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Biology, environmental and nutritional modulation of skin mucus alkaline phosphatase in fish: A review

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ABSTRACT

Alkaline phosphatase (AP) is a major, recently recognized component of innate immunity. The intestinal AP (IAP) isoform plays a pivotal role in controlling gastrointestinal and systemic inflammation in terrestrial mammals. This is so essentially through detoxification (by dephosphorylation) of proinflammatory microbial components that can no longer be recognized by so-called toll-like receptors, thus preventing cellular inflammatory cascade activation. A unique feature of fish is the presence of AP in skin and epidermal mucus (skin mucus AP) but its actual functions and underlying mechanisms of action are presently unknown. Here, we gather and analyse knowledge available on skin mucus AP in order to provide a holistic view of this important protective enzyme. Our main conclusions are that skin mucus AP is responsive to biotic and abiotic factors, including nutrients and bioactive feed components, prebiotics and probiotics. Importantly, both skin mucus AP and IAP appear to correlate, thus raising the interesting possibility that skin mucus AP be used as a proxy for IAP in future nutritional studies. Blood serum AP also seems to correlate with skin mucus AP, though biological interpretation for such relationship is presently unknown. Finally, the precise isoform/s of AP present in skin should be identified and underlying molecular mechanisms of skin mucus AP actions deciphered.

1. Introduction

There is an increasing demand worldwide for fish farming production and also for ornamental fish. There is also increasing pressure of the public to reduce prophylactic (and therapeutic) use of antibiotics and chemical substances in animal rearing and food industry, including fish [1]. This has led to new investigations on so-called « natural substances and plant bioactives » as sustainable alternatives for keeping animals healthy.

In recent years, chronic low-grade inflammation has been identified as a strong driver of chronic non-communicable inflammatory diseases in humans [2]. In production animals, inflammation participates in energy balance [3] and associated immune responses represent a metabolic cost limiting production efficiency [4]. Among evolutionary conserved endogenous defense systems, intestinal alkaline phosphatase (IAP) has been identified unequivocally as one major enzyme for keeping gut (and systemic) inflammation under control [5–8].

Skin mucus AP is considered as part of fish mucosal immune system, though its actual mechanisms of action are poorly understood [9]. Data suggest that skin mucus AP displays antimicrobial activities and participate in fish defense against water pathogens. Its use as an indicator of stress has also been suggested [10]. Importantly, the AP found in fish intestine was also demonstrated to combate pro-inflammatory compounds and to inhibit the generation and tissue infiltration of neutrophils at sites of inflammation [11–13]. In connection with the present review, and given the similarities in e.g. hydromineral and related hormonal physiology between the gut and the skin of mammalian and aquatic species [14], it is tempting to speculate that fish skin mucus-associated AP enzyme contributes to dephosphorylate and, therefore, detoxify pro-inflammatory microbial compounds present in water, this in order to protect the organism and keep skin inflammation under control.

Another strong motivation for the present review is that food and many nutritional compounds are major stimulators of intestinal AP (IAP) in both terrestrial mammals and fish [5,6,8,13]. As the different AP isoforms, regardless of their tissular localisation and specificity, all belong to a single AP « system » aimed at controlling inflammation everywhere at mucosal interfaces and in the body [8]. Therefore, our present hypothesis is that skin (mucus) AP is also responsive to nutritional factors. The publications reviewed here collectively support this hypothesis.

In summary, skin mucus AP is modulated by a number of biotic and

Abbreviations: AP, alkaline phosphatase; IAP, intestinal alkaline phosphatase; Skin mucus AP, skin mucus alkaline phosphatase

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abiotic factors, including nutrients and food components which are reviewed below.

2. Biology of AP and importance in innate immmunity and inflammation

AP has long been recognised as an important component of innate immunity though its precise immunological roles remained largely unknown for many decades. Major advances have been made, especially since late 90's on the IAP isoform [5,6,8,15]. A among the many physiological properties of (I)AP, the most important appears to be its ability to dephosphorylate pro-inflammatory bacterial components such as lipopolysaccharides (LPS), flagellins, DNA CpG motifs, and also pro-inflammatory endogenous moieties such as phosphorylated adenosine (ATP, ADP, AMP) and uridine (UDP) when they are released extracellularly after cellular integrity alterations subsequent to wound or infection. These are viewed by the immune system as danger signals and strong induced of inflammatory responses. Other isoforms, including tissue non-specific AP (TNAP, previously called bone-liver-kidney AP) and placental AP are also able to dephosphorylate such pro-inflammatory compounds [8]. In terms of molecular mechanisms, dephosphorylated compounds are no longer recognised by their toll-like receptors (e.g. TLR4 for LPS; TLR5 for flagellin; TLR9 for microbial DNA CpG) and, thus cannot elicit cellular pro-inflammatory responses involving the NF-κB activation pathway, gene activation and release of pro-inflammatory cytokines (e.g. IL-8) [5,6].

