive controls (1X Pen-Strep and 5 µg Ciprofloxacin) exhibited a rather broad-spectrum antibacterial action with varying strengths against all 16 bacterial strains tested. This is not surprising as these commercial antibiotics, with concentrations as

low as 5 µg, are pure compounds that have been well studied and tested for their antimicrobial activities, but the mucus extracts are still at screening stage and might require further purification, isolation, and characterisation. Despite having higher concentration (FD extracts ranging from 41.12 to 77.23 µg/ml and AP extracts ranging from 2031.69 to 2473.25 µg/ml), it does not entail greater antibacterial activities than that of the positive controls in the study. This agreed with the study by Elavarasi and coworkers (2013) where protein concentration of walking catfish extract was lower than that of Mozambique tilapia yet exhibiting better bactericidal activities. It strongly suggests that the protein concentration is not a simple measure of the antibacterial activity of fish skin mucus extract. Although high protein contents increase the chance of having more antibacterial proteins in the extracts, it could indicate presence of more contaminants that are inert and do not contribute to any activity (Al-Rasheed et al., 2018), which could ultimately dilute the effect of any active compound.

Variation in antibacterial activity was observed not only among the active extracts within the fish species, but also between species in all the extracts. For both species, FDC and FDS demonstrated activities against both Gram-positive and Gram-negative bacteria but both acidic extracts (FDA and APA) managed to inhibit Gram-negative bacteria only. The acidic extracts might contain specialised antimicrobial peptides (AMPs) that would selectively act against Gram-negative bacteria only. The presence of outer membrane in Gram-negative bacteria warranted different killing mechanisms by AMPs than that in Gram-positive bacteria. Generally, AMPs possess net cationic charge and amphipathic structures. These two properties enable attraction of negatively charged bacterial membranes and promote interaction and absorption within bacteria (Nissen-Meyer & Nes, 1997). Such interactions might lead to agglutination of bacterial cells which in turn cause fatal cell

damage (Guan et al., 2008). Thus, the active compounds present in FDA and APA might interact with the outer membrane of Gram-negative bacterial strains tested, resulting in specific activity against Gram-negative strains.

Other than that, selectivity was also observed in fish species. *Salmonella braenderup* was only sensitive to FDA of *Barbodes sealei* while *Pseudomonas aeruginosa* was only sensitive to FDS and APA of *Barbodes evertti*. The results agreed with several studies (Kumari et al., 2011, 2019) that stated antibacterial properties of the fish epidermal mucus varied even among closely related species due to different ecological and physiological conditions such as salinity, pH, handling stress, maturity, and growth stage. Furthermore, Dhanaraj et al. (2009) reported the different antibacterial strength of the epidermal mucus extract from five snakehead species that belong to the same genus *Channa* (i.e. *C. striatus*, *C. micropeltes, C. marulius*, *C. punctatus* and *C. gachua*). In addition, Shephard (1994) and Esteban (2012) both stated that the composition of fish epidermal mucus was species dependent and varied among the fish species.

On top of that, the sensitivity of bacterial strains towards fish epidermal mucus might influence the results as well. Lee et al. (2020) systematically reviewed four bacterial strains, namely *Escherichia coli* (n=40), *Pseudomonas aeruginosa* (n=27), *Staphylococcus aureus* (n=27), *Klebsiella pneumoniae* (n=25), as the most sensitive to fish epidermal mucus extracts from more than half of 47 fish species reported. However, in present study, *E. coli* and *S. aureus* were resistant to all mucus extracts tested and only *P. aeruginosa* was sensitive to the acidic extract from both fish species. It is believed that the difference in the mucus composition might influence their expression of immune components that resulted in varying

antibacterial effects in terms of fish species, extraction methods and bacterial strains of different sensitivity.

Notably, FDA of *B. sealei*, with a relatively low protein concentration (3.57 ± 0.50 μg/ml), was able to inhibit two bacterial strains as well. In present study, the preparation of FDA involved the combination of acetic acid solvent and short-minute heat. The combination was reported to target cationic, low molecular weight proteins to obtain the extract enriched with acid-soluble proteins and peptides (Subramanian et al., 2008a; Manikantan et al., 2016). Due to the nature of being hydrophilic and thermally stable, the solubility of cationic proteins and peptides could be increased more effectively when treated with heat in low concentration of acetic acid for a brief period (Nigam et al., 2015). It could selectively inactivate proteolytic enzyme activity that might cause degradation of these cationic peptides as well (Cole & Ganz, 2000). It was suggested that these acid-soluble proteins are responsible for the defensive role in exhibiting broad-spectrum potent antibacterial activities (Hancock & Diamond, 2000; Brinchmann, 2016). Unlike crude and aqueous extracts, acidic extraction would produce insoluble pellets that would be then omitted from the experiment. This is consistent with the present Bradford results where 30 – 60 % protein was lost after acidic extraction in APA of both *B. sealei* and *B. everetti* (Refer to Table 4.2). Thus, the peptides remaining in the FDA were suspected to be purer without interference from other proteolytic enzymes and were able to exhibit a certain degree of antibacterial activity despite losing high amounts of protein contents during extraction.

Additional experiments such as the MIC tests were also performed to further characterise the antibacterial activity of the active mucus extracts. Although there were reports on the antibacterial activity of fish epidermal mucus against *Salmonella* sp., this is

the first report of minimal inhibitory activities against *Salmonella braenderup* to the best of our knowledge. However, the MIC values obtained for both species (200–350 µg/ml) contradicted the experiments by Vennila et al. (2011) in which the acidic mucus extract of marine stingray inhibited the development of another *Salmonella* sp with much lower MIC values (16-32 µg/ml). In addition, MIC values of APA of *Barbodes everetti* fell within a higher range (468.31–654.68 µg/ml) in inhibiting the growth of *Pseudomonas aeruginosa* and were not in the same range with recent studies. For instance, Rao et al. (2015) reported acidic mucus extract of bagrid catfish inhibited the bacterial growth at the concentration as low as 23.91 µg/ml while Subramanian et al. (2008a) extracted the skin mucus from several distinct species such as brook trout, haddock and hagfish using acidic solvent and achieved the MIC range of 21–273 µg/ml against other strains of *Pseudomonas aeruginosa*. Interestingly, aqueous mucus extracts of four other carp species (Tyor & Kumari, 2016; Kumari et al., 2019) were reported to inhibit *P. aeruginosa* at MIC value of 50 µg/ml. This confirmed again the protein contents of fish skin mucus were not positively correlated with the antibacterial activity exhibited and might vary in different fish species and different extraction methods of their epidermal mucus.

Preliminary antibacterial screening in present study suggested that acidic and aqueous mucus extracts from freshwater fish species in Borneo have potential to demonstrate antimicrobial activity and could be a good source of antimicrobial compounds. Antimicrobial proteins present in mucus extracts could be responsible for bacterial defence purposes as protein was regarded as the major component of fish mucus. More studies are required in order to purify and characterise the antibacterial components in fish skin mucus.

5.3 Antimicrobial Proteins (AMP) in APA of two *Barbodes* species

AMP study was pioneered by several researchers in 1980s in the light of the discoveries of insect cecropins (Steiner et al., 1981), human α-defensins (Selsted et al., 1985) and amphibians magainins (Zasloff, 1987). The database of identified antimicrobial proteins (AMP) has been growing steadily in the past twenty years. To date, over 3000 antimicrobial peptides have been isolated and described in myriads of living species. Although more than three quarters are found in animals (Wang et al., 2016), only about 5 % are fish peptides (Masso-Silva & Diamond, 2014). The activity exhibited by the epidermal mucus of *Barbodes sealei* and *Barbodes everetti* could be ascribed to these fish AMPs. By using the proteomic technology such as LC-MS/MS, major proteins of the active epidermal mucus extracts in the study were successfully identified. In comparison with relevant existing literature, four proteins from two protein groups namely histone proteins – Histone H2A, Histone H2B, Histone H4 and heat shock proteins - Heat shock protein 70 were reported to be antibacterial.

5.3.1 Histone Proteins

Histones are highly conserved, ubiquitous proteins that can be found in the nuclei of all eukaryotes. This family of protein comprises linker histones (H1 and H5) and core histones (H2A, H2B, H3 and H4) which are responsible for the formation of nucleosomes. Traditionally, the primary functions of histones are thought to provide structural support for DNA and regulate gene transcriptions (Parseghian & Luhrs, 2006). Over the years, as more and more studies were conducted, histones have grown to be one of the most promising sources of AMPs.

The interaction between histones and bacteria was first defined over 60 years ago when Hirsch's discovery in 1958 established the bactericidal effect of histones against *E. coli* K12. However, little attention was paid to this discovery due to lack of theoretical concepts at that time that could possibly explain the detailed mechanisms of such interactions. It was not until late 1990s when researchers began to relate the antibacterial properties of histones with fish immunity and characterised them in various fish species (Park et al., 1998; Robinette et al., 1998; Noga et al., 2001; Richards et al., 2001; Fernandes et al., 2002, 2003). Furthermore, the findings of Robinette and Noga (2001) reported the histone levels in channel catfish were profoundly repressed in the absence of disease. This showed that histone might serve as a useful indicator to monitor and assess fish health.

Core histone H2A is a potent antibacterial agent, whether as a full-length protein or peptide fragments derived from it (Doolin et al., 2020). Purified full-length H2A from skin exudates of rainbow trout were active against several Gram-positive bacteria at a maximum concentration of 16 µg / ml (Fernandes et al., 2002). It was stated the antibacterial activity of intact H2A could be attributed to protein reconstitution into a planar lipid bilayer without forming ion channels which perturbed the bacterial cell membrane. Thus, absence of stable ion channels during reconstitution suggested that pore-forming properties are not responsible for the activity exhibited. On top of that, several truncated N-terminal fragments of H2A from various aquatic organisms also exhibited broad-spectrum antibacterial activity. Such peptide fragments include abhisin from disk abalone (De Zoysa et al., 2009), buforins from several amphibians and clam species (Li et al., 2007; Cho et al., 2009; Muñoz-Camargo et al., 2018), hipposin from Atlantic halibut (Birkemo et al., 2003), parasin I from Japanese common catfish (Park et al., 1998), and several undesignated fragments from shrimps, crabs and fishes (Patat et al., 2004; Chen et al., 2015; Ma et al., 2017; Sruthy et al., 2019). In most

cases, the fragments were generated via proteolytic cleavage. In addition, Cho et al. (2002a, 2002b) further described the regulation mechanisms of parasin-I which were mediated by two specific enzymes namely cathepsin D and matrix metalloproteinase 2.

