

Label-Free Shotgun Proteomics Approach to Characterize Muscle Tissue from Farmed and Wild European Sea Bass (*Dicentrarchus labrax*)

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Abstract Sea bass represents one of the main fish products in the market. Most of it comes from farming and is bred in different conditions with respect to the wild fish. Differences may thus be expected. In this study, a proteomic profile of farmed and wild sea bass samples was performed, employing a fractionation strategy where peptide samples were first separated by 2D chromatography. The peptides were finally analyzed by shotgun proteomics workflow combined to tandem MS. The chosen fractionation approach was successful allowing to greatly improve the fish muscle protein characterization and detect some interesting differences between wild fish and farmed sea bass. Sixty-nine proteins were overexpressed in farmed fish samples, whereas 182 proteins were underexpressed. Some of these proteins could be related to the breeding conditions and the diet with which fishes were fed, thus providing some interesting results for assessing food quality based on a comprehensive proteomic study.

Keywords European sea bass · Shotgun proteomics · Label free quantification · Bidimensional chromatography · Muscle tissue protein

Introduction

Recently, aquaculture has become central in meeting the increased fish demand; according to the Organization for Economic Cooperation and Development (OECD) and the Food and Agriculture Organization (FAO), in 2022 the world of aquafarming will be increased by 35%, while traditional fishing only by 5%, due to population growth, rising incomes, and increasing urbanization (FAO 2016). During farming, fish species are subjected to a plethora of stimuli and environmental constraints such as different diets, feeding regimens, crowding, and different water qualities (temperature, salinity), with respect to wild fish species. The

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nutritional characterization of aquaculture products represents an important step toward a quality certification process of seafood that informs the consumer about the origin, handling practices, and processing history of the products, thus guaranteeing safety requirements and nutritional properties (Addis 2013). Under a nutritional perspective, studies on fatty acid and lipid compositions between wild and aqua-farmed individuals have been playing a major role in the past years leading to the publication of many papers in the literature, which highlight that omega-3 fatty acids were naturally more concentrated in wild fish, whereas their presence in farmed fish was lower in favor of omega-6 and oleic acid, as an evident consequence of food supplementation (Alasalvar et al. 2002; Costa et al. 2017).

In the last years, the study of protein content has also become particularly important for the characterization of food products and to assess food quality and safety (Sauer and Luge 2015; Capriotti et al. 2016). Proteomics can be exploited for seafood as well, in order to distinguish fish species, trace their origin, and compare the farmed product to the wild one. In the case of fish proteomics, studies were usually focused on muscle tissues to provide a global identification of the protein fingerprint, and may serve to identify novel proteins suitable as biomarkers about the different biological, physiological, and ecological aspects of aquaculture, which may be advantageous in optimizing quality and safety in aquatic species (fish physiology and growth, flesh quality, food safety, and aquatic environmental monitoring) (Addis et al. 2010; Tedesco et al. 2014).

Several economically relevant farmed species, such as Atlantic cod, Mediterranean sea bass, and gilthead sea bream, were characterized at the proteomic muscle level in order to provide useful information on the influence exerted by aquaculture, size, diet, crowding, postmortem processing, and other factors (Gebriel et al. 2010; Addis 2013; Piovesana et al. 2016a). In most of the above mentioned works, a gel-based strategy was usually employed as the approach for comparative proteomic analysis, whereby differences in protein abundances were determined by comparing stained protein spot volumes followed by identification of proteins by mass spectrometry (MS). However, this method is limited in sensitivity, has a low dynamic range, and is inefficient when analyzing proteins with very high or low molecular mass. Gel-free shotgun proteomics is a good alternative approach for the identification and quantification of proteins in large-scale studies (Domon 2006; Fercha et al. 2013).

For these reasons, in this work, a shotgun proteomics approach, based on an in-solution tryptic digestion followed by an off-line bidimensional liquid chromatography (2D-LC) coupled to label-free quantitative tandem mass spectrometry (MS), has been applied for the proteome characterization of sea bass muscle samples. Sea bass was chosen because it is one of the European and Italian aquaculture's main fish

species, which provides 92% of global production, corresponding to an economical income of EUR 304 million (2007) [http://ec.europa.eu/fisheries/marine_species/farmed_fish_and_shellfish/seabass_en]. To the best of our knowledge, this is the first time that a gel-free, label-free shotgun proteomic approach has been employed to investigate the change in the muscle proteins of sea bass in response to different life conditions. A qualitative and quantitative characterization of protein profiles obtained from farmed and wild sea bass was performed.

Materials and Methods

Fish Samples

Five specimens of farmed and wild sea bass (*Dicentrarchus labrax*) each, with the same developmental stage (weight range 274–452 g), were obtained from the sea of Syracuse (Mediterranean Sea). Farmed animals were furnished from Sicilian aquafarm “Acqua Azzurra di Pachino” located in the neighborhood of Syracuse (Sicily, Italy); they were bred in offshore cages and fed with flours and a mixture of vegetable and fish oils. The environmental data related to the breeding site were the following: pH = 8.36, salinity = 37.6 psu, temperature = 20.2 °C, average dissolved oxygen = 5.41 mg mL⁻¹, size of the offshore cage = 25 × 10 × 1 m. Wild fish were captured in the same geographical area. Animals were killed by euthanasia which consisted of a two-step process, with initial anesthesia to the point of loss of equilibrium, followed by a chemical method to cause brain death. For tissue sampling, a fragment of lateral muscle was collected from apparently healthy fish after skin removal. To reduce fish individual variability and obtain subsamples representative of the whole population, individual muscle tissue samples were pooled and homogenized, and finally stored at -80 °C until use. Organism handling and subsequent procedures complied with European laws (European Community Council Directive of November 24, 1986–86/609/EEC), and all the efforts were made to minimize possible animal suffering.

