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Skin mucus metabolomics provides insights into the interplay between diet and wound in gilthead seabream (*Sparus aurata*)

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ABSTRACT

The molecular processes underlying skin wound healing in several fish species have been elucidated in the last years, however, metabolomic insights are scarce. Here we report the skin mucus metabolome of wounded and non-wounded gilthead seabream (*Sparus aurata*) fed with silk fibroin microparticles, a functional additive considered to accelerate the wound healing process. The three experimental diets (commercial diet enriched with 0 mg (control), 50 mg or 100 mg of silk fibroin microparticles Kg^{-1}) were administered for 30 days and thereafter, a skin wound was inflicted. Skin mucus was collected on day 30 of feeding and 7 days post-wounding and subjected to metabolomic analysis by Ultra Performance Liquid Chromatography coupled with a high-resolution quadrupole-orbitrap mass spectrometry. The most enriched metabolite class was amino acids and derivatives, followed by nucleotides, nucleosides and analogues and carbohydrates and their derivatives. Metabolomic profiles revealed that the diet had a more profound effect than wounding in skin mucus. Metabolic pathway analysis of significantly affected metabolites revealed perturbations in the aminoacyl t-RNA biosynthesis in the skin. In particular, skin wound resulted in a decreased methionine level in mucus. Further, silk fibroin supplementation increased methionine level in skin mucus, which correlated with several wound morphometric parameters that characterized the epithelial healing capacity in seabream. The results provided new insight into the physiological consequences of skin wounds and how these processes could be influenced by dietary manipulation.

1. Introduction

The fish skin is a metabolically active organ due to its diverse functions, from thermoregulation, sensory functions, hormone regulation to glandular secretions and protection against a large variety of biological, chemical and physical stressors in the aquatic environment. It possesses a fascinating multitude of specialized cells, including lymphocytes, goblet cells, club cells, keratocytes, and sensory cells that exhibit enormous metabolic reaction capacities to meet various external influences [1]. The skin is considered the first line of defence from external aggressions and its role immunity is emphasized by the presence of skin-associated lymphoid tissue (SALT) that upon infection or damage generates signal molecules that activate the processes of inflammation, thus orchestrating the immune response [2].

The chemical and physical quality of the skin is an important indicator of the health of fish. Poor skin health has become a significant problem in modern aquaculture, partly attributed to increased stocking density, poor water quality and harsh mechanical handling. The prevalence of skin lesions, abrasions, and ulcerations has become a serious welfare problem in aquaculture. In particular, the presence of wounds causes physiological disturbances [3–8] and increases the susceptibility of fish to infections [9,10]. In previous studies, we have shown that a superficial wound on the skin of gilthead seabream (*Sparus aurata*) could affect the biological composition of the epithelial mucosa [3,7,11–14]. In recent years, we have made significant advancements in understanding the mechanisms underlying wound healing in fish, including the molecules and dynamic processes involved in inflammation, re-epithelialization, new tissue formation, and remodelling [4,12,15–18].

Mucus is a glycopolymeric biophysical feature of fish mucosa that performs numerous functions, including immunity, osmoregulation, protection against abrasion, protection against environmental toxicants,

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parental feeding, and chemical communication [19–22]. As an immune defence structure, mucus contains several molecules involved in the orchestration of immune response, including lytic enzymes, antioxidants, immunoglobulins, complement, lectins, and antimicrobial peptides [19,21]. Changes in the biological composition of skin mucus have been studied in relation to alterations in diet [23,24], treatment [25–27], and infection [28–30]. The results point to the important role of bioactive skin mucus molecules in the physiological countermeasures to stressors and in maintaining homeostasis under challenging conditions [3,7]. Biological changes in skin mucus to a stressor have been studied by either a small panel of molecules (i.e., colorimetric assays, ELISA) or by a large array of proteins (i.e., 2D gel electrophoresis, shotgun proteomics), both offering different levels of resolution on mucosal responses.

The application of *-omics* technologies (i.e., transcriptomics, proteomics and metabolomics) has facilitated a holistic study of a biological system at the level from metabolites to proteins and their responses to stimuli of different nature. Metabolomics is the systematic study of all chemical processes related to metabolites, providing the characteristic chemical fingerprints produced by specific cellular processes by studying their small-molecule metabolite profiles [31], thus, a powerful tool for identifying new biomarkers of an organism's physiological and health status. A few studies have applied metabolomics to understand the metabolic profile of wounds and the role metabolites play in the wound-healing process, though mostly in mammalian models [32]. Wound-induced metabolites control the activation and proliferation of cells by serving as signalling molecules, post-translational modifications of enzymes and thereby affecting their function, and synthesis and remodelling of the structural organization of the extracellular matrix (ECM) [33]. To our knowledge, no studies have been performed in fish on the metabolic profile of skin mucosa during experimental wounding. Dissection of the metabolome will allow us to identify metabolic signatures of wounds that will not only elucidate wound healing mechanisms in teleosts fish but also facilitate the discovery of new biomarkers for skin wounds in farmed fish.

The present paper reports the skin mucus metabolome of gilthead seabream (*Sparus aurata*), an economically valuable marine fish, as elucidated by high-throughput metabolomics. In particular, we explored whether dietary manipulation could influence the metabolomic signatures of skin mucus following wound infliction. To this end, we fed the model fish a diet containing silk fibroin microparticles, a feed additive shown to influence wound healing in the skin of *S. aurata* [12]. Silk is a biocompatible polymer with high stability formed by fibroin and sericin, in addition to its low immunogenicity and biodegradability [34–36]. These characteristics have made it an easily modifiable biomaterial widely used in tissue regeneration engineering in different formats, from microparticles, gels, and matrices [37–39]. It is known that silk fibroin in mammals has good adhesion and favours cell growth, proliferation and migration of different cell types involved in the tissue healing process. We have recently documented such properties in seabream [12]. In this study, we used the silk fibroin from *Bombix mori* due to its cultural and traditional importance in Murcia, Spain.

2. Materials and methods

2.1. Experimental animals & ethical considerations

Specimens (43.3 ± 10.6 g mean body weight) of the hermaphroditic protandrous seawater teleost gilthead seabream (*S. aurata* L.) were obtained from a local farm (Mazarrón, Spain) and randomly assigned to flow-through seawater aquaria in the Marine Fish Facilities at the University of Murcia (250 L, flow rate 900 L h^{-1}), under the following parameters: 28‰ salinity, 20 °C and artificial photoperiod (12L:12D). During quarantine and holding before the trial, the fish were fed a commercial diet at a rate of 1.5% body weight day^{-1}.

All animal handling and manipulations described in this study were in accordance with the guidelines of Directive 2010/63/EU of the European Union and were approved by the Ethical Committee of the University of Murcia (Spain) (protocol code A13150104). In support of the 3R (Replacement, Reduction & Refinement) principles in fish research, we utilize metadata from a previous study to further explore the biological significance of some results in this paper [12]. We confirm that no data have been duplicated in this study.

2.2. Silk fibroin microparticles

The silk fibroin microparticles (SF) were produced at 97% purity from the silk cocoon of the silkworm *Bombyx mori* using ionic liquids and high-power ultrasounds, according to Lozano-Perez et al. [40].

2.3. Experimental diets

Commercial feed (protein 56%, fats 15%, ash 9.2%, calcium 2.5%, fiber 1.2%, phosphorus 1.5% and sodium 0.8% (for more details see Table S1 in Supplementary Material) was pulverized and mixed with different concentrations of silk microparticles to produce three experimental diets: non-supplemented diet (control), 50 mg of silk fibroin microparticles Kg^{-1} supplemented diet (SF50), and 100 mg of silk fibroin microparticles Kg^{-1} supplemented diet (SF100) (Fig. 1). Then the pellet was remade with a diameter of 2 mm and a length of 5 mm, with a cold extrusion screw-press machine.

2.4. Experimental design and sampling

The experimental set-up was previously reported in Albaladejo-Riad et al., 2022 [12]. Briefly, the study used six tanks with 6 fish per tank. The initial weight of the fish was recorded. Two replicate tanks were assigned to each diet group. The experimental diets were fed to the fish at a rate of 1.5% of their body weight day^{-1} for a period of 30-day. On day 30, the skin mucus of three fish from each tank (non-wounded) was collected by gently massaging the lateral skin of the left side in a cephalic-caudal direction using a cell scraper (18 mm, Corning), avoiding the anal periphery for contamination [41]. Thereafter, the fish were euthanized with 100 mg L^{-1} of clove oil (Guinama®) (Fig. 1) and the final weight was recorded. This approach prevented the interference of clove oil to the biochemical profile of skin mucus. Fish were not fed for 24 h prior to sample collection. Skin mucus samples were vortexed and centrifuged (300×*g*, 10 min, 4 °C). Supernatants were collected, suspended in 1X PBS (Fisher BioReagents) and stored at −80 °C until use in. The remaining fish (three from each tank) were anesthetized with 20 mg L^{-1} clove oil and experimentally wounded (8 mm diameter and 2 mm depth) in the middle of the left flank (below the lateral line) with a circular biopsy punch (Integra™ Miltex™). Immediately after the wounding procedure, the wounded fish were returned to their respective tank and continued to receive their corresponding diet for the next seven days. Infliction of superficial wounds did not affect the feeding behavior of the experimental fish. On day 37, the remaining fish (wounded) were also sampled for skin mucus, as described above (Fig. 1).

To study the effects of the diet on growth, growth performance [Weight gain rate (%) = (Total final weight – Total initial weight)/Total initial weight × 100] and feed utilization [Feed efficiency = (Total final weight – Total initial weight)/feed consumed] were calculated (Table S2).

