

Antimicrobial Action of Epidermal Mucus Extract of *Clarias gariepinus* (Burchell, 1822) Juveniles-Fed Ginger Inclusion in Diet

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Abstract

The antimicrobial activity of epidermal mucus extract of *C. gariepinus* juveniles-fed ginger inclusion in diet was investigated and compared with the activity of epidermal mucus extract of *C. gariepinus* juveniles (control) without ginger in diet. This study demonstrates the antimicrobial role of ginger in improving protection of fish against bacterial infection as shown by the higher zones of inhibition observed for epidermal mucus extract of fish-fed ginger in diet as compared with control. Zones of inhibition for epidermal mucus of treatment fish were 30.7 mm, 29.8 mm, 26.3 mm and 19.3 mm for *Bacillus*, *Escherichia*, *Staphylococcus* and *Streptococcus* species respectively. Though these values were not significantly ($P > 0.05$) higher than those obtained for the control fish with zones of inhibition of 25 mm, 11.2 mm, 9.0 mm and 7.3 mm for *Bacillus*, *Escherichia*, *Staphylococcus* and *Streptococcus* species respectively, the higher values recorded for the treatment fish shows that ginger inclusion in fish diet had an antibiotic effect against isolates of bacteria in fish samples from cultured ponds. The addition of ginger in *C. gariepinus* diet is encouraged as its action is indicative of the potentials of ginger in preventing emergence of resistant bacteria and improving the antimicrobial role of fish mucus and therefore the quality of *C. gariepinus*.

Keywords: antimicrobial, *Clarias gariepinus*, epidermal mucus, ginger

1. Introduction

1.1 The Problem

All fish live in microbe-rich environment and are vulnerable to invasion by pathogenic and opportunistic micro-organisms. The environment of fish being aquatic is very challenging with fish in constant interaction with a wide range of pathogenic and non-pathogenic micro-organisms (Subramanian, Mackinnon, & Ross, 2007). Environmental degradation due to pollution of natural water bodies and poor culture conditions of some culture fish ponds have elicited the presence of these pathogenic agents (Nwabueze, 2011, 2012) with bacteria being one of the most common micro-organisms of farmed catfishes.

1.2 Importance of the Problem

Fish is sometimes overwhelmed by these pathogenic agents and sometimes succumb to infections. In culture ponds, especially in the tropics, a number of antimicrobial agents have been used to combat several diseases of fish. In recent times though, an increase in the antibiotic resistant strains of some of these micro-organisms have been observed. Antimicrobial activity has been demonstrated in fish mucus of several fish species, yet this activity seems to vary from fish species to species such as rock fish (*Sebastodes schlegelii*), rainbow trout (*Oncorhynchus mykiss*) and tilapia (*Tilapia hornorum*) and can be specific toward certain bacteria (Noya, Magarinos, Toranzo, & Lamas, 1995). *Clarias gariepinus* is a valuable farmed food fish in Nigeria. Its high nutrition and importance in aquaculture have encouraged a lot of researches in the improvement of the quality of the fish species.

1.3 Relevant Scholarship

Increase in antibiotic resistance of some microbial strains has lead to investigations on the use of traditional plants for their antibacterial and medicinal values (Bhalodia & Shukla, 2011). Fishes produce mucus from the epidermal cell layer for protection against some of these pathogenic agents in the aquatic medium. Fish epidermal mucus has been reported to be a component of innate immunity playing an important role in the

prevention of colonization by parasites, bacteria and fungi (Muroga, Higashi, & Keitoku, 1987; Kanno, Nakai, & Maroga, 1989; Ebran, Julien, Orange, Auperin, & Molle, 2000). Fish epidermal mucus has also been known to contain a variety of biologically active compounds and antimicrobial peptides that are constitutively expressed to provide immediate protection of fish from potential pathogenic microbes and parasites (Hjelmeland, Christe, & Raa, 1983). Kasai, Ishikawa, Komata, Fukuchi, Chiba, Nozaka, Nakamura, Sato and Miura (2009) reported an antibacterial protein l-amino acid oxidase (LAAO) from the epidermal mucus of flounder *Platichthys stellatus* which exerted antibacterial activity against *Staphylococcus epidermidis*, *S. aureus* and methicillin-resistant *S. aureus*. It has also been noted that this antibacterial protein exerted antibacterial activity in a variety of animal fluids such as snake venom (Du & Clemetson, 2002), fish epidermal mucus and extract (Jung, Mai, Iwamoto, Arizono, Fujimoto, Sakamaki, & Yonehara, 2000; Kitani et al., 2007; Kitani, Kikuchi, Zhang, Ishizaki, Shimakura, Shiomi, & Nagashima, 2008) body surface mucus of the giant African snail (Obara, Otsuka-Fuchino, Sattayasai, Nonomura, Tsuchiya, & Tamiya, 1992). It is therefore important to consider ways of improving fish health by using biological agents to prevent infections rather than using drugs to provide cure. Ginger plant is a spice that has been noted for its medicinal values as an analgesic, sedative, antipyretic and antibacterial agent (Patrick-Iwuanyanwu, Wegwu, & Ayalogu, 2007). Ginger also has anti-microbial, anti-oxidative and seasoning qualities.

