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Enteritis induction by soybean meal in *Totoaba macdonaldi* diets: Effects on growth performance, digestive capacity, immune response and distal intestine integrity

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ABSTRACT

The aim of the present study was to investigate the effects of increasing levels of dietary soybean meal (SBM) with constant taurine supply in the induction of enteritis in juvenile *Totoaba macdonaldi*. Four isoproteic (48.5%) and isolipidic (8.6%) diets were formulated to include increasing levels of a mixture of soybean meals (SBM); (soy protein concentrate and soybean meal at a ratio of 1:4) at 0%, 22%, 44% and 64% replacing fishmeal in a diet containing 1% taurine. Upon completion of the 56-day feeding trial, SBM caused marked dose-dependent responses in growth performance and digestive physiology processes. Severe enteritis symptoms in the distal intestine and liver were found when SBM was included above 22%. SBM dose-dependent impairments in digestive functions were found in digestive enzyme activity for trypsin, chymotrypsin, L-aminopeptidase, total alkaline proteases, and amylase. Interleukin (*il-8*) expression patterns showed an inflammatory response during the first four weeks in the presence of the higher levels of SBM (44% and 64%) suggesting an impaired immunological response. However, after 8 weeks no immunological inflammatory response was observed, but a severe atrophy of the intestine could still be revealed. Results indicate a detrimental status of the digestive physiology of totoaba fed SBM-based diets at inclusion levels above 22%. Thus, suggesting that SBM should be cautiously used in totoaba feed formulations.

1. Introduction

Fish meal (FM) sparing and replacement is still a great concern in aquaculture research. Alternative vegetable sources, in particular soybean meal (SBM), which has the highest protein content among the plant-based ingredients, has been pointed out as one of the most promising alternative protein sources. Not surprisingly, the use of SBM has been implemented and adapted into husbandry nutrition of several species over the years. In the aquaculture industry, defatted-SBM has been considered a viable alternative to replace at least part of FM in marine fish feeds due to high availability and 40–48% crude protein with a constant amino acid profile at low cost (Gatlin et al., 2007). However, increase in SBM in certain carnivorous fish diet is related to the occurrence of enteritis (Bakke-McKellep et al., 2000; Krogdahl et al., 2003), defined as non-infectious inflammation of distal intestine (Baeverfjord and Krogdahl, 1996) which can be reversed by eliminating dietary SBM (Bakke et al., 2010).

Additionally, SBM contains a high level of antinutritional factors for fish (i.e. protease inhibitors, saponins, lectins, phytic acid, alkaloids, oligosaccharides, glucosinolates, antigens), associated with a damage of the mucosal integrity, decreased pancreatic and brush-border enzymes, loss nitrogen in the faeces, thyroid hormone suppressors, lower mineral absorption, reduced palatability and suppression of the immune system (Francis et al., 2001; Krogdahl et al., 2010; Krogdahl and Bakke-McKellep, 2015). From all, saponins were suggested to induce enteritis in Atlantic Salmon *Salmo salar* (Krogdahl et al., 2015). Distal intestine associated with enteritis exhibited the following histological features: shortening of mucosal folds (MF), reduction in the number of

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supranuclear vacuoles (SNV) of the enterocytes, thickening of the lamina propria (LP), enlargement of the connective tissue, increased number of goblets cells (GC) in the epithelium and infiltration of inflammatory cells in connective tissue and LP (van den Ingh et al., 1991; Baeverfjord and Krogdahl, 1996).

There is a lack of nutritional studies evaluating soy products in diets of *Totoaba macdonaldi,* which is a marine carnivorous species with high potential of commercial aquaculture (Juárez et al., 2016). A previous study investigating dietary soybean protein concentrate (SPC) reported improved growth and feed efficiency with acceptable haematological parameters in totoaba fed diets containing 30 to 60% of SPC (López et al., 2015). Despite, a hepatic damage was reported in fish fed SPC-based diets, regardless SPC dietary level. Likewise, Bañuelos-Vargas et al. (2014) concluded that since taurine is an important modulator of the intermediate metabolism of the liver, when it is present at 1% in SPC-based diets no damages of enteritis could be noticed. In agreement, Trejo-Escamilla et al. (2016) reported that up to 34% of SPC could be included in totoaba diets containing 1.5% taurine without affecting growth. However, to our knowledge no data has been published evaluating the overall performance of the fish, the effect on digestive physiology and immunological responses.

As SBM is a lower-cost protein alternative to FM, in the present work we evaluate the effect of graded levels of SBM on growth performance, digestive capacity, molecular expression, distal intestine and liver integrity in juveniles of *Totoaba macdonaldi*, that may result in enteritis with detrimental outcomes in fish of commercial size.

2. Materials and methods

2.1. Diet formulation

Four isoproteic (485 g crude protein (CP) kg^{-1} diet) and isolipidic (86 g crude lipid (CL) kg^{-1} diet) diets were formulated to replace FM (69% protein content, Maz Industrial SA de CV, Mazatlán Sinaloa, México) protein at 0, 25, 50 and 75%, with a mixture of SPC and soybean meal (SBM) at 1:4 ratio, Alimentos COLPAC, Sonora, México and NutriVance™, Midwest Ag Enterprices, Inc. MN, USA and referred as SBM from this point forward. Soybean meal contained 48% CP. Soy protein concentrate contained 60% protein. Additionally, all diets included 1 g kg^{-1} of krill oil (Biogrow, ProAqua, México) as attractant and 10 g kg^{-1} of taurine (Insumos Nubiot, SA de CV, México) (Table 1). Diets were mixed (Robot-Coupe, model R10, USA), pelleted at 5 mm in a meat grinder (Tor-Rey, Model M32–5, Mexico) and dried at 60 °C in a forced air oven for 24 h. Once dried, diets were packaged and stored at −20 °C until used for the feeding trial. Four essential amino acids (lysine, methionine, threonine and arginine; EVONIK, Degussa, México) were supplemented to reach equal levels found in the control diet (0% SBM). Although dietary treatments were formulated to replace FM protein at 0, 25, 50 and 75% with a mix of soybean meals, the actual SBM mixture content for each treatment was 0, 22, 44 and 64% (Table 1).

2.2. Experimental design, animals and facilities

Juvenile totoaba were reared from eggs, of hormone-induced spawns, at the Marine Fish Culture Laboratory at the Centro de Investigación Científica y de Educación Superior de Ensenada (CICESE), Mexico. Forty-eight fish (71.7 ± 35.7 g; mean ± SE) were randomly stocked into twelve 450-L cylindrical blue fiberglass tanks (four fish per tank) connected to a closed recirculation system composed of a compacted bead bed filter coupled with a fluidized biofilter (media kaldnes) and a heat pump (Titan 1 1/2 hp. Aqualogic, USA) with a daily water renewal of 5%. Water quality was monitored daily, with mean values for temperature = 23.3 ± 1.1 °C, dissolved oxygen = 5.5 ± 0.4 mg L^{-1} with an oxygen saturation up to 80%, salinity = 35.2 ± 1.0‰ and a water flow of 2.5 L min^{-1}. Every three days the ammonia, nitrite and nitrate levels were measured (Api Pharmaceutic Aquarium Kit) to keep values < 1.0 mg L^{-1}, 0.5 mg L^{-1} and < 80 mg L^{-1}, respectively. Fish were kept under natural photoperiod between September and November of 2016 (31°87 N, 116°66 W).

Table 1
Ingredient formulation (g kg^{-1}) and proximate composition of diets used to fed *T. macdonaldi* containing increasing levels of SBM. Dietary formulation is presented as fed basis and proximate composition in g kg^{-1} on a dry matter basis.