2.5. Liquid Chromatography–mass spectrometry

Skin mucus samples (N = 3, pooled of 2 fish per replicate) were reconstituted in 200 μL Eluent A and transferred to an HPLC vial. Sample analysis was conducted by MS-Omics ApS as follows: the analysis was carried out using a UPLC system (Vanquish, Thermo Fisher Scientific) coupled with a high-resolution quadrupole-orbitrap mass spectrometer (Q Exactive™ HF Hybrid Quadrupole-Orbitrap, Thermo Fisher

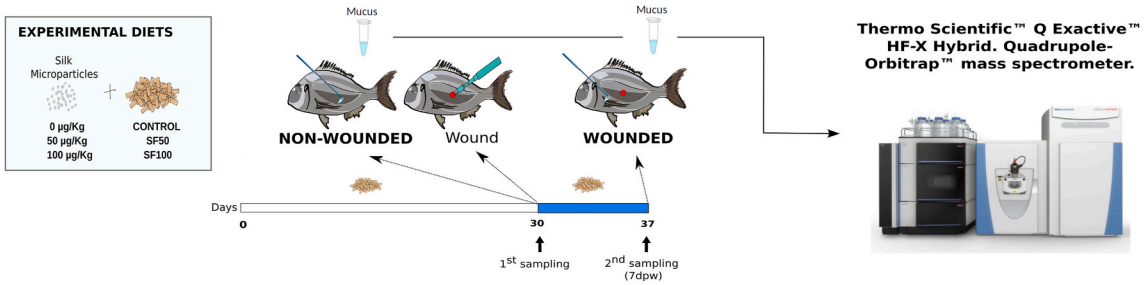

Fig. 1. Schematic representation of the experimental design. Gilthead seabream fed different experimental diets for 30 days [control (non-supplemented diet), SN50 (control diet enriched with 50 mg of silk fibroin microparticles Kg-1), or SN100 (control diet enriched with 100 mg of silk-fibroin microparticles Kg-1)]. Half of the fish were sampled at day 30 of the trial (non-wounded). The remaining fish were wounded (with a biopsy punch of 8 mm of diameter) and continued to be fed the same diet. At 7 days after wounding (wounded), fish were sampled. Samples of skin mucus were treated and analyzed by Liquid Chromatography-HF Mass Spectrometry.

Scientific). An electrospray ionization interface was used as an ionization source. Analysis was performed in negative and positive ionization mode. The UPLC was performed using a slightly modified version of the protocol described by Doneanu et al. [42]. Peak areas were extracted using Compound Discoverer 3.1 (Thermo Scientific). Data processing was carried out in MZmine 2 [43] followed by curation utilizing a custom-made in-house protocol of MS-Omics ApS. Compound identification was performed at two levels; Level 1: identification by retention times (compared to authentic in-house standards), accurate mass (with an acceptable deviation of 3 ppm), and MS/MS spectra, Level 2a: identification by retention times (compared to authentic in-house standards), accurate mass (with an acceptable deviation of 3 ppm).

2.6. Database search and metabolomic analysis

The 111 metabolites obtained from levels 1 and 2a were categorized along with their chemical taxonomy down to the sub-class level, according to different databases such as the Human Metabolome Database (HMDB) [44], Reactome [45], PubChem [46], MetaCYC [47], PathBank [48] and Kyoto Encyclopedia of Genes and Genomes (KEGG) [49–51].

We selected 37 metabolites that changed in the skin mucus as a consequence of at least one of the factors studied, and then topological pathway analysis was performed in MetaboAnalyst 5.0 [52]. Topological pathway analysis was performed using the Hypergeometric test as the enrichment method, the relative centrality of the interrelationship was selected, and zebrafish (*Danio rerio*) metabolome was used as the reference metabolome.

2.7. Statistical analyses

Statistical differences among the fish feed the 3 diets (Control, SF50 and SF100) were assessed by One-way ANOVA and followed by the Tukey or Games Howell test, depending on the homogeneity of the variables, and a Student's T-analysis was performed to compare between wounded and non-wounded fish groups. For analyses to determine the joint effect of the diet factor and the injury factor, as well as their possible interaction, a Two-way ANOVA followed by a Tukey's multiple comparisons test was performed. The normality of the variables was confirmed by the Shapiro–Wilk test and homogeneity of variance by the Levene test. Pearson's correlation analysis was performed to identify pairwise associations (positive or negative) between the different parameters, according to the Gaussian distribution of the samples. The significance level was 95% in all cases ($p < 0.05$). The computer application SPSS analyzed all the data for Windows® (version 25.0, SPSS Inc.) or GraphPad Prism for Windows® (version 8.0).

3. Results

3.1. General biochemical profile of the skin mucus metabolome

A total of 111 metabolites were obtained with annotation levels 1 and 2a (http://hdl.handle.net/10201/126625), which were classified by classes - 43 corresponded to amino acids, peptides and analogues, 10 carbohydrates and conjugated carbohydrates, 13 nucleotides, nucleosides and analogues, 5 fatty acids and derivatives, 5 purines and derived from purines, 3 indoles and derivatives, 3 pyrimidines and derivatives, 3 organosulphonic acids and derivatives and 23 metabolites corresponded to other minority classes in which no more than 2 metabolites were found, such as benzoids, ketoacids and derivatives or ammonium salts (Fig. 2).

3.2. Metabolites affected by diet, wound, or their combination

Statistical analysis showed that 37 metabolites out of 111 changed significantly in skin mucus ($p < 0.05$) due to diet and/or wound effect (Table S3). A greater number of metabolites changed ($p > 0.05$) due to the effect of diet alone (i.e., 12 metabolites) than to the presence of a skin wound (i.e., 4 metabolites). There were 5 metabolites that changed significantly due to the effect of these two factors (Fig. 3A). Two-way ANOVA showed interactions between the two factors studied (Table S3). Specifically, 8 metabolites (i.e., 2-oxoglutaric acid, 4-acetamidophenol, 5-methoxytryptophan, creatinine, glycyl-leucine, hexose dimer, N8-acetylspermidine and N-acetyl-glucosamine) changed as a consequence of diet, wounding and the interaction of these two factors. In addition, 5 metabolites (i.e., 2-deoxycytidine, glycine, phenylalanine, trigonelline and tyrosine) changed due to diet and the interaction between the factors. Besides this, 3 metabolites (i.e., 2-deoxyuridine, guanine and methanesulfonic acid) changed because of skin wounding and the interaction between the two factors (Fig. 3A).

The alterations in the proportion of the class of metabolites whose concentration changed as a result of skin wound, diet or the interaction of both factors are presented in Fig. 3B. Of the metabolites that changed due to skin wound, 20% were amino acids, peptides and analogues, 15% were nucleotides, nucleosides and analogues and 15% were carbohydrates. On the other hand, the proportion of metabolites affected due to the diet comprised 50% amino acids, 7% nucleotides and 17% carbohydrates. Finally, changes related to the interaction of both factors were distributed as 25% amino acids, 12% nucleotides and 18% carbohydrates.

3.3. Metabolic pathways affected by diet, wound and their interactions

Next, we explored the associations of the 37 selected metabolites in different pathways involved in the metabolism of amino acids, fatty acids, carbohydrates, nucleotides and others (Fig. 4). We found 21

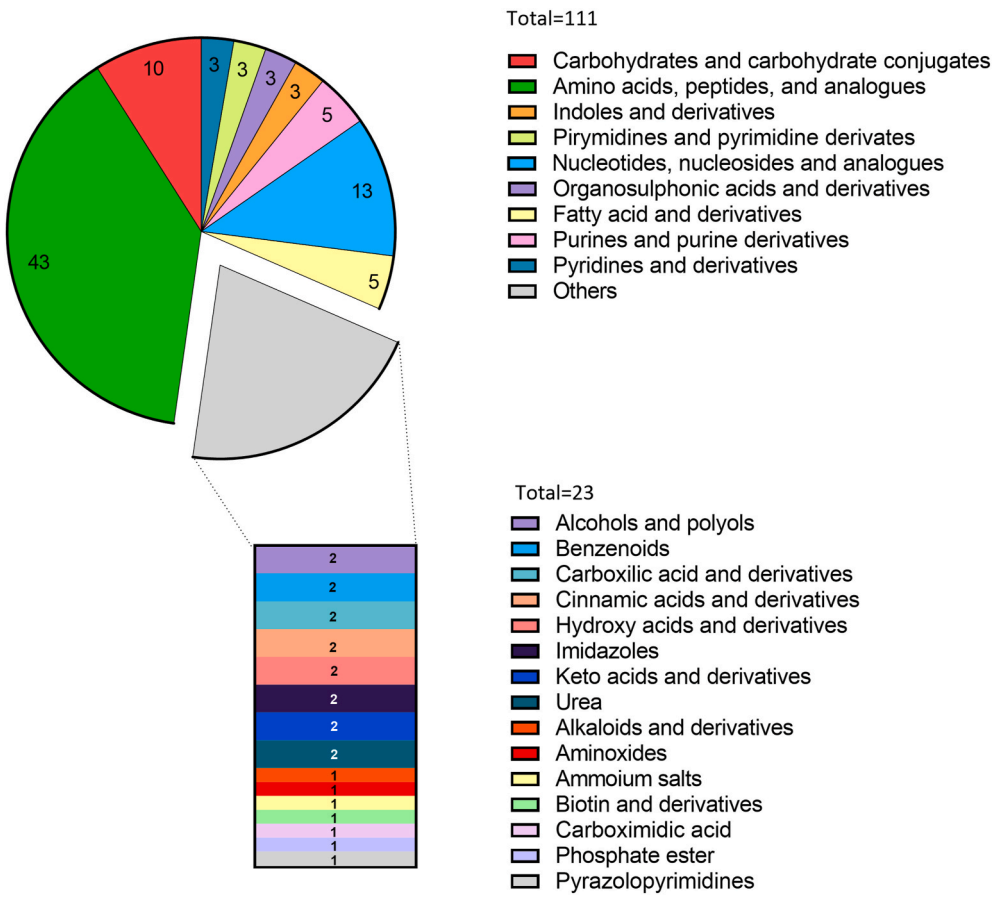

Fig. 2. Classification of the 111 metabolites found in the skin mucus of *Sparus aurata* by Liquid Chromatography-HF Mass Spectrometry. The metabolites were classified according to different databases, see Materials and Methods for the details on these databases.

