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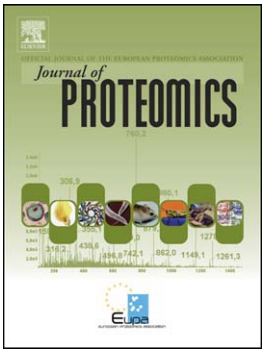

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Differential proteome profile of skin mucus of gilthead seabream (*Sparus aurata*) after probiotic intake and/or overcrowding stress

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Abbreviations: ACTB, actin beta; APOA1, apolipoprotein A-1; ARFGAP, ADP-ribosylation factor GTPase-activating protein; C3, complement component 3; GST, glutathione S-transferase; IL-1, interleukin-1; IMPA, inositol monophosphate; KRT, keratin; LEI, leucocyte elastase inhibitor; LYZ, lysozyme; NCC, nonspecific cytotoxic cell; NCCRP, nonspecific cytotoxic cell receptor protein; NK, natural killer; NKEF, natural killer cell enhancing factor; PFN, profilin; PRDX, peroxiredoxin; TPIA, triosephosphate isomerase A.

Keywords: gilthead seabream (*Sparus aurata*), mucosal immunity, proteome, skin, probiotic Pdp11, overcrowding.

Abstract

Gilthead seabream (*Sparus aurata* L.) is the major cultured fish species in the Mediterranean area. High density stocking causes stress and increases the impact of diseases leading to economic losses. Probiotics could represent a solution to prevent diseases through several mechanisms such as improving the immune status and/or mucosal microbiota or competing with pathogens. The probiotic *Shewanella putrefaciens*, also known as Pdp11, was firstly isolated from the skin of healthy gilthead seabream. Our study focuses on the skin mucus proteome after dietary probiotic Pdp11 intake in fish maintained under normal or overcrowding conditions. 2-DE of skin mucus followed by LC-MS/MS analysis was done for each experimental group and differentially expressed proteins were identified. The results showed differentially expressed proteins especially involved in immune processes, such as lysozyme, complement C3, natural killer cell enhancing factor and nonspecific cytotoxic cell receptor protein 1, whose transcript profiles were studied by qPCR. A consistency between lysozyme protein levels in the mucus and lysozyme mRNA levels in skin were found. Further research is necessary to unravel the implications of skin mucosal immunity on fish welfare and disease.

Biological significance

The present work reveals the proteomic changes, which are taking place in the skin mucus of stressed and non-stressed gilthead seabream after Pdp11 probiotic intake. The study contributes to improve the knowledge on skin mucosal immunology of this relevant farmed fish species. Furthermore, the paper shows for the first time how a suitable proteomic methodology, in this case 2-DE followed by LC-MS/MS is useful to perform a comparative study with a non-invasive technique of skin mucus of gilthead seabream.

1. Introduction

Gilthead seabream (*Sparus aurata* L.) is a hermaphroditic protandrous marine teleost which has a strong impact on the European aquaculture industry, mainly distributed in Atlantic and Mediterranean seas [1]. Due to intensive fish farming, large number of diseases could emerge, commonly in relation to stress problems, i.e. overcrowding [2]. In the recent years, to avoid or diminish both fish diseases and/or stress problems, probiotics have arisen as promising tools. Probiotics may act as sources of nutrients, improving fish health by exclusion competition with pathogenic bacteria and modulating immune parameters [3–5], and consequently prevent stress problems and pathogen infections in fish. In this sense, *Shewanella putrefaciens*, also known as Pdp11, is a gilthead seabream probiotic isolated from the skin of healthy specimens, which was tested as potential probiotic [6,7], getting some benefits after dietary intake such as an increase of survival against vibriosis [8] and stimulation of systemic immune parameters [9–11].

Recent advances in proteomics research methods have been used for identification and quantification of proteins [12]. These methodologies have been successfully used to evaluate the proteome in fish after administration of dietary supplements or under stress factors [13–15]. However, most of them are based on liver proteomes. Nowadays, the characterization of mucosal surfaces are taking importance, mainly fromm the immunological point of view, since skin mucus is the first barrier of defence in fish [16] and can be studied with non-invasive techniques. Thus, many molecules involved in immunity have been reported in skin mucus [17]. Due to this, in the last years, several skin/epidermal mucus proteomes have been studied in fish, including European sea bass [18] and gilthead seabream [19,20]. For example, it has been observed changes in protein composition after infection [21,22], handling stress [23] or parental care [24]. However, so far, very little is known about the regulatory mechanisms of dietary probiotics and/or overcrowding stress conditions at the proteomic level in the fish skin mucus.

The aim of this work was to study the differentially expressed proteins present in skin mucus after probiotic feeding under overcrowding stress, identified using 2-DE followed by LC-MS/MS, and next study the changes of transcript levels of four of these molecules (*c3*, *nkefb*, *nccrp1* and *lyz*) in skin of gilthead seabream, which will contribute to a better understanding of

changes in mucosal immunity as well as checking if probiotic Pdp11 could improve fish health of stressed fish.

2. Materials and methods

2.1 Animal care

Forty specimens of gilthead seabream (*S. aurata*) (104.2±7.4 and 116.2±5.1 g of initial and final mean body weight, respectively), obtained from a local farm (Murcia, Spain), were kept in running seawater aquaria (flow water 900 l h^{-1}) at 28‰ salinity, 22ºC and a photoperiod of 12h light: 12h dark. All the fish handling procedures were approved by Bioethical Committee of the University of Murcia.

2.2 Probiotic diet

Bacteria cells of *S. putrefaciens* were grown in tryptic soy broth (TSB; Sigma-Aldrich) agar plates for 24 h at 25ºC after which one colony was inoculated in tubes containing 5 mL of TSB supplemented with 1.5% NaCl (TSBs). After 24 h of incubation at 22ºC and 200 rpm, the number of probiotic bacteria present per millilitre of TSB was measured by using a Particle Counter (Beckman Coulter). Bacteria were washed twice in phosphate buffer saline (PBS) and added with water into a crushed commercial diet (Optibream D4, Skretting) to a final concentration of 10^8 cfu g^{-1}, mixed and re-pelleted again. The same process without adding the probiotic was used for the control diets.

2.3 Experimental design

Fish were weighted and measured in order to calculate diet and density ratios, and divided into 4 different tanks. Two groups received the commercial diet and the other two a Pdp11-supplemented diet at a rate of 1% biomass once per day. A tank of each dietary group was maintained at a density of 5 kg m^{-3}, considered low density, while the other was maintained at a density of 20 kg m^{-3}, considered high density or overcrowding condition for gilthead seabream [25]. Fish were maintained under these experimental circumstances for 15 and 30 days until sampling.

