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Correlation Between Three Freshwater Fish Skin Mucus Antiproliferative Effect and Its Elemental Composition Role in Bacterial Growth

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Abstract

Fish skin mucus acts as an immunological barrier that prevents entry of pathogens. However, there are gaps in the knowledge of microbes inhabiting skin mucus constituents to invade the host and induce pathogenecity. Hence, in the present study, skin and skin mucus of three freshwater fishes *Cyprinus carpio, Labeo rohita, Cirrhinus mrigala* was analyzed to compare histology and mucus activity against cancer cells. The skin mucus elemental composition and its influence on bacterial growth were also investigated. Histological examination of fish skin showed the presence of mucus secreting cells and differences in the distribution of cells were clearly seen depending on fish species. The cytotoxic potential of lyophilized skin mucus against human lung adenocarcinoma cells revealed a higher percentage of cell death at 1000 µg mL^{-1} in *C. mrigala* skin mucus when comparing other two fish species. Elemental analysis of lyophilized skin mucus through Field Emission Scanning Electron Microscope coupled with Energy Dispersive X-ray (FESEM-EDX) confirmed the presence of carbon, nitrogen, oxygen and sulfur in *C. carpio* and *C. mrigala.* In the case of *L. rohita*, the element sulfur was absent. The results of bacterial growth in autoclaved skin mucus demonstrated an initial reduction in bacterial population and gradually increased over time. Initial reduction in bacteria might be due to the presence of inhibitory molecules in fish skin mucus. Subsequently, the bacteria utilize the elemental composition of skin mucus as a nutrient source to increase their growth. Study findings suggest that the presence of bioactive compounds in lyophilized skin mucus hinder the proliferation of cancer cells. Nevertheless, after autoclaving the skin mucus components, it supports the growth of bacteria due to the absence of immune molecules. The present study represents the knowledge of skin mucus composition, which could be explored further to understand how pathogens overcome the skin mucus barrier.

Introduction

In the animal kingdom, mucus secretion occurs almost universally and it is engaged in multifarious life processes (Gould *et al*., 2019). Fish is one of the organisms that has managed to survive in the presence of millions of microorganisms in their environment (Jeffries *et al*., 2014). Fish developed a complex immune defense system to encounter biotic and abiotic factors (Lazado & Skov, 2019). The mucus is an important innate immune system of fish and an adhesive mass of lubricating form which is viscous, gelatinous and porous property (Fæste *et al*., 2020). Fish skin mucus has many physiological and mechanical functions that protects the host from the surrounding environment by preventing the access of entry for many pathogens (Esteban, 2012). The mucus layer helps in controlling the movement of water and solutes across the skin of fish and significant in respiration, osmotic regulation, protection from abrasions, communication, entanglement of particulate

materials etc (Magnadóttir, 2006; Tort *et al*., 2003). The skin mucus components aid in maintaining the health of fish (Patel & Brinchmann, 2017). It is considered as a repository for numerate immune components (Nigam *et al*., 2012). It contains lysozymes, C-reactive proteins, immunoglobulins, alkaline phosphatase, complements, lectins, different types of proteases including trypsin, metalloproteases and cathepsin, carbonic anhydrase, calmodulin, hemolysin and antimicrobial peptides (Abolfathi *et al*., 2020; Supriya Dash *et al*., 2014; Rakers *et al*., 2013; Ren *et al*., 2015; Salinas, 2015). The amino acids such as threonine present in mucus helps in sustaining the mucosal integrity and immune functions (Ashraf *et al*., 2020; Mao *et al*., 2011).

The mucus provide variety of immune responses and components depending on species habit, habitat and distinctive sorts of nourishment (Kumari *et al*., 2019). The composition and rheological properties are important for the fish to maintain its mucosal immunity (Guardiola *et al*., 2015). Each fish community has its own mucus composition; by virtue of factors that are secreted are species specific and ambience in which they live (Reverter *et al*., 2018). Endogenous factors such as developmental stage and sex; exogenous factors such as infections and stress influence the fish mucus composition (Dash *et al*., 2018; Esteban & Cerezuela, 2015). The secretion of mucus is higher in freshwater species than marine species due to exchange of water across fish skin (Shephard, 1994; Tort *et al*., 2003). Although having structural and functional differences in the mucus present in skin, gill and gut, all share similar characteristic features with type I mucosal surfaces of mammals (Iwasaki, 2007).

The fish common carp (*Cyprinus carpio*) generally inhabits freshwater and is distributed in almost all the countries as a potential candidate for commercial aquaculture in Asian and European continents (Rahman, 2015). Rohu (*Labeo rohita*) and mrigal (*Cirrhinus mrigala*) fish are the most important among three Indian major carps used in polyculture systems because of its great commercial importance (Khan *et al*., 2011). The rohu fish is geographically broad species in tropical freshwater of India and nearby nations with variations in growth parameters. While, mrigal fish is an indigenous freshwater fast growing major carp broadly distributed in the inland waters of Indian subcontinent (Iqbal-Mir *et al*., 2015).

