Terminal carbohydrates abundance, immune related enzymes, bactericidal activity and physico-chemical parameters of the Senegalese sole (*Solea senegalensis*, Kaup) skin mucus

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a b s t r a c t

Recently, interest in mucosal surfaces, more specifically fish skin and its secreted mucus, has greatly increased among immunologists. The abundance of terminal carbohydrates, several enzymes (proteases, lysozyme, peroxidase, alkaline phosphatase, esterases and ceruloplasmin), bactericidal activity against fish pathogenic and non-pathogenic bacteria and several physico-chemical parameters (protein concentration, pH, conductivity, redox potential, osmolarity, density and viscosity) in the skin mucus of Senegalese sole (*Solea senegalensis*, Kaup) have been evaluated. Present results evidence the abundance of *N*-acetylneuraminic acid, mannose, glucose and *N*-acetyl-galactosamine in skin mucus. The levels of lysozyme, proteases, esterases and alkaline phosphatase were very similar (from 20 to 30 Units mg $^{-1}$ protein). However, 93 Units mg $^{-1}$ protein were detected of ceruloplasmin and only 4.88 Units mg $^{-1}$ protein of peroxidase. Skin mucus of *S. senegalensis* showed high bactericidal activity against the tested pathogen bacteria but weak activity against non-pathogenic bacteria. Finally, a clear relationship between mucus density and temperature was detected, while viscosity showed a direct shear- and temperature-dependent behaviour. These results could be useful for better understanding the role of the skin mucus as a key component of the innate immune system, as well as, for elucidating possible relationships between biological and physico-chemical parameters and disease susceptibility.

1. Introduction

Mucosal surfaces of fish are important physical barriers, whose main function is to protect the systemic environment of the body against bacterial invasion. The surface of most epithelia are covered by a complex viscous secretion called mucus, which covers the epithelial cells [1] and is secreted by various epithelial cell types [2,3]. Mucus is a viscous colloid gel that forms an adherent layer covering the entire body of fish. Due to this localization, mucus represents an interface between the environment and the interior milieu, making it of vital importance for aquatic animals [2]. In fish, mucosa-associated lymphoid tissue (MALT) is mainly present in skin, gill and gastrointestinal tract. The composition and functional characterization of fish skin mucus have recently received significant interest since it contains many defence mechanisms (including important enzymes) that constitute the first line of defence against a broad spectrum of pathogens present in the aquatic environment. Moreover, mucus plays a critical role in these defence mechanisms, acting as a natural, dynamic, physical, chemical, and biological barrier [4,5] which allows the exchange of nutrients, gases, odorants and hormones, among other substances [6]. One of the most

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important functions of fish mucus is its implication in the immune response and disease resistance. Even though the species has been extensively studied in the last two decades from an immunological point of view [7e11], there is little available information on the skin mucus of Senegalese sole (*Solea senegalensis*), a high-value flatfish with great potential for future farming at commercial scale [12] due to its high market value [13].

Fish skin mucus is mainly composed of water (approx. 95%) and mucins, which are glycoproteins conjugated with a large content of high molecular weight oligosaccharides [14,15]. Mucins are strongly adhesive, and play a major role in the defence of the mucosae because they form a matrix in which a diverse range of antimicrobial molecules are found [16]. Furthermore, mucins are thought to be responsible for providing viscoelastic and other rheological properties to the mucosal layers [17]. Carbohydrate side chains constitute up to 80% of the total mucin mass [17,18] and give an elongated and rigid structure to the molecules, which contributes to their mechanical properties [19]. Apart from functioning in the protection and lubrication of epithelial surfaces, the physico-chemical properties of skin mucus are also crucial for many other functions attributed to fish skin, such as respiration, ionic and osmotic regulation, reproduction, locomotion, excretion and communication [2,20].

The immunological or defensive function of fish epidermal mucus is the result of its physical and biochemical properties. Firstly, epidermal mucus is continuously replaced and renewed and the mucus layer thickness and composition prevents pathogen adherence to the underlying tissues and provides a medium in which antibacterial mechanisms may act [21,22]. Secondly, besides being a physical barrier, fish epidermal mucus serves as a repository of numerous innate immune components such as cytokines, glycoproteins, lysozyme, immunoglobulins, complement proteins, lectins, C-reactive protein, flavoenzymes, proteolytic enzymes and antimicrobial peptides [2,23e26], which exert inhibitory or lytic activity against pathogens [27,28]. Interestingly, while some components only have a defensive purpose [18], others may also act by modifying the organization and properties of the gel structures [17]. While these immune components present in fish mucus have been studied in many fish species, no studies have correlated them with the skin mucus physico-chemical parameters. Thus, most of the studies available only approach the mucus immune molecules and their properties from a biological standpoint. Taking all the above into account, the aim of the present study was to evaluate terminal carbohydrates abundance, several enzymes related to immunity, the bactericidal activity and different physico-chemical parameters in skin mucus of Senegalese sole (*Solea senegalensis*, Kaup). Findings concerning biological and physico-chemical parameters are discussed from an integrative point of view, focusing on the crucial role of skin mucus in fish mucosal immunity.

