Proteomic evaluation of potenciated sulfa treatment on gilthead sea bream (*Sparus aurata* L.) liver

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Abstract

Potentiated sulfa drugs are a combination of sulfonamides and pyrimidine potentiators, such as trimethoprim. They are currently used against bacterial pathogens in Mediterranean marine fish farming, including gilthead sea bream (*Sparus aurata*). The present work aimed studying the potential hepatotoxicity of a combination of sulfadiazine (SDZ) and trimethoprim (TMP) in gilthead sea bream juveniles after oral administration, at the recommended ratio of 5:1 (SDZ/TMP), for 10 days. Difference Gel Electrophoresis Technology (DIGE) coupled with MS was used to identity possible markers of hepatotoxicity of this treatment. The results obtained show significant changes in the expression of 41 proteins by treatment ($p \leq 0.02$). Among these proteins, 14 increased in abundance, and 27 decreased respect to the control group. Spots showing differential expression respect to the control were identified by PMF and/or LC-MS/MS and database research. Proteins like apolipoprotein A-I and fatty acid binding protein (lipid metabolism and transport, and antioxidant role), phosphogucomutase 1 (carbohydrate metabolism), elongation factor 1-alpha (protein biosynthesis and antioxidant role), mitochondrial aldehyde dehydrogenase (oxidation regulation activity and antioxidant role), ypbc-32-D06 (aminotransferases), were differentially expressed in treated fish. These proteins have not been associated before to potentiated sulfa effect; however, they are frequently found expressed differentially as a characteristic cellular/tissue stress response under different experimental conditions, making difficult their use as specific biomarkers for this treatment.

1. Introduction

It is known that gilthead sea bream (*Sparus aurata*) is an economically very valuable fish species in the Mediterranean. Bacterial outbreak is the major cause of fish diseases and mortalities in Mediterranean cage fish farming (Rigos and Troisi 2005; Rigos and Katharios 2009), causing important losses. The main species causing important bacterial infections in gilthead sea bream are *Vibrio anguillarum* serotype 1b, and *Photobacterrium damsela* subsp. *piscida* (formerly *Pausterella*). Disease outbreaks in aquaculture are normally confronted with mass therapy, usually orally administered via incorporation of drugs into the feed. Antibacterial agents such as sulfonamides have been used to treat and control bacterial diseases in cultured fish for decades, essentially because of their low cost and efficacy in common infections. Sulfonamides mainly represented by sulfadiazine (SDZ) are a large range of structurally related synthetic compounds that are derivatives of sulphanilamide. Nowadays, sulfonamides are used in combination with pyrimidine potentiators or diaminopyrimidines such as trimethoprim (TMP) or ormetoprim (OMP), and are frequently called potentiated sulfonamides or, simply, potentiated sulfa. They have a broad spectrum of bacterial activity, and their combined efficacy is greater that the sum of the two separate drugs. This is because the sulfonamides are structural analogues and competitive antagonists of para-aminobenzoic acid (PABA). As such, they block normal bacterial use of PABA for the synthesis of dihydrofolic acid (folic acid), and the particular pyrimidine potentiators (TMP or OMP) used in combination, inhibit the next enzyme in the sequence (dihydrofolate reductase) to prevent the formation of tetrahydrofolic acid (folinic acid), which is required for the synthesis of DNA. This two inhibitory actions in the same pathway produce a bactericidal rather than bacteriostatic effect. Folic acid is not synthetized in fish, but it is instead a dietary requirement. This allows for selective toxicity to bacterial cells over fish cells.

Currently, potentiated sulfa are regularly used against bacterial infections in Mediterranean euryhaline fish farming (Rigos and Troisi, 2005), and the combination sulfadiazine and trimethoprim (SDZ/TMP) at a ratio of 5:1 is one of the most used treatments in Mediterranean aquaculture, although there are not specific data on the pharmacokinetic properties of sulfadiazine and trimethoprim. The recommended dose in fish is 25 and 5 mg kg^{-1} fish for SDZ and TMP respectively, for 5 -10 days presented on medicated food (Scott 1993; EMEA 1997).

Previous studies on fish have shown that potentiated sulfa is rapidly absorbed and distributed throughout body fish tissues after oral administration, although there are differences in the comportment of the two antibiotics (Horsberg et al. 1997; Samuelsen et al. 1997; Kosoff et al. 2007). In Atlantic salmon *(Salmo salar)* held in seawater, the pharmacokinetics of SDZ/TMP after oral administration is fitted to one-compartment model. TMP is absorbed from the intestine quickly and completely and the peak plasma concentration (Tmax) is reached in 12h, whereas SDZ is absorbed slowly and the Tmax is found 24h post administration, with a bioavailability of 100% for TMP and 46% for SDZ (Hormazabal and Rogstad 1992; Horsberg et al. 1997). TMP concentrations are higher in tissues that in plasma, while SDZ is found at higher concentration in plasma for much longer time, and it is distributed slowly and uniformly through the tissues and body fluids. Both antibiotics have been shown to accumulate in tissues such as plasma, muscle, liver, skin and the uveal track of the eye, and TMP has also been shown to bind to tissues containing melanin (Bergsjo et al. 1979; Hormazabal and Rogstad 1992). The accumulation of other diaminopyrimides (such as OMP) in fish has been related to its binding to specific proteins including probably those involved in haematopoiesis, that are located in head kidney and spleen; and OMP and/or metabolites have been detected in all tissues relative to plasma, mainly in the excretory tissues (i.e. liver and trunk kidney), head kidney, spleen, muscle and skin (Pakas et al. (1990). In fish sulfonamides are metabolized primarily in the liver, but metabolism also occurs in other tissues, and acetylation and glucuronidation are the major pathways. Biliary excretion is the main route of elimination of both drugs, although renal excretion has been also reported in fish (Bergsjø et al., 1979).

