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REVIEW

The antibacterial activity of fish skin mucus with various extraction solvents and their *in-vitro* evaluation methods

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Abstract Epidermal mucus is known to facilitate fish survival due to its multifunctional barrier with various cellular and humoral components, especially to combat invading pathogens. The research on the antibacterial properties of fish skin mucus has been expanding over the years, with more refined protocols being developed and more fish species being investigated. The vast information generated from these researches requires a systematic and comprehensive review, to provide consistent knowledge and guidelines for researchers to effectively apply in their research in future. This article reviewed the protocols relating to the fish epidermal mucus collection and extraction. The collection of mucus was further categorised into destructive and non-destructive ways, and the extraction methods were divided into three major types, namely aqueous, organic and acidic. The commonly- used antibacterial bioassays (i.e. agar disk diffusion, agar well diffusion, broth micro-dilution, bacterial cell growth curves, etc.) of the fish mucus against a total of 46 bacterial species were also discussed. Despite the methodological variation, slight modifications of the standardized protocols are warranted for a better experimental approach. The antibacterial effectiveness (bactericidal activity) of fish epidermal mucus extracts was exhibited by 47 fish species from three out of the five classes, which suggests their great potential in aquaculture and possibly in human-health applications. To acquire a deeper understanding of fish epidermal mucus, it is imperative to conduct purification and characterization of its antimicrobial components.

Keywords Bactericidal activity . Epidermal mucus . Fresh and seawater fish.

Introduction

As the largest group of vertebrates on earth, comprising 33,230 species recorded worldwide (Froese and Pauly 2018), fish come in a vast array of shapes, sizes and structures of body parts. Their remarkably diverse biological adaptations, in terms of morphology, physiology or behaviour, permit them to occupy and utilize an equally diverse type of aquatic habitats. Fish can be found in various aquatic environments, ranging from mountain, lake to deep oceans, with a wide range of abiotic variables such as temperature, salinity and oxygen content (Videler 2011).

Despite their highly adaptiveness and flexibility, fish have higher risks of developing skin diseases compared to terrestrial vertebrates, due to their intimate contact with their aquatic environments. This is because the said habitats are an ideal medium for the thriving of pathogenic micro-organisms such as bacteria, virus and fungi (Magnadottir 2010). Under normal conditions, the fish can defend themselves against these potential invaders through a complex system of innate defence mechanisms (Arellano et al. 2004; Mozumder 2005). The innate immune system in fish is divided into physical barriers (such as

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scales on the body and cutaneous mucus surfaces of skin, gills and guts, which act as the first line of defence against infection (Esteban 2012), cellular (phagocytic cells and the non-specific cytotoxic cells) and humoral components (Magnadóttir 2006; Subramanian et al. 2007).

Therefore, skin mucus is considered an important immunological factor in fish. Fish skin mucus is multi-functional, acting as a natural, physical, biochemical, dynamic, and semi-permeable barrier that enables the exchange of nutrients, water, gases, odorants, hormones, and gametes (Alexander and Ingram 1992; Shephard 1994; Mokhtar 2017). However, its primary function is to act as the mechanical protective barrier that impedes the entry of foreign pathogens such as bacteria, virus and fungi into the body. The slime layer also serves as a chemical barrier, containing certain enzymes and antibodies that can kill these invading pathogenic organisms at the early stage (Rottmann et al. 1992). Naturally, their skin mucus would continuously encounter, monitor and regulate myriads of pathogens that are always present in the aquatic environment. Recent reports have revealed that the anti-microbial peptides (AMPs) and other immune relevant proteins found in fish skin mucus play a vital role in their survival, by combating pathogenic infections (Najafian and Babji 2012; Cordero et al. 2015; Reverter et al. 2018). A growing body of research on the anti-microbial properties of fish skin mucus on human and fish pathogens and reports of their mucosal AMPs suggest a potential future clinical application in both human and farm animal diseases. As humans increasingly depend on fish exploitation, the true potential of fish skin mucus should be properly evaluated. In this review, we focused on the literature available (n=38) on fish skin mucus studies, with various laboratory methodologies applied to different fish species for *in vitro* antibacterial screening of their mucus extracts. The papers were excluded in the cases of ambiguity in fish identity (species name was not mentioned, common names used apply to more than one species, etc.). Owing to the new challenge of combating multi-drug-resistant bacteria, it is vital to develop a deeper understanding of the current screening strategies available and the true potential of their antibacterial effect, to ensure a more accurate approach for those who would make an attempt at this field of research in future. In this review, the techniques to collect and extract fish skin mucus were discussed in detail. The antibacterial effects of the fish epidermal mucus extracts on various Gram-positive and Gram-negative bacteria were summarised and grouped, based on the taxonomy of the fish species.