On the other hand, core histone H2B and H4 as whole proteins were reported to express antibacterial activity as well. In 1993, Hiemstra et al. first purified antimicrobial H2B from murine macrophages. For the following decade, more researchers isolated H2B from gills, skin, and surface mucus of several fish species (Robinette et al., 1998; Noga et al., 2001; Bergsson et al., 2005) as well as skin of Schlegel's green tree frog (Kawasaki et al., 2003) and haemocytes of Pacific white shrimp (Patat et al., 2004) that inhibited the growth of many pathogenic bacterial strains. Interestingly, potent activity exhibited by H2B against fish pathogen *Aeromonas hydrophilia* indicates it might have a critical role in fish immunity (Robinette et al., 1998). Although research on core histone H4 was comparatively scarce, this histone purified from shrimp haemocytes (Patat et al., 2004) and secretion of human sebocytes (Lee et al., 2009) have been briefly reported to be exhibiting potent activity against several Gram-positive and Gram-negative bacteria as well (Knappe et al., 2009). On the other hand, Lee et al. (2009) reported the enhancer role of histone H4 in increasing antimicrobial effect of sebum free fatty acids. Other than just being specialised AMPs, this finding provides a different perspective on the alternative role of histones against bacterial infections.

Although the antibacterial mechanisms involved remain largely unexplored, recent findings from these histone-derived AMPs suggest their activity may involve membrane permeabilization or disruption and pore-forming (Doolin et al., 2020) which is a common mechanism of most AMPs. This is because histones share similar traits as most AMPs such

as small (<100 amino acid), cationic nature, alpha-helical structure, and high abundance of hydrophobic amino acid. Further in-vivo studies are required to reveal how histones interact with invading pathogens on fish body surface to fully elucidate their role in skin defence.

5.3.2 Heat Shock Proteins

Heat shock proteins (HSPs) are one of the highly conserved and constitutively expressed stress-response proteins which are found in a wide range of organisms, including fish (Morimoto & Santoro, 1998; Demeke & Tassew, 2016). Apart from heat stress, the up regulation of HSPs could be induced by other stress stimuli such as acidosis, hypoxia, ischaemia, microbial damage, or protein degradation (Roberts et al., 2010). In general, HSPs are grouped according to their molecular masses namely low molecular weight heat shock proteins (>47 kDa), Hsp70 (68–73 kDa) and Hsp90 (85–90 kDa).

Hsp70 play significant roles in fish health relating to the development of the specific or non-specific immune responses to bacterial and viral infections. Its antibacterial significance was first revealed by Forsyth and his team in 1997 with increased Hsp70 level over a 63-day period observed in coho salmons when infected by *Renibacterium salmoninarum*. In addition, Roberts et al. (2010) reported that elevated Hsp70 synthesis in salmon and gilthead seabream enhanced by a chemical inducer named TEX-OE® increased their survivability substantially when subjected to Vibrio challenge. Furthermore, platy fish managed to survive from *Yersinia ruckeri* infections when treated with intra-coelomal injection of two bacterial HSPs namely DnaK and GroEL, proteins equivalent to Hsp70 and Hsp60 integrated with a non-lethal heat shock (Ryckaert et al., 2010). Although the mode of action involved continues to elude researchers, these findings confirmed the importance of bactericidal role for Hsp70 in fish.

Interestingly, in 2013, Taniguchi et al. (2013) demonstrated the antibacterial properties of Hsp70-18 and the mechanism of action was elucidated. Hsp70-18 (Amino acid sequence: DNRMVNHFVQEFKRKHKK), a potent octadecapeptide derived from Hsp70 of rice (*Oryza sativa* L. *japonica*), inhibit the growth of a Gram-negative *Porphyromonas gingivalis* ATCC 33277. This short α-helical cationic peptide contains four lysine, two arginine, and two histidine residues that might be responsible for its antibacterial activity. Other than that, a close correlation was observed between the degree of cell membrane disruption and the strength of antibacterial activity. Therefore, the antibacterial activity could be attributed to the membrane disruption induced by Hsp70-18. Nevertheless, further research is warranted to fully understand the contribution of each cationic amino acid in Hsp70-18 to its antibacterial activity as well as the mechanistic steps involved between Hsp70-18 and the cell membranes.

5.3.3 Review on Present Study

In present study, it is believed that the antibacterial proteins identified from APA of two *Barbodes* species namely Histone H2A, Histone H2B, Histone H4 and Heat shock protein 70 are majorly responsible for the in vitro activity exhibited. Notably, the growth of *Pseudomonas aeruginosa* ATCC 27853 was inhibited by APA of *Barbodes everetti* only which possess one additional antibacterial protein - Histone H2B. It is plausible to assume that this protein might have a greater influence on that activity demonstrated. However, the actual role of each antibacterial protein remains unknown, and it is inconclusive whether the activity observed is due to the interactions of the antibacterial protein with other protein or each protein can act as a sole antimicrobial agent.

Future experiments should focus on purifying and isolating the proteins of interest to fully elucidate their biological and biochemical roles as well as the mechanistic details in relation to their antimicrobial activity. Precise characterisation of the proteins could aid in predicting their function in a more relevant way which might serve as useful indicators for fish health monitoring.

CHAPTER 6

CONCLUSION AND RECOMMENDATIONS

6.1 Conclusion

Although the antibacterial properties of fish epidermal mucus have been well-established over the past decades, native freshwater fish species from Borneo remain under-studied and could be an interesting subject to be investigated for novel source of biologically active compounds. Chong et al. (2005) and Manivasagan et al. (2009) reported the predominantly proteinaceous properties of epidermal mucus in various fish species and most antibacterial components identified were proteins or peptides. Present studies compared different extractions with different concentrating methods for *Barbodes sealei* and *Barbodes everetti* to find out the process with the best recovery. Overall, the protein content of AP mucus obtained were much higher than that of FD mucus, but FD mucus extracts exhibited a significantly higher protein recovery rate for both species. The difference of protein contents between AP and FD mucus could be due to the different levels of stress experienced by each fish specimen. Although the strategies to prevent protein losses remain unclear, it is believed that the preparation of FD mucus extracts involved a lower number of transfers compared to AP mucus which resulted in minimal loss of proteins.

Present study successfully demonstrated varying antibacterial activities from four out of five epidermal mucus extracts (FDC, FDS, FDA and APA) of both *Barbodes sealei* and *Barbodes everetti* against four bacterial strains namely *Listeria monocytogenes* ATCC 7644, *Pseudomonas aeruginosa* ATCC 27853, *Salmonella braenderup* ATCC BAA 664, and *Vibrio cholera*. However, high protein contents did not entail greater antibacterial activities. High protein contents could mean presence of more contaminants that are inert which might

weaken the antibacterial effect. Interestingly, FDA of *B. sealei* managed to exhibit antibacterial activity with lower protein contents compared to other extracts. The proteins remaining in the FDA were believed to be purer without interference from other proteolytic enzymes and were able to exhibit a certain degree of antibacterial activity despite losing high amounts of protein contents during extraction.

Proteins predominantly existing in the active epidermal mucus extracts in the study were identified and compared with relevant existing literature. Amongst all the proteins identified, four proteins from two protein groups namely histone proteins – Histone H2A, Histone H2B, Histone H4 and heat shock proteins - Heat shock protein 70 were reported to be antibacterial elsewhere. In general, the activity of the proteins could be related to permeabilization, or disruption of the bacterial membranes, just like most AMPs. Nevertheless, fractionation, purification, and characterisation of these proteins in the active mucus extracts are needed to further understand their mechanisms of action involved in fish epidermal mucus.

Present study has further established the antimicrobial importance of fish epidermal mucus and provide interesting new avenues of research in exploring the antimicrobial potential of fish epidermal mucus. It is a low-cost and sustainable source that could be a good candidate for the isolation of new biologically active compounds. Although several antibacterial proteins had been identified from the mucus extracts, it is inconclusive that the activity exhibited was exclusively due to these proteins as the extracts might contain other secondary metabolites with antibacterial properties.

6.2 Limitations

In this study, there are some limitations to be acknowledged and further addressed: First of all, low survivability of wild fish in laboratory conditions. Instead of commercially available fish that are common in the aquaculture industry, fish species caught in the wild could be difficult to care for and even more challenging for long-term maintenance. Present study managed to capture other fish species such as *Barbodes kuchingensis*, *Channa Lucius* and *Clarias leiacanthus* but they could not survive the acclimation period; Secondly, the inconsistencies on mucus protein concentrations and low protein recovery (high protein loss). The protein concentrations of FD mucus extract were far too low compared to those of AP mucus extracts which made FD samples unfit (too diluted) to be subjected to other analysis such as SDS-PAGE and LC-MS/MS. However, AP samples with higher protein concentrations involved concentrating method using ammonium sulphate precipitation that incurred more than 70% of protein loss which indicates that the composition of the AP mucus extracts might not be fully represented; Thirdly, the primary roles, perhaps in host defence mechanism against bacterial infection, of all the proteins identified from active epidermal mucus samples remained unclear. Although proteomic technology such as LC-MS/MS can easily identify the proteins within the mixtures, it is still impossible to tell the function of each protein solely from their names, as most proteins hits belong to computationally annotated (unreviewed) database called "UniProtKB/TrEMBL" instead of high quality manually-annotated (reviewed) and non-redundant database named "UniProtKB/Swiss-Prot" which contain experimental results, computed key features and scientific conclusions extracted from published literatures.

