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ASSESSMENT OF BACTERICIDAL ROLE OF EPIDERMAL MUCUS OF MAJOR CARPS AGAINST PATHOGENIC MICROBIAL STRAINS

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ABSTRACT:

This research evaluated the antimicrobial potential of mucus from major carps, specifically *Cirrhinus mrigala* and *Labeo rohita*, against *Klebsiella pneumoniae* and *Escherichia coli.* Fish weighing 300-350 grams were stoked and acclimated for 15 days, fed with commercially available diet at 4% of their body weight. Fish were treated with $KMnO_4$ solution to avoid any infection before mucus collection and collected mucus was stored at 4°C. Mucus extracts were screened for antibacterial potential using the agar well diffusion method, measuring antibacterial effects by the zone of inhibition (ZOI) in mm. *L. rohita* secreted more mucus than *C. mrigala.* The mucus appearance of *L. rohita* was highly viscous, while less viscous *in C. mrigala.* In *L. rohita mucus,* maximum antimicrobial efficiency was observed. Results showed greater efficacy in limiting the growth of *E. coli* with zone of inhibition (16mm). Mucus is a key defense against disease. Fish skin mucus can serve as an alternative to antibiotics for use in aquaculture and potentially for human application. As a natural product, it may help reduce problems associated with antibiotic resistance.

KEYWORDS*:* Major Carps, Antibacterial Activity, *Klebsiella Pneumoniae*, *Escherichia Coli*, And Mucous

1. INTRODUCTION

Water is essential for the survival of all life forms and supports human welfare (existence, food production, economic development). It constitutes about 3% of the world's total supply, but only 0.5% of this freshwater is accessible. Pakistan is one of the most affected countries by severe water pollution. Pakistan's drinking water is frequently contaminated with harmful metals and microorganisms, often exceeding the World Health Organization's (WHO) safety standards. Poor water quality management and monitoring pose a significant health risk to the community (Noor *et al*., 2023).

Fish are more vulnerable to diseases in a swarming, mostly artificial environment. Fish with immature immune systems are more susceptible to illness during their initial stages. As a result, disease outbreaks are a problem for the aquaculture sector, decreasing global aquaculture productivity. Copper exposure also induces stress in the fish gills (Hoseini and Al Sulivany, 2024). The most often-used method of treating illnesses caused by pathogenic microbes is the administration of drugs, chemicals, and antibiotics, which are employed to manage these illnesses. Fish health is negatively impacted by the buildup of these substances and drugs in the environment, which also damages the aquatic environment (Kumar *et al* al., 2022).

Microbes can have both positive and negative effects on the health of humans and other living things. Antibiotics may eradicate microorganisms, but the more often they are exposed, the more resistant they become. Microbes can modify and change the chemicals employed against them, resulting in antibiotic resistance. The need for novel antimicrobials increases when bacteria resistance increases. Because they come into regular touch with dangerous bacteria in aquatic habitats, fish can develop immunological defense systems. Fish contain a variety of antibacterial substances distributed throughout their body as a component of their defense mechanisms (Hussain *et al*., 2023). Fish are more vulnerable to diseases in a swarming, mostly artificial environment. Fish with immature immune systems are more susceptible to illness during their initial stages. Higher salinities adversely impact physiological health and growth performance (Owis *et al*., 2024). As a result, disease outbreaks are a problem for the aquaculture sector, decreasing global aquaculture productivity. The most often-used method of treating illnesses caused by pathogenic microbes is the administration of drugs, chemicals, and antibiotics, which are employed to manage these illnesses. Fish health is negatively impacted by the buildup of these substances and drugs in the environment, which also damages the aquatic environment (Kumar *et al*., 2022).

Skin mucous acts as the immune system's first primary protection from bacteria encountered in water. The mucus layer on the surface of the skin serves as both a physical and chemical barrier to keep out harmful microorganisms (Hussain and Sachan, 2023). Fish skin mucus can be utilized as an alternative of antibiotics which perhaps could be employed in aquaculture and also for humans. Being a natural product, therefore, it could help in reducing the problems of antibiotic resistance (Qamer *et al*., 2023). Mucus on the skin of fish assists in preventing bacteria, fungi, and parasites from colonizing their bodies. A wide variety of polypeptides with antibacterial properties are found in skin secretions. Mucus contains bioactive chemicals that provide fish with instant defense against possible infections. These substances include lysozyme, proteolytic enzymes, lectins, flavoenzymes, C-reactive proteins, immunoglobulins, a protein called A-1, and antibacterial

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peptides (Ranjini *et al.,* 2020). Innate immunity is a crucial survival strategy for fish in their early stages of life. Afterward, through various receptor proteins, innate immunity plays an instructive function in acquired immune responses (Kumar *et al*., 2022). Innate immune components, along with immunoglobulins, give fish skin mucus its antibacterial strength (Bhatnagar and Budhalia, 2022).