Fish skin mucus collection methods

The methods of collecting fish mucus varies from one study to the other (see Table 1). In general, they are divided into destructive and non-destructive methods. In both the methods, the fish were starved for 24 hours prior to mucus collection in most studies (Subramanian et al. 2008; Wei et al. 2010; Elavarasi et al. 2013; Ramesh 2013; Patil et al. 2015; Rao et al. 2015; Tyor and Kumari 2016; 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 euthanising with a lethal dose of anaesthesia such as tricaine methanesulphate (Gabriella et al. 2014). The 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 2M of Sodium hydroxide, NaOH (pH 11.5) solution for 25 minutes after placing the fish in 3-amino-benzoic acid ethyl ester (0.6g/L) for five minutes (Al-Arifa et al. 2011). Since the alkali-treated mucus collections have chemical residues that might affect its antibacterial activities, the mucus samples collected would be neutralised to normal pH (7.5) by adding 2N 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. 2008; Al-Arifa et al. 2011; Guardiola et al. 2014a, b; Rao et al. 2015) or clove oil (Guardiola et al. 2017) were introduced into the fish in a sub-lethal dose.

Table 1 Destructive and non-destructive methods for fish mucus collection.

Type	Methods	Condition	References
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 blow to the head until death.	Hiwarale et al. (2016)
	Freeze to death	Fish transported to laboratory and kept at -20°C until death.	Bragadeeswaran et al. (2011)
	Chemical euthanization	Fish anesthetized and euthanized at a lethal dose of Tricaine methanesulphate (MS-222) at 0.1 g/L.	Gabriella et al. (2014)
Non-Destructive	Hypothermic stress	Fish kept in enclosed container with water placed in freezer for one hour at -20°C or add ice gradually until insensitive to human touch.	Al-Rasheed et al. (2018); Katra et al. (2016); Kumari et al. (2011); Nigam et al. (2015)
	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 minutes. Neutralisation of mucus by 2N 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).	Al-Arifa et al. (2011); Guardiola et al. (2017); Guardiola et al. (2014a, b); Rao et al. (2015); Subramanian et al. (2008)
No Treatment	Mucus was collected directly without any prior treatment.		Balasubramanian et al. (2012); Elavarasi et al. (2013); Haniffa et al. (2014); Hellio et al. (2002); Islam et al. (2014); Kuppulakshmi et al. (2008); Loganathan et al. (2011, 2013); Magarinos et al. (1995); Manikantan et al. (2016); Nwabueze (2014); Patil et al. (2015); Ramesh (2013); Subhashini et al. (2013); Tyor and Kumari (2016); Wei et al. (2010)

Lastly, there are a significant number of studies that did not induce any non-destructive stress prior to mucus collection (Magarinos et al. 1995; Hellio et al. 2002; 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; Nwabueze 2014; Haniffa et al. 2014; Islam et al. 2014; Patil et al. 2015; Tyor and Kumari 2016; Manikantan et al. 2016). 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; Al-Arifa et al. 2011; Loganathan et al. 2011, 2013; Varghese and Arathy 2011; Vennila et al. 2011; Anbuchezhian et al. 2011; Bragadeeswaran et al. 2011; Balasubramanian et al. 2012; Subhashini et al. 2013; Elavarasi et al. 2013; Nwabueze 2014; Gabriella et al. 2014; Haniffa et al. 2014; Islam et al. 2014; Patil et al. 2015; Manikantan et al. 2016; Tyor andKumari 2016; Al-Rasheed et al. 2018). It was reported that the physico-chemical 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 (Magarinos et al. 1995; Fernández-Alacid et al. 2018), and cell scraper (Guardiola et al. 2014a, b, 2017; Nigam et al. 2015; 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 minutes, and finally collecting the mucus by scrubbing or moving back and forth to slough off the mucus (Subramanian et al. 2008; Wei et al. 2010; Ramesh 2013; Hisar et al. 2014; 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 (Guardiola 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. 2019b).