In general the toxicity related with sulfonamides in both human and animals, is crystalluria (renal toxicity); however, the lower doses of sufonamide used in the potentiated sulfa combinations makes nephrotoxicity less probable to occur than with sulphonamide administrated alone (The United States Pharmacological Convention, INC, 2007). Although there are not many reports on the specific toxic side effects of potentiated sulfa in fish, different immunomodulatory effects have been associated with the use of this antibacterial drugs. Enhanced leucocyte proliferation *in vitro* but not *in vivo* has been shown in rainbow trout (*Oncorhynchus mykiss*) after treatment with different sulfonamides combined with TMT (Grondel et al. 1986; Lunden and Bylund 2002). Saglam and Yonar (2009) found decreases in haematological (haematocrit and leucocrit values, and haemoglobin), and immunological parameters (total plasma protein and total Ig levels) in the same fish species treated with sulfamerazine. This study also showed that sulfamerazine had an immunosuppressive effect on fish because of a decrease in NBT(+) cell levels. By contrast, Lunden and Bylund (2000)

did not find neither negative effect on the immune system of rainbow trout after oral administration of SDZ/TMP in a ratio 5:1, nor in the survival. Yildiz and Altunay (2011) reported the effect of sulfamethoxazole and TMP on the physiologial stress and immunological response of gilthead sea bream and sea bass *(Dicentrarchus labrax)*. This study concluded that the treatment produces the activation of the classical acute stress response with a transient increase in plasma cortisol and glucose after treatment, and a decreased during the recovery period, for both species. Regarding to innate immune markers measured (haematocrit, lysozyme activity, C-reactive protein and ceruloplasmin), the authors concluded that it is difficult to consider the inmunosuppression in gilthead sea bream and sea bass.

There are not previous studies about the capacity of the SDZ/TMP to produce liver injury at molecular level in cultured euryhaline fish after a routinely antibacterial treatment. At present, proteomic technology approach has the potential to find the protein alterations indicative of the mode of action of chemicals without previous knowledge of their mode of action, and it may be useful to discover new biomarkers of hepatotoxicity in animals, including fish (Varó et al., 2010). Thus, the present study aimed at testing the potential hepatotoxicity effects of SDZ/TMP on gilthead sea bream juveniles after oral administration at the recommended ratio of 5:1 for 10 days. The liver was investigated as the principal target of toxicity due to the role of this organ in energetic and xenobiotic metabolism of drugs, such as SDZ/TMP. To this end the effect of this treatment was evaluated thought a proteomic approach, using 2-D differential gel electrophoresis (2D-DIGE), and proteins of interest were identified by mass spectrometry analysis and database search**.** Unveiling of the liver protein pattern could give useful insight to detect early hepatotoxicity effects at molecular level in response to a routinely (standard) SDZ/TMP treatment used in the control of bacterial fish diseases. The proteomic results were confirmed by real-time RT-PCR of selected proteins, and the study was also complemented with the histological analysis of liver.

2. Materials and Methods

2.1 Animals and sample preparation

Juveniles of gilthead seabream (*S. aurata*) (35±9 g) were purchased from a local commercial aquaculture farm (Acuicola Marina SL, Burriana, Castellón, Spain), and kept in the facilities of the aquarium plant of the University of Valencia. Fish were placed in several 2000 L fibreglass tanks (120 fish per tank) filled with seawater (32

‰), supplied with continuous aeration under room temperature (19 ± 0.5 ºC) in a closed circuit, and acclimated for 1 week before starting the experiment.

After acclimation, the test fish groups were fed medicated dry food pellet with SDZ/TMP at the recommended concentration ratio of 5:1, equivalent to a dose of 30 mg Kg^{-1} fish d^{-1}, for 10 days (daily feeding ratio 1% body weight). The control fish groups were administered unmedicated pelleted feed at the same feeding schedule. The experimental feeds were made at the Fish Nutrition and Pathology Laboratory, Institute of Aquaculture, of the Hellenic Center for Marine Research in Athens (Greece). Commercial feed (Biomar) with the following composition: fish meal (35%), soya bean meal (20%), wheat meal (15%), rapeseed meal (2.7%), wheat gluten (5%), corn gluten (10%), fish oil (13%), premix (0,3%), were grounded, mixed with the drug, and prepared as dry pellets suitable for the size of the fish. The same basic procedure was followed for the preparation of the unmedicated diet.

At the end of treatment, eight fish from each experimental group were anaesthetized with clove oil (20mg L^{-1}) and sacrificed before livers being dissected out. The samples were quickly frozen in liquid nitrogen and stored at -80º C until analysed. Handling of the fish was done according to national and institutional regulations for animal experimentation.

2.2 2D difference gel electrophoresis (2D-DIGE): protein sample preparation and protein labelling

Liver tissues used in the 2D-DIGE analyses were processed as previously described (Varó et al., 2010). Briefly, individual livers were homogenized with the aid of the grinding kit system (General Electric Healthcare) in nine volumes of DIGE buffer (7 M urea, 2 M thiourea, 4% CHAPS, 30 mM Tris) and 100 μL mL^{-1} of the Complete Mini (Roche) as protease inhibitor. The solubilized proteins were separated from non-solubilized cellular components by centrifugation (20,000 g x 20 min). Proteins present in the supernatants were precipitated using the 2D Clean-up kit (GE Healthcare, Uppsala, Sweden) according to the manufacturer's instructions and resolublized in DIGE buffer. The pH of protein extract was adjusted to 8.5 by adding 50 mM NaOH, and protein concentration was determined using the Bradford Biorad Protein Assay (RcDc kit) with BSA (bovine serum albumin) as standard.

Liver proteins extracted from control (n=8) and treated (n=8) fishes were randomly labelled with Cy3 or Cy5. For DIGE minimal labelling, 50 μg of protein sample was mixed with 400 pmol CyDye (GE healthcare) by vortexing and incubated on ice in the dark for 30 min. The labelling reaction was stopped by the addition of 1 μL 10 mM

lysine followed by incubation on ice for a further 10 min. The internal standard sample was prepared by pooling 25 µg of protein from each liver sample studied, and by labelling by Cy2 as described above. Combinations of a Cy3 and a Cy5 labelled sample were then mixed with Cy2-labeled internal standard, and DTT (65 mM final concentration) and ampholytes (1% final concentration, pH = 3-10) were added to the mixture before running the first dimension.

2.3 Gel electrophoresis (2D-DIGE gel) and image capture

The 16 liver protein samples were analysed on a total of 8 analytical 2-D gels. IPG strips (24 cm, pH = 3-11NL) were rehydrated in 8 M urea, 4% CHAPS, DeStreak (12 µL mL^{-1}), and ampholytes (1% final concentration, pH = 3-10) overnight at room temperature. Cy-labelled samples were applied onto IPG rehydrated strips via anodic cup loading, and IEF was performed on a Ettan IPGphor II horizontal electrophoresis system (Amersham Biosciences) at 20 °C using the following program: step 1:300 V 4 h, gradient to 1000 V 6h, gradient to 8000 V 3 h; step 2: 8000 V until reached 32000 V h.