Fish epidermal mucus serves a variety of functions, including reducing pathogen attacks and preventing mechanical abrasion. Because of its multifunctional barrier made up of many cellular and humoral components, epidermal mucus is known to help fish survive, especially when it comes to fighting against invasive diseases. Over time, additional fish species have been studied, and more sophisticated techniques have been created in the ongoing study of the antibacterial characteristics of fish skin mucus (Lee *et al*., 2020).

Skin mucus' composition contains three distinct types of cells: sacciform cells, goblet cells, and club cells. Glycoproteins and mucus granules are produced by goblet cells, which are widely distributed on the surface of the gills as well as all exterior surfaces. Club cells mostly release proteinaceous components, whereas sacciform cells combine their fluids with those produced by goblet cells (Tiralongo *et al*., 2020). Glycoproteins, known as mucins, are the main macromolecules that produce gels in fish mucus. For injuries to heal, mucin is essential. Mucin is made up of a protein backbone to which threonine and serine residues are joined by O-glycans (Benktander *et al*., 2021).

2. MATERIALS AND METHODS

Experimental Fish

For the experiment, fish (*C. mrigala* and *L. rohita* weighing between 300 to 350 grams were procured from the Fish Hatchery on Satyana Road Faisalabad and stocked into cemented tanks.

Acclimatization

Prior to the trial, fish were acclimatized to laboratory conditions for 15 days and fed with a commercial diet containing 32% of CP at 4% of their body weight. Physiochemical parameters (pH, DO, and Temperature) were monitored and controlled daily (APHA, 1998).

Table 1:Control Diet Composition.

Feed Ingredients (g)	Control diet
Fish meal	25
Sunflower meal	18
Gluten	50
Wheat bran	1.7
Rice bran	1.7
Vitamin premix	1.3
Canola oil	2.3
Total	100g

Mucus Collection

At the end of the trial, from the six fish three of them randomly were picked out. Without anesthetizing, fish mucus was collected. Using a sterile spatula, mucus was carefully scraped off the dorsal side of the body, working from the head to the tail in an anterior-posterior manner. One millimeter of mucus was amassed periodically for twenty minutes in five attempts from each fish of the two species. Mucus was individually extracted from each fish species into sterilized tubes (Balasubramanian et al., 2012). The samples were then centrifuged at 5000rpm for 20 minutes, and the supernatant was then stored at 40C to inhibit the growth of bacteria (Kumari *et al*., 2019).

Preparation Of Mucus Extract

The acidic extract of fish mucus was manufactured by applying an ameliorated protocol of Subramanian et al. A mixture of fish mucus, and 3% acetic acid was prepared using 1ml of mucus combined with 0.5mL of acetic acid. A 1:0.5 mixture of fish mucus and 3% acetic acid was prepared. The samples were subsequently placed in a boiling water bath for 5 minutes. The acidic mixture was chilled, vortexed for 30 seconds, and then centrifuged at 18,000 rpm for 35 mins at 4^0C. The supernatant was then collected into sterilized Eppendorf tubes using a micropipette. The mucus was purified with a syringe having a 0.45µm filter. The combined filtrate was put into sterile Eppendorf tubes and refrigerated at 4^0C (Lee *et al*., 2020).

Antibiotic Preparation

Gentamicin was used as a positive control for the antibacterial assay. A 5 mg/mL gentamicin solution was prepared by dissolving 5 mg of gentamicin with 1 ml of sterile water and vortexing the mixture for 15 minutes (Devi et al., 2019).

Pathogenic Bacteria Strains

Table 2 provides detailed information on human and fish bacterial pathogens utilized in the study, including their respective sources.

Table 2: Human and fish bacterial pathogens and their sources

Test bacterial strains	Sources
(K.pneumoniae) CNMC *(E. coli)* CNMC	Biotechnology Lab, Bioinformatics Department GC University Faisalabad.