6.3 Future Recommendations

Future work should aim to study more fish species to have a clearer picture on how fish combat microbial infections. It is of utmost importance to understand the biological needs of the fish species studied so a simulated stress-free environment with the optimum temperature, pH, and salinity could be set up that could substantially increase the fish survivability.

The protein concentrations of the mucus extracts and their recovery could be maximised and optimised by applying different steps from FD and AP extracts in the study. For instance, the optimum ratio of water to fish number for mucus collection is 1:1 (as in AP extracts) which could induce a higher amount of stress to the fish specimens and ensure higher amount of protein contents and concentrating the mucus extracts using freeze-drying (as in FD extracts) to ensure higher protein recovery. Besides, there are many other stressors such as hypothermic stress, alkali stress, salt stress and anaesthetic stress (Lee et al., 2020) that might aid in improving the mucus secretions in a non-destructive way. Other than that, other protein quantification methods such as Lowry protein assay and bicinchoninic acid method can be carried out to provide a possibly more accurate depiction of the protein contents in different extracts.

The proteome of fish epidermal mucus is known to be non-invasive powerful tools that allow scientists to conduct research without needing to dissect the fish. To the best of our knowledge, the present study revealed many uncharacterised or unidentified proteins in fish epidermal mucus for the first time which could be interesting subjects to be studied. Functional analysis on the proteomics data that involves mining of biological information databases should be carried out in order to predict protein function which could be of great

help in understanding diseases or discovering drug targets that would ultimately benefit the aquaculture industry and human healthcare.

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APPENDICES

Appendix A: BSA standard assay data for (a) FD mucus extracts and (b) AP mucus extracts

Tube No.		Blank	1	2	3	4	5
BSA Protein Standard (µg/200 µl)		0	4	8	12	16	20
Stock (µl)		0	4	8	12	16	20
Diluent (µl) – Saline		200	196	192	188	184	180
Bradford Reagent (ml)		1	1	1	1	1	1
Absorbance at 595nm	Replicate 1	0	0.126	0.288	0.465	0.55	0.636
	Replicate 2	0	0.098	0.252	0.406	0.525	0.651
	Replicate 3	0	0.134	0.272	0.421	0.485	0.620
	Mean ± Standard Deviation	0 ± 0	0.119 ± 0.019	0.271 ± 0.018	0.431 ± 0.031	0.517 ± 0.033	0.636 ± 0.016

(a)

Tube No.		Blank	1	2	3	4	5	6
BSA Protein Standard (µg / 100 µl)		0	2	4	6	8	10	12
Stock (µl)		0	2	4	6	8	10	12
Diluent (µl) – Distilled water		100	98	96	94	92	90	88
Bradford Reagent (ml)		1	1	1	1	1	1	1
Bradford Reagent (ml)		1	1	1	1	1	1	
Absorbance at 595nm	Replicate 1	0	0.115	0.139	0.203	0.271	0.322	0.418
	Replicate 2	0	0.118	0.135	0.212	0.275	0.338	0.396
	Replicate 3	0	0.113	0.115	0.164	0.224	0.296	0.374
	Mean ± Standard Deviation	0 ± 0	0.115 ± 0.003	0.130 ± 0.013	0.193 ± 0.026	0.257 ± 0.028	0.319 ± 0.021	0.396 ± 0.022

(b)

Appendix B: Protein concentrations of fish epidermal mucus and their extracts for (a) dH_2O mucus and (b) saline mucus

	*Protein Concentration of Freeze-dried (FD) dH_2O Mucus																			
	Before Freeze-drying					After Freeze-drying														
	dH_2O Mucus					Crude Extract - FDC					Aqueous Extract - FDS					Acidic Extract - FDA				
	1	2	3	x̄	σ	1	2	3	x̄	σ	1	2	3	x̄	σ	1	2	3	x̄	σ
Barbodes sealei																				
Volume (ml)	50	50	50	50	0	1	1	1	1	0	1	1	1	1	0	1	1	1	1	0
Specimen no(s)	25	25	25	25	0	8.33	8.33	8.33	8.33	0	8.33	8.33	8.33	8.33	0	8.33	8.33	8.33	8.33	0
A_{595}	0.054	0.043	0.048	0.048	0.006	0.581	0.468	0.468	0.506	0.065	0.31	0.286	0.321	0.306	0.018	0.032	0.026	0.027	0.028	0.003
µg/200 µl	1.51	1.17	1.32	1.33	0.17	17.77	14.28	14.28	15.45	2.01	9.41	8.67	9.75	9.27	0.55	0.83	0.64	0.67	0.71	0.10
µg/ml	7.53	5.83	6.60	6.66	0.85	88.86	71.42	71.42	77.23	10.07	47.04	43.33	48.73	46.37	2.76	4.14	3.21	3.36	3.57	0.50
Total protein (µg)	376.54	291.67	330.25	332.82	42.50	88.86	71.42	71.42	77.23	10.07	47.04	43.33	48.73	46.37	2.76	4.14	3.21	3.36	3.57	0.50
µg/specimen	15.06	11.67	13.21	13.31	1.70	10.66	8.57	8.57	9.27	1.21	5.64	5.20	5.85	5.56	0.33	0.50	0.39	0.40	0.43	0.06
[1]Recovery	-	-	-	-	-	70.80%	73.46%	64.88%	69.71%	4.39%	37.48%	44.57%	44.27%	42.11%	4.01%	3.30%	3.30%	3.06%	3.22%	0.14%
Barbodes everetti																				
Volume (ml)	50	50	50	50	0	1	1	1	1	0	1	1	1	1	0	1	1	1	1	0
Specimen no(s)	25	25	25	25	0	8.33	8.33	8.33	8.33	0	8.33	8.33	8.33	8.33	0	8.33	8.33	8.33	8.33	0
A_{595}	0.053	0.048	0.04	0.047	0.007	0.267	0.292	0.256	0.272	0.018	0.412	0.311	0.377	0.367	0.051	0.456	0.371	0.364	0.397	0.051
µg/200 µl	1.475	1.321	1.074	1.29	0.20	8.080	8.852	7.741	8.22	0.57	12.556	9.438	11.475	11.16	1.58	13.914	11.290	11.074	12.09	1.58
µg/ml	7.377	6.605	5.370	6.45	1.01	40.401	44.259	38.704	41.12	2.85	62.778	47.191	57.377	55.78	7.91	69.568	56.451	55.370	60.46	7.90
Total protein (µg)	368.827	330.247	268.519	322.53	50.60	40.401	44.259	38.704	41.12	2.85	62.778	47.191	57.377	55.78	7.91	69.568	56.451	55.370	60.46	7.90
µg/specimen	14.753	13.210	10.741	12.90	2.02	4.848	5.311	4.644	4.93	0.34	7.533	5.663	6.885	6.69	0.95	8.348	6.774	6.644	7.26	0.95
[1]Recovery	-	-	-	-	-	32.86%	40.21%	43.24%	38.77%	5.34%	51.06%	42.87%	64.10%	52.68%	10.71%	56.59%	51.28%	61.86%	56.58%	5.29%

(a)

- indicates not applicable; *Applicable to equation $y = 0.0324\,x + 0.0052$ where x = µg/200 µl and $y = A_{595}$; Statistical means and standard deviations are denoted by $\bar{}$ and σ respectively; [1]Recovery = (µg/specimen after freeze-drying)/(µg/specimen before freeze-drying) x 100 %