1.4 Hypothesis

This study examines the antimicrobial activity of epidermal mucus extract of *C. gariepinus*-fed ginger inclusion in diet, to know if ginger can enhance protection in fish as expressed by the epidermal mucus extract of cultured *C. gariepinus*.

2. Materials and Methods

2.1 Study Area

The antibiotic activity of epidermal mucus extract of *C. gariepinus* (average weight of 40.6 ± 0.1 g and total length of 17.2 ± 0.4 cm) juveniles-fed ginger inclusion in diet was investigated from January to May, 2013 at the Department of Fisheries and Faculty of Agriculture Research Laboratories of Delta State University, Asaba Campus, Asaba, Nigeria.

2.2 Acclimation and Experimental Tanks

Sixty juvenile fish were obtained from the Faculty of Agriculture Research Farm and acclimated for 7 days in stock tank during which time fish were fed commercially available feed at 4% body weight. Stock tank ($45\text{ cm} \times 45\text{ cm} \times 90\text{ cm}$) was well aerated and contained 50 litres of borehole water, tank water temperature was 26.4°C and tank water was changed twice. Ten apparently healthy fish samples each distributed into two smaller tanks ($40\text{ cm} \times 40\text{ cm} \times 60\text{ cm}$) containing 25 litres of water and labelled, A (control) and B (Treatment) with two replicates of A_1, A_2 and B_1, B_2 making up a total of 60 *C. gariepinus* fish were used.

2.3 Preparation of Experimental Diet

Ginger root was bought from a local market in Asaba, back was peeled off and 100 g ginger was cut into bits and sun-dried for 5 days and later ground into powder form using an electric blender, sieved and stored in container away from moisture and direct sun light according to Stoll (2000) until further use. Fish in experimental tanks were fed formulated diet with fish in the treatment tanks B, B_1 and B_2 having an addition of 0.5% (0.025 kg) inclusion of ginger in fish diet (Table 1). Fish were fed for 4 months after which the epidermal mucus extract of fish in control and treatment tanks were collected and examined for their antimicrobial activities.

Table 1. Composition of experimental diets

Ingredients	Control diet composition (kg)	Treatment diet composition (kg)	Concentration of diets (%)
Groundnut cake	0.78	0.75	15.0
Soya bean	0.78	0.75	15.0
Fish meal	0.78	0.75	15.0
Vitamin C	0.026	0.025	0.50
Bone meal	0.21	0.20	4.0
Premix	0.10	0.10	2.0
White maize	0.52	0.50	10.0
Wheat (rice bran)	0.52	0.50	10.0
Palm oil	1.46	1.4	28
*Ginger	-	0.025	0.50

*Added only for fish in treatment tanks.

2.4 Isolation and Identification of Bacteria Isolates

Bacterial isolates were obtained from epidermal mucus of apparently healthy 6 months old live *C. gariepinus* fish weighing about 480 g, harvested from a culture pond in Asaba. Mucus samples were carefully and aseptically scraped from the dorsal surface of fish skin using a sterile soft rubber spatula. The aqueous mucus scrapings were pooled and cultured on Sabouraud Dextrose agar at 37 °C for 24 h. Distinct growth colonies observed were sub cultured into sterile Petri dishes with Nutrient agar by streaking method to obtain pure isolates which were incubated aerobically at 37 °C for 24 h. A growing edge of distinct isolates was picked from pure cultures and streaked with inoculating loop onto sterile Petri dish with Nutrient agar as the growth medium for each of the isolates. Isolates were identified using routine Gram Staining Techniques and Biochemical Characterization according to MacFaddin (1980). Bacterial isolates obtained were *Staphylococcus*, *Streptococcus*, *Escherichia* and *Bacillus* species. Each pure colony was suspended in physiological saline (0.85% NaCl) and standardized to correspond to 1.5×10^8 CFU/ml of each bacterial isolates.

2.5 Preparation of Plate Inoculums

Fresh sterile cotton-tipped swap was dipped into suspension of pure bacterial isolates and was used to inoculate sterile Mueller-Hinton Agar plates by streaking and ensuring even distribution of the inoculums. This was done for each bacterial isolates.