After IEF, the strips were reduced in equilibration buffer (Tris 50 mM, urea 6 M and glycerol 30% (v/v), 2% SDS (w/v)) containing 2% DTT, for 15 min at room temperature; followed by alkylation in equilibration buffer containing 2.5% iodoacetamide, for 15 min at room temperature. Then, the strips were transferred to the second dimension 12.5% acrylamide SDS-PAGE gels (25 cm x 21 cm x 1 mm) made between low fluorescence glass plates, and overlaid with 0.5% low melting agarose. The gels were run in Ettan Dalt Six Unit (GE Healthcare) electrophoresis system at 2 W per gel for 1 h and 15 W per gel for 6 h.

After electrophoresis, the 2-D gels were scanned directly in a Typhoon™ 9400 Variable Mode Imager to visualize the labelled proteins. Excitation and emission wavelengths were chosen specifically for each of the dyes according to manufacturer's recommendations (GE Healthcare).

2.4 Data analysis

Fluorescence images were analyzed using DeCyder™ V. 6.5 and DeCyder™ EDA software V.1.0 as described in Varó et al. (2010). Briefly, the intra-gel images were processed by DeCyder-DIA (Differential In-gel Analyses) software module to co-detect and differentially quantify the protein spots in the images, with the threshold set to 2 standard deviations. Then, the DeCyder-BVA (Biological Variation Analysis) was

applied to inter-gel matching, and differences in average ratios of protein expression were analysed by the Student´s t- test ($p \leq 0.02$). Finally, EDA software was used for multivariate statistical analysis of data. Principal Components Analysis (PCA) was carried out following the nonlinear iterative partial least squared method, including only proteins present in at least 80% of the spot maps and applying a t-test filter ($p \leq 0.02$). A hierarchical cluster analysis was performed using the same protein selection criteria.

2.5 Protein identification by mass spectrometry (MALDI, MS/MS) analysis

Proteins of interest were manually excised from analytical gels and digested with sequencing grade trypsin (Promega) as described elsewhere (Shevchenko et al. 1996), and subject to PMF (MALDI) and/or LC-MS/MS analyses.

The digestion mixture was dried in a vacuum centrifuge, resuspended in 7 µL of 0.1% TFA (trifluoroacetic acid, Sigma), and 1 µL was spotted onto the MALDI target plate. After the droplets were air-dried at room temperature, 0.5 µL of matrix (5 mg mL^{-1} CHCA (α-cyano-4-hydroxycinnamic acid, Sigma) in 0.1% TFA-ACN/H2O (1:1, v/v) was added and allowed to air-dry at room temperature. The resulting 576 fractions were analyzed in a 4700 Proteomics Analyzer (Applied Biosystems, Foster City, USA) in positive reflection mode (2000 shots every position). Five of the most intense precursors (according to the threshold criteria: minimum signal-to-noise: 10, minimum cluster area: 500, maximum precursor gap: 200 ppm, maximum fraction gap: 4) were selected for every position for the MS/MS analysis. And, MS/MS data was acquired using the default 1 kV MS/MS method.

The MS and MS/MS information was sent to MASCOT via the GPS software (Applied Biosystems). Database search on Swiss-Prot and NCBI databases was performed using MASCOT search engine (Matrix-Science). Searches were done with tryptic specificity allowing one missed cleavage and a tolerance on the mass measurement of 100 ppm in MS mode and 0.8 Da for MS/MS ions. Carbamidomethylation of Cys was used as a fixed modification and oxidation of Met and deamidation of Asn and Gln as variable modifications.

The samples without a positive identification were analysed by LC-MS/MS. Peptide separation by LC-MS/MS was performed using an Ultimate nano-LC system (LC Packings) and a QSTAR XL Q-TOF hybrid mass spectrometer (MDS Sciex-Applied Biosystems). Samples (5 µL) were delivered to the system using a FAMOS autosampler (LC Packings) at 40µL min^{-1}, and the peptides were trapped onto a PepMap C18 pre-column (5 mm 300 µm i.d.; LC Packings). Peptides were then eluted onto the PepMap C18 analytical column (15 cm 75 µm i.d.; LC Packings) at 200 nL

min^{-1} and separated using a 55 min gradient of 15–50% CAN. The QSTAR XL was operated in information-dependent acquisition mode, in which a 1-s TOF MS scan from 400–2000 *m/z*, was performed, followed by 3-s product ion scans from 65–2000 *m/z* on the three most intense doubly or triply charged ions.

The MS/MS information was sent to MASCOT via the MASCOT DAEMON software (Matrix-Science). The search parameters were defined as for MS-MS/MS analysis.

2.6 Quantitative RT-PCR of selected proteins

Total RNA was prepared from the same liver used for the proteomic analysis using the RNAspin MiniRNA isolation kit (GE HealthCare), according to the manufacturer´s instructions, and stored at -80 ºC. The purity and quantity of extracted RNA were measured using the Experion System (Bio-Rad). Two hundred ng of total RNA was reverse transcribed into cDNA using the High-Capacity cDNA reverse transcription kit (Applied Biosystems), with a final reaction volume of 20 µL. The RT conditions were: 10 min at 25 ºC, 120 min at 37 ºC and 5 sec at 85 ºC. The cDNA samples were stored at -20 ºC until use.

Transcript measurements were made by real-time PCR using an iCycler IQ Real-time Detection System (Bio-Rad, Hercules, CA, USA), following the procedure described by Calduch-Giner et al. (2003) RT reactions were conveniently diluted and 7.5 µL were used for PCR reactions in a 25 µL volume. Each PCR-well contained a SYBR Green Master Mix (Bio-Rad) with specific primers at a final concentration of 0.9 µM (see Table 1). The housekeeping gene α-tubulin was used as an internal control to normalize the data and the efficiency of PCR reactions for target and reference genes varied between 88% and 95%, respectively. The dynamic range of standard curves spanned five orders of magnitude, and the amount of product in a particular sample was determined by interpolation of the cycle threshold (Ct) value. The specificity of reaction was verified by analysis of melting curves. Fluorescence data acquired during the extension phase were normalized to α-tubulin by the delta-delta method (Livak and Schmittgen, 2001), using data in control fish as reference values. No changes in α-tubulin expression were found in response to treatment.

