



## Short communication

## NK-lysin from skin-secreted mucus of Atlantic salmon and its potential role in bacteriostatic activity

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## ABSTRACT

NK-lysin, despite being a direct effector of cytotoxic T and natural killer cells, is an antimicrobial peptide (AMP) with known antibacterial function in vertebrates and so in fish. Its presence has been described in different tissues of teleost fish. One of the strongest antimicrobial barriers in fish is skin-secreted mucus; however, this mucus has been found to contain only a small number of AMPs. The present study describes for the first time the constitutive expression of NK-lysin in Atlantic salmon (*Salmo salar*) mucus produced by the skin, recording the AMP at a higher concentration than in serum with greater bacteriostatic activity. Hepcidin may be involved to a greater extent in systemic responses since it was expressed to a higher degree in serum which was more potent for alternative complement and peroxidase activities.

## 1. Introduction

In teleost fish, mucous membranes limiting digestive, respiratory or external surfaces of the body use a defensive function based on antimicrobial activity by entrapping and destroying foreign agents in order to keep them out of the organism [1]. The differentiated cells responsible for secreting mucus are the mucous goblet cells. This secreted mucus is mobile and dynamic and, in the case of skin, covers the fish's external epidermal surface and is composed of a high amount of water along with molecules that vary depending on the fish species [2]. In recent years, certain studies have assessed the presence of antimicrobial peptides (AMPs) with antimicrobial activity such as histones, high-density lipoproteins, pleurocidin or defensins in skin or even mucous goblet cells, pointing to their presence in skin-secreted mucus [3–5]. NK-lysin is an effector of cytotoxic T cells and a potent AMP that is widespread in vertebrates. It also possesses an effective antimicrobial function [5–8]. In fish, its antibacterial activity has been widely demonstrated [8–12]. For example, NK-lysin from Japanese flounder, tongue sole or yellow croaker showed bactericidal activity against different strains of Gram + and/or Gram – bacteria species that are known to be fish and non-fish pathogenic [13]. However, its transcriptomic detection in skin seems species-dependent since it is not expressed in all fish species [6,10,11]. NK-lysin expression at the peptide level has not yet been studied in mucus. The present study analyses the antimicrobial function of mucus and serum from Atlantic salmon (*Salmo salar*) and

reports, for the first time, the presence of NK-lysin in these fish's mucus, which may be involved in its local bacteriostatic activity, while hepcidin appears to be more related to systemic responses.

## 2. Material and methods

Healthy smolts of Atlantic salmon (*Salmo salar*; 58 ± 13 g body weight) provided by Centrovet (Chile) were bred at the Pontificia Universidad Católica de Valparaíso (PUCV; Chile) with a photoperiod of 16:8 h light:dark, temperature of 14 ± 1 °C, and water salinity of 32‰. The specimens were fed with a commercial dry pellet diet (Skretting) at 1% of body weight per day. Temperature, mortality and food intake were recorded daily. The specimens were anesthetized with 20 µL/100 L of benzocaine (Richmond) before handling. The blood and skin mucus were obtained as elsewhere [14]. Briefly, blood was obtained from the caudal peduncle and the serum samples by centrifugation (10,000 × g, 10 min, 4 °C). Skin mucus was collected by soft scrapping of the dorso-lateral skin taking care to avoid any contamination; this was followed by centrifugation (2000 × g, 10 min, 4 °C). Antimicrobial activity was tested in the serum and mucus samples adjusted to 300 µg of protein per reaction, previously measured by Pierce BCA Protein Assay Kit (ThermoFisher) in accordance with the manufacturer's instructions. The haemolytic activity of the complement was assayed using a previously described protocol with slight modifications [15]. Concisely, mouse red blood cells (MRBC) were used as targets. Mice were

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ethanated with 10 mg/kg xilacine and 150 mg/kg ketamine (Richmond) and completely bled by cardiac puncture. Blood was gently deposited on the wall of a glass flask with autoclaved metal balls slowly oscillating until the fibrin clot was completely formed. The defibrinated blood was carefully extracted from the flask without touching the clot and stored at 4 °C until use. Equal volumes of MRBC suspension in phenol red-free Hank's buffer containing  $Mg^{+2}$  (Panreac) and EGTA (Sigma) were mixed with serially diluted samples. Samples were incubated (90 min, 22 °C) and centrifuged (400 × g, 5 min, 4 °C). The results were expressed in ACH<sub>50</sub> units as the titre at which 50% haemolysis is produced [15]. Bacteriostatic activity was determined by evaluating the inhibition on the bacterial growth curves of *Aeromonas salmonicida* and *Vibrio anguillarum* [16]. Peroxidase activity was measured using a previously described method [17]. Briefly, samples were incubated with 100 µl of 10 mM 3,3',5,5'-Tetramethylbenzidine solution containing 0.015% H<sub>2</sub>O<sub>2</sub> as substrate and the change of colour was then measured. One unit was defined as the amount producing an absorbance change (450 nm) of 1 and the activity expressed as mg<sup>-1</sup> of protein [17]. Protease activity was determined as the percentage of hydrolysis of azocasein by 4 mg/ml of proteinase K [18]. Total anti-protease activity was determined as the percentage of inhibition of the hydrolysis of azocasein by 4 mg/ml of proteinase K [19]. The constitutive levels of NK-lysin and hepcidin were analysed by indirect ELISA, as elsewhere with slight modifications [20]. The molecules of interest were identified in the Atlantic salmon using mouse and rabbit polyclonal antibodies that detect NK-lysin from European sea bass (*Dicentrarchus labrax*) and hepcidin from rainbow trout (*Oncorhynchus mykiss*), respectively [20]. The specificity of antibodies was verified in extracts of proteins of Atlantic salmon head-kidney by Western blot [20]. The bands observed showed the expected molecular weights (Supplementary data). The NK-lysin (NP\_001134582) detected corresponds to the mature peptide of 12 kDa [10] and the hepcidin (AAO85553.1) was 9.4 kDa, as previously described [20]. Data is presented as mean ± standard error of the mean (SEM; n = 6) and significance of the differences between mucus and serum was analysed by the Student's *t*-test (*p* < 0.05).