2. Materials and methods

2.1. Fish care and maintenance

Thirty adult specimens of Senegalese sole (26.7 ± 4.6 g mean body weight) were obtained in June 2014 from the stocks of the Spanish Oceanographic Institute of (IEO, Santander, Spain). All fish were kept in seawater aquaria (150 L) covered with a dark net (50 lx) and connected to an open circulating system (35.4 g L^{-1} salinity, 16.6 C ± 0.5 C) with a renovation rate of 300% day^{-1}. Fish were fed a commercial pellet diet (18% total lipids and 55% crude protein, Skretting, Burgos, Spain) at a rate of 2% body weight day^{-1} eight times a day.

2.2. Skin mucus collection and storage

Fish were anesthetized prior to sampling with clove oil (40 ppm, Guinama®, Valencia, Spain). Skin mucus samples were collected according to the method described by Guardiola et al. [29]. Briefly, the ocular side and blind side surfaces of naïve specimens were gently scraped using a cell scraper, with sufficient care to avoid contamination by blood and/or urine-genital and intestinal excretions. In order to obtain sufficient mucus for all the assays, samples of mucus were pooled in the following way. To determine the mucus glycosylation pattern, enzymes activities and bactericidal activity, 3 pools were established, each with samples of mucus from 10 fish. Then, each resulting pool was homogenized with 1 volume of Tris-buffered saline (TBS, 50 mM TriseHCl, pH 8.0, 150 mM NaCl). The homogenates were vigorously shaken and centrifuged (500 g, 10 min, 4 C) and the supernatant was lyophilized following freezing at 80 C. Lyophilized skin mucus powder was dissolved in Milli-Q water, and the undissolved mucus portion was isolated by centrifugation (500 g, 10 min, 4 C). The protein concentration in each sample was determined by Bradford's dye binding method [30], using bovine serum albumin (BSA, Sigma) as standard. Then, the proteins in skin mucus samples were adjusted to 500 mg protein ml^{-1} with Milli-Q water, as in Guardiola et al. [26]. Finally, the samples were aliquoted and stored at 20 C until use.

To analyze the physico-chemical parameters, one pool with samples from 30 fish was established and directly stored at 80 C until use.

2.3. Determination of the terminal glycosylation pattern

The glycosylation pattern was determined by lectin ELISA in skin mucus as described previously [31]. Thus, 10 mg $well^{-1}$ of mucus sample were placed in flat-bottomed 96-well plates in triplicate and coated overnight at 4 C with the use of 50 mM carbonate-bicarbonate buffer (pH 9.6). Samples were rinsed three times with PBS-T [20 mM phosphate saline buffer (PBS) and 0.05% Tween 20, pH 7.3], blocked for 2 h at room temperature with blocking buffer (3% BSA in PBS-T) and rinsed again with PBS-T. Samples were then incubated for 1 h with 20 mg per well of biotinylated lectins (Table 1), washed and incubated with streptavidin conjugated to horseradish-peroxidase (HRP, 1:1000; Life Technologies) for 1 h. After exhaustive rinsing with PBS-T the samples were developed using 100 ml of a 0.42 mM solution of 3,3′,5,5'- tetramethylbenzidine hydrochloride (TMB, Sigma), prepared daily in a 100 mM citric acid/sodium acetate buffer (pH 5.4) containing 0.01% H_2O_2. The reaction was allowed to proceed for 10 min and stopped by the addition of 50 ml of 2M H_2SO_4. The plates were read at 450 nm in a plate reader (FLUOstar Omega, BMG Labtech). Negative controls consisted of samples without skin mucus or without lectins, whose optical density (OD) values were subtracted for each sample value. Data are presented as the OD at 450 nm for the skin mucus and lectin used.

2.4. Enzymatic activities

2.4.1. Lysozyme activity

Lysozyme activity was measured according to the turbidimetric method described by Parry et al. [32] with some modifications. One hundred microliters of skin mucus diluted 1/2 with 10 mM PBS, pH 6.2, were placed in flat-bottomed 96-well plates in triplicate. To each well, 100 ml of freeze-dried *Micrococcus lysodeikticus* in the above buffer (0.3 mg ml^{-1}, Sigma) was added as lysozyme substrate. The reduction in absorbance at 450 nm was measured after 0 and 15 min at 22 C in a plate reader. One unit of lysozyme activity was defined as a reduction in absorbance of 0.001 min^{-1}. The units

Table 1
Lectins used in this study, their acronym and sugar-binding specificities.

Acronym	Lectin source	Sugar binding specificity
BSL I	*Bandeiraea simplicifolia*	a-D-galactose, *N*-acetyl-a-D-galactosamine
PNA	*Arachis hypogaea*	b-D-galactose
UEA I	*Ulex europeaus*	a-L-Fucose
Con A	*Canavalia ensiformis*	a-D-mannose, a-D-glucose
WFA	*Wisteria floribunda*	*N*-acetyl-D-galactosamine
WGA	*Triticum vulgaris*	*N*-acetyl-b-D-glucosamine, *N*-acetylneuraminic acid
LEA	*Lycopersicon esculentum*	*N*-acetyl-b-D-glucosamine

of lysozyme present in skin mucus were obtained from a standard curve made with hen egg white lysozyme (HEWL, Sigma) and the results were expressed as U mg ^{1} mucus proteins.

