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10 *The Open Marine Biology Journal,* 2014, *8,* 10-16

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Comparative Study of Antibacterial and Haemolytic Activities in Sea Bass, European Eel and Blackspot Seabream

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Abstract: Sea bass (*Dicentrarchus labrax*, Moronidae), European eel (*Anguilla anguilla*, Anguillidae) and blackspot seabream (*Pagellus bogaraveo*, Sparidae) were studied to check their mucus, blood sera and tissue samples for antibacterial and agglutinating activity against a variety of Gram negative and positive bacteria. Samples were also examined for their haemolytic properties against sheep red blood cells.

The highest antibacterial activity was detected in the blood sera of blackspot seabream and European eel (against *Vibrio alginolyticus*) and in the kidneys of sea bass (against *Photobacterium damselae* subsp. *piscicida*). Haemolytic properties against sheep red blood cells were observed in the mucus of sea bass and blackspot seabream, as well as in the sera of eel and sea bass. The sera of sea bass and eel showed also agglutinating activity against *Pseudomonas aeruginosa* and *Staphylococcus aureus*; the mucus of sea bass was able to agglutinate isolates of *Vibrio fluvialis, V. alginolyticus* and *A. hydrophila*.

The study suggested that the mucus secretions, biological fluids and organs of the examined fish species can be regarded as an interesting source of bioactive compounds with antibacterial and haemolytic properties.

Keywords: Antibacterial properties, disease resistance, haemolytic activity, mucus, serum, Teleosts, tissues.

1. INTRODUCTION

Like other organisms living in aquatic environments, fish possess complex defense mechanisms to protect them from a wide range of pathogenic and non-pathogenic microorganisms. The immune system of fish is physiologically similar to that of higher vertebrates, although the main difference is that fish are free-living organisms already from early embryonic stages of life and depend on their innate immune system for survival, in contrast to higher vertebrates [1]. Key innate immune components of fish include the mucus layer on the skin, gills and gastrointestinal tract, and constituents of the blood such as phagocytes and natural killer cells. Particularly, skin mucus and gills are recognised as the first physical barriers to infections [2-4]. The mucus layer, which covers the fish surface and is secreted by the goblet or mucus cells in the epidermis, plays an important role against the skin colonization by bacteria, fungi and parasites. It represents not only a physical but also a biochemical barrier between fish and its aquatic environment, since it contains a variety of biologically active substances involved in fish innate immunity such as lysozyme, lectins, immunoglobulins, C-reactive protein, apolipoprotein A-I and antimicrobial peptides which protect fish from potential pathogens [2, 5, 6]. The mucosal barrier of the skin is an extremely important barrier to diseases, being fish constantly immersed in media containing potentially harmful agents [5].

The study of the mechanisms involved in the natural defence of fish against bacterial pathogens is of particular significance for fish welfare, since a direct relationship between the fish ability to counteract diseases (i.e. through own antibacterial properties) and health status has been established [7, 8]. So, the characterization of the antibacterial defense systems in these organisms is required in order to get insights on the complex mechanisms that regulate immunity and diseases. Previous studies have documented that secretions and tissues of Teleost fish possess antibacterial properties towards Gram-negative and positive microorganisms [9-11], and haemolytic properties [2]; nevertheless, the little availability of comparative studies on different species in the pertinent literature makes this subject a topic of growing interest. Moreover, search for natural antibacterial agents, able to control infectious diseases, is gaining importance in recent years [12], in relation to the

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1874-4508/14

widespread occurrence of antibiotic resistance phenomena [13]. In this context, a study has undertaken on samples of mucus, blood serum and organs (kidney and spleen) of three Teleost species (sea bass, *Dicentrarchus labrax* Linnaeus), European eel (*Anguilla anguilla* Linnaeus) and blackspot seabream (*Pagellus bogaraveo* Brünnich), to explore the occurrence of antibacterial properties against some potentially pathogenic bacteria. Moreover, blood sera of the same species were also examined for the possible lytic capacity against sheep red blood cells, to better characterize the non-specific immune defense abilities of such Teleosts, which are still little known.

The selection of the three different fish species examined in the present study was performed according to the following criteria: sea bass is the most common species reared in Mediterranean aquaculture, European eel represents an important economic resource for Italian fish farming, being the most common cultured fish after trout and carp, whereas blackspot seabream is considered as a candidate species for Mediterranean aquaculture diversification [14].