2.4 Mucus and tissues samples

Five fish per group were anesthetized with 100 mg l^{-1} MS222 prior to sampling. Mucus was gently scraped off from the skin surface, avoiding blood, urine and faeces during collection [26]. Mucus was transferred into tubes of 15 ml and stored at -80 ºC until use. Skin tissue was collected in QIAzol lysis reagent (Qiagen) and stored at -80 ºC for subsequent RNA extraction.

2.5 Mucus protein purification

Pooled mucus samples were obtained from five individual fish. These pooled samples in triplicate for each group were solubilised with 1 mM DTT and 1.5 mM EDTA, which serves to act as a mild mucolytic agent [27]. Next, after two rounds of sonication for 6 seconds followed by cooling for 1 min, samples were centrifuged at 20,000 g for 30 min at 4 ºC. The supernatant containing the soluble mucus proteins was desalted with proteomic grade water (G Biosciences) using centrifugal filters of 3 kDa (VWR) by spinning 3 times at 14,000 g at 4 ºC with 0.2 ml of ice cold water each time. The dialysed protein solution was further purified by 2D clean-up kit (Bio-Rad) following the manufacturer's instructions.

2.6 2DE

The samples obtained after the 2D clean-up process were suspended in 2D lysis buffer (Bio-Rad) containing 7 M urea, 2 M thiourea, 1% (w/v) ASB-14, 40 mM Tris base, 0.001% bromophenol blue and 50 mM DTT (Sigma-Aldrich) and 0.5% (v/v) Biolytes 3-10 ampholyte (Bio-Rad). The protein content of solubilised samples was estimated using Qubit protein assay (Life Technologies). Two hundred µg proteins for each sample were rehydrated in 17 cm 3-10 IPG strips (Bio-Rad) and isoelectric focusing (IEF) was carried out using protean IEF cell (Bio-Rad). After IEF, the electro-focused IPG strips were reduced and alkylated for 15 min each in equilibration buffer containing 6 M urea (Sigma Aldrich), 0.375 M Tris-HCl pH 8.8 (Bio-Rad), 2% (w/v) SDS (Sigma-Aldrich), 20% (v/v) glycerol (Merck) with 0.2% (w/v) DTT (Sigma-Aldrich) or 0.3% (w/v) iodoacetamide (Bio-Rad), respectively. The equilibrated strips were loaded on 12.5% polyacrylamide gels to perform SDS-PAGE [28], running on PROTEAN II system (Bio-Rad). The gels were stained overnight with SYPRO® Ruby Protein Gel Stain (Life Technologies) according to the supplier's protocol. Gel image documentation was carried out using ChemiDocTM XRS imaging system (Bio-Rad). Raw pictures were analysed using

PDQuest Advanced software version 8.0.1 (Bio-Rad) including detection of spots, normalization using local regression, spot matching and differential expression analysis. The coefficient of variation (CV) intra-pools was also analysed for each spot of interest in each treatment group and showed in Table 3. Protein spots were considered as differentially expressed when expression level was at least 1.5-fold different compared to the control group and when the differences were detected as significant at $p<0.01$ by two tailed Student's t-test.

2.7 LC-MS/MS analysis

Spots from SYPRO-stained gilthead seabream skin mucus 2D gels (n=3) were picked, excised and subjected to in-gel reduction, alkylation, and tryptic digestion using 2–10 ng/µl trypsin (V511A; Promega) as described elsewhere [29]. Peptide mixtures containing 0.1% formic acid were loaded onto a nanoACQUITY UltraPerformance LC (Waters), containing a 5 µm Symmetry C18 Trap column (180 µm × 20 mm; Waters) in front of a 1.7 µm BEH130 C18 analytical column (100 µm × 100 mm; Waters). Peptides were separated with a gradient of 5–95% acetonitrile, 0.1% formic acid, with a flow of 0.4 µl min^{-1} eluted to a Q-TOF Ultima mass spectrometer (Micromass/Waters). The samples were run in data dependent tandem mass spectrophotometry (MC/MC) mode. Peak lists were generated from MS/MS by the ProteinLynx Global server software (version 2.2; Waters) and submitted to MASCOT search engine (version 2.5.1) and searched against NCBInr with the following parameters: maximum one missed cleavage by trypsin, peptide mass tolerance 100 ppm, MS/MS ion tolerance set to 0.1 Da, carbamidomethylation of cysteine selected as fixed modification and methionine oxidation as variable modification. Protein hits not satisfying a significance threshold ($p<0.05$) or with low sequence coverage were further searched against Swissprot and vertebrate EST (expressed sequence tags) databases, taxonomy *Actinopterygii*.

2.8 Primer design

Primers were designed by OligoPerfect™ Designer (Life Technologies) from *S. aurata* sequences which are available in NCBInr database. Details regarding oligonucleotide primers and their attributes are given in Table 1.

2.9 Gene expression analysis

The mRNA levels corresponding to four differentially expressed immune-related proteins in the skin of the experimental fish were analysed by real-time PCR (qPCR). RNA was extracted from 50 mg of skin from five specimens of gilthead seabream for each treatment group and control group using QIAzol lysis reagent method (Qiagen) as described elsewhere [30]. Quality of total RNA was checked on 1% agarose gel, followed by the quantification using the Qubit® RNA assay kit and Qubit® 2.0 fluorometer (Life Technologies). The complementary DNA (cDNA) was synthetised from 1 µg of RNA using QuantiTec Reverse Transcription Kit (Qiagen). Ten times diluted cDNA was used to conduct qPCR on ABI PRISM 7500 instrument (Applied Biosystems) using SYBR Green PCR Core Reagents (Applied Biosystems) and the $2^{-\Delta\Delta Ct}$ method [31]. Reaction mixtures [containing 10 µl of 2 x SYBR Green supermix, 5 µl of primers (0.6 µM each) and 5 µl of cDNA template] were incubated (10 min, 95ºC), followed by 40 cycles of 15 s at 95 ºC, 1 min at 60 ºC, and finally 15 s at 95 ºC, 1 min at 60ºC and 15 s at 95 ºC. Fluorescence was measured at the end of each cycle. Subsequently, a melt curve analysis (60–95 ºC) was performed on every sample in the plate to ensure the specificity of primers and the absence of primer secondary structures. Each plate subjected to qPCR contained a negative control for cDNA template (water) as well as a control for reverse transcription. No amplification product was observed in negative controls and neither primer-dimer formation nor secondary structures were observed in any case. Additionally, a three-fold serially diluted cDNA (pooled) was included for each plate of each gene to evaluate the efficiency of qPCR reaction based on standard curve method, using the formulae $E = 10^{(-1/slope)} - 1 \times 100$. All qPCR reactions were carried out in duplicate and quantification cycle (Ct) values of each gene (target) were converted into relative quantities. Normalization factors were calculated as the geometric mean of relative quantities of reference genes *elongation factor 1 alpha* (*ef1a*) and *ribosomal protein s18* (*rps18*) using the BestKeeper© algorithm [32].