2.4.2. *Peroxidase activity*

The peroxidase activity in skin mucus samples was measured according to Quade and Roth [33]. Briefly, 30 ml of skin mucus were diluted with 120 ml of Hank's buffer (HBSS) without Ca^{b2} or Mg^{b2} in flat-bottomed 96-well plates. As substrates, 50 ml of 20 mM TMB and 5 mM H_2O_2 were added. The colour-change reaction was stopped after 2 min by adding 50 ml of 2 M sulphuric acid and the OD was read at 450 nm in a plate reader. Standard samples without skin mucus samples were used as blanks. One unit was defined as the amount producing an absorbance change of 1 and the activity expressed as U mg ^{1} mucus proteins.

2.4.3. *Alkaline phosphatase activity*

Alkaline phosphatase activity was measured by incubating an equal volume of skin mucus samples with 4 mM *p*-nitrophenyl liquid phosphate (Sigma) in 100 mM ammonium bicarbonate buffer containing 1 mM $MgCl_2$ (pH 7.8, 30 C), as described by Ross et al. [34]. The OD was continuously measured at 1 min intervals over 3 h at 405 nm in a plate reader. The initial rate of the reaction was used to calculate the activity. One unit of activity was defined as the amount of enzyme required to release 1 mmol of *p*-nitrophenol product in 1 min and the activity expressed as U mg ^{1} mucus proteins.

2.4.4. *Esterase activity*

Esterase activity was determined according to the method of Ross et al. [34]. An equal volume of skin mucus sample was incubated with 0.4 mM *p*-nitrophenyl myristate as substrate in 100 mM ammonium bicarbonate buffer containing 0.5% Triton X-100 (pH 7.8, 30 C). The OD and activity was determined as above.

2.4.5. *Protease activity*

Protease activity was quantified using the azocasein hydrolysis assay according to the method of Ross et al. [34]. Briefly, an equal volume of skin mucus was incubated with 100 mM ammonium bicarbonate buffer containing 0.7% azocasein (Sigma) for 19 h at 30 C. The reaction was stopped by adding 4.6% trichloro acetic acid (TCA) and the mixture was centrifuged (10,000 g, 10 min). The supernatants were transferred to a 96-well plate in triplicate containing 100 ml well ^{1} of 0.5 N NaOH, and the OD was read at 450 nm using a plate reader. Skin mucus was replaced by trypsin (5 mg ml ^{1}, Sigma) as positive control (100% of protease activity), or by buffer as negative control (0% activity).

2.4.6. *Antiprotease activity*

Total antiprotease activity was determined by the ability of skin mucus to inhibit trypsin activity [35]. Because antiprotease activity in skin mucus is very low samples were adjusted to 2 mg ml ^{1} of mucus protein (instead of 0.5 mg ml ^{1}) [29]. Briefly, 10 ml of skin mucus samples were incubated (10 min, 22 C) with the same volume of a trypsin solution (5 mg ml ^{1}). After adding 100 ml of 100 mM ammonium bicarbonate buffer and 125 ml of 0.7% azoca- sein, samples were incubated (2 h, 30 C) and, following the addition of 250 ml of 4.6% TCA, a new incubation (30 min, 30 C) was carried out. The mixture was then centrifuged (10,000 rpm, 10 min) being the supernatants transferred to a 96-well plate in triplicate containing 100 ml well ^{1} of 0.5 N NaOH, and the OD was read at 450 nm using a plate reader. For a positive control, buffer replaced skin mucus (100% protease and 0% antiprotease activity), and for a negative control, buffer replaced the trypsin (0% protease and 100% antiprotease activity). The percentage of inhibition of trypsin activity for each sample was calculated.

2.4.7. *Ceruloplasmin oxidase activity*

The ceruloplasmin oxidase activity was measured according to Dunier et al. [36]. Briefly, equal volumes of skin mucus and 0.1% *para*-phenylenediamine in acetate buffer, pH 5.2, and 0.02% sodium azide were incubated. The kinetic of the increase in absorbance was followed at 550 nm for 15 min, and 1 unit was defined as an increase of OD of 0.001 min ^{1}.

2.5. *Bactericidal activity*

Three marine pathogenic bacteria (*Vibrio harveyi* Lg 16.00, *V. anguillarum* (CECT 4344) and *Photobacterium damselae* subsp. *Piscicida* Lg *41.00*) and three non-pathogenic bacteria [*Escherichia coli* (CECT 434), *Bacillus subtilis* (CECT 35) and *Shewanella putrefaciens* Pdp11] were used to determine the bactericidal activity present in skin mucus samples. *V. harveyi* and *P. damselae* subsp. *piscicida*, were isolated from diseased farmed *Solea senegalensis* [37,38], whereas *S. putrefaciens* Pdp11 was isolated from the skin of healthy specimens of gilthead seabream (*Sparus aurata*) [39], respectively. All bacteria were grown in agar plates at 25 C in a suitable medium: tryptic soy (TSB, Sigma) for *V. harveyi*, *V. anguillarum*, *P. damselae* and *S. putrefaciens*, Luria (LB, Sigma) for *E. coli* and nutrient broth (NB) (Conda) for *B. subtilis*. Then, fresh single colonies of 1e2 mm were diluted in 5 ml of appropriate liquid culture medium and cultured on an orbital incubator at 200e250 rpm in orbital shaker for 16 h at 25 C.