The difference in gene expression levels between control and treated fish was calculated by two-tailed independent Student t-test using SPSS statistics software v 17.0. A p value ≤ 0.05 was considered statistically significant**.**

2.7 Histology

For histological analyses subsamples from the same liver used for the proteomic study were processed following standard procedures. The samples were immediately fixed in buffered formalin at 4°C for 24 h. Then, they were transferred to 70% alcohol and kept there until processing. Finally, samples were dehydrated in alcohol, wax-embedded, sliced into 6 µm thick sections and stained with the hematoxylin and eosin technique for histological evaluation.

3. Results

A representative 2D-DIGE-gel image of the liver proteins profile from treated *versus* control fish is shown in Figure 1. Proteins over the range of pH applied in this experiment (pH = 3-11NL) and with a molecular weight from approximately 10 to 250 kDa were resolved, and 3994 spots in the master gel were detected using the DeCyder BVA software. Each protein spot was assigned an average ratio (i.e. change in expression level due to SDZ/TMP treatment) and *p*-value (t-student) to indicate the level of significance. Forty-one proteins were differentially expressed in the livers of the treated fish (standardized average volume ratio ≥ 1.3, t-student ≤ 0.02). Among these protein spots, 14 were up-regulated and 27 down-regulated by potentiated sulfa (SDZ/TMP) treatment. The positions of those differentially expressed protein spots in the 2D-DIGE are also shown in Figure 1**.** The PCA and hierarchical cluster analyses of data are shown in Figure 2. The PCA results obtained indicated that two components are enough to cluster the different experimental groups (control *vs* treated), with a clear separation in the first component (PC1) between them (Fig 2A). The pattern analyses showed a clustering in a hierarchical way, where protein maps corresponding to control group formed a cluster separated from those corresponding to the SDZ/TMP treated group (Fig 2B)

The 2D-DIGE analyses (spot nº, protein name, theoretical MW/pI, accession number, p-value, and the average ratio) and the results of protein identification by PMF and/or LC-MS/MS and database research are listed in Table 2. Ten of these forty-one differentially expressed protein spots were successfully identified after database searching in the public access data bases and the in-house developed sea bream EST database (only spot 3850). Most of them correspond to protein sequences that have previously been described in fish. The rest of the protein spots differentially expressed were in too low amount of protein after in-gel trypic digestion to obtain a good peptide mass spectrum for identification using MASCOT search. Table 2 also shows the

biological process involved, according to Gene Ontology (UniProtKB GO).

The analyses of biological processes of the identified proteins in terms of GO, revealed that the up-regulated proteins are related to carbohydrate metabolism (phosphogucomutase 1: PGM1, spot 1253), translational elongation activity (elongation factor 1-alpha: EF1α, spot 1597), and oxyreductase activity (mitochondrial aldehyde dehydrogenase: ALDH2, spot 1543). Another up-regulated spot (spot 1902) was identified in EST database as ypbc-32-D06 (Yellow perch control brain library *Perca flavescens* cDNA), which is associated with amino acid metabolic processes. As for the down-regulated spots, a first group of three down-regulated proteins is involved in the cholesterol metabolism/lipid transport (apolipoprotein A-I: apoA-I, spots 3076, 3993 and 3992). Another spot corresponds to a protein related with lipid transport (fatty acid binding protein FABP, spot 3850), and a last one to a binding protein (USCH1C-binding protein, spot 2223). Besides, potentiated sulfa treatment induced down-regulation in one protein of unknown function (spot 3762).

The relative transcript hepatic expression levels of PGM1, EF1α, ALDH2, FABP, and Apo A-I are shown in Figure 3. The relative gene expressions apparently increase as a result of the potentiated sulfa treatment, even though there were not significant differences between control and treated fish ($p > 0.05$).

In the histological analyses structural or morphological differences were not observed between controls and potentiated sulfa treated livers, and no pathological alterations were found in the livers from fish treated with potentiated sulfa (Fig 4).

4. Discussion

Proteomic approach is an efficient method to screen for differences in protein expression as well as to identify new proteins in a particular tissue, which can be associated with specific conditions such as drug treatment, diseases or nutritional status. In this study, 2D DIGE analysis coupled with MS protein identification was performed in order to identify the hepatic proteins differentially expressed in response to standard potentiated sulfa treatment in gilthead sea bream. Although the same proteomic approach has been used to analyse the effects of ivermectin (antiparasitic drug) in this species (Varo et al. 2010), this is the first attempt of such approach to examine the potential hepatotoxicity of antibacterial drugs, such as SDZ/TMP in sea bream.

The 2D DIGE results showed that 41 spots were differentially expressed in response to SDZ/TMP treatment, and 10 of these were positively identified using MS/MS. The differences in the expression levels of 41 proteins allow discriminating

treated fish from control fish according to the cluster analyses. Considering the changes in the liver proteome profile, together with the lack of hepatic damage, it is likely that this set of proteins indicates a transient or preliminary hepatotoxicity, and that these proteins could be considered as early PES (altered protein expression signatures) associated to SDZ/TMP treatment. This result is similar to our previous study on gilthead sea bream treated with ivermectin, where the changes in protein expression of 36 spots allowed to separate the medicated from the control group of fish, and thus, it was considered as a very early PES of ivermectin routine treatments for this species (Varó et al., 2010). Moreover, the fold changes found here in the protein spots were comparable to those obtained with invermectin.

Using PMF and/or LC-MS/MS and database research we were able to identify positively only 10 of the 41 proteins differentially expressed by SDZ/TMP treatment, because of identification of proteins unrepresented in databases from incomplete or non-sequenced organism remains challenging (Liska and Shevchenko 2003; Waridel et al. 2007). The proteins identified are involved in the metabolism of carbohydrates (PGM1), metabolism and transport of lipids (apoA-I, FABP), protein biosynthesis and binding (EF1α, USH1C-), oxidation regulation activity (ALDH2), as well as in amino acid metabolic process (ypbc-32-D06) (Table 2), in agreement with the metabolic role of the liver. It should be noted that these proteins are different to those differentially expressed by invermectin treatment, with the exception of apo A-I (Varó et al., 2010). However, these proteins have been previously identified in fish, highlighting the fact that most of them are similar or belong to some of the most frequently detected proteins in proteomic studies independently of the experimental conditions or tissue analysed (Wang et al. 2009).