Fishes are considered as an unexplored source of pharmaceutical products, therapeutics etc. (Alshammari *et al*., 2019; Patel *et al*., 2020). Bioactive peptides from fish showed antimicrobial, antiproliferative effects and considered as lead compounds for the development of nutraceuticals (Ashraf *et al*., 2020). Reports indicated the anticancer properties of different fish skin mucus viz, round goby (*Neogobius melanostomus*) and *C. carpio* (Alijani Ardeshir *et al*., 2020), flathead grey mullet (*Mugil cephalus*, Balasubramanian *et al*. 2016), marine catfish (*Tachysurus dussumieri*, Arulvasu *et al*. 2012), Japanese eel (*Anguilla japonica*, Kwak *et al*. 2015). Even though the composition of fish skin mucus prevents the adherence of pathogens and provide medium for antibacterial mechanisms (Cone, 2009; Tort *et al*., 2003), pathogens override skin mucosal system and induce disease in fish. Thus, it is necessary to understand the possibilities of pathogens that could survive the skin mucus and cause pathogenecity. Gupta *et al*. (2017) reported that microorganisms utilize and recycle elements especially carbon, oxygen, nitrogen etc and these elements play structural and functional roles in microbes. The elements are considered as the driving force for the adaptation of microbes (Merchant & Helmann, 2012). In our previous research findings, it was reported that the fish skin mucus of *C. carpio*, *L. rohita* and *C. mrigala* showed bactericidal activity against *Proteus vulgaris* and *Pseudomonas aeruginosa* (Sridhar *et al*., 2020). Concurrently, Shoemaker and LaFrentz (2015) reported that *Flavobacterium columnare* utilize skin mucus of sex reversed hybrid tilapia (*Oreochromis niloticus* × *O. aureus*) as a nutrient source and increase its virulence factors towards the host. Energy Dispersive X-ray (EDX) spectroscopy analysis reveals the elemental composition of fish skin mucus since microbes utilize elements such as carbon (C), nitrogen (N) and sulfur (S) from the surrounding environment to maintain their internal metabolisms (Xu *et al*., 2015). Understanding the role of fish skin mucus and its components provide information for insights to biological mechanisms of pathogens interactions. Therefore, in this context, based on the economical importance and versatility, three freshwater species *C. carpio*, *L. rohita* and *C. mrigala* were selected for this study. As our previous research already demonstrated the antibacterial activity of skin mucus, this study was focused to display their cytotoxic properties and also to analyze the autoclaved skin mucus influence towards bacterial cells. In addition, the elemental composition of skin mucus was investigated to understand the anomaly within the species and their role in bacterial growth. This study hypothesized that microorganisms could utilize fish skin mucus for its growth in the absence of immune molecules.

Materials and Methods

Fish Collection and Maintenance

The healthy live freshwater fish: *C. carpio*, *L. rohita* and *C. mrigala* were collected from Nathan fish farm (Thanjavur, Tamil Nadu, India). They were packed in aerated polyethylene bags and transported carefully to the Aquarium Facility at Bharathidasan University (Tiruchirappalli, Tamil Nadu, India) without giving maximum stress. In aquarium facility, the freshwater was treated with biological filters and ultraviolet then transferred to circular water tanks (2,000 L) and left for four days undisturbed before introducing fish. Then the collected fish were stocked in the above mentioned water tanks. The water temperature was maintained at

30±2°C, pH at 7.29±0.07 and the tanks were continuously aerated (0‰ salinity). The photoperiod was 12 h light: 12 h dark. The health status of the fish was inspected carefully by visual observation. The individual fish was observed for its swimming patterns, abnormal behaviors and for any lesions. Fish with lesions and abnormal behaviors were removed from the tank. Fish were fed with commercial pellet diet twice a day *ad libitum*. Fish were allowed to acclimatise for 2 weeks and monitored daily before starting the experiment.

Skin Collection

All applicable guidelines for the care and use of animals outlined by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India were strictly adhered while maintaining and handling the fish. For skin collection, briefly, healthy fish (*n*=1) from each species: *C. carpio*, *L. rohita* and *C. mrigala* were separated in small plastic containers and sacrificed by deepening anesthesia using clove oil. The fish were taken from the containers and placed in surgical table. The fish skin was wiped cleanly with filter paper to remove the water, mucus, clove oil residues and any other suspended particles. Then, the skin samples from dorsal side were excised using dissection kit.

Skin Histology

After excising skin samples, they were washed with phosphate buffered saline (PBS) and transferred to tubes containing 4% formaldehyde for 24 h for fixation. Then, the fixative solution was removed and the skin samples were washed with 70% ethanol. The samples were dehydrated in increased concentrations of ethanol, 70% for 24 h, 80% for 30 min, 96% for 1 h and finally in 100% for 1 h. Then, the skin samples were embedded in paraffin. The blocks containing skin samples were trimmed and sections were done with the help of microtome. The sectioned samples were stained with hematoxylin-eosin. The stained slides were studied under light microscope (Carl Zeiss, Axioscope2plus) and images were taken at 400 × magnification.

Skin Mucus Collection

The skin mucus was collected from healthy fish species (*n* = 6) according to the method of Subramanian *et al*. (2008). The fish were starved for one day prior to skin mucus collection. To slough off the skin mucus, the fish were transferred into a sterile polyethylene bag and gently moved back and forth and care was taken to avoid the contamination of blood and excretions. Skin mucus samples obtained from each fish species were pooled and homogenized separately. The homogenate was centrifuged at 1500×*g* for 10 min at 4 ºC to remove the insoluble particles and the supernatants were collected and separated into two parts. The first part was stored to investigate its cytotoxic properties and also to determine that microbes use skin mucus as a source of nutrient. The second part was lyophilized and stored at -20ºC for the analysis of elemental composition.

Cell Culture and MTT Assay

Human lung adenocarcinoma cell line (A549) was obtained from National Centre for Cell Science (NCCS), Pune, India. The cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM, HiMedia, Mumbai) supplemented with 10% fetal bovine serum (FBS, Invitrogen), calcium chloride and 2% streptomycin (Gibco) at 37°C in a 5% CO_2 environment. All experiments were performed using cells from passage 15 or less. The stock solutions were prepared by dissolving fish skin mucus samples in sterile double distilled water. The stock solutions were diluted separately with media to get various concentrations (0-1000 µg mL^{-1}). Then, 200 µL of samples were added to the wells containing A549 cells (5 × 10^3 per well). After 24 h of incubation, the DMEM medium was replaced with fresh medium and treated with DMSO containing different concentrations of skin mucus samples from each fish species separately. Untreated cells were served as control. The cells were incubated for 24 h. After the incubation period, 20 µL of MTT solution (5 mg mL^{-1} in PBS) was added to each well and the plates were incubated at 37°C for 4 h. The formed purple formazan crystals were dissolved in 100 µL of DMSO and the absorbance was read at 570 nm using microplate reader (Bio-Rad, Hercules, CA, USA). Data were collected for three replicates each and used to calculate the respective mean. The percentage of inhibition was plotted against concentration.