The antimicrobial activity present in skin mucus was determined by evaluating the effect on the bacterial growth curves using the method of Sunyer and Tort [40] with some modifications. Aliquots of 100 ml of each of the bacterial dilutions (1/10) were placed in flat-bottomed 96-well plates and cultured with an equal volume of *Solea senegalensis* skin mucus. The OD of the samples was measured at 620 nm at 30 min intervals for 24 h at 25 C. Samples without bacteria were used as blanks (negative control) while samples without mucus were used as positive controls (100% growth or 0% bactericidal activity). All microbial tests and controls were performed in triplicate.

2.6. Physico-chemical parameters

Protein concentrations present in skin mucus samples were determined by Bradford's method [30]. The pH measurements were made using a pH & ION-Meter GLP 22þ (Crison). Conductivity measurements were carried out at 25 C using a Crison Conductimeter microCM 2200 and compared with the reference solution (ETALON CONDUCTIVITE 97 10 of 1413 mS cm 1; Crison). Redox potential values were obtained from potentiometric measurements made with an electrode system consisting of a Crison 52e67 platinum electrode and an Orion Ag/AgCl double-junction reference electrode (Orion 90e02) connected to a homemade high-impedance data acquisition 16-channel box connected to a personal computer by USB (Universal Serial Bus). Redox standard solutions of 124 and 250 mV (Fluka) were used as reference.

Osmolarity was measured using a VAPRO vapor pressure osmometer (model 5520). Density measurements were made in a densimeter (MDA5000M Anton Paar) at 12, 17 and 22 ± 0.1 C. In this instrument the sample is introduced into a U-shaped borosilicate glass tube that is excited to vibrate at a characteristic frequency. The density was determined from the frequency changes caused by the presence of the sample. In order to estimate the reproducibility of the measurements made by the densimeter, six different repetitions were performed for each sample and resulted with a constant standard deviation (0.002 g ml 1).

2.7. Mucus viscosity

For viscosity analysis, skin mucus samples were thawed at room temperature in ice, briefly vortexed and centrifuged (7000 g, 5 min) (Minispin, Eppendorf). The supernatants were collected and measured for their viscosity, and the remaining mucus was stored at 20 C for later analysis. Viscosity measurements were made in two different devices for low and high shear rates. For lower shear rates, we used a rheometer (Anton Paar MCR 102) with a plate-plate measurement system (plate model PP50, Anton Paar; plate-plate distance 0.8 mm) set at 12, 17 and 22 ± 0.1 C using approximately 1.6 ml aliquots of sample. To obtain a characteristic profile, the viscosity was measured over a range of shear rates (11.5, 23, 46 and 115 s 1), each rate being measured for 200 s, recording the viscosity value each second. During analysis, the 20 firsts points recorded were removed in order to eliminate artefacts at the beginning of the measurement. In all cases viscosity did not show any systematic deviation from an average value. The results presented in this work are averages and standard deviations from these measurements. To obtain stable recordings, mucus samples were allowed to equilibrate for 5 min after each measurement before a new measurement was made.

A microviscometer (Lovis 2000 ME, Anton Paar) was used to obtain the 400 þshear rates results. Samples were treated as above and aliquots of 1 ml were used for measurement at 12, 17 and 22 ± 0.1 C. This instrument is based on the Rolling Ball Principle, according to which a ball rolls through a closed capillary tube filled with the sample and inclined at a given angle. By changing the angle, the sample can be submitted to different shear rates. In these experiments all shear rates were slightly above 400 s 1. Three inductive sensors in the tube determine the rolling time of the ball between defined marks. The sample viscosity is directly proportional to the rolling time.

2.8. Statistical analysis

The results are expressed as mean ± standard error (SE). Data were statistically analysed by one-way analysis of variance (ANOVA) to determine differences between each carbohydrate and among the different levels of bactericidal activity with respect to the positive control. 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 and a non-parametric Kruskal-Wallis test, followed by a multiple comparison test, was used when data did not meet parametric assumptions. Statistical analyses were conducted using SPSS 19 and differences were considered statistically significant when P 0.05.

3. Results

3.1. Skin mucus terminal glycosylation pattern

All the tested terminal sugar residues were present in the skin mucus samples, the levels depending on the lectin studied (Fig. 1). Based on the highest absorbance, lectin binding to skin mucus followed the order: WGA > Con A > BSL I > WFA > UEA I ~ PNA > LEA. This binding pattern suggests that terminal carbohydrates abundance in Senegalese sole skin mucus is, in decreasing order, *N*-acetylneuraminic acid, mannose, glucose, *N*-acetyl-galactosamine, fucose, galactose and *N*-acetyl-glucosamine residues. No significant differences were detected between Con A and BSL I and between UEA I and PNA binding to skin mucus samples (Fig. 1).

3.2. Enzyme activities

Lysozyme, peroxidase, alkaline phosphatase, esterase, protease, antiprotease and ceruloplasmin activities were detected in the skin mucus of Senegalese sole (Table 2). While some levels of enzymes were very similar (lysozyme, esterase and alkaline phosphatase), ceruloplasmin showed higher activity and peroxidase a lower level Moreover, protease showed a higher percentage of activity than antiprotease (Table 2).