Fish mucus extraction methods

Various solvents were used in the extraction of the fish mucus samples. Principally, the extraction solvents were divided into three major types, i.e. aqueous, acidic and organic extracts (refer to Table 2).

Aqueous extract

For aqueous extracts, the most widely used solvent was physiological saline (0.85% NaCl). The mucus samples were mixed thoroughly with an equal amount of sterilised physiological saline (Magarinos et al. 1995; Kuppulakshmi et al. 2008; Manivasagan et al. 2009; Dhanaraj et al. 2009; Bragadeeswaran et al. 2011; Loganathan et al. 2011; Balasubramanian et al. 2012; Nwabueze 2014; Gabriella et al. 2014; Haniffa et al. 2014; Islam et al. 2014; Tyor and Kumari 2016; Kumari et al. 2019) or phosphate-buffered saline (Vennila et al. 2011), which were either directly used for antibacterial screening or pre-centrifuged first at room temperature at various centrifugation speeds, to obtain the supernatant for antimicrobial studies. There were four other aqueous solvents used in the extraction of fish skin mucus, such as 100 mM ammonium bicarbonate (Anbuchezhian et al. 2011; Elavarasi et al. 2013; Al-Rasheed et al. 2018), Tris-buffered Saline

Table 2 Fish mucus extraction methods and solvents used.

Types	Extraction Solvents	Conditions/Key Steps	References
Aqueous	Physiological Saline (0.85% NaCl)	Mucus to solvent ratio = 1:1. Centrifugation (15 min, 5000 rpm, 25 °C). Sterile filtration with 0.45 µm syringe filter (optional).	Balasubramanian et al. (2012); Bragadeeswaran et al. (2011); Dhanaraj et al. (2009); Gabriella et al. (2014); Haniffa et al. (2014); Islam et al. (2014); Kumari et al. (2019); Kuppulakshmi et al. (2008); Loganathan et al. (2011); Magarinos et al. (1995); Manivasagan et al. (2009); Nwabueze (2014); Tyor and Kumari (2016)
	Phosphate-buffered Saline (PBS)	Mucus to solvent ratio = 1:1. No centrifugation.	Vennila et al. (2011)
	100mM Ammonium Bicarbonate	Mucus to solvent ratio = 1:1. Centrifugation (30 min, 30000 x g, 4 °C).	Al-Rasheed et al. (2018); Anbuchezhian et al. (2011); Elavarasi et al. (2013)
	Tris-buffered Saline (TBS)	Mucus to solvent ratio = 1:1. Centrifugation (10 min, 500 x g, 4 °C).	Guardiola et al. (2014a, b, 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); Katra et al. (2016); Kumari et al. (2011); Nigam et al. (2015); Ramesh (2013); Rao et al. (2015); Subhashini et al. (2013); Wei et al. (2010)
Organic	Ethanol and Dichloromethane	Mucus to solvent ratio = 1:1. Centrifugation (30 min, 30000 x g, 4 °C).	Hellio et al. (2002); Katra et al. (2016); Manikantan et al. (2016); Rao et al. (2015); Subhashini et al. (2013); Subramanian et al. (2008); Vennila et al. (2011); Wibowo et al. (2015)
	Acetone and Methanol	Mucus to solvent ratio = 1:1. Centrifugation (15 min, 5000 rpm, 25 °C).	Varghese and Arathy (2011)
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); Wei et al. (2010)
	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).	Rao et al. (2015); Subramanian et al. (2008); Vennila et al. (2011)
	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).	Bragadeeswaran and Thangaraj (2011); Kumari et al. (2019); Loganathan et al. (2013); Patil et al. (2015); Tyor and Kumari (2016); Wei et al. (2010)