In the present study apoA-I was down-regulated as result of SDZ/TMP treatment. This is the major component of high-density lipoprotein (HDL), which is known to be involved in reverse cholesterol transport from tissues to the liver, and in lipid metabolism. Moreover, apoA-I has been reported to play other roles in fish, related with antimicrobial activity (Johnston et al. 2008), innate immunity (Villarroel et al. 2007), or osmotic regulation (Chen et al. 2009). The apoA-I responsible for these functions, however, is more abundantly expressed in several other tissues such as epidermis, gills and intestinal mucosa, than in the liver. The same decreased expression of apoA-I was found in the liver of gilthead sea bream after 10 days ivermectin treatment at the recommended dose of 0.2 mg /kg fish (Varó et al., 2010). Again, similarly, reduced expression of apoA-I has been observed in fish liver when transferred from freshwater to brackish water (Chen et al., 2009), or when fed 3-thia fatty acids (Kleveland et al. 2006). Likewise, a decrease in apoA-I abundance was found in plasma and muscle of

Atlantic salmon (*Salmon salar*) in response to crowding stress (Veiseth-Kent et al. 2010).

Another protein down-regulated in the treated fish was fatty acid binding protein (FABP). FABPs are members of the superfamily of lipid-binding proteins. The main role of all the FABP family members is regulation of fatty acid uptake and intracellular transport. They are involved in the transport and storage of lipids, as well as cholesterol and phospholipid metabolism (Chmurzynska 2006). Also, FABPs are related to metabolic and immune response pathways (Furuhashi and Hotamisligil 2008), and to antioxidant functions (Wang et al. 2005). Previous studies on fish have reported changes in the expression of liver FABP in response to stress. Wang et al. (2008) found in the liver of goldfish (*Crassius auratus*), that one of the two spots identified as FABP (paralogs) decreased, whereas the other increased in the stressful environment. Reduced expression of liver FABP in Senegalese sole (*Solea senegalensis*) affected by hyperoxia stress has been related to protein oxidative damages (Salas-Leiton et al. 2009). However, the increase in the level of liver FABP found in gilthead sea bream and Senegalese sole subjected to handling and crowing stress, was associated with an increase in lipid mobilization, in order to respond to the increased energetic requirements due to stress, as well as in preventing oxidative damage (Alves et al. 2010; Cordeiro et al. 2012).

The down regulation of apoA-I and FABP found in the liver of fish treated with SDZ/TMP suggests a reduction in lipid metabolism and transport, and probably in their capacity to protect liver from oxidative stress induced by xenobiotics like potentiated sulfa. This fact might be important in the development of fatty livers in potentiated sulfa treated fish after prolonged treatments or higher doses, since no alteration was found in the livers of treated fish, which is in agreement with previous studies involving other compounds like hydrazine and ivermectin (Waterfield et al. 1997; Kleno et al. 2004; Varo et al. 2010). In addition, in livers of FABP deficient mouse it has already shown a shift in lipid distribution in favour of cholesterol, cholesterol esters, and phospholipids, as well as potentiated hepatic cholesterol accumulation in cholesterol-fed females, supporting that liver FABP is involved in the physiological regulation of cholesterol metabolism, which is important for the maintenance of animal homeostasis (Martin et al. 2003; Martin et al. 2006).

Likewise, it was found that potentiated sulfa (SDZ/TMP) induced an up-regulation of phosphoglucomutase 1 (PGM1). This is a protein related to carbohydrate metabolism, catalysing the reverse conversion of glucose 1-phosphate into glucose 6-phosphate. An increase in hepatic PGM1 has been found in gilthead sea bream fed maslinic acid, indicating a stimulation of glycogen metabolism (Rufino-Palomares et al.,

2011). An up-regulation of PGM1 has also been obtained in fish as response to long-term anoxia (Wulff et al. 2008). Another study done on cytotrophoblasts, reported an increase in PGM1 as response to hypoxia (Hoang et al. 2001). Our results may suggest that the treatment with SDZ/TMP produce an activation of carbohydrate metabolism as classical stress response in fish, since glucose is required to cope with increased energy demand of stress responding tissues as liver. This is consistent with an increase in plasma glucose levels found in gilthead sea bream and sea bass exposed to a combination of sulfamethoxazole and TMP (Yildiz and Altunay 2011).

Another protein that increased in the liver of treated fish was elongation factor 1-alpha (EF1α). This protein is very abundant in eukaryotes, comprising 1 to 10% of total cellular protein content. In gilthead sea bream EF1α has an ubiquitous and uniform distribution in tissues (Nowell et al. 2000). This protein plays a central role in protein biosynthesis. It is the aminoacyl–transfer RNA binding factor in peptide chain elongation, as it promotes the GTP-dependent binding protein of aminoacyl-tRNA to the A-site of ribosomes during protein synthesis. In addition, EF1α has been related to the mobilization of the ubiquitin-proteasome pathways and protein degradation (Buckley et al. 2006; Gonen et al. 1994) in the cell apoptosis process in response to oxidative stress (Chen et al. 2000; Duttaroy et al. 1998), as well as in response to thermal stress (Ibarz et al. 2010; Buckley et al. 2006). In the present study the overexpression of hepatic EF1 α found in fish treated may be related with a higher degree of liver protection from oxidative stress caused by potentiated sulfa (SDZ/TMP), since this protein is required for protein biosynthesis. This agrees with previous results demonstrating an increase in hepatic EF1 α associated to oxidative stress and apoptosis in gilthead sea bream as response to cold (Ibarz et al. 2010). An increase in gill EF1 α has also been shown in the goby (*Gillichthys mirabilis*) exposed to heat stress, and was related to the mobilization of the ubiquitin-proteasome pathways, whereas the repression of muscle EF1 α was linked to a minor need for ubiquitylation and degradation of damaged proteins in this tissue (Buckley et al. 2006).

An increase in mitochondrial aldehyde dehydrogenase (ALDH2) in the liver of treated fish was also observed. This protein belongs to the aldehyde dehydrogenase family (ALDHs), and has an oxidoreductase activity. The ALDH2 catalyses the conversion of acetaldehyde into acetic acid. This enzyme is known to play a major role in acetaldehyde oxidation in vivo (Vasiliou et al. 2000). In fish, previous studies showed ALDHs enzymes to be involved in detoxification processes (Nilsson 1988; Pretti et al. 2001). The increase in the levels of ALDH2 found in treated fish could reflect an increase in their capacity to process aldehydes and, therefore to protect the liver from

oxidative stress. In fact, a down regulation of ALDHs enzymes in fish liver has been related to a diminution of the capacity to protect this organ from oxidative stress (Cordeiro et al. 2012), and also with a non-induction of detoxification processes (Rufino-Palomares et al. 2011).