Mucus as a Source of Nutrient

The utilization of mucus components as a source of nutrient by bacteria were analyzed *in vitro*. The ability of bacterial isolates to grow in fish skin mucus was examined in 96-well polystyrene round bottom microtiter plates. Modified method of Papadopoulou *et al*. (2017) was adopted for the *in vitro* growth of bacteria in fish skin mucus. Briefly, the clinical isolates (*Escherichia coli*, *Staphylococcus aureus*, *Aeromonas hydrophila* and *Vibrio cholerae*) were grown in nutrient agar (Sigma-Aldrich, Bengaluru, India) plates at 28±2°C overnight. Then fresh single colonies were diluted in 5 mL of nutrient broth (Sigma-Aldrich) and cultured for 12 h at 28°C on an orbital incubator at 150-200 rpm. The first part of the supernatant mucus was autoclaved at 121°C for 15 min. Bacterial suspensions and mucus samples were serially diluted 2-fold with PBS. Then, 100 µL of bacterial dilutions and equal volume of autoclaved skin mucus were placed 96-well microtiter plates. Positive wells contained 100 µL of bacterial

suspension and 100 µL of nutrient broth. Negative wells contained 100 µL of autoclaved skin mucus and 100 µL of nutrient broth. Experiment was done in triplicates. The plates were incubated at 28±2°C. At an interval of every 1 h, the OD was measured at 600 nm for 12 h using microplate reader (Synergy HT, Bio-Tek Instruments, Inc., Winooski, VT, USA).

Characterization of Lyophilised Fish Skin Mucus

The morphology and elemental analysis of lyophilized fish skin mucus samples were investigated by Field Emission Scanning Electron Microscope (FE-SEM, Carl Zeiss, Germany) coupled with EDX. The prepared lyophilized fish skin mucus samples were sputter-coated with gold (Emitech, SC7620).

Data Analysis

Data obtained from *in vitro* bacterial growth and MTT assay were subjected to one-way ANOVA, and the results were presented as means ± standard deviations of three experimental replicates. By using GraphPad Prism software (GraphPad software Inc., California, USA), the data obtained were graphically represented.

Results and Discussion

Skin Histology

The epidermal layer which consisted of mucus secreting cells and superficial epithelial cells were observed in the histological sections of all the three experimental fish species skin samples (Figure 1). The mucus secreting cells were developed and interspersed among the epithelial cells. These cells secrete the mucus and provide the mucosal surface all over the body of the fish. The numbers of mucus secreting cells varied depending on fish species. The differences in the number and distribution of mucus cells on epidermis might be the adaptation of scooping habits of each species (Yang *et al*., 2019). Pittman *et al*. (2013) observed the skin histology of Atlantic salmon (*Salmo salar*) and suggested that the mucus cell size and density on the epidermal region was influenced by diet, sex, strain and season. The mucus cells of high cellular activity and hyperchromatic with several mitotic cells were observed in South American lungfish (*Lepidosiren paradoxa*) skin by Romano *et al*. (2019). The increase in mucus cells also indicate the stress conditions of a fish (Vatsos *et al*., 2010). It is highly valuable of linking mucus cells with environmental stress and somatic health (Dang *et al*., 2020).

Cell Cytotoxicity

The cytotoxic activity of *C. carpio*, *L. rohita* and *C. mrigala* skin mucus were analysed against A549 human lung adenocarcinoma cell line. The *in vitro* cytotoxic activity results revealed a dose dependent moderate activity on A549 cells (Figure 2). The IC_{50} value represents the sample concentration that resulted in 50% of cell survivability. The IC_{50} values of the skin mucus samples against A549 cells followed the order of *C. mrigala*>*C. carpio*>*L. rohita*, the values were 735, 855 and 875 µg mL^{-1} respectively. Alijani Ardeshir *et al*. (2020) reported the IC_{50} value of *C. carpio* for breast adenocarcinoma cell line (MCF-7) and prostate cancer cell line (LNCaP) was found to be 1000 µM and proposed the reason of cell death was induced apoptosis by P53 gene up-regulation and cell cycle arrest at G1 phase.

The increasing concentration of fish skin mucus was directly proportional to the inhibition of A549 cells. In the present study, the *C. mrigala* fish skin mucus showed higher percentage of cell death at 1000 µg mL^{-1}, while *L. rohita* and *C. carpio* showed decreased cell death percentage at the same concentration. Balasubramanian *et al*. (2016) showed the activity of flathead grey mullet (*M. cephalus*) skin mucus against Laryngeal cancer cell line at the concentration of 1000

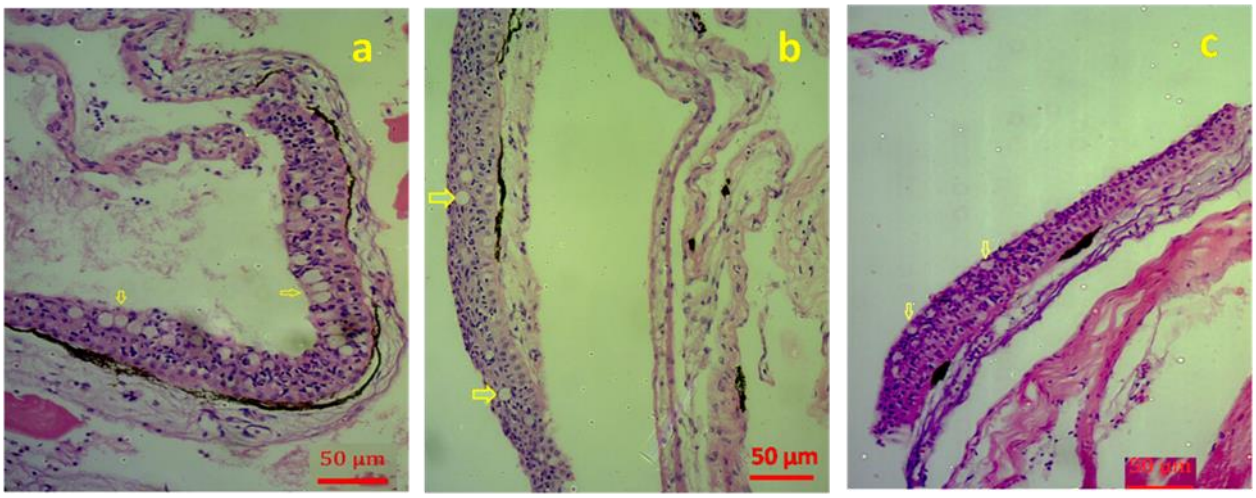

Figure 1. Histological observations of epidermal layer of a) *Cyprinus carpio*; b) *Labeo rohita*; and c) *Cirrhinus mrigala*. Yellow arrow shows mucus secreting cells. Scale bars: 50 µm.