	*Protein Concentration of Ammonium-sulphate-precipitated (AP) Saline Mucus																								
	Before Extraction					Before Ammonium Sulphate Precipitation										**After Ammonium Sulphate Precipitation									
	Saline mucus					Aqueous Extract					Acidic Extract					Aqueous Extract - APS					Acidic Extract - APA				
	1	2	3	x̄	σ	1	2	3	x̄	σ	1	2	3	x̄	σ	1	2	3	x̄	σ	1	2	3	x̄	σ
Barbodes sealei																									
Volume (ml)	100	100	100	100	0	50	50	50	50	0	50	50	50	50	0	1	1	1	1	0	1	1	1	1	0
Specimen no(s)	100	100	100	100	0	50	50	50	50	0	50	50	50	50	0	50	50	50	50	0	50	50	50	50	0
A_{595}	0.727	0.641	0.643	0.670	0.049	0.726	0.614	0.630	0.657	0.061	0.247	0.317	0.317	0.294	0.040	1.085	0.853	0.969	0.969	0.116	0.935	1.067	0.837	0.946	0.115
µg/100 µl	23.06	20.27	20.33	21.22	1.59	23.03	19.39	19.91	20.77	1.97	7.47	9.75	9.75	8.99	1.31	34.68	27.15	30.92	30.92	3.77	29.81	34.10	26.63	30.18	3.75
µg/ml	230.58	202.66	203.31	212.19	15.94	230.26	193.90	199.09	207.75	19.67	74.74	97.47	97.47	89.89	13.12	2774.55	2171.95	2473.25	2473.25	301.30	2384.94	2727.79	2130.39	2414.37	299.79
Total protein (µg)	23058.44	20266.23	20331.17	21218.61	1593.67	11512.99	9694.81	9954.55	10387.45	983.36	3737.01	4873.38	4873.38	4494.59	656.08	2774.55	2171.95	2473.25	2473.25	301.30	2384.94	2727.79	2130.39	2414.37	299.79
µg/specimen	230.58	202.66	203.31	212.19	15.94	230.26	193.90	199.09	207.75	19.67	74.74	97.47	97.47	89.89	13.12	55.49	43.44	49.46	49.46	6.03	47.70	54.56	42.61	48.29	6.00
[1]Recovery	-	-	-	-	-	99.86%	95.67%	97.92%	97.82%	2.09%	32.41%	48.09%	47.94%	42.82%	9.01%	24.07%	21.43%	24.33%	23.28%	1.60%	20.69%	26.92%	20.96%	22.85%	3.52%
[2]Recovery	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	24.10%	22.40%	24.85%	23.78%	1.25%	63.82%	55.97%	43.71%	54.50%	10.13%
Barbodes everetti																									
Volume (ml)	100	100	100	100	0	50	50	50	50	0	50	50	50	50	0	1	1	1	1	0	1	1	1	1	0
Specimen no(s)	100	100	100	100	0	50	50	50	50	0	50	50	50	50	0	50	50	50	50	0	50	50	50	50	0
A_{595}	0.625	0.712	0.726	0.688	0.055	0.592	0.707	0.720	0.673	0.070	0.511	0.422	0.518	0.484	0.054	0.927	0.799	0.671	0.799	0.128	1.025	0.738	1.007	0.923	0.161
µg/100 µl	19.747	22.571	23.026	21.78	1.78	18.68	22.41	22.83	21.31	2.29	16.05	13.16	16.27	15.16	1.74	29.55	25.40	21.24	25.40	4.16	32.73	23.42	32.15	29.43	5.22
µg/ml	197.47	225.71	230.26	217.81	17.77	186.75	224.09	228.31	213.05	22.87	160.45	131.56	162.73	151.58	17.38	2364.16	2031.69	1699.22	2031.69	332.47	2618.70	1873.25	2571.95	2354.63	417.55
Total protein (µg)	19746.75	22571.43	23025.97	21781.39	1776.64	9337.66	11204.55	11415.58	10652.60	1143.65	8022.73	6577.92	8136.36	7579.00	868.82	2364.16	2031.69	1699.22	2031.69	332.47	2618.70	1873.25	2571.95	2354.63	417.55
µg/specimen	197.47	225.71	230.26	217.81	17.77	186.75	224.09	228.31	213.05	22.87	16045.45%	13155.84%	16272.73%	151.58	17.38	47.28	40.63	33.98	40.63	6.65	52.37	37.46	51.44	47.09	8.35
[1]Recovery	-	-	-	-	-	94.57%	99.28%	99.15%	97.67%	2.68%	81.26%	58.29%	70.67%	70.07%	11.50%	23.94%	18.00%	14.76%	18.90%	4.66%	26.52%	16.60%	22.34%	21.82%	4.98%
[2]Recovery	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	25.32%	18.13%	14.89%	19.45%	5.34%	32.64%	28.48%	31.61%	30.91%	2.17%

(b)

- indicates not applicable; *Applicable to equation y = 0.0308 x + 0.0168 where x = µg/100 µl and y = A_{595}; **Total protein concentration was calculated by further multiplying x with the dilution factor of 8; Statistical means and standard deviations are denoted by ¯ and σ respectively; [1]Recovery = (µg/specimen after extraction)/(µg/specimen before extraction) x 100 %; [2]Recovery = (µg/specimen after ammonium sulphate precipitation)/(µg/specimen before ammonium sulphate precipitation) x 100 %

Appendix C: Detailed inhibition zone diameter of freeze-dried mucus extracts against selected bacterial strains

Bacterial Strain	Inhibition Zone Diameter – IZD (mm)																			
	Crude Extract - FDC					Aqueous Extract - FDS					Acidic Extract - FDA					1X Pen-Strep (+ve control)				
	1	2	3	x̄	σ	1	2	3	x̄	σ	1	2	3	x̄	σ	1	2	3	x̄	σ
									Barbodes sealei											
Gram-positive																				
Bacillus cereus ATCC 33019	6	6	6	6	0	6	6	6	6	0	6	6	6	6	0	21.91	21.32	21.19	21.47	0.38
Listeria monocytogenes ATCC 7644	7.09	7.19	7.12	7.13	0.05	6.59	7.54	8.82	7.65	1.12	7.43	7.53	8.26	7.74	0.45	25.83	27.88	27.65	27.12	1.12
Staphylococcus aureus ATCC 25933	6	6	6	6	0	6	6	6	6	0	10.34	8.70	9.58	9.54	0.82	6.93	7.16	6.78	6.96	0.19
Gram-negative																				
Escherichia coli O157: H7	6	6	6	6	0	6	6	6	6	0	6	6	6	6	0	14.17	12.31	8.23	11.57	3.04
Pseudomonas aeruginosa ATCC 27853	6	6	6	6	0	6	6	6	6	0	10.96	11.37	10.63	10.99	0.37	15.27	15.42	15.71	15.47	0.22
Salmonella braenderup ATCC BAA 664	6	6	6	6	0	6	6	6	6	0	10.84	12.40	10.23	11.16	1.12	14.83	15.75	15.03	15.20	0.48
Salmonella typhimurium	6	6	6	6	0	6	6	6	6	0	6	6	6	6	0	19.18	18.14	18.04	18.45	0.63
Vibrio cholerae	7.39	7.57	7.48	7.48	0.09	7.39	7.27	7.48	7.38	0.11	8.64	13.07	12.56	11.42	2.42	22.75	28.65	19.29	23.56	4.73
									Barbodes everetti											
Gram-positive																				
Bacillus cereus ATCC 33019	6	6	6	6	0	6	6	6	6	0	6	6	6	6	0	22.53	22.77	23.56	22.95	0.54
Listeria monocytogenes ATCC 7644	6.47	7.59	6.73	6.93	0.59	6.41	6.83	6.72	6.65	0.22	6.60	7.80	8.83	7.74	1.13	24.15	23.74	22.41	23.43	0.91
Staphylococcus aureus ATCC 25933	6	6	6	6	0	6	6	6	6	0	10.42	7.73	7.87	8.67	1.51	10.42	7.70	7.37	8.50	1.67
Gram-negative																				
Escherichia coli O157: H7	6	6	6	6	0	6	6	6	6	0	6	6	6	6	0	9.29	9.63	11.35	10.09	1.10
Pseudomonas aeruginosa ATCC 27853	6	6	6	6	0	9.03	8.10	9.15	8.76	0.58	10.72	11.21	11.32	11.08	0.32	17.69	18.06	16.05	17.27	1.07
Salmonella braenderup ATCC BAA 664	6	6	6	6	0	6	6	6	6	0	10.21	7.79	10.38	9.46	1.45	14.88	17.92	15.14	15.98	1.69
Salmonella typhimurium	6	6	6	6	0	6	6	6	6	0	9.41	8.31	8.23	8.65	0.66	12.74	14.33	15.89	14.32	1.58
Vibrio cholerae	8.05	6.78	7.32	7.38	0.64	8.39	7.54	7.32	7.75	0.57	10.02	9.73	10.63	10.13	0.46	20.27	19.89	21.00	20.39	0.56

IZD includes 6 mm disc diameter; Measurement (mm) = 6 indicates there is no observable zone of inhibition; Statistical means and standard deviations are denoted by x̄ and σ respectively

Appendix D: Detailed inhibition zone diameter of negative controls of freeze-dried mucus extracts against selected bacterial strains

Bacterial Strain	Inhibition Zone Diameter – IZD (mm)																			
	Negative control of FDC					Negative control of FDS					Negative control of FDA					1X Pen-Strep (+ve control)				
	1	2	3	x̄	σ	1	2	3	x̄	σ	1	2	3	x̄	σ	1	2	3	x̄	σ
Gram-positive																				
Bacillus cereus ATCC 33019	6	6	6	6	0	6	6	6	6	0	6	6	6	6	0	21.91	21.32	21.19	21.47	0.38
Listeria monocytogenes ATCC 7644	6	6	6	6	0	6	6	6	6	0	8.14	7.86	7.93	7.98	0.15	25.83	27.88	27.65	27.12	1.12
Staphylococcus aureus ATCC 25933	6	6	6	6	0	6	6	6	6	0	9.53	7.42	8.80	8.58	1.07	6.93	7.16	6.78	6.96	0.19
Gram-negative																				
Escherichia coli O157: H7	6	6	6	6	0	6	6	6	6	0	6	6	6	6	0	14.17	12.31	8.23	11.57	3.04
Pseudomonas aeruginosa ATCC 27853	6	6	6	6	0	6	6	6	6	0	8.86	9.42	9.20	9.16	0.28	15.27	15.42	15.71	15.47	0.22
Salmonella braenderup ATCC BAA 664	6	6	6	6	0	6	6	6	6	0	7.47	7.09	7.32	7.29	0.19	14.83	15.75	15.03	15.20	0.48
Salmonella typhimurium	6	6	6	6	0	6	6	6	6	0	8.17	7.67	7.95	7.93	0.25	19.18	18.14	18.04	18.45	0.63
Vibrio cholerae	6	6	6	6	0	6	6	6	6	0	12.61	14.03	12.41	13.02	0.88	22.75	28.65	19.29	23.56	4.73

IZD includes 6 mm disc diameter; Measurement (mm) = 6 indicates there is no observable zone of inhibition; Statistical means and standard deviations are denoted by x̄ and σ respectively

Appendix E: Detailed inhibition zone diameter of ammonium-sulphate-precipitated mucus extracts against selected bacterial strains (with negative controls) for (a) *Barbodes sealei* and (b) *Barbodes everetti*