The qPCR data of target genes (*nkefb* and *nccrp1*) were analysed by one-way analysis of variance (ANOVA) followed by Tukey's test post-hoc analysis. Normality of the data was previously assessed using a Shapiro–Wilk test and homogeneity of variance was also verified using the Levene test. A non-parametric Kruskal–Wallis H test was used when data did not

meet parametric assumptions, concretely for *c3* and *lyz* genes. Data are expressed as relative gene expression of each target gene (mean ± SEM). Statistical tests were performed using SPSS software v19.0 (SPSS) and the differences of means were considered significant at $p<0.05$.

3. Results and discussion

In our experience working with proteomic tools [33,34] and taking into account previous proteomic studies [13,14,24], 2-DE followed by coupled tandem LC-MS/MS provides good resolution for comparatives studies. Furthermore, the development of fluorescent dyes from SYPRO group get great performance (in terms of linear range and limit of detection) [35], compared with other classical dyes such as silver or Coomassie brilliant blue.

A range from 431 to 452 spots was detected in total. Next, an exhaustive analysis, adjusting and optimizing the quantification of spots, and discarding spots that were not consistent, was carried out. The present study shows several differentially expressed proteins (through 22 identified spots) in the skin mucus from gilthead seabream specimens (Tables 2 and 3): fed with commercial diet and non-stressed (control, Figure 1A), fed with probiotic and non-stressed (Figure 1 B), fed commercial diet and stressed by overcrowding (Figure 1C), fed probiotic diet and stressed by overcrowding (Figure 1 D). The intra-group variability of differentially expressed spots was in a range between 0.5% and 9.3% (Table 3). In addition, a literature-based comparison with 2-DE techniques was performed in order to see the variability of these proteins in gilthead seabream and how they are conserved across fish species (Table 4).

3.1 Effects of Pdp11 and/or overcrowding stress on structural proteins

Structural proteins are a major group in seabream mucus proteins and include several isoforms of actins, keratins, tubulins, tropomyosin 4-2, cofilin-2 and filamin-A-like [19], and some of them are modulated in the present study (Tables 2 and 3). First, actin beta (ACTB; spots 7 and 21) was found differentially expressed in both experimental times (Table 3). At 15 days, the levels of ACTB (around 42kDa) in skin mucus were decreased in seabream specimens under overcrowding stress compared to the control group. However, a different ACTB (around 35kDa) was found up-regulated almost 2-fold after probiotic dietary administration in non-stressed specimens compared to the control group. ACTB is among the most common structural

proteins in the skin mucus and seems to be a normal protein with some functionality in this surface rather a product of the epidermal cell disruption [36]. In fact, the presence of its proteolytic products, as in our study, has been related to some stressful situations and these products could enhance the immune response [23]. Moreover, the decrease in the 42 kDa isoform after overcrowding stress could also indicate that this isoform is fragmented in several products different from the 35 kDa isoform, a hypothesis that should be further evaluated.

Keratin, type I cytoskeletal 50 kDa (KRT; spot 14) protein was also found over-expressed in seabream specimens under overcrowding stress after 30 days compared to the control group (Table 3). At this point, profilin (PFN; spot 5) was also increased in fish fed the probiotic diet under overcrowding stress compared to the control group. These data suggest a role different than the strictly structural, at least in the epidermal mucus. For example, KTRs of fish mucus have shown some antibacterial activity due to their pore-formation ability [37], and this points to the increase of this immune activity in seabream specimens. In fact, seabream exposed to heavy metal stressors showed significant increments in the bactericidal activity of the skin mucus and altered protein profiles [38]. These data together suggest that the altered structural proteins in the skin mucus might have some unknown biological role at present, which merits further evaluation.

3.2 Effects of Pdp11 and/or overcrowding stress on metabolism proteins

The presence of some proteins involved in the cellular metabolism in seabream mucus is also regulated by dietary probiotic administration and/or overcrowding stress (Tables 2 and 3). Apolipoprotein A-1 (APOA1; spot 4) was over-expressed with 2.31-fold increase after probiotic diet both in non-stressed group and overcrowding stressed group compared to the control group at 30 days (Table 3). Despite of primary role of APOA1 in the reverse cholesterol transport, its presence in the skin mucus and over-expression after probiotic Pdp11 administration suggest not only an extracellular role of antimicrobial activity against pathogens in gilthead seabream, as occurs in striped bass [39], but also an improving of the antimicrobial properties inferred by the Pdp11 probiotic diet. In fact, this protein has been shown to be increased in infected Atlantic salmon and Atlantic cod and showed bactericidal activity [22,34,36,40].

The predicted 14-3-3 protein (spot 22) was over-expressed in probiotic fed fish 1.58-fold but under-expressed in stressed fish (0.55-fold) and stressed and Pdp11-fed fish (0.38-fold), compared to the control group at 15 days (Table 3). Despite the fact that this protein was previously identified in skin mucus of gilthead seabream [19], to our knowledge this is the first time that over-expression has been found after any probiotic diet, Pdp11 in this study. In addition, this protein has also reported in skin mucus of teleosts such as European sea bass [18] or Atlantic cod [34] but further studies are needed to clarify its implication in skin mucus.

NADP-dependent isocitrate dehydrogenase (spot 18) was over-expressed more than 5-fold in both overcrowding groups compared to the control group at 30 days. At cytosolic level, isocitrate dehydrogenase catalyses oxidative decarboxylation of isocitrate to 2-oxoglutarate and require NAD^+ or $NADP^+$, producing NADH and NADPH, respectively [41], playing a critical role protecting cells against oxidative stress [42]. At extracellular level, this protein is for the first time reported in fish mucus, however it has been previously described in human cervical mucus [43].

Inositol monophosphate (IMPA; spot 6) and triosephosphate isomerase A (TPIA; spot 9) were under-expressed in overcrowding groups compared to the control group at 30 days (Figures 1 C and D). In certain agreement with our data, *impa* gene was up-regulated in European eel and Nile tilapia fish species stressed by seawater change [44]. At proteome level, this protein was identified in skin mucus of Atlantic cod after infection [22], suggesting a role in disease protection on fish.