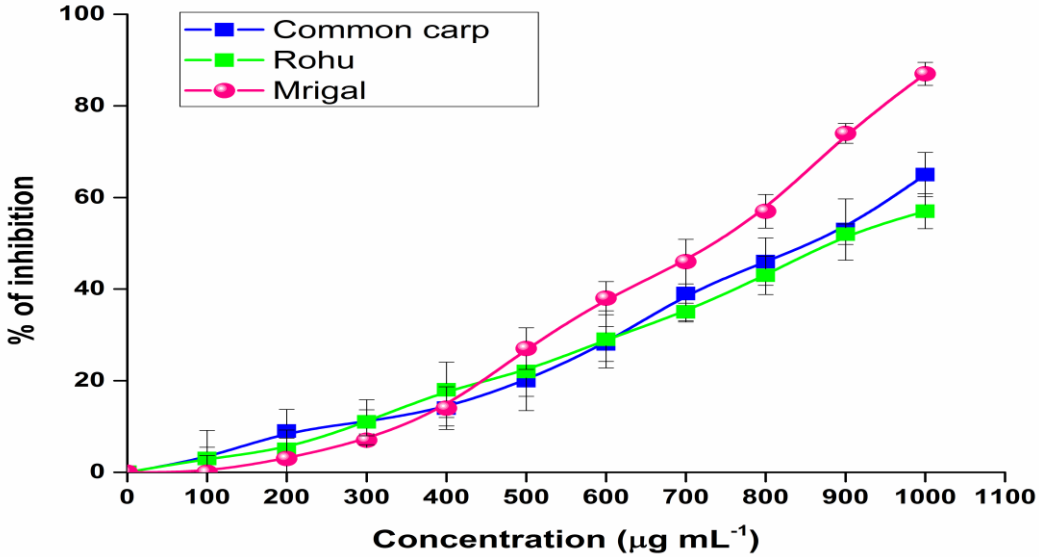

Figure 2. Cytotoxicity assay (MTT) of skin mucus of rohu (*Labeo rohita*), common carp (*Cyprinus carpio*) and mrigal (*Cirrhinus mrigala*) against A549 human lung adenocarcinoma cell line after 24 h exposure. Data were expressed as mean ± standard deviations (*n* = 3).

µg mL^{-1} and suggested higher quantity of lysine in skin mucus caused the lysis in cancer cells. Kwak *et al*. (2015) demonstrated that *A. japonica* skin mucus triggered apoptotic cell death and inhibited cell proliferation in human leukemic K562 cells in a dose dependent manner and showed higher cell death at 500 µg mL^{-1}. Recent research is focused on animal based protein molecules for the treatment of many infectious diseases and the cytotoxic properties of fish mucus against cancer cells indicating its potentiality of developing pharmaceutical/antitumoral strategies (Álvarez *et al*., 2016; Reverter *et al*., 2018). The skin mucus from common stingray (*Dasyatis* pastinaca) against acute leukemia cells (HL60) evident dose dependent inhibition of proliferation up to the concentration of 1500 µg mL^{-1} and exhibited no toxicity towards non-tumoral cells and that substantiated the capability of fish skin mucus as a cytotoxic agent (Fuochi *et al*., 2017).

Mucus as a Source of Nutrient

The skin mucus of experimental fishes were analysed for the growth of bacteria: *E. coli, S. aureus, A. hydrophila* and *V. cholerae*. Initially, the skin mucus suppressed the bacterial population then gradual increase in growth was observed over the time of incubation in this study (Figures 3-5). The fish *C. carpio*, *L. rohita* and *C. mrigala* skin mucus depicted similar results with the bacteria tested except *V. cholerae*, which did not show drastic reduction in the initial hour. The suppression of growth in bacteria revealed the presence of inhibitory molecules in the skin mucus when comparing the growth of bacteria without the presence of mucus. The antibacterial activity of fish skin mucus were reported by several authors (Adel *et al*., 2018; Balasubramanian *et al*., 2012; Bragadeeswaran *et al*., 2011; Fuochi *et al*., 2017; Kumari *et al*., 2019; Kumari *et al*., 2011; Kuppulakshmi *et al*., 2008; Mahadevan *et al*., 2019; Nigam *et al*., 2017; Sridhar *et al*., 2020; Wang *et al*., 2019; Wei *et al*., 2010) and indicate that the skin mucus innate immune components are crucial for its activity against microbes.

In the present study, after a period of time (1 h), the bacteria surpass the inhibitory molecules and surge their population. Similarly, Shoemaker and LaFrentz (2015) examined the growth of *F. columnare* in autoclaved skin mucus of sex reversed hybrid tilapia and indicated that autoclaving caused denaturing of proteins and reduced the antibacterial properties of mucus which in turn supported the growth of *F. columnare*. The protein denaturation occurs due to prolong duration of heat and pressure in autoclave. Papadopoulou *et al*. (2017) showed a slight decline in the number of *F. psychrophilum* cells at 1 and 2 h post inoculation in filtered and autoclaved skin mucus of rainbow trout (*Oncorhynchus mykiss*) and suggested that bacteria use glycoproteins and other proteins in the fish skin mucus for their growth and multiplication. Different mechanisms are followed by pathogens to adhere on skin mucus as requirement for successful infection in fish (Benhamed *et al*., 2014). Adherence of bacteria to the mucus is a significant property for colonization (Adnan *et al*., 2017). Bacteria colonize on the outer mucus layer by degrading mucus glycans as an energy source (Johansson *et al*., 2008). The bacteria take the host mucus as nutrients, as it provides carbon and nitrogen sources (Bakshani *et al*., 2018). By using mucus as a carbon source, bacteria enhance its survivability, which in turn leads to multiplication and extend its colonies (Benhamed *et al*., 2014). The infection of fish depends on health status of the fish, environmental conditions and the composition of mucus (Chen *et al*., 2008). Ohneck *et al*. (2018) observed that bacteria, *Acinetobacter baumannii* recognize mucins in the mucus