Experimental diets	0% SBM	22% SBM	44% SBM	64% SBM
Ingredients (g kg^{-1} DM)				
Sardine meal (69%CP)[a]	619.2	464.4	309.6	154.8
Soybean meal (48%CP)[b]	0.0	163.4	327.0	490.5
Soy protein concentrate (60%CP)[c]	0.0	54.5	109.0	163.5
Starch	218.2	146.8	73.9	0.4
Sardine oil[a]	52.0	58.8	65.7	72.5
Gelatin	60.0	60.0	60.0	60.0
Rovimix for carnivorous fish[d]	25.0	25.0	25.0	25.0
Stay-C[d]	4.0	4.0	4.0	4.0
Taurine[e]	10.0	10.0	10.0	10.0
Methionine[f]	2.7	3.9	5.1	6.3
Lysine[f]	0.0	2.6	5.3	7.9
Arginine[f]	2.9	0.9	0.0	0.0
Threonine[f]	1.9	1.6	1.3	1.0
Attractant (krill oil)[g]	1.0	1.0	1.0	1.0
Sodium benzoate	2.0	2.0	2.0	2.0
Choline chloride	1.0	1.0	1.0	1.0
BHT	0.1	0.1	0.1	0.1
Proximate composition (g kg^{-1} DM)				
Dry matter	989 ± 1.0	991 ± 0.4	975 ± 1.6	988 ± 1.0
Crude protein	488 ± 1.7	486 ± 3.4	488 ± 4.0	484 ± 6.4
Crude fat	88 ± 1.3	87 ± 1.3	85 ± 0.4	84 ± 0.9
Ash	147 ± 1.1	131 ± 0.7	118 ± 0.2	96 ± 0.9
NFE[h]	277 ± 3.7	296 ± 3.2	309 ± 4.0	336 ± 5.7

[a] Maz Industrial SA de CV, Mazatlán, Sinaloa, México.
[b] Alimentos COLPAC, Sonora, México.
[c] NutriVance™ Midwest Ag Enterprises, Inc. MN, USA (U.S. Soybean Export Council).
[d] Rovimix; Stay-C DSM, Guadalajara, México.
[e] Insumos NUBIOT SA de CV, México.
[f] Free aminoacids donated by EVONIK, Degussa, México.
[g] Biogrow, Proveedora de Insumos Acuícolas, SA de CV, Mazatlán, Sinaloa, México.
[h] Nitrogen free extract; NFE (%) = 100 − (% crude protein + % total lipid + % ash).

Each dietary treatment was randomly assigned into triplicate experimental units. Fish were hand-fed daily to apparent satiation at 08:30, 12:00 and 16:00 h during 8 weeks. Daily, all uneaten feed was removed within an hour of feeding and dry weighed to determine the most accurate feed consumption rates possible.

2.3. Sampling

All fish were measured (mm, SL) and weighted (g) at the beginning of the feeding trial and then every fifteen days. Mean individual weight of each experimental unit was determined dividing the bulk weight by the number of individuals, performance response indices and somatic indices were calculated as follows:

Thermal growth coefficient (TGC) = [(final weight ⅓ − initial weight ⅓) / ($T_{°C}$ × D_{days})] × 1000.
Feed Conversion Ratio (FCR) = total feed consumed/ wet weight gained.
Condition Factor (CF) = final body weight × (body length)^3 × 100 (Hardy and Barrows, 2002).
Protein Efficiency Ratio (PER) = weight gain/ protein intake.
Feed Intake = FI (% day^{-1}) = 100 × (total amount of the feed

consumed / ((initial body weight + final body weight) / 2) / days).
HSI (Hepatosomatic Index) = (hepatopancreas weight / body weight) × 100.
VSI (Viscerosomatic Index) = (viscera weight / body weight) × 100.
ISI (Intestinal Somatic Index) = (intestine weight / body weight) × 100.

For analytical analyses samplings were performed at 4 and 8 weeks, using one fish from each tank (three fish per treatment). Fish were fasted for 18 h, and then humanely slaughtered with a cut in the spine next to the skull, following CICESE's animal ethics protocols. Intestine and liver were individually dissected, cleaned to remove any mesenteric tissue or fat, and weighed. Distal intestine was located between the intestinal constriction and the anus. For histological samples, distal intestine from each fasted fish was cut 8–10 mm of the central section, then washed with distilled water and stored in a 4% formaldehyde solution with phosphate buffer for 24 h and in 70% ethanol until analysis. Samples for gene expression (i.e., only from the distal intestine) were immediately stored in RNA later (Ambion) for 24 h and then immediately frozen at −80 °C for subsequent molecular analyses.

Samples for digestive enzyme activity were taken only at the end of the experiment (8 weeks). The stomach, pyloric caeca and intestine were dissected and stored separately from each experimental unit. The entire digestive tract from fasted fish was extracted and placed on a cold plate (using gel ice) and each digestive organ dissected. Each organ was weighed separately and stored at −80 °C until analysis. The hepatosomatic index was calculated at 4 and 8 weeks, while visceral and intestinal indexes only at 8 weeks. Liver samples for histology were taken at the end of the experiment (8 weeks) following the same procedure describe for the intestine fixation.

2.4. Analytical methods

Proximate analyses of the experimental diets were performed in triplicate and reported on a dry matter basis according to standard procedures of the AOAC (2005). Moisture content was determined after achieving constant dry weight at 60 °C for 48 h. For total ash, samples were incinerated in a muffle furnace at 550 °C for 8 h. Crude protein (N × 6.25) was estimated by the micro-Kjeldahl method. Crude lipid was determined gravimetrically by Soxhlet extraction method with petroleum ether. The nitrogen-free extract including soluble and insoluble carbohydrates was calculated by difference NFE (%) = 100 − (% crude protein + % crude lipids + % ash).

2.5. Intestine and liver histology

For each fish sample, the distal intestine was divided into three sections and gradually dehydrated in ethanol, clarified in benzene and embedded in paraffin. The same procedure was used for the liver tissue. Subsequently, a complete intestinal annular ring from each fish (three per treatment) was cut into three sections ($n = 9$) and placed into one slide for histological measurements using three replicates. Transversal sections of 6–7 μm were cut using a rotary microtome (Leica RM2245), stained with hematoxylin and eosin (H&E). Additionally, intestine samples were stained using alcian blue 8GX (C.I. 74240, Sigma A3157) to visualized goblet cells (GC). Slides were evaluated by blind examination (i.e., sample treatment unknown to the observer) in light microscope (Leica DMLS), pictures were taken with a digital camera (Leica DFC450) and processed and measured using the image program LAS CORE (Version 4.3.0 Leica).

To illustrate the degree of enteritis, 30 measurements were made to determine the number of mucosal folds (MF) by annular ring, length of complete MF, enlargement of the lamina propria (LP), sub-epithelium mucosa (SM) and enterocyte height (EH). Based on the results obtained at the end of the experiment using the set of first histological slides, thinner cuts were made at 3 μm from the same dehydrated samples (8 weeks) to allow observing for higher detail the damage caused by the SBM inclusion. Liver histology alterations in terms of nuclear displacement, cytoplasm vacuolization and infiltration of peripancreatic fat was assessed at the end of the experiment, based on the characterization system used in Caballero et al. (2004).

2.6. Gene expression quantification

Intestine samples were preserved in RNAlater (Ambion) and individually processed for total RNA extraction using the NucleoSpin® RNA kit (Macherey-Nagel). Genomic DNA (gDNA) was removed via on-column DNase digestion at 37 °C for 30 min using rDNase (RNase-free) included in the kit. A micropistill was used to homogenize the tissue before the extraction. RNA quantity and quality was measured by gel electrophoresis and spectrophotometrically (Nanodrop® LITE, Thermo Fisher Scientific INC., Wilmington, USA). Only RNA samples with OD260nm-OD280nm ratios between 1.90 and 2.10 were used for expression quantification.

Total RNA (500 ng) was reverse-transcribed in a 20 μL reaction using the High-Capacity cDNA Reverse Transcription kit (Applied Biosystems; Carlsbad CA, USA) in a Verity 96 well thermal cycler (Applied Biosystems). The reverse transcription program consisted in 10 min at 25 °C, 120 min at 37 °C, 5 min at 85 °C and finally kept at 4 °C. qRT-PCR reactions were performed with 1 ng of cDNA, sense and antisense primers (200 nM each, indicated in Table 2) and SYBR® Select Master Mix (Applied Biosystems). *18S* was used as the internal reference gene. Reactions were conducted in 10 μL, in MicroAmp® Fast Optical 96-well reaction plates (Applied Biosystems) covered with MicroAmp® Optical Adhesive Film (Applied Biosystems).

Relative gene quantification was calculated by the ΔΔCT method (Livak and Schmittgen, 2001) using automated threshold and walking baseline for determining the CT values. PCR conditions were: an initial denaturation and polymerase activation step during 10 min at 95 °C; 40 cycles of denaturing for 15 s at 95 °C, annealing and extension for 45 s at 60 °C; and a final melting curve from 60 °C to 95 °C for 20 min to check for primer-dimer artefacts. Optimization of qRT-PCR conditions was made on primer annealing temperature (60 °C), primer concentration (200 nM) and template concentration (five 1:10 dilution series from 10 ng to 100 fg of input RNA). Nucleotide sequences for *18S* and *il-8* are available in GenBank under accession numbers, HM754483 and KU847777 respectively.

2.7. Digestive enzyme activity

Digestive enzyme extracts were obtained using sections of the whole digestive system and cutting them into pieces and latter homogenized in 5 mL of cold distilled water for 2 min with a tissue grinder

Table 2
Primers pairs used for q-PCR Primer sequences, amplicon sizes in base pairs (bp), reaction efficiencies (E) and Pearson's coefficients of determination (R^2) are indicated.