3. Differences between terrestrial mammals and fish for skin mucus and AP

The skin of terrestrial mammals does not harbour goblet cells nor produces skin mucus [16]. Normal mammalian skin contains very low levels of AP activity compared with the intestine, the liver of the kidney [17]. This AP activity is of the TNAP isoform, does exist in soluble and membrane-bound forms and is restricted to the capillary zone of mammalian skin dermis, with no AP activity in the epidermis [17]. In diseased mammalian skin (e.g. in psoriasis), AP activity is strongly increased and persisting, precisely around dermal capillaries of the skin proliferative zone [18].

Although different publications described AP localization in fish skin, the more exhaustive and semi-quantitative account has been provided recently in catfish (*Heteropneustes fossilis*) submitted to cutaneous wound and acidic water stress [19]. In summary, AP staining was transiently detected by histochemistry in skin basal cells (strong staining, between 60 h and 30 days after wound), epithelial cell middle layer (strong, 18 hr-30 d), epithelial outer layer (moderate, 72 hr-30 d) in response to skin healing, with kinetics depending on skin site. Very nice pictures of AP staining in skin epithelial rodlet cells in carp (*C. carpio*) were also published [20]. These cells are not present in the epidermis of fish maintained in control conditions but they appear after stress [20]. They may be reasonably the source of AP found in epidermal mucus. AP staining was never observed in skin goblet cells in this study, contrasting with earlier reports e.g. in carp (*Cyprinus carpio*) or trout (*Onchorynchus mykiss)* [20,21]. Transient AP staining was also noted in subepithelial tissues and cells, including fibroblasts (5–15 days) and dermal cells (5–20 days) [19]. Finally, which AP gene and enzyme isoforms are present in skin have not been documented. In one report in channel catfish (*Ictalurus punctatus*), Iap gene was indicated in the gene catalog investigated but samples of gill and skin were analysed together so that conclusion regarding skin AP gene cannot be drawn specifically [22].

4. Methodologies for collecting fish skin mucus and assaying AP activity

Most publications on fish skin mucus report an initial 24 h-fasting imposed to fish, most often followed by a sub-lethal anesthesia with either tricaine methane sulfonate (MS-222) (100 mg L^{-1} water) or clove powder (5 mg L^{-1}) according to the protocol published by Ross and co-workers [23]. Three publications appeared to somewhat diverge from this protocol: no anesthesia [24], or anesthesia with higher concentration of ML-222 (200 mg L^{-1} water) [25] or much higher clove powder concentration (500 mg L^{-1} water) [26]. Importantly, different anesthetic agents (MS-222, clove powder, 2-phenoxyethanol) did not appear to differ between them regarding their effects on skin mucus AP activity at 1 h or 24 h post-anesthesia [27].

Fish skin mucus itself is either collected on whole body surface area by keeping fish is a polyethylene bag containing a given volume (usually 10 mL) of salted water (50 and 100 mM NaCl for freshwater and seawater fish, respectively) and being gently shaked for 1–2 min or collected partially on skin dorsal or dorso-lateral surface areas by gentle scraping with a sterile plastic or rubber spatula. This is so for limiting mucus contamination with feces, sperm or scales. After mucus is collected, the liquid preparations are centrifuged (e.g. between 1500 × *g* and 10,000 × *g* for 10–15 min at +4 °C) and the supernatant is aliquoted, freeze-dried and stored at −70 to −80 °C. When the whole mucus is collected, it is so in salted water but when it is partially collected by skin scraping, it is usually put into a tris-buffed saline (TBS, 50 mM Tris-HCl, pH 8.0, 150 mM NaCl). Sometimes the supernatant is further filtered using Whatman paper (No. 1) [28] or Millipore filter (0.45 μm) [29].

Regarding assays, protein concentration in thawed mucus powder reconstituted in solution, when determined is carried out using the Lowry [30] or the Bradford [31] methods. Skin mucus AP activity is always determined using the synthetic substrate *para*-nitrophenyl phosphate which is dephosphorylated into *para*-nitrophenol following the action of mucus AP. This product is assayed kinetically by measuring sample absorbance at 405 nm. Research groups use either laboratory-made reagents or commercial kits but the protocols are probably very similar. A typical protocol is as follows: freeze-dried mucus is reconstituted with 100 mM ammonium bicarbonate buffer containing 1 mM MgCl2, at pH 7.8 at 30 °C for 15 min [23]. The working pH and temperature can differ slightly (pH 7.8 and 37 °C). One (international) unit of AP activity is defined as the amount of enzyme required for releasing 1 μmol of *para*-nitrophenol in a minute. Data are finally expressed as total (IU.L^{-1}) or specific (IU.mg^{-1} protein) activity concentration. This methodology for AP activity determination using the mentioned synthetic substrate is also used in mammalian and fish intestine [5,6]. Importantly, a number of publications provide both mucus protein concentrations and AP total activity concentrations whereas others report only AP specific activity concentrations. This may be a problem because quite often both mucus protein and AP activity concentrations do vary with the treatment under study whereas the AP total activity to mucus protein concentration ratio may not be affected by treatments.