The predicted aldose reductase-like (spot 10) was under-expressed in fish fed the probiotic diet under overcrowding stress after 30 days, compared to the control group (Figure 1 D; Table 3). Aldose reductase catalyses the NADPH-dependent conversion of glucose to sorbitol [45], and it has been associated to oxidative stress [46], which may suggest a putative role against overcrowding stress but more information is needed to confirm this.

Glutathione S-transferase (GST; spots 17 and 20) was over-expressed in both probiotic fed groups at 15 days, as well as over-expressed in overcrowding groups at 30 days, compared to the control group (Table 3). GST is a family of proteins which are involved in biotransformation

of compounds including toxic substances and oxidative stress products, transport of ligands, and regulation of signalling pathways [47]. It was reported that GST levels increased after exposure to pyrethroids [48]. In addition, GST omega 1 was over-expressed in skin mucus of Atlantic cod after infection [22]. According with these studies, after 30 days of overcrowding stress the oxidative stress by GST over-expression in skin mucus of gilthead seabream could increase.

ADP-ribosylation factor GTPase-activating protein (ARFGAP; spot 16) was over-expressed in probiotic diet fed group, and under-expressed in both overcrowding stressed groups, compared to the control group, at 30 days (Table 3). ARFGAP has been associated with Golgi organization and actin cytoskeletal organization, mediating cell adhesion [49]. In relation with that, probiotic diet promotes over-expression of beta actin (1.95-fold) as well as ARFGAP over-expression (1.39-fold) in skin mucus of gilthead seabream. Similarly, ACTB and ARFGAP showed under-expression in skin mucus of gilthead seabream under overcrowding stress at 15 and 30 days.

Overall, our data show some important regulation of seabream skin mucus metabolic proteins after feeding a probiotic and/or after overcrowding stress. However, the implications of these proteins in the surface mucus should be further ascertained in order to relate them, as some studies suggest, with other different roles such as immunity.

3.3 Effects of Pdp11 and/or overcrowding stress on immune-related proteins

Another group of proteins with known immune functions have been found to be regulated in our study (Tables 2 and 3). First, C3 (spots 12 and 13) was over-expressed in both probiotic fed groups at both trial times, and overcrowding stress group at 30 days (Table 3). The highest over-expression was found in specimens fed Pdp11 probiotic diet with 1.95-fold and 1.74-fold at 15 and 30 days, respectively (Figure 1 B). In previous studies, complement activity was undetected in the skin mucus of seabream [20,50], but it was reported in . At gene level, a *c3* up-regulation was found after yeast dietary administration in gilthead specimens [51] whilst bathing of turbot with acid lactic probiotics also up-regulated its transcription in several tissues [52]. Taking this into account, our results at protein level of C3 over-expression in skin mucus of seabream after Pdp11 probiotic diet may indicate beneficial effects in the key component C3 in skin mucosal

immunity, which acts against pathogens. Furthermore, the two identified spots with a little difference in MW could indicate isoforms which are influenced not only by probiotic intake but also by overcrowding stress at 30 days.

Lectins are a group of sugar binding proteins which are involved in both innate and adaptive immunity [53], including pathogen recognition and neutralization [54]. Furthermore, a diversity of lectins was reported in fish skin mucus [55]. On one hand, in our study, a C-type lectin (spot 1) was identified as over-expressed in both overcrowding stressed fish, reaching 2.61-fold in the group fed with probiotics under stress conditions, compared to the control group (Table 3; Figure 1 D). On the other hand, F-type lectin (spot 8) was over-expressed after probiotic diet and/or overcrowding stress, with the maximum protein expression (2.79-fold) in the skin mucus of seabream specimens under both treatments (Table 3). The fact of finding the maximum up-regulation in Pdp11 probiotic diet groups could suggest the improvement of skin mucosal immunity by Pdp11 probiotic effect and no negative impact of the tested stress. In this regard, skin mucus of seabream specimens under stress caused by exposure to heavy metals showed increased levels of F-lectin as detected by western blotting [38].

Leucocyte elastase inhibitor (LEI; spot 2) was over-expressed in the skin mucus of specimens fed with probiotic, stressed or not, but and under-expressed in those under overcrowding stress, compared to the control group (Table 3). LEIs are inhibitory proteases regulating tissue destruction and inflammation, commonly associated to several skin diseases [56]. Our results suggest that Pdp11 may avoid deleterious effects in skin. In addition, it was found that LEI has antimicrobial properties in mucosal fluids [57] and is up-regulated by cytokines such as interleukin-1 (IL-1) and tumor necrosis factor (TNF) [58]. Based on this, LEI under-expression in overcrowding stress fish may suggest an increased susceptibility of skin to infections.

Nonspecific cytotoxic cell receptor protein 1 (NCCRP1) is the receptor of NCC, evolutionary precursors of mammalian NK cells, which are involved in innate cell-mediated cytotoxicity on fish, including gilthead seabream [59]. It was reported that *nccrp1* is up-regulated by overcrowding stress [8]. Besides, other study with probiotics has demonstrated up-regulation of

nccrp1 after *in vitro* incubation in fish leucocytes [60]. NCCRP1 (spot 3) was over-expressed in the overcrowding groups fed with probiotic or with control diets after 30 days (Table 3).

Peroxiredoxin 2 (PRDX2) or natural killer enhancing factor B (NKEF-B; spot 11) was over-expressed in overcrowding stress groups at 30 days, fed with the Pdp11 probiotic or not, compared to the control group,. Additionally, a predicted peroxiredoxin 6-like (spot 19) was under-expressed in overcrowding stress group at 15 days (Table 3). PRDXs are a family of antioxidant enzymes which act protecting cells from oxidative damage [61] but also show an important role in immunity since extracellular NKEF-A or PRDX1 acts as "endogenous" danger signal by binding to danger signal sensors [62]. We suggest a similar mechanism in skin mucus of gilthead seabream under overcrowding stress since NKEF showed almost 9.7 and 4.8-fold increase and also because recently, not only in gilthead seabream but also in European sea bass, it has been demonstrated *nkefa* and *nkefb* up-regulation after infection [63].

Last, LYZ (spot 15) was over-expressed in fish fed probiotic diet and/or under overcrowding stressed groups, compared to the control group, at 30 days (Table 3). In the present study, the theoretical and practical MW and *pI* converge with 20.3kDa and 6.9, respectively. LYZ, widely known in fish, is an enzyme which hydrolyses N-acetylmuramic acid and N-acetylglucosamine which are constituents of the peptidoglycan layer of bacterial cell walls [64]. For this reason, LYZ is a key molecule in skin mucosal immunity, conferring protection against pathogens. In agreement with our results it has been demonstrated that different stressors, including overcrowding stress, increase lysozyme levels in rainbow trout [65]. Furthermore, some probiotics such as *Bacillus* ssp. or *Lactobacillus* ssp. increase LYZ levels in fish (review in [66]). Our results demonstrated that dietary Pdp11 increases the LYZ expression in skin mucus of gilthead seabream even under overcrowding stress situation which could be considered very interesting results for fish farmers.