Gene (*symbol*)	Fwd sequence (5′ - 3′)	Rev sequence (5′ - 3′)	Size (bp)	E	R^2
18S	CGGTTCTATTTTGTGGGTTTTC	CTTTCGCTTTCGTCCGTCTT	126	0.98	1.00
il8	CCTGAGAAGTCTGGGAGTCG	GGAGTTGGGAGGGATGATCT	103	1.00	0.99

Table 3
Growth performance of *T. macdonaldi* fed diets containing different levels of SBM after 8 weeks of experimental procedure. Values are presented as means ± standard deviation. Also, pooled standard error (PSE) is given from three replicates per treatment and *P* values resulting from one-way ANOVA test are also provided. Different letters as super indices represent significant different values ($P < .05$) within the same row.

	0% SBM	22% SBM	44% SBM	64% SBM	PSE	*P* value
TGC[1]	1.61 ± 0.13^{a}	1.39 ± 0.14^{ab}	1.30 ± 0.05^{ab}	1.21 ± 0.19^{b}	0.06	0.029
FCR[2]	0.82 ± 0.03^{b}	1.03 ± 0.07^{a}	1.06 ± 0.09^{a}	1.11 ± 0.07^{a}	0.04	0.004
CF[3]	1.70 ± 0.06^{a}	1.52 ± 0.09^{b}	1.51 ± 0.02^{b}	1.50 ± 0.02^{b}	0.03	0.007
PER[4]	2.50 ± 0.09^{a}	2.00 ± 0.13^{b}	1.94 ± 0.18^{b}	1.87 ± 0.11^{b}	0.08	0.002
FI (% day^{-1})[5]	0.87 ± 0.05	0.84 ± 0.04	0.98 ± 0.15	0.95 ± 0.07	0.03	0.263

[1] TGC (Thermal Growth Coefficient) = [(final weight$^{1/3}$ − initial weight$^{1/3}$)/(T°C × D)] × 1000.
[2] FCR (Feed Conversion Ratio) = total feed consumed / wet weight gained.
[3] CF (Condition Factor) = final body weight × (body length)^{3} × 100 (Hardy and Barrows, 2002).
[4] PER (Protein Efficiency Ratio) = weight gain / protein intake.
[5] FI, Feed Intake = FI (% day^{-1}) = 100 × (total amount of the feed consumed / ((initial body weight + final body weight) / 2) / days).

(POLYTRON® PT 1200, Kinematica AG, Switzerland) and centrifuged at 14000 rpm for 45 min at 4 °C (5417R, Eppendorf, USA). Supernatant was collected and stored in 0.5 mL aliquots at −80 °C. Aliquots once thawed were used within 48 h and kept refrigerated at 4 °C. Total activity per organ was estimated for the stomach, pyloric caeca and intestine (U organ^{-1}).

The soluble protein was determined using Bradford method (BIO RAD Protein assay; Hercules, CA, USA) with bovine serum albumin (BSA) as standard. Each enzymatic assay includes a blank sample using distilled water instead of the homogenate and a positive control with commercial enzymes at concentration of 1 mg mL^{-1}. The methods were adapted to perform the spectrophotometric measurements on a plate reader Varioskan Flash (Thermo Scientific) and the data were processed in the software SkanIt RE 2.4.5.

Trypsin activity was measured according to the method of Erlanger et al. (1961). In summary, using 1 mM of BAPNA (Nα-benzoyl-DL-arginine-p-nitroanilide hydrochloride, Sigma B-4875) as substrate in 500 μL of DMSO. The reaction conditions were 50 mM tris-HCl buffer, 20 mM $CaCl_2$, pH 8.2 at 37 °C by 30 min. The reaction was stopped with 30% of acetic acid and the absorbance was recorded at 410 nm after 10 min of stabilization. Chymotrypsin activity was determined by the method of Hummel (1959), as modified by Applebaum et al. (2001), using 0.56 mM of BTEE (N-Benzoyl-L-tyrosine ethyl ester, Sigma 13,110-F) as substrate in 100 mM Tris-HCl buffer, 25 mM $CaCl_2$, pH 7.8 and methanol 2.5% (*v*/v) at 37 °C. The reaction was recorded every minute during 30 min at 256 nm in a 96-well quartz plate.

Leucine aminopeptidase (LAP) was determined by the method of Apple (1974), using 1.2 mM L-leucin-P-nitroanilide (Sigma, L-9125) as substrate in 50 mM HCl-Tris buffer, pH 8.0 at 37 °C. The reaction was incubated during 30 min and stopped with 30% of acetic acid and the absorbance was recorded at 405 nm after 10 min of stabilization. Total alkaline proteinase activity was measured by the method of Sarath et al. (1989) using 2% of azocasein (Sigma, A2765) as substrate in 50 mM HCl-Tris buffer, 10 mM $CaCl_2$, 9.0 pH at 37 °C. The reaction was incubated during 10 min and stopped with 10% trichloroacetic acid. The samples were centrifuged (14,000 rpm, 5 min, 4 °C) and the absorbance of supernatant recorded at 440 nm.

Amylase activity was measured by the method described in Worthington Biochemical Corporation (1993); using 1% starch (Sigma, S9765) as substrate. The starch was mixed in 20 mM sodium phosphate buffer, 6 mM NaCl and 6.9 pH. The homogenate and the buffer + substrate were mix at intervals of time, incubate at 25 °C and at exactly three minutes, 1% dinitrosalicylic acid was added as color reagent. Immediately after, all tubes were incubated in a boiling water bath for 5 min. Finally let cool to room temperature and mixed well to read absorbance at 540 nm. A standard maltose curve was used to calculate by regression equation the micromoles of maltose released in each sample. The following formula was used to calculate the amylase activity; Unit mg^{-1} = (micromole maltose released / mg enzyme in reaction mixture × 3 min). All the samples were diluted 10 times to a final volume reaction of 1.2 mL to allow readings within the range of the standards. Lipase activity was estimated according the method of Gjellesvik et al. (1992) using 0.56 mM of 4-Nitrophenyl myristate (Sigma 70124) as substrate dissolved in 0.5 mL DMSO. The reaction conditions were 150 mM Tris-HCl buffer, 15 mM sodium taurocholate, pH 8.5 at 37 °C. The reaction was recorded every minute by 30 min at 405 nm.

Pepsin activity (i.e., acid proteolytic activity) was estimated according to Sarath et al. (1989), using as substrate 1% of haemoglobin (Spectrum Chemical, HE120) in 200 mM, pH 2 at 37 °C. The reaction was incubated during 10 min and stopped with 5% trichloroacetic acid. The samples were centrifuged (14,000 rpm, 5 min, 4 °C) and the absorbance of supernatant was recorded at 280 nm.

In all cases one unit of enzyme activity was defined as the amount of enzyme required to cause an increase of 1 unit of absorbance per minute (Lazo et al., 2000).

2.8. Statistical analyses

Significant differences in performance indices, somatic indices, enzyme activities and gene expression levels were analyzed by one-way ANOVA, followed by post-hoc Tukey rank test. Prior to statistical analysis, percentage data were arcsine transformed. The effect of SBM inclusion and time of MF number and length, EH, LP, SM and *Il-8* expression was analyzed by two-way ANOVA. For all cases statistical significance was set at $P < .05$. Statistical analysis was performed using the software STATISTICA 8.0™ (StatSoft, Inc. USA).

3. Results

3.1. Growth performance and somatic indexes

At 8 weeks growth and production performance of juvenile totoaba was significantly affected by dietary SBM inclusion level (Table 3). Overall, fish fed SBM-free diets outperformed those fed SBM-based diets and increasing dietary SBM affected TGC, FCR, CF, PER and HSI. For example, fish fed 64% SBM diet resulted in significantly ($P < .05$) lower TGC (1.18), higher FCR (1.11) and lower CF (1.50) compared to those fed 0% SBM diet (TGC = 1.57, FCR = 0.82, CF = 1.70). All SBM-based diets yielded significantly lower PER (1.94–2.00) in comparison with SBM-free diet (2.50).

At 4 weeks, a significant reduction in the hepatosomatic index was observed in fish fed the 44% and 64% SBM inclusion diets. At 8 weeks the HSI tended to decrease with increasing levels of dietary SBM (HSI = 1.48–0.87) for Control (0% SBM) and 44% SBM, respectively; whereas no significant differences ($P > .05$) were found for VSI and ISI at this time (Table 4).

Table 4
Somatic indexes of *T. macdonaldi* fed diets containing different levels of SBM at 8 weeks and HSI at 4 weeks. Values are presented as means ± standard variation. Also, pooled standard error (PSE) is given from three replicates per treatment and *P* values resulting from one-way ANOVA test is also provided. Different letters as super indices represent significant different values ($P < .05$) within the same row.