Collectively, the published methodological information shows that the vast majority of publications use relatively standardized protocols for skin mucus collection and protein and AP activity assays, though two approaches are equally used for mucus sampling (total versus partial collection). Dilutions protocols may differ among publications, thus making absolute comparisons across publications for mucus protein concentrations and total AP activity concentrations difficult.

5. Variability in skin mucus AP across fish species

Skin mucus AP activity has been reported in nearly 30 fish species, data being expressed in activity units per liter (Fig. 1) [26–28,32–44] or per mg of mucus protein (Fig. 2) [23–25,45–56].

The most striking feature of these data is their high variability across fish species (means ± SD = 44 ± 50 U L^{-1}, min. = 2 and max. = 194 U L^{-1}; and 10 ± 14 U mg^{-1} protein, min. = 0 and max. = 45 U mg^{-1} protein, respectively), despite the fact of an

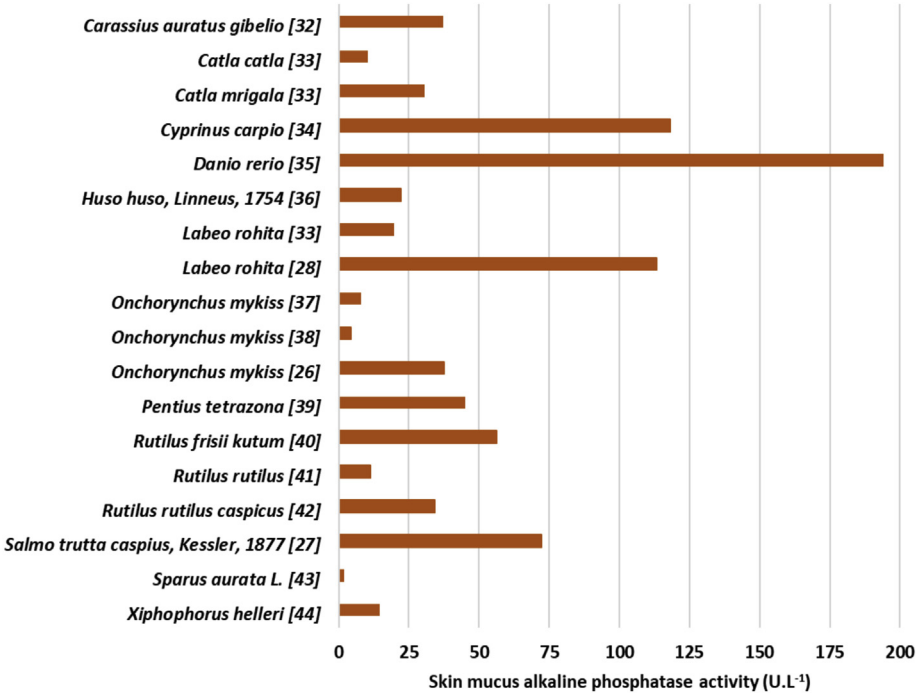

Fig. 1. Skin mucus alkaline phosphatase activity values in fish (data expressed in $U.L^{-1}$) [26–28,31–43].

expression of AP specific enzyme activity. One publication suggests Skin mucus AP to be somewhat higher in seawater compared to freshwater in rainbow trout (*Onchorynchus mykiss*) [25].

6. Non-nutritional biotic and abiotic factors

Regarding biotic factors, wound healing appears as a strong inducer of skin mucus AP activity in various fish species [19,21]. Parasitic infection with the sea louse *Lepophtheirus salmonis* was also able to dramatically increase (3–5 fold) skin mucus AP activity in Atlantic salmon (*Salmo salar*) when the parasitic load was sufficiently high (200 lice per fish) [23]. Bacterial infection by *Aeromonas salmonicida*, responsible for furunculosis resulted in increased AP activity (transiently) and gene expression after infection in salmon (*Salmo salar* L.) [53], though such variations appeared relatively independent one from the other. Also crowding stress strongly stimulated skin mucus AP activity (+168%) with stocking levels increasing from 40 to 80 fish per tank in tiger barb (*Pentius tetrazona*) [39]. Contrasting with this, skin mucus AP decreased (−27%) in turbot (*Scophthalmus maximus*) with increasing initial stocking density (1500, 2200 and 3100 fish per tank) [57].