Fish Protein Extraction, Quantification, and Digestion

Fish muscle proteins were extracted by the freezing and grinding method (Piovesana et al. 2016b). Briefly, 1 g of sea bass muscle sample was ground to fine powder with mortar and pestle with the aid of liquid nitrogen. Then, 500 mg of the powder was treated with 10 mL of cold lysis buffer consisting of 8 mol L⁻¹ urea in 50 mmol L⁻¹ Tris-HCl (pH 8) and 0.1% (v/v) protease inhibitor cocktail (Sigma-Aldrich). The sample was vortexed for 10 min, then centrifuged at 9400×g at 4 °C for 10 min to sediment cell debris, and finally transferred into

a new tube. Protein concentration was determined by the Bradford assay (Sigma-Aldrich, St. Louis, MO, USA) following the instructions and using bovine serum albumin standard. The results are shown in Table 1.

Three experimental replicates for farmed and wild fish pooled samples were performed, resulting in a total of six protein extractions.

For digestion, 1000 µg protein aliquots was diluted to 1 µg µL⁻¹ with 8 mol L⁻¹ urea in Tris-HCl buffer, then treated with 200 mmol L⁻¹ dithiothreitol in Tris-HCl buffer and 200 mmol L⁻¹ iodoacetamide in Tris-HCl. Digestion was performed with trypsin (enzyme to protein ratio, 1:20), diluting the urea concentration to 1 mol L⁻¹ for tryptic overnight digestion. Digestion was quenched with trifluoroacetic acid (TFA), and then samples were desalted using solid phase extraction (SPE) C18 cartridges (Bond Elut 1cc LRC-C18, Varian, Palo Alto, CA, USA) conditioned with acetonitrile (ACN) and rinsed with 0.1% TFA. Peptides were eluted from the SPE column with 500 µL ACN/H₂O (50/50, v/v) containing 0.1% TFA, and were dried in a SpeedVac SC250 Express (Thermo Savant, Holbrook, NY, USA).

High-pH Reversed Phase Liquid Chromatography Fractionation

Samples were reconstituted in 200 µL of a solution consisting of 20 mmol L⁻¹ ammonium formate (pH 10). High-pH reversed phase liquid chromatography was performed using a polymeric reversed phase (PLRP-S) column (4.6 × 250 mm, 5 µm, Agilent Technologies, Santa Clara, CA, USA) coupled with a PLRP-S guard cartridge (5 × 3 mm, Agilent Technologies, Santa Clara, CA, USA), and the detector was set at a wavelength of 214 nm. The sample was eluted at 1 mL min⁻¹ by 20 mM ammonium formate (pH 10) as mobile phase A and ACN/20 mM ammonium formate (pH 10) (90/10, v/v) as mobile phase B. The gradient started from 100% A, while B was increased to 40% in 40 min; finally, B was increased to 100% in 3 min and kept for 4 min. Finally, B was brought back to 0% within 2 min. The column was re-equilibrated for 20 min.

Twenty-four fractions were collected from 2 to 60 min and pooled into 12 fractions, using the mixing strategy described in Fig. 1 (Fr1 + Fr1, Fr2 + Fr2, etc.).

Fractions were lyophilized with SpeedVac and stored at -80 °C prior to MS analysis.

LC-MS/MS Analysis

Peptide mixtures were separated by RP chromatography using the Dionex UltiMate 3000 (Dionex Corporation, Sunnyvale, CA, USA). The LC system was connected to a hybrid linear ion trap-Orbitrap mass spectrometer (Orbitrap Elite, Thermo Scientific, Bremen, Germany) by a nanoelectrospray ion source. Peptide mixtures were enriched on a 300 µm i.d. × 5 mm Acclaim PepMap C18 (5 µm particle size, 100 Å pore size) µ-precolumn (Dionex), employing a premixed mobile phase ddH₂O/ACN 98/2 (v/v) (from loading pump) containing 0.1% (v/v) HCOOH at a flow-rate of 10 µL min⁻¹. Then, peptide mixtures were separated with an EASY-Spray column (PepMap 100 C18 2 µm particle size, 100 Å pore size, 75 µm × 50 cm) at 200 nL min⁻¹. LC gradient was optimized to detect the largest set of peptides, using H₂O/HCOOH (99.9/0.1, v/v) as phase A and ACN/HCOOH (99.9/0.1, v/v) as phase B. After an isocratic step at 1% B for 5 min, B was linearly increased to 5% within 2 min and then to 35% within 90 min; afterwards, phase B was increased to 90% within 3 min and was maintained at 90% for 20 min to rinse the column. Finally, B was lowered to 1% over 1 min and the column re-equilibrated for 45 min (166 min total run time). Full-scan and MS/MS analyses of eluting peptides were performed in the *m/z* range of 380–1400 Da and 30,000 (full width half maximum at *m/z* 400) resolution for the full scan. A data-dependent mode acquisition was enabled, in top 10 mode, rejecting +1 and unassigned charge states, and fragmenting precursor ions by HCD at normalized collision energy of 35%, and an isolation window of 2 *m/z*. To minimize redundant spectral acquisitions, dynamic exclusion was enabled with a repeat count of 1 and a repeat duration of 30 s with exclusion duration of 70 s.