Bacterial Strain	Inhibition Zone Diameter – IZD (mm)																								
	Aqueous Extract - APS					Negative control of APS					Acidic Extract - APA					Negative control of APA					5 µg Ciprofloxacin (+ve control)				
	1	2	3	x̄	σ	1	2	3	x̄	σ	1	2	3	x̄	σ	1	2	3	x̄	σ	1	2	3	x̄	σ
Gram-positive																									
Bacillus cereus ATCC 33019	6	6	6	6	0	6	6	6	6	0	12.18	11.05	13.25	12.16	1.10	16.06	14.25	14.43	14.91	1.00	24.03	25.21	23.70	24.31	0.79
Listeria monocytogenes ATCC 7644	6	6	6	6	0	6	6	6	6	0	6	6	6	6	0	6	6	6	6	0	19.92	20.75	20.99	20.55	0.56
Staphylococcus aureus ATCC 25923	6	6	6	6	0	6	6	6	6	0	7.94	8.48	8.76	8.39	0.42	8.70	8.02	7.77	8.16	0.48	20.36	19.88	20.79	20.34	0.46
Gram-negative																									
Aeromonas hydrophila PRP 012	6	6	6	6	0	6	6	6	6	0	8.36	10.01	8.88	9.08	0.84	11.83	7.95	9.71	9.83	1.94	16.69	17.05	18.38	17.37	0.89
Escherichia coli O157:H7	6	6	6	6	0	6	6	6	6	0	10.22	9.45	7.79	9.15	1.24	10.49	9.83	10.06	10.13	0.34	22.21	20.20	21.54	21.32	1.02
Klebsiella pneumoniae PRP 010	6	6	6	6	0	6	6	6	6	0	8.79	8.62	9.45	8.95	0.44	7.56	7.91	10.08	8.52	1.37	14.96	14.03	13.84	14.28	0.60
Pseudomonas aeruginosa ATCC 27853	6	6	6	6	0	6	6	6	6	0	8.03	8.42	10.65	9.03	1.41	8.74	7.51	8.33	8.19	0.63	26.28	24.32	25.11	25.24	0.99
Salmonella braenderup ATCC BAA 664	6	6	6	6	0	6	6	6	6	0	10.91	10.63	10.64	10.73	0.16	9.10	8.44	9.55	9.03	0.56	31.04	26.96	30.14	29.38	2.14
Salmonella enteritidis ATCC 13036	6	6	6	6	0	6	6	6	6	0	11.40	9.47	9.08	9.98	1.242	11.84	9.33	8.14	9.77	1.89	26.14	24.83	25.34	25.44	0.66
Salmonella typhi ATCC 14028	6	6	6	6	0	6	6	6	6	0	9.18	6.86	10.73	8.92	1.948	10.07	8.20	7.69	8.65	1.25	23.94	22.90	19.88	22.24	2.11
Salmonella typhimurium	6	6	6	6	0	6	6	6	6	0	9.77	11.00	11.12	10.63	0.74	9.48	9.50	9.47	9.48	0.02	19.44	21.14	20.58	20.39	0.87
Shigella boydii ATCC 9207	6	6	6	6	0	6	6	6	6	0	9.08	7.00	7.94	8.01	1.04	8.31	9.22	9.49	9.01	0.62	16.11	16.35	17.34	16.60	0.65
Shigella flexneri ATCC 12022	6	6	6	6	0	6	6	6	6	0	8.82	9.27	9.96	9.35	0.57	11.07	11.89	11.29	11.42	0.42	13.85	14.39	13.97	14.07	0.28
Shigella sonnei ATCC 25931	6	6	6	6	0	6	6	6	6	0	7.94	10.30	11.07	9.77	1.63	8.98	10.43	10.91	10.11	1.01	25.12	26.83	27.00	26.32	1.04
Vibrio cholerae	6	6	6	6	0	6	6	6	6	0	7.09	7.21	8.20	7.50	0.61	8.80	7.40	8.70	8.30	0.78	26.90	26.49	23.98	25.79	1.58
Yersinia enterocolitica	6	6	6	6	0	6	6	6	6	0	6	6	6	6	0	6	6	6	6	0	29.18	29.82	28.28	29.09	0.77

(a)

IZD includes 6 mm disc diameter; Measurement (mm) = 6 indicates there is no observable zone of inhibition; Statistical means and standard deviations are denoted by ¯ and σ respectively

Bacterial Strain	Inhibition Zone Diameter – IZD (mm)																								
	Aqueous Extract - APS					Negative control of APS					Acidic Extract - APA					Negative control of APA					5 µg Ciprofloxacin (+ve control)				
	1	2	3	x̄	σ	1	2	3	x̄	σ	1	2	3	x̄	σ	1	2	3	x̄	σ	1	2	3	x̄	σ
Gram-positive																									
Bacillus cereus ATCC 33019	6	6	6	6	0	6	6	6	6	0	12.35	11.18	13.94	12.49	1.39	16.06	14.25	14.43	14.91	1.00	24.03	25.21	23.70	24.31	0.79
Listeria monocytogenes ATCC 7644	6	6	6	6	0	6	6	6	6	0	6	6	6	6	0	6	6	6	6	0	19.92	20.75	20.99	20.55	0.56
Staphylococcus aureus ATCC 25923	6	6	6	6	0	6	6	6	6	0	8.08	7.52	9.10	8.23	0.80	8.70	8.02	7.77	8.16	0.48	20.36	19.88	20.79	20.34	0.46
Gram-negative																									
Aeromonas hydrophila PRP 012	6	6	6	6	0	6	6	6	6	0	9.54	10.14	9.33	9.67	0.42	11.83	7.95	9.71	9.83	1.94	16.69	17.05	18.38	17.37	0.89
Escherichia coli O157:H7	6	6	6	6	0	6	6	6	6	0	10.04	9.03	9.86	9.64	0.54	10.49	9.83	10.06	10.13	0.34	22.21	20.20	21.54	21.32	1.02
Klebsiella pneumoniae PRP 010	6	6	6	6	0	6	6	6	6	0	9.34	8.16	10.30	9.27	1.07	7.56	7.91	10.08	8.52	1.37	14.96	14.03	13.84	14.28	0.60
Pseudomonas aeruginosa ATCC 27853	6	6	6	6	0	6	6	6	6	0	9.54	10.16	10.42	10.04	0.45	8.74	7.51	8.33	8.19	0.63	26.28	24.32	25.11	25.24	0.99
Salmonella braenderup ATCC BAA 664	6	6	6	6	0	6	6	6	6	0	12.61	10.60	11.52	11.58	1.01	9.10	8.44	9.55	9.03	0.56	31.04	26.96	30.14	29.38	2.14
Salmonella enteritidis ATCC 13036	6	6	6	6	0	6	6	6	6	0	10.78	9.60	8.54	9.64	1.12	11.84	9.33	8.14	9.77	1.89	26.14	24.83	25.34	25.44	0.66
Salmonella typhi ATCC 14028	6	6	6	6	0	6	6	6	6	0	10.02	10.30	10.50	10.27	0.24	10.07	8.20	7.69	8.65	1.25	23.94	22.90	19.88	22.24	2.11
Salmonella typhimurium	6	6	6	6	0	6	6	6	6	0	9.16	6	9.95	8.37	2.09	9.48	9.50	9.47	9.48	0.02	19.44	21.14	20.58	20.39	0.87
Shigella boydii ATCC 9207	6	6	6	6	0	6	6	6	6	0	9.85	9.20	7.84	8.96	1.03	8.31	8.22	9.49	8.67	0.62	16.11	16.35	17.34	16.60	0.65
Shigella flexneri ATCC 12022	6	6	6	6	0	6	6	6	6	0	9.89	9.09	10.11	9.70	0.54	11.07	11.89	11.29	11.42	0.42	13.85	14.39	13.97	14.07	0.28
Shigella sonnei ATCC 25931	6	6	6	6	0	6	6	6	6	0	8.65	9.43	12.22	10.10	1.88	8.98	10.43	9.91	9.77	1.01	25.12	26.83	27.00	26.32	1.04
Vibrio cholerae	6	6	6	6	0	6	6	6	6	0	6.92	6.26	8.86	7.35	1.35	8.80	7.40	8.70	8.30	0.78	26.90	26.49	23.98	25.79	1.58
Yersinia enterocolitica	6	6	6	6	0	6	6	6	6	0	6	6	6	6	0	6	6	6	6	0	29.18	29.82	28.28	29.09	0.77

(b)

IZD includes 6 mm disc diameter; Measurement (mm) = 6 indicates there is no observable zone of inhibition; Statistical means and standard deviations are denoted by ¯ and σ respectively

Appendix F: Protein identified via LC-MS/MS from the active mucus extract (APA) of (a) *Barbodes sealei* and (b) *Barbodes everetti*