Preparation Of Bacteria

All bacterial strains were cultivated for 24 hours at 37°C in a nutritional broth culture (0.5% peptone, distilled water, 0.5% NaCl, pH 6.8, 0.3% beef extract) to prepare them for antimicrobial assays. In Muller Hinton agar, the concentration of the bacterial suspensions was set at 108 colony-forming units (108 CFU/ml) (Kumari *et al*., 2019). Pure bacterial strain was added to the nutrient broth, a 24-hour incubation period was conducted at 37°C. Following the 24-hour incubation period, the inoculum was calculated at 600 nm using a spectrophotometer, and colony-forming units were adjusted based on the McFarland standard (107 CFU/ml).

Agar Well Diffusion Assay

The mucus was measured by using the agar well diffusion method (Al-Rasheed et al., 2018).

Antibacterial Test

The nutrient agar was liquefied using distilled water to produce the nutrient broth media. This media was then sterilized in an autoclave at 121ºC for 15 minutes. Sterilized Falcon tubes were filled with 5 ml of this sterile broth. A single bacterial strain was inoculated onto nutrient agar plates. A center well on each plate was filled with gentamicin, a known antibiotic (positive control).Four surrounding wells contained the extracted mucus Samples. The final well was filled with 3% acetic acid, a negative control. Plates were incubated at 37ºC for 24 hours. The diameter of the zone of inhibition around each well was measured to assess the antimicrobial activity of the mucus samples following the Clinical and Laboratory Standards Institute (CLSI) guidelines from 2012.

Assessment Of Minimum Inhibitory Concentrations (MIC)

The MIC is the lowest drug concentration that stops bacterial growth. Determined by broth dilution method.

Overnight cultures of *E. coli* and *K. pneumoniae* isolates were grown and adjusted to a turbidity equivalent to a 0.5McFarland standard. One hundred microliters of Mueller Hinton broth were added to each of the twelve wells in a 96-microplate. 100µl of crude mucus from *L. rohita* was added to 1st well.

The mucus samples were serially diluted. A volume of 100µl of bacterial inoculum was added to each well up to the 12th well. The 11th well served as a positive control, containing only broth, and the 12th well was the negative control, containing bacterial suspension and broth. The microplates were incubated at 37 ˚C for 18-24 hours (Alekish *et al*., 2018). Following incubation, the viability of bacterial cells was determined by measuring absorbance at 650 nm using an ELISA reader.

Evaluation Of Minimum Bactericidal Concentrations (Mbcs)

MBC was determined following the protocol outlined by Sevinc and Hanley (2010). This involved sub-culturing 100µl of broth dilutions that showed no visible growth in broth, followed by incubation for 24-48 hours at 37°C. The lowest concentration displaying no evident growth on the broth plates was considered as the MBC

3. RESULTS

The trial aimed to investigate the antibacterial efficacy of the epidermal mucus of *C. mrigala* and *L. rohita* against common pathogenic bacteria.

Study Of Quantity And Physical Appearance Of Mucus

The carp species, including *L. rohita* and *C. mrigala*, produced varying amounts of mucus, with their appearance differing among species. The highest amount of mucus was collected from *L. rohita* as compared to *C. mrigala*. Fish skin mucus from major carp *L. rohita* was highly viscous and sticky, while the mucus from *C. mrigala* was bubbly and adhesive in texture, though less thick.

Table 3: Amount of mucus extracted from two major carp during a one-day scrapping session (5 trials at consistent intervals of 20 minutes).

Fish Species	Mucus amount Collected (ml)					
	No. of Attempts					
	1st	2nd	3rd	4th	5th	Total
C. mrigala (Fish1)	0.25	0.15	0.20	0.25	0.50	1.8ml
(Fish2)	0.30	0.20	0.25	0.30	0.60	1.6ml
(Fish3)	0.20	0.15	0.25	0.15	0.15	1.5ml
L. rohita (Fish1)	0.50	0.60	0.25	0.50	0.50	2.5ml
(Fish 2)	0.50	0.25	0.20	0.35	0.20	1.5ml
(Fish 3)	0.25	0.25	0.15	0.15	0.20	1.5ml

Antibacterial Activity of Major Carp's Mucus

To determine their antibacterial potential, the bacterial strains selected were Gram-negative (*E. coli* and *K. pneumoniae*). Our findings demonstrated the greatest zone of inhibition with Carp mucus against all isolates.