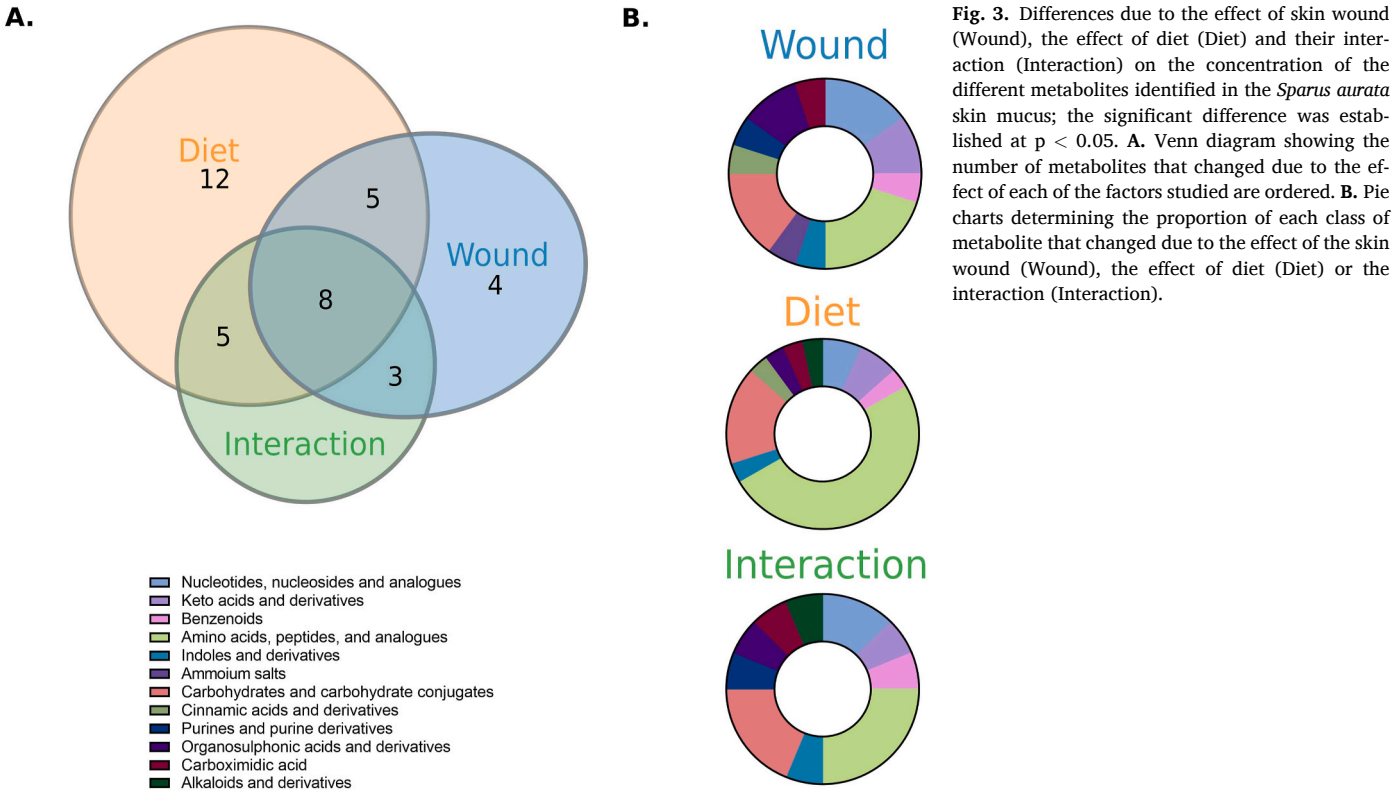

Fig. 3. Differences due to the effect of skin wound (Wound), the effect of diet (Diet) and their interaction (Interaction) on the concentration of the different metabolites identified in the *Sparus aurata* skin mucus; the significant difference was established at $p < 0.05$. **A.** Venn diagram showing the number of metabolites that changed due to the effect of each of the factors studied are ordered. **B.** Pie charts determining the proportion of each class of metabolite that changed due to the effect of the skin wound (Wound), the effect of diet (Diet) or the interaction (Interaction).

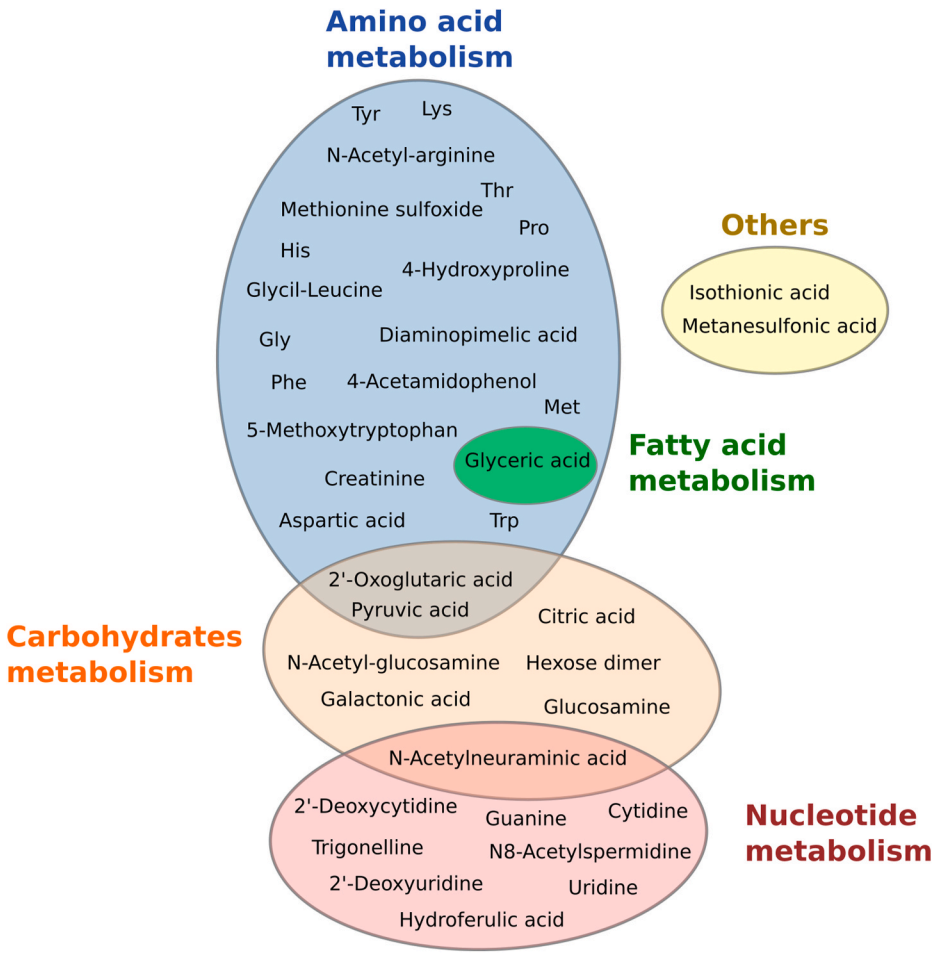

Fig. 4. Venn diagram illustrating the metabolites whose concentration changed in skin mucus of *Sparus aurata*, organized according to their involvement in the different metabolic pathways classified by class.

metabolites involved in amino acid metabolism, 18 of which were exclusively associated with this group, including key amino acids (i.e, Tyr, Lys, Th, Pro, His, Trp, Met and Phe) and their derivatives (i.e., N-acetylarginine, methionine sulfoxide, 4-hydroxyproline, glycyl-leucine, 4-acetamidophenol, 5-methoxytryptophan and creatinine, diaminopimelic acid and aspartic acid). The other 3 metabolites were also involved in fatty acid metabolism (i.e, glyceric acid) or carbohydrate metabolism (i.e, 2′-oxoglutaric acid and pyruvic acid). We found 8 metabolites involved in carbohydrate metabolism, 5 of which (i.e., N-acetylglucosamine, citric acid, hexose dimer, glucosamine and galactonic acid) did not appear in any other metabolic pathways, whereas 1 metabolite (i.e., N-acetylneuraminic acid) could also be found in nucleotide metabolism pathway. 9/37 metabolites were involved in nucleotide metabolism, 8 of which (i.e., 2′-deoxycytidine, guanine, cytidine, trigonelline, N8-acetylspermidine, 2′-deoxyuridine, uridine and hydroferulic acid) were only found in this group. Isothionic acid and methanofulphonic acid are involved in pathways classified as cofactor and vitamin metabolism (Fig. 4).

Topological analyses ($p < 0.05$) of the metabolic pathways showed that wound and diet significantly affected the metabolites involved in aminoacyl-tRNA biosynthesis (Fig. 5). Both factors equally affected the pathway associated with alanine, aspartate and glutamate metabolism (Fig. 5). In addition, both factors also modified the nucleic acid pathways (i.e, Pyrimidine metabolism) and carbohydrate metabolism (i.e, Glyoxylate and dicarboxylate metabolism & TCA cycle). Wound only affected the D-glutamine and D-glutamate metabolism pathway ($p > 0.05$), whereas diet alone affected glycine, serine and threonine metabolism, phenylalanine metabolism, and phenylalanine, tyrosine and

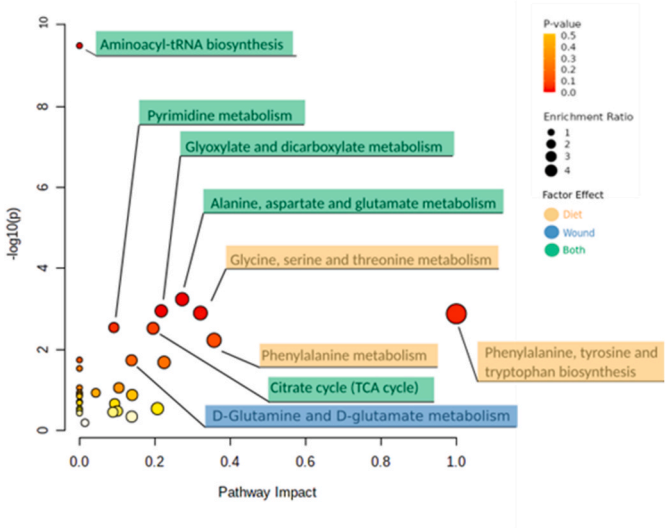

Fig. 5. Metabolic pathways involved according to topological pathway analyses based on the metabolites found in *Sparus aurata* skin mucus whose concentration changed ($p > 0.05$) due to the effect of diet (yellow), the effect of skin wound (blue) or both (green).

tryptophan metabolism.