db|UniqueIdentifier|EntryName	Protein name	Organism name	Gene name
BAND 1			
tr|B5X872|B5X872_SALSA	Actin, cytoplasmic 1	*Salmo salar*	ACTB
tr|A0A2U9B6V2|A0A2U9B6V2_SCOMX	Putative histone H2B type 2-E-like	*Scophthalmus maximus*	SMAX5B_012257
tr|A0A0P7UMM5|A0A0P7UMM5_SCLFO	Actin, cytoplasmic 2-like	*Scleropages formosus*	Z043_118570
tr|A0A3N0Y8D6|A0A3N0Y8D6_ANAGA	Histone H4	*Anabarilius grahami*	DPX16_9602
tr|A0A3Q2G9X8|A0A3Q2G9X8_CYPVA	Histone H3	*Cyprinodon variegatus*	NA
tr|A0A2I4CE20|A0A2I4CE20_9TELE	histone H3-like	*Austrofundulus limnaeus*	LOC106527799
tr|A0A3B1J9L7|A0A3B1J9L7_ASTMX	GLOBIN domain-containing protein	*Astyanax mexicanus*	NA
tr|A0A3B4VLV2|A0A3B4VLV2_SERDU	IF rod domain-containing protein	*Seriola dumerili*	NA
tr|M3ZFR9|M3ZFR9_XIPMA	Uncharacterized protein	*Xiphophorus maculatus*	NA
tr|A0A3P8QTL6|A0A3P8QTL6_ASTCA	Uncharacterized protein	*Astatotilapia calliptera*	NA
tr|A0A3P9J2C2|A0A3P9J2C2_ORYLA	IF rod domain-containing protein	*Oryzias latipes*	NA
tr|A0A4U5UXM5|A0A4U5UXM5_COLLU	Gelsolin Actin-depolymerizing factor	*Collichthys lucidus*	D9C73_013700
tr|A6QL59|A6QL59_DANRE	Histone H2A	*Danio rerio*	hist1h2a6
tr|A0A498NJJ0|A0A498NJJ0_LABRO	Putative threonine-rich GPI-anchored glyco isoform X2	*Labeo rohita*	ROHU_004713
tr|A0A2R8Q0V6|A0A2R8Q0V6_DANRE	Keratin 4	*Danio rerio*	krt4
tr|A0A146ZCQ4|A0A146ZCQ4_FUNHE	Histone H2B	*Fundulus heteroclitus*	NA
tr|A0A3G9CN67|A0A3G9CN67_CYPCA	Glutathione S-transferase omega	*Cyprinus carpio*	NA
tr|A0A146UBU9|A0A146UBU9_FUNHE	Histone H2B (Fragment)	*Fundulus heteroclitus*	NA
tr|A0A3Q3VLK8|A0A3Q3VLK8_MOLML	Transmembrane protein 132D	*Mola mola*	TMEM132D
tr|F1QJS8|F1QJS8_DANRE	Si:dkey-65b12.6 (Fragment)	*Danio rerio*	si:dkey-65b12.6
tr|A0A060VZ29|A0A060VZ29_ONCMY	IF rod domain-containing protein	*Oncorhynchus mykiss*	GSONMT00081034001
tr|H3DDD8|H3DDD8_TETNG	Guanine nucleotide binding protein (G protein), alpha 15 (Gq class), tandem duplicate 4	*Tetraodon nigroviridis*	NA
tr|Q76IL7|Q76IL7_DANRE	Pol-like protein	*Danio rerio*	ORF2
tr|A0A484DCQ2|A0A484DCQ2_PERFV	UmuC domain-containing protein	*Perca flavescens*	EPR50_G00053750
tr|A0A3B3HRV0|A0A3B3HRV0_ORYLA	Ig-like domain-containing protein	*Oryzias latipes*	NA
tr|A0A3Q2GKU3|A0A3Q2GKU3_CYPVA	Uncharacterized protein	*Cyprinodon variegatus*	ACTB
BAND 2			
tr|A0A3N0XEC2|A0A3N0XEC2_ANAGA	Gelsolin	*Anabarilius grahami*	DPX16_20242
tr|A0A498M1X6|A0A498M1X6_LABRO	Coiled-coil domain-containing 18-like isoform X1	*Labeo rohita*	ROHU_028433
tr|A0A3Q3IH74|A0A3Q3IH74_MONAL	Uncharacterized protein	*Monopterus albus*	NA
tr|A0A3Q1IYL6|A0A3Q1IYL6_ANATE	Uncharacterized protein	*Anabas testudineus*	NA
tr|A0A0R4IQ11|A0A0R4IQ11_DANRE	Scinderin-like a	*Danio rerio*	scinla

tr|A0A444U3J4|A0A444U3J4_ACIRT	Gelsolin	*Acipenser ruthenus*	EOD39_8512
tr|A0A1S3PAL0|A0A1S3PAL0_SALSA	gelsolin-like	*Salmo salar*	LOC106584171
tr|G3PSP8|G3PSP8_GASAC	Scinderin like b	*Gasterosteus aculeatus*	NA
tr|A0A2I4CLF4|A0A2I4CLF4_9TELE	gelsolin-like isoform X1	*Austrofundulus limnaeus*	LOC106529836
tr|W5UTS7|W5UTS7_ICTPU	Gelsolin	*Ictalurus punctatus*	GSN
tr|A0A3Q0SV12|A0A3Q0SV12_AMPCI	Scinderin like b	*Amphilophus citrinellus*	NA
tr|A0A3B4DAN9|A0A3B4DAN9_PYGNA	Uncharacterized protein	*Pygocentrus nattereri*	NA
tr|A0A3Q2V687|A0A3Q2V687_HAPBU	Uncharacterized protein	*Haplochromis burtoni*	NA
tr|B5X872|B5X872_SALSA	Actin, cytoplasmic 1	*Salmo salar*	ACTB
tr|A0A3P8XCQ1|A0A3P8XCQ1_ESOLU	Uncharacterized protein	*Esox lucius*	NA
tr|A0A3B3DPM0|A0A3B3DPM0_ORYME	Uncharacterized protein	*Oryzias melastigma*	NA
tr|A0A3B3RPQ6|A0A3B3RPQ6_9TELE	Uncharacterized protein	*Paramormyrops kingsleyae*	NA
tr|A0A1S3QZ97|A0A1S3QZ97_SALSA	gelsolin-like	*Salmo salar*	LOC106598932
tr|A0A4U5UXM5|A0A4U5UXM5_COLLU	Gelsolin Actin-depolymerizing factor	*Collichthys lucidus*	D9C73_013700
tr|A0A3B4BU39|A0A3B4BU39_PYGNA	Uncharacterized protein	*Pygocentrus nattereri*	NA
tr|A0A2U9B6V2|A0A2U9B6V2_SCOMX	Putative histone H2B type 2-E-like	*Scophthalmus maximus*	SMAX5B_012257
tr|A0A3B1J9L7|A0A3B1J9L7_ASTMX	GLOBIN domain-containing protein	*Astyanax mexicanus*	
tr|A0A0P7UMM5|A0A0P7UMM5_SCLFO	Actin, cytoplasmic 2-like	*Scleropages formosus*	Z043_118570
tr|A0A3B3THM5|A0A3B3THM5_9TELE	Histone H4	*Poecilia latipinna*	NA
tr|A0A3Q2G9X8|A0A3Q2G9X8_CYPVA	Histone H3	*Cyprinodon variegatus*	NA
tr|A0A2I4CE20|A0A2I4CE20_9TELE	histone H3-like	*Austrofundulus limnaeus*	LOC106527799
tr|H2L816|H2L816_ORYLA	AA_TRNA_LIGASE_II domain-containing protein	*Oryzias latipes*	LOC101171337
tr|A0A060Y244|A0A060Y244_ONCMY	Uncharacterized protein	*Oncorhynchus mykiss*	GSONMT00038203001
tr|A6QL59|A6QL59_DANRE	Histone H2A	*Danio rerio*	hist1h2a6
tr|A0A2D0SST5|A0A2D0SST5_ICTPU	alpha-2-macroglobulin-like	*Ictalurus punctatus*	LOC108277478
tr|A0A146QB50|A0A146QB50_FUNHE	Histone H2A type 2-A (Fragment)	*Fundulus heteroclitus*	NA
tr|A0A437C175|A0A437C175_ORYJA	Uncharacterized protein	*Oryzias javanicus*	OJAV_G00232420
BAND 3			
tr|A0A3N0Z785|A0A3N0Z785_ANAGA	Heat shock cognate 71 kDa protein	*Anabarilius grahami*	DPX16_10733
tr|A0A1U9X9S4|A0A1U9X9S4_CHACN	Hsc70	*Chanos chanos*	NA
tr|W5KA74|W5KA74_ASTMX	Uncharacterized protein	*Astyanax mexicanus*	NA
tr|A0A1I9LXI2|A0A1I9LXI2_ANGMA	Heat shock cognate 70	*Anguilla marmorata*	hsc70
tr|A0A3Q3AR85|A0A3Q3AR85_KRYMA	Uncharacterized protein	*Kryptolebias marmoratus*	NA
tr|A0A3P8WYY7|A0A3P8WYY7_CYNSE	Uncharacterized protein	*Cynoglossus semilaevis*	NA
tr|A0A146NKP1|A0A146NKP1_FUNHE	Heat shock cognate 71 kDa protein	*Fundulus heteroclitus*	NA
tr|Q6QIS4|Q6QIS4_PIMPR	Heat shock cognate 70 kDa protein	*Pimephales promelas*	HSP70
tr|A0A2U9B4I2|A0A2U9B4I2_SCOMX	Heat shock cognate 71 kDa protein	*Scophthalmus maximus*	SMAX5B_004559
tr|A0A3B4CQA3|A0A3B4CQA3_PYGNA	Uncharacterized protein	*Pygocentrus nattereri*	NA
tr|A0A3P9AI26|A0A3P9AI26_ESOLU	Uncharacterized protein	*Esox lucius*	NA
tr|A0A3B5AG78|A0A3B5AG78_9TELE	Uncharacterized protein	*Stegastes partitus*	NA
tr|A0A3Q3MLV6|A0A3Q3MLV6_9TELE	Uncharacterized protein	*Mastacembelus armatus*	NA