Regarding the environmental pollutants heavy metals, gilthead seabream (*Sparus aurata*) specimens waterborne exposed to arsenic trioxide (5 mM), cadmium chloride (5 mM) and methylmercury chloride (0.04 mM) for 2, 10 and 30 days exhibited complex skin mucus AP responses, usually with first an increase between 2 and 10 days and then a decrease at 30 days from/to initial levels (for mercury) or futher decrease (inhibition, with arsenic and cadmium) [56]. More recently, the toxicity of polymethylmethacrylate nanoplastics (0–2 mg L^{-1}) was investigated in European seabass (*Dicentrarchus labrax)* [58]. Skin mucus AP levels were decreased threefold at the highest dose, thus suggesting impaired innate immunity following exposure to these compounds [50].

Three anaesthetic agents commonly used in aquaculture practice (MS-222, 2-phenoxyethanol and clove oil) all increased skin mucus AP activity by 40–50% compared to controls in rainbow trout (*O. mykiss*) 24 h post-anesthesia [27].

Regarding abiotic factors, water salinity is suggested to stimulate skin mucus AP activity because its activity was detected in rainbow trout (*O. mykiss*) and coho (*O. kisutch*) and Atlantic (*S. salar*) salmons reared in seawater but not in freshwater [25]. Acidic water stress delayed AP staining in skin migrating epidermis cells [19]. Seasonal variations in skin mucus AP activity were also documented [52]. This may have reflected changes in associated factors, e.g. feed availability and possibly water temperature, though no significant correlation with the latter was found by the authors [52]. Skin mucus AP varies according to circadian cycles with an important (−40%) activity decrease during the night in permit (*Trachinotus falcatus*) [59]. Again, this may partly reflect changes in other factors, e.g. water temperature and feed intake.

7. Influence of nutrients, plants and plant extracts

Feed intake and nutrition appear to be strong modulators of skin mucus AP, as already observed for IAP in the intestine of terrestrial mammals and fish [5,6,8,13]. One study reported a strong upregulation of *Iap* gene in channel catfish (*I. punctatus*) after 7 days of starvation, but samples included gill and skin mixed together [22], as mentioned above.

Fourteen studies on this topic have been identified in the Web of Science. Increasing skin mucus AP by supplementing diets with nutrients or plants or plant components was successful (significant) in 80% of the published studies (Fig. 3).

Again, the variability in skin mucus AP responses is very high (mean ± SD = +75% of control values ± 89%). Four studies, with garlic (*Allium sativum,* range tested: 0–15 g kg^{-1} feed) in Caspian roach (*Rutilus*), myrtle (*Myrtus communis* L., 0–15 g kg^{-1}) in rainbow trout (*O. mykiss*), vitamin C (0–2 g kg^{-1}) in Caspian roach (*Rutilus*) and ginger (*Zingiber officinale*, 0–50 g kg^{-1}) in rainbow trout (*O. mykiss*) reported very strong skin mucus AP responses with activity increases > 100% of control values within studies (Fig. 3) [38,41,42,60]. Three other studies reported strong skin mucus AP activity increases (comprised between +50 and + 100% of control values) with date palm (*Phoenix dactylifera* L., Tunisian Degla variety) fruit water-soluble extracts (0–200 ml kg^{-1}) in common carp (*C. carpio*), peppermint (*Mentha piperita*, 0–30 g kg^{-1}) in Caspian white fish (*Rutilus frisii kutum*) and with ginger (*Z. officinale*, 0–10 g kg^{-1}) in rohu (*Labeo rohita*) [28,34,40]. This was so following