(TBS) (Guardiola et al. 2014a, b, 2017), normal distilled water (Hellio et al. 2002; Wei et al. 2010; Ramesh 2013; Subhashini et al. 2013; Rao et al. 2015; Katra et al. 2016) and triple distilled water (Kumari et al. 2011; Nigam et al. 2015). Unlike as in using physiological saline, the samples using these aqueous solvents were homogenized or stirred for two to three hours at 4°C before centrifugation (30 minutes, 30,000 × g, 4°C). Further, in some of the studies, the collected supernatants were filtered with Whatman filter paper (Hellio et al. 2002; Wei et al. 2010; Subhashini et al. 2013; Katra et al. 2016) or 0.45 µm syringe filter (Al-Rasheed et al. 2018).

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. 2008; Vennila et al. 2011; Subhashini et al. 2013; Rao et al. 2015; Katra et al. 2016; Manikantan et al. 2016). In general, the collected fish mucus was first suspended by stirring in 95% ethanol (1 mg/ml) and centrifuged at high speed (30 minutes, 30,000 × g, 4°C). The resultant pellet was re-extracted several times in the same way. The alcoholic extracts were combined and evaporated under vacuum. Next, distilled water was added to dissolve the pellets and partitioned with an equal amount of dichloromethane (CH_2Cl_2) which produced two distinct phases (aqueous and organic). The step would be repeated three or more times to maximise the protein recovery from the mucus extracts. The aqueous phases were collected, lyophilised, and re-suspended in absolute ethanol, filtered and concentrated under vacuum before use. The organic phases were collected, dried for 24 h under sodium sulphate (Na_2SO_4), filtered and concentrated under vacuum. Lyophilised aqueous and organic phases were dissolved in pure water and 5% dimethylsulfoxide (DMSO), respectively. DMSO is a popular chemical frequently used to inhibit enzyme activity that causes protein degradation. Both phases were stored at -40°C or below before use. Besides, other organic compounds such as acetone and methanol (Varghese and Arathy 2011) were also used in the extraction of mucus, by mixing an equal amount of mucus before subjecting to low-speed centrifugation (15 minutes, 25°C); clear supernatants were then concentrated in a rotary vacuum evaporator for antibacterial studies. Different approaches of extraction method through ethanol precipitation was reported elsewhere (Wibowo et al. 2015), where one volume of water-soluble fraction of fish skin mucus was mixed with three volumes of cooled ethanol and left at -20°C for 2 hours. The collection of precipitate was made by low-speed centrifugation. As organic solvents are highly volatile, the extracts are mostly left to dry under vacuum, in order to concentrate the samples instead of freeze-drying them.

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 several studies (Wei et al. 2010; Kumari et al. 2011; Nigam et al. 2015), the lyophilised fish skin mucus (10mg/ml) was first resuspended in distilled water, which was then mixed with an equal amount of 3% acetic acid or 0.1% trifluoroacetic acid, homogenized at 4°C and centrifuged (15 minutes, 10,000 × g, 4°C). Supernatants were collected separately and stored at –20°C. A total of 10% acetic acid was used elsewhere (Subramanian et al. 2008; Vennila et al. 2011; Rao et al. 2015) with a slightly different extraction protocol. Prior to homogenization and centrifugation (35 minutes, 18,000 × g, 4°C), the acid-mucus mixture was placed in a boiling water bath for 5 minutes and then cooled in ice. After centrifugation, the supernatant collected was then partially purified, in which the resulting elute was then concentrated or freeze-dried before being re-suspended in water for antimicrobial assay. Wei et al. (2010) resorted to further purification of the mucus extracts with a 0.22 µm syringe filter, while Al-Rasheed et al. (2018) prepared the mucus extracts by mixing 1% acetic acid with the fish mucus in the ratio of 1:4, followed by three-minute water bath boiling, homogenization and centrifugation (35 min, 25,000 × g, 4°C). The supernatants were then filtered with both Whatman No.1 filter paper and 0.45 µm syringe filter.