2. MATERIALS AND METHODOLOGY

2.1. Fish Specimens

The individuals of sea bass, European eel and blackspot seabream examined during the present study were obtained from a commercial Sicilian fish farm and reared at the Experimental Aquaculture Plant of the CNR-IAMC, Messina, Italy. Throughout the experimental period, fish were fed a commercial dry diet for carnivorous fish (Trouvit pellets, TROUW NUTRITION SpA, Verona, Italy), administered until satiation. Each tank was supplied with a constant aerated flow of seawater. The water temperature was 24-25 °C, pH was 8.2 and dissolved oxygen was 7-8 mg L^{-1}. Photoperiod was kept natural.

2.2. Collection and Treatment of the Biological Samples

Before sampling, fish were anesthetized and euthanized with a lethal dose of MS-222 (tricaine methanesulfonate, 0.1g L^{-1}, Sigma-Aldrich, Milan, Italy). Samples of skin mucus, blood serum, kidney and spleen were collected from three individuals of each species under study. Mucus was collected from each fish by gently scraping with a sterile spatula from the dorsal surface of the body, avoiding the ventral side to avoid intestinal and sperm contamination. It was stored in sterile Eppendorf microcentrifuge tubes and mixed with an equal amount of sterilized physiological saline (0.85% NaCl). Precipitates present in the suspension were removed by centrifugation at 6000 x g and the supernatant was collected and stored at -20°C until analysis.

Blood samples were drawn separately from the caudal vein of each individual; small volumes were collected in heparinised (14 International Units mL^{-1}) tubes and centrifuged; the obtained plasma was stored at - 80°C for further antibacterial activity assay.

From each fish, spleen and kidney samples were also removed using a sterile scalpel, homogenized in physiological saline (1:10, w/v) and stored at -80°C until analysis. From each fish, amounts of 0.5 mL of mucus and blood sera were collected, whereas for spleen and kidney the quantity removed from each fish was 50 milligrams.

All the methodologies applied in this study were standardized to make the comparisons among different fish and organs feasible and reproducible.

2.3. Antibacterial Activity Measurements

Antibacterial activity of mucus and blood sera was determined against a collection of target strains of human pathogens and environmental bacteria. The target bacterial strains used in this study belonged to a collection of the Department of Biomedical Sciences and Morphological and Function Images (Prof. Delia, Dr. Laganà, University of Messina, Italy). Strains of Gram-negative (*Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Salmonella typhi*, *Vibrio fluvialis*, *V. alginolyticus*, *V. anguillarum*- strains 975 and 953, *V. parahaemolyticus*) and Gram-positive bacteria (*Enterococcus faecium*, *Staphylococcus aureus*, *Staphylococcus epidermidis*) as well as some fish pathogens were considered (*Aeromonas hydrophila*, *Photobacterium damselae subsp. piscicida*).

The antibacterial activity was measured using the standard disc diffusion method, according to the procedure indicated by Bauer *et al*. [15], with some modifications. All isolates were cultured in Brain Heart Infusion broth (Oxoid, Rodano, Milan, Italy), adjusted to correspond to a final concentration of 10^8 cell mL^{-1} with a standard of McFarland density. Plates of Tryptic Soy agar (Oxoid) added with 1% NaCl were inoculated with 50 µL of each broth culture by spreading the agar surface using a sterile cotton swab. Sterile discs of absorbent paper (diameter: 6 mm) were imbibed with 20 µL of each sample or homogenate and then placed on the surface of each plate with the help of a sterilized forceps. Sterile water was used as the control for the antibacterial activity assays, showing no inhibition of bacterial growth. After incubation at 22 or 35 °C for 24 h (depending on the optimal temperature for each bacterial species), the diameters of the zones of inhibition of bacterial growth were measured, considering the edges of the clear zone, using a precision calliper (Mitutoyo, Andover, UK).

2.4. Haemolytic activity Assay

The haemolytic activity of blood sera and mucus was measured on Columbia Blood agar base (Oxoid) plates added with 5% sheep red blood cells (Microbiol s.n.c., Macchiareddu, Cagliari, Italy) and incubated at 30°C for 24 h. The diameter of haemolysis produced after incubation was measured using a precision ruler.

2.5. Agglutinating Activity Assay

The agglutinating activity of serum and mucus samples was assayed by mixing some drops of each sample with each broth culture on the surface of a glass slide. The appearance, after some seconds of gentle manual agitation of the slide, of aggregates of bacterial cells was considered as a positive result.

Table 1. Antibacterial activity of samples of mucus, blood sera and tissues homogenates from European eel, blackspot seabream and sea bass.
The mean diameters of inhibition ± standard deviation (in cm) measured on plates of Tryptic Soy Agar added with 1.5% NaCl are reported.