3.4 Gene expression profile

The analysis of four selected genes (*c3*, *nkefb*, *nccrp1* and *lyz*) in the skin of gilthead seabream was performed by qPCR (Fig. 2). Strikingly, neither *c3*, *nkefb* nor *nccrp1* gene expression was significantly regulated by probiotic and/or overcrowding stress and these results were not

correlated to the regulations observed at protein level. This could indicate differential half-lives for mRNA and proteins, regulations out of the transcriptional control for these proteins or that the mRNA studied and the proteins detected are not the same isoforms. Furthermore, the option that mucus proteins are not produced by epidermal cells can be also considered but needs further confirmation. Our results are in an agreement with a previous study which demonstrated that overcrowding stress did not modify *c3* mRNA levels in liver from gilthead seabream [67]. To our knowledge, there is no available information about probiotic or stress regulation of *nkefb* and *nccrp1*. Strikingly, *lyz* gene expression showed up-regulation in probiotic and/or overcrowding stressed groups compared to the control group, at 15 days (Figure 2D), although the highest increase of *lyz* gene expression was found in probiotic fed under non-stressed conditions, around 8-fold, compared to the control group. Present results suggest that Pdp11 probiotic diet may confer protection in skin mucosal immunity through an increase of lysozyme, not only at protein level in mucus but also at gene expression level in skin of gilthead seabream.

4. Conclusions

Functional and nutritional feedings such as probiotics are taking importance in aquaculture in order to prevent infections. The great interest of this study was to find out the molecules which are involved in the mechanism of protection at a mucosal level, as well as studying if Pdp11 is useful against overcrowding stress. Molecules such as F-type lectin, C3, LEI, NCCRP1 and LYZ, through over-expression by Pdp11 intake could be directly involved in mucosal protection, and consequently in fish well-being. Moreover, overcrowding stress had positive (such as in the case of C3 and LEI) or little negative impact on the skin mucus proteomic profile, which was even improved by probiotic feeding, suggesting that the skin mucus is not affected in a negative way by overcrowding. And last but not least, in the case of LYZ there is positive correlation between mRNA levels and protein expression in skin mucus suggesting a local synthesis of one of the most important antimicrobial protein. All these data together strongly reveal and support that dietary Pdp11 improves the gilthead seabream skin mucosal immunity.

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Figure legends

Figure 1. Representative 2-DE gels of skin mucus of *S. aurata* for each experimental group: commercial diet (A), probiotic diet (B), overcrowding stress (C) and overcrowding stress and probiotic diet (D). All the four gels were generated from samples at 30 days of treatment in triplicates. Skin mucus proteins were isoelectrically focused on 17 cm IPG strips (*pI* 3–10) and subjected to 12.5% SDS-PAGE. The 2DE gels were stained with SYPRO® Ruby protein gel stain and the spots identified in (A–D) were annotated using the data from LC-MS/MS. The spot numbers represented in gels correspond to the protein identities mentioned in Table 2.

Figure 2. Gene expression mRNA levels of four selected genes *c3* (A), *nkefb* (B), *lyz* (C) and *nccrp1* (D) in skin from *S. aurata* were analysed. Control groups are represented with red bars, probiotic diet groups with blue bars, overcrowding stressed group with green bars, and probiotic diet and overcrowding stressed group with orange bars. Transcripts were quantified by qPCR and normalised using the geometric average of the reference genes elongation factor 1 alpha (*ef1a*) and ribosomal protein S18 (*rps18*). The values are presented as mean ± SEM (n = 5). For the statistical analysis, ANOVA and Tukey's post-hoc test was performed in the case of *nkef* and *nccrp1* (Levene's test values were p=0.052 and p=0.232, respectively); for *c3* and *lyz* Kruskal-Wallis H test was performed (Levene's test values are p=0.000 in both genes). The asterisks indicate significant difference ($p<0.05$) in expression levels of each treatment group compared to the control group at the same time points.

Table 1. Information of primers used for qPCR study.

Gene symbol / Accession number	AS [a]	R^2 [b]	E (%) [c]	AT(°C) [d]	Sequence (5' → 3')
c3 CX734936	147	0.978	109	60	F: ATAGACAAAGCGGTGGCCTA R: GTGGGACCTCTCTGTGGAAA
nkefb GQ252680	99	0.998	96	60	F: CAAGCAGTAAATGTGAAGGTC R: GATTGGACGCCATGAGATAC
lyz AM749959	280	0.986	95	60	F: CCAGGGCTGGAAATCAACTA R: CCAACATCAACACCTGCAAC
nccrp1 AY651258	100	0.995	93	60	F: ACTTCCTGCACCGACTCAAG R: TAGGAGCTGGTTTTGGTTGG
ef1a AF184170	115	0.987	113	60	F: TGTCATCAAGGCTGTTGAGC R: GCACACTTCTTGTTGCTGGA
rps18 AM490061	109	0.937	117	60	F: CGAAAGCATTTGCCAAGAAT R: AGTTGGCACCGTTTATGGTC

a) Amplicon size (bp)
b) Pearson's coefficients of determination
c) Reaction efficiency (%).
d) Annealing temperature (°C) at 50mM Na+.

Table 2. Details of the differentially expressed protein spots in skin mucus of *S. aurata* after dietary probiotic administration and/or overcrowding stress.