### 3. Results and discussion

The functional results showed great differences in the antimicrobial activities between serum and mucus (Fig. 1). The alternative complement activity was easily detected in serum but not in mucus (Fig. 1A). Prior to this study, alternative complement activity in fish skin mucus had only been reported in gilthead seabream (*Sparus aurata*) [21]. However, the protein C3 had been detected in the skin mucus of several fish species [22,23]. The pivotal role of skin-secreted mucus in fish defence against pathogenic bacteria has been widely demonstrated, suggesting that antibacterial function follows pathways different to that of haemolytic alternative complement. Indeed, the antibacterial function of skin mucus has been demonstrated in fish species such as ayu (*Plecoglossus altivelis*) and turbot (*Scophthalmus maximus*) [24,25]. Moreover, in turbot the removal of skin mucus and the subsequent infection with *Vibrio anguillarum* resulted in increased mortality rates (from 50% in naïve fish to 100% in fish with removed skin mucus) [25]; or resulted in higher susceptibility to bacterial infection, as with common carp (*Cyprinus carpio*) [26]. Interestingly, our results showed higher bacteriostatic activity, by means of a decrease in bacterial growth, in mucus than in serum after the incubation with *A. salmonicida* or *V. anguillarum* (Fig. 1B and C). This is in agreement with the relevance of mucus in the fight against bacteria. Moreover, the *in vivo* infection with *A. salmonicida* triggered enhancement in activities which are directly or indirectly involved in immune responses such as lysozyme, alkaline phosphatase, superoxide dismutase, peroxide, glutamate pyruvate transaminase and glutamic oxalacetic transaminase activities, 2 days post infection [27]. These data are in accordance with what occurs in other fish species, such as gilthead seabream, where bacterial

inhibition after incubation of several fish pathogenic bacteria with mucus was greater than with serum [14]. Enzymatic activity in epidermal mucus, including peroxidase, protease and anti-protease, may also play a relevant role in fish immunity. For example, peroxidases are enzymes with important microbicidal function which eliminate H<sub>2</sub>O<sub>2</sub> and maintain redox balance, and whose role in mucus has been studied in a number of fish species [28]. In the present study, peroxidase activity in the Atlantic salmon was higher in serum (Fig. 1D) as demonstrated previously with older fish [27], whilst the opposite occurred in gilthead seabream [14]. This indicates different paths of action depending on the species. Despite the directly and indirectly protective role of proteases, they are able to activate and enhance pathways related to the innate immune system such as complement, antibacterial peptides and immunoglobulins [29–31]. Therefore, protease and anti-protease activities were also studied. Our data showed no differences between sample types in anti-protease activity (Fig. 1E) while protease activity was undetected in serum and in mucus (data not shown). The variances in antimicrobial function in serum and skin mucus, which have been demonstrated previously [27], are directly correlated with their protein composition. Moreover, it is commonly understood that skin-secreted mucus is composed of a wide range of innate immune-related compounds [22,23,32]. However, although some AMPs have been detected in skin mucus for a small number of fish species [5], their presence and their mechanisms of action in mucus are still poorly understood. Strikingly, in Atlantic salmon we detected NK-lysin for the first time in mucus, with content levels 4.0 fold higher than in serum (Fig. 1F). This coincides with the greater bacteriostatic activity observed. NK-lysin antibacterial activity has been demonstrated in several fish species against Gram - bacteria species [8,10,12], such as those used in the present study. Moreover, gene coding for NK-lysin is constitutively expressed in the skin of tilapia (*Oreochromis niloticus*), where mucus-secreting goblet cells are located [9]. Furthermore, over-expression of NK-lysin results in lower bacterial loads and fish survival [8,10,12]. Taking all this data into account, the present results point to a potential antibacterial role of NK-lysin in skin mucus, though further study is needed to shed more light on this issue. At the same time, hepcidin was found at a higher level in the serum, as is the case with complement and peroxidase activities, suggesting its involvement in other antimicrobial pathways.