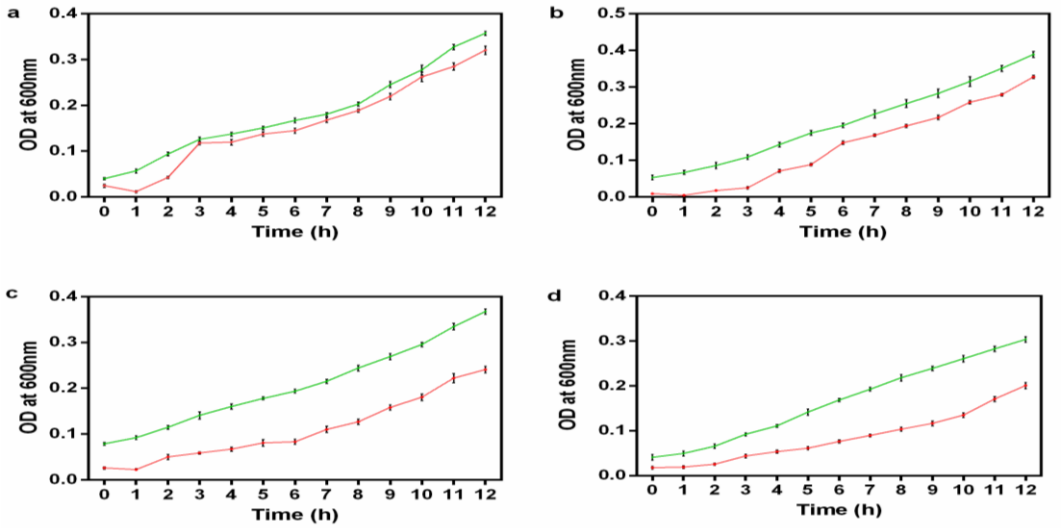

Figure 3. Growth of a) *Escherichia coli*; b) *Staphylococcus aureus*; c) *Aeromonas hydrophila* and d) *Vibrio cholerae* for 12 h with *Cyprinus carpio* skin mucus (red line); with nutrient broth (green line). Data were expressed as mean ± standard deviations (*n* = 3).

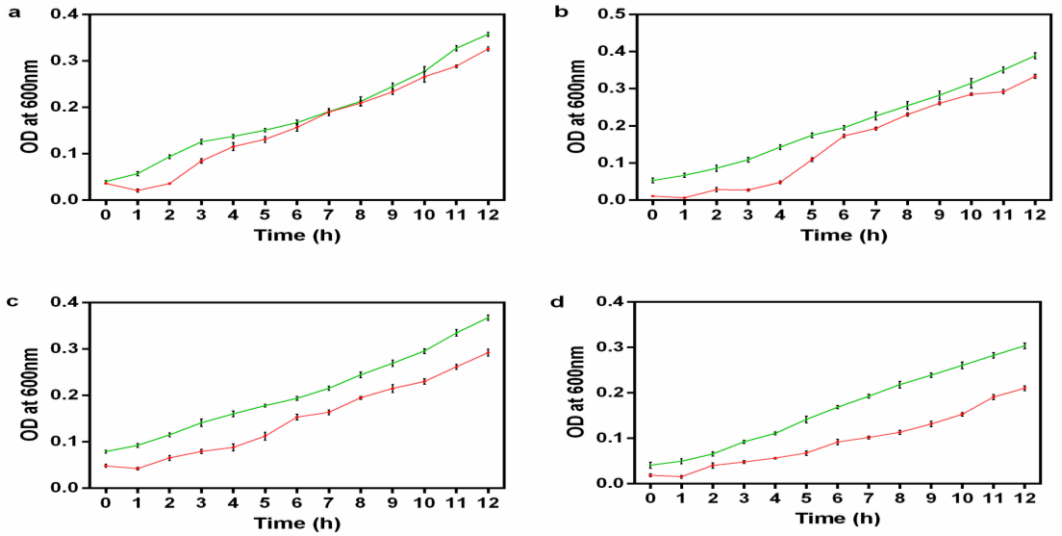

Figure 4. Growth of a) *Escherichia coli*; b) *Staphylococcus aureus*; c) *Aeromonas hydrophila* and d) *Vibrio cholerae* for 12 h with *Labeo rohita* skin mucus (red line); with nutrient broth (green line). Data were expressed as mean ± standard deviations (*n* = 3).