3.4. Effects of wounding on aminoacyl t-RNA biosynthesis pathway

We studied the aminoacyl t-RNA biosynthesis pathway in depth because it was the main metabolic pathway that was significantly affected by both the wound and diet (Fig. 6). Ten amino acids were involved in this pathway, and all of them were upregulated, except for methionine, which was downregulated in *S. aurata* mucus after skin wounding (Fig. 6).

Using metadata from a previous study conducted by our group on the same gilthead seabream specimens [12], we performed a correlation analysis between the 10 metabolites significantly affected in the aminoacyl t-RNA biosynthesis pathway and different parameters characterizing the wound, including wound perimeter, area, roundness and RNA:DNA ratio (Fig. 7). We found that methionine concentration in skin mucus was positively correlated with wound area ($r > 0.6$; $p > 0.05$) and perimeter ($r > 0.6$; $p > 0.05$). On the other hand, phenylalanine, glycine and tyrosine negatively correlated with wound area.

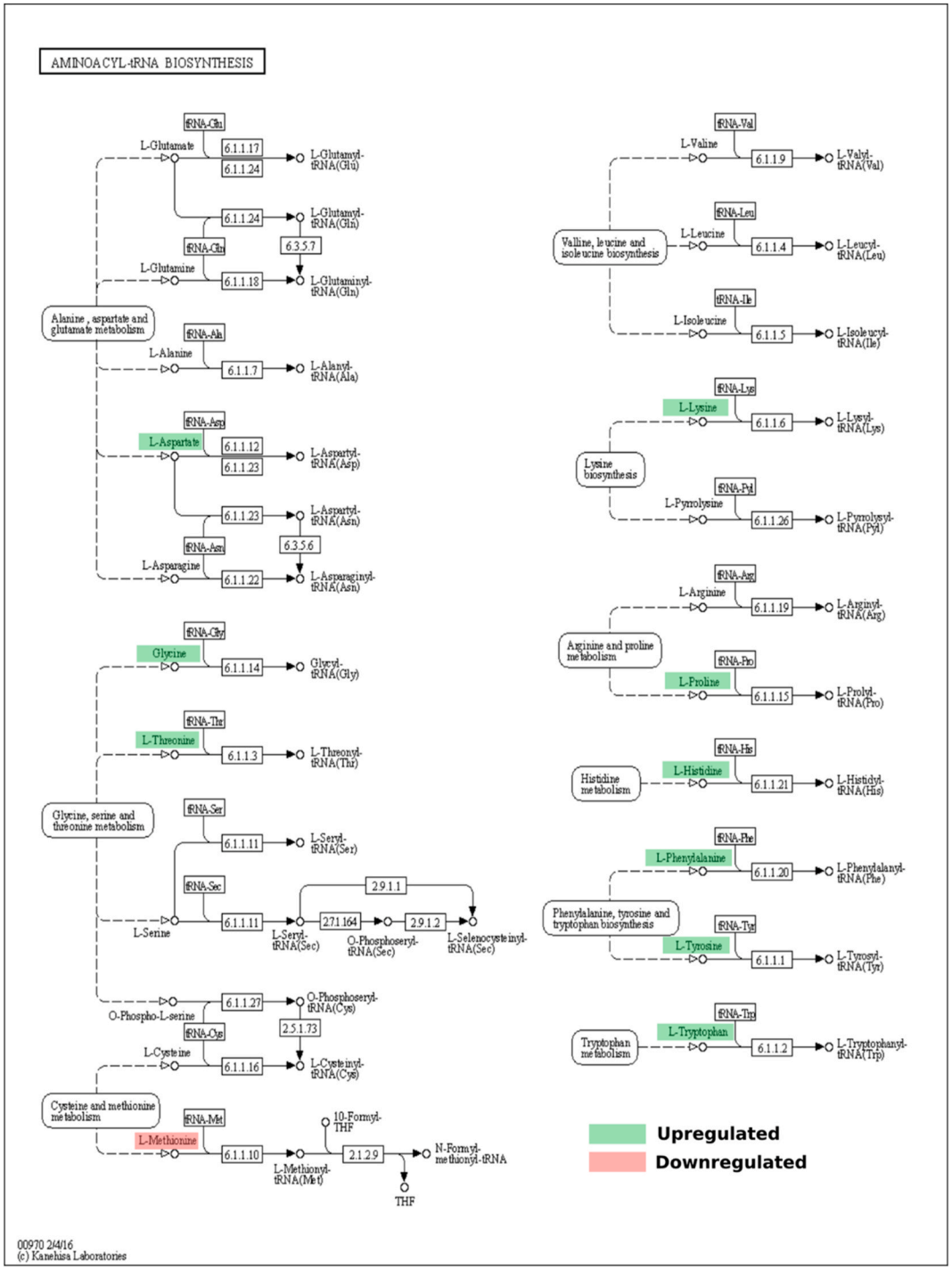

Fig. 6. Aminoacyl t-RNA biosynthesis pathway representing skin mucus metabolites affected by the presence of a skin wound in *Sparus aurata*. Green color indicates upregulated metabolites, while red shows downregulation. The pathway map was downloaded from KEGG PATHWAY Database (https://www.genome.jp/kegg/pathway.html).

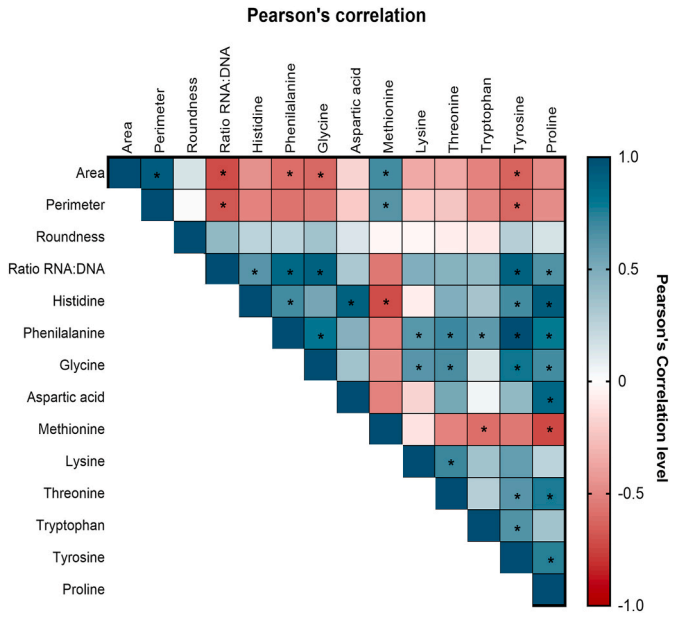

Fig. 7. Correlation matrix of the different wound morphometric parameters (as reported in Albaladejo-Riad et al., 2022) and the metabolites that changed in the skin mucus of *Sparus aurata* following wounding. The color scale represents the level of correlation - blue and red colors indicate positive and negative correlation, respectively. Asterisks indicate significant differences between non-wounded and wounded fish (correlation test; $p < 0.05$).

3.5. Potential involvement of skin mucus methionine in the interaction of wound and diet

We further explored the potential involvement of methionine in the diet-wound interaction using the metadata from the previous study [12]. In wounded fish fed a diet supplemented with silk fibroin microparticles, a higher level of methionine was observed in the skin mucus compared with wounded fish fed a control diet (Fig. 8A). Further, correlation analyses were performed with wound histomorphometric results from the previous study [12]. There was a positive correlation between wound area and roundness ($r > 0.7$; $p > 0.05$), and between the dietary silk fibroin microparticles concentration and the skin mucus methionine concentration ($r > 0.8$; $p > 0.05$). Additionally, there was a negative correlation ($r < -0.5$; $p > 0.05$) between histomorphometric parameters (i.e. wound area and roundness), and skin mucus methionine concentration and dietary of silk fibroin microparticles concentration (Fig. 8B).

4. Discussion

The skin mucus is an important biophysical barrier of the fish mucosa, and ensuring its integrity and functionality is crucial for the overall health of the organism. The chemical composition of skin mucus can be attributed to i) proteins transported by the classical delivery of extracellular material through the Golgi complex, ii) proteins (synthesized in the cytosol) and other molecules delivered to the mucus layers via transport routes directly over the cell membrane either by transporters or through channels or other non-classical mechanisms such as membrane vesicles like exosomes and microvesicles, iii) dead/dividing epidermal cells and, or iv) the commensal microbiota community [19]. The composition of this biological matrix depends on several factors, such as temperature [26], infection and stress status [53], diet [11,12], and injury [8], among others.

In recent years, the chemical and physical properties of skin mucus have been used to monitor and understand the health of farmed fish [27,53]. Although *omics* technologies have been applied to fish research for some years, metabolomics is an emerging and underexplored area. Metabolomics has evolved from a simple tool for biomarker identification to a technology for discovering active drivers of biological processes [54]. Here we present the first study describing the metabolome of the gilthead seabream skin mucus and how its profile is influenced by wound, diet and their interactions. The identified metabolites and metabolic pathways shed light on the underlying physiological processes in the skin due to wounding and dietary manipulation.