SN[a)]	Protein name	Organism AN[b)]	pI/MW[c)]	S/C[d)]	M_p/U_p[e)]	Peptide sequence and e-value[f)]
S1	C-type lectin	*S. aurata* CB177017	7.2/28.8	59/3	1/1	**CFFMTPDK** ($4.9*10^{-2}$)
S2	Leukocyte elastase inhibitor	*S. aurata* FM146914	8.8/28.3	80/12	2/2	**ADAPYALSVANR** ($7.7*10^{-3}$) **DVQDDVHSSFAQLLGELNK** ($5.2*10^{2}$)
S3	Nonspecific cytotoxic cell receptor protein-1	*S. aurata* AAT66406	5.0/26.6	59/12	2/2	**DTPPPEPQLSDVPR** (3.2*10) **EVSYVFSGYGPGVR** (1.2*10)
S4	Apolipoprotein A1	*S. aurata* O42175	5.2/29.6	50/13	3/3	**IQANVEETK** ($1.2*10^{2}$) **TLLTPIYNDYK** (1.7*10) **AVNQLDDPQYAEFK** (3.0*10)
S5	Profilin	*S. aurata* FM146227	9.6/21.3	337/46	7/7	**EGGIWSASDMFK** (1.8) **GITPDEIK** (9.8*10) **ALYAGTEGPGNGSIVNLAGIK** ($1.7*10^{-4}$) **VITLVTMK** (1.3*10) **NTVMSESSPLVIGFFK** ($4.3*10^{-6}$) **TGLVIGLGKPGFR** (3.5*10) **SVGVTVESTTSQLK** (5.8)
S6	Inositol monophosphate	*Oreochromis mossambicus* AFY10067	5.3/31.2	284/30	7/1	**SSTVDLVTK** ($2.9*10^{-2}$) EEFGEGTHCFIGEESVAK ($1.8*10^{-4}$) EAGGILLDVDGGPFDLMSR ($1.5*10^{-3}$) IFSTMQK (5.2) IIIGSLKEEFGEGTHCFIGEESVAK($1.8*10^{2}$) ELEFGVVYSCLEDK ($1.3*10^{-2}$) SIIISEHGTDR (9.0)
S7	Beta actin	*O. mossambicus* P68143	5.3/42.1	176/15	5/5	**SYELPDGQVITIGNER** ($1.5*10^{-9}$) **QEYDESGPSIVHR** ($2.1*10^{-3}$) **EITALAPSTMK** ($3.4*10^{-4}$) **GYSFTTTAER** ($6.9*10^{-4}$) **DLTDYLMK** ($1.5*10^{-2}$)
S8	F-type lectin	*Oplegnathus fasciatus* BAK38714	5.7/31.0	144/8	2/2	**APTGENLALQGK** ($5*10^{-7}$) **IGDSLENNGNNNPR** ($1.4*10^{-2}$)
S9	Triose phosphate isomerase A	*Danio rerio* Q1MTI4	4.9/29.2	33/5	1/1	**GAFTGEISPAMIK** ($3.9*10^{-3}$)
S10	PREDICTED: aldose reductase-like	*Haplochromis burtoni* XP_005915666	6.2/36.0	201/13	6/5	**AAISAGYR** (4.6) **TILGFNR** (1.8) **TPAQVLIR** ($2.8*10^{-3}$) **AIGISNFNK** ($9.7*10^{-1}$) **KTPAQVLIR** (8.1) REDLFIVSK ($1.1*10^{-1}$)
S11	Peroxiredoxin 2	*Oncorhynchus mykiss* Q91191	7.0/22.3	46/5	1/1	**QITINDLPVGR** ($1.9*10^{-4}$)
S12	Complement c3	*S. aurata* ADM13620	8.1/187	138/2	5/5	**LPYSAVR** (2.8) **SVPFIIIPMK** (3.0*10) **DSSLNDGIMR** ($1.1*10^{-1}$) **VVPQGVLIK** ($3.5*10^{-1}$) **IVTLDPANK** (2.4*10)
S13	Complement c3	*S. aurata* ADM13620	8.1/187	110/1	3/3	**DSSLNDGIMR** ($2.3*10^{-2}$) **VVPQGVLIK** (5.0) **IVTLDPANK** ($1.1*10^{-1}$)
S14	Keratin, type I cytoskeletal 50 kDa	*Carassius auratus* Q90303	5.1/49.7	34/6	2/2	**SQMTGTVNVEVDAAPQEDLSR** (2.4) **ATMQNLNDR** ($3.0*10^{-3}$)
S15	Lysozyme	*S. aurata* CAO78618	6.9/20.3	217/31	4/4	**SDGLGYTGVK** ($1.3*10^{-2}$) **YGIDPAIIAAIISR** ($1.6*10^{-7}$) **GGIAAYNFGVK** ($2.8*10^{-1}$) **NVQTVAGVDVGTNHGDYSNDVVAR** ($9.1*10^{-1}$)
S16	ADP-ribosylation factor GTPase-activating protein	*Carassius auratus* AM930069	9.4/23.6	60/9	1/1	**GMDTAITKQISGADGGASR** ($2.0*10^{-2}$)
S17	Glutathione S-transferase	*S. aurata* AAQ56182	8.5/24.8	120/11	3/3	**LAAYYNR** (1.8) **MWEGYLQK** (8.0) **MFEGLTLQQK** ($4.5*10^{-4}$)
S18	NADP-dependent isocitrate	*S. aurata* AGU38793	7.2/38.1	79/10	3/3	**AGSVVEMQGDEMTR** (1.2) **ATDFVVPGPGK** ($5.2*10^{-1}$)

	dehydrogenase					**LIDDMVAQAMK** ($1.4*10^{-2}$)
S19	PREDICTED: peroxiredoxin-6-like	*Astyanax mexicanus* XP_007259536	5.8/24.8	59/4	1/1	**VIDSLQLTAKK** ($1.3*10^{-3}$)
S20	Glutathione S-transferase	*S. aurata* AFV39802	6.9/25.5	286/31	6/4	**FTGILGDFR** ($4.1*10^{-2}$) **MTEIPAVNR** ($3.4*10^{-2}$) **TVMEVFDIK** ($3.5*10^{-2}$) **LLSDGDLMFQQVPMVEIDGMK** ($2.6*10^{-1}$) AILNYIAEK (2.5) VLSGQIYLVGGK ($4.5*10^{-6}$)
S21	Beta actin	*Morone saxatilis* AAA53024	5.1/31.7	92/15	3/3	**VAPEEHPVLLTEAPLNPK** ($1.6*10^{-2}$) **GYSFTTTAER** ($3.3*10^{-1}$) **SYELPDGQVITIGNER** ($4.2*10^{-2}$)
S22	14-3-3 protein	*S. aurata* AM957903	4.6/26.9	114/9	2/2	**DSTLIMQLLR** ($6.7*10^{-1}$) **EVLGLLDDYLIPK** ($2.3*10^{-3}$)

a) Spot number.
b) Accession number according with NCBI and SwissProt databases.
c) Theoretical isoelectric point and molecular weight (kDa).
d) Total score and coverage (%)
e) Total matched peptides (Mp)/total unique peptides (Up).
f) Unique peptides are in bold. Expect value (e-value) is noted for each peptide sequence.