Others

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 (Wei et al. 2010; Bragadeeswaran and Thangaraj 2011; Loganathan et al. 2013; Tyor and 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).

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).

Antibacterial assays

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 OD measurement.

Agar disk diffusion

Agar disk diffusion was the most commonly used method for antimicrobial susceptibility testing in fish skin mucus studies (Magarinos et al. 1995; Kuppulakshmi et al. 2008; Dhanaraj et al. 2009; Wei et al. 2010; Anbuchezhian et al. 2011; Kumari et al. 2011; Loganathan et al. 2011, 2013; Bragadeeswaran and Thangaraj 2011; Varghese and Arathy 2011; Vennila et al. 2011; Bragadeeswaran et al. 2011; Balasubramanian et al. 2012; Subhashini et al. 2013; Elavarasi et al. 2013; Nwabueze 2014; Gabriella et al. 2014; Hisar et al. 2014; Islam et al. 2014; Nigam et al. 2015; Patil et al. 2015; Wibowo et al. 2015; Katra et al. 2016; Hiwarale et al. 2016). This method was adopted following the standardized 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 standardized inoculum (0.5 McFarland standard or 10^8 CFU ml^{-1}) 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 hours), the diameters of clear inhibition zones exhibited by positive extract would be measured, which is also known as Inhibition zone diameter (IZD). Some studies also allowed the pre-diffusion of the extracts on the agar plates prior to incubation. Normally, the temperature of diffusion is set at around 4°C and the incubation period is 1 hour. Most studies used Mueller Hinton Agar (MHA) or Tryptic Soy Agar (TSA) to perform the susceptibility tests for various Gram-positive and Gram-negative bacteria, while other fastidious bacteria may require selective media and agar for incubation and susceptibility test (Magarinos et al. 1995).

Agar well diffusion

Agar well diffusion was also widely used to evaluate the antimicrobial activity of fish skin mucus (Hellio et al. 2002; Manivasagan et al. 2009; Al-Arifa et al. 2011; Ramesh 2013; Haniffa et al. 2014; Manikantan et al. 2016; Tyor and Kumari 2016; Al-Rasheed et al. 2018). This method is quite similar to the procedure used in the disk diffusion method, but for the usage of filter paper discs. At first, the agar plate surface is inoculated by spreading a volume of the microbial inoculum over the entire surface. 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. Then, agar plates are incubated under suitable conditions (at 37°C for 16-20 hours). Similar to the agar disk diffusion method, the positive results are presented in IZD.

Broth micro-dilution

Broth micro-dilution was also used to access the antibacterial activity of fish skin mucus (Subramanian et al. 2008; 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. Each well is then inoculated with a standardized volume of bacterial inoculum, which is diluted into a final suspension density of 2 x 10^4 mL. After mixing well, the plate is incubated under suitable conditions (at 37°C for 18-24 hours). 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.

Others

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). At first, the bacterial suspension in MH broth was diluted to obtain a final concentration of 7.5 x 10^5 CFU/mL. The fish skin mucus was then added in decreasing concentrations. Inoculated bottles with different concentrations of mucus were incubated, while aliquots of each dilution were taken for cell counting every 60 min for 22 hours. The bacterial growth curves containing each mucus concentration were compared with negative control. Besides, the bactericidal activity of fish skin mucus was also determined by evaluating its effect on the bacterial growth curves (Guardiola et al. 2014a, b, 2017; Sanahuja et al. 2019a). The procedures were principally similar with different incubation conditions (i.e. 25°C for 24 hours) and shorter time intervals (30 min) to collect the OD value. 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.

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 number 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 miniaturization 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.

Antibacterial properties of fish skin mucus

This review section reported 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). It is to be noted that no review was made of the other two fish classes, namely Cephalaspidomorphi (lampreys) and Sarcopterygii (lobe-finned fishes), due to the absence of skin mucus antibacterial studies. A broad spectrum of antibacterial activity against a total of 46 bacterial species, including 13 Gram-positive bacteria (see Table 3), one acid-fast bacteria, and 32 Gram-negative bacteria (See Table 4) was reported here.