4.1. Skin mucus metabolomics – biochemical profiling in a minimally invasive way

A prominent advantage of using skin mucus as a biological matrix to assess health is that sample collection is minimally invasive and, if performed correctly, does not subject the animal to rough handling. Although no water samples were collected during the trial, pellet degradation was avoided, preventing the diffusion of molecules/chemicals (e.g. free amino acids, carbohydrates etc) into the skin mucus. The most enriched metabolite class, comprising about thirty-nine percent of the metabolites profiled in the gilthead seabream skin mucus metabolome, were free amino acids and their derivatives. This metabolomic composition was similar in the skin mucus of other species such as Atlantic salmon (*Salmo salar*) [55,56], fathead minnow (*Pimephales*

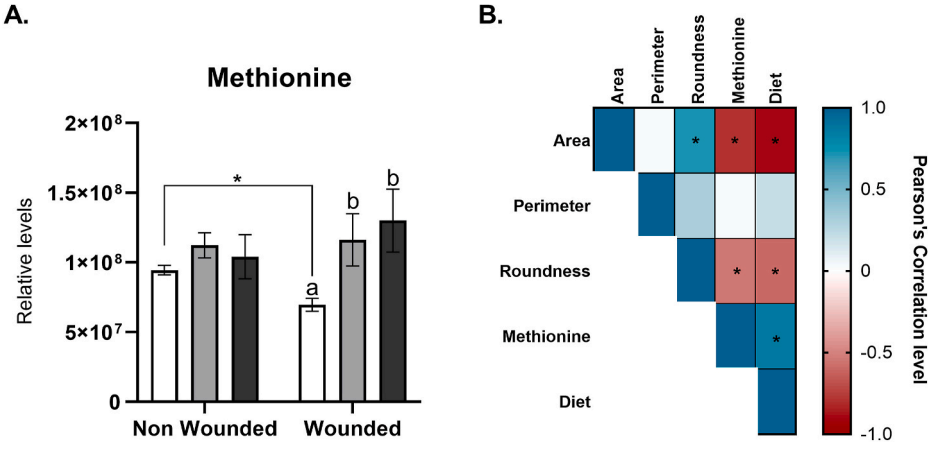

Fig. 8. A. Relative methionine concentration in *Sparus aurata* skin mucus fed different experimental diets: control (non-supplemented diet; white), SF50 (control diet enriched with 50 mg of silk fibroin microparticles Kg-1; grey), and SF100 (control diet enriched with 100 mg of silk-fibroin microparticles Kg-1; black) after 30-day of feeding (Non-wounded) or 7 days post-wounding (Wounded). Error bars in columns denote standard error of means ($n = 6$). Different letters denote significant differences among the diet groups in either non-wounded or wounded group, and asterisk (*) indicates significant difference in a diet group between non-wounded or wounded ($p < 0.05$). **B.** Correlation matrix between different wound morphometric parameters versus skin mucus methionine that changed in wounded *Sparus aurata* due to supplementation with silk fibroin microparticles. The color scale represents the Pearson's correlation value where blue and red colors indicate positive and negative correlation, respectively. Asterisks indicate correlation of the morphometric wound parameters and the level of silk fibroin of the diet (correlation test; $p < 0.05$).

promelas) [57], rainbow trout (*Oncorhynchus mykiss*) [58] and goldfish (*Carassius auratus*) [59]. The results of previous studies showed that amino acids in skin mucus played several roles in inter and intraspecific interactions, such as chemical communication, antimicrobial activity or UV protection [19]. These amino acids found in the skin mucus have different origins, such as from the proteins secreted via the classical cellular pathways [60,61], release of cytosolic content of dead/dividing epithelial cells (63) or by-products of the skin microbiota [21]. The present study could not elaborate on the contribution of each of these sources to the amino acid profiles in skin mucus. Nonetheless, characterization of the skin mucus metabolome of seabream provides a new dimension to the biochemistry of skin mucosa and how the fish skin mucosal surface responds to factors associated with husbandry. The significantly represented metabolites identified in the skin mucus are important in shedding light on the physiological processes following perturbation due to interventions or stimuli.

A previous study showed that anaesthetics could influence the skin mucus metabolome of fish [62]. Hence, we collected the skin mucus before euthanasia to avoid this confounding factor. The application of metabolomics to study fish skin mucus is in its infancy. Several practical considerations should be investigated in future research, for example, how collection protocols and materials influence the metabolomic profiles. Standardization of sample handling and processing will be beneficial for comparative studies.

4.2. Skin mucus metabolome is influenced by diet and wound

Diet is a factor that can significantly influence the chemical composition of fish mucus [63]. In the present study, fish were fed diets enriched with silk fibroin, a natural protein that has shown low immunogenicity, high biocompatibility and biodegradability, hence used in tissue engineering in different forms (e.g., micro- or nanoparticles, gels, etc.) [64,65]. Silk fibroin is a polymer formed mainly from 48% glycine, 32% alanine, 11% serine, 4.5% tyrosine, 2% valine, and others in minor concentrations [66]. Moreover, it has been shown to have a positive effect on tissue regeneration [37,65]. In our previous work, we reported a decrease in peroxidase activity and IgM levels in the skin mucus of fish (*S. aurata*) fed diets with high silk fibroin content [12].

Skin mucus from gilthead seabream-fed silk fibroin showed an increase in the abundance of amino acids, such as histidine, proline, threonine, lysine, and glycine, among others. The next most enriched metabolite class after amino acids was the group of nucleotides, nucleosides and analogues, which accounted for 11.7% of the documented metabolites, mirroring the metabolic profile of the skin mucus of the fathead minnow (*Pimephales promelas*) [57]. The primary source of this class in skin mucus is epithelial cells, either dead or dividing cells [67]. There are not many studies on how the metabolic profile of fish epithelial mucus changes due to a specific type of diet, however, the metabolic profiles of muscle or liver provide information on the influence of nutritional changes. The topological analyses of metabolic pathways were in agreement with results described in studies detailing the metabolomic consequence of a high-protein diet – i.e., protein metabolism and glycolysis pathways were affected in muscle [68], while tricarboxylic acid metabolism and glycogenolysis in liver [69].

The effects of skin wound had more significant impacts on nucleotides, nucleosides and analogues in skin mucus, generally, an increased concentration was documented. Providing support to the changes in the abundance of these metabolites in the skin mucus following wounding, a sister study showed an increase in RNA:DNA ratio seven days after a wound was experimentally inflicted, which was implicated in cell proliferation during the wound healing process [12]. To our knowledge, this is the first study describing changes in the metabolic profile of fish skin mucus due to wounding.

The metabolic pathway aminoacyl-tRNA biosynthesis was documented to be altered significantly by wounding, as reflected by the differentially affected metabolites identified in skin mucus. The definition of the genetic code by correctly pairing amino acids with their cognate tRNAs, and faithful translation of the genetic code during protein synthesis is mediated by aminoacyl-transfer RNA (tRNA) synthetases [70]. The enzymes involved in aminoacyl-tRNA synthesis are responsible for binding amino acids to tRNAs, participating in different processes involved in the healing process - transcription, translation, splicing, inflammation, angiogenesis, cell proliferation, tissue remodelling and apoptosis [71,72]. We believe this mechanism is also at play in the skin of gilthead seabream during wound healing - the production of the key molecules in this pathway by metabolically active cells on the skin likely ensures that physiological processes during wound healing develop with the correct genetic regulatory makeup. In addition, molecules in this pathway function as cytokines, which have been implicated in regulating cell proliferation [71,73]. Furthermore, it has been observed that secretion of these tRNA synthetases could increase fibroblast proliferation and collagen synthesis, as well as increase the release of tumor necrosis factor α (*TNF-α)* and interleukin 8 (*il-8*) by macrophages present in a wound [73]. We have previously demonstrated significant modulation of the expression of *tnf-α* and genes involved in cell proliferation [*fibronectin 1α* (*fn1α*) or *sonic hedgehog* (*shh*)] and extracellular matrix generation [*keratin 2* (*krt2*) or *matrix metallopeptidase 9* (*mmp9*)] in the skin surrounding a wound in gilthead seabream fed diets supplemented with silk fibroin. In addition, these molecular changes were implicated in increased angiogenesis [12], a crucial process in wound healing. Therefore, the results of the previous work and the new metabolic insights generated in the present study implicated the involvement of the aminoacyl-transfer RNA pathway in the wound healing process influenced by dietary manipulations in sea bream.

4.3. Methionine may have a key role in wound healing mediated by silk fibroin

Methionine plays an important function in many physiological processes as an essential amino acid and a precursor of other amino acids such cysteine and taurine [74]. It is considered to be the main and fastest source of the sulfur group of proteins to produce other amino acids [75,76]. Methionine is an initiator of amino acid in eukaryotic protein synthesis [74] and participates in connective tissue synthesis [77]. Methionine was the amino acid in the aminoacyl tRNA biosynthesis pathway that exhibited distinct changes in skin mucus, where superficial wounding decreased its concentration. Although methionine is a minor amino acid in *Bombyx mori* silk fibroin, silk fibroin supplementation, regardless of inclusion level, increased methionine level in skin mucus. To our knowledge, this is the first study that reports a decrease in methionine concentration in wounded fish. Methionine cannot be synthesized *de novo* by fish, thus modern farmed fish diets are supplemented with methionine. Therefore, the decrease in methionine concentration might indicate that wounding limited the ability of fish to utilize the methionine present in the diet. The increase in methionine in groups fed with fibroin suggests that either silk fibroin supplied additional methionine or silk fibroin stimulated utilization of methionine in the diet or could be both. The concentration of silk fibroin in the diet and of methionine in skin mucus of wounded fish demonstrated a positive correlation, while in non-wounded fish this profile was not evident. Methionine has been shown to accelerate wound healing in terrestrial organisms [77–80]. Therefore, we speculate that fibroin in the diet affected the mobilization of methionine to speed up the production of proteins and accelerate wound healing in sea bream. Fish receiving diets with a higher silk fibroin content exhibited faster wound healing rate (less wound area) and irregular wound shape (less roundness), and these parameters correlated with methionine in skin mucus. These chemical and phenotypic associations further corroborate that methionine likely participated as a regulator of cellular processes involved in wound healing in sea bream. In a work by Tamayo et al. [77] in rats, methionine was implicated as one of the primary amino acids involved in wound

healing. It was found that dietary protein deficiency delayed the rate of wound healing and, when methionine was added to a protein-deficient diet, the morphological pattern of wound healing returned to normal. Methionine, together with ascorbic acid, are considered the main components of a base substance on which tropocollagen molecules are converted into larger and more stable forms of collagen by physicochemical bonds [81], and this may partly explain the regulatory function of methionine in the wound healing process of gilthead seabream. The influence of silk fibroin supplementation in the diet that considerably affected skin mucus methionine level lent support to the potential of this dietary additive in improving skin health of gilthead seabream.