	0% SBM	22% SBM	44% SBM	64% SBM	PSE	*P* value
HSI%[1] at 4 weeks	1.71 ± 0.32^{a}	1.23 ± 0.33^{ab}	0.99 ± 0.15^{b}	1.04 ± 0.11^{b}	0.10	0.026
HSI%[1] at 8 weeks	1.48 ± 0.30^{a}	1.35 ± 0.28^{a}	0.87 ± 0.16^{b}	1.06 ± 0.29^{ab}	0.07	0.003
VSI%[2]	4.50 ± 0.39	4.18 ± 0.45	3.58 ± 0.46	3.57 ± 0.51	0.20	0.946
ISI%[3]	0.85 ± 0.10	0.83 ± 0.15	0.80 ± 0.10	0.71 ± 0.14	0.00	0.881

[1] HSI (Hepatosomatic Index) = (hepatopancreas weight / body weight) × 100.
[2] VSI (Viscerosomatic Index) = (viscera weight / body weight) × 100.
[3] ISI (Intestinal somatic Index) = (intestine weight / body weight) × 100.

3.2. Morphology of the distal intestine and liver

Fish fed the control diet with 0% SBM, did not show any signs of morphological damage or change in the DI at 4 or 8 weeks (Table 5. Fig. 1A). After 4 weeks, fish fed with SBM develop the typical characteristics associated of mucosal inflammation and intestinal damage and increased as the level of SBM increased in diet. SBM decreased enterocyte height with concomitant reduction of the SNV and nuclei displacement toward apexes of enterocyte, decreased the number and length of MF and increased the coalescence of the MF (Fig. 1B), increased the enlargement of LP (Fig. 1E to H) and SM (Fig. 1I to L). Few cellular infiltrations of leucocyte and eosinophilic granulocytes were also observed. Additionally, as the SBM level increased in the diet evident anatomical changes related to atrophy of the MF was observed, as well as, the hypertrophy and high hyperplasia of the GC toward the apexes of MF (Fig. 1C and D).

At the end of the experiment on 8 weeks, the severity of the histological changes increased over time in fish fed with SBM (Fig. 2). SBM inclusion in the diet reduced the number of MF from 55 and 51 to 46 MF in 22% and 44% SBM inclusion levels, respectively. This reduction in number of MF increased the intermucosal fold space. Fish fed with SBM presented an enlargement of the apical zone of the MF with lymphocyte infiltration and vascular congestion (Fig.2F). The 44% and 64% SBM inclusion levels resulted in the atrophic process of the MF associated with a reduced number of cells such as GC, decreased in the length of the MF and a subsequent loss of absorption surface. A decrease in the EH was observed in particular in the 22% SMB inclusion level, from 25 to 20.5 μm and an evident reduction of the SNV to almost extinction in 44% and 64% of SBM diets. Although, the width of the LP decreased in comparison to the 4 weeks, the number of LP of fish fed with SBM are greater than fish fed SBM-free diet. Contrarily to the 8 weeks, the SM decreased in fish fed with increasing levels of SBM at 8 weeks, with few foci of inflammatory cells such lymphocytes and eosinophilic granulocytes that migrate into the LP (Fig. 3).

The liver histology at 8 weeks in fish fed with 0% SBM diet showed large vacuoles of lipid with displacement of the nucleus of the hepatocytes from the central position and reduction of the sinusoid space (Fig. 4A–D), characteristic of a fatty liver, typical of this specie (Perez-Velazquez et al., 2017). Fish fed with increasing levels of SBM in the diets resulted in a reduction in cytoplasmic vacuolization with displacement of the nucleus to the central position and increase of the sinusoid space (Fig. 4E–H). At the 44% and 64% of SBM inclusion levels, larges lipid vacuoles were revealed (Fig. 4G and H). The increase of SBM in the diet increased infiltration of lipid vacuoles in the pancreatic tissue and in 44 and 64% inclusion levels large accumulation of vacuoles replaced part of the pancreatic tissue (Fig. 4I and L). Likewise, as dietary SBM content increased, the pancreatic acini increased in eosinophilic coloration (Fig. 4I and L).

3.3. Gene expression

Relative expression patterns of Interleukin (*il8*) are shown in Fig. 5. At 4 weeks relative *il8* expression exhibited a peak in fish fed the 22% SBM diet (2.00), whereas the expression level of fish fed the 0%, 44% and 64% SBM diets remained with significantly lower expression levels (0.87, 0.75, and 1.02, respectively). At 8 weeks, the highest expression level was found in fish fed the 44% SBM diet (1.10). Nevertheless, no significant differences were found ($P > .05$) among SBM inclusion levels (0.93, 0.82, and 0.54 for 0%, 22%, and 64%, respectively). An interaction between time (4 and 8 weeks) and diet (SBM inclusion levels) was found at 4 weeks ($P < .05$) were interleukin (*il8*) relative expression levels of fish fed the 22% SMB diet resulted in significantly higher levels compared to the other SMB inclusion levels.

3.4. Enzymatic activity

The protease activity present in the intestine; trypsin, chymotrypsin, and total alkaline proteases, revealed a gradual decrease as SBM increase in the diet. Higher values for fish fed with 0% SBM compared to the other treatments, while the activity of these enzymes in the pyloric caeca fail to show significant differences among treatments (Fig. 6). The same trend was observed for amylase activity ($P < .05$) in both organs. Although, L-aminopeptidase did not result in significant differences in total activity among treatments, a considerable decrease in intestinal activity was observed in the 44% and 64% SBM inclusion levels. The highest lipase activity occurred in the pyloric caeca and tended to increase in the intestinal region with increasing levels of SBM in the diet (Fig. 6). In relation to acid digestive enzymes, pepsin activity did not show any significant differences ($P = .751$) among treatments (Fig. 7).

4. Discussion

The present work evaluated the effect on the growth performance, digestive capacity, as well as the progression of histological changes of the distal intestine and the expression levels of an immune system reference gene in juvenile totoabas fed with SBM.

Fish performance resulted in a negative dose-dependent effect with dietary SBM inclusion. A gradually reduction in TGC is consistent with decreasing nutrient utilization and a lower PER with higher FCR. This trend was also observed in other species fed with SBM in their diets such as cobia *Rachycentron canadum* (Chou et al., 2004), silvery-black porgy *Sparidentex hasta* (Yaghoubi et al., 2016), spotted rose snapper *Lutjanus guttatus* (Silva-Carrillo et al., 2012) and Mediterranean yellowtail, *Seriola dumerili* (Tomas et al., 2005). In totoaba, this is the first work reporting the effect of SBM in the intestinal integrity assessed by histology and digestive enzyme activity. A recent studies with the same species, using SPC reported that diets containing up to 30% FM replacement exhibited no adverse effects (Trejo-Escamilla et al., 2016), whereas a 60% replacement of SPC resulted in a significantly reduced growth (López et al., 2015).

A reduction in growth performance is often associated with a lower feed intake when plant protein inclusion increases in diet (NRC, 2011). Some authors have suggested that reduce FI is due to the poor palatability of SBM caused by the bitter taste of saponins (Bureau et al.,

Table 5
Distal intestine measurements to illustrate the degree of enteritis in *T. macdonaldi* fed diets containing different SBM levels. Values are presented as means ± standard deviation (SD) of three replicates. P values resulting from two-way ANOVA test.

Time	4 weeks				8 weeks				Anova7				
SBM	0%	22%	44%	64%	0%	22%	44%	64%	Time (weeks)	Diet (%)			Int[6]
									4 vs 8	0	22	44	64	
MF number[1]	54.6 ± 4.9	55.3 ± 1.5	51.6 ± 1.3	46.3 ± 2.3	51.9 ± 1.0	46.1 ± 2.8	46.4 ± 6.0	46.4 ± 3.8	>	a	ab	ab	b	NS
MF length[2]	501.2 ± 24.5	528.7 ± 78.9	401.3 ± 26.1	383.0 ± 50.2	562.0 ± 65.3	590.9 ± 81.3	458.2 ± 30.7	435.3 ± 43.3	<	a	a	b	b	NS
EH[3]	25.2 ± 5.2	25.3 ± 2.6	18.1 ± 0.9	17.9 ± 0.5	21.3 ± 1.6	20.5 ± 0.8	17.6 ± 0.6	15.2 ± 1.0	>	a	a	b	b	NS
LP[4]	10.0 ± 0.6	16.8 ± 0.6	16.8 ± 2.0	24.0 ± 2.8	8.2 ± 1.4	11.7 ± 1.5	15.6 ± 1.1	17.5 ± 1.1	>	c	b	b	a	NS
SM[5]	25.4 ± 3.2y	26.7 ± 4.2zy	35.5 ± 5.6z	37.3 ± 5.6z	18.2 ± 1.0z	14.7 ± 2.6zy	13.3 ± 1.6y	14.4 ± 0.5y	>	*				*

a, b, c, for parameters with a significant effect of diet and no interaction, values without a common letter are different (a indicated the highest value; $P < .05$).
z, y, for parameters with a significant interaction, differences in diets are compared within each time (one-way ANOVA, Tukey's test), values without a common superscript are different ($P < .05$).
7 NS, non-significant; *, $P < .05$. For variables with a significant effect of time ($P < .05$) and no interaction, < or > indicates whether the values measured at 4 weeks was less than or greater than that measured at 8 weeks.
[1] MF number = mucosal fold number.
[2] MF length = mucosal fold length.
[3] EH = enterocyte height.
[4] LP = lamina propria.
[5] SM = sub-epithelial mucosa.
[6] Int = Interaction.