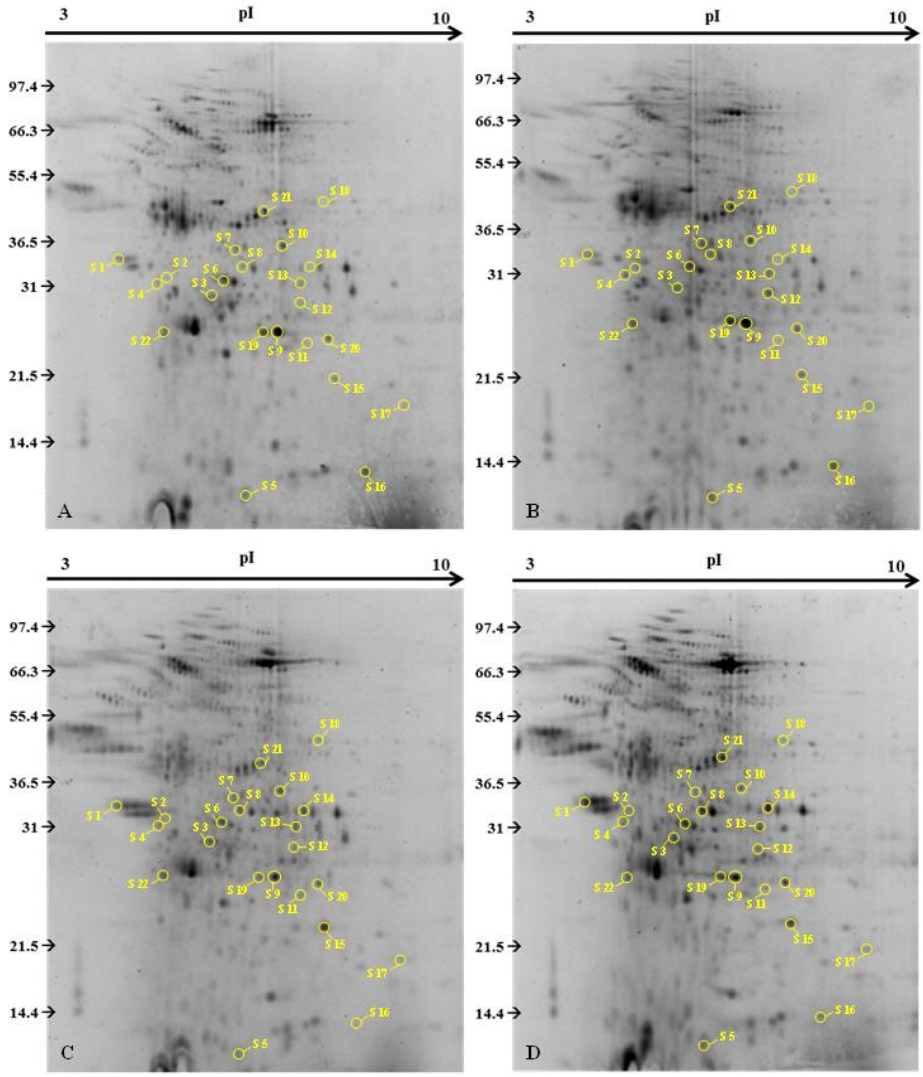
Table 3. List of proteins that are differentially expressed in skin mucus of *S. aurata* after dietary probiotic administration and/or overcrowding stress for 15 and 30 days. ↑ and ↓ indicate over- and under-expression of the proteins at $p<0.01$, respectively. Coefficient of variation (CV) in percentage (%) from different pools (n=3) is represented in brackets.

Spot	Protein name	Fold change relative to control group + CV intra-groups		
		Probiotic	Overcrowding	Probiotic + Overcrowding
Fifteen days				
S12	Complement c3 (C3)	↑ 1.95 (2.8%)		↑ 1.6 (3.9%)
S19	Peroxiredoxin 6-like (PRDX6)		↓ 0.63 (1.8%)	
S20	Glutathione S-transferase (GST)	↑ 1.97 (6.3%)		↑ 4.20 (3.3%)
S21	Beta-actin (ACTB)		↓ 0.56 (2.1%)	
S22	14-3-3 (YWHAB)	↑ 1.58 (5.2%)	↓ 0.55 (3.3%)	↓ 0.38 (1.7%)
Thirty days				
S1	C-type lectin (CLEC)		↑ 1.58 (3.6%)	↑ 2.63 (3.2%)
S2	Leucocyte elastase inhibitor (LEI)	↑ 2.18 (4.1%)	↓ 0.64 (1.4%)	↑ 2.36 (1.9%)
S3	Nonspecific cytotoxic cell receptor protein 1 (NCCRP-1)	↑ 3.27 (0.8%)	↑ 2.56 (5.8%)	↑ 2.05 (2.8%)
S4	Apolipoprotein A-1 (APOA1)	↑ 2.31 (1.6%)		↑ 1.67 (2.5%)
S5	Profilin (PFN)			↑ 1.85 (8.2%)
S6	Inositol monophosphate (IMPA)		↓ 0.39 (3.7%)	↓ 0.51 (4.9%)
S7	Actin beta (ACTB)	↑ 1.95 (7.2%)		
S8	F-type lectin (FBL)	↑ 1.54 (4.3%)	↑ 1.59 (2.1%)	↑ 2.79 (3.6%)
S9	Triose phosphate isomerase A (TPIA)		↓ 0.43 (1.9%)	↓ 0.57 (3.4%)
S10	Aldose reductase-like (AR)			↓ 0.41 (0.5%)
S11	Natural killer cell enhancing factor b (NKEF2)		↑ 9.69 (3.9%)	↑ 4.79 (5.6%)
S13	Complement c3 (C3)	↑ 1.74 (2.3%)	↑ 1.52 (3.5%)	↑ 1.64 (2.5%)
S14	Keratin, type I cytoskeletal 50 kDa (KRT1)		↑ 3.40 (5.0%)	↑ 4.31 (4.8%)
S15	Lysozyme (LYZ)	↑ 1.27 (6.1%)	↑ 4.58 (2.3%)	↑ 2.80 (1.8%)
S16	ADP-ribosylation factor GTPase-activating protein (ARFGAP)	↑ 1.39 (5.5%)	↓ 0.01 (2.7%)	↓ 0.14 (9.3%)
S17	Glutathione S-transferase (GST)		↑ 1.54 (3.1%)	↑ 1.58 (4.2%)
S18	NADP-dependent isocitrate dehydrogenase (IDH)		↑ 5.12 (4.4%)	↑ 5.20 (3.9%)

Table 4. List of differentially expressed proteins in the present study: literature-based comparison about presence of these proteins in skin mucus of other fish species after 2-DE spot detection.