Database Search for Protein Identification and Quantification of Muscle Tissue

Data analysis was accomplished by using MaxQuant software (<http://www.coxdocs.org/doku.php?id=:maxquant:start> version 1.5.1.2) with Andromeda search engine (Cox and Mann 2008) against the UniProt database manually restricted to proteins of *Eupercaria* order (202,482 sequences, <http://www.uniprot.org/taxonomy/1489922>), also searching against the reverse sequences from the same database as generated by MaxQuant and a database of common contaminants.

Table 1 Quantification results from Bradford assay

Replicates	Farmed samples			Wild samples		
	1	2	3	1	2	3
	8.78 ± 0.38	8.72 ± 0.30	8.69 ± 0.29	5.70 ± 0.15	5.74 ± 0.10	5.75 ± 0.12

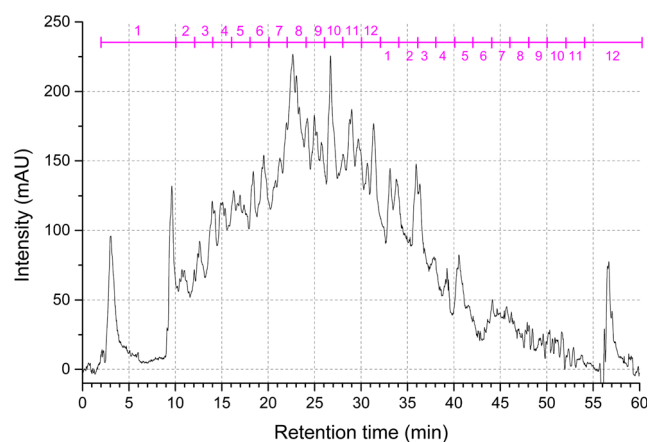


Fig. 1 Fractionation pooling strategy of sea bass sample tryptic hydrolysates

We set the digestion mode to fully tryptic and a maximum of two missed cleavages. Carbamidomethylation of cysteine (57.02146 Da) was set as a fixed modification, while oxidation of methionine (15.99491 Da) as well as acetylation of protein N-termini (42.01056 Da) were set as variable modifications. As no labeling was performed, multiplicity was set to 1. Candidate peptides were required to have a length of at least six amino acids. The fragment ion tolerance was set to 20 ppm for FTMS spectra. A first search with a precursor ion tolerance of 20 ppm was used to recalibrate raw data based on all peptide-spectrum matches (PSMs). After recalibration, the data were searched with a precursor ion tolerance of 6 ppm; the “Second peptides” option was enabled to identify co-fragmented peptide precursors. We used “Match between runs” with an alignment time window of 20 min and a match time window of 0.7 min to transfer identifications between raw files of the same and closed fractions (± 1 fraction). Using the classical target-decoy approach with a concatenated database of reversed peptide sequences, data were filtered using a PSM, peptide, and protein false discovery rate (FDR) of 1%. Protein groups were required to have at least one unique peptide. For quantification, we used unique and razor peptides. Relative label-free quantification (LFQ) of these proteins was carried out using the MaxLFQ algorithm (Cox et al. 2014) with fast-LFQ, and with the following parameters: LFQ minimum ratio count set to 1, and the LFQ average number of neighbors to 4.

Data Analysis

Data analysis was carried out using the freely available Perseus software (version 1.5.5.3). After loading the “proteingroups.txt” file produced by MaxQuant, hits to the reverse database, contaminants (like trypsin or Keratins), and proteins only identified with modified peptides were deleted. We used for statistical analysis only the LFQ intensities (the non-normalized intensities are reported in Electronic

Supplementary Table S1); values were base 2 logarithmized, samples were first grouped in triplicates, and identifications were filtered for proteins having at least two valid values in at least one replicate group, respectively. Missing values were substituted with a new distribution with a downshift of 1.8 and a width of 0.3 standard deviations. This kind of numbers simulate signals of low abundance proteins under the assumption that they are biased toward the detection limit of the MS measurement (Deeb et al. 2012). The total matrix thus created enabled statistical analysis. We created a volcano plot to show protein distribution and significance of differently expressed proteins; moreover, a gene ontology (GO) analysis was performed using STRAP (Bhatia et al. 2009).

Results and Discussions

To establish the relationship between seafood quality and safety and the breeding conditions adopted for fish, a large-scale study for the characterization of the protein expression profile of muscle tissue in farmed sea bass was performed and results compared to wild individuals caught in the same area of the Mediterranean Sea. To this purpose, 10 pooled and homogenized muscle tissue samples (5 farmed and 5 wild) were examined. A previous study performed on farmed sea bass using electrophoresis and MS revealed some differences in muscle protein expression, with nine proteins differently expressed, among wild and farmed individuals (Monti et al. 2005). Despite this, however, the gel-based strategy does not allow a global quantification of the entire proteome of fish muscle. In confirmation of this, in our previous work, where a shotgun proteomics method was developed and then applied to the characterization of gilthead sea bream edible muscle, 155 differently expressed proteins were identified (Piovesana et al. 2016a).