### 4. Conclusion

To summarize, the results show differences in innate immune function between the skin-secreted mucus and serum of Atlantic salmon. Furthermore, NK-lysin was detected for the first time in the mucus, where it was expressed at higher loads than in the serum, a result that correlated with the higher bacteriostatic activity found. Hepcidin appears predominantly in the serum. All these data may indicate different mechanisms of action of the two AMPs, suggesting greater involvement of NK-lysin in local responses in skin-secreted mucus while hepcidin may be more related to systemic responses. However, further study is required to clarify the specific roles of these AMPs in the antimicrobial response of Atlantic salmon.

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### Appendix A. Supplementary data

Specificity test of polyclonal mouse anti-NK-lysin from European sea bass and rabbit anti-hepcidin from rainbow trout in Atlantic salmon head-kidney samples. SDS-PAGE shows head-kidney (HK) protein

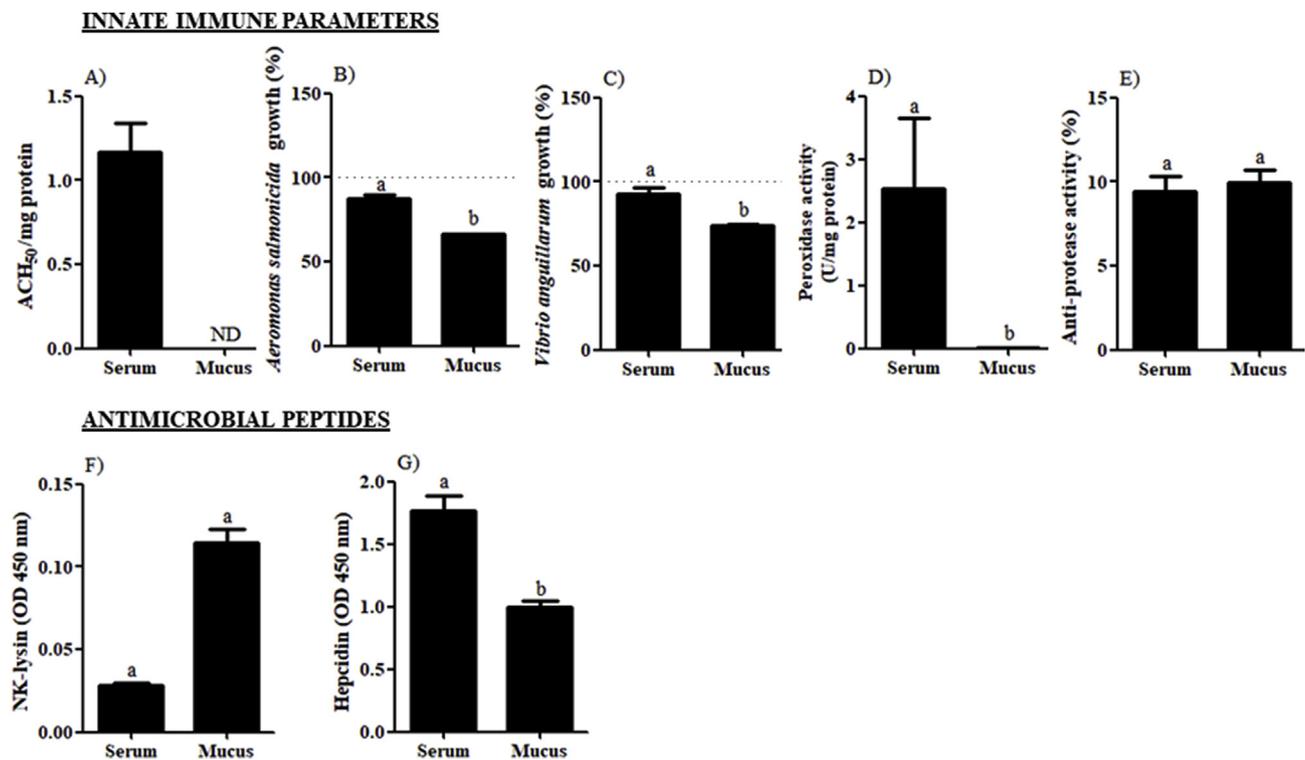


Fig. 1. Atlantic salmon innate immune parameters (A–E) and antimicrobial peptide content (F,G) in serum and skin-secreted mucus. Haemolytic activity of complement (A), bacteriostatic activity against *Aeromonas salmonicida* (B) and *Vibrio anguillarum* (C), peroxidase (D) and anti-protease (E) activities, NK-lysin (F) and hepcidin (G). Data represent the mean  $\pm$  standard error of the mean ( $n = 6$ ). Different letters denote statistical differences between serum and mucus according to the Student's  $t$ -test ( $P \leq 0.05$ ). ND, non-detected.

profile. Western blot shows the corresponding bands with the proper molecular weight for NK-lysin and hepcidin peptides in salmon.

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