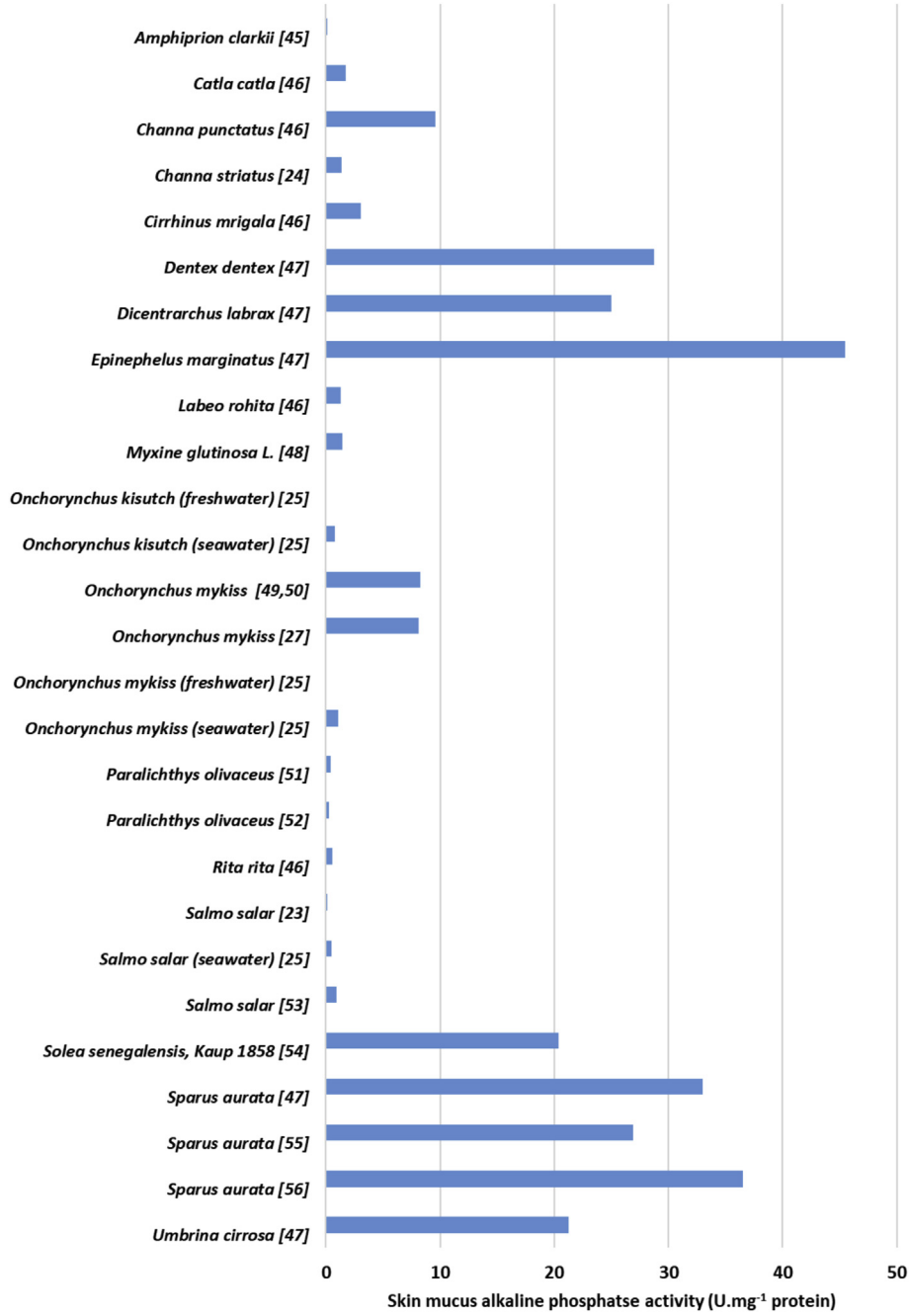

Fig. 2. Skin mucus alkaline phosphatase activity values in fish (data expressed in U.mg^{-1} protein) [23–25,45–56].

intraperitoneal injection of aloin (*Aloe barbadensis,* 1 mg kg^{-1} body weight) in Indian major carp (*L. rohita*) [61]. Four studies reported modest (inferior to +50% of control values) but significant increases in skin mucus AP with stakhouse (*Graciliara gracilis*, 0–10 g kg^{-1}) in zebrafish (*D. rerio*), peppermint (*M. piperita*, 0–30 g kg^{-1}) in Caspian brown trout (*S. trutta caspius*), a seaweed mixture (Ergosan, a mixture of two brown sea weeds, *Laminaria digitata* and *Ascophyllum nodosum*, 5 g kg^{-1}) and stinging nettle (*Urtica dioica*, 0–30 g kg^{-1}) in rainbow trout (*O. mykiss*) [28,35,37,49]. Finally, two studies reported no significant effect of plants on fish skin mucus AP: fenugreek (*Trigonella foenum graecum*) fed at a single dose (50 g kg^{-1}) to gilthead seabream (*S. aurata*) [43] and common guava (*Psidium guajava* L.) leaves (0–10 g kg^{-1}) fed to common carp (*C. carpio*) [62].

Collectively, these data indicate that different fish species respond to dietary supplementation with nutrients (vitamin C) and various plants or plant extracts with increasing their skin level of AP. The magnitude of such responses depends on supplement concentration in diets and can be very large (> 100% basal levels) with some of them.

8. Influence of prebiotics, probiotics and microbial components

Thirteen studies have been published in this category. Overall, increasing skin mucus AP by supplementing diets with prebiotics, probiotics or microbial components appears successful (significant) in 52% of the published studies. This is somewhat lower than with nutrients, plants or plant extracts. The variability in skin mucus AP responses is also very high here (mean increase of +44% of control values ± 49% in skin mucus AP activity).