tr|A0A3B4UD02|A0A3B4UD02_SERDU	Uncharacterized protein	*Seriola dumerili*	NA
tr|V9PTF2|V9PTF2_SCHPR	Heat shock protein Hsc70	*Schizothorax prenanti*	Hsc70
tr|Q6PGX4|Q6PGX4_DANRE	Heat shock cognate 70	*Danio rerio*	hsc70
tr|A0A2P1K697|A0A2P1K697_MYLPI	Glucose-regulated protein 78	*Mylopharyngodon piceus*	NA
tr|A0A0P7U8Q6|A0A0P7U8Q6_SCLFO	Heat-Shock Cognate 70kd Protein (Fragment)	*Scleropages formosus*	Z043_117667
tr|A0A3Q3B3S9|A0A3Q3B3S9_KRYMA	Uncharacterized protein	*Kryptolebias marmoratus*	NA
tr|A0A1S3MI49|A0A1S3MI49_SALSA	heat shock 70 kDa protein-like	*Salmo salar*	LOC106572869
tr|A0A3N0Z6I9|A0A3N0Z6I9_ANAGA	78 kDa glucose-regulated protein	*Anabarilius grahami*	DPX16_9564
tr|A0A3B3SXK8|A0A3B3SXK8_9TELE	Uncharacterized protein	*Paramormyrops kingsleyae*	NA
tr|A0A3Q1HXW4|A0A3Q1HXW4_ANATE	Uncharacterized protein	*Anabas testudineus*	NA
tr|A0A3B3ZFX4|A0A3B3ZFX4_9GOBI	Heat shock cognate 70	*Periophthalmus magnuspinnatus*	NA
tr|A0A3P9MHS2|A0A3P9MHS2_ORYLA	Uncharacterized protein	*Oryzias latipes*	NA
tr|A0A3B5KIU0|A0A3B5KIU0_TAKRU	Uncharacterized protein	*Takifugu rubripes*	LOC101075813
tr|A0A172LPZ7|A0A172LPZ7_TACFU	Heat shock protein 5 (Fragment)	*Tachysurus fulvidraco*	NA
tr|A8CEI1|A8CEI1_POERE	HSP70 protein	*Poecilia reticulata*	NA
tr|A0A2U9C1L7|A0A2U9C1L7_SCOMX	Inducible heat shock protein 70	*Scophthalmus maximus*	SMAX5B_014757
tr|A0A3B3CHS0|A0A3B3CHS0_ORYME	Uncharacterized protein	*Oryzias melastigma*	NA
tr|A0A315W4Q1|A0A315W4Q1_GAMAF	AdoHcyase_NAD domain-containing protein	*Gambusia affinis*	CCH79_00010700
tr|B5X872|B5X872_SALSA	Actin, cytoplasmic 1	*Salmo salar*	ACTB
tr|Q8JHD1|Q8JHD1_CARAU	Serotransferrin	*Carassius auratus*	TF
tr|A0A3Q1EUS7|A0A3Q1EUS7_9TELE	Heat shock protein family A (Hsp70) member 2	*Acanthochromis polyacanthus*	HSPA2
tr|A0A444U3J4|A0A444U3J4_ACIRT	Gelsolin	*Acipenser ruthenus*	EOD39_8512
tr|A0A0P7UMM5|A0A0P7UMM5_SCLFO	Actin, cytoplasmic 2-like	*Scleropages formosus*	Z043_118570
tr|A0A3N0XEC2|A0A3N0XEC2_ANAGA	Gelsolin	*Anabarilius grahami*	DPX16_20242
tr|A0A3B1J9L7|A0A3B1J9L7_ASTMX	GLOBIN domain-containing protein	*Astyanax mexicanus*	NA
tr|A0A3Q2G9X8|A0A3Q2G9X8_CYPVA	Histone H3	*Cyprinodon variegatus*	NA
tr|A0A3N0XQ52|A0A3N0XQ52_ANAGA	Gelsolin	*Anabarilius grahami*	DPX16_19757
tr|A0A2I4CE20|A0A2I4CE20_9TELE	histone H3-like	*Austrofundulus limnaeus*	LOC106527799
tr|A0A2U9B6V2|A0A2U9B6V2_SCOMX	Putative histone H2B type 2-E-like	*Scophthalmus maximus*	SMAX5B_012257
tr|A0A3B3THM5|A0A3B3THM5_9TELE	Histone H4	*Poecilia latipinna*	NA
tr|A0A498LU05|A0A498LU05_LABRO	Complement C3-like protein	*Labeo rohita*	ROHU_010504
tr|I3J0M2|I3J0M2_ORENI	Centrosomal protein 350	*Oreochromis niloticus*	NA
tr|A0A3B4DAN9|A0A3B4DAN9_PYGNA	Uncharacterized protein	*Pygocentrus nattereri*	NA
tr|Q52RN6|Q52RN6_RACCA	Heat shock protein 70 (Fragment)	*Rachycentron canadum*	NA
tr|A0A3Q2XJ85|A0A3Q2XJ85_HIPCM	ATPase_AAA_core domain-containing protein	*Hippocampus comes*	NA
tr|Q8UVE7|Q8UVE7_CYPCA	Serotransferrin	*Cyprinus carpio*	NA
tr|A0A3B4VEP6|A0A3B4VEP6_SERDU	AA_TRNA_LIGASE_II_ALA domain-containing protein	*Seriola dumerili*	AARS
tr|A0A3B4BU39|A0A3B4BU39_PYGNA	Uncharacterized protein	*Pygocentrus nattereri*	NA
tr|W5NC62|W5NC62_LEPOC	Uncharacterized protein	*Lepisosteus oculatus*	NA
tr|A0A498MTM3|A0A498MTM3_LABRO	IF rod domain-containing protein	*Labeo rohita*	ROHU_021778

tr|A6QL59|A6QL59_DANRE	Histone H2A	*Danio rerio*	hist1h2a6
tr|A0A4U5UXM5|A0A4U5UXM5_COLLU	Gelsolin Actin-depolymerizing factor	*Collichthys lucidus*	D9C73_013700
tr|A0A060XJW5|A0A060XJW5_ONCMY	Uncharacterized protein	*Oncorhynchus mykiss*	GSONMT00034728001
tr|A0A3B3HRV0|A0A3B3HRV0_ORYLA	Ig-like domain-containing protein	*Oryzias latipes*	NA
tr|A0A3Q2GKU3|A0A3Q2GKU3_CYPVA	Uncharacterized protein	*Cyprinodon variegatus*	NA
tr|A0A2D0QRC8|A0A2D0QRC8_ICTPU	leucine-rich repeat and IQ domain-containing protein 1	*Ictalurus punctatus*	lrriq1
tr|Q5SEP6|Q5SEP6_GRASX	Histone H3 (Fragment)	*Grammistes sexlineatus*	NA
tr|A0A498LHW8|A0A498LHW8_LABRO	Echinoderm microtubule-associated-like 2 isoform X1	*Labeo rohita*	ROHU_011891
BAND 4			
tr|A0A096VJY6|A0A096VJY6_EPICO	Heat shock cognate protein 70	*Epinephelus coioides*	hsc70
tr|A0A498LX76|A0A498LX76_LABRO	Major vault	*Labeo rohita*	ROHU_029253
tr|A0A0A1HAN6|A0A0A1HAN6_9TELE	Hemopexin	*Carassius carassius*	Wap65-1
tr|B5X872|B5X872_SALSA	Actin, cytoplasmic 1	*Salmo salar*	ACTB
tr|A0A3Q2G9X8|A0A3Q2G9X8_CYPVA	Histone H3	*Cyprinodon variegatus*	NA
tr|A0A2I4CE20|A0A2I4CE20_9TELE	histone H3-like	*Austrofundulus limnaeus*	LOC106527799
tr|A0A2U9B6V2|A0A2U9B6V2_SCOMX	Putative histone H2B type 2-E-like	*Scophthalmus maximus*	SMAX5B_012257
tr|A0A3B3THM5|A0A3B3THM5_9TELE	Histone H4	*Poecilia latipinna*	NA
tr|G3KG82|G3KG82_MISMI	Warm-temperature-acclimation-associated 65-kDa protein	*Misgurnus mizolepis*	WAP65-1
tr|A0A060XVS1|A0A060XVS1_ONCMY	WD_REPEATS_REGION domain-containing protein	*Oncorhynchus mykiss*	GSONMT00038488001
tr|A0A1A7XBL5|A0A1A7XBL5_9TELE	WD repeat domain 1	*Iconisemion striatum*	WDR1
tr|A0A0P7UMM5|A0A0P7UMM5_SCLFO	Actin, cytoplasmic 2-like	*Scleropages formosus*	Z043_118570
tr|A0A0F8APN0|A0A0F8APN0_LARCR	Alpha-1-antitrypsin	*Larimichthys crocea*	EH28_09619
tr|A0A3B3HRV0|A0A3B3HRV0_ORYLA	Ig-like domain-containing protein	*Oryzias latipes*	NA
tr|A0A3Q2GKU3|A0A3Q2GKU3_CYPVA	Uncharacterized protein	*Cyprinodon variegatus*	NA
tr|A0A4U5UXM5|A0A4U5UXM5_COLLU	Gelsolin Actin-depolymerizing factor	*Collichthys lucidus*	D9C73_013700
tr|Q5SEP6|Q5SEP6_GRASX	Histone H3 (Fragment)	*Grammistes sexlineatus*	NA
tr|A0A0F8ACS7|A0A0F8ACS7_LARCR	Hemoglobin subunit alpha-1	*Larimichthys crocea*	EH28_05226
sp|Q6PHG2|HEMO_DANRE	Hemopexin	*Danio rerio*	hpx
tr|A0A1A7ZD11|A0A1A7ZD11_NOTFU	Abelson helper integration site 1	*Nothobranchius furzeri*	AHI1
tr|A0A146QB50|A0A146QB50_FUNHE	Histone H2A type 2-A (Fragment)	*Fundulus heteroclitus*	NA
tr|A6QL59|A6QL59_DANRE	Histone H2A	*Danio rerio*	hist1h2a6
tr|A0A2D0QRC8|A0A2D0QRC8_ICTPU	leucine-rich repeat and IQ domain-containing protein 1	*Ictalurus punctatus*	lrriq1
tr|A0A0R4IQ11|A0A0R4IQ11_DANRE	Scinderin-like a	*Danio rerio*	scinla
tr|A0A437C175|A0A437C175_ORYJA	Uncharacterized protein	*Oryzias javanicus*	OJAV_G00232420
BAND 5			
tr|A0A498MKB6|A0A498MKB6_LABRO	L-lactate dehydrogenase	*Labeo rohita*	ROHU_026592
tr|A0A1A7Z665|A0A1A7Z665_9TELE	L-lactate dehydrogenase	*Iconisemion striatum*	LDHB
tr|A0A3Q2XWT5|A0A3Q2XWT5_HIPCM	L-lactate dehydrogenase	*NA*	NA
tr|I3IZU4|I3IZU4_ORENI	L-lactate dehydrogenase	*Oreochromis niloticus*	LOC100694281
tr|A0A2U9B6V2|A0A2U9B6V2_SCOMX	Putative histone H2B type 2-E-like	*Scophthalmus maximus*	SMAX5B_012257

tr|A0A3Q2G9X8|A0A3Q2G9X8_CYPVA	Histone H3	*Cyprinodon variegatus*	NA
tr|A0A3B3THM5|A0A3B3THM5_9TELE	Histone H4	*Poecilia latipinna*	NA
tr|A0A2I4CE20|A0A2I4CE20_9TELE	histone H3-like	*Austrofundulus limnaeus*	LOC106527799
tr|A0A3B1J9L7|A0A3B1J9L7_ASTMX	GLOBIN domain-containing protein	*Astyanax mexicanus*	NA
tr|A6QL59|A6QL59_DANRE	Histone H2A	*Danio rerio*	hist1h2a6
tr|A0A146QB50|A0A146QB50_FUNHE	Histone H2A type 2-A (Fragment)	*Fundulus heteroclitus*	NA
tr|A0A3B3HRV0|A0A3B3HRV0_ORYLA	Ig-like domain-containing protein	*Oryzias latipes*	NA
tr|A0A3Q2GKU3|A0A3Q2GKU3_CYPVA	Uncharacterized protein	*Cyprinodon variegatus*	NA
tr|Q5SEP6|Q5SEP6_GRASX	Histone H3 (Fragment)	*Grammistes sexlineatus*	NA

(a)

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Unique Identifier - Primary accession number of the UniProtKB entry.