Control demonstrated more excellent resistance against the *E. Coli*. The zone of inhibition for *C. mrigala* was greater in *E. coli* than in *K. pneumoniae*. The skin mucus of *C. mrigala* demonstrated a more substantial effect in inhibiting the growth of Gram-negative bacteria *E. coli* and *K. pneumoniae*, with inhibition zones measuring 14mm in diameter for *E. coli* and 12mm for *K. pneumoniae. However*, positive control showed more excellent resistance against *K. pneumoniae.*

Table 4: Zone of inhibition of different pathogenic microbial strains by using *C. mrigala* and *L. rohita.*

Groups	Diameter of Zone of Inhibition(mm)	
	Gram (-ve)	
	E.coli	*K. pneumonia*
L. rohita	16mm	14mm
C. mrigala	15mm	12mm
Gentamicin (+ve control)	20mm	22mm

Gram-negative Bacterium *E. coli*

Mucus from major carp *L. rohita* mucus indicated the most potent antibacterial activity against *E. coli* with a zone of inhibition measuring 16mm in diameter, while *C. mrigala* displayed a zone of inhibition of 15mm in diameter. Gentamicin was employed as the positive control and displayed a 20mm zone of inhibition. Mucus from *L. rohita* demonstrated more potent antibacterial activity against *E. coli*, with a zone of inhibition of 16mm. However, this was lower than the positive control, measuring 20mm. The negative control,3% Tryptic Soya Broth (TSB), showed no results.

Gentamicin recorded the highest zone of inhibition at 20mm, while the lowest values were observed for *L. rohita* and *C. mrigala*, measuring 16mm and 15mm, respectively (Table 5

Table 5**:** Zone of inhibition of *E. coli* by using *C. mrigala* and *L. rohita*

Zone of inhibition against *E. coli*			
Samples	Z1	Z2	Z3
L. rohita	16mm	13mm	17mm
C. mrigala	15mm	12mm	14mm
Gentamicin	20mm	20mm	20mm

Table 6: Comparison of means Zone of inhibition by carp mucus against *E. coli*

E. coli		
Fish Mucus	Concentration	Inhibition Zone (mm)

L. rohita	2:1(mucus: 3% TSB)	15.33 ± 2.08^{A}
C. mrigala	2:1(mucus: 3% TSB)	13.66 ± 1.52^{A}
Control (Gentamicin)	50µL	20 ± 0.09^{A}

The means were compared using Tuckey's Test in SPSS; the means sharing a similar letter in uppercase are statistically non-significant (P>0.05). Non-significant variation (P<0.01) was observed in *L. rohita* when means were compared (Table 6).

Gram-Negative Bacterium *Klebsiella Pneumonia*

Mucus from major carp (*L. rohita*) demonstrated the highest antimicrobial activity against *K. pneumoniae*, with a growth inhibition zone measuring 14mm in diameter, while *C. Migala* showed an inhibition zone of 12mm in diameter. Gentamicin was used as a positive control and was more effective against *K. pneumoniae*, with a growth inhibition zone diameter of 22mm.

Table 7: Zone of inhibition of *K. pneumoniae* by using *C. mrigala* and *L. rohita*

Zone of inhibition against *Klebsiella pneumonia*			
Samples	**Z1**	**Z2**	**Z3**
L. rohita	14mm	16mm	13mm
C. mrigala	12mm	10mm	13mm
Control	22mm	22mm	22mm

Gentamicin (control) had the highest zone of inhibition at 22mm, while the lowest values for *L. rohita* and *C. mrigala* were 14mm and 12mm, respectively (Table 7).

Table 8: Comparison of means of Zone of inhibition by carp mucus against *K. pneumoniae*

Klebsiella pneumonia		
Fish Mucus	Concentration	Inhibition Zone (mm)
L. rohita	2:1(mucus: 3% TSB)	14.333 ± 1.52^{A}
C. mrigala	2:1(mucus: 3%TSB)	11.66 ± 1.52^{A}
Control	50µL	22 ± 0.10^{A}

The means were compared using Tuckey's Test in SPSS; the means sharing a similar letter in uppercase are statistically non-significant (P>0.05). Non-significant variation (P<0.01) was observed in *L. rohita* when means were compared (Table 8)

Mic Assay

The skin mucus extract of *L. rohita* demonstrated minimum inhibitory concentration (MIC) at 50µl/ml against *K. pneumoniae*, and *E. coli* was 25µl/ml.

The minimum mucus concentration ranging from 25µl/ml to 50µl/ml was effective in inhibiting the growth of the selected bacterial pathogens (Table 9).

Table 9: MIC of *L. rohita* against all bacterial strains

L. rohita (MIC)	
Bacterial Strains	MIC(µl/ml)
K. pneumoniae	50 µl/ml
E. coli	25µl/ml

Mbc Assay

The *L. rohita* mucus extract demonstrated a minimum bactericidal concentration (MBC) of greater than 100 µl/ml against *K .pneumoniae and E. coli*, specifically at 100 µl/ml.