Two studies with prebiotics have been published (Fig. 4). A commercial fermentable raw fiber concentrate (Vitacel®, 10 g kg^{-1}) had a

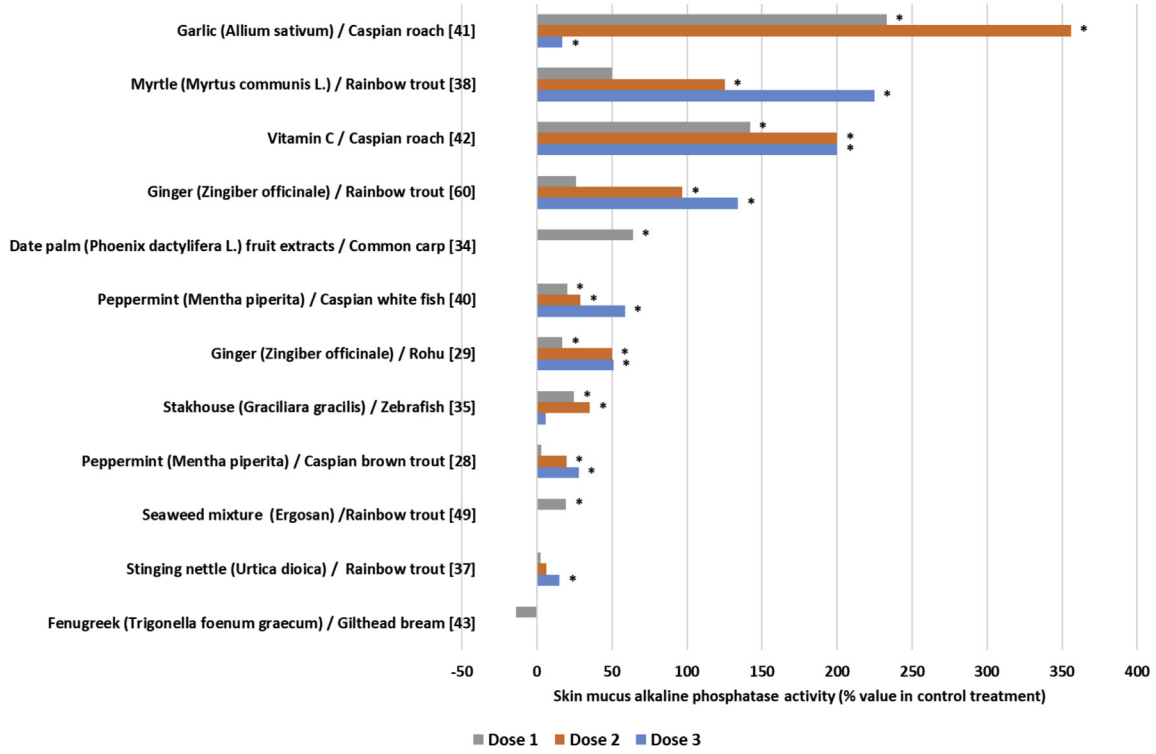

Fig. 3. Influence of nutrients, plants or plant extracts on skin mucus alkaline phosphatase activity in fish (data expressed in percentage of control values without any dietary supplementation; * significant effect of supplementation at $p < 0.05$ within studies) [28,29,34,35,37,38,40–43,49,60].

strong (> 100% basal value) stimulatory effect on skin mucus AP in Caspian white fish (*R. frisii kutum*) [63]. Conversely, galactooligosaccharides (Vivinal-GOS®, 0–20 g kg^{-1} feed) obtained through the enzymatic conversion of lactose had no significant effect on skin mucus AP in goldfish (*Carassius auratus gibelio*) [32].

Probiotics have been studied alone or in combination with prebiotics or plants or plant extracts (Fig. 4). Two studies reported very strong (> 100% control values) effects on skin mucus AP. One was with fermentable fiber (Vitacel®, 10 g kg^{-1}) associated with a probiotic mixture (PrimaLac®, commercial probiotic mixture of *Lactobacillus acidophilus, L. casei, Enterococcus faecium and Bifidobacterium bifidum*, 1 g kg^{-1}) in Caspian white fish (*R. frisii kutum*) [32]. Most of this effect