We found an increase in the spot 1902 identified in EST database as ypbc-32-D06 (Yellow perch control brain library *Perca flavescens* cDNA). A new search using BLASTx showed a sequence producing significant alignments of this EST with the protein annotation: PREDICTED: aspartate aminotransferase, cytoplasmic-like (AAT_like) (NCBI Reference Sequence: XP_003454182) described in tilapia (*Oreochromis niloticus*). AAT_like is a family that belongs to pyridoxidal phosphate (PLP)-dependent transaminase enzyme, and catalyses the reversible transfer of a α-amino group between aspartate and glutamate and, as such, is an important enzyme in amino acid metabolism. Aspartate aminotransferase, like alanine aminotransferase, is a key enzyme in the protein to carbohydrate metabolism. It has a wide distribution in both mammalian and fish tissues (Eze 1983; Gaudet et al. 1975). The enzyme is found in the liver, heart, skeletal muscle, kidneys, brain, and red blood cells, and changes in aminotransferases activities are frequently used as biomarker to determine fish liver damage (De la Torre et al. 2005; Prashanth and Neelagund 2008; Inyang et al. 2010). The up-regulation of ypbc-32-D06 found in liver of treated fish may indicate the activation of aspartate aminotransferase enzyme, which could be suggesting an increased participation of proteins in the energy production during treatment, as described De Smet and Blust (2001) in carp (*Cyprinus carpio*) exposed to cadmium, since no morphological alterations were observed in the liver of fish treated with potentiated sulfa. In the same way, a down regulation of a mitochondrial precursor of aspartate aminotransferase found in gilthead sea bream under cold stress was related to a lower capacity for amino acid catabolism and interconversion in cold and fasting fish (Ibarz et al. 2010).

The overexpression of theses proteins involved in energy production (PGM1 and ALDH2) and protein biosynthesis (EF1α) could be related to an increased energy demand to cope with the xenobiotic stress caused by potentiated sulfa treatment in order to maintain homeostasis, as well as with a stimulation of antioxidant defences.

In addition, analysis of mRNA coding for 4 of the proteins identified from those differentially expressed in control and treated fish did not reveal significant differences, although they showed higher values in treated fish. The lack of correlation found between mRNA expression and proteomic analysis is in agreement with the results of Wang et al (2008), who suggested that discrepancy between the abundance of cognate proteins and RNA molecules is frequently observed.

In conclusion, this study provides a proteomic analysis of the liver protein expression in response to a routine 10 days potentiated sulfa treatment in gilthead sea bream. A set of 41 proteins was differentially expressed in response to treatment. These were able to separate medicated and control groups of fish, and may be considered as PES for possible hepatotoxicity of potentiated sulfa in this species. The liver proteins found to have different expression in treated fish are involved in several well-known processes related to metabolic stress, including amino acid, carbohydrate and lipid metabolism, as result of transient metabolic and energetic adjustments with a lack of liver injury, due probably to the short period of time of the potentiated sulfa treatment. These results highlight that the proteins identified are generally expressed differentially as characteristic cellular/tissue stress response under different experimental conditions, and their use as biomarkers for this treatment should be done with caution.

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Figure legends

Fig. 1. Representative 2D-DIGE gel of soluble liver proteins extracted from gilthead sea bream (*S. aurata*). 3-10NL pH range were used for IEF. Protein spots differentially expressed and identified are numbered as in table 2. In green down-regulated spots and in red up-regulated spots.

Fig. 2. Multivariate analyses of liver proteomic data**.** (A) Principal Component Analysis (PCA) and hierarchical cluster analyses (B) of the proteins differentially expressed. PCA and the dendrogram after hierarchical analysis show a good separation of the spots maps corresponding to the different experimental groups (control in blue *vs* treated in red).

Fig. 3. Relative liver gene expression of PGM (phosphoglucomutase 1), EF (elongation factor 1-alpha), ALDH2 (mitochondrial aldehyde deshydrogenase), FABP (fatty acid binding protein), and apoA-I (apolipoprotein A-1) in control and potentiated sulfa treated fish. Values are means ± sd (n = 4-6). Means were compared by t-test with a p-value of 0.05.

Fig. 4. Histological sections from the liver of (A) control and (B) potentiated sulfa treated gilthead seabream (*S. aurata*) fish.

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Table 1. Forward and reverse primers used in the real-time quantitative PCR assay.

Gene	Accession number		Primer sequence	Position
Apolipoprotein A-1	AF013120	F	GAA TAC AAG GAG CAG ATG AAG CAG ATG	664-690
		R	TGG TGA CGG AGG CAG CGA TG	808-789
Mitochondrial aldehyde dehydrogenase	HQ228168	F	CCA TCC CAG CAC CCA ACA CC	135-154
		R	CCT CAT CAG CCT CAG CCA CTT G	284-263
Phosphoglucomutase 1	HQ228169	F	ACT CCG CCG TCA ACT GTG TC	265-284
		R	CCA GGT CAG CAG CGT AGG TC	345-326
Fatty acid-binding protein	HQ228170	F	AAA TGG TTG AGG CTT TCT GTG CTA C	48-72
		R	ATC GCT ACT GTC GGC TTG GTG	177-157
Elongation Factor 1	AF184170	F	CCC GCC TCT GTT GCC TTC G	560-578
		R	CAG CAG TGT GGT TCC GT	694-674
α-Tubulin	AY326430	F	GAC ATC ACC AAT GCC TGC TTC	514-534
		R	GTG GCG ATG GCG GAG TTC	647-630

Table 2 – Protein identities differentially expressed in the gilthead seabream (*Sparus aurata*) liver after treatment with SDZ/TMP for 10 days. p-value represents the significance level of t-test performed by the image analysis software DeCyder of up or down regulated spots.