5. Conclusions

The skin is a metabolically active organ, characterized by a distinct layer of mucus. The chemical composition of this glycopolymeric layer is largely attributed to several specialized cells in the skin that can be regulated by intrinsic and extrinsic factors. The present study uncovered new insights into the wound healing process in gilthead seabream skin through metabolomic analysis of skin mucus. The skin mucus metabolomic profiles revealed that the most predominant metabolite classes were amino acids and derivatives (most affected by the diet) followed by nucleotides, nucleosides and derivatives (most affected by the wound), which have been implicated as crucial molecular drivers of protein metabolism and cell proliferation. Both diet and wound have been found to affect a number of metabolites involved in the aminoacyl-tRNA biosynthesis pathway, a group of molecules implicated in many activities related to the wound healing process. In the present study, the potential of methionine as a biomarker for wound healing was identified. The influence of silk fibroin as an additive to accelerate wound healing in fish was further expounded, as its supplementation was shown to alter the level of methionine in the skin mucus of wounded fish. Moreover, increased in methionine level has been shown to be associated with several morphometric features of the wound healing process. The present study contributes to a better understanding of the interaction of wound and diet in fish from a metabolomic perspective and is expected to support the development of preventive health measures targeting skin health in farmed fish. In addition, the opportunity provided by skin mucus as a biological matrix for metabolomic profiling is an advancement for improved and minimally invasive welfare monitoring in farms.

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CRediT authorship contribution statement

Nora Albaladejo-Riad: conceptualized the trial and the analysis, performed the experiments and collected the samples, analyzed, visualized and interpreted the data, wrote the first draft of the manuscript. **Cristóbal Espinosa-Ruiz:** performed the experiments and collected the samples, reviewed the manuscript. **María Ángeles Esteban:** acquired the funding, conceptualized the trial, interpreted the results, supervised the students, edited and reviewed the manuscript. **Carlo C. Lazado:** acquired the funding, conceptualized the project and the analysis, interpreted the data, supervised the student, edited and reviewed the manuscript..All authors contributed to the writing and review of the final version of the manuscript.

Declaration of competing interest

The authors declare that the research was conducted without any commercial or financial relationships that could be construed as a potential conflict of interest. Mention of trade names and service providers is solely for reporting and does not imply recommendations from the University of Murcia or Nofima.

Data availability

The data set is deposited in a public repository. Link is provided in the manuscript.

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Appendix A. Supplementary data

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References

[1] M. Ángeles Esteban, R. Cerezuela, Fish mucosal immunity: skin, in: Mucosal Heal. Aquac., Elsevier, 2015, pp. 67–92, https://doi.org/10.1016/B978-0-12-417186-2.00004-2.
[2] J. Wayne Streilein, Circuits and signals of the skin-associated lymphoid tissues (SALT), J. Invest. Dermatol. 85 (1985) S10–S13, https://doi.org/10.1111/1523-1747.ep12275413.
[3] C. Espinosa-Ruíz, M.Á. Esteban, Wound-induced changes in antioxidant enzyme activities in skin mucus and in gene expression in the skin of gilthead seabream (*Sparus aurata* L.), Fishes 6 (2021) 15, https://doi.org/10.3390/fishes6020015.
[4] L. Sveen, C. Karlsen, E. Ytteborg, Mechanical induced wounds in fish – a review on models and healing mechanisms, Rev. Aquacult. 12 (2020) 2446–2465, https://doi.org/10.1111/raq.12443.
[5] Z. Chen, D. Ceballos-Francisco, F.A. Guardiola, M.Á. Esteban, Influence of skin wounds on the intestinal inflammatory response and barrier function: protective role of dietary *Shewanella putrefaciens* SpPdp11 administration to gilthead seabream (*Sparus aurata* L.), Fish Shellfish Immunol. 99 (2020) 414–423, https://doi.org/10.1016/j.fsi.2020.02.022.
[6] Z. Chen, D. Ceballos-Francisco, F.A. Guardiola, D. Huang, M.Á. Esteban, The alleviation of skin wound-induced intestinal barrier dysfunction via modulation of TLR signalling using arginine in gilthead seabream (*Sparus aurata* L), Fish Shellfish Immunol. 107 (2020) 519–528, https://doi.org/10.1016/j.fsi.2020.11.017.
[7] D. Ceballos-Francisco, H. Cordero, F.A. Guardiola, A. Cuesta, M.Á. Esteban, Healing and mucosal immunity in the skin of experimentally wounded gilthead seabream (*Sparus aurata* L), Fish Shellfish Immunol. 71 (2017) 210–219, https://doi.org/10.1016/j.fsi.2017.10.017.
[8] Y.S. Svendsen, J. Bøgwald, Influence of artificial wound and non-intact mucus layer on mortality of Atlantic salmon (*Salmo salar* L.) following a bath challenge with *Vibrio anguillarum* and *Aeromonas salmonicida*, Fish Shellfish Immunol. 7 (1997) 317–325, https://doi.org/10.1006/fsim.1997.0087.
[9] A.B. Olsen, H. Nilsen, N. Sandlund, H. Mikkelsen, H. Sørum, D.J. Colquhoun, *Tenacibaculum sp*. associated with winter ulcers in sea-reared Atlantic salmon *Salmo salar*, Dis. Aquat. Org. 94 (2011) 189–199, https://doi.org/10.3354/dao02324.
[10] M.D. Fast, N.W. Ross, A. Mustafa, D.E. Sims, S.C. Johnson, G.A. Conboy, D. J. Speare, G. Johnson, J.F. Burka, Susceptibility of rainbow trout *Oncorhynchus mykiss*, Atlantic salmon *Salmo salar* and coho salmon *Oncorhynchus kisutch* to experimental infection with sea lice *Lepeophtheirus salmonis*, Dis. Aquat. Org. 52 (2002) 57–68, https://doi.org/10.3354/dao052057.
[11] Z. Chen, D. Ceballos-Francisco, F.A. Guardiola, M.Á. Esteban, Dietary administration of the probiotic *Shewanella putrefaciens* to experimentally wounded gilthead seabream (*Sparus aurata* L.) facilitates the skin wound healing, Sci. Rep. 10 (2020), 11029, https://doi.org/10.1038/s41598-020-68024-z.
[12] N. Albaladejo-Riad, C. Espinosa Ruiz, M.Á. Esteban, Dietary administration of silk microparticles improves the epidermal and dermal regeneration after a skin wounding in gilthead seabream (*Sparus aurata* L.), Fish Shellfish Immunol. 124 (2022) 92–106, https://doi.org/10.1016/j.fsi.2022.03.049.
[13] H. Cordero, M. Brinchmann, A. Cuesta, M. Esteban, Chronic wounds alter the proteome profile in skin mucus of farmed gilthead seabream, BMC Genom. 18 (2017) 939, https://doi.org/10.1186/s12864-017-4349-3.
[14] Z. Chen, D. Ceballos-Francisco, F.A. Guardiola, D. Huang, M.Á. Esteban, Skin wound healing in gilthead seabream (*Sparus aurata* L.) fed diets supplemented with