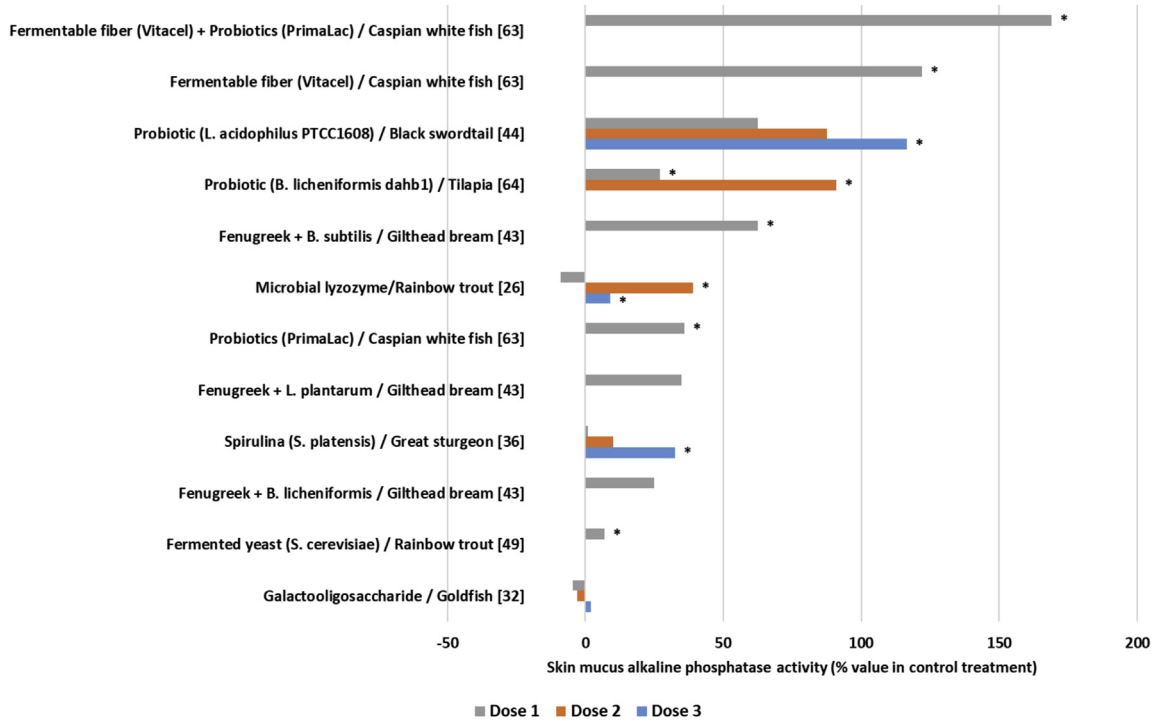

Fig. 4. Influence of probiotics and prebiotics on skin mucus alkaline phosphatase activity in fish (data expressed in percentage of control values without any dietary supplementation); * significant effect of supplementation at p < 0.05 within studies) [26,32,36,43,44,49,63,64].

was due to fermentable fiber as reported above, the probiotic mixture alone accounting for a moderate (+36%) but significant skin mucus AP increase [63]. The second study was with *L. acidophilus* (strain PTCC1608, 0–6x10^8 CFU. kg^{-1}) in black swordtail (*Xyphophorus helleri*) [44]. Two other probiotics generated strong (+50% to +100% of control values) skin mucus AP responses, namely *B. Licheniformis* (dahb1 strain, 0–10^7 CFU. kg^{-1}) in Mozambique tilapia (*Oreochromis mossambicus*) [64] and *B. subtilis* (20 g kg^{-1}) associated with fenugreek (that had no effect *per se*, see above; 50 g kg^{-1}) in gilthead bream (*S. aurata*) [43]. By contrast, the probiotics *L. plantarum* (20 g kg^{-1}) and *B. licheniformis* (20 g kg^{-1}) associated with fenugreek (50 g kg^{-1}) had no effect on skin mucus AP in this study [43]. Spirulina (*S. platensis*, 0–100 g kg^{-1}) and microbial lysozyme (0–15 g kg^{-1}) displayed moderate stimulatory effects on skin mucus AP in great sturgeon (*Huso* Linnaeus, 1754) and rainbow trout (*O. mykiss*), respectively [26,36]. Finally, fermented yeast (*Saccharomyces cerevisiae*, 5 g kg^{-1}) displayed small (7%) but significant stimulation on skin mucus AP in rainbow trout (*O. mykiss*) [49].

Taken together, these data indicate that a number of probiotics and some prebiotics have the potential to stimulate skin mucus AP *in different fish species. Again, responses are variable and seem to depend on dietary supplement dosage, and strain for probiotics.*

9. Links between skin and intestinal alkaline phosphatase activities

Numerous studies in terrestrial mammals indicate that IAP isoform plays a major role in the control of local and systemic inflammation by detoxifying (by dephosphorylation) microbial pro-inflammatory compounds (e.g. LPS, flagellin, CpG motifs), besides its many roles in various facets of gut physiology [5,6,8]. Futhermore, many diseases have been recently characterized with altered IAP activity (or gene expression) [6,8]. Supply of exogenous (e.g. bovine intestinal or human recombinant chimeric intestinal-placental) AP has proven successful in preventing or treating such diseases, at least in animal models.

Fish skin faces the same challenges as the intestine in terms of physical, chemical and microbiological threats and one could speculate on the possibility that both IAP and skin mucus AP would vary in a concerted way in response to the same stressors. At present, only one study reported data on both skin mucus AP and IAP in Caspian white fish (*R. frisii kutum*) fingerlings [63]. Interestingly, these data appear positively correlated one to each other (Fig. 5), thus suggesting that skin mucus AP may vary as IAP with nutrition and also be used as a proxy for IAP, in a noninvasive perspective. However, more data are needed before drawing firm conclusions and establishing predictive equations for IAP based on skin mucus AP.