Class Actinopterygii

Ray-finned fishes are the most diverse class of vertebrates, comprising about 99% of freshwater and marine species in the world (Rastogi 2007). The majority of the fish species (43 out of 47) reviewed are grouped

Table 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 and Thangaraj (2011); Gabriella 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					Kumari et al. (2019); Tyor & Kumari (2016)
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. (2008)
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						Dhanaraj et al. (2009); Kumari et al. (2011); Kuppulakshmi et al. (2008)
Channa striatus	Striped snakehead	B,F			+ a,b		+ b	+ b			+ b,c			+ b		+ b	Dhanaraj et al. (2009); Haniffa et al. (2014); Loganathan et al. (2013); Ramesh (2013); Wei et al. (2010)
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						Gabriella et al. (2014); Guardiola et al. (2014a); Magarinos et al. (1995)
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)

Table 3 continued

Class Order *Species*	Common name	Habitat	Gram-positive bacteria 1	2	3	4	5	6	7	8	9	10	11	12	13	14*	Gabriella et al. (2014) References
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]				Magarinos 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. (2008)
Siluriformes																	
Arius caelatus	Engraved catfish	M									+[b]						Bragadeeswaran et al. (2011)
Arius maculatus	Spotted catfish	M													+[b]		Anbuchezhian et al. (2011); Manivasagan et al. (2009)
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]						Elavarasi et al. (2013); Loganathan et al. (2011); Patil et al. (2015); Varghese and Arathy (2011)
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)
Myxini																	
Myxiniformes																	
Myxine glutinosa	Atlantic hagfish	M										+[a]					Subramanian et al. (2008)

*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 -

under Actinopterygii, which consists of seven orders, i.e. Anguilliformes, Cypriniformes, Gadiformes, Perciformes, Pleuronectiformes, Salmoniformes, Siluriformes.

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 and Thangaraj 2011; Gabriella 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).

Order Cypriniformes

Cyprinidae is the largest and most diverse fish family in Cypriniformes. There was a total of eight species reviewed, 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. boydi*. 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 and 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.

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. 2008) 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

Table 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,c, d		Bragadeeswaran and Thangaraj (2011); Gabriella 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																		Kumari et al. (2019); Tyor & Kumari (2016)
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. (2008)
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						Dhanaraj et al. (2009); Kumari et al. (2011); Kuppulakshmi et al. (2008)

Table 4 continued

Class			Gram-negative bacteria																															
Order *Species*	Common name	Habitat	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	References
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			Gabriella et al. (2014); Guardiola et al. (2014a); Magarinos et al. (1995)
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			Gabriella et al. (2014)
Sparus aurata	Gilthead seabream	M				+b					+b												+b				+b				+b				Guardiola et al. (2014a,b); Magarinos et al. (1995)
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													Magarinos 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. (2008)
Siluriformes																																			
Arius caelatus	Engraved catfish	M																	+b									+b				+b			Bragadeeswaran et al. (2011)
Arius maculatus	Spotted catfish	M				+b			+b					+b							+b				+b		+b								Anbuchezhian et al. (2011); Manivasagan et al. (2009)
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)

Table 4 continued

Class			Gram-negative bacteria																													
Order *Species*	Common name	Habitat	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	References
Clarias gariepinus	African sharptooth catfish	F					+ 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				+ 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)
Myxini																																			
Myxiniformes																																			
Myxine glutinosa	Atlantic hagfish	M		+ a		+ a				+ a				+ a						+ a														+ a	Subramanian et al. (2008)

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*

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.

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 snakehead 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.*

Tilapia are among the most important commercial cichlids (Family: Cichlidae) found in the world. In this review, 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 15.96µg/ml, which the others exhibited greater resistance against the tilapia mucus with MBC value of 31.91 µg/ml. In another study (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 physiological saline (Gabriella et al. 2014),sterile seawater (Magarinos 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. In this review, 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 (Gabriella et al. 2014) showed activity only against one Gram-negative bacteria – *V. parahaemolyticus,* while the Gilthead seabream (Magarinos et al. 1995; Guardiola et al. 2014b, a) 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).