3.3. Bactericidal activity

The bactericidal activity of skin mucus from *S. senegalensis* against both pathogenic and non-pathogenic bacteria was determined (Fig. 2). As regard pathogenic bacteria, the bacteriolytic activity of the mucus had a significantly higher capacity to kill firstly *P. damselae*, then *V. harveyi* and finally *V. anguillarum* bacteria (in decreasing order of bactericidal capacity), compared with the results obtained for positive controls of each bacteria. In the case of the non-pathogenic bacteria (*E. coli*, *B. subtilis* and *S. putrefaciens)*

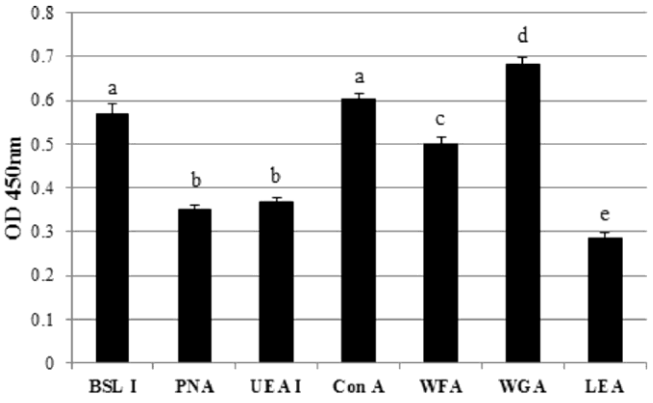


Fig. 1. Lectin binding (Con A, WGA, BSL I, WFA, LEA, UEA 1 and PNA) (OD 450 nm) to carbohydrates present in skin mucus of *Solea senegalensis* specimens. Bars represent the mean of 3 pools of 10 fish ± S.E. Different letters denote significant differences between carbohydrates abundance (P 0.05). See Table 1 for lectin specificity.

Table 2
Enzyme activities in skin mucus of *Solea senegalensis* specimens. Data represent the mean of 3 pools of 10 fish ± S.E.

Enzymes evaluated	Species
	Solea senegalensis
Lysozyme (U mg ^{1} protein)	30.18 ± 0.30
Peroxidase (U mg ^{1} protein)	4.88 ± 0.34
Phosphatase alkaline (U mg ^{1} protein)	20.38 ± 2.71
Esterase (U mg ^{1} protein)	26.15 ± 4.21
Protease (%)	28.73 ± 0.67
Antiprotease (%)	7.41 ± 0.97
Ceruloplasmin (U mg^{1} protein)	93.81 ± 6.14

Table 4
Density measurements (g mL ^{1}) of skin mucus of *Solea senegalensis* specimens. Data represent the mean of a pool of 30 fish ± S.E.

Samples	Density measurements (g mL ^{1})		
	12 C	17 C	22 C
Solea senegalensis	1.009 ± 0.0017	1.0083 ± 0.0016	1.0071 ± 0.0016
Water	0.9995 ± 0.002	0.9988 ± 0.002	0.9978 ± 0.002

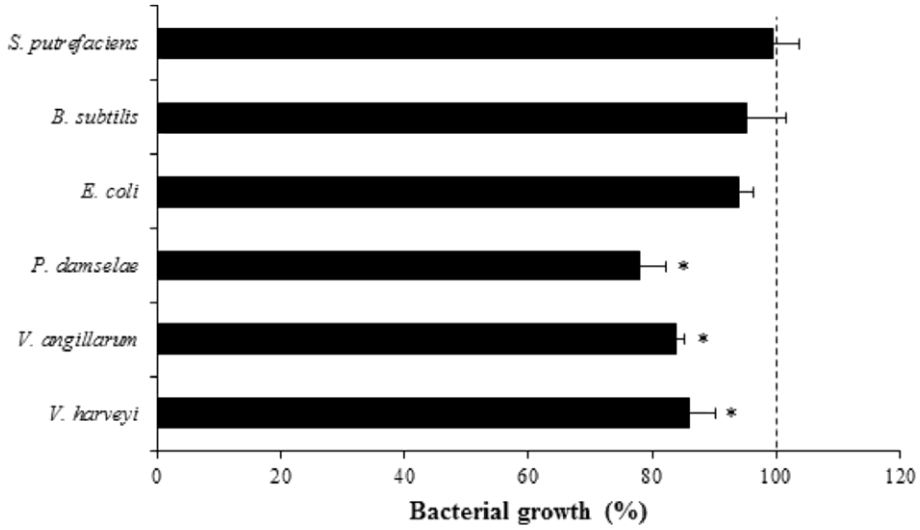


Fig. 2. Bacterial growth (%) in skin mucus of *Solea senegalensis* specimens. Bars represent the mean of 3 pools of 10 fish ± S.E. Asterisks denote significant differences between positive control (100% growth) and bacteria incubated with skin mucus sample (P ◆ 0.05).

the viability was unaffected by the presence of fish skin mucus, although the bacterial growth was slightly reduced (Fig. 2).

3.4. Physico-chemical parameters

The protein concentration, pH, conductivity, redox potential, osmolarity (Table 3) and density (Table 4) were measured in the skin mucus of *S. senegalensis* with the following results: protein concentration, 0.85 mg ml ^{1}; pH, 7.2; conductivity, 17.8 mS cm ^{1}; redox potential, 177.3 mV; and osmolarity, 413 mmol kg ^{1} (Table 3).

As expected, the density measurements followed a similar pattern between the different temperatures tested, although a correlated decrease was observed with increasing temperature (Table 4).

3.5. Mucus viscosity

Skin mucus viscosity of *S. senegalensis* followed a non-Newtonian behaviour, more specifically a shear-thinning effect (Fig. 3), the values reflecting an expected decrease in viscosity with temperature accompanied by an increasing shear rate. Generally, these differences diminished with increasing shear rate until only slight differences in skin mucus viscosity were observed at the temperatures tested when the samples were submitted to the maximum shear rate assayed (400 s ^{1}).