In view of this data, the analytical strategy chosen for this proteomic investigation was a gel-free shotgun analysis by 2D-LC coupled to high-resolution MS/MS acquisition. In fact, this type of approach is robust and allows an increased high throughput with respect to gel-based methods; besides, the upstream fractionation greatly extends the dynamic range of investigated proteins, leading to a larger number of identified and quantified proteins.

Typically, in proteomic experiments the final fractionation step used for peptide separation is nano-flow LC using low pH C18 RP chromatography. There are several first-dimension methods that are commonly combined with RP to further increase peptide separation and identification efficiency.

It is well known that peak capacity can be greatly enhanced in 2D-LC. Indeed, from a theoretical point of view, it has been demonstrated that the maximum peak capacity of such a system is given by the product between the peak capacities of each separation dimension. What is certain is that, irrespective

of the mode of operation of the system, in order to reach the theoretical maximum peak capacity, the two dimensions of the 2D-LC system must be truly orthogonal, i.e., they have to be based on two completely different separation mechanisms (D'Attoma et al. 2012).

Recently, multidimensional chromatography using a combination of high pH RP and low pH RP chromatography was very promising for its high degree of orthogonality, since the pH change between the two fractionation methods causes the hydrophobicity of certain amino acids to change, resulting in a shift of peptide retention times in each dimension (Stein et al. 2013). For these reasons, high pH RP chromatography and low pH RP chromatography were coupled off-line for 2D separations of muscle fish peptides to improve the dynamic range of the analysis and increase the number of protein identifications.

The experiments were carried out in triplicate, and for each sample, the 24 collected fractions were pooled into 12 to reduce the hydrophobicity overlap (Fig. 1). Each collected fraction was injected in the nano-HPLC-MS/MS system for a total of 36 technical replicates for farmed and wild samples, each.

The total number of peptides identified in every collected fraction from the 2D separation was used as the prime indicator of separation efficiency. As an example, the number of identified peptides for each fraction of the first experimental replicate for the farmed sample is reported in Fig. 2a and in Electronic Supplementary Table S1. From such overview, it was possible to assess that the employed first dimension fractionation was generally successful, as all fractions provide between 1700 and 2700 peptides. To exclude excessive overlapping of eluting peptides during fractionation and to evaluate the efficiency of the separation of the first dimension, the distribution of peptides identified across the 12 fractions was considered. Most of the peptides, exactly 70%, were found in only one fraction. The number of peptides found in two or more fractions was also generally very low, with a percentage lower than 16% (Fig. 2b and data reported in Electronic Supplementary Table S1). Other factors were evaluated, namely, the intensity and retention time distribution of peptides in every collected fraction; as shown in Fig. 2c, peptide intensity was around 10^6 in every collected fraction, further supporting that the fractionation approach was successful. Finally, Fig. 2d shows the retention time distribution, from which it was possible to see as 50% of identified peptides were eluted between 40 and 80 min.

The entire experiment, taking into consideration both types of samples (farmed and wild), provided a total of 1,850,829 MS/MS spectra, permitting the identification of 12,536 peptides with a 0.01 FDR and 2301 protein groups. Among them, 147 protein groups were found in only one biological replicate. Ruling them out, the number of protein identifications was reduced to 2154 (Electronic Supplementary Table S1). In each experiment, an average number of about 2000 protein

groups were identified, and 1639 of them (76.1% of the total) were shared across all six experiments. The complete list of identified proteins and quantitative values provided by MaxQuant are reported in Electronic Supplementary Table 1. We decided to accept protein identifications also with a single unique peptide, after a manual spectrum check, to provide a more comprehensive protein profile description. This is a common issue in proteomic studies of non-sequenced organisms, because protein assignment is performed for the most part by homology against databases which comprise protein sequences for several species (Forné et al. 2010; Capriotti et al. 2013a, b). However, even a single unique peptide may identify proteins with a high score and can be used for protein identification.

To evaluate the reproducibility of the LFQ between biological replicates, Pearson correlation was calculated on a scatter plot of filtered intensities. The LFQ between biological replicates for each state was highly reproducible as highlighted by a Pearson correlation, ranging between 0.92 and 0.95, which demonstrates the reliable degree of precision of the strategy used for sea bass investigation (Fig. 3 and Supplementary Table S1).

To test the statistical significance of protein differences, the missing values were imputed (Electronic Supplementary Table S1) and a volcano plot was obtained, showing that 251 proteins were significantly (p value < 0.05) changed in abundance (Fig. 4 and Electronic Supplementary Table S2). A p value threshold of 0.05 ($-\log[p \text{ value}] = 1.301$) and a $\log_2$ ratio higher than +1, or lower than -1, were chosen: this second threshold indicated a protein twofold change, chosen to define proteins with higher or lower abundance, respectively. Using this stringent statistical criterion, a total of 69 and 182 proteins were significantly overexpressed and underexpressed in farmed samples, respectively.

For a better understanding of the biological context of differently regulated proteins, GO analysis was used to get additional insight into the biological significance. GO annotation of differently expressed proteins was performed by STRAP (Bhatia et al. 2009) and the results reported for biological processes, molecular function, and cellular components (Fig. 5 and Supplementary Table 2).