Protein	Reported in skin mucus?	Fish species	Reference
14-3-3	Yes	*Dicentrarchus labrax*	[18]
		Gadus morhua	[34]
		Sparus aurata	[19]
ACTB	Yes	*D. labrax*	[18]
		G. morhua	[34]
		S. aurata	[19]
APOA1	Yes	*D. labrax*	[18]
		G. morhua	[34]
		S. aurata	[19,20]
		Salmo salar	[22]
AR	No	*S. aurata*	This study
ARFGAP	No	*S. aurata*	This study
C3	Yes	*D. labrax*	[18]
CLEC	Yes	*Anguilla japonica*	[55]
FBL	Yes	*D. labrax*	[18]
GST	Yes	*D. labrax*	[18]
		G. morhua	[34]
		S. aurata	[19]
		S. salar	[22]
IDH	No	*S. aurata*	This study
IMPA	Yes	*G. morhua*	[34]
		S. aurata	[20]
KRT1	Yes	*S. aurata*	[20]
LEI	Yes	*D. labrax*	[18]
		G. morhua	[34]
LYZ	Yes	*D. labrax*	[18]
		G. morhua	[34]
		S. aurata	[19]
NCCRP-1	No	*S. aurata*	This study
NKEF2	Yes	*S. aurata*	[19,20]
PFN	Yes	*D. labrax*	[18]
		S. aurata	[20]
PRDX6	Yes	*S. salar*	[22]
TPIA	Yes	*S. aurata*	[18]

Figure 1



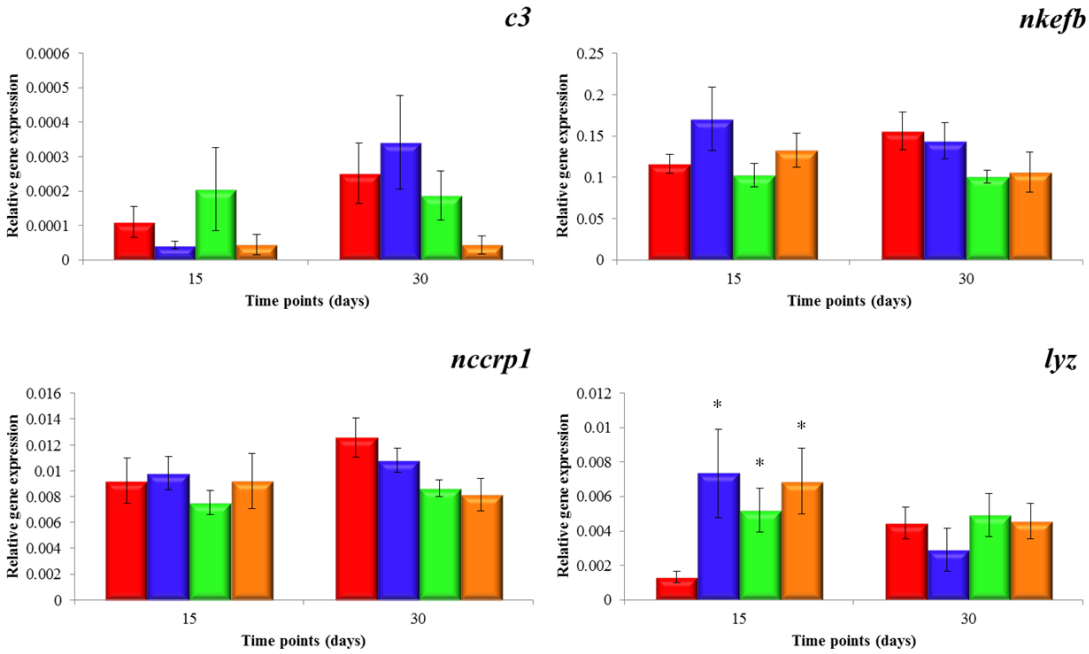
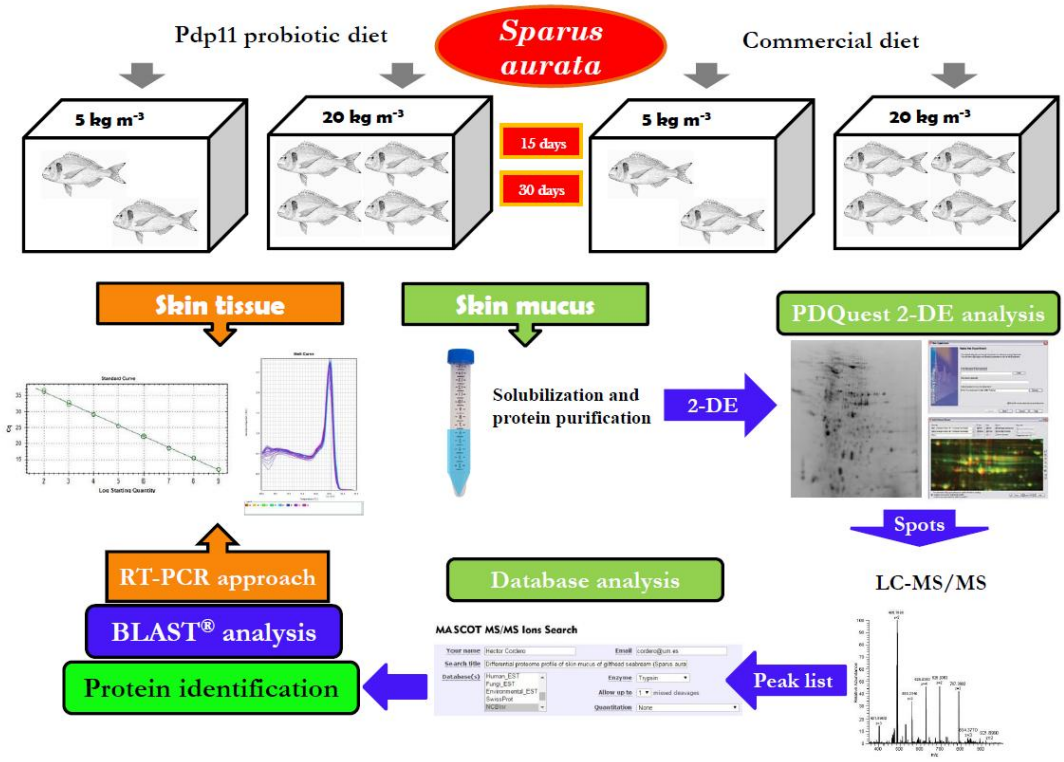


Figure 2



Graphical abstract

Highlights

-A comparative study based on 2-DE followed by LC-MS/MS was performed in skin mucus.

-Skin mucus proteome was altered by Pdp11 intake and/or overcrowding stress in *S. aurata*.

-Structural, metabolic, immune and stress proteins were identified.

-Pdp11 probiotic promotes the over-expression of most of the proteins in skin mucus.

-Lysozyme is up-regulated and over-expressed in skin and skin mucus, respectively.