2.6 Collection and Sterilization of Fish Epidermal Mucus

Mucus from the control and experimental fish samples was carefully scraped from the anterior to the posterior of the dorsal body using a sterile soft rubber spatula. Mucus scrapings were pooled from the ten experimental and also pooled from the ten control fish separately. Mucus samples were then thoroughly mixed with equal quantity of sterilized physiological saline (0.85% NaCl) and centrifuged at 5000 rpm for 15 minutes according to Kuppulakshmi, Prakash, Gunasekaran, Manimealai and Sarojini (2008). The supernatant was collected and stored at 4 °C and were used to prepare the antibiotic disc for the antimicrobial studies.

2.7 Preparation of Antibiotic Disc

Sensitivity disc was prepared employing pipette delivery to impregnate 20 µl of the epidermal mucus extract of control and treatment *C. gariepinus* fish onto a disc using disc diffusion method (Cavalieri et al., 2005). Discs were firmly pressed down to ensure complete level contact with agar.

2.8 Sensitivity Testing

After the introduction of the disc, the plate was allowed to incubate at 37 °C for 24 h. Zones of inhibitions were measured to the nearest millimetres. Sensitivity testing was carried out on epidermal mucus extract of fish from control and treatment tanks on the bacterial lawn for the different isolates. A clear zone of inhibition (plaque) indicates absence of bacterial growth while no discernable plaque around the disc, means that the bacteria are growing normally. The presence of a plaque means sensitivity while the absence of a plaque means resistance.

2.9 Statistical Analysis

Student ‘t’ test statistic was employed to analyze data collected for zones of inhibition of the four different strains of bacteria isolated from the fish epidermal mucus from both the control and treatment tanks. Differences between means were considered significant when $P < 0.05$.

3. Results

Bacterial isolates used for this study were *Staphylococcus*, *Streptococcus*, *Escherichia* and *Bacillus* species. All bacteria isolates were sensitive to epidermal mucus extract of fish from both control and treatment experiments. Zones of inhibition of epidermal mucus extract of control and treatment fish against bacterial isolates are presented in Table 2. The resulting zones of inhibition for epidermal mucus of treatment fish were 30.7 mm, 29.8 mm, 26.3 mm and 19.3 mm for *Bacillus*, *Escherichia*, *Staphylococcus* and *Streptococcus* species respectively. While for the control fish values of 25 mm, 11.2 mm, 9.0 mm and 7.3 mm were zones of inhibition for *Bacillus*, *Escherichia*, *Staphylococcus* and *Streptococcus* species respectively. *Bacillus* species was observed to be more sensitive to the activity of ginger than the other isolates. *Streptococcus* species was the least sensitive. The same trend was also observed for epidermal mucus of the control fish.

Zones of inhibition were observed to be higher in the epidermal mucus of fish-fed ginger inclusion in diet as compared with epidermal mucus of control fish without ginger in diet. Zones of inhibition of the four different strains of bacteria isolated from fish epidermal mucus from both the control and treatment tanks were analyzed separately. Results of analysis however show that there were no significant difference ($P > 0.05$) in the zones of inhibition of epidermal mucus of control fish as compared with the epidermal mucus of treatment fish (Table 3). In this study, the mucus extracted from *C. gariepinus* (control fish) showed inhibitory effect on selected bacteria isolates. However, *C. gariepinus*-fed ginger in diet had more inhibitory effect on the selected bacteria isolates.

Table 2. Zones of inhibition of epidermal mucus of *C. gariepinus* against bacteria isolates

S/n	Bacterial Isolates	Zones of Inhibition for Control and Experimental fish mucus (mm)			
		Experimental Tanks (Control)	Epidermal mucus of Control fish	Experimental Tanks (Treatment)	Epidermal mucus of Treatment fish
1	<i>Staphylococcus</i>	Tank A	8.8	Tank B	26.4
		Tank A ₁	9.2	Tank B ₁	25.7
		Tank A ₂	9.1	Tank B ₂	26.1
2	<i>Streptococcus</i>	Tank A	7.8	Tank B	18.5
		Tank A ₁	7.1	Tank B ₁	20.2
		Tank A ₂	6.9	Tank B ₂	19.3
3	<i>Escherichia</i>	Tank A	11.0	Tank B	30.3
		Tank A ₁	11.1	Tank B ₁	28.7
		Tank A ₂	11.4	Tank B ₂	30.0
4	<i>Bacillus</i>	Tank A	24.7	Tank B	31.3
		Tank A ₁	26.1	Tank B ₁	30.8
		Tank A ₂	24.3	Tank B ₂	29.9

Table 3. Results of analysis of zones of inhibition of epidermal mucus of control and treatment fish

S/n	Bacteria isolates	T Statistics	T Critical
1	<i>Staphylococcus</i>	- 53.5674	4.302653
2	<i>Streptococcus</i>	- 16.9336	4.302653
3	<i>Escherichia</i>	- 37.5034	4.302653
4	<i>Bacillus</i>	- 10.266	4.302653

T test: Paired Two Sample Means (two-tailed).