Order Pleuronectiformes

Flatfishes are one of the popular bottom-feeding fishes in commercial fisheries. One of the many families reviewed 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 (Magarinos 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 and Magarinos 1997) and may greatly impact commercial fisheries.

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. 2008) 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.

Order Siluriformes

Siluriformes (catfishes) was among the most studied order for antibacterial research on its epidermal mucus. In this review, 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 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) were reported more extensively.

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 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 17mm). Notably, different strengths of inhibition was 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 and 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.

Class Elasmobranchii
Order Myliobatiformes

Three stingray species (Order: Myliobatiformes; Family: Dasyatidae), namely *Dasyatis pastinaca* (common stingray), *D. sephen* (cowtail stingray) and *Himantura gerrardi* (whitespotted whipray) were also reviewed 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.* Typhi 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.

Class Myxini
Order Myxiniformes

The only hagfish species studied in this review, *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. 2008) 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 in this review (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.

Conclusions

This review highlights the antibacterial effects of different extracts from the epidermal mucus in 47 fish species, as demonstrated by various methodologies. In general, both commercially and non-commercially valued fish showed a broad-spectrum of antibacterial activities against pathogenic micro-organisms, including bacteria associated with nosocomial infections (*Pseudomonas aeruginosa, Staphylococcus aureus, Klebsiella pneumoniae, Proteus mirabilis, Serratia marcescens*) and multi-drug resistant bacteria (MRSA), as well as common fish pathogens (*Photobacterium damselae* subsp. *piscicida* and *V. anguillarum*). Among all the studies relating to fish species, *Channa* and *Clarias* species are the best studied genera and appeared to possess broad antibacterial actions against both Gram-positive and Gram-negative bacteria. Out of the 47 fish species reviewed, Gram-negative *Escherichia coli* was the most sensitive towards the epidermal mucus extracts of fish (n= 40), followed by *Pseudomonas aeruginosa* (n=31) and *Klebsiella pneumoniae* (n=25), while Gram-positive *Staphylococcus aureus* was the most sensitive to fish skin mucus (n=27). Therefore, it is recommended that a preliminary screening be conducted against these bacteria, prior to any in-depth analysis of antibacterial effect of fish skin mucus.

Although most reviewed studies demonstrated the antibacterial effectiveness of fish skin mucus, they only conducted disk-diffusion tests, which can be made more informative by incorporating quantitative tests such as MIC and bacterial growth curves, to determine the effective concentration of the mucus extracts for antibacterial activity. With more antimicrobial susceptibility testing methods continuing to be developed, it is important to adopt the fundamental rules of microbiology to strictly abide by the standards of CLSI and EUCAST. In addition, provided that the methodologies used to collect and extract the desired components from the fish mucus could be largely different from study to study, it is of utmost importance for the researchers to seek the most appropriate way, prior to further analysis. However, when dealing with natural products such as fish skin mucus, slight modifications on the standardized protocols are warranted from time to time, as minor methodological adaptations to standardized protocols could ensure a more accurate experimental approach and allow other researchers to compare results with higher reliability.

The most potent antibacterial activity exhibited by a wide range of fish species indicates that the components in their skin mucus could provide the key defence against pathogenic infections. We recommend the purification and characterization of isolated antimicrobial components (e.g. proteins) from the fish epidermal mucus, as they might be candidates for potent therapeutic agents, and contribute greatly to the aquaculture industry and possibly human health-related applications.

List of abbreviations
IZD: Inhibition Zone Diameter
MIC: Minimum Inhibitory Concentration
MBC: Minimal Bactericidal Concentration

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Authors' contributions YL reviewed and summarised all the recent studies and drafted most of the manuscript. LMB and BS also helped to draft the manuscript. YLC refined the writing style and language as well as helped to draft the manuscript. All authors read and approved the final manuscript.

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