Spot nº	Protein name	Theo. MW/pI	Accession no.	2D-DIGE		Protein identification								Biological process[a]	Species
						PMF (MALDI TOFTOF)				LC MSMS					
				p-value	Av. Ratio (fold induction)	Score #	matched peptides	%seq cov	MSMS peptides	Score #	Nº peptides	%seq cov			
Up															
1253	Phosphoglucomutase 1	61375/5,74	gi|32766553 (Q7SXW7)	0,0022	1,5	164 (1)	13	28	4					Carbohydrate metabolic process	Danio rerio
1597	Elongation factor 1-alpha	50780/9,23	gi|5923899 (Q9PUG6)	0,0090	1,4	140(1)	13	36	3					Translational elongation	Sparus aurata
1543	Aldehyde dehydrogenase, mitochondrial precursor	57271/5,93	gi|209154764	0,0100	1,4					111	3	6		Oxidation reduction	Salmo salar
1902	ypbc-32-D06 (**)	32305/5,60	gi|226825511	0,0068	1,45					54(1)	2	7		(*)Aspartate aminotransferases	Perca flavences
Down															
3076	Apolipoprotein A-1	296015/5,04	gi|2511712	0,00020	-1,6					361(1)	7	38		Cholesterol metabloic process/lipid transport/lipopr o-tein metabolic process	Sparus aurata
	Apolipoprotein A-1		gi|2511712												
	Apolipoprotein A-1		gi|2511712												
3994		296015/5,21	gi|2511712	0,0088	-1,8	95(1)	13	76	2						
3084		296015/5,04	gi|2511712	0,0039	-1,8					81(1)	3	13			
3850	Fatty acid binding protein	14922/6,59	gi|4538320 (Q05423)	0,0024	-1,4	114(1)	4	38	3					Transport	Gallus gallus
2223	USH1C-binding protein 1	227951,98/6,12	gi|81879044 (Q8R370)	0,0088	-1,5					91(2)	2	2		n.i	Mus musculus
3850	Fatty acid binding protein	14922/6,59	gi|4538320 (Q05423)	0,0024	-1,4	114(1)	4	38	3					Transport	Gallus gallus
3762	Unnamed protein product	85031/9,32	gi|47228876	0,0002	-1,5					47(1)	1	8		n.i	Tetraodon nigroviridis

Accession nº: NCB nº (UniProt nº)
Protein identification: (1)PMF= (MALDI), (2) MSMS =(Q-Star)
([a]) Biological process according to Gene Ontology (UNiProtKB GO); n.i., non indientified; (*) BLAST NCBI fro gene; (**) EST data base

Fig. 1

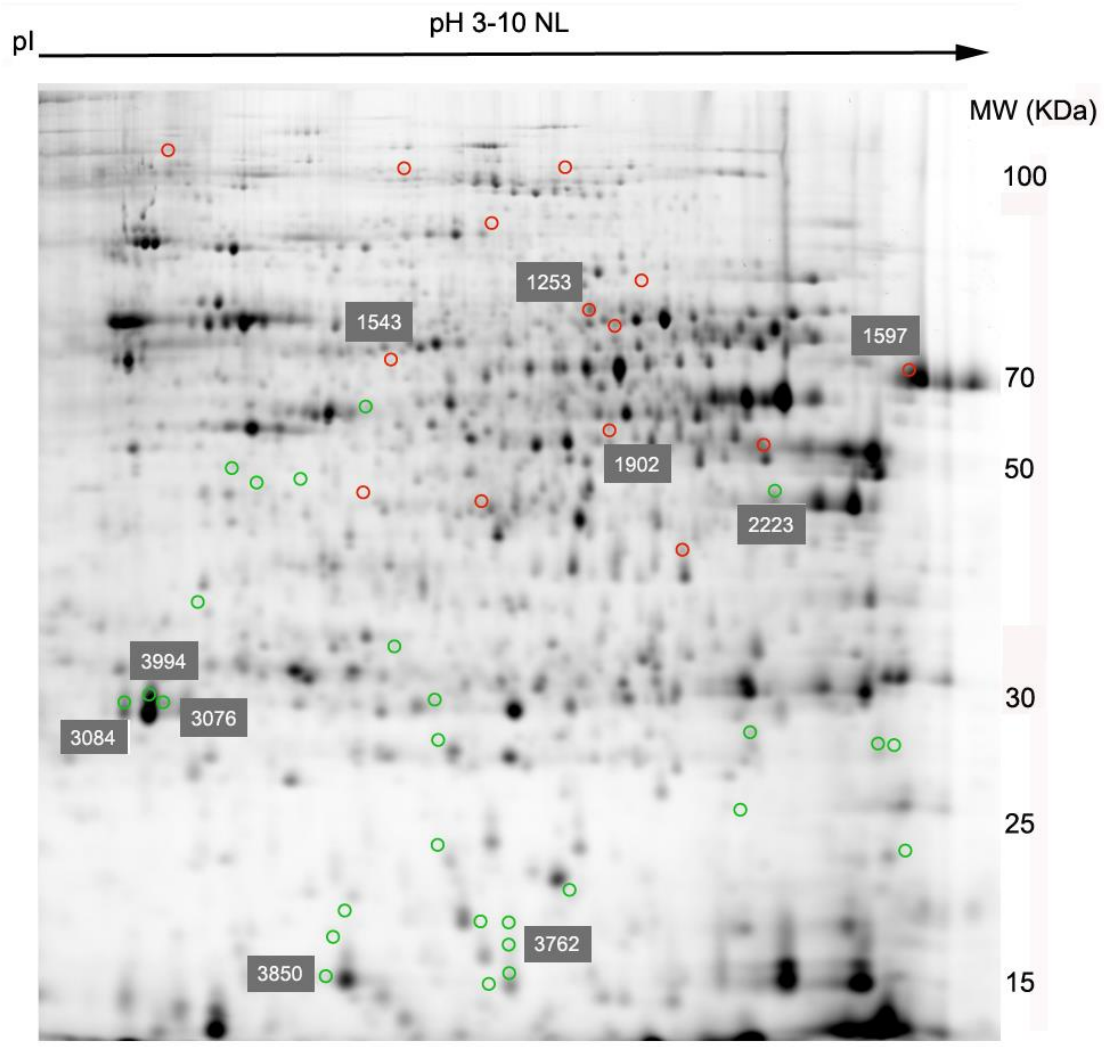
pI
pH 3-10 NL
MW (KDa)
100
70
50
30
25
15
1253
1543
1597
1902
2223
3994
3076
3084
3762
3850

Fig. 2 (A)