1998; Chikwati et al., 2012). In the present study, feed intake fails to show significant differences among dietary treatments, indicating good palatability of the diets even at the highest level of SBM. It is likely that the addition of krill oil as attractant, and the balanced supplementation of amino acids in all diets mitigate the adverse effects of palatability of the diets. Similarly, no significant differences in feed intake were reported for gilthead seabream even when fed with a diet containing 30% SBM (Robaina et al., 1995), red seabream *Pagrus major* with 56% of SBM (Kader et al., 2012) and in *Seriola dumerili* feed intake increase in diets with 40% and 50% SBM (Tomas et al., 2005).

Histological evaluation of the intestines of marine fish fed vegetable ingredients helps assess in greater detail the effects on the intestinal health at the different inclusion levels, and in the overall welfare of farmed fish. The current findings demonstrate that dietary SBM, even at relatively low levels of inclusion (i.e., 22%), impaired not only growth and feed efficiency, but also and more importantly, the morphology and function of the digestive tract. Based on the histopathological alterations previously reported for the DI in the literature, our findings suggest that totoaba suffered enteritis that lead to a mucosal atrophy. The DI inflammation causing morphological changes of the intestinal mucosa seems to depend on the SBM inclusion level, and the number of days feeding on the SBM. At 4 weeks, fish fed SBM diets showed a visible inflammation process which was more severe in the higher inclusion levels, notably the hyperplasia of GC (densely grouped in 44% and 64% SBM diets) in the apexes of the MF, wider LP and SM, shorted MF and EH with high reduction of SNV, almost to extinction. In species with different feeding habits, Urán et al. (2008a, 2008b) reported similar damage in Atlantic salmon *Salmo salar* and omnivorous common carp *Cyprinus carpio* even with only 7 days feeding with a 20% SBM diet. Additionally, in Atlantic salmon the inflammatory process tends to worsen with time (at 20 days), whereas for the common carp after 4 weeks a recovery from a case of enteritis was observed. In the present study, totoaba fed with SBM for 8 weeks showed an increased in the severity of intestinal damages. Fish fed with SBM diets exhibited some more branched folds in the intestine that had not been observed at 4 weeks (Fig. 2B, C, G). The most severe cases were found in fish fed with 44% and 64% SBM diets that showed a markedly reduce number and length of MF, increase fold fusion, revealing a clear atrophy. However, in some samples, few foci of eosinophils granulocytes in the SM with migration into the LP (Fig. 3) and complete loss of SNV with reduction of the enterocyte height was revealed.

The same inflammatory alteration in the DI was reported for the turbot *Scophthalmus maximus* from 26% to 54% of SBM at 8 weeks (Gu et al., 2016), Atlantic salmon from 10% to 45% of SBM at 60 days (Krogdahl et al., 2003), gilthead sea bream from with 30% of SBM at 80 days (Bonaldo et al., 2008) and rainbow trout (*Oncorhynchus mykiss*) with 45% SBM during 18 weeks (Heikkinen et al., 2006). Nevertheless, species such as Atlantic halibut *Hippoglossus hippoglossus* (Grisdale-Helland et al., 2002), channel catfish *Ictalurus punctatus* (Evans et al., 2005) European sea bass *Dicentrachus labrax* (Bonaldo et al., 2008) or turbot *Psetta maxima* (Bonaldo et al., 2011) did not exhibit any inflammatory response of intestinal mucosa when fed diets with 20% to 40% inclusion of SBM. This suggests the totoaba has a high sensitivity to dietary SBM.

The structural changes observed in the DI at 8 weeks are related to a MF atrophy process. Mukherjee and Nagarsheth (2015), defined mucosal atrophy as anatomical changes in the intestinal mucosa; such as reduced number of cells, decreased surface area and shortened villous height with a subsequent loss of intestinal function (MacDonald, 1992; Mukherjee and Nagarsheth, 2015; Shaw et al., 2012). These processes may alter nutrient transport in the intestinal epithelia. Additionally, this atrophic process resulting in loss of tissue mass in the DI is reflected in the reduction of the intestinal somatic Index (ISI) clearly documented at 8 weeks. Moreover, during this sampling date, it was notice that the intestine was flaccid and thinner in particular with the intermediate and high SBM inclusion levels. The integrity of the mucosal barrier is

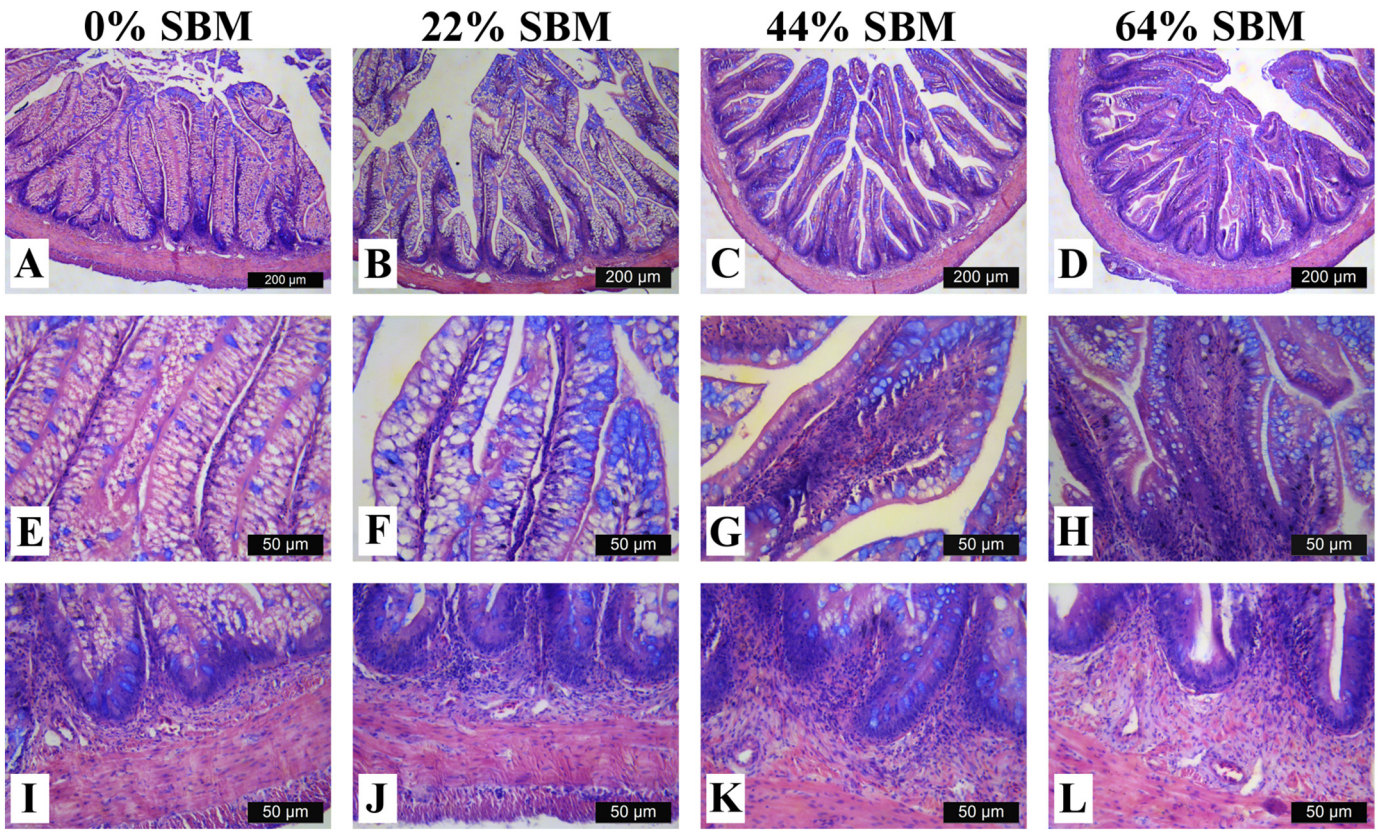

Fig. 1. Light microscopic images depicting morphological changes in distal intestine associated to inflammatory process in *T. macdonaldi* fed with 0% SBM (A, E, I), 22%SBM (B, F, J), 44% SBM (C, G, K) and 64% SBM (D, H, L) at 4 weeks. Mucosal folds length trend to decreases as SBM inclusion level increases (A to D, Bar = 200 µm); reduction of the supranuclear vacuoles, markedly increase in the width of lamina propria and hyperplasia of goblets cells (blue dots) in relation with SBM inclusion level (E to H, Bar = 50 µm); sub-epithelial mucosa directly enlarged as SBM inclusion levels increased (I to L, Bar = 50 µm). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