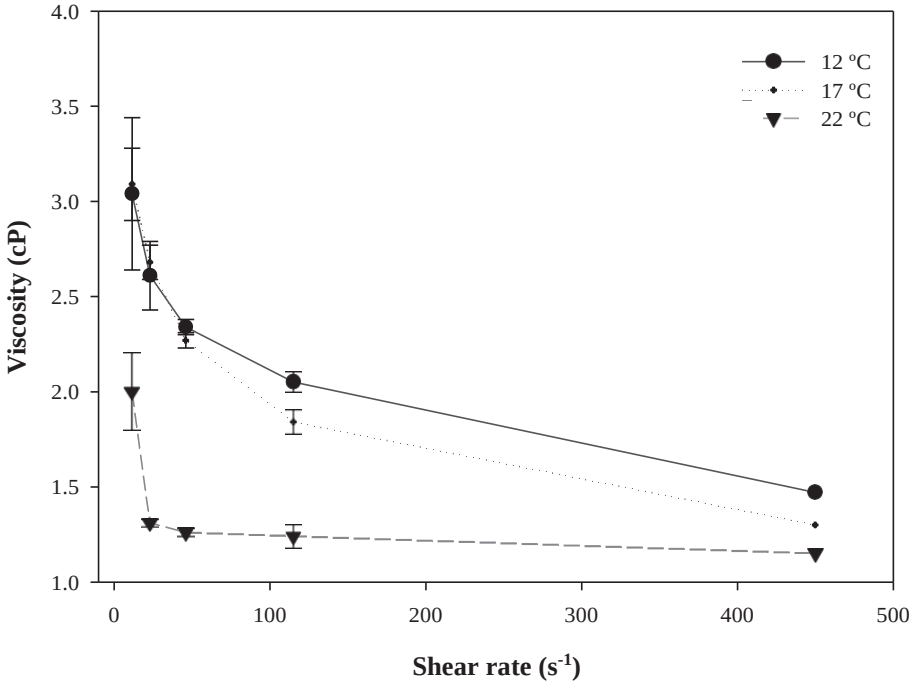
Table 3
Physico-chemical parameters of skin mucus of *Solea senegalensis* specimens. Data represent the mean of a pool of 30 fish ± S.E.

Parameters measured	Species
	Solea senegalensis
Protein (mg ml $^{1)}$	0.85 ± 0.02
pH	7.2
Conductivity (mS cm ^{1})	17.8 ± 0.2
Redox potential (mV)	177.3 ± 1.5
Osmolarity (mmol kg ^{1})	413 ± 1.08

4. Discussion

Fish skin surfaces are in constant interaction with a wide range of non-pathogenic and pathogenic microorganisms present in the aquatic environment, and consequently they are provided with a very effective physico-chemical and biological barrier [4,16], formed of a mucus layer, which constitutes one of the first lines of defense. Mucus has specific physico-chemical properties and includes a very diverse range of innate and adaptive immune factors [25,41]. In the present work, the terminal carbohydrates abundance, immune related enzymes, bactericidal activity and physico-chemical parameters of the Senegalese sole (*Solea senegalensis*, Kaup) skin mucus were studied.

Mucins are the most abundant proteins present in fish mucus, where they are very glycosylated. These proteins are responsible for many of the mechanical properties of the mucus such as viscosity and its gel-like elasticity [19]. Besides, they play an important role in the recognition of foreign particles and organisms, which is the first step in the initiation of defensive responses [42]. The carbohydrate residues tested in the present work were selected because they have been described to be related with the infection progress



T (ºC)	Shear rates (s^{-1})				
	11.5	**23**	**46**	**115**	**400+**
12	3.04 ± 0.40	2.61 ± 0.18	2.34 ± 0.04	2.05 ± 0.05	1.47 ± 0.01
17	3.09 ± 0.19	2.68 ± 0.09	2.27 ± 0.04	1.84 ± 0.06	1.31 ± 0.01
22	2.01 ± 0.20	1.31 ± 0.02	1.26 ± 0.02	1.24 ± 0.06	1.15 ± 0.01

Fig. 3. Mucus viscosity (cP) in relation to shear rate (s ¹) in skin mucus of *Solea senegalensis* specimens at 12, 17 and 22 ± 0.1 C. Standard deviation for 400 þ shear rate results is denoted as 0.01 cP because the reproducibility of the measurement is within the precision used for the rest of the data. Data represent the mean of a pool of 30 fish ± S.E. of the technical replicates. T, temperature.