For the group of biological processes, the categories with the most abundantly overexpressed proteins in farmed sea bass samples were the cellular and developmental processes, with 41 and 11%, respectively (Fig. 5a); with respect to their molecular function, most overexpressed proteins belonged to the category of catalytic activity (34%), binding (37%), and structural molecule activity (15%, Fig. 5b).

As far as the underexpressed proteins are concerned, the main biological process categories were regulation (23%), cellular process (37%), and metabolic process (10%, Fig. 5a). As before, the mainly represented molecular functions were catalytic activity (35%) and binding (50% Fig. 5b). For

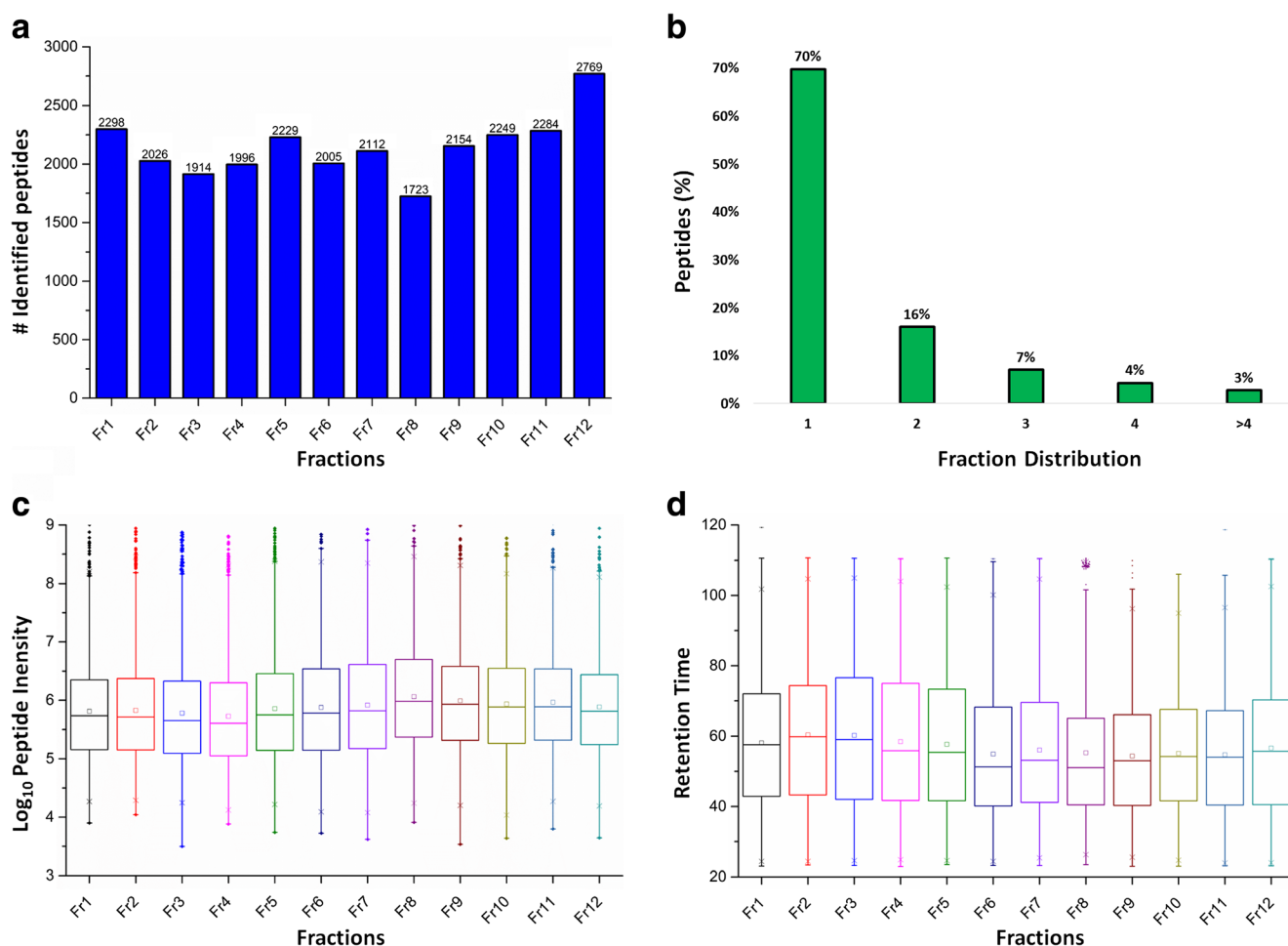


Fig. 2 **a** Number of peptides identified in every first dimension fraction. **b** Histogram showing fractionation efficiency. **c** Boxplot showing the distribution of peptide intensity in each collected fraction. **d** Boxplot

showing the distribution of the retention time of peptides identified in each collected fraction

the cellular component, most of these proteins belonged to cytoplasm, cytoskeleton, nucleus, macromolecular complex, and mitochondria, and all these proteins were found underexpressed in farmed samples (Fig. 5c).

The obtained GO categories and type of identified proteins were in line compared to previous works (Monti et al. 2005; Addis et al. 2010; Terova et al. 2014), but we found a larger number of differently expressed proteins due to the use of a 2D-LC fractionation approach coupled to a high resolving power LTQ Orbitrap elite mass spectrometer.