MUCUS	*V.*	*V.*	*V.*	*S. aureus*	*Ps.*
	fluvialis	*alginolyticus*	*parahaemolyticus*		*aeruginosa*
eel	0.80 ± 0.30	0.80 ± 0.20	0.80 ± 0.30	0.80 ± 0.20	/
sea bream	/	/	0.80 ± 0.25	/	/
sea bass	0.80 ± 0.20	0.80 ± 0.35	0.87 ± 0.30	0.80 ± 0.25	0.80 ± 0.25
BLOOD SERA	*V. anguillarum*	*V. anguillarum*	*V.*	*V.*	*Ps.*
	(strain 975)	(strain 953)	*alginolyticus*	*parahaem.*	*aeruginosa*
eel	/	/	0.95 ± 0.20	/	0.90 ± 0.40
sea bream	0.80 ± 0.20	/	1.50 ± 0.50	1.00 ± 0.30	/
sea bass	/	0.80 ± 0.25	0.87 ± 0.25	/	/
TISSUES	*P. damselae*	*E. coli*	*V.*	*Ps.*	
			alginolyticus	*aeruginosa*	
eel - kidney	0.90 ± 0.50	0.75 ± 0.20	/	0.85 ± 0.35	
eel - spleen	/	0.65 ± 0.20	/	0.90 ± 0.40	
sea bream - kidney	/	0.75 ± 0.20	0.90 ± 0.40	0.70 ± 0.20	
sea bass - kidney	1.13 ± 0.40	/	0.77 ± 0.25	0.93 ± 0.40	
/, no reaction					

2.6. Statistical Analysis

Results were reported as the mean value ± standard deviation obtained from three individuals for each species. Each assay was repeated three times to assess the reproducibility of the results. Normality of the data was previously assessed using a Shapiro Wilk test and homogeneity of variance was also verified using the Levene test. Non-normally distributed data were log-transformed prior to analysis. Statistical differences among the obtained data were assessed by One way Analysis of Variance (ANOVA), according to the Fisher's method [16]. Only the P values <0.05 were considered as statistically significant. The software Sigma Stat version 3.0 was used for the analysis.

3. RESULTS

3.1. Antibacterial Activity Measurements

The results of the antibacterial activity assays performed on the samples of mucus, blood sera and tissues homogenates are reported in Table **1**. To compare the antibacterial activity of the various samples across the species, all the samples were tested against the same bacteria. For convenience, only the positive results are shown in the Table.

Regarding the samples of mucus, both in eel and sea bass a broad spectrum of antibacterial activity against *V. fluvialis*, *V. parahaemolyticus* and *V. alginolyticus* was recorded. The two fish species showed similar levels of activity against *V. fluvialis* and *V. alginolyticus*, as also confirmed by the ANOVA results (Table **2**). In sea bass, the activity of mucus against *V. parahaemolyticus* did not differ significantly from that of eel. *S. aureus* was similarly sensitive to the mucus of sea bass and eel. The mucus of blackspot seabream showed antibacterial activity against *V. parahaemolyticus* comparable to that of eel.

Blackspot seabream sera showed a strong antibacterial activity against *V. alginolyticus* and *V. parahaemolyticus*; the activity against *V. alginolyticus* was not significantly different from those measured in both eel and sea bass sera.

Table 2. Results of Analysis of Variance (ANOVA) performed among species and among samples. The Probability values of the comparisons between groups are reported; asterisks indicate statistically significant differences. n.d.= not determined.

ANOVA AMONG SPECIES				
MUCUS				
	V. fluvialis	***V. alginol.***	***V. parahaemol.***	***S. aureus***
eel/sea bass	0.774	0.542	0.749	0.715
eel/seabream			0.976	
seabream/sea bass			0.728	
BLOOD SERA				
		V. alginol.		
eel/sea bass		0.687		
eel/seabream		0.152		
seabream/sea bass		0.123		
TISSUES				
	P.damselae	*E. coli*	*V. alginol.*	*Ps. aerugin.*
eel kidney/eel spleen		0.573		0.878
eel kidney/seabream kidney		n.d.		0.554
eel kidney/sea bass kidney	0.568			0.807
seabream kidney/sea bass kidney			0.658	0.423
eel spleen/seabream kidney		0.573		0.482
eel spleen/sea bass kidney				0.931
ANOVA AMONG SAMPLES				
Eel				
	V. alginolyticus			
mucus *vs*. sera	0.410			
	Ps. aeruginosa			
sera *vs*. kidney	0.878			
sera *vs*. spleen	n.d.			
kidney *vs*. spleen	0.878			

Table 2. contd…

ANOVA AMONG SAMPLES				
	V. fluvialis	***V. alginol.***	***V. parahaemol.***	***S. aureus***
Seabream				
	V. parahaemolyticus			
mucus vs sera	0.425			
Sea bass				
	V. alginolyticus			
mucus vs kidney	0.910			
	Ps. aeruginosa			
mucus vs kidney	0.658			

Eel sera also exhibited high antibacterial activity against *Ps. aeruginosa*. Also sea bass sera possessed antibacterial activity against *V. alginolyticus*.