[42,44]. The abundance of terminal carbohydrates in the skin mucus of Senegalese sole agreed with previous data recorded for skin mucus of different fish species including catfish, gilthead seabream, European sea bass, shi drum and dusky grouper [26,45]. However, our results point to the greater abundance of *N*-acetylneuraminic acid, mannose, glucose and *N*-acetyl-galactosamine, and lower amounts of fucose, galactose and *N*-acetyl-glucosamine. Curiously, these results are contrary to those found in the skin mucus of common dentex, where all the evaluated terminal carbohydrates were detected at very low levels [26]. Some of these carbohydrate residues have been implicated in many roles related to the immune system, while the relationships between other sugars and immunity has not yet been established. For example, *N*-acetyl-galactosamine is involved in cell-to-cell communication, which is important for both normal systemic function and disease processes [46]. *N*e acetylneuraminic acid is associated with infection resistance, because it provides a negative charge to the mucin molecules, reducing bacterial binding [1,47]. The role in infection progression of *N*-acetylneuraminic acid was also demonstrated in the gilthead seabream gut mucosa, where a dramatic decrease in the expression of this carbohydrate was observed as infection by an intestinal parasite (*Enteromyxum leei*) progressed [43]. Future studies are needed to understand the precise role of all terminal carbohydrates present in fish skin mucus as well as their possible role in pathogen adhesion and/or invasion, but the importance of the present results lies in the fact that they help understand the progression of microbial infestation in fish.

Many studies have suggested that enzymes in the epidermal mucus also play important roles in fish immunity. For example, many enzymes have been identified in the skin mucus of several fish species as displaying biostatic and biocidal activities although each of them acts in a specific way [26,48,49]. In the skin mucus of Senegalese sole the levels of some of these enzymes (e.g. lysozyme, protease, esterase and alkaline phosphatase) are very similar. Interestingly, the values obtained for lysozyme (a powerful antimicrobial agent) in the skin mucus of Senegalese Sole are lower than those observed in the skin mucus of olive flounder [49], but very similar to those found in the mucus of other fish species such as gilthead seabream, European sea bass, shi drum, common dentex, dusky grouper and rainbow trout [26,50]. Proteases act in pathogen recognition and elimination, signal processing and cell homeostasis. The proteases in the skin mucus of Senegalese Sole presented similar values to the values observed in gilthead seabream, European sea bass and dusky grouper, but lower than those in the skin mucus of shi drum, common dentex and rainbow trout [26,49]. Alkaline phosphatase and esterase enzyme activities in the

skin mucus of Senegalese sole showed a similar pattern to that found in the skin mucus of other teleosts [26] but higher than those found in rainbow trout and olive flounder [49,50]. The skin mucus of Senegalese sole had a low quantity of antiproteases (pathogens can produce analogous proteases to subvert the host immune response, and so hosts are provided with antiproteases to attack such proteases) and peroxidase but a high quantity of ceruloplasmin compared with the values found in the mucus of other fish species. Perhaps, antiprotease production remains is low in order to keep the high levels of mucus proteases active. Ceruloplasmin is a multicopper oxidase involved in the acute phase reaction to stress, while its role in iron homeostasis and protection against free radical-initiated cell injury has been widely documented [51]. Cerulosplasmin levels have been determined in the mucus from fish previously exposed to stressors (e.g. pathogens and environmental contaminants) [52–54]. Surprisingly, the ceruloplasmin activity detected in the skin mucus of Senegalese Sole was even higher than those previously mentioned, so that further studies are needed to explain the high levels of this enzyme recorded in this fish species. Overall, the results seem to indicate that, while most of the enzymes related to immunity are present in fish skin mucus, the quantity of each one is dependent not only on the fish species but also on the previous immune history. Further characterization is necessary to increase our knowledge of these enzymes and the precise role they play in mucosal immunity.

The prevention of invasion by aquatic microorganisms is mediated by many immune system compounds, including not only enzymes but also antibacterial peptides and polypeptides. The present study evaluates the overall bactericidal activity of the skin mucus because this could be considered more important than the results obtained for individual enzymatic activities bearing in mind that there are still numerous molecules present on fish mucus that have not been characterized [6]. Many studies have revealed that the skin mucus of several fish species has strong anti-bacterial activity against a broad range of microbial pathogens [26,55–58]. In agreement with this, the skin mucus of Senegalese sole also showed significant bactericidal activity against fish pathogenic bacteria while the viability of non-pathogenic bacteria was non-significantly affected by incubation with skin mucus of this fish species. However, the present data contrast with those observed in a previous study in which a high level of direct lytic activity of the skin mucus was observed against the same non-pathogenic bacteria used in the present work [59]. In this previous study the levels of enzymatic activities such as lysozyme, protease and especially alkaline phosphatase and esterase were higher than the levels of these same activities in the mucus of *Solea senegalensis*. This fact could explain the higher survival of these microorganisms in this mucus. However, further studies will characterize new molecules present in fish skin mucus with bactericidal activity, such as antimicrobial peptides, which could be very useful for identifying new natural substances with similar activities to those of antibiotics but with fewer adverse effects.

This study has evaluated different physico-chemical parameters of the Senegalese sole skin mucus. The viscosity of the skin mucus followed a non-Newtonian behaviour, whereby mucus exhibited greater viscosity at low shear rates than at high ones. This behaviour has also been described in the literature for the skin mucus of seawater-reared specimens but to a much lower extent in the same species reared in freshwater [60–62]. The shear rates used in this study attempted to imitate different velocities of fish swimming and some of them have already been used in laboratory swimming trials for several species, including Senegalese sole [62,63]. However, viscosity may also be related with other physico-chemical parameters measured in the present work. Previous studies found that high protein and low osmolality and density values were correlated with increased skin mucus viscosity (in European sea bass and dusky grouper) and *vice versa* (in gilthead seabream and common dentex) [62]. Comparing the values of those studies and those of the present study, the skin mucus of Senegalese sole presented the lowest protein concentration, osmolality and density values while its mucus viscosity was the highest of all of them [62]. Therefore, it seems that the viscosity of the skin mucus of this flatfish does not follow the same pattern as that described for the previously mentioned fish species. On the other hand, species with similar skin mucus data as regard protein content, osmolarity, pH and redox potential (e.g. gilthead seabream and shi drum) exhibited a negative correlation between viscosity and conductivity values [62]. However, although the values of both parameters were higher in the skin mucus of Senegalese than those obtained in skin mucus of dusky grouper (which has the similar protein concentration, pH and redox potential) [62], it seems that the differences found in the viscosity values may be due to the differences in osmolality and conductivity.