crucial in maintaining tissue homeostasis against pathogens and feed antigens, the mucus secreted from GC provides the first layer of intestinal protection and the integrity of mucosal barrier depends on cellular proliferation to replace damaged cells (Sahlmann et al., 2013). These morphological changes have been suggested to be related to a cellular turnover with a concomitant increase in cell migration and apoptosis (Bakke-McKellep et al., 2007; Sahlmann et al., 2015). Zhou et al. (2017) reported that fish fed with SBM were more susceptible to diseases when challenged with different pathogens. This inflammation of the DI is typically associated with the presence of soy saponins (Krogdahl et al., 2015) that bind to membrane cholesterol of intestinal epithelial cells forming holes and changing its permeability, facilitating the entry of pathogens into the enterocytes. Additionally, Buttle et al. (2001) reported that lectins such as agglutinins, are a contributing factor to the pathological damage in the distal intestine in Atlantic salmon and rainbow trout. Lectins bind to the intestinal brush border membrane resulting in a disruption of the folds integrity with sloughing of mucosa and cellular infiltration into the lamina propria.

Histological analysis of totoaba liver confirms a dose-dependent damage of this organ with SBM inclusion in the diets. Totoaba is considered a moderately lean fish and have their main energy reserves in the liver, which is reflected in a liver with a high fat content (Perez-Velazquez et al., 2017). The clear reduction in fat vacuoles as SBM inclusion increased; agrees with the pattern found for the HSI. In the sharpsnout seabream *Diplodus puntazzo* a similar trend in HSI was observed (Hernández et al., 2007). This reduction in the liver fat content has been reported in fishes under starving conditions (Barreto-Curiel et al., 2017; Kjær et al., 2009; Speakman and Mitchell, 2011). In addition, the increase of fat vacuoles and HSI at 64%, may be due to the fact that after consuming the lipid reserves of the liver, a process of catalysis is started on muscle tissue, where once again the nutrients circulate to be deposited again in the liver as reported by Barreto-Curiel et al. (2017).

Additionally, the highest levels of SBM in the diets caused an accumulation of peripancreatic fat in the fish pancreas. We do not know for certain if the presence of high levels of peripancreatic fat, could led to a reduction in pancreas functionality. However, a reduction in trypsin, chymotrypsin and amylase activity was observed possibly resulting in lower protein and carbohydrate digestion in the lumen of the intestine (Haard et al., 1996). We are aware that fasting could lead to a reduction of enzyme activity in the intestine. Nonetheless, all samples were taken at the same time and the objective of the present study was to compare among dietary SBM inclusion levels and not the postprandial activity. The reduction of enzyme activity could be due to the lower nutrient absorption, the chronic state of intestinal inflammation or potentiated by the peripancreatic fat resulting in a possible reduction of pancreas functionality. The reduction of the trypsin and chymotrypsin observed in this work could be explained by the presence of protease inhibitors in the SBM (Francis et al., 2001). However, both products used in the present study should be free of trypsin inhibitors according the manufacturer. Nonetheless, other antinutritional factors present in the SBM could lead to a disruption of the normal digestive capacity. For example, it is possible that the lower trypsin activity could be associated to a lower enterokinase production by the enterocytes from the atrophied intestine to activate trypsiongen. Additionally, is interesting to note that the higher SBM levels in the diet the greater eosinophilyc granules in the pancreas (i.e., zymogenes), suggesting that the pancreas is compensating for the lower protease activity observed in fish fed the higher SBM diets by producing more zymogens.

In the present study, lipase activity increased with higher levels of

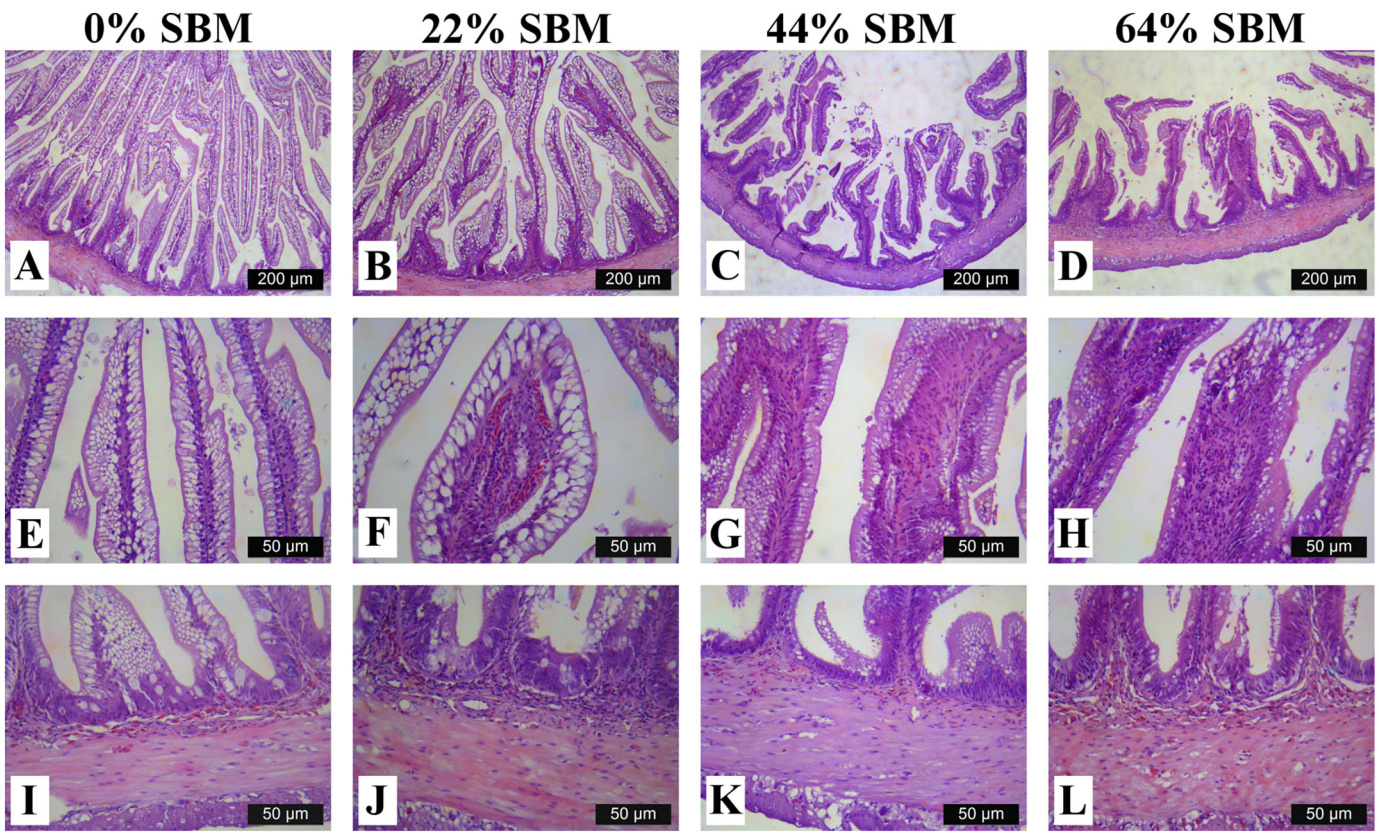

Fig. 2. Light microscopic images depicting morphological changes in distal intestine associated to inflammatory process in *T. macdonaldi* fed with 0% SBM (A, E, I), 22%SBM (B, F, J), 44% SBM (C, G, K) and 64% SBM (D, H, L) at 8 weeks. Mucosal folds number and length trend to decreases as SBM inclusion level increases (A–D, Bar = 200 µm) and MF of 44% and 64% showed an atrophic process; increase in the width of LP with a reduction in supranuclear vacuoles in relation with SBM inclusion level (E–F, Bar = 50 µm); sub-epithelial mucosa contrary to 4 weeks, were shrink as SBM inclusion levels increase (I–L, Bar = 50 µm).