None of the examined sera showed antibacterial activity against bacterial strains of human origin, both Gram-negative (*E. coli, K. pneumoniae*) and positive (*E. faecium, S. aureus*) tested in this study(data not shown in Table **1**).

Considering the homogenates of kidney samples, a great antibacterial activity was recorded for the kidney of sea bass against *P. damselae* subsp. *piscicida*, *Ps. aeruginosa* and *V. alginolyticus* (Table **1**). Also the kidney of eel showed good levels of antibacterial activity against *P. damselae* subsp. *piscicida* and *Ps. aeruginosa*, being weakly active against *E. coli* too. The spleen of eel exhibited a good antibacterial activity against *Ps. aeruginosa* and only a weak activity against *E. coli*. The kidney of blackspot seabream showed antibacterial activity against *V. alginolyticus, E. coli* and *Ps. aeruginosa,* but the measured values did not differ significantly from those of sea bass kidney and eel spleen (Table **2**).

3.2. Haemolytic Activity Assay

The results of the haemolytic activity assay performed on mucus and blood sera are shown in Table **3**. Positive results against sheep red blood cells were found for the sera of sea bass and eel; a weak hemolytic activity was also observed for the mucus of sea bass and blackspot seabream. No significant differences were found comparing the halos of haemolysis yielded by the mucus of blackspot seabream (showing the lowest diameter) with those produced by the sera of sea bass and eel (showing the highest ones) (F= 5.769, P= 0.074 and 4.537, P= 0.10, respectively).

Table 3. Haemolytic properties of mucus and blood sera. The mean diameters of lysis ± standard deviation (in cm) obtained on Columbia agar plates added with 5% sheep blood red cells are reported.

A. anguilla - serum	1.35 ± 0.40
D. labrax - serum	1.30 ± 0.30
D. labrax - mucus	1.00 ± 0.30
P. bogaraveo - mucus	0.80 ± 0.20

3.3. Agglutinating Activity Assay

The agglutinating activity assayed on mucus and blood sera samples yielded the results summarized in Table **4**. The sera of sea bass and eel showed an agglutinating ability against *Ps. aeruginosa* and *S. aureus*, while none of them was able to agglutinate different species of *Vibrio* (*V. fluvialis, V. alginolyticus, V. anguillarum, V. parahaemolyticus*) or *A. hydrophila*. A good agglutinating ability was also recorded for the mucus of sea bass, against *V. fluvialis, V. alginolyticus* and *A. hydrophila.*

4. DISCUSSION

The bacteria used for the present study have been selected because these microorganisms are likely to be spread in the habitat where the examined fishes live. The results obtained in the present study indicate that fish mucus, sera and kidneys from sea bass, blackspot seabream, and European eel possess both antibacterial and haemolytic properties and therefore they could be regarded as an interesting source of active biocompounds. The antibacterial activity is expressed above all against some Gram-negative bacterial strains widespread in aquatic environments (*Vibrio* spp., *A. hydrophila, Ps. aeruginosa, P. damselae* subsp. *piscicida*), while it is weak or absent against other strains of marine (*V. anguillarum*) or human origin (*E. coli, E. faecium, K. pneumoniae*, with the exception of *S. aureus*). The highest antibacterial properties are observed in blackspot

Table 4. Agglutinating properties of mucus and blood sera against the target bacteria.

Bacterial Strains			
	***A. anguilla* - serum**	***D. labrax* - serum**	***D. labrax* - mucus**
Proteus mirabilis	-	-	-
Vibrio fluvialis	-	-	+
Vibrio alginolyticus	-	-	+
Vibrio anguillarum	-	-	-
Vibrio parahaemolyticus	-	-	-
Aeromonas hydrophila	-	-	+
Pseudomonas aeruginosa	+	+	-
Staphylococcus aureus	+	+	/
Escherichia coli	-	(+)	-
Klebsiella pneumoniae	-	-	-