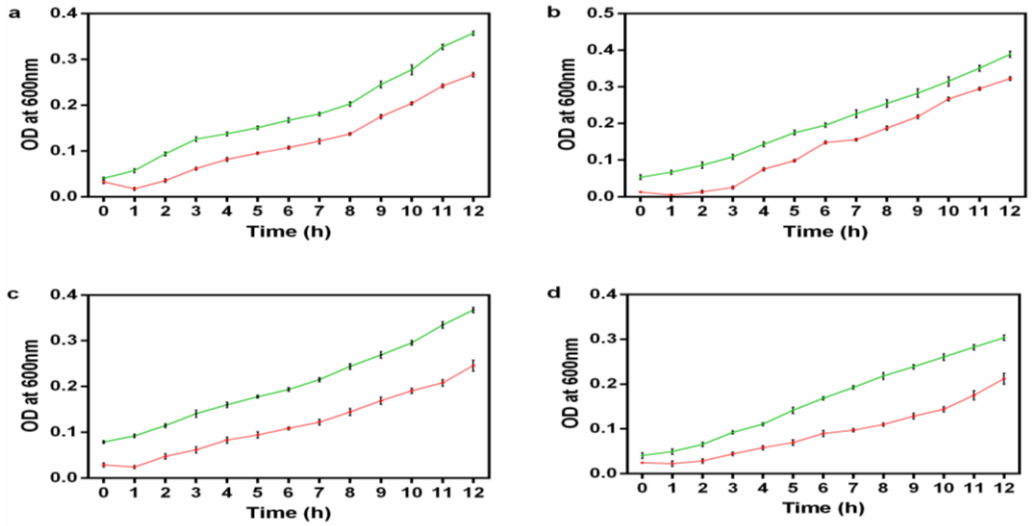

Figure 5. Growth of a) *Escherichia coli*; b) *Staphylococcus aureus*; c) *Aeromonas hydrophila* and d) *Vibrio cholerae* for 12 h with *Cirrhinus mrigala* skin mucus (red line); with nutrient broth (green line). Data were expressed as mean ± standard deviations (*n*=3).

as signal and utilized the nutrients to promote host-pathogen interactions to increase and influence the virulence factors that lead to the pathogenesis of bacterial infections.

Characterization of Fish Skin Mucus

The FE-SEM micrographs of lyophilized fish skin mucus were shown in Figure 6. The results showed different morphologies of fish skin mucus topographical microstructures like spherical, thin layered and agglomerated depending on fish species. More importantly, these microstructures provide structural support (Balaji *et al*., 2020). In this study, the *C. carpio* skin mucus showed self-assembled spherical like morphological structures (Figure 6a & b), while *L. rohita* fish skin mucus showed thin-layered structure (Figure 6c & d). Figure 6 (e & f) showed the aggregated, thin-layered like morphology in *C. mrigala* fish skin mucus.

Furthermore, skin mucus samples were analysed for elemental analysis with mapping. EDX spectrum (Figure 7-9) clearly revealed the elemental mapping of fish skin mucus. The elemental analysis revealed major signals such as carbon (C), oxygen (O), nitrogen (N) and sulfur (S), which are the constituents of proteins (Misran & Jaafar, 2019). The *C. mrigala* and *C. carpio* showed sulfur peak signal, but it was absent in the case of *L. rohita*. Other peaks such as chlorine (Cl), potassium (K), sodium (Na), although not in higher percentage, also recorded from the EDX spectrum. The gold (Au) peak observed in the fish skin mucus was due to the usage of Au as surface coating agent. Variations in the elemental signals in the current study were due to the species difference and metabolic pathways. The freshwater fish actively takes sodium and chloride through mucosal surfaces (Glover *et al*., 2013), which accounts for Na and Cl peaks observed in this study. The skin mucus has iron regulatory functions that help fish for long term survival (Elsheikh, 2013). The elementary mapping of *C. carpio* skin mucus signals as shown in Figure 7 (c-f) depicts C-53%, N-29%, O-12% and S-6%. Figure 8 (c-e) showed the peaks of C-71.57%, N-20.59% and O-7.84% from *L. rohita* skin mucus. Figure 9 (c-f) indicated the elementary mapping of *C. mrigala* skin mucus and obtained signals were C-43%, N-49.3%, O-5.2% and S-2.5%. The elements C and N are the macronutrients for microbes (Diaz & Savage, 2007). The macronutrients are essential for microbes to maximize the growth (Reyna-Gómez *et al*., 2019). The bacterial growth was assessed by macronutrients and bacteria utilize carbon for cell structure, nitrogen for protein synthesis and sulfur is a constituent for amino acids (Vintiloiu *et al*., 2012). These elemental compositions in the skin mucus contributed the bacterial growth in the present study.