A minimum concentration of mucus ranging from 100µl/ml to > 100µl/ml was effective in killing the bacterial pathogens ultimately (Table 10).

Table 10: MBC of *Labeo rohita* against bacterial strains

***Labeo rohita* (MBC)**	
Bacterial Strains	MBC(µl/ml)
K. pneumoniae	> 100 µl/ml
E. coli	100µl/ml

4.DISCUSSION

The antimicrobial characteristics of fish epidermal mucus have long been known. However, previous investigations have focused on how it affects marine strains of bacteria rather than examining how it affects human health. Fish might be an excellent resource for producing novel and effective antibacterial chemicals. The current study exposed that all the examined major carp species, *C. mrigala*, and *L. rohita*, secreted large amounts of mucus, with the volume of secretion varying between the two species. Qamer *et al*., (2023) demonstrated that carp mucus plays an important role in protection of fish against the invasion of pathogens.

Observations indicate that body weight and length significantly influence the secretion of mucus in fish. These findings align with the research conducted by Kumari *et al*. (2019), showing that large volumes of mucus were produced by the three carp species under examination, *Hypophthalmichthys nobilis*, *Cyprinus carpio*, and *Ctenopharyngodon idella*, with the amount of secretion varying between them. According to Wang *et al*. (2019), fish skin mucus functions as an effective physicochemical barrier containing immunoglobulins, protease, glycoproteins, lysozyme and antimicrobial peptides (AMP). In fish, epidermal mucus contains lysozyme and protease, two antibacterial enzymes that play an essential role in immune function. Significantly, the levels of lysozyme and protease in the skin mucus of *A. clarkii* are much lower than those observed in other marine fish species like *Dicentrarchus labrax*, *Epinephelus marginatus*, *Sparus aurata*, and *Umbrina cirrosa*. Habitat and genetic adaptations might influence enzyme function. Nigam *et al.* (2017) reported that fish skin mucus extracts derived from the investigated fish species exhibited antibacterial potency against a diverse range of pathogenic bacteria as evidenced by inhibition zones measuring between 6 to 12 mm. Variations in inhibition zone reported for the same fish species by various researchers could be attributed to variations in the ecological conditions of the habitat or differences in the methods used for antibacterial assays. Nevertheless, the zone of inhibition values observed in our study for the same microbes were more significant. As in our studies, *L. rohita* mucus extract showed a zone of inhibition measuring 16 mm, meaning it was effective at preventing bacterial growth to a moderate extent. Gentamicin, a standard antibiotic, exhibited a 20mm zone of

inhibition. In comparison, the mucus extract from *C. mrigala* showed the minor zone of inhibition at 15 mm, indicating it was the least effective against *E. coli*. Against *K. pneumonia*, mucus from *C. mrigala* showed an inhibition zone at 12 mm while demonstrating antibacterial activity was less effective compared to mucus from *L. rohita* and the positive control.

Lirio *et al*. (2019) claim that antibacterial ingredients work in a non-specific manner, causing target bacteria's cell membranes to become porous, allowing cell contents to seep out, and finally causing cell death. Thus, it seems sensible to speculate that the potent antibacterial activity shown may be explained by the pore-forming characteristics of antimicrobial chemicals in skin mucus. According to the study, *L. rohita* shows more antibacterial activity than *C. mrigala* because the mucus of *L. rohita* contains more antibacterial compounds. These findings align with the study by Ali *et al.* (2023), which showed that acidic skin mucus extract from *C. catla*, *C. idella*, and *L. rohita* exhibit high bactericidal activity, suggesting that these fish produce mucus with significant antibacterial properties. It also indicates that the skin mucus can be developed as an affordable remedy and used as an antimicrobial agent with a lower chance of resistance. Bhatnagar *et al*. (2021) found that epidermal mucus plays a crucial role in the natural defense mechanisms. This research further confirmed that fish epidermal mucous is a valuable source of antibacterial compounds.

CONCLUSION

All in all, the study shows that fish mucus has the potential to be an antibacterial source for both human and fish pathogens. Being a natural product, fish mucus could potentially combat antibiotic resistance and offer a cost-effective solution. The effective use of antimicrobial substances found in fish epidermal mucus may help address the global problem of antibiotic resistance, which is 62 endangering technological advances and lifespans. Thus, the fish skin mucus can be used to treat bacterial illnesses in both humans and fish instead of using antibiotics.

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