+, positive reaction; (+), weak positive reaction; -, negative reaction

seabream sera against *V. alginolyticus* and in sea bass kidney against *P. damselae* subsp. *piscicida*. The detection of antibacterial activity in the skin secretions and organs of the examined species underline their role in the mechanisms of innate immune defense, as known for other fish species [17-19]. On the other hand, the antimicrobial properties of skin secretions of many fish species are widely known. Several studies have previously demonstrated the antimicrobial property of epidermal mucus in common carp, *Cyprinus carpio* [20]; queen parrot fish, *Scarus vetula* [21]; hagfish, *Myxine glutinosa* [22]; catfish, *Arius maculatus* [23]. Antibacterial activity against *S. typhi, K. pneumoniae, S. aureus, E. coli, V. cholerae* was shown by the epidermal mucus secretion of marine stingrays (*Dasyatis sephen* and *Himantura gerrardi*) [24]. The mucus and epidermal extract of Tinfoil barb fish were able to inhibit the growth of *Bacillus cereus*, *S. aureus*, *Shigella boydii* and *E. coli* [25]. Bioactive molecules (lysozyme, alkaline phosphatase and proteases, esterase, peroxidase) are constitutively present in both skin mucus and serum of gilthead seabream, *Sparus aurata* [19].

In European eel, the presence of antibacterial and haemolytic activity has been previously documented in the mucus [26]; particularly, positive reactions against *Ps. aeruginosa, V. parahaemolyticus, E. coli, S. aureus, S. typhi, S. paratyphi, K. pneumoniae, K. oxytoca, Proteus mirabilis* and *Lactobacillus vulgaris* were reported. In the present study, the eel mucus was found active against *V. parahaemolyticus, V. fluvialis, V. alginolyticus* and *S. aureus*; compared to mucus, higher antibacterial activity against *Ps. aeruginosa* and *V. alginolyticus* was recorded in eel sera.

In addition, the mucus of sea bass and blackspot seabream, together with the sera of eel and sea bass examined in this study, show haemolytic properties. Also Bragadeeswaran *et al*. [27] found that the mucus of two fish species (*Cynoglossus arel* and *Arius caelatus*) has not only high antimicrobial activity against human pathogens (*V. cholerae, V. parahaemolyticus, S. aureus*) but also high haemolytic activity. A similar result was reported in the mucus of two other fish species, *Channa punctatus* and *Cirrhinus mrigala* [28]. Also Uthayakumar *et al*. [29] observed that the mucus secretion of *Mastacembelus armatus* possesses a potent haemolytic activity against sheep and cow blood cells. Previously, Hellio *et al.* [30] reported in the mucus of bony fishes the presence of some antimicrobial agents which bind with microbes and destroy the blood cells, resulting in haemolysis. It has to be noted, however, that although antibacterial compounds have been found in many fish species, it is difficult to perform quantitative comparisons among several studies, since the expression of antibacterial proteins may be affected by a different solubility in the aqueous or acidic medium of extraction. Significant variations in the relative levels of epidermal mucus enzymes involved in the innate immune system of different fish species (i.e. lysozyme, cathepsin B, proteases and alkaline phosphatase) were also reported [11], although it is still unclear whether they could depend on evolutionary or genetic adaptation of the organisms to environmental factors. Also the response of haemolytic reactions may differ considerably depending on the used erythrocytes (i.e. sheep, chicken or goat).

Comparing the samples tested for antibacterial properties in this study, mucus, blood sera and kidney of sea bass exhibit antibacterial activity against *V. alginolyticus*. However, no significant differences among the diameters of inhibition were observed. In blackspot seabream, both kidney and blood sera show antibacterial activity against this same bacterium. Similar levels of antibacterial activity against *Ps. aeruginosa* are found in eel spleen and blood sera. In gilthead seabream, skin mucus is reported to have a stronger bactericidal activity than serum against fish pathogens [19].

Another interesting result obtained in the present study is the detection in sea bass mucus of agglutinating activity against *V. fluvialis, V. alginolyticus* and *A. hydrophila*, being all these microorganisms widely distributed in marine and

brackish environments, this finding led us to hypothesize that sea bass could be less susceptible than eel and blackspot seabream to infectious diseases (i.e. vibriosis) sustained by these bacteria.

CONCLUSION

The detection of antibacterial and haemolytic properties in the biological secretions and tissues of the examined fish species stresses the need for their further characterization. Further efforts are necessary for the chemical purification and isolation of active antimicrobial compounds. Extraction of antimicrobial peptides could provide not only a good and sustainable way for the utilization of fish by-products like mucus and skin, but it could also contribute in advancing current understanding of the function of these substances in the mucosal defense mechanisms of fish, in order to establish their possible applications as natural therapeutic compounds against infectious diseases.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

ACKNOWLEDGEMENTS

Declared none.

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Received: May 12, 2014 Revised: July 18, 2014 Accepted: July 22, 2014