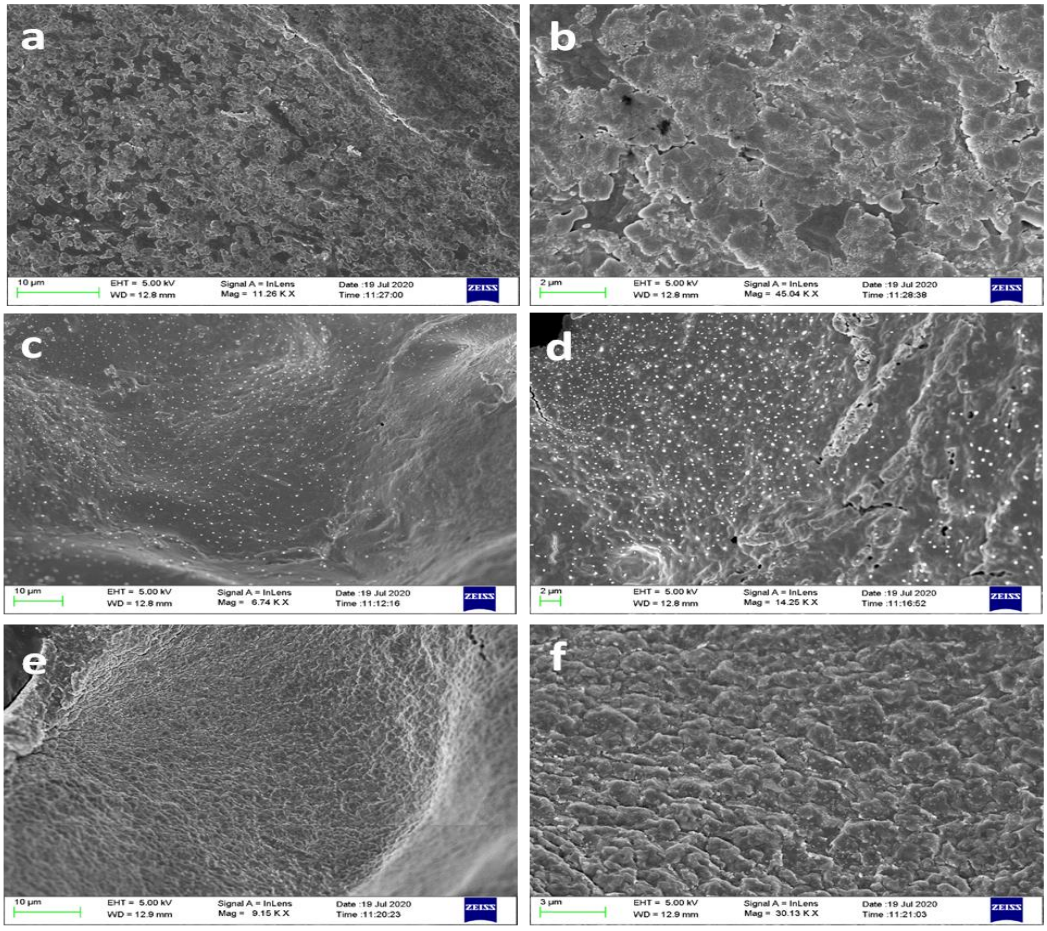

Figure 6. Topographical architecture of lyophilized fish skin mucus analysed through FE-SEM. Representative images of *Cyprinus carpio* (a &b), *Labeo rohita* (c & d) and *Cirrhinus mrigala* (e & f).

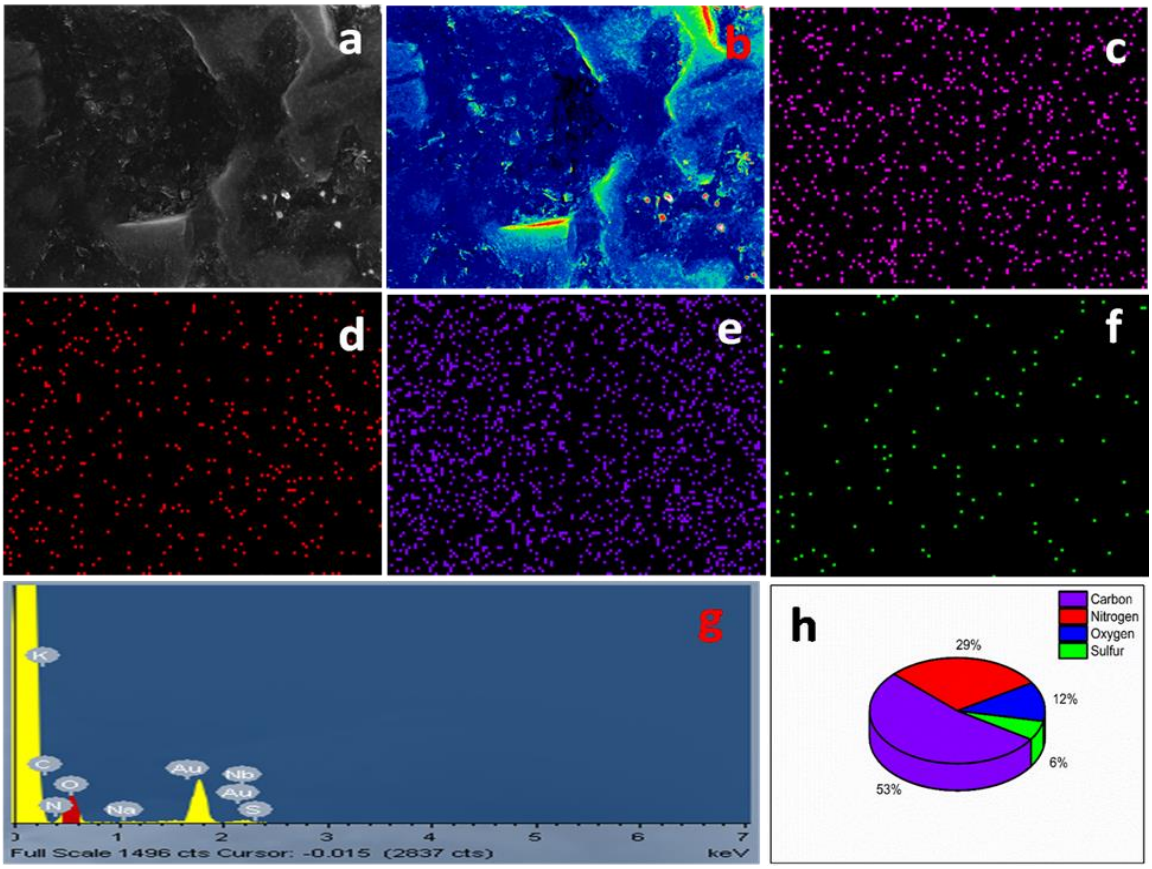

Figure 7. FE-SEM elemental mapping composition of lyophilized skin mucus of *Cyprinus carpio*. a) electron image, b) secondary electron image and analogous elemental mapping of the element, c) carbon, c) nitrogen, e) oxygen, f) sulfur, g) EDX spectrum of *Cyprinus carpio* lyophilized skin mucus, and H) atomic percentage of various elements.

Conclusion

The current study demonstrated that the bacteria can able to utilize nutrients from mucus as it contains carbon and nitrogen source for their growth. The present results suggest that the increased production of mucus during stressful conditions may contain less number of active immune molecules. If high density of pathogens adheres to the fish skin during abnormal conditions internally or externally, could invade the fish mucosal system and cause infections. Further studies in analyzing the compounds responsible for bacterial adherence on fish skin mucus provide biological insights on host-pathogen interactions.

Ethical Statement

Not applicable.

Funding Information

Not applicable.

Authors' Contribution

AS: Investigation, Methodology, Writing - original draft, review & editing. DBM: Investigation, Visualization. SP: Investigation, Resources, Data curation. RKS: Investigation, Resources. TR: Conceptualization, Project administration, Supervision, Validation, Writing - review & editing.