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ProteinName - Recommended name of the UniProtKB entry

Organism Name - Scientific name of the organism of the UniProtKB entry.

Gene Name - First gene name of the UniProtKB entry; NA - Gene name is not available.

db|UniqueIdentifier|EntryName	Protein name	Organism name	Gene name
BAND 1			
tr|A0A3N0XEP2|A0A3N0XEP2_ANAGA	Collagen alpha-1(I) chain	Anabarilius grahami	DPX16_16217
tr|A0A2U9B6V2|A0A2U9B6V2_SCOMX	Putative histone H2B type 2-E-like	Scophthalmus maximus	SMAX5B_012257
tr|A0A3Q2G9X8|A0A3Q2G9X8_CYPVA	Histone H3	Cyprinodon variegatus	NA
tr|A0A3B3THM5|A0A3B3THM5_9TELE	Histone H4	Poecilia latipinna	NA
tr|A0A1A8QZN3|A0A1A8QZN3_9TELE	Collagen, type I, alpha 1b (Fragment)	Nothobranchius rachovii	COL1A1B
tr|A0A2I4CE20|A0A2I4CE20_9TELE	histone H3-like	Austrofundulus limnaeus	LOC106527799
tr|A0A3P8Y1R8|A0A3P8Y1R8_ESOLU	Histone H2B	Esox lucius	HIST1H2BA
tr|A0A146QB50|A0A146QB50_FUNHE	Histone H2A type 2-A (Fragment)	Fundulus heteroclitus	NA
tr|A6QL59|A6QL59_DANRE	Histone H2A	Danio rerio	hist1h2a6
sp|Q6PHG2|HEMO_DANRE	Hemopexin	Danio rerio	hpx
BAND 2			
tr|A0A3N0XEC2|A0A3N0XEC2_ANAGA	Gelsolin	Anabarilius grahami	DPX16_20242
tr|A0A498M1X6|A0A498M1X6_LABRO	Coiled-coil domain-containing 18-like isoform X1	Labeo rohita	ROHU_028433
tr|A0A147APP2|A0A147APP2_FUNHE	Gelsolin	Fundulus heteroclitus	NA
tr|A0A0R4IQ11|A0A0R4IQ11_DANRE	Scinderin-like a	Danio rerio	scinla
tr|A0A1S3PAL0|A0A1S3PAL0_SALSA	gelsolin-like	Salmo salar	LOC106584171
tr|A0A498LLC5|A0A498LLC5_LABRO	Gelsolin-like protein	Labeo rohita	ROHU_011763
tr|A0A3N0XQ52|A0A3N0XQ52_ANAGA	Gelsolin	Anabarilius grahami	DPX16_19757
tr|A0A3Q0SV12|A0A3Q0SV12_AMPCI	Scinderin like b	Amphilophus citrinellus	NA
tr|A0A1S3QZ97|A0A1S3QZ97_SALSA	gelsolin-like	Salmo salar	LOC106598932
tr|A0A2I4CLF4|A0A2I4CLF4_9TELE	gelsolin-like isoform X1	Austrofundulus limnaeus	LOC106529836
tr|A0A2D0S0P8|A0A2D0S0P8_ICTPU	gelsolin-like	Ictalurus punctatus	LOC108272108
tr|A0A4U5UXM5|A0A4U5UXM5_COLLU	Gelsolin Actin-depolymerizing factor	Collichthys lucidus	D9C73_013700
tr|A0A2U9BKW1|A0A2U9BKW1_SCOMX	Scinderin-like protein	Scophthalmus maximus	SMAX5B_006415
tr|A0A498P385|A0A498P385_LABRO	Alpha-2-macroglobulin-like protein	Labeo rohita	ROHU_000906
tr|A0A0P7V3N2|A0A0P7V3N2_SCLFO	Keratin, type II cytoskeletal 8-like (Fragment)	Scleropages formosus	Z043_111930
tr|A5PMZ3|A5PMZ3_DANRE	Scinderin-like b	Danio rerio	scinlb
tr|A0A4U5U528|A0A4U5U528_COLLU	Keratin, type I cytoskeletal 17	Collichthys lucidus	D9C73_003377
tr|A0A2U9B6V2|A0A2U9B6V2_SCOMX	Putative histone H2B type 2-E-like	Scophthalmus maximus	SMAX5B_012257
tr|A0A3Q2G9X8|A0A3Q2G9X8_CYPVA	Histone H3	Cyprinodon variegatus	NA
tr|A0A0P7VFE6|A0A0P7VFE6_SCLFO	Keratin, type I cytoskeletal 13-like	Scleropages formosus	Z043_108950
tr|A0A3B5AM04|A0A3B5AM04_9TELE	IF rod domain-containing protein	Stegastes partitus	NA
tr|A0A3P8UNJ3|A0A3P8UNJ3_CYNSE	IF rod domain-containing protein	Cynoglossus semilaevis	NA
tr|A0A3Q2XJ85|A0A3Q2XJ85_HIPCM	ATPase_AAA_core domain-containing protein	Hippocampus comes	NA
tr|A0A3Q1H2L9|A0A3Q1H2L9_ANATE	IF rod domain-containing protein	Anabas testudineus	NA
tr|A0A2D0RZT6|A0A2D0RZT6_ICTPU	keratin, type I cytoskeletal 18-like	Ictalurus punctatus	LOC108272218
tr|F6P9S6|F6P9S6_DANRE	Si:ch211-243g18.2	Danio rerio	si:ch211-243g18.2
tr|A0A0P7WQ36|A0A0P7WQ36_SCLFO	Thread biopolymer filament subunit alpha-like (Fragment)	Scleropages formosus	Z043_115770

BAND 3			
tr|A0A2U9B6V2|A0A2U9B6V2_SCOMX	Putative histone H2B type 2-E-like	Scophthalmus maximus	SMAX5B_012257
tr|A0A3Q2G9X8|A0A3Q2G9X8_CYPVA	Histone H3	Cyprinodon variegatus	NA
tr|C1JCB0|C1JCB0_HYPNO	Transferrin variant B (Fragment)	Hypophthalmichthys nobilis	NA
tr|A0A0R4IHQ0|A0A0R4IHQ0_DANRE	Serotransferrin	Danio rerio	Tfa
tr|A6QL59|A6QL59_DANRE	Histone H2A	Danio rerio	hist1h2a6
tr|A0A146QB50|A0A146QB50_FUNHE	Histone H2A type 2-A (Fragment)	Fundulus heteroclitus	NA
tr|A0A2U9CDM0|A0A2U9CDM0_SCOMX	Uncharacterized protein (Fragment)	Scophthalmus maximus	SMAX5B_017760
BAND 4			
tr|E1U3C1|E1U3C1_CTEID	Warm temperature acclimation-related 65kDa protein (Fragment)	Ctenopharyngodon idella	NA
tr|Q90323|Q90323_CYPCA	Serine protease inhibitor	Cyprinus carpio	CP9
tr|A0A060DFM8|A0A060DFM8_GAMAF	Heat shock protein 70 (Fragment)	Gambusia affinis	NA
tr|A0A2I4JRC7|A0A2I4JRC7_PARDA	Hemopexin	Paramisgurnus dabryanus	WAP65-2
sp|Q6PHG2|HEMO_DANRE	Hemopexin	Danio rerio	Hpx
tr|A0A2U9B6V2|A0A2U9B6V2_SCOMX	Putative histone H2B type 2-E-like	Scophthalmus maximus	SMAX5B_012257
tr|G3KG82|G3KG82_MISMI	Warm-temperature-acclimation-associated 65-kDa protein	Misgurnus mizolepis	WAP65-1
tr|A0A3B3THM5|A0A3B3THM5_9TELE	Histone H4	Poecilia latipinna	NA
tr|A0A2K8J325|A0A2K8J325_CYPCA	Warm temperature acclimation-related 65 kDa protein 2	Cyprinus carpio	wap65-2
tr|A0A3Q2G9X8|A0A3Q2G9X8_CYPVA	Histone H3	Cyprinodon variegatus	NA
tr|A0A2I4CE20|A0A2I4CE20_9TELE	histone H3-like	Austrofundulus limnaeus	LOC106527799
tr|A6QL59|A6QL59_DANRE	Histone H2A	Danio rerio	hist1h2a6
tr|A0A3P8W4E4|A0A3P8W4E4_CYNSE	BEN domain-containing protein	Cynoglossus semilaevis	NA
tr|F6P9S6|F6P9S6_DANRE	Si:ch211-243g18.2	Danio rerio	si:ch211-243g18.2
BAND 5			
tr|A0A2U9B6V2|A0A2U9B6V2_SCOMX	Putative histone H2B type 2-E-like	Scophthalmus maximus	SMAX5B_012257
tr|A0A146QB50|A0A146QB50_FUNHE	Histone H2A type 2-A (Fragment)	Fundulus heteroclitus	NA

(b)

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