A deep and complete characterization of muscle proteome is crucial to highlight many aspects of fish aquaculture, growth, diet, food safety, seafood authentication and quality, traceability, and shelf-life. Monti et al. (2005) evaluated the impact of farming on sea bass fish samples, carrying out a proteomic approach based on a protein separation with SDS-PAGE followed by LC-MS/MS analysis; their results showed a significant alteration of expression of just nine proteins. Also, Addis et al. studied the proteome profile of sea bass muscle samples by two-

dimensional gel electrophoresis and tandem mass spectrometry, identifying a total of 49 protein spots (Terova et al. 2014). Both studies could detect a smaller number of differently expressed proteins with respect to our 251 identified proteins. In particular, several interesting variations in protein abundance were especially detected in structural proteins and glycolytic enzymes. The overexpression or underexpression of structural proteins, such as myosin-6, titin, tropomyosin, collagen, tubulin, caveolin, musculoskeletal embryonic nuclear protein 1, cytoskeletal protein, myosin light chain C, tropomyosin alpha-4 chain, and troponin T, are related to dietary feed (data are shown in Fig. 4). In fact, tropomyosin was found to be increased in abundance in the β -glucan-treated rainbow trout, while five spots that were downregulated with dietary β -glucan supplementation were identified as different forms of myosin: myosin light polypeptide, myosin light chain 1, fast myosin light chain 2, and myosin heavy chain (Ghaedi et al. 2016). Higher expression or lower expression of the abovementioned proteins may suggest that farmed sea bass

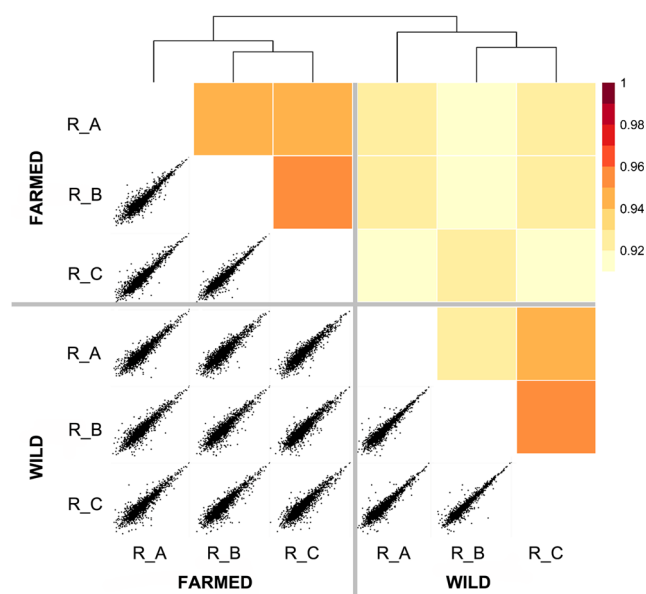


Fig. 3 Comparison of LFQ quantification of proteins between farmed and wild biological replicates (reported as R_A, R_B and R_C); the heatmap shows the Pearson correlations and correlation plot for different replicates (for interpretation of the color references in this figure legend, the reader is referred to the web version of this article)

were fed with feed containing β -glucan which regulates the muscle growth of fish through an intermediate pattern

of hyperplasia and hypertrophy. Consistent with these findings, growth enhancement of fish species through changes in muscle structural proteins has been reported in other feeding experiments (Tahmasebi-Kohyani et al. 2012; Alami-Durante et al. 2014; Ghaedi et al. 2016).

Another interesting example of the effect of diet on farmed sea bass species is represented by the overexpression of tubulin beta chain in the farmed samples, a protein that was already found upregulated in lean salmon when their diet was based on vegetable oil (Morais et al. 2012).

Proteins found significantly downregulated in farmed species are likely implicated in protection from oxidative stress (hemopexin-like protein and peroxiredoxin-1), involved in signaling (2-peptidylprolyl isomerase A, PPIA) and actin-based motility (myosin light chain, MYL). This latest data was in contrast with the results obtained for two genetically characterized groups of Atlantic salmon (*Salmo salar*) (Morais et al. 2012). The feed given to the farmed species analyzed in this study could be a possible explanation of the above observation, since it consisted of a mixture of fish oil and vegetable oil.

Malic enzyme and ribosomal protein families (L5, L14, L27, and S27) were found overexpressed in farmed species. Such result can be related to the type of diet of farmed fishes. In fact, a percentage superior to 4% of dietary conjugated linoleic acid affects protein expression increasing malic