Conflict of Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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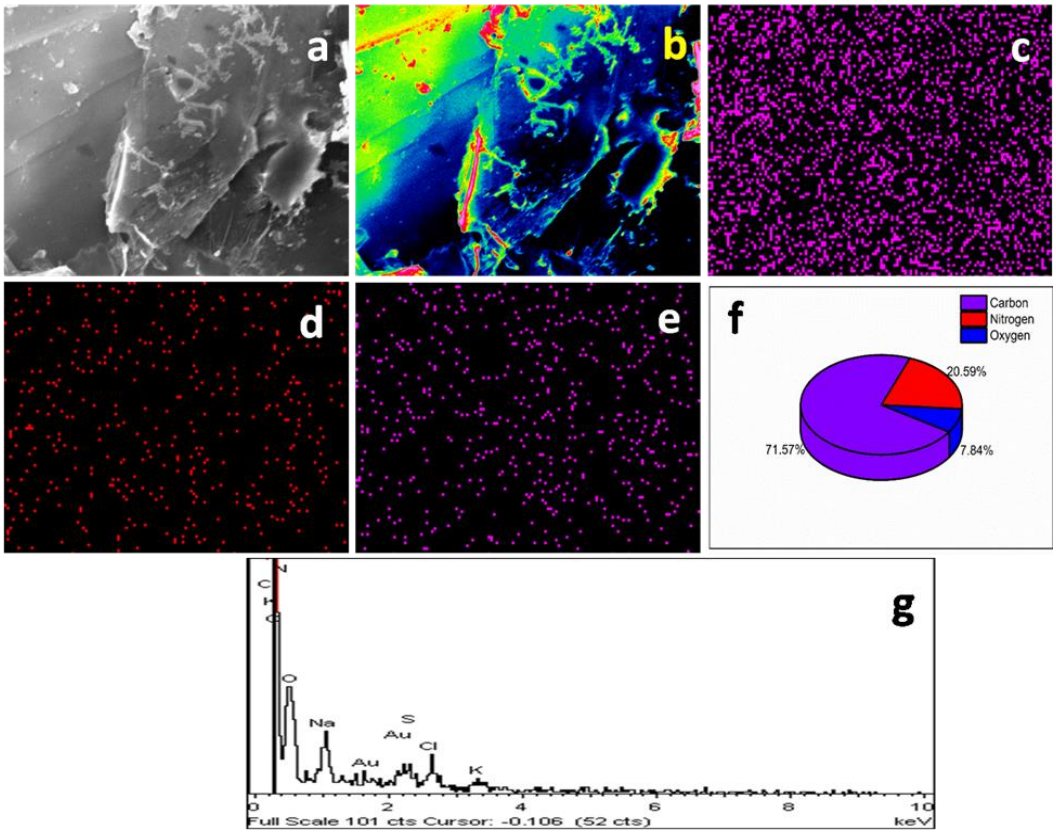

Figure 8. FE-SEM elemental mapping composition of lyophilized skin mucus of *Labeo rohita*. a) electron image, b) secondary electron image and analogous elemental mapping of the element, c) carbon, d) nitrogen, e) oxygen, f) atomic percentage of various elements, and g) EDX spectrum of *Labeo rohita* lyophilized skin mucus.

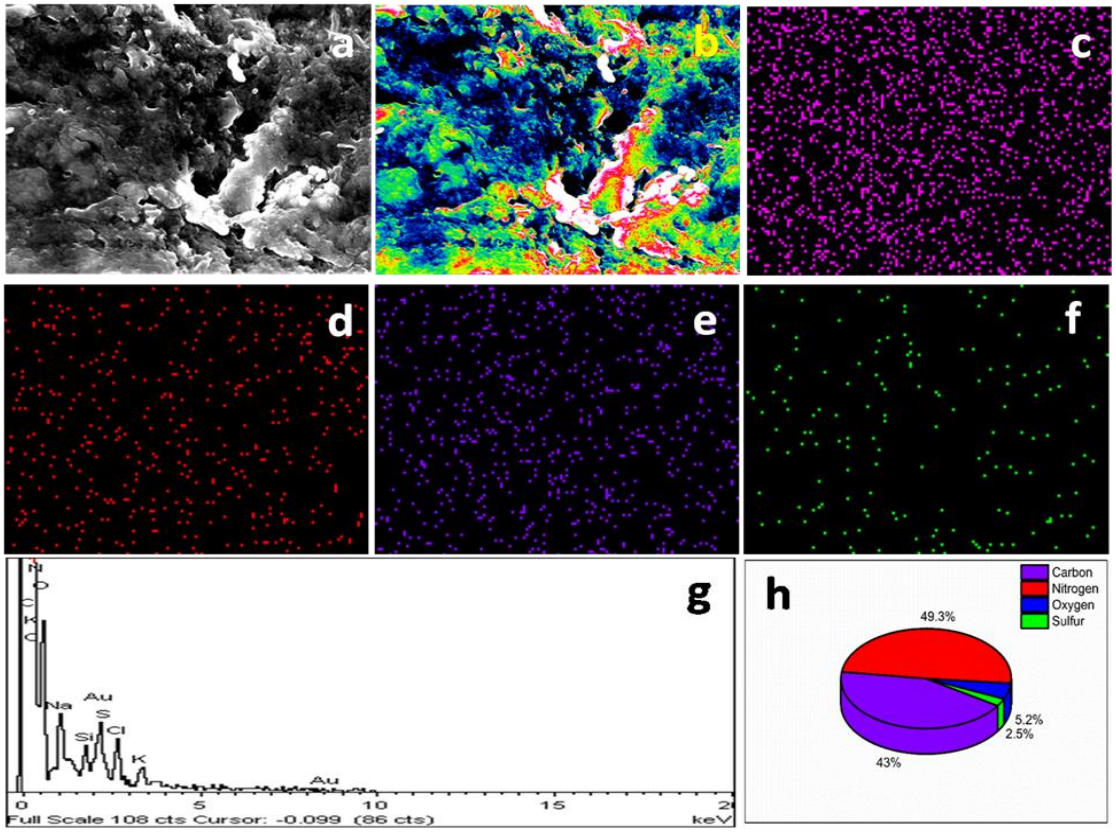

Figure 9. FE-SEM elemental mapping composition of lyophilized skin mucus of *Cirrhinus mrigala*. a) electron image, b) secondary electron image and analogous elemental mapping of the element, c) carbon, d) nitrogen, d) oxygen, f) sulfur, g) EDX spectrum of *Cirrhinus mrigala* lyophilized skin mucus, and h) atomic percentage of various elements.

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