4. Discussion

The role of epidermal mucus as an important component of fish innate immunity was demonstrated in this study with fish epidermal mucus being a potential source of antimicrobial activity for specific fish pathogens. This fact is evidenced by the clear zones of inhibition observed for the epidermal mucus of the control fish signifying absence of bacterial growth thereby indicating that fish mucus has antimicrobial properties which prevented bacterial growth. This fact could be attributed to a complex system of innate defence mechanisms enabling fish epidermal mucus to have a potential broad spectrum-antimicrobial activity. Balasubramanian, Baby, Arul, Prakash, Senthilraja and Gunasekaran (2012) noted that despite an intimate contact with high concentrations of pathogens (bacteria and viruses) in their environment, the fish can still maintain a healthy system under normal conditions. Fish skin is a complex limiting structure providing mechanical, chemical and immune protection against injury and pathogenic micro-organisms (Fontenot & Neiffer, 2004). Many researchers have proved that mucus exhibits good resistance to invading pathogens (Fletcher, 1978; Ingram, 1980; Austin & McIntosh, 1988; Fouz, Devaja, Gravningen, Barija, & Tranzo, 1990). Fish mucus layer confers an innate immune protection against pathogen entry. Mucus covering fish surfaces exposed to water acts as an innate and adaptive first line of defence against pathogen entry (Shephard, 1994).

In fish, the epidermal mucus is considered a key component of innate immunity. The composition and rate of mucus secretion has been observed to change in response to microbial exposure or to environmental fluctuations (Ellis, 2001). Raj et al. (2011) reported that skin mucus removal and epidermal lesions in *Cyprinus carpio* in invitro experiments enhanced the entry of CyHV-3 virus while the presence of skin mucus of *Cyprinus carpio* conferred protection against the entry of the virus. Numerous studies on innate immunity in fish have shown that fish epidermal mucus can inhibit the growth of some bacteria and therefore may have a potential source of novel antimicrobial components in it (Wei, Xavier, & Marimuthu, 2010). Kasai et al. (2009) observed inhibition of growth of *Staphylococcus epidermidis* and *S. aureus* and noted that the proliferation of *S. epidermidis* in particular was strongly suppressed, the effect being most marked among all the bacteria strains studied.

The antibacterial activity of fish mucus may be due to the presence of antibacterial glycoproteins which are able to kill bacteria by forming large pores in the target membranes (Ebran, Julien, Orange, Saglio, Lemaitre, & Molle, 1999). Fish mucus is believed to play an important role in the prevention of colonization by parasites, bacteria and fungi and thus act as a chemical defence barrier (Gobinath & Ravichandran, 2011).

Results show an antibiotic effect of ginger against isolates of bacteria in fish samples from cultured ponds. The antimicrobial role of ginger in improving protection of fish against bacterial infection was shown by the higher zones of inhibition observed for epidermal mucus extract of fish-fed ginger in diet when compared with epidermal mucus extract of control fish. Dugenci, Arda and Candan (2003) have shown that the rainbow trout fish fed with diets containing aqueous extracts of mistletoe (*Viscum album*), nettle (*Urtica dioica*), and ginger (*Zingiber officinale*) exhibited significant non-specific immune responses. Pandy (2013) also reported that all medicinal plants are able to stimulate only non-specific immune responses and suggested that vaccines might be a better way to prevent deadly diseases and as such the plants could be used as vaccine adjuvant. In addition, Idris, Omojowo, Omojasola, Adetunji and Ngwu (2010) while working on the effect of different concentrations of ginger on smoked-dried *C. gariepinus*, found that ginger reduced the free fatty acid values, trimethylamine values as well as reduced the fungi load of processed fish. The antimicrobial action of ginger has also been reported by Patel, Thaker and Patel (2011) in invitro studies using ginger in combination with honey against *Staphylococcus* isolates. Ginger definitely has antimicrobial properties and its use in the prevention of emergence of resistant bacteria is of high benefit.

5. Conclusion

This study has demonstrated the antibacterial role of fish mucus as shown by the higher zones of inhibition observed for mucus extract of fish-fed ginger in diet as against inhibition zones of mucus extract of fish without ginger in diet. The addition of ginger in *C. gariepinus* diet is encouraged as its action is indicative of the potentials of ginger in preventing emergence of resistant bacteria. Ginger inclusion in fish diet is beneficial in improving the antimicrobial role of fish mucus and hence the quality of *C. gariepinus*.

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