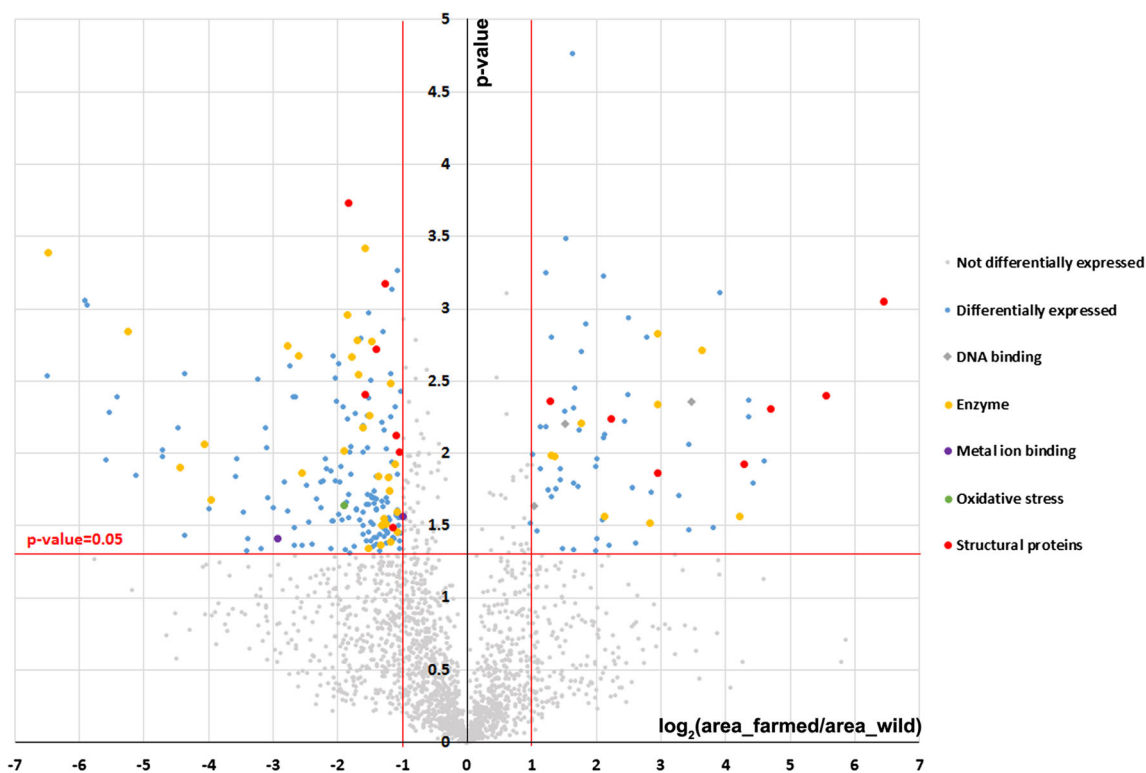


Fig. 4 Volcano plot showing protein distribution and significance; light gray dots: proteins identified with no significant difference; blue dots: proteins which showed a different expression; dark gray circles: DNA-

binding proteins; yellow dots: metabolic enzyme proteins; purple dots: metal ion binding; green dots: oxidative stress; red dots: structural proteins (color figure online)