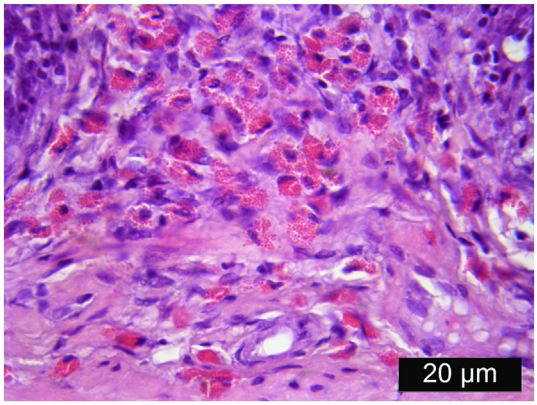

Fig. 3. Foci of inflammatory infiltrations (eosinophilic granulocytes) at 8 weeks due to the inclusion of SBM in *T. macdonaldi* diet.

soybean meal in the diets. This is in contrast to the results reported for other species such as the silvery-black porgy (Yaghoubi et al., 2016). In the latter study the decrease in lipase activity was associated with a low taurine content from the soybean meal diets, which limited the production of bile salts and therefore lipid digestion. Nonetheless, all diets in the present study were supplemented with taurine (1% of diet) to maintain adequate liver function. Krogdahl et al. (2010), reports that the content of fibers, phytosterols, phytoestrogens and saponins in plant ingredients affect the re-absorption of bile salts and increases the amount of bile in the intestine, which may stimulate the secretion and activity of the lipase. This could help explain the increase in lipase activity found in the intestine in the present study in fish fed the higher levels of SBM.

L-Aminopeptidases (LAP) located in the intestinal brush border are enzymes known to carry out intestinal membrane digestion (Tibaldi et al., 2006), were significantly affected by SBM in the diet. These enzymes in combination with other intestinal membrane bound enzymes (i.e., alkaline phosphatases) are important for the absorption of the nutrients to keep the homeostasis (NRC, 2011). Reduction of activity of these intestinal enzymes could be explained by the atrophy degree with the tissue disruption observed in the histological analysis accordingly the SBM inclusion levels. van den Ingh et al. (1991) reported an intestinal microvilli reduction in *Salmo salar* when SBM was included in the diet. Previous reports using SBM in fish diets, concluded that a reduction in enzyme activity was the result of epithelial cells lost in the intestinal tract associated with a proliferation of immature cells (Bakke-McKellep et al., 2007; Chikwati et al., 2013). Additionally, it has been suggested that under an infection processes and inflammatory antigenic conditions, the intestine suffers a reduction of enzyme activity in the mucosal tissue (Krogdahl et al., 2003; MacDonald, 1992). In the present study it was possible to observe an early immunological reaction at 4 weeks in the treatment with 22% SBM, with a clear decrease in intestinal functions according to SBM inclusion levels. However, due to the lack of additional immune reaction at higher SBM levels or at 8 weeks, it is suggested that the inflammation process occurs during the first days, triggering the following processes until the cell is completely exhausted leading to a clear intestinal damage. Therefore, differences with earlier works with the same species (López et al., 2015; Trejo-Escamilla et al., 2016) could be associated with the type soybean used at high supplementation levels of SBM. Bureau et al. (1998) reported that soybean sensitivity is species-specific and depends on the tolerance to some of the antinutritional factors present in the SBM such as saponins and other anti-nutritional components as observed in chinook

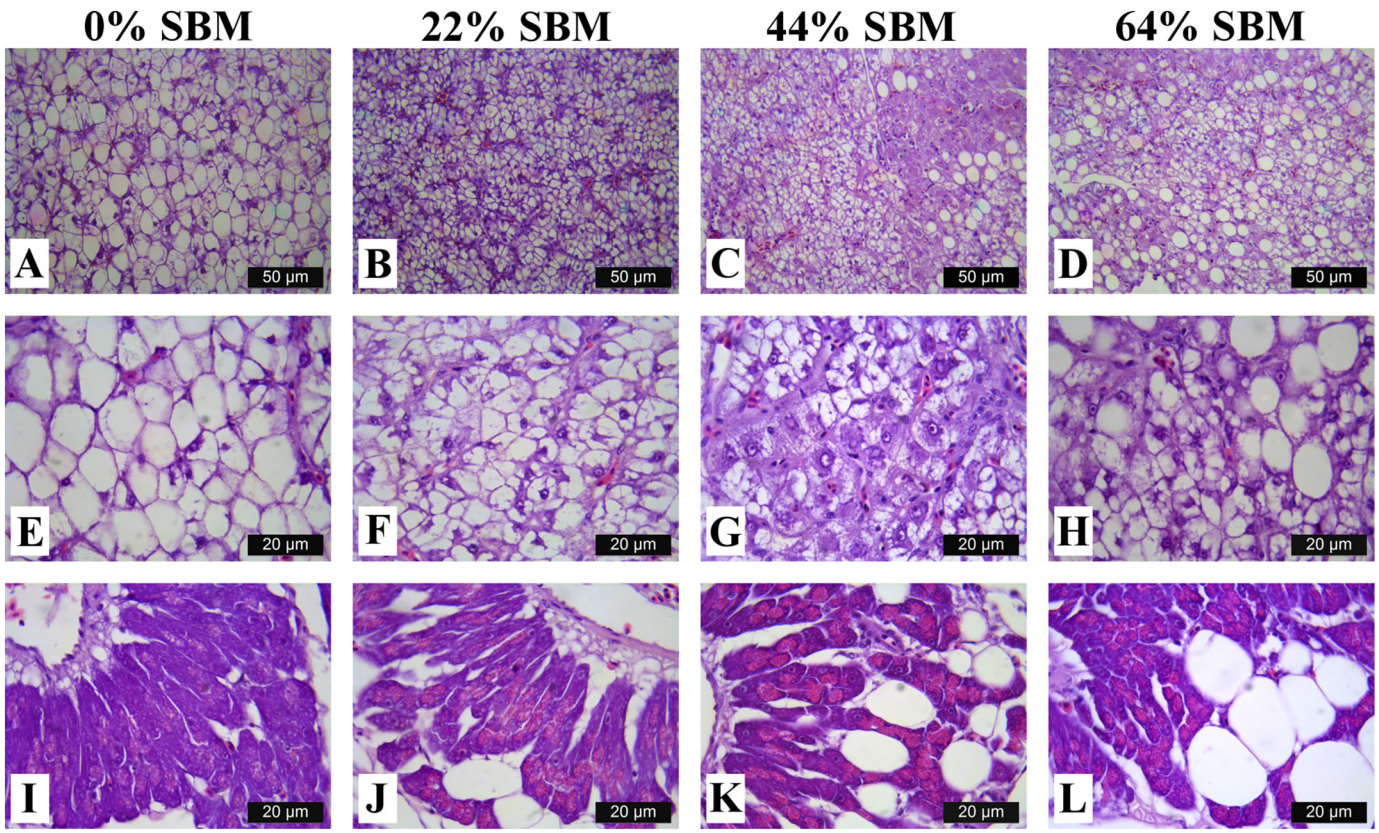

Fig. 4. Light microscopic images depicting morphological changes in *T. macdonaldi* liver fed with increasing levels of SBM in diet at day 56. A reduction in cytoplasmic vacuoles (Fig. 4 A–D, Bar = 50 µm) with displacement of the nucleus to the central position and increase of the sinusoid space (Fig. 4 E to H, 20 µm) is observed as SBM inclusion level increases. The increase of SBM in the diet increased infiltration of lipid vacuoles in the pancreatic tissue (Fig. 4. I to L 20 µm) and in 44 and 64% large accumulation of vacuoles replaced part of the pancreatic tissue (Fig. 4. I and L,). Likewise, the eosinophilic coloration increased in the pancreas acini in SBM diets (Fig. 4. J, K, and L).

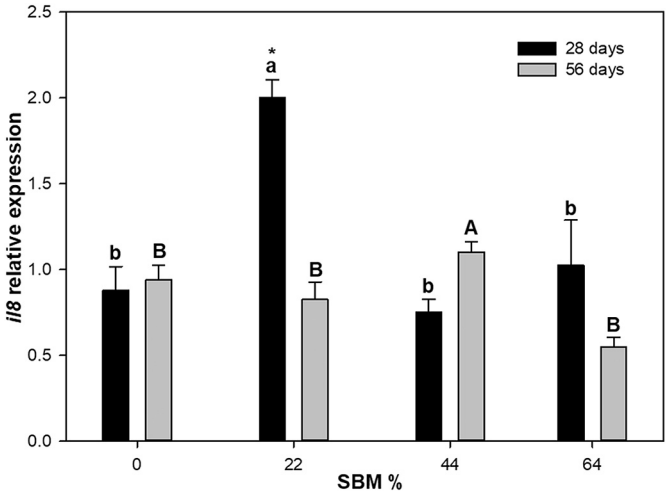

Fig. 5. Interleukin (*il8*) relative expression in *T. macdonaldi* 4 and 8 weeks, fed diets containing different SBM levels. Different letters represent significantly different values ($P < .05$) within the same day ($n = 3$), lowercase for 4 weeks and uppercase for 8 weeks. * shows the interaction between age and SBM inclusion level.

salmon and rainbow trout. However, the results from the present work suggest that the inclusion of SBM produces functional damages in the digestive system and physiological affectations in totoaba even at a relative low inclusion level (i.e. 16% SBM in combination with 5.5% SPC).