In general, our results confirm previously published results on the viscosity of the fish mucus, where a positive correlation between viscosity and osmolality was observed [60,64]. However, higher mucus osmolality does not entail greater mucus viscosity unless a certain change in the fluid structure is produced. Contrarily, a negative correlation between both viscosity and density parameters with temperature were obtained in the skin mucus of Senegalese Sole, in agreement with previous results in other fish species [62]. Furthermore, the variation in mucus and water densities with temperature was essentially identical, which can be considered a reliable index that no significant structural changes were produced in the components of the skin mucus in the range of temperature studied.

Values of viscosity and bactericidal activity against pathogenic bacteria obtained in the skin mucus of Senegalese sole can be compared with those previously obtained in our laboratory using the same bacteria and mucus samples from five different species of teleosts [26,62]. Overall, the results suggested that low skin mucus viscosity values (for example, in gilthead seabream) maintain high levels of bactericidal activity, while the opposite was observed for the samples of Senegalese sole skin mucus studied in the present work. Whether the viscosity of skin mucus is related to its bactericidal capacity or is only related to the reduction of the ability of pathogens to penetrate and cross the mucus barrier has never been studied. Therefore, more studies should be carried out to better understand the relation between these parameters and the behaviour of this surface when confronted by important fish pathogens.

The protein concentration found in the skin mucus of Senegalese Sole was very similar to the levels found in other species such as gilthead seabream, shi drum, common dentex and dusky grouper, although lower than the levels found in European sea bass and olive flounder [26,49]. These results seem to demonstrate that the small variations recorded in this parameter are perhaps due to interspecific and even intraspecific differences. As regard pH, conductivity and the redox potential, they are all clearly interrelated by the amount of ions, which typically have an aqueous solution [62]. In this way, each organism can adopt different forms depending on the amount of protons present in the medium and conductivity can be strongly affected by the presence of $H^{þ}$ or OH , since one measure of the ability of a material to allow electrical current to pass freely depends on the atomic and molecular structure of the material. The redox potential is a measure of the activity of the electrons and the tendency of the chemical species to accept them, which is related to the pH and oxygen content, whilst the pH is a measure of proton activity [62]. The present results obtained for the pH and redox potential follow a similar pattern to that found in the

skin mucus of other teleosts species, while the conductivity of the skin mucus of Senegalese Sole was higher than that observed in the same species, in some cases double the level [62]. Increased knowledge of these parameters could have important practical implications since all of them bear some relation with bacterial adherence and invasion. For example, a previous study demonstrated a similar adhesion pattern in all the bacterial strains tested at alkaline pH, adhesion being strongest in the pH range of 8.2–8.5; however, at slightly acid or neutral pH values, a differential pattern of bacterial adhesion to mucus was obtained for the different strains used [65].

Finally, osmolality may indicate great ion gradients in skin mucus. Ion gradients between the surrounding water and mucus would offer a reduced ion gradient to the plasma, thereby reducing the cost of ion transport [66]. By contrast, Roberts and Powell [60] observed slight differences in osmolality between three seawater fish species, which may indicate small ion gradients. Taking into account that the seawater osmolality measured by us was 1106 ± 3.2 mmol kg^{-1}, we could consider that skin mucus of Senegalese sole as hypo-osmotic to the surrounding water (similar results were obtained for European sea bass and dusky grouper). However, the skin mucus of gilthead seabream, shi drum and common dentex was categorized as iso-osmotic [62]. Hence, in the case of iso-osmotic mucus, the energy destined to ionic transport seems to be irrelevant, unlike in the case of hyper-osmotic mucus [60].

In conclusion, several physico-chemical and biological parameters of the skin mucus of Senegalese sole were determined to obtain more knowledge about the biology and function of this defensive barrier. For this purpose, we studied different terminal carbohydrate residues (related with the initiation of defensive responses and bacterial binding), several enzymes (chosen for their implication in immunity) and several physico-chemical parameters interconnected with viscosity, all factors which reduce the ability of pathogens to penetrate the fish surface. The differences recorded in these parameters among different fish species suggest that mucosal immunity is very complex and is not based on individual components. Furthermore, different fish species may have developed different mucosal defence strategies based on different repertoires of physico-chemical parameters and/or substances. Nevertheless, a close relation between some parameters (e.g. viscosity and bactericidal activity) seems to be a general rule in fish mucus; in other words, skin mucus with low viscosity values has a high percentages of bactericidal activity and *vice versa*. In fact, it is conceivable that high viscosity in the skin mucus increases the barrier effect for pathogens, and so any bactericidal capacity would not be so acute. Further studies on the characterization and relationship of these parameters with disease susceptibility should be carried out to increase our knowledge of the biology and function of this essential barrier in Senegalese sole, which could have important applications for the fish-farming industry.

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