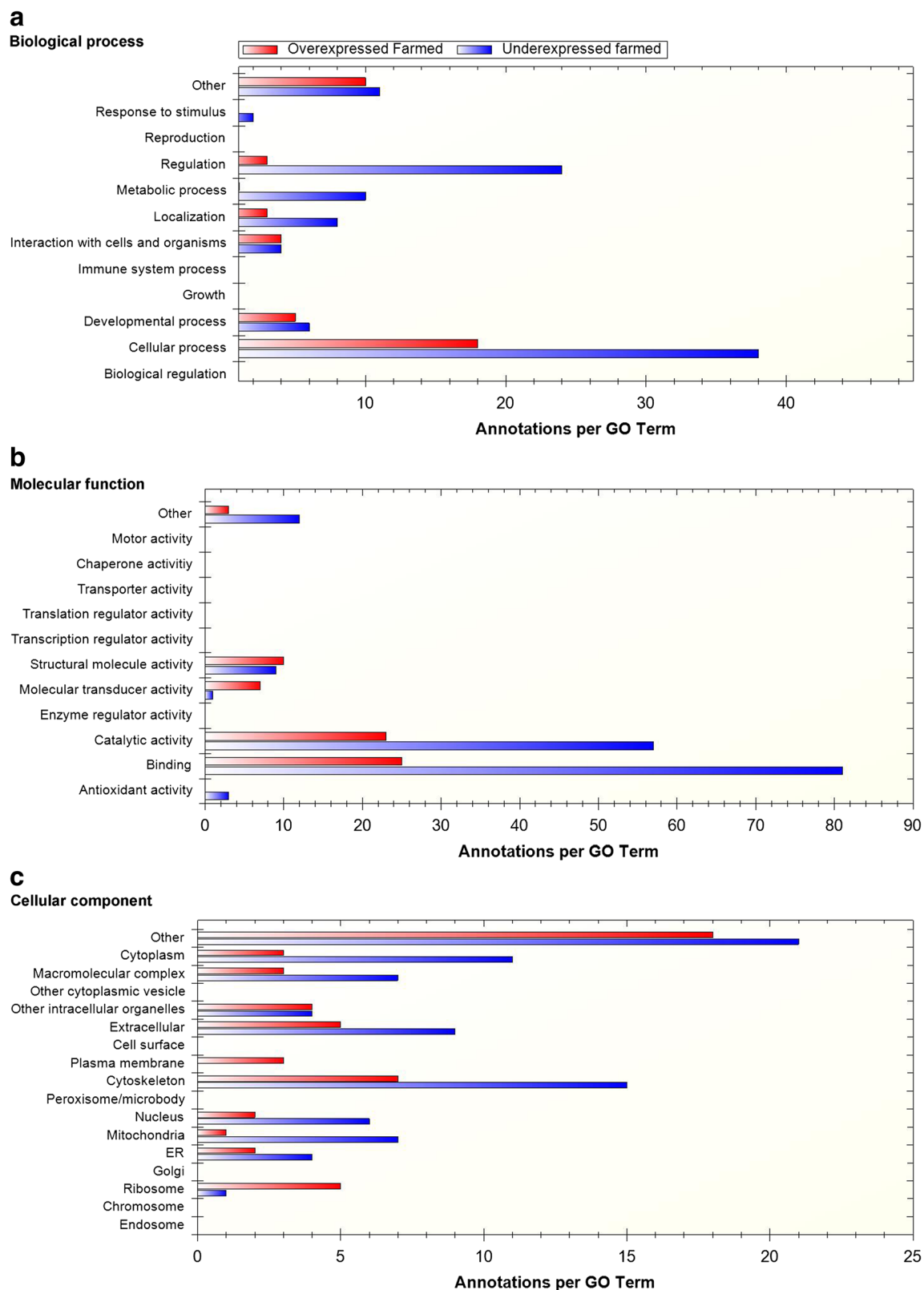


Fig. 5 Gene ontology characterization of the proteins which resulted in overexpression (*red*) and underexpression (*blue*) of farmed fish samples (color figure online)

enzyme concentration (Diez et al. 2007), as well as the ribosomal protein family, which was found overexpressed when

fishes were fed with a plant protein replacement rather than a high marine protein diet (Tacchi et al. 2012).

Other interesting variations in protein expression were detected both in individual proteins, such as parvalbumin, and in proteins related to environmental change or acclimation.

Parvalbumin, for example, is a small calcium-binding protein already recognized as one of the major fish allergens detected in most fish species (Kuehn et al. 2014). In the present work, the protein expression in sea bass farmed species decreased, a trend which agreed to a previous work performed on the same fish (Monti et al. 2005) but did not agree to our previous work on gilthead sea bream, since the protein was found upregulated (Piovesana et al. 2016a). The different trend could be ascribed to fish development (Focant et al. 2003). In the common carp, for example, muscle from smaller fish exhibits higher level of parvalbumin and has shorter relaxation time (Brownridge et al. 2009). We postulated that changes in parvalbumin expression are an important part of the adaptive change in muscle mechanical properties in response to varying functional demands and environmental change. This hypothesis was also supported by a study on two different fish species showing that parvalbumin levels are 20 times higher in cod than in swordfish, thus confirming that different protein expression is correlated to fish size (Griesmeier et al. 2010).

Hemopexin is the mammalian homolog of WP65, an acclimation-related protein, whose expression depends on water temperature of offshore cages. Our results were in line with a study in which the level of expression was lowest in fish farmed at an average temperature of 20 °C, namely, the same temperature at which our samples were bred (Kikuchi et al. 1995; Addis et al. 2010).

Another interesting protein family is made by metabolic enzymes, such as aldolase, phosphofructokinase, phosphoglycerate mutase, pyrophosphatase, nucleoside diphosphate kinase, methyltransferase family, and glutathione S-transferase; these metabolic proteins were all differentially expressed and are reported in yellow in Fig. 4. There was not a specific trend but rather a variability in expression levels that was also documented by other authors in association with development and exercise in zebrafish and trout (Bosworth et al. 2005; Hamelin et al. 2006; Morzel et al. 2006; Reddish et al. 2008). In particular, proteins showing changes in expression levels were related to the nucleotide and small molecule metabolism, including nucleoside-diphosphate kinase, elongation factor 1- α , and ribosomal S12 subunit, all undergoing decreases and indicating a general decrease in protein synthesis.

In summary, our results showed several differences in muscle proteome between farmed and wild species, highlighting that farming could lead to muscle atrophy.

Conclusion

In this study, a comprehensive characterization of the farmed sea bass muscle proteome was performed and results compared to

the wild sea bass. In order to maximize protein identification, a 2D-LC fractionation approach of peptides recovered from the tryptic digestion of muscle proteins was chosen. The coupling of an upstream high pH RP fractionation of peptides to the conventional low pH RP directly coupled to high-resolution tandem MS proved a successful approach. Peptides were evenly distributed among the collected fractions, with 70% of them isolated in a single fraction. The study provided 69 upregulated proteins and 182 downregulated proteins in farmed samples, showing some interesting variations related to the breeding conditions and diet.

The detailed characterization accomplished in this study may now set the ground for studies aimed at developing novel molecular methods and protocols for seafood authentication, monitoring of growth dynamics, traceability, and quality of maricultured sea bass.

Raw Data Repository

The MS proteomics data have been deposited to the ProteomeXchange Consortium (Vizcaino et al. 2016) via the PRIDE partner repository with the dataset identifier PXD006029.

Compliance with Ethical Standards

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Conflict of Interest Riccardo Zenezini Chiozzi declares that he has no conflict of interest. Anna Laura Capriotti declares that she has no conflict of interest. Chiara Cavaliere declares that she has no conflict of interest. Giorgia La Barbera declares that she has no conflict of interest. Carmela Maria Montone declares that she has no conflict of interest. Susy Piovesana declares that she has no conflict of interest. Aldo Laganà declares that he has no conflict of interest.

Ethical Approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

Informed Consent Not applicable.

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