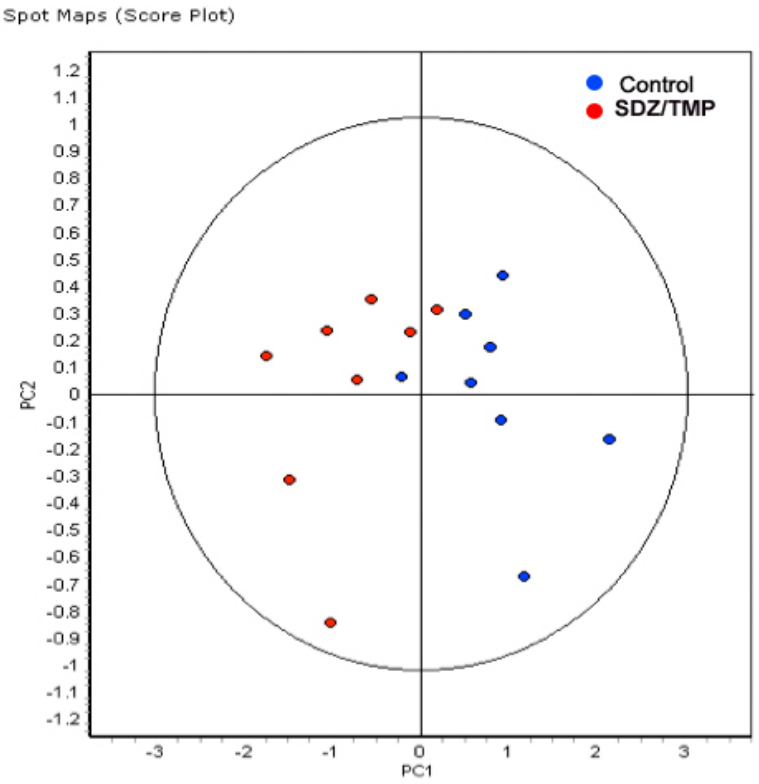
Spot Maps (Score Plot)
Control
SDZ/TMP
PC2
PC1
1.2
1.1
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
-0.1
-0.2
-0.3
-0.4
-0.5
-0.6
-0.7
-0.8
-0.9
-1
-1.1
-1.2
-3
-2
-1
0
1
2
3

Fig. 2 (B)

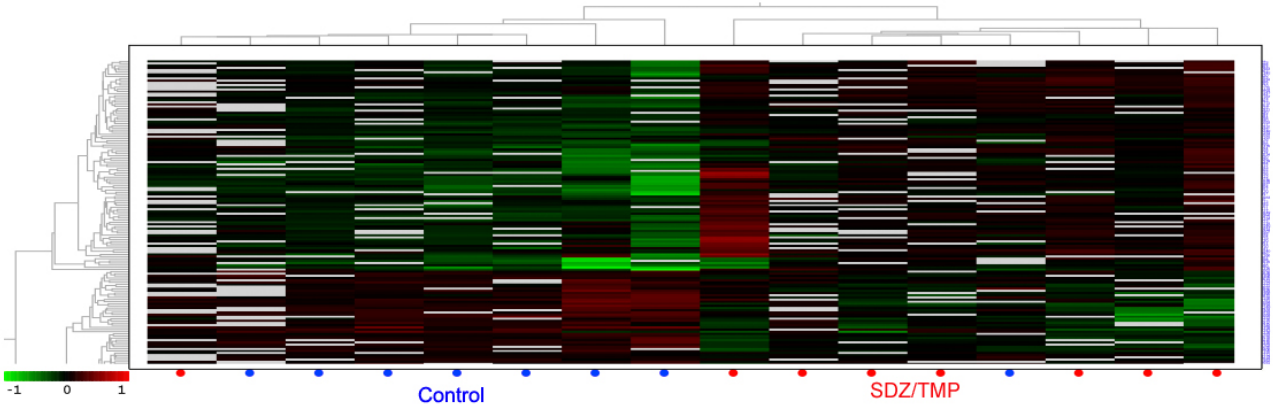
-1
0
1
Control
SDZ/TMP

Fig 3

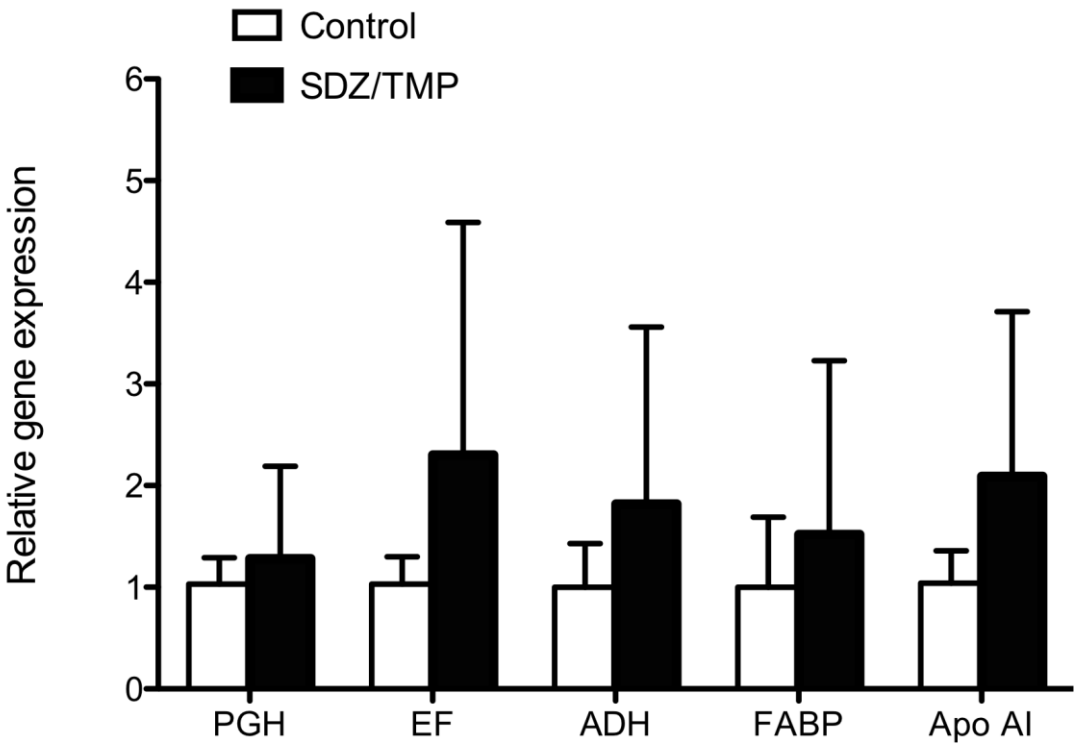
Control
SDZ/TMP
Relative gene expression
0
1
2
3
4
5
6
PGH
EF
ADH
FABP
Apo AI

Fig. 4