Interleukin-8 (*il-8*) is one of the main immune-relevant cytokines produced during an inflammatory reaction to induce wound healing during an inflammatory process (Li and Yao, 2013; Lilleeng et al., 2009). Therefore, the relative expression level of *il-8* is a good indicator of an inflammatory response. As expected and in agreement with the literature, we observed a significant increase in *il-8* expression in fish fed SBM-based diets at 4 weeks, including at the lower inclusion level (22% SBM), indicating a response in the posterior intestine as a result of a possible acute reaction (Bonaldo et al., 2015; Gu et al., 2016; Lilleeng et al., 2009; Nordrum et al., 2000; Perera and Yúfera, 2017; Urán et al., 2008b). Previous studies have reported that DI changes appear to facilitate a subsequent sensitization, proliferation and mobilization of T cells (Lilleeng et al., 2009; Sahlmann et al., 2013). The expression levels of the *il-8* are typically associated to the stimulatory factor (pathogens / stressors) during the beginning of the immune response. However, if the factor is extreme or sustained, the T cell population might be exhausted and become physically depleted (Klenerman et al., 2002). Therefore, the reduction in expression levels of *il-8*, demonstrate the negative effect of dietary SBM, producing a typical immune response under the presence of a permanent negative stimulus (i.e., antinutritional factors or pathogens), regardless of the inclusion level at 8 or 4 weeks at higher levels of SBM. Nonetheless, the reduction in expression levels, in response to the higher SBM inclusion level, could be a consequence of the degree of damage in the DI, as verified through histological analysis (atrophy). It is possible that the reduction in *il-8* expression within the DI could be a result of the increased epithelial exfoliation and tissue damage as reported for many species (Bakke-McKellep et al., 2007; Gu et al., 2016; Lilleeng et al., 2007, 2009; Sahlmann et al., 2013). Interestingly, several authors have reported that chronic and acute stress in fish affects the welfare indicators and energy metabolism (Barcellos et al., 1999; Santos et al., 2010; Van Weerd and Komen, 1998), but

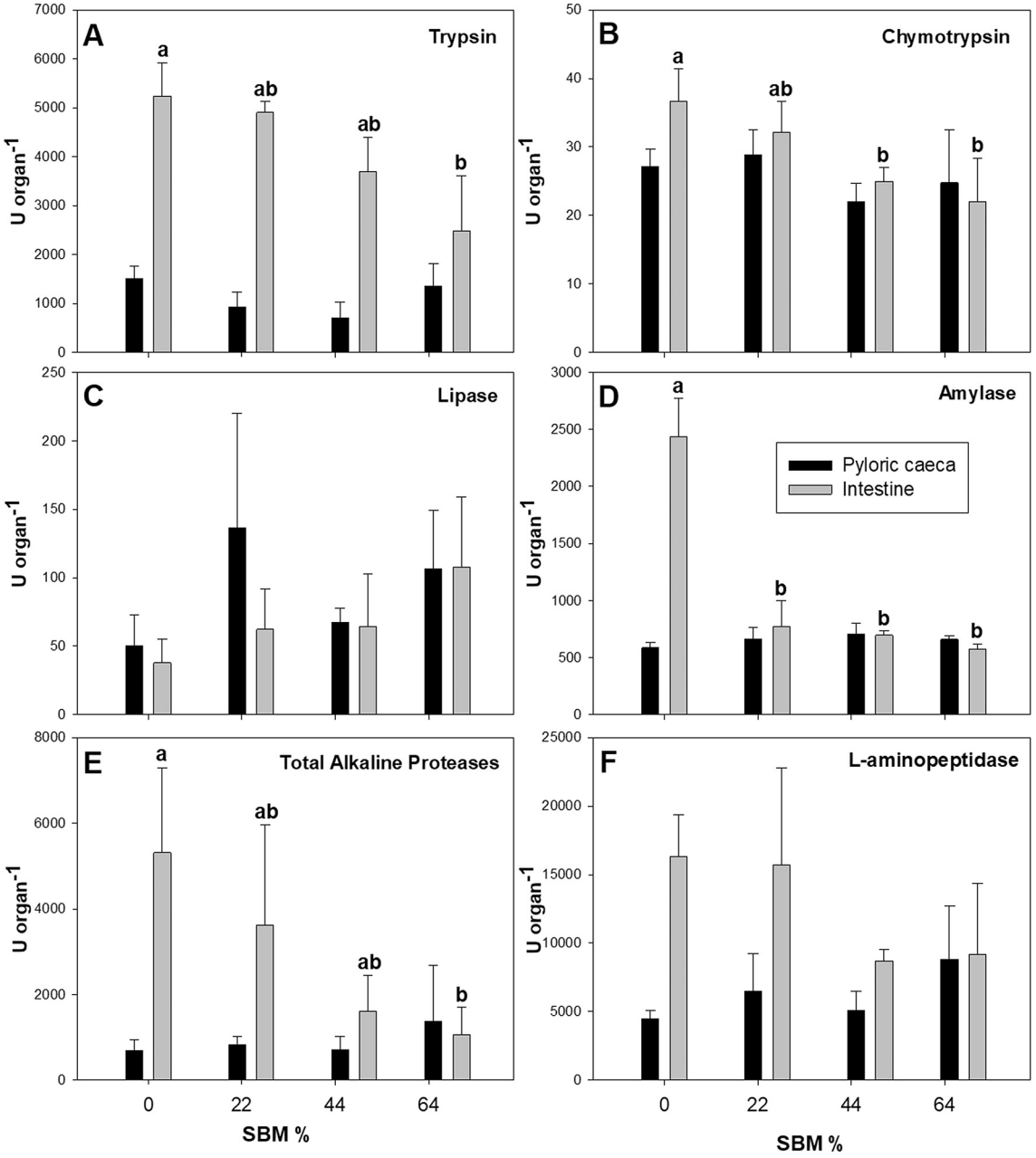

Fig. 6. Total enzyme activity (U organ $^{-1}$) per pyloric caeca and intestine for trypsin (A), chymotrypsin (B), lipase (C), amylase (D), total alkaline proteases (E) and L-aminopeptidase (F) in *T. macdonaldi* at 8 weeks fed diets containing different SBM levels. Different letters represent significantly different values ($P < .05$) within the same organ (n = 3).

above all, there is an negative immunological effect which can trigger an increase in infectious diseases during the time the negative stimulus is present (Pickering and Pottinger, 1989; Snieszko, 1974). As a result of the above morphological changes and a likely chronic immunological response, with a concomitant failure of the functionality of the intestine, which consequently affects fish performance.

Based on the overall performance of the fish and through the histological analysis from the distal intestine performed in the present study, we suggest the antinutritional factors in SBM are related to adverse effects found in this work, such as the reduction of the digestive enzyme activity, early inflammation reaction, tissue disruption of distal intestine, reducing nutrient absorption, late atrophy of the intestinal mucosa, with growth reduction.

In conclusion, the present study characterizes the limitations of SBM inclusion in *T. macdonaldi* diets, noticeably affecting the structure and physiology of distal intestine (i.e. at histological and molecular level) as well as the overall digestive enzyme activity and consequently impairing growth when included at the intermediate (44% SMB) and higher (64% SBM) levels evaluated. Additionally, our findings demonstrated a state of intestinal atrophy in totoaba caused by the exposure of high dietary SBM inclusion levels during the time. Therefore, the present work directly suggests that SBM should be cautiously used in totoaba feeds.

Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

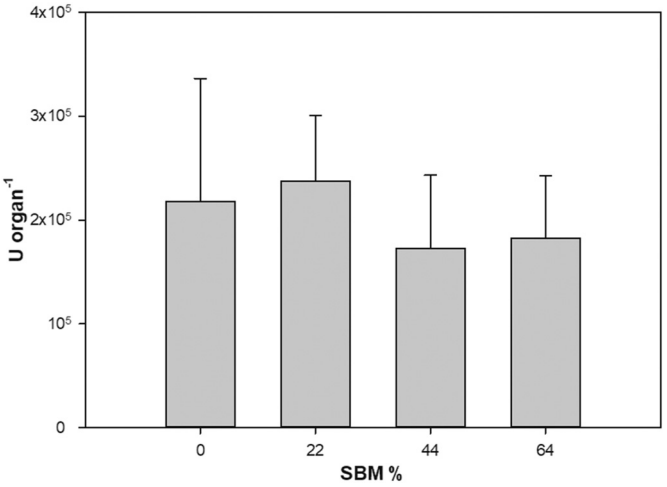

Fig. 7. Acid protease (pepsin) activity with different SBM inclusion levels in diet in *T. macdonaldi* at 8 weeks fed diets containing different SBM levels.

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