arginine, Fish Shellfish Immunol. 104 (2020) 347–358, https://doi.org/10.1016/j.fsi.2020.06.026.
[15] L.R. Sveen, G. Timmerhaus, A. Krasnov, H. Takle, S. Handeland, E. Ytteborg, Wound healing in post-smolt Atlantic salmon (*Salmo salar* L.), Sci. Rep. 9 (2019) 3565, https://doi.org/10.1038/s41598-019-39080-x.
[16] D.K. Fontenot, D.L. Neiffer, Wound management in teleost fish: biology of the healing process, evaluation, and treatment, Vet. Clin. North Am. Exot. Anim. Pract. 7 (2004) 57–86, https://doi.org/10.1016/j.cvex.2003.08.007.
[17] R. Richardson, M. Metzger, P. Knyphausen, T. Ramezani, K. Slanchev, C. Kraus, E. Schmelzer, M. Hammerschmidt, Re-epithelialization of Cutaneous Wounds in Adult Zebrafish Uses a Combination of Mechanisms at Play during Wound Closure in Embryonic and Adult Mammals, Development, 2016, https://doi.org/10.1242/dev.130492.
[18] R. Richardson, K. Slanchev, C. Kraus, P. Knyphausen, S. Eming, M. Hammerschmidt, Adult zebrafish as a model system for cutaneous wound-healing research, J. Invest. Dermatol. 133 (2013) 1655–1665, https://doi.org/10.1038/jid.2013.16.
[19] M. Reverter, N. Tapissier-Bontemps, D. Lecchini, B. Banaigs, P. Sasal, Biological and ecological roles of external fish mucus: a review, Fishes 3 (2018) 41, https://doi.org/10.3390/fishes3040041.
[20] S. Dash, S.K. Das, J. Samal, H.N. Thatoi, Epidermal mucus, a major determinant in fish health: a review, Iran. J. Vet. Res. 19 (2018) 72–81. http://www.ncbi.nlm.nih.gov/pubmed/30046316.
[21] M.F. Brinchmann, Immune relevant molecules identified in the skin mucus of fish using -omics technologies, Mol. Biosyst. 12 (2016) 2056–2063, https://doi.org/10.1039/C5MB00890E.
[22] K.L. Shephard, Functions for fish mucus, Rev. Fish Biol. Fish. 4 (1994) 401–429, https://doi.org/10.1007/BF00042888.
[23] J.M. García-Beltrán, A.T. Mansour, A.S. Alsaqufi, H.M. Ali, M.Á. Esteban, Effects of aqueous and ethanolic leaf extracts from drumstick tree (*Moringa oleifera*) on gilthead seabream (*Sparus aurata* L.) leucocytes, and their cytotoxic, antitumor, bactericidal and antioxidant activities, Fish Shellfish Immunol. 106 (2020) 44–55, https://doi.org/10.1016/j.fsi.2020.06.054.
[24] A.T. Mansour, L. Miao, C. Espinosa, J.M. García-Beltrán, D.C. Ceballos Francisco, M.Á. Esteban, Effects of dietary inclusion of *Moringa oleifera* leaves on growth and some systemic and mucosal immune parameters of seabream, Fish Physiol. Biochem. 44 (2018) 1223–1240, https://doi.org/10.1007/s10695-018-0515-z.
[25] F.A. Guardiola, M. Dioguardi, M.G. Parisi, M.R. Trapani, J. Meseguer, A. Cuesta, M. Cammarata, M.A. Esteban, Evaluation of waterborne exposure to heavy metals in innate immune defences present on skin mucus of gilthead seabream (*Sparus aurata*), Fish Shellfish Immunol. 45 (2015) 112–123, https://doi.org/10.1016/j.fsi.2015.02.010.
[26] I. Sanahuja, L. Fernández-Alacid, S. Sánchez-Nuño, B. Ordóñez-Grande, A. Ibarz, Chronic cold stress alters the skin mucus interactome in a temperate fish model, Front. Physiol. 9 (2019), https://doi.org/10.3389/fphys.2018.01916.
[27] C.C. Lazado, G. Timmerhaus, M.W. Breiland, K. Pittman, S. Hytterød, Multiomics provide insights into the key molecules and pathways involved in the physiological adaptation of atlantic salmon (*Salmo salar*) to chemotherapeutic-induced oxidative stress, Antioxidants 10 (2021) 1931, https://doi.org/10.3390/antiox10121931.
[28] N.W. Ross, K.J. Firth, A. Wang, J.F. Burka, S.C. Johnson, Changes in hydrolytic enzyme activities of naïve Atlantic salmon *Salmo salar* skin mucus due to infection with the salmon louse *Lepeophtheirus salmonis* and cortisol implantation, Dis. Aquat. Org. 41 (2000) 43–51, https://doi.org/10.3354/dao041043.
[29] V.S. Raj, G. Fournier, K. Rakus, M. Ronsmans, P. Ouyang, B. Michel, C. Delforges, B. Costes, F. Farnir, B. Leroy, R. Wattiez, C. Melard, J. Mast, F. Lieffrig, A. Vanderplasschen, Skin mucus of *Cyprinus carpio* inhibits cyprinid herpesvirus 3 binding to epidermal cells, Vet. Res. 42 (2011) 92, https://doi.org/10.1186/1297-9716-42-92.
[30] B. Rajan, J. Lokesh, V. Kiron, M.F. Brinchmann, Differentially expressed proteins in the skin mucus of Atlantic cod (*Gadus morhua*) upon natural infection with *Vibrio anguillarum*, BMC Vet. Res. 9 (2013) 103, https://doi.org/10.1186/1746-6148-9-103.
[31] R. Rivera, N. Garrido, Metabolomics, in: Oxid. Antioxidants Impact Oxidative Status Male Reprod, Elsevier, 2019, pp. 277–285, https://doi.org/10.1016/B978-0-12-812501-4.00025-0.
[32] M. Ashrafi, Y. Xu, H. Muhamadali, I. White, M. Wilkinson, K. Hollywood, M. Baguneid, R. Goodacre, A. Bayat, A microbiome and metabolomic signature of phases of cutaneous healing identified by profiling sequential acute wounds of human skin: an exploratory study, PLoS One 15 (2020), e0229545, https://doi.org/10.1371/journal.pone.0229545.
[33] S.A. Eming, P.J. Murray, E.J. Pearce, Metabolic orchestration of the wound healing response, Cell Metabol. 33 (2021) 1726–1743, https://doi.org/10.1016/j.cmet.2021.07.017.
[34] D. Chouhan, B.B. Mandal, Silk biomaterials in wound healing and skin regeneration therapeutics: from bench to bedside, Acta Biomater. 103 (2020) 24–51, https://doi.org/10.1016/j.actbio.2019.11.050.
[35] M.F. Maitz, C. Sperling, T. Wongpinyochit, M. Herklotz, C. Werner, F.P. Seib, Biocompatibility assessment of silk nanoparticles: hemocompatibility and internalization by human blood cells, Nanomed. Nanotechnol. Biol. Med. 13 (2017) 2633–2642, https://doi.org/10.1016/j.nano.2017.07.012.
[36] T.K. Mwangi, R.D. Bowles, D.M. Tainter, R.D. Bell, D.L. Kaplan, L.A. Setton, Synthesis and characterization of silk fibroin microparticles for intra-articular drug delivery, Int. J. Pharm. 485 (2015) 7–14, https://doi.org/10.1016/j.ijpharm.2015.02.059.
[37] M.A. Tomeh, R. Hadianamrei, X. Zhao, Silk fibroin as a functional biomaterial for drug and gene delivery, Pharmaceutics 11 (2019) 494, https://doi.org/10.3390/pharmaceutics11100494.
[38] T. Wongpinyochit, P. Uhlmann, A.J. Urquhart, F.P. Seib, PEGylated silk nanoparticles for anticancer drug delivery, Biomacromolecules 16 (2015) 3712–3722, https://doi.org/10.1021/acs.biomac.5b01003.
[39] I. V Bessonov, Y.A. Rochev, A.Y. Arkhipova, M.N. Kopitsyna, D. V Bagrov, E. A. Karpushkin, T.N. Bibikova, A.M. Moysenovich, A.S. Soldatenko, I.I. Nikishin, M. S. Kotliarova, V.G. Bogush, K. V Shaitan, M.M. Moisenovich, Fabrication of hydrogel scaffolds via photocrosslinking of methacrylated silk fibroin, Biomed. Mater. 14 (2019), 034102, https://doi.org/10.1088/1748-605X/ab04e0.
[40] A.A. Lozano-Pérez, M.G. Montalbán, S.D. Aznar-Cervantes, F. Cragnolini, J. L. Cenis, G. Víllora, Production of silk fibroin nanoparticles using ionic liquids and high-power ultrasounds, J. Appl. Polym. Sci. (2014), https://doi.org/10.1002/app.41702 n/a-n/a.
[41] F.A. Guardiola, A. Cuesta, M. Arizcun, J. Meseguer, M.A. Esteban, Comparative skin mucus and serum humoral defence mechanisms in the teleost gilthead seabream (*Sparus aurata*), Fish Shellfish Immunol. 36 (2014) 545–551, https://doi.org/10.1016/j.fsi.2014.01.001.
[42] C.E. Doneanu, W. Chen, J.R. Mazzeo, O.R. Ds, UPLC/MS monitoring of water-soluble vitamin bs in cell culture media in minutes, water appl, Notes 1–7 (2011).
[43] T. Pluskal, S. Castillo, A. Villar-Briones, M. Orešič, MZmine 2: modular framework for processing, visualizing, and analyzing mass spectrometry-based molecular profile data, BMC Bioinf. 11 (2010) 395, https://doi.org/10.1186/1471-2105-11-395.
[44] D.S. Wishart, D. Tzur, C. Knox, R. Eisner, A.C. Guo, N. Young, D. Cheng, K. Jewell, D. Arndt, S. Sawhney, C. Fung, L. Nikolai, M. Lewis, M.-A. Coutouly, I. Forsythe, P. Tang, S. Shrivastava, K. Jeroncic, P. Stothard, G. Amegbey, D. Block, D.D. Hau, J. Wagner, J. Miniaci, M. Clements, M. Gebremedhin, N. Guo, Y. Zhang, G. E. Duggan, G.D. MacInnis, A.M. Weljie, R. Dowlatabadi, F. Bamforth, D. Clive, R. Greiner, L. Li, T. Marrie, B.D. Sykes, H.J. Vogel, L. Querengesser, HMDB: the human metabolome database, Nucleic Acids Res. 35 (2007) D521–D526, https://doi.org/10.1093/nar/gkl923.
[45] M. Gillespie, B. Jassal, R. Stephan, M. Milacic, K. Rothfels, A. Senff-Ribeiro, J. Griss, C. Sevilla, L. Matthews, C. Gong, C. Deng, T. Varusai, E. Ragueneau, Y. Haider, B. May, V. Shamovsky, J. Weiser, T. Brunson, N. Sanati, L. Beckman, X. Shao, A. Fabregat, K. Sidiropoulos, J. Murillo, G. Viteri, J. Cook, S. Shorser, G. Bader, E. Demir, C. Sander, R. Haw, G. Wu, L. Stein, H. Hermjakob, P. D'Eustachio, The reactome pathway knowledgebase 2022, Nucleic Acids Res. 50 (2022) D687–D692, https://doi.org/10.1093/nar/gkab1028.
[46] S. Kim, J. Chen, T. Cheng, A. Gindulyte, J. He, S. He, Q. Li, B.A. Shoemaker, P. A. Thiessen, B. Yu, L. Zaslavsky, J. Zhang, E.E. Bolton, PubChem in 2021: new data content and improved web interfaces, Nucleic Acids Res. 49 (2021) D1388–D1395, https://doi.org/10.1093/nar/gkaa971.
[47] R. Caspi, T. Altman, R. Billington, K. Dreher, H. Foerster, C.A. Fulcher, T. A. Holland, I.M. Keseler, A. Kothari, A. Kubo, M. Krummenacker, M. Latendresse, L. A. Mueller, Q. Ong, S. Paley, P. Subhraveti, D.S. Weaver, D. Weerasinghe, P. Zhang, P.D. Karp, The MetaCyc database of metabolic pathways and enzymes and the BioCyc collection of Pathway/Genome Databases, Nucleic Acids Res. 42 (2014) D459–D471, https://doi.org/10.1093/nar/gkt1103.
[48] D.S. Wishart, C. Li, A. Marcu, H. Badran, A. Pon, Z. Budinski, J. Patron, D. Lipton, X. Cao, E. Oler, K. Li, M. Paccoud, C. Hong, A.C. Guo, C. Chan, W. Wei, M. Ramirez-Gaona, PathBank, A comprehensive pathway database for model organisms, Nucleic Acids Res. 48 (2020) D470, https://doi.org/10.1093/nar/gkz861. –D478.
[49] M. Kanehisa, KEGG: Kyoto Encyclopedia of genes and Genomes, Nucleic Acids Res. 28 (2000) 27–30, https://doi.org/10.1093/nar/28.1.27.
[50] M. Kanehisa, M. Furumichi, Y. Sato, M. Ishiguro-Watanabe, M. Tanabe, KEGG: integrating viruses and cellular organisms, Nucleic Acids Res. 49 (2021), https://doi.org/10.1093/nar/gkaa970. D545–D551.
[51] M. Kanehisa, Toward understanding the origin and evolution of cellular organisms, Protein Sci. 28 (2019) 1947–1951, https://doi.org/10.1002/pro.3715.
[52] Z. Pang, G. Zhou, J. Ewald, L. Chang, O. Hacariz, N. Basu, J. Xia, Using MetaboAnalyst 5.0 for LC–HRMS spectra processing, multi-omics integration and covariate adjustment of global metabolomics data, Nat. Protoc. 17 (2022) 1735–1761, https://doi.org/10.1038/s41596-022-00710-w.
[53] F.A. Guardiola, A. Cuesta, M.Á. Esteban, Using skin mucus to evaluate stress in gilthead seabream (*Sparus aurata* L.), Fish Shellfish Immunol. 59 (2016) 323–330, https://doi.org/10.1016/j.fsi.2016.11.005.
[54] M.M. Rinschen, J. Ivanisevic, M. Giera, G. Siuzdak, Identification of bioactive metabolites using activity metabolomics, Nat. Rev. Mol. Cell Biol. 20 (2019) 353–367, https://doi.org/10.1038/s41580-019-0108-4.
[55] L. Ivanova, H. Tartor, S. Grove, A. Kristoffersen, S. Uhlig, Workflow for the targeted and untargeted detection of small metabolites in fish skin mucus, Fishes 3 (2018) 21, https://doi.org/10.3390/fishes3020021.
[56] C.C. Lazado, L.-F. Pedersen, K.H. Kirste, M. Soleng, M.W. Breiland, G. Timmerhaus, Oxidant-induced modifications in the mucosal transcriptome and circulating metabolome of Atlantic salmon, Aquat. Toxicol. 227 (2020), 105625, https://doi.org/10.1016/j.aquatox.2020.105625.
[57] D.R. Ekman, D.M. Skelton, J.M. Davis, D.L. Villeneuve, J.E. Cavallin, A. Schroeder, K.M. Jensen, G.T. Ankley, T.W. Collette, Metabolite profiling of fish skin mucus: a novel approach for minimally-invasive environmental exposure monitoring and surveillance, Environ. Sci. Technol. 49 (2015) 3091–3100, https://doi.org/10.1021/es505054f.
[58] D.M. Kallert, S. Ponader, S. Adelt, P. Kaese, R. Geyer, W. Haas, M. El-Matbouli, Analysis of rainbow trout *Oncorhynchus mykiss* epidermal mucus and evaluation of semiochemical activity for polar filament discharge in *Myxobolus cerebralis*