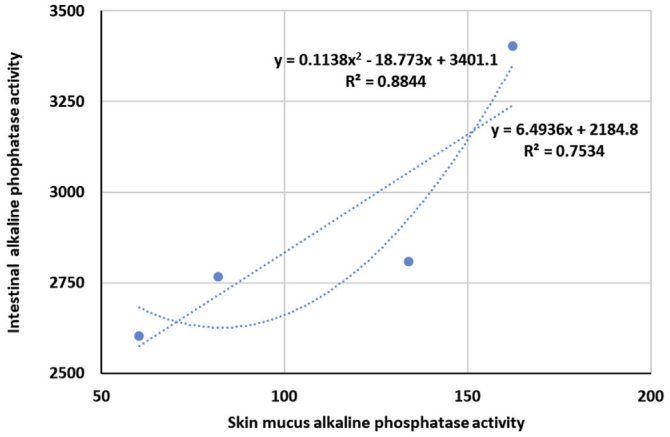

Fig. 5. Correlations between intestinal alkaline phosphatase and skin mucus alkaline phosphatase activities in Caspian white fish (*Rutilus frisii kutum*) (enzyme activities expressed in absorbance units at 405 nm) [63].

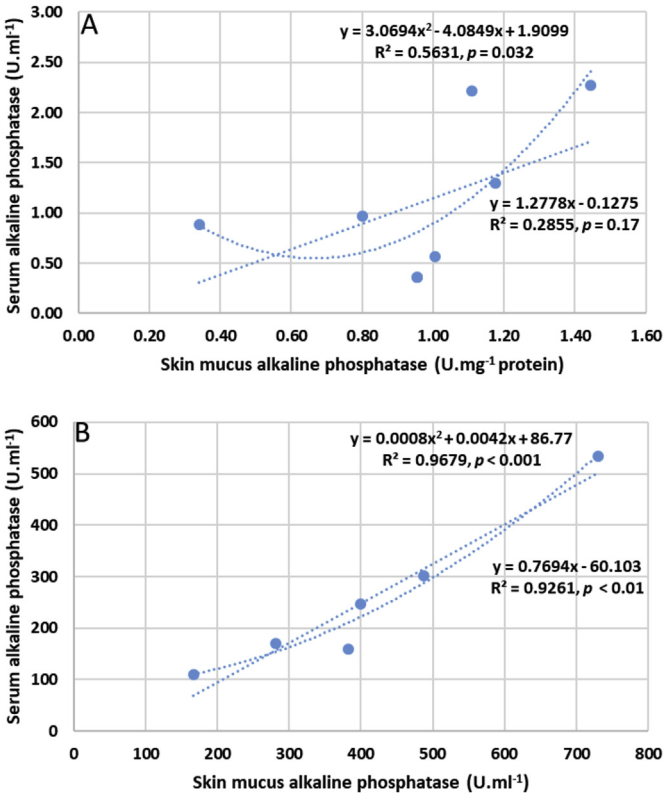

Fig. 6. Correlations between skin mucus and blood serum alkaline phosphatase activities in Atlantic salmon (*Salmo salar* L.) (A) and Mozambique tilapia (*Oreochromis mossambicus*) (B) [53,63].

10. Links between skin and blood serum alkaline phosphatase

In one of our reviews on IAP, we reported that blood serum AP is mostly a marker of inflammation in different clinical settings [6]. Therefore, it does not reflect increased IAP activity which is synonymous with intestinal and body protection [8]. In fish, published data suggest a positive correlation between serum AP and skin mucus AP (Fig. 6) [53,63].

Here, the actual physiological meaning of such a relationship is questioned. Indeed, in terrestrial mammals the proportion of IAP isoform in blood serum is low (< 10%) and increased serum AP often reflects higher systemic inflammation (see Ref. [6]). Therefore, additional controlled experiments (e.g. with fish models of inflammation) are needed for investigating such links between skin (and intestinal) AP and blood serum AP in order to understand the actual biological meaning of such relationships in fish.

11. Conclusions and perspectives

A unique feature of fish is to produce AP enzyme in their epidermis and to release it in the skin mucus. It is undoubtedly a component of innate immunity aimed at preventing skin aggression by environmental microbes and toxic substances. Although the precise functions have not been documented for fish skin mucus AP, we suggest that it may act as an epithelial anti-inflammatory mechanism aimed at dephosphorylating (and thus detoxifying) pro-inflammatory microbial compounds present in the water. As reviewed here, skin mucus AP is modulated by environmental factors and also nutritional factors and dietary supplementation, making it a convenient way to maintain AP activity at a high level. Many bioactive plants or plant extracts, probiotics and prebiotics can successfully boost skin mucus AP activity, though results may depend on dietary supplement dosage, and strain for probiotics.

Future developments beside increasing the number of studies with dietary supplements should include: identification of AP genes present in skin epidermis and AP enzyme isoforms released in the mucus; identification of the underlying mechanisms and biological interpretations; and confirmation of relationships between skin mucus AP and IAP activities as well as between skin mucus AP and blood serum AP activities in the context of a whole AP system and inflammation.

Declarations of interest

None.

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