actinospores, J. Fish. Biol. 77 (2010) 1579–1598, https://doi.org/10.1111/j.1095-8649.2010.02785.x.
[59] P. Saglio, B. Fauconneau, Free amino acid content in the skin mucus of goldfish, *Carassius auratus* L.: influence of feeding, Comp. Biochem. Physiol. Part A Physiol. 82 (1985) 67–70, https://doi.org/10.1016/0300-9629(85)90705-4.
[60] C.E.L. Chua, Y.S. Lim, M.G. Lee, B.L. Tang, Non-classical membrane trafficking processes galore, J. Cell. Physiol. 227 (2012) 3722–3730, https://doi.org/10.1002/jcp.24082.
[61] K.C. Kim, J.I. Rearick, P. Nettesheim, A.M. Jetten, Biochemical characterization of mucous glycoproteins synthesized and secreted by hamster tracheal epithelial cells in primary culture, J. Biol. Chem. 260 (1985) 4021–4027. http://www.ncbi.nlm.nih.gov/pubmed/3980465.
[62] L. Ivanova, O.D. Rangel-Huerta, H. Tartor, M.C. Gjessing, M.K. Dahle, S. Uhlig, Fish skin and gill mucus: a source of metabolites for non-invasive health monitoring and research, Metabolites 12 (2021) 28, https://doi.org/10.3390/metabo12010028.
[63] F.E. Reyes-López, A. Ibarz, B. Ordóñez-Grande, E. Vallejos-Vidal, K.B. Andree, J. C. Balasch, L. Fernández-Alacid, I. Sanahuja, S. Sánchez-Nuño, J.P. Firmino, L. Pavez, J. Polo, L. Tort, E. Gisbert, Skin multi-omics-based interactome analysis: integrating the tissue and mucus exuded layer for a comprehensive understanding of the teleost mucosa functionality as model of study, Front. Immunol. 11 (2021), https://doi.org/10.3389/fimmu.2020.613824.
[64] M. Pollini, F. Paladini, Bioinspired materials for wound healing application: the potential of silk fibroin, Materials 13 (2020) 3361, https://doi.org/10.3390/ma13153361.
[65] Y.G. Chung, K. Algarrahi, D. Franck, D.D. Tu, R.M. Adam, D.L. Kaplan, C. R. Estrada, J.R. Mauney, The use of bi-layer silk fibroin scaffolds and small intestinal submucosa matrices to support bladder tissue regeneration in a rat model of spinal cord injury, Biomaterials 35 (2014) 7452–7459, https://doi.org/10.1016/j.biomaterials.2014.05.044.
[66] K. Shimura, A. Kikuchi, K. Ohtomo, Y. Katagata, A. Hyodo, Studies on silk fibroin of Bombyx mori. I. Fractionation of fibroin prepared from the posterior silk gland, J. Biochem. 80 (1976) 693–702, https://doi.org/10.1093/oxfordjournals.jbchem.a131328.
[67] J. De Bersaques, Purine and pyrimidine metabolism in human Epidermis**From the department of dermatology, university of gent, Belgium, J. Invest. Dermatol. 48 (1967) 169–173, https://doi.org/10.1038/jid.1967.27.
[68] K. Cheng, L. Wagner, A.A. Moazzami, P. Gómez-Requeni, A. Schiller Vestergren, E. Brännäs, J. Pickova, S. Trattner, Decontaminated fishmeal and fish oil from the Baltic Sea are promising feed sources for Arctic char (*Salvelinus alpinus* L.)—studies of flesh lipid quality and metabolic profile, Eur. J. Lipid Sci. Technol. 118 (2016) 862–873, https://doi.org/10.1002/ejlt.201500247.
[69] S. Zhang, X. Zeng, M. Ren, X. Mao, S. Qiao, Novel metabolic and physiological functions of branched chain amino acids: a review, J. Anim. Sci. Biotechnol. 8 (2017) 10, https://doi.org/10.1186/s40104-016-0139-z.
[70] X. Wang, T. Pan, Stress response and adaptation mediated by amino acid misincorporation during protein synthesis, Adv. Nutr. An Int. Rev. J. 7 (2016) 773S–779S, https://doi.org/10.3945/an.115.010991.
[71] S.G. Park, K.L. Ewalt, S. Kim, Functional expansion of aminoacyl-tRNA synthetases and their interacting factors: new perspectives on housekeepers, Trends Biochem. Sci. 30 (2005) 569–574, https://doi.org/10.1016/j.tibs.2005.08.004.
[72] Z. Gao, E. Johansen, S. Eyers, C.L. Thomas, T.H. Noel Ellis, A.J. Maule, The potyvirus recessive resistance gene, sbm1 , identifies a novel role for translation initiation factor eIF4E in cell-to-cell trafficking, Plant J. 40 (2004) 376–385, https://doi.org/10.1111/j.1365-313X.2004.02215.x.
[73] S.G. Park, H. Shin, Y.K. Shin, Y. Lee, E.-C. Choi, B.-J. Park, S. Kim, The novel cytokine p43 stimulates dermal fibroblast proliferation and wound repair, Am. J. Pathol. 166 (2005) 387–398, https://doi.org/10.1016/S0002-9440(10)62262-6.
[74] J.T. Brosnan, M.E. Brosnan, R.F.P. Bertolo, J.A. Brunton, Methionine: a metabolically unique amino acid, Livest. Sci. 112 (2007) 2–7, https://doi.org/10.1016/j.livsci.2007.07.005.
[75] M.B. Williamson, H.J. Fromm, Effect of cystine and methionine on healing of experimental wounds, Exp. Biol. Med. 80 (1952) 623–626, https://doi.org/10.3181/00379727-80-19712.
[76] M.B. Williamson, H.J. Fromm, Utilization of sulfur amino acids during healing of experimental wounds, Exp. Biol. Med. 83 (1953) 329–333, https://doi.org/10.3181/00379727-83-20350.
[77] R. Perez-Tamayo, M. Ihnen, The effect of methionine in experimental wound healing; a morphologic study., Am. J. Pathol. 29 (n.d.) 233-49. http://www.ncbi.nlm.nih.gov/pubmed/13030690.
[78] H.J. Fromm, R.C. Nordlie, Redistribution of methionine and cystine during experimental wound healing, Biochim. Biophys. Acta 31 (1959) 357–364, https://doi.org/10.1016/0006-3002(59)90008-3.
[79] S.A. Localio, M.E. Morgan, J.W. Hinton, The biological chemistry of wound healing; the effect of dl-methionine on the healing of wounds in protein-depleted animals, Surg. Gynecol. Obstet. 86 (1948) 582–590, 18910940.
[80] S.A. Localio, L. Gillette, J.W. Hinton, The biological chemistry of wound healing; the effect of di-methionine on the healing of surface wounds, Surg. Gynecol. Obstet. 89 (1949) 69–73. http://www.ncbi.nlm.nih.gov/pubmed/18131960.
[81] R.V. DeVito, Healing of wounds, surg, Clin. North Am. 45 (1965) 441–459, https://doi.org/10.1016/S